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Caroline Rose

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biology)

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Temporal Changes in the Microbial Community of A PAH-Contaminated Soil During Bench-Top Bioremediation

TITRE DE LA THÈSE / TITLE OF THESIS

P. White

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

A. Poulain

J. Blais

I. Lambert

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Temporal Changes in the Microbial Community of a PAH-Contaminated Soil during Bench-Top Bioremediation

Caroline Gayle Rose, B.Sc.

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Department of Biology
Faculty of Science
University of Ottawa

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ABSTRACT

Contamination of soils with PAHs, substances that can pose serious environmental and human health risks, is a serious problem. Removal of risk requires effective and sustainable methods to decrease or eliminate toxicological hazard. Bioremediation is a sustainable option that can be accomplished by a variety of means. However, due to the limitations of classical culturing methods, there is a paucity of information regarding the composition of soil microbial communities, and moreover, the identity of organisms involved in contaminant catabolism. Newer, molecular techniques directly examine metagenomic DNA or RNA, and provide opportunities to investigate the genetic constitution of soil samples without the need for culturing. 16S rRNA has proven to be a valuable tool in the identification of soil microbes due to its highly conserved nature throughout the bacterial kingdom, in addition to inclusion of variable regions that are unique to a particular organism, or closely related group of organisms. This study used analysis of 16S rRNA gene sequences to examine the temporal changes in the soil microbial community in a PAH-contaminated soil during bioremediation. These results were compared with previously observed temporal changes in the chemical composition of the same soil during bioremediation. Interplay between the temporal changes of soil microbial community and the temporal changes of the PAH concentration was observed. The PAH concentration decreased an average of 77% across all treatments and reactors. Soil micro-organisms whose growth during treatment corresponded with a decline in PAH concentration included several known PAH-degraders such as *Achromobacter*, *Acidovorax*, *Pseudomonas*, *Rhodanobacter* and *Stenotrophomonas*. The metagenomic approach employed in this study can be used to evaluate innovative bioremediation methods, and moreover, identify members of the microbial community that are critical for the catabolism of PAHs.

RÉSUMÉ

La contamination des sols par les HAP, des substances qui peuvent poser des risques sérieux pour l'environnement et la santé des humains, est un grave problème. L'exclusion du risque exige que l'on emploie des méthodes efficaces et durables afin de diminuer ou d'éliminer le risque de toxicité. À ce titre, la biorestauration représente une option viable qui peut être mise en œuvre par divers moyens. Cependant, vu les limites des méthodes de mise en culture classiques, on manque d'information au sujet de la composition des communautés microbiennes du sol et de l'identité des organismes en cause dans le catabolisme des contaminants. De nouvelles techniques moléculaires permettent d'examiner directement les données métagénomiques sur l'ADN et l'ARN et offrent des occasions d'étudier la constitution génétique des échantillons de sol sans avoir à recourir à la mise en culture. L'ARNr 16S s'est avéré un outil précieux dans l'identification des microorganismes du sol par-ce qu'il s'agit d'une région hautement conservée chez les bactéries. De plus, la séquence comprend des régions variables spécifiques d'organismes donnés ou de groupes d'organismes étroitement apparentés. Dans cette étude, nous avons utilisé l'analyse des séquences de l'ARNr 16S pour vérifier, dans un sol contaminé aux HAP, les variations temporelles dans les communautés microbiennes du sol durant le processus de biorestauration. Nous avons comparé ces résultats avec des variations temporelles observées précédemment dans la composition chimique du même sol durant un processus de biorestauration. Nous avons observé une interaction entre les variations temporelles des communautés microbiennes du sol et les variations temporelles des concentrations en HAP. Ces dernières ont diminué en moyenne de 77 % par suite de tous les traitements et dans tous les bioréacteurs. Les microorganismes du sol dont la croissance durant les traitements s'est accompagnée d'une réduction des concentrations en HAP comprenaient plusieurs espèces, notamment des genres *Achromobacter*, *Acidovorax*, *Pseudomonas*, *Rhodanobacter* et *Stenotrophomonas*, connues pour leur capacité à dégrader les HAP. L'approche métagénomique employée dans cette étude peut être utilisée pour évaluer des méthodes de biorestauration innovatrices ainsi que pour identifier les espèces de la communauté microbienne qui sont cruciales dans le catabolisme des HAP.

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If we knew what we were doing, it wouldn't be called Research.
-Albert Einstein

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LIST OF ABBREVIATIONS

α	alpha
ACS	American Chemical Society
ASE	accelerated solvent extraction
bp	base pair(s)
BLAST2	Basic Local Alignment Search Tool 2
β	beta
B[*a*]P	benzo[*a*]pyrene
BPDE	benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide
°C	temperature in degrees Celsius
CCME	Canadian Council of Ministers of the Environment
CEPA	Canadian Environmental Protection Act
C/N/P	Carbon/Nitrogen/Phosphorous ratio
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1 (example)
dATP	deoxyadenosine triphosphate (nucleotide)
dCTP	deoxycytidine triphosphate (nucleotide)
ddNTP	dideoxynucleotide triphosphate
dNTP	deoxynucleotide triphosphate
gGTP	deoxyguanosine triphosphate (nucleotide)
gTTP	deoxythymidine triphosphate (nucleotide)
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
dO_2	dissolved oxygen
FASTA	FAST-All, works with different alphabets (nucleotide and protein)
γ	gamma
*g	G-force
HMW	high molecular weight
HPLC	high pressure liquid chromatography
IARC	International Agency for Research on Cancer
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kb	kilobase pair(s)
k_{ow}	octanol-water partition coefficient (ratio of the concentration of a chemical in octanol and in water at equilibrium and at a specified temperature)
λ	lamda (wavelength in nm)
LWM	low molecular weight
MPa	megapascal(s)
μ	mu, micro (10^{-6})
mEH	microsomal epoxide hydrolase
mM	milliMolar
mPA	millipascal(s)
M	Molar (moles per litre)

mol.	one mole has 6.0221415×10^{23} atoms or molecules of the pure substance being measured
MspI	type-2 restriction enzyme from *Moraxella* sp.
ng	nanogram(s)
nm	nanometre(s)
N-	nitrogen functional group
O-	oxygen functional group
-OH	hydroxide group
O.T.U.	operational taxonomic unit(s)
Pa	Pascal(s)
PAC	polycyclic aromatic compound
PAH	polycyclic aromatic hydrocarbon
pH	negative logarithm of the molar concentration of dissolved hydrogen ions (H+)
ppm	parts per million
psi	pounds per square inch
PVC	polyvinyl chloride
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	rotations per minute
S-	sulphur functional group
SIP	stable-isotope probe
S.O.C.	super optimal broth with catabolite repression
Tm	DNA melting temperature, °C (temperature at which a DNA double helix dissociates into single strands)
USEPA	United States Environmental Protection Agency
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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1.0 INTRODUCTION

Contaminated soil can pose serious environmental and human health risks. According to a status report of the Commissioner of the Environment and Sustainable Development to the House of Commons released in March 2008 there are currently about 17,800 known or suspected federally-owned contaminated sites in Canada [1]. This includes abandoned mines north of 60° latitude, areas that surround leaking petroleum storage tanks, airports, government laboratories, harbours and ports, landfills, light stations, military sites and training facilities, and Aboriginal reserve lands [1]. Moreover, site contamination is a worldwide problem, and many governments are seeking sustainable methods of remediating contaminated sites, and thus, reducing the risks of adverse health and environmental effects.

Large portions of contaminated sites, including almost 50% of the federally-owned contaminated sites in Canada, contain petroleum and/or polycyclic aromatic hydrocarbons (PAHs). PAHs are composed of two or more fused aromatic rings, and are an unfortunate by-product of incomplete natural or industrial combustion [2-5]. Many PAHs are known cancer-causing agents [2, 3, 6, 7] and are listed in Canadian Environmental Protection Act's (CEPA) Priority Substances List [3, 8] (see Figure 1.1).

Many different remediation techniques are currently being employed to reclaim contaminated environments (see Section 1.3 below). Bioremediation is a sustainable and economically feasible remediation solution. Bioremediation, or soil remediation via biologically-driven processes, can be accomplished *in-situ* [9-12] or *ex-situ* [9, 13]. The approach uses natural processes to break down and mineralize

contaminants, ideally to water, carbon dioxide and other inorganic compounds [9, 12]. Some soil microbes are able to live in contaminated soils, and selected individuals have been observed to have the capacity to mineralize and degrade toxic contaminants from both anthropologic and natural sources [6, 10, 14].

Current understanding of the structure of soil microbial communities is rather limited, and detailed information regarding community structure and the nature of the interactions and metabolic relationships between members of the soil microbial community is largely unavailable. Consequently, rational and scientifically sound manipulation of microbes in soil to permit or enhance the remediation of environmental contamination is challenging, and, for the most part, ineffective. More detailed information regarding the structure and diversity of microbial communities inhabiting contaminated environments is required in order to determine the roles of various organisms in site remediation, and moreover, permit exploitation of selected members of the microbial community to augment site remediation and reclamation [2, 10, 15].

1.1 CONTAMINATED SOIL ENVIRONMENTS

Soil contamination is a global environmental issue, and many countries now keep inventories of their contaminated sites [9, 16]. Table 1.1 below, which is adapted from White and Claxton (2004), provides a brief summary of the number of contaminated sites in several industrialized countries.

Table 1.1 **Inventory of contaminated land sites in selected industrialized countries, adapted from White and Claxton (2004) [9].**

Country	Total area (km^2)	Population (millions)	Number of contaminated sites
Canada	**9,976,140**	**31.6**	**15,000-40,000[a]**
United States	9,656,345	228.4	45,516
Germany[b]	356,910	82.8	202,880
Denmark	43,090	5.3	37,000
Sweden	449,960	8.9	7,000
Norway	323,900	4.5	2,121
Estonia	45,227	1.4	1,565

[a]Values for Canada are estimates from the Ministry of Environment or The Federal Contaminated Sites and Solid Waste Landfills Inventory.
[b]Potentially contaminated current and former military sites not included.

1.1.1 ANTHROPOGENIC ACTIVITIES CAUSING SOIL CONTAMINATION

Land contamination results from a vast array of anthropogenic activities. The activities include, but are not limited to, direct industrial discharge and waste disposal [13] as well as land fill and deep burial activities [9]. An example of a site where direct release contributed to severe contamination is Rock Bay harbour in Victoria, British Columbia, where by-products of coal gasification (i.e., coal tar and petroleum waste) were disposed on site and released into the harbour. Natural phenomena also contribute to land contamination via, for example, volcanic eruptions. Such events can also be associated with flooding and landslides, which can alter the spatial distribution of contaminants in the soil environment [13].

1.2 POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

Polycyclic aromatic hydrocarbons are a class of very stable, cyclic organic molecules that are made up of only carbon and hydrogen [5, 17]. However, ring substitution can take place to produce heterocyclic compounds (e.g., N- S- or O-heterocyclics). PAHs in soil are often associated with petroleum spills, sites formerly used for wood preservation (i.e., coal-tar creosote), and old gasworks sites [7, 11]. As already noted, many PAHs are known to be toxic, mutagenic and carcinogenic [5-7, 18]. PAHs have been observed to bioaccumulate in a variety of organisms (e.g., bivalve molluscs) [8, 19], and thus, can pose a considerable health risk to a variety of organisms, including humans.

PAH contamination comes from a variety of sources, and contamination of soil with PAHs is widespread in Canada. Moreover, due to their relatively persistent

nature they can be found in soil long after first deposited [2, 3, 6, 18]. Sources include vehicular combustion, biomass combustion, coke production, coal gasification, aluminum refining, steel refining and founding, and petroleum refining [3, 4, 20]. One of the primary sources of PAH contamination is manufactured (coal) gas [3, 4, 7]. A key by-product of manufactured gas, also known as coal gas or town gas, is coal tar, which was often discarded on site. Coal tar is a known animal carcinogen, and contains a wide range of PAHs and other polycyclic aromatic compounds (PACs), some of which have been highlighted under CEPA.

1.2.1 CHEMISTRY OF PAHS

Health Canada (under CEPA) and the US Environmental protection Agency (USEPA) [8, 21] outline 16 priority PAHs that are routinely monitored at contaminated sites. The chemical structures of the 16 priority PAHs are shown in Figure 1.1, and pertinent physical-chemical information on the 16 priority PAHs is summarized in Table 1.2, including information on carcinogenicity from the International Agency for Research on Cancer (IARC). Low molecular weight PAHs (2- to 3-rings), which are generally more labile, have been found to be acutely toxic [19], whereas the higher molecular weight PAHs (4 or more rings), which are more refractory to soil particles, are often mutagenic and carcinogenic [19].

1.2.1.1 PAH MIXTURES

With few exceptions, PAHs in environmental media (i.e., air, soil, sediment) usually occur as complex mixtures (e.g., coal tar and coal-tar creosote) [3]. From a risk assessment perspective this presents a challenge since mixtures likely contain a

variety of non-carcinogenic and carcinogenic PAHs of varying potencies. Potencies of PAHs may be both known and unknown [3, 22]. Moreover, the behaviour, environmental persistence and toxicity of a PAH mixture may differ significantly from that expected for individual priority PAHs [22]. As such, the toxicological hazard posed by a PAH mixture may involve multiple modes of action, and the development of rational soil quality guidelines for PAHs constitutes a significant challenge [3].

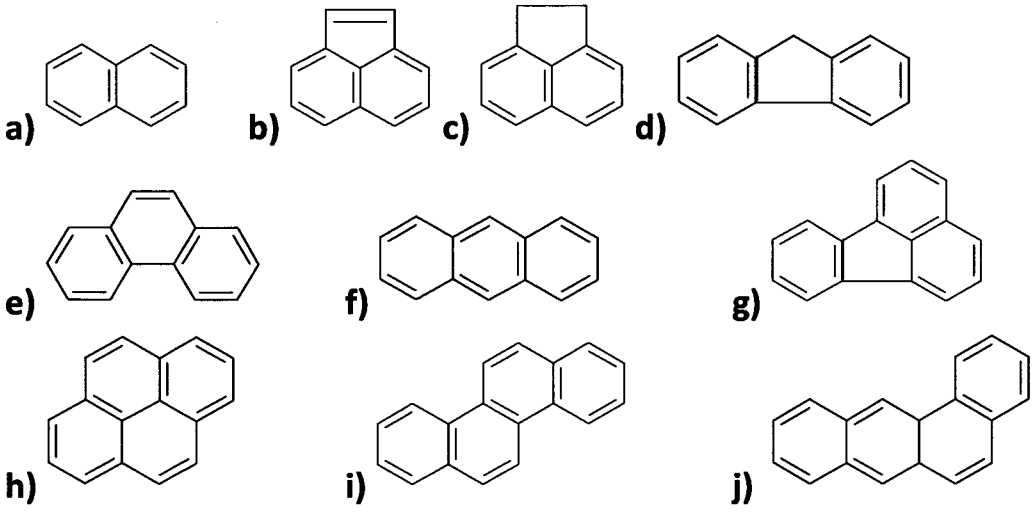
1.2.2 HUMAN HEALTH HAZARDS OF PAH CONTAMINATION

Excluding occupational exposures, total daily PAH intake for humans can vary from 0.2 to about 20 μg/day. Exposure is primarily through food consumption (e.g. charbroiled and smoked food) and cigarette smoking [3]. High occupational exposures can occur in aluminum production, iron and steel founding, fossil fuel processing, and wood impregnation facilities. Workers involved in roofing, road paving, or activities that involve exposure to diesel exhaust are exposed to PAHs [23]. Exposure and toxicological hazard need to be determined in order to accurately evaluate the risk of adverse health effects associated with environmental contaminants such as PAHs. For the carcinogenic PAHs, the goal is to eliminate human exposure, or to reduce exposure as much as possible [3].

Although several PAHs are noteworthy mutagens, they generally cannot bind to DNA and induce mutations unless they are converted to metabolized by-products (e.g. PAH diol-epoxides) [3]. In mammals, this conversion requires microsomal mixed function oxidases such as cytochrome P450 isozymes 1A1 and 1A2 [3]. The

cytochrome P450 isozymes constitutes a large family of enzymes involved in drug, xenobiotic chemical, and steroid metabolism [23]. Metabolic activation by cytochrome P450 isozymes involves the insertion of oxygen at one or more sites within the outer carbon skeleton of a particular PAH [23, 24]. The resulting metabolites are more water soluble, have increased reactivity, and are able to covalently bond with DNA to form adducts [23, 25, 26]. Mutations can arise if adducts are not repaired and replication based on the damaged DNA template contributes to an increased chance of a permanent sequence change (i.e., mutation) [24]. Adduct formation involving benzo[*a*]pyrene (B[*a*]P) is described by Nock *et al.* [25] and is shown in Figure 1.2. B[*a*]P is initially metabolized by CYP1A1 or CYP1B1 to form B[*a*]P-7,8-epoxide, which is subsequently hydrolyzed by microsomal epoxide hydrolase (mEH) to form B[*a*]P-7,8-dihydrodiol. CYP1A1, CYP1B1, or CYP3A4 then metabolize the product to form B[*a*]P-7,8-dihydrodiol-9,10-epoxide or BPDE, a highly reactive diol-epoxide. BPDE can covalently bind to DNA to create a PAH[BPDE]-DNA adduct which can induce mutation(s), principally in the form of A to T or G to T transversions. Transversions are formed by PAHs activated to radical cations by one-electon oxidation catalyzed by cytochrome P450 and form mainly depurinating adducts with DNA. The loss of depurinating adducts leaves behind apurinic sites which can lead to preferential insertion of deoxyadenosine opposite that site resulting in a transversion upon DNA replication [27].

Figure 1.1 Chemical structures of the 16 priority PAHs.



2, 3, and 4 ring compounds easily degraded by bacteria

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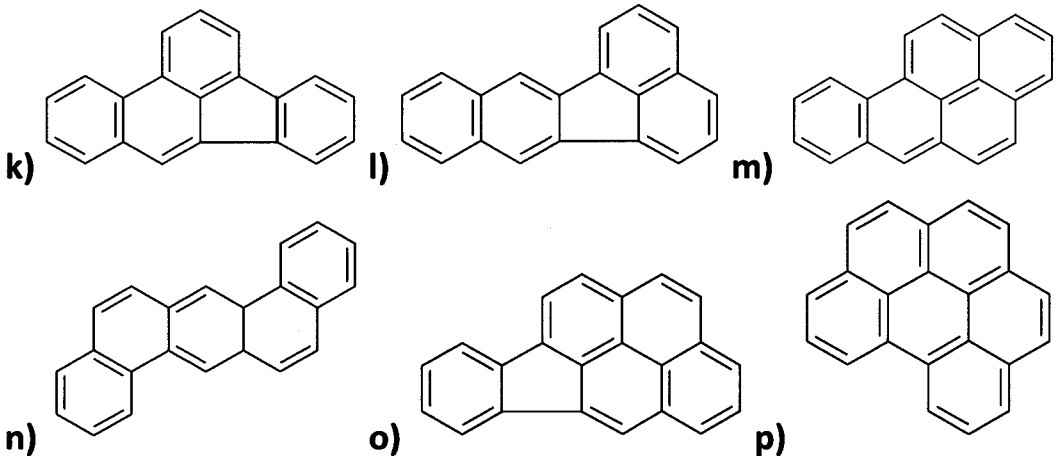
5 and 6 ring compounds more difficult to degrade



a) naphthalene
b) acenaphthylene
c) acenaphthene
d) fluorene
e) phenanthrene
f) anthracene
g) fluoranthene
h) pyrene
i) chrysene
j) benzo[*a*]anthracene
k) benzo[*b*]fluoranthene
l) benzo[*k*]fluoranthene
m) benzo[*a*]pyrene
n) dibenz[*a,h*]anthracene
o) indeno[1,2,3-*cd*]pyrene
p) benzo[*ghi*]perylene

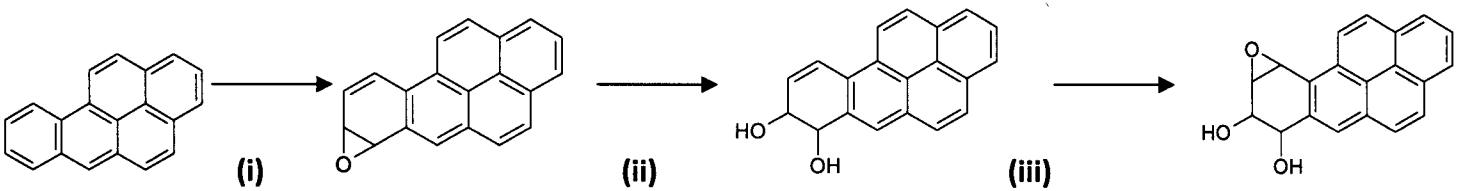
Table 1.2 **Physical-chemical information and carcinogenicity of 16 priority PAHs.**

