Bioavailability and Biodegradation of Organic Xenobiotic Recalcitrant Polycyclic Aromatic Hydrocarbons (PAHs) in Different Soil Environments

By

Jamshid Jazestani

Department of Bioresource Engineering
Faculty of Agricultural and Environmental Sciences
McGill University, Montreal, Quebec, Canada

February 2011

A Thesis Submitted to the Faculty of Graduate Studies and Research of McGill University in Partial Fulfilment of the Requirements of the

Degree of

Doctor of Philosophy

ABSTRACT

Bacterial isolates from soils contaminated with a large range of organic pollutants were tested for their ability to metabolise selected recalcitrant PAHs as their sole energy and carbon source. Plating of soil microorganisms isolated from 12 petroleum-contaminated soils on solid medium, supplemented with the PAH phenanthrene as sole carbon source, yielded 228 phenanthrene-degrading bacterial strains, of which 60 were further characterized. Southern DNA-DNA hybridization identified 14 of the 60 colonies as potentially competent in naphthalene degradation (*ndoB* Positive). The 14 bacterial isolates were further screened for their capacity to mineralize six radiolabelled PAHs serving as their sole carbon and energy source. Under such conditions, bacterial isolate strain S65 cumulatively mineralized 61% of phenanthrene, 61% of pyrene, and 24% of fluoranthene over a 9-day period, but could not degrade naphthalene, anthracene or fluorene.

Three different characterisation approaches were applied to isolate S65 (designated ***Mycobacterium* sp. Strain S65** in **Genbank**): (***i***) micro/macro-morphological — phase-contrast microscopy showed S65 to be a Gram variable rod-coccus; S65 produced creamy yellow circular colonies that were catalase-positive, (***ii***) molecular — HPLC analysis of Strain S65's cell wall mycolic acids indicated it was a novel *Mycobacterium*, (***iii***) molecular — species confirmed by others' 16S rRNA gene sequence analysis. Strain S65's mineralization kinetics also showed it to be a new and unique PAH-degrading *Mycobacterium*.

Strain S65's capability and capacity to mineralize ^{14}C-labelled phenanthrene or pyrene, allowed to age in sterile farm soil microcosms for 0, 30, 180 or 360 days prior to inoculation with S65, was quantified on the basis of cumulative $^{14}CO_2$ evolution 1, 3, 6, or 9 days after inoculation, and expressed as a percent of the initial PAH concentration. For each ageing period, a factorial combination of four soil types (Sandy-Loam, Sand, Clay, Clay-Loam)**,** two levels of soil organic matter (high, low), and two concentrations of PAH substrate (phenanthrene, 250 or 500 mg L^{-1}; pyrene, 500 or 1000 mg L^{-1}), was replicated six times, with three replicates receiving 1.0×10^7 CFU of live S65 and 3 'negative' controls receiving heat-killed S65. Un-inoculated soil microcosm controls

were implemented, as well as 'positive' controls in liquid growth medium. Results were analysed by ANOVA.

Strain S65 required a 3-day lag to initiate the degradation of non-aged (day 0) phenanthrene, but proceeded immediately with pyrene. Cumulative mineralization of phenanthrene from day 3 to day 9 at either initial PAH concentration was greater in sandy-loam soil with low organic matter than in clay-loam soil with high organic matter (18.5-20.0% *vs.* 13.5-14.5% on day 0; 13.0-14.5% *vs.* 10.5-11.5% after 30 days' ageing). Cumulative mineralization of pyrene reached 18.5% and 19%, respectively (for initial concentrations of 500 or 1000 mg L^{-1}) after 24 hours, close to the maximum reached at 9 days (no time lag occurred). After 9 days, for both initial concentration, pyrene mineralization was greater in sandy-loam soil with low organic matter than clay-loam soil with high organic matter (20.0-21.0% *vs.* 14.5-15.5% on day 0; 13.25-14.0% *vs.* 11.5-12.5% after 30 days' ageing). Overall mineralization declined as ageing progressed; in particular, for 180- and 360-day aged PAHs, cumulative mineralization dropped below 10%, well below rates for shorter ageing times. This suggests that as their ageing in the soil medium proceeds, phenanthrene and pyrene become less accessible to degradation.

This multidisciplinary study highlights the fact that *Mycobacterium* sp. Strain S65 shows a great potential to be useful in biodegradation of high molecular weight PAHs., and strongly supports the general concept of ***Mycobacteria*** having a significant role in PAHs-biodegradation, bioremediation, and biodetoxification in contaminated environments.

RÉSUMÉ

La capacité d'isolats bactériens, provenant de sols contaminés avec une grande variété de polluants organiques, à métaboliser une sélection de HAPs rémanents comme source unique d'énergie et de C, fut évaluée. La mise en culture de microbes du sol provenant de 12 sols contaminés par des substances pétrolières sur une gélose avec un ajout de phénanthrène, représentant l'unique source de C, identifia 228 souches bactériennes pouvant décomposer la phénanthrène. Les 14 colonies pouvant dégrader la naphthalène (*ndoB*+) furent triés selon leur capacité à minéraliser six HAPs. Sur 9 jours la minéralisation cumulative de phénanthrène ou de pyrène par l'isolat S65 fut de 61%, et 24% pour la fluoranthène. Cet organisme ne put minéraliser la naphthalène, l'anthracène ou la fluorène.

L'isolat S65 (designé ***Mycobacterium* sp. isolat S65** dans **Genbank**) fut caractérisé: (***i***) micro/macro-morphologique — la microscopie montra que S65 était un bâtonnet-coccus, gram-variable, aux colonies d'un jaune crémeux et catalase-positif, (***ii***) moléculaire — une analyse par CLHP des acides mycoliques de la paroi cellulaire de l'isolat S65 indiqua qu'il s'agissait d'une nouvelle espèce de *Mycobacterium*, (***iii***) moleculaire — le genre fut confirmé par d'autres par une analyse génétique. La cinétique de minéralisation des HAPs par l'isolat S65 confirma aussi qu'il représentait une espèce nouvelle et unique de *Mycobacterium,* compétente dans la dégradation des HAPs.

La capacité de minéralisation de l'isolat S65 et le taux de minéralisation de pyrène et de phénanthrène radiomarqués ayant subi un vieillissement pour 0, 30, 180 ou 360 jours dans un microécosystème en sol agricole avant leur inoculation avec l'isolat S65, furent quantifiées par l'évolution cumulée de $^{14}CO_2$, 1, 3, 6, or 9 jours après l'inoculation. Pour chaque vieillissement, une combinaison factorielle de quatre types de sol (loam sableux, sableux, argileux, loam argileux**)**, deux niveaux de matière organique (MO) du sol (élevé, bas), et deux concentrations de substrat HAP (phénanthrène, 250 ou 500 mg L^{-1}; pyrène, 500 ou 1000 mg L^{-1}) furent évalués. Trois des six réplicats reçurent 1.0×10^7 CFU de S65 vivant et trois témoins 'negatifs' reçurent des cellules thermostérilisées. Des microécosystèmes n'ayant reçu aucune inoculation, ainsi que des

témoins ‘positifs’ en culture liquide s’ajoutèrent à ceux-ci. Les données furent soumises a une ANOVA.

L’isolat S65 nécessita 3 jours avant de commencer à dégrader la phénanthrène non-vieilli (jour 0), mais commença immédiatement avec le pyrène. La minéralisation du phénanthrène du jour 3 au jour 9, pour toutes concentrations de HAP, fut plus élevée pour le loam sableux avec peu de MO que pour le loam argileux avec beaucoup de MO (18.5-20.0% *vs.* 13.5-14.5% sans vieillissement, jour 0; 13.0-14.5% *vs.* 10.5-11.5% après 30 jours de vieillissement).

La minéralisation cumulative de pyrène atteignit 18.5% et 19% (500 ou 1000 mg L^{-1}, respectivement) après 24 hr, près du niveau maximum atteint à 96 hr (i.e., sans aucun délai). Pour quelque concentration de HAP que ce soit, la minéralisation cumulative de pyrène du jour 3 au jour 9 fut plus élevée pour le loam sableux avec peu de MO que pour le loam argileux avec beaucoup de MO (20.0-21.0% *vs.* 14.5-15.5% sans vieillissement, jour 0; et 13.25-14.0% *vs.* 11.5-12.5% après 30 jours de vieillissement). La minéralisation diminua avec la progression du vieillissement. Pour les HAP vieillis 180 ou 360 jours, la minéralisation cumulé diminua en deçà de 10%, soit plus bas que pour les vieillissements plus courts. Donc, lorsque que leur vieillissement dans le sol avance, la phénanthrène et le pyrène deviennent moins accessibles à la dégradation.

L’isolat S65 du genre *Mycobacterium* sp. démontre un potentiel utilitaire vis à vis la biodégradation de HAPs de poids moléculaire élevé, et soutient l’idée que les ***Mycobacteries*** ont un rôle dans la biodégradation, biorestauration et biodétoxication des HAPs dans les environnements contaminés.

ACKNOWLEDGMENTS

I wish to express my heartfelt gratitude and sincere appreciation to my thesis supervisor, ***Shiv O. Prasher***, James McGill Professor and Chair of the ***Department of Bioresource Engineering at McGill University***, for his leadership, his immense and continuous guidance, and his deep knowledge in various disciplines, which contributed to this project. I admire his humour, understanding, unfailing patience, kindness, goodness, moral support and for the confidence, he showed in me for my research work, especially during the tough periods of my Ph.D. project. What a wonderful road trip! It was an experience that I will never forget. My sincere appreciation is also extended to Ms. ***Sunita Prasher*** for her warm hospitality, caring, and consideration of students.

I would sincerely like to acknowledge James McGill Professor ***Chandra A. Madramootoo***, for his excellent teaching abilities, research skills and his leadership as Dean of the Faculty of Agricultural and Environmental Sciences of McGill University. I also admire his intimate and modest relationship with everyone on campus and for his unfailing moral support.

I wish to express my appreciation and sincere gratitude to Professor ***Michael Ngadi*** (thesis internal examiner) and Professor ***Ali Madani*** (thesis external examiner) for their precious comments and valuable peer reviewing of this research work.

I would also like to express my deep gratitude to my M.Sc. supervisors: Emeritus Professor ***Robert S. Broughton*** and Professor ***Suzelle Barrington***. My sincere appreciation is also extended to Professors ***G.S.V. Raghavan***, ***Eric Norris***, ***Angus Mackenzie***, ***Edward McKyes***, Professor Emeritus ***Robert Kok*** and Professor ***Robert Bonnell*** for their friendship, excellent teaching abilities and research skills, which provided a whole lots of credits for them and also for being kind and helpful to all students.

I would sincerely like to express my sense of appreciation to a lovely person, Ms. ***Susan Gregus*** (administrative assistant) for her kindness, goodness, and unfailing support to all students. I also thank Ms. ***Trish Singleton*** and Ms. ***Abida Subhan*** (secretaries) for their departmental services.

I am deeply grateful for the financial support provided by the ***Scholarships, Student Aid & International Student Services of McGill University.*** I wish to express my appreciation and sincere gratitude to Ms. ***Judy Stymest***, for her leadership as Director of Scholarships, Student Aid & International Student Services of McGill University, her vision and understanding in helping students to overcome their financial problems and, in particular, for providing me student aid during the difficult periods of my graduate studies.

I wish to express my appreciation and sincere gratitude to Ms. ***Aghdas Zamani*** from McGill University for her precious assistance, insightful inputs into microbiological experiments, peer reviewing of manuscripts, kindness, goodness, and unfailing moral support.

I would like to thank my dear friends, Professor ***Venkatesh Meda*** and Professor ***Chantal Hamel*** for their precious assistance, insightful inputs, kindness, goodness, and unfailing moral support.

I sincerely wish to acknowledge my dear friend Doctor ***Yousef Karimi-Zindashty*** for his precious assistance, insightful inputs, peer reviewing and unfailing moral support.

I would like to express my sincere gratitude to my dear friend Doctor ***Georges T. Dodds*** for his valuable editorial work, peer reviewing, and French translation of the abstract (Résumé) of this multi-disciplinary Ph.D. thesis.

I would like to acknowledge the financial support provided by the ***National Research Council of Canada's Biotechnology Research Institute*** (***NRC-BRI***) for the initial part of this research work. I also wish to extend my sincere appreciation to Doctor ***Charles W. Greer*** and Doctor ***Jalal Hawari***, from ***NRC-BRI***, for valuable supervision and insightful guidance during the first part of the microbial research work.

I also wish to express my gratitude to my dear friends and researchers at ***NRC-BRI*** during the early stages of microbial work, particularly Professor ***Lyle Whyte*** (also, for his peer reviewing of one manuscript), ***Gesine*** (***Ina***) ***Wisse***, ***Nathalie Fortin***, Doctor. ***Laleh Yerushalmi***, Doctor ***David Juck***, Doctor ***Real Roy***, ***Anca Mihoc***, ***Sylvie Sanschagrin***, ***Claude Masson***, and ***Serge Delisle*** for being generous, helpful, and for their insights in this research work.

I sincerely acknowledge the valuable guidance and moral support of Professors ***Willliam D. Marshall***, the late ***James P. Smith***, and ***Brian T. Driscoll*** (also, for his peer reviewing of one manuscript) for their generosity and allowing me to access and use their laboratories, facilities, chemicals, and for their scientific insights in this study.

I greatly acknowledge Professor ***Caroline Begg*** and Ms. ***Helene Lalande*** for their invaluable help in selection and analysis of different farm soils. I would like to acknowledge Professors ***Urs Kuhnlein*** and ***David Zadworny***, for allowing me to access their laboratories and using their radioactive facilities during the ageing of PAHs substrates, as well as Professor ***Leroy Phillip*** for valuable guidance before my comprehensive exam.

I also wish to express my sincere gratitude to Professor ***Paula Ribeiro*** for her generosity, and allowing me to access to the radioactive laboratory and using the Liquid Scintillation Counter for analysis of microbial mineralization data. I also wish to express my gratitude to Ms. ***Louise Thibert*** from the ***Laboratoire de Santé Publique du Québec*** (***LSPQ-Bactériologie***) and the ***Institut National de Santé Publique du Québec*** in Montreal (Quebec, Canada), for her laboratory help, particularly her valuable insights regarding the analysis of Mycolic Acid Esters.

A special thanks go to Professor ***Martin Chénier***, Doctor ***Ramanbhai Patel*** and Doctor ***Jayashree Ramaswamy*** for their peer reviewing of two manuscripts and to Ms. ***Manon Leduc*** from ***École Polytechnique de l'université de Montréal***, for providing different PAHs-contaminated soils.

I also wish to express my sincere gratitude to my dear friends Professors ***Valérie Orsat***, ***Grant Clark***, ***Jacques-André Landry***, ***Jan Adamowski*** and Doctores ***Emilia Antecka***, ***Raman Bassi***, ***Reza Mehmannavaz***, ***Bano Mehdi***, ***Abdirashid A. Elmi***, ***Timothy John Rennie***, ***Marc-David Andrade***, ***Pradeep Kumar Goel***, ***Malek Amiali***, ***Sadia Ehsan***, ***Azfar Hussain*** and graduate students ***ShuangYe Lucia Qiao***, ***Eman Elsayed***, ***Golmar Golmohammadi***, ***Jamshid Rahimi***, ***Lylia Khennache***, ***Hernan Rey-Sanchez S***. and ***Jamie Troini*** (Network Administrator) for their friendship and assistance throughout the course of my graduate studies. There are also so many other people to thank: many people whom I had the pleasure of meeting and the list goes on. Thank you all for your kindness and friendship.

I would finally like to express my great appreciation and sincere gratitude to my precious and lovely members of my family for their understanding, caring, continuous and unconditional love, kindness, goodness, unfailing patience and all kind of support throughout my life. This appreciation extends in particular to my lovely children ***Azar Jazestani*** (my daughter) and ***Behruze Jazestani*** (my son), to their kind and good mother, my long time comrade ***Homa Alizadeh***, and her respectful extended family. I further acknowledge my brother ***Bahman Jazestani*** and his beautiful daughter ***Shiva Jazestani***, for being too good and generous to me and for immense, unfailing, and continuous financial and spiritual support, especially in difficult times. Special thanks are extended to my kind and gorgeous sister ***Khorshid Jazestani*** and her humorous son ***Pooyan Najaf***, for their kindness in providing care to the elders of the family, and lovely ***Jamie Roy*** for his goodness, kindness, and moral support. I would finally like to express my appreciation to my dear late ***parents*** and ***aunts*****,** and my living ***aunt***. You all have always been there for me, and have loved and encouraged me to follow my desires and goals. I wish to acknowledge with gratitude my indebtedness to the members of my family for their kindness, goodness, humour, understanding, unfailing patience, time, consideration, and all kind of help and support throughout the course of my life. This research work would not have been possible without your generosity, assistance, companionship, the very capable spiritual support, and unconditional love and affection provided by all of you to let my dreams come true.

DEDICATION

I sincerely wish to dedicate this thesis to those people who, through their vision, commitment, and humanity ***have***, from the dimmest ages, presently ***do***, and ***will***, as long as our planet sustains life, struggle for freedom, equality, and justice; in particular those who contribute to the protection and respect of all natural resources, species, individuals, ideas and beliefs, leading to a holistic ecological view wherein humans, other species and the environment act as a single system, maintaining an equilibrated global ecosystem and sustaining the balance of life on our beautiful planet.

It is also with heartfelt wishes that I further dedicate this piece of work to my family: my lovely children ***Azar Jazestani*** (my daughter) and ***Behruze Jazestani*** (my son), for their understanding, sacrifices and unconditional love; their mother and my long time comrade ***Homa Alizadeh***, for her kindness and goodness; my respectful and generous brother ***Bahman Jazestani***, for his unique manner of brotherly care, kindness and goodness; my gorgeous sister ***Khorshid Jazestani***, for her goodness and kindness; and my dear late ***parents*** and ***aunts***, and my living ***aunt***, for their unconditional love and affection, as well as the unique way in which they parented and guided me. They also taught me to enjoy and appreciate the fields of science and art, and encouraged me to set high standards in my life. This research work represents little compensation for the unconditional love, affection, time, support, encouragement, understanding, generosity, and assistance they have so freely given me. Cordially, I wish to express my great appreciation and sincere gratitude to all of you. Lastly, I am so delighted and it is with great joy and pride that I would humbly like to write:

This thesis is as much yours as it is mine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FORMAT OF THESIS

This thesis is submitted in the form of original papers suitable for journal publications. The thesis format has been approved by the Graduate and Postdoctoral Studies Office, McGill University, and follows the conditions outlined in the "Thesis Preparation and Submission Guidelines, I. Thesis Preparation, C. Manuscript-based thesis" which are as follows:

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following:

(a) A table of contents;
(b) a brief abstract in both English and French;
(c) an introduction which clearly states the rational and objectives of the research;
(d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper);
(e) a final conclusion and summary;
(f) a thorough bibliography; and
(g) appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.**"**

CONTRIBUTION OF AUTHORS

These manuscripts (research works) have been submitted for publication:

1. **Jamshid Jazestani** and Shiv O. Prasher. 2011. ***Isolation of Recalcitrant PAHs-degrading Bacterial Strains from Different Contaminated Soil Environments and Mineralization of Selected PAHs Compounds***. This manuscript has been submitted for publication in ***Environmental Pollution*** in 2011.
2. **Jamshid Jazestani** and Shiv O. Prasher. 2011. ***Characterization of Recalcitrant PAH-degrading Bacterial Isolates from Soils Contaminated with a Range of Organic Pollutants***. This manuscript has been submitted for publication in ***World Journal of Microbiology*** in 2011.
3. **Jamshid Jazestani** and Shiv O. Prasher. 2011. ***Biodegradation of Soil-aged ^{14}C-labelled Phenanthrene by Mycobacterium sp. Strain S65 in Different Soil Environments***. This manuscript has been submitted for publication in ***Biological Engineering*** in 2011.
4. **Jamshid Jazestani** and Shiv O. Prasher. 2011. ***Microbial Degradation of Soil-aged ^{14}C-labelled Pyrene by Mycobacterium sp. Strain S65 in Different Soil Environments***. This manuscript was published in ***Biological Engineering*** 3(3): 127-149.

The research work reported here was performed and completed by the candidate **Jamshid Jazestani** under the supervision of professor Shiv O. Prasher Chair of the Department of Bioresource Engineering, Macdonald Campus of McGill University**.** The necessary funds for this research project were provided by Biotechnology Research Institute (BRI) of National Research Council of Canada via Drs. Charles W. Greer and Jalal Hawari and McGill University via professor Shiv O. Prasher.

All experimental work was done by the candidate, as well as all the data analysis and writing of the articles and thesis. The authorship of all articles (**Chapters 3** to **6**) is **Jamshid Jazestani** and Shiv O. Prasher.

LIST OF ACRONYMS AND ABBREVIATIONS

The most commonly used symbols, abbreviations and nomenclatures in this research work follow as:

(ACS)	American Chemical Society
(ASABE)	American Society of Agricultural and Biological Engineers
(ATCC)	American Type Culture Collection
(ATSDR)	Agency for Toxic Substances and Disease Registry
(**BRI**)	Biotechnology Research Institute
(C) /(°C)	Chemical Concentration/ Centigrade
(CAWQ)	Canadian Association on Water Quality
(CEC)	Cation Exchange Capacity
(CFU g^{-1})	Colony-Forming Unit/gram
(CL-PEST)	Chlorinated Pesticides
(CoEECs)	Contaminants of Emerging Environmental Concern
(CSBE)	The Canadian Society for Bioengineering
(DDL)	Diffuse Double Layer
(DDT)	Dichlorodiphenyltrichloroethane
(DTA)	Direct (Eco)Toxicity Assessment
(DNA)	Deoxyribonucleic Acid
(dpm)	Disintegrations Per Minute
(E)	Enzyme
(EBA)	Environmental Bioavailability
(e.g.)	For Example
(ERA)	Ecological Risk Assessment
(ES)	Enzyme Substrate
(FDA)	Food and Drug Administration
(g)	Gram
(HACs)	Heterocyclic Aromatic Compounds
(HMW)	High Molecular Weight

(HOCs) Hydrophobic Organic Compound

(HPLC) High Performance Liquid Chromatography

(i.e.) That is

(Kg) Kilogram

(K_{ow}) Octanol-Water Partition Coefficients

(L) Liter

(LMW) Low Molecular Weight

(LSPQ) Laboratoire de Santé Publique du Québec

(μg) Micro Gram

(μL) Micro Liter

(mg) Milligram

(MGP) Manufactured Gas Plants

(mL) Milliliter

(mm) Millimeter

(mg cm^{-3}) Milligrams Per Cubic Centimeter

(mg kg^{-1}) Milligrams Per Kilogram

(MPCA) Minnesota Pollution Control Agency

(MW) Molecular Weight

(NABEC) Northeast Agricultural & Biological Engineering

(NAP) National Academics Press

(NAPLs) Nonaqueous-Phase Liquids

(ndoB) Catabolic Gene Probe (*ndoB)*: (*i.e.* Regarding, using PCR and hybridization analysis, the strong hybridization of the *ndoB* gene with DNA extracted from substrate-degrading isolates using as probe the catabolic gene *ndoB,* encoding the enzyme naphthalene 1,2-dioxygenase.)

(nm) Nanometer

(NPF) National Psoriasis Foundation

(NRC) National Research Council

(NRCC) National Research Council of Canada

(OCPs) Organochlorine Pesticides

(OD) Optical Density

(P) Products

(PACs) Polycyclic Aromatic Compounds

(PAHs) Polycyclic Aromatic Hydrocarbons

(PCBs) Polychlorinated Biphenyls

(PCDDs) Dibenzo-p-dioxins

(PCDFs) Dibenzo Furans

(**PCE)** Perchloroethylene

(PCR) Polymerase Chain Reaction

(PNAs) Polynuclear Aromatic Hydrocarbons

(POPs) Persistent Organic Pollutants

(ppb) Parts-Per-Billion

(ppm) Parts-Per-Million

(ppt) Parts-Per-Trillion

(PROC ANOVA) Analysis of variance procedure in SAS program

(Redox) Oxidation-Reduction Reaction

(RNA) Ribonucleic Acid

(RT-PCR) Reverse Transcriptase Polymerase Chain Reaction

(S) Substrate

(TCE) Trichloroethylene

(TNT) Trinitrotoluene

(UAM) Unit of Atomic Mass

(U.K.) United Kingdom

(UNEP) United Nations Environment Program

(U.S.A.) United States of America

(USEPA) U.S. Environmental Protection

(USGS) U.S. Geological Survey

(UST) Underground Storage Tank

(UV) Ultraviolet (UV radiation)

(V) volume

(W) Weight

(ZPC) Zero Point Charge

GLOSSARY OF TERMS

The most commonly used terminologies in Bioavailability, Biodegradation, Bioremediation, and Biodetoxification disciplines are as follows:

Abiotic Losses:

Refers to contaminant losses that do not result from biological activity.

Ageing:

The time that a contaminant is resident in soil prior to the introduction of microorganisms for the purpose of biodegradation or prior to the introduction of conditions conducive to desorption.

Bioaugmentation:

Generally involves the process of introducing microorganisms of sufficient biomass into a contaminated site such that the fate of the targeted chemical, or chemicals, is significantly affected.

Bioavailability:

The term bioavailability is relatively new, bioavailability as a concept has a long history in toxicology, pharmacology, crop science, and nutritional science. Common to all off these contexts is uptake by living organisms. More specifically in the environmental arena, it represents the ability of a compound to be accessed by microorganisms for transformation.

Bioavailability Process:

The application of an understanding of the bioavailability process within the environmental arena has occurred very recently, and it involves such contextual issues as solubility, mass transfer, mobility, and reaction in addition to uptake by living organisms. These processes affect whether contaminants in soils and sediments are bioavailable to humans, animals, microorganisms, and plants. Bioavailability processes are defined as the individual physical, chemical, and biological interactions that determine the exposure of microorganisms, plants, animals, and human to chemicals associated with soils and sediments. Bioavailability processes are embedded within existing human health and ecological risk frameworks.

Bioconversion, Biodegradation, and Biotransformation:

Are also used interchangeably to refer to the partial microbial breakdown of organic contaminants.

Biodegradation:

Can be defined as the transformation of a substance through the action of biological agents, especially microorganisms. In general, biodegradation is the process of decay initiated by microorganisms. However, in a stricter sense biodegradation has come to signify the complete microbial breakdown, or mineralization, of complex materials into simple inorganic constituents, such as carbon dioxide, water, ammonia, chloride, and sulphate.

Biotic Losses:

Contaminant losses that is due to microbial breakdown or complete mineralization.

Biotransformation:

Alteration of the structure of a compound by an organism.

Carcinogen:

A chemical capable of inducing cancer.

Colony:

A macroscopically visible growth of microorganisms on a solid culture medium.

Colony-Forming Unit (CFU):

The cell or aggregate of cells which gives rise to a single colony in the plate-culture technique.

Cometabolism:

Biotransformation of a compound by a microorganism which is incapable of utilizing the compound as a source of energy or growth.

Crude Oil:

It is composed mainly of hydrocarbons, but also minor amounts of sulphur, oxygen, and nitrogen as well as trace amounts of metals (*e.g.*, nickel and vanadium) are present.

Detoxification:

Reduction in the hazardous nature of a compound.

Enrichment:

Procedure for isolating microorganisms that are capable of using particular chemicals for their growth.

Enrichment Culture:

A culture of microorganisms capable of degrading a contaminant(s) due to prior contact.

Environmental Forensics:

It is the systematic and scientific evaluation of physical, chemical and historical information for the purpose of developing defensible scientific and legal conclusions regarding the source or age of a contaminant release into the environment. Within this general definition, the science of environmental forensics has evolved into a global scientific discipline with numerous applications.

Genus (*plural*: Genera):

A group of very closely related species.

Incubation:

In microbiology, the subjecting of cultures of microorganisms to conditions (especially temperatures) favourable to their growth.

Incubation Period:

The period during which microorganisms inoculated into a medium are allowed to grow.

Inoculation:

The artificial introduction of microorganisms or other substances into the body or into a culture medium.

Inoculum:

The substance, containing microorganisms or other material that is introduced in inoculation.

Mineralization:

The complete microbial conversion (biodegradation) of an organic compound (contaminant) to simple inorganic constituents, such as carbon dioxide (CO_2), water (H_20), ammonia (NH_3), chloride (chloride ion: Cl^{-}), and sulphate (sulphate ion: SO_4^{2-}) and remaining biomass.

Organic Xenobiotics:

Synthetic products (*i.e.*, organic chemicals) that are not formed by natural biosynthetic processes (not products of biosynthesis).

Petroleum:

The word *petroleum* means "rock oil" from the Greek words *petros* (rock) and *elaion* (oil). Petroleum is a complex mixture of hydrocarbons that exist naturally in gaseous (natural gas), liquid (crude oil), and solid (asphalt) states. It is derived from a variety of organic materials that are chemically converted over long periods of time (hundreds of million of years) under different geological and thermal conditions.

Persistent Organic Pollutants (POPs):

They are organic contaminants that are resistant to degradation. These can remain in the environment for long periods and have the potential to cause adverse environmental effects.

Recalcitrance:

Resistance of a compound to biodegradation, resulting in its persistence in the environment.

Redox Conditions:

Chemical conditions of oxidation-reduction reaction

Spread-Plate Method:

A procedure for separating cells and obtaining colonies by spreading inoculum on a sterile agar surface with a bent glass rod.

Surfactants:

The term surfactant is a blend of surface acting agent. Surfactants are usually organic compound that are amphiphilic, meaning they contain both hydrophobic groups (their "tails") and hydrophilic groups (their "heads"). Therefore, they are soluble in both organic solvents and water. Surfactants reduce the surface tension of water by adsorbing at the liquid-gas interface. They also reduce the interfacial tension between oil and water by absorbing at the liquid-liquid interface. The use of surfactants can enhance the biodegradation rate.

Recalcitrant Molecules:

The low-and high-molecular-weight substances that thus resist biodegradation.

CHAPTER 1

General Introduction and Rationale

1.1 Statement and Nature of the Problem

Since the time of the earliest civilization some environmental problems have occurred. The quantities of contaminants were, in general, small and localized in time and space. It was generally felt that the world was so vast that it could not be permanently affected by human activities (Miller, 1995). With the development of a worldwide industrial society, widespread pollution of the earth has become a reality (Boehm, 2006). Particularly, ever since the industrial and agricultural revolution, humans have been discharging contaminants into natural ecosystems which not only have an adverse impact on these systems but also human health as well (Majumar *et al.*, 1995; Bhandari, 2009).

All over the world, ever-increasing amounts of organic *xenobiotic* (*i.e.*, organic chemicals that are not products of biosynthesis) are released into the environment and consequently create the hazardous waste sites that pose risks to human health and the environment (World Health Organization, 1998; Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006; Plattenberg, 2007; Stenuit *et al.*, 2008). This release may be a direct consequence of the industrial, agricultural, or domestic usage of these chemicals. Often, it is a result of accidents or negligence. In the past century, the ensuing pollution of the atmosphere, of seas and oceans, of surface water and of subsurface porous formations (soils, aquifers) has become the cause of major societal concern in all over the globe (Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Obbard *et al.*, 2007; Diaz, 2008).

Among these, polycyclic aromatic hydrocarbons (PAHs) are potentially mutagenic and carcinogenic substances which occur at various concentrations in natural media such as the atmosphere, soil, water, sediment and plants. PAHs inherited both from natural and anthropogenic processes are persistent organic pollutants (POPs) due to their chemical stability and resistance to biodegradation (Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Li *et al*., 2006; Singh and Tripathi, 2007; Diaz, 2008).

One of the main reason for the prolonged persistence of hydrophobic hydrocarbons in the environment is their low water solubility which increases their sorption to soil particles and limit their availability to biodegrading microorganisms (Cerniglia, 1993; Sherma, 1993; Alexander, 1999; Dabestani and Ivanov, 1999; Philip *et al.*, 2005; Prichard *et al.*, 2006; Thwaites *et al.*, 2007; Diaz, 2008; Desai *et al.*, 2008).

The 21st century has exposed the widespread occurrence of a new category of contaminants which have attracted the attention of citizens, scientists and engineers, researchers, state and federal agencies, environmental groups, industrial and commodity groups and regulators: contaminants of emerging environmental concern "CoEECs" (Bhandari, 2009). These contaminants are predominantly unregulated anthropogenic chemicals that occur in air, soil, water, food, and human/animal tissues in trace concentrations, are persistent in the environment, and are capable of perturbing the physiology of target receptors. The USGS has focused on a subset of CoEECs (U.S. Geological Survey, 2008):

1. ***Antibiotics*** (Human and Veterinary) — *i.e. Tetracyclines*, *Fluoroquinolones*, *Macrolides*;
2. ***Other Human Pharmaceuticals*** — *i.e. prescription*, *Non- Prescription*;
3. ***Sex and steroidal Hormones*** — *i.e. Biogenics*, *Pharmaceuticals*, *Sterols*;
4. ***Household and Industrial Chemicals*** — *i.e. Insecticides*, *Antioxidants*, *PAHs*, *Fire Retardants*, *Others*.

Considered by some (Tuli, 2007; Naidu and Bolan, 2008) as an inevitable evil of our progress and modernization, environmental pollution — the release of chemical, physical, biological or radioactive contaminants into the environment — is a global issue due to direct contamination from growing industrialized centres, application of pesticides, herbicides and insecticides, and indirect contamination resulting from long-range atmospheric transport that distributes persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs), around the world (Meijer *et al.*, 2003; Singh and Ward 2004; Boehm, 2006).

The contamination of soils and groundwater by toxic and/or hazardous organic pollutants is a widespread environmental problem and the removal of hydrophobic organic compounds (HOCs) from them is becoming a major concern (Zhou and Zhu, 2007). Singh and Ward (2004) presented an excellent overview of biotechnology and bioremediation that reported their viewpoints on the large-scale manufacturing, processing and handling of chemicals, which have led to serious surface and subsurface soil contamination with a wide variety of hazardous and toxic hydrocarbons. Many of the chemicals, which have been synthesized in great volume, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), trichloroethylene (TCE) and others, differ substantially in chemical structure from natural organic compounds and are designated as *xenobiotics* because of their relative recalcitrance to biodegradation in different environments (Dominguez, 2008). More specifically, the high molecular weight PAHs (*i.e.* having four or more fused rings) are toxic and recalcitrant to biodegradation (Alexander, 1999; Baveye *et al.*, 1999; Singh and Ward 2004).

The POPs including organochlorine pesticides (OCPs), PAHs, and PCBs are of significant concern due to their potential toxicity and prevalence in a wide range of environmental media, even at remote geographical locations (Obbard *et al.*, 2007). High molecular weight PAHs (having four or more fused rings) are particularly recalcitrant to biodegradation. The latter compounds, the products of incomplete combustion of natural organic materials and hydrocarbons, occur in the soil environment as a result of naturally ignited forest fires (WHO, 1998, Alexander, 1999, USEPA, 2000, Singh and Ward, 2004, Boehm, 2006, Plattenberg, 2007; Stenuit *et al.*, 2008). However, intensification of energy-related and other industrial processes with associated production of wastes and by-products, rich in PAHs, has led to serious soil contamination of many industrial sites.

This research work focuses on one group of POPs - the PAHs - among the worst hazardous, recalcitrant, and xenobiotic compounds released daily to the environment, which are known for their strong mutagenic, carcinogenic and toxic properties (Environment Canada, 1994; WHO, 1998; Alexander, 1999; USEPA, 2000; Conte *et al.*, 2001; Luning Prak and Pritchard, 2002a, 2002b; National-Research-Council, 2002; Douben, 2003; Singh and Ward, 2004; Aitken and Long, 2004; Philp *et al.*, 2005; Buscot and Varma 2005; Boehm, 2006; Prichard *et al.*, 2006; Thwaites *et al.*, 2007; Singh and

Tripathi, 2007; Diaz, 2008; Stenuit *et al.*, 2008; Bhandari, 2009).

In summary, the PAHs represent a large family of organic compounds that are considered environmental contaminants. The PAHs are mainly derived from incomplete fossil fuel combustion and oil-related discharges to the environment (Obbard *et al.*, 2007). The PAHs have received much attention, both as pollutants and for their potential for biodegradation. Because of their widespread distribution, PAHs have been pollutants of great concern for many years (Philp *et al.*, 2005). These compounds can be present in quantities that threaten environmental, human, and animal health. As a class of compounds, the PAHs have been classified as carcinogens, mutagens, and immuno-suppressants (Sherma 1993; Environment Canada, 1994; WHO, 1998; Alexander, 1999; Dabestani and Ivanov 1999; USEPA, 2000; Conte *et al.*, 2001; Luning Prak and Pritchard, 2002a, 2002b; National-Research-Council, 2002; Douben, 2003; Singh and Ward, 2004; Aitken and Long, 2004; Philp *et al.*, 2005; Buscot and Varma 2005; Prichard *et al.*, 2006; Boehm, 2006; Thwaites *et al.*, 2007; Singh and Tripathi, 2007; Stenuit *et al.*, 2008; Diaz, 2008; Desai *et al.*, 2008; Bhandari, 2009).

1.2 Our Interest in PAHs

The interest in PAHs remains firmly present from a regulatory and academic perspective (Alexander, 1999). While much focus has been on human health-related issues, ecotoxicological aspects gradually come to the fore. Since the publication of the IPCS monograph (WHO, 1998), much work has been done. It is therefore appropriate to concentrate on environmental aspects (WHO, 1998; Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006; Plattenberg, 2007; Stenuit *et al.*, 2008).

Nevertheless, a prevailing reality of PAH contamination in the environment is that the biologically-mediated removal of PAHs tends to be incomplete, even if aggressive bioremediation approaches are used (Alexander, 1999, Singh and Ward, 2004, Boehm, 2006, and Diaz, 2008). Aitken and Long (2004) reported that we have learned from numerous well-controlled laboratory studies that virtually all of the PAHs we are concerned with are biodegradable, and that the organisms capable of transforming or degrading PAHs are essentially ubiquitous. We have also learned that the association of PAHs with nonaqueous compartments can decrease their rates of degradation.

Yet, in any given contaminated system, do we really know whether limitations in bioavailability actually control the extent of degradation of the most recalcitrant compounds? What variables do we need to control if we want to improve the extent of PAH degradation in active bioremediation processes? What processes and mechanisms should we include if we want to predict rates of natural attenuation for PAH contamination in the subsurface? Surprisingly, there are no simple answers to these questions, but they are the ones we need to ask as a basis for developing improved approaches to bioremediation of PAH-contaminated systems (Environment Canada, 1994; WHO, 1998; Alexander, 1999; USEPA, 2000; National-Research-Council, 2002; Douben, 2003; Singh and Ward, 2004; Aitken and Long, 2004; Philp *et al.*, 2005; Buscot and Varma 2005; Boehm, 2006; Prichard *et al.*, 2006; Thwaites *et al.*, 2007; Singh and Tripathi, 2007; Diaz, 2008; Stenuit *et al.*, 2008).

1.3 Role of Soil Microorganisms on Sustainability of Ecosystems

Global scale processes in which soil microorganisms are active participants include energy flow, organic matter decomposition, and biogeochemical cycling (Scow, 2000). Soil microorganisms carry out key functions in the global cycling of carbon, nitrogen, sulfur and other elements. In this context, soil microorganisms also contribute to the sustainability of ecosystems, *e.g.*, by mineralizing pesticides on agricultural fields, organic pollutants at industrial contaminated sites and urban wastes in treatment plants. On the other hand, soil microbial activities can have detrimental ecological effects, as indicated by production of greenhouse gases such as methane or nitrous oxides (Tebbe, 2005).

Microbial degradation can be the dominant process in the removal of organic xenobiotics (*e.g.*, PAHs) in the environment, but photooxidation and volatilization may also be at work. Biodegradation kinetics of individual PAH compounds by pure and mixed microbial communities have been reported by several researchers (Mueller *et al.*, 1989; Ye *et al.*, 1996; Kanaly and Harayama 2000). Investigations revealed that contaminated sites are often polluted by complex mixtures of PAHs (Guha *et al.*, 1999; Leblond *et al.*, 2001). USEPA (1989) reported that bioremediation has proven to be a remarkably good remediation approach in over 135 Superfund and Underground Storage

Tank (UST) sites, as well as many other sites contaminated with complex mixtures of PAHs. Nevertheless, the efficacy of bioremediation technology to treat contaminated sites is still challenged (Mohan *et al.*, 2006; Desai *et al.*, 2008).

1.4 Project General Objectives

Assessing the bioavailability of organic xenobiotic (*i.e.* organic chemicals that are not products of biosynthesis) recalcitrant compounds (*i.e.* selected PAHs) in the different soil environments is the main objective of this research work and is currently the objective of considerable attention from scientists, environmental activists, and policy makers. To accomplish this *goal*, more *specific research objectives*, pertaining to bioavailability and fate of organic xenobiotic recalcitrant selected PAHs in the different soil environments was undertaken to:

1. Isolate bacterial strains suitable for bioremediations from soils contaminated with many different organic pollutants, particularly pentachlorophenol (PCP) and polycyclic aromatic hydrocarbons (PAHs).
2. Screen microorganisms in PCP/PAH-contaminated soils using molecular hybridization with gene probes for known bacterial catabolic pathways.
3. Investigate if bacterial strains isolated from different contaminated soils can host biodegradation pathways and to quantify their mineralization ability with respect to selected PAHs compounds.
4. Identify the most suitable microorganisms for bioremediation and characterize them at the micromorphological, biochemical and genetic levels so as to optimize their natural biodegradative capacities.
5. Investigate competent strains' biodegradative behaviour on aged PAHs in sterilized farm soils differing in their particle size distribution and organic matter content. Bioaugmentation experiments will also be carried out. The mineralization capability and capacity (by $^{14}CO_2$ evolution) of the single selected highly competent *Mycobacterium* sp. Strain S65 (GenBank) was assessed for soil-aged ^{14}C-labelled phenanthrene or pyrene.

1.5 Organization of Thesis

This thesis presents new leading-edge research on environmental biodegradation, specifically the destruction of organic xenobiotic compounds (*i.e.* selected PAHs) by microorganisms (*i.e. Mycobacterium* sp. Strain S65) in different soil environments. The body of this dissertation consists of nine chapters and six appendices. The organization of chapters and appendices is as follows:

CHAPTER 1**:** titled ***"General Introduction and Rationale"*** provides the general introduction and literature related to the aforementioned objectives, development of problem (statement and nature of the problem, our interest in PAHs) at hand, the objectives and associated tasks of the research, the organization of the thesis, and scope of the research project.

CHAPTER 2**:** titled ***"Review of the Literature"*** supports the overall research objectives and reinforces the need to address different issues as listed: Polycyclic aromatic hydrocarbons (PAHs), isolation of pollutant-degrading bacteria from the environment, soil bioremediation, bioavailability and fate of organic compounds and PAHs in environment, biodegradation of organic compounds and (PAHs), growth and enzyme kinetics in microbial processes for biodegradation, bioremediation strategies, and finally sorption phenomenon (contaminant-soil interaction and mechanisms of the sorption).

CHAPTER 3**:** titled ***"Isolation of Recalcitrant PAHs-degrading Bacterial Strains from Different Contaminated Soil Environments and Mineralization of Selected PAHs Compounds"*** describes how PAHs-degrading bacterial strains from nineteen different contaminated sites were isolated and tested for their ability to biodegrade selected PAHs substrates in different sterilized soil microcosms environments contaminated with ^{14}C PAHs substrates.

CHAPTER 4*: titled *"Characterization of Recalcitrant PAHs-degrading Bacterial Isolates from Soils Contaminated with a Range of Organic Pollutants" investigates whether bacterial strains isolated from soils contaminated with a large range of organic pollutants, including PCP and PAHs, are able to break down selected PAHs, and furthermore characterise this capacity at the micromorphological and molecular-genetic level. Molecular biology and biotechnology techniques are used to optimize their natural biodegradative capacities.

CHAPTER 5*: titled *"Biodegradation of Soil-aged ^{14}C-labelled Phenanthrene by Mycobacterium sp. Strain S65 in Different Soil Environments" investigates whether bioaugmentation using *Mycobacterium* sp. Strain S65 as a remediator of PAH on four sterilized farm soils containing different levels of soil organic matter content, in microcosm environments, contaminated with ^{14}C aged phenanthrene (PAH), enhances biodegradation of this specific substrate.

CHAPTER 6*: titled *"Microbial Degradation of Soil-aged ^{14}C-labelled Pyrene by Mycobacterium sp. Strain S65 in Different Soil Environments" investigates whether bioaugmentation using *Mycobacterium* sp. Strain S65 as a remediator of PAH on four sterilized farm soils containing different levels of soil organic matter content, in microcosm environments, contaminated with ^{14}C aged pyrene (PAH), enhances biodegradation of this specific substrate

CHAPTER 7*: titled *"General Summary and Concluding Remarks" provides the general summary, statements of the general conclusions and concluding remarks of this Ph.D. research work**.**

CHAPTER 8*: titled *"Contributions to Knowledge and Recommendations for Future Research" presents a statement of originality, contributions to knowledge of this research work, and recommendations for future research.

***CHAPTER 9*: titled *"References"* presents the literature cited in this thesis.**

APPENDIX A*: *List of Methods and Protocols used in the Soil-Nutrient Analysis

APPENDIX B*: *Different Bacterial Growth Media

APPENDIX C*: *A Protocol for Pyrene Mineralization by Isolated Bacteria

APPENDIX D*: *A Protocol for Preparation of Radioactive (^{14}C) Substrates

APPENDIX E*: *A Protocol for Viable Cultural Bacterial Population

APPENDIX F*: *A Protocol for Colony Lifting and Hybridization

1.6 Scope of the Research Project

Since this research project is based on a multidisciplinary approach, therefore, so many different microbial and chemical experiments and assays should be conducted in fulfilment of the requirements for the overall objectives of this thesis. Consequently, there is a set of conceptual assumptions and externally-imposed limiting conditions because of complexity of the microbial world and biosystems. Therefore, it seems quite appropriate to render transparent, right at the beginning, some of the implicit assumptions and limiting conditions embodied in this research project.

This study is composed of four major parts:

- Isolation of PAH-degrading bacterial strains and mineralization of PAHs;
- Characterization of PAHs-degrading bacterial strain S65;
- Mycobacterium sp. Strain S65 degradation of soil-aged phenanthrene;
- Mycobacterium sp. Strain S65 degradation of soil-aged pyrene.

The nature of contaminants targeted for degradation was limited to PAHs, and the microorganisms involved were limited to the twelve contaminated soil environments. The following limitations may still be taken into consideration:

1. The twelve contaminated soils were collected from different wood-preserving plants, oil refineries, and various petroleum hydrocarbons-contaminated sites.

2. Work was conducted to isolate bacterial strains from soil contaminated with many different organic pollutants such as the wood treatment chemicals PCP and creosote containing oils and PAHs.

3. One study investigated whether bacterial strains isolated from different contaminated soils hosted particular biodegradation pathways and to quantify the mineralization ability of the isolated bacterial strains for selected PAH compounds in broth-medium and γ-ray sterilized farm soils. The limited number of contaminated soils sampled means that other competent organisms could exist elsewhere.

4. This research project investigated the bioavailability of organic contaminants (*i.e.* PAHs) in four specific farm soil environments. Altogether, the bioavailability of six selected PAHs compounds were investigated with two concentrations for each substrate.

5. The six selected PAHs substrates are naphthalene (2 fused-benzene rings), anthracene, phenanthrene, and fluorene (3 fused-benzene rings), fluoranthene and pyrene (4 fused-benzene rings), at concentration ranges of 250 or 500 mg kg^{-1} for phenanthrene and 500 or 1000 mg kg^{-1} for pyrene.

6. A standard aerobic microcosm methodology was adopted for biodegradation experiments, so this may not represent what goes on in a full open soil ecosystem.

7. Microbial degradation of phenanthrene and pyrene aged in soil microcosms for 0, 30, 180 or 360 days by *mycobacterium* (designated: *Mycobacterium* sp. Strain S65 in Genbank) was investigated in four soil environments

8. Emphasis was neither placed on the fate and transport of parent compounds nor on that of byproducts or metabolites from mineralization or bioaugmentation.

Moreover, this will open a window to view the beauty of the landscape of this research work in the wider and deeper context of microbial behaviour and bioavailability and fate of organic compounds, biodegradation, bioremediation, and some mechanisms of sorption/desorption phenomenon of PAHs in the soil as a natural ecosystem.

CHAPTER 2

Review of Literature

2.1 Introduction

The value of our limited natural resources continues to appreciate as they are exploited to support and indulge the human species. Over the past century our industries have produced thousands of chemicals to enhance our overall quality of life (Li *et al.*, 2007), and a rapid growth of the world population has occurred. These compounds have allowed us to increase agricultural productivity, improve animal health, and boost human longevity. The improper use and disposal of some of these chemicals, however, has also resulted in adverse human health impacts and environmental problems (Bhandari, 2009).

Thus, ever-growing demands for energy, security, food, healthcare and consumables have placed unprecedented pressure on our ecosystem and accentuated the need for sustainable management of the environment. Contamination of natural resources, arising from both natural (biogenic and geochemical) and anthropogenic sources (Young *et al.*, 1997; Philp *et al.*, 2005; Bhandari, 2009) affects soils and groundwater, and remains a major global ecological concern in the 21st century. It cannot be underemphasized that the health of our soil and groundwater is intimately tied to our well-being and to the wellness of other species that share our ecosystem (Bhandari *et al.*, 2007). Current estimates of the percentage of cancers caused by environmental contaminants are in the range of 1-5% (Stewart and Kleihues, 2003), though some studies suggest these values could be substantially higher. If contaminants are not properly treated and removed from the environment, there is the possibility of disrupting the food chain and harm human beings (Newby and Howard, 2005).

A huge range of industries, including most notably the oil and gas industry, has contributed to the problem. There has been a large increase in the diversity of organic compounds that are produced industrially and have been carelessly released into the environment. Most organic substances are potential pollutants as they enter groundwater, surface water, or soil, where their degradation can cause a depletion of dissolved oxygen, or they can have directly toxic effects, thereby directly harming living

organisms and ecosystems (Perk, 2006). These organic compounds include petroleum and its derivatives, polycyclic aromatic hydrocarbons (PAHs), and chlorinated hydrocarbons. Most polluting organic compounds are man-made and industrially produced, although a number of these chemicals also occur naturally, produced by biochemical synthesis, incomplete decomposition of organic matter, volcanic eruptions, forest fires, or lightning (Perk, 2006) .

Contamination of soils and water from improper disposal of hazardous industrial and municipal wastes has long been recognized as an environmental issue of public concern, requiring regulatory activities, and scientific investigation. Poor disposal systems have resulted in the occurrence of in excess of 80,000 potentially contaminated sites in Australia (Natusch, 1997) and over 3 million such sites in Asia (Naidu *et al.*, 2003). In the United States, there are estimated to be about 400,000 waste disposal sites where soil and groundwater contamination is deemed to be of sufficient extent and magnitude that some type of remedial action is warranted to protect public health or to minimize adverse environmental and ecological impacts (USEPA, 2004; Naidu and Bolan 2008). Contaminants can have a range of long and short-term impacts on human and animal health through various exposure pathways including ingestion of contaminated soil, inhalation of vapours, gases or dust, skin contact, ingestion through food (plants and animals) and drinking contaminated surface or groundwater. For these reasons, a restricted use or remediation of contaminated sites is often recommended (USEPA, 1994; Naidu and Bolan 2008).

In successive works, Luthy *et al.*, (1994) and Loehr and Webster (1997) reported that recalcitrant compounds in soil and sediments may be biodegraded by microorganisms to a residual concentration that no longer decreases with time or that only decreases slowly over years with continued treatment. Bosma *et al.*, (1997) noted that further reductions are seemingly limited by the availability of the recalcitrant compounds to microorganisms. Attempts have been made to increase this availability through the use of surfactants, but results have varied (Putcha and Domach, 1993 and Auger *et al.*, 1995). Additionally, as contaminants age, they become less available compared to freshly contaminated materials. As a consequence of binding with soils and sediments and subsequently slow release rates, residual recalcitrant compounds may be

significantly less leachable by water and less toxic as measured by uptake tests (Alexander, 1995; Kelsey *et al.*, 1997; Talley, 2006).

In North America, in Western Europe, and to a lesser extent all over the world, major concerns about pollution by organic xenobiotics has given rise to significant remediation efforts, beginning in the seventies. Various strategies were developed for contaminated soils, sediments and waters (Baveye *et al.*, 1999). In the last two decades, bioremediation both *ex*- and *in situ*, has also emerged as a major contender in this field, in large part because of its cost-competitiveness. Unfortunately, in spite of an initially widespread enthusiasm for bioremediation, it has in many cases failed to deliver adequate results in the (usually too short) time frames allowed for clean-up, and its adoption rate has begun to stagnate. Baveye *et al.*, (1999) also noted that this disappointing performance appears to be related to the entrapment of organic xenobiotics within the soil or sediment matrix, and the resulting low availability of these compounds to microorganisms. Alexander (1999) and Baveye *et al.*, (1999) reported on a related problem which is that, occasionally, the effect of bioremediation strategies does not seem to last very long; in a number of well-documented cases, xenobiotics concentrations in groundwater were back to their original level (above regulatory standards) barely a year after bioremediation had been declared successful. In the midst of the on-going debate on the causes and practical consequences of the limited bioavailability of contaminants, some researchers have argued that current regulatory standards for the concentrations of organic xenobiotics in subsurface environments should be lowered. This proposal has caused significant controversy among scientists (Baveye *et al.*, 1999).

PAHs are recognized as a worldwide environmental contamination problem because of their intrinsic chemical stability, high resistance to various transformation processes, and toxicity property. Because of the wide distribution of the PAHs in the environment, human exposure to PAHs is likely to occur from dermal contact, ingestion of particles, inhalation of airborne dust, or bioaccumulation in the food chains. Therefore, their remediation is considered indispensable for environmental clean up and human health (Hwang *et al.*, 2007).

There are a number of published papers and handbooks of principles and applications on contaminants, modern environmental microbiology, biodegradation and

bioremediation science and technologies, chemical bioavailability, bioremediation and detoxification of hazardous recalcitrant organic xenobiotics compounds in different environments. Norris *et al.*, (1994); Baker and Herson (1994); Skipper and Turco, (1995); Tate, (1995); Crawford and Crawford, (1996); Alexander, (1999); Baveye *et al.*, (1999); Valdes, (2000); Scow, (2000); USEPA, (2000); Conte *et al.*, (2001); National Research Council "NRC", (2002); Head *et al.*, (2003); Singh and Ward, (2004); Atlas and Philp, (2005); Sylvia *et al.*, (2005); Talley, (2006); Wang, (2007); Pawley, (2007); Van Elsas *et al.*, (2007); Singh and Tripathi, (2007); Diaz, (2008); Naidu and Bolan (2008); Wang *et al.*, (2009) and Bhandari, (2009) are some of the more critical and complete reviews.

Being based on a multi-disciplinary approach that includes biological engineering, environmental microbiology, cell and molecular biology, organic and inorganic chemistry, and soil and water engineering, the material presented in this thesis, as a whole, presents a very complete coverage of the theoretical and practical aspects of the (limited) bioavailability of 6 selected hazardous recalcitrant organic xenobiotic compounds (*i.e.* 6 out of 16 USEPA priority pollutant PAHs in different soil environments (Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Stokes *et al*., 2005; Singh and Tripathi, 2007 - Table 2.1).

2.2 Chapter Organization

This chapter is divided into twelve sections addressing the assimilative capacity of soils, microorganisms and natural attenuation, particularly in the light of management of pollutants in the ground, and in the light of sustainable development and land use.

2.3 Polycyclic Aromatic Hydrocarbons (PAHs)

The PAHs are also known as polycyclic aromatic (*i.e*., bearing carbon and hydrogen in fused-ring structures) compounds, polyaromatic hydrocarbons or polynuclear aromatics. To date, more than 100 different PAHs have been identified and a few hundred PAH-derivatives are known (Alexander, 1999; Singh and Ward, 2004; Grosenheider *et al.*, 2005; Perk, 2006; Singh and Tripathi, 2007; - Figure 2.1). Small amounts of PAHs are common in the environment. Most are harmless or mildly toxic, but some PAHs are carcinogenic (Minnesota Pollution Control Agency "MPCA" of U.S.A., 1998; USEPA,

2000). These molecules are found in coal and petroleum, but they are also products of incomplete combustion (burning), of either natural or anthropogenic origin. Anthropogenic sources to the environment are more abundant than natural sources and include burning of wood, coal, oil and gas, garbage, or other organic substances like tobacco or charbroiled meat. The most important natural sources are forest fires and volcanoes, (National Research Council of USA, 1983; Alexander, 1999; Valdes, 2000; USEPA, 2000; National Research Council, 2002; Singh and Ward, 2004; Atlas and Philp, 2005; Boehm, 2006; Singh and Tripathi, 2007).

Some PAHs are manufactured and are used in medicine or to make dyes, plastics, or pesticides. Pure PAHs usually exist as colourless, white, or pale yellow-green solids. PAHs are commonly divided into two groups, depending upon their physical and chemical properties: low-molecular-weight (LMW) PAHs, containing three or fewer aromatic rings, and high-molecular-weight (HMW) PAHs, containing more than three aromatic rings. Most PAHs are semi-volatile and the volatility generally increases with decreasing molecular weight. PAHs containing six or more benzene rings are barely volatile. The USEPA has defined a subset of 16 PAHs that are the most important from an environmental perspective (Alexander, 1999; Valdes, 2000; USEPA, 2000; National Research Council, 2002; Singh and Ward, 2004; Atlas and Philp, 2005; Boehm, 2006; Perk, 2006 – Table 2.1 and Figure 2.1).

2.3.1 Origin (Natural and Anthropogenic) Sources of PAHs in the Environment

Hundreds of PAH compounds present in nature have been identified and named (Bjorseth, 1983, 1985, Sander and Wise, 1997, Boehm, 2006). PAHs are produced by natural and anthropogenic processes. Similar compounds are introduced by both sets of processes, and these similarities, along with key diagnostic tools to differentiate sources, must be carefully understood and considered in any PAH environmental forensics investigation (Boehm, 2006).

The PAHs have been released into the environment from three main sources: biosynthetic (biogenic), geochemical and anthropogenic (National Research Council 1983). Anthropogenic sources are of two types: one is the result of accidental spillage or intentional dumping of such materials as creosote, coal tar and petroleum products, while

the other type is derived from the incomplete combustion of organic matter, such as wood burning, municipal incineration, automobile emissions and industrial discharges. The latter sources of PAHs are the current focus of a number of environmental clean up programs and consequently form the basis for the development of effective bioremediation technologies. Atmospheric PAH depositions are usually occurring from very dispersed sources, but cover significant areas of land surface. PAH concentrations from these sources are typically quite low in soil and they are absorbed strongly to soil particles (Singh and Tripathi, 2007). Moreover, another type of broad, non-point source introduction of PAHs into soils is through land treatment procedures. For example, in U.S. and Europe, sewage sludge is applied to agricultural land as fertilizer and this has been shown to contain significant concentrations of PAHs (Wild *et al.*, 1990 a,b; Singh and Tripathi, 2007).

The PAHs consist of a class of chemicals with two or more fused benzene rings in linear, angular or clustered arrangements (Sims and Overcash, 1983; Su *et al.*, 2008). PAHs are predominantly distributed in nature as components of surface waxes on leaves, plant oils, cuticles of insects and lipids of microorganisms (Millero and Sohn, 1991). Petroleum and coal provide the largest source of mononuclear and polynuclear compounds. Studies in terrestrial and aquatic (marine) environments show that PAHs can also occur from geochemical origin. They are formed whenever organic substrates are exposed to high temperatures. The aromatic rings so formed are more stable than their precursors, usually alkylated benzene rings (Singh and Tripathi, 2007).

PAH's ubiquitous environmental occurrence stems from their many sources, both natural and anthropogenic. As a rule, PAHs are produced by combustion, which can be natural (*e.g.*, forest fires, volcanoes) or anthropogenic (*e.g.*, combustion in automobiles). The main recognized sources of steady PAHs contribution are industrial processing and combustion processes, including incineration and transportation. PAHs are present in heavy petroleum products such as fuel oils and crude oils, and as a major component in creosote. These substances have been shown to be widely distributed in air, soil, water, plants, aquatic sediments and animals (Environment Canada, 1993 and 1994; Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006).

Natural sources of *petrogenic* PAHs arise from oil seepages and erosion of petroliferous shales (NRC, 1985; Bence *et al.*, 1996; Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006) while natural sources of PAHs from combustion or pyrolysis (*i.e. pyrogenic* sources) include PAHs from incomplete (*i.e.* insufficient oxygen availability) combustion of wood and biomass via forest and grass fires (Hites *et al.*, 1977; Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

While diagenesis and biosynthesis are solely natural processes, anthropogenic sources of PAHs arise through a multitude of pathways. Anthropogenic (pollution) related PAHs inputs can result in similar, but not identical, PAH compounds or assemblages of PAHs to those of natural origin. Anthropogenic inputs of PAH arise from the release into the environment of petrogenic PAHs through accidental acute petroleum spillages and through chronic non-point source and point source inputs such as urban (storm water) runoff and municipal waste treatment plant discharges. The most common and ubiquitous sources of anthropogenic PAHs, however, are those associated with pyrogenic inputs (NRC, 1985; Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006).

One of the most widespread categories of pyrogenic PAH inputs relates to the high temperature combustion of motor (automobile), bunker (shipping), and power plant (coals and petroleum) fuels (Bjorseth, 1985; Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007). The combustion processes introduce large amounts of PAHs globally, but these are more concentrated in urban areas. Residential burning of wood is one of the largest sources of atmospheric pyrogenic PAHs. Other important sources in indoor air include environmental tobacco smoke, unvented radiant and convective kerosene space heaters, and gas cooking and heating appliances. Stationary sources account for about 80% of total annual PAH emissions. Mobile sources (vehicular exhaust) are often the major atmospheric sources in urban or suburban areas (ATSDR, 1995; Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

Pollutant pyrogenic inputs also arise from the high temperature processing of fossil fuels, such as coal tar and tar products (*e.g.*, creosote) formed from coals (Emsbo-

Mattingly and Boehm, 2003; Singh and Ward, 2004; Boehm, 2006) and from fugitive emissions released from aluminium smelters, with most of these emissions being released from smelters that use the Horizontal Stud Soderberg process (Naes and Oug, 1998). Other uses of these products provide other potential entry routes. For example, the use of creosote in the treatment of wood has resulted in the association of wood treatment facilities with potential release of creosote-related (pyrogenic) PAHs (Alexander, 1999; USEPA, 2000; Walker *et al.*, 2004).

What primarily differentiates the origins of petrogenic and pyrogenic PAHs, whether *natural sources* of PAHs or *anthropogenic sources* is the temperature of formation. During the generation of PAHs, the degree of alkylation in a given PAH assemblage is inversely proportional to the temperature of formation (Blumer, 1976; Alexander, 1999; Singh and Ward, 2004; Boehm, 2006). Because the degree of alkylation and the resulting distribution of PAHs depend on the temperature of formation, the characteristic compositional profiles of these different sources can be used to help distinguish among different sources of PAHs in the environment (Alexander, 1999; USEPA, 2000; Walker *et al.*, 2004; Singh and Ward, 2004; Boehm, 2006).

2.3.2 Assemblages of PAHs

Once they are produced by petrogenic and pyrogenic processes, PAHs may be introduced into the environment through a number of pathways (Figure 2.2**;** Boehm, 2006).

These chemicals can enter the environment on local, regional, and global scales (Neff, 1979; Neff *et al.*, 2005). Point sources, such as municipal or industrial outfalls, are on the local scale, and are generally made up of mixtures of PAHs that are combustion- or oil-related. Non-point sources (*e.g.* rainfall runoff or atmospheric deposition) are found on regional scales, and are made up of PAHs from multiple primary sources. Wide-field atmospheric deposition is a global source that distributes primarily pyrogenic PAHs (Ohkouchi *et al.*, 1999) to remote regions of the earth. Airborne transport of PAHs on soot particles from forest fires and the combustion of coal and oil have been established as a major mechanism for the distribution and delivery of PAHs to soils and sediments on the regional and global scales.

The source(s) of PAH in coastal and urban sediments was first investigated in the early to mid-1970s. PAHs in sediments were shown to originate in both non-alkylated and alkylated PAH combinations (Giger and Blumer, 1974; Youngblood and Blumer, 1975), rather than individual, non-alkylated PAH. These petrogenic and pyrogenic source categories could be readily distinguished on the basis of their alkyl PAH distributions (Youngblood and Blumer, 1975; Lee *et al.*, 1977; Laflamme and Hites, 1978). The petrogenic and pyrogenic PAH can be derived from both "point" and "non-point" sources.

Furthermore, Boehm (2006) showed that in the latter half of the 19th and first half of the 20th centuries, gas-manufacturing plants produced gas from oil and coal, resulting in residues collectively referred to as "coal tar". These coal tars contain large quantities (up to 70-80% by weight) of pyrogenic PAHs as a result of the high temperature processing of coal in the plants. Coal tars were largely dominated by 2- and 3-ringed compounds (*e.g.*, naphthalene, anthracene) in fresh material and 3- to 6-ringed PAHs in more weathered material. These PAHs have entered the environment via groundwater flow and runoff to coastal rivers and sediments.

Boehm (2006) and Bohem *et al.,* (2001) also reported that, other point sources might include direct or indirect discharges from former or existing industrial facilities (*e.g.*, petroleum handling, aluminium smelting, manufactured gas production, tar distillation, rail yards, *etc*.) as well as loading/unloading facilities (*e.g.*, creosote pilings), marinas, discharge canals, and storm water outfalls. Storm water runoff from paved urban and suburban roadways and developments, as they are discharged into surface waters, serve as local, point sources for PAH that are derived from 'non-point' sources, for example, atmospheric (combusted) particulates and fugitive (dripped and leaked) petroleum washed from the surrounding urban roadways, parking lots, vegetation, and structures during rainfall events. Other non-point sources of PAH can include recreational boat traffic, commercial ship traffic, general runoff (*i.e.* not entering from a specific outfall location), and direct atmospheric particulate deposition to the waterway.

Boehm (2006) concluded that, understanding how and where PAHs enter the environment is important in conducting environmental forensic studies and especially in determining the PAH background into which emissions from a specific PAH source are added. On a global basis and in areas remote from urban influence, PAHs from pyrogenic

processes transported over large distances are the principal source of background concentrations — more important than petrogenic PAH inputs — though the levels tend to be very low. On more localized scales, background PAH concentrations are generally much higher, and PAHs from urban runoff together with combustion-related PAH inputs are very important contributors to most receiving environments. In selected geologically active environments, oil seeps and erosion from oil source rocks and coal result in elevated concentrations from natural sources of PAHs. These background concentrations of PAH are of particular significance when the potential effects (*i.e.* incremental addition) of PAHs from new oil and gas exploration projects or effects of oil spills are being evaluated as part of new project plans or environmental impact and damage assessments.

2.3.3 Human Exposure

With respect to humans, PAHs are generally associated with chronic risks, which are often the result of exposure to complex mixtures of aromatic compounds rather than to low levels of a single compound. Toxic effects include DNA adducts and cancer. In general, the heavier (4-, 5-, and 6-ring) PAHs have greater carcinogenic potential than the lighter (2- and 3-ring) PAHs (ATSDR, 2006 and Perk, 2006). Human exposure to PAHs is mainly via the inhalation of contaminated air in the work environment and outdoors, and the ingestion of grilled or charred meats or contaminated water, cow's milk, or other foodstuffs (*e.g.*, cereals, vegetables, and fruits) (Perk, 2006).

For U.S. residents, the greatest PAH exposure is through the ingestion of food, but this can vary depending on lifestyle. Other important exposure pathways are through active and passive inhalation of the compounds in tobacco smoke, wood smoke and contaminated air, as well as dermal contact with coal tar in cosmetics and shampoos. Human daily exposure is estimated to be 0.207 µg from air, 0.027 µg from water, and 0.16 to 1.6 µg from food. These estimates may be as much as twice as high in smokers (ATSDR-U.S., 1995; Grosenheider *et al.*, 2005).

The PAHs are stored mostly in the kidneys, liver and fat. In long-term PAH exposure scenarios, cancer, cataracts, kidney and liver damage, and jaundice may develop. However, in humans most PAHs are released in faeces and urine within a few days (ATSDR, 1995). Increased levels of PAH metabolites were found in the urine of

individuals occupationally and therapeutically exposed to coal tar, a substance that is high in PAHs, compared to unexposed subjects (Clonfero *et al.*, 1990). The U.S. Food and Drug Administration (FDA) state that over-the-counter products with coal tar concentrations between 0.5% and 5% are safe. The FDA maintains that there is no evidence that coal tar products cause cancer (National Psoriasis Foundation, 2005).

2.3.4 PAHs in Different Environments

In the environment, PAHs can volatilize, photolyze, oxidize, biodegrade, bind to suspended particles, or accumulate in aquatic organisms. The bulk of PAHs in the environment is tied to organic matter in soil. Sorption of PAHs to soil and sediments increases with increasing soil organic carbon and with increasing surface area of the sorbent particles (ATSDR, 1990 and 1995).

2.3.4.1 In Air

PAHs enter the atmosphere mostly as releases from volcanoes, forest and peat fires, fossil fuels combustion, coke and asphalt production, waste incineration, and aluminium smelting. In air, PAHs occur mostly attached to dust particles smaller than 1-2 μm. Over a period of days to weeks, PAHs can break down by reacting with sunlight and other airborne chemicals. They are also removed from the atmosphere by deposition, as a result of which they reach the Earth's surface. The lighter molecular weight PAHs can be revolatilised and redistributed before they are redeposited. PAH levels in air, soil, and water are particularly enhanced in urbanized regions. PAHs are ubiquitous in the environment, even in remote areas, due to long-range atmospheric transport. In addition to atmospheric deposition, the source of PAHs to terrestrial environment include release from creosote-treated products, spills of petroleum products, and application of compost (Perk, 2006).

Most PAHs, — whether they are the result of natural or anthropogenic processes — will be released into the air. Residential burning of wood is the largest anthropogenic source; however, on a local scale other emission sources can be dominant. Urban air contains as much as 5 times higher PAH concentrations (0.15-19.3 ng m^{-3}) than rural air (0.02-1.2 ng m^{-3}). Winter concentrations are 5-10 times higher than summer

concentrations due to the difference in temperature, sunlight radiation, and an increase in heating emissions (ATSDR, 1995). An estimated 8,900- 26,800 Mg of PAHs are emitted annually in the U.S. (Baek *et al.*, 1991; USEPA, 1998).

Most PAHs in air are associated with particulate matter, with some in the gaseous phase (Yang *et al.*, 1991). The photo degradation of PAHs in air occurs generally over days to weeks. Indoor air has a potentially increased level of PAHs due to tobacco smoke, unvented radiant and convective kerosene space heaters, gas cooking and heating appliances. However, it is common for outdoor PAH levels to be ten times higher than indoor (Hoffmann and Hoffmann, 1993).

2.3.4.2 In Terrestrial Plants and Animals

The PAH contents of terrestrial animals may be substantially greater than the PAH contents of the soil on which they live, though the PAH content of terrestrial plants is often independent from those in soil because PAH compounds are poorly soluble and poorly available in soils (ATSDR, 2006 and Perk, 2006). A major source of PAHs in plants is the accumulation of airborne PAHs on plant leaves; plant uptake of PAHs via the root system is generally negligible. The process of accumulation is affected by a variety of factors. It increases with decreasing ambient temperature, and with increasing leaf surface area and lipid concentration in plant tissues. Volatile PAHs with a lower molecular weight are primarily subject to the form of dry gaseous deposition, whereas non-volatile PAHs mainly accumulate on plant surfaces in the form of dry particulates (Bakker, 2000). However, PAHs are potentially hazardous to plants due to photo-enhanced toxicity in the presence of ultraviolet (UV) or other types of solar radiation (Perk, 2006).