Compound (CAS number)	Molecular weight (g/mol) [a or c]	Log K_{ow} [a or c]	Water solubility at 25°C (mg/L) [a or c]	Melting point (°C) [a or c]	Vapour pressure at 25°C (mPa) [a or c]	Human Carcinogenicity[b] (IARC classification)
Acenaphthene[a] (83-32-9)	154.2	4.3	3.4	95.0	594.0	Not classifiable (3)
Acenaphthylene[c] (208-96-8)	152.1	4.0	3.9	91.8	9.1×10^{-4}[de]	Not classifiable (3)
Anthracene[a] (120-12-7)	178.2	4.5	4.5×10^{-2}	216.0	25.0	Not classifiable (3)
Benz[*a*]anthracene[a] (56-66-3)	228.0	5.6	5.7×10^{-3}	162.0	14.7×10^{-3}	Possible (2b)[f]
Benzo[*b*]fluoranthene[a] (205-99-2)	252.3	6.0	1.4×10^{-2}	168.0	0.1×10^{-5} to 0.133 at 20°C	Possible (2b)[f]
Benzo[*k*]fluoranthene[a] (207-08-9)	252.3	6.0	4.3×10^{-3}	217.0	2.8×10^{-9}	Possible (2b)[f]
Benz [*a*]pyrene[a] (50-32-8)	252.3	6.0	3.8×10^{-3}	179.0	0.34×10^{-6}	Yes (1)[f]
Benzo[*ghi*]perylene[c] (191-24-2)	276.3	6.5	2.6×10^{-4}	273.0	1.0×10^{-10}	Not classifiable (3)
Chrysene[c] (218-01-9)	228.2	5.1	2.8×10^{-3}	254.0	6.3×10^{-7}	Possible (2b)[f]
Dibenz[*a,h*]anthracene[c] (53-70-3)	278.3	6.8	5.0×10^{-4}	262.0	1.0×10^{-10} (at 20°C)	Probable (2a)[f]
Fluoranthene[a] (206-44-0)	202.2	5.1	2.6×10^{-1}	111.0	1328.0	Not classifiable (3)
Fluorene[a] (86-73-7)	166.0	4.1	2.0	116.5	94.7	Not classifiable (3)
Indeno[1,2,3-*cd*]pyrene[a] (193-39-5)	276.0	6.4	5.3×10^{-4}	164.0	1.3×10^{-5}	Possible (2b)[f]
Naphthalene[a] (91-20-3)	128.1	3.5	3.2×10^{1}	80.5	11,960.0	Possible (2b)
Phenanthrene[a] (85-01-8)	178.2	4.5	1.29	101.0	90.7	Not classifiable (3)
Pyrene[a] (129-00-0)	202.2	4.9	1.4×10^{-1}	156.0	9.1×10^{-6}	Not classifiable (3)

[a]Government of Canada, 1994 [28]
[b]IARC, 2009 [29]
[c]George and Mumtaz, 1995 [30]
[d]Irwin, 1997 [31]
[e]USEPA, 2009 [32]
[f]Known or strongly suspected to act as a carcinogen in humans and other mammals. CCME, 2008 [3]

Figure 1.2 **(A) Metabolism of benzo[a]pyrene by cytochrome P450 and (B) PAH[BPDE]-DNA adduct formation with deoxyadenosine (dA), deoxyguanosine (dG) adducts are also common.**

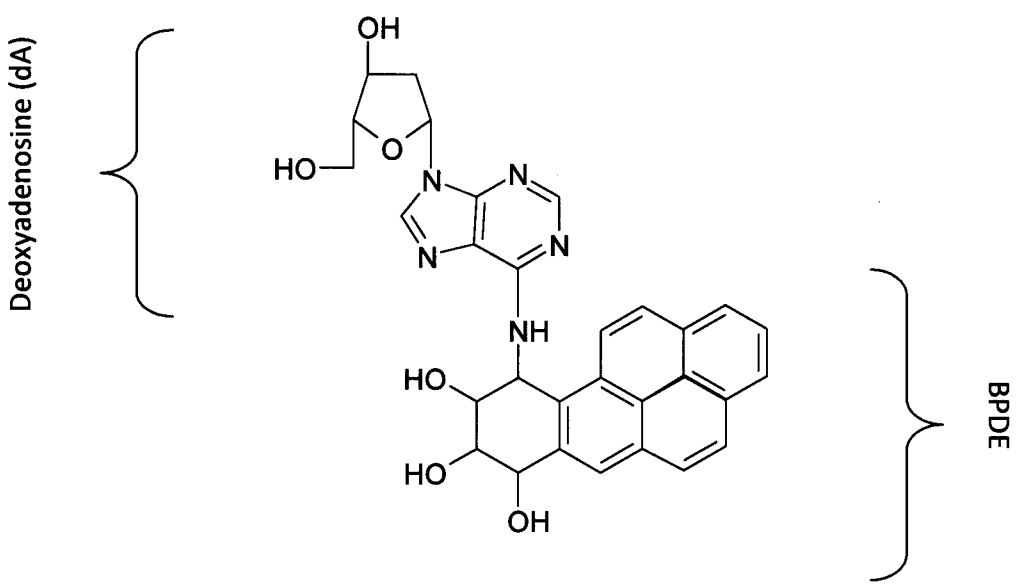
(A)



Benzo[*a*]pyrene 7,8-epoxide | Benzo[*a*]pyrene-dihydrodiol | Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE)

(i) CYP1A1 or CYP1B1; (ii) mEH; (iii) CYP1A1, CYP1B1 or CYP3A

(B)



1.3 REDUCTION OR ELIMINATION OF RISK

Reduction or elimination of risk posed by a contaminated site requires the reduction and/or removal of a particular hazard. Decreasing or removing risk can be achieved through the employment of several different techniques ranging from the restriction of site access through the erection of physical barriers (i.e., elimination of exposure), to site remediation and hazard removal. There are a plethora of remediation techniques that are employed in order to reclaim contaminated land. Remediation techniques can be broken down into five main categories: physical, chemical, thermal, solidification, and biological [9, 13]. Before any site remediation is attempted it is important to evaluate the hazard and risk associated with the contamination, and to determine the extent of remediation required.

1.3.1 PHYSICAL REMEDIATION

Physical remediation techniques include excavation, entombment, cover, soil washing, flushing, soil vacuum extraction, electro-reclamation, and particle size separation [9, 13]. Excavation involves the preparation of the contaminated soil for on- and off-site treatments. To avoid dispersion of contaminants an entombment technique is employed to encase the soil. Cover remediation literally involves covering the contaminants with materials such as asphalt or clean soil. Soil washing and flushing separate the contaminants from the soil and reduce the concentration of contaminants. Soil vacuum extraction extracts contaminants from unsaturated soil by injecting clean air or allowing air to pass into the unsaturated zone causing a mass migration of chemicals from the soil water into the soil air. Electro-

reclamation can be accomplished *in-situ* and involves electrically charging the soil with a direct current. Particle size separation uses gravity to separate particles.

1.3.2 CHEMICAL REMEDIATION

Chemical remediation techniques include neutralization, oxidation, photolysis, precipitation, reduction, carbon adsorption and ion exchange [9, 13]. Neutralization is a detoxification and immobilization process used to decrease the corrosiveness and reactivity of acid- and alkali-containing systems. Oxidation involves the detoxification of oxidizable contaminants. Photolysis is used in the detoxification of dioxins and nitrated contaminants. Precipitation is used to separate, reduce volume and immobilize metals and certain anions by the formation of insoluble precipitates. Reduction is used in the detoxification of chromium, silver and mercury. Carbon adsorption and ion exchange are separation and immobilization techniques.

1.3.3 THERMAL REMEDIATION

Thermal remediation techniques use high temperatures to reduce the volume, destroy and/or detoxify contaminants and include fluidized bed, rotary kiln, and pyrolysis [9, 13]. Fluidized bed remediation is a thermal process used to reduce volume and detoxify halogenated and non-halogenated organics and organic cyanides. Rotary kiln is a volume reducing and detoxifying process. Pyrolysis is a remediation technique used for soils that are not suitable for conventional incineration and soils containing volatile metals or recoverable residues.

1.3.4 SOILDIFICATION AND STABILIZATION REMEDIATION

Solidification and stabilization remediation techniques include cement solidification, pozzolanic solidification, thermoplastic stabilization, and vitrification [9, 13]. Cement solidification is a storage and immobilization technique accomplished by mixing soil and cement to form a hardened matrix. Pozzolanic solidification is based on the reaction of lime with fine-grained siliceous material. Thermoplastic stabilization involves sealing the soil in a matrix such as asphalt bitumen or polyethylene. Vitrification is used to store and immobilize inorganics and some organics.

1.3.5 BIOLOGICAL REMEDIATION

Biological remediation uses microbes to breakdown organic molecules into simpler compounds (i.e., CO_2 and H_2O) and/or to detoxify contaminants. It is limited by the degree of contamination in the soil, in the sense that the soil can not be biocidal [13]. Biological techniques can be divided into *in-situ* or *ex-situ* processes and will be explored further in Section 1.4.

1.4 BIODEGRADATION

Bioremediation can be a sustainable and economical option for the removal of hazardous PAHs in contaminated soils [12, 15], and it can be conducted *ex-situ* or *in-situ* [9]. The more commonly practiced bioremediation techniques can be sub-divided into four *ex-situ* categories: biopile, composting, land farming, and bioslurry; and three *in-situ* categories: attenuated bioremediation, passive remediation, and phytoremediation [9].

Biopile treatment requires contaminated soil to be excavated, piled into heaps, incubated for extended amounts of time under natural conditions and supplemented with air, nutrients, minerals, and water in order to enhance degradation of contaminants [9, 33, 34]. Organisms can be added to the soil heaps and are selected based on their ability to degrade a given contaminant [9, 33]. Composting involves supplementing the soil with manure and/or dry plant material in order to provide a source of organic carbon and to enhance the concentration of nutrients (e.g., nitrogen, phosphorus) [9, 34, 35]. Land farming involves tilling the contaminated soil with uncontaminated soil, followed by incubation and monitoring [9, 34, 36]. Tilling and subsequent irrigation occur at pre-determined intervals to enhance the remediation capacity of indigenous soil organisms [9]. Bioslurry [9, 20, 34] involves mixing soil with water, nutrients and air and incubating the slurry in a bioreactor under controlled conditions [9, 20, 24, 34].

Slurry bioremediation permits control of environmental conditions (e.g., oxygen, water content, humic content, nutrients) in order to provide conditions that favour microbial growth and degradation [9, 12]. The depletion rates of a contaminant in a slurry system depends on the degradation activity of the microorganisms available in that system, and the results of the remediation exercise reflect the capacity to mineralize and decontaminate under the slurry conditions [12].

The *in-situ* methods eliminate the need to transport large amounts of soil, or build large structures to house the soil, and involve the use of indigenous fauna or augmented indigenous fauna to degrade environmental contamination [9]. This is

achieved without site excavation and techniques are generally considered to be passive such that they are non-invasive and require little to no intervention [37]. Attenuated bioremediation employs the injection of compounds (e.g., compounds that release oxygen for aerobic remediation or hydrogen for anaerobic remediation) to accelerate the degradation of contaminants by native microbes [9, 38, 39]. Natural attenuation processes include a variety of physical, chemical and biological phenomena that will reduce the mass, toxicity, mobility, volume or concentration of a contaminant in soil [37, 40]. This treatment option can also involve the introduction of organisms selected for their ability to degrade particular substances. For example *Dehalococcoides sp.* accelerates the degradation of trichloro- and tetrachloroethene to ethylene [9, 41]. Phytoremediation employs plants to degrade, extract, transform, or sequester contaminants [9].

Bioremediation techniques can also be divided into bioaugmentation and natural attenuation. Bioaugmentation involves assisting or reinforcing natural biological process that break down organic matter by introducing cultured organisms or nutrients that augment the capacity to mineralize a particular substance or class of substances [42]. Natural attenuation is a strategy that relies on natural processes to achieve site-specific remediation objectives within a reasonable time frame [40]. Biodegradation, which is an integral part of both natural attenuation and bioaugmentation, involves the microbial breakdown of organic contaminants into smaller compounds through metabolic or enzymatic processes [15, 43].

1.5 MICROBIAL MINERALIZATION OF SOIL CONTAMINANTS

It has been estimated that there are approximately 4 x 10^3 to 10^4, perhaps as many as a million, bacterial species per gram (*g*) of soil [44-46]. Soil microbes perform a multitude of functions that contribute to processes such as soil formation, toxin removal, and elemental cycling of carbon, nitrogen and phosphorus, and soil health is dependent on the diversity of the soil microbial community [44, 47]. However, environmental stressors such as pesticides and herbicides can alter microbial populations in soil, therefore adversely impacting soil health and microbial diversity, an integral soil property that permits adaptation through the creation of new strains via mutation and genetic transfer [44, 48].

The role of specific elements of the microbial community in controlling temporal changes in the chemical or toxicological profile of contaminated soils is poorly understood. Effective degradation requires knowledge about the key microbial players, and the mineralization reactions that are occurring during treatment [15, 49]. Moreover, assessing the temporal changes in microbial community structure during bioremediation may provide information about the types of organisms that are critical for PAH mineralization and hazard removal. The main goal of bioremediation is to mineralize organic pollutants into CO_2 and H_2O, or to transform them into benign metabolites [50]. For use in bioremediation, a bacterial PAH-degrader should ideally mineralize PAHs and be capable of using PAHs as a sole carbon and energy source [19].

PAHs are relatively persistent in soils, and degradation *in-situ* is often slow due to their stable chemical structures. However, environmental persistence can also be attributed to nutrient availability, bioavailability of PAHs in soil (i.e., sorption to particles), temperature, oxygen availability, and presence of PAH-degrading microorganisms [11]. Microbial degradation of low molecular weight PAHs (two- and three-ringed) has been comprehensively studied [15, 51, 52]. More recent work has shown a variety of isolated microorganisms capable of metabolizing PAHs with up to four-rings [15, 53]. See Table 1.3 for information on common soil microbes with known PAH-degrading ability. Much work is being carried out, using both culture-dependent and independent approaches, to achieve a better understanding of soil microbial communities and their capacity to mineralize PAHs [37, 54]. Many microbial species have an array of enzymatic pathways that can metabolize both low and high molecular weight PAHs [37, 55]. Co-metabolism is often required for higher molecular weight PAHs (i.e., four or more rings) [37].

Table 1.3 Selected soil microbes with known PAH-degrading ability.

Genera	PAH	Reference
Achromobacter	Phenanthrene	Long *et al.*, 2009 [56]
Acidovorax	Phenanthrene	Singleton *et al.*, 2009 [57]; Seo *et al.*, 2009 [50]
Alcaligenes	Fluoranthene	Ringelberg *et al.*, 2001 [15]; Seo *et al.*, 2009 [50]; Toledo *et al.*, 2006 [58]
	Phenanthrene	Kiyohara *et al.*, 1981 [59]; Kiyohara *et al.*, 1990 [60]
	Naphthalene	Toledo *et al.* 2006 [58]
Pseudomonas	Fluoranthene	Ringelberg *et al.*, 2001 [15]; Leys *et al.*, 2003 [61]
	Naphthalene, phenanthrene, fluorene	Seo *et al.*, 2009 [50]; Leys *et al.*, 2003 [61]
	Naphthalene, phenanthrene	van Hamme *et al.*, 2003 [37]
Rhodanobacter	Benzo[*a*]pyrene[a]	Kanaly *et al.*, 2002 [62]
Sphingomonas	Fluorene, phenanthrene[b], anthrancene[b], fluoranthene[b]	Schuler *et al.*, 2007 [4]
	Naphthalene, fluorene, phenanthrene, anthrancene, benzo[*b*]fluorene	Leys *et al.*, 2003 [61]
	Phenanthrene, anthrancene, benzo[*b*]fluoranthene, naphthalene, fluoranthene, pyrene	van Hamme *et al.*, 2003 [37]
Stenotrophomonas	Pyrene, fluoranthene, benzo[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene	Seo *et al.*, 2009 [50]; Juhasz *et al.*, 2000 [63]

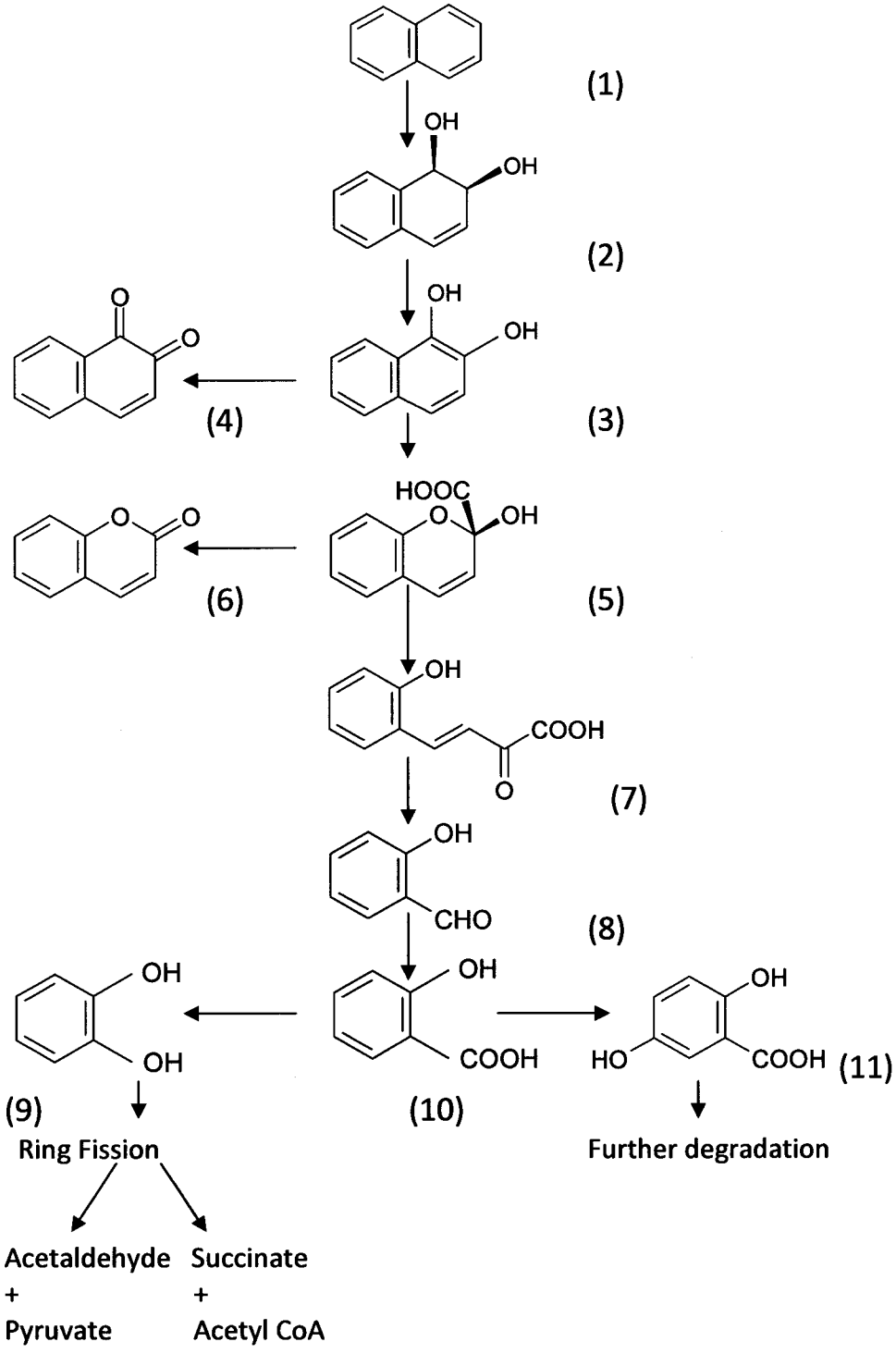
[a]Known to increase bioavailability

[b]Degraded by co-metabolism with pyruvate

Naphthalene is the simplest and most soluble PAH, and is often used as a model to investigate the ability of microorganisms to mineralize PAHs and predict breakdown pathways for larger PAHs [50]. Figure 1.3 provides a detailed outline of the proposed steps involved in the breakdown of naphthalene by bacteria such as *Alcaligenes*, *Pseudomonas* and *Sphingomonas* (adapted from Seo *et al.*, 2009) [50]. The process is described as follows [50]: naphthalene dioxygenase (i.e., *cis*-naphthalene-dehydrogenase) attacks the aromatic ring to form *cis*-(1R,2S)-dihydroxy-1,2-dihyronaphthalene, which is then dehydrogenated to 1,2-dihydroxynaphthalene by a *cis*-dihydrodiol dehydrogenase. 1,2-dihydroxynaphthalene is metabolized to salicylate via 2-hydroxy-2*H*-chromene-2-carboxylic acid, *cis-o*-hydroxybenzalpyruvate, and 2-hydroxy-benzaldehyde. The salicylate is decarboxylated to catechol, and can be further metabolized by ring fission.

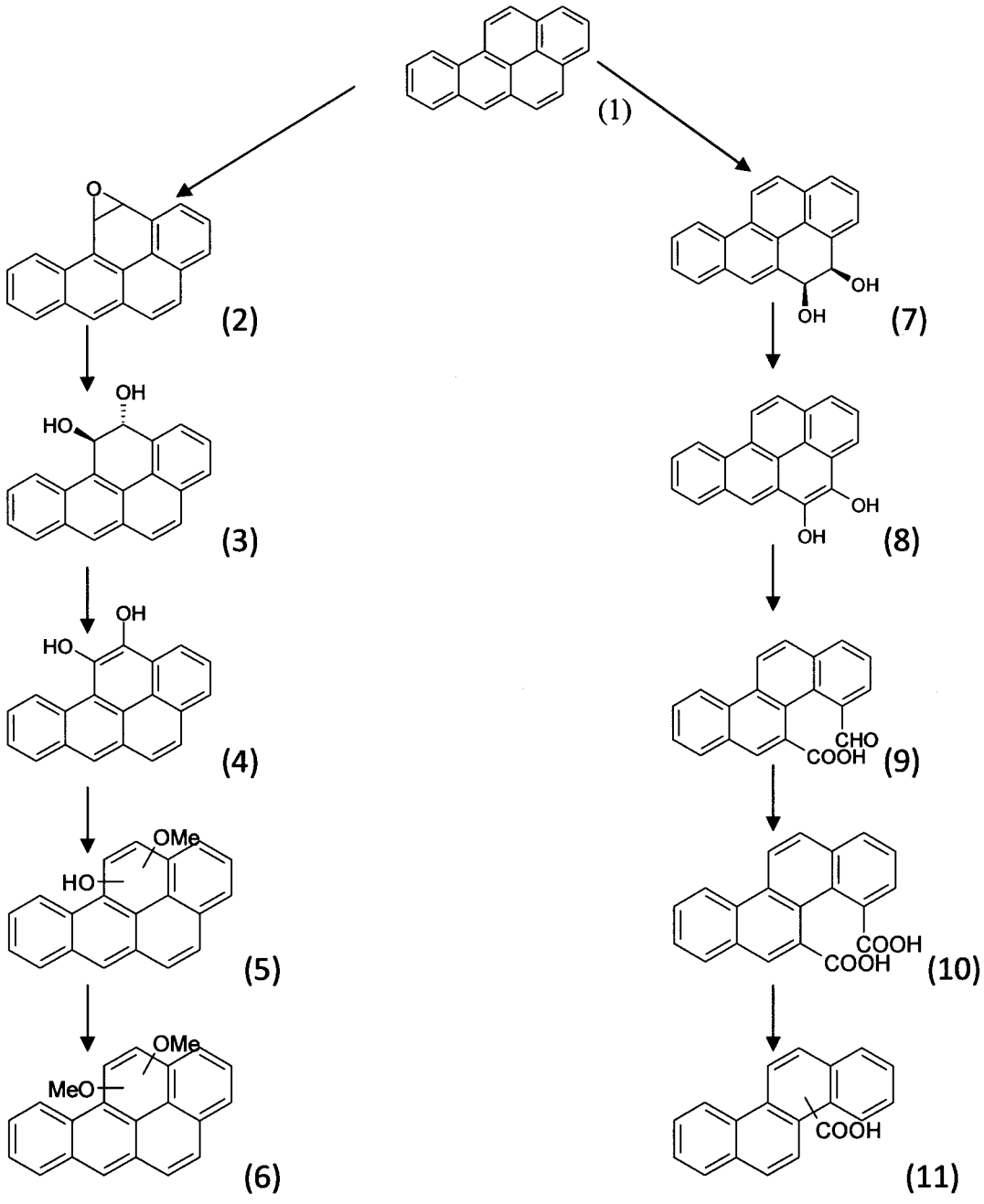
Benzo[*a*]pyrene (B[*a*]P) is one of the most potent carcinogenic PAHs [3, 50]. Several species, including *Mycobacterium* sp., *Sphingomonas paucimobilis*, and *Stenotrophomonas maltophilia*, have been observed to have the ability to degrade it [50, 63, 64]. However, the information available to date is still limited. High molecular weight PAHs are more persistent and their characteristics may complicate catabolism by bacterial assemblages [50]. Several catabolic pathways for B[a]P have been proposed by Seo *et al.* 2009 [50], some of which are shown in Figure 1.4. In each instance the initial step involves the breaking of one of the double bonds in an aromatic ring to form either an epoxide or a dihydrodiol.

Figure 1.3 Bacterial metabolism of naphthalene [50].



Compound names: (1) Napthalene; (2) *cis*-(1R, 2S)-Dihydroxy-1,2-dihyronaphthalene; (3) 1,2-Dihydroxynaphthalene; (4) 1,2-Naphthoquinone; (5) 2-Hydroxy-*2H*-chromene-2-caboxylic acid; (6) Coumarin; (7) *cis*-*o*-Hydroxybenzalpyruvate; (8) 2-Hydroxybenzaldehyde; (9) Catechol; (10) Salicylate; (11) Gentisate.

Figure 1.4 Bacterial metabolism of benzo[*a*]pyrene [50].



Compound names: (1) Benzo[*a*]pyrene; (2) benzo[*a*]pyrene-11,12-epoxide; (3) benzo[*a*]pyrene trans-11,12-dihydrodiol; (4) 11,12-dihydroxybenzo[*a*]pyrene; (5) hydroxymethoxy-benzo[*a*]pyrene; (6) dimethoxybenzo[*a*]pyrene; (7) benzo[*a*]pyrene cis-4,5-dihydrodiol; (8) 4,5-dihydroxybenzo[*a*]pyrene; (9) 4-formylchrysene-5-carboxylic acid; (10) 4,5-chrysene-dicarboxylic acid; (11) chrysene-4 or 5-carboxylic acid.

1.6 THESIS SAMPLES

A recent study by Whynot (2009) [24] employed a bench-scale bioslurry to examine changes in the mutagenic activity and chemical composition of soil from a former coal gasification site in Rock Bay, Victoria, British Columbia under three different treatments scenarios over the course of a 90 day incubation. Historically, industrial activities at Rock Bay harbour included a tannery, sawmills, a coal gasification plant, a propane tank farm, a concrete batch plant and an asphalt plant. The major source of contamination was the coal gasification plant, and as a result, the main contaminants of concern are petroleum hydrocarbons and PAHs. Analyses of soil samples collected at Rock Bay revealed levels of priority PAHs in the range of 475 to 12,600 μg/g dry soil (geometric mean = 2730 μg/g dry soil) [24].

1.6.1 SLURRY SAMPLES

The first experimental bioslurry treatment involved incubation of a 20% w/v slurry under controlled conditions (i.e., constant temperature, dO_2, and pH) to monitor the basal rate of PAH degradation. Dissolved oxygen was maintained at 100% saturation since biodegradation of PAHs occurs much more rapidly in an aerobic environment [3, 37], and aerobic microbial degradation of PAHs in soil is generally the most important process accounting for intermediate and long-term changes in contaminant levels over time [3, 17]. Two subsequent treatments examined degradation following a nutrient adjustment of both macro and micro nutrients (C/N/P ratio of approximately 100/10/1), and an augmentation with

nutrients as well as an exogenous culture of hydrocarbon degrading bacteria, respectively.

The results obtained showed a temporal decrease in the concentration of selected priority PAHs, and a simultaneous rise in mutagenic activity/hazard assessed using the Salmonella reverse mutation test. The latter change has generally been attributed to toxic intermediates that form during the breakdown, metabolism and mineralization of priority PAHs and related substances [7, 9, 15, 55]. Toxic intermediates can include compounds such as oxy-PAHs that have increased mobility, and are more readily taken up by organisms then parent PAHs [23, 25, 65]. Thus, the use of bioassays, in addition to priority chemical analysis, can be effective for tracking temporal changes in the hazard of a PAH-contaminated soil during bioremediation. Slurry samples were collected during the aforementioned treatment, and preserved for eventual analysis of temporal changes in the soil microbial community. This thesis investigated these changes, and the relationships between the microbial changes and noteworthy changes in contaminant concentration.

1.6.2 ANALYSIS OF SOIL MICROBIAL COMMUNITIES

Soil microbial communities are diverse and complex [37, 54, 66]. Variability can occur within minute distances and estimations indicate thousands to a million different species of single celled prokaryotic organisms can be found in a few grams of soil [44, 46, 67]. Moreover, individual members of soil communities are known to form intimate relationships with one another. As a result of these relationships,

culturing of soil organisms via conventional laboratory methods is extraordinarily difficult [49, 68, 69]. Traditional broth and plate cultures have previously been used to describe the soil microbial communities. However, traditional culturing methods are unable to reliably identify all the organisms present in soil communities, since they cannot take into accounts all the specific needs of each organism [49, 54, 70]. As a result, it is estimated that only 0.1% to 1% [71-73] of the soil microbial community has been reliably and accurately described.

Newer, molecular techniques have proven to be more effective in accurately characterizing the individual microorganisms present in soil [10, 66, 68, 70, 74, 75]. Molecular techniques circumvent the limitation of the aforementioned techniques by working directly with metagenomic DNA or RNA [10, 69, 76, 77], with phylogenetic relationships being based on genetic constitution. A metagenome represents a mixture of genomes of multiple organisms (e.g. mixed microbial genomes) extracted from an environmental sample [73]. Isolation of genetic material directly from the environment completely circumvents the need to culture, and permits a more comprehensive genetic identification. Analysis of rDNA (i.e., ribosomal DNA) of the soil microbial community has been previously described as an effective way of characterizing microbial communities in soil [15, 75]. The approach involves analysis of the community by characterizing highly conserved genes of the bacterial kingdom, such as those in the 16s rRNA gene [74, 78-83].

1.6.2.1 16S rRNA

The 16S rRNA gene is highly conserved throughout the Bacterial domain, and is directly linked to the phylogeny of microorganisms [10, 74, 82, 84]. The 16S rDNA contains both highly conserved regions that are found in all living organisms, as well as variable regions that are unique to specific organisms or closely related groups of organisms, and thus, can be used as a phylogenetic tool [69, 74, 82, 85, 86]. Depending on the degree of variability, phylogenetic ranking can be done to the genus, species and strain level via DNA sequence analysis [66, 86, 87]. Metabolically active cells contain a higher number of ribosomes so it can be assumed that the rRNA extracts from soil would predominantly reflect the diversity of the metabolically active members of a given soil community [10, 85].

1.6.3 OBJECTIVES AND HYPOTHESIS

1.6.3.1 OBJECTIVES

- Isolate total metagenomic DNA from soil. Amplify, clone, and sequence 16S rDNA.

- Identify members of the soil community by analysing rDNA sequences using the Ribosomal Database and Operational Taxonomic Units (O.T.U.s).

- Compare temporal changes in bacterial community structure to changes in PAH content, and identify genera that appear to play a role in the PAH mineralization.

1.6.3.2 HYPOTHESIS

If the analysis of 16S rDNA sequences in metagenomic samples of DNA from soil permits temporal analysis of community structure changes during a bioslurry treatment of PAH-contaminated soil, then observed interplay between microbial community structure changes and previously recorded changes in PAH concentration will yield insight into the elements of the microbial community (i.e., bacterial genera) that are determinants of PAH mineralization.

2.0 METHODS

2.1 SOIL PREPARATION

2.1.1 SOIL COLLECTION

Soil samples used in this study were obtained from an earlier bioremediation study that monitored the changes in mutagenic activity and chemical composition over time. The samples were collected on behalf of Health Canada from a Transport Canada owned site in British Columbia (Rock Bay, Victoria). Historically, the Rock Bay site had many different industries including a coal gasification plant and a propane tank farm. Much of the soil contamination can be attributed to the coal gasification plant that was in operation between the 1860s and the 1950s. Coal tar, which is a waste product of the coal gasification process, is the primary source of contamination in the Rock Bay harbour area [88]. Soil was taken from areas where black veins of coal tar were visible to the naked eye. Soil samples were double bagged inside a sealed plastic bucket in order to prevent vapour release. The buckets were stored in darkness at 4°C until needed.

2.1.2 SOIL PREPARATION FOR BIOREACTOR TREATMENT

Soil was removed from the plastic storage bucket and manually homogenized using stainless steel tool in a fume hood. Debris larger than 1cm in diameter was removed manually. The soil was then introduced into sterile BioFlo 110 bioreactors (see below) [12]. In total, for each treatment, 1 kg of soil was added to 5 L of sterile distilled water so that the final slurry concentration of each bioreactor was 20% (w/v) soil in water at the initiation of each treatment.

2.2 BIOREACTOR OPERATION

2.2.1 PREPARATION

Two BioFlo benchtop bioreactors, power-control units and probes used during the experiments were obtained from New Brunswick Scientific, Edison, NJ. They were prepared as follows: the reactors were disassembled and washed in a Lancer automatic dishwasher (Model# 1B014066) using an acidic rising agent (25% acetic acid) and laboratory detergent (Decon Laboratories Inc.) The internal components of the apparatus were rinsed with hexane and acetone in a fume hood and then rinsed thoroughly with sterile Milli-Q® laboratory water (Millipore Corp., Bedford, MA). The bioreactors were reassembled and 5 L of Milli-Q water added. To ensure a closed system, all seals were coated with silicone vacuum grease prior to assembly. Sterile PVC tubing (Fisher Scientific Ltd.) was prepared and added to all addition ports using no-leak quick connectors (TekniScience Inc.) Probes to monitor pH and dissolved oxygen were inserted to the appropriate ports and the entire system was sterilized at 121°C for 30 minutes in a Steris SV-120 Scientific Prevacuum sterilizer. Once cooled, the sterilized bioreactors were connected to the power control unit, and the system was allowed to equilibrate to operating conditions. Air flow through a 0.2 μm millipore filter, which was provided by a Gas and Cryo Equipment Zero Air Generator (model #ZAG-150-50), was set at 10 L/min, and directed into the slurry through an aspirator located at the bottom of the vessel. Exhaust produced by the bioreactors was first passed through a cold water condenser and a 0.2 μm Millipore™ Millex™ filter (Fisher Scientific Ltd.), and then passed into a container of

activated charcoal. The internal impellers rotated at 400 rpm for the duration of each treatment and were set 7.5 cm apart on the agitation motor's shaft. The temperature for each reaction vessel was set at 25°C. This temperature was maintained by the combination of internal cold water cooling coil and external heating by an electric heating blanket. The temperature probed was inserted into the appropriate port, and once the temperature had stabilized, the pH probe was connected and calibrated using an external pH meter. The reactor pH was adjusted using 0.5 M HCl or 0.5 M NaOH which was added to the system as required by a peristaltic pump. The dissolved oxygen probe (Mettler Toledo InPro 6800 series O_2 sensor) was connected and calibrated at pH 7 and 25°C. Saturation (100% dissolved oxygen) was set after the system had equilibrated for 24 hours. The span value (0% dissolved oxygen) was set after disconnecting the probe from the controller system and obtaining a steady input value on the calibration screen. The level controller probe was inserted in to the appropriate port and set to the "wet off" mode.

All input tubes (sterile Milli-Q water, 0.5 M HCl, and 0.5 M NaOH) were primed until drops were observed entering the vessel. The prepared soil was then introduced into the vessel via an access port, and the level controller was adjusted to the top of the slurry. The entire vessel was then blanketed with four layers of black landscaping fabric, each layer preventing 98% light penetration.

A software control program was created prior to the initial treatment. The same program was used for each of the later treatments to control the physical parameters and operation of the two bioreactors. The physical parameters were set

to a temperature of 25°C, a pH range between 6.5 and 8.0, and an agitation rate of 400 rpm. The software recorded temperature, agitation rate, dO_2, pH, and pump operation every 6 minutes over the course of each 90 day treatment.

2.2.2 OPERATION

2.2.2.1 BIOREACTOR TREATMENTS

Soil taken from the Rock Bay site was subjected to 90 day remediation runs using duplicate BioFlo 110 modular benchtop fermentors (described above). The soil slurry was sampled at 15-day intervals. Three different treatments were examined. The first was a control condition (Treatment A) with constant volume, aeration, pH, temperature, light and agitation [37]. These control conditions applies to all treatments. The second run (Treatment B) involved the addition of nutrients and micro-nutrients, which were added to the slurry every 30 days commencing after the first sampling on day 0. The third run (Treatment C) involved the addition of nutrients and an exogenous microbial culture with known hydrocarbon degrading capacity added every 30 days commencing on day 0 after sampling.

The reagents for Treatments B and C were from a Medina contaminated soil clean up kit (Medina Agricultural Products Co. Inc.., Hondo, TX), which included Bio-D (used in Treatments B and C), a microbial activator (used in treatments B and C), and a hydrocarbon degrading microbial seed (used in Treatment C). A C/N/P ratio of approximately 100/10/1 was maintained by the addition of Bio-D, which is a proprietary mixture of sugars, humic acid and other organic material providing macronutrients. The Bio-D nutrient that was added to treatments B and C contains

(per gram): 21.0 mg ammonia-N, 21.0 g nitrate-N, 144.3 mg organic-N, 28.0 g ortho phosphate, 18.3 mg potassium and 31.4 mg humic material. The microbial activator is an algae-based fermentation extract that contains essential micronutrients. The hydrocarbon degrading microbial seed is a dehydrated culture of microbes that had been formulated for the specific use in treatment of oil field and refinery wastes. The composition of the exogenous culture (including both the number of species present in the mixture and the concentration of organisms in the mixture) was not provided by the manufacturer.

The addition of 3.1 mL of the Bio-D and 1.55 mL of the microbial activator solution to treatments B and C began on day 0 of each experimental run, and continued every 30 days afterwards. The addition of 0.75 g of the exogenous microbial seed was added concurrently to the slurry during treatment C. The microbial seed was soaked in 20 mL of sterile milli-Q water for 15 minutes before being introduced into the slurry.

2.2.2.2 SAMPLE COLLECTION

Sample collection occurred every 15 days for the duration of each treatment, beginning at day 0. At each time point 4 x 25mL samples were collected in sterile, glass BioFlo 110 sample vials (Fisher Scientific Ltd.). Vials were properly labelled and stored at 4°C in the dark until needed for mutagenic activity or chemical analyses. One sample was transferred under a laminar flow hood to a sterile 50 mL conical plastic Falcon tube and 7.5 mL of 50% glycerol was added. The sample was

thoroughly vortexed to ensure homogenous glycerol dispersal, and stored at -80°C for use in microbial community analyses.

2.3 ANALYSIS OF SOIL PAHS BY HPLC

Sample handling and extraction as well as quantification of PAHs were carried out by Chris Whynot using HPLC. Details of methods emplyed are available are described in detail in his thesis [24].

2.4 SOIL DNA ISOLATION

Microbial community DNA was isolated from soil using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). The kit allows for isolation of soil DNA using both chemical and mechanical means of lysing bacterial cells. The MoBio kit has been previously used in similar studies with published results [89, 90]. The kit removed some of the humic contaminants that could adversely affect downstream procedures such as the polymerase chain reaction. The protocol outlined by Mo Bio Laboratories for maximum yields was used, and is outlined in detail in section 2.4.2.

2.4.1 SOLUTIONS

All solutions, bead tubes and spin filters used in the isolation of soil DNA were obtained from MoBio Laboratories and were included in the UltraClean Soil DNA Isolation Kit. The solutions and their roles in DNA isolation are listed in Table 2.1.

2.4.2 SOIL DNA ISOLATION PROTOCOL

Soil slurries frozen at –80°C with 7.5 mL of 50% glycerol were thawed at room temperature and vortexed briefly to ensure homogeneity. Soil slurry aliquots of 2 mL were collected from each sample in to the bead tube (components were removed) and centrifuged in an Eppendorf Centrifuge 5415C at 2039xg for 15 seconds to pellet the particulate matter. The supernatant was transferred to a new, sterile microcentrifuge tube and spun at 8154xg for 2 minutes to pellet suspended bacteria and supernatant was discarded. The two pellets were then combined into the bead tube using sterile toothpicks, and the bead tube components were added. The bead tubes were briefly vortexed. Sixty microlitres of the S1 solution was added to the bead tubes and the tubes inverted several times to mix. Two hundred microlitres of the IRS solution was subsequently added. The bead tubes were fastened horizontally to a 24-tube vortex adapter (MoBio Laboratories Inc., Carlsbad, CA) and vortexed at the maximum speed for 10 minutes. Mechanical lysis was introduced at this stage by randomly shaking the beads such that they collided with the bacterial cells causing them to break open. The tubes were centrifuged for 30 seconds at 8154xg to pellet beads, cell debris, humic acids and soil. The supernatant was transferred to a clean microcentrifuge tube, where 250 µL of the S2 solution was added. The mixture was vortexed for 5 seconds before incubating at 4°C for 5 minutes. The tubes were centrifuged at 8154xg for 1 minute to pellet the residues of humic acids, cell debris, and proteins. The supernatant was removed and added to a new, sterile microcentrifuge tube and 1.3 mL of the S3 solution was added. The

tubes were vortexed for 5 seconds. Approximately 700 µL of the mixture was loaded on to the silica membrane of a spin filter and centrifuged at 8154xg for 1 minute. The flow through was discarded and the process repeated until all of the supernatant had passed through the spin filter (about three iterations per samples). Three hundred microlitres of S4 solution was added to the spin filter and centrifuged for 30 seconds at 8154xg. The flow through was discarded and the tube centrifuged for 1 minute at 8154xg to remove any remaining S4 solution. The spin filter was then carefully placed into a new, clean microcentrifuge tube and 50 µL of S5 solution was added to the centre of the silica filter membrane and the tube centrifuged for 30 seconds. The spin filter was discarded and the DNA stored at -20°C until needed.

Table 2.1 Solutions included in the UltraClean Soil DNA Isolation kit [91].

Solution	Action
S1	SDS-based detergent that aids in cell lysis by solubilising the fatty acids and lipids associated with the cell membrane of several organisms
IRS	A proprietary reagent designed to precipitate humic acids and other PCR inhibitors. Must be used if the DNA will be used in a PCR reaction
S2	Contains a protein precipitation reagent required for the removal of contaminating proteins that may interfere with downstream processing and analysis.
S3	A DNA binding salt solution. DNA will bind to the silica membrane of the spin filter in the presence of high salt concentrations.
S4	Ethanol based wash solution used to clean the DNA bound to the silica filter membrane in the spin filter. It will remove residues of salt, humic acids and other contaminants.
S5	An elution buffer that releases the DNA from the spin column.

2.5 SOIL DNA PURIFICATION

Samples from Treatments B and C required additional purification. The additional humics resulted in poor PCR results with inconsistent product amplification. The subsequent ligation reaction was also affected by these humic substances. Two additional purification steps were needed, and these are described below in Section 2.5.1 and 2.5.2.

2.5.1 REMOVAL OF HUMIC CONTAMINANTS FROM DNA USING A PVPP MATRIX (PRIOR TO AMPLIFICATION)

Some of the isolated DNA samples were slightly discoloured despite the humic precipitating step provided in the Mo Bio kit. Polyvinylpolypyrrolidone (PVPP) has been described as a useful tool for removing additional contaminating substances from DNA isolated from soil [92]. PVPP removes humic acid contaminants by absorption to the insoluble polymer [93]. There are several preparatory steps required prior to the use PVPP.

2.5.1.1 PREPARATION OF A 3 M HCL SOLUTION

A sterile 3 M solution of hydrochloric acid (HCl) was made by diluting 250 mL of 12 M HCl into 750 mL of sterile distilled water. The solution was sterilized at 121°C for 20 minutes.

2.5.1.2 PREPARATION OF A 20 mM POTASSIUM PHOSPHATE SOLUTION

A 20 mM (0.02 M) solution of potassium phosphate was prepared by combining two separate solutions of K_2HPO_4 and KH_2PO_4. A 200 mM (0.2 M) solution of each was made, and the solutions combined until pH 7.4 was reached [93]. To obtain pH

7.4, 19.8 mL of 0.2 M KH_2PO_4 was added to 80.2 mL of 0.2 M K_2HPO_4 [94]. The final solution was diluted to 4 L so the resulting concentration was 20 mM. The solution was sterilized at 121°C for 20 minutes.