2.3.4.3 In Surface Water

The most important source of PAHs in surface water is from the deposition of airborne PAHs. Other sources include municipal wastewater discharge, urban storm water runoff, runoff from coal storage areas, effluents from wood treatment plants and other industries, oil spills, and petroleum processing. In a localized environment any of the above sources can be dominant. Background levels of PAHs in drinking water range from 4 to

24 ng L^{-1}. As in air, PAHs are mostly associated with particles and only one third of PAHs in water are in the dissolved phase. The degradation of PAHs in water and soil occurs primarily though microbial metabolism within weeks or months. Losses can also occur through volatilization and sorption to settling particles (Grosenheider *et al.*, 2005).

PAHs tend to adsorb onto sediments and soil particles. PAH levels are usually much higher in sediments than in surface water, *i.e.* in the range of μg kg^{-1} (ppb) rather than ng kg^{-1} (ppt). Nevertheless, PAH toxicity in aquatic environments is mainly associated with the more soluble compounds, particularly two-ring compounds such as naphthalene (Perk, 2006).

2.3.4.4 In Soil

Soil, like water, receives most PAHs from atmospheric deposition after local and long-range transport (ATSDR, 1995). Other sources include sludge disposal from public sewage treatment plants, automotive exhaust, tire and asphalt wear, irrigation with coke oven effluent, leachate from bituminous coal storage sites, and use of compost-based fertilizers. PAHs can be lost from soil through microbial breakdown, photo oxidation, volatilization, crop uptake and leaching (Smith *et al.*, 1996). Large variations in soil PAH concentrations are shown by data from several publications which were pooled together in an Agency for Toxic Substance and Disease Registry Publication (1995). Soil contamination by PAHs may also inhibit root growth. Microorganisms can break down PAHs in soil or water after a period of weeks to months, but some PAHs are resistant to decay. Microorganisms degrade low molecular weight PAHs more easily than high molecular weight PAHs (Perk, 2006).

2.3.5 Physico-Chemical Characteristics

The PAHs are usually coloured, crystalline solids, with high melting and boiling points (from 68 to 439°C and from 218 to 596 °C, respectively). Their molecular weight varies from a little over 100 to more than 350 UAM (*i.e.* unit of atomic mass). They generally have a low vapor pressure (10^{-17} to 10^{-3} kPa at 25°C), are not very soluble in water (< 1 μg L^{-1} to 1 mg L^{-1}). The high-molecular-weight (HMW) species have especially low

water solubility. PAHs exhibit a great adsorption affinity for particle surfaces, particularly for binding to organic carbon in soils. The PAHs comprising less than four aromatic rings are, as a general rule, more volatile and more soluble in water than those that have a larger number of aromatic rings. Lipophilic and electrically neutral PAHs pass easily through cellular membranes. This characteristic becomes important in the course of toxicological studies (Environment Canada, 1993).

The PAHs most commonly encountered in the environment contain two (naphthalene) to seven (coronene) fused benzene rings, though PAHs with greater number of rings are also found (Sander and Wise, 1997; Alexander, 1999; Singh and Ward, 2004). The "ultimate" PAH is graphite, an inert material comprised of planes of fused benzene rings. Like all hydrocarbons, PAHs contain only hydrogen and carbon. However, closely related compounds called heterocyclic aromatics, or polycyclic aromatic compounds (PACs), in which an atom of nitrogen, oxygen, or sulphur replaces one of the carbon atoms in a ring, are commonly found with PAHs from most sources. Dibenzothiophene, for example, is a sulphur-containing heterocycle. PACs often occur with aliphatic (straight chain) hydrocarbons attached to the rings at one or more points (Alexander, 1999; USEPA, 2000; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007). These compounds are referred to as "branched" or "alkylated" PAHs.

PAH derivatives include PAHs having an alkyl or other radical attached to ring; heterocyclic aromatic compounds (HACs) include PAHs having any one-carbon atom in a ring replaced by a nitrogen, oxygen, or sulphur atom. Pure PAHs are usually coloured, crystalline solids at ambient temperature. The physical properties of PAHs vary with their molecular weight and structure. Except for naphthalene, they have very low to low water solubilities, and low-to-moderately high vapor pressures. Their octanol-water partition coefficients (K_{ow}) are relatively high, indicating a relatively high potential for adsorption to suspended particulates in the air and in the water, and for bioconcentration in organisms (Environment Canada, 1994).

The PAHs have been placed on the USEPA (EPA) priority pollutants list due to their toxicity and, in some cases, carcinogenicity. Sixteen PAHs are listed as priority pollutants by the USEPA, including benzo[a]pyrene (BaP) (Juhasz and Naidu, 2000). Soil and sediment are the major sinks for their accumulation, with PAHs contamination

ranging from 5 μg kg^{-1} soil in an undeveloped area to 1.79 g kg^{-1} at a spillage in an oil refinery (Kotterman *et al.*, 1998). The cleanup criteria for PAHs in soil vary widely within and between nations (Su *et al.*, 2008).

PAHs with up to five rings are biodegradable (Cerniglia, 1992); however, the bioremediation of contaminated sites is often limited by the low bioavailability of higher molecular weight PAH associated with soils and sediments (Wilson *et al.*, 1993). This is primarily due to the tendency of these compounds to partition into the organic matter content of soils or sediments or into nonaqueous-phase liquids (NAPLs) where they are thought to be inaccessible to intracellular enzymes. Desorption of PAHs from the soil matrix is affected by the length of time the contaminants have been in contact with the matrix, the rate at which the PAHs are removed from the water phase, and the diffusion rate of PAHs within the matrix (Pignatello *et al.*, 1996).

2.4 Soil Contamination

The assessment of soil quality regarding chemical pollutants is complex. The main objective of soil quality assessment has usually been human health protection. Recently, criteria for the protection of ecosystems have been incorporated and ecotoxicological analyses are recommended to estimate the risk of soil contaminants to ecological receptors in soils (Calow, 1993; Stephenson *et al.*, 2002; Loibner *et al.*, 2003; Robidoux *et al.*, 2004; Fernadez and Tarazona, 2008). The ecotoxicological assessment of soils is mostly based on the toxicity test with selected organisms. Two complementary approaches are available: (**i**) identification of toxicity thresholds for each relevant pollutant, thresholds that are based on the evaluation of effects of chemical substances on selected organisms representing relevant ecological receptors, and (**ii**) toxicity assays are performed directly with the contaminated media (*i.e.* soil, water, sediment) (Peterson *et al.*, 1990; Torstensson, 1993; Torslov *et al.*, 1997; Fernadez and Tarazona, 2008).

From the perspective of remediation, the soil environment can be divided into two zones: shallow surface soils (upper 0.3 to 1.0 m of the soil) and subsurface (vadose) soils. The former represent the region of the environment typically included in the agronomic definition of soils. They are easily modified and are generally more amenable to remediation than deeper vadose soils (Alexander, 1999; Singh and Ward, 2004; Boehm,

2006; Singh and Tripathi, 2007). Vadose soils are those soils, which lie between the surface soils and the water table or aquifer.

2.5 Isolation of Pollutant-Degrading Bacteria from the Environment

Bacteria, filamentous fungi, yeast, cyanobacteria, diatoms, and eukaryotic algae have the enzymatic capacity to oxidize PAHs that range in size from naphthalene to benzo(a)pyrene. Prokaryotic organisms, bacteria and cyanobacteria use different biodegradation pathways than eukaryotes, fungi and algae but both involve molecular oxygen. Bacteria employ dioxygenases to incorporate two oxygen atoms into the substrate to form dioxyethanes, which are then oxidized to cis-dihydrodiols and then to dihydroxy products. The rate-limiting step in the biodegradation of PAHs is the initial ring oxidation, the genes for which are localised on plasmids. The bacterial oxidation pathway of terminal ring cleavage appears to apply for all PAH compounds such as naphthalene, phenanthrene, pyrene and anthracene, etc. (Alexander, 1999; Baveye, 1999).

The importance of the role of microorganisms in the catabolism of both naturally occurring and man-made organic molecules has been the subject of numerous studies. The majority of aromatic ring-hydroxylating dioxygenases have been identified from isolated microorganisms capable of growth on specific aromatic hydrocarbons. Methods for their isolation typically include selective enrichment and subsequent plating of subcultures on minimal medium containing the aromatic substrate (Greer *et al.*, 1990 and 1993; Valdes, 2000; Parales and Resnick, 2004). Substrates can be supplied in the vapor phase (Gibson, 1976 and 2000), incorporated into the agar media or sprayed as an insoluble layer onto the plate surface (Kiyohara *et al.*, 1982; Greer *et al.*, 1990 and 1993; Valdes, 2000; Parales and Resnick, 2004), depending on the solubility and vapor pressure of the compound. The source of inocula selected for enrichment often includes contaminated environments with a history of previous exposure to environmental pollutants such as creosote, gasoline, or refined petroleum products. A number of colorimetric indicators have been established for detecting the activities of dioxygenases or of downstream enzymes required for aromatic hydrocarbon degradation. These include the well-established conversion of indole to indigo (Ensley *et al.*, 1983), a reaction catalyzed by many aromatic hydrocarbon dioxygenases, and the conversion of indole

carboxylic acids to indigo by certain aromatic acid dioxygenases (Eaton and Chapman, 1995). The detection of ring-fission products formed from catechols derived through the oxidation of parent hydrocarbon substrates (*e.g.*, dibenzothiophene, dibenzofuran, and biphenyl) has been used to identify coupled activities of ring-hydroxygenating dioxygenases, *cis*-dihydrodiol dehydrogenases, and *meta* ring-cleavage dioxygenases (Kodama *et al.*, 1973).

Approaches involving hybridization probing for aromatic hydrocarbon dioxygenase genes can be employed for screening libraries prepared directly from the environment, or from enriched pure cultures. Such methods were successfully applied in the discovery of hydrolases (Gray *et al.*, 2003). Several challenges exist for the application of these techniques to multicomponent aromatic hydrocarbon dioxygenases. Activity-based screening requires that each protein be present and sufficiently expressed for detection of activity. Homology-based hybridization can limit the identification of genetic diversity in favor of homologue rediscovery. Hybridization of multiple components can be complicated by the "mosaic" genetic organization of the encoding genes (Kim and Zylstra, 1999) and the application of substrate-independent discovery techniques ultimately requires a demonstration of new activity. An alternative approach was used to identify novel PAH degradation genes by screening the genomic DNA of isolates for lack of hybridization to standard dioxygenase gene probes (Zylstra *et al.*, 1997).

2.6 Bioremediation

Alexander (1999) stated that bioremediation is a strategy or process that uses microorganisms, plants, microbial or plant enzymes to detoxify contaminants in the soil and other environments. This concept includes biodegradation, which refers to the partial, and sometimes total, transformation or detoxification of contaminants by microorganisms and plants. Mineralization is a more restrictive term for the complete conversion of an organic contaminant to its inorganic constituents by a single species or a consortium of microorganisms. Cometabolism is another more restrictive term referring to the transformation of a contaminant without it providing carbon or energy for the degrading microorganisms. These definitions are all used in the context of converting contaminants

to less toxic intermediates or mineralizing them to their inorganic forms (*e.g.*, CO_2, NH_4^+, and PO_4^{-3}).

Bioremediation has been considered as an attractive decontamination strategy due to relatively low cost and small impact to the environment (Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007). For environments heavily contaminated with petroleum fuels, the use of bioremediation is limited, since it is only applicable after the mass of petroleum is reduced by physical and/or chemical means (Ron and Rosenberg, 2002; Meguro *et al.*, 2005). One reason for this limited use of bioremediation would be that a high concentration of petroleum is lethal to bacteria or at least suppresses bacterial activities. Nevertheless, bacteria that can grow under these conditions have been found (Watanabe *et al.*, 2000; Cheung and Kinkle, 2001), suggesting that they may have the ability to resist high concentrations of petroleum. Since bacteria generally have narrow substrate ranges, they should exclude non-catabolizable substrates from cells for their survival. We can assume that molecular mechanisms found in laboratory isolates of solvent-resistant bacteria may also operate in bacteria inhabiting petroleum-contaminated environments, although no ecological evidence exists to support this idea (Meguro *et al.*, 2005).

2.6.1 Soil Bioremediation

Soil bioremediation represents the managed or spontaneous microbiological processes used to degrade or transform hydrocarbon contaminants concentrations in various types of soils and sediments to less toxic or nontoxic forms that no longer pose an unacceptable risk to the environment or human health (USEPA, 1994; Linz and Nakles, 1997; Talley, 2006). Nevertheless, hydrocarbons that remain in treated soils and sediments still might not meet stringent regulatory levels, even if they represent site-specific, environmentally acceptable endpoints (NRC, 1997). This unresolved issue of the availability of residual hydrocarbon contaminants is the focus of many studies. However, there is a great need to understand contaminant soil-sediment interactions and their effect on bioavailability and toxicity (NRC, 1997). This is especially true for recalcitrant compounds. The adherence and slow release of recalcitrant compounds from soils and sediments is an obstacle to remediation (NRC, 1994) and is challenging our concepts about cleanup standards and

risks (Alexander, 1995 and 1999; Talley, 2006). This is particularly the case for biological treatment of recalcitrant compounds, in which one of the most important site-specific factors is the availability of the compounds held within solids and how this affects treatment rates and acceptable toxicological endpoints (Alexander, 1999; Talley, 2006). Nevertheless, biotechnology has the potential to play an important role in the development of treatment processes for contaminated soil. As with any microbial process, optimizing the environmental conditions in bioremediation processes is central goal in order that the microbial, physiological and biochemical activities are directed towards biodegradation of the target contaminants. However, understanding the biochemical and physiological aspects of bioremediation processes will provide us with the requisite knowledge and tools to optimize these processes to control key parameters and to make the processes more reliable (Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

2.6.2 Advantages and Disadvantages of Bioremediation

Biological degradation of organic waste can, and usually does, result in complete destruction of the contaminants and elimination of future liability to the responsible party. As the number of contaminated sites increase and the number of options such as use of landfills decreases or become more expensive, bioremediation has become more popular and subsequently more widely used (Bourquin, 1994). However, as the popularity of using biological treatment increases, the pressures for performance beyond developed capabilities also increase. It has long been realized that microorganisms are capable of metabolizing naturally-occurring organic compounds. Nevertheless, many problem environmental sites also have *xenobiotic* or synthetic compounds which may or may not be biodegradable (Bourquin, 1994; Alexander, 1999; Baveye, 1999; Talley, 2006; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

Bioremediation is often less hazardous to cleanup personnel and less expensive because contaminants can be treated on site. Bioremediation is a natural process and therefore often has lower environmental impact because no wastes are generated. On the other hand, bioremediation has a number of disadvantages. For example, low bioavailability of a contaminant would limit the application of bioremediation

(Alexander, 1999; Baveye, 1999). The worst-case scenario is for those sites contaminated with not one compound or even one class of compound, but with a mixture of inorganic and organic compounds. At such sites, one compound might be biodegradable by microorganisms while another might be toxic to microorganisms; one compound might require aerobic conditions for biodegradation while another might require anaerobic conditions; one compound might require neutral to alkaline conditions for biodegradation while another might require acidic condition. Our knowledge of modelling and manipulating biological, chemical and physical interactions where sites have complex mixture of chemicals is still extremely limited (Alexander, 1995 and 1999; Talley, 2006; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

Bourquin (1994) reported that another difficulty facing the increased commercialization of bioremediation is the lack of reliability in laboratory to field technology transfer as well as the inability to transfer a technology from one field site to another. Of course, biological systems are dynamic and are constantly changing with their environment. The result is that today, most treatability studies and field scale up experiments are done on a site-specific basis. Furthermore, the public views bioremediation favourably, and many agencies worldwide are promoting its use for the restoration of sites damaged by environmental contaminants (Alexander, 1999; Harayama, 2001; Gormmen and Verstraete, 2002; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

2.7 Bioavailability

While the concept of bioavailability is widely accepted, the processes that control it are poorly understood. This latter situation results from an incomplete understanding of basic processes in aquatic systems and soil environments, which are further exacerbated by the numerous and sometimes conflicting qualitative definitions of bioavailability used by investigators from different scientific disciplines (Alexander, 1991 and 1999; Singh and Ward, 2004; Boehm, 2006; Wang, 2007). To avoid confusion with the term bioavailability, as employed in pharmacology and toxicology, the term environmental bioavailability should be employed. Environmental bioavailability (EBA) constitutes the fraction of the environmentally available compound, which an organism accumulates

when processing a given medium. Environmental availability can be defined as the portion of the total material in a compartment(s) of the environment that is involved in a process or group of processes and is subject to physical, chemical, and biological modifying influences. This represents the total pool of material that is potentially available to organisms and represents the broadest sense of the concept (Alexander, 1991, 1995, and 1999; Singh and Ward, 2004; Boehm, 2006; Wang, 2007; Singh and Tripathi, 2007).

Bioavailability can be viewed as a special case of environmental availability in which organisms are involved as a target rather than as part of a process. Thus bioavailability, by necessity, incorporates not only the characteristics of the chemical and its environmental speciation, but also the behaviour and physiology of the organism. The availability of organic contaminants to microbial destruction in a soil system has been identified as one of four research priorities in the field of bioremediation. More specifically, there is a need to determine the factors that govern the availability of organic contaminants for bioremediation and to devise ways to overcome the restrictions imposed on bioavailability by these factors. Conceptualizing the bioavailability phenomena logically precedes any attempts at making future predictions or embarking on a particular preventive or remedial course of action (Alexander, 1991, 1995, and 1999; Van Hamme, 2004).

2.7.1 Bioavailability of Environmental Contaminants

The amount of a chemical in the terrestrial environment that is bioavailable depends on a variety of factors including the properties of both the chemical and the environmental media (Naidu *et al.*, 2008). Soil plays a very significant role in reducing the potential bioavailability of contaminants in the environment. Nevertheless, bioavailability is impacted not only by soil type, but also contact time (*i.e.* ageing), which reduces the concentration of a chemical available for uptake by organisms or which causes toxicity (Alexander, 1995 and 1999; Vig *et al.*, 2002). There are number of physico-chemical and biological processes occurring in soil systems, which have a significant impact on the bioavailability of chemicals (Naidu *et al.*, 2003; Van Hamme, 2004). The primary soil factors controlling the potential bioavailability of all contaminants are soil pH, cation or

anion exchange capacity (depending upon available charged sites on soil surfaces), clay content, and soil organic matter (Alexander, 1995 and 1999; Vig *et al.*, 2002; Naidu *et al.*, 2003 and 2008).

The most important factor for efficient microbial degradation of chemical contaminants to occur is that the contaminants must be bioavailable to the degrading microorganisms. The biodegradation rate of a contaminant depends on the rate of contaminant uptake and mass transfer. Bioavailability of a contaminant in soil is influenced by a number of factors such as desorption, diffusion and dissolution. The decrease in bioavailability due to long-term contamination of soil, often referred to as ageing or weathering, is a result of chemical oxidation reactions and slow chemical diffusion of the contaminant into small pores incorporating contaminants into the organic matter. However, use of chemical or bio-surfactants during the biodegradation process helps overcome bioavailability problems (Aronstein and Alexander, 1993; Van Haumme *et al.*, 2003; Singh and Ward, 2004). The molecular structure of the contaminant and its hydrophobicity may also affect the pollutant uptake by the microorganisms. Indeed, the cells may also have active or selective systems for transporting the contaminants into the cell. Given that many of these contaminants have low solubility in aqueous media, understanding mechanisms of their uptake by the degrading microbes and developing strategies to promote or accelerate their accession represent important aspects of effective bioremediation processes (Singh and Ward, 2004).

2.7.2 Factors Affecting Bioavailability of PAHs

Al-Bashir (1991) reported that there are several factors governing the bioavailability of the PAH compounds to the microbial population and the possible effects they may have on the biodegradation rate of PAHs. Some of the most important factors considered are: physical state, dissolution rate, solubility, molecular weight, adsorption by solid matter, the effect of surface active agents (surfactants), initial concentration and cell membrane permeability.

2.7.3 Factors Affecting Biodegradation Rate of PAHs

The rate of biodegradation may be altered by the degree of contamination. At hazardous waste sites, biodegradation half-life may be longer since other contaminants at the site may be toxic to degrading microorganisms (Cerniglia, 1984). Several factors influence the rate of PAHs biodegradation and their degree of recalcitrance (Cerniglia 1984; Alexander, 1991 and 1999). Microbial metabolism is the major process for degradation of PAHs in soil environments. Photolysis, hydrolysis and oxidation are not considered important processes for the degradation of PAHs in soils. The rate and extent of degradation of PAHs in soil are affected by environmental factors, characteristics of the microbial population and the physico-chemical properties of the PAHs. Environmental factors that may influence the rate of PAHs degradation in soil include temperature, pH, oxygen concentration, PAH concentrations and contamination history of soil, soil type, moisture, nutrients, and other substances that may act as substrate cometabolites. The size and composition of microbial populations can, in turn, be affected by these factors. For example, in low pH soils, fungi are dominant over bacteria, and thereby control microbial degradation in these environments (Sims and Overcash, 1983; Boehm, 2006; Wang, 2007). These factors can be categorized in three domains:

1. PAH-related: type, concentration, ring number, solubility, lipophility;
2. Environment-related: sediment type, soil type, organic content, nutrient status, salinity, soil-to-water ratio, temperature, pH, redox potential;
3. Bacteria-related: types, population, distribution, and previous exposure.

2.7.4 Susceptibility of PAHs

Susceptibility of PAHs to microbial degradation is inversely correlated with the number of rings in the PAH; therefore, naphthalene is more easily degraded than anthracene or phenanthrene. The decreased degradation of three-, four-, and five-ring PAHS is largely a result of the low water solubility and hence low bioavailability of these compounds. Several studies have investigated the formation of PAHs, characterization, analysis, possible metabolic pathways, occurrence in different environments and their toxicity. However, more investigations should be focused on their fate in the terrestrial

environment, susceptibility to microbial attack and the effect of the several environmental factors on their persistence and accumulation in soil (Alexander, 1999).

2.8 Fate of Organic Compounds and PAHs in Environment

2.8.1 Sources of PAH in the Environment

Most of the PAHs in soil are believed to result from atmospheric deposition after local and long-range transport. The presence of PAHs in the soil of regions remote from any industrial activity supports this contention. Other potential sources of PAHs in soil include sludge disposal from public sewage treatment plants, automobile exhaust, and irrigation with coke oven effluent, and use of soil compost and fertilizers (Santodonato, 1981).

2.8.2 Transport and Partitioning

With respect to the global movements of PAHs, Sullivan and Mix (1985) reported that PAHs released to the atmosphere are subject to short- and long-range transport and are removed by wet and dry deposition. In surface water, PAHs can volatilize photodegrade, oxidize, biodegrade, bind to particulates, or accumulate in aquatic organisms. In sediments, PAHs can biodegrade or accumulate in aquatic organisms. PAHs in soil can biodegrade or accumulate in plants; PAHs can enter groundwater and be transported within an aquifer.

Transport and partitioning of PAHs in the environment are determined to a large extent by physical/chemical properties such as water solubility, vapor pressure, Henry's Law constant, Octanol-water Partition Coefficient (K_{ow}), and Organic Carbon Partition Coefficient (K_{oc}). In general, PAHs have low water solubility. The Henry's Law constant is the partition coefficient that expresses the ratio of the chemical's concentrations in air and water at equilibrium, and is used as an indicator of a chemical's potential to volatilize. The K_{oc} indicates the chemical's potential to bind to organic carbon in soil and sediment. The K_{ow} is used to estimate the potential for an organic chemical to move from a water to a lipid phase, and has been correlated with bioconcentration in aquatic organisms. Some of the transport and partitioning characteristics (*e.g.*, Henry's Law

constant, K_{oc} values, and K_{ow} values) of the 15 PAHs are roughly correlated to their molecular weights.

The (K_{oc}) indicates the chemical's potential to bind to organic carbon in soil and sediment. The low molecular weight PAHs (*i.e.* naphthalene, anthracene, and phenanthrene) have K_{oc} values in the range of 10^3 to $10^{4,}$ which indicates a moderate potential to be adsorbed to organic carbon in soils and sediments. The medium molecular weight compounds (*i.e.* pyrene) has K_{oc} values in the 10^4 range. High molecular weight PAHs (e.g., Benz(a)anthracene, Benzo(b)fluoranthene, etc.) has K_{oc} values in the range of 10^5 to 10^6 which indicates a stronger tendency to adsorb to organic carbon (Sullivan and Mix, 1985).

Sorption of PAHs to soil and sediments increases with increasing organic carbon content and is also directly dependent on particle size. PAHs may also volatilize from soil. Volatilization of low molecular weight PAHs from soil may be substantial (Coover and Sims, 1987).

2.8.3 Transformation and Degradation

Microbial metabolism is the major process for degradation of PAHs in soil environments. Photolysis, hydrolysis, and oxidation are not considered important processes for the degradation of PAHs in soils. However, the rate and extent of degradation of PAHs in soil are affected by environmental factors, characteristics of the microbial population, and the physical and chemical properties of the PAHs (Sims and Overcash, 1983).

Environmental factors that may influence the rate of PAH degradation in soil include temperature, pH, oxygen concentration, PAH concentration and contamination history of soil, soil type, moisture, nutrients, and other substances that may act as substrate co-metabolites. The size and composition of microbial populations can in turn be affected by these factors. For example, in low pH soils, fungi are dominant over bacteria, and thereby control microbial degradation in these environments. While the pathways of microbial degradation are well known for anthracene, and phenanthrene, degradation pathways for other PAHs are largely unknown. Metabolism of PAHs by bacteria includes the formation of cis-dihydrodiols through dioxetane intermediates,

whereas in fungi (and mammalian systems) trans-dihydrodiols are produced through arene oxide intermediates (Sims and Overcash, 1983).

2.8.4 Other Important Factors Affecting Fate

Salinity of the environment may be an important factor in the fate and biodegradation of PAHs. Biodegradations of these compounds are generally not affected by ambient salinity. However, some reports have shown a positive correlation between salinity and rate of mineralization of some PAHs. In hypersaline environments, a lower rate of mineralization has been observed and it is due to reduction in microbial metabolic rate. Salinity may affect PAH-particle interactions and solubility of the compounds (Kastner *et al*., 1998; Mahro *et al*., 1994).

PAH mineralization is related to the length of incubation time, temperature, molecular weight of the hydrocarbon, and previous exposure to PAH or related contaminants. Environmental factors such as aerobic conditions, moisture, non-toxic loading rates, indigenous acclimated organisms, nutrients and degradable organic matter could influence biodegradation of PAHs contaminated soils significantly. The soil moisture requirement for microbial activity ranges from 25 to 85% of water holding capacity and from 30 to 90% for optimum PAH degradation. Microorganisms generally require a soil pH of 5.5 to 8.5 and a pH of 7.0 or 7.5 to 7.8 for optimum PAH degradation (Alexander, 1999; Baveye, 1999).

The soil type can greatly affect the degree of PAH biodegradation, even under the same optimum growth conditions. PAHs in some soil have been determined to be unbiodegradable, probably because the material is highly sorbed and is no longer available. Soil organic matter would slow biodegradation of PAHs that are otherwise readily metabolized. Phenanthrene sorbs to soil constituents, the extent of which is directly related to the percent organic matter in the soil. This may explain why mineralization of the compound occurs more slowly in soil than in liquid media. Fulvic acid has been shown to decrease mineralization of pyrene, apparently due to its toxicity to microbes and possible sorption of the compound, making it less bioavailable. Other studies have found that the biodegradation of PAHs was increased significantly by the

addition of compost, which stimulated the mineralization and fixation of PAHs in the soil (Mahro *et al*., 1994).

The environmental fate of PAHs includes volatilization as well as biotic and abiotic transformations. Volatilization is important only for two-ring PAHs, such as naphthalene, whereas it is the biotic mechanism which is responsible for removal of PAHs with three or more rings. Bioremediation is the process whereby biodegradative abilities of microorganisms are harnessed or exploited to remove or detoxify environmental pollutants (Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008). However, the rate and extent of biodegradation of PAHs in soils and sediments is affected by multiple factors (Table 2.2). The major factor limiting the bioremediation of soils and sediments contaminated with PAHs is the poor availability of these hydrophobic contaminants to microorganisms (Mihelcic *et al.*, 1993; Hughes *et al.*, 1997; Buscot *et al.*, 2005; Labana *et al.*, 2007).

2.8.5 Mass Balance

There are three possible outcomes for a chemical present at a specific location in the environment at a particular time:

1. the chemical can remain in that location,
2. can be carried elsewhere by a transport process,
3. or can be eliminated through transformation into another chemical.

This very simple observation is known as mass balance or mass conservation. Mass balance is a concept around which an analysis of the fate and transport of any environmental chemical can be organized; mass balance also serves as a check on the completeness of knowledge of a chemical's behaviour (Hemond and Fenchner-Levy, 2000).

2.9 Biodegradation

Biodegradation can be defined as the biologically catalyzed reduction in complexity of chemicals. In the case of organic compounds, biodegradation frequently, although not necessarily, leads to the conversion of much of the C, N, P and S, and other elements in the original compounds to inorganic products. Such a conversion of an organic substrate to inorganic products is known as mineralization. Ultimate biodegradation is a term sometimes used as a synonym for mineralization. Thus, in the mineralization of organic forms of C, N, P, or S, CO_2 or inorganic forms of N, P, S are released by the organism and enter the surrounding environment (Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

While plant and animal respiration are mineralization processes that destroy numerous organic molecules drawn from living organisms, the mineralization of synthetic chemicals by biological processes appears to largely be the result of microbial activity. Indeed, frequently microorganisms are the sole means, biological or non-biological, of converting synthetic chemicals to inorganic products. Nevertheless, few non-biological reactions in nature bring about comparable changes. It is because of their ability to mineralize anthropogenic compounds that microorganisms play a large role in soils, waters, and sediments (Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

Alexander (1994, 1999) reported that several conditions must be satisfied for biodegradation to take place:

1. An organism must exist that has the necessary enzymes to bring about the biodegradation. The mere existence of an organism with appropriate catabolic potential is necessary but not sufficient for biodegradation to occur.
2. That organism must be present in the environment containing the chemical. Although some microorganisms are present in essentially every environment near the earth's surface, particular environments may not contain an organism with the necessary enzymes.

3. The chemical must be accessible to the organism having the requisite enzymes. Many chemicals persist even in environments containing the biodegrading species simply because the organism does not have access to the compound that it would otherwise metabolize. Inaccessibility may result from the substrate being in a different microenvironment from the organism, in a solvent not miscible with water or sorbed to solid surfaces.

4. If the initial enzyme bringing about the degradation is extracellular, the bonds acted upon by that enzyme must be exposed for the catalyst to function. This is not always the case because of sorption of many organic molecules.

5. Should the enzymes catalyzing the initial degradation be intracellular, that molecule must penetrate the surface of the cell to the internal sites where the enzyme acts. Alternatively, the products of an extracellular, reaction must penetrate the cell for the transformation to proceed further.

Because the population or biomass of bacteria or fungi acting on many synthetic compounds is initially small, conditions in the environment must be conducive to allow for proliferation of the potentially active microorganisms (Alexander, 1973). However, there are also several environmental conditions which affect the degradation of organic contaminants in soil (Wilson and Jones, 1993 - **Table 2.3**).

2.9.1 Microbial Catabolism of Environmental Pollutants

The microbial degradation process involves the breakdown of organic compounds either through biotransformation into less complex metabolites or through mineralization into inorganic minerals, H_2O, CO_2 (*i.e.* in aerobic) or CH_4 (*i.e.* in anaerobic). Both bacteria and fungi have been extensively studied for their ability to degrade a range of environmental pollutants including recalcitrant PAHs, halogenated hydrocarbons and nitroaromatic compounds. Moreover, the biochemical pathways/enzymes required for the initial transformation stages are often specific for particular target environmental contaminants, converting them to metabolites which can be assimilated into more ubiquitous central bacterial pathways. However, the extent and rate of biodegradation depend on many factors including pH, temperature, oxygen, microbial population, degree

of acclimation, accessibility of nutrients, chemical structure of the compound, cellular transport properties, and chemical partitioning in a growth medium. Nevertheless, in bioremediation processes, it is generally an objective to exploit microbial technology to accelerate the rate of pollutant removal (Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

Biodegradation is frequently equated with the catabolism of particular organic compounds by a single microbial strain. However, it is becoming increasingly apparent that biodegradation in the natural environment is carried out by mixed microbial communities, and even under laboratory conditions it has been shown that a mixed group of microorganisms may be more effective than any of the component strains acting alone. An organism in the natural environment will find only one organic compound at its disposal. However, most of our information about degradative pathways rests on studies made on single microbial strains isolated by elective subculture for their ability to grow on a particular organic compound. These isolates have provided a rich source of experimental material (Clarke, 1984 and Alexander, 1973, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

2.9.2 Microbial Metabolism

Microbial metabolism is the main force in organic compound transformation or degradation; however, some organic compounds are resistant to microbial degradation and persist longer in the environment. Some others are only transformed to intermediate products, occasionally of higher toxicity. Therefore, it is essential to have a general understanding of the action of microorganisms and the fate of each organic compound in the environment. Only then will it be possible to have a better view and a work strategy towards resolving problems of organic compound pollution (Clarke, 1984 and Alexander, 1973, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

Microbial metabolism is the major process for degradation of PAHs in soil environments. Photolysis, hydrolysis, and oxidation are not considered important processes for the degradation of PAHs in soils. The rate and extent of degradation of PAHs in soil are affected by environmental factors, characteristics of the microbial

population, and the physical and chemical properties of the PAHs. Environmental factors that may influence the rate of PAHs degradation in soil include temperature, pH, oxygen concentration, PAH concentrations and contamination history of soil, soil type, moisture, nutrients, and other substances that may act as substrate cometabolites. The size and composition of microbial populations can in turn be affected by these factors. For example, in low pH soils, fungi are dominant over bacteria, and thereby control microbial degradation in these environments (Sims and Overcash, 1983; Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

2.9.2.1 Outcomes of PAH Microbial Metabolism

Bacterial metabolism of any given PAH has the following possible outcomes:

1. growth of an organism on the PAH substrate;
2. mineralization of the PAH substrate in the absence of detectable growth; and
3. transformation of the PAH substrate to non-mineral, terminal products.

Any of these outcomes might be included in the ambiguous term "degradation," which is often used to describe the removal of a particular compound from a system whether the mechanism is known or not. Either of the latter two outcomes could also be described as cometabolism. Since different metabolic capabilities are involved, we try to distinguish among the three outcomes by referring to them as growth, mineralization, and either transformation or incomplete metabolism, respectively (Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

2.9.3 Microbial Transformation

The degradation of organic compounds through microbial metabolic processes is considered to be the primary mechanism of biological transformation. Since microorganisms can proliferate in almost any environment because of their remarkable powers of mutation and adaptation, there appear to be great potential for acquiring degradative capabilities when exposed to organic compounds. However, the following

processes are involved in the microbial transformation of organic compounds (Bollage and Liu, 1990; Alexander, 1973, 1994, 199; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008):

1. biodegradation, where an organic compound can serve as a substrate for growth,
2. cometabolism, in which the organic compound is transformed by metabolic reactions but does not serve as an energy source for the microorganism,
3. polymerization or conjugation, in which organic compound molecules are linked together with other organic compounds, or with naturally occurring compounds,
4. accumulation, in which the organic compound is incorporated into microorganism, and
5. secondary effects of microbial activity, in which the organic compound is transformed because of changes in the pH, redox (oxidation-reduction reaction) conditions, reactive products, etc., in the environment, as caused by the microorganisms.

The microbial transformation of an organic compound may involve more than one type of mechanism, and under different conditions, various products can be derived from the same initial compound depending on the environmental parameters. The transformation processes can be mediated by one organism or can result from the combined effects of several organisms (Bollage and Liu, 1990; Alexander, 1973, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

2.9.4 Complete Biodegradation (Mineralization)

The most interesting and environmentally valuable aspect of organic compound transformation by microbes is the complete biodegradation (*i.e.* mineralization) of a certain organic molecule. If an organic compound can be used in such a way by one or more interacting microorganism, it will be metabolized into CO_2 and other inorganic components, and microorganisms can obtain their requirements for growth and energy from the organic molecules. From an environmental point-of-view, the complete metabolism of an organic compound is desired, if one is interested in avoiding the

generation of potentially hazardous intermediate products. There are many organic compounds whose complete decomposition could be demonstrated under certain conditions by adequately labelling them with ^{14}C-carbon (Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

2.9.5 Biodegradation of PAHs

Microbial transformation is a major environmental process affecting the fate of PAHs in both terrestrial and aquatic ecosystems. The microbial degradation of PAHs, having two or three rings, is well documented, but in the last decade, a number of bacteria that metabolize larger PAH molecules, have also been isolated. Biological technologies are now being explored for their potential in the remediation of contaminated sites. However, their successful application demands a broader understanding of the biochemical pathways by which PAHs are degraded, both individually and in mixtures. An extensive literature describing the degradation of individual PAHs by microorganisms which are able to utilize them as sole sources of carbon and energy, does exist. These studies have yielded fundamental information about the biodegradability of individual compounds (Cerniglia, 1992; Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

The rates of biodegradation of PAHs are highly variable and are dependent only on PAH structure, but also on the physico-chemical parameters of the site as well as the number and types of microorganisms present. PAHs sorbed to organic matter in soils and sediments, and the rate of their desorption strongly influences the rate at which microorganisms can degrade the pollutants (Shuttleworth and Cerniglia, 1995 and Singh and Tripathi, 2007). Nevertheless, much of the research is focused on techniques to enhance the bioavailability and consequently the degradation rate of PAHs at polluted sites. Degradation products of PAHs are, however, not necessarily less toxic than the parent compounds. Therefore, toxicity assays need to be incorporated into the procedures used to monitor the effectiveness of PAH bioremediation (Shuttleworth and Cerniglia, 1995 and Singh and Tripathi, 2007).

Aerobic bacteria have been extensively studied for use in remediation processes and both enzymological and genetic studies are being carried out for the purpose of

effective biodegradation. PAHs are degraded by microorganisms either in metabolism or co-metabolism (Greer, 1993; Habe and Omori, 2003;). Co-metabolism is very important for degradation of mixtures of PAHs and high molecular weight PAHs. In contrast, several two-, three-, and four-ring PAHs have been known to be growth substrates for bacteria (Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006).

On biodegradability of PAHs, Juhasz and Naidu, (2000) reported that, numerous genera of micro-organisms have been observed to oxidise PAHs (Table 2.4). While there is a great diversity of organisms capable of degrading the low molecular weight PAH, such as naphthalene, acenaphthene and phenanthrene, relatively few genera have been observed to degrade the high molecular weight-PAHs, such as the BaP. Juhasz and Naidu (2000) also reported on degradation of some four- and five- ring PAHs (Table 2.4).

2.9.6 Surfactants (Surface Active Agents)

The hydrophobic nature of many organic contaminants often limits the capacity of microorganisms, which generally exist in aqueous environments, to assimilate and degrade these compounds. Hydrocarbon-degrading bacteria produce a variety of biosurfactants, either associated with the cell surface or secreted into the extracellular medium (Makkar and Cameotra, 2002). However, production of biosurfactants by fermentation, as a possible amendment for bioremediation, appears to be more costly, so the use of commercially-produced biosurfactants products in bioremediation is arguably uneconomic. Hence, chemical surfactants, which are much cheaper to produce, have a potential role. Properly chosen, chemical surfactants may enhance biodegradation (Jayashree and Vasudevan, 2007). However, both enhancements and the inhibition of biodegradation of hydrocarbons have been observed (Mulligan *et al.*, 2001; Singh and Ward, 2004).

Singh and Tripathi (2007) reported that a variety of synthetic surfactants, both ionic and non-ionic have been shown to increase the bioavailability of PAHs as well as other hydrophobic contaminants and have contributed to our understanding of the mechanisms by which surfactants increase solubility. However, some synthetic surfactants can actually inhibit PAH biodegradation via toxic interactions, stimulation of surfactant degraders, or sequestration of PAHs into surfactant micelles. Microbially-

produced surfactants represent a promising alternative to chemical surfactants. The isolation of microorganisms producing biosurfactants, when grown on PAHs, has been reported (Prabhu and Phale, 2003). However, biosurfactants have been shown to have many of the positive effects of synthetic surfactants, but without their drawbacks. Nevertheless, they are biodegradable and non-toxic, and many biosurfactants do not produce true micelles, thus facilitating direct transfer of the surfactant-associated PAHs to bacteria (Singh and Tripathi, 2007).

2.10 Growth and Enzyme Kinetics in Microbial Processes for Biodegradation

2.10.1 Concepts of Enzymes

Copeland (2000) reported that life depends on a well-orchestrated series of chemical reactions. However, many of these reactions proceed too slowly on their own, to sustain life. Hence, nature has designed catalysts, which we now refer to as enzymes, to greatly accelerate these chemical reactions. The catalytic power of enzymes facilitates life processes in essentially all life-forms from viruses to man. Many enzymes retain their catalytic potential after extraction from the living organism, and it did not take long for mankind to recognize and exploit the catalytic power of enzyme for commercial purposes. In fact the earliest known references to enzymes are from ancient texts dealing with the manufacture of cheeses, breads, and alcoholic beverages, and for tenderizing of meats. Today enzymes continue to play key roles in many food and beverage manufacturing processes and in microbial processes for bioremediation of environmental contaminants (Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

2.10.2 What is an Enzyme?

Enzymes are a type of protein present in, and essential to, all living things. They have a number of functions in the living cell, but ultimately, they help to convert food into energy and new material for the growth and repair of the organism in which they function. Enzymes act as biological catalysts; they increase the rate of chemical reaction without undergoing any permanent change themselves. They are not consumed in the

reaction and therefore continue to catalyze a reaction as long as the proper reactants, usually called substrates, are available (Mathewson, 1998; Copeland, 2000; Singh and Ward, 2004; Boehm, 2006).

2.10.3 Structure of Enzymes

The basic properties of enzymes are determined by their protein nature. All proteins are composed of amino acids (there are about 20 that occur in nature). Each amino acid consists of an amino function ($-NH_2$) and a carboxylic acid function (COOH) attached to the same carbon atom.

2.10.4 Enzyme Activity

Enzymes are proteins with the special ability to catalyze, or speed up the rate of chemical reactions under the rather mild condition found in living organisms. For example, sucrose can be hydrolyzed, or split into its component sugars, glucose and fructose, by being heated in acid. This requires relatively harsh conditions that would not be suitable for sucrose conversion in most living things. In living organisms, the conversion of sucrose is brought about by an enzyme called *invertase*. Not only can the enzymatic reaction proceed under mild conditions, the rate of the enzymatic reaction is over 5×10^{10} times faster than that of the acid-heat reaction (Mathewson, 1998; Copeland, 2000; Singh and Ward, 2004; Boehm, 2006).

Heterotrophilic microorganism use organic compounds as both carbon and energy sources for growth. This is accomplished by enzymes acting in an organized and sequential manner, and for many simple growth substrates, such as glucose, the catabolic pathways are well understood. When a compound is the sole carbon source for growth, the catabolic pathway has to provide all the intermediates for biosynthesis and generate the energy-yielding reactions that are essential for biosynthesis and maintenance of cellular activities (Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

2.10.5 Chemical Kinetics

Chemical reactions can take place with or without enzymes. In the absence of an enzyme, chemical reactions occur, but they depend on a number of factors. First, the reactants must find and meet each other. This is normally the result of random collisions of reactant molecules in some medium such as water. Second, not just any collision is sufficient to result in the formation of a product. The molecules must collide with sufficient energy and in the proper orientation so that they form a complex, sometimes called a reactive intermediate. Often, when collisions occur, the orientation of the molecules is not correct and there is not enough energy involved to rearrange the bonds, so the reactive intermediate cannot develop and no new products are formed. The rearrangement of bonds requires, among other things, the input of energy. This energy of activation represents an "energy barrier." The reactant molecules must collide with at least this amount of energy to overcome the barrier so that the intermediate can be formed and the reaction can proceed to the formation of products (Mathewson, 1998; Copeland, 2000; Singh and Ward, 2004; Boehm, 2006; Diaz, 2008).

2.10.6 Enzyme Kinetics

Copeland (2000) explained that the kinetics of enzyme reactions is similar to those for non-enzymatic reactions except that enzymes have a unique way of accelerating the reaction. They act as catalysts by bringing the reactants together so as to promote the chemical reaction. First there is the rapid combination of the enzyme (E) with the reactant molecule or substrate (S) to form a reactive intermediate, In this case, the enzyme-substrate (ES) complex. This complex then breaks down into products (P) and releases the enzyme, unchanged, to combine with another substrate molecule.

One important concept in enzyme kinetics is reaction order, defined as zero-order, first-order, second-order, or third-order based on the rate of reaction under defined conditions where only the concentration of reactants varies and the enzyme concentration is held constant. In a zero-order reaction, the rate of reaction is independent of reactant (substrate) concentration. In first-, second-, and third-order reactions, the rate is dependent on one, or two, or three reactants, respectively. It is important to keep this in

mind when assaying enzyme activity. The substrate concentration must be kept very high relative to the enzyme concentration so that the reaction is zero-order with respect to substrate. Under such conditions (*i.e.*, at constant pH, temperature, *etc.*), the reaction is independent of substrate concentration and depends on enzyme concentration only (Mathewson, 1998; Copeland, 2000; Singh and Ward, 2004; Boehm, 2006).

2.10.7 Microbial Growth

Alexander (1999) stated that microorganisms use natural occurring and many synthetic chemicals for their growth. They use these molecules as a source of C, energy, N, P, S, or another element needed by the cells. Most attention has been focused on the acquisition of C and energy to sustain the growth of bacteria and fungi. For the synthetic substrates that are extensively degraded, the molecule is simply another organic substrate from which the population can obtain the needed elements or the energy required for biosynthetic reactions.

A common research procedure that relies on the ability of microorganisms to use organic compounds as sources of C and energy for growth is known as the enrichment-culture technique. The method is based on the selective advantage gained by an organism that is able to use a particular test compound as a C and energy source in a medium containing inorganic nutrients but no other sources of C and energy. Under these conditions, a species that is able to grow by utilizing that chemical will multiply. Few other bacteria and fungi will proliferate in this medium. However, species that use products excreted by the populations acting on the added organic nutrient will also flourish, and thus the final isolation of a microorganism in pure culture requires plating on agar medium so that individual colonies can be selected. That agar medium is also made selective by having a single source of C and energy. Repeated transfer of the enrichment through solutions that contain the test compound and inorganic nutrients further increases the degree of selectivity before plating because organic materials and unwanted species from the original environmental sample are diluted by the serial transfers (Greer, 1993; Alexander, 1994, 1999; Talley, 2006; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

The enrichment-culture technique has been the basis for the isolation of pure cultures of bacteria and fungi that are able to use a large number of organic molecules as C and energy sources. However, attempts to obtain microorganisms that are able to grow on a variety of other organic compounds have met with failure. Many of the failures can be attributed to misuse of the technique or errors in the approach of the investigator; for example, sometimes the concentration of the organic nutrient may be too low or too high so that microorganisms fail to develop because of toxicity. In other instances, the failure results from the absence from the selective medium of the growth factors essential for the organisms, which degrade the compound. Nevertheless, when the failure to isolate a microorganism by enrichment culture agrees with the persistence of the chemical in nature, it is likely that microorganisms do not use the compound as a source of C and energy (Greer *et al.*, 1993; Alexander, 1994, 1999; Singh and Ward, 2004; Boehm, 2006; Diaz, 2008).

As a rule, mineralization of organic compounds is characteristic of growth-linked biodegradation, in which the organism converts the substrate to CO_2, H_2O, cell components, and products typical of the usual catabolic pathways.

2.10.8 Microbial Kinetics

A knowledge of the kinetics of biodegradation is essential to the evaluation of the persistence of organic pollutants and to assessing exposure of humans, animals, and plants (Alexander, 1999). Once degradation of a chemical commences, the amount disappearing with time and the shape of the disappearance curve will be a function of the compound in question, its concentration, the organisms responsible, and a variety of environmental factors. Information on kinetics is extremely important because it characterizes the concentration of the chemical remaining at any time, permits prediction of the levels likely to be present at some future time, and allows assessment of whether the chemical will be eliminated before it is transported to a site at which susceptible humans, animals, or plants may be exposed. Such knowledge is thus essential for the assessment of the potential risk associated with exposure of susceptible individuals and species to the chemical.

Moreover, Alexander (1999) explained that research on kinetics has focused on two topics: (**i**) the factors affecting the rate of substrate transformation (temperature, pH, soil moisture, and C sources), (**ii**) the shapes of the degradation curves and evaluating which pattern of decomposition best fits the metabolism of given chemicals in a microbial culture, laboratory microcosms, or, occasionally, in the field.

However, the study of kinetics of biodegradation in natural environment is often empirical, reflecting the rudimentary level of knowledge about microbial population and activity in these environments. Kinetic models, such as the power rate model, where the change in substrate concentration is expressed as a power of the substrate concentration, provide a basis for the comparison of different curves, but it gives no insight into the reasons for the shapes and may often have no predictive value. Investigators rarely state whether the model they are using has a theoretical basis or is simply empirical, or whether kinetic constants have any physical meaning or are only fitting parameters (Bazin *et al.*, 1976; Alexander, 1999). An example of empirical approach is the power rate model (Hamaker, 1972; Alexander, 1999).

$$\frac{dC}{dt} = kC^n \tag{2.1}$$

Where,

C is the substrate concentration,

t is time,

k is the rate constant for chemical disappearance, and

n is a fitting parameter.

This model can be fitted to substrate-disappearance curves by varying n and k until a good fit is achieved. From this equation, it is evident that the rate is proportional to a power of the substrate concentration. The power-rate law provides a basis for comparison of different curves, but it gives no insight into the reasons for the shapes. Therefore, often it may have no predictive value. Moreover, investigators interested in kinetics do not always state whether the model they are using has a theoretical basis or is

simply empirical, or whether constants in an equation have physical meaning or are only fitting parameters (Bazin *et al.*, 1976; Alexander, 1999).

Nevertheless, an appropriate introduction to the kinetics of biodegradation is to consider a pure culture of a single bacterial population that is growing on and degrading a single, soluble organic chemical, and to assume that no barrier exists between the substrate and the cells (Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007).

2.10.9 Processes Linked to Growth

Alexander (1999) stated that microorganisms use natural occurring and many synthetic chemicals for their growth. They use these molecules as a source of C, energy, N, P, S, or another element needed by the cells. As a rule, mineralization of organic compounds is characteristic of growth-linked biodegradation, in which the organism converts the substrate to CO_2, H_2O, cell components, and products typical of the usual catabolic pathways (Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

Biodegradation of a particular organic substrate may be carried out by microorganisms that are (Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008):

1. growing at the expense of that substrate and using it as a source of C, energy, or possibly another nutrient element needed for proliferation;
2. growing at the expense of another organic nutrient that is used as a source of C, energy, or both but metabolizing the substrate of interest (although not using it to supply building blocks for cell synthesis); or
3. not growing as they metabolize the chemical of concern.

2.10.10 Microbial Destruction of Organic Pollutants

Biodegradation of individual compounds has been the subject of active concern for more than 40 years. The initial interest was in the fate and persistence of pesticides in soils; however, the field has expanded enormously in recent years to encompass a wide variety

of chemicals and a broad array of issues. Moreover, technologies have been developed that markedly enhance biodegradation or that result in microbial destruction of organic pollutants that otherwise would persist at the sites of contamination. These bioremediation technologies have led to the clean-up of many polluted ground waters and soils, and they have fostered the development of a new bioremediation industry. The concept of bioavailability should be identified as the key area of study involved in biodegradation processes and bioremediation treatments (Alexander, 1999;Ward, 2004; Boehm, 2006).

Petroleum hydrocarbons and petroleum products are common pollutants. Microorganisms have been found that are capable of utilizing these compounds as carbon and energy sources. Generally, harmful organic compounds can be catabolised into less toxic intermediate byproducts or completely mineralized into water and carbon dioxide by microorganisms. The process by which organisms degrade contaminants in the environment is called biodegradation, and is considered a safe, economic, and efficient way to clean up a polluted area (Providenti *et al.*, 1993; Singh and Ward, 2004; Boehm, 2006; Talley, 2006; Singh and Tripathi, 2007).

2.11 Bioremediation Strategies

Two main types of bioremediation strategies have been developed for soils: bioaugmentation and biostimulation (Greer *et al.*, 1993).

2.11.1 Bioaugmentation

Bioaugmentation is the process by which biodegradative microorganism, are added to the soil to enrich the total bacterial population and thus increase the rate of biodegradation of the pollutant. Bioaugmentation, however, is an effective method to decrease the time necessary to degrade the contaminants, if the biodegradative indigenous population is too small to significantly biodegrade a contaminant in a particular environment (Providenti *et al.*, 1993). Therefore, if bioaugmentation is to be used successfully, there must be reliable methods, which allow the indigenous, and the added bacteria to be monitored, and to some extent, controlled.

Singh and Ward (2004) concluded that several laboratory and field investigations have indicated that inoculation with selected and acclimated microorganisms (*i.e.* bioaugmentation) did not significantly enhance rates of oil biodegradation over those achieved by nutrient enrichment of the natural microbial population (*i.e.* biostimulation).

In contrast, bioaugmentation has been effectively used in agriculture and wastewater treatment (Jasper, 1994). Moreover, in bioremediation, there are examples of successes, where the introduction of competent microorganisms appeared to enhance the bioremediation rate and where the indigenous population appeared non-competent (Riggle, 1995). Bioaugmentation has also been beneficial in treating some recalcitrant compounds such as 2,4,6-TNT, carbon tetrachloride and PCP (Edghill, 1995). However, the importance of using microorganisms that have demonstrated long survival times in a particular soil environment may be an important dimension in this approach. Nevertheless, the use of very high microbial numbers in bioaugmentation ($> 10^7$/g soil) has been shown to be effective where bioavailability was not the limiting factor (Pearce *et al.*, 1995; Singh and Ward, 2004).

2.11.2 Biostimulation

Biostimulation involves adding nutrients or chemicals to the soil in order to stimulate indigenous bacterial growth and consequently, the rate of biodegradation. While there is some concern that the chemicals or nutrients added could have some adverse effects on the environment, biostimulation has often been the preferred strategy because it is easier to control and to monitor the movement of the added chemicals than to predict how microorganisms added in a bioaugmentation strategy will function when released in to the environment.

Unterman *et al.*, (2000) described a hierarchy of technology choices for bioremediation. Where the degrading population exists in the contaminated zone but, nutrients or other conditions are not sufficient to promote microbial activity. Oxygen is often the most limiting substrate, which may be supplied by the introduction of air, O_2, H_2O_2 or manganese peroxide. The process may also be accelerated by the addition of other nutrients, typically sources of nitrogen and phosphorus. For remediation of carbon

contaminants, added co-substrates may promote growth and may be especially important where the contaminant is degraded by co-metabolism (Singh and Ward, 2004).

2.12 Sorption Phenomenon

2.12.1 Mechanism of the Sorption

The term sorption includes both adsorption and absorption. Adsorption and absorption are generally very difficult to distinguish as separate processes in most situations. Adsorption is generally defined as the process by which dissolved compounds become physically and chemically bound to colloidal surfaces.

2.12.2 Contaminant-Soil Interaction Mechanisms

2.12.2.1 Sorption

The general term sorption as defined by Young *et al.*, (1992) is used to indicate the process in which the solutes (ions, molecules, and compounds) are partitioned between the liquid phase and the soil particle interface. Of the various phenomena that can contribute to sorption, chemical interactions constitute the major subject of interest in contaminant-soil interactions. When it is difficult to fully distinguish between the mechanisms of physical adsorption, chemical adsorption and precipitation, the term sorption is used to indicate the general transfer of material to the interfaces.

In regard to contaminant-soil interaction, the adsorption reactions which occur are influenced by which contaminant solutes in solution become attached to the surface of soil (solids) particles through mechanisms which seek to satisfy the forces of attraction from the soil solids (surfaces). These processes are governed by the surface properties of the soil solids (inorganic and organic), and the chemistry and physical-chemistry of the contaminant leachate and its constituents *e.g.*, cations, anions and non-ionic molecules. The net energy of interaction due to adsorption of a solute ion or molecule onto a soil constituent surface is the result of both short range chemical forces such as covalent bonding, and long range forces such as electrostatic forces. Sorption may be physical or chemical (often referred to as physisorption and chemisorption) depending on the strength of forces causing the sorption process and their energies (Young *et al.*, 1992).

2.12.2.2 Physical Adsorption

Young *et al.,* (1992) reported that physical adsorption occurs when the contaminants in the soil solution (aqueous phase, pure water) are attracted to the soil constituent's surfaces because of the unsatisfied charges (attractive forces) of the soil particles. Cations and anions are specifically or non-specifically adsorbed by the soil solids. Ions can interact in both the diffuse double layer (DDL) and in the Stern layer. Those (ions) that interact in the DDL by reducing the zeta potential are generally referred to as indifferent ions. With reference to indifferent contaminant ions, they are non-specific, in that they do not reverse the sign. Some researchers also reported that the term non-specific adsorption referred to outer-sphere surface complexation of ions by the functional groups exposed on soil particles.

In nonspecific cation adsorption, the ions are held primarily by electrostatic forces. The replacement of exchangeable cations *i.e.* cation exchange, involves those cations associated with the negative charge sites on clay soil solids through largely electrostatic forces. Ion exchange reactions occur in the various soil constituents *i.e.* clay minerals and non-clay mineral soil fractions. In general, one can consider non-specific adsorption as adsorption occurring as a result of electrostatic attraction (Young *et al.*, 1992).

2.12.2.3 Chemical Adsorption

Young *et al.*, (1992) also defined that chemical adsorption refers to high affinity, specific adsorption which generally occurs in the inner Helmholtz layer through covalent bonding. In specific cation adsorption, the ions penetrate the coordination shell of the structural atom and are bonded by covalent bonds via O and OH groups to the structural cations. The valence forces are of the type which binds atoms to form chemical compounds of definite shapes and energies. The ions have the ability to influence the sign of zeta potential, and are referred to as the potential determining ions. The interaction mechanism in chemical adsorption is difficult to distinguish from electrostatic positive adsorption except for their higher adsorption energies for chemical adsorption.

Reactions can be either endothermic or exothermic, and usually involve activation energies in the process of adsorption, *i.e.* the energy barrier between the molecule being adsorbed and the soil solid surface must be overcome if a reaction is to occur. Strong chemical bond formation is often associated with high exothermic heat of reaction. By and large, the first layer is chemically bonded to the surface and additional layers are held by Van Der Waals forces (Young *et al.*, 1992).

Adsorption of anions by soil particles can occur as specific adsorption *e.g.*, ligand exchange reaction where anion displacement of OH^- or H_2O occurs and becomes a ligand in the coordination of the structural cations. The hydrous oxides and hydroxides of Fe and Al are typical examples of soil materials which allow for a specific adsorption of anions to occur. The adsorption of anions can also be non-specific *e.g.*, Cl^- and NO_3^-, which are held by electrostatic or Coulombic forces, and is generally confined to pH-dependent charge particle surfaces at pH values below the zero point charge (ZPC) (Young *et al.*, 1992).

2.12.3 Organic Compounds as Adsorbates

In aqueous systems, sorption of non-ionisable organic compounds is primarily through solute-partitioning (dissolution) into the soil organic phase. The fact that non-ionisable organic compounds sorb into organic matter through partitioning is very well documented (Means *et al.*, 1980).

Chiou *et al.*, (1982 and 1988) observed that the sorption (partitioning) of nonionic compounds from water onto soil yields linear isotherms and exhibit no obvious competition between solute molecules. In wet soils, the relative contribution of the mineral fraction is minimal probably due to the strong dipole-interaction between water molecules and soil minerals which renders this fraction of the soil inaccessible or inert to nonionic organic solutes. In contrast, the sorption of organic compounds from non-polar organic solvents or the vapour phase on dry sub-saturated soils is competitive and yields isotherms with distinctive curvatures. In the latter case, sorption is controlled mainly by mineral adsorption, which predominates over uptake by partitioning into organic matter.

2.12.4 Soil Constituents as Adsorbents

Sand, silt and clay are the main groupings of soil particles (passing through a 2 mm round-hole sieve) based on upper and lower particle size limits. The relative distribution of these fractions determines soil texture and subsequently the amounts of surface available for physical interactions and chemical reactions. Sand (2.00-0.05 mm) and silt (0.05-0.002) consist mainly of primary and secondary minerals. The more refractory minerals (such as quartz) dominate the sand fraction and softer primary minerals (such as feldspar and muscovite mica) along with secondary minerals dominate the silt fraction. Clay minerals (<0.002 mm) are of secondary origin and include layered-silicate, oxides and hydrous oxide minerals (Millar *et al.*, 1965; Young *et al.*, 1992).

2.13. Concluding Remarks

Given the significant environmental risks associated with PAHs, a thorough understanding of the multidisciplinary aspects delved on in the literature with respect to such compounds' chemistry, interaction with soils and susceptibility to biodegradation, must inform the design and focus of the present study. Specific and original research objectives must reflect a knowledge of prior studies and their limitations. The literature suggests that contaminated sites such as airports, car dumps, etc. may harbour bacterial strains competent in PAH biodegradation. Such strains can be specifically selected for, given our present knowledge of bacterial PAH-degradation pathways and associated genes. The importance of soil constituents and contaminant ageing, highlighted in the literature, can inform the design of experiments focusing on parameters particularly important in modulating the extent and rate of bioavailability, biodegradation, bioremediation and biodetoxification mechanisms.

Table 2.1. Selected Properties of the 16 USEPA Priority Pollutant PAHs at 25°C

Compound[a]	log (K_{ow})[b]	C_{sat} (mg L^{-1})[b,c]	TEF[d]	Carcinogenic Potency[e]	Threshold of PAHs contaminants[f]
Naphthalene (NAP)	3.37	31.0	0.001	-	40
Acenaphthylene (ACY)	4.0	16.1	0.001	-	840
Acenaphthene (ACE)	3.92	3.80	0.001	-	1300
Fluorene (FLU)	4.18	1.90	0.001	-	350
Phenanthrene (PHN)	4.57	1.10	0.001	-	40
Anthracene (ANT)	4.54	0.045	0.01	-	28
Fluoranthene (FLA)	5.22	0.26	0.001	-	40
Pyrene (PYR)	5.18	0.132	0.001	-	250
Chrysene (CHR)	5.65	0.002	0.01	0.001	19
Benz[a]anthracene (BaA)	5.91	0.011	0.1	0.1	NA[g]
Benzo[b]fluoranthene (BbF)	5.80	0.0015	0.1	0.1	19
Benzo[k]fluoranthene (BkF)	6.0	0.0008	0.1	0.01	19
Benzo[a]pyrene (BaP)	6.04	0.0038	1	1	1.9
Benzo[g,h,i]perylene (BgP)	6.50	0.00026	0.01	-	40
Dibenz[a,h]anthracene (DBA)	6.75	0.0006	5	1	1.9
Indeno[1,2,3-c,d]pyrene (INP)	7.66	0.062	0.1	0.1	19

[a]Compounds listed in bold are classified by USEPA as probable human carcinogens.
[b]Data are apparent consensus values from Mackay *et al.*, (1992). Aqueous solubilities can vary depending on the method used. Some recent measurements differ significantly from the value shown (De Maagd *et al.*, 1998), while others support them (Reza *et al.*, 2002).
[c]Aqueous solubility
[d]Toxicity equivalency factor proposed by Nisbet and LaGoy (1992).
[e]USEPA (1993).
[f]Threshold of PAHs contaminants in Soil in Ontario, Canada (μg g^{-1} of soil)
[g]NA: Not analyzed
Source: (Singh and Ward, 2004)

Table 2.2: Factors of Affecting Bioremediation of PAH-contaminated Sites

Physico-Chemical Factors	Biological Factors	Environmental Factors
➢ Physical/Chemical Properties of PAHs (Number of Rings, log K_{ow}) ➢ Organic Content of Soil ➢ Structure/Particle Size of Soil ➢ Presence of Contaminants	➢ Characteristics of the Microbial Population (Diversity, Genetic/Catabolic Potential)	➢ Temperature ➢ Moisture ➢ pH ➢ Sorption ➢ Degree of Contamination

Source: (Labana *et al.*, 2007)

Table 2.3. Environmental Conditions Affecting the Degradation of Organic Contaminants in Soil

Parameter	Conditions Required for Microbial Activity	Optimum Values for PAH Degradation
Soil moisture	25-85% of water-holding capacity	30-90%
Soil pH	5.5-8.5	7.5-7.8
Oxygen	aerobic, minimum air-filled pore content space of 10%	10 - 40% O_2
Nutrient	N and P for microbial growth C:N:P 120:10:1	salt concentration <4% C:P 800: 1, C:N 60: 1
Temperature (°C)	15-45	24 - 30

Source: (Wilson and Jones, 1993)

Table 2.4. Polycyclic Aromatic Hydrocarbon (PAH) Oxidized by Different Species of Bacteria

Organisms	References
Naphthalene	
Acinetobacter calcoaceticus, Alcaligenes denitrificans, Mycobacterium sp. *Pseudomonas* sp. *P. putida, P. fluorescens, Sp paucimobilis, Brevundimonas vesicularis, Burkholderia cepacia, Comamonas testosteroni, Rhodococcus* sp., *Corynebacterium renale, Moraxella* sp., *Streptomyces* sp., *B. cereus, P.marginalis, P. stutzeri, P. saccharophilia, Neptunomonas naphthovorans, Cycloclasticus* sp.	Ryu *et al.* (1989), Weissenfels *et al.* (1990, 1991), Kelly *et al.* (1991), Dunn and Gunsalus (1973), Davies and Evans (1964), Foght and Westlake (1988), Jeffrey *et al.* (1975), Mueller *et al.* (1990), Kuhm *et al.* (1991), Walter *et al.* (1991), Dua and Meera (1981), Tagger *et al.* (1990), Garcia - Valdes *et al.* (1988), Trower *el al.* (1988), Grund *et al.* (1992), Bams1ey (1975), Bamsley (1983), Yang *et al.* (1994), Burd and Ward (1996), Allen *et al.* (1997), Stringfellow and Aitken (1995), Filonov *et al.* (1999), Hedlund *et al.* (1999), Geiselbrecht *et al.* (1998), Foght and Westlake (1996), Goyal and Zylstra (1996)
Anthracene	
Beijernickia sp., *Mycobacterium* sp., *P. putida, Sphingomonas. paucimobilis, Rhodococcus* sp., *Flavobacterium* sp., *Arthrobacter* sp., *P. marginalis, Cycloclasticus* sp., *P. fluorescens, Sp. yanoikuyae, Acinetobacter calcoaceticus, Gordona* sp., *Sphingomonas* sp., C. *testosteroni, Cycloclasticus pugetii Bu. cepacia,*	Colla *et al.* (1959), Akhtar *el al.* (1975), Jerina *et al.* (1976), Evans *et al.* (1965), Ellis *et al.* (1991), Weissenfe1s *et al.* (1991), Foght and Westlake (1988), Walteretal. (1991), Mueller *et al.* (1990), Savino and Lollini (1977), Tongpim and Pickard (1996), Burd and Ward (1996), Geiselbrech *et al.* (1998), Foght and Westlake (1996), Kim *et al.* (1997), Lal and Khanna (1996), Mahro *et al.* (1995), Goyal and Zyltra (1996), Dyksterhouse *et al.* (1995), Allen *et al.* (1999)
Phenanthrene	
Aeromonas sp., *A. faecalis, A. denitrificans, Arthrobacter polchromogenes,Beijernickia* sp., *Micrococcus* sp., *Mycobacterium* sp., *P. putida, Sp. Paucimo ilis, Rhodococcus* sp., *Vibrio* sp., *Nocardia* sp., *Flavobacterium* sp., *Streptomyces* sp., S. *griseus, Acinetobacter* sp., *P. aeruginosa, P. stutzeri, P. saccharophila, Stenotrophomonas maltophilia,*	Kiyohara *et al.* (1976, 1982, 1990), Weissenfels *et al.* (1990, 1991), Keuth and Rehm (1991), Jerina *et al.* (1976), Colla *et al.* (1959), West *et al.* (1984), Kiyohara and Nagao (1978), Heitkamp and Cerniglia (1988), Guerin and Jones (1988, 1989), Treccani *et al.* (1954), Evans *et al.* (1965), Foght and Westlake (1988), Mueller *et al.* (1990), Sutherland *et al.* (1990), Ghosh and Mishra (1983), Savino and Lollini (1977),

Table 2.4. (Cont.) Polycyclic Aromatic Hydrocarbon (PAH) Oxidized by Different Species of Bacteria

Organisms	References
Phenanthrene (cont.)	
Cycloclasticus sp., *P. fluorescens, Acinetobacter calcoaceticus,* C. *testosteroni, Acidovorax* al. *delafieldii, Gordana* sp., *Cycloclasticus pugetii, Sp. yanoikuyae, Agrobacterium* sp., *Bacillus* sp., *Burkholderia* sp., *Sphingomonas* sp., *Pseudomonas* sp., *Rhodotorula glutinis, Nocardioides* sp., *Flavobacterium gondwanense, Halomonas meridiana*	Trower *et al.* (1988), Barnsley (1983), Yang *et al.* (1994), Kohler *et al.* (1994), Stringfellow and Aitken (1995), Boonchan (1998), Juhasz (1998), Geiselbrecht *et al.* (1998),Foght and Westlake (1996), Kastner *et al.* (1998), Lal and Khanna (1996), Shuttleworth and Cerniglia (1996), Mahro *et al.* (1995), Goyal and Zylstra (1996), Dyksterhouse *et al.* (1995), Allen *et al.* (1999), Aitken *et al.* (1998), Romero *et al.* (1998), Iwabuchi *et al.* (1998), Churchill *et al.* (1999), Juhasz (1991)
Fluoranthene	
A. denitrificans, Mycobacterium sp., *P. putida, Sp. paucimobilis, Bu. cepacia, Pseudomonas* sp., *Strenotrophomonas maltophilia, Acinetobacter calcoaceticus, Acidovorax delafieldii, Gordona* sp., *Sphingomonas* sp., *P. saccharophilia, Pasteurella* sp.	Kelly and Cerniglia (1991), Walter *et al.* (1991), Weissenfels *et al. Rhodococcus* sp. (1991), Foght and Westlake (1988), Barnsley (1975), Mueller *et al.* (1990), Ye *et al.* (1996), Kelly *et al.* (1993), Boonchan (1998), Juhasz (1998), Lal and Khanna (1996), Shuttleworth and Cerniglia (1996), Mahro *et al.* (1995), Churchill *et al.* (1999), Chen and Aitken (1999)
Pyrene	
A. denitrificans, Mycobacterium sp., *Rhodococcus* sp., *Sp. paucimobilis, Strenotrophomonas maltophilia, Acinetobacter calcoaceticus, Gordona* sp., *Sphingomonas* sp., *P. putida, Bu. cepacia, P. saccharophilia*	Heitkamp *et al.* (1988), Walter *et al.* (1991), Weissenfels *et al.* (1991), Grosser *et al.* (1991), Schneider *et al.* (1996), Ye *et al.* (1996), Boonchan (1998), Juhasz (1998), Lal and Khanna (1996), Mahro *et al.* (1995), McNally *et al.* (1999), Jimemez and Bartha, Churchill *et al.* (1999),Chen and Aitken (1999)

Source: (Juhasz and Naidu, 2000)

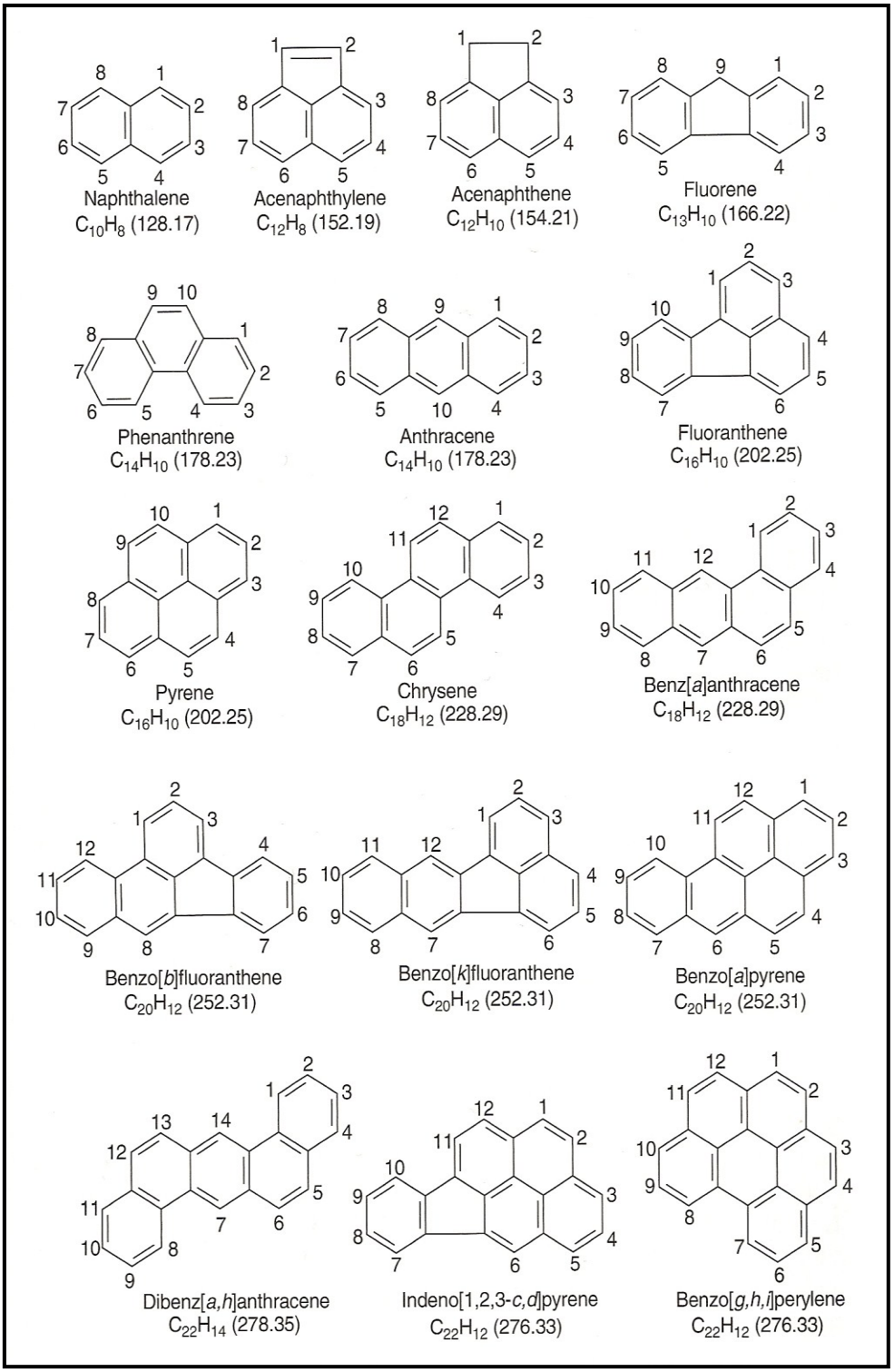

Figure 2.1. Structures, Chemical Formulas, and Molecular Weights of the 16 US Environmental Protection Agency (USEPA) Priority Pollutant PAHs

Source: (Singh and Ward, 2004)

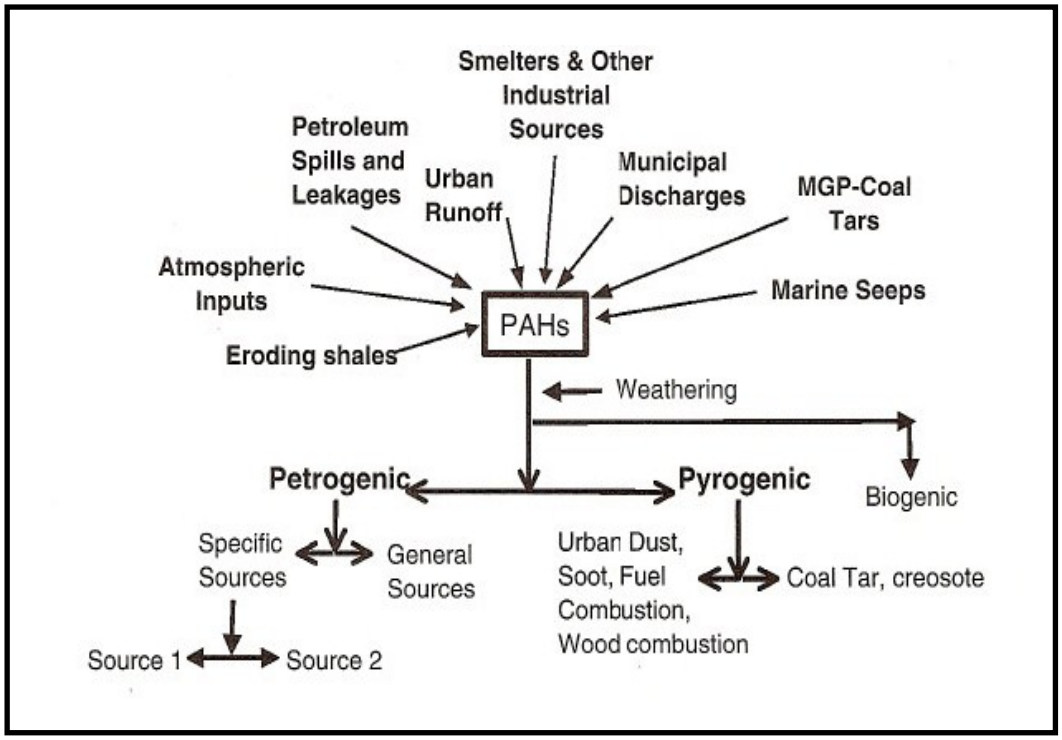

Figure 2.2. Generalized PAH Forensics Flow Chart.