2.5.1.3 PREPARATION OF PVPP FOR DNA CLEANUP

Seventy five grams of PVPP was added to 1 L of 3 M HCl and the suspension was allowed to sit at room temperature for 12 to 16 hours. The suspension was then separated using a vacuum flask and filer of a pore size of approximately 20-25 µm (Fisher Scientific Ltd.). The PVPP was re-suspended in a 1L solution of 20 mM potassium phosphate (pH 7.4) and mixed by stirring for 1 to 2 hours. The PVPP was then filtered again and re-suspended in 20 mM potassium phosphate, and this process repeated until pH 7.0 was reached. The final 10% (w/v) solution was sterilized at 121°C for 20 minutes.

2.5.1.4 PVPP SPIN COLUMNS

Bio-spin chromatography columns (BioRad Laboratories), caps and 1.5 mL microcentrifuge tubes were sterilized at 121°C for 30 minutes. An aliquot of 300 µL of well mixed 10% PVPP solution was added to a sterile spin column, and the column placed in a sterile 1.5 mL microcentrifuge tube [92]. The spin column was then centrifuged at 11,300xg for 1 minute [95] (Mini spin plus Eppendorf). The catch tube was emptied and this step was repeated to dry the PVPP matrix. The entire volume of isolated DNA (50 µL) was then added and centrifuged for 1 minute at 11,300xg. A small volume of DNase/RNase-free water was then added to the PVPP matrix and re-spun to ensure all DNA products were obtained in the catch tube. At this point the

product should appear optically clear indicating that the humic contaminants have been removed. The purified samples were properly labelled and stored that -20°C until needed.

2.5.2 PHENOL/CHOLOFORM EXTRACTION (POST AMPLIFICATION)

Although the PCR amplification step was aided by PVPP purification, the ligation reactions (section 2.7.2) were still adversely affected by contaminating substances. An additional phenol/chloroform step was introduced to eliminate any remaining contaminants (e.g., humic substances) that could interfere with the ligation of the soil DNA into the pCR®II-TOPO® vector.

2.5.2.1 PHENOL/CHLOROFORM EXTRACTION

485 µL of TE buffer pH 8.0 was added to the PCR amplified DNA (15 µL), and the entire mixture was transferred to sterile, polypropylene microcentrifuge tubes. Five hundred microlitres of phenol:chloroform (1:1) was added to the DNA and the tubes were inverted 60X by hand. The tubes were then centrifuged at 850xg for 4 minutes in an IEC Micromax RF Refrigerated Microcentrifuge (Thermo Electron Corporation, Model# 120). The aqueous top layer (containing DNA) was then removed and transferred to sterile, polypropylene microcentrifuge tubes. The organic layer was discarded. The extraction was repeated a total of three times. An equal volume (500 µL) of chloroform:isoamyl alcohol (1:1) was added and the tubes were inverted 60X by hand. The tubes were centrifuged at 850xg for two to three minutes. The aqueous top later was removed and transferred to a sterile, polypropylene microcentrifuge tubes. A 0.07X volume of 3 M sodium acetate pH 5.2 and 2.5X

volume of cold 95% ethanol was then added to precipitate DNA. Further precipitation was achieved by incubating the tubes at -20°C for at least 30 minutes. DNA was pelleted by centrifugation at 15,800xg for 5 minutes. The supernatant was discarded and the pellet was washed with 70% cold ethanol (approximately 1 mL). The DNA was re-pelleted by centrifugation at 15,800xg for 2 minutes. The supernatant was discarded and the majority of the ethanol was removed by air drying. Pellet drying was aided by placing the tubes in a Speed Vac SVC100 (Savant Instruments Inc, Farmingdale, NY, model # SVC100D). The pellets were re-suspended in 20 µL of TE pH 8.0. Re-suspension was aided by mixing the tubes in a thermomixer (Eppendorf, Hamberg, Germany) at 50°C and 1400 rpm for more than one hour. The samples were then properly labelled and stored at 4°C until needed.

2.6 PCR AMPLIFICATION OF ISOLATED DNA (PCR1)

2.6.1 REAGENTS AND PRIMERS

All reagents were obtained from Roche Diagnostics unless otherwise stated. Custom primers were synthesized by Sigma-Genosys. The custom primers were designed to amplify only the 16s rDNA from the bacterial genome. It has been cited in the literature that due to the conserved nature of the 16S gene primer sequences can be developed to target regions on either side of the gene in order to isolate and amplify it [96, 97]. The 8f primer is widely used for 16S rDNA amplification [98, 99]. The 1389r primer is a slightly altered version of the 1387r primer developed by Marchesi *et al.* (1998) and was found to form dimers less frequently then the former [100, 101]. Their sequences are summarized in Table 2.2.

2.6.2 PCR1 PROTOCOL

Each reaction tube contained 1 µL of PCR reaction buffer (10X-concentrated containing 100 mM Tris-HCl, 500 mM KCl, 20 mM $MgCl_2$, and adjusted to pH 8.3), 0.8 µL of DMSO (Sigma-Aldrich), 0.1 µL of *Taq (*0.4 U/µL), 0.2 µL of dNTPs (10 mM each dATP, dTTP, dGTP and dCTP), 0.1 µL of each primer (0.2 µM), 1 µL of template DNA and 16.7 µL of PCR quality water (Qiagen Inc.) for a total of 20 µL. Two separate sub-mixes were prepared for the amplification and were combined just prior to running the reaction. Sub-mix A contained the PCR buffer, DMSO, *Taq* and 8.1 µL of PCR quality water for a total of 10 µL per reaction tube. Sub-mix B contained dNTPs, primers and 8.6 µL of PCR quality water for a total of 9 µL per reaction tube. Once the two sub-mixes were combined, 1 µL of purified template DNA (DNA from the soil isolations) was added to the reaction mixture for a total of 20 µL per reaction tube. The mixture was then spun down briefly at no more than 86xg using a Sorvall® Legend RT Centrifuge (Fisher Scientific Ltd.).

The reaction cycle was as follows:

1 minute at 94˚C
30 cycles of: 94˚C for 30 seconds
56˚C for 30 seconds
72˚C for 2 minutes
10 minutes at 72˚C
Hold at 4˚C
Amplified DNA was stored at -20˚C until needed.

Table 2.2 **Sequences of primers used to amplify the 16S rRNA gene region from the isolated soil DNA.**

Primer	Sequence	Tm (°C)
Forward, 8F	5′ AGAGTTTGATCCTGGCTCAG	61.0
Reverse, 1389R	5′ ACGGGCGGTGTGTACAA	63.9

2.6.3 0.8 % AGAROSE GEL ELECTROPHORESIS

Once amplified, 5 µL of the product with 2 µL of loading buffer (3 mL glycerol, 25 mg bromophenol blue, dH_2O to 10 mL) was run on a 0.8% agarose gel placed in a Maxi 20 Electrophoresis System (VWR International). A 1 kb DNA ladder (1.0 µg/µL, Invitrogen) was used as a scale. Samples were run at 120 V for approximately 1 hour to ensure sufficient band migration. Successful PCR runs were indicated by a product seen on the gel as a single, discrete 1400 bp band. The 0.8% agarose gel was made with 400 mL of 1X TBE buffer and 3.2 g of UltraPure agarose (Invitrogen). Before pouring the gel, 40 µL of ethidium bromide (EtBr, 10 mg/mL, 0.2 g EtBr in 20 mL sterile dH_2O) was added to permit visualization of DNA under UV light. A G:Box with GeneSnap GeneTools (Syngene (a division of Synoptics), Cambridge, England) were used to visualized DNA bands and record images.

2.7 CLONING REACTION

The cloning step is integral in the separation and identification of individual soil micro-oganisms. Isolated and amplified soil DNA was first ligated into the cloning vector pCR®II-TOPO®. The pCR®II-TOPO® vector contains a multicloning site that is located in the *lacZ* gene. Insertion of DNA into the cloning site results in the inactivation of the *lacZ* gene. Ideally, each vector will ultimately contain an approximately 1400 bp segment from one 16S rRNA gene region obtained from a single organism. The vector was cloned into chemically competent TOP 10F' *E .coli* cells. Each resulting colony represents the 16S rRNA gene region from a single soil bacterium. Colonies that contain the 16S rRNA gene region were detected by

culturing in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). The TOPO TA Cloning® kit was obtained from Invitrogen [102]. All other reagents and solutions were obtained from Invitrogen unless otherwise stated.

2.7.1 VECTORS AND STRAINS

The plasmid vector (pCR®II-TOPO®) is supplied linearized with single 3'-thymidine (T) overhangs for TA (thymidine-deoxyadenosine) Cloning®, and with topoisomerase I covalently bonded to the vector thus creating an "activated" vector. A map of the vector features is shown in Figure 2.1. The TOP10F' strain of *Escherichia coli* over expresses the *Lac* repressor (*lacI*^{q} genotype). In order to obtain expression from the *lac* promoter, isopropyl-β-D-1-thiogalactopyranoside (IPTG) must be added to culture plates. The strain contains the F episome and can be used for single-strand rescue of plasmid DNA containing an f1 origin [102].

2.7.2 LIGATION REACTION

Taq polymerase, described in Section 2.6, has non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' end of PCR products. This combined with the vector's overhanging 3' deoxythymidine (T) residues allowed the amplified foreign DNA to ligate efficiently to the vector [102].

As described by Invitrogen [102], Topoisomerase I (*Vaccinia* virus) is able to bind to duplex DNA at specific sites and cleaves the phosphodiester backbone after the 5'-CCCTT sequence in one strand. The energy produced is conserved by the formation of a covalent bond between the 3' phosphate of the cleaved strand and the tyrosyl residue of topoisomerase I. The topoisomerase is released by the reverse

reaction of the phosopho-tyrosyl bond being attacked by the 5' hydroxyl of the original cleaved strand.

2.7.2.1 PROTOCOL

Half volumes of the manufacturer's procedure were used [102]. For chemically competent *E. coli* cells, 2 µL of fresh PCR product was added to 0.5 µL of provided salt solution and 0.5 µL of the linearlized vector. The use of fresh PCR product is crucial due to the tendency of the A-overhang to degrade over time. If degradation occurred, the addition of a small amount of *Taq* to the PCR product and incubation at 72°C for 30 minutes was employed to "re-tail" the DNA. The final volume of the ligation reaction was 3 µL. The mixture was incubated at room temperature for 30 minutes and placed on ice or stored at -20°C until needed.

2.7.3 TRANSFORMATION PROTOCOL

Half (1.5 µL) of the ligation mixture was added to 25 µL of One Shot Chemically Competent TOPO F' *E. coli* and mixed gently. The mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds in a water bath (Jouan Inc., Winchester, VA), and then immediately transferred to ice. An aliquot of 200 µL of room temperature S.O.C. (Super Optimal broth with Catabolite repression) was added to the mixture, the entire mixture transferred to a culture tube and incubated with shaking at 37°C for one hour (Max$^{Q™}$mini, Barnstead International, Dubuque, IA, model# SHKE4450). S.O.C. medium contains 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, and 10 mM magnesium per one litre. During the incubation, media plates were warmed

to 37°C (CGA Precision Scientific, Chicago, IL, model# 6M). An aliquot of 100 µL of the transformed cell mixture was diluted by adding it to 900 µL of 0.85% sterile saline solution and the diluted mixture plated on several pre-warmed media plates. The plates were then inverted and incubated overnight at 37°C.

2.7.4 MEDIA

Luria-Bertani (LB) plates were prepared by adding 0.5% LB broth base (Life Technologies, Paisley, Scotland), 0.75% agar (Becton, Dickinson and Co., Sparks, MD), and 0.64% NaCl (EMD Chemicals Inc., Gibbstown, NJ) to dH_20. The media mixture was sterilized at 121°C for 30 minutes. Once cooled, kanamycin (50 µg/mL, Sigma-Aldrich) was added to the media in a laminar flow hood. Plates were poured by hand at a volume of 8mL per plate, and allowed to set. Once set, 40 µL X-Gal (40 mg/mL in DMSO (Sigma-Aldrich)) and 40 µL IPTG (100 mM in dH_20 (Sigma-Aldrich)) were spread onto each plate for use in the X-Gal assay.

2.7.4.1 X-GAL ASSAY

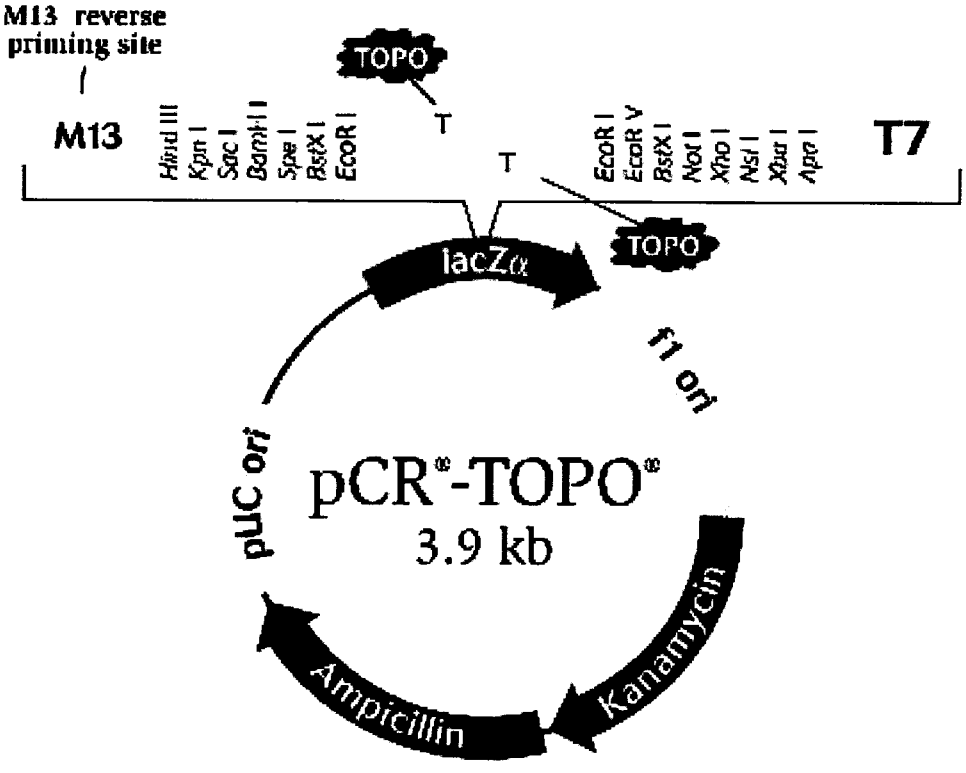
The *lac Z* gene encodes the β-galactosidase enzyme which cleaves 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) to yield galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product [94]. The appearance of the blue precipitate is indicative of the strength of activation of the *lac Z* gene. The TOPO TA vector contains a portion of the *lac Z* gene that complements the incomplete *lac Z* gene found in the chemically competent *E. coli* cells. When complementation occurs, the β-galactosidase enzyme is produced, X-gal is cleaved and a blue precipitate is formed.

When foreign DNA is inserted into the vector it will be unable to create a *lac Z* complement and no enzyme will be produced resulting in white/colourless colonies.

2.7.4.2 SELECTION OF COLONIES

Colonies containing the insert (i.e., white/colourless colonies) were picked using sterile toothpicks and transferred into 20 µL of sterile water in a 96-well plate. These plates were stored at 4°C until ready for amplification and further analysis. Toothpicks were transferred onto a media plate described in Section 2.7.4 and incubated overnight at 37°C to ensure colonies colour. Once successfully amplified, 20 µL of sterilized 40% UltraPure glycerol (Invitrogen) was added to the samples. The 96-well plates were properly labelled and stored at -20°C to -80°C until needed.

Figure 2.1 **Illustration of the pCR®II-TOPO® vector [102].**



2.8 PCR AMPLIFICATION FOR DIGESTION (PCR2)

2.8.1 REAGENTS AND PRIMERS

All reagents were obtained from Roche Diagnostics unless otherwise stated. Primers were synthesized by Sigma-Genosys. The primers target the multicloning region of the TOP10F' cells. The sequences are outlined in Table 2.3.

2.8.2 PCR2 PROTOCOL

Each reaction tube contained 1 µL of PCR reaction buffer (10X-concentrated containing 100 mM Tris-HCl and 500 mM KCl), 0.4 µL of $MgCl_2$, 0.4 µL of DMSO (Sigma-Aldrich), 0.3 µL of each primer (0.3 µM), 0.2 µL of dNTPs (10 mM each dATP, dTTP, dGTP and dCTP), 0.08 µL of *Taq* (0.4 U/µL), 1 µL of picked colony stock and 6.32 µL of PCR quality water (Qiagen, Inc.) for a total of 10 µL per reaction mixture. The mixture was spun down briefly at no more than 86xg using a Sorvall® Legend RT Centrifuge. The reaction mixture was incubated for 10 minutes at 94°C prior to the start of the PCR programme to destroy nucleases and release DNA.

The reaction cycle was as follows:

10 minute at 94°C
35 cycles of: 94°C for 30 seconds
56°C for 30 seconds
72°C for 2 minutes
10 minutes at 72°C
Hold at 4°C
Amplified DNA was then stored at -20°C until needed.

Table 2.3 Sequence of primers used to amplify DNA in transformed TOP10F' cells based on the priming regions of the TOPO vector [102].

Primer	Sequence	Tm (°C)
pUC forward	5' CTCACTATAGGGCGAATTGGG	64.3
pUC reverse	5' GCTATGACCATGATTACGCCA	64.6

2.9 RESTRICTION DIGEST

Once amplified, the PCR products were digested using a restriction enzyme so that variations in captured and cloned sequences could be visualized using agarose gel electrophoresis.

2.9.1 DIGESTIVE ENZYME

The restriction digest used was MspI (New England BioLabs Ltd.). The recognition sequence for MspI enzyme is CCGG, and the enzyme will cut double stranded DNA after the first C. All reagents are from New England BioLabs unless otherwise stated.

2.9.2 PROTOCOL

The reaction mixture contained 25 µL of dH2O, 50 µL of 10X NEBuffer 4 restriction buffer (1X NEBuffer 4 contains 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM Dithiothretiol, pH 7.9), and 25 µL of MspI (10 U/µL) for a total volume of 100 µL. The reaction mixture was vortexed briefly to thoroughly mix contents, and 1 µL of the reaction mixture was pipetted into each of the PCR products in the 96-well plate. Plates were tapped gently to mix and then spun down briefly at no more than 86xg using a Sorvall® Legend RT Centrifuge. The plates were then placed in a 37°C water bath for at least 2 hours, and no more than 16 hours.

2.9.3 3% AGAROSE GEL ELECTROPHORESIS

Digested samples with loading buffer were loaded on a 3% agarose gel (1x TBE) and placed in a Maxi 20 Electrophoresis System (VWR). Samples were run at 60 V

for approximately 3 hours to ensure good band separation. A 100 bp DNA ladder (1.0 μg/μL, Invitrogen) was used to approximate the size of the DNA band fragments.

2.10 DNA SEQUENCING

Samples that produced banding patterns of interest were sequenced by DNA LandMarks Inc. (Saint-Jean-sur-Richelieu, QC). Between 5-10 μL of culture solution stored in glycerol were cultured in approximately 80 μL of nutrient broth (0.05% bacto-tryptone (Becton, Dickinson and Company, Sparks, MD), 0.1% bacto-yeast (Difco Laboratories, Detroit, MI) and 0.05% NaCl (EMD Chemicals Inc., Gibbstown, NJ) with 50 μg/mL of kanamycin) at 37°C for at least 2 days. When the samples appeared turbid, 20 μL of sterile 10% UltraPure glycerol (Invitrogen) was added. Samples were then shipped to DNA LandMarks Inc. for sequencing.

2.11 ANALYSIS OF SEQUENCING RESULTS

Sequencing results were supplied in FASTA format, which was simply uploaded to the Ribosomal Database Project (RDP) for sample identification using the Seqmatch tool. The Seqmatch tool works by identifying 7-base oligomers shared between the sequence of interest and a given RDP sequence. The resulting Seqmatch score is the number of these unique 7-base oligomers shared between the two sequences divided by the lowest number of unique oligos in either of the two sequences [103, 104]. Up to 20 of the top Seqmatch scores can be presented. Identities are shown from the domain down to the species and strain level. For each

sample, the top five matches were recorded and the top match (with the highest Seqmatch score, preferably over 0.85) was declared the identity of the sample

In some instances a single sample's sequence came back in two halves. In this case the sequences needed to be manually assembled. This involved determining the reverse complement of one half and finding the area of overlap with the other half. Once a full sequence of approximately 1400bp was obtained it was submitted to the RDP for identification.

3.0 RESULTS

Recovery of total metagenomic DNA from the contaminated Rock Bay soil was accomplished with relative ease. This was confirmed through amplification of the 16S rRNA gene region using 16S targeted primers. Amplification products produced a discrete band when electrophoretically run on a 0.8% agarose gel. Using further cloning, amplification and restriction digestion, the diversity of the soil microbial community was observed. Identification of selected sample sequences was obtained by uploading sequences to the RDP database. Samples that were not sequenced were identified by their Operational Taxonomix Units (O.T.Us), and confirmation was accomplished by aligning known sequences that had similar O.T.U.s using BLAST2.

Once the total metagenomic DNA had been recovered and identified, the temporal changes occurring within each reactor during treatment could be assessed. Observed temporal changes in the soil microbial community were compared with previously observed temporal changes in PAH concentration in an effort to examine temporal correspondence in microbial community structure and soil contamination. Such comparisons should yield insights into the elements of the microbial community that are determinants of PAH mineralization.

3.1 TEMPORAL CHANGES IN PAH CONCENTRATION DURING BIOSLURRY TREATMENTS

The reduction of total PAHs for each treatment is summarized in Table 3.1. It is important to note that the initial concentration of total PAHs is not the same between treatments.