Source: (Boehm, 2006).

PREFACE TO CHAPTER 3

In Chapter 2, the following topics were covered: introduction, polycyclic aromatic hydrocarbons (PAHs), isolation of pollutant-degrading bacteria from the environment, soil bioremediation, bioavailability and fate of organic compounds and PAHs in environment, biodegradation of organic compounds and PAHs, growth and enzyme kinetics in microbial processes for biodegradation, bioremediation strategies, and sorption phenomenon (*i.e.* contaminant-soil interaction mechanisms and mechanism of the sorption). The comprehensive literature review revealed the fact that the development of efficient, economical, and environmentally sustainable technology for the bioremediation of PAHs-contaminated soil environments, are necessarily related to the isolation of bacterial strains capable of mineralizing PAHs.

In the next chapter, isolation of PAHs-degrading bacterial strains from soils contaminated with a range of different organic pollutants, such as the wood treatment chemicals pentachlorophenol (PCP) and creosote containing oils and PAHs was conducted. A further goal was to investigate if bacterial strains isolated from different contaminated soils bore biodegradation pathways for six recalcitrant selected PAHs substrates. The work included an assessment of the bioavailability of PAHs from nineteen contaminated soils, collected from different contaminated sites, and mineralization studies for six recalcitrant selected PAHs substrates.

This manuscript has been submitted for publication.

Jazestani, J. and S. O. Prasher, 2011. Isolation of Recalcitrant PAHs-Degrading Bacterial Strains from Different Contaminated Soil Environments and Mineralization of Selected PAHs Compounds. (submitted for publication in ***Environmental Pollution***).

CHAPTER 3

Isolation of Recalcitrant PAHs-Degrading Bacterial Strains from Different Contaminated Soil Environments and Mineralization of Selected PAHs Compounds

3.1 ABSTRACT

Bacterial strains were isolated from nineteen different petroleum-contaminated soil environments, previously characterized in terms of their relative fractions of available (or potentially available) contaminants suitable as microbial substrates. Various strains' abilities to biodegrade six polycyclic aromatic hydrocarbons (PAHs), namely naphthalene (2 fused-benzene rings), anthracene, phenanthrene, fluorene (3 fused-benzene rings), fluoranthene and pyrene (4 fused-benzene rings), were investigated.

Using serial dilution and spread-plate techniques on solid MSM-agarose (DNA-grade, Bio-Rad) medium, containing phenanthrene (PAH) as sole carbon source, 228 phenanthrene-degrading bacterial strains were isolated from 12 petroleum-contaminated soil samples. Out of the 228 strains, 60 positive colonies, namely those presenting a clear zone in the phenanthrene precipitate, were selected and purified for further characterization. All 60 positive colonies were routinely subcultured at room temperature (25^{o}C) on YTS_{1000} - agarose plates.

A DNA-DNA (Southern) Hybridization Technique was used to identify 14 colonies, among the 60 isolated colonies, which exhibited a potential for naphthalene-degradation (ndoB Positive). To further screen for the biodegradation capacity of the fourteen isolated bacterial strains, standard aerobic liquid serum bottle microcosms were set up for quantitative radioactive tracer analysis of the biodegradation and cumulative percent mineralization of the six PAHs compounds.

One of the PAH-degrading bacterial strains (strain S65**)**, isolated from a jet-fuel contaminated site at the Sept-Iles airport (Sept-Iles, Quebec, Canada), on the north shore of the St. Lawrence river, was able to mineralize selected PAH compounds. Strain S65 was routinely subcultured at room temperature (25^{o}C) on YTS_{1000} - agarose nutrient agar plates. The PAHs mineralization experiments with Strain S65 showed high cumulative percent mineralization for Pyrene (61%), Phenanthrene (61%), and Fluoranthene (24%),

as sole carbon and energy sources, but there was no significant degradation of naphthalene, anthracene or fluorene.

Key Words: Bioavailability, Biodegradation, Isolation, Mineralization, PAHs, Naphthalene, Anthracene, Phenanthrene, Fluorene, Fluoranthene, and Pyrene.

3.2 INTRODUCTION

Chemicals play a vital role in our everyday life and are manufactured for either material comfort or for enhanced agricultural production (*e.g.*, pesticides and herbicides). However, over the last several decades, substantial amounts of industrial chemicals have been released into the environment. It is important to emphasize that a large number of these compounds, especially those structurally related to natural compounds, are often easily degraded by microorganisms. On the other hand, significant portions of these chemicals are man-made (*xenobiotics*) and have little, if any, structural similarity with naturally-occurring compounds. Xenobiotics are metabolized very slowly; if at all, and thus, tend to accumulate in the environment. Many of these compounds exhibit some degree of toxicity and, consequently, contribute strongly to environmental pollution problems (Chaudry and Chapalamadugu, 1991; Alexander, 1999; Alexander and Alexander, 2000; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008; Desai *et al.*, 2008).

Environmental pollution is a global issue due to direct contamination from growing industrialized centres, and movement of gaseous or particulate-bound contaminants through the atmosphere. This research work focuses on one group of POPs - the PAHs - among the most hazardous and recalcitrant xenobiotic compounds released daily to the environment. These are known for their strong mutagenic, carcinogenic and toxic properties (Environment Canada, 1994; WHO, 1998; Alexander, 1999; Alexander and Alexander, 2000; USEPA, 2000; Conte *et al.*, 2001; Luning Prak and Pritchard, 2002a, 2002b; National-Research-Council, 2002; Douben, 2003; Singh and Ward, 2004; Aitken and Long, 2004; Philp *et al.*, 2005; Buscot and Varma, 2005; Boehm, 2006; Prichard *et al.*, 2006; Thwaites *et al.*, 2007; Singh and Tripathi, 2007; Diaz, 2008; Desai *et al.*, 2008; Stenuit *et al.*, 2008).

The PAHs represent a large family of organic compounds that are considered environmental contaminants. The PAHs consist of a class of chemicals with two or more fused benzene rings in linear, angular or cluster arrangements. Among the most abundant environmental pollutants, the aromatic compounds are of major concern because of their persistence and toxicity. PAHs are ubiquitous in nature, being found throughout the

environment in air, water and soil (Zhang *et al.*, 1997). PAHs are one of the significant contaminant groups threatening environmental sustainability and human health. Although most PAH compounds are biologically degradable, biodegradation rates for PAHs in the environment are frequently hindered by their low solubility and sorption to soil particles. The PAHs are mainly derived from incomplete fossil fuel combustion and oil-related discharges to the environment (Obbard *et al.*, 2007). They are also produced during waste incineration, or as by-products of industrial processes, such as coal gasification and petroleum refining, and often released in large quantities into the environment. There are more than 100 different PAHs which occur as complex mixtures, rarely as a single compound (Finlayson-pitts and Pitts, 1997; Alexander, 1999; Singh and Ward, 2004; Boehm, 2006; Singh and Tripathi, 2007; Diaz, 2008).

PAHs are hydrophobic organic pollutants that have been placed on the United States Environmental Protection Agency's (USEPA) priority pollutants list due to their toxicity and, in some cases, carcinogenicity. PAHs with up to five rings are biodegradable (Cerniglia, 1992) however, the bioremediation of contaminated sites is often limited by the low bioavailability of higher molecular weight PAHs associated with soils and sediments (Wilson *et al.*, 1993). This is primarily due to the tendency of these compounds to partition into the organic matter content of soils or sediments or into nonaqueous-phase liquids (NAPLs) where they are thought to be inaccessible to intracellular enzymes (Pignatello *et al.*, 1996). Desorption of PAHs from the soil matrix is affected by the length of time the contaminants have been in contact with the matrix, the rate at which the PAHs are removed from the water phase, and the diffusion rate of PAHs within the matrix (Pignatello *et al.*, 1996).

This study sought to isolate bacterial strains from soils contaminated with a range of different organic pollutants, such as the wood treatment chemicals pentachlorophenol (PCP) and creosote containing oils and PAHs. A further goal was to investigate if bacterial strains isolated from different contaminated soils bore biodegradation pathways for six selected PAHs substrates. The work included an assessment of the bioavailability of PAHs from twelve of nineteen contaminated soils, collected from different contaminated sites, and mineralization studies for six selected PAHs substrates.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals

For preparation of YTS_{250A}, YTS_{250B}, YTS_{1000A}, YTS_{1000B}, MSM_A, and MSM_B media, and other assays, chemicals such as Yeast Extract, Tryptone, Starch, Granulated Agar, Ultra-Pure Agarose, Glycerol, KOH, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Co(NO_2)_2.6H_2O$, $AlK(SO_4)_2.12H_2O$, Cu SO_4, Zn $SO_4.7H_2O$, Mn SO_4. H_2O, Fe $SO_4.7H_2O$, Na_2MoO_4. $2H_2O$, $Ca(NO_3)_2.4H_2O$, H_2SO_4, toluene ($C_6H_5CH_3$), acetone (CH_3COCH_3), hexane (C_6H_{14}), HPLC grade methanol (CH_3OH), and ethanol (C_2H_5OH) were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada), and Sigma-Aldrich Chemical Company (Oakville, Ontario, Canada). All chemicals and solvents were of ACS Reagent grade or better and were used as received.

3.3.2 Radiochemicals

Naphthalene, Anthracene, Phenanthrene, Fluorene, Fluoranthene, and Pyrene labelled (*i.e.* hot) and unlabeled (*i.e.* cold) substrates were provided (radiochemically pure), by the manufacturer (Sigma-Aldrich, Oakville, Ontario, Canada; Tables 3.1 and 3.2), and stored under refrigerated conditions (4°C) until use.

3.3.3 Soil Sampling

Twelve contaminated soils were collected by NRC-BRI from different wood-preserving plants, oil refineries, and various petroleum hydrocarbons-contaminated sites proved to be rich in potentially PAH degradation competent organisms/strains (Table 3.3). The soil sampling procedures were conducted from the surface to a depth of about 0.3 m. Each sample consisted of a composite of five sub samples from an area 3 m × 3 m. The sampling points were chosen randomly. Soil samples were collected with a shovel which was scrubbed with a brush to remove large particles, and then shovel and brush were cleaned with acetone and hexane. They were finally rinsed with de-mineralized water after each sampling point, in order to avoid cross-contamination. Each soil sample was sieved on-site (8 mm), and soil samples then transferred to sealed plastic bags. They were immediately transferred to the laboratory and stored at 4°C, in order to limit evaporation

and biological activity. Then, the soil was gradually thawed at ambient temperature for bioaugmentation, chemical and microbial community analysis.

3.3.4 Characterization of Contaminated Soils

A characterization study was undertaken to evaluate the soil conditions were evaluated at several sites in Quebec, Canada, along with one site in California. The details of the designation of contaminated soils, their origin, characteristics, type of contaminants and site information are presented below and in Table 3.3.

Côte St-Paul/St-Ambroise **(Soils P-1, P-5, TR-2A, TR4)**

The overburden at the Côte St-Paul/St-Ambroise site was observed to consist of brown gravely sand, dry, with 1% brick and asphalt content.

Sample TR2 (0-0.3 m) indicated concentrations for C_{10}-C_{50} petroleum hydrocarbons, benzo(a)anthracene, benzo(b,j,k) fluoranthene and benzo(a)pyrene within the B-C range of MEF criteria. Benzo(a)anthracene, chrysene, benzo(b,j,k) fluoranthene and benzo(a)pyrene were within the B-C range of the MEF criteria for the composite sample TR1. All seven soils (P-1, P-5, TR-2A, and TR-4) were characterized based on the report generated and analyses conducted at the BRI.

Sept-Iles **(Soil S)**

Soil from the Sept-Iles airport site (Sept-Iles, QC, Canada), on the north shore of the St. Lawrence River, showed a hydrocarbon contamination level of roughly 4.2 g kg^{-1} of dry soil. The contaminant was found to resemble diesel oil. The soil seemed to have undergone a certain alteration with time. It seemed deficient in nutrients, because the addition of nutrients had a temperature dependent effect of diminishing $C_{10} - C_{50}$ hydrocarbons.

Lac-des-Loups **(Soil L)**

The soil on the site of the old Lac-des-Loups airport (QC, Canada) was contaminated by hydrocarbons at levels of 1.2 g kg^{-1}. The contaminant was similar to diesel oil used as a standard in chemical analyses (altered 50%). It seemed to have undergone a chemical and

biological alteration with time. Also, the soil seemed deficient in nutrients, because the addition of nutrients caused a strong decrease in $C_{10} - C_{50}$ hydrocarbons.

***Farnham* (Soil F)**

The microbial analysis of eight soils, sampled at different locations on the Canadian Pacific (CP) Farnham site, revealed that the total heterotrophic microbial populations were quite low in the saturated zone. The population levels were in the range of 10^3 colony forming units (cfu) per gram of wet soil. In the two vadose zone samples the population was significantly higher at 10^5 to 10^6 cfu g^{-1} of wet soil. The purpose of these samples was to verify the soil conditions in general. These soils could already be impacted by hydrocarbons. The other samples were located within the plume and on its edges and showed a range of oil and grease concentrations between 5.0 and 69.0 g kg^{-1}.

***Sawmill* (Soils 14 and 65)**

PAH-PCP-contaminated soils (soil 14 and soil 65) were obtained from a sawmill site located in southwest Quebec, Canada. These sandy soils had been, over a 10-year period (1960-1970), contaminated with PAH (1.7 g kg^{-1}) and PCP (0.5 g kg^{-1}) by an annual fall flushing of a condenser tank into the ground.

***Utility Pole Storage Area* (Soil 16)**

The PAH-PCP-contaminated soil 16 was collected from a utility pole storage area in Pointe-aux-Trembles, QC, Canada. This silty loam soil had been contaminated over a 30-year period with PAH (35-50 mg kg^{-1}) and PCP (80 mg kg^{-1}) by the leaching of PAH-PCP technical-grade solutions from treated wood poles.

***Sablier Thouin* (193)**

Samples of the TPH-PAH-contaminated soil 193 were obtained from a contaminated site Sablière Thouin, Mascouche, QC, Canada. This silty soil ($d_p < 2$ mm) was contaminated with allopathic hydrocarbons (TPH = 40.0 mg kg^{-1}) and polycyclic aromatic hydrocarbons (PAH = 630 mg kg^{-1}). Soil 193 was characterized based on the reports

generated and analyses conducted at the Centre de Recherché BIOPRO, Department de Genie Chimique, Ecole Polytechnique in Montreal, Quebec, Canada.

Port Tlueneme **(Soils 5)**

Diesel fuel and heavy oil (TPH and PAH)-contaminated soil 5 was obtained from a contaminated site located in Port Tlueneme, California, U.S.A, Department of Defence, National Test Site. These sandy soils were contaminated with total petroleum hydrocarbons (TPH) of 420 mg kg^{-1} and no value for polycyclic aromatic hydrocarbons. The initial soil characteristics are listed as: (pH=7.2; EC=1.0 (mmhos cm^{-1}); NO_3^--N = 0.4 (mg kg^{-1}); NH_4^+-N = 1 (mg kg^{-1}); Bray P = 6 (mg kg^{-1}); OM = 2.1 (%); Sand = 59 (%); Silt = 26 (%); Clay = 15 (%).

3.3.5 Culture Media

Mineral salts medium (MSM-agarose, DNA-grade, Bio-Rad; 1.5% w/v: granulated agar/distilled water), and (MSM-broth, DNA-grade, Bio-Rad; and no granulated agar), and final pH=7.0 for both media, YTS_{250} µg L^{-1} - agarose (containing 0.25g each of yeast extract, tryptone, starch in 1.5% w/v: granulated agar/distilled water, pH 7.0), and YTS_{250} µg L^{-1} - broth (containing 0.25 g L^{-1} each of yeast extract, tryptone, starch in distilled water, pH 7.0, and no granulated agar) media, designated MSM_A, MSM_B, YTS_{250A}, YTS_{250B}, respectively, were used in all bacterial isolation experiments. YTS_{1000} µg L^{-1} - agarose (containing 1.0g each of yeast extract, tryptone, starch in 1.5% w/v: granulated agar/distilled water, pH 7.0), and YTS_{1000} µg L^{-1} - broth (containing 1.0g each of yeast extract, tryptone, pH 7.0, and no granulated agar), media, designated YTS_{1000A} and YTS_{1000B}, respectively, and MSM-broth, (DNA-grade, Bio-Rad; and no granulated agar, and final pH = 7.0) medium were used in all mineralization experiments. Quantification of the total and PAHs degrading bacteria were performed by counts of colony forming units (CFU) on appropriate solid media. Total counts were evaluated on MSM_A containing phenanthrene (PAH) as sole carbon source plates (MSM_{A+PAH}), while PAHs degraders were cultured on similar plates containing YTS_{250} also supplemented with phenanthrene (PAH) as sole carbon source ($YTS_{250+PAH}$). All media were sterilized by autoclaving at 121^oC for 20 minutes.

3.3.6 Bacterial Isolation Procedures

Pure cultures were isolated from different contaminated soil samples. Colonies that had clear zones were isolated and purified by transferring three times on MSM_A and YTS_{250A} plates by following procedures:

3.3.6.1 Bacterial Enumeration

The viable bacteria in soil (CFU g^{-1}) were determined by serial dilution and spread-plate techniques. An aliquot of soil (5 g) was aseptically added to pre-weighed sterile test tubes containing 2.5 g of glass beads (3 mm diameter). The tubes were re-weighed and sterile tetrasodium pyrophosphate buffer (0.1% w/v: $Na_4P_2O_7.10H_2O$ in distilled water, pH 7.0) was added at a 3:1 buffer: soil w/w ratio. The mixture was vortexed for several minutes before preparing a 1:10 dilution series in MSM_B. A 0.1 mL aliquot from the dilution series was spread-plated in triplicate onto MSM_A containing phenanthrene (PAH) as sole carbon source plates (MSM_{A+PAH}), and the plates were incubated at ambient temperature (25^oC) for at least one week before counting colonies.

3.3.6.2 Isolation of Phenanthrene-Degrading Bacteria

The phenanthrene-degrading bacteria were isolated from contaminated soil samples by serial dilution and spread-plate techniques using solid MSM_{A+PAH} and $YTS_{250A+PAH}$ media. Phenanthrene was added to the MSM_A and YTS_{250A} plates as 0.5 ml of a 2.5 g L^{-1} phenanthrene solution in acetone. The solution was spread using a sterile glass rod and the solvent was allowed to evaporate overnight before use. Phenanthrene crystallized onto the surface of the agarose and did not interfere with subsequent plate spreading. Positive colonies which produced a clear zone in the phenanthrene precipitate were isolated and subcultured onto $YTS_{250A+PAH}$ plates (Greer *et al.*, 1990 and 1993; Whyte *et al.*, 1999; Chénier *et al.*, 2003).

3.3.6.3 Preparation of Dilution Series

According to the protocols followed at the BRI Environmental Microbiology Lab., several chunks of stored contaminated soil (4^oC) were taken out and a 5 g aliquot was

taken as a good representative of the soil sample. The soil was transferred to a pre-weighed sterile culture tube (25 mm × 150 mm) containing approximately 2.5 g of glass beads (3 mm diameter), and the tube, beads and soil weighed once more to accurately determine the wet weight of the original soil sample. Buffer [tetrasodium pyrophosphate: 0.1% w/v $Na_4P_2O_7.10H_2O$ in distilled water (pH 7.0)] was added to a final 3:1, buffer: soil w/w ratio, thereby giving a 1:4 dilution of the sample. Once the sample was in contact with diluent, it was put on ice, diluted and plated immediately. The dilution and plating of the samples were done in one shot. All dilution tubes were stored on ice during their preparation and subsequently while plates were being spread.

The tubes were vortexed for 2 minutes at high speed and 1 mL immediately transferred to a fresh tube containing 9 mL of sterile dilution solution. Care was taken to ensure that the tube mouths were flamed briefly each time a tube was opened. The tube was vortexed for approximately 15-30 seconds, and then 1 mL of the diluted solution was immediately transferred to a fresh tube that contained 9 ml of sterile dilution solution using a new sterile pipette each time. The above step was repeated after vortexing until a dilution of series of 1:10 was prepared (from the glass beads tubes) from approximately 10^{-1} to 10^{-5} depending on which dilution was plated. To be on the safe side (when plating from a site for the first time), four dilutions were plated (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}).

3.3.6.4 Spread Plate Technique

Petri plates were labelled and contained appropriate nutrient media according to sample number, dilution tube (10^{-1} to 10^{-5}), and date. The dilution tube was vortexed briefly (10-15 seconds) just prior to removing sample to be plated. For triplicate plates with a well-diluted suspension (*i.e.* one cannot see particles in suspension) 0.5 mL of the appropriate dilution was removed and 0.1 mL was carefully applied to the surface of three plates of medium, making sure not to pierce or damage the surface of the agar. The pipette was held as vertical as possible while distributing the 0.1 mL to the plates, to ensure that the three 0.1 ml volumes distributed were as similar as possible.

The plates were later put onto the centre of the turntable one at a time and liquid was spread over the entire surface using a sterile glass spreader, soaked in alcohol (95%), ignited just prior to each spreading, and allowing the flame to extinguish itself. The

spreader was cooled before using it. The glass rod was moved slightly on the surface while the turntable was turning to try to create a uniform covering on the surface of the plate. It was important to use media plates that had been stored at room temperature for 3 days prior to spreading. This ensured a dry surface that would take up the liquid quite rapidly. Spreading was continued until the surface of the plate was dry, indicated by the sticking of the glass rod to the surface. If the plate was not spread until they were dry, the colonies would not be countable as they tend to run together in the liquid remaining on the surface. The glass spreader was re-sterilized between each plating. After all plates were spread in this fashion, they were incubated in an inverted position at 25^{o}C.

Counting of colonies on each plate was performed twice, once after approximately one week of incubation and again after about two weeks of incubation. When counting a permanent ink marker was used to mark a colony to avoid recounting it. At the second counting the slow growing colonies were identified by a different colour. The most statistically significant counts ranged between 30 and 300 colonies. The numbers of colonies were recorded on the plates along with the corresponding dilution.

3.3.6.5 Culture Preservation

The primary aim of culture preservation is to maintain the organism alive, uncontaminated, and without variation or mutation, that is, to preserve the culture in a condition that is as close as possible to the original isolate. Many methods have been used to preserve bacteria, but not all species respond in a similar manner to a given method. In fact, some strains of the same species give variable results with the same procedure. The success or failure of any preservation method also depends on the use of the proper medium and cultivation procedure and on the age of the culture at the time of preservation (Greer *et al.*, 1990 and 1993; Gherna, 1994; Whyte *et al.*, 1999; Chénier *et al.*, 2003).

3.3.6.6 Subculturing

The traditional method of preserving bacterial culture is by periodic serial transfer to fresh medium. The interval between such transfers varies with the organism, the medium

used, and the external conditions. Some bacteria must be transferred every other day, whereas others need to be transferred only after several weeks or months. The major disadvantages of the serial-transfer technique are the risks of contamination, transposition of strain numbers or designations (mislabelling), selection of variants or mutants, and possible loss of culture, as well as the required storage space. Therefore, subculturing is not recommended as a long-term preservation method.

3.3.6.7 Deep Freezing

The deep-freezing procedure involves the freezing of a bacterial suspension in the presence of a cryoprotective agent such as glycerol to prevent cellular damage during the freezing process. Many bacterial species have been successfully preserved for several years using this method. Therefore bacterial species were deep-frozen based on following procedure.

First, YTS_{250B} medium was prepared. Three millilitres of YTS_{250B} medium were poured into each of two sterilized snap plastic tubes (12 mL). A few isolate colonies were taken with a flamed loop and transferred to each of the tubes. The tubes were then incubated in shaking incubator at room temperature (25^oC) for one week. When the colonies were grown, each 3 mL of culture was transferred to two 1.5 mL conical ependorf tubes and centrifuged for five minutes. The supernatant was removed from the tubes and replaced with 375 μL of fresh YTS_{250B} medium, the pellet resuspended with a micropipette to achieve an increased cell concentration. Two concentrated cultures (2×375 μL = 0.75 mL) were then transferred into a 2 mL cylindrical ependorf tube. Then, 0.75 mL of glycerol solution (40%) was added, resulting in a solution with final glycerol concentration of 20%. The tube was tightened, immediately placed in dry ice, and then labelled, dated and stored (at -80^oC).

3.3.6.8 Colony Hybridization and Radioactive Gene Probing Techniques

The phenanthrene-degrading isolates were tested as to whether they were genotypical competent in naphthalene biodegradation, and if so to characterize this capacity at the molecular level. The phenanthrene-degrading isolates were transferred onto nylon membranes and examined with probes for the *ndoB* naphthalene catabolism trait by a

DNA-DNA Southern Hybridization (Figure 3.1). The *ndoB* gene is one of the genes encoding naphthalene dioxygenase from *Pseudomonas putida* (ATCC 17484), which is involved in the biodegradation pathway of naphthalene. Bacterial colonies were lifted onto membranes, the cells were lysed, allowing the bacterial DNA to be denatured and fixed to the membranes according to the standard protocol in BRI (Figure 3.2). Membrane DNA was allowed to hybridize with radioactive gene probes and the number of probe-positive bacteria was determined via autoradiography (Figure 3.3). The results indicate the relative numbers of bacteria possessing the catabolic pathway.

3.3.6.9 Mineralization Experiment

To screen for the biodegradation potential of the 60 bacterial isolates, a standard aerobic microcosm methodology was adopted to target the utilization of radioactive tracer analysis to quantify biodegradation and percent mineralization of selected PAHs compounds. The schematic of a typical serum bottle microcosm set up is presented in (Figure 3.4).

The mineralization assay is regularly used in biodegradation studies to monitor the rate of biodegradation by microorganisms in pure culture or in microcosm analysis. Microcosms are self-contained mini-environments in which biodegradative microorganisms are incubated with a specific ^{14}C-labelled contaminant. There is a KOH-based CO_2 trap within the microcosm. During the process of mineralization (*i.e.* complete breakdown into CO_2 and H_2O), the KOH absorbs the radioactive CO_2. The KOH solution is then analyzed for its level of radioactivity in a scintillation counter machine. Values obtained in disintegrations per minute (dpm) were converted to mg substrate mineralized or could be expressed as a percentage of substrate mineralized over time.

Liquid microcosm studies using 20 mL of the final culture liquid (MSM_B and YTS_{250B}, DNA-grade, Bio-Rad; and no granulated agar, and final pH=7.0) medium were performed to monitor and screen biodegradation activity of the 60 bacterial isolates (microbial populations) in the contaminated medium at ambient temperatures (25^oC). The liquid mediums in microcosms were spiked with ^{14}C-labelled selected PAH substrates and the rate of $^{14}CO_2$ evolution was monitored by liquid scintillation counter machine. The treatments and negative controls (abiotic) were run in triplicate.

Aerobic liquid microcosm studies were performed in 120 mL serum bottles (Greer *et al.*, 1993; Whyte *et al.*, 1999). To inoculate each serum bottles (microcosms), a 1.0 mL aliquot of the culture (OD_{600} of 0.1) with a final cell density of 1.0×10^7 cfu mL^{-1} was added so as to have 20 mL of final culture liquid (MSM_B and YTS_{250B}). All serum bottles were slowly agitated on a shaker (150 rpm) at ambient temperature (25°C). Treatments and heat-killed abiotic controls (negative controls) were run alongside one another, each in triplicate. The abiotic culture controls were prepared by autoclaving the culture at 121°C, twice for 30 minutes at a 24-hour interval. A 5 mL glass test-tube, containing 1 mL of 0.5N KOH and serving as a CO_2 trap was placed inside each serum bottle.

The inoculated MSM_B and YTS_{250B} media were spiked with 100µL ^{14}C-labelled selected PAHs solutions (Sigma-Aldrich, Oakville, Ontario, and Canada; Tables 3.1 and 3.2) using a 100µL glass syringe such that approximately 1.0×10^5 dpm of substrate was present in each serum bottle. Then, the 120 mL serum bottles (microcosms) were tightly sealed with Teflon-coated rubber stoppers and aluminium crimps. Furthermore, air was supplemented by flushing the serum bottle's headspace with 3 mL of air before each sampling; to insure that all of the substrate could be oxidized (*i.e.* mineralized: complete breakdown into CO_2 and H_2O).

Aliquots of the CO_2-bearing 0.5N KOH solution (1 mL) were routinely removed at intervals using a long stainless steel needle attached to a 5 mL syringe. The trapped tube was rinsed with 1 mL of fresh 0.5N KOH solution. This was then removed and added to the first aliquot, resulting in a 2 mL sample. An additional 1 mL of fresh 0.5N KOH solution was added to the trapped tube to capture further CO_2 generated by the mineralization of the ^{14}C-labelled selected PAH substrates.

The 2 mL CO_2-bearing 0.5N KOH aliquots were each mixed with 18 mL of scintillation cocktail (Fisher Scientific, Whitby Ontario, and Canada) and placed in a 20 mL scintillation glass vial and was shaken for 5 seconds. The 20 mL KOH solution was then analyzed for its level of radioactivity in a scintillation counter. The rate of $^{14}CO_2$ evolution was derived from the liquid scintillation counts. Values obtained in disintegrations per minute (dpm) were converted to mg substrate mineralized or could be expressed as the percentage of introduced substrate [^{14}C]PAHs mineralized over time and recovered as [^{14}C]CO_2. This sampling regime (The frequency of sampling: 3 day interval)

continued for period of 14 days, until no further mineralization occurred (*i.e.* mineralization rate reached a plateau).

3.3.6.10 Culture Collection Organization

Of the 60 positive colonies (PAHs-degrading microorganisms), 14 were *ndoB* positive, and one isolate bacterial strain (strain S65) which mineralized selected PAHs were all used to organize a bacterial culture collection. This culture collection was created to maintain a bacterial GenBank for the purpose of selection of the genes involved in PAHs-biodegradation and further research work in the bioremediation area.

3.3.7 Statistical Analysis

Analysis of the cumulative percentage of PAH mineralisation was performed using PROC ANOVA in SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC, USA). The design consisted of a factorial combination of 3 replicates and 6 PAHs combined with 5 repeated measures in time. A threshold of significance of $P \leq 0.05$ was used.

3.4 RESULTS AND DISCUSSION

The results of potentially PAH-degrading microorganisms from 12 different petroleum-contaminated soil types from various sites are presented in (Table 3.3). The 228 phenanthrene-degrading bacteria were isolated from contaminated soil samples by serial dilution and spread-plate techniques using solid MSM_{A+PAH} and $YTS_{250+PAH}$ media, containing phenanthrene (PAH) as a sole carbon source. Out of 228 isolates, 60 positive isolate bacterial strains produced a very clear zone in the phenanthrene precipitate.

3.4.1 Bacterial Identification Procedures

3.4.1.1 Characterization of Bacterial strains

The positive colonies (phenanthrene-degrading microorganisms; Table 3.4) were micromorphologically characterized based on standard methods (Chan *et al.*, 1993). The

further identification studies will characterize the bacterial isolates, in particular, a single PAH-mineralization-competent strain S65, at macro- and micro- morphological, as well as biochemical and genetic levels.

To proceed in the full bacterial identification processes, three different approaches were applied to the characterization of bacterial isolates:

(**i**) phase-contrast microscopy was used to determine the properties of the bacterial isolates at the micromorphological level. Gram stain and optimal growth temperature studies was also carried out;

(**ii**) analysis of the cell wall mycolic acids by high-performance liquid chromatography (HPLC) with UV detection was performed with the original growth on YTS_{1000} as well as on Lowenstein-Jensen agar media at 30°C.

(**iii**) The HPLC pattern obtained for Strain S65 was compared with all the reference profiles currently held for *Mycobacterium sp.* by the Laboratoire de Santé Publique du Quebec-Bacteriologie (LSPQ-Bacteriologie), at the Institut National de Santé Publique du Quebec in Montreal (QC, Canada). At this phase, the HPLC pattern obtained indicated whether the Strain S65 would be a novel, PAH-degrading *Mycobacterium* or not. Furthermore, the HPLC pattern obtained was confirmed by the result of identification procedures of the strain S65, using other's 16S rRNA gene sequence analysis.

3.4.1.2 Gene Probes for Polycyclic Aromatic Hydrocarbons (PAHs)

To do a screening of the bacterial soil population, the positive colonies (phenanthrene-degrading microorganisms) that developed on the solid media were probed with a known catabolic gene probes (*ndoB*). The 14 isolate bacterial strains were selected as *ndoB* probe-positive (Table 3.5). The 14 positive colonies were recognized as phenanthrene- and naphthalene-degrading microorganisms and used in further experiments to quantify their capacity to mineralize pyrene.

3.4.2 Microcosm Study (Mineralization Activity)

Liquid serum bottle microcosm's experiments were performed in order to quantify the mineralization ability of the 14 positive colonies from the bacterial culture collection (Table 3.4). One of the PAH-degrading microorganism strains, isolated from a jet-fuel contaminated site at the Sept-Iles airport (Sept-Iles, Quebec, and Canada), on the north shore of the St. Lawrence River, which is identified as Strain S65, was capable of mineralizing pyrene, phenanthrene, and to a lesser extent fluoranthene, but not naphthalene, anthracene and fluorine, was selected for further characterization.

The Strain S65 utilized Fluoranthene (24% mineralization, Figure 3.5), Phenanthrene (61% mineralization, Figure 3.6), and Pyrene (61% mineralization, Figure 3.7), as sole carbon and energy sources, but no significant degradation of Naphthalene, Anthracene and Fluorene (Figure 3.8). Results of ANOVA are presented in Table 3.6.

Degradation of organic pollutants by microorganisms has been studied for many decades. Over the past few years, an extensive database has been developed on the environmental biodegradation of PAHs by a wide variety of bacteria, fungi and algae. This has resulted in a remarkable understanding of the biochemical pathways and molecular genetics involved in the catabolism of a relatively small number of intensively studied pollutants by a relatively small group of microorganisms. However, bioremediation, which exploit the catabolic versatility of microorganisms to accelerate the degradation of environmental pollutants, is an important industry in alleviating environmental contamination (Labana *et al.*, 2007).

Bioavailability *i.e.,* the ability of a compound to be freely transported across the cell membrane for intercellular or available for extracellular metabolism, may be the most important factor in determining the feasibility of bioremediation of PAHs, In most cases, mass transfer limitations prevent the full exploitation of the microbial derivative potential (Bosma *et al.*, 1997). Limited bioavailability is due to low water solubility and consequently the tendency to partition onto soil mineral surfaces and to sorb strongly to the soil or sediment matrix (Harms and Bosma, 1997). Several mechanisms work together to influence bioavailability, and different mechanisms predominate in any given situation, however, they are still not fully understood. It is usually assumed that the water-dissolved fraction of chemicals is the only one available to microorganisms.

Therefore, degradation rates are dependent on the mass transfer rates of PAHs from solid or soil bound phase to the aqueous phase and desorption of PAHs from soil is considered as the controlling factor in their biodegradation (Volkering *et al.*, 1992; Pignatello and Xing, 1996; Labana *et al.*, 2007).

3.5 CONCLUDING REMARKS

Twelve different petroleum-contaminated soil environments, previously characterized in terms of their relative fractions of available (or potentially available) contaminants suitable as microbial substrates, yielded a number of bacterial strains. Various strains' abilities to biodegrade six polycyclic aromatic hydrocarbons (PAHs), namely Naphthalene (2 fused-benzene rings), Anthracene, Phenanthrene, Fluorene (3 fused-benzene rings), Fluoranthene and Pyrene (4 fused-benzene rings), were investigated.

Applying serial dilution and spread-plate techniques on solid MSM-agarose medium containing phenanthrene (PAH) as sole carbon source, 228 phenanthrene-degrading bacterial strains were isolated. Out of the 228 strains, 60 positive colonies were selected and purified for further characterization. A DNA-DNA (Southern) Hybridization Technique was used to identify 14 colonies, among the 60 isolated, which exhibited a potential for naphthalene-degradation (ndoB Positive). To further screen for the biodegradation capacity of the fourteen isolated bacterial strains, standard aerobic liquid serum bottle microcosms were set up for quantitative radioactive tracer analysis of the biodegradation and cumulative percent mineralization of the six PAHs compounds.

A single PAH-degrading bacterial strain (S65), isolated from a jet-fuel contaminated site at the Sept-Iles airport (Sept-Iles, Quebec, Canada), was competent in mineralizing selected PAH compounds. After 9 days' incubation, Strain S65 showed high cumulative percent mineralization for Pyrene (61%), Phenanthrene (61%) and Fluoranthene (24%), as sole carbon and energy sources, but no significant degradation of Naphthalene, Anthracene or Fluorene.

In view of diminishing the significant amounts of the three compounds (Fluoranthene, Phenanthrene and Pyrene), that were available to strain S65, the data strongly suggest that it might eventually, after further studies, allow prediction of the bioavailability of PAHs in contaminated soils in light of the bacterial mineralization

process. Bioremediation can be viewed as an extension of the metabolism that occurs within an microorganism, when it utilizes the organic pollutant as a sole source of carbon and energy for its growth. However, bioaugmentation with these microorganisms (more specifically strain S65) may provide an innovative and practical decontamination process for PAH-contaminated sites.

Table 3.1: Radioactive Selected PAH Substrates Used in Mineralization Studies

Chemical Name	Chemical Formula	Molecular weight(g)	No. of Fused Benzene Rings	Unlabelled	Labelled (95% Radiochemical Purity)		
				Grade	Solvent	^{14}C Location	Specific Activity (mCi $mmol^{-1}$)
Naphthalene	$C_{10}H_8$	128.2	2	99+% Scintillation Grade	Methanol	[-1-^{14}C]	2-10
Anthracene	$C_{14}H_{10}$	178.2	3	99+% Scintillation Grade	Toluene	[1,2,3,4,4A,9A-^{14}C]	10-30
Phenanthrene	$C_{14}H_{10}$	178.2	3	> 96% HPLC Grade	Methanol Toluene	[-9-^{14}C]	5-15 and 40-60
Fluorene	$C_{13}H_{10}$	166.2	3	99+% HPLC Grade	Methanol Toluene	[-9-^{14}C]	5-15
Fluoranthene	$C_{16}H_{10}$	202.3	4	99+% HPLC Grade	Methanol	[-3-14C]	40-60
Pyrene	$C_{16}H_{10}$	202.3	4	99% Purity for GC	Methanol, Toluene, and Acetone	[-4,5,9,10-^{14}C]	40-60

Source: (Sigma-Aldrich, Oakville, Ontario, and Canada)

Table 3.2: Physical Properties of Selected PAH Substrates Used in Mineralization Studies

Compound	Chemical Formula	Molecular weight (g)	Log K_{ow}	Water Solubility at 25 °C (mg/L)	Melting Point (°C)	Boiling Point (°C)	Vapour Pressure at 25 °C (mPa)
Naphthalene	$C_{10}H_8$	128.2	3.5	31.7	80.5	218	11960
Anthracene	$C_{14}H_{10}$	178.2	4.5	0.045	216	340	25
Phenanthrene	$C_{14}H_{10}$	178.2	4.5	1.29	101	340	90.7
Fluorene	$C_{13}H_{10}$	166.2	4.18	1.98	116.5	295	94.7
Fluoranthene	$C_{16}H_{10}$	202.3	5.1	0.26	111	375	1328
Pyrene	$C_{16}H_{10}$	202.3	4.9	0.135	156	375	91.3×10^6

Source: (Sigma-Aldrich, Oakville, Ontario, and Canada)

Table 3.3: List of Potentially PAHs-Degrading Bacterial Isolates

Number	Contaminated Soil	Number of solate Bacteria	Dilution Factor	CFU g^{-1} soil	Number of Frozen Culture at (¯80 ^{o}C)
1	Soil (5)	6	10^{-4}	4.79×10^{-7}	6
2	Soil (P-1)	7	10^{-4}	2.64×10^{-7}	7
3	Soil (P-5)	7	10^{-4}	2.75×10^{-7}	7
4	Soil (TR-4)	25	10^{-5}	7.43×10^{-8}	25
5	Soil (TR-2A)	17	10^{-4}	2.16×10^{-7}	17
6	Soil (L)	27	10^{-3}	9.61×10^{-6}	27
7	Soil (S)	39	10^{-3}	11.92×10^{-6}	39
8	Soil (F)	23	10^{-3}	11.00×10^{-6}	23
9	Soil (14)	14	10^{-3}	8.01×10^{-6}	14
10	Soil (16)	17	10^{-3}	9.64×10^{-6}	17
11	Soil (65)	19	10^{-3}	9.76×10^{-6}	19
12	Soil (193)	27	10^{-3}	3.41×10^{-6}	27
TOTAL		228			

Table 3.4: Colony Characteristics

- **Colonies are identified on the basis of the soil from which they were isolated.**
- **The medium used for growth of all isolated colonies was YTS_{250A}.**
- **Age of cultures was 7 days.**
- **S.: Slightly**

Soil	Colony	Shape			Colour	Size	Surface Appearance		
		Form	Elevation	Margin		Diameter (mm)	Smooth	Rough	Mucoid
S16	1	Circular	S. Convex	Entire	White	~2.0		Dull	
S16	2	Irregular	S. Convex	Undulate	Yellowish	~1.0		Dull	
S16	3	Irregular	Convex	Undulate	Yellowish	~1.0			Slimy
S16	4	Irregular	S. Convex	Undulate	White	~4.0		Dull	
S16	5	Irregular	S. Convex	Undulate	White	~2.0		Dull	
S5	A4	Circular	Convex	Entire	Yellow	~1.0			Slimy
TR-2A	1	Circular	S. Convex	Entire	White	~2.0			Slimy
TR-2A	2	Circular	S. Convex	Entire	White	~1.0			Slimy
TR-4	1	Circular	S. Convex	Entire	White	~2.0		Dull	
TR-4	2	Circular	Convex	Entire	Yellowish	~2.0		Dull	
TR-4	3	Circular	S. Convex	Entire	White	<1.0	Shiny		
TR-4	4	Circular	S. Convex	Entire	White	<1.0	Shiny		
L	1	Circular	S. Convex	Entire	White	<1.0	Shiny		
F	1	Circular	S. Convex	Entire	Yellow	~1.0			Slimy
F	2	Circular	S. Convex	Entire	Yellow	~1.0			Slimy
F	3	Circular	S. Convex	Entire	White	~2.0			Slimy
F	4	Circular	S. Convex	Entire	Yellowish	~1.0			Slimy
S193	1	Circular	S. Convex	Entire	Yellowish	<1.0			Slimy
S193	2	Circular	S. Convex	Entire	White	<3.0		Dull	
S193	3	Circular	S. Convex	Entire	White	<3.0		Dull	
S193	4	Circular	S. Convex	Entire	White	<3.0		Dull	
S193	5	Circular	S. Convex	Entire	White	<3.0		Dull	
S193	6	Circular	S. Convex	Entire	White	~1.0		Dull	
S193	7	Circular	S. Convex	Entire	Yellowish	<1.0			Slimy
S193	8	Circular	S. Convex	Entire	White	~1.0		Dull	
S193	9	Circular	S. Convex	Entire	Yellow	~1.0			Slimy
S65	1	Circular	S. Convex	Entire	Yellowish	<1.0			Slimy
S65	2	Circular	S. Convex	Entire	Yellowish	<1.0			Slimy

Table 3.4: (contd.): Colony Characteristics

Soil	Colony	Shape			Colour	Size	Surface Appearance		
		Form	Elevation	Margin		Diameter (mm)	Smooth	Rough	Mucoid
S65	3	Circular	S. Convex	Entire	Yellowish	<1.0			Slimy
S65	4	Circular	S. Convex	Entire	Yellowish	<1.0			Slimy
S65	5	Circular	S. Convex	Entire	Yellow	<1.0			Slimy
S65	6	Circular	S. Convex	Entire	Yellow	<1.0			Slimy
S14	1	Circular	S. Convex	Entire	White	<1.0		Dull	
S14	2	Circular	S. Convex	Entire	White	<1.0		Dull	
S14	3	Circular	S. Convex	Entire	White	<1.0		Dull	
S14	4	Circular	S. Convex	Entire	Yellow	<1.0			Slimy
S14	5	Circular	S. Convex	Entire	Yellow	~1.0			Slimy
S14	6	Circular	S. Convex	Entire	Yellow	~1.0		Dull	
S	1	Circular	S. Convex	Entire	Yellow	~1.0		Dull	
S	2	Circular	S. Convex	Entire	Yellow	~1.0		Dull	
S	3	Circular	S. Convex	Entire	Yellow	~1.0	~1.0	Dull	
S	4	Circular	S. Convex	Entire	Yellow	~1.0		Dull	
S	5	Irregular	S. Convex	Undulate	White	~3.0		Dull	
S	6	Irregular	S. Convex	Undulate	White	~3.0		Dull	
S	7	Circular	S. Convex	Entire	Yellow	<2.0		Dull	
S	8	Circular	S. Convex	Entire	Yellowish	<3.0		Dull	
S	9	Irregular	S. Convex	Undulate	White	<3.0		Dull	
S	10	Circular	S. Convex	Entire	Yellow	<2.0		Dull	
S	11	Circular	S. Convex	Entire	Yellow	<2.0		Dull	
S	12	Circular	S. Convex	Entire	Yellow	<2.0		Dull	
S	13	Circular	S. Convex	Entire	Yellow	<2.0		Dull	
S	14	Circular	S. Convex	Entire	Yellowish	~3.0		Dull	
S	15	Circular	S. Convex	Entire	Yellowish	~3.0		Dull	
S	16	Circular	S. Convex	Entire	Yellow	<2.0		Dull	
S	17	Circular	S. Convex	Entire	Yellowish	~3.0		Dull	
S	18	Circular	S. Convex	Entire	Yellowish	~3.0		Dull	
S	19	Circular	S. Convex	Entire	Yellowish	~3.0		Dull	
S	20	Circular	S. Convex	Entire	Yellow	<2.0		Dull	
S	21	Circular	S. Convex	Entire	Yellow	<2.0		Dull	
S	22	Irregular	S. Convex	Undulate	White	~3.0		Dull	

Table 3.5: Degradation Potential of Isolated Bacteria

(a) Colonies are identified on the basis of the soil from which they were isolated.
(b) The medium used for growth of all isolated colonies was YTS_{250A}.
(c) Age of cultures was 7 days.

Soil	Colony	% Mineralization				Phenanthrene	ndoB
		Pyrene	Phenanthrene	Fluoranthene	Naphthalene	Clearance	
S16	1	(-)	(-)	(-)	(-)	(+)	(-)
S16	2	(-)	(-)	(-)	(-)	(+)	(-)
S16	3	(-)	(-)	(-)	(-)	(+)	(-)
S16	4	(-)	(-)	(-)	(-)	(+)	Strong (+)
S16	5	(-)	(-)	(-)	(-)	(+)	Strong (+)
S5	A4	(-)	(-)	(-)	(-)	(+)	(-)
TR-2A	1	(-)	(-)	(-)	(-)	(+)	(-)
TR-2A	2	(-)	(-)	(-)	(-)	(+)	(-)
TR-4	1	(-)	(-)	(-)	(-)	(+)	(-)
TR-4	2	(-)	(-)	(-)	(-)	(+)	(-)
TR-4	3	(-)	(-)	(-)	(-)	(+)	(-)
TR-4	4	(-)	(-)	(-)	(-)	(+)	(-)
L	1	(-)	(-)	(-)	(-)	(+)	(-)
F	1	(-)	(-)	(-)	(-)	(+)	(-)
F	2	(-)	(-)	(-)	(-)	(+)	(-)
F	3	(-)	(-)	(-)	(-)	(+)	(-)
F	4	(-)	(-)	(-)	(-)	(+)	Strong (+)
S193	1	(-)	(-)	(-)	(-)	(+)	(-)
S193	2	(-)	(-)	(-)	(-)	(+)	Strong (+)
S193	3	(-)	(-)	(-)	(-)	(+)	Strong (+)
S193	4	(-)	(-)	(-)	(-)	(+)	Strong (+)
S193	5	(-)	(-)	(-)	(-)	(+)	Strong (+)
S193	6	(-)	(-)	(-)	(-)	(+)	(-)
S193	7	(-)	(-)	(-)	(-)	(+)	(-)
S193	8	(-)	(-)	(-)	(-)	(+)	(-)
S193	9	(-)	(-)	(-)	(-)	(+)	(-)
S65	1	(-)	(-)	(-)	(-)	(+)	(-)
S65*	2	Strong (+)	Strong (+)	Strong (+)	Weak (+)	(+)	Strong (+)

Table 3.5 (contd.): Degradation Potential of Isolated Bacteria

Soil	Colony	% Mineralization				Phenanthrene Clearance	ndoB
		Pyrene	Phenanthrene	Fluoranthene	Naphthalene		
S65	3	(-)	(-)	(-)	(-)	(+)	(-)
S65	4	(-)	(-)	(-)	(-)	(+)	(-)
S65	5	(-)	(-)	(-)	(-)	(+)	(-)
S65	6	(-)	(-)	(-)	(-)	(+)	(-)
S14	1	(-)	(-)	(-)	(-)	(+)	(-)
S14	2	(-)	(-)	(-)	(-)	(+)	(-)
S14	3	(-)	(-)	(-)	(-)	(+)	(-)
S14	4	(-)	(-)	(-)	(-)	(+)	(-)
S14	5	(-)	(-)	(-)	(-)	(+)	(-)
S14	6	(-)	(-)	(-)	(-)	(+)	(-)
S	1	(-)	(-)	(-)	(-)	(+)	(-)
S	2	(-)	(-)	(-)	(-)	(+)	(-)
S	3	(-)	(-)	(-)	(-)	(+)	Strong (+)
S	4	(-)	(-)	(-)	(-)	(+)	(-)
S	5	(-)	(-)	(-)	(-)	(+)	Strong (+)
S	6	(-)	(-)	(-)	(-)	(+)	Strong (+)
S	7	(-)	(-)	(-)	(-)	(+)	(-)
S	8	(-)	(-)	(-)	(-)	(+)	(-)
S	9	(-)	(-)	(-)	(-)	(+)	(-)
S	10	(-)	(-)	(-)	(-)	(+)	(-)
S	11	(-)	(-)	(-)	(-)	(+)	(-)
S	12	(-)	(-)	(-)	(-)	(+)	(-)
S	13	(-)	(-)	(-)	(-)	(+)	(-)
S	14	(-)	(-)	(-)	(-)	(+)	(-)
S	15	(-)	(-)	(-)	(-)	(+)	(-)
S	16	(-)	(-)	(-)	(-)	(+)	Weak (+)
S	17	(-)	(-)	(-)	(-)	(+)	(-)
S	18	(-)	(-)	(-)	(-)	(+)	(-)
S	19	(-)	(-)	(-)	(-)	(+)	Strong (+)
S	20	(-)	(-)	(-)	(-)	(+)	(-)
S	21	(-)	(-)	(-)	(-)	(+)	(-)
S	22	(-)	(-)	(-)	(-)	(+)	Strong (+)

*** Strain S65 (2)** is a ***Mycobacterium*** **sp**.

Table 3.6. Significance of Single Factor Effects and Interactions on the Mineralization of Selected PAHs in Broth YTS_{250B}.

Sources	Probability
R	NS
C	**
IT	**
C*IT	**
R*C	**
R*IT	NS

R: Replicate; **C**: Chemicals; **IT**: Incubation time.

NS: Non-Significant**,** ($P > 0.05$); *, Significant ($P \leq 0.05$); **, Highly Significant ($P \leq 0.01$)

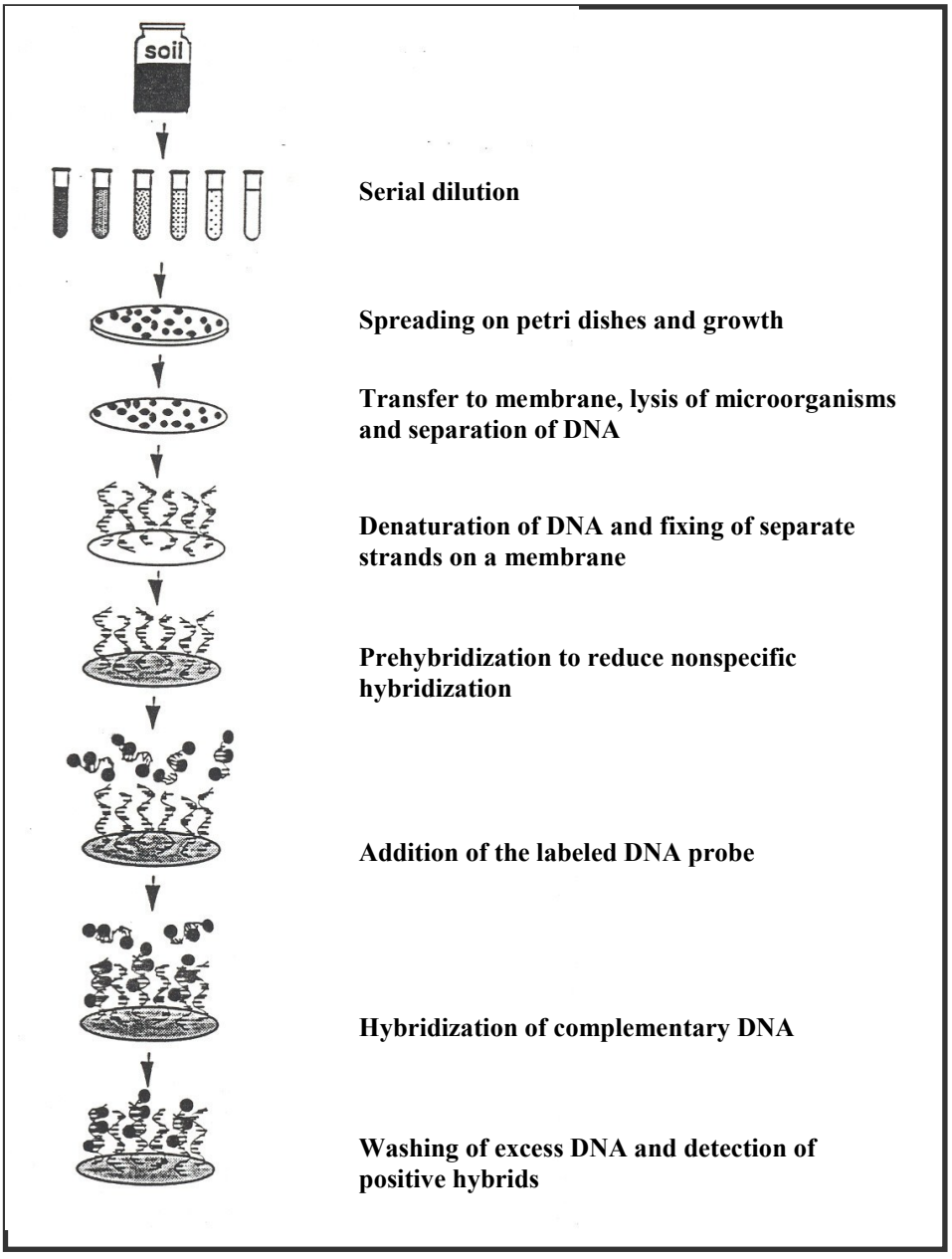

Figure 3.1: Procedure for Genetically Examining PCP/PAH Contaminated Soil for Potential Degrader Bacteria by a DNA-DNA (Southern) Hybridization Technique.

SOURCE: National Research Council of Canada's Biotechnology Research Institute (NRC-BRI), 2000

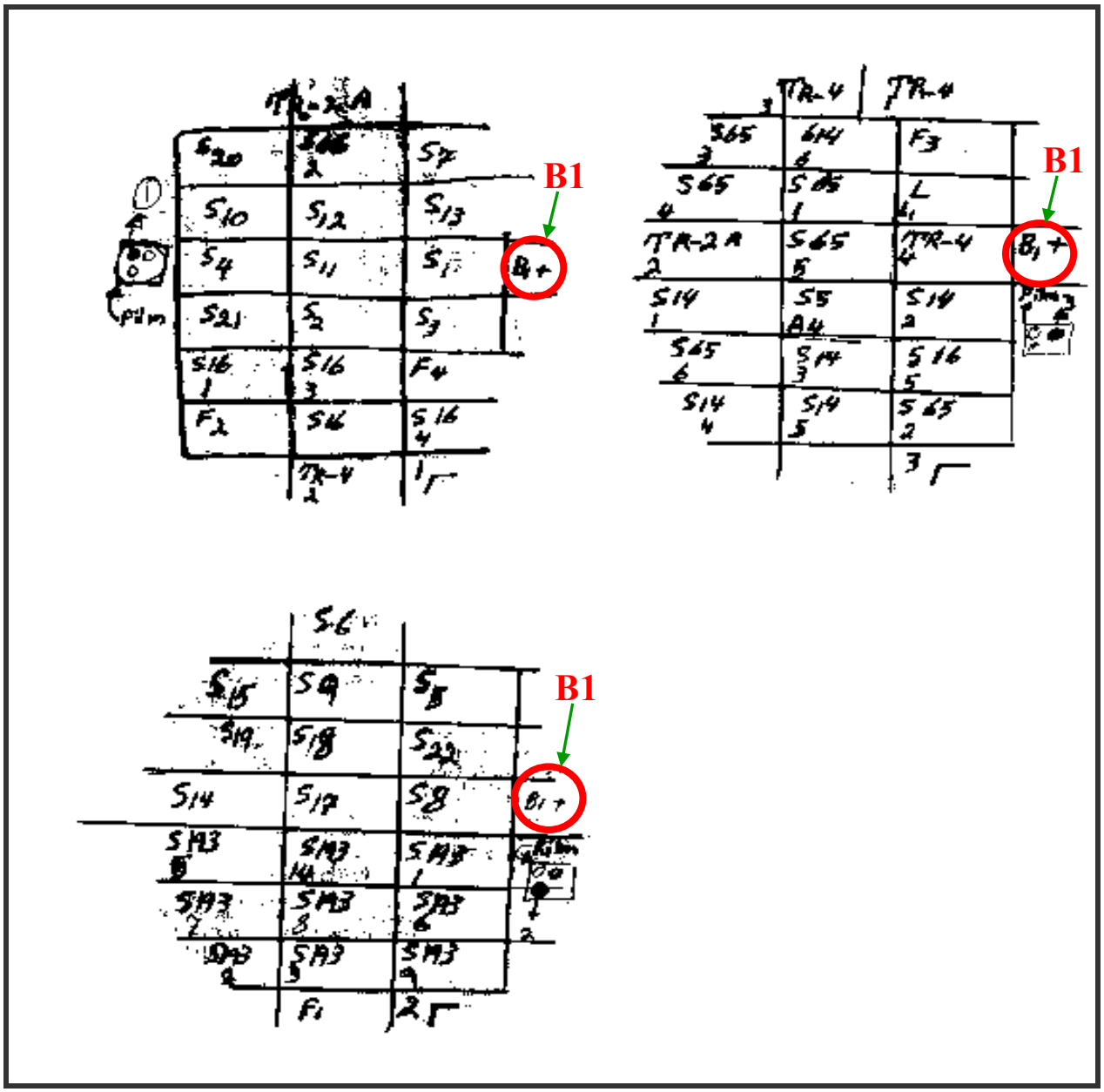

Figure 3.2: Transferring Strains on Nylon Membrane- B1 is positive control.

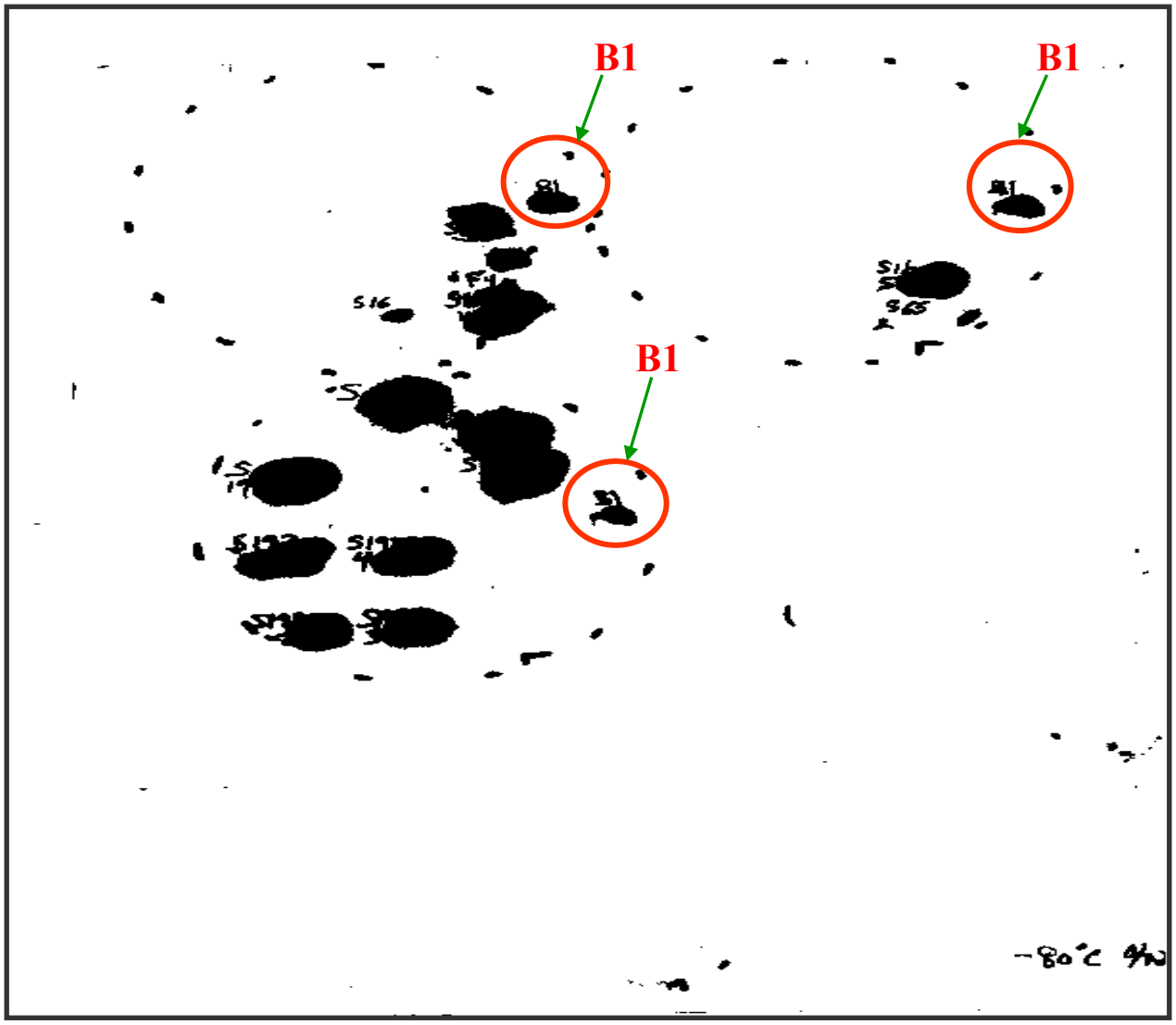

Figure 3.3: Positive Colonies on Film Membrane - B1 is positive control.

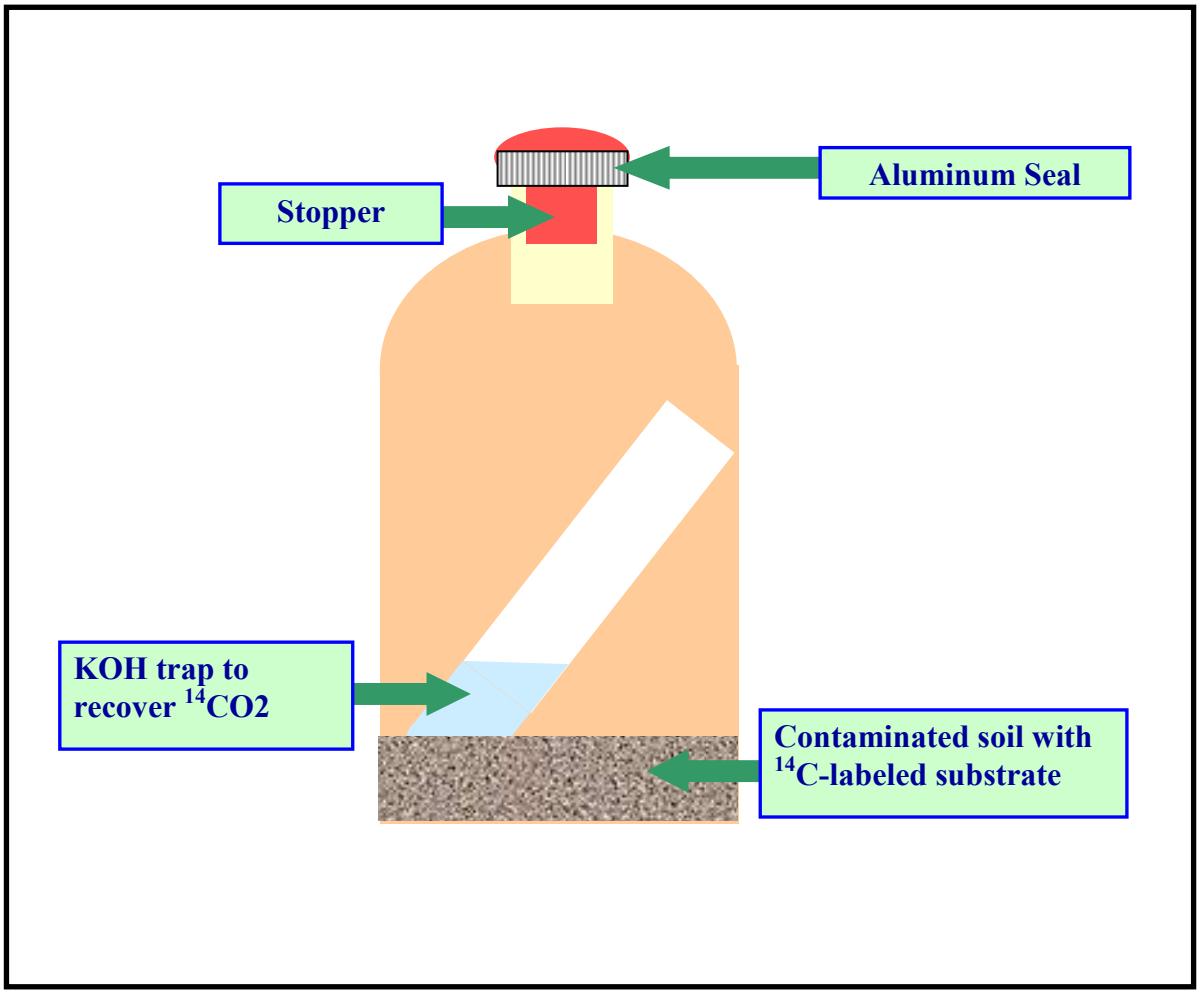

Figure 3.4: Schematic of a Microcosm Serum Bottle

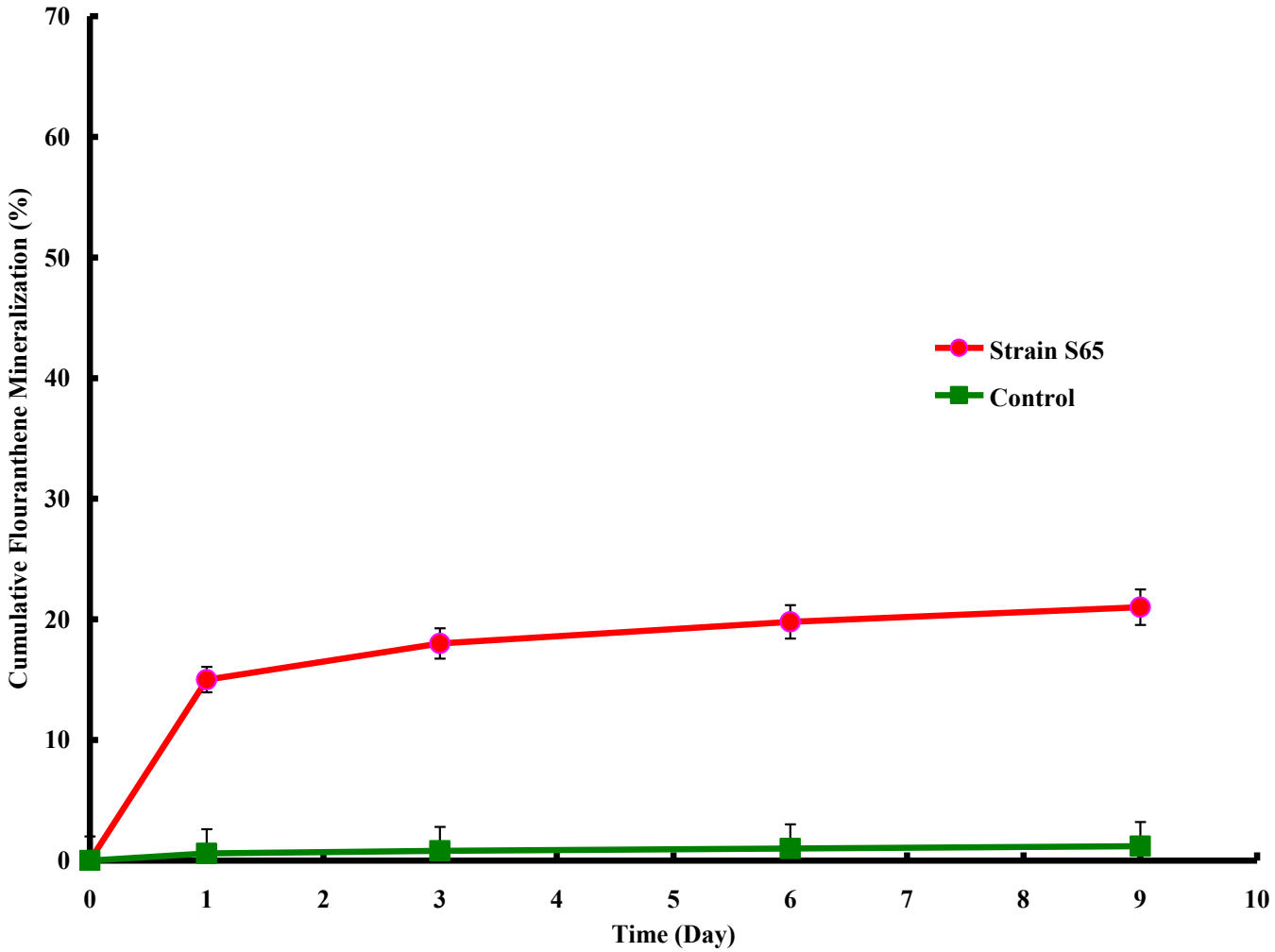

Figure 3.5: Mineralization of Fluoranthene by Isolate Bacterial Strain S65 in YTS_{250B}
Control: autoclaved bacteria at 121^0C
Data points are the average of triplicate samples.
Error bars represent one standard deviation.

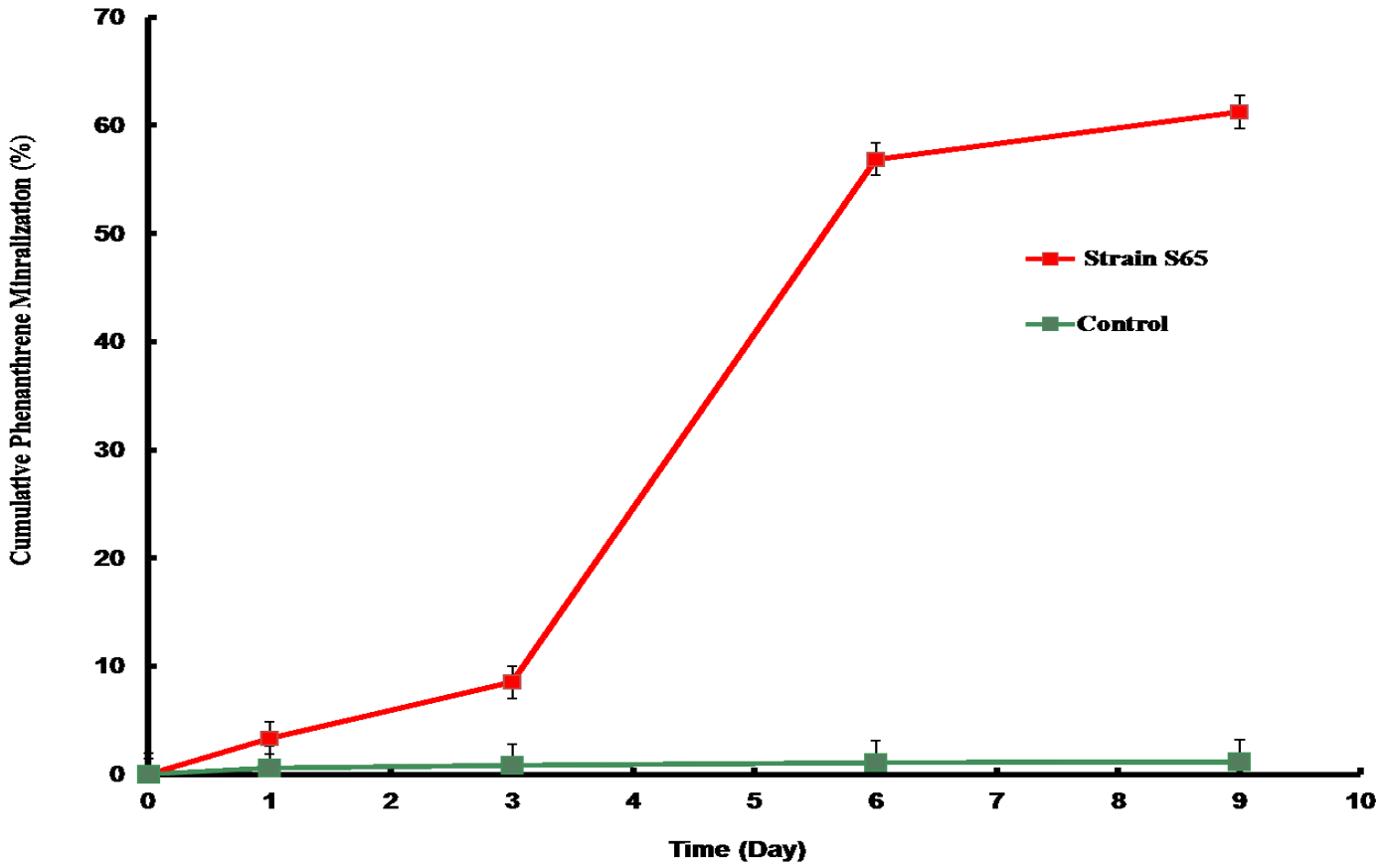

Figure 3.6: Mineralization of Phenanthrene by Isolate Bacterial Strain S65 in YTS_{250B}
Control: autoclaved bacteria at 121^0C
Data points are the average of triplicate samples.
Error bars represent one standard deviation.

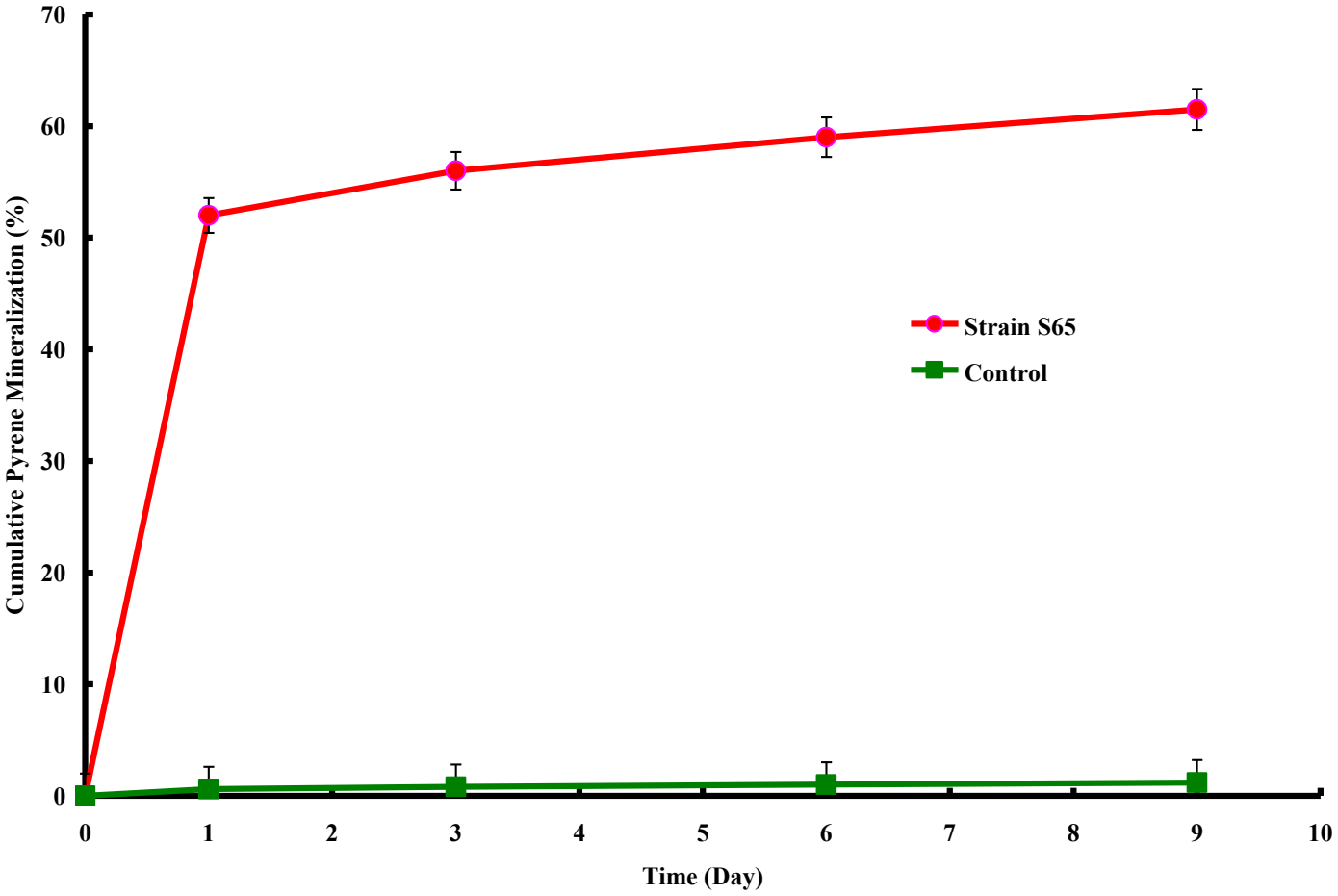

Figure 3.7: Mineralization of Pyrene by Isolate bacterial Strain S65 in YTS_{250B}
Control: autoclaved bacteria at 121^0C.
Data points are the average of triplicate samples.
Error bars represent one standard deviation.

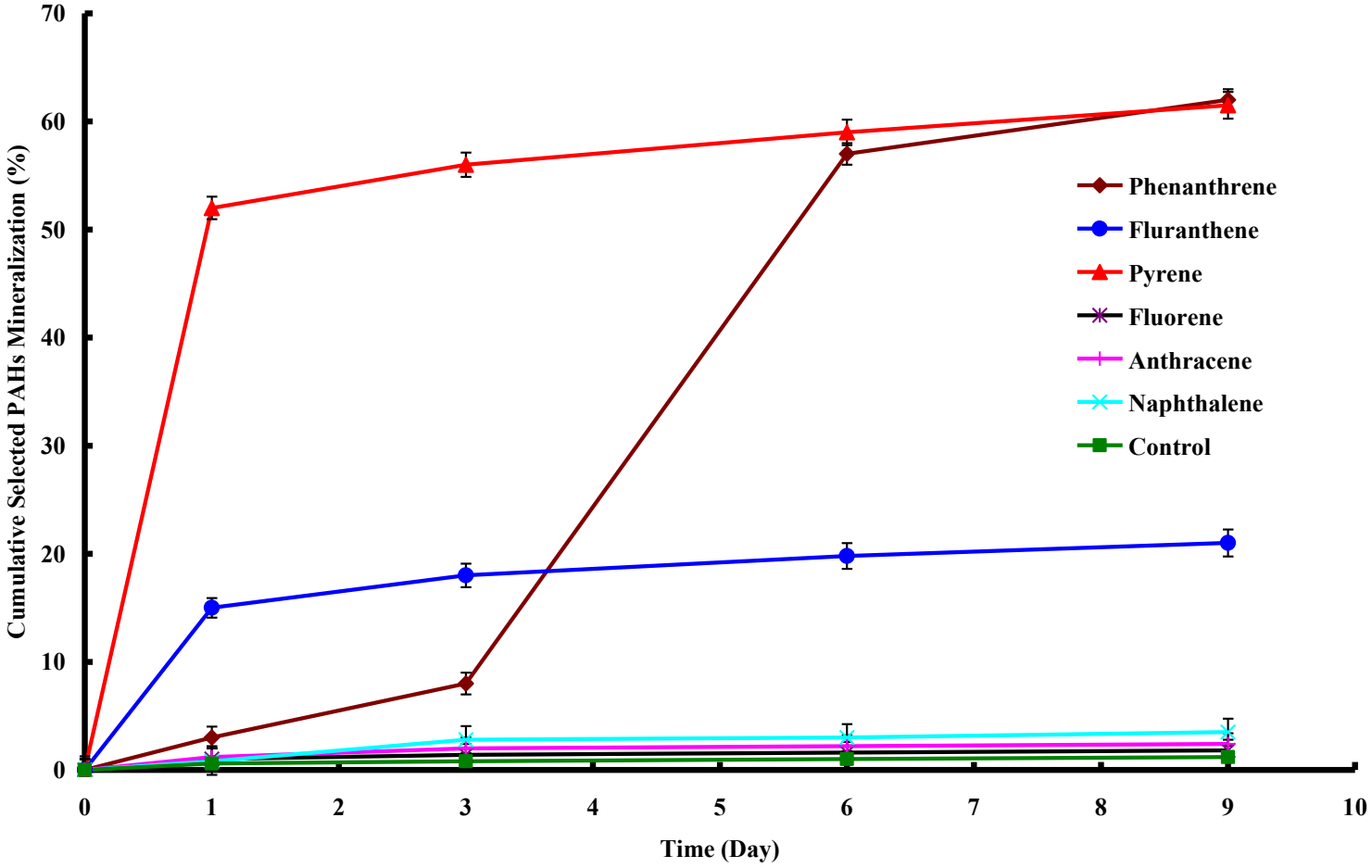

Figure 3.8: Mineralization of Selected PAHs by Isolate bacterial Strain S65 in YTS_{250B}
Control: autoclaved bacteria at 121^0C.
Data points are the average of triplicate samples.
Error bars represent one standard deviation.

PREFACE TO CHAPTER 4

In Chapter 3, twelve contaminated soils were collected from different contaminated sites. The soil sampling was done from the surface to a depth of about 0.3 m. Each sample consisted of a composite of five sub-samples from an area 3 m × 3 m and was immediately transferred to the laboratory and stored at 4°C. Then, the soil was gradually thawed at ambient temperature for bioaugmentation, chemical and microbial community analysis. Furthermore, the following assays were conducted: culture media, bacterial isolation procedures, bacterial enumeration, isolation of phenanthrene-degrading bacteria, preparation of dilution series, spread plate technique, culture preservation, sub-culturing, deep-freezing, colony hybridization and radioactive gene probing techniques, mineralization experiment, and culture collection organization. In view of the diminishing amounts of the three compounds (Fluoranthene, Phenanthrene and Pyrene), that were available to strain S65, the data suggest that it might eventually, after further studies, allow prediction of the bioavailability of PAHs in contaminated soils in light of the bacterial mineralization process. Therefore, bioaugmentation with these organisms may provide an innovative and practical decontamination process for PAHs-contaminated sites.

In Chapter 4, the overall goal was to characterize bacterial isolates obtained as described in Chapter 3, in particular, a single PAH-mineralization-competent strain S65, at macro- and micro- morphological, as well as biochemical and genetic levels.

This manuscript has been submitted for publication.

Jazestani, J. and S. O. Prasher, 2011. Characterization of Recalcitrant PAHs-degrading Bacterial Isolates from Soils Contaminated with a Range of Organic Pollutants. (submitted for publication in ***World Journal of Microbiology***).

CHAPTER 4

Characterization of Recalcitrant PAHs-degrading Bacterial Isolates from Soils Contaminated with a Range of Organic Pollutants

4.1 ABSTRACT

Bacterial strains isolated from soils contaminated with a large range of organic pollutants, including the wood treatment chemicals pentachlorophenol (PCP) and creosote-containing oils and polycyclic aromatic hydrocarbons (PAHs), were tested for their ability to breakdown selected recalcitrant PAHs — compounds with minimal water solubility and low bioavailability in soils — as a sole source of carbon and energy in MSM-broth and YTS-broth, respectively.

Bacterial strains were characterized at the micromorphological and molecular-genetic level. Molecular biology and biotechnology techniques were used to optimize their natural biodegradative capacities. A culture collection was created to maintain a bacterial GenBank for the purpose of selection of the genes involved in PAHs-biodegradation and for further research work in the bioavailability and bioremediation areas. All bacterial species were preserved at $^{-}80^{\circ}C$ in a culture collection stored at the Environmental Microbiology Laboratory, Biotechnology Research Institute (BRI), National Research Council of Canada, Montreal, Quebec, Canada.

Isolated and partially characterized from a jet-fuel contaminated site at the Sept-Iles airport (Sept-Iles, QC, Canada), bacterial strain "S65" (**GenBank designation: *Mycobacterium* sp. Strain S65**), was able to mineralize selected recalcitrant PAHs-compounds. In aerobic liquid microcosms Strain S65 rapidly consumed, as its sole carbon and energy sources, Pyrene (61% cumulative mineralization), Phenanthrene (61% cumulative mineralization), and to a lesser extent Fluoranthene (24% cumulative mineralization), but was not able to significantly degrade Naphthalene, Anthracene or Fluorene. Strain S65's mineralization kinetics showed this *Mycobacterium* to be a new and unique member of the xenobiotic-degrading mycobacteria.

Key Words: Bioavailability, Biodegradation, Characterization, Isolation, Mineralization, *Mycobacterium* sp., PAHs.

4.2 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread contaminants that are of environmental concern due to their carcinogenic and mutagenic properties (Uyttebroek *et al.*, 2007). PAHs are components of creosote and are mainly produced as a result of incomplete combustion of organic material such as fossil fuels and during petroleum refining, coke production and wood preservation. PAHs are ubiquitous organic pollutants in nature. As suspected carcinogens these ubiquitous organic pollutants have been the subjects of numerous scientific studies, and their biodegradation has been the subject of several reviews (Alexander, 1973; Bjorseth, 1983; Al-Bashir 1991; Alexander, 1999; Juhasz and Naidu, 2000; Singh and Ward, 2004; Boehm, 2006). A general form of PAHs is $C_{4n+2}H_{2n+4}$, where n is the number of rings. As the number of rings increases, the compounds become more difficult to degrade owing to their decreasing volatility, solubility and increased sorption (thus greater association with non-aqueous phases, renders it more difficult to degrade). They are also degraded in a manner similar to single-ring aromatics since PAHs are degraded one ring at a time. Nevertheless, simple aromatics must be present to induce the enzymes necessary for PAH degradation. If they are not, limited biodegradation of the PAHs up to five rings may take place. However, understanding dissolution and sorption characteristics are necessary to study the natural attenuation of PAHs (Yong and Mulligan, 2004).

Biologically-mediated removal of PAHs from the environment tends to be incomplete, even when aggressive bioremediation approaches are used (USEPA, 2000). This seems inconsistent with the fact that, under controlled laboratory conditions, virtually all PAHs have been found to be biodegradable by organisms that are essentially ubiquitous in the environment. Thus, in any given contaminated system, it is unclear whether the extent of degradation of the most recalcitrant compounds is limited by PAH or simple aromatic compounds' bioavailability, or what variables must be altered to enhance PAH degradation. Clearly, such questions must be addressed if one is to improve the bioremediation of PAH-contaminated system**s** (Singh and Ward, 2004; Yong and Mulligan, 2004).

Were one to isolate a naturally-occurring microorganism capable of PAH degradation, its taxonomic identity would be of prime interest. The task of identifying microbial isolates was formerly undertaken mainly through comparison of an accurate morphological and phenotypical description of the isolate to similar descriptions of type strains. Traditional identification keys, such as those in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) arrange bacteria into groups based upon phenotypic characteristics (*i.e.* cell and colony morphology, Gram stain reaction, oxygen requirements, ability to metabolise different carbon sources), assuming that microbes which share many characteristics are more closely related than those that share few. However, species that are distantly related may exhibit similar phenotypes simply because they have adapted to similar environments or through prokaryotes' unique ability to exchange genetic material. As a result, perfect matches are rare and a judgment must often be made regarding a most probable rather than an absolute identification.

Recognizing these shortcomings, microbiologists sought to develop a classification scheme that accurately represents the manner in which microbes may have evolved. The underlying principle of this approach was that nucleotide sequences were expected to be more similar in species that were most closely related and increasingly divergent in species that were more distantly related. Ribosomal RNA genes were selected as the basis for comparison because they are present in all cellular organisms, are moderately sized, and contain highly conserved regions as well as variable regions. In the 1980s, Woese *et al.* (1985) and Woese (1987) showed that the phylogenetic relationships of bacteria could be determined by comparing the genes coding for the 5S, 16S and 23S ribosomal subunits and the areas between them. The portion of bacterial DNA now most commonly used for taxonomic purposes is the 16S rRNA gene (Bottger 1989; Woese *et al.*, 1990; Kolbert and Persing, 1999; Garrity and Holt, 2001; Tortoli, 2003; Harmsen and Karch, 2004). A comparison of 16S rRNA gene sequences allows differentiation between microorganisms at the genus level across all major phyla of bacteria, in addition to classifying strains at the species and subspecies-level.

Among the soil microorganisms known to be capable of breaking down PAHs are the *mycobacteria* (Holt *et al.*, 1994). The *mycobacteria*, the group often treated as the family *Mycobacteriaceae*, contains a single genus, *Mycobacterium*. These are slender,

straight or slightly curved, rod-shaped bacteria (0.2-0.7 × 1.0-10 μm), which can undergo filamentous or mycelium-like growth (Holt *et al.*, 1994). They are characteristically acid-fast, usually weakly Gram-positive, aerobic, non-motile, non-sporing, slow-growing, living free in soil and water or as pathogens of vertebrates. Colonies are often pink, orange, or yellow, especially when exposed to light. They are generally catalase positive, arylsulfatase positive, and lysozyme resistant. Given their generally slow growth rates and often highly-specific nutrient requirements, mycobacteria require specialized methods of study.

The *Mycobacteriaceae* have traditionally been considered as a group apart, but there is considerable evidence that they are closely allied to the genera *Corynebacterium* and *Nocardia.* The three genera are sometimes referred to as the CNM group, to which the genus *Rhodococcus* should be added. Species of *Mycobacterium* may be confused with other related genera. The genera most easily confused with *Mycobacterium* are shown by Bergey (1994) (Table 4.1). These genera differ from *Mycobacterium* in cell wall type and phospholipids. The property of acid-fastness, due to waxy materials in the cell walls, is particularly important for recognizing mycobacteria. These bacteria are commonly described as acid-alcohol-fast, implying that after staining they resist decolourization with acidified alcohol as well as with strong mineral acids. Some other bacteria, however, are partially acid-fast, and may be readily decolourized by alcohol although they may resist decolourization by weak acid. Artefacts may occur that retain the stain if the technique is not carefully performed. It should be noted that spores may be acid-fast, and occasionally waxy substances in tissues or other materials may cause confusion, and the degree of acid-fastness varies with technique and cultural condition (Bergey, 1994). While the more rapidly growing *Mycobacterium* species (visible colonies within 7 days at optimum temperature on nutritionally rich media) can be identified reasonably easily, slower growing species (> 7 days to form visible colonies) often form complexes of very similar species, rendering them difficult to distinguish (Sneath *et al.*, 1986).

HPLC is probably the single most used analytical technique today, surpassing even gas chromatography for the separation and analysis of mixtures. This is mainly due to the great versatility of the technique, arising from the fact that both stationary phase

and mobile phase interactions may be used to alter the system's selectivity. The art of eluent additive chromatography, whereby a small amount of an ionic or complexing species may be added to the eluent to interact specifically with certain types of solute, has been developed to the extent that is now routine (Gilbert, 1987). HPLC separations are now a fundamental analytical tool for the vast majority of testing laboratories. Over the last few years HPLC has expanded in two important ways. First, it becomes even more prevalent in the pharmaceutical, nutraceutical (natural products), and protein/peptide characterization areas. Second, along with this, mass spectrometry has become more widespread as a detector of choice (Sadek, 2002). HPLC of mycolic acid esters has been demonstrated to be a rapid and reliable method for identification of many *Mycobacterium* species. Mycolic acids are high molecular weight (20 to 90 carbon atoms) α-substituted, β-hydroxy fatty acids found in the cell wall of several bacterial genera: *Corynebacterium, Rhodococcus, Gordonia, Skermania, Dietzia, Nocardia, Tsukamurella*, and *Mycobacterium*. The genus *Mycobacterium* can usually be easily distinguished from the other nocardioform Actinomycetes genera because its mycolates are more complex and of higher molecular weight (LSPQ-Bacteriologie, 2003).

The 16S rRNA gene sequencing can be routinely used for identification of the generally slow-growing and difficult-to-identify mycobacteria. Consequently, they were an important group of organisms in early studies establishing the usefulness of 16S rRNA gene sequencing for clinical and environmental microbiology (Boddinghaus *et al.*, 1990; Kirschner *et al.*, 1993). Recent studies comparing the identification of mycobacteria by 16S rRNA gene sequencing or phenotypic methods confirmed the greater accuracy of 16S rRNA gene sequencing in identification of mycobacteria to the species level (Cloud *et al.*, 2002; Cook *et al.*, 2003). Thus, for the identification of novel mycobacterial species, 16S rRNA gene sequence analysis is the most accurate method available. The 16S rRNA gene sequence has been determined for a large number of mycobacterial strains. GenBank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene. This means that there are many previously deposited sequences against which to compare the sequence of an unknown bacterium.

The current project's overall goal was to isolate bacterial strains capable of biodegrading one or more of six selected recalcitrant PAHs — compounds with minimal water solubility and low bioavailability in soils — from contaminated soil sites. The specific objective was to characterize these bacterial isolates, in particular, PAH-mineralization-competent strain S65, at macro- and micro- morphological, as well as biochemical and genetic levels.

4.3 MATERIALS AND METHODS

4.3.1 Bacteria and Contaminated Soils

The twelve contaminated soils were collected from different wood-preserving plants, oil refineries, and various petroleum hydrocarbons-contaminated sites. The soil sampling was done from the surface to a depth of about 0.3 m. Each sample consisted of a composite of five sub samples from an area 3 m × 3 m and was immediately transferred to the laboratory and stored at 4°C. Then, the soil was gradually thawed at ambient temperature for bioaugmentation, chemical and microbial community analysis. The details of contaminated soils, their origin, characteristics, type of contaminants and site information were reported by Jazestani and Prasher (2003b). Bacterial strains were isolated from the above-mentioned 12 petroleum-contaminated soil environments, previously characterized in terms of their relative fractions of available (or potentially available) contaminants suitable as microbial substrates. The various strains' abilities to biodegrade selected recalcitrant PAHs-compounds, namely Naphthalene (2 fused-benzene rings), Anthracene, Phenanthrene, Fluorene (3 fused-benzene rings), Fluoranthene and Pyrene (4 fused-benzene rings), was investigated (Jazestani and Prasher, 2003b).

4.3.2 Chemicals

For preparation of YTS_{250A}, YTS_{250B}, YTS_{1000A}, YTS_{1000B}, MSM_A, and MSM_B media, and other assays, chemicals such as Yeast Extract, Tryptone, Starch, Granulated Agar, Ultra-Pure Agarose, Glycerol, KOH, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Co(NO_2)_2.6H_2O$, $AlK(SO_4)_2.12H_2O$, $CuSO_4$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $FeSO_4.7H_2O$,

Na_2MoO_4. 2 H_2O, $Ca(NO_3)_2$ .4 H_2O, H_2 SO_4, toluene ($C_6H_5CH_3$), acetone (CH_3COCH_3), hexane (C_6H_{14}), HPLC grade methanol (CH_3OH), and ethanol (C_2H_5OH) were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada), and Sigma-Aldrich Chemical Company (Oakville, Ontario, Canada). All chemicals and solvents were of ACS Reagent grade or better and were used as received.

4.3.3 Culture Media

Culture media are the different substances that satisfy the nutritional needs of microorganisms. The following media were made and used in various assays in this research work. Mineral Salts Medium (MSM-agarose, DNA-grade, Bio-Rad; 1.5% w/v: agarose /distilled water), and MSM-broth **(**DNA-grade, Bio-Rad; and no agarose), and final pH=7.0, for both media, YTS_{250} µg L^{-1} – granulated agar (0.25 g each of yeast extract, tryptone, starch in 1.5% w/v: granulated agar/distilled water, and pH 7.0), and YTS_{250} µg L^{-1} - broth (containing 0.25 g L^{-1} each of yeast extract, tryptone, starch in distilled water, pH=7.0) media, designated MSM_A, MSM_B, YTS_{250A}, YTS_{250B}, respectively, were used in all bacterial isolation experiments (Greer *et al.*, 1990). YTS_{1000A} µg L^{-1} - granulated agar (containing 1.0g each of yeast extract, tryptone, starch in 1.5% w/v: granulated agar/distilled water, and pH 7.0), and YTS_{1000} µg L^{-1} - broth (containing 1.0g each of yeast extract, tryptone, and pH 7.0) media, designated YTS_{1000A} and YTS_{1000B}, respectively, and MSM-broth, medium were used in all mineralization experiments (Greer *et al.*, 1993).

Quantification of the total and PAHs-degrading bacteria was performed by counts of colony forming units (CFU) on appropriate solid media. Total counts were evaluated on MSM_A containing phenanthrene (PAH) as sole carbon source (MSM_{A+PAH}), while PAHs degraders were cultured on similar plates containing YTS_{250} also supplemented with phenanthrene (PAH) as sole carbon source ($YTS_{250+PAH}$). Lowenstein-Jensen agar medium (LSPQ-Bacteriologie) was used for analysis of the cell wall mycolic acids by HPLC with UV detection. All media were sterilized by autoclaving at 121^oC for 20 minutes.

4.3.4 Pure and Mixed Cultures

To determine the characteristics of a microorganism, it should first be cultivated in pure culture, such that all cells in the population are identical in the sense that they have arisen from the same parent cell. Microbiologically speaking, microorganisms in nature normally exist in a mixed culture, with many different species occupying the same environment. Therefore, one must first separate, or isolate, the different species contained in a *specimen*. Once microorganisms have been isolated in pure cultures, it is necessary to keep the cultures alive for some period of time in order to study them (Pelczar *et al.*, 1993).

Consequently, pure cultures were isolated from different contaminated soil samples. Colonies that had clear zones were isolated and purified by transferring three times on MSM_A and YTS_{250A} plates. Isolated strains were routinely sub-cultured at ambient temperature (25°C) on YTS_{1000} - nutrient agar plates (*i.e.* 1.0 g L^{-1} each of yeast extract, tryptone, and starch in 1.5% (w/v) agar and pH 7.0) and stored at refrigeration temperature (4°C).

4.3.5 Determination of the Viable Culturable Bacterial Population

According to the methods reported by Greer *et al.*, (1990), viable bacteria in soil (CFU g^{-1}) were determined by serial dilution and spread-plate techniques. A similar approach has been followed in this study. An aliquot of soil (5 g) was aseptically added to pre-weighed sterile test tubes containing 2.5 g of glass beads (3 mm diameter). The tubes were re-weighed and sterile tetra-sodium pyrophosphate buffer (0.1% w/v: $Na_4P_2O_7.10H_2O$ in distilled water, and pH 7.0) was added to a 3:1 buffer: soil w/w ratio. The mixture was vortexed for 2 minutes at high speed, before preparing a 1:10 dilution series in MSM_B. A 0.1 mL aliquot from the dilution series was spread-plated in triplicate onto MSM_A containing phenanthrene (PAH) as sole carbon source (MSM_{A+PAH}), and the plates were incubated at ambient temperature (25°C) for at least one week before counting colonies (Greer *et al.*, 1993).

4.3.6 Preparation of Dilution Series

According to the protocols followed at the Environmental Microbiology Laboratory of Biotechnology Research Institute (BRI) at National Research Council of Canada in Montreal (QC, Canada); several chunks of stored contaminated soil (4°C) were taken out and a representative 5 g aliquot was taken. The soil was transferred to a pre-weighed sterile culture tube (25 mm × 150 mm) containing approximately 2.5 g of glass beads (3 mm diameter), and the tube, beads and soil weighed once more to accurately determine the wet weight of the original soil sample. Buffer (tetra-sodium pyrophosphate buffer 0.1% w/v: $Na_4P_2O_7.10H_2O$ in distilled water, and pH 7.0) was added to a final 3:1, buffer: soil w/w ratio, thereby giving a 1:4 dilution of the sample.

Once the sample was in contact with diluent, it was put on ice, diluted and plated immediately. The dilution and plating of the samples were done together. All dilution tubes were stored on ice during their preparation and subsequently while plates were being spread. The tubes were vortexed for 2 minutes at high speed, and 1 mL immediately transferred to a fresh tube containing 9 ml of sterile dilution solution. The tube was vortexed for approximately 15-30 seconds, and then 1 mL of the diluted solution was immediately transferred to a fresh tube that contained 9 ml of sterile dilution solution using a new sterile pipette each time. The above steps were repeated after vortexing until a dilution of series of 1:10 was prepared (from the glass beads tubes) from approximately 10^{-1} to 10^{-5} depending on which dilution was plated. To be on the safe side (when plating from a site for the first time), four dilutions were plated (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}).

4.3.7 Bacterial Enumeration Using Spread Plate Technique

Petri plates were labelled and contained appropriate nutrient media according to sample number, dilution tube (*e.g.*, 10^{-1} to 10^{-4}), and date. The dilution tube was vortexed briefly (10-15 seconds) just prior to removing the sample to be plated. For triplicate plates with a well-diluted suspension (one cannot see particles in suspension) 0.5 mL of the appropriate dilution was removed and 0.1 mL was carefully applied to the surface of each of three plates of medium, making sure not to pierce or damage the surface of the agar.

The pipette was held as vertical as possible while distributing the 0.1 mL to the plates, to ensure that the three 0.1 mL volumes distributed were as similar as possible.

The plates were later put onto the centre of the turntable one at a time and liquid was spread over the entire surface using a sterile glass spreader, soaked in alcohol (95%), ignited just prior to each spreading, and allowing the flame to extinguish itself. The spreader was cooled before using. The glass rod was moved slightly on the surface while the turntable was turning to try to create a uniform covering on the surface of the plate. It was important to use media plates that had been stored at room temperature for 3 days prior to spreading.

This ensured a dry surface that would take up the liquid quite rapidly. Spreading was continued until the surface of the plate was dry, indicated by the sticking of the glass rod to the surface. If the plate was not spread until they were dry, the colonies would not be countable, as they would tend to run together in the liquid remaining on the surface. The glass spreader was re-sterilized between periods of plating. After all plates were spread in this fashion, they were incubated in an inverted position at ambient temperature (25°C). Counting of colonies on each plate was performed twice, once after approximately one week of incubation and again after about two weeks of incubation. For counting permanent ink marker was used to mark a colony to avoid re-counting it. At the second counting the slow growing colonies were identified by a different colour. The most statistically significant counts ranged between 30 and 300 colonies. The numbers of colonies were recorded on the plates along with the corresponding dilution and date.

4.3.8 Isolation and Cultivation of Pure Culture Experiment

Samples of topsoil (0-0.3 m depth) from wood-preserving plants, oil refineries, and various petroleum-hydrocarbon-contaminated sites were collected (Jazestani and Prasher, 2003b). Each location's sample represented a composite of five sub-samples obtained with 3 m × 3 m quadrat. Samples were transferred to the laboratory cold-room and stored at 4°C, and later slowly allowed to warm to permit bioaugmentation for chemical and microbial community analyses.

MSM_A, MSM_B, YTS_{250A}, YTS_{250B}, YTS_{1000A}, YTS_{1000B}, were used in bacterial isolation experiments (Greer *et al.*, 1990). The nutrients of prepared media were obtained

from Sigma-Aldrich, Oakville, Ontario, and Canada. Furthermore, YTS_{250A}, YTS_{250B}, YTS_{1000A}, YTS_{1000B}, and MSM_B, were also used in mineralization experiments (Greer *et al.*, 1993). Quantification of total and PAH-degrading bacteria was performed by counts of colony forming units (CFU) on appropriate solid media. Total counts were evaluated on MSM_A plates containing phenanthrene (PAH) as sole carbon source (MSM_{A+PAH}), while PAHs degraders were cultured on similar plates of phenanthrene-supplemented YTS_{250} ($YTS_{250+PAH}$). Lowenstein-Jensen agar medium (LSPQ-Bacteriologie, 2003) was used for analysis of cell wall mycolic acids. All media were sterilized by autoclaving at 121°C for 20 minutes.

Greer *et al.* (1993) have reported that phenanthrene-degrading bacteria were isolated from contaminated soil samples by serial dilution and spread-plate techniques using solid MSM_{A+PAH} and $YTS_{250A+PAH}$ media. Phenanthrene was added to the MSM_A and YTS_{250A} plates as 0.5 mL of a 2.5 g L^{-1} phenanthrene solution in acetone. The solution was spread using a sterile glass rod and the solvent was allowed to evaporate overnight before use. Phenanthrene crystallized onto the surface of the agarose and did not interfere with subsequent plate spreading. Positive colonies which produced a clear zone in the phenanthrene precipitate were isolated and sub-cultured onto $YTS_{250A+PAH}$ plates. Of the 228 bacterial strains, 60 positive colonies were selected and purified for further characterization. All 60 positive colonies were routinely sub-cultured at ambient temperature (25°C) on YTS_{1000A} plates (Greer *et al.*, 1990). Phenanthrene degradation competent colonies were probed by DNA-DNA (Southern) hybridization for a gene known to confer the ability to degrade naphthalene (*ndoB*).

4.3.9 Colony Hybridization and Radioactive Gene Probing Techniques

The phenanthrene-degrading isolates were tested as to whether they were genotypical competent in naphthalene biodegradation, and if so, to characterize this capacity at the molecular level. The phenanthrene-degrading isolates were transferred onto nylon membranes and examined with probes for the *ndoB* naphthalene catabolism trait by DNA-DNA Southern Hybridization. The *ndoB* gene is one of the genes encoding naphthalene dioxygenase from *Pseudomonas putida* (ATCC 17484), and is involved in the biodegradation pathway of naphthalene.

Bacterial colonies were lifted onto membranes, the cells were lysed, allowing the bacterial DNA to be denatured and fixed to the membranes according to the standard protocol**.** Membrane DNA was allowed to hybridize with radioactive gene probes and the number of probe-positive bacteria was determined via autoradiography. The results indicate the relative numbers of bacteria possessing the catabolic pathway (Jazestani and Prasher, 2003b).

To further screen these strains for their PAH-biodegradative capacity, standard aerobic liquid serum bottle microcosms were set up for quantitative radioactive tracer analysis of the biodegradation and cumulative percent mineralization of the selected recalcitrant PAHs-compounds with minimal water solubility and low bioavailability in soils, as a sole source of carbon and energy in $YTS_{250B.}$

4.3.10 Mineralization Experiment of Recalcitrant Selected PAHs Compounds

The mineralization assay is regularly used in biodegradation studies to monitor the rate of biodegradation by microorganisms in pure culture or in microcosm analysis. Microcosms are self-contained mini-environments in which biodegradative microorganisms are incubated with a specific ^{14}C-labelled contaminant. There is a KOH-based CO_2 trap within the microcosm. During the process of mineralization (i.e. complete breakdown into CO_2 and H_2O), the KOH absorbs the radioactive CO_2. The KOH solution is then analyzed for its level of radioactivity in a scintillation counter machine. Values obtained in disintegrations per minute (dpm) were converted to mg substrate mineralized or could be expressed as a percentage of substrate mineralized over time.

Liquid microcosm studies using 20 mL of final culture liquid (MSM_B and YTS_{250B}, DNA-grade, Bio-Rad; and no granulated agar, and final pH=7.0) medium were performed to monitor and screen biodegradation activity of the 14 bacterial isolates (microbial populations) in the contaminated medium at ambient temperatures (25^oC). The liquid mediums in microcosms were spiked with ^{14}C-labelled selected PAH substrates and the rate of $^{14}CO_2$ evolution was monitored by liquid scintillation counter machine. The treatments and negative controls (abiotic) were run in triplicate. The details of the experiment are outlined below.

A standard aerobic microcosm methodology was adapted to monitor and screen the 14 bacterial isolates (*i.e.* to assess the capacity of a given biomass) for PAHs-biodegradative competence. Aerobic liquid microcosm studies were performed in 120 mL serum bottles (Greer *et al.*, 1993; Whyte *et al.*, 1999). To inoculate each serum bottles (microcosms), a 1.0 mL aliquot of the culture (OD_{600} of 0.1) with the final cell density of 1.0×10^7 cfu mL^{-1} was added, so as to have 20 mL of final culture liquid (MSM_B and YTS_{250B}). All serum bottles were slowly agitated on a shaker (150 rpm) at ambient temperature (25°C). Treatments and heat-killed abiotic controls (negative controls) were run alongside one another, each in triplicate. The abiotic culture controls were prepared by autoclaving the culture at 121°C, twice for 30 minutes at a 24-hour interval. A 5 mL glass test-tube, containing 1 mL of 0.5N KOH and serving as a CO_2 trap was placed inside each serum bottle.

The inoculated MSM_B and YTS_{250B} media were spiked with 100µL ^{14}C-labelled selected PAHs solutions (Sigma-Aldrich, Oakville, Ontario, and Canada - Tables 3.1 and 3.2 in Chapter 3) using a 100µL glass syringe, such that approximately 1.0×10^5 dpm of substrate was present in each serum bottle. Then, the 120 mL serum bottles (microcosms) were tightly sealed with Teflon Coated Rubber Stoppers and Aluminium Crimps. Furthermore, oxygen was supplemented by flushing the serum bottle's headspace with 3 mL of air before each sampling; to insure that all of the substrate could be oxidized (*i.e.* mineralized: complete breakdown into CO_2 and H_2O).

Aliquots of the CO_2-bearing 0.5N KOH solution (1 mL) were routinely removed at intervals using a long stainless steel needle attached to a 5 mL syringe. The trapped tube was rinsed with 1 mL of fresh 0.5N KOH solution. This was then removed and added to the first aliquot, resulting in a 2 mL sample. An additional 1 mL of fresh 0.5N KOH solution was added to the trapped tube to capture further CO_2 generated by the mineralization of the ^{14}C-labelled selected PAHs.

The 2 mL CO_2-bearing 0.5N KOH aliquots were each mixed with 18 mL of scintillation cocktail (Fisher Scientific, Whitby Ontario, and Canada) and placed in a 20 mL scintillation glass vial and was shaken for 5 seconds. The 20 mL KOH solution was then analyzed for its level of radioactivity in a scintillation counter. The rate of $^{14}CO_2$ evolution was derived from the liquid scintillation counts. Values obtained in

disintegrations per minute (dpm) were converted to mg substrate mineralized or could be expressed as the percentage of introduced substrate [^{14}C]PAHs mineralized over time and recovered as [^{14}C]CO2. This sampling regime (The frequency of sampling: 3 day interval) continued until no further mineralization occurred (*i.e.* mineralization rate reached a plateau).

One of the PAH-degrading microorganismal strains, isolated by (Jazestani and Prasher, 2003b) from a jet-fuel contaminated site at the Sept-Iles airport (Sept-Iles, Quebec, and Canada), on the north shore of the St. Lawrence river, was capable of mineralizing pyrene, phenanthrene, and to a lesser extent fluoranthene, but not naphthalene, anthracene or fluorine, was designated Strain S65, and selected for further characterization.

4.3.11 Micromorphological Characterization of Bacterial Isolates

Bacteria are transparent when viewed in their natural state; it is necessary to stain them to study their morphology in detail. Although, there are many different types of bacterial stains, the Gram stain is the most common stain used routinely in the microbiology laboratories **(**Rowland *et al.*, 1994**).** The Gram stain has great practical value for identifying and classifying bacteria (Alexander, 2005).

4.3.12 Preparation of Gram Stain

To prepare a Gram stain, a smear of bacterial cells was first stained with crystal violet, a dye that imparted a deep purple colour to the cells. After applying an iodine solution, which formed a complex with the crystal violet in the cytoplasm of the cell, the smear was carefully decolorized with 95% ethanol. Ethanol removes the crystal violet-iodine complex from the cytoplasm of Gram-negative bacteria, but not from the cytoplasm of Gram-positive bacteria, because of differences in the molecular structure of the peptidoglycan layer of the cell wall. The smear was then counterstained with a red dye called safranin. However, the counterstain makes Gram- negative bacteria appear pink, while Gram-positive bacteria appear dark purple.

4.3.13 Determination of Micromorphological Characteristics

Micromorphological properties of the 60 positive bacterial colonies (phenanthrene-degrading microorganisms) were determined by phase-contrast microscopy based on standard methods reported by Chan *et al*. (1993) and Murray *et al*. (1994). For the morphological characteristics of the cell, Gram stain method and the streak isolation technique were applied to study the shape (form, elevation, margin), colour, size (diameter), surface appearance (smooth, rough, and mucoid), and structural details of a bacterial cell. Bacterial colony's diameter were also measured in millimetres and recorded. A colony can be described in terms of its surface texture (smooth, rough, rugose, and granular), viscosity (butyrous, membranous, brittle, and viscid), contour (smooth, jagged), reflective/transmittive properties (opaqueness, translucence, opalescence, iridescence, dullness, glossiness), and colour (white, yellow, pink, etc.). The pigment may be soluble, meaning that it diffuses from colonies into the medium, or non-soluble remaining confined to the colony. Sixty bacterial isolates (positive colonies) were routinely sub-cultured at ambient temperature (25°C) on YTS_{1000A}. Plates were carefully examined and kept under the ventilated hood to prevent any contamination. Sub-cultured plates were then stored at refrigeration temperature (4°C).

4.3.14 Optimal Growth Temperature Study

Temperature has a great influence on the growth of microorganisms. Therefore, all the processes of growth are dependent on chemical reactions that are affected by temperature. Microorganisms can grow over a rather broad range of temperatures. At the most favourable temperature for growth, the number of cell divisions per hour, is called the growth rate. The principle of the growth phenomenon indicates that growth behaviour is the result of a series of integrated, enzyme-based chemical reactions. However, the temperature at which a species of microorganism grows most rapidly is the optimum growth temperature (Pelczar *et al.*, 1993).

An assay was conducted to determine the optimum growth temperature for *Mycobacterium* sp. Strain S65. To inoculate each of 250-mL Erlenmeyer flasks, a 1.0 mL aliquot of the culture (OD_{600} of 0.1) with a cell density of 10^7 cfu g^{-1} of soil was added to

have 49-mL of YTS_{1000B} culture medium. Erlenmeyer flasks were loosely closed and then incubated at different temperatures (*i.e.* 25°C, 30°C, and 35°C). Liquid cultures were set up in triplicate for each temperature. The optical density (OD) was monitored at 600 nm by spectrophotometer until the cultures reached the stationary phase.

4.3.15 HPLC Characterisation of Mycolic Acid Esters

HPLC of mycolic acid esters has been demonstrated to be an accurate, reliable, and analytical method for identification of many *Mycobacterium* species, and in distinguishing them from the other nocardio-form actinomycetes. The standard method employed by the LSPQ-Bacteriologie Laboratory of the Institut National de Santé Publique du Québec in Montreal was used (LSPQ-Bacteriologie, 2003). Analysis of the cell wall mycolic acids by HPLC with UV detection was performed with cells grown at 30°C on both YTS_{1000A} medium as well as cells sub-cultured on Lowenstein-Jensen agar medium at 30°C.

4.3.16 UV-VIS Detector

The ultraviolet-visible spectrophotometer is the most widely used detector for HPLC analysis. The basis of UV-VIS detection is the difference in the absorbance of light by the analyte and the solvent. A number of functional groups absorb strongly in the ultraviolet, including aromatic compounds, carbonyl compounds, such as esters, ketones, aldehydes, alkenes, and amides (Larson *et al*., 1997).

4.3.17 Experimental Procedures

One or two loop-fulls of cells grown on solid medium were suspended in a methanolic potassium hydroxide solution and saponified by heating. After acidification and extraction with chloroform, free mycolic acids were derivatized to UV-absorbing *p*-bromophenacyl esters. A high molecular weight internal-standard (HMW) was added, and the sample, injected. The UV-absorbing mycolic acid esters were separated on a reverse-phase C_{18} ultrasphere XL analytical cartridge column (4.6 mm by 7 cm; particle size, 3 μm; Beckman Instruments, Inc., San Ramon, California.) at 35 ± 1°C by a methanol-methylene chloride gradient elution and detected by UV spectrophotometry.

Furthermore, chromatographic separation and detection was performed with a modular system composed of a Waters automated gradient controller (model 680; Millipore Corporation, Milford, Mass.) with model 501 and 510 pumps, a Waters model 715 Ultra Wisp sample processor, and a diode array detector (model 1040A; Hewlett-Packard Co., Avondale, Pa.) set at 254 nm. A Hewlett-Packard integrator (model 3392A) and a Nelson analytical data system consisting of a model 950 Intelligent Interface and PC Integrator Software (model 2100, version 5.0; Perkin-Elmer Nelson Systems Inc., Cupertino, California.) were used for data collection and analysis as well as the plotting of the chromatograms.

4.3.18 HPLC Pattern Identification

Pattern recognition was by visual comparison of sample results with mycolic acid patterns from reference species of known *Mycobacteria*, in-house and published data. An extract prepared from the reference strain of *Mycobacterium* ATCC 13950T was used as a positive control as well as a comparative strain and provides an external standard peak naming reference, with the high-molecular weight standard. HPLC patterns can be identified to the species or group level by visual or mathematical means. A pattern atlas derived from a multicenter study of more than 350 strains, representing 23 species, illustrates species patterns. The standardized method recommends a visual comparison of an unknown sample's HPLC pattern to an atlas of reference strain patterns in combination with the use of peak height ratios

4.3.19 Basics of Sequencing and Standard Procedures for 16S rDNA Sequencing

The procedures to perform 16S rRNA gene sequence analysis for bacterial identification in a routine microbiology laboratory are briefly outlined in Table 4.2. Table 4.2 also shows the approximate time required for each step of the ABI dye terminator method. Nevertheless, the 16S rRNA gene sequence analysis of *Mycobacteria* seemed to be the most accurate method available for the accurate identification of species in the database and the taxonomic placement (Clarridge, 2004).

4.4 RESULTS AND DISCUSSION

4.4.1 Gene Probes for PAHs

To do a screening of the bacterial soil population, the positive colonies (phenanthrene-degrading microorganisms) that developed on the solid media were probed with a known catabolic gene probe (*ndoB*). The analysis of gene probes identified 14 *ndoB* positive colonies among the 60 phenanthrene degradation-competent bacterial strains. Therefore, these 14 isolate bacterial strains were selected as *ndoB* probe-positive and used in further experiments to quantify their capacity to mineralize the other selected recalcitrant PAHs-compounds. The results are shown in Table 3.5 (in Chapter 3).

4.4.2 Isolation and Culture Preservation of PAH-Degrading Bacteria

Once microorganisms (PAH-degrading Bacteria) have been isolated in pure culture, it is necessary to keep the culture alive for some period of time in order to study them for further identification. The primary aim of culture preservation is to maintain the organism alive, uncontaminated, and without variation or mutation, that is, to preserve the culture in a condition that is as close as possible to the original isolate.

Sub-culturing is the traditional method of preserving bacterial culture by periodic serial transfer to fresh medium. Then isolate bacterial strains were routinely sub-cultured and stored at refrigeration temperature (4°C). The major disadvantages of the serial-transfer technique are the risks of contamination, transposition of strain numbers or designations (mislabelling), selection of variants or mutants, and possible loss of culture, as well as the required storage space. Therefore, sub-culturing is not recommended as a long-term preservation method (Gherna, 1994).

The deep-freezing procedure involves the freezing of a bacterial suspension in the presence of a cryo-protective agent such as glycerol to prevent cellular damage during the freezing process. Many bacterial species have been successfully preserved for several years using this method. However, one may want to preserve the cultures for long-term applications. Likewise, for long-term storage all the isolate bacterial strains were conserved by deep freezing at $^{-}80^{\circ}C$ in a big culture collection (designated: Jamshid Jazestani culture collection) in Environmental Microbiology Laboratory, at

Biotechnology Research Institute (BRI) of National Research Council of Canada in Montreal for future research purposes (Jazestani and Prasher, 2003b).

4.4.3 PAH Degradation Competence of Strain S65

Compared to the 59 other strains tested for their ability to break down selected recalcitrant PAHs compounds with different chemical structures (linear, angular, and cluster) and varying numbers of fused-benzene rings (2 to 4), Strain S65 showed the great cumulative percentage of PAHs mineralization. This strain was isolated and characterized partially from a jet-fuel contaminated site at the Sept-Iles airport (Sept-Iles, QC, Canada), on the north shore of the St. Lawrence river, was able to mineralize selected recalcitrant PAHs-compounds. It utilized Fluoranthene to a limited extent (24% cumulative mineralization), rapidly utilized Phenanthrene (61% cumulative mineralization) as well as Pyrene (61% cumulative mineralization), as sole carbon and energy sources, but gave no significant degradation of Naphthalene, Anthracene and Fluorene (Figure 3.8 in Chapter 3), and Jazestani and Prasher (2003b). Strain S65 was routinely sub-cultured at ambient temperature (25°C) on YTS_{1000} - nutrient agar plates (*i.e.* 1.0 g L^{-1} each of yeast extract, tryptone, and starch in 1.5% (w/v) agar and pH 7.0) and stored at refrigeration temperature (4°C).

Analysis of mineralization results and degradation pathway of selected PAHs showed that degradation of Phenanthrene (angular and three fused-benzene rings) started with a 3-day lag phase and accumulative phenanthrene mineralization continued to reach to 61% (Figure 3.8 in Chapter 3). But, Pyrene (cluster and four fused-benzene rings) degradation pathway was quite different. Quickly, after the inoculation of bacterium in pyrene serum bottles, Strain S65 rapidly utilized the substrate without any lag phase and cumulative pyrene mineralization increased to 50% in the first sampling day (24 hours), and by the second sampling (9 days) reached 61% (Figure 3.8 in Chapter 3). Then the sampling regime continued until no further mineralization occurred (mineralization rate reached a plateau after 9 days). Mineralization results of Fluoranthene (linear and three fused-benzene rings) showed that Strain S65 degraded the substrate as the sole carbon and energy sources with lesser enthusiasm and therefore, accumulative Fluoranthene

mineralization got to a lower peak of 24% (Figure 3.8 in Chapter 3). No lag phase was observed for the Fluoranthene pathway degradation.

Microbial PAHs-degradation has been reported in several studies, confirming the ability of bacteria to utilize PAHs. A *Mycobacterium* sp. PYR-1 was isolated from oil-contaminated sediments and was found to be capable of mineralizing pyrene as well as several other PAHs (Heitkamp *et al.*, 1988a) when growing on peptone, yeast extract and soluble starch media. But, it was not capable of growth on pyrene as a sole source of carbon and energy. Another strain of *Mycobacterium* sp. was also isolated from soil at an abandoned coal gasification site (Boldrin *et al.*, 1993). This organism grew on phenanthrene, pyrene and fluoranthene and degraded fluorene co-metabolically. In another study, Grosser *et al.* (1991) reported on the mineralization of pyrene in soils collected from abandoned coal gasification plants. One organism capable of mineralizing pyrene was also isolated in pure culture and identified as a *Mycobacterium* sp. PAH 135. The strain could grow on pyrene in mineral salt medium (MSM), providing the medium was supplemented with cofactors, including peptone, yeast extract and soluble starch. The initial products of pyrene degradation and a pyrene catabolic pathway were not determined. Furthermore, Walter *et al.* (1991) indicated that a *Rhodococcus* species has been isolated from contaminated soils with the ability to use pyrene as sole sources of carbon and energy. Other research was conducted by Dean-Ross and Cernilia, (1996) on pyrene-degradation by *Mycobacterium flavescens*. The strain of *Mycobacterium flavescens* was isolated from polluted sediments. It was capable of utilizing pyrene as a sole source of carbon and energy. It is also noteworthy that all of these strains, including the strain reported here, are gram-positive organisms; five out of the six are members of the genus *Mycobacterium*.

Nevertheless, Strain S65 did not show any significant ability to degrade low-molecular-weight (LMW) PAHs including Naphthalene, Anthracene and Fluorene (Figure 3.8 in Chapter 3). However, *Mycobacterium* sp. Strain S65 showed remarkable ability in the rapid utilization of cluster (Pyrene) and angular PAHs (Phenanthrene and Fluoranthene), rather than linear (Naphthalene, Anthracene and Fluorene) PAH molecules as sole carbon and energy sources.

Since PAHs are practically insoluble in water and thermodynamically stable, they

are more resistant to microbial degradation, the solubilisation of these substrates is very important for the uptake of substrates into microbial cells. Therefore, little information exists on the microbial degradation of PAHs. In this situation S65 could play very important role in degradation of PAHs.

4.4.4 Micromorphological Characterization of Bacterial Isolates

Micromorphological properties of 60 bacterial isolates, grown on YTS_{1000A}, were determined by phase-contrast microscopy method (Jazestani and Prasher, 2003b). An advantage of this technique was its ability to show cell structure without using dyes or killing the organism. The Gram stain procedure, revealed a broad distinction of bacterial groups. It also permitted a study of the form, size, and structural details of the bacterial cells.

The obtained macromorphological characteristics of the bacterial isolates are detailed in Table 3.5 (in Chapter 3). A colony can be described in terms of its surface texture (smooth, rough, rugose, and granular), viscosity (butyrous, membranous, brittle, and viscid), contour (smooth, jagged), reflective/transmittive properties (opaqueness, translucence, opalescence, iridescence, dullness, glossiness), and colour (white, yellow, pink…). The pigment may be soluble, meaning that it diffuses from colonies into the medium, or non-soluble – remaining confined to the colony. Degradation-competent strain S65 was found to be Gram variable, catalase-positive, with a rod-coccus morphology, and produced creamy yellow, slightly convex, slimy, circular colonies; with an entire margin and a diameter of less than 1 mm after 7 days on YTS_{1000A} medium.

4.4.5 Optimal Growth Temperature

The growth can be divided in three distinct phases. The lag phase (or pre-exponential phase) is characterized by a slow rate of cell division. The exponential phase represents the period during which cell division is maximal. Typically, the numbers of cells in the culture will double every 40-60 minutes in the exponential phase. Finally, the cells reach the plateau phase, during which the growth rate is reduced as a result of nutrient depletion under the high cell density (Laboratory Exercises in Microbiology and Immunology, 2001). These growth phases are affected by temperature. For any particular microbe, the

three important temperatures are the minimum, optimum, and maximum growth temperatures. These are known as the *cardinal temperatures* of a species of microorganism. However, the optimum temperature for a microbial species does not necessarily lie midway between the minimum and maximum temperature. Instead, it is nearer the upper limit of the temperature range, because the rate of enzyme reactions increases with increasing temperature until a point where the enzymes are damaged by heat and cells stop growing (Pelczar *et al.*, 1993).

Determination of the optimum growth temperature of *Mycobacterium* sp. Strain S65 was performed at temperatures of 25°, 30°, and 35°C in YTS_{1000B} medium. The optimal growth temperature of Strain S65 was 30°C, while Strain S65 was unable to grow at 35°C (Figure 4.1). This indicates that Strain S65 cannot survive in the human body and thus is unlikely to be a human pathogen. Bacterial strains that are pathogenic to humans grow best at body temperature, which is 37°C (Pelczar *et al.*, 1993).

Comparatively, shepard (1965) found that *Mycobacterium leprae* had an optimum growth temperature of about 20°C in mice and did not grow at 35°C. Young *et al.* (2005) grew several species of *Mycobacterium,* only some of which thrived at 37°C. In the present study, exponential growth continued until 150 hours, when cells grown at 25°C and 30°C had just reached the stationary phase. While significantly but only marginally greater strain S65 cell accumulation occurred at 30°C than at 25°C over the first 6 days, by 150 hours both populations had reached the stationary phase, and their numbers were not significantly different. For the sake of experimental simplicity, ambient temperature (25°C) was chosen for carrying out the mineralization experiments.

4.4.6 Mycolic Acid Esters of Strain S65

The HPLC patterns obtained for this unknown strain of *Mycobacterium* did not match any of current reference profiles of LSPQ-Bacteriologie 2003, from Institut National de Santé Publique du Québec in Montreal for *Mycobacterium* sp. The diagnostic peaks were located from ± 4 minutes to ± 8 minutes in absolute retention time. The patterns obtained from the growth collected on the two media were very similar, but the peaks were better defined with the Lowenstein-Jensen agar medium growth, most probably because more biomass was available. It indicated to be the only one cluster of peaks with 3 zones with

higher peaks. The HPLC chromatograms of the pattern obtained for this unknown strain of *Mycobacterium* are shown in Figures 4.2 and 4.3, respectively. Overall, it can be concluded from HPLC analysis of mycolic acid esters that Strain S65 was a novel PAH-degrading *Mycobacterium*.

4.4.7 16S rRNA Gene Sequence Analysis for Identification of Strain S65

The rationale of molecular identification is linked to the detection within the genome of highly conserved regions harbouring hyper-variable sequences. The gene encoding the 16S rRNA has been for many years and still is the primary target of molecular taxonomic studies with several other genomic regions playing a minor role. The 16S rRNA is an approximately 1,500-nucleotides sequence encoded by the 16S ribosomal DNA (rDNA). The latter is a highly conserved gene in which regions common to all living beings exist while nucleotide variations are concentrated in specific areas. The 16S rRNA gene sequence is about 1550 bp long and is composed of both variable and conserved regions. The gene is large enough with sufficient inter-specific polymorphisms of 16S rRNA gene to provide distinguishing and statistically valid measurements. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1550-bp region), and the sequence of the variable region in between is used for the comparative taxonomy (Chen *et al.*, 1989; Relman, 1999). Although 500 and 1500 bp are common lengths to sequences and compare, sequences in databases can be of various lengths. Standard procedures for 16S rRNA Gene Sequence Analysis for identification of strain S65 (Clarridge, 2004) were employed by Sho *et al*., (2004) and consequently, the sequence published in **GenBank** (as ***Mycobacterium* sp. Strain S65**) showed strain S65 to be closely allied to other *Mycobacterium* species/strains.

4.5 CONCLUDING REMARKS

The PAH-degrading bacterial strain "S65" (**GenBank designation: *Mycobacterium* sp. Strain S65**), isolated and characterized partially by Jazestani and Prasher (2003b) from a jet-fuel contaminated site at the Sept-Iles airport (Sept-Iles, QC, Canada), on the north shore of the St. Lawrence river, was able to mineralize selected PAH compounds. Strain

S65 was routinely sub-cultured at ambient temperature (25°C) on YTS_{1000A} and stored at refrigeration temperature (4°C).

The Strain S65 was able to rapidly utilized pyrene (61% cumulative mineralization) and phenanthrene (61% cumulative mineralization) as sole carbon and energy sources. The Strain S65 was also able to degrade fluoranthene to a limited extent (24% cumulative mineralization), but showed no significant ability to degrade naphthalene, anthracene and fluorene. Most importantly, the mineralization kinetics of the strain S65 revealed that this mycobacterium is unique and might be a new member of the xenobiotic-degrading mycobacteria.

Bacterial isolates were characterized at the micromorphological, biochemical**,** genetic levels and molecular biology. Biotechnology techniques were used to optimize their natural biodegradative capacities. Following up to this research, all the isolate bacterial strains were conserved to deep freezing at -80°C in a culture collection (**designated: Jamshid Jazestani Culture Collection**) in Environmental Microbiology Laboratory, at Biotechnology Research Institute (BRI) of National Research Council of Canada in Montreal for future research. Once the Strain S65 was isolated in pure culture, it was necessary to keep the culture alive for some period of time in order to use it for mineralization experiments and further identification. Therefore, Strain S65 was routinely sub-cultured and stored at refrigeration temperature (4°C).

The optimum growth temperature study of Strain S65 was conducted at different temperatures and optimal growth temperature occurred at 30°C. This fact indicated that the environmental strain S65 cannot survive in human body. Therefore, it is not pathogenic for humans. Characterization of *Mycobacterium* sp. strain S65 was done with three different approaches:

(**i**) phase-Contrast Microscopy method was applied to determine the properties of the bacterial isolates at the micromorphological level. *Mycobacterium* sp. strain S65 was found to be gram-stain variable, catalase-positive, with a rod-coccus morphology, and produced creamy yellow circular colonies.

(**ii**) analysis of the Cell Wall Mycolic Acids method by HPLC with UV detection was performed according to the standardized method (LSPQ-Bacteriologie, 2003). The analysis was performed with the original growth on YTS_{1000A} medium as well as with the growth obtained with the subculture on Lowenstein-Jensen agar medium at 30°C.

(**iii**) the HPLC patterns obtained for this previously unknown isolate bacterial strain did not match any of current reference profiles of LSPQ-Bacteriologie, from Institut National de Santé Publique du Québec in Montreal, Quebec, and Canada for *Mycobacterium* sp. The analysis of the obtained results revealed that, this isolated bacterial strain (***Mycobacterium* sp. Strain S65**) was a novel *Mycobacterium* (a PAHs-degrading bacterium). Standard procedures for 16S rRNA Sequencing (Clarridge, 2004) were employed by Sho *et al*., (2004) to characterize this strain at the biochemical and genetic levels. The analysis of the observed results showed that this bacterium belonged to the **genus *Mycobacterium***. and consequently, the sequence published in **GenBank** (as ***Mycobacterium* sp. Strain S65**) showed strain S65 to be closely allied to other *Mycobacterium* species/strains.

Table 4.1: Differentiation of *Mycobacterium* from other Genera

Characteristics	*Mycobacterium*	*Nocardia*	*Rhodococcus*	*Corynebacterium*
Morphology	Rods, occasionally Branched filaments; rarely aerial mycelium	Mycelium, later fragmenting into rods and cocci; usually some aerial mycelium	Scanty mycelium, fragmenting into irregular rods and cocci; no aerial mycelium	Pleomorphic rods, often club-shaped; commonly in angular and palisade arrangement
Rate of growth: time to visible colonies	2-60 days	1-5 days	1-3 days	1-2 days
Degree of acid-fastness	Usually strongly acid-fast	Often partially acid-fast	Often partially acid-fast	Sometimes weakly acid-fast
Acid-alcohol-fast	At least some cells in young cultures are acid-alcohol-fast	Negative	Negative	Negative
Degree of Gram staining	Weak	Usually strong	Usually strong	Strong
Production of arylsulfatase	Positive, sometimes slow	Uncommon	Negative	Negative
Reaction to penicillin	Usually resistant	Resistant	Sensitive	Sensitive

Source: (Bergey, 1994)

Table 4.2: Procedures and Time to Perform 16S rRNA Gene Sequence Analysis for Bacterial Identification in a Routine Clinical Microbiology Laboratory

Step	Procedure	Time (hands-on)	Waiting time (machine time)[a]
1	Harvest. Organism can be harvested from any plot or broth if it is a pure culture, e.g., antibiotic susceptibility plate. The age of the culture is not important. One or two 0.01 loops full are sufficient. Save for processing as a batch.	3–5 min each	
2	Extraction of DNA.	0.5 h	10 min and 3min
3	PCR amplification.	0.5 h	2.0 h
4	Analysis of the PCR product. Loading, running, and examining gel.	20 min	1 h
5	Purification of PCR products.	1 h	
6	Cycle sequencing.	30 min	3.0 h
7	Purification of PCR products.	1 h	
8	Sequencing of the 16S rRNA gene. Load capillary tray; allows to run during time away, e.g., overnight or while doing something else.	1 h	2.5 h
9	Analysis time. It takes 5 min or less to edit the sequence if the operator, software, and runs are good.	5–15 min/sample	
10	Assignment of a name. If the organism is in the database, it takes 1 min; if it is a novel organism and several databases must be searched and sequences compared in detail, 15–30 min. At this point, correlation with phenotypic characteristics and clinical presentation is also done.	Not counted for this analysis	
11	Reporting of results.	30 min	
	Total labour time, based on integrating and completing three runs of 20 samples per wk.	60 samples/40 h	1 sample/40 min

[a] Based on ABI 3100 instrument.