3.1.1 TREATMENT A

For reactor 1 the largest reduction in total PAH concentration occurred between days 45 and 60 (58.2%). The smallest reduction in total PAH concentration occurred between days 15 and 30 (3.3%). The total PAH concentration was rapidly reduced between days 0 to 15 and days 30 to 60. Slower reduction occurred between days 15 to 30 and 60 to 90.

In reactor 2 the largest reduction in total PAH concentration occurred between days 30 and 45 (30.8%). The smallest reduction in total PAH concentration occurred between days 45 and 60 (6.8%). The total PAH concentration was rapidly reduced between days 0 to 45 compared to a slower reduction rate between days 45 to 90.

3.1.2 TREATMENT B

The largest reduction in total PAH concentration in reactor 1 was observed between days 45 and 60 (30.0%). The smallest reduction in total PAH concentration occurred between days 30 and 45 (2.1%). The total PAH concentration was rapidly reduced between days 0 to 15, declined at a slower rate between days 30 to 45 and resumed rapid decline for the remainder of the treatment.

The largest reduction in total PAH concentration in reactor 2 was observed between days 0 and 15 (54.9%). The smallest reduction in total PAH concentration occurred between days 45 and 60 (1.3%). The total PAH concentration was rapidly reduced between days 0 to 15, followed by a slow rate of reduction for the remainder of the treatment.

3.1.3 TREATMENT C

In reactor 1 the largest reduction in total PAH concentration occurred between days 75 and 90 (44.2%). The smallest reduction in total PAH concentration occurred between days 15 and 30 (4.2%). The total PAH concentration was reduced at a relatively consistent rate for the duration of the treatment.

In reactor 2 the largest reduction in total PAH concentration occurred between days 15 and 30 (36.7%). The smallest reduction in total PAH concentration occurred between days 30 and 45 (6.0%). The total PAH concentration rapidly declined between days 0 to 30, compared to the slower rate of reduction that occurred for the remainder of the treatment.

Table 3.1 **Temporal changes in the concentration of total PAHs (μg per g dry soil) in slurry samples from reactors 1 and 2 for each treatment, adapted from Whynot (2009) [24].**

	Reactor	Day 0	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90
Treatment A	Reactor 1	12631	9759	9440	6496	2718	2028	1897
	Reactor 2	4209	6024	5234	3379	4119	3292	2737
Treatment B	Reactor 1	760.2	569	468	460.5	322	258	216
	Reactor 2	1352.5	630	574	559	553	436	263
Treatment C	Reactor 1	474	389	375	286	269	238	132
	Reactor 2	604	416	264	247	226	159	112

3.2 MICROBIAL COMMUNITY ANALYSES

As described in Sections 2.8 to 2.11, samples that produced banding patterns of interest were sequenced and the resulting FASTA files were uploaded to the Ribosomal Database Project (RDP) in order to identify the bacteria. Identification was based on matching 7-base oligomers shared between the 16S rDNA sequence of interest and a given RDP sequence [103, 104]. Samples that were not sequenced were identified by hand, based on their operational taxonomic unit (O.T.U.) and the sequence results. The O.T.U. is the banding pattern that the samples produced when digested with the restriction enzyme MspI end were electrophoretically separated on 3% agarose. Selected samples had their DNA sequences aligned using BLAST2 to confirm their identities [105]. Some samples were given the same identity by RDP2, but had distinctly different O.T.U. patterns. Alignments were done to ensure different O.T.U. patterns could be identified as the same organism, as well as to compare organisms observed in different treatments and in the exogenous microbial consortia added in Treatment C. Sequence that produced similarity of >96% when aligned were considered to be in the same genus.

All treatments started with relatively similar representations of microbial genera. However, because a soil sub-sample was taken from the storage container prior to the start of each treatment there are variations across treatments due to the physical, chemical and biological heterogeneity of the soil. These differences can also be attributed to the general nature of soil communities. Variability can occur across minute distances and estimations indicate thousands to a million different

species of single celled prokaryotic organisms can be found within a few grams of soil [67].

3.2.1 TREATMENT A

3.2.1.1 MICROBIAL GENERA DETECTED IN REACTOR 1

In reactor 1 of Treatment A there are a total of 24 different bacterial genera that occur during the 90-day treatment. The results are summarized in Table 3.2, and the changes observed are illustrated in Figure 3.1. The results are recorded as the percentage of total clones, that is, the number of clones that carried a particular organism's 16S rRNA gene region divided by the total number of clones harvested for a given sampling point for that reactor and treatment. A colour code for the presentation of the soil microbial genera in all graphs in Section 3.2 can be found in Appendix 1.

Day 0 showed the least amount of observed species richness, compared to the other two time points in Treatment A. There were only two classes of the *Proteobacteria* phylum: *Beta-* and *Gammaproteobacteria*. They are closely related according to 16S rRNA analyses, however, their physiologies are very diverse and there is no obvious metabolic pattern [106]. Within the *Betaproteobacteria* class four genera were observed: *Achromobacter*, *Acidovorax*, *Variovorax*, and an unknown *Rhodocyclaceae* genus denoted '35' (based on O.T.U. results). Within the *Gammaproteobacteria* class three genera were observed: *Pseudomonas*, *Lysobacter*, and *Rhodanobacter.*

Day 45 showed a substantial increase in the observed species richness. A total of 19 different genera representing five different phyla were observed. Novel phyla seen at day 45 are *Actinobacteria*, *Bacteriodetes*, *Firmicutes* and *Planctomycetes*. However, none of these phyla dominated the percentage of total clones as much as the *Proteobacteria*. The *Alphaproteobacteria* class also appears at this time point. *Alphaproteobacteria* includes mostly oligotrophic proteobacteria that are capable of growing at low nutrient levels [106]. The *Phenylobacterium* genus was observed with a percentage of total clones of 7.4%.

Day 90 shows a slight decrease in the observed species richness, with 16 genera observed. A novel phylum, the *Chloroflexi*, appears in addition to the ones already mentioned for day 45. However, there is only a single clone to represent this phylum. The genus *Rhodanobacter* (of the *Xanthomonadaceae* family) increases gradually throughout the treatment peaking at day 90 (7.2%).

Table 3.2 Temporal changes in the genera present in reactor 1 of Treatment A.

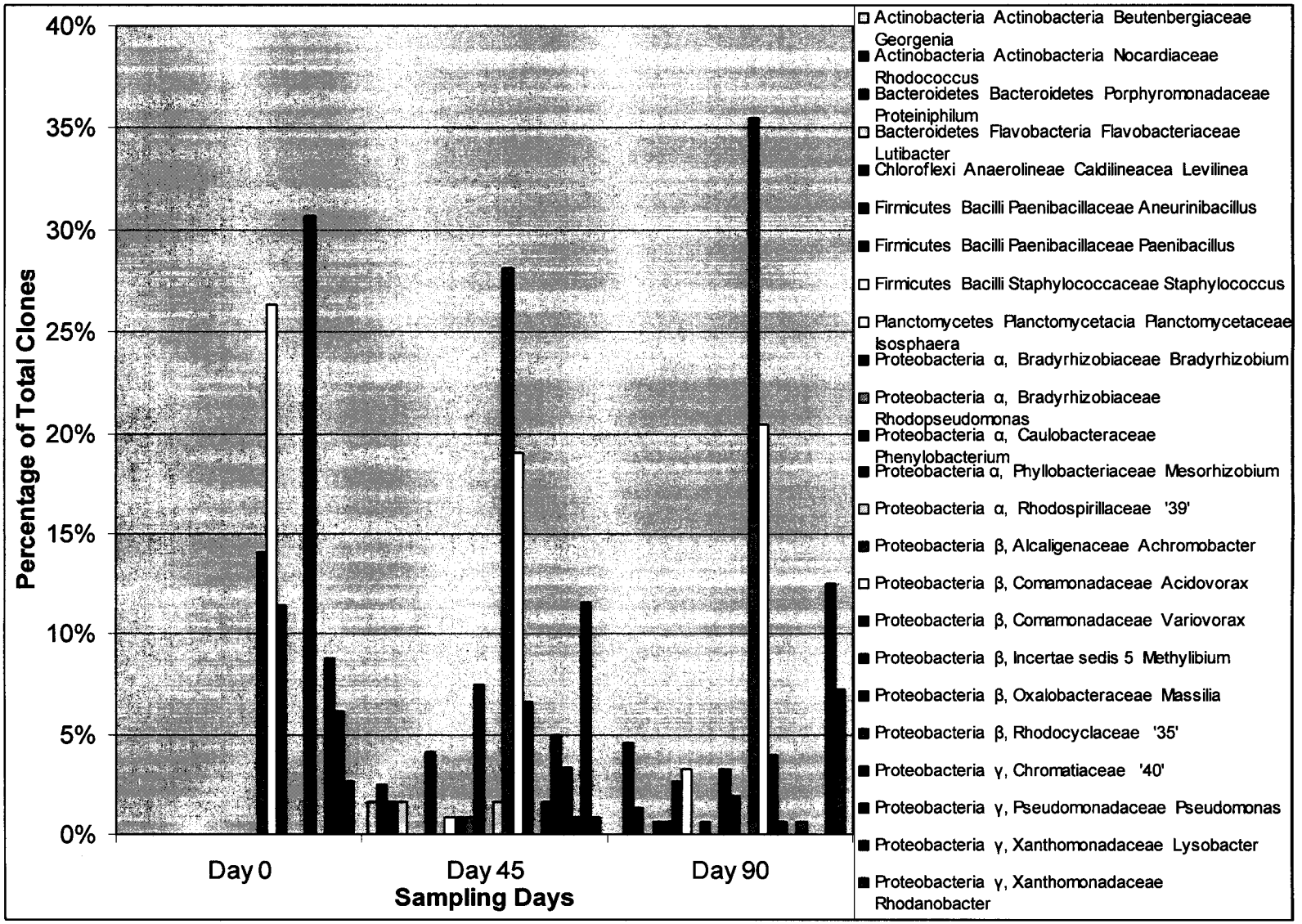
Phylum	Class	Family	Genus	Day 0 (%)[a]	Day 45 (%)	Day 90 (%)
Actinobacteria	*Actinobacteria*	*Beutenbergiaceae*	*Georgenia*	0.0	1.7	0.0
		Nocardiaceae	*Rhodococcus*	0.0	2.45	4.6
Bacteroidetes	*Bacteroidetes*	*Porphyromonadaceae*	*Proteiniphilum*	0.0	1.7	1.3
	Flavobacteria	*Flavobacteriaceae*	*Lutibacter*	0.0	1.7	0.0
Chloroflexi	*Anaerolineae*	*Caldilineacea*	*Levilinea*	0.0	0.0	0.6
Firmicutes	*Bacilli*	*Paenibacillaceae*	*Aneurinibacillus*	0.0	0.0	0.6
			Paenibacillus[b]	0.0	4.1	2.6
		Staphylococcaceae	*Staphylococcus*	0.0	0.0	3.2
Planctomycetes	*Planctomycetacia*	*Planctomycetaceae*	*Isosphaera*	0.0	0.8	0.0
Proteobacteria	*Alphaproteobacteria*	*Bradyrhizobiaceae*	*Bradyrhizobium*	0.0	0.8	0.7
			Rhodopseudomonas	0.0	0.8	0.0
		Caulobacteraceae	*Phenylobacterium*	0.0	7.4	3.3
		Phyllobacteriaceae	*Mesorhizobium*	0.0	0.0	2.0
		Rhodospirillaceae	*'39'*	0.0	1.67	0.0
	Betaproteobacteria	*Alcaligenaceae*	***Achromobacter***	**14.0**	**28.1**	**35.5**
		Comamonadaceae	***Acidovorax***	**26.3**	**19.0**	**20.4**
			Variovorax	**11.4**	**6.6**	**4.0**
		Incertae sedis 5	*Methylibium*	0.0	0.0	0.7
		Oxalobacteraceae	*Massilia*	0.0	1.7	0.0
		Rhodocyclaceae	***'35'***	**30.7**	**5.0**	**0.7**
	Gammaproteobacteria	*Chromatiaceae*	*'40'*	0.0	3.3	0.0
		Pseudomonadaceae	*Pseudomonas*	8.8	0.8	0.0
		Xanthomonadaceae	***Lysobacter***	**6.1**	**11.3**	**12.5**
			Rhodanobacter	2.6	0.8	7.2
			Total Number of Clones Sampled	**114**	**121**	**152**

Highlighted cells denote percentage of total clones of approximately 10.0% for at least one of the three sampling days.

[a]percentage of total clones.

[b]*Paenibacillaceae, Paenibacillus*.

Figure 3.1 Temporal changes in the genera present in reactor 1 of Treatment A.

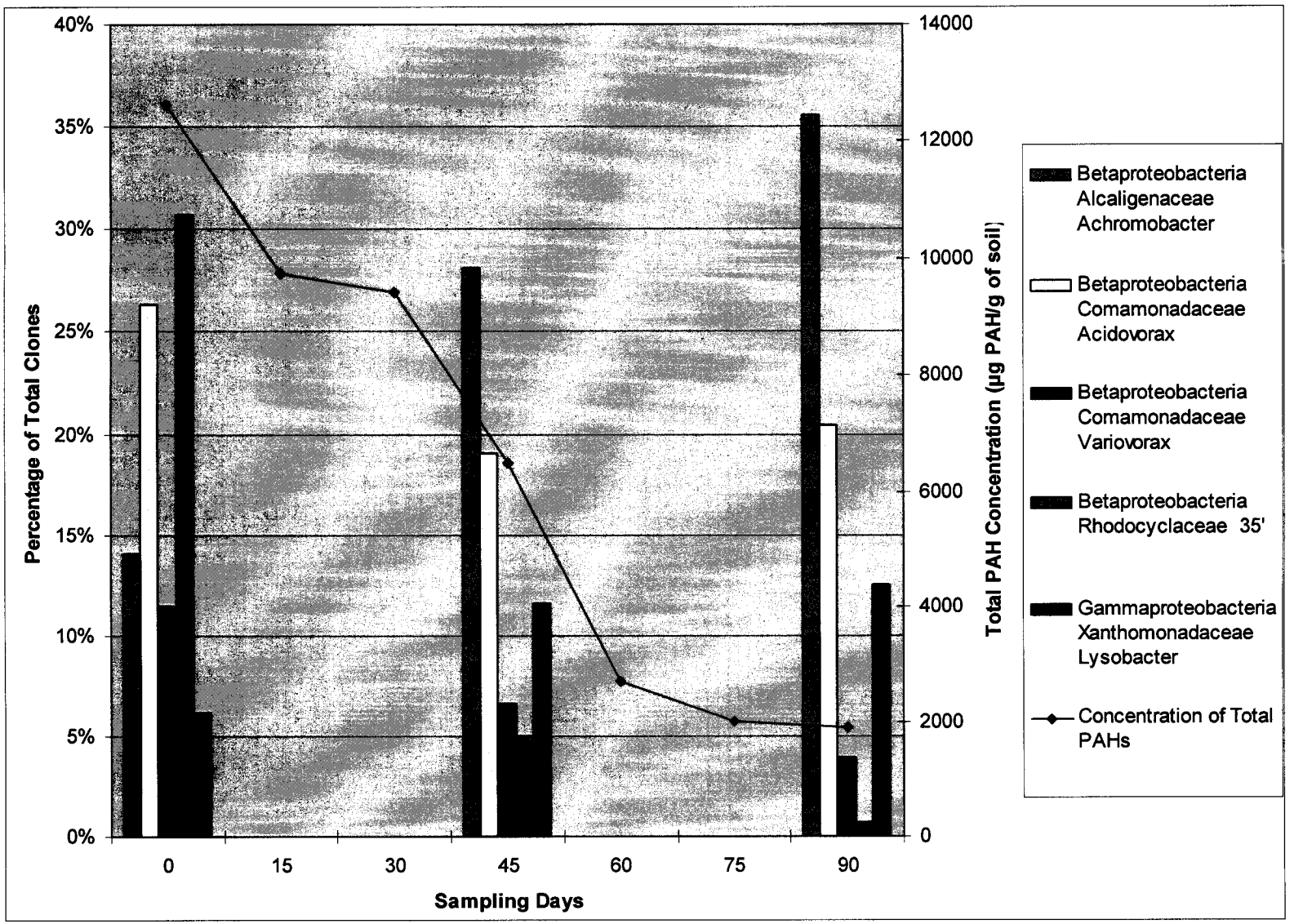


3.2.1.2 COMPARISON BETWEEN THE TEMPORAL CHANGES IN BACTERIAL COMMUNITY & TEMPORAL CHANGES IN PAH CONCENTRATION OBSERVED IN REACTOR 1

The decline in the concentration of total PAHs throughout each treatment corresponded with numerous temporal changes in the soil microbial community. Figure 3.2 illustrates temporal changes in the prevalent microbial genera total PAH concentration. There are five prevalent genera observed in reactor 1 that each represent approximately 10.0% of the total clones for at least one of the sampling days (shown as shaded cells in Table 3.2). The prevalent genera, which are within the *Proteobacteria* phylum, specifically in the *Beta* and *Gammaproteobacteria* classes, are *Achromobacter*, *Acidovorax*, *Variovorax*, *Rhodocyclaceae* '35', and *Lysobacter*. The PAH-degrading ability of selected members of the prevalent genera has already been summarized in Table 1.3.

The *Achromobacter* genus was observed to increase over the course of treatment. The *Acidovorax* genus showed an initial decrease before increasing slightly towards the end of treatment. The *Variovorax* genus was found to decrease steadily over the course of a 90-day treatment. The *Rhodocyclaceae* '35' genus was found to decrease drastically over the course of a 90-day treatment. The *Lysobacter* genus increased gradually over the course of a 90-day treatment.

Figure 3.2 **Comparison of temporal changes in the prevalent genera to temporal changes in total PAH concentration for reactor 1 of Treatment A.**



3.2.1.3 MICROBIAL GENERA DETECTED IN REACTOR 2

The results obtained for reactor 2 of Treatment A showed a total of 18 different microbial genera present throughout the three time points sampled. The results are shown in Table 3.3 and the changes observed are illustrated in Figure 3.3. There were only two classes present at Day 0: *Beta-* and *Gammaproteobacteria*, including six genera, *Achromobacter*, *Acidovorax*, *Variovorax*, *Rhodocyclaceae*'35', *Lysobacter*, and *Stenotrophomonas*.

Day 45 observations showed a slight increase in the observed species richness of reactor 2. There were a total of 13 different genera representing three different classes. The *Alphaproteobacteria* were observed at this time point, in addition to the *Beta* and *Gammaprotebacteria*. Three different genera from the *Alphaproteobacteria* appear at this time point, but none in significant numbers. These included *Bradyrhizobium* (2.5%), *Phenylobacterium* (1.6%), and *Rhodospirillaceae* '39' (4.1%). A novel genus of *Betaproteobacteria* also appears at this point – the *Methylibium* (4.9%).

Day 90 shows a slight increase in the observed species richness in reactor 2 (i.e, 17 genera). Three new phyla appear at this time point: *Acidobacteria*, *Firmicutes,* and *Verrumcomicrobia*. However, there is only a single genus to represent each of these phyla, none of which achieve a percentage of total clones greater than 3.0%.