Source: (Clarridge, 2004)

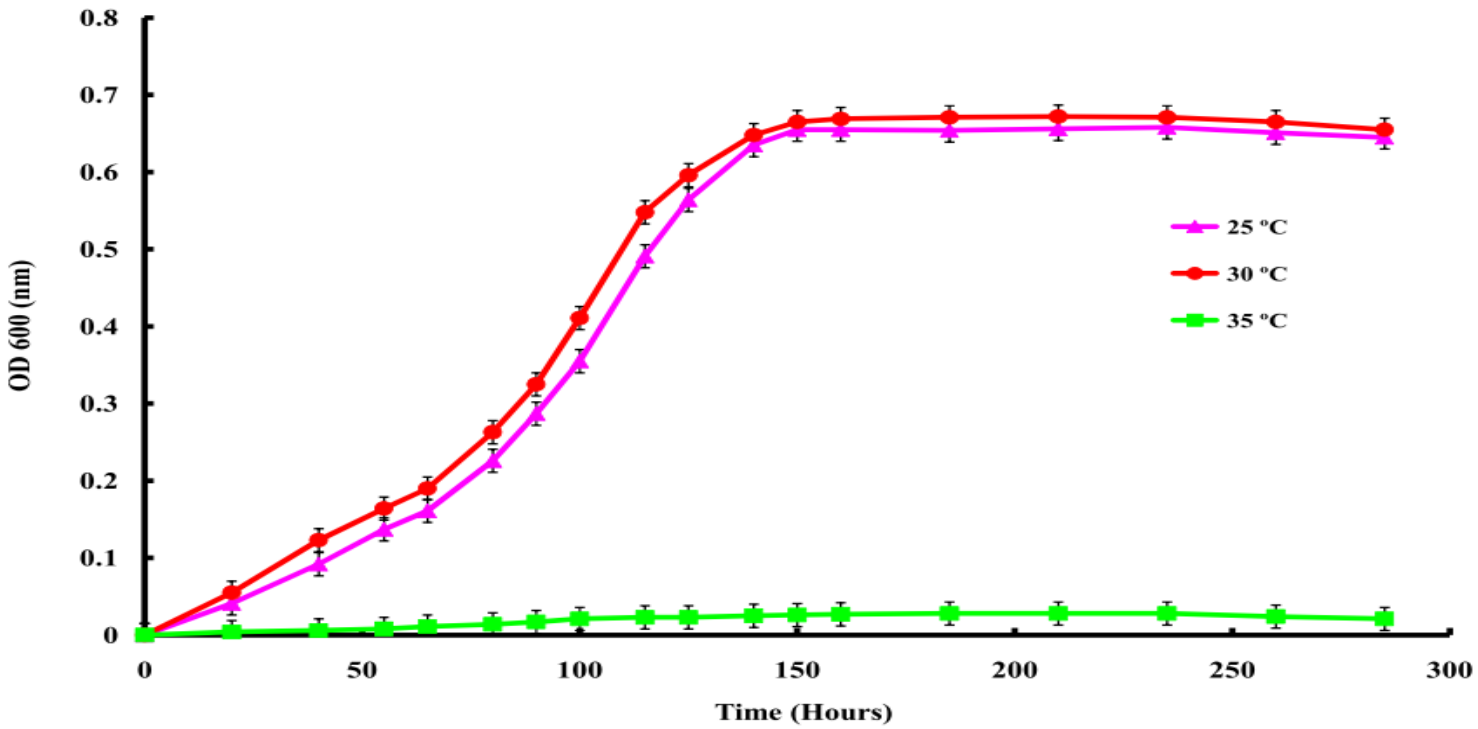

Figure 4.1: ***In vitro*** **growth curves of** ***Mycobacterium*** **sp. Strain S65 in YTS_{1000B}, and pH 7.0 at Different Temperatures.**

S65 was inoculated into YTS_{1000B} in triplicate at three temperatures. The rate of growth was measured by absorbance as the measurement of optical density (OD) at 600 nm.
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

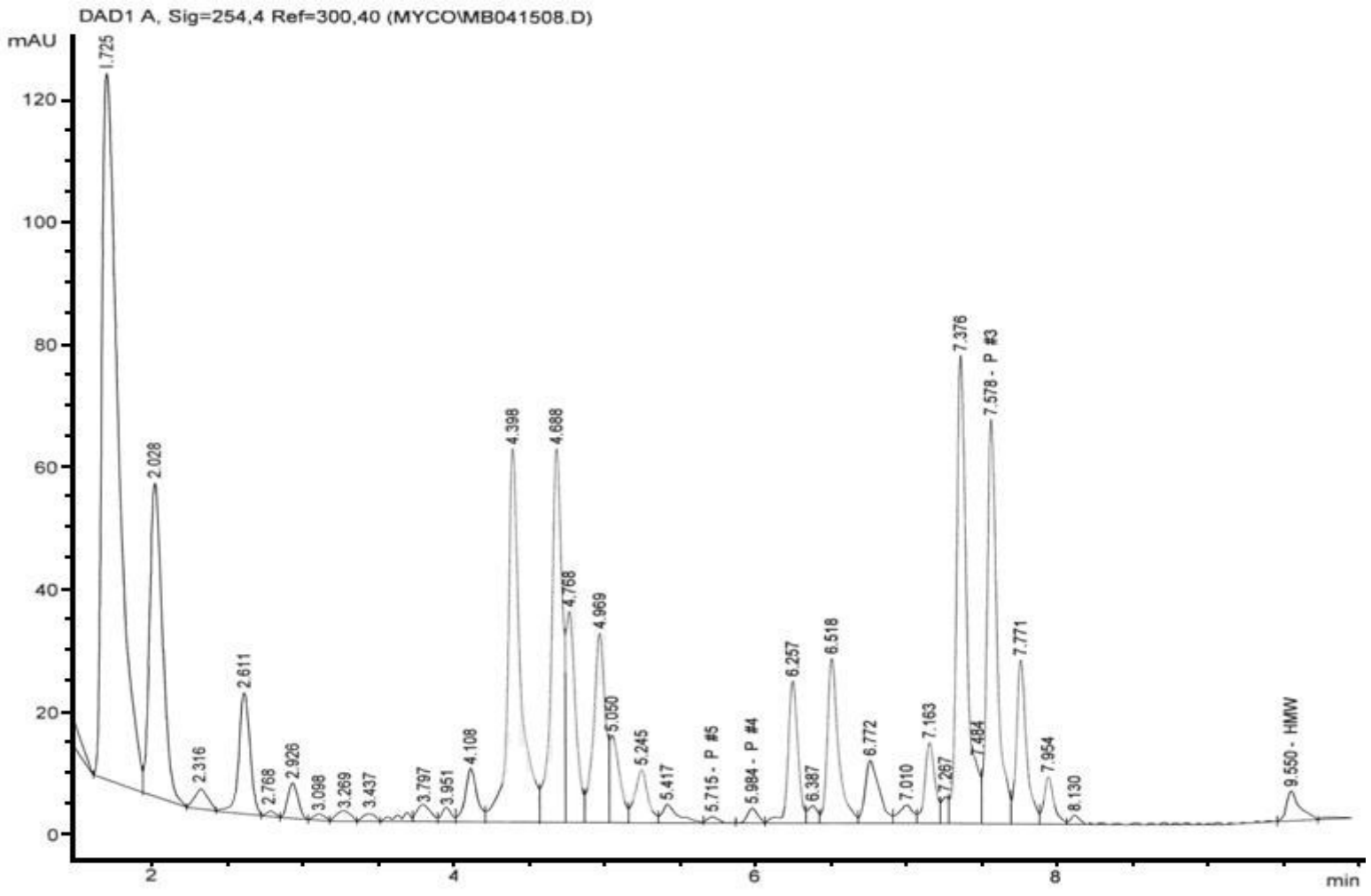

Figure 4.2: Analysis of the Cell Wall Mycolic Acids of the environmental isolate of *Mycobacterium Sp.* Strain S65 with the growth on Lowenstein-Jensen medium by high-performance liquid chromatography (HPLC) and UV detection.

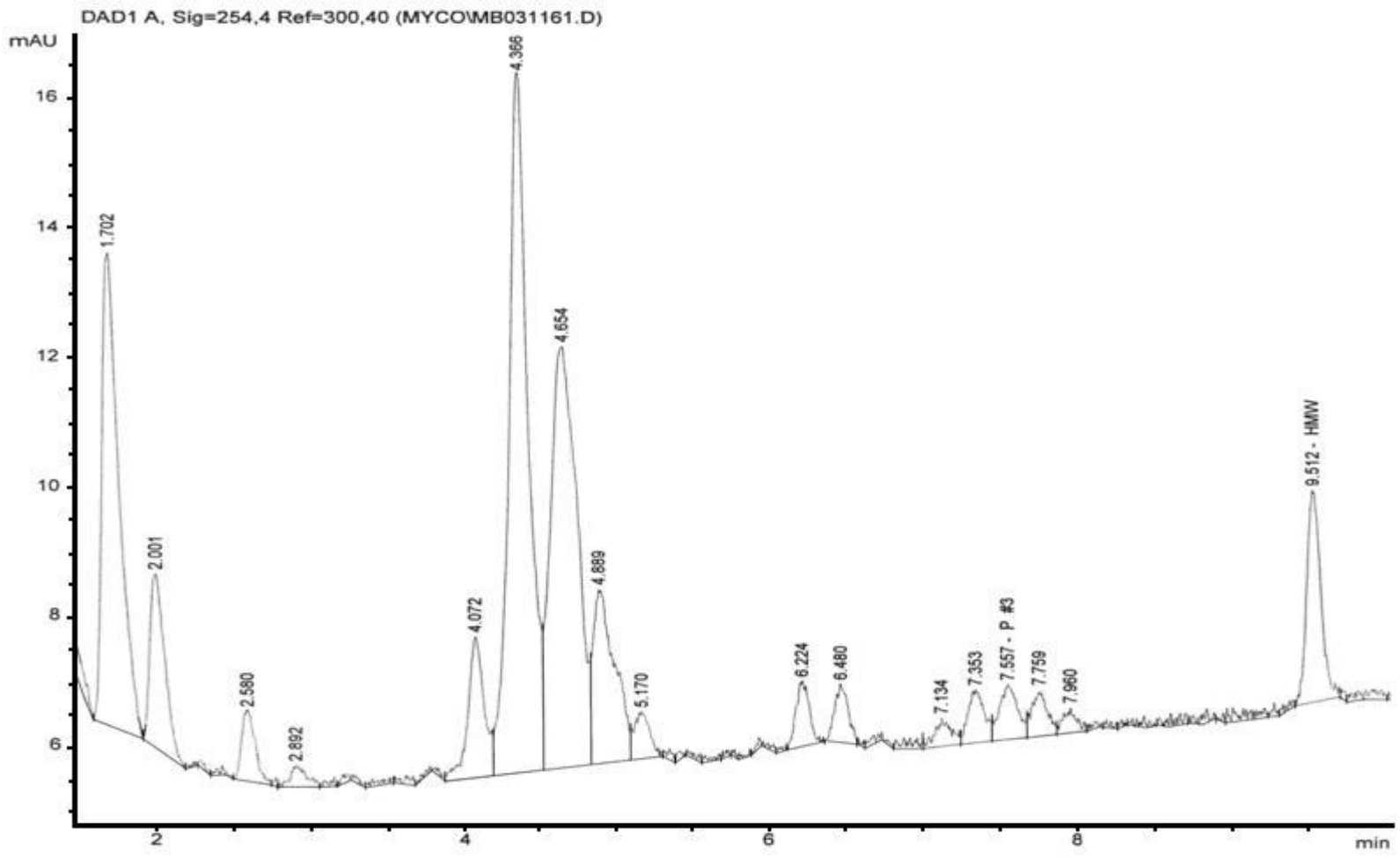

Figure 4.3: Analysis of the Cell Wall Mycolic Acids of the environmental isolate of *Mycobacterium Sp.* Strain S65 with the original growth on YTS_{1000} medium by high-performance liquid chromatography (HPLC) and UV detection.

PREFACE TO CHAPTER 5

In Chapter 4, different experiments were conducted to characterize bacterial strains at the micromorphological and molecular-genetic level. Molecular biology and biotechnology techniques were used to optimize their natural biodegradative capacities. The isolated and partially characterized bacterial strain S65 (**GenBank designation: *Mycobacterium* sp. Strain S65**), was previously able to mineralize selected recalcitrant PAHs-compounds in aerobic liquid microcosms. Strain S65 rapidly consumed as its sole carbon and energy sources, Pyrene (61% cumulative mineralization), Phenanthrene (61% cumulative mineralization), and to a lesser extent Fluoranthene (24% cumulative mineralization). Strain S65's mineralization kinetics showed this *Mycobacterium* to be a new and unique member of the xenobiotic-degrading mycobacteria. However, experimental investigations for utilization of selected aged ^{14}C-labelled PAHs in different soil environments were needed to complete the bioavailability studies.

Therefore, in Chapter 5, assessment of bioavailability of aged ^{14}C-labelled **phenanthrene** (a PAH) in different soil environments was determined by introducing the potentially active microorganism (*i.e.*, *Mycobacterium* sp. Strain S65), as an bioaugmentation agent, into different soil microcosms.

This manuscript has been submitted for publication in ***Biological Engineering*** which is published by American Society of Agricultural and Biological Engineers (ASABE).

Jazestani, J. and S. O. Prasher, 2011. Biodegradation of Soil-Aged ^{14}C-labelled Phenanthrene by *Mycobacterium* sp. Strain S65 in Different Soil Environments. ***Biological Engineering*** (submitted for publication).

CHAPTER 5

Biodegradation of Soil-Aged ^{14}C-labelled Phenanthrene by Mycobacterium sp. Strain S65 in Different Soil Environments

5.1 ABSTRACT

Isolated and characterized from a jet-fuel contaminated airport site (Sept-Îles, QC, Canada), a polycyclic aromatic hydrocarbon (PAH) degrading *Mycobacterium* (designated**: *Mycobacterium* sp. Strain S65** in **Genbank)**, was found to be competent in mineralizing selected recalcitrant PAH compounds of minimal water solubility and low bioavailability in soils when grown in yeast extract-tryptone-soluble starch medium. Based on $^{14}CO_2$ evolution, Strain S65 was capable of 61% cumulative mineralization of phenanthrene over 14 days in aerobic liquid microcosms.

Four types of γ-ray sterilized farm soil (Sandy-Loam, Sand, Clay, Clay-Loam) microcosms were spiked with ^{14}C-labelled phenanthrene and allowed to age for 0, 30, 180 or 360 days prior to inoculation with live *Mycobacterium* sp. Strain S65. Treatment microcosms' sterility prior to inoculation as well as that of control non-inoculated phenanthrene-bearing microcosms were tested by serial dilution and spread-plate techniques. A factorial combination of four soil types, two levels of soil organic matter (high and low) and two concentrations of phenanthrene (500 mg L^{-1} and 250 mg L^{-1}), with six replicates per treatment, 3 live and 3 heat-killed (total 192 microcosms at 48 microcosms per inoculation date), served as the experimental design. Aerobic liquid microcosms served as positive controls (treated *vs.* heat-killed × concentrations × aging time × replicates, $n = 2 \times 2 \times 4 \times 3 = 48$), and sterile controls (soils × aging time × replicates, $n = 4 \times 4 \times 3 = 48$) ran simultaneously, resulting in 288 microcosms being used for phenanthrene.

The evolved $^{14}CO_2$, trapped in a KOH solution over 1, 3, 6, or 9 days was measured by scintillation counter, and cumulative mineralization expressed as a percent of initial phenanthrene.

Mycobacterium sp. Strain S65 required a 3-day lag phase to begin to degrade non-aged phenanthrene in soil (Day 0). Cumulative mineralization of phenanthrene added at

concentrations of 500 and 250 mg L^{-1} reached a maximum of 20.0% and 18.5% respectively in sandy-loam soil with low organic matter, but only reached 14.5% and 13.5% respectively in clay-loam soil with high organic matter. Thus phenanthrene mineralization was greatest in the low organic matter, sandy soil. Sampling continued until no further mineralization occurred, indicating an end to further mineralization. Soil-aged phenanthrene was metabolized at a moderate rate by *Mycobacterium* sp. Strain S65; whereas non-aged phenanthrene was mineralized at a greater rate (61% cumulative mineralization) in the aerobic liquid microcosm.

For initial additions of 500 and 250 mg L^{-1} phenanthrene, followed by 30 days of ageing, *Mycobacterium* sp. Strain S65 maximum cumulative mineralization of this compound reached 14.5% and 13.0%, respectively, in low organic matter sandy-loam soil, but only 11.5% and 10.5% in high organic matter clay-loam soil. For 180 and 360-day aged phenanthrene, cumulative phenanthrene mineralization remained below 10%, a significant drop from less aged phenanthrene-soil microcosms, suggesting that as in-soil ageing proceeds phenanthrene becomes less accessible to degradation. Nonetheless, *Mycobacterium* sp. Strain S65 appears to have the potential to be useful in bioremediation and biodetoxification of high molecular weight PAHs-contaminated sites.

Key Words: *Mycobacterium* sp. Strain S65, Microbial Degradation, Bioavailability, Biodegradation Kinetics, Bioremediation, Bioaugmentation, Isolation, Mineralization, Ageing, PAHs, Phenanthrene and Soil Environments.

5.2 INTRODUCTION

The PAHs have been the subject of scientific study for many years, and a number of excellent reviews have been published on PAHs biodegradation (Environment Canada, 1994; Alexander, 1999; Alexander and Alexander, 2000; Singh and Ward, 2004; Hwang *et al.*, 2007). PAHs are persistent organic pollutants (POPs) widely distributed in the ecosphere. They are composed of two or more fused benzene rings, and pose serious risks to human health owing to their carcinogenic potential (Hamdia *et al.*, 2007). Other four-ringed PAHs as well as five-ringed compounds such as benzanthracene and benzo[α]pyrene are both genotoxic and carcinogenic (Verschueren 1983; Dibble *et al.*, 1990; Dean-Ross *et al.*, 2002). Nevertheless, as a class of compounds, PAHs have been classified as carcinogens, mutagens, and immunosuppressants (Hap *et al.*, 2006). The metabolism of PAHs in the human body produces epoxide compounds with mutagenic and carcinogenic properties and cases of lung, intestinal, liver, pancreas and skin cancers have been reported (Samanta *et al.*, 2002; Santos *et al.*, 2008). The United States Environmental Protection Agency (USEPA) has designated 16 PAHs as being environmentally important and representative of PAHs as a whole (Table 2.1 and Figure 2.1 in Chapter 2). They are classed in the USEPA's Priority Pollutant List (Singh and Ward, 2004; Liu *et al.*, 2007).

Due to their high hydrophobicity and solid–water distribution ratio, PAHs in soil tend to interact with the non-aqueous phase and organic matter; consequently, becoming less available for future microbial degradation (Kastner *et al.*, 1998; Johnsen *et al.*, 2005). The breakdown of PAHs in soil may result from the combined metabolism of microbial communities or from catabolism by individual strains (Juhasz *et al.*, 1997). The PAHs of most concern with respect to the environment and human health, are a class of neutral, non-polar organic compounds varying in size and consisting of two or more fused benzene rings in linear, angular, or cluster arrangements. These compounds are all hydrophobic, as illustrated by their relatively high octanol-water partition coefficients (K_{ow}) and low solubility in water (Table 2.1 in Chapter 2). Hydrophobicity increases with an increase in molecular size, with aqueous solubilities declining from the low mg L^{-1} range for two- or three-ring compounds to 1 μg L^{-1} or less for five- and six-ring

compounds (Singh and Ward, 2004). Exposure of animals to PAHs occurs through breathing, ingestion and dermal exposure.

Biodegradation of PAHs is the process in which these compounds are transformed as a result of biological activity. The most important mechanism for PAHs biodegradation is metabolic dissimilation or mineralization, which can cause the complete degradation to biomass (bacterial cell), CO_2 and H_2O. Other degradation mechanisms include partial transformation through specific oxidative (often fungal) enzymes, co-metabolic transformation, and detoxification. For biodegradation to occur, it is essential that two factors be present: (a) sufficient microbial degrading capacity (microorganisms) and (b) bioavailable substrate (PAH). For mineralization, the presence of an appropriate electron acceptor is also required (Volkering and Breure, 2003).

Bioremediation is one of the most promising methods of removal of PAHs from contaminated environments. A wide array of bacteria isolated from contaminated sites has been shown to be capable of degrading a range of PAHs when present singly (Atlas and Cerniglia, 1995). Studies using single PAHs, however, do not reflect the true complexity of PAH degradation in natural environments where the compounds are present in multi-component mixtures. When present in mixtures, PAHs have the capacity to influence the rate and extent of biodegradation of other components of the mixture. In some cases, these interactions may be positive, resulting in an increase in biodegradation of one or more components, while in other cases negative effects have been observed (Tiehm and Fritzsche, 1995). To elucidate the extent of such interactions, the biodegradation of PAHs alone and in two-component and three-component mixtures was studied using bacteria isolated from contaminated sediments (Bouchez *et al.*, 1995; Stringfellow and Aitken, 1995; Trzesicka-Mlynarz and Ward, 1995; Dean-Ross *et al.*, 2002).

Technologies employing biological treatments aimed at detoxifying PAH-contaminated soils have seen a broad and rising usage over the last few years and are viewed as being both cost-effective and environment-friendly (Sasek *et al.*, 2003). However, bioremediation is not a panacea, and its technical use depends on several factors such as the type, concentration and bioavailability of contaminants, organic and inorganic nutrients supply, and contaminated site history. Two processes have been found

to increase the activity of microorganisms during bioremediation: (**i**) biostimulation, or the addition of nutrients and/or of a terminal electron acceptor, increases the limited activity of indigenous microbial populations, and (**ii**) bioaugmentation, which involves the addition of external microbial strains (indigenous or exogenous), which have the ability to degrade the target toxic molecules (Odokuma and Dickson, 2003 and Hamdia et al., 2007). These two processes can occur simultaneously in case of amendments using active organic residues that may contain microbial strains capable of metabolizing pollutants.

Assessing the mineralization potential of a selected recalcitrant-PAHs-degrading bacterium, *Mycobacterium* sp. Strain S65, as a bioremediation agent in different sterilized farm soil environments was the primary goal of this research work. This assessment was done under conditions varying in soil organic matter (low *vs.* high), and at two phenanthrene concentrations (250 mg L^{-1} and 500 mg L^{-1}). This study also sought to investigate the effect of in-soil ageing time (0, 30, 180, and 360 days), on the phenanthrene biodegradation process.

5.3 MATERIALS AND METHODS

5.3.1 Soil Sources

Six different agricultural farm soils from the farm site of the Macdonald Campus of McGill University in Ste-Anne-de-Bellevue, Quebec, Canada ranging in organic matter content from low to high were selected and extensively characterized. Four of these soils were used for this study, as we chose the soils with the lowest and highest organic matter content (Table 5.1).

5.3.1.1 Soil Sampling and Characteristics

Each soil sample consisted of a composite of five, 0.3 m deep sub-samples, drawn from a 3 m × 3 m area. Samples were immediately transferred to the laboratory and stored at 4°C. For bioaugmentation, physical, chemical and microbial community analysis, soil samples were thawed at ambient temperature, then air-dried, and subsequently passed through a U.S. standard sieve (2 mm mesh). Soil physical and chemical characteristics,

textural class, and experimental water demand, were determined in the McGill University Soil Science Laboratory (Tables 5.1, 5.2 and Figure 5.1).

5.3.1.2 Soil Sterilization

Aliquots (30 g) of one of four soils types were placed in sealed bags, then sterilized with 2.5 Mrad of (γ)-irradiation from a ^{60}Co source at the Institut Armand-Frappier of Laval, Quebec. At all times dried soil samples were maintained at 4°C at either the Biotechnological Research Institute or McGill University, Montreal, Quebec, Canada.

5.3.2 Chemicals

For preparation of growth media and other assays, chemicals such as Yeast Extract, Tryptone, Starch, Granulated Agar, Ultra-Pure Agarose, Glycerol, KOH, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Co(NO_2)_2.6\ H_2O$, $AlK(SO_4)_2.12\ H_2O$, $CuSO_4$, $Zn\ SO_4.7\ H_2O$, $MnSO_4.\ H_2O$, $FeSO_4.7\ H_2O$, $Na_2MoO_4.\ 2\ H_2O$, $Ca(NO_3)_2\ .4\ H_2O$, H_2SO_4, toluene ($C_6H_5CH_3$), acetone (CH_3COCH_3), hexane (C_6H_{14}), HPLC grade methanol (CH_3OH), and ethanol (C_2H_5OH) were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada), and Sigma-Aldrich Chemical Company (Oakville, Ontario, Canada). All chemicals and solvents were of ACS reagent grade or better and were used as received.

5.3.3 Radiochemicals

Phenanthrene ($C_{14}H_{10}$, FW: 178.2, 3 fused-benzene rings) isomeric with anthracene has a log octanol: water partition coefficient (log K_{ow}) of 4.5, a water solubility at 25°C of 1.29 mg L^{-1}, melting point at 101°C, and vapor pressure at 25°C = 90.7 mPa (Sigma-Aldrich Catalogue, 2009). Uniformly ring-labelled [-9-^{14}C] phenanthrene at 5-15 mCi $mmol^{-1}$ in methanol, and 40-60 mCi $mmol^{-1}$ in toluene were provided (radiochemically pure), by the manufacturer (Sigma-Aldrich, Oakville, Ontario, Canada; Tables 3.1, 3.2 in Chapter 3, and 5.3), and stored under refrigerated conditions (4°C) until use.

5.3.4 Culture Media

The following growth media were used: YTS_{250A}, YTS_{250B}, YTS_{1000A}, YTS_{1000B}, MSM_A, and MSM_B, where A and B represent the presence or absence of agar/agarose, *i.e.* a solid medium or a broth**.** Mineral salts medium (MSM_A, Ultra-Pure Agarose, DNA-grade, Bio-Rad; 1.5% w/v: granulated agar/distilled water, Furukawa and Chakrabarty, 1982), and (MSM_B, DNA-grade, Bio-Rad; and no granulated agar, and final pH = 7.0 for both media), YTS_{250A} (containing 250 mg L^{-1} each of yeast extract, tryptone, and starch in 1.5% w/v: granulated agar/distilled water, pH = 7.0), and YTS_{250B} (containing 250 mg L^{-1} each of yeast extract, tryptone, starch in distilled water, pH 7.0, and no granulated agar) media, were used in all bacterial isolation experiments (Greer *et al.*, 1990). YTS_{1000A} (containing 1000 mg L^{-1} each of yeast extract, tryptone, starch in 1.5% w/v: granulated agar/distilled water, pH = 7.0), and YTS_{1000B} (containing 1000 mg each of yeast extract, tryptone, pH 7.0, and no granulated agar), media, and MSM-broth**, (**DNA-grade, Bio-Rad; and no granulated agar, and final pH=7.0) medium were used in all mineralization experiments (Greer *et al.*, 1993).

Quantification of the total and PAH-degrading bacteria was performed by counts of colony forming units (CFU) on appropriate solid media. Total counts were evaluated on MSM_A containing phenanthrene (PAH) as sole carbon source plates (MSM_{A+PAH}), while PAHs degraders were cultured on similar plates containing YTS_{250} also supplemented with phenanthrene (PAH) as sole carbon source ($YTS_{250+PAH}$). All media were sterilized by autoclaving at 121^{o}C for 20 minutes.

5.3.5 Bacterial Strains

The *Mycobacterium* strains isolated a collection of nineteen contaminated soils obtained from different wood-preserving plants, oil refineries, and various petroleum hydrocarbons-contaminated sites were extensively characterized. The areas and soils were designated as follows:

Parc Pitt and *Côte St-Paul/St-Ambroise*: Soil (P-1), Soil (P-2), Soil (P-5), Soil (TR-1), Soil (TR-2), Soil (TR-2A), Soil (TR-4), *Sept-Iles*: (Soil S), *Lac-des-Loups*: (Soil L), *Farnham*: (Soil F), *Borden*: (Soil B), *Sawmill*: (Soils 14 and 65), *Utility pole storage area*: (Soil 16), *Sablier Thouin*: (Soils 53, 131, 193), and *Port Tlueneme*: (Soils 5-6). The

contaminated soils' origin, characteristics, contaminants and other site information are reported in Jazestani and Prasher (2003b). Some 228 bacterial strains were isolated, grown and subsequently subcultured at ambient (25°C) temperatures on a range of media, depending on the application they were to be put to. A culture collection was routinely sub-cultured at ambient (25°C) temperature on YTS_{1000A} and stored at 4°C. A single strongly positive colony (*Mycobacterium* sp. Strain S65) from amongst the 228 isolates, was shown to mineralize selected recalcitrant PAHs in aerobic liquid (in MSM_B and YTS_B media) microcosm experiments, and was consequently further characterized.

5.3.6 Culture Preservation and Creating a Bacterial Genbank

To preserve cultures (*i.e.*, deep freezing at -80°C), each of two sterilized Eppendorf tubes (12 mL) received 3 mL of YTS_{250B} medium. A few isolated colonies of each strain were drawn from plates and transferred to the each of the tubes. After a week's incubation at ambient temperature (25°C) on a shaker, each 3 mL of culture was transferred to two 1.5 mL conical Eppendorf tubes and centrifuged for five minutes. The supernatant was removed from the tubes and replaced with 375 μL of fresh YTS_{250B} medium. The pellet was then resuspended and two high cell concentration cultures (2×375 μL = 0.75 mL) were then transferred into a 2 mL cylindrical eppendorf tube. Then, 0.75 mL of glycerol solution (40%) was added, resulting in a solution with a final glycerol concentration of 20%. The closed tube was labeled and dated, then immediately placed in dry ice and stored at $^{-}80^{\circ}C$ (Jazestani and Prasher, 2003a, b).

5.3.7 Optimal Growth Temperature Study

For any particular microbe, the three important or 'cardinal' temperatures are the minimum, optimum and maximum growth temperatures (Pelczar *et al.*, 1993). However, the optimum temperature for a microbial species does not necessarily lie midway between the minimum and maximum temperatures. Instead, it tends to be nearer the upper limit of the temperature range, because the rate of enzyme reactions increases with increasing temperature until a point where the enzymes are damaged by heat and cells stop growing (Pelczar *et al.*, 1993). The growth can be divided in three distinct phases: (**i**) the lag phase (or pre-exponential phase), characterized by a slow rate of cell division, (**ii**)

the exponential phase, when cell division is maximal and typically, the microbial population (*i.e.* the number of cells in the culture) will double every 40-60 minutes, and (**iii**) the plateau or stationary phase, during which the growth rate is reduced as a result of nutrient depletion in the medium and high cell density (Laboratory Exercises in Microbiology and Immunology, 2001).

An assay was conducted to determine *Mycobacterium* sp. Strain S65's optimum growth temperature. In each of nine 250-mL Erlenmeyer flasks (3 temperatures × 3 replicates), a 1.0 mL aliquot of the *Mycobacterium* culture ($OD_{600\ nm}$ of 0.1) was added to 49-mL of YTS_{1000B} so as to attain a final cell density of 1.0×10^7 cfu mL^{-1}. Erlenmeyer flasks were loosely closed and incubated aerobically at different temperatures (*i.e.*, 25°C, 30°C, or 35°C). Optical density (OD) at 600 nm was monitored by using spectrophotometry (nephelometry) until the cultures reached the stationary phase.

5.3.8 Inoculation of *Mycobacterium*

An aqueous suspension of *Mycobacterium* sp. Strain S65 was prepared from cells drawn from 7-day-old, low nutrient medium, YTS_{250} plates which had been incubated at 25°C. Strain S65 was then grown in YTS_{1000B} until it reached the exponential growth stage. The culture was harvested and washed twice with an equal volume (v/v) of distilled water. The culture was then used to inoculate both aerobic soil (treatment) and liquid (positive control) serum bottles (microcosms) for mineralization screening experiments, after different PAH substrate ageing times.

5.3.9 Screening of *Mycobacterium* Isolate for Mineralization Activity in Aerobic Liquid Microcosms

A standard aerobic liquid microcosm methodology was adopted to monitor and screen *Mycobacterium* sp. Strain S65 in low nutrient YTS_{250B} medium for phenanthrene-PAH degradation capacity (kinetics) and served as a positive control. Liquid aerobic microcosm studies were performed in 120 mL serum bottles (Greer *et al.*, 1993; Whyte *et al.*, 1999; Chénier *et al.*, 2003).

To inoculate each serum bottles (microcosm), a 1.0 mL aliquot of the culture ($OD_{600\ nm}$ of 0.1) with the final cell density of 1.0×10^7 cfu mL^{-1} was introduced into

YTS_{250B} to a final volume of 20 mL. All serum bottles were slowly agitated on a shaker (150 rpm) at ambient temperature (25°C). All aerobic liquid microcosm (treatments) and heat-killed abiotic controls (negative controls) were run alongside one another, each in triplicate. Heat-killed controls consisted of cultures that were pre-grown for 7 days under conditions identical to those of the corresponding experimental cultures and then killed by autoclaving. Therefore, the abiotic culture controls were prepared by autoclaving the culture at 121°C, twice for 30 minutes at a 24 hours interval. A 5 mL glass test-tube, containing 1 mL of 0.5N KOH as a CO_2 trap was placed inside each serum bottle. The inoculated YTS_{250B} medium (cell culture) was spiked with aliquots of 100 µL ^{14}C-labelled selected substrate solution (*i.e.* ^{14}C-labelled phenanthrene-PAH in methanol solution; Sigma-Aldrich, Oakville, Ontario, Canada, Table 3.1 in Chapter 3) using a 100 µL glass syringe, such that approximately 1.0×10^5 dpm of substrate was present in each serum bottle. Then, the 120 mL serum bottles (microcosms) were tightly sealed with Teflon coated rubber stoppers and aluminium crimps. To insure that all of the substrate (PAH parent compound) could be oxidized (*i.e.* mineralized: complete breakdown into CO_2, H_2O and bacterial cells); oxygen was supplemented by flushing the serum bottle's headspace with 3 mL of air before each sampling.

Aliquots of the CO_2-bearing 0.5N KOH solution (1 mL) were routinely removed at intervals using a long stainless steel needle attached to a 5 mL syringe. The wall of trap tube was rinsed with 1 mL of fresh 0.5N KOH solution. This was then removed and added to the first aliquot, resulting in a 2 mL sample. An additional 1 mL of fresh 0.5N KOH solution was added to the trap tube to capture further CO_2 generated by the mineralization of the phenanthrene-PAH.

The 2 mL CO_2-bearing 0.5N KOH aliquots were each mixed with 18 mL of scintillation cocktail (Fisher Scientific, Whitby Ontario, Canada) and placed in a 20 mL scintillation glass vial and shaken for 5 seconds. The 20 mL KOH solution was then analysed for its level of radioactivity in a scintillation counter machine. The rate of $^{14}CO_2$ evolution was derived from the liquid scintillation counts. Mineralization results (Values) obtained in disintegrations per minute (dpm) were converted to mg substrate mineralized or could be expressed as a percentage of initially introduced substrate [^{14}C]phenanthrene mineralized over time and recovered as [^{14}C]CO_2. This sampling regime (*i.e.* the

frequency of sampling: 3 day interval) continued until no further mineralization occurred (*i.e.* cumulative mineralization reached a plateau).

5.3.10 Screening of *Mycobacterium* Isolate for Mineralization Activity in Aerobic Soil Microcosms

In further step, the same serum bottle microcosm protocol was adopted for soil microcosms, to assess the mineralization capability of environmental *Mycobacterium* sp. Strain S65 based on $^{14}CO_2$ evolution resulting from the mineralization of ^{14}C-labelled aged phenanthrene-PAH in four different farm soil media, previously sterilized with 2.5 Mrad of gamma (γ)-irradiation from a ^{60}Co source. The soils ranged in soil organic matter content from low to very high across all soil texture types, and were spiked with one of two concentrations of phenanthrene (*i.e.* 250 mg L^{-1} or 500 mg L^{-1}). Quantitative radioactive tracer analysis of the biodegradation and cumulative percent mineralization of the phenanthrene-PAH compound in different soil environments, as well as liquid microcosms were determined by liquid scintillation counting, as described above.

5.3.11 Mineralization and Ageing Procedures of ^{14}C-labelled Phenanthrene (PAH) in Different Soil Environments

A standard aerobic soil microcosm methodology was conducted to monitor and screen the bacterial isolate, *Mycobacterium* sp. Strain S65 in different soil environments instead of *in vitro* cell cultures, for its capacity to degrade ^{14}C-labelled phenanthrene. The mineralization experiment was performed in triplicate.

Aliquots of 30 g sterilized farm soils were dispensed into 120 mL serum bottles (soil microcosms). A 5 mL glass test-tube, containing 1 mL of 0.5N KOH as a CO_2 trap was then placed inside each serum bottle. The soil microcosm were spiked with aliquots of 100 µL ^{14}C-labelled selected substrate solution (*i.e.* ^{14}C-labelled phenanthrene-PAH in methanol solution; Sigma-Aldrich, Oakville, Ontario, Canada, Table 3.1 in Chapter 3) using a 100 µL glass syringe, such that approximately 1.0×10^5 dpm of substrate was present in each serum bottle. Then, the 120 mL serum bottles were tightly sealed with Teflon coated rubber stoppers and aluminium crimps. Given that ^{14}C's half-life is about 5730 years the loss of radioactivity (decay) over the longest incubation (360 days) was

negligible (0.0087% or 873 dpm) and was not taken into account in mineralization calculations.

To inoculate each serum bottles at each ageing time, an aliquot of 1.0 mL culture ($OD_{600\ nm}$ of 0.1) with the final cell density of 1.0×10^7 cfu mL^{-1} was introduced into soil microcosms which had previously received ^{14}C-labelled phenanthrene-PAH solution. To maintain the optimum microbial activity, the field capacity and actual moisture values of each soil were used to calculate the volume of moisture that had to be added to the various soil microcosms at each ageing time to achieve the experimental conditions of moisture content (soil water saturation), namely 110% of soil field capacity (Cattneo *et al.*, 1997).

Aerobic soil microcosms (serum bottles) were then placed in customized boxes and statically incubated (without agitation) in a dark radioactivity containment room for four sets of ageing times at ambient temperature (25°C). All aerobic soil microcosms (treatments: organic matter × soil type × concentrations × aging time × replicates, $n = 2 \times 2 \times 2 \times 4 \times 3 = 96$), heat-killed abiotic controls (negative controls: same as treatments $n = 96$), aerobic liquid microcosms [positive controls: (treatment *vs.* heat-killed) × concentrations × aging time × replicates, $n = 2 \times 2 \times 4 \times 3 = 48$], and sterile controls (organic matter × soil type × aging time × replicates, $n = 2 \times 2 \times 4 \times 3 = 48$) were run simultaneously. Thus, for phenanthrene, 288 microcosms were used.

Heat-killed controls consisted of cultures that were pre-grown for 7 days under conditions identical to those of the corresponding experimental cultures and then killed by autoclaving. Therefore, the abiotic culture controls were prepared by autoclaving the culture at 121°C, twice for 30 minutes on two consecutive days (*i.e.* at a 1 day interval). To insure that all of the substrate could be oxidized (*i.e.* mineralized: complete breakdown into CO_2 and H_2O); air was supplemented by flushing the serum bottle's headspace with 3 mL of air before each sampling.

Furthermore, aliquots of the CO_2-bearing 0.5N KOH solution (1 mL) were routinely removed at intervals using a long stainless steel needle attached to a 5 mL syringe. The wall of the trap tube was rinsed with 1 mL of fresh 0.5N KOH solution. This was then removed and added to the first aliquot, resulting in a 2 mL sample. An

additional 1 mL of fresh 0.5N KOH solution was added to the trap tube to capture further CO_2 generated by the mineralization of the Phenanthrene-PAH.

The 2 mL CO_2-bearing 0.5N KOH aliquots were each mixed with 18 mL of scintillation cocktail (Fisher Scientific, Whitby Ontario, Canada) and placed in a 20 mL scintillation glass vial and shaken for 5 seconds. The 20 mL KOH solution was then analyzed for its level of radioactivity in a scintillation counter machine. The rate of $^{14}CO_2$ evolution was derived from the liquid scintillation counts. Values obtained in disintegrations per minute (dpm) were converted to mg substrate mineralized or could be expressed as a percentage of introduced substrate [^{14}C]phenanthrene mineralized over time and recovered as [^{14}C]CO_2.

This sampling regime (*i.e.* frequency of sampling: 72 hours interval) for treatments, negative, and positive controls continued until no further mineralization occurred (*i.e.* cumulated mineralization reached a plateau). The laboratory experiment ran for 360 days during which the effects of four sets of ageing times (*i.e.* days: 0, 30, 180, and 360), four different type of soils, and four different percentages of soil organic matter content on phenanthrene mineralization were studied. The experimental design for aerobic soil and liquid microcosm study of ^{14}C-labelled aged phenanthrene mineralization for each ageing time is shown in Table 5.3.

5.3.12 Screening of Sterile Control Soil Microcosms

To monitor and screen for the possibility of growth of any other competitive bacterial strain alongside *Mycobacterium* sp. Strain S65 in the controlled soil microcosms experiment, a sterile control microcosms study was performed. Four sets of soil microcosms were prepared from the same experimental farm soils, which were previously sterilized with 2.5 Mrad of gamma (γ)-irradiation from a ^{60}Co source.

Aliquots of 30 g of four different soils were introduced into 120 mL serum bottles, each in triplicate for each ageing time**,** for a total of 48 microcosm bottles (*i.e*. 4 soil/OM combinations × 4 ageing time × 3 replicate). The 120 mL serum bottles (microcosms) were then tightly sealed with Teflon-coated rubber stoppers and aluminium crimps. To insure that all of the sterile control soil microcosms were aerobic; air was supplemented by flushing the serum bottle's headspace with 3 mL of air before each

sampling of the main aerobic soil microcosms experiment. A bacterial enumeration assay was performed to determine the possible presence of viable microorganisms in soils (CFU g^{-1}) at each ageing time, by using serial dilution and spread-plate techniques (Jazestani and Prasher, 2003a, b).

5.3.13 Determination of the Likely Viable Culturable Bacterial Population

Serial dilution and spread-plate techniques served to assess the possible presence of viable microorganisms in soils (CFU g^{-1}). An 5 g aliquot of soil was aseptically added to pre-weighed sterile test tubes containing 2.5 g of 3 mm-diameter glass beads. The tubes were re-weighed and sterile tetra-sodium pyrophosphate buffer (0.1% w/v: $Na_4P_2O_7.10H_2O$ in distilled water, and pH = 7.0) was added in a 3:1 buffer: soil (w/w) ratio. Vortexed for several minutes the mixture was then used to prepare a 1:10 dilution series in MSM_B medium. A 0.1 mL aliquot from the dilution series was spread-plated in triplicate onto MSM medium containing phenanthrene (PAH) as sole carbon source (MSM_{A+PAH}), and the plates were incubated at ambient temperature (25°C) for at least 7 days prior to counting colonies (Greer *et al.*, 1990, 1993).

5.3.14 Statistical Analysis

Analysis of the cumulative percentage of phenanthrene mineralisation was performed using PROC ANOVA in SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC, USA). The design consisted of a factorial combination of 3 replicates, 2 phenanthrene concentrations, 4 aging times (0, 30, 180 and 360 days) and 4 soil types, with 5 repeated measures in time. A threshold of significance of $P \leq 0.05$ was used.

5.4 RESULTS AND DISCUSSION

5.4.1 Soil Sampling and Characteristics

Detailed soil physical characteristics, including textural class (soil particles size distribution), nutrient content, chemical characteristics, and water holding capacity of the soil samples to determine experimental water demand were made in the Soil Science Laboratory of McGill University (Tables 5.1, 5.2 and Figure 5.1).

5.4.2 Culture Preservation and a Bacterial Genebank

Of the 228 positive isolate bacterial strains, 60 strong positive colonies (PAHs-degrading microorganisms) were selected; of which 14 were *ndoB* positive and one isolate bacterial strain (Strain S65) mineralized selected recalcitrant PAHs. All 60 strong isolates were used to generate a bacterial culture collection. These strains were grown and maintained by subculturing on MSM_A, MSM_B, YTS_{250A}, YTS_{250B}, YTS_{1000A} and YTS_{1000B} media, at ambient temperature (25^oC). Deep-freezing was used to preserve the 60 isolates. Bacterial suspensions were then prepared in the presence of a cryoprotective agent such as glycerol to prevent cellular damage during the freezing process. All bacterial species were successfully preserved and stored at $^{-}80^{\circ}C$ at the Environmental Microbiology Laboratory of Biotechnology Research Institute (BRI), of the National Research Council of Canada, Montreal, Quebec, Canada (Jazestani and Prasher, 2003a, b). This culture collection was created to maintain a bacterial GenBank for the purpose of selection of the genes involved in recalcitrant PAHs-biodegradation and further research work in the bioremediation area.

One of the potential bioremediation isolate, *Mycobacterium* sp. Strain S65, was also grown and maintained by subculturing on MSM_A, MSM_B, YTS_{250A}, YTS_{250B}, media. This strain was used in all aged phenanthrene (PAH) contaminated aerobic soil (solid) and liquid microcosm experiments.

5.4.3 Optimal Growth Temperature

Determination of the optimum growth temperature of *Mycobacterium* sp. Strain S65 was performed at temperatures of $25^{\circ}C$, $30^{\circ}C$, and $35^{\circ}C$ in YTS_{1000B} medium. Optimal growth temperature of Strain S65 occurred at $30^{\circ}C$, and Strain S65 was unable to grow at $35^{\circ}C$ (Figure 5.2), indicating that Strain S65 cannot survive in the human body, and is thus unlikely to be a human pathogen. Bacterial strains that are pathogenic to humans grow best at about body temperature ($37^{\circ}C$; Pelczar *et al.*, 1993). Growth continued until 150 hours, when cells grown at $25^{\circ}C$ to $30^{\circ}C$ had just reached the stationary phase. Although optimal growth temperature was $30^{\circ}C$, the growth was not significantly different at room

temperature of 25°C. Thus for practical reasons all the experiments were conducted at 25°C

5.4.4 Biodegradation Potential of *Mycobacterium* sp. Strain S65 in Liquid Microcosms

Prior measurements of mineralization activity in aerobic liquid microcosms had shown that *Mycobacterium* sp. Strain S65 rapidly consumed, as its sole carbon and energy source, Pyrene (61% cumulative mineralization), Phenanthrene with three days lag (61% cumulative mineralization), and to a lesser extent Fluoranthene with no lag (24% cumulative mineralization), but not Naphthalene, Anthracene or Fluorene (Figure 3.8 in Chapter 3).

5.4.5 Biodegradation Potential of *Mycobacterium* sp. Strain S65 in Soil Microcosms

The mineralization ability of the *Mycobacterium* sp. Strain S65 for soil-aged phenanthrene was determined in standard aerobic soil (solid) serum bottle microcosms at ambient temperature 25°C for 360 days. The sampling regime continued until no further mineralization occurred (cumulative mineralization reached a plateau). Analysis of mineralization results for non-aged (Day 0) phenanthrene revealed that pyrene mineralization followed similar kinetics in liquid and soil microcosms, with a 3-day lag phase before mineralization began. Results of ANOVA are presented in Table 5.4.

For non-aged microcosms (Day 0), the compound only started to mineralize after a 3-day lag phase. Thereafter, phenanthrene mineralization by *Mycobacterium* sp. Strain S65 continued, until, for additions of 250 mg L^{-1} and 500 mg L^{-1}, it reached a cumulative maximum at 9 days of 18.5% and 20.0%, respectively in sandy-loam low organic matter soil, and 13.5% and 14.5% in clay-loam high organic matter soil (Figures 5.3 and 5.4). While *Mycobacterium* sp. Strain S65 metabolized the soil-housed compound at a moderate rate, in aerobic liquid microcosms it was metabolized at a much higher rate (61% cumulative mineralization).

Mineralization results for 30-day aged phenanthrene (Day 30) showed that *Mycobacterium* sp. Strain S65 consumed phenanthrene (initially containing 250 or 500 mg L^{-1} Day 0 level, respectively), to a greater extent (cumulative 14.5% and 13.0%, %) in

sandy-loam low organic matter soil, than the cumulative values of 11.5% and 10.5% %, for clay-loam high organic matter soil (Figures 5.5 and 5.6) for the same respective concentrations.

For phenanthrene soil-aged for 180 days, *Mycobacterium* sp. Strain S65 utilized this compound (initially containing 250 or 500 mg L^{-1}, Day 0 level, respectively), but phenanthrene mineralization declined to a cumulative 7.0% and 8.35%, respectively in sandy-loam low organic matter soil, and to a cumulative 5.0% and 6.25% in clay-loam high organic matter soil, for the same respective concentrations.

Lastly, after 360 days' ageing of the phenanthrene, *Mycobacterium* sp. Strain S65 was capable of utilizing the compound (initially containing 250 or 500 mg L^{-1} day 0 levels, respectively), but cumulative phenanthrene mineralization declined to its lowest extent of 4.25% and 5.5%, respectively, in sandy-loam low organic matter soil, and 2.5% and 4.0% in a clay-loam high organic matter soil for the same respective concentrations.

Persistent organic compounds availability to soil microorganisms often declines with time. Slowly biodegradable organic compounds in soils decline with time, but at a steadily declining rate, such that after several years little or no further loss can be detected. Because microorganisms present in field soils can metabolize such compounds, the chemicals appear to become less bioavailable with time. Soil sequestration of organic molecules with ageing, making them somehow less available over time, occurs with a number of organic pollutants (Alexander, 1995).

In practical bioremediation practices in a PAHs-contaminated soil site, despite the decline in bioavailability of aged compounds, a portion remains in a form that can be metabolized by microorganisms and, if the substance is toxic, a part is still present in a form that can do harm. Hence, remediation is still warranted by some means, and bioremediation is feasible. That the microbiological treatment is effective in reducing the total concentration and the bioavailability of the aged compounds is evident from a laboratory investigation in which several PAHs were aged in sterile soil, and the uptake of the aged PAHs by other species of higher organism (earthworms) was determined before ageing, after ageing but before inoculation, and after the addition of PAH-utilizing bacteria to soil containing the aged or freshly added hydrocarbon (Tang *et al.*, 1998; Alexander, 1999; Alexander and Alexander, 2000;). However, the decline in availability

of the compound to bacteria and earthworms as a result of ageing as well as the combined effect of the processes of sequestration and biodegradation explains why the amount of PAH taken up by the organisms is reduced. It is also noteworthy that a small amount of hydrocarbon was still found in the worm tissue even after ageing and bioremediation. Similar effects have been observed with plants (Alexander, 1999; Alexander and Alexander, 2000).

PAHs are one of the more prominent groups of persistent compounds. Among many of the less readily metabolized compounds that remain in soil years after their first introduction, a bi- or multiphasic rate of disappearance is evident. In the first few months, the degradation rate is rapid, but then it slows dramatically to a point that little is lost succeeding years. This may reflect the sequestering of the compound in a manner that makes it less available to microorganisms, that is, the so-called "ageing" of the molecule. It is now evident that many compounds are subject to a time-dependent sequestration in soil, including PAHs, chlorinated hydrocarbons, and chemicals of several structural classes (Alexander, 1999). From the viewpoint of actual field bioremediation, the frequent observations that bioremediations do not reduce the levels of target pollutants below regulatory levels may be misleading because the amount that remains, which is determined by vigorous extraction of the soil, may be largely unavailable and thus pose little risk. The bioremediation, therefore, may have met its actual objectives even if the analytical procedure suggests a failure.

The results of this study revealed that the environmental *Mycobacterium* sp. Strain S65 successfully mineralized phenanthrene (PAH) in liquid microcosms. But, in contrast, *Mycobacterium* sp. Strain S65 performed relatively poorly as a bioaugmentation agent for the microbial degradation of soil-aged ^{14}C-labelled phenanthrene in different soil environments. There were a number of possible reasons for this, including ageing, insufficiency of the added PAH to stimulate the microbial strain to degrade the chemical(s), uneven distribution of microorganism within the soil during the inoculation process and possibly uneven distribution of the added PAH within the unmixed soil, strong adsorption of the PAH to soil particles as well as other environmental parameters, which could interact to reduce the survival power and the ability of the strain S65 to perform as an appreciable bioremediator.

This study suggests that more research should be carried out to assess the potential of *Mycobacterium* sp. Strain S65 in bioremediation, and on rendering its biodegradation of PAHs more efficient. To overcome the problem of hydrophobicity and limited availability of PAHs, surfactants could play a major role in soil remediation. The use of surfactants has been shown to enhance the biodegradation rate of recalcitrant compounds in soil (Aronstein and Alexander, 1993; Cookson, 1995; Deziel *et al*., 1996). For example, the use of the surfactant polyoxyethylene sorbitan monooleate (Tween 80) as a soil amendment remarkably enhanced the rate of biodegradation of endosulfan by *Pseudomonas aeruginosa* (Jayashree and Vasudevan, 2007).

5.4.6 Assessment of Possible Viable Culturable Microbial Population in Sterile Control Soils

Serial dilution and spread-plate techniques served to assess the possible presence of a viable culturable microorganism population in different sterile soils (CFU g^{-1}). Plates incubated at ambient temperature (25°C) for one week were routinely examined by dissecting microscope to detect and enumerate microbial colonies. No microbial colonies were observed, indicating that no competitive microbial strain, which might contribute to the mineralization of ^{14}C-labelled phenanthrene, was present alongside *Mycobacterium* sp. Strain S65, throughout the entire ageing process.

5.4.7 Bioavailability and other Factors that Limit PAHs-Biodegradation

A bioaugmentation study was carried out for 360 days to serve to assess the degradation kinetics for *Mycobacterium* sp. Strain S65 in different soil environments. The mineralization experiment indicated that, in spite of a sufficient quantity of phenanthrene (250 or 500 mg L^{-1}), which were well above Canadian regulatory criteria (40 mg L^{-1}), for soil, the inoculated degrader organism was less competent in degrading phenanthrene in soil environments than in liquid microcosms (YTS_{250} broth medium).

This may in part be related to the fact that the 100 µL (1.0×10^5 dpm ^{14}C-labelled substrate) of added phenanthrene is applied in a localized manner to aliquot of 30 g of soil where it can adsorb strongly to the soil particles. Given that the soil in the

microcosms cannot be mixed, given experimental limitations, the phenanthrene is limited to a certain area which may be more or less accessible to the microorganism.

Due to the strong relationship of PAHs with the organic fractions of contaminated soil and sediment, which may however be potential nutrient sources for the microorganism, little of the PAH mass is in the aqueous phase in such systems. Consequently, the greater portion of PAH mass is not directly available to microorganisms. As the PAHs in the aqueous phase are depleted, continuing degradation requires mass transfer from the interior of a non-aqueous phase to a location accessible to microbial cells. Therefore in such cases, rates of desorption from the non-aqueous phase(s) would drive overall rates of biodegradation. Nevertheless, bioremediation involves the use of biological systems, typically relying on microbial processes, and availability of given compounds to degrader organisms, in order to remove a target contaminant from soil. However, there are factors other than bioavailability which may limit the biodegradation of the PAHs. These factors include: (**i**) low numbers of organisms capable of degrading the PAHs; (**ii**) inherently slow enzyme activity for a given compound; (**iii**) slow rates of degradation exacerbated by competition among substrates; (**iv**) depletion of growth substrates; and (**v**) the accumulation of inhibitory products (Singh and Ward, 2004).

Nevertheless, transformation and fate of contaminants in soils as controlled by both the properties of the soil and the nature and type of contaminants. However, bioavailability is impacted not only by soil type, but also contact time (*i.e.* ageing), which reduces the concentration of chemical available for uptake by organisms or which causes toxicity (Alexander, 1995, 1999; Alexander and Alexander, 2000; Vig *et al.*, 2002). Soil properties, including pH, organic matter and clay contents, surface charge, soil solution composition, and contaminant physico-chemical properties, including ionic and oxidation state, molecular structure, polarity, aqueous solubility, and lipophilicity, are responsible for controlling the behaviour of contaminants. These properties in turn affect the fraction of contaminants that may be bioavailable in a given soil. However, this is further impacted upon by the duration of contact between contaminants and soils, with bioavailability declining with ageing (Alexander, 1995, 1999; Alexander and Alexander, 2000; Vig *et al.*, 2002; Naidu *et al.*, 2003, 2008; Naidu and Bolan, 2008).

In addition to these factors, limitations in oxygen, other electron acceptors or other nutrients might also explain limitations in PAHs-biodegradation (Madsen *et al.*, 1996). However, Wick *et al.*, (2001) stated that "limited bioavailability occurs when the capacity of the microbial biomass to consume a substrate exceeds the capacity of its environment to deliver the substrate."

Bioavailability may be the most important factor in determining the feasibility of bioremediation of PAHs, as, in most cases, mass transfer limitations prevent the full exploitation of microbial degradative potential (Bosma *et al.*, 1997). Several mechanisms work together to influence bioavailability, and different mechanisms predominate in any given situation, however, they are still not fully understood. It is usually assumed that the water-dissolved fraction of chemicals is the only one available to microorganisms. Therefore, degradation rates are dependent on the mass transfer rates of PAHs from the solid or soil-bound phase to the aqueous phase, and desorption of PAHs from soil is considered as the controlling factor in their biodegradation (Volkering *et al.*, 1992; Pignatello and Xing, 1996; Labana *et al.*, 2007).

5.5 CONCLUDING REMARKS

This research work was conducted to assess the mineralization capability of the environmental ***Mycobacterium* sp. Strain S65** based on $^{14}CO_2$ evolution resulting from the mineralization of soil-aged ^{14}C-labelled recalcitrant PAH substrates (specifically soil-aged ^{14}C-labelled phenanthrene) in different farm soils environments which were previously sterilized with 2.5 Mrad of gamma (γ)-irradiation from a ^{60}Co source.

The experimental design consisted of a factorial combination of four types of soil (Sandy-Loam, Sand, Clay, Clay-Loam), two levels of soil organic matter (high and low), and two concentrations of phenanthrene (250 and 500 mg L^{-1}), with six replicates per treatment (48 microcosms per inoculation date). Cumulative PAHs mineralization percentages were determined by radioactive tracer analysis. The experiment ran for 360 days during which the effects of ageing were assessed at four times (0, 30, 180, and 360 days).

Soil-aged ^{14}C-labelled phenanthrene's bioavailability in different soil environments was determined in the presence of *Mycobacterium* sp. Strain S65,

introduced as a bioaugmentation agent, into soil microcosms. Phenanthrene's degradation in soil microcosms, particularly the three day lag period, followed a similar pattern as was seen in liquid microcosms. Gamma-ray sterilized farm soil microcosms were spiked with 1.0×10^5 dpm ^{14}C-labelled phenanthrene and allowed to age for 0, 30, 180 or 360 days before inoculation.

With no ageing effect (Day 0), phenanthrene mineralization did not begin immediately, the *Mycobacterium* sp. Strain S65 only utilized the substrate after a 3-day lag phase. Phenanthrene (initially at 250 mg L^{-1} or 500 mg L^{-1}) mineralization continued thereafter, reaching a cumulative maximum of 18.5% and 20.0% after 9 days, respectively in sandy-loam soil with low organic matter, but reaching a lower cumulative maximum 13.5% and 14.5% after 9 days in clay-loam soil with high organic matter. The sampling regime continued until no further mineralization occurred (cumulative mineralization reached a plateau). The non-aged soil-borne compound was metabolized at moderate rate, whereas, in contrast, the non-aged compound in aerobic liquid microcosms was mineralized to a much greater extent (61% cumulative mineralization).

For 30-day aged phenanthrene, *Mycobacterium* sp. Strain S65 gave a maximum cumulative mineralization of 13.0% and 14.5% after 9 days respectively for initial additions of 250 and 500 mg L^{-1} phenanthrene in sandy-loam soil with low organic matter, but only reached maximum cumulative mineralization of 10.5% and 11.5% after 9 days in clay-loam soil with high organic matter. For 180 and 360-day aged phenanthrene, *Mycobacterium* sp. Strain S65 utilized the phenanthrene, but cumulative phenanthrene mineralization declined to less than 10%.

Overall, the high clay-high organic matter soil showed the lowest degradation rates. This is likely the result of the greater number of potential PAH adsorption binding sites on clay particles and on organic matter, than on larger soil particles such as those found in a low organic matter sand soil. Our results indicating that less PAH degradation occurred in high organic matter soils, is confirmed by previous studies (Manilal and Alexander, 1991; Weissenfels *et al.*, 1992; Johnsen *et al.*, 2005).

As a further basis of comparison, mineralization of non-aged phenanthrene by *Mycobacterium* sp. Strain S65 in YTS_{250B} liquid medium (*i.e.* positive control) and heat-killed *Mycobacterium* (*i.e.* negative control), were also measured over the same

incubation period of mineralization treatments. Serial dilution and spread-plate techniques served to assess the possible presence of a viable culturable microorganism population (CFU g^{-1}) in different sterile soil microcosm bottles (*i.e.* sterile control). No microbial colonies were observed, indicating that no competitive microbial strain, which might contribute to the mineralization of ^{14}C-labelled phenanthrene (PAH), was present alongside *Mycobacterium* sp. Strain S65. All three controls were applied in mineralization experiments throughout the entire ageing process.

On this conclusion rests a visionary point of view, which will enlighten future strategies regarding the bioavailability, biodegradation, bioremediation, and biodetoxification of ever-increasing amounts of organic xenobiotic (*i.e.* organic chemicals that are not products of biosynthesis). Greer (2008) has opined that such studies should investigate innovative techniques to use microorganisms to degrade organic pollutants and thus cleanse the Earth of man's indiscriminate pollution. To reach these lofty goals we must identify and profile the microorganisms that are naturally present, given that we are only familiar with about 1% of microorganisms that are actually out there. Greer further elaborates that his and fellow bioremediation scientists' work should look at the entire genomic content of a gram of soil, rather than the genome of a particular organism. This assesses the potential of whole microbial communities and identifies those which could help in remediation. This study strongly supports the concept of there being a significant role of *Mycobacteria* in PAHs-biodegradation, bioremediation, and biodetoxification in contaminated environments.

Table 5.1: Detailed Chemical Characteristics of Soils

Soil Number	Soil Sample	pH	EC	MEHLICH Extraction				Total N	Organic		2 M KCl Extraction		
				P	K	Ca	Mg		C	Matter	$N\text{-}NH_4$	$N\text{-}NO_3$	Moisture Content
			$mS\ m^{-1}$	$mg\ kg^{-1}$	$mg\ kg^{-1}$	$mg\ kg^{-1}$	$mg\ kg^{-1}$	%	%	%	$mg\ kg^{-1}$	$mg\ kg^{-1}$	%
1	Sandy-Loam LOM	7.34	206	141	78	2285	72	0.1	1.2	2.2	4.1	12.25	10.93
2	Sand HOM	7.42	171	246	86	2266	106	0.24	4.4	7.6	9.0	4.72	27.55
3	Clay LOM	6.65	301	54	320	4774	838	0.28	3.4	5.8	14.4	14.69	37.78
4	Clay-Loam HOM	5.91	327	10	92	2566	422	0.41	5	8.6	15.8	0.90	28.84
5	Sand VLOM	7.8	39.4	13	21	358	45	0	0.1	0.2	1.2	0.52	6.04
6	LoamVHOM	4.8	385	20	63	1471	160	0.4	6.6	11.3	22.2	0.63	33.00

		Cation Exchange Capacity based on a $BaCl_2$ Extraction in cmol(+) /kg								
		Ca	Mg	K	Na	Mn	Fe	Al	CEC	% B.S.
									$cmol(+)\ kg^{-1}$	
1	Sandy-Loam LOM	13.8	0.86	0.251	0.023	0.015	0	0	15.0	99.9
2	Sand HOM	15.9	1.43	0.373	0.033	0.025	0	0	17.8	99.9
3	Clay LOM	36.4	11.68	0.52	0.538	0.241	0	0	49.4	99.5
4	Clay-Loam HOM	27.7	7.07	0.452	0.061	0.329	0	0	35.6	99.1
5	Sand VLOM	1.4	0.02	0.074	0.003	0.015	0	0	1.5	99.0
6	Loam VHOM	16.2	3.3	0.237	0.057	1.69	0	0.591	22.1	89.9

HOM: High organic matter, **LOM:** Low organic matter, **VHOM:** Very high organic matter, **VLOM:** Very low organic matter

Table 5.2: Soil Physical Characteristic and Textural Class

Soil Number	Soil Type	Clay (%)	Sand (%)	Silt (%)	Textural Class
1	Sandy - Loam (low organic matter)	16.2	62.0	21.8	Sandy - Loam
2	Sand (high organic matter)	4.1	86.6	9.3	Sand
3	Clay (low organic matter)	50.0	15.1	34.9	Clay
4	Clay - Loam (high organic matter)	25.0	39.0	36.0	Clay - Loam
5	Sand (very low organic matter)	0.5	98.5	1.0	Sand
6	Loam (very high organic matter)	11.5	49.0	39.5	Loam

Table 5.3: Experimental Design for Aerobic Soil and Liquid Microcosm Study of ^{14}C-Labelled Phenanthrene Mineralization for each Ageing Time

Concentration (PAH)	250 ppm						500 ppm						Radiochemical ^{14}C-Labelled Phenantrene (dpm)	Bacterial Strain *Mycrobacterium* sp. Strain S65		
T&C	Treatment			Control (-)			Treatment			Control (-)				Volume (mL)	OD_{600}	CFU g^{-1}
Soil1	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1 x 10^5	1	0.1	1 x 10^7
Soil2	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1 x 10^5	1	0.1	1 x 10^7
Soil3	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1 x 10^5	1	0.1	1 x 10^7
Soil4	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1 x 10^5	1	0.1	1 x 10^7
YTS_{250B}: C (+)	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1 x 10^5	1	0.1	1 x 10^7

PAH: Polycyclic Aromatic Hydrocarbons
T: Treatment
C: Negative control (-): Heat-killed (sterilized by autoclaving at 121^{o}C for 20 minutes, *i.e.* abiotic controls)
Soil 1: Sandy - Loam low organic matter (**LOM**)
Soil 2: Sand high organic matter (**HOM**)
Soil 3: Clay low organic matter (**LOM**)
Soil 4: Clay - Loam high organic matter (**HOM**)
Concentration (**ppm**)**:** part per million (*i.e.* mg L^{-1})
YTS_{250B}: Positive Control: **C** (+): YTS_{250} µg L^{-1} - broth (containing 0.25 g L^{-1} each of yeast extract, tryptone, starch in distilled water, pH 7.0, and no granulated agar) medium
Dpm: Disintegrations Per Minute (level or values of radioactivity for mineralization analysis)
OD_{600}: Optical Density of a Given Culture at Wave Length of 600 nm as measured by Spectrometry
CFU g^{-1}: Colony-Forming Unit of Microorganism per Gram of Soil

Table 5.4. Significance of Single Factor Effects and Interactions on Cumulative Phenanthrene Mineralization.

Source	Probability
R	NS
S	**
C	**
AT	**
IT	**
S*AT	**
S*C	*
AT*C	*
S* IT	**
C* IT	**
AT * IT	**

R: Replicate; **S**: Soil, **C**: Phenanthrene Concentration, **AT**: Aging Time, **IT**: Incubation Time

NS: Non-Significant**,** ($P > 0.05$); *, Significant ($P \leq 0.05$); **, Highly Significant ($P \leq 0.01$)

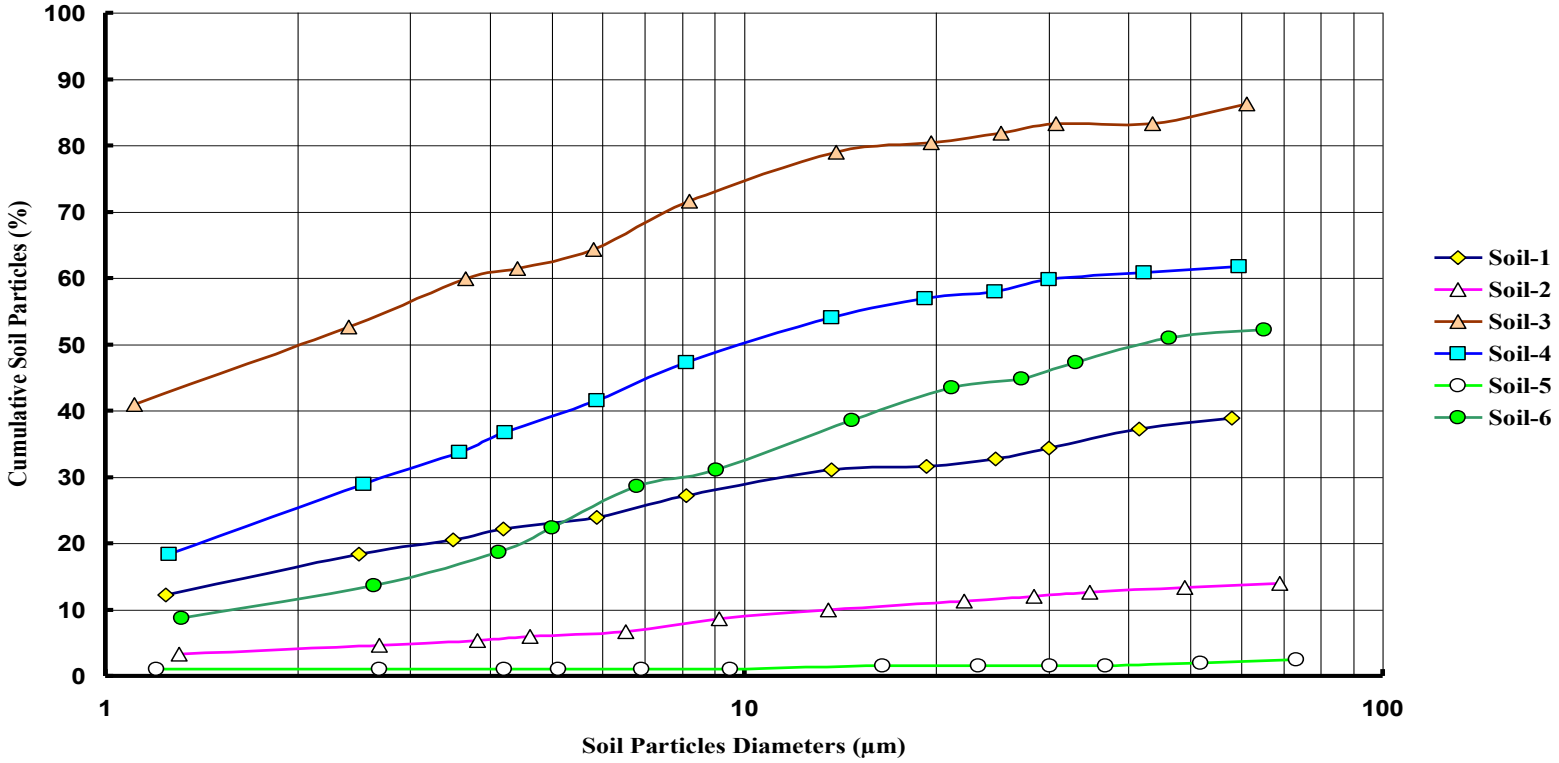

Figure 5.1: Soil Particles Size Distribution

Soil-1: Sandy - Loam low organic matter (**LOM**)
Soil-2: Sand high organic matter (**HOM**)
Soil-3: Clay low organic matter (**LOM**)
Soil-4: Clay - Loam high organic matter (**HOM**)
Soil-5: Sand very low organic matter (**VLOM**)
Soil-6: Loam very high organic matter (**VHOM**)

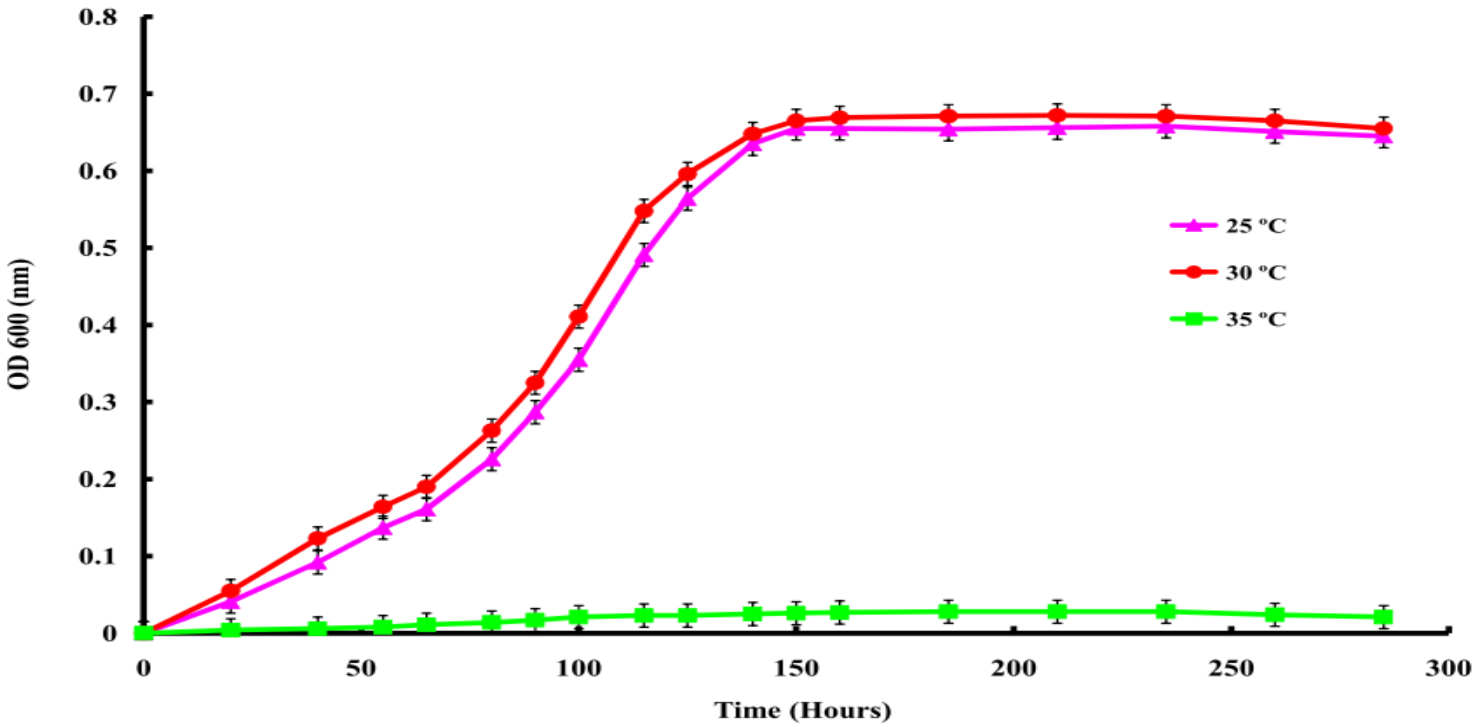

Figure 5.2: ***In vitro*** **growth curves of** ***Mycobacterium*** **sp. Strain S65 in YTS_{1000B} and pH 7.0 at Different Temperatures.**

S65 was inoculated into YTS_{1000B} in triplicate at three temperatures. The rate of growth was measured by absorbance as the measurement of optical density (OD) at 600 nm.
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

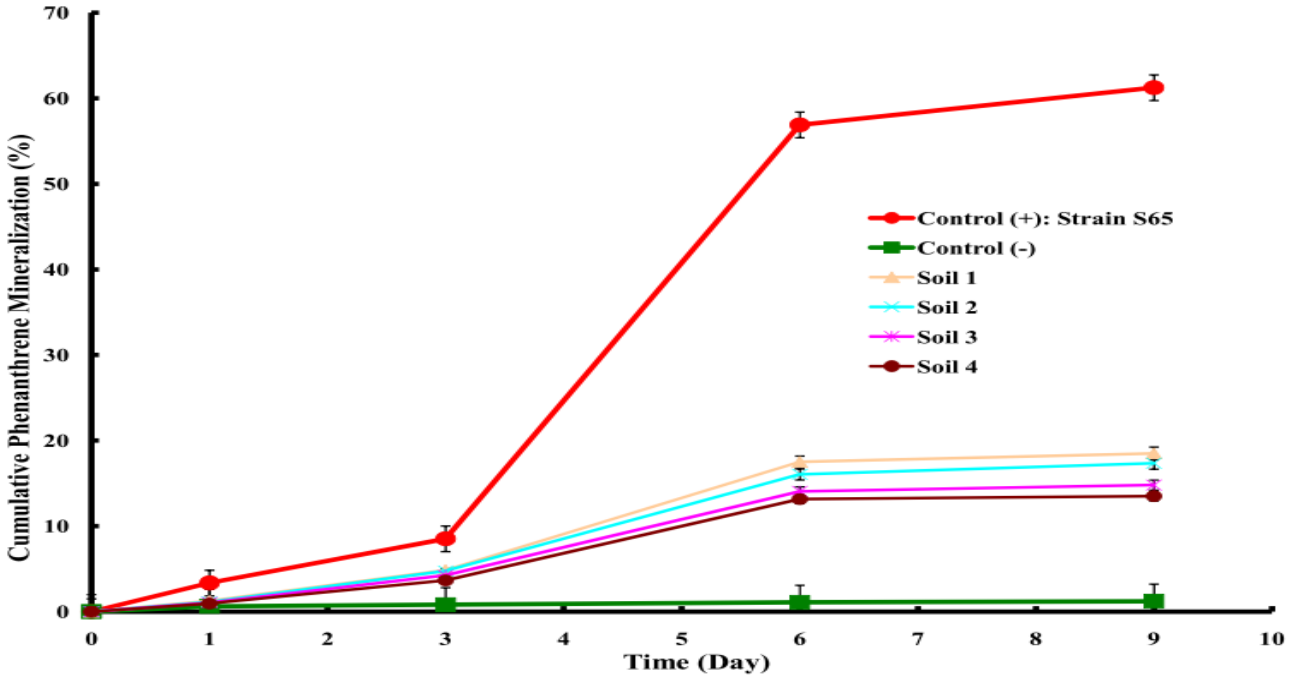

Figure 5.3: Mineralization of Phenanthrene [250 mg L^{-1} (ppm) concentration] by *Mycobacterium* sp. strain S65 in Different Soil Environments at Day (0)

Soil 1: Sandy-Loam low organic matter; Soil 2: Sand high organic matter
Soil 3: Clay low organic matter; Soil 4: Clay-Loam high organic matter
Control (-): Autoclaved *Mycobacterium* sp. strain S65 at 121°C
Control (+): *Mycobacterium* sp. strain S65 in YTS_{250}-Broth medium
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

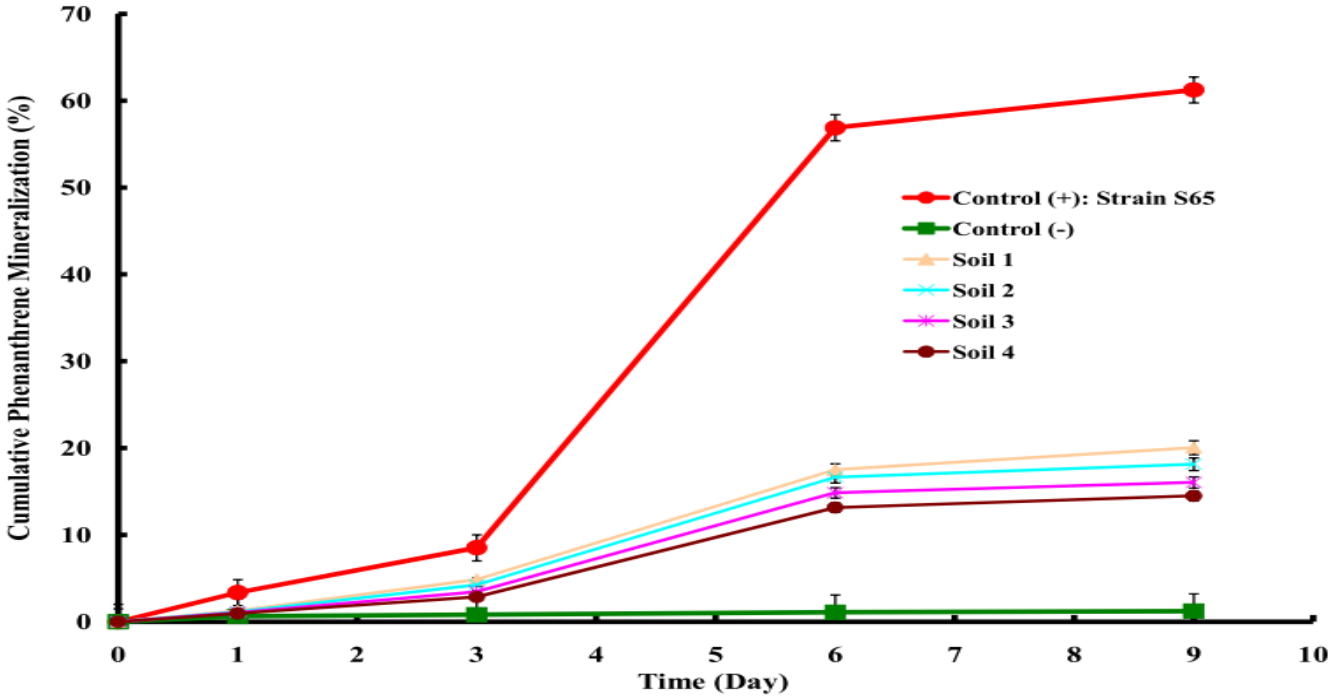

Figure 5.4: Mineralization of Phenanthrene [500 mg L^{-1} (ppm) concentration] by *Mycobacterium* sp. strain S65 in Different Soil Environments at Day (0)

Soil 1: Sandy-Loam low organic matter; Soil 2: Sand high organic matter
Soil 3: Clay low organic matter; Soil 4: Clay-Loam high organic matter
Control (-): Autoclaved *Mycobacterium* sp. strain S65 at 121°C
Control (+): *Mycobacterium* sp. strain S65 in YTS_{250}-Broth medium
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

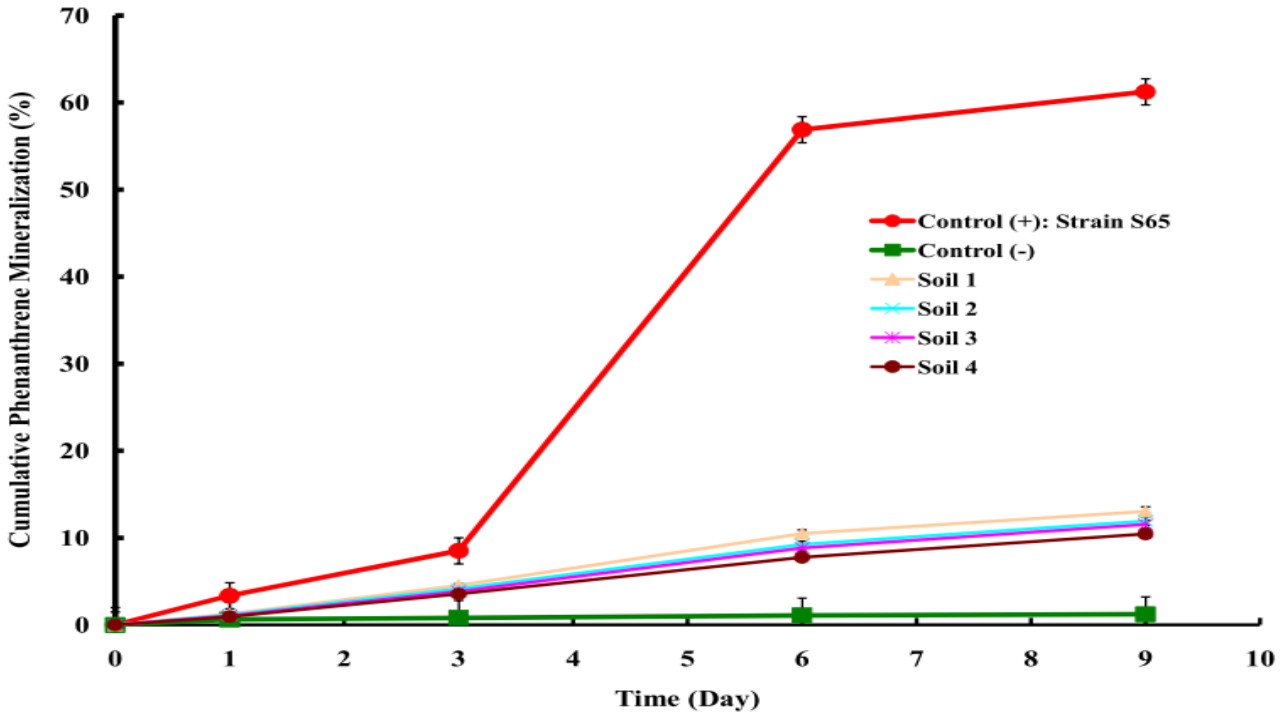

Figure 5.5: Mineralization of Phenanthrene [250 mg L^{-1} (ppm) concentration] by *Mycobacterium* sp. strain S65 in Different Soil Environments at Day (30)

Soil 1: Sandy-Loam low organic matter; Soil 2: Sand high organic matter
Soil 3: Clay low organic matter; Soil 4: Clay-Loam high organic matter
Control (-): Autoclaved *Mycobacterium* sp. strain S65 at 121°C
Control (+): *Mycobacterium* sp. strain S65 in YTS_{250}-Broth medium
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

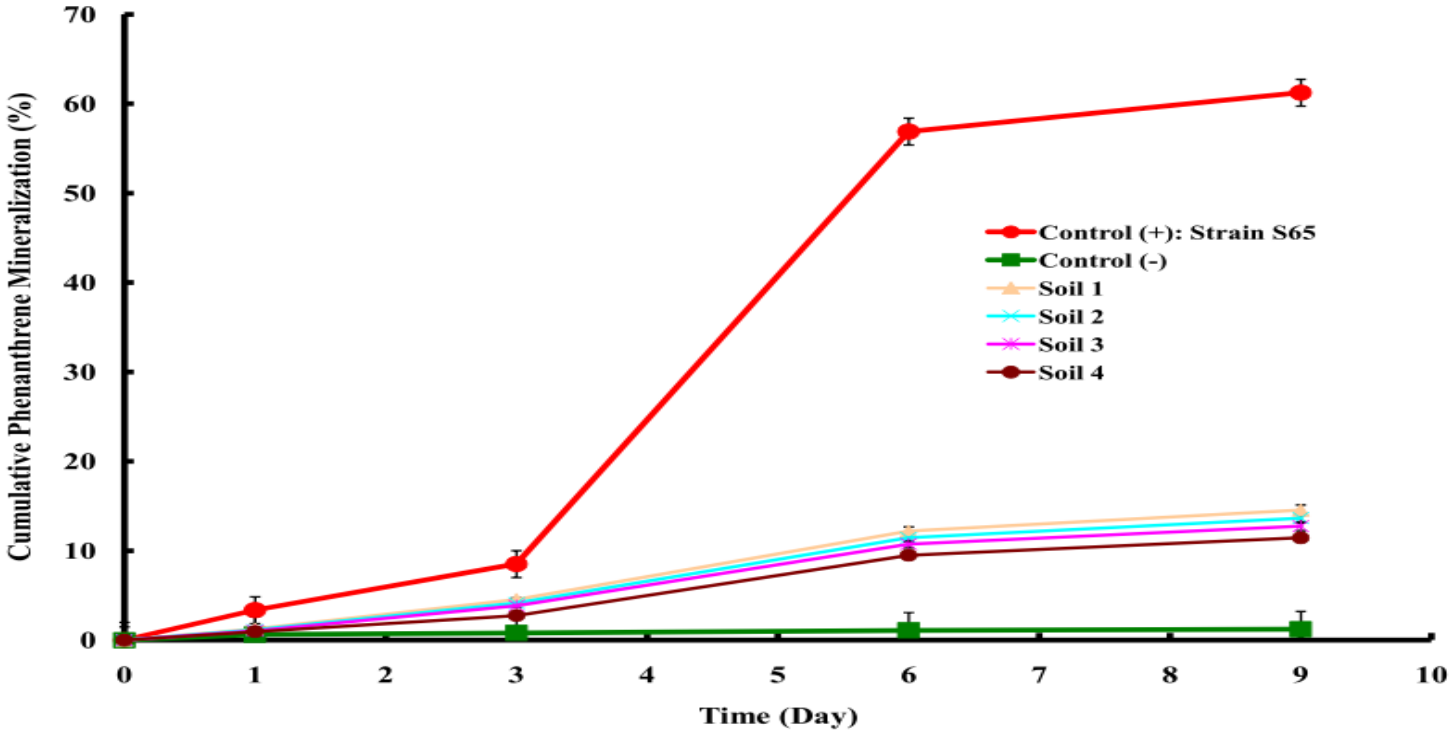

Figure 5.6: Mineralization of Phenanthrene [500 mg L^{-1} (ppm) concentration] by *Mycobacterium* sp. strain S65 in Different Soil Environments at Day (30)

Soil 1: Sandy-Loam low organic matter; Soil 2: Sand high organic matter
Soil 3: Clay low organic matter; Soil 4: Clay-Loam high organic matter
Control (-): Autoclaved *Mycobacterium* sp. strain S65 at 121°C
Control (+): *Mycobacterium* sp. strain S65 in YTS_{250}-Broth medium
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

PREFACE TO CHAPTER 6

In Chapter 4, different experiments were conducted to characterise bacterial strains at the micromorphological and molecular-genetic level. Molecular biology and biotechnology techniques were used to optimize their natural biodegradative capacities. The isolated and partially characterized bacterial strain S65 (**GenBank designation: *Mycobacterium* sp. Strain S65**), was able to mineralize selected recalcitrant PAHs-compounds in aerobic liquid microcosms. Strain S65 rapidly consumed as its sole carbon and energy source, Pyrene (61% cumulative mineralization), Phenanthrene (61% cumulative mineralization), and to a lesser extent Fluoranthene (24% cumulative mineralization). Strain S65's mineralization kinetics showed this *Mycobacterium* to be a new and unique member of the xenobiotic-degrading mycobacteria. However, experimental investigations for utilization of selected aged ^{14}C-labelled PAHs in different soil environments were needed to complete the bioavailability studies.

In Chapter 5, assessment of bioavailability of aged ^{14}C-labelled **phenanthrene** (a PAH) in different soil environments was determined by introducing the potentially active microorganism (*i.e.***,** *Mycobacterium* sp. Strain S65), as a bioaugmentation agent, into different soil microcosms.

In Chapter 6, assessment of bioavailability of aged ^{14}C-labelled **pyrene** (a PAH) in different soil environments was investigated as well by introducing the potentially active microorganism (*i.e.*, *Mycobacterium* sp. Strain S65), as a bioaugmentation agent, into soil microcosms.

This manuscript was published in ***Biological Engineering*** which is published by American Society of Agricultural and Biological Engineers (ASABE).

Jazestani, J. and S. O. Prasher. (2011). Microbial Degradation of Soil-Aged 14C-Labeled Pyrene by an Environmental *Mycobacterium* sp. Strain S65 in Different Soil Environments. ***Biological Engineering***. 3(3): 127-149.

CHAPTER 6

Microbial Degradation of Soil-aged ^{14}C-labelled Pyrene by Mycobacterium sp. Strain S65 in Different Soil Environments

6.1 ABSTRACT

A polycyclic aromatic hydrocarbon (PAH) degrading ***Mycobacterium*** (designated: ***Mycobacterium*** **sp. Strain S65** in **Genbank)**, previously isolated and characterized from a jet-fuel contaminated site at the Sept-Iles, QC, Canada airport, was found to be competent in mineralizing selected recalcitrant PAH compounds of minimal water solubility and low bioavailability in soils when grown in yeast extract-tryptone-soluble starch medium. Based on $^{14}CO_2$ evolution, Strain S65 was capable of 61% cumulative mineralization of pyrene over 14 days in aerobic liquid microcosms.

The experimental design consisted of a factorial combination of four types of soil (Sandy-Loam, Sand, Clay, Clay-Loam) with two levels of soil organic matter (high and low), and two concentrations of pyrene (1000 mg L^{-1} and 500 mg L^{-1}), with six replicates per treatment (48 microcosms per inoculation date). One set of 48 aged microcosms were inoculated after each ageing period (0, 30, 180 or 360 days), with three replicates per treatment (*i.e.* 24 microcosms), each receiving 1.0×10^7 cfu of live *Mycobacterium* sp. Strain S65 (*i.e.* treatment) and remaining three replicates per treatment receiving heat-killed *Mycobacterium* (*i.e.* negative control), for an overall total of 192 microcosms. The $^{14}CO_2$ evolved over 1, 3, 6, or 9 days and trapped in a KOH solution was measured by scintillation counter, and cumulative mineralization was calculated as a percent of initial pyrene. As a further basis of comparison, mineralization of non-aged pyrene in culture broth was also measured over the same incubation period, serving as a positive control: (treatment *vs.* heat-killed × concentrations × aging time × replicates, $n = 2 \times 2 \times 4 \times 3 = 48$). Serial dilution and spread-plate techniques served to assess the possible presence of a viable culturable microorganism population (CFU g^{-1}**)** in different sterile soils after different incubation times (*i.e.*, sterile control, soils × ageing time × replicate, $n = 4 \times 4 \times 3 = 48$). No microbial colonies were observed on solid media, indicating that no

culturable competitive microbial strain, which might contribute to the mineralization of ^{14}C-labelled pyrene, was present at any time during the ageing process.

Immediately after inoculation and without any lag phase (Day 0), *Mycobacterium* sp. Strain S65 metabolized non-aged pyrene present in the soil. Cumulative mineralization of pyrene (added at concentrations of 1000 and 500 mg L^{-1}) reached 19% and 18.5% respectively after 24 hours, and 21% and 20% after 9 days in sandy-loam soil with low organic matter. In constrast, in clay-loam soil with high organic matter, cumulative pyrene mineralization was only 15.5% and 14.5% respectively after 9 days, distinctly less mineralization than in sandy-loam soil with low organic matter. Sampling continued until no further mineralization occurred (cumulative mineralization reached a plateau). Soil-aged pyrene was metabolized at moderate rate by *Mycobacterium* sp. Strain S65; whereas non-aged pyrene was mineralized at a greater rate (61% cumulative mineralization) in an aerobic liquid microcosm.

For initial additions of 1000 and 500 mg L^{-1} pyrene, followed by 30 days of ageing, *Mycobacterium* sp. Strain S65 showed a maximum cumulative mineralization of 14% and 13.25% respectively after 9 days in sandy-loam soil with low organic matter, but only showed 12.5% and 11.5% cumulative mineralization after 9 days in clay-loam soil with high organic matter. For 180 and 360-day aged pyrene, cumulative pyrene mineralization was less than 10%, a significant drop from less aged pyrene-soil microcosms. This suggests that as the ageing proceeds in the soil medium, the pyrene becomes less accessible for degradation. Nonetheless, this study supports the idea that *Mycobacterium* sp. Strain S65 has the potential to be useful in bioremediation and biodetoxification of high molecular weight PAHs-contaminated sites.

Key Words: *Mycobacterium* sp. Strain S65, Microbial Degradation, Bioavailability, Biodegradation Kinetics, Bioremediation, Bioaugmentation, Isolation, Mineralization, Ageing, PAHs, Pyrene and Soil Environments.

6.2 INTRODUCTION

In the last century, anthropogenic practices such as industrial processing, petroleum spills and incomplete combustion of fossil fuel have led to an accumulation of polycyclic aromatic hydrocarbons (PAHs) in the environment. PAH contamination is a growing environmental concern (Simpson *et al.*, 1996). Many industrial sites have been contaminated by toxic hydrophobic organic contaminants (HOCs), such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Liu *et al.*, 2007).

The PAHs have been the subject of scientific study for many years, and a number of excellent reviews have been published on PAHs biodegradation (Environment Canada, 1994; Alexander, 1999; Alexander and Alexander, 2000; Singh and Ward, 2004; Hwang *et al.*, 2007). These chemicals represent a large family of organic compounds that are considered environmental contaminants. PAHs are widespread organo-pollutants in ecosystems and can be present in quantities that threaten environmental and human health. PAHs are of concern because some PAHs constitute a significant group of chemical carcinogens (Sherma, 1993 and Dabestani and Ivanov, 1999; Santos *et al.*, 2008). Hence, as a class of compounds, PAHs have been classified as carcinogens, mutagens, and immunosuppressants (Hap *et al.*, 2006).

The PAHs of most concern with respect to the environment and human health are a class of neutral, non-polar organic compounds varying in size and consisting of two or more fused benzene rings in linear, angular, or cluster arrangements. These compounds are all hydrophobic, as illustrated by their relatively high octanol-water partition coefficients (K_{ow}) and low solubility in water (Table 2.1 in Chapter 2). Hydrophobicity increases with an increase in molecular size, with aqueous solubilities declining from the low mg/L range for two- and three-ring compounds to 1 $\mu g\ L^{-1}$ or below for five- and six-ring compounds (Singh and Ward, 2004).

Due to the possible acute and chronic toxicity, primarily associated with the lower molecular weight (LMW)-PAHs, and the potential carcinogenicity associated with the higher molecular weight (HMW)-PAHs, the United States Environmental Protection Agency (USEPA) has placed 16 PAHs on their Priority Pollutant List, given their environmental importance and as representative of PAHs as a class of compounds (Table 2.1 and Figure 2.1 in Chapter 2; Singh and Ward, 2004; Liu *et al.*, 2007). Polycyclic

aromatic hydrocarbons (PAHs) are known to be genotoxic. The International Agency for Research on Cancer (IARC) has identified 15 PAHs, including 6 of the 16 USEPA-regulated PAHs, as "reasonably anticipated to be human carcinogens based on sufficient evidence of carcinogenicity in experimental animals" (National Toxicology Program, 2001). The USEPA classified 7 PAHs as group B2 "probable human carcinogens" (USEPA, 1993).

Microbial degradation is believed to be one of the major processes involved in the clean up of PAH-contaminated sites (Danne *et al.*, 2001). Bacterial biodegradation of lower molecular weight PAHs is well characterized, but less information is available on the degradation of higher molecular weight, although they do serve as growth substrates for a number of soil bacteria (Aitken *et al.*, 1998; Danne *et al.*, 2001; Said *et al.*, 2008). The aerobic degradation of PAHs in soil and sediments has been documented (Tam *et al.*, 2002; Guo *et al.*, 2005; Yu *et al.*, 2005; Chang *et al.*, 2008) and biodegradation kinetics of individual PAH compounds by pure and mixed microbial communities have also been discussed by several researchers (Boldrin *et al.*, 1993; Ye *et al.*, 1996; Kanaly and Harayama, 2000).

However, contaminated sites are commonly polluted by complex mixtures of PAHs (Guha *et al.*, 1999; Leblond *et al.*, 2001). Nevertheless, mounting scientific evidence (Loehr and Webster, 1997; Smith *et al.*, 1995) suggests that bioremediation can be applied to provide organic chemical stabilization in conjunction with their ultimate conversion to environmentally benign products such as CO_2, H_2O, and bacterial cell matter.

Bioremediation is a remediation approach in over 135 superfund and underground storage tank (UST) sites, as well as many other sites contaminated with complex mixtures of PAHs (USEPA, 1989). Yet, the efficiency of bioremediation still remains limited (Mohan *et al.*, 2006; Desai *et al.*, 2008). Nevertheless, when it is determined that the presence of such contaminants at a site creates a situation of unacceptable risk to public health and the environment, remedial action is required to reduce the respective chemical concentrations to acceptable levels or to minimize exposure. In this regard, several research groups and governmental institutes have reported that, *in-situ* bioremediation technology is widely used in a manner to achieve both of these goals because it is

potentially less disruptive to the environment and less expensive than other treatment options such as soil washing, or excavation followed by incineration or land disposal (NRC, 1993 and 1994; USEPA, 1995; NATO, 1997).

The primary goal of this research work was to determine the potential mineralization of recalcitrant selected PAHs with degrading bacterium, *Mycobacterium* sp. Strain S65, as a bioremediation agent in different sterilized farm sand and clay soil environments, varying in soil organic matter content from low to very high percentages, and with two concentrations of pyrene (*i.e.* 1000 mg L^{-1} and 500 mg L^{-1}). The second objective of this study was to investigate the effects of four ageing times (*i.e.* Days: 0, 30, 180, and 360), of pyrene in the biodegradation process.

6.3 MATERIALS AND METHODS

6.3.1 Soil Sources

Six different agricultural farm soils from the farm site of the Macdonald Campus of McGill University in Ste-Anne-de-Bellevue, Quebec, Canada, ranging in organic matter content from low to high, were selected and extensively characterized. Four of these soils were used for this study, as we chose the soils with the lowest and highest organic matter content (Table 5.1 in Chapter 5).

6.3.1.1 Soil Sampling and Characteristics

Soil sampling was conducted from the surface to a depth of about 0.3 m. Each soil sample consisted of a composite of five sub samples from an area 3 m × 3 m and was immediately transferred to the laboratory and stored at 4°C. Four of six soils had been chosen, ranging in soil organic matter from low to high. The soils were then gradually thawed at ambient temperature for bioaugmentation, physical, chemical and microbial community analysis. Before use, the soil samples were air-dried, and passed through a U.S. standard sieve (2 mm mesh). Then, a detailed analysis of soil physical and chemical characteristics, textural class, and experimental water demand, were made in the Soil Science Laboratory of McGill University. Results are shown in Tables 5.1 and 5.2 and Figure 5.1 in Chapter 5.

6.3.1.2 Soil Sterilization

Aliquots (30 g) of one of four soils types were placed in sealed bags, then sterilized with 2.5 Mrad of (γ)-irradiation from a ^{60}Co source at the Institut Armand-Frappier of Laval, Quebec..Prior to and after gamma-irradiation, soil samples were stored under refrigerated conditions (4°C) for experimental purposes, at the Biotechnological Research Institute, and McGill University, Montreal, Quebec, and Canada.

6.3.2 Chemicals

For preparation of YTS_{250A}, YTS_{250B}, YTS_{1000A}, YTS_{1000B}, MSM_A, and MSM_B media, and other assays, chemicals such as Yeast Extract , Tryptone, Starch, Granulated Agar, Ultra Pure Agarose, Glycerol, KOH, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Co(NO_2)_2.6 H_2O$, $AlK(SO_4)_2.12 H_2O$, $CuSO_4$, $ZnSO_4.7 H_2O$, $MnSO_4. H_2O$, $FeSO_4.7 H_2O$, $Na_2MoO_4. 2 H_2O$, $Ca(NO_3)_2 .4 H_2O$, H_2SO_4, toluene ($C_6H_5CH_3$), acetone (CH_3COCH_3), hexane (C_6H_{14}), HPLC-grade methanol (CH_3OH), and ethanol (C_2H_5OH) were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada) and Sigma-Aldrich Chemical Company (Oakville, Ontario, Canada). All chemicals and solvents were of ACS Reagent grade or better and were used as received.

6.3.3 Radiochemicals

Pyrene ($C_{16}H_{10}$, FW: 202.3, 4 fused-benzene rings) has a log octanol: water partition coefficient (log K_{ow}) of 4.9, a water solubility at 25°C of 0.135 mg L^{-1}, melting point at 156 °C, and vapor pressure at 25°C = 91.3×10^{-6} mPa (Sigma_Aldrich Catalog, 2009). Uniformly ring-labelled [-4,5,9,10-^{14}C] pyrene at 40-60 mCi $mmol^{-1}$ was provided in methanol (radiochemically pure), by Sigma-Aldrich (Oakville, Ontario, Canada); Tables 3.1 and 3.2 in Chapter 3, Table 6.1), and stored under refrigerated conditions (4°C) until use.

6.3.4 Culture Media

All media were sterilized by autoclaving at 121°C for 20 minutes. Mineral salts medium (MSM-agarose, Ultra Pure Agarose, DNA-grade, Bio-Rad; 1.5% w/v: granulated

agar/distilled water, pH = 7.0; Furukawa and Chakrabarty, 1982), and (MSM-broth, DNA-grade, Bio-Rad; and no granulated agar, pH=7.0), YTS_{250} agar (250 mg L^{-1} each of yeast extract, tryptone, and starch in 1.5% w/v: granulated agar/distilled water, pH 7.0), and YTS_{250} broth (containing 250 mg L^{-1} each of yeast extract, tryptone, starch in distilled water, pH 7.0, and no granulated agar) media, designated MSM_A, MSM_B, YTS_{250A}, YTS_{250B}, respectively, were used in all bacterial isolation experiments (Greer *et al*., 1990). YTS_{1000} agar (containing 1000 mg L^{-1} each of yeast extract, tryptone, starch in 1.5% w/v: granulated agar/distilled water, pH = 7.0), and YTS_{1000} broth (containing 1000 mg L^{-1} each of yeast extract, tryptone, pH = 7.0, and no granulated agar), media, designated YTS_{1000A} and YTS_{1000B}, respectively, and MSM-broth, (DNA-grade, Bio-Rad; and no granulated agar, and final pH = 7.0) medium were used in all mineralization experiments (Greer *et al.*, 1993).

Colony forming units (CFU) counts served to quantify total and specifically PAH-degrading bacteria on the appropriate solid media. Total counts were evaluated on MSM_A containing phenanthrene (PAH) as sole carbon source plates (MSM_{A+PAH}), while PAHs degraders were cultured on similar plates containing YTS_{250} also supplemented with phenanthrene (PAH) as sole carbon source ($YTS_{250+PAH}$).

6.3.5 Bacterial Strains

The *Mycobacterium* strain used in this research work was previously isolated from a soil samples drawn from a collection of nineteen contaminated soils obtained from different wood-preserving plants, oil refineries, and various petroleum hydrocarbons-contaminated sites and thereafter extensively characterized. The area and soils were designated as follows:

Parc Pitt* and *Côte St-Paul/St-Ambroise: Soil (P-1), Soil (P-2), Soil (P-5), Soil (TR-1), Soil (TR-2), Soil (TR-2A), Soil (TR-4). ***Sept-Iles***: (Soil S), ***Lac-des-Loups***: (Soil L), ***Farnham***: (Soil F), ***Borden***: (Soil B), ***Sawmill***: (Soils 14 and 65), *Utility pole storage area*: (Soil 16), ***Sablier Thouin***: (Soils 53, 131, 193), and ***Port Tlueneme***: (Soils 5-6). The details of contaminated soils, their origin, characteristics, type of contaminants and site information were reported by Jazestani and Prasher (2003b).

The 228 PAH-degradation competent bacterial strains isolated were grown and maintained on MSM_A, MSM_B, YTS_{250A}, YTS_{250B}, YTS_{1000A} and YTS_{1000B} media by subculturing, at ambient 25°C temperature and storage at 4°C for further research purposes. Of the 228 strains, one strong positive colony, *Mycobacterium* sp. Strain S65, previously shown to mineralize selected PAHs in aerobic liquid (MSM_B and YTS_B media) microcosm experiments was selected.

6.3.6 Culture Preservation and Creating a Bacterial Genbank

Culture preservation method serves to maintain the organism alive, uncontaminated, and without variation or mutation, that is, to preserve the culture in a condition as close as possible to the original isolate. The method was used to create and maintain a bacterial collection.

In this assay, three millilitres of YTS_{250B} medium were poured into each of two sterilized snap plastic tubes (12 mL). A few isolate colonies were taken with a flamed loop and transferred to the each of the tubes. The tubes were then incubated in a shaking incubator at ambient temperature (25°C) for one week. When the colonies were grown, each 3 mL of culture was transferred to two 1.5 mL conical eppendorf tubes and centrifuged for five minutes. The supernatant was removed from the tubes and replaced with 375 µL of fresh YTS_{250B} medium, and the pellet resuspended with a micropipette to achieve an increased cell concentration. Two concentrated cultures (2×375 µL = 0.75 mL) were then transferred into a 2 mL cylindrical eppendorf tube. Then, 0.75 mL of glycerol solution (40%) was added, resulting in a solution with a final glycerol concentration of 20%. The tube was tightened, immediately placed in dry ice, and then labelled, dated and stored at ⁻80°C (Jazestani, and Prasher, 2003a, b).

6.3.7 Optimal Growth Temperature Study

Temperature has a great influence on the growth of microorganisms. Therefore, all the processes of growth are dependent on chemical reactions that are affected by temperature. Microorganisms can grow over a rather broad range of temperatures. The number of cell divisions per hour is called the growth rate. The growth behaviour is the result of a series of integrated, enzyme-based chemical reactions. However, the temperature at which a

species of microorganism grows most rapidly is the optimum growth temperature (Pelczar *et al.*, 1993).

For any particular microbe, the three important temperatures are the minimum, optimum, and maximum growth temperatures. These are known as the cardinal temperatures of a species of microorganism. However, the optimum temperature for a microbial species does not necessarily lie midway between the minimum and maximum temperatures. Instead, it tends to be nearer the upper limit of the temperature range, because the rate of enzyme reactions increases with increasing temperature until a point where the enzymes are damaged by heat and cells stop growing (Pelczar *et al.*, 1993).

The growth can be divided in three distinct phases: (**i**) the lag phase (or pre-exponential phase), characterized by a slow rate of cell division, (**ii**) the exponential phase, when cell division is maximal and typically, the microbial population (*i.e.* the number of cells in the culture) will double every 40-60 minutes, and (**iii**) the plateau or stationary phase, during which the growth rate is reduced as a result of nutrient depletion in the medium and high cell density (Laboratory Exercises in Microbiology and Immunology, 2001).

The optimum growth temperature was determined for *Mycobacterium* sp. Strain S65. In each of nine 250-mL Erlenmeyer flasks (3 temperatures × 3 replicates), a 1.0 mL aliquot of the *Mycobacterium* culture ($OD_{600\ nm}$ of 0.1) was added to 49-mL of YTS_{1000B} so as to attain a final cell density of 1.0×10^7 cfu mL^{-1}. Erlenmeyer flasks were loosely closed and incubated aerobically at different 25°C, 30°C, or 35°C). Optical density (OD) at 600 nm was monitored by nephelometry until the cultures reached the stationary phase.

6.3.8 Inoculation of Mycobacterium

Mycobacterium sp. Strain S65 cells in aqueous suspension were prepared from cells drawn from 7-day-old, low nutrient medium, YTS_{250} plates incubated at 25°C. Strain S65 was then grown in YTS_{1000B} until cells reached the exponential growth. The culture was harvested and washed twice with an equal volume (v/v) of distilled water. The culture was then used to inoculate both aerobic soil (treatment) and liquid (positive control) serum bottles (microcosms) for mineralization screening experiments, after different PAH substrate ageing times.

6.3.9 Screening of *Mycobacterium* Isolate for Mineralization Activity in Aerobic Liquid Microcosms

To study the pyrene-PAH degradation capacity (kinetics) of *Mycobacterium* sp. Strain S65 in low nutrient YTS_{250B} medium and maintain a positive control, a standard aerobic liquid microcosm (120 mL serum bottles) methodology was adopted (Greer *et al.*, 1990 and 1993; Whyte *et al.*, 1999; Chénier *et al.*, 2003).

Each serum bottle (microcosm), was inoculated by adding a 1.0 mL aliquot of *Mycobacterium* culture (1.0×10^7 cfu mL^{-1} or $OD_{600\ nm}$ of 0.1) to 19 mL of YTS_{250B}. Serum bottles were agitated on a shaker (150 rpm) at ambient temperature (25°C). All aerobic liquid microcosm (treatments) and heat-killed abiotic controls (negative controls) were run in parallel, each in triplicate. Cultures pre-grown for 7 days under conditions identical to those of the corresponding experimental cultures and then killed by autoclaving served as heat-killed controls. Abiotic culture controls were prepared by twice autoclaving the culture at 121°C for 30 minutes, at a 24 hours interval. A 5 mL glass test-tube, containing 1 mL of 0.5N KOH as a CO_2 trap was placed inside each serum bottle. Using a 100 µL glass syringe, the cell culture was spiked with 100 µL ^{14}C-labelled pyrene in methanol ($\approx 1.0 \times 10^5$ dpm per bottle; Table 3.1 in Chapter 3). The 120 mL serum bottles (microcosms) were then tightly sealed with Teflon-coated rubber stoppers and aluminium crimps. To insure that all of the substrate (PAH parent compound) could be mineralized (*i.e.* completely oxidized to CO_2 and H_2O); air was supplemented by flushing the serum bottle's headspace with 3 mL of air before sampling.

Using a long stainless steel needle attached to a 5 mL syringe, aliquots of the CO_2-bearing 0.5N KOH solution (1 mL) were routinely drawn. The wall of the CO_2 trap was rinsed with 1 mL of fresh 0.5N KOH solution, which was then removed and added to the first aliquot, resulting in an overall sample of 2 mL. An additional 1 mL of fresh 0.5N KOH solution was added to the trap to capture further CO_2 generated by the pyrene mineralization.

The CO_2-bearing 0.5N KOH aliquots (2 mL) were each mixed with 18 mL of scintillation cocktail (Fisher Scientific, Whitby Ontario, and Canada), placed in a 20 mL glass scintillation vials, shaken for 5 seconds. Radioactivity levels were assessed by

liquid scintillation counting. The rate of $^{14}CO_2$ evolution was derived from the liquid scintillation counts. Disintegrations per minute (dpm), representative of the cumulative mineralization which had occurred, were converted to mg substrate mineralized or could be expressed as a percentage of initially introduced substrate [^{14}C] pyrene mineralized over time and recovered as [^{14}C]CO_2. This 3 day-interval sampling regime continued until no further mineralization occurred.

6.3.10 Screening of *Mycobacterium* Isolate for Mineralization Activity in Aerobic Soil Microcosms

In a further study, the same screening standard aerobic serum bottle microcosm protocol was adopted for soil microcosms, as previously described for liquid microcosms, to assess the mineralization capacity of *Mycobacterium* Strain S65 of ^{14}C-labelled pyrene aged in different farm soil media, previously sterilized with 2.5 Mrad of gamma (γ)-irradiation from a ^{60}Co source, based on $^{14}CO_2$ evolution.

Mineralization of aged pyrene by *Mycobacterium* Strain S65, was investigated in four farm soil environments, varying in soil organic matter content from low to very high percentages and factorially combined with four soil types (Sandy-Loam, Sand, Clay, Clay-Loam) and two initial pyrene concentrations (*i.e.*, 1000 mg L^{-1} and 500 mg L^{-1}). Aerobic soil microcosms (serum bottles) were then placed in customized boxes and statically incubated (without agitation) in a dark radioactivity containment room for four sets of ageing times at ambient temperature (25°C). Quantitative radioactive tracer analysis of the biodegradation and cumulative percent mineralization of pyrene in different soil environments as well as liquid microcosms were determined in a liquid scintillation counter.

6.3.11 Mineralization and Ageing Procedures of ^{14}C-labelled Pyrene (PAH) in Different Soil Environments

A standard aerobic soil microcosm methodology was conducted to monitor and screen the bacterial isolate, *Mycobacterium* Strain S65 in different soil environments rather than *in vitro* cell cultures, for aged ^{14}C-labelled pyrene degradation capacity (kinetics). The mineralization experiment was performed in triplicate.

Sterilized farm soil aliquots (30 g) were placed in 120 mL serum bottle microcosms, and a 5 mL glass test-tube, containing 1 mL of 0.5N KOH was then placed inside each serum bottle to serve as a CO_2 trap. Using a glass syringe, the soils aliquots were spiked with 100 μL ^{14}C-labelled pyrene in methanol solution (Table 3.1 in Chapter 3), such that approximately 1.0×10^5 dpm of substrate was present in each serum bottle. Then, the 120 mL serum bottles (microcosms) were tightly sealed with Teflon-coated rubber stoppers and aluminium crimps. Loss of radioactivity due to decay (^{14}C $t_{½}$ = 5730 years) was negligible even over the longest incubation (360 days) (0.0087% or 873 dpm) and was not taken into account in mineralization calculations.

Subsequent to each PAH-ageing period, serum bottles were inoculated with a 1.0 mL aliquot of *Mycobacterium* culture (OD_{600} of 0.1), resulting in a final cell density of 1.0×10^7 cfu mL^{-1}. To maintain the optimum microbial activity, the field capacity and actual moisture values of each soil were used to calculate the volume of moisture that had to be added to the various microcosms at each ageing time to achieve the experimental condition of moisture content (soil water saturation), namely 110% of soil field capacity (Cattneo *et al.*, 1997).

Aerobic serum bottle microcosms were incubated at 25°C, without agitation, in the dark for one of four ageing times. All aerobic soil microcosms (treatments: organic matter × soil type × concentrations × aging time × replicates, $n = 2 \times 2 \times 2 \times 4 \times 3 = 96$), heat-killed abiotic controls (negative controls: same as treatments $n = 96$), aerobic liquid microcosms [positive controls: (treatment *vs.* heat-killed) × concentrations × aging time × replicates, $n = 2 \times 2 \times 4 \times 3 = 48$], and sterile controls (organic matter × soil type × aging time × replicates, $n = 2 \times 2 \times 4 \times 3 = 48$) were run simultaneously. Thus, for pyrene, 288 microcosms were used.

Cultures that were pre-grown for 7 days under conditions identical to those of the corresponding experimental cultures, then killed by autoclaving (121°C) twice for 30 minutes, 1 day apart served as heat-killed controls. To insure that all of the substrate could be mineralized (*i.e.* completely oxidized to CO_2, and H_2O); air was supplemented by flushing the serum bottle's headspace with 3 mL of air before each sampling.

Aliquots of the CO_2-bearing 0.5N KOH solution (1 mL) were routinely removed at intervals using a long stainless steel needle attached to a 5 mL syringe. The wall of the

trap was rinsed with 1 mL of fresh 0.5N KOH solution. This was then removed and added to the first aliquot, resulting in a 2 mL sample. An additional 1 mL of fresh 0.5N KOH solution was added to the trap to capture further CO_2 generated by the mineralization of the pyrene-PAH.

The 2 mL CO_2-bearing 0.5N KOH aliquots were each mixed with 18 mL of scintillation cocktail (Fisher Scientific, Whitby Ontario, and Canada) and placed in a 20 mL scintillation glass vial and shaken for 5 sec. The 20 mL KOH solution was then analyzed for its level of radioactivity in a scintillation counter. The rate of $^{14}CO_2$ evolution was derived from the liquid scintillation counts. Values obtained in disintegrations per minute (dpm) were converted to mg substrate mineralized or could be expressed as a percentage of introduced substrate [^{14}C] pyrene mineralized over time and recovered as [^{14}C]CO_2.

This 74 hr interval sampling regime for treatments, negative, and positive controls continued until no further mineralization occurred (*i.e.* cumulative mineralization reached a plateau). The laboratory experiment ran for 360 days during which the effects of four sets of ageing times (0, 30, 180, or 360 days), factorially combined with two types of soil (sandy and clay), and two levels of soil organic matter content (high/low) on pyrene mineralization were studied. This experimental design is shown in Table 6.1.

6.3.12 Screening of Sterile Control Soil Microcosms

A sterile control microcosms study was undertaken to monitor and screen for the possibility of growth of any other competitive bacterial strain alongside *Mycobacterium* sp. Strain S65 in the controlled soil microcosms experiment. Four sets of soil microcosms were prepared from the same gamma (γ)-irradiated sterile experimental farm soils.

Four different types of soil aliquots (30 g) were introduced into 120 mL serum bottles, each in triplicate for each ageing time, for a total of 48 microcosm bottles (*i.e.* 4 soil/OM combinations × 4 ageing time × 3 replicate). The 120 mL serum bottles (microcosms) were then tightly sealed with Teflon-coated rubber stoppers and aluminium crimps. For the main aerobic soil microcosms, oxygen was supplemented by flushing the serum bottle's headspace with 3 mL of air before each sampling, to insure that all of the sterile control soil microcosms were aerobic. The possible presence of viable

microorganisms in soils (CFU g^{-1}) at each ageing time, was assessed by using serial dilution and spread-plate techniques (Jazestani and Prasher, 2003a, b).

6.3.13 Determination of the Likely Viable Culturable Bacterial Population

The possible presence of viable microorganisms in soils (CFU g^{-1}) was determined by serial dilution and spread-plate techniques. An aliquot of soil (5 g) was aseptically added to pre-weighed sterile test tubes containing 2.5 g of glass beads (3 mm diameter). The tubes were re-weighed and sterile tetra-sodium pyrophosphate buffer (0.1% w/v: $Na_4P_2O_7.10H_2O$ in distilled water, and pH 7.0) was added in a 3:1 buffer: soil w/w ratio. The mixture was vortexed for several minutes before preparing a 1:10 dilution series in MSM_B medium. A 0.1 mL aliquot from the dilution series was spread-plated in triplicate onto MSM medium containing pyrene (PAH) as sole carbon source (MSM_{A+PAH}), and the plates were incubated at ambient temperature (25°C) for at least one week before counting any possible colonies (Greer *et al.*, 1990 and 1993; Whyte *et al.*, 1999; Chénier *et al.*, 2003).

6.3.14 Statistical Analysis

Analysis of the cumulative percentage of pyrene mineralisation was performed using PROC ANOVA in SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC, USA). The design consisted of a factorial combination of 3 replicates, 2 phenanthrene concentrations, 4 aging times (0, 30, 180 and 360 days) and 4 soil types, with 5 repeated measures in time. A threshold of significance of $P \leq 0.05$ was used.

6.4 RESULTS AND DISCUSSION

6.4.1 Soil Sampling and Characteristics

Soil textural class (soil particles size distribution), nutrient content, chemical characteristics, and water holding capacity (used to determine experimental water demand) were determined in the Soil Science Laboratory of McGill University (Tables 5.1, 5.2 and Figure 5.1 in Chapter 5).

6.4.2 Culture Preservation and a Bacterial Genbank

Sixty of the 228 positive isolate bacterial strains selected were strongly positive (PAHs-degrading microorganisms). Of these, 14 were *ndoB* positive and one isolate bacterial strain (Strain S65) mineralized several selected PAHs. All 60 strong isolates were used to create a bacterial culture collection. All strains were successfully preserved and stored at $^{-}80^{\circ}C$ at the Environmental Microbiology Laboratory of Biotechnology Research Institute (BRI), at National Research Council of Canada, Montreal, Quebec (Jazestani and Prasher, 2003a and b). This culture collection was created to maintain a bacterial gene bank for the purpose of selection of the genes involved in recalcitrant PAHs-biodegradation and further research work in the bioremediation area.

6.4.3 Optimal Growth Temperature

The optimum growth temperature of *Mycobacterium* sp. Strain S65 was determined at temperatures of $25^{\circ}C$, $30^{\circ}C$, and $35^{\circ}C$ in YTS_{1000B} medium. *Mycobacterium* Strain S65 grew best at $30^{\circ}C$, but could not grow at $35^{\circ}C$ (Figure 5.2 in Chapter 5), indicating that Strain S65 is highly unlikely to be an internal human pathogen. Bacterial strains that are pathogenic for human grow best at body temperature ($37^{\circ}C$) (Pelczar *et al.*, 1993). At $25^{\circ}C$ to $30^{\circ}C$ growth continued until 150 hours, at which point when cells had just reached the stationary phase (for practical reasons, all experiments were conducted at $25^{\circ}C$).

6.4.4 Biodegradation Potential of *Mycobacterium* sp. Strain S65 in Liquid Microcosms

Mycobacterium sp. Strain S65's mineralization activity in aerobic liquid microcosms indicated that it rapidly utilized, as its sole carbon and energy source, pyrene (61% cumulative mineralization), phenanthrene with three days lag (61% cumulative mineralization), and to a lesser extent fluoranthene with no lag (24% cumulative mineralization). However, it did not metabolized naphthalene, anthracene or fluorene (Figure 3.8 in Chapter 3).

6.4.5 Biodegradation Potential of Mycobacterium sp. Strain S65 in Soil Microcosms

The extent of soil-aged pyrene's mineralization by *Mycobacterium* sp. Strain S65 was determined in standard aerobic soil (solid) serum bottle microcosms at ambient temperature (25°C) over a 360 day pre-inoculation ageing period (Figures 6.1 to 6.4). Analysis of mineralization results for non-aged (Day 0) pyrene revealed that pyrene mineralization followed similar kinetics in liquid and soil microcosms, with no significant lag time before mineralization began. Results of ANOVA are presented in Table 6.2.

For initial pyrene concentrations of 500 and 1000 mg L^{-1}, cumulative pyrene mineralization reached 18.5% and 19%, respectively after 24 hours, and reached a cumulative maximum mineralization of 20% and 21% after 9 days in sandy-loam soil with low organic matter. In contrast, in clay-loam soil with high organic matter, the cumulative maximum mineralization reached only 15.5% and 14.5% respectively (Figure 6.1 and 6.2). The sampling regime continued until no further mineralization occurred (cumulative mineralization reached a plateau). While soil non-aged pyrene was metabolized by *Mycobacterium* strain S65 at a moderate rate, the non-aged compound in aerobic liquid microcosms was mineralized at a much greater rate (61% cumulative mineralization).

Mineralization results for 30-day aged pyrene showed that *Mycobacterium* sp. Strain S65 mineralized pyrene (initial concentration of 500 and 1000 mg L^{-1} — Day 0 level), respectively, to a greater extent (cumulative mineralization of 13.25% and 14%) in sandy-loam soil with low organic matter , than in clay-loam soil with high organic matter (cumulative mineralization of 11.5% and 12.5%, respectively) (Figure 6.3 and 6.4).

For pyrene soil-aged for 180 days, *Mycobacterium* strain S65 used this compound (initially containing 500 or 1000 mg L^{-1} — Day 0 level), but pyrene mineralization declined to a cumulative 7.0% and 7.5%, respectively, in sandy-loam soil with low organic matter, and a cumulative 4.0% and 4.5%, respectively, in clay-loam soil with high organic matter.

Mycobacterium sp. Strain S65 was capable of using 360 days-aged pyrene substrate (initially containing 500 or 1000 mg L^{-1} — Day 0 levels); however, cumulative pyrene mineralization declined to its lowest extent, with only 5.75% and 6.0%

mineralization in sandy-loam soil with low organic matter, and only 3.35% and 3.5%, respectively, in clay-loam soil with high organic matter.

Organic compounds that persist in the soil often become increasingly less available to microorganisms. The rates of disappearance from field soils of slowly biodegradable organic compounds decline with time, and after several years, little or no further loss can be detected. Because microorganisms present in these field soils are able to metabolize those compounds, the chemicals appear to become less bioavailable with time. This ageing process, which represents a sequestration of the molecules to make them somehow no longer available, occurs with a number of organic pollutants (Alexander, 1995; Alexander and Alexander, 2000). Despite the decline in bioavailability of aged compounds, some remain in a form that can be metabolized by microorganisms and, if the substance is toxic, a portion remains in a potentially harmful form. However, the decline in availability of the compound as a result of ageing as well as the combined effect of the processes of sequestration and biodegradation explains why the amount of PAH taken up by the soil biota is reduced (Alexander, 1999; Alexander and Alexander, 2000).

Among many of the less readily metabolized compounds that remain in soil years after their first introduction, PAHs are one of the more prominent groups, a bi- or multiphasic rate of disappearance is evident. The degradation rate in the first few months is rapid, but quickly slows to a point where little is lost in succeeding years. This may reflect the sequestering of the compound in a manner that makes it less available to microorganisms, that is, the so-called “ageing” of the molecule (Alexander, 1999; Alexander and Alexander, 2000). Frequent observations that bioremediation did not reduce levels of target pollutants below regulatory levels may be misleading because the amount that remains, determined by vigorous extraction of the soil, may be largely unavailable and thus pose little risk. Therefore, bioremediation may actually have met its objectives even if the analytical procedure suggests the contrary.

In the present study, *Mycobacterium* sp. Strain S65 was shown to successfully mineralize pyrene (PAH) in liquid microcosms; however it performed relatively poorly as a bioaugmentation agent for the microbial degradation of soil-aged ^{14}C-labelled pyrene in different soil environments. A number of reasons may account for this: substrate ageing,

insufficient PAH to stimulate the microbial strain to degrade it, uneven distribution of microorganism within the soil during the inoculation process and similarly a possibly uneven distribution of the added PAH within the unmixed soil, strong adsorption of the PAH to soil particles, as well as other environmental parameters, which could interact to reduce the ability to survive and the ability of the strain S65 to perform as significant bioremediator.

The true potential of *Mycobacterium* sp. Strain S65 in PAH bioremediation, and the optimization of this process should be the focus of further study. Surfactants could play a major role in overcoming problems of hydrophobicity and limited availability of PAHs. Indeed, surfactants have been shown to enhance the biodegradation rate of recalcitrant compounds in soil (Aronstein and Alexander, 1993; Cookson, 1995; Deziel *et al*., 1996). The use of the surfactant polyoxyethylene sorbitan monooleate (Tween 80) as a soil amendment greatly enhanced the rate of biodegradation of endosulfan by *Pseudomonas aeruginosa* (Jayashree and Vasudevan, 2007).

6.4.6 Assessment of Possible Viable Culturable Microbial Population in Sterile Control Soils

The possible presence of a viable culturable microorganism population in different sterile soils (CFU g^{-1}) was assessed by serial dilution and spread-plate techniques undertaken at ambient temperature (25^{o}C). No microbial colonies were observed, indicating that no competitive microbial strain, which might contribute to the mineralization of ^{14}C-labelled Pyrene (PAH), was present alongside *Mycobacterium* sp. Strain S65 throughout the entire ageing process.

6.4.7 Bioavailability and other Factors that Limit PAHs-Biodegradation

Degradation kinetics of pyrene by *Mycobacterium* sp. Strain S65 in different soil environments was investigated in a bioaugmentation study carried over a 360 day period. In spite of a sufficient quantity of pyrene (500 and 1000 mg L^{-1}), well above Canadian regulatory criteria (250 mg L^{-1}) for soil, the inoculated degrader organism was less competent in degrading pyrene in soil environments than in liquid microcosms (YTS_{250} broth medium).

The labelled pyrene was applied in a localized manner to aliquot of 30 g of soil where it could adsorb strongly to the soil particles. As the soil in the microcosms cannot conveniently be mixed, the pyrene may be limited to a certain area which may be more or less accessible to the microorganism.

Bioremediation involves the use of biological systems, generally relying on microbial processes, and availability of given compound to degrader organisms, in order to remove a target contaminant from the soil. As PAHs strongly interact with the organic fractions of contaminated soil and sediment, which, however, represents a potential nutrient source for the microorganism, little of the PAH mass remains in the aqueous phase in such systems. Consequently, the greater portion of PAH mass is not directly available to microorganisms. As the PAHs in the aqueous phase are depleted, continuing degradation requires mass transfer from the interior of a non-aqueous phase to a location accessible to microbial cells. Therefore, in such cases rates of desorption from the non-aqueous phase(s) will dictate overall biodegradation rates.

However, factors beyond bioavailability may limit the biodegradation of the PAHs. These factors can include: (**i**) low numbers of organisms capable of degrading the PAHs; (**ii**) inherently slow enzyme activity for a given compound; (**iii**) slow rates of degradation exacerbated by competition among substrates; (**iv**) depletion of growth substrates; and (**v**) the accumulation of inhibitory products (Singh and Ward, 2004).

Both the properties of the soil and the nature and type of contaminants control the transformation and fate of contaminants in soils. The quantity of a chemical that remains bioavailable in the terrestrial environment depends on a variety of factors including the properties of both the chemical and the environmental media (Naidu *et al.*, 2008). However important the soil's influence is, bioavailability is also affected by contact time (*i.e.* ageing), which reduces the concentration of chemical available for uptake by organisms or which causes toxicity (Alexander, 1995, 1999; Vig *et al.*, 2002). Soil pH, organic matter and clay contents, surface charge, soil solution composition, and contaminant physico-chemical properties, including ionic and oxidation state, molecular structure, polarity, aqueous solubility, and lipophilicity, are responsible for controlling the behaviour of contaminants. These properties in turn affect the fraction of contaminants that may be bioavailable in a given soil. However, this is further impacted

upon by the duration of contact between contaminants and soils, with bioavailability declining with ageing (Alexander, 1995, 1999; Vig *et al.*, 2002; Naidu *et al.*, 2003, 2008; Naidu and Bolan, 2008).

A major environmental process, microbial transformation affects the fate of PAHs in both terrestrial and aquatic ecosystems. Only in the last decade have bacteria capable of metabolizing larger PAH molecules been isolated. While biological technologies are now being explored for their potential in the remediation of contaminated sites, their successful application demands a broader understanding of the biochemical PAH-degradation pathways, both individually and in mixtures. The degradation of individual PAHs by microorganisms which are able to utilize them as sole source of carbon and energy is well documented. These studies have yielded fundamental information about the biodegradability of individual compounds (Cerniglia, 1992). The rates of biodegradation of PAHs are highly variable and are dependent not only on PAH structure, but also on the physico-chemical parameters of the site as well as the number and type of microorganisms present. PAHs are sorbed to organic matter in soils and sediments, and the rate of their desorption strongly influences the rate at which microorganisms can degrade them (Shuttleworth and Cerniglia, 1995). Enhancing the bioavailability and consequently the degradation rate of PAHs at polluted sites receives much of the focus of current studies. However, PAH degradation products are not necessarily less toxic than their parent compounds; therefore, toxicity assays must be included in monitoring the effectiveness of PAH bioremediation (Shuttleworth and Cerniglia, 1995). PAHs are degraded by microorganisms either through metabolism or co-metabolism. Co-metabolism is very important for degradation of mixtures of PAHs and high molecular weight PAHs. In contrast, several two-, three- and four-ring PAHs have been known to be growth substrates for bacteria (Habe and Omori, 2003).

Innovative bioremediation method for heavily polluted soils have investigated processes based on microbial activation through degradation and mineralization of PAHs (Baveye *et al.*, 1999; Alexander, 1999; Alexander and Alexander, 2000; Douben, 2003; Singh and Ward, 2004; Aitken and Long, 2004; Philp *et al.*, 2005; Boehm, 2006; Prichard *et al.*, 2006; Thwaites *et al.*, 2007; Singh and Tripathi, 2007; Diaz, 2008). Exploiting the catabolic versatility of microorganisms to accelerate the degradation of environmental

pollutants, bioremediation is an important industry in alleviating environmental contamination (Labana *et al.*, 2007).

Limitations in oxygen, other electron acceptors or nutrients may also contribute to limitations in PAHs-biodegradation (Madsen *et al.*, 1996). However, Wick *et al.*, (2001) stated that "Limited bioavailability occurs when the capacity of the microbial biomass to consume a substrate exceeds the capacity of its environment to deliver the substrate."

6.5 CONCLUDING REMARKS

The mineralization capability of the environmental ***Mycobacterium*** **sp. Strain S65,** evaluated on the basis of $^{14}CO_2$ evolved during the mineralization of soil-aged ^{14}C-labelled pyrene was investigated in different initially γ-ray sterilized farm soils environments.

The present study's experimental design consisted of a factorial combination of four types of soil (Sandy-Loam, Sand, Clay, Clay-Loam), two levels of soil organic matter (high or low), and two concentrations of pyrene (1000 mg L^{-1} and 500 mg L^{-1}), with six replicates per treatment (48 microcosms per inoculation date). Cumulative PAHs mineralization percentages were determined by radioactive tracer analysis. The experiment ran for 360 days during which the effects of ageing were assessed at four times (0, 30, 180, or 360 days).

Assessment of bioavailability of soil-aged ^{14}C-labelled pyrene to degradation in different soil environments was determined in the presence of *Mycobacterium* sp. Strain S65, was introduced as a bioaugmentation agent, into soil microcosms. The pattern of pyrene degradation in liquid microcosms, particularly the lack of a lag period, was similar to what occurred in soil microcosms. Gamma-ray sterilized farm soil microcosms were spiked with 1.0×10^5 dpm ^{14}C-labelled pyrene, which was allowed to age for up to 360 days. An aliquot of 1.0 mL culture ($OD_{600\ nm}$ of 0.1) with a final cell density of 1.0×10^7 cfu mL^{-1} was introduced into soil microcosms after the required ageing period.

For non-aged (Day 0) pyrene mineralization began immediately, *Mycobacterium* sp. Strain S65 rapidly utilized the substrate without any lag phase. Pyrene (at initial concentration of 500 and 1000 mg L^{-1}) cumulative mineralization increased to 18.5% and 19%, respectively, within 24 hours, and finally reached 20% and 21%, after 9 days in

sandy-loam soil with low organic matter. In contrast, in clay-loam soil with high organic matter, cumulative mineralization occurred to a lesser extent, reaching only, 14.5% and 15.5% after 9 days. The sampling regime continued until no further mineralization occurred (cumulative mineralization reached a plateau). The non-aged soil-borne compound was metabolized at a moderate rate, whereas, in contrast, the non-aged compound in aerobic liquid microcosms was mineralized to a much greater extent (61% cumulative mineralization).

For 30-day aged pyrene (at initial concentrations of 500 and 1000 mg L^{-1}), *Mycobacterium* sp. Strain S65 performed maximum cumulative mineralization of 13.25 and 14%, respectively after 9 days in sandy-loam soil with low organic matter, but only reached 11.5% and 12.5% cumulative mineralization after 9 days in clay-loam soil with high organic matter. For 180 and 360-day aged pyrene, *Mycobacterium* sp. Strain S65 utilized the pyrene, but cumulative mineralization declined to less than 10%.

In general, the high clay and high organic matter soil showed the lowest degradation rates. This is likely the result of the greater number of potential PAH adsorption binding sites on clay particles and on organic matter, than on larger soil particles such as those found in a low organic matter sandy soil. The observation that less PAH degradation occurs in high organic matter soils, is confirmed by several studies (Manilal and Alexander, 1991; Weissenfels *et al.*, 1992; Johnsen *et al.*, 2005).

Mineralization of non-aged pyrene by *Mycobacterium* sp. Strain S65 in YTS_{250B} liquid medium (*i.e.* positive control) and heat-killed *Mycobacterium* (*i.e.* negative control), were also measured over the same incubation period of mineralization treatments. The possible presence of a viable culturable microorganism population (CFU g^{-1}) in different sterile soil microcosm bottles (*i.e.* sterile control) was assessed by serial dilution and spread-plate techniques. No competitive microbial strain, which might contribute to the mineralization of ^{14}C-labelled pyrene (PAH), was present alongside *Mycobacterium* sp. Strain S65. All three controls were applied in mineralization experiments throughout the entire ageing process.

Transformation and fate of contaminants in soils is controlled by both the properties of the soil and the nature and type of contaminants. The amount of a chemical in the terrestrial environment that is bioavailable depends on a variety of factors including

the properties of both the chemical and the environmental media (Naidu *et al.*, 2008). Soil plays a very significant role in reducing the potential bioavailability of contaminants in the environment. However, bioavailability is impacted not only by soil type, but also contact time (*i.e.* ageing), which reduces the concentration of chemical available for uptake by organisms or which causes toxicity (Alexander, 1995, 1999; Vig *et al.*, 2002). Soil properties, including pH, organic matter and clay contents, surface charge, soil solution composition, and contaminant physico-chemical properties, including ionic and oxidation state, molecular structure, polarity, aqueous solubility, and lipophilicity, are responsible for controlling the behaviour of contaminants. These properties in turn affect the fraction of contaminants that may be bioavailable in a given soil. However, this is further impacted upon by the duration of contact between contaminants and soils, with bioavailability declining with ageing (Alexander, 1995, 1999; Vig *et al.*, 2002; Naidu *et al.*, 2003, 2008; Naidu and Bolan, 2008).

Bioavailability *i.e.,* the ability of a compound to be freely transported across the cell membrane for intercellular or available for extracellular metabolism, may be the most important factor in determining the feasibility of bioremediation of PAHs. In most cases, mass transfer limitations prevent the full exploitation of the microbial degradative potential (Bosma *et al.*, 1997). Limited bioavailability is due to low water solubility and consequently the tendency to partition onto soil mineral surfaces and to sorb strongly to the soil or sediment matrix (Harms and Bosma, 1997). Several mechanisms work together to influence bioavailability, and different mechanisms predominate in any given situation, however, they are still not fully understood. It is usually assumed that the water-dissolved fraction of chemicals is the only one available to microorganisms. Therefore, degradation rates are dependent on the mass transfer rates of PAHs from solid or soil bound phase to the aqueous phase and desorption of PAHs from soil is considered as the controlling factor in their biodegradation (Volkering *et al.*, 1992; Pignatello and Xing, 1996; Labana *et al.*, 2007).

Moreover, this conclusion will be followed up by further research, and support future strategies regarding the bioavailability, biodegradation, bioremediation, and biodetoxification of ever-increasing amounts of organic xenobiotics which are constantly released into the environment.

Greer (2008) has opined that such studies seek to find ways to use microorganisms to degrade organic pollutants and thus to clean up man's indiscriminate pollution using these microorganisms. To do this, we must identify and profile the microorganisms that are naturally there since we are only familiar with about 1% of what is actually out there. He went on to elaborate that his work looked at the entire genomic content of a gram of soil, rather than the genome of a particular organism. This assesses the potential of whole microbial communities and identifies those which could help in remediation. Lastly, it is noteworthy that this research work strongly supports the significant role of *Mycobacteria* in PAHs-biodegradation, bioremediation, and biodetoxification in contaminated environments.

Table 6.1: Experimental Design for Aerobic Soil and Liquid Microcosm Study of ^{14}C-Labelled Pyrene Mineralization for each Ageing Time

Concentration (PAH)	500 ppm						1000 ppm						Radiochemical ^{14}C-Labelled Pyrene (dpm)	Bacterial Strain *Mycrobacterium* sp. Strain S65		
T&C	Treatment			Control (-)			Treatment			Control (-)				Volume (mL)	OD_{600}	CFU g^{-1}
Soil1	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1×10^5	1	0.1	1×10^7
Soil2	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1×10^5	1	0.1	1×10^7
Soil3	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1×10^5	1	0.1	1×10^7
Soil4	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1×10^5	1	0.1	1×10^7
YTS_{250B}: C (+)	T1	T2	T3	C1	C2	C3	T1	T2	T3	C1	C2	C3	1×10^5	1	0.1	1×10^7

PAH: Polycyclic Aromatic Hydrocarbons
T: Treatment
C: Negative control (-): Heat-killed (sterilized by autoclaving at 121^{o}C for 20 minutes, *i.e.* abiotic controls)
Soil 1: Sandy - Loam low organic matter (**LOM**)
Soil 2: Sand high organic matter (**HOM**)
Soil 3: Clay low organic matter (**LOM**)
Soil 4: Clay - Loam high organic matter (**HOM**)
Concentration (**ppm**)**:** part per million (*i.e.* mg L^{-1})
YTS_{250B}: Positive Control: **C** (+): YTS_{250} µg L^{-1} - broth (containing 0.25 g L^{-1} each of yeast extract, tryptone, starch in distilled water, pH 7.0, and no granulated agar) medium
Dpm: Disintegrations Per Minute (level or values of radioactivity for mineralization analysis)
OD_{600}: Optical Density of a Given Culture at Wave Length of 600 nm as measured by Spectrometry
CFU g^{-1}: Colony-Forming Unit of Microorganism per Gram of Soil

Table 6.2. Significance of Single Factor Effects and Interactions on Cumulative Pyrene Mineralization.