Table 3.3 **Temporal changes in the genera present in reactor 2 of Treatment A.**

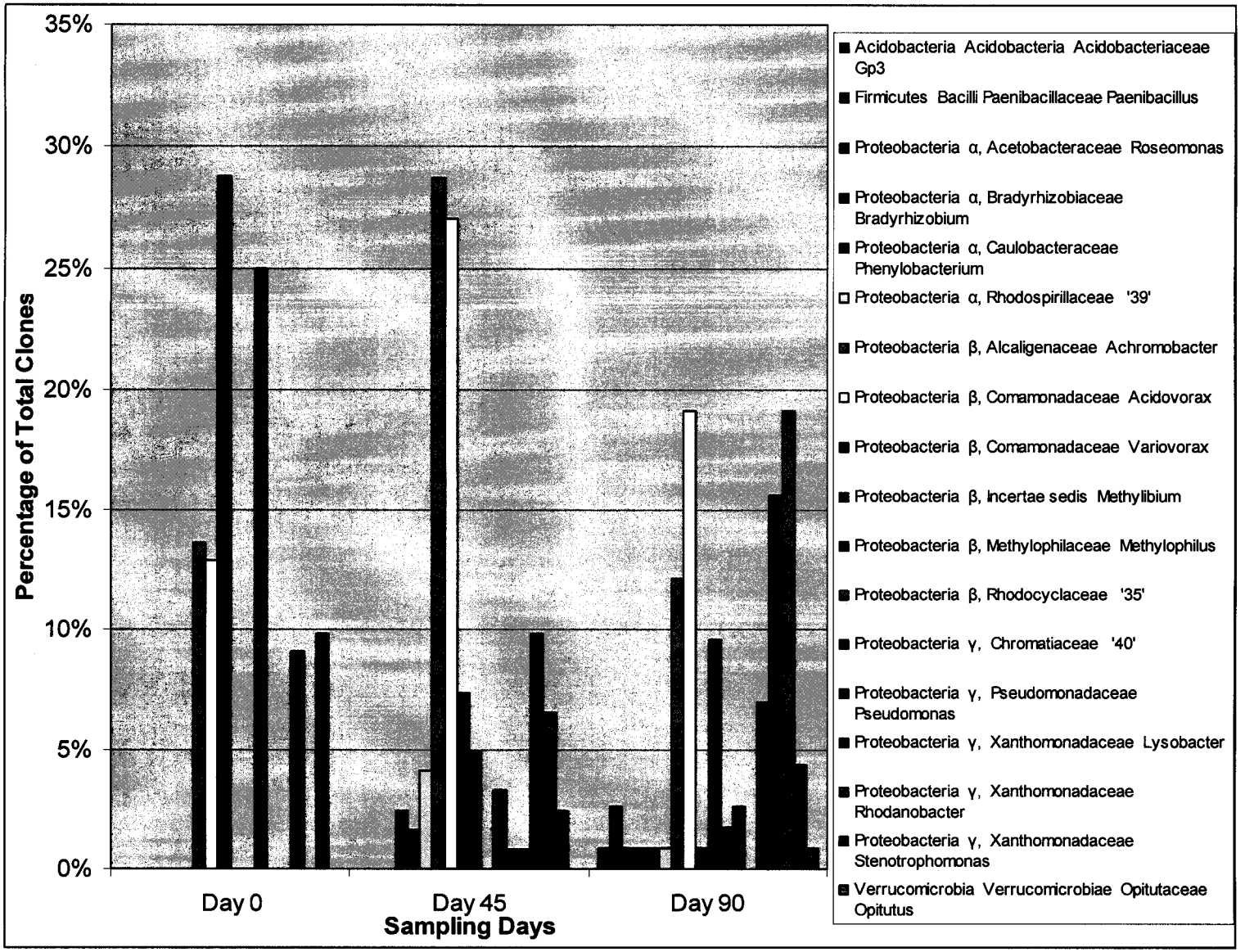
Phylum	Class	Family	Genus	Day 0 (%)[a]	Day 45 (%)	Day 90 (%)
Acidobacteria	*Acidobacteria*	*Acidobacteriaceae*	*Gp3*	0.0	0.0	0.9
Firmicutes	*Bacilli*	*Paenibacillaceae*	*Paenibacillus*[b]	0.0	0.0	2.6
Proteobacteria	*Alphaproteobacteria*	*Acetobacteraceae*	*Roseomonas*	0.0	0.0	0.9
		Bradyrhizobiaceae	*Bradyrhizobium*	0.0	2.5	0.9
		Caulobacteraceae	*Phenylobacterium*	0.0	1.6	0.9
		Rhodospirillaceae	*'39'*	0.0	4.1	0.9
	Betaproteobacteria	*Alcaligenaceae*	***Achromobacter***	**13.6**	**28.7**	**12.1**
		Comamonadaceae	***Acidovorax***	**12.9**	**27.1**	**19.1**
			Variovorax	**28.8**	**7.4**	**0.9**
		Incertae sedis 5	*Methylibium*	0.0	4.9	9.6
		Methylophilaceae	*Methylophilus*	0.0	0.0	1.7
		Rhodocyclaceae	***'35'***	**25.0**	**3.3**	**2.6**
	Gammaproteobacteria	*Chromatiaceae*	*'40'*	0.0	0.8	0.0
		Pseudomonadaceae	*Pseudomonas*	0.0	0.8	7.0
		Xanthomonadaceae	***Lysobacter***	**9.1**	**9.8**	**15.6**
			Rhodanobacter	**0.0**	**6.6**	**19.1**
			Stenotrophomonas	**9.9**	**2.5**	**4.4**
Verrucomicrobia	*Verrucomicrobiae*	*Opitutaceae*	*Opitutus*	0.0	0.0	0.9
			Total Number of Clones Sampled	**131**	**122**	**115**

Highlighted cells denote percentage of total clones of approximately 10.0% for at least one of the three sampling days.

[a]percentage of total clones.

[b]*Paenibacillaceae, Paenibacillus*.

Figure 3.3 **Temporal changes in the genera present in reactor 2 of Treatment A.**

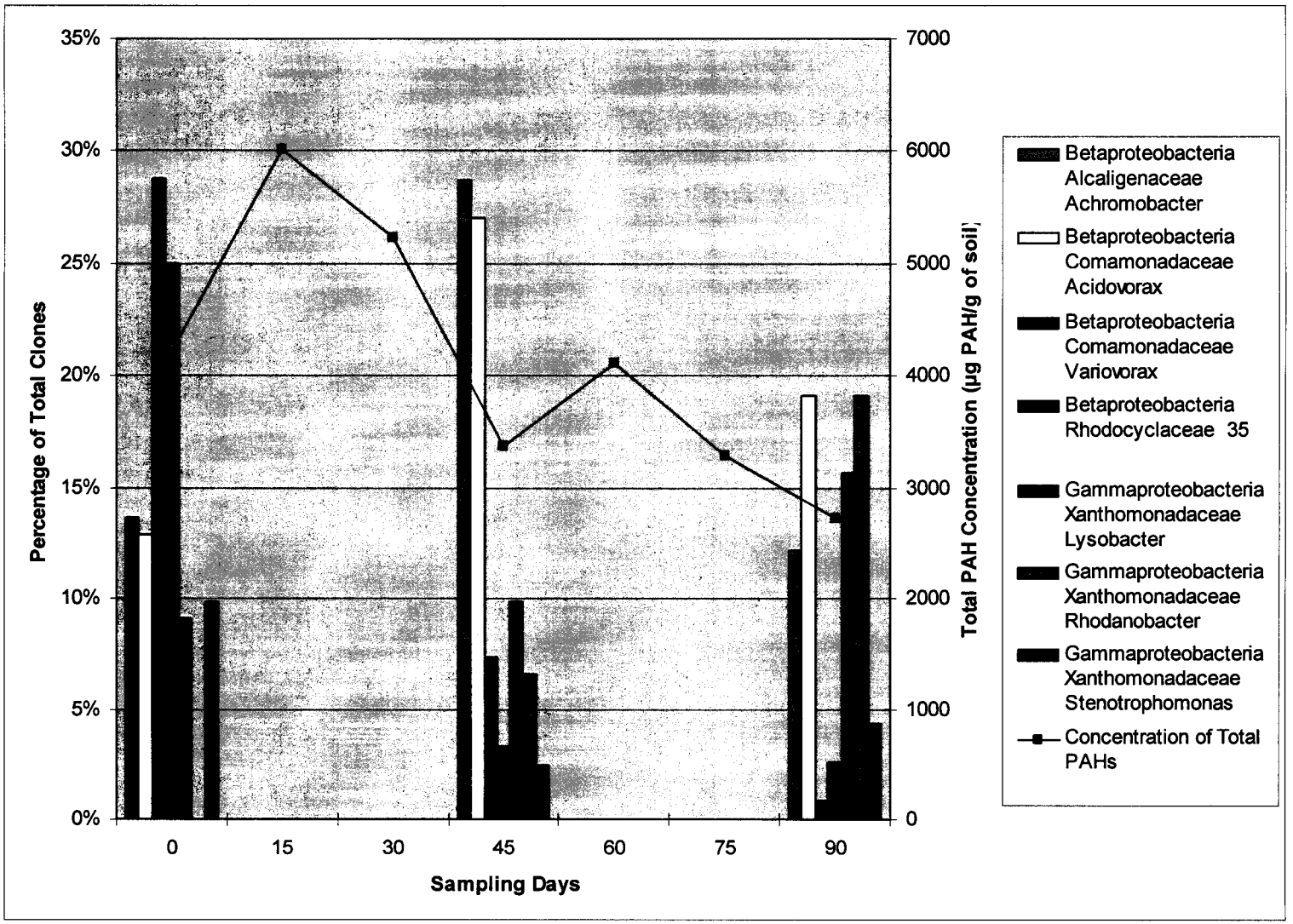


3.2.1.4 COMPARISON BETWEEN THE TEMPORAL CHANGES IN BACTERIAL COMMUNITY & TEMPORAL CHANGES IN PAH CONCENTRATION OBSERVED IN REACTOR 2

Seven prevalent genera were observed in reactor 2 of Treatment A, and they are denoted with shaded cells in Table 3.3. The prevalent genera are *Achromobacter, Acidovorax, Variovorax, Rhodocyclaceae* '35', *Lysobacter, Rhodanobacter,* and *Stenotrophomonas*. They are within the *Proteobacteria* phylum, specifically in the *Beta-* and *Gammaproteobacteria* classes. The temporal correspondence between changes in the microbial community and changes in PAH concentration are illustrated in Figure 3.4.

The *Achromobacter* genus was observed to increase between day 0 and 45 and then decrease towards the end of treatment. The *Acidovorax* genus was observed to increase between day 0 and 45 and then decrease towards the end of treatment. The *Variovorax* genus was found to decrease steadily over the course of a 90-day treatment. The *Rhodocyclaceae* '35' genus was found to decrease drastically over the course of a 90-day treatment, most notably between day 0 and 45. The *Lysobacter* genus was observed to increase gradually over the course of a 90-day treatment. The *Rhodanobacter* genus increased throughout Treatment A and there were no clones observed at the start of the treatment. The *Stenotrophomonas* genus decreased between day 0 and 45 and increased slightly towards the end of the treatment.

Figure 3.4 **Comparison of the temporal changes in the prevalent genera of soil organisms to the temporal changes in total PAH concentration for reactor 2 of Treatment A.**



3.2.1.5 COMPARISONS BETWEEN THE REACTORS OF TREATMENT A

Although the starting populations of both rectors are fairly similar, there are some differences in the percentage of total clones for several of the genera present. The percentage of total clones of the *Acidovorax* genus in reactor 1 (26.3%) is almost double that of reactor 2 (12.9%), whereas the *Variovorax* genus shows the opposite, i.e., they are half as abundant in reactor 1 (11.4%) compared to reactor 2 (28.8%). The *Stenotrophomonas* genus in reactor 2 showed a relatively high relative abundance (9.9%), whereas they were absent in reactor 1. This observation can most likely be attributed to the spatial heterogeneity of the starting material.

Over the course of the 90-day treatment the *Achromobacter* population in reactor 1 continuously showed a rise in the percentage of total clones, whereas in reactor 2 the population peaks at day 45, and then begins to decrease. The *Acidovorax* genus behaves inversely between the two reactors. In both reactors the *Variovorax* and *Rhodocyclaceae* '35' genera decrease over the course of the 90-day treatment. *Lysobacter* was observed to increase steadily in both reactors.

The phyla detected in each reactor over the course of the treatment are notably different. Reactor 1 results indicate the appearance of the phyla *Actinobacteria*, *Bacteriodetes*, *Chloroflexi*, and *Planctomycetes* after day 0. Reactor 2 results indicate the appearance of both the *Acidobacteria* and *Verrucomicrobia* phyla after day 0. Although none of these phyla account for a large portion of clones, the differences nonetheless highlight noteworthy differences in community composition of the two reactors.

3.2.2 TREATMENT B

3.2.2.1 MICROBIAL GENERA PRESENT IN THE TOTAL SOIL COMMUNITY OF REACTOR 1.

Treatment B, described in Section 2.2.2.1, involved the addition of macro and micro nutrients to the bench-top bioreactor. The results for reactor 1 of Treatment B revealed a total of 28 different microbial genera across the three time points sampled. The results are shown in Table 3.4 and the changes observed are visualized in Figure 3.5.

The Day 0 results revealed only eight genera within the *Beta-* and *Gammaproteobacteria* classes: *Achromobacter*, *Acidovorax*, *Variovorax*, *Rhodocyclaceae*'35', *Pseudomonas*, *Lysobacter*, *Rhodanobacter*, and *Stenotrophomonas*.

The day 45 results for Treatment B showed a significant increase in the observed species richness of the slurry. Results showed a total of 18 different genera representing six different classes. New classes seen at day 45 include *Acidobacteria*, *Spingobacteria*, *Bacilli*, and *Alphaproteobacteria*. The *Bradyrhizobium* genus was observed at 7.9% of total clones.

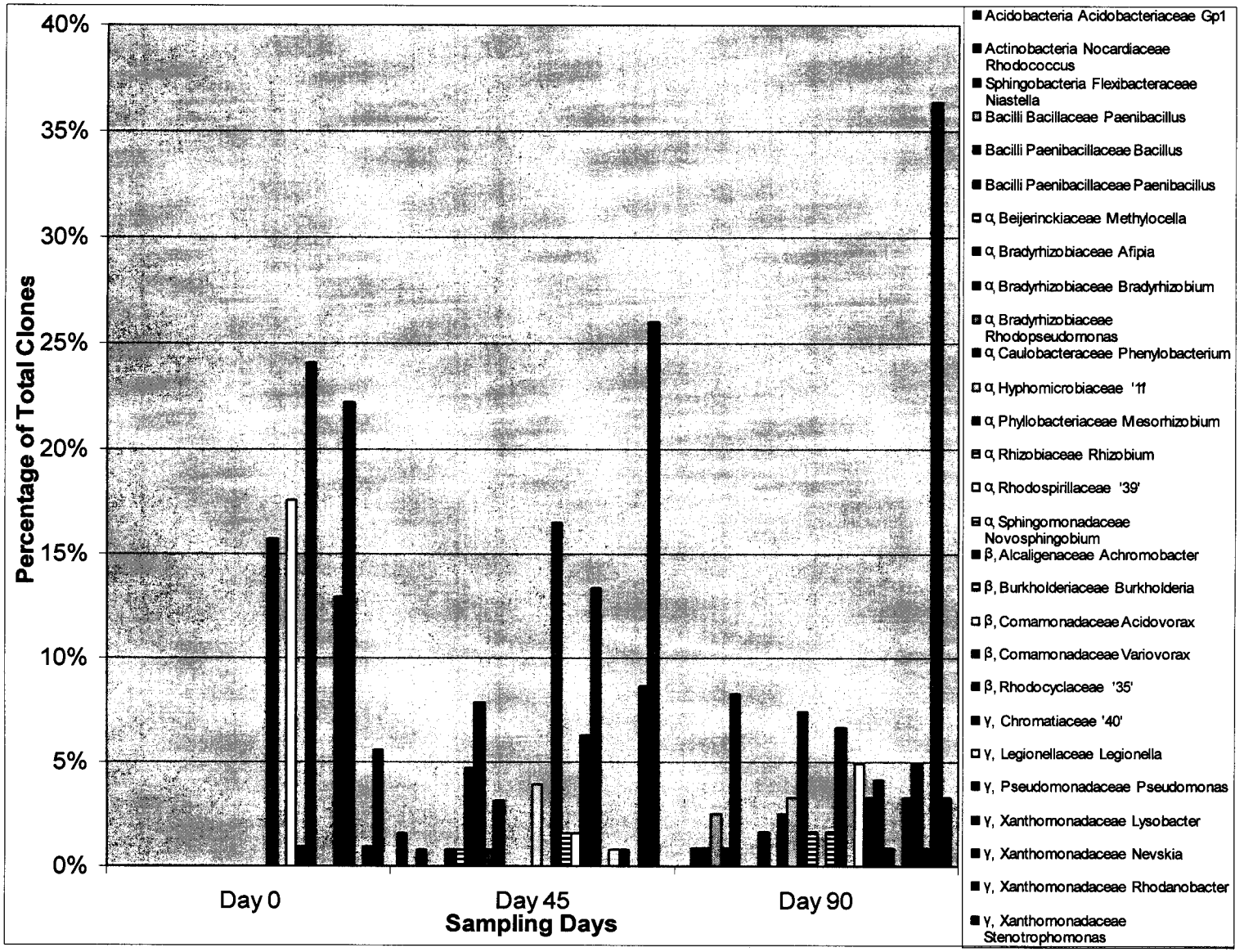
The Day 90 results showed a slight increase in the observed species richness of reactor 1, and a total of 21 genera. Two genera that showed significant increases in the percentage of total clones at day 90 are the *Paenibacillus* (of the family *Paenibacillaceae*) (8.3%) and the *Mesorhizobium* (7.4%).

Table 3.4 Temporal changes in the genera present in reactor 1 of Treatment B.

Phylum	Class	Family	Genus	Day 0 (%)[a]	Day 45 (%)	Day 90 (%)
Acidobacteria	*Acidobacteria*	*Acidobacteriaceae*	*Gp1*	0.0	1.6	0.0
Actinobacteria	*Actinobacteria*	*Nocardiaceae*	*Rhodococcus*	0.0	0.0	0.8
Bacteroidetes	*Sphingobacteria*	*Flexibacteraceae*	*Niastella*	0.0	0.78	0.8
Firmicutes	*Bacilli*	*Bacillaceae*	*Paenibacillus*[b]	0.0	0.0	2.5
		Paenibacillaceae	*Bacillus*	0.0	0.0	0.8
			Paenibacillus[c]	0.0	0.8	8.3
Proteobacteria	*Alphaproteobacteria*	*Beijerinckiaceae*	*Methylocella*	0.0	0.8	0.0
		Bradyrhizobiaceae	*Afipia*	0.0	4.7	0.0
			Bradyrhizobium	0.0	7.9	1.7
			Rhodopseudomonas	0.0	0.8	0.0
		Caulobacteraceae	*Phenylobacterium*	0.0	3.6	2.5
		Hyphomicrobiaceae	*Hyphomicrobium*	0.0	0.0	3.3
		Phyllobacteriaceae	*Mesorhizobium*	0.0	0.0	7.4
		Rhizobiaceae	*Rhizobium*	0.0	0.0	1.7
		Rhodospirillaceae	*'39'*	0.0	3.9	0.0
		Sphingomonadaceae	*Novosphingobium*	0.0	0.0	1.7
	Betaproteobacteria	*Alcaligenaceae*	***Achromobacter***	**15.7**	**16.5**	**6.6**
		Burkholderiaceae	*Burkholderia*	0.0	1.6	0.0
		Comamonadaceae	***Acidovorax***	**17.6**	**1.6**	**5.0**
			Variovorax	1.0	6.3	3.3
		Rhodocyclaceae	***'35'***	**24.1**	**13.4**	**4.1**
	Gammaproteobacteria	*Chromatiaceae*	*'40'*	0.0	0.0	0.8
		Legionellaceae	*Legionella*	0.0	0.8	0.0
		Pseudomonadaceae	***Pseudomonas***	**13.0**	**0.8**	**3.3**
		Xanthomonadaceae	***Lysobacter***	**22.2**	**0.0**	**5.0**
			Nevskia	0.0	8.7	0.8
			Rhodanobacter	**0.9**	**26.0**	**36.4**
			Stenotrophomonas	5.56	0.0	3.3
			Total Number of Clones Sampled	**108**	**127**	**121**

Highlighted cells denote percentage of total clones of approximately 10.0% for at least one of the three sampling days; [a]percentage of total clones; [b]*Bacillaceae, Paenibacillus*; [c] *Paenibacillaceae, Paenibacillus*.

Figure 3.5 **Temporal changes in the genera present in reactor 1 of Treatment B.**

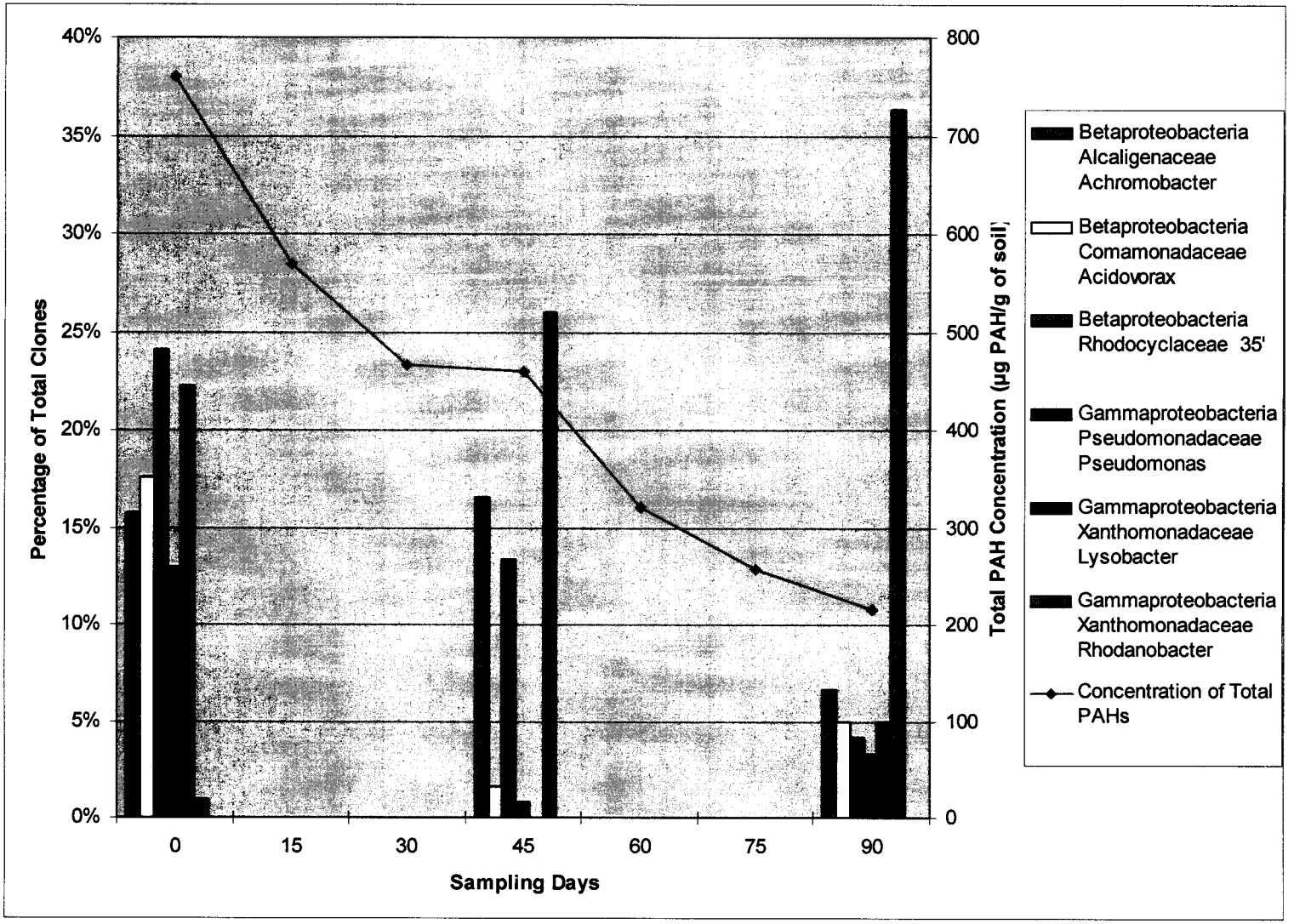


3.2.2.2 COMPARISON BETWEEN THE TEMPORAL CHANGES IN BACTERIAL COMMUNITY & TEMPORAL CHANGES IN PAH CONCENTRATION OBSERVED IN REACTOR 1.

Six prevalent genera were observed in reactor 1 of Treatment B and they are denoted with shaded cells in Table 3.4. These prevalent genera, *Achromobacter*, *Acidovorax*, *Rhodocyclaceae* '35', *Pseudomonas*, *Lysobacter*, and *Rhodanobacter*, are all within the *Beta* and *Gammaproteobacteria* classes. The temporal correspondence between the changes in PAH concentration and changes in the relative abundance of the prevalent clones are illustrated in Figure 3.6.

The *Achromobacter* were observed to increase slightly at the start of the treatment, but decreased significantly between day 45 and 90. The *Acidovorax* were observed to decrease drastically at the start of treatment, but then increased slightly towards day 90. The *Rhodocyclaceae* '35' decreased gradually over the course of the 90-day treatment. The *Lysobacter* disappeared by day 45, but then increased slightly towards the end of treatment. The *Rhodanobacter* is the only prevalent genus that increased throughout Treatment B. The *Pseudomonas* genus decreased significantly between day 0 and 45, but then increased slightly towards the end of treatment.

Figure 3.6 **Comparison of the temporal changes in the prevalent genera of soil organisms to the temporal changes in total PAH concentration for reactor 1 of Treatment B.**



3.2.2.3 MICROBIAL GENERA DETECTED IN REACTOR 2.

The results obtained indicate that reactor 2 of Treatment B contained a total of 27 different microbial genera throughout the three time points sampled. The results are shown in Table 3.5, and the changes observed are illustrated in Figure 3.7.

Day 0 showed the lowest amount of observed species richness with eleven genera present. The results showed four classes at the start of the run: *Alpha-*, *Beta-* and *Gammaproteobacteria*, and the *Actinobacteria*. Within the *Actinobacteria* a single clone from the *Georgina* genus was detected. There were two genera present within the *Alphaproteobacteria*: the *Caulobacter* and *Phenylobacterium*. Within the *Betaproteobacteria* class four genera were observed: *Achromobacter*, *Acidovorax*, *Variovorax*, and *Rhodocyclaceae* '35'. The results showed four genera from the *Gammaproteobacteria* class: *Pseudomonas*, *Lysobacter*, *Rhodanobacter*, and *Stenotrophomonas*.

Day 45 showed a slight increase in the observed species richness with a total of 16 different genera representing six different classes. Novel classes seen at day 45 include *Spingobacteria* and *Bacilli*. The results for the *Sphingobacteria* class showed a single genus in reactor 2 - *Niastella*. Likewise, the results for the Bacilli class showed a single genus - the *Paenibacillus* (of the *Paenibacillaceae* family).

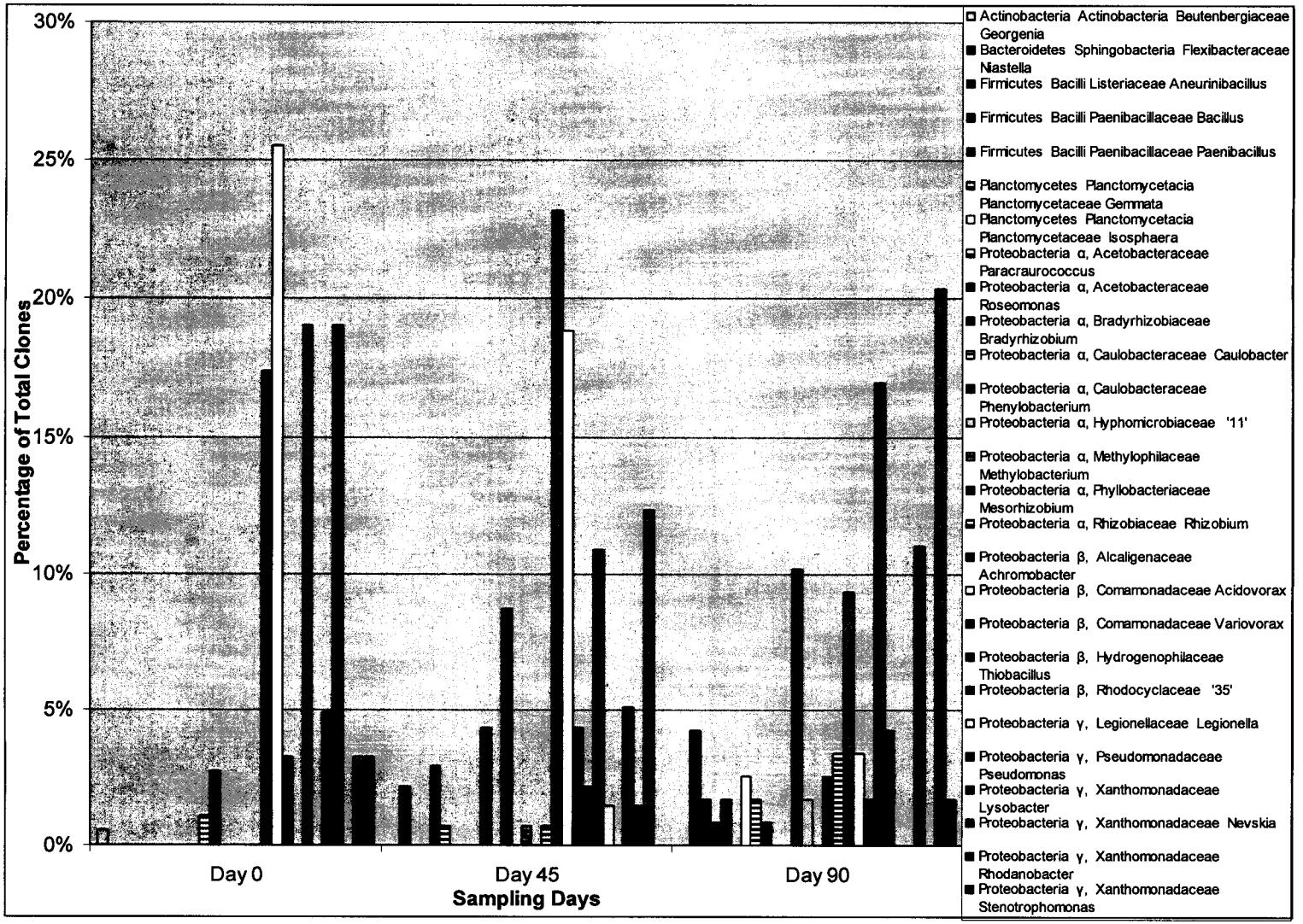
Day 90 showed a minute increase in the observed species richness, with a total of 19 genera detected. The *Thiobacillus*, a genus not previously seen in reactor 1 of Treatment B or Treatment A, represents a large amount (17.0%) of the total clones at day 90.

Table 3.5 Temporal changes in the genera present in reactor 2 of Treatment B.

Phylum	Class	Family	Genus	Day 0 (%)[a]	Day 45 (%)	Day 90 (%)
Actinobacteria	*Actinobacteria*	*Beutenbergiaceae*	*Georgenia*	0.5	0.0	0.0
Bacteroidetes	*Sphingobacteria*	*Flexibacteraceae*	*Niastella*	0.0	2.2	4.2
Firmicutes	*Bacilli*	*Listeriaceae*	*Aneurinibacillus*	0.0	0.0	1.7
		Paenibacillaceae	*Bacillus*	0.0	0.0	0.9
			Paenibacillus[b]	0.0	2.9	1.7
Planctomycetes	*Planctomycetacia*	*Planctomycetaceae*	*Gemmata*	0.0	0.7	0.0
			Isosphaera	0.0	0.0	2.5
Proteobacteria	*Alphaproteobacteria*	*Acetobacteraceae*	*Paracraurococcus*	0.0	0.0	1.7
			Roseomonas	0.0	0.0	0.9
		Bradyrhizobiaceae	*Bradyrhizobium*	0.0	4.4	0.0
		Caulobacteraceae	*Caulobacter*	1.1	0.0	0.0
			Phenylobacterium	**2.7**	**8.7**	**10.7**
		Hyphomicrobiaceae	*'21'*	0.0	0.0	1.7
		Methylobacteriaceae	*Methylobacterium*	0.0	0.7	0.0
		Phyllobacteriaceae	*Mesorhizobium*	0.0	0.0	2.5
		Rhizobiaceae	*Rhizobium*	0.0	0.7	3.4
	Betaproteobacteria	*Alcaligenaceae*	***Achromobacter***	**17.4**	**23.2**	**9.3**
		Comamonadaceae	***Acidovorax***	**25.5**	**18.8**	**3.4**
			Variovorax	3.3	4.4	1.7
		Hydrogenophilaceae	***Thiobacillus***	**0.0**	**2.2**	**17.0**
		Rhodocyclaceae	***'35'***	**19.0**	**10.9**	**4.2**
	Gammaproteobacteria	*Legionellaceae*	*Legionella*	0.0	1.5	0.0
		Pseudomonadaceae	*Pseudomonas*	4.9	0.0	0.0
		Xanthomonadaceae	***Lysobacter***	**19.0**	**5.1**	**11.0**
			Nevskia	0.0	1.5	0.0
			Rhodanobacter	**3.3**	**12.3**	**20.3**
			Stenotrophomonas	3.3	0.0	1.7
			Total Number of Clones Sampled	**184**	**138**	**118**

Highlighted cells denote percentage of total clones of approximately 10.0% for at least one of the three sampling days; [a]percentage of total clones; [b]*Paenibacillaceae, Paenibacillus*.

Figure 3.7 **Temporal changes in the genera present in reactor 2 of Treatment B.**

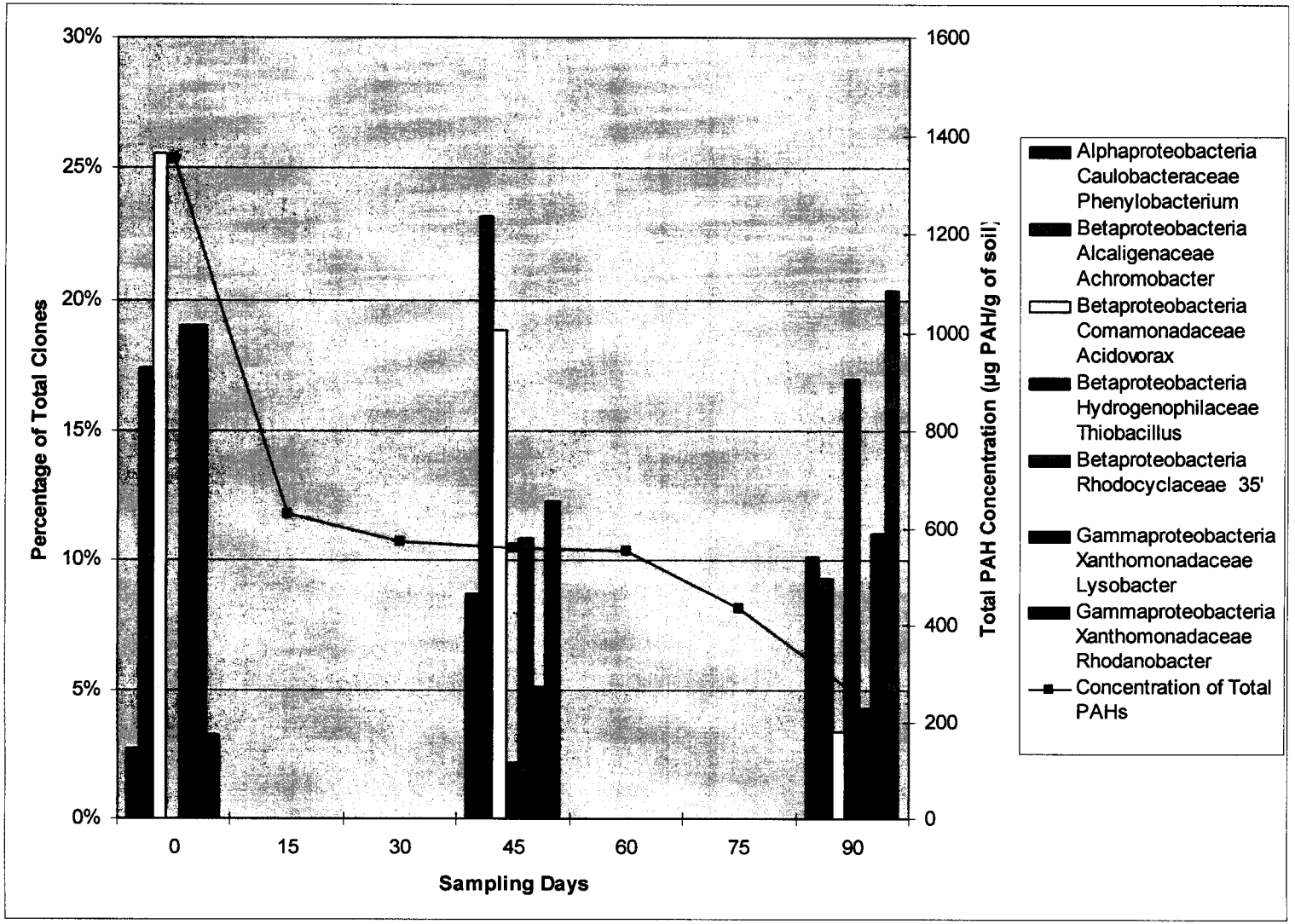


3.2.2.4 COMPARISON BETWEEN THE TEMPORAL CHANGES IN BACTERIAL COMMUNITY & TEMPORAL CHANGES IN PAH CONCENTRATION OBSERVED IN REACTOR 2.

There were seven prevalent genera denoted with shaded cells in Table 3.5: the *Phenylobacterium*, *Achromobacter*, *Acidovorax*, *Thiobacillus*, *Rhodocyclaceae* '35', *Lysobacter*, and *Rhodanobacter*. General trends of the prevalent genera will be discussed below. These results are illustrated in Figure 3.8 along with the temporal changes in PAH concentration.

The *Phenylobacterium* were observed to increase gradually over the course of the 90-day treatment. The *Achromobacter* increases towards day 45, and then decrease significantly towards the end of the treatment. The *Acidovorax* and *Rhodocyclaceae* '35' genera decrease gradually throughout the course of the 90-day treatment. The *Lysobacter* genus decreases at the beginning of treatment, but recovers slightly towards the end of treatment. The *Rhodanobacter* genus increases gradually throughout the course of the treatment. The *Thiobacillus* genus represented 17.0% of the total clones at day 90.

Figure 3.8 **Comparison of the temporal changes in the prevalent genera of soil organisms to the temporal changes in total PAH concentration for reactor 2 of Treatment B.**



3.2.2.5 COMPARISON BETWEEN THE REACTORS OF TREATMENT B

There are several noteworthy differences observed between the two reactors of Treatment B. Although the starting populations are similar, there were differences in the genera present, as well as in the percentage of total clones for those genera. In reactor 2 two additional classes were observed in comparison with reactor 1. The *Actinobacteria* and the *Alphaproteobacteria* each had a genus present at day 0. The *Pseudomonas* genus was not consistent in percentage of total clones at day 0 for either reactor. In reactor 1 of Treatment B the *Pseudomonas* represented 13.0% of total clones, whereas the relative abundance in reactor 2 was substantially smaller at 4.9%. The *Phenylobacterium* genus was not well represented in reactor 1, although in reactor 2 it gradually increased throughout the treatment. The *Thiobacillus* genus was observed in reactor 2, and it was not previously observed in Treatment A. At the termination of Treatment B the *Thiobacillus* represented over 15% of the total clones. However, they were not detected in reactor 1.

There are several noteworthy similarities between the two reactors of Treatment B. Over the course of the 90-day treatment it observations for both reactors showed that the *Achromobacter* genus increased in the percentage of total clones between days 0 and 45, and then decreased between days 45 and 90. In both reactors the *Lysobacter* saw the opposite shift. The *Rhodocyclaceae* population of both reactors decreased gradually over the course of a 90-day treatment. The relative abundance of *Rhodanobacter* genus increased gradually, in both reactors, over the course of the treatment.

3.2.3 TREATMENT C

3.2.3.1 MICROBIAL GENERA DETECTED IN REACTOR 1.

A total of 24 genera were observed in reactor 1 of Treatment C across the three time points sampled. The results are summarized in Table 3.6, and the changes observed are illustrated in Figure 3.9.

The results obtained for Day 0 of Treatment C showed the least amount of observed species richness compared with the other time points, as well as to the starting points of the other treatments. Three classes were detected at the start of Treatment C: the *Alpha-*, *Beta-*, and *Gammaproteobacteria*. The *Pseudmonas* genus represented 98.1% of the total clones of reactor 1 at the commencement of treatment.

Several novel classes were observed at day 45 - the *Actinobacteria*, *Bacilli* and *Plantomycetes*. There was a large increase in the observed species richness, with 18 genera observed, as well as a large shift in the percentage of total clones (e.g., *Pseudomonas* genus decreased to 2.4%). Several genera not previously observed at day 0 were detected at day 45, and showed high percentages of total clones. These include *Achromobacter* (14.3%), *Rhodanobacter* (11.1%), and *Stenotrophomonas* (15.9%).

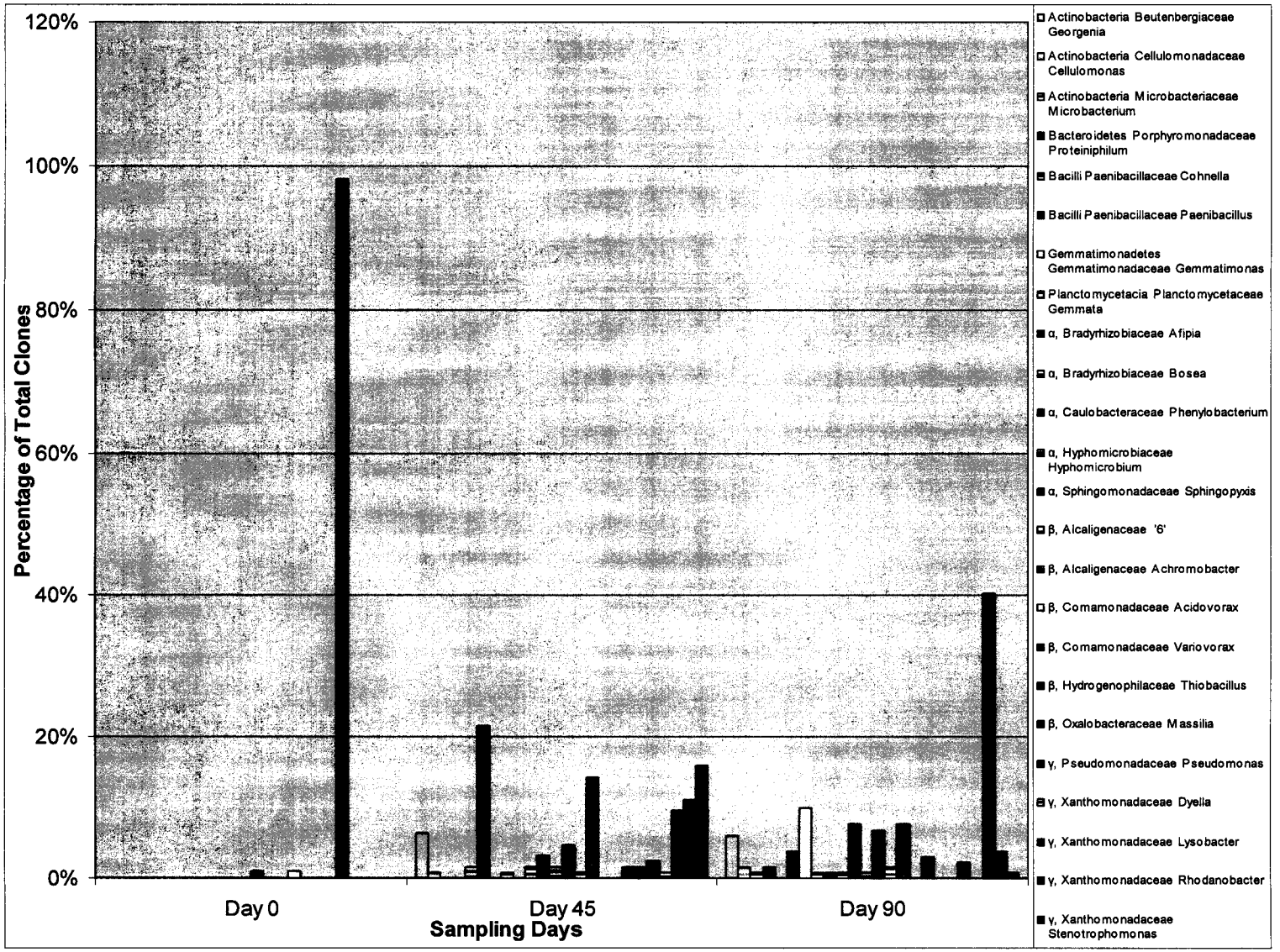
At the termination of Treatment C there was only a slight rise in the observed species richness of reactor 1, with 19 distinct genera detected. There was another large shift in the percentages of total clones and two genera appeared at this time point: *Gemmatimonas* (9.9%) and *Lysobacter* (37.9%).

Table 3.6 Temporal changes in the genera present in reactor 1 of Treatment C.