Sources	P
S	**
C	**
R	NS
AT	**
IT	**
S*AT	**
S*C	**
AT*C	NS
S* IT	**
C* IT	*
AT * IT	**

R: Replicate; **S**: Soil, **C**: Pyrene Concentration, **AT**: Aging Time, **IT**: Incubation Time

NS: Non-Significant**,** ($P > 0.05$); *, Significant ($P \leq 0.05$); **, Highly Significant ($P \leq 0.01$)

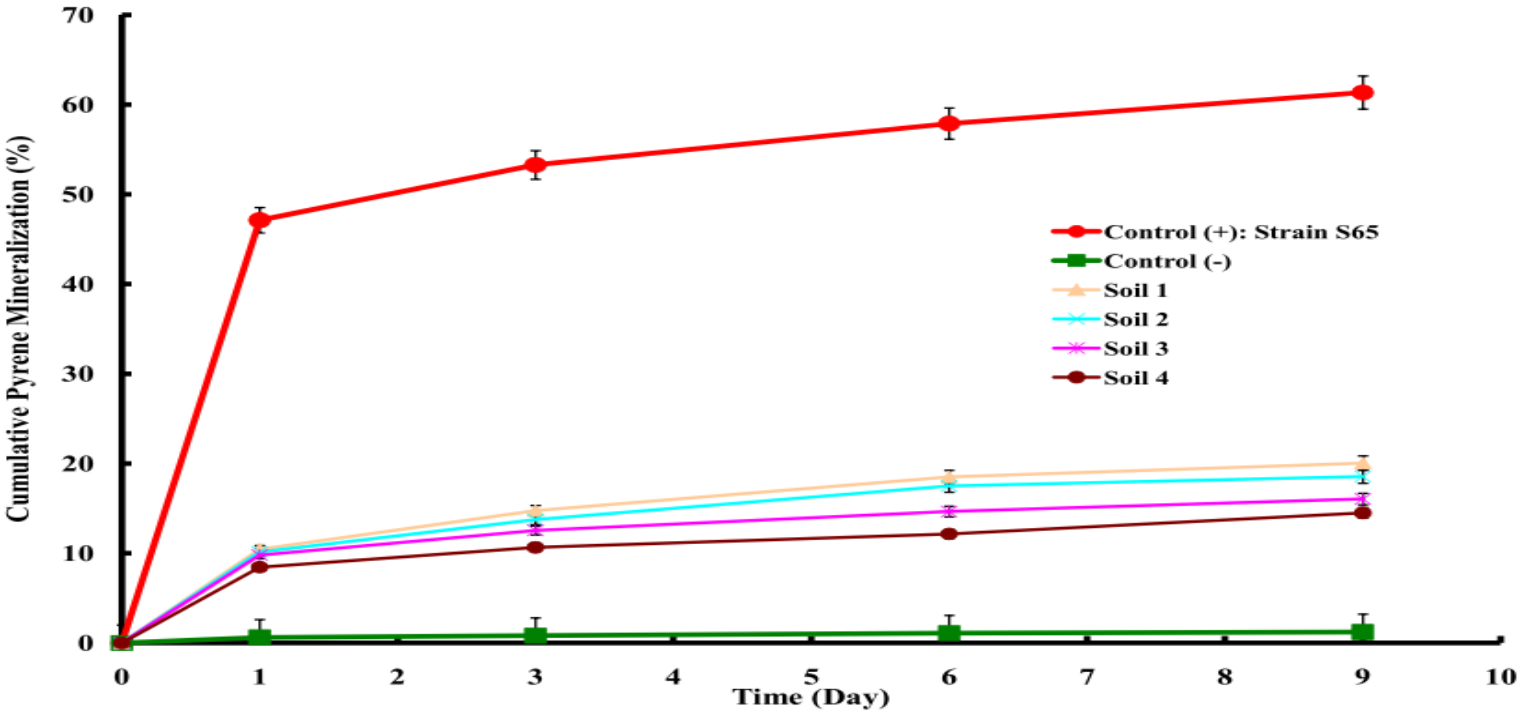

Figure 6.1: Mineralization of Pyrene [500 mg L^{-1} (ppm) concentration] by *Mycobacterium* sp. strain S65 in Different Soil Environments at Day (0)

Soil 1: Sandy-Loam low organic matter; Soil 2: Sand high organic matter
Soil 3: Clay low organic matter; Soil 4: Clay-Loam high organic matter
Control (-): Autoclaved *Mycobacterium* sp. strain S65 at 121 °C
Control (+): *Mycobacterium* sp. strain S65 in YTS_{250}-Broth medium
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

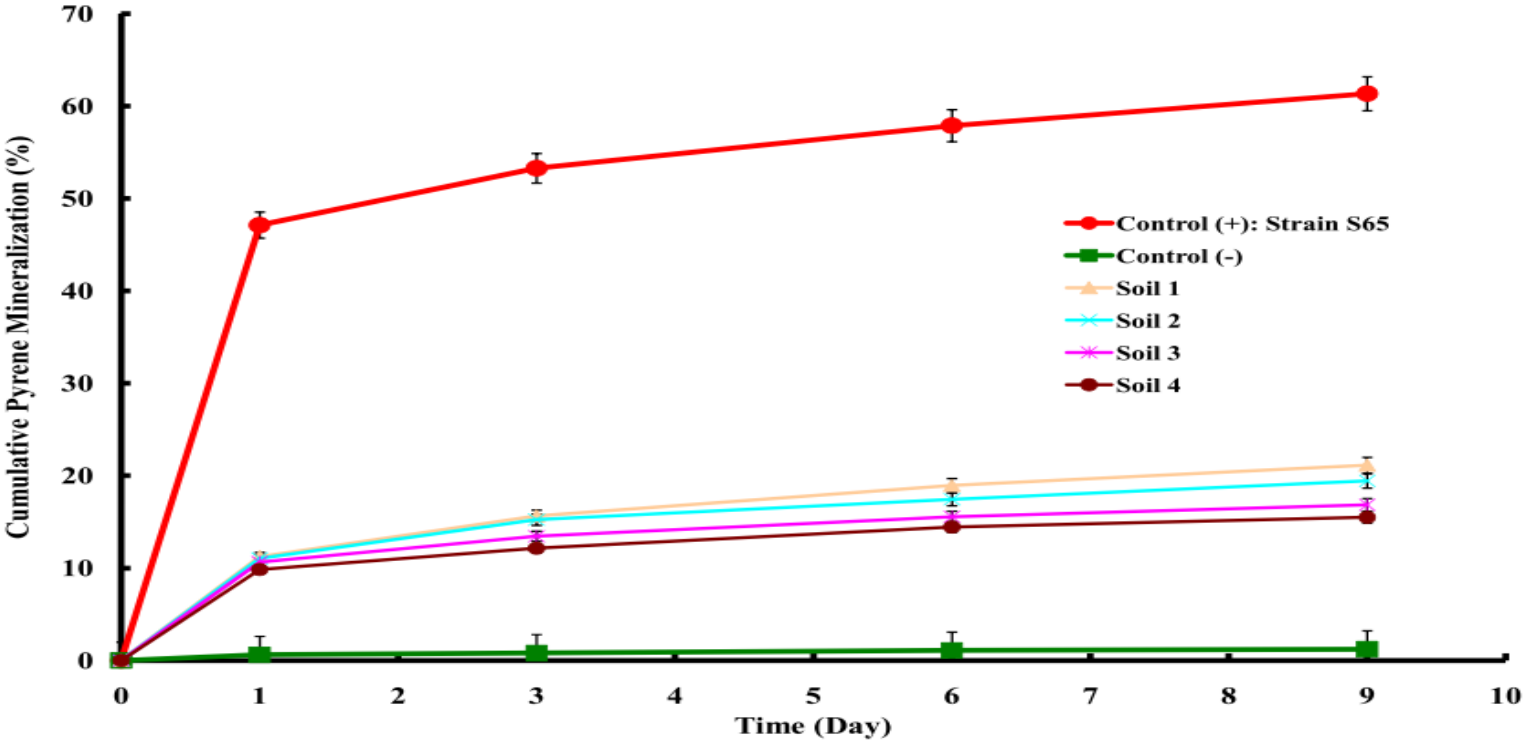

Figure 6.2: Mineralization of Pyrene [1000 mg L^{-1} (ppm) concentration] by *Mycobacterium* sp. strain S65 in Different Soil Environments at Day (0)

Soil 1: Sandy-Loam low organic matter; Soil 2: Sand high organic matter
Soil 3: Clay low organic matter; Soil 4: Clay-Loam high organic matter
Control (-): Autoclaved *Mycobacterium* sp. strain S65 at 121°C
Control (+): *Mycobacterium* sp. strain S65 in YTS_{250}-Broth medium
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

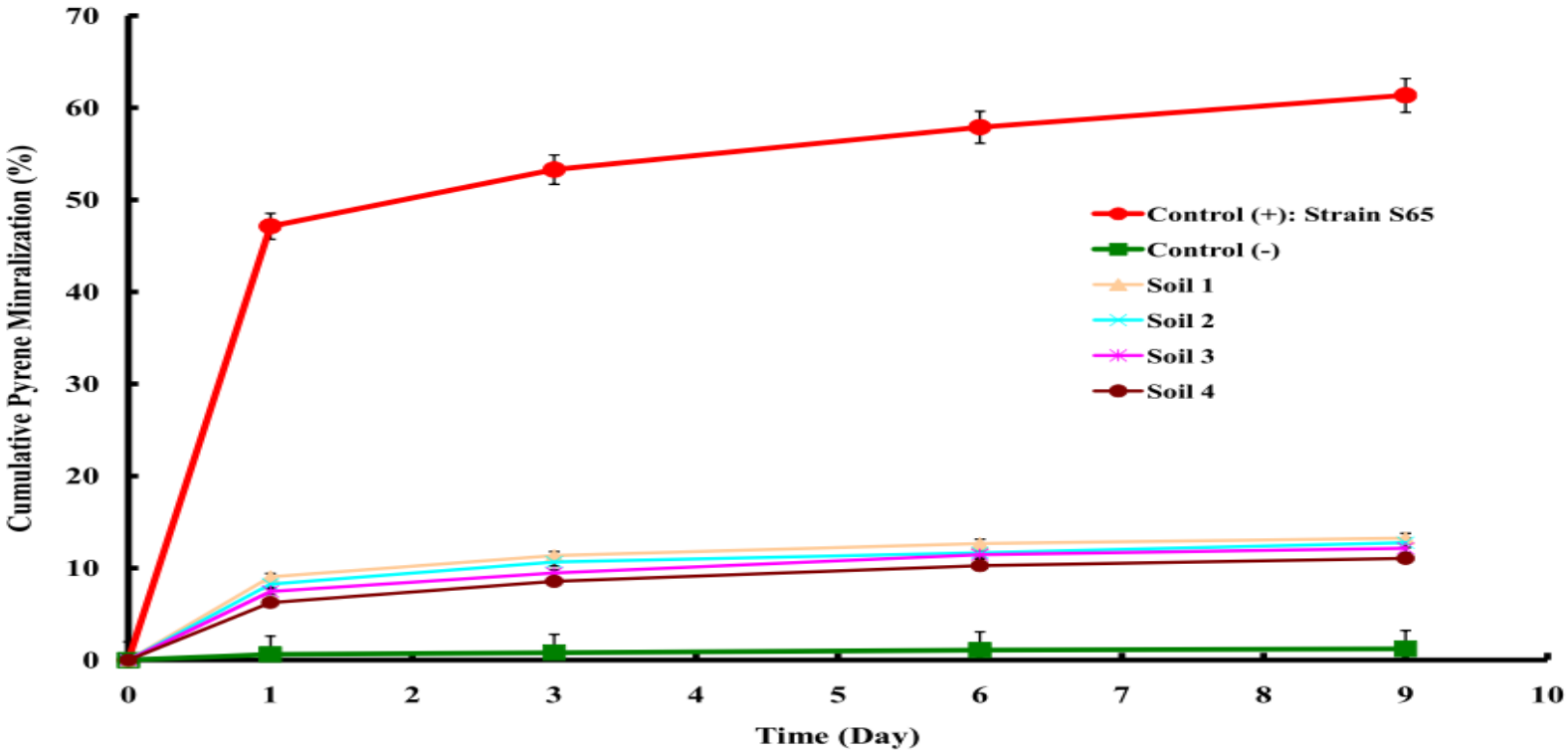

Figure 6.3: Mineralization of Pyrene [500 mg L^{-1} (ppm) concentration] by *Mycobacterium* sp. strain S65 in Different Soil Environments at Day (30)

Soil 1: Sandy-Loam low organic matter; Soil 2: Sand high organic matter
Soil 3: Clay low organic matter; Soil 4: Clay-Loam high organic matter
Control (-): Autoclaved *Mycobacterium* sp. strain S65 at 121°C
Control (+): *Mycobacterium* sp. strain S65 in YTS_{250}-Broth medium
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation.

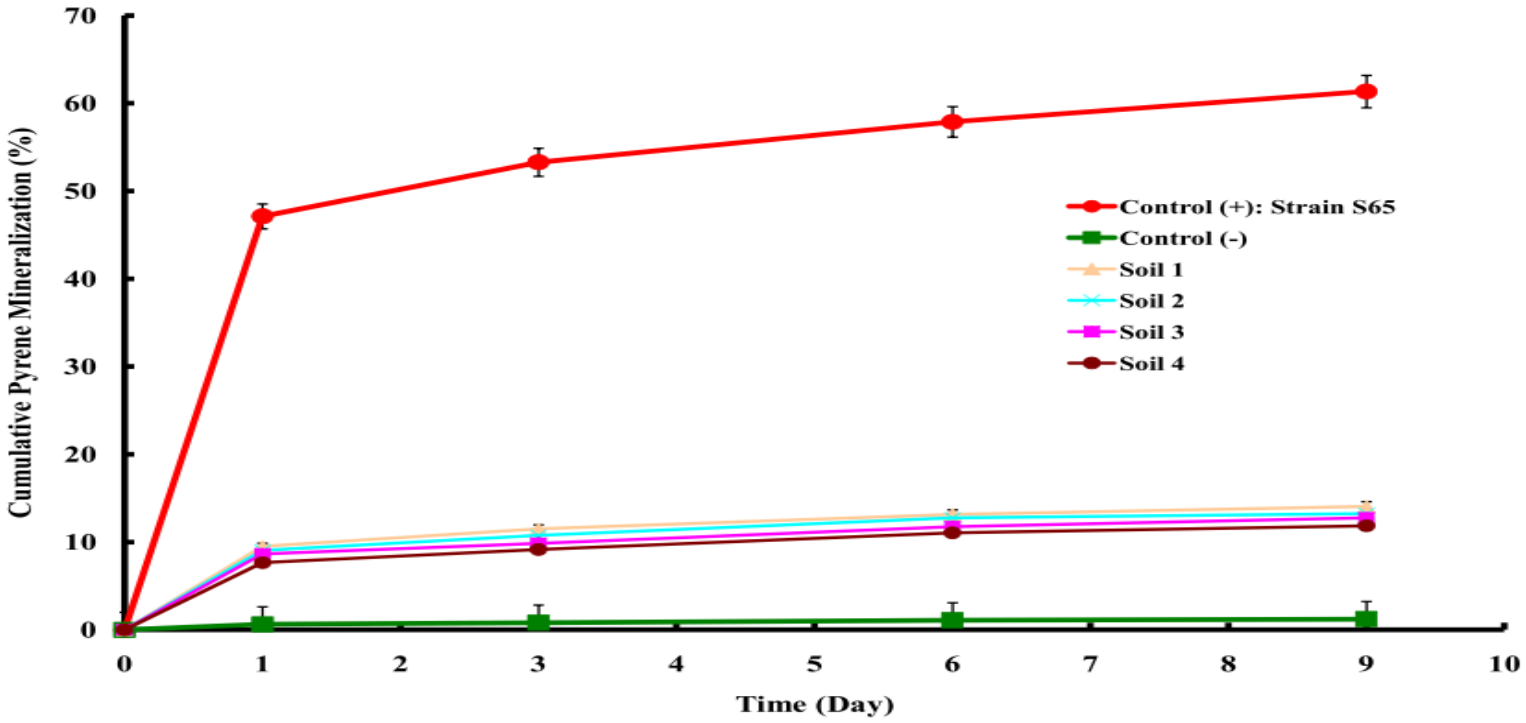

Figure 6.4: Mineralization of Pyrene [1000 mg L^{-1} (ppm) concentration] by *Mycobacterium* sp. strain S65 in Different Soil Environments at Day (30)

Soil 1: Sandy-Loam low organic matter; Soil 2: Sand high organic matter
Soil 3: Clay low organic matter; Soil 4: Clay-Loam high organic matter
Control (-): Autoclaved *Mycobacterium* sp. strain S65 at 121°C
Control (+): *Mycobacterium* sp. strain S65 in YTS_{250}-Broth medium
Data points are the average of triplicate samples.
Error bars represent ± one standard deviation

CHAPTER 7

General Summary and Concluding Remarks

This chapter provides the general summary, statements of the general conclusions and concluding remarks of this Ph.D. research work.

7.1. General Summary

The ***"Bioavailability and Biodegradation of Organic Xenobiotic Recalcitrant Polycyclic Aromatic Hydrocarbons (PAHs) in Different Soil Environments"*** was the main objective of this research work. Many experiments were carried out and different experimental conditions were considered, in an attempt to analyze the collected data and evaluate and determine the effects that these conditions had on the bioavailability. To accomplish this goal, the more specific research objectives, pertaining to the bioavailability and fate of selected organic *xenobiotic* recalcitrant PAHs compounds in the different soil environments were undertaken as follows:

1. Research work was conducted to isolate bacterial strains (*i.e.* to isolate the more suitable microorganisms for bioremediation and biodetoxification) from soil contaminated with many different organic pollutants such as the wood treatment chemicals pentachlorophenol (PCP) and creosote containing oils and PAHs. These strains are currently maintained in a culture collection.

2. Work was carried out to screen the PCP/PAH-contaminated soils using molecular techniques (*e.g.*, hybridization with gene probes derived from known bacterial catabolic pathways) to determine if the native microbial population contains biodegradation genes for the degradation of selected PAHs compounds.

3. Investigations were made to determine if bacterial strains isolated from different contaminated soils can host biodegradation pathways and to quantify the mineralization ability of the isolates for selected PAHs compounds.

4. Characterization (*i.e.* to identify the most suitable microorganisms for bioremediations and biodetoxification) of isolated bacterial strains was done at the micromorphological, biochemical and genetic levels in order to optimize their natural biodegradative capacities. Precise analysis of the Phase-Contrast Microscopy and Cell Wall Mycolic Acids by HPLC with UV Detection methods were carried out. The patterns of the Cell Wall Mycolic Acids obtained was finally confirmed by the identification procedures of novel mycobacterial species, using 16S rRNA Gene Sequence Analysis. Finally strain S65 was completely characterized (*i.e.* GenBank designation: *Mycobacterium* sp. Strain S65).

5. Research was done to investigate biodegradative behaviour after ageing of phenanthrene in different γ-ray sterilized farm soils, containing different levels of soil organic matter content. Bioaugmentation experiments were carried out. Based on potential to mineralize various PAHs in previous experiments and $^{14}CO_2$ evolution, the mineralization capability and capacity of this PAH degrading organism (*Mycobacterium* sp. Strain S65) for soil-aged ^{14}C-labelled phenanthrene was assessed.

6. Investigations of biodegradative behaviour after ageing of pyrene in different γ-ray sterilized farm soils (Sandy-Loam, Sand, Clay, Clay-Loam), containing different levels of soil organic matter content were made. Bioaugmentation experiments were carried out. Based on potential to mineralize various PAHs in previous experiments and $^{14}CO_2$ evolution, the mineralization capability and capacity of this PAH degrading organism (*Mycobacterium* sp. Strain S65) for soil-aged ^{14}C-labelled pyrene was also assessed.

7.2. General Conclusions

Regarding to severity of environmental problems: having a General Environmental Protection Agenda, which recommends applied solutions for real-world environmental protection, is a necessity, which requires preparing and publishing a priority substances list that identifies substances, including chemicals, effluents, and wastes, that may be harmful to the environment or constitute a danger to human and animal health. Consequently, the urgent elimination and removal (*i.e.*, Environmental Cleanup) of a wide range of hazardous pollutants (*e.g.*, PAHs) and wastes from the environment in an holistic ecologically responsible, safe, rapid, and cost-effective way, should be a priority for governments and environmental management agencies and more importantly should be an absolute requirement to promote a sustainable development of our society with a low environmental impact.

Moreover, this conclusion will be followed up by future research and support future strategies regarding the bioavailability, biodegradation, bioremediation, and biodetoxification of ever-increasing amounts of organic xenobiotics (*i.e.* organic chemicals that are not products of biosynthesis) which are constantly released into the environment..

7.3. Concluding Remarks

There are several general conclusions that may be drawn from this research work presented here, therefore, these will be outlined in an order reflecting the chronological order of the study presented in the main chapters of this thesis.

General conclusions from this research fall into four main areas:

(i) Isolation of PAH-degrading bacterial strains and mineralization of PAHs

Twelve different previously characterized petroleum-contaminated soil environments, yielded 228 phenanthrene-degrading bacterial strains. Of 228

isolates, 60 produced a clear zone in a phenanthrene precipitate on MSM_{A+PAH} and $YTS_{250+PAH}$ plates.

Various strains' abilities to biodegrade polycyclic aromatic hydrocarbons (PAHs), namely naphthalene (2 fused-benzene rings), anthracene, phenanthrene, fluorene (3 fused-benzene rings), fluoranthene and pyrene (4 fused-benzene rings), was tested. Of the 60 positive colonies (PAHs-degrading microorganisms), 14 were *ndoB* probe-positive, The 14 positive colonies were recognized as phenanthrene and naphthalene-degrading microorganisms and used in further experiments to quantify their capacity to mineralize pyrene.

Furthermore, all the 60 positive colonies of the bacterial GenBank were micromorphologically characterized based on standard methods and served to form the material making up a bacterial GenBank for the purpose of selection of the genes involved in PAH-biodegradation. These would serve in further research work in bioavailability, biodegradation, bioremediation and biodetoxification research projects. One bacterial isolate (**Strain S65**) which mineralized selected PAHs was chosen for further characterization.

(ii) Characterization of PAHs-degrading bacterial strain S65

Strain “S65” was isolated from a jet-fuel contaminated site (Sept-Iles airport, Sept-Iles, QC, Canada). In aerobic liquid microcosms, Strain S65 rapidly mineralized Phenanthrene or Pyrene as sole carbon and energy source (61% cumulative mineralization, each), and to a lesser extent Fluoranthene (24% cumulative mineralization), but was not able to significantly degrade Naphthalene, Anthracene or Fluorene. Strain S65’s mineralization abilities indicated it to be a new and unique member of the xenobiotic-degrading *Mycobacteria*.

This was further confirmed by (***i***) micromorphological observations: Strain S65 was found to be Gram variable, catalase-positive, with rod-coccus morphology, and produced creamy yellow circular colonies, (***ii***) analysis of the strain S65 Cell Wall Mycolic Acids by HPLC: Strain S65’s HPLC peak pattern while similar to reference profiles currently held for *Mycobacterium* ***sp.*** by local

public health laboratories did not exactly match any. (***iii***) and external 16S rRNA Gene Sequence Analysis testing: this was distinct among mycobacterial species.

The optimal growth temperature for Strain S65 (YTS_{1000B} medium) was 30°C, but it was unable to grow at 35°C, indicating that it is unlikely to be a human pathogen. Strain S65 was given **GenBank designation, *Mycobacterium* sp. Strain S65**.

In view of the remarkable mineralization of fluoranthene, phenanthrene and pyrene by *Mycobacterium* sp. Strain S65 in liquid culture, it might be hoped that the microorganism could eventually serve as a bioassay for the bioavailability of PAHs in contaminated soils. Furthermore, bioaugmentation with this organism was predicted to provide an innovative and practical decontamination process for PAHs contaminated sites.

(iii) *Mycobacterium* sp. Strain S65 degradation of soil-aged phenanthrene

A bioassay of the bioavailability of soil-aged ^{14}C-labelled phenanthrene in different soil environments using *Mycobacterium* sp. Strain S65 was undertaken in soil microcosms. Sterilized farm soil microcosms were spiked ^{14}C-labelled phenanthrene and allowed to age for up to 360 days, at which point they were inoculated with Strain S65.

The pattern of phenanthrene biodegradation by Strain S65 in non-aged soil microcosms followed the same three-day lag period as occurred in liquid microcosms. Phenanthrene mineralization continued thereafter for 9 days, reaching a cumulative maximum of 20% in sandy-loam soil with low organic matter, but only 14% in clay-loam soil with high organic matter.

Metabolism of non-aged soil-borne phenanthrene was moderate in comparison to that in aerobic liquid microcosms (20% vs. 61% cumulative mineralization). For 30-day aged phenanthrene, *Mycobacterium* sp. Strain S65 gave a maximum cumulative mineralization of roughly 14% in sandy-loam low organic matter soil, but only 11% in clay-loam high organic matter soil. For 180 and 360-day aged phenanthrene, *Mycobacterium* sp. Strain S65 utilized the phenanthrene, but cumulative mineralization was less than 10%. High clay-high

organic matter soil showed the lowest degradation rates, likely due to the greater number of potential PAH adsorption binding sites on clay particles and organic matter. Liquid medium live culture (positive) and heat-killed (negative) Mycobacterium S65 soil microcosm controls were run in parallel to the aging experiment.

(iv) *Mycobacterium* sp. Strain S65 degradation of soil-aged pyrene

A bioassay of the bioavailability of soil-aged ^{14}C-labelled pyrene in different soil environments using *Mycobacterium* sp. Strain S65 was undertaken in soil microcosms. Sterilized farm soil microcosms were spiked ^{14}C-labelled pyrene and allowed to age for up to 360 days, then at the end of each aging time, they were inoculated with *Mycobacterium* sp. Strain S65.

The lack of a lag period in pyrene degradation in liquid microcosms, also occurred in soil microcosms, indicating that *Mycobacterium* sp. Strain S65 rapidly utilized the substrate. Cumulative pyrene mineralization reached 18.5% to 19.0% within 24 hours, and 20.0 to 21.0% after 9 days in sandy-loam soil with low organic matter. However, in clay-loam soil with high organic matter, 9 day cumulative mineralization was lower at 14,5 to 15.5%.

Metabolism of non-aged soil-borne pyrene was moderate in comparison to that in aerobic liquid microcosms (21% vs. 61% cumulative mineralization). For 30-day aged pyrene, *Mycobacterium* sp. Strain S65 gave a maximum cumulative mineralization of pyrene of 13.5% in sandy-loam soil with low organic matter, but only about 12% in clay-loam soil with high organic matter. For 180 and 360-day aged pyrene, *Mycobacterium* sp. Strain S65 utilized the pyrene, but cumulative pyrene mineralization was less than 10%. High clay and high organic matter soil showed the lowest degradation rates, likely due to the greater number of potential PAH adsorption binding sites on clay particles and organic matter. Positive control (live *Mycobacterium* S65 in liquid media) and negative control (heat-killed *Mycobacterium* S65 in liquid media) were performed in microcosms in parallel to the aging experiments.

Lastly, it is noteworthy that based on the encouraging data and analysis of the precise results obtained in this study, this research strongly supports the concept of there being a significant role for *Mycobacteria* in PAHs-biodegradation, bioremediation, and biodetoxification in contaminated environments.

CHAPTER 8

Contributions to Knowledge and Recommendations for Future Research

8.1 Statement of Originality

To the best of author's knowledge, this cutting-edge research work was one of the first study of its kind. This study delved deeply into the details of one of the most critical global environmental issue:

"Bioavailability and Biodegradation of Organic Xenobiotic Recalcitrant Polycyclic Aromatic Hydrocarbons (PAHs) in Different Soil Environments"

More specifically, this thesis included the design, investigation and analysis of critical aspects of the bioavailability and fate of organic xenobiotic recalcitrant PAHs. Also, it evaluated novel techniques to comprehensively and precisely assess mineralization and biodegradation of PAHs in different soil environments, in view of their use in bioremediation and biodetoxification management of contaminated sites.

More generally, this thesis explored such timely issues as global contamination, application of science and biotechnology, environmental cleanup, and views for a holistic ecological protection.

Lastly, and more importantly, the author intended to conclude and draw upon ideas and beliefs which lead to a holistic ecological view wherein humans, other species and the environment act as a single system, maintain an equilibrium in ecosystems and sustain the balance of life on our beautiful planet.

8.2 Contributions to Knowledge

This research project investigated the ***Bioavailability and Biodegradation of Organic Xenobiotic Recalcitrant Polycyclic Aromatic Hydrocarbons (PAHs) — compounds with minimal water solubility and low bioavailability in soils — in Different Soil Environments*** in terms of the (**i**) relative fractions of contaminants that are either

unavailable, available (or potentially available) for microbial utilization, and the (**ii**) rates at which these fractions are utilized.

Bacterial strains isolated from soils contaminated with a large range of organic pollutants, were tested for their ability to break down selected organic *xenobiotic* recalcitrant PAHs. A total of 228 phenanthrene-degrading bacterial strains were preserved in a first culture collection. Of these, 60 strains were preserved in a separate GenBank Culture Collection held at $^{-}$80°C for future researches in bioremediation and biodetoxification projects at contaminated sites. Testing for their mineralization capacity towards selected organic *xenobiotic* recalcitrant PAHs, along with their genetic makeup, allowed one to narrow down the 14 most widely competent strains.

A single isolate, strain S65 was found to cumulatively mineralize, in liquid culture, Phenanthrene 61%, Pyrene 61%, and to a lesser extent Fluoranthene 24%, but not Naphthalene, Anthracene or Fluorene**,** indicating that Strain S65's was a new and unique member of the PAHs-degrading *Mycobacteria*. Analysis of Strain S65's Cell Wall Mycolic Acids by HPLC showed that it did not match any reference profile currently held in local (Quebec) public health laboratories. Along with an external 16S rRNA Gene Sequence Analysis, Strain S65 was further confirmed as being a unique and novel *Mycobacterium.*

Work in soil microcosms with PAHs, aged for different periods (0 - 360 days) prior to inoculation with Strain S65 (**designated**: ***Mycobacterium* sp. Strain S65** in **Genbank**), showed that, as in liquid cultures, degradation of non-aged phenanthrene only started after a 3-day lag. With phenanthrene concentrations of 250 mg L^{-1} and 500 mg L^{-1}, cumulative mineralization reached a maximum at 9 days of 18.5% and 20.0%, respectively, in sandy-loam soil with low organic matter, and 13.5% and 14.5% in clay-loam soil with high organic matter. Thus, with the shorter PAH-aging periods (0-day and 30-day aged), soils with high clay and high organic matter content showed the lowest degradation rates, likely due to the greater number of potential PAH adsorption binding sites on clay particles and organic matter. Comparatively, cumulative pyrene mineralization reached a maximum of 20% and 21% (500 or 1000 mg L^{-1}, respectively) in sandy-loam soil with low organic matter, and 14.5% and 15.5% in clay-loam soil with high organic matter. Similar to the results obtained in the liquid culture, the

mycobacterium started degrading pyrene without any lag period. The effect of clay content and soil organic matter content, however, was the same as for phenanthrene.

For the longer 180- and 360-day aged pyrene and phenanthrene, cumulative mineralization was less than 10%, a significant drop from microcosms with lesser-aged PAHs. Therefore, longer ageing appears to have a detrimental effect on mineralization.

This work strongly supports the role of *Mycobacterium* sp. Strain S65 in PAHs-biodegradation, bioremediation, and biodetoxification of contaminated environments.

8.3 Recommendations for Future Research

The crucial situation of hazardous substrates associated with recalcitrant PAHs pollution has created awareness in research communities around the globe to develop methods to remove PAHs contaminants from contaminated sites. Nevertheless, to permit a more comprehensive assessment, it is recommended that these areas of further research should be pursued in the future:

1. Understanding the mechanisms and factors governing the bioavailability and fate of hydrophobic organic compounds in different soil, sediment and water environments are key aspects for the eventual remediation of contaminated sites.

2. One of the main reasons for the prolonged persistence of hydrophobic hydrocarbons in the environment is their low water solubility which increases their sorption to soil particles and limit their availability to biodegrading microorganisms. On the other hand, the sorption–desorption are known to be the two most critical processes affecting fate and transport of PAHs. Also, both clay minerals and soil organic matter are important determinants of these processes. Therefore, sorption–desorption experiments should be conducted to investigate the adsorptive behaviour of PAHs in different soil, sediment, and water environments.

3. Special attention should be given to different approaches that can be used to assess the bioavailability of PAHs.

4. Studies on laboratory species should be used to evaluate the potential effects of PAH exposure in wild species.

5. More research should be conducted for the assessment of exposure of human to PAHs and their molecular, biochemical and cellular actions, as well as their organism-level and ecological effects.

6. To better characterized the effects of PAHs in soil, sediment, water, and in the air on human and animal's lives.

It would be valuable to pursue the advice of Doctor Charles W. Greer (2008) regarding the use of Genomic Techniques such as DNA Sequencing, PCR Amplification, Micro-Array Screening and Bioinformatics Databases, in identifying more suitable microorganisms for biodegradation, bioremediation, and biodetoxification of organic *xenobiotic* recalcitrant chemicals in contaminated environments. These cutting-edge techniques can be used to identify and profile microorganismal communities that can help in bioremediation. The view of there being a significant role of *Mycobacteria* in PAH biodegradation, bioremediation and biodetoxification in contaminated environments is strongly supported by this study.

CHAPTER 9

References

Aitken, M.D. and T.C. Long. (2004). Biotransformation, Biodegradation, and Bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs). In: Singh, A., and O.P. Ward. (Eds.). *Biodegradation and Bioremediation*. Springer-Verlag, Berlin Heidelberg. Printed in Germany.

Aitken, M.D. W.T. Stringfellow, R.D. Nagel, C. Kazunga and S.H. Chen. 1998. Characteristics of phenanthrene-degrading bacteria isolated from soils contaminated with polycyclic aromatic hydrocarbons. Can. J. Microbiol. 44: 743-752.

Al-Bashir, B. (1991). Biodegradation of Polycyclic Aromatic Hydrocarbons in Soil/Water Systems. *A master thesis submitted to McGill University*, Montreal, Quebec, Canada.

Alexander, D.B. (2005). Bacteria and archaea. In: Hartel, P.G.; D.M. Sylvia; J.J. Fuhrmann and D.A. Zuberer. (Eds.). *Principles and Applications of Soil Microbiology*. 2nd edition. Upper Saddle River, NJ: Prentice-Hall, U.S.A.

Alexander, M. (1973). Biotechnology Report: Nonbiodegradable and Other Recalcitrant Molecules. *Biotechnology Bioengineering*. 15(4): 611-647.

Alexander, M. (1991). Research Needs. *Environmental Science and Technology.* 25:1972-73.

Alexander, M. (1995). How Toxic are Toxic Chemicals in Soil? *Environ. Sci. Technol.* 29: 2713-2717.

Alexander, M. (1999). *Biodegradation and Bioremediation*. Published by the Academic Press, 525 B Street, Suite 1900, San Deigo, California 92101-4495, U.S.A.

Alexander, R.R and M. Alexander. (2000). Bioavailability of Genotoxic Compounds in Soils. *Environ. Sci. Technol.* 34: 1589-1593.

Aronstein, B.N. and M. Alexander. (1993). Effect of a Non-Ionic Surfactant Added to the Soil Surface on the Biodegradation of Aromatic Hydrocarbons Within the Soil. *Appl. Microbiol. Biotechnol.* 39: 386-390.

Atlas, R.M. and C.E. Cerniglia. (1995). Bioremediation of Petroleum Pollutants. *BioScience*. 45: 332-339.

Atlas, R.M. and J.C. Philp. (Eds.). (2005). *Bioremediation: Applied Microbial Solutions for Real-World Environmental Cleanup*. ASM Press. American Society for Microbiology, 1752 N Street, N.W. Washington, D.C. 20036-2904, U.S.A.

ATSDR, (1995). U.S. Agency for Toxic Substances and Disease Registry. *Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs)*: U.S. Department of Health and Human Services, Public Health Service. Atlanta, GA. U.S.A.

ATSDR, (2006). U.S. Agency for Toxic Substances and Diseases Registry. *Toxicological Profile for Information Sheet*. Atlanta, GA. U.S.A. Available Online: http://www.atsdr.cdc.gov/toxprofiles/[verified 15 April, 2006].

ATSDR. (Agency for Toxic Substances and Disease Registry U.S.). (1990). Public *Health Statement: Polycyclic Aromatic Hydrocarbons (PAHs)*. U.S. Department of Health and Human Services, Public Health Service. Atlanta, GA., U.S.A.

ATSDR. (Agency for Toxic Substances and Disease Registry U.S.). (1995). *Toxicological Profile for: Polycyclic Aromatic Hydrocarbons (PAHs).*U.S. Department of Health and Human Services, Public Health Service. Atlanta, GA., U.S.A.

Auger, R.L., A.M. jacbson and M.M Domach. (1995). Aqueous Phase Fluorescence Quecheing Technique for Measuring Naphthalene Partition Coefficients. *Environ. Sci. Technol.* 29: 1273-1278.

Baek, S.; R.A. Field and M.E. Goldstone. (1991). A Review of Atmospheric Polycyclic Aromatic Hydrocarbons: Sources, Fate and Behavior. *Water, Air and Soil Pollution.* 60: 279-300.

Baeyer, Ph.; J.-C. Block and V.V. Goncharuk. (1999). *Bioavailability of Organic Xenobiotics in the Environment: Practical Consequences for the Environment*. Kluwer Academic Publishers. P.O. Box 17, 3300 AA Dordrecht. The Netherlands.

Baker, K.H. and D.S. Herson. (1994). *Bioremediation*. McGraw-Hill, Inc. U.S.A.

Bakker, M.I. (2000). *Atmospheric Deposition of Semi-Volatile Organic Compound to Plants*. Ph.D. Thesis, (Uttecht: Research Institute of Toxicology, Uttecht University).

Baveye, Ph.; J.-C. Block; V.V. Goncharuk. (Eds.). (1999). *Bioavailability of Organic Xenobiotics in the Environment: Practical Consequences for the Environment*. Kluwer Academic Publishers. 101 Philip Drive, Norwell. MA., 02061, U.S.A.

Bazin, N.J.; P.T. Saunders and J.I. Prosser. (1976). CRC Cirt. *Rev. Microbiol.* 4: 463-498.

Bence, A.E.; K.A. Kvenvolden and M.C. Kennicutt, II. (1996). "Organic Geochemistry Applied to Environmental Assessments of Prince William Sound, Alaska, after the Exxon Valdez Oil Spill-A Review." *Organic Geochemistry.*24:7-42.

Bergey, D.H. (1994). In: Holt, J.G.; N.R. Krieg; P.H.A. Sneath; J.T. Staley and S.T. Williams. (Eds.). *Bergeh's Manual of Determinative Bacteriology*. Ninth Edition. William & Wilkins. 428 East Preston Street. Baltimore, MD. 21202, U.S.A.

Bhandari, A. (2009). Contaminants of Emerging Environmental Concern. In: Bhandari, A.; R.Y. Surampalli; C.D. Adams; P. Champagne; S. K. Ong; R.D. Tyagi; and T.C. Zhang. (Eds.). Contaminants of Emerging Environmental Concern. American Society of Civil Engineers. 1801 Alexander Bell Drive, Reston, Virginia, 20191-4400. U.S.A.

Bhandari, A.; R.Y. Surampalli; P. Champagne; S.K. Ong; R,D. Tyagi and I.M.C. Lo. (Eds.) (2007). *Remediation Technologies for Soils and Groundwater*. American Society of Civil Engineers (ASCE). 1801 Alexander Bell Drive, Reston, Virginia, 20191-4400. U.S.A.

Bjorseth, A. (1985). Sources of Emissions of PAH. In: Bjorseth, A. and T. Ramdahl, (eds.), *Handbook of PolycyclicAromatic Hydrocarbons.* Marcel Dekker, Inc., 507. New York, NY., U.S.A., Pp.I-20.

Bjorseth, A. (Ed.). (1983). *Handbook of PAH.* Marcel Dekker, Inc., 507. New York, NY., U.S.A.

Blumer, M. (1976). Polycyclic Aromatic Hydrocarbons in Nature. *Scientific American.* 234: 35-45.

Boddinghaus, B., T. Rogall, T. Flohr, H. Blocker and E.C. Bottger. (1990). Detection and identification of mycobacteria by amplification of rRNA. *Journal of Clinical Microbiology* 28(8):1751-1759.

Boehm, P.D. (2006). Polycyclic Aromatic Hydrocarbons (PAHs). In: Morrison, R.D. and B.L. Murphy. (Eds.). *Environmental Forensics Contaminant Specific Guide*.

Academic Press is an Imprint of Elsevier. 30 Corporate Drive, Suite 400, Burlington, MA. 01803, U.S.A.

Boehm, P.D.; D.S. Page; W.A. Burns, A.E. Bence, P.J. Mankiewicz and J.S. Brown. (2001). Resolving the Origin of the Petrogenic Hydrocarbon Background in Prince William Sound, Alaska. *Environmental Science and Technology.* 35: 471-479.

Boldrin, B.; A. Tiehm and C. Fritsche. (1993). Degradation of Phenanthrene, Fluorene, Fluoranthene, and Pyrene by a *Mycobacterium* sp. *Appl. Environ. Microbiol*. 59: 1927-1930.

Bollage, J.M. and S.Y. Liu. (1990). Pesticides in the Soil Environment: Processes, Impacts, and Modeling. *Soil Science Society of America*, Inc. U.S.A.

Bosma, R.M.P., P.J.M. Middeldorp, G. Schraa and A.J.B. Zehnder. (1997). Mass Transfer Limitation of Biotransformation: Quantifying Bioavailability. *Environ. Sci. Technol.* 31: 248-252.

Bottger, E.C. (1989). Rapid Determination of Bacterial Ribosomal RNA Sequences by Direct Sequencing of Enzymatically Amplified DNA. *Federation of European Microbiological Societies Microbiology Letters*. 53(1-2):171-176.

Bouchez, M.; D. Blanchet and J.-P. Vandecasteele. (1995). Degradation of Polycyclic Aromatic Hydrocarbons by Pure Strains and by Defined Strain Associations: Inhibition Phenomena and Cometabolism. *Appl. Microbiol. Biotechnol*. 43: 156-164.

Bourquin, A.W. (1994). Special Issue on Bioremediation. *Biodegradation*. 4(4). Kluwer Academic Publishers.

Buscot, F. (2005). What are Soils? In: Buscot, F. and A. Varma. (Eds.). *Microorganisms in Soils: Roles in Genesis and Functions*. Springer-Verlag, Berlin Heidelberg. Printed in Germany.

Buscot, F. and A. Varma. (2005). *Microorganisms in Soils: Roles in Genesis and Functions*. Springer-Verlag, Berlin Heidelberg. Printed in Germany.

Calow, P. (1993). Handbook of Ecotoxicology. Volume (1). 1st Edition. Oxford: Blackwell Scientific Publications.

Cattaneo, M.V.; C. Masson and C.W. Greer. (1997). The Influence of Moisture on Microbial Transport, Survival and 2,4-D Biodegradation with a Genetically Marked *Burkholderia Cepacia* in Unsaturated Soil Columns. *Biodegradation*. 8: 87-96.

Cerniglia, C.E. (1984). Microbial Metabolism of Polycyclic Aromatic Hydrocarbons. *Adv. Appl. Microbiol.* 30: 31-71.

Cerniglia, C.E. (1992). Biodegradation of Polycyclic Aromatic Hydrocarbons. *Biodegradation*. 43:156-168.

Cerniglia, C.E. (1993). Biodegradation of PAHs. *Curr. Opin. Biotechnol.* 4:331-338.

Chan, E.C.S.; M.J. Pelczar Jr. and N.R. Krieg. (1993). *Laboratory Exercises in Microbiology*. 6th Edition. McGraw-Hill, Inc., New York, NY., U.S.A.

Chang, B.-V.; I.T. Chang and S.Y. Yuan. (2008). Anaerobic Degradation of Phenanthrene and Pyrene in Mangrove Sediment. *Bull. Environ. Contam. Toxicol.* 80: 145–149.

Chaudry, G.R. and S. Chapalamadugu. (1991). Biodegradation of Halogenated Organic Compounds. *Microbiol. Rev.* 55: 59-79.

Chen, K.; H. Neimark; P. Rumore and C.R. Steinman. (1989). Broad-Range DNA Probes for Detecting and Amplifying Eubacterial Nucleic Acids. *Federation of European Microbiological Societies Microbiology Letters*. 48(1): 19-24.

Chénier, M.R.; D. Beaumier; R. Roy; B.T. Driscoll; J.R. Lawrence and C.W. Greer. (2003). Impact of Seasonal Variations and Nutrient Inputs on Nitrogen Cycling and Degradation of Hexadecane by Replicated River Biofilms. *Applied and Environmental Microbiology*. 69(9): 5170–5177.

Cheung, P.Y. and B.K. Kinkle. (2001). *Mycobacterium* Diversity and Pyrene Mineralization in Petroleum-Contaminated Soils. *Appl. Environ. Microbiol.* 67: 2222–2229.

Chiou, C.T.; D.E. Klle and R.L. Malcolm. (1988). Sorption of Vapors of some Organic Liquids on Soil Humic-acid and its Relation to Partitioning of Organic Compounds in Soil Organic Matter. *Environ. Sci. Technol.* 22: 298-303.

Chiou, C.T.; D.W. Schmedding and M.Manes. (1982). Partitioning of Organic Compounds in Octanol-Water Systems. *Environ, Sci. Technol.* 16: 4-10.

Clarke, P.H. (1984). The Evolution of Degradative Pathways. In: Gibson, D.T. (Ed.). *Microbial Degradation of Organic Compounds*. Marcel Dekker, Inc. 270 Madison Avenue, New York, NY. U.S.A.

Clarridge, J.E. (2004). Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases**.** *Clinical Microbiology Reviews*, 17(4): 840-862.

Clonfero, E.; F.J. Jongeneelen and M. Zordan. (1990). Biological Monitoring of Human Exposure to Coal Tar. Urinary Mutagenicity Assays and Analytical Determination of Polycyclic Aromatic Hydrocarbon Metabolites in Urine. In: Vainio, H., M. Sorsa and A.J. McMichael (Eds.). Complex Mixtures and Cancer Risk. International Agency for Research on Cancer. *IARC Sci. Publ.* 104: 215-222. Lyon, France.

Cloud, J.L.; H. Neal; R. Rosenberry; C.Y. Turenne; M. Jama; D.R. Hillyard and K.C. Carrol. (2002). Identification of *Mycobacterium* sp. by Using a Commercial 16S Ribosomal DNA Sequencing Kit and Additional Sequencing Libraries. *Journal of Clinical Microbiology*. 40(2): 400-406.

Conte, P.; A. Zena; G. Pilidis and A. Piccolo. (2001). Increased Retention of Polycyclic Aromatic Hydrocarbons in Soils Induced by Soil Treatment with Humic Substances. *Environ. Pollut.* 112: 27-31.

Cook, V.J.; C.Y. Turenne; J. Wolf; R. Pauls and A. Kabani. (2003). Conventional Methods Versus 16S Ribosomal DNA Sequencing for Identification of Nontuberculous Mycobacteria: Cost Analysis. *Journal of Clinical Microbiology*. 41(3): 1010-1015.

Cookson, JR. J.T. (1995). Bioremediation Engineering: Design and Application. *McGraw-Hill, Inc.* A Book from McGraw-Hill Environmental Engineering Books of Interest. Printed in the United States of America.

Coover, M.P. and R.C. Sims. (1987). The Effects of Temperature on Polycyclic Aromatic Hydrocarbon Persistence. *Haz Waste Haz Mat.* 4: 69-82.

Copeland, R.A. (2000). Enzymes. *A Practical Introduction to Structure, Mechanism and Data Analysis.* John Wiley & Sons, Inc. 605 Third Avenue, New York, NY., 10158-0012, U.S.A.

Crawford, R.L. and D.L. Crawford. (1996). *Bioremediation Principles and Applications*. Cambridge University Press, New York, NY., U.S.A.

Danne, L.L., I. Harjono, G.J. Zylstra, M.M. Häggblom. (2001). Isolation and Characterization of Polycyclic Aromatic Hydrocarbon-degrading Bacteria Associated with the Rhizosphere of Salt Marsh Plants. *Appl Environ Microbiol* 67: 2683–2691.

Dabestani, R. and I.N. Ivanov. (1999). A Compilation of Physical, Spectroscopic and Photophysical Properties of PAHs. *Photochem Photobiol.* 70:10–34.

Dean-Ross, D. and C.E. Cerniglia. (1996). Degradation of Pyrene by *Mycobacterium flavenscens*. *Applied and Environmental Microbiology*. 46(3): 307-312.

Dean-Ross, D., J.Moody; C.E. Cerniglia. (2002). Utilization of Mixtures of Polycyclic Aromatic Hydrocarbons by Bacteria Isolated from Contaminated Sediment. *FEMS Microbiol. Ecol*. 41(1): 1–7.

De Maagd, P.G.J., D.T.E.M. ten Hulscher, , H. van den Heuvel, , A. Opperhuizen, D.T.H.M. Sijm. (1998). Physicochemical Properties of Polycyclic Aromatic Hydrocarbons: Aqueous

Solubilities, n-octanol/water Partition Coefficients, and Henry's Law Constants. *Environ.Toxicol. Chem*.17, 251–257.

Desai, A.M.; R.L. Autenrieth; P. Dimitriou-Christidis and T.J. McDonald. (2008). Biodegradation kinetics of Select Polycyclic Aromatic Hydrocarbon (PAH) Mixtures by *Sphingomonas paucimobilis* EPA505. *Biodegradation*. 19:223–233.

Deziel, E.; G. Paquette; R. Villemur; F. Lepine and J.G. Bisaaillon. (1996). Biosurfactant Production by a Soil *Pseudomonas* Strain Growing on Polysyclic Aromatic Hydrocarbons. *Appl. Environ. Microbiol.* 62:1908-1912.

Diaz, E. (Ed.). (2008). *Microbial Biodegradation: Genomics and Molecular Biology*. Caister Academic Press. Norfolk, U.K.

Dibble, A.S.; C. Cheng and C.A.H. Biggar. (1990). Polycyclic Aromatic Hydrocarbon Carcinogens. In: Pariza, M.W.; H.U. Aeschbacher; J.S. Fenton and S. Sato. (Eds.). *Mutagens and Carcinogens in the Diet*. pp. 109-127. *Wiley-Liss*. New York, NY., U.S.A.

Dominguez, J.B. (Ed.). (2008). *Soil Contamination Research Trends*. Nova Science Publishers, Inc. New York. NY., U.S.A.

Douben, P.E.T. (Ed.). (2003). *PAHs: An Ecotoxicological Perspective*. John Wiley & Sons, Ltd. Unilever Colworth R&D, Safety and Environmental Assurance Centre. Sharnbrook, Bedford, U.K.

Eaton, R.W. and P.J. Chapman. (1995). Formation of Indigo and Related Compounds from Indolecarboxylic Acids by Aromatic Acid-Degrading Bacteria: Chromogenic Reactions for Cloning Genes Encoding Dioxygenases that Act on Aromatic Acids. *J. Bacteriol.* 177: 6983-6988.

Edghill, R.U. (1995). Removal of Pentachlorophenol from Soil by *Arthrobacter* Strain ATCC 33790. In: Hinchee R.E.; J. Frederickson and B.C. Alleman. (Eds.). *Bioaugmentation for Site Remediation*. Pp.85-90. Batelle Press, Columbus, OH. U.S.A.

Emsbo-Mattingly, S. and P.D. Boehm. (2003). Identifying PAHs from Manufactured Gas Plant Sites. *Technical Report No. 1005289*. Electric Power Research Institute, Palo Alto, California, U.S.A.

Ensley, B.D. and D.T. Gibson. (1983). Naphthalene Dioxygenase: Purification and Properties of a Terminal Oxygenase Component. *J. Bacteriol.* 155: 505-511.

Environment Canada. (1994). Polycyclic Aromatic Hydrocarbons. In: Canadian Environmental Protection Act. *Priority Substances List Assessment Report*. Government of Canada; Environment Canada and Health Canada. National Printers (Ottawa) Inc., Canada.

Environment Canada. (1993). Canadian Environmental Protection Act. PAH Emission into the Canadian Environment-1990. *Report Prepared for Environment Canada, Quebec region.* Montreal, Quebec, Canada.

Fernadez, M.D. and J.V. Tarazona. (2008). Complementary Approaches for Using Ecotoxicity Data in Soil Pollution Evaluation: Risk Based Versus Direct Toxicity Assessments. In: Domingues, J.B., (Ed.). *Soil Contamination Research Trends*. Nova Science Publishers, Inc. New York. NY., U.S.A.

Finlayson-pitts, B.J. and J.N. Jr. Pitts. (1997). Tropospheric Air Pollution: Ozone, Airborne Toxic, PAHs and Particles. *Science.* 276: 1045-1052.

Furukawa, K. and A.M. Chakrabarty. (1982). Involvement of Plasmids in Total Degradation of Chlorinated Biphenyls. *Applied and Environmental Microbiology*. 44(3): 619-626.

Garrity, G.M. and J.G. Holt. (2001). The Road Map to the Manual. In: Garrity, G.M. (Ed.). *Bergey's Manual of Systematic Bacteriology*. Springer-Verlag. New York, NY., U.S.A.

Gherna, R.L. (1994). Culture Preservation. In: *Methods for General and Molecular Bacteriology*. American Society for Microbiology, 1325, Massachusetts Ave., N.W. Washington, D.C., 20005, U.S.A.

Gibson, D.T. (1976). Initial Reaction to the Bacterial Degradation of Aromatic Hydrocarbons. *Zentralbl. Bakt. Hyg. I. Abt. Orig. B.* 162: 157-168.

Gibson, D.T. and R.E. Parales. (2000). Aromatic Hydrocarbons Dioxygenases in Environmental Biotechnology. *Curr. Opin. Biotechnol*. 11: 236-243.

Giger, W. and M. Blumer. (1974). Polycyclic Aromatic Hydrocarbons in the Environment: Isolation and characterization by Chromatography, Visible, Ultraviolet, and Mass Spectrometry. *Analytical Chemistry.* 46: 1663-1671.

Gilbert, M.T. (1987). *High-Performance Liquid Chromatography (HPLC)*. Techno House. Bristol, U.K.

Gormmen, R. and W. Verstraete. (2002). Environmental Biotechnology: The Ongoing Quest. *J. Biotechnol.* 98:113-1232.

Gray, K.A.; T.H. Richardson; D.E. Robertson; P.E. Swanson and M.V. Subramanian. (2003). Soil-Based Gene Discovery: A New Technology to Accelerate and broaden Biocatalytic Applications. *Adv. Appl. Microbiol.* 52:1-27.

Greer, C.W. (2008). Interview with Dr. Charles Greer Environmental Microbiology Group and Research Program Leader. Posted on Site of: National Research Council of Canada's Biotechnology Research Institute (NRC-BRI). 6100, Royal Mount Avenue, Montreal, Quebec and Canada, H4P 2R2. www.bri.nrc.gc.ca/rd /environment/envmicrobiology/activities.

Greer, C.W., L. Masson, Y. Comeau; R. Brousseau and R. Samson. (1993). Application of Molecular Biology Techniques for Isolating and Monitoring Pollutant-Degrading Bacteria. *Water Pollution Research Journal of Canada*. 28(2): 275-287.

Greer, C.W.; Hawari, J. and R. Samson. (1990). Influence of Environmental Factors on 2,4-dichlorophenoxyacetic Acid Degradation by *Pseudomonas cepacia* Isolated from Peat. *Arch. Microbiol.* 154: 317-322.

Grosenheider, K.E.; P.R. Bloom; T.R. Halbach and M.R. Johnson. (2005). A Review of the Current Literature Regarding Polycyclic Aromatic Hydrocarbons in Asphalt Pavement. *University of Minnesota Conference*. U.S.A.

Grosser, R.J.; D. Warshawsky and J.R. Vestal. (1991). Indigenous and Enhanced Mineralization of Pyrene, benzo[α]pyrene, and Carbozole in Soils. *Appl. Environ. Micrrobiol.* 57:3462-3469.

Guha, S.; C.A. Peters and P.R Jaffe. (1999). Multisubstrate Biodegradation Kinetics of Aphthalene, Phenanthrene, and Pyrene Mixtures. *Biotechnol. Bioeng*. 65:491–499.

Guo, C.L; H.W. Zhou; Y.S. Wong and N.F.Y. Tam. (2005). Isolation of PAH-Degrading Bacteria from Mangrove Sediments and their Biodegradation Potential. *Mar. Pollut. Bull*. 51: 1054–1061.

Habe, H. and T. Omori. (2003). Genetics of Polynuclear Aromatic Hydrocarbon Metabolism in Diverse Aerobic Bacteria. *Biosci. Biotechnol. Biochem.* 67: 225-243.

Hamaker, J.W. (1972). In: Goring, C.A.I. and J.W . Hamaker. (Eds.). *Organic Chemicals in the Soil Environment.* Pp. 253-340. Dekker, New York, U.S.A.

Hamdia, H.; S. Benzartib, L. Manusadzianasc; I. Aoyamaa and N. Jedidid. (2007). Bioaugmentation and Biostimulation Effects on PAH Dissipation and Soil Ecotoxicity under Controlled Conditions. *Soil Biology & Biochemistry*. 39: 1926–1935.

Hap, P.; J. Jones-Meehan; C. Nestler; L. D. Hansen; W. Straube; W. Jones; J. Hind, and J.W. Talley. (2006). Polycyclic Aromatic Hydrocarbons (PAHs): Improved Land Treatment with Bioaugmentation. Chapter 7. In: J.W. Talley (Ed.), Bioremediation of Recalcitrant Compounds. *Published by CRC Press, Taylor & Francis Group*. 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487-2742. U.S.A.

Harayama, S. (2001). Environmental Biotechnology. *Curr.Opin. Biotechnol.* 12: 229-230.

Harms, H. and Bosma, T.N.P. (1997). Mass Transfer Limitation of Microbial Growth and Pollutant Degradation. *J. Ind. Microbiol. Biotechnol.* 18: 97-105.

Harmsen, D. and H. Karch. (2004). 16S rDNA for Diagnosing Pathogens: A Living Tree. *American Society for Microbiology News.*70 (1): 19-24.

Head, I.M.; I. Singleton and M.G. Milner. (Eds.). (2003). *Bioremediation A Critical Review*. Horizon Scientific Press. P.O. Box 1. Wymondham, Norfolk, NR18 0EH, England.

Heitkamp, M.A.; W. Franklin and C.E. Cerniglia. (1988a). Microbial Metabolism of Polycyclic Aromatic Hydrocarbons: Isolation and Characterization of a Pyrene Degrading Bacterium. *Applied and Environmental Microbiology*. 54(10): 2549-2555.

Hemond, H.F. and E.J. Fechner-Levy. (2000). Chemical Fate and Transport in the Environment. *Academic Press.* A Harcourt Science and Technology Company. 525 B Street, Suite 1900, San Diego, California, 92101-4495, U.S.A.

Hites, R.A; R.E. Laflamme and J.W. Farrington. (1977). Sedimentary Polycyclic Aromatic Hydrocarbons: A historical Record. *Science.* 198: 829-831.

Hoffmann, D. and I. Hoffmann. (1993). Tobacco Smoke as a Respiratory Carcinogen. In: Hirsch, A.; M. Goldberg and J-P. Martin. *Prevention of respiratory diseases*. Pp. 497-532. New York, NY. Marcel Dekker, Inc., U.S.A.

Holt, J.G.; N.R. Krieg; P.H.A. Sneath; J.T. Staley and S.T. Williams. (Eds.). (1994). *Bergey's Manual of Determinative Bacteriology*. 9th Edition. William & Wilkins. 428 East Preston Street. Baltimore, MD. 21202, U.S.A.

http://www.cdc.gov/ncidod/publications/hplc.pdf (2006/05/04).

Hughes, J.B.; D.M. Beckles; S.D. Chandra and C.H. Ward. (1997). Utilization of Bioremediation Processes for the Treatment of PAH Contaminated Sediments. *J. Ind. Microbiol. Biotechnol.* 18: 152-160.

Hwang, H-M; H. Xiaoke and Z. Xueheng. (2007). Enhanced Bioremediation of Polycyclic Aromatic Hydrocarbons by Environmentally Friendly Techniques. *Journal of Environmental Science and Health Part C*. 25:313–352.

Jasper, D.A. (1994). Bioremediation of Agricultural and Forestry Soils with Symbiotic Microorganisms. *Aust. J. Soil Res.* 32:1301-1319.

Jayashree, R. and N. Vasudevan. (2007). Effect of Tween 80 Added to the Soil on the Degradation of Endosulfan by *Pseudomonas aeruginosa*. *Int. J. Environ. Sci. Tech.* 4(2): 203-210.

Jazestani, J. and S.O. Prasher. (2003a). Characterization of Recalcitrant PAH-degrading Bacterial Isolates from Soils Contaminated with a Range of Organic Pollutants. This Paper was Presented at the Annual CSBE Conference, and this Final Manuscript has been also Submitted for Publication in *World Journal of Microbiology* (2011).

Jazestani, J. and S.O. Prasher. (2003b). Isolation of Recalcitrant PAHs-degrading Bacterial Strains from Different Contaminated Soil Environments and Mineralization of Selected PAHs. This Paper was Presented at the Annual CSBE Conference, and this Final Manuscript has been also Submitted for Publication in *Environmental Pollution (2011)*.

Johnsen, A.R.; L.Y. Wick and H. Harms. (2005). Principles of Microbial PAH-Degradation in Soil. *Environmental Pollution*. 133: 71–84.

Juhasz, A.L. and R. Naidu. (2000). Bioremediation of High Molecular Weight Polycyclic Aromatic Hydrocarbons: A Review of the Microbial Degradation of Benzo[a]pyrene. *International Biodeterioration & Biodegradation.* 45: 57-88.

Juhasz, A.L.; M.L. Britz and G.A. Stanley. (1997). Degradation of benzo[a]pyrene, dibenz[a,h]anthracene and Coronene by *Burkholderia cepacia*. *Water Science and Technology*. 36: 45–51.

Kanaly, R.A and S. Harayama. (2000). Biodegradation of Highmolecular-weight PAHs by Bacteria. *J. Bacteriol*. 182:2059–2067.

Kastner, M.; M. Breuer-Jammali and B. Mahro. (1998). Impact of Inoculation Protocols, Salinity and pH on the Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) and Survival of PAH-Degrading Bacteria Introduced into Soil. *Applied and Environmental Microbiology*. 64: 359–362.

Kelsey, J.W.; B.D. Kotteler and M. Alexander. (1997). Selective Chemical Extractants to Predict Bioavailability of Soil-Aged Organic Chemicals. *Environ. Sci. Technol.* 31: 214-217.

Kim, E. and G.J. Zylstra. (1999). Functional Analysis of Genes Involved in Biphenyl, Naphthalene, Phenanthrene, and M-Xylene Degradation by *Sphingomonas yanoikuyae* B1. *J. Ind. Microbiol. Biotechnol.* 23: 294-302.

Kirschner, P.; B. Springer; U. Vogel; A. Meier; A. Wrede; M. Kiekenbeck; F.C. Bange and E.C. Bottger. (1993). Genotypic Identification of Mycobacteria by Nucleic Acid Sequence Determination. Report of a 2-Year Experience in a Clinical Laboratory. *Journal of Clinical Microbiology*. 31(11): 2882-2889.

Kiyohara, H.; K. Nagao and K. Yana. (1982). Rapid Screen for Bacteria Degrading Water-Insoluble, Solid Hydrocarbons on Agar Plates. *Appl. Environ. Microbiol.* 43: 454-457.

Kodama, K.; K. Umehara; K. Shimizu; S. Nakatani; Y. Minoda and K. Yamada. (1973). Identification of Microbial Products from Dibenzothiophene and its Proposed Oxidation Pathway. *Agric. Biol. Chem.* 37: 45-50.

Kolbert, C.P. and D.H. Persing. (1999). Ribosomal DNA Sequencing as a Tool for Identification of Bacterial Pathogens. *Current Opinion in Microbiology*. 2(3): 299-305.

Kotterman. M.J.J.; E.H. Vis; and J.A. Field. (1998). Successive Mineralization Detoxification of benzo(a)pyrene by the White Rot Fungus *Bjerkandera* sp. Strain BOS55 and Indigenous Microflora. *Appl. Environ. Microbiol*. 64: 2853–2858.

Labana, S.; M. Kapur; D.K. Malik; D. Prakash and R.K. Jain. (2007). Diversity, Biodegradation and Bioremediation of PAHs. In: Singh, S.N. and R.D. Tripathi. *Environmental Bioremediation Technologies*. Springer-Verlag, Berlin Heidelberg. Printed in Germany.

Laboratory Exercises in Microbiology and Immunology. (2001). *Department of Microbiology and Immunology*. McGill University, Montreal, Quebec, and Canada.

Laflamme, R.E. and R.A Hites. (1978). The Global Distribution of Polycyclic Aromatic Hydrocarbons in Recent Sediments. *Geochim. Cosmochim. Acta.* 42: 289-303.

Larson, J.R.; J.E. Tingstad and J.K. Swadesh. (1997). Introduction. In: Swadesh, J. (Ed.). *HPLC Practical and Industrial Applications*. CRC Press Inc. Boca Raton, FL., U.S.A.

Leblond, J.D.; T.W. Schultz and G.S. Sayler. (2001). Observations on the Preferential Biodegradation of Selected Components of Polyaromatic Hydrocarbon Mixtures. *Chemosphere*. 42:333–343.

Lee, M.L.; G.P. Prado; J.B. Howard and R.A. Hites. (1977). Source Identification of Urban airborne PAHs by GC/MS and High Resolution MS. *Biomedical Mass Spectrometry*. 4: 182-186.

Li, A.; S. Tanabe; G. Jiang; J. P. Giesy; and P.K.S. Lam. (2007). *Persistent Organic Pollution in Asia: Sources, Distributions, Transport and Fate*. Elsevier Ltd. Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands Linacre House, Jordan Hill, Oxford, 0X2-8DP, U.K.

Li, Q.Q.; A. Loganath; Y.S. Chong and J.P. Obbard. (2006). Persistent Organic Pollutants and Adverse Health effects in Humans. *Jornal of Toxicology & Environmental Health*. 69: 1987-2005.

Linz, D.G. and D.V. Nakles. (Eds.). (1997). *Environmentally Acceptable Endpoints in Soil*. American Academy of Environmental Engineers, Annapolis, M.D., U.S.A.

Liu, L.; J.A. Tindall and M.J. Friedel. (2007). Biodegradation of PAHs and PCBs in Soils and Sludges. *Water, Air and Soil Pollution*. 181:281–296.

Loehr, R,C. and M.T. Webster. (1997). Effects of Treatment on Contaminant availability, Mobility, and Toxicity. In: Linz, D.G. and D.V. Nakles. (Eds.). *Environmentaly acceptable endpoints in Soil*. American Academy of Environmental Engineers, Anapolis, MD. U.S.A.

Loibner, A.P.; O.H.J. Szolar; R. Braun; D. Hirmann. (2003). Ecological Assessment and Toxicity Screening in Contaminated Land Analysis. In: Thompson, k.C.; C.P. Nathanail. (Eds.). *Chemical Analysis of Contaminated Land*. 229-267. Blackwell Publishing Ltd. Oxford, U.K.

LSPQ-Bacteriologie (Laboratoire de Santé Publique du Quebec-Bacteriologie) of Institute National de Sante Publique du Quebec. (2003). *Current Reference Profiles LSPQ-Bacteriologie*. Request Number: 03A681040. 20045. Sainte-Anne-de-Bellevue, Quebec: Institute National de Sante Publique du Quebec.

Luning Prak, D.J. and P.H. Pritchard. (2002a). Degradation of Polycyclic Aromatic Hydrocarbons dissolved in Tween 80 Surfactant Solutions by *Sphingomonas paucimobilis* EPA 505. *Can. J. Microbiol.* 48:151-158.

Luning Prak, D.J. and P.H. Pritchard. (2002b). Solubilization of Polycyclic Aromatic Hydrocarbons Mixtures in Micellar Nonionic Surfactant Solutions. *Water Res.* 36: 3463-3472.

Luthy, R.G.; D.A. Dzombak; C.A. Peter; S.B. Roy; A. Raamaswami; D.V. Nakles and B.R. Nott. (1994). Remediation Tar-Contaminated Soils at Manufactured Gas Plant Sites. *Environ. Sci. Technol.* 28: 266A-227A.

Mackay, D. (2001). *Multimedia Environmental Models: The Fufacity Approach*. 2nd Edition, Lewis/CRC Press. 2000 N.W. Corporate Blvd. Boca Raton, Florida, 33431, U.S.A.

Mackay D., W. Y. Shiu and K.C.Ma (1992) Illustrated Handbook of Physical and Environmental Fate for Organic Chemicals. Volume II: Polynuclear Aromatic Hydrocarbons, Polychlorinated Dioxins and Dibenzofurans. Lewis Publishers. Boca Raton, Florida. pp. 1-367. In: IPCS.

Madsen, E.L.; C.L. Mann and S. Bilotta. (1996). Oxygen Limitation and Ageing as Explanation for the Persistence of Naphthalene in Coal-Tar Contaminated Surface Sediments. *Environ. Toxicol. Chem.* 15:1876-1882.

`Mahro, B.; A. Eschenbach; M. Kaestner and G. Schaefer. (1994). Investigation of Possibilities for Targeted Stimulation of Biogenic Mineralization and Humification of PAHs in Soils. *Biol. Abwasserreinig.* 4: 55-68.

Majumar, S.K.; E.W. Miller and F.J. Brenner. (Eds.). (1995). Environmental Contaminants, Ecosystems and Human Health. *The Pennsylvania Academy of Science*. Easton, PA. 18042, U.S.A.

Makkar, R.S. and S.S. Cameotra. (2002). An Update on the Use of Unconventional Substrates for Biosurfactant Production and their New Applications. *Appl. Microbiol. Biotechnol.* 58:428-434.

Manilal, V.B. and M. Alexander. (1991). Factors affecting the microbial degradation of phenanthrene in soil. *Applied Microbiology and Biotechnology*. 35: 401-405.

Mathewson, P.R. (1998). Enzymes. *American Association of Cereal chemists.* 3340 Pilot Knob Road. St. Paul, Minnesota, 55121-2097, U.S.A.

Means, J.C.; S.G. Wood; J.J Hassett and W.L. Banwart. (1980). Sorption of Polynuclear Aromatic Hydrocarbons by Sediments and Soils. *Environ. Sci. Technol.* 14: 1524-1528.

Meguro, N; Y. Kodama; M.T. Gallegos and K. Watanabe. (2005). Molecular Characterization of Resistance-Nodulation-Division Transporters from Solvent- and Drug-Resistant Bacteria in Petroleum-Contaminated Soil. *Applied and Environmental Microbiology.* 71(1): 580–586

Meijer, S.N.; W.A. Ockenden; A. Sweetman; K. Breivik; J.O. Grimalt and K.C. Jones. (2003). Global Distribution and Budget of PCBs and HCB in Background Surface Soils: Implications for Sources and Environmental Processes. *Environ. Sci. Technol.* 37: 667-672.

Mihelcic, J.R.; D.R. Lueking; R.J. Mitzell and J.M. Stapleton. (1993). Bioavailability of Sorbed and Separate-Phase Chemicals. *Biodegradation*. 4:141-153.

Millar, C.E.; L.M. Turk and H.D. Foth. (1965). *Fundamentals of Soil Science. 4th Edition.* John Wiley and Sons. NY. U.S.A.

Miller, E.W. (1995). Environmental Hazards: Survey and Perspective. In: Majumdar, S.K.; E.W. Miller and F.J. Brenner. (Eds.). *Environmental Contaminants, Ecosystems and Human Health*. A publication of the Pennsylvania Academic of Science. p1-14. Easton, PA. 18042 , U.S.A.

Millero, F.J. and M.L. Sohn. (1991). *Chemical Oceanography*. CRC. Press. Boca Raton, Pp 531. FL. U.S.A.

Minnesota Pollution Control Agency of U.S.A. (1998). Fact Sheet PAH List. http://www.pca.state.mn.us/programs/pubs/qa-pahs.pdf .

Mohan, S.V; T. Kisa; T. Ohkuma; A.K Robert and Y. Shimizu. (2006). Bioremediation Technologies for Treatment of PAH-contaminated Soil and Strategies to Enhance Process Efficiency. *Rev. Environ. Sci. Biotechnol.* 5:347–374.

Mueller, J.G; P.J. Chapman; B.O. Blattmann and P.H. Pritchard. (1989). Action of a Fluoranthene-Utilizing Bacterial Community on PAH Components of Creosote. *Appl. Environ. Microbiol.* 55:3085–3090.

Murry, R.G.E.; R.N. Doetsch and C.F. Robinow. (1994). Determinative and Cytological Light Microscopy. In: Gerhardt, P.; R.G.E. Murry; W.A. Wood and N.R. Krieg. (Eds.). *Methods for General and Molecular Bacteriology.* American Society for Microbiology Press. Washington, D.C., U.S.A.

Mulligan, C.N.; R.N. Yong and B.F. Gibbs. (2001). Surfactant-Enhanced Remediation of Contaminated Soil: A Review. *Eng. Geol.* 60:371-380.

Naes, K. and E. Oug. (1998). The Distribution and Environmental Relationships of Polycyclic Aromatic Hydrocarbons (PAHs) in Sediments from Norwegian Smelter-affected fjords. *ChemosPhere.* 36: 561-576.

Naidu, R. and N.S. Bolan. (2008). Contaminant Chemistry in Soils: Key Concepts and Bioavailability. In: Naidu, R.; N.S. Bolan; M. Megharaj; A.L. Juhasz; S.K. Gupta; B.E. Clothier; and R. Schulin. (Eds.). *Cheical Bioavailability in Terrestrial Environments.* Elsevier. Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands Linacre House, Jordan Hill. Oxford OX2-8DP, N.K.

Naidu, R.; N.S. Bolan; and D.C. Adriano. (Eds.). (2003). Bioavailability, Toxicity and Risk Relationships in Ecosystems: The Path Ahead. In: *Bioavailability and its Potencial Role in Risk Assessment*. pp.331-339. Scince Publishers, New Yourk, NW, U.S.A.

Naidu, R.; N.S. Bolan; M. Megharaj; A.L. Juhasz; S.K. Gupta; B.E. Clothier and R. Schulin. (2008). Chemical Bioavailability in Terrestrial Environment. In: Naidu, R. (Ed.). *Chemical Bioavailabilityin Terrestrial Environment*. Elsevier Ltd. Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands. Linacre House, Jordan Hill, Oxford, 0X2 8DP, U.K.

National Research Council (NRC). (2002). *Bioavailability of Contaminants in Soils and Sediments: Processes, Tools, and Applications*. National Academics Press. Washington, D.C., U.S.A.

National Research Council (NRC). (1985). *Oil in the Sea: Inputs, Fates, and Effects*. National Academy Press. p. 601, National Academics Press. Washington, D.C., U.S.A.

National Research Council (NRC). (1983). *Polycyclic Aromatic Hydrocarbons: Evaluation of Sources and Effects*. ES/l-ES/7., National Academics Press. Washington, D.C., U.S.A.

National Psoriasis Foundation (NPF). (2005). Cancer Warnings Coming on Coal Tar Products. http://health-report.co.uk/psoriasis_cancer_warnings.html.

NATO. (1997). *Perspective in Bioremediation: Technologies for Environmental Improvement*. NATO Advanced Research Workshop on Biotechnological Remediation of Contaminated Sites. Kluwer Academic Publishers, Dordrecht. The Netherlands.

Natusch, J. (1997). *Application and Development of Contaminated Site Remediation Technologies in Astrialia*. ANZAC Fellowship Report to Department of Internal Affairs, Wellington, New Zealand and Department of Forreign Affairs and Trade, Canberra, Australia.

Neff, J.M. (1979). Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. *Applied Science Publishers Ltd.* p. 262, London, U.K.

Neff, J.M.; S.A. Stout and D.G. Gunster. (2005). Ecological Risk Assessment of Polycyclic Aromatic Hydrocarbons in Sediments: Identifying Sources and Ecological Hazard. *Integrated Environmental Assessment.* 1: 22-33.

Newby, J.A., and V. Howard. (2005). Review. Environmental Influences in Cancer Aetiology. Journal of Nutritional and Enviromental Medicine. 15(2/3): 56-114.

Nisbet. I.C.T., P.K. LaGoy. (1992). Toxic Equivalency Factors _TEFs. For Polycyclic Aromatic Hydrocarbons-PAHs.. *Regul Toxicol Pharmacol*. 16:290-300.

Norris, R.D.; R.E. Hinchee; R. Brown; P.L. McCarty and L. Semprini; J.T. Wilson and D.H. Kampbell; M. Reinhard; E.J. Bouwer; R.C. Borden; T.M. Vogel; J.M. Thomas and C.H. Ward. (1994). *Handbook of Bioremediation*. R.S. Kerr Environmental research laboratory. Lewis publishers. CRC Press, Inc., U.S.A.

NRC (National Research Council). (1994). *Alternatives for Ground Water Clean-up*. National Research Council. National Academy Press. Washington D.C., U.S.A.

NRC (National Research Council). (1997). Contaminated Sediments in Ports and waterways, cleanup strategies and Technologies, *National Research Council Report*. National Academy Press, Washington D.C., U.S.A.

NRC (National Research Council). (1993). *In-situ Bioremediation: When Does It Work?* National Research Council. National Academy Press. Washington D.C., U.S.A.

NRC (National Research Council). (1985). *Oil in the Sea: Inputs, Fates, and Effects*. National Academy Press. p. 601, National Academics Press. Washington, D.C., U.S.A.

Obbard, J.P.; O. Ward and S. Bayen. (2007). Persistent Organic Pollutants in Singapore's Marine Environment. In: Krupa, S.V. (Ed.). *Persistent Organic Pollutants in Asia Sources, Distributions, Transport and Fate*. Elsevier Ltd. The Netherlands Linacre House, Jordan Hill, Oxford, 0X2 8DP, U.K.

Odokuma, L.O. and A.A. Dickson. (2003). Bioremediation of a Crude Oil Polluted Tropical Rain Forest Soil. *Global Journal of Environmental Sciences*. 2: 29–40.

Ohkouchi, N.; K. Kawamura and H. Kawahata. (1999). Distributions of Three-to Seven Ring Polynuclear Aromatic Hydrocarbons on the Deep Seafloor of the Central Pacific. *Environmental Science and Technology.* 33: 3086-3090.

Parales, R.E. and S.M. Resnick. (2004). Aromatic Hydrocarbon Dioxygenases. In: Singh, A. and O.P. Ward. (Eds.). *Biodegradation and Bioremediation.* Soil Biology. Volume (2). Springer-Verlag, Berlin, Heidelberg. Printed in Germany.

Pawley, L.E. (Ed.). (2007). *Learning-Edge Environmental Biodegradation Research*. Nova Science Publishers, Inc., New York, NY., U.S.A.

Pearce, K.; H.G. Snyman; R.A. Oellermann and A. Gerber. (1995). Bioremediation of Petrolium-Contaminated Soil. In: R.E. Hinchee; J. Frederckson and B.C. Alleman (Eds.). *Bioaugmentation for Site Remediation*. Pp. 71-76. Battel Press, Columbus, OH. U.S.A.

Pelczar, M.J.; E.C.S. Chan and N.R. Krieg. (1993). *Microbiology Concepts and Applications*. McGraw-Hill, Inc., New York, NY., U.S.A.

Perk, M.V.D. (2006). *Soil and Water Contamination from Molecular to Catchment Scale*. Taylor & Francis Group plc, London, U.K.

Peterson, S.A.; J.C. Greene; W.E. Miller. (1990). Toxicological Assessment of Hazardous Waste Samples Extracted with Deionized water or Sodium Acetate Leaching Media. In: Friedman, D. (Ed.). Waste Testing and quality assurance.

American Society for Testing and Materials. ASTM STP 1062. 2: 107-129. Philadelphia, U.S.A.

Philp, J.C.; S.M. Bamforth; I. Singleton and R.M. Atlas. (2005). Environmental Pollution and Restoration: A Role for Bioremediation. In: Atlas, R.M. and J. Philp. (Eds.). *Bioremediation: Applied Microbial Solutions for Real-World Environmental Cleanup*. ASM Press. American Society for Microbiology. 1752 N Street N.W., Washington, D.C. 20036-2904, U.S.A.

Pignatello, J.J. and B. Xing. (1996). Mechanisms of Slow Sorption of Organic Chemicals to Natural Particles. *Environ. Sci. Technol.* 30: 1-11. and 25: 372-379.

Plattenberg, R.H. (2007). *Environmental Pollution: New Research*. Nova Science Publishers, Inc. New York, NY., U.S.A.

Prabhu, Y. and P.S. Phale. (2003). Biodegradation of Phenanthrene by *Pseudomonas* sp. Strain pp2:Novel Metabolic Pathway, Role of Biosurfactant and Cell Surface Hydrophobicity in Hydrocarbon Assimilation. *Appl. Microbiol.Biothecnol.* 61:342-351.

Prichard, H.; J. Jones-Meehane; C. Nestler; L.D. Hansen; W. Straube; W. Jones; J. Hind and J.W. Talley. (2006). Polycyclic Aromatic Hydrocarbons (PAHs): Improved Land Treatment with Bioaugmentation. In: Talley, j.w. (Ed.). *Bioremediation of Recalcitrant Compounds*. CRC Press, Talor & Fracis Group. 6000, Broken Sound Parkway N.W., Suit 300. Boca Raton, FL. 33487-2742, U.S.A.

Providenti, M.A.; H. Lee and J.T. Trevors. (1993). Selected Factors Limiting the Microbial Degradation of Recalcitrant Compounds. *Journal of Industrial Microbiology and Biotechnology.* 12(6): 379-395.

Putcha, R.V. and M.M. Domach. (1993). Flurescence Monitoring of Polycyclic Aromatic Hydrocarbon Biodegradation and the Effect of Surfactants. *Environ. Prog.* 12: 81-85.

Relman, D.A. (1999). The Search for Unrecognized Pathogens. Science. 284(5418): 1308-1310.

Reza, J., A. Trejo, L.E.Vera-Avila. (2002). Determination of the Temperature Dependence of Water Solubilities of Polycyclic Aromatic Hydrocarbons by a

Generator Column-on Linesolid Phase Extraction-liquid Chromatographic Method. *Chemosphere.* 47: 933–945.

Riggle, D. (1995). Successful Bioremediation with Compost. *Biocycle.* 36:57-59.

Robidoux, P.Y.; P. Gong; M. Sarrazin; G. Bardai; L. Paquet; J. Hawari; C. Dubois; G.L. Sunahara. (2004). Toxicity Assessment of Contaminated Soils from an Antitank firing Range. *Ecotoxicology and Environmental Safety*. 58: 300-313.

Ron, E.Z. and E. Rosenberg. (2002). Biosurfactants and Oil Bioremediation. *Curr. Opin. Biotechnol.* 13: 249–252.

Rowland, S.S.; S.R. Walsh; L.D. Teel and A.M. Carnahan. (1994). *Pathogenic and Clinical Microbiology*: *A Laboratory Manual*. Lippincott/Williams & Wilkins. Philadelphia, PA., U.S.A.

Sadek, P.C. (2002). *The HPLC Solvent Guide*. Second Edition. John Wiley and Sons. New York, NY., U.S.A.

Said, O.B.; M.S.G. Urriza; M.E. Bour; M. Dellali; P. Aissa and R. Duran. (2008). Characterization of Aerobic Polycyclic Aromatic Hydrocarbon-degrading Bacteria from Bizerte Lagoon Sediments, Tunisia. *Journal of Applied Microbiology*. 104: 987–99.

Samanta, S.K.; O.V. Singh and R.K. Jain. (2002). Polycyclic Aromatic Hydrocarbons: Environmental Pollution and Bioremediation. *Trends in Biotechnology*. 20: 243–248.

Sander, L.C. and S. Wise. (1997). Polycyclic Aromatic Hydrocarbons Structure Index. *National Institute of Standards and Technology (NIS1), Special Publication 922.* Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, MD., U.S.A.

Santodonato, J. (1981). Polycyclic Organic Matter. *J, Environ. Pathol Toxicol.* 5: 1-364.

Santos, E.C.; R.J.S. Jacques; F.M. Bento; M.C.R. Peralba; P.A. Selbach; E.L.S. Sa and F.A.O. Camargo. (2008). Anthracene Biodegradation and Surface Activity by an Iron-Stimulated *Pseudomonas* sp.. *Bioresource Technology*. 99: 2644–2649.

Sasek, V.; J.A. Glaser and P.H. Baveye. (2003). The Utilization of Bioremediation to Reduce Soil Contamination: Problems and Solution. *Nato Science Series, IV. Earth*

and Environmental Sciences. Kluwer Academic Publishers, Dordrecht. The Netherlands.

Scow, K.M. (2000). Soil Microbiology. In: Lederberg, J.; M. Alexander; R. Hull and S.G. Oliver. (Eds.). *Encyclopedia of Microbiology*. 2nd Edition. 4: 321-335.

Shepard, C.C. 1965. Temperature Optimum of *Mycobacterium leprae* in Mice. *J Bacteriol*. 90(5): 1271–1275.

Sherma, J. (1993). *Handbook of chromatography*. CRC Press, Inc., Boca Raton, FL., U.S.A.

Sho, M.; C. Hamel and C.W. Greer. (2004). Two Distinct Gene Clusters Encode Pyrene Degradation in Mycobacterium sp. strain S65. FEMS Microbiology Ecology 48: 209–220.

Shuttleworth, K.L. and C.E. Cerniglia. (1995). Environmental Aspects of Polycyclic Aromatic Hydrocarbon Biodegradation. Appl. Biochem. 54:291-302.

Simpson, C.D; A.A Mosi; W.R Cullen and K.J Reimer. (1996). Composition and Distribution of Polycyclic Aromatic Hydrocarbons in Surficial Marine Sediments from Kitimat Harbour, Canada. *Sci. Total. Environ.* 181: 265–278.

Sims, R.C. and M.R. Overcash. (1983). Fate of Polynulear Aromatic Compounds (PNAs). In: Soil-plant Systems. *Res. Rev.* 88: 1-68.

Singh, A. and O.P. Ward. (2004). Biotechnology and Bioremediation-An Overview. In: Singh, A. and O.P. Ward. (Eds.). *Biodegradation and Bioremediation*. Springer-Verlag, Berlin Heidelberg. Printed in Germany.

Singh, S.N. and R.D. Tripathi. (2007). *Environmental Bioremediation Technologies*. Springer-Verlag, Berlin Heidelberg. Printed in Germany.

Skipper, H.D. and R.F. Turco. (1995). *Bioremediation: Science and Applications*. SSSA Special Publication Number 43. Madison, Wisconsin, U.S.A.

Smith, D.J.T.; E.C. Edelhauser and R.M. Harrison. (1996). Polynuclear Aromatic Hydrocarbon Concentrations in Road Dust and Soil Samples Collected in the United Kingdom and Pakistan. *Environ. Technol.* 16: 45-53.

Smith, J.R.; R.M. Tomicek; P.V. Swallow; R.L. Weightman; D.V. Nakles and M. Hebling. (1995). Definition of Biodegradation Endpoints for PAH Contaminated Soils, Using a Risk-Based Approach. In: Kostecki, P.T.; E.J. Calabrese and M.

Bonazountas. (Eds.). Amherst Scientific Publishers. *Hydrocarbon Contaminated Soils*. 4: 521–572. Amherst, MA., U.S.A.

Sneath, P.H.A.; N.S. Mair; M.E. Sharpe and J.G. Holt. (Eds.). (1986). The Proteobacteria. *Bergey's Manual of Systematic Bacteriology*. Vol. 2. William & Wilkins. 428 East Preston Street. Baltimore, MD. 21202, U.S.A.

Stenuit, B.; L. Eyers; S.N. Agathos and I. George. (2008). High-throughput Approaches to Analyze Waste Biotreatment in Confined Environments. In: Diaz, E. (Ed.). *Microbial Biodegradation: Genomics and Molecular Biology*. Caister Academic Press. Norfolk, U.K.

Stephenson, G.L. ; R.G. Kuprrman ; G.L. Linder ; S. Visser. (2002). Toxicity Test for Assessing Contaminated Soils and Ground Water. In : Sunahara, G.L. ; A.Y. Renoux ; C. Thellen ; C.L. Gaudet ; A. Pilon (Eds.). *Environmental Analysis of Contaminated Sites*. John Wiley and Sons. Ltd. 25-43. New York, U.S.A.

Stewart, B.W, and Kleihues, P. (Eds.). (2003). World Cancer Report. Lyon: IARC Press.

Stokes, J.D.; G.L. Paton and K.T. Semple. (2005). Behavior and Assessment of Bioavailability of Organic Contaminants in Soil: Relevance for Risk Assessment and Remediation. *Soil Use Manage.* 21: 475-486.

Stringfellow, W.T. and M.D. Aitken. (1995). Competitive Metabolism of Naphthalene, Methylnaphthalenes and Fluorene by Phenanthrene-Degrading *Pseudomonads*. *Appl. Environ. Microbiol*. 61: 357-362.

Su, D., P. Li, X. Wang, F. Stagnitti, X. Xiong. (2008). Biodegradation of Benzo[a]pyrene in Soil by Immobilized Fungus. *Environmental Engineering Science*, 25: (8).

Sullivan, T.J. and M.C. Mix. (1985). Persistence and Fate of Polynuclear Aromatic Hydrocarbons Deposited on Slash Burn Sites in the Cascade Mountains and Coast Range of Oregon, USA. *Arch. Environ. Contam. Toxicol.* 14: 187-192.

Sylvia, D.M.; J.F. Fuhrmann; P.G. Hartel and D.A. Zuberer. (2005). *Principles and Applications of Soil Microbiology*. Pearson Education Inc., Upper Saddle River, New Jersey, 07 458, U.S.A.

Talley, J.W. (2006). Introduction to Recalcitrant Compounds. In: Talley, J.W. (Ed.). *Bioremediation of Recalcitrant Compounds*. CRC Press. Taylor and Francis Group. 6000 Broken Sound Parkway N.W., Suit 300, Boca Raton, FL. 33487-2742, U.S.A.

Tam, N.F.Y; C.L. Guo; K.W.Y. Yau; Y.S. Wong. (2002). Preliminary Study on Biodegradation of Phenanthrene by Bacteria Isolated from Mangrove Sediments in Hong Kong. *Mar. Pollut. Bull.* 4: 316–324.

Tang, J.; M.J. Carroquino; B.K. Robertson and M. Alexander. (1998). Combined Effect of Sequestration and Bioremediation in Reducing the Bioavailability of Polycyclic Aromatic Hydrocarbons in Soil. *Environ. Sci. Technol.* 32: 3586-3590.

Tate, R.L. (1995). Soil Microbiology. John Wiley and Sons, Inc., 605 Third Avenue, New York, NY., 10158-0012, U.S.A.

Tebbe, C.C. (2005). Marker Genes in Soil Microbiology. In: Buscot, F. and A. Varma. (Eds.). *Microorganisms in Soils: Roles in Genesis and Functions*. Springer-Verlag, Berlin Heidelberg. Printed in Germany.

Thwaites, J.M.; R.L. Farrell; S.D. Duncan; R.T. Lamar and R.B. White. (2007). Fungal-Based Remediation: Treatment of PCP Contaminated Soil in New Zealand. In: Singh, S.N. and R.D. Tripathi. (Eds.). *Environmental Bioremediation Technologies*. Springer - Verlag, Berlin Heidelberg. Printed in Germany.

Tiehm, A. and C. Fritzsche. (1995). Utilization of Solubilized and Crystalline Mixtures of Polycyclic Aromatic Hydrocarbons by a *Mycobacterium* sp. *Appl. Microbiol. Biotechnol*. 42: 964-968.

Torslov, J.; L. Samsoe-Peterson; J.O. Rasmussen; P. kristensen. (1997). Use of Waste Products in Agriculture. *Environmental Project 366*. Copenhagen, Denmark: Danish Environmental Agency.

Torstensson, L. (1993). *Guidelines: Soil Biological Variables in Environmental Hazard Assessment*. Report No. 4262. Stockholm, Sweden: Swedish Environmental Protection Agency.

Tortoli, E. (2003). Impact of Genotypic Studies on Mycobacterial Taxonomy: The New Mycobacteria of the 1990s. *Clinical Microbiology Reviews*. 16(2): 319-354.

Trzesicka-Mlynarz, D. and O.P. Ward. (1995). Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) by a Mixed Culture and its Component Pure Cultures, Obtained from PAH-Contaminated Soil. *Can. J. Microbiol*. 4: 470-476.

Tuli, R. (2007). Foreword. In: Singh, S.N. and R.D. Tripathi. (Eds.). *Environmental Bioremediation Technologies*. Springer - Verlag, Berlin Heidelberg. Printed in Germany.

Unterman, R.; M.F. DeFlaum and R.J. Steffan. (2000). Advanced In Situ Bioremediation: A Hierarchy of Technology Choices. In: Rehm, H-J and G. Reed. (Eds). Biotechnology. 11(b), Second Edition. Wiley-VCH, Weinheim.

USEPA. (1989). The Superfund Innovative Technology Programme: Technology Profiles. EPA Report No. 540/5–89/01. U.S. EPA Risk Reduction Engineering Laboratory Office of Research and Development. Cincinnati, OH., USA. In: Wilson, S.C and K.C Jones. (1993). Bioremediation of Soil Contaminated with PAHs: A review. *Environ. Pollution.* 81:229–249.

USEPA. (1993). *Provisional Guidance for Quantitative Risk Assessment of Polycyclic Aromatic Hydrocarbons (PAHs), EPA/600/R-93/089*. USEPA, Washington D.C., U.S.A.

USEPA. (1994). Assessment and Remediation of Contaminate Sediments (ARCS) Program, Final Summary Report, EPA-905-S-94-001, EPA, Chicago. U.S.A.

USEPA. (1995). *Symposium on Bioremediation of Hazardous Wastes. EPA/600/R-95/076*. US Environmental Protection Agency, Washington D.C., U.S.A.

USEPA. (1998). 1990 Emissions Inventory of Section 112(c)(6) Pollutants: POM, 2,3,7,8-TCDD/ 2,3,7,8-TCDF, PCB's, Hexachlorobenzene, Mercury and Alkylated Lead. *Final Report USEPA*. Emission Factor and Inventory Group (MD-14) and Visibility and Ecosystem Protection Group (MD-15). http://www.epa.gov/ttn/atw/112c6/final2.pdf.

USEPA. (2000). A Resource for MGP Site Characterization and Remediation. Expedited Site Characterization and Source Remediation at Former Manufactured Gas Plant Sites. *EPA-542-R-00-005*. US Environmental Protection Agency, Washington, D.C., U.S.A.

USEPA. (2004). EPA's Superfund. http://www.epa.gov/superfund/ news/30 years.htm.

Uyttebroek, M.; S. Vermeir; P. Wattiau; A. Ryngaert and D. Springael. (2007). Characterization of Cultures Enriched from Acidic Polycyclic Aromatic

Hydrocarbon-Contaminated Soil for Growth on Pyrene at Low pH. *Applied and Environmental Microbiology*. 73(10): 3159–3164.

Valdes, J.J. (Ed.). (2000). *Bioremediation*. Kluwer Academic Publishers. 101 Philip Drive, Norwell, MA., 02061, U.S.A.

Van Elsas, J.D.; J.K. Jansson and J.T. Trevors. (Eds.). (2007). *Modern Soil Microbiology*. CRC Press. Taylor and Francis Group. 6000 Broken Sound Parkway N.W., Suit 300, Boca Raton, FL. 33487-2742, U.S.A.

Van Hamme, J.D. (2004). Bioavailability and Biodegradation of Organic Pollutants: A Microbial Perspective. In: Singh, A. and O.P. Ward. (Eds.). *Biodegradation and Bioremediation*. Springer-Verlag, Berlin Heidelberg. Printed in Germany.

Van Hamme, J.D.; E.T. Wong; H. Dettman; M.R. Gray, and M.A. Pickard. (2003). Dibenzyle Sulfide Metabolism by White Rot Fungi. *Appl. Environ. Microbiol.* 69: 1320-1324.

Verschueren, K. (1983). *Handbook of Environmental Data on Organic Chemicals*. Van Nostrand Reinhold. New York, NY., U.S.A.

Vig, K. M. Megharaj; N. Sethunathan and R. Naidu. (2002). Bioavailability and Toxicity of Cadmium to Microorganisms and their Activities in Soil: A Review. *Adv. Environ. Res.* 8: 121-135.

Volkering, F. and A.M. Breure. (2003). Biodegradation and General Aspects of Bioavailability; In: Douben, P.E.T. (Ed.). *PAHs: An Ecotoxicological Perspective*. John Wiley & Sons, Ltd. West Sussex, U.K.

Volkering, F.; A.M. Breure; A. Sterkenburg and J.G. Van Andel. (1992). Microbial Degradation of Polysyclic Aromatic Hydrocarbons: Effect of Substrate Availability on Bacterial Growth Kinetics. *Appl. Microbiol. Biotechnol.* 36: 548-552.

Walker, S.E.; R.M. Dickhut and C. Chisholm-Brause. (2004). Polycyclic Aromatic Hydrocarbons in a Highly Industrialized Urban Estuary: Inventories and Trends. *Environmental Toxicology and Chemistry.* 23: 2655-2664.

Walter, U.; M. Beyer; J. Klein and H.J. Rehm. (1991). Degradation of Pyrene by *Rhodococcus* sp. UW1. *Appl. Microbiol. Biotechnol.* 34:671-676.

Wang, B.Y. (Ed.). (2007). *Environmental Biodegradation Research Focus*. Nova Science Publishers, Inc., New York, NY., U.S.A.

Wang, L.K.; N.K. Shammas and Y-T. Hung. (2009). *Advances in Hazardous Industrial Waste Treatment*. CRC Press. Taylor and Francis Group. 6000 Broken Sound, Parkway N.W., Suit 300, Boca Raton, FL. 33487-2742. U.S.A.

Watanabe, K.; K. Watanabe; Y. Kodama; K. Syutsubo and S. Harayama. (2000). Molecular Characterization of Bacterial Populations in Petroleum-Contaminated Groundwater Discharged from Underground Crude-Oil-Storage Cavities. *Appl. Environ. Microbiol.* 66: 4803–4809.

Weissenfels, W.P.; H.J. Kiever and J. Langhoff. (1992). Adsorption of Polycyclic Aromatic Hydrocarbons (PAHs) by Soil Particles: Influence on Biodegradation and Biotoxicity. *Applied. Microbiol. Biotchnol.* 36: 689-696.

World Health Organization (WHO). (1998). *Environmental Health Criteria 202: Selected Non-heterocyclic Polycyclic Aromatic Hydrocarbons.* p. 883. World Health Organization. Geneva, Swisarland.

Whyte, L.G.; L. Bourbonniere; C. Bellerose and C.W. Greer. (1999). Bioremediation Assessment of Hydrocarbon-Contaminated Soils from the High Arctic. *Bioremediation Journal*. 3(1): 69-79.

Wick, L.Y.; T. Colangelo and H. Harms. (2001). Kinetics of Mass Transfer-limited Bacterial Growth on Solid PAHs. *Environ. Sci. Technol*. 35: 354-361.

Wild, S.R.; K.C. Jones; K.S. Waterhouse and S.P. McGrath. (1990a). Organic Contaminants in an Agricultural Soil with a History of Sewage Sludge Amendments: PAHs. *Environ. Sci. Tech.* 24:1706-1711.

Wild, S.R.; S.P. McGrath and K.C. Jones. (1990b). The PAH Content of Archive Sewage Sludge. *Chemosphere*. 20:703-716.

Wilson, S.C. and K.C. Jones. (1993). Bioremediation of Soil Contaminated with Polynuclear Aromatic Hydrocarbons (PAHs): A Review. *Environ. Pollut.* 81: 229-249.

Woese, C.R. (1987). Bacterial Evolution. *Microbiological Reviews*. 51(2): 221-271.

Woese, C.R.; E. Stackebrandt; T.J. Macke and G.E. Fox. (1985). A Phylogenetic Definition of the Major Eubacterial Taxa. *Systematic and Applied Microbiology*. 6: 143-151.

Woese, C.R.; O. Kandler and M.L. Wheeler. (1990). Towards a Natural System of Organisms: Proposal for the Domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences*. (USA.). 87(12): 4576-4579.

Yang, S.Y.N.; D.W. Connell and D.W. Hawker. (1991). Polycyclic Aromatic Hydrocarbons in Air, Soil and Vegetation in the Vicinity of an Urban Roadway. *Sci. Total. Environ.* 102: 229-240.

Ye, D.; M.A. Siddiqi; A.E. Maccubbin; S. Kumar and H.C Sikka. (1996). Degradation of PAHs by *Sphingomonas paucimobilis*. *Environ. Sci. Technol.* 30:136–142.

Yong, R.N. and C.N. Mulligan. (2004). *Natural Attenuation of Contaminants in Soil*. CRC Press. Lewis Publishers. Boca Raton, FL., U.S.A.

Young, J.S., E. Gormley, and E.M.H. Wellington. (2005). Molecular Detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in Soil. *Appl. Environ. Microbiol*. 71(4): 1946-1952.

Young, P.J.; S. Pollard and P. Crowcroft. (1997). Overview: Context, Calculating Risk and Using Consultants, p.1-24. In: Hester, R.E. and R.M. Harrison. (Eds.). *Contaminated Land and Its Reclamation*. Thomas Telford Publishing, London, U.K.

Young, R.N.; A.M. O. Mohammed and B.P. Warkentin. (1992). Principles of Contaminant Transport in Soils. In: *Developments in Geotechnical Engineering.* 73. Elsevier. Science Publishers, NY., U.S.A.

Youngblood, W.W. and M. Blumer. (1975). Polycyclic Aromatic Hydrocarbons in the Environment: Homologous Series in Soils and Recent Marine Sediments. *Geochimica et Cosmochimica Acta.* 39: 1303-1314. Table 11-1. Concentrations of Individual and Total PAHs in a Typical Crude Oil, Coal, Coal tar, and Creosote. Concentrations are mg kg^{-1}. From: Neff, *et al.*, (1998).

Yu, S.H; L. Ke; W.S. Wong and N.F.Y. Tam. (2005). Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) by a Bacterial Consortium Enriched from Mangrove Sediments. *Environ. Int.* 31: 149–154.

Zhang, Y.M.; W.J. Maier and R.M. Miller. (1997). Effect of Rhamnolipids on the Dissolution, Bioavailability and Biodegradation of Phenanthrene. *Environ. Sci. Technol.* 31:2211-2217.

Zhou, W. and L. Zhu. (2007). Efficiency of Surfactant-Enhanced Desorption for Contaminated Soils Depending on the Component Characteristics of Soil-Surfactant-PAHs System. *Environmental Pollution*. 147: 66–73.

Zylstra, G.J.; E. Kim and A.K. Goyal. (1997). Comparative Molecular Analysis of Genes for Polycyclic Aromatic Hydrocarbon Degradation. *Genet. Eng.* 19: 257-269.

APPENDICES

This section provides six informative appendices which present the details of the experimental procedures employed in this study.

APPENDIX A: List of Methods and Protocols Used in the Soil-Nutrient Analysis

APPENDIX B: Different Bacterial Growth Media

APPENDIX C: A Protocol for Pyrene Mineralization by Isolated Bacteria

APPENDIX D: A Protocol for Preparation of Radioactive (^{14}C) Substrate

APPENDIX E: A Protocol for Viable Cultural Bacterial Population

APPENDIX F: A Protocol for Colony Lifting and Hybridization

Notice: All methodologies presented in appendices are extracted and modified from experimental procedures, which are used either at McGill University or at the National Research Council of Canada's Biotechnology Research Institute (NRC-BRI).

APPENDIX A

List of Methods and Protocols used in the Soil-Nutrient Analysis

A1: Protocol for Soil Test Methods

1. pH

1:1 or 1:2 soil-to-solution ratio using water (as specified).

Hendershot, W.H.; H. Lalande, and M. Duquette. (1993). Soil Reaction and Exchangeable Acidity. Chapter 16: In Soil Sampling and Methods of Analysis. M.R. Carter (Ed.). *Can. Soc. Soil Sci. Lewis Publishers*. pp 141-143.

2. Percentage of Carbon (C)

A wet oxidation of organic matter using an acid dichromate solution heated at 150^{o}C for 45 minutes. The estimation of organic carbon is done by a back titration, using an indicator, of the remaining dichromate with a ferrous ammonium sulphate solution.

Tiessen H. and J.O. Moir. (1993). Total and Organic Carbon. Chapter 21: In Soil Sampling and Methods of Analysis. M.R. Carter (Ed.). *Can. Soc. Soil Sci. Lewis Publishers*. pp 190-191.

3. Percentage of Organic Matter by Loss on Ignition (LOI)

A (previously heated to 105^{o}C for 24 hours) sample is burned at 360^{o}C for 4 hours. The difference in weight between the two steps is attributed to loss of organic matter expressed as a percent.

Schulte, E.E.; C. Kaufmann and J.B. Peter. (1991). The Influence of Sample Size and Heating Time on Soil Weight Loss-on-Ignition. *Comm. Soil Sci. and plant Anal.*, Vol. 22: pp 159-168.

4. Available P, K, Ca, Mg, Na, Al, Cu, Zn, Mn and Fe

A multi-element extraction using the Mehlich III solution (a mixture of acetic acid, ammonium nitrate, ammonium fluoride, nitric acid and EDTA). A colorimetric technique was used for the determination of P (by: Lachat Instrument – Flow Injection Analysis) and the determination of the metals was done using an Atomic Absorption Spectrophotometer – Perkin-Elmer 2380.

Tran, Sen T. and R.R. Simard. (1993). Mehlich III – Extractable Elements. Chapter 6: In Soil Sampling and Methods of Analysis. M.R. Carter (Ed.). *Can. Soc. Soil Sci. Lewis Publishers*. pp 43-49.

5. Extractable Ammonium and Nitrates in soils

A 2M KCl extraction was performed using a ratio of 1:10 soil-to-solution and shaken for 1 hour. The filtrate was analyzed by colorimetry for the determination of N as NH_4 and N as NO_3 on a Multi-Channel Lachat Auto-Analyser.

Maynard, D.G. and Kalra Y.P. (1993). Nitrate and Exchangeable Ammonium Nitrogen. Chapter 4: In Soil Sampling and Methods of Analysis. M.R. Carter (Ed.). *Can. Soc. Soil Sci. Lewis Publishers*. pp 25-32.

Lachat Instruments. QuickChem Method 13-107-06-1-A Lachat Instruments, 6645 West Mill Road, Milwaukee, WI 53218, U.S.A.

Lachat Instruments. QuickChem Method 10-107-06-2-C. Lachat Instruments, 6645 West Mill Road, Milwaukee, WI 53218, U.S.A.

6. Total N in Soils

A soil sample was digested using a digestion mixture composed of K_2SO_4, $CuSO_4$ and Se (ratio of 100:10:1) in H_2SO_4 at 350 ^oC for 3 hours. The volume was made up to 100 mL and the solution measured colorimetrically for N as NH_4.

McGill W.B. and Figueiredo C.T. (1993). Total Nitrogen. Chapter 22: In Soil Sampling and Methods of Analysis. M.R. Carter (Ed.). *Can. Soc. Soil Sci. Lewis Publishers*. pp 201-211.

Lachat Instruments. QuickChem Method 13-107-06-2-B Lachat Instruments, 6645 West Mill Road, Milwaukee, WI 53218, U.S.A.

7. Cation Exchange Capacity and Percentage of Base Saturation (B.S.)

A summation of the major cations (Ca, Mg, K, Na, Fe, Al, Mn, Zn) present in an unbuffered extractant of 0.1 M $BaCl_2$. The percentage of B.S. was calculated as the sum of the base cations (Ca, Mg, K, and Na) over the total CEC. Cation concentrations were determined on a Perkin-Elmer Atomic Absorption Spectrophotometer PE-2380.

Hendershot, W.H.; H. Lalande, and M. Duquette. (1993). Ion Exchange and Exchangeable Cations. Chapter 19: In Soil Sampling and Methods of Analysis. M.R. Carter (Ed.). *Can. Soc. Soil Sci. Lewis Publishers*. pp. 168-170.

8. Ammonium and Nitrates in Natural Waters

The ammonium of the solution was heated with salicylate and hypochlorite in an alkaline phosphate buffer. The green colour was measured colorimetrically at 660 nm on a flow injection instrument. Nitrates were measured using a reducing procedure that

transformed them to nitrites in a copperized cadmium column. The magenta colour was measured colorimetrically at 520 nm on a Lachat flow injection instrument.

Lachat Instruments. QuickChem Method 10-107-06-2-C (ammonium) and 10-107-04-1 C(nitrate). Lachat Instruments, 6645 West Mill Road, Milwaukee, WI 53218, U.S.A.

9. Total N and P in Tissue

The tissue was digested in sulphuric acid and peroxide with the addition of catalysts (lithium and selenium) at 340 C for about three hours. The content was diluted to 100 mL and analysed colorimetrically for N and P.

Parkinson, J.A. and S.E. Allen. (1975). A Wet Oxidation Procedure Suitable for the Determination of Nitrogen and Mineral Nutrients in Biological Material. *Comm. Soil Sci. and plant Anal.*, Vol. 6 (1). pp 1-11.

Lachat Instruments. QuickChem Method 13-115-01-1-B. Phosphorus and 13-107-06-2-A for Total N. Lachat Instruments, 6645 West Mill Road, Milwaukee, WI 53218, U.S.A.

10. Total P in Natural Water

Persulfate was added to the solution to oxidise the organic P and the solution was digested in autoclave. The digested solution was analysed on the flow injection instrument at 880 nm following a complexation with ammonium molybdate.

Technique No. CPQ 104E2. Laboratoire Régional – Québec, Centre Saint-Laurent. Environnement Canada. Mars, (1994).

Lachat Instruments. QuickChem Method 10-115-01-1-A. Phosphorus. Lachat Instruments, 6645 West Mill Road, Milwaukee, WI 53218, U.S.A.

11. Total N in Natural Water

The solution was digested in an autoclave after the addition an alkaline persulfate solution. The content of $N\text{-}NO_3$ was measured colorimetrically at 520 nm following a step in a reduction cadmium column.

Cabrera, M.L. and M.H. Beare. (1993). Alkaline Persulfate Oxidation for Determining Total Nitrogen in Microbial Biomass Extracts. *Soil Sci. Soc. Am. J.*, Vol. 57: pp 1007-1012.

Lachat Instruments. QuickChem Method 10-107-04-1-C. Phosphorus. Lachat Instruments, 6645 West Mill Road, Milwaukee, WI 53218, U.S.A.

12. Particle Size Distribution (Hydrometer Method)

The hydrometer was used to measure the density of the material in suspension. Taking the reading at specific intervals according to settling times of grain size gave the particle size distribution.

Sheldrick, B.H. and C. Wang. (1993). Particle Size Distribution. Chapter 47: In Soil Sampling and Methods of Analysis. M.R. Carter (Ed.). *Can. Soc. Soil Sci. Lewis Publishers*. pp 507-509.

Carter, M.R. and E.G. Gregorich, (Eds.). (2008). Soil Sampling and Methods of Analysis. *Canadian Society of Soil Science*. CRC Press, Talor & Francis Group. 6000 Broken Sound Parkway, N.W, Suit 300. Boca Raton, FL., 33487-7742, U.S.A.

A2: Protocol for Determining the Cation Exchange Capacity (CEC) and Exchangeable Cations of Soil by Silver Thiourea Method at pH of the Soil

Reagents

Silver Thiourea Solution, 0.01 M silver and 0.1 M thiourea (AgTU extractant):
To 1 L thiourea, 0.2 M solution, add 500 mL water. Homogenize. Then slowly add 500 mL of $AgNO_3$, 0.04 M solution, under strong stirring.

Procedure

1. Crush 5 g of soil to pass a 0.5 mm sieve.
2. Weigh 1 g of this sample into 50 mL centrifuge tube. Include two blanks and a reference sample.
3. Pipette 40 mL of the AgTU extractant into the tube and close this with a cap or rubber stopper.
4. Shake for 4 hours on a reciprocating shaking machine.
5. Centrifuge.
6. Measure Ag in the clear supernatant.

Measurement

1. Pipette 2 mL extract into 100 mL volumetric flask, add 5 mL nitric acid, 1M, and make to volume with water.
2. Measure Ag by Atomic Absorption Spectrophotometer (Varian AA-975 Series, Varian Techtron Pty .Ltd. Mulgrave, Australia) using an Ag hollow cathode lamp at a wavelength of 328.1 nm.

Calculations

$$CEC(me/100g\ soil) = \frac{(b-a)\times 1.85\times mcf}{s}$$

Where:

a = ppm Ag in 5Ox Diluted Soil Extract;

b = ppm Ag in 5Ox Diluted Blank Extract;

s = Air-Dry Sample Weight in gram;

mcf = Moisture Correction Factor.

Source: (Van Reeuwijk**,** L.P. 1987)

A3. Protocol for Determining the Organic Carbon Content of Soil. Walkley-Black Procedure

Reagents

1. Potassium dichromate ($K_2Cr_2O_7$), 1N.
2. Concentrated Sulphuric Acid (H_2S0_4), 95-98%.
3. Concentrated Phosphoric Acid (H_3PO_4), 85%.
4. Barium Diphenylamine Sulphonate, 0.16%.
5. Ferrous Sulphate ($FeSO_4.7H_2O$), 0.5N.

Procedure

1. Grind 10 g of soil to pass through 0.5 mm sieve.
2. Transfer 1 g into 500 mL Erlenmeyer flask.
3. Add 10 mL of 1N $K_2Cr_2O_7$ swirling gently. Include two blanks.
4. Carefully add 20 mL of concentrated H_2S0_4 swirling gently and then mix vigorously for 1 min.
5. Let stand for 30 min. and then add 200 mL of water and 10 mL of phosphoric acid.
6. Add 1 mL of indicator and titrate with ferrous sulphate while stirring. At the end point the colour changes to green.

Calculations

The carbon content of the soil:

$$\%C = M \times \frac{V1 - V2}{s} \times 0.39 \times mcf$$

Where:

M = molarity of ferrous sulphate solution from blank titration.

V1 = mL ferrous sulphate solution required for blank.

V2 = mL ferrous sulphate solution required for sample.

s = weight of air-dry sample (g).

mcf = moisture correction factor.

Sumner, M.E., Editor-in Chief. (2000). In: *Hand Book of Soil Science*. CRC Press, LLC. 2000 Corporate Blvd., N.W., Boca Raton, FL., 33431, U.S.A.

APPENDIX B

Different Bacterial Growth Media

(B1)

YTS (Yeast extract, Tryptone, Starch) - Broth : 1 L Preparation

250 mg	Yeast extract
250 mg	Tryptone
250 mg	Starch

Notice: Mix in 1 L tap water and adjust pH to 6.5 then autoclave.

(B2)

YTS (Yeast extract, Tryptone, Starch) – Agar (Agar granulated): 1 L Preparation

250 mg	Yeast extract
250 mg	Tryptone
250 mg	Starch

Notice: Mix in 1 L tap water and adjust pH to 6.5 then, for plates add 15-g agar granulated to 1 L solution, dissolve and autoclave.

(B3)

Minimal Salts Medium (MSM) - Agarose (Ultra-Pure Agarose)

Stock	**Formula Weight (ml L^{-1})**	**Volume Added (mM)**	**Final Concentration**
1 M KH_2PO_4	136.09	6.4	6.4
1 M K_2HPO_4	174.18	12.99	12.99
1 M $(NH_4)_2SO_4$	132.13	8.33	8.33
0.5 M $MgSO_4.7H_2O$	246.47	0.79	0.395
Trace Metals	1.0		

Source: (Furukawa and Chakrabarty, 1982)

(B4)

Trace Metals Composition

Chemical	Formula Weight Added	mg/100 mL (mM)	Final Concentration
$Co(NO_2)_2.6\ H_2O$	291.05	29.11	1
$AlK(SO_4)_2.12\ H_2O$	474.39	47.44	1
$Cu\ SO_4$	159.60	15.96	1
$Zn\ SO_4.7\ H_2O$	287.54	287.50	10
$Mn\ SO_4.\ H_2O$	169.01	169.00	10
$Fe\ SO_4.7\ H_2O$	278.02	278.02	10
$Na_2MoO_4.\ 2\ H_2O$	241.95	48.20	2
$Ca(NO_3)_2\ .4\ H_2O$	236.15	236.15	10

Notice: Acidified to pH ~ 2 with H_2SO_4. Mix in 1 L Distilled Water then, for plates add 15-g agarose (ultra pure agarose) to 1 L solution, dissolve and autoclave. This is a Chlorine Free Medium (*i.e.*, use Distilled Water).

APPENDIX C

A Protocol for Pyrene Mineralization by Isolated Bacteria

Objective

The objective of the microcosm test was to assess the capacity of a given biomass (from an indigenous soil population) to mineralize completely into CO_2 a given contaminant or substrate (Pyrene). Mineralization tests were carried out by introducing all the elements of the test, including the radio-labelled substrate, to a closed system consisting of a serum bottle equipped with a KOH trap. The KOH solution trapped the ^{14}C- CO_2 mineralized from the carbon-labelled substrate, which was then measured by scintillation counting. Values obtained in disintegrations per minute (dpm) were converted to a rate of substrate mineralized which can be expressed as a mg or percentage of substrate mineralized over time (ex.: mg day^{-1} or % day^{-1}).

Materials and Methods

Microcosm Set-Up

A standard aerobic microcosm methodology was adopted for this mineralization study using mineral salt medium [(MSM -broth) + 50 mg L^{-1} Yeast extract] and pyrene added as the sole source of carbon and energy. Fourteen bacterial isolates, chosen based on phenanthrene clearance and probing for the presence of the ndoB gene were inoculated into microcosms.

Chemicals

Both labelled and non-labelled pyrene were obtained from Sigma Chemical Co. (Oakville, Ontario, Canada). [4.,5,9,10-^{14}C]Pyrene (10-30 mCi $mmol^{-1}$, Radiochemical purity: ≥95%).

Materials

What should be prepared for this study includes:

1. 45 serum bottles (125 mL) with Teflon-lined rubber.
2. 45 KOH tubes (glass).
3. A 100 μL glass syringe to add the radioactive substrate.
4. A 3 mL and a 1 mL plastic syringe with a long stainless steel needle for the addition of KOH.
5. Pipettes and syringes for addition of solutions.
6. Solvent (acetone, methanol, hexane or any proper solvent).
7. Stock solution for KOH (0.5 M).
8. Diluted radioactive solution that will give 100 000 dpm (0.045 μCi) per 20 μL or per microcosm.
9. Stock solution for cold substrate.
10. Scintillation liquid (cocktail) with dispenser.
11. Scintillation counter trays.
12. Scintillation counter.
13. Spectrophotometer

Note: Everything must be autoclaved to meet aseptic conditions

General Procedure

Step 1. Growth of cultures

1. Prepare of 2 L of mineral salt medium [(MSM-broth) + 50 mg L^{-1} Yeast extract].

2. Prepare of 17 (14 flasks for inoculation and 3 for control) erlenmeyer flasks (250 mL).
3. Adding 50 mL of medium [(MSM-broth) + 50 mg L^{-1} Yeast extract] into each of the 17 flasks.
4. Inoculate each of the 14 tubes with 3-5 colonies from each of the bacterial isolates.
5. Close the flasks loosely and incubate them with shaking at room temperature (25 ^{o}C) for 3-7 days.
6. Measure the optical density (O.D.) of the cultures by using spectrophotometer at 600 nm.
7. Dilute cultures to the desired optical density (0.02-0.05), by transferring concentrated cultures to fresh medium [(MSM-broth) + 50 mg L^{-1} Yeast extract].

Step 2. Preparation of the microcosms

8. Make 14 sets of triplicate microcosm for each of the bacterial isolates [14 (bacterial isolates) × 3 = 42 microcosms].
9. Make one set of triplicate microcosms for control [Only (MSM-broth) + 50 mg L^{-1} Yeast extract, pyrene, and no culture].
10. Inoculate each of triplicate microcosms with 20 mL of final culture liquid [(MSM-broth) + 50 mg L^{-1} Yeast extract + Culture).
11. Add 20 mL [(MSM-broth) + 50 mg L^{-1} Yeast extract, no culture], into triplicate control microcosms.
12. Add calculated amount of Pyrene (hot +cold), into all 45 microcosms.
13. Insert the KOH tubes (glass), into each serum bottle.
14. Inject 1.0 mL KOH solution (0.5 M) into each KOH tube (trap tube).
15. Cap the bottles.
16. Incubate radioactive microcosms at ambient temperature (25 ^{o}C).

Step 3. Sampling

The frequency of sampling depended on the substrate studied, varying from every few hours to once a week. Sampling should be performed until the mineralization rate reaches a plateau.

17. Preparation of scintillation vials: one for each serum bottle plus one for the background measurement.

18. Identification all of the caps and placing them in the racks for the scintillation counter.

19. Have one needle in the KOH solution and one installed on the syringe for the sampling itself.

20. Flush the serum bottle's headspace with air (or pure O2 for high activity). Addition of oxygen can be necessary to insure complete mineralization of the substrate.

21. Pump 1.0 mL of fresh KOH solution and put it in the background vial. Add 1.0 mL of KOH and 18 mL of scintillation cocktail. Shake for 5 second.

22. Insert a needle on the syringe, insert it in the KOH tube of the first bottle to sample, and pump all of the KOH.

23. Remove the syringe (not the needle), put the KOH in the corresponding vial, plug the syringe on the KOH needle, pump 1.0 mL, put it in the vial, and add 18 mL of scintillation cocktail, shake 5 second.

24. Plug the syringe on the KOH needle, pump 1.0 mL, plug the syringe in the needle that is in the KOH tube, put the KOH, remove the needle. Go to next bottle.

25. When finished, replace the bottles in the shaker, leave the needles in the hood or wash them in Contrad Solution. Clean everything and proceed to the scintillation counter.

APPENDIX D

A Protocol for Preparation of Radioactive (^{14}C) substrate

Radio-labelled substrate can come in any phase: solid, liquid or gas. It will usually be too concentrated to inject directly into the microcosms. Therefore, a dilution is needed. Each radio-labelled substrate has its own activity and a specific calculation must be made to obtain a solution with roughly 100 000 dpm per 100 µL.

A vial with 1 µL of the pure radioactive product should be placed in 20 mL of scintillation cocktail, mixed, and the mixture should then be counted. From that number, one can calculate the required dilution factor to obtain 100 000 dpm in 100 or 20 µL (*ex.*: 1 µL gives 5000 dpm, *i.e.* 100 µL would give 500 000; therefore, the dilution needed is 5 fold to obtain 100 000 dpm).

The calculated labelled (*i.e.* hot) and unlabeled (*i.e.* cold) substrates are mixed together in acetone (or any proper solvent) solution to make a work solution, *e.g.*, for ~ 10 ppm naphthalene one may add 26 µL Stock-Solution in 1974 µL MeOH containing 10 mg mL^{-1} cold naphthalene, which will be added to each sample. The following procedures for the preparation of work solutions for naphthalene, phenanthrene, and pyrene were as follows:

1. Preparation of ^{14}C-Naphthalene Work Solution:

Molecular Weight: 128.1 (128.1g mol^{-1})
100 µCi
1.6 mg
Specific Activity = 8.1 mCi $mmole^{-1}$ (1 mmol = 128.1 mg)
1 µCi = 2.2×10^6 dpm
100 000 dpm = 0.045 µCi

Stock Solution: This solution should be prepared as follow: added 1 mL MeOH, without cold Naphthalene.

1 μL **Stock Solution** = 392 149 dpm (as counted in the scintillation counter machine)

Work Solution: This solution should be prepare as follows:

Having:

1.6 mg (1.6 mg / 100 μCi) → 0.00072 mg / 0.045 μCi 0.72 μg / 100 000 dpm

→ 0.72 μg / 20 g soil in a microcosm represents ~ 36 ppb

Now, we need ~ 10 ppm of substrate and for that, we will have to add cold naphthalene. However, for ~ 10 ppm naphthalene: 26 μL Stock-Solution in 1974 μL MeOH containing 10 mg mL^{-1} cold naphthalene.

Therefore, 20 μL of this work solution should be able to provide 100 000 dpm of substrate naphthalene with concentration of 10 ppm in a 20 g soil microcosm.

2. Preparation of ^{14}C-Phenanthrene Work Solution:

Molecular Weight: 178.2 (178.2 g mol^{-1})

100 μCi

Specific Activity = 46.9 mCi $mmole^{-1}$ (1 mmol = 178.2 mg)

1 μCi = 2.2×10^6 dpm

100 000 dpm = 0.045 μCi

Stock Solution: The solution should be prepared as followed for Naphthalene and Phenanthrene.

Work Solution: This solution should be prepared as follow:

If 46.9 mCi (46900 μCi) contains 178.2 mg,

0.045 μCi contains 0.000171 mg

0.171 μg of Phenanthrene in 20 mL liquid microcosm represents ~ 8.5 ppb

Now, for example to prepare a 100 ppm Working-Solution of Phenanthrene, the same procedure as for Naphthalene (*i.e.* labelled Phenanthrene in MeOH containing cold substrate) should be followed.

3. Preparation of ^{14}C-Pyrene-4,5,9,10 Work Solution:

Molecular Weight: 202.3 (202.3 g mol^{-1})
50 μCi in 111 μL methanol (MeOH)
Specific Activity = 58.7 mCi $mmole^{-1}$ (1 mmol = 202.3 mg)
1 μCi = 2.2×10^6 dpm
100 000 dpm = 0.045 μCi

Stock Solution: The solution should be prepared as followed for Naphthalene.

In order to prepare 50 μCi of labelled Pyrene in a large volume, 900 μL aliquot of the methanol (MeOH) should be added to the original vial containing ^{14}C-Pyrene (*i.e.* 50 μCi of Pyrene in 111 μL MeOH).

Work Solution: This solution should be prepared as follow:

In order to prepare this solution, 2 μL aliquot of the labelled Pyrene prepared Stock-Solution was added to 20 mL scintillation cocktail in a 20 mL scintillation vial and was counted in the scintillation counter. The result was:
1 μL of Stock-Solution contained 220 000 dpm activity (*i.e.* 2 μL = 220,000 dpm).

Since,

1 μCi = 2.2×10^6 dpm

50 μCi / 1000 μL × 2.2×10^6 dpm = 110,000 dpm μL^{-1}

Furthermore, for spiking our 125 mL microcosm bottle, 100,000 dpm of labelled substrate (Pyrene) in about 20 μL, a required volume which can be injected with precision into each microcosm. Therefore, for spiking ~ 50 microcosm bottles, 1 mL aliquot of that Work-Solution which contains 100,000 dpm in each 20 μL of it is required.

Therefore,

100,000 dpm / 20 μL = *x* dpm / 1000 μL

The value for *x* is:

x dpm = 5,000,000 dpm

We then get the exact value of required aliquot of Stock Solution**:**

5,000,000 dpm / 110,000 dpm / μL = 45.5 μL

Lastly, at this stage, 45.5 μL aliquot of the Stock Solution was added to 954.5 μL of the solvent (MeOH) thus preparing the Work Solution.

4. For preparation of [1,2,3,4,4A,9A-^{14}C]Anthracene, [-9-^{14}C]Fluorene, and [-3-^{14}C]Fluoranthene Work-Solutions, the same procedures were applied.

APPENDIX E

A Protocol for Viable Cultural Bacterial Population

Overview

Under natural conditions microbial populations contain many different species—not only different species of bacteria, but also species of yeast, moulds, algae, and protozoa. There may be several kinds of viruses present as well. Frequently it is important to identify how many and what kinds of microorganisms are present in a particular environment. For example, microbiologists routinely use one test to determine the safety of public drinking water on the basis of the presence or absence of the bacterium *Escherichia coli*. Safe drinking water does not contain this organism, which is part of the normal microbial population living in the intestine.

Likewise, one may want to determine the total number and kinds of species in a sample of stream water or in a sample of soil, in order to understand how populations of microorganisms interact in a particular environment. However, a microbiologist needs to identify and quantify these microorganisms as part of a proper medical diagnosis, and similarly these tasks are needed when undertaking the bioremediation of recalcitrant complex molecules in contaminated environments. Consequently, for these reasons microbiologists must be able to isolate, enumerate, and identify the microbes in a specific sample of material (Pelczar *et al.*, 1993).

Viable Plate-Count Technique

Spread Plate Technique is a procedure for separating cells and obtaining colonies by spreading inoculum on a sterile agar surface with a bent glass rod (Pelczar *et al.*, 1993). One of the routine procedures for determining the bacterial content of many different materials is the *Plate-Count Technique*. A typical plate-count procedure is a combination of a *Serial Dilution* and the use of a suitable growth medium to detect colonies from

aliquots of the dilutions. This procedure is based upon the assumption that each viable cell will develop into a colony; hence, the number of colonies on the plate reveals the number of individual organisms contained in the sample that were capable of growing under the specific conditions of incubation and culture (Chan *et al.*, 1993).

Nevertheless, a clump of cells would also give rise to one colony. This is the reason that it is necessary to mix dilutions well in order to break up any clumps present. Since this is difficult to do with some species of bacteria, microbiologists use the term *Colony-Forming Units* (CFU) instead of colonies in a quantitative plate count. The plate count is an indirect measurement of cell concentration and provides results based on viable cells, that is, cells able to divide and form colonies. This is why the procedure is called the *Viable Plate-Count Technique*. When the number of viable bacteria in a sample is reported, it is expressed as the "number of bacteria (colonies) mL^{-1} or g^{-1}" or "number of CFU mL^{-1} or g^{-1}" of the sample (Chan *et al.*, 1993).

Objective

The goal of this detailed and precise protocol is the determination of the viable culturable bacterial population of a sample, following dilution and inoculation onto an appropriate solid nutrient medium, which are routinely used in biodegradation, bioavailability, and bioremediation research projects.

A. Preparation of Dilution Series

A.1 Protocol for Soil Samples

For Core Samples

The two methods are:

1. In the laminar flow hood, aseptically remove one of the covers of the core sample tube and with a sterile spatula (soaked in alcohol, ignited and cooled down) remove about 10 mm of soil from the end of the sample. Re-sterilize the spatula and aseptically remove an aliquot of the soil sample from the centre of the core sample

(do not take anything touching the side of the tube). During the length of the project, the same end of the core sample should be taken each time (*i.e.* either the top or the bottom, usually identified by different coloured covers).

2. In addition, the soil from the sample tube could be transferred into a plastic sterile bag by hitting the core liner with a hammer and mixing the core sample.

For Samples from a Jar or a Pail

With a jar, the sampling can be done as above in the laminar flow hood, taking extra care not to take soil touching the side of the jar. With a pail, it is more difficult to keep everything sterile. The pail cannot be put in the laminar flow hood. It should be put beside the flow hood and the sample removed as aseptically as possible with a sterile spatula from the centre of the pail. It is advisable to remove the surface soil to the side of the pail before taking a sample.

When taking soil samples, it is better to take several small chunks of soil here and there than one big one. Taking many small samples to make up the 5 g is more representative of the soil. Try to not be biased by the soil appearance, try to blindly take the soil without discriminating. The soil should be transferred to a pre-weighed sterile culture tube (25 mm × 150 mm) containing approximately 2.5 g of glass beads (3 mm diameter).

Weight of Soil

A good weight of soil to use is usually between 4 and 6 g (more than that and you will run out of space in the first dilution tube for good mixing, less than that and you may not have something representative of the sample). Obviously, it depends on the amount of soil available. If you are sampling a small microcosm, many times you will not be able to remove 5 g each times; you might have to settle for 1-2 g.

1. Once the soil sample is in the glass bead tube (*i.e.* 2.5 g per tube), re-weight the tube to accurately determine the wet weight of the original soil sample, and add exactly 3 times the weight of soil in volume of dilution solution [*Sterile Saline***:** 0.85% W/V NaCl in water for water samples or *Tetrasodium Pyrophosphate*: 0.1% W/V $Na_4P_2O_7.10\ H_2O$ in water (pH = 7.0) for soil samples] thereby giving a 1:4 dilution of the sample. Once the sample is in contact with diluent, diluted and plated should be put into ice right away. If they are many samples to be done, it is acceptable to take the soils out of a few samples (4-6 at maximum) in one shot, keeping the soils in their glass beads tubes on ice without liquid. The dilution and plating of a sample should be done in one shot. When diluent is added to the sample, it should be diluted and plated right away. Do not leave samples over lunchtime sitting on ice. Prepare only the ones you can do before an interruption like lunch.

Notice: All dilution tubes should be stored on ice during their preparation and subsequently, while plates are being spread.

2. Vortex the tube for exactly 2 minutes at high speed, then immediately transfer 1 mL to a fresh tube containing 9 mL of sterile dilution solution. Ensure that tube mouths are flamed briefly each time a tube is opened.

3. Vortex the tube for approximately 15-30 seconds, then immediately transfer 1 mL into a fresh tube containing exactly 9 mL of sterile dilution solution using a new sterile pipette each time.

4. Vortex and repeat step three above, until a 1:10 dilution series has been prepared (from the glass beads tube) from approximately 10^{-1} to 10^{-5} depending on which dilution will be plated. For a moderately contaminated soil, we usually plate 10^{-2}, 10^{-3} and 10^{-4} on non-selective medium and 10^{-1}, 10^{-2} and 10^{-3} on selective medium. To be on the safe side (when plating from a site for the first time) it might be advisable to plate 4 dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). If the soil is not contaminated and very rich in organic matter, it might be better to plate higher dilutions (10^{-3}, 10^{-4}, and 10^{-5}). If you

know that the soil was amended with bioaugmented nutrients, the dilution series should be adjusted accordingly.

A.2 Protocol for Water Samples

The protocol (vortexing, *etc.*) is essentially the same for water samples except that the first 1:4 dilution is not done, unless, you judge that the water sample contains a significant amount of suspended solids. The samples usually come in 4 L jugs that will need to be shaken vigorously for 2 minutes (instead of vortexing) before the first 1 mL could be removed.

B. Spread Plate Technique

The proper procedures are outlined as:

1. Label Petri plates containing appropriate nutrient media with sample number, dilution tube (*e.g.*, 10^{-2}, 10^{-3}, and 10^{-4}) and date.

2. Vortex dilution tube briefly (10-15 seconds) just prior to removing sample to be plated.

3. For triplicate plates, if you are working with a well-diluted suspension (*i.e.* you cannot see particles in suspension) remove 0.3 mL of the appropriate dilution, and carefully apply 0.1 mL to the surface of each of three plates of medium. Ensure that the surface of the agar is not pierced or otherwise damaged. Do not touch surface of the agar with the pipette (it will damage the surface). If you work with soil dilutions at low dilution or with water containing visible suspended solids, remove 0.4-0.5 mL of the suspension for triplicate plates. Hold the pipette as vertical as possible while distributing the 0.1 mL to the plates. The extra volume is to ensure that the three 0.1 mL volumes distributed are as similar as possible (you will note that the last 0.1 mL accumulates solids while coming down the pipette sides). Another way would be to pipette separately three times 0.1 mL (this would be better, but takes a lot of time). Please note that the Pipettor (*i.e.* Pipetman or Eppendorf brands) with sterile tips

could work with clear suspensions and tips block easily with soil particles, but you have to ensure that you are not touching the side of the tube with the pipettor while removing the sample.

4. Put the plates, one at a time, onto the centre of the turntable and spread the liquid over the entire surface using a sterile glass spreader (soaked in alcohol, ignited just prior to each spreading, and allowing the flame to extinguish itself). Let the spreader cool before using it. The glass rod should be moved slightly on the surface, while the turntable is turning to try to create a uniform covering on the surface of the plate. If the plates will be used for colony lifts and probing, it is advisable to try spread within the size of a membrane (*i.e.* 82 mm – a Petri dish is ~ 100 mm wide), and do not spread too much on the side of the dish – spare a 2-3 mm portion on the side of the dish.

Notice: It is important to use media plates that have been stored inverted at room temperature for 3 days prior to spreading. This ensures a dry surface that will take up the liquid quite rapidly. Spreading should continue until the surface of the plate is dry, indicated by sticking of the glass rod to the surface (It could also be verified by looking at the plate with an angle to see reflected light). If the plate was not spread until dry, the colonies will not be "countable", because they will run together in remaining liquid on the surface.

5. Dip the glass spreader in alcohol (95% ethanol is better since no water will be left after flaming), ignite and wait until all alcohol has burned off. Proceed to the next plate. If too much alcohol was on the glass rod prior to flaming, allow it to cool somewhat before proceeding.

6. When all plates of medium have been spread in this fashion, they should be incubated in an inverted position at the appropriate temperature (usually room temperature).

7. Counting of colonies on each plate should be performed at least twice: once after approximately one week of incubation (depending on incubation temperature), and second time, after about 2 weeks of incubation. To prevent recounting, you should use a marker (Permanent ink) to mark the colony. Therefore, at subsequent counting, the slow growing colonies can be identified by a different colour. You should note that, the most statistically significant counts are in the range of 30 to 300 colonies.

8. Record the number of colonies on the plates and the corresponding dilution. Do not forget in your calculations the first ¼ dilution done (one weight of soil + three volume of diluent). If colony lifts and hybridizations are to be performed, it is better to use plates with higher numbers of colonies on them (*i.e.* 100 to 600) for lifting in order to have a better chance to obtain significant numbers (probe-positive colonies may represent a very small percentage (*i.e.* below 0.1% sometimes) of the total colonies on the plate.

Guideline: If you have the population of the first column (Table Appendix - E.1), the other columns will show the number of colonies you will have on the plates of a given dilution. You should note that, the first tube for soil is a dilution 1:4 and 0.1 mL is plated. However, with Table Appendix - E.1, if you estimate that, you have between 10^{-5} and 10^{-7} CFU g^{-1} of soil, you can see which dilutions you should plate to have 10^{-2} to 10^{-3} CFU $plate^{-1}$.

Table A.E.1: Preparation of Dilution Series

CFU g^{-1} of Soil	Plate No. Dilu.	Plate Dilu. 10^{-1}	Plate Dilu. 10^{-2}	Plate Dilu. 10^{-3}	Plate Dilu. 10^{-4}	Plate Dilu. 10^{-5}	Plate Dilu. 10^{-6}	Plate Dilu. 10^{-7}	Plate Dilu. 10^{-8}	Plate Dilu. 10^{-9}
4E+10	1 E+09	1 E+08	1 E+07	1 E+06	1 E+05	1 E+04	1 E+03	1 E+02	10	1
4E+09	1 E+08	1 E+07	1 E+06	1 E+05	1 E+04	1 E+03	1 E+02	10	1	
4E+08	1 E+07	1 E+06	1 E+05	1 E+04	1 E+03	1 E+02	10	1		
4E+07	1 E+06	1 E+05	1 E+04	1 E+03	1 E+02	10	1			
4E+06	1 E+05	1 E+04	1 E+03	1 E+02	10	1				
4E+05	1 E+04	1 E+03	1 E+02	10	1					
4E+04	1 E+03	1 E+02	10	1						
4E+03	1 E+02	10	1							
4E+02	10	1								
4E+01	1									

APPENDIX F

A Protocol for Colony Lifting and Hybridization

1. Nylon filter placed on plate containing colonies making sure not to move the filter once it has touched any colony. Let it gently fall onto the plate and become wet.

2. Blot the filter gently with a Kleenex or Kimwipe and make three different marks using a wax pencil on the edge of the filter to aid in alignment of the colonies with the developed autoradiograph. Identification of the membrane with sample number, date and number of colonies needs to be marked as well.

3. Carefully lift the filter off of the plate with tweezers making sure not to shift or twist the membrane.

4. Place the filter (colonies facing up) on ~ 2.0 mL of lysis and denaturation solution (0.5 M NaOH) for 5 minutes, on a cafeteria tray. Make sure when placing and removing filters that you do not transfer filters overtop of another one to prevent portion of colonies from falling off and contaminating other filters.

5. Remove the filter and blot it on 3 MM Whatman paper for 1 minute.

6. Place filter on ~ 2.0 mL neutralization solution (1 M Trish, pH = 7.5) for 1 minute and then blot on a second 3MM Whatman filter paper for 1 minute.

7. Place filter on ~ 2.0 mL high salt solution (0.5 M Tris-HCl, pH = 7.5, 1.5 M NaCl) for 5 minutes and transfer to a third 3MM Whatman paper.

Notice: The two different methods for binding the DNA to the nylon membranes are following as:

A. Let the membranes air dry for 30 minutes and then bake them at 80°C under vacuum for 30 minutes.

B. While the membranes are still wet, place them in U.V. Crosslinker, press the Auto Crosslink button and then start. Let the membranes air.

Notice: After binding the DNA to the membranes, the filters should be placed in a plastic bag, sealed, and stored at - 20°C or - 80°C.

Removal of Bacterial Debris Assay

Soak the filters in 6 X SSC, 0.05% Triton X-100 for 30-60 minutes. Scrape bacterial debris off filter using a razor blade held at a 45° angle and dragging it gently across the filter.

Prehybridization and Hybridization Assays

Prehybridize and hybridize the membranes according to standard protocols.

Radioactive Labelling of DNA Probe Assay

Radioactive labelling of DNA fragments using the Multiprime Kit by Amersham. The following components are added with the enzyme being added last:

Purified PCR fragment, 35-40 ng;

- CTP, GTP and TTP solutions, 4 μL of each;
- 10 × buffer, 5 μL;
- Primer/BSA mix, 5 μL;
- [alpha]^{32}P-ATP (4500 Ci mmole^{-1}), 5 μL;

- Klenow fragment (1000 U mL^{-1}), 2 μL;
- Autoclaved Zenopure water to a final volume of 50 μL.

The purified PCR fragment is boiled 2 minutes prior to its addition to the reaction mixture to ensure single strandedness. All reagents are added except the ^{32}P-ATP and Klenow outside of the radioactive area. The ^{32}P-ATP is added and then the Klenow fragment is added last and the total incubation time is 1 hour at 37°.

Colony Hybridization Assay

The procedures for colonies no bigger than 2-3 mm are listed as follows:

- Lay filter on plate, transfer onto fresh plate and leave over night at 37°;
- With tweezers, carefully lift up filter and float face up on 2.5 mL 0.5 M NaOH, for 5 minutes, R.T. (lysis + denaturing);
- Blot on facial tissues for ~ 1 minute;
- Float filter on 1.5 ml of 1 m tris-hcl, ph 7.5 for 1 minute, r.t. (neutralizing);
- Blot again as before;
- Float filter on 1.5 m tris-hcl, ph 7.5, nacl 1.5 m, for 5 minutes, r.t.;
- Blot again as before;
- Air dry for 30 minutes;
- Bake filter for 30 minutes at 80° (with vacuum for nitrocellulose filters);
- Soak filter in 6 × ssc, 0.05% triton until excess bacterial debris can be scraped off, for approximately 30 minutes to one hour; and then, scrap off cell debris with blade while filters are in the solution;
- Prehybridize and hybridize according to any standard procedure.