Phylum	Class	Family	Genus	Day 0 (%)[a]	Day 45 (%)	Day 90 (%)
Actinobacteria	*Actinobacteria*	*Beutenbergiaceae*	*Georgenia*	0.0	6.4	6.1
		Cellulomonadaceae	*Cellulomonas*	0.0	0.8	1.5
		Microbacteriaceae	*Microbacterium*	0.0	0.0	0.8
Bacteroidetes	*Bacteroidetes*	*Porphyromonadaceae*	*Proteiniphilum*	0.0	0.0	1.5
Firmicutes	*Bacilli*	*Paenibacillaceae*	*Cohnella*	0.0	1.6	0.0
			Paenibacillus[bc]	0.0	21.4[c]	3.8
Gemmatimonadetes	*Gemmatimonadetes*	*Gemmatimonadaceae*	***Gemmatimonas***	**0.0**	**0.0**	**9.9**
Planctomycetes	*Planctomycetacia*	*Planctomycetaceae*	*Gemmata*	0.0	0.8	0.8
Proteobacteria	*Alphaproteobacteria*	*Bradyrhizobiaceae*	*Afipia*	0.0	0.0	0.8
			Bosea	0.0	1.6	0.8
		Caulobacteraceae	*Phenylobacterium*	0.0	3.2	7.9
		Hyphomicrobiaceae	*Hyphomicrobium*	0.0	1.6	0.8
		Sphingomonadaceae	*Sphingopyxis*	1.0	4.8	6.8
	Betaproteobacteria	*Alcaligenaceae*	*'6'*	0.0	0.8	1.5
			Achromobacter	**0.0**	**14.3**	**7.6**
		Comamonadaceae	*Acidovorax*	0.0	0.0	3.0
			Variovorax	1.0	0.0	0.0
		Hydrogenophilaceae	*Thiobacillus*	0.0	1.6	0.0
		Oxalobacteraceae	*Massilia*	0.0	1.6	0.0
	Gammaproteobacteria	*Pseudomonadaceae*	***Pseudomonas***	**98.1**	**2.4**	**2.3**
		Xanthomonadaceae	*Dyella*	0.0	0.8	0.0
			Lysobacter	**0.0**	**9.5**	**40.2**
			Rhodanobacter	**0.0**	**11.1**	**3.8**
			Stenotrophomonas	**0.0**	**15.9**	**0.8**
			Total Number of Clones Sampled	**104**	**126**	**132**

Highlighted cells denote percentage of total clones of approximately 10.0% for at least one of the three sampling days; [a]percentage of total clones; [b]*Paenibacillaceae*, *Paenibacillus*; [c]according to alignment results samples identified as *Paenibacillus* by RDP showed a 90% similarity indicating that they are not within the same genus.

Figure 3.9 **Temporal changes in the genera present in reactor 1 of Treatment C.**



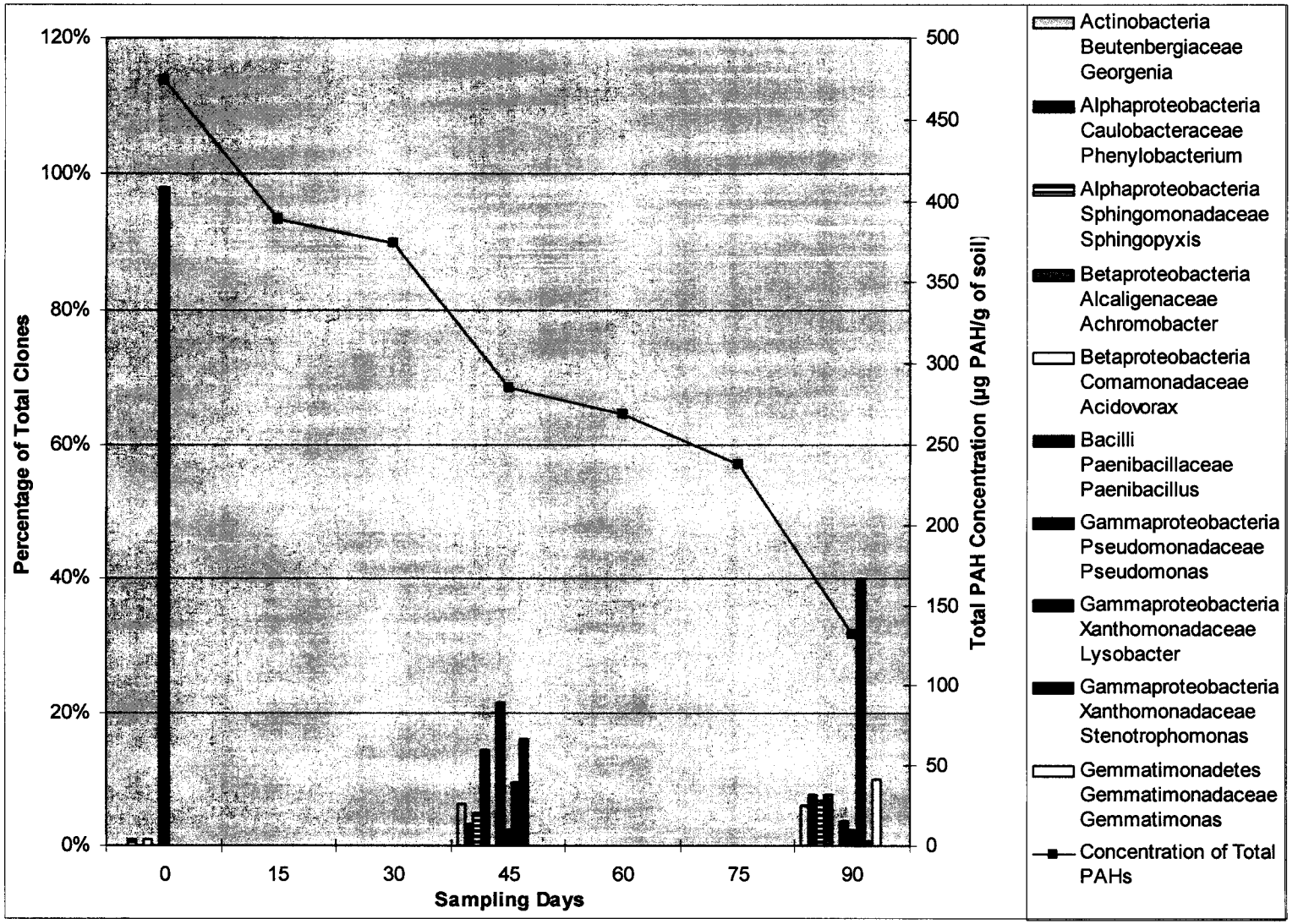
3.2.3.2 COMPARISON BETWEEN THE TEMPORAL CHANGES IN BACTERIAL COMMUNITY & TEMPORAL CHANGES OF PAH CONCENTRATION OBSERVED IN REACTOR 1.

There were six prevalent genera for reactor 1 of Treatment C, and they are denoted with shaded cells in Table 3.6. The prevalent genera are *Gemmatimonas, Achromobacter, Pseudomonas, Lysobacter, Rhodanobacter,* and *Stenotrophomonas.* These genera come from a wider range of classes compared to the other two treatments. However, due the dominance seen in some genera throughout this treatment the top 5 genera for each time point will be shown in addition to the prevalent genera. The results are illustrated in Figure 3.10 along with the temporal changes in PAH concentration.

The *Pseudomonas* genus had the highest percentage of total clones compared to any other genera, however, they disappeared almost completely as treatment progressed. The *Achomobacter, Lysobacter, Rhodanobacter,* and *Stenotrophomonas* genera are not observed until day 45 were they increase significantly. The *Gemmatimonas* genus was not observed until day 90 of Treatment C.

It is important to note than when sequence alignments were performed to confirm O.T.U. identities, different O.T.U. patterns that were identified to be the *Paenibacillus* genus by the Ribosomal Database showed only 90% similarity according to BLAST2. When alignments were performed for other taxa, similarity within a genus was found to be consistently above 96%. A low similarity score, such as that observed for Paenibacillus, is generally associated with sequences that were within the same class but different families.

Figure 3.10 **Comparison of the temporal changes in the prevalent genera of soil organisms as well as the top 5 genera (in terms of total clones) at each time point to the temporal changes in total PAH concentration for reactor 1 of Treatment C.**



3.2.3.3 MICROBIAL GENERA DETECTED IN REACTOR 2.

The results obtained for reactor 2 of Treatment C revealed a total of 29 different microbial genera throughout the three time points sampled. The results are summarized in Table 3.7, and the changes observed are illustrated in Figure 3.11. The results obtained showed that Day 0 of Treatment C had the least amount of observed species richness compared with the two other time points. There were four phyla observed in the soil at day 0: *Actinobacteria, Chloroflexi, Firmicutes,* and *Proteobacteria*. Though the percentage of total clones for most of the genera observed at this time points is quite low, it should be noted that the starting microbial community is markedly different than for Treatments A and B. Similar to the percentage of total clones that was observed for reactor 1 of Treatment C the *Pseudomonas* was the prevalent organism (87.0%).

At day 45 of Treatment C there was an increase observed in the observed species richness of the soil microbial community and 15 genera were detected. The largest notable shift was that the dominating *Pseudomonas* completely disappeared and the *Rhodanobacter* increased to 54.2 % of total clones.

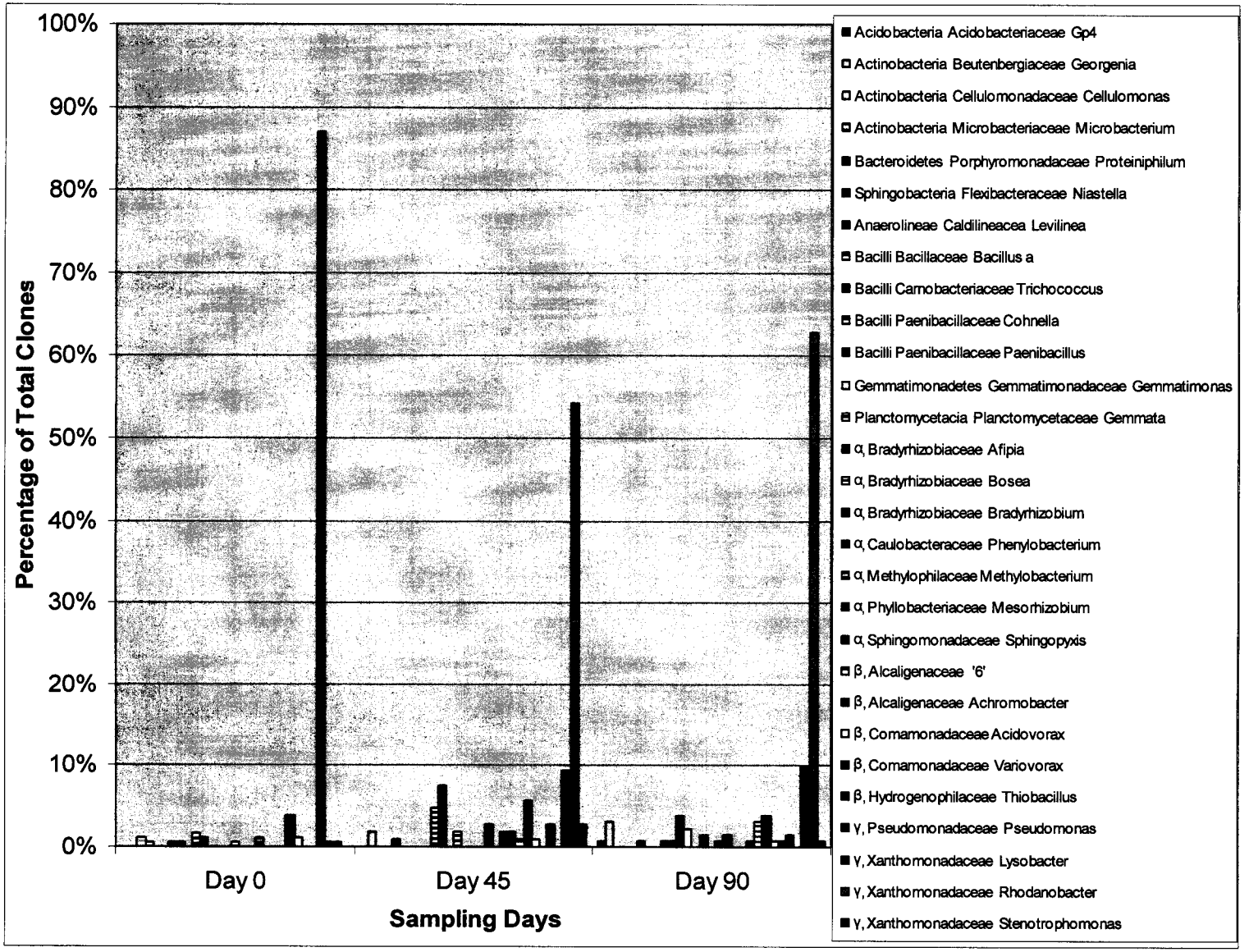
By the termination of the treatment at day 90 the observed species richness of the community increased to 19 distinct genera. The *Rhodanobacter* were observed to increase slightly, and the *Lysobacter* increased to 9.9%. The *Pseudomonas* remained undetectable at the termination of treatment.

Table 3.7 Temporal changes in the genera present in reactor 2 of Treatment C.

Phylum	Class	Family	Genus	Day 0 (%)[a]	Day 45 (%)	Day 90 (%)
Acidobacteria	*Acidobacteria*	*Acidobacteriaceae*	*Gp4*	0.0	0.0	0.8
Actinobacteria	*Actinobacteria*	*Beutenbergiaceae*	*Georgenia*	0.0	1.9	3.0
		Cellulomonadaceae	*Cellulomonas*	1.1	0.0	0.0
		Microbacteriaceae	*Microbacterium*	0.5	0.0	0.0
Bacteroidetes	*Bacteroidetes*	*Porphyromonadaceae*	*Proteiniphilum*	0.0	0.9	0.0
	Sphingobacteria	*Flexibacteraceae*	*Niastella*	0.0	0.0	0.8
Chloroflexi	*Anaerolineae*	*Caldilineacea*	*Levilinea*	0.5	0.0	0.0
Firmicutes	*Bacilli*	*Bacillaceae*	*Bacillus a*	0.5	0.0	0.0
		Carnobacteriaceae	*Trichococcus*	0.0	0.0	0.8
		Paenibacillaceae	*Cohnella*	1.6	4.7	0.8
			Paenibacillus[1]	1.1	7.5	3.7
Gemmatimonadetes	*Gemmatimonadetes*	*Gemmatimonadaceae*	*Gemmatimonas*	0.0	0.0	2.3
Planctomycetes	*Planctomycetacia*	*Planctomycetaceae*	*Gemmata*	0.0	1.9	0.0
Proteobacteria	*Alphaproteobacteria*	*Bradyrhizobiaceae*	*Afipia*	0.0	0.0	1.5
			Bosea	0.5	0.0	0.0
			Bradyrhizobium	0.0	0.0	0.8
		Caulobacteraceae	*Phenylobacterium*	0.0	2.8	1.5
		Methylobacteriaceae	*Methylobacterium*	1.1	0.0	0.0
		Phyllobacteriaceae	*Mesorhizobium*	0.0	1.9	0.0
		Sphingomonadaceae	*Sphingopyxis*	0.0	1.8	0.8
	Betaproteobacteria	*Alcaligenaceae*	*'6'*	0.0	0.9	3.0
			Achromobacter	3.8	5.6	3.8
		Comamonadaceae	*Acidovorax*	1.1	0.9	0.8
			Variovorax	0.0	0.0	0.8
		Hydrogenophilaceae	*Thiobacillus*	0.0	2.8	1.5
	Gammaproteobacteria	*Pseudomonadaceae*	***Pseudomonas***	**87.0**	**0.0**	**0.0**
		Xanthomonadaceae	***Lysobacter***	**0.5**	**9.3**	**9.9**
			Rhodanobacter	**0.5**	**54.2**	**62.9**
			Stenotrophomonas	0.0	2.8	0.8
			Total Number of Clones Sampled	**185**	**107**	**132**

Highlighted cells denote percentage of total clones >9.85% for at least one of the three sampling days; [a] percentage of total clones; [b] *Paenibacillaceae, Paenibacillus*.

Figure 3.11 **Temporal changes in the genera present in reactor 2 of Treatment C.**

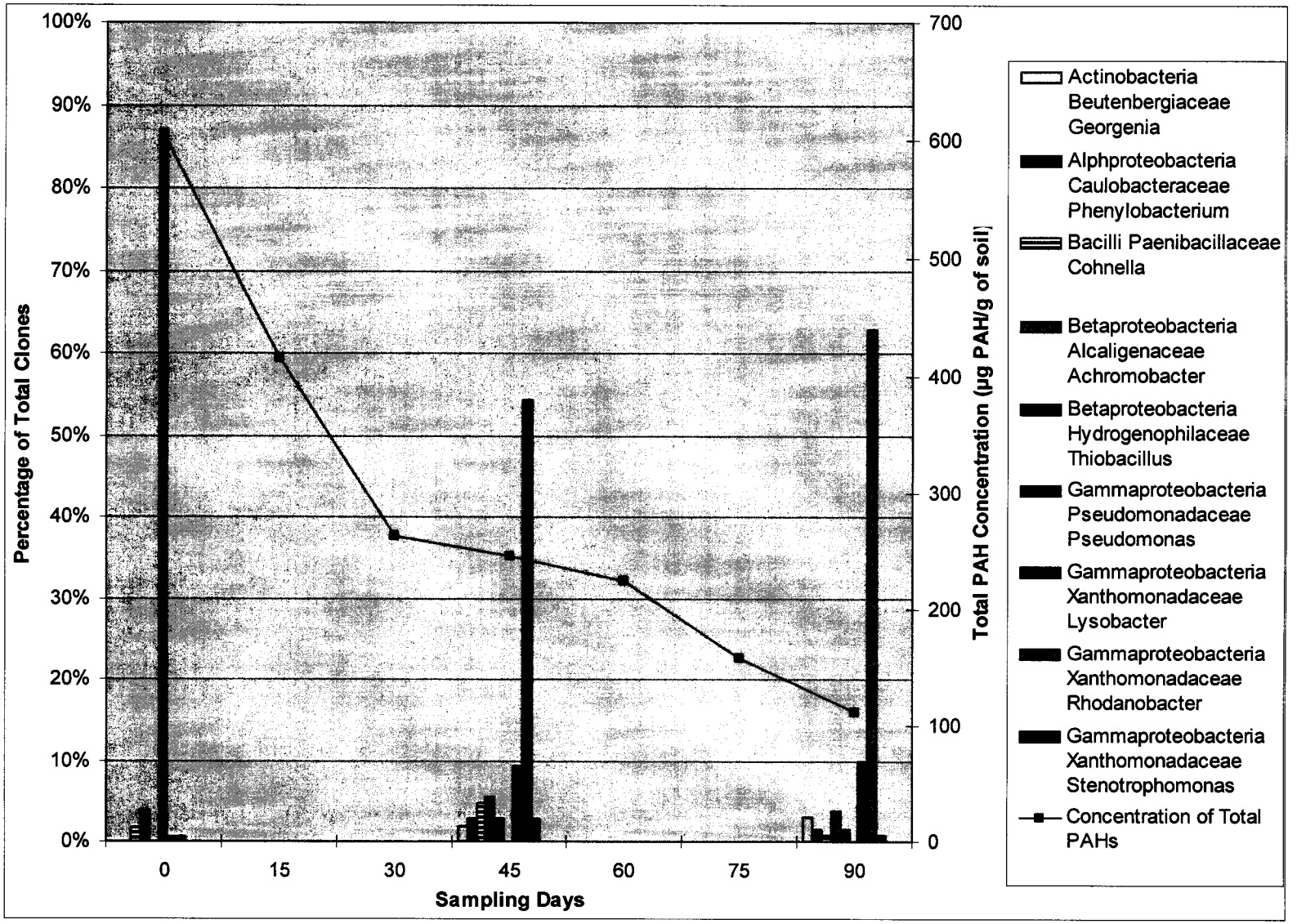


3.2.3.4 COMPARISON BETWEEN THE TEMPORAL CHANGES IN BACTERIAL COMMUNITY & TEMPORAL CHANGES OF PAH CONCENTRATION OBSERVED IN REACTOR 2.

There were three prevalent genera observed, they are denoted with shaded cells in Table 3.7. The prevalent genera were *Pseudomonas*, *Lysobacter,* and *Rhodanobacter*. They are all within the *Gammaproteobacteria* class. However, due the dominance seen in some genera throughout this treatment the top 5 genera for each time point will be shown in addition to the prevalent genera. These results are illustrated in Figure 3.12 along with the temporal changes in PAH concentration.

Similar to reactor 1 of Treatment C, the *Pseudomonas* genus showed a much higher percentage of total clones compared to the other genera present, and completely disappeared as the treatment progressed. The *Lysobacter* only represented a minute fraction of the microbial community at the initiation of treatment; however, they increased toward the end of treatment. Similar to the *Lysobacter*, the *Rhodanobacter* represented a small fraction of the total soil community at the start of treatment. Throughout the course of treatment however, the *Rhodanobacter* increased significantly to represent a large majority of the total microbial community.

Figure 3.12 **Comparison of the temporal changes in the prevalent genera of soil organisms as well as the top 5 genera (in terms of total clones) at each time point to the temporal changes in total PAH concentration for reactor 2 of Treatment C.**



3.2.3.5 COMPARISON BETWEEN THE REACTORS OF TREATMENT C

The most notable difference between the reactors of Treatment C is that reactor 1 has six dominating genera, whereas reactor 2 had only three. Reactor 1 had a genus from *Gemmatimonadetes*, in addition to the *Beta-* and *Gammaproteobacteria* classes. Reactor 2 had only genera from *Gammaproteobacteria*. Another significant difference between the two reactors is the behaviour of the *Lysobacter* and *Rhodanobacter* genera. In both reactors the *Lysobacter* genus increased throughout the 90-day treatment and both showed similar initial percentages of total clones. However, the *Lysobacter* population of reactor 1 was significantly higher in percentage of total clones (37.9%) compared to reactor 2 (9.9%). The changes in total clones for the *Rhodanobacter* genus also differ significantly between the two reactors. In reactor 1, the percentage of total clones peaked at day 45 with 11.1%, before decreasing to 3.8% at day 90. In reactor 2, the percentage of total clones for *Rhodanobacter* increased throughout the course of treatment finishing at day 90 with 62.9%.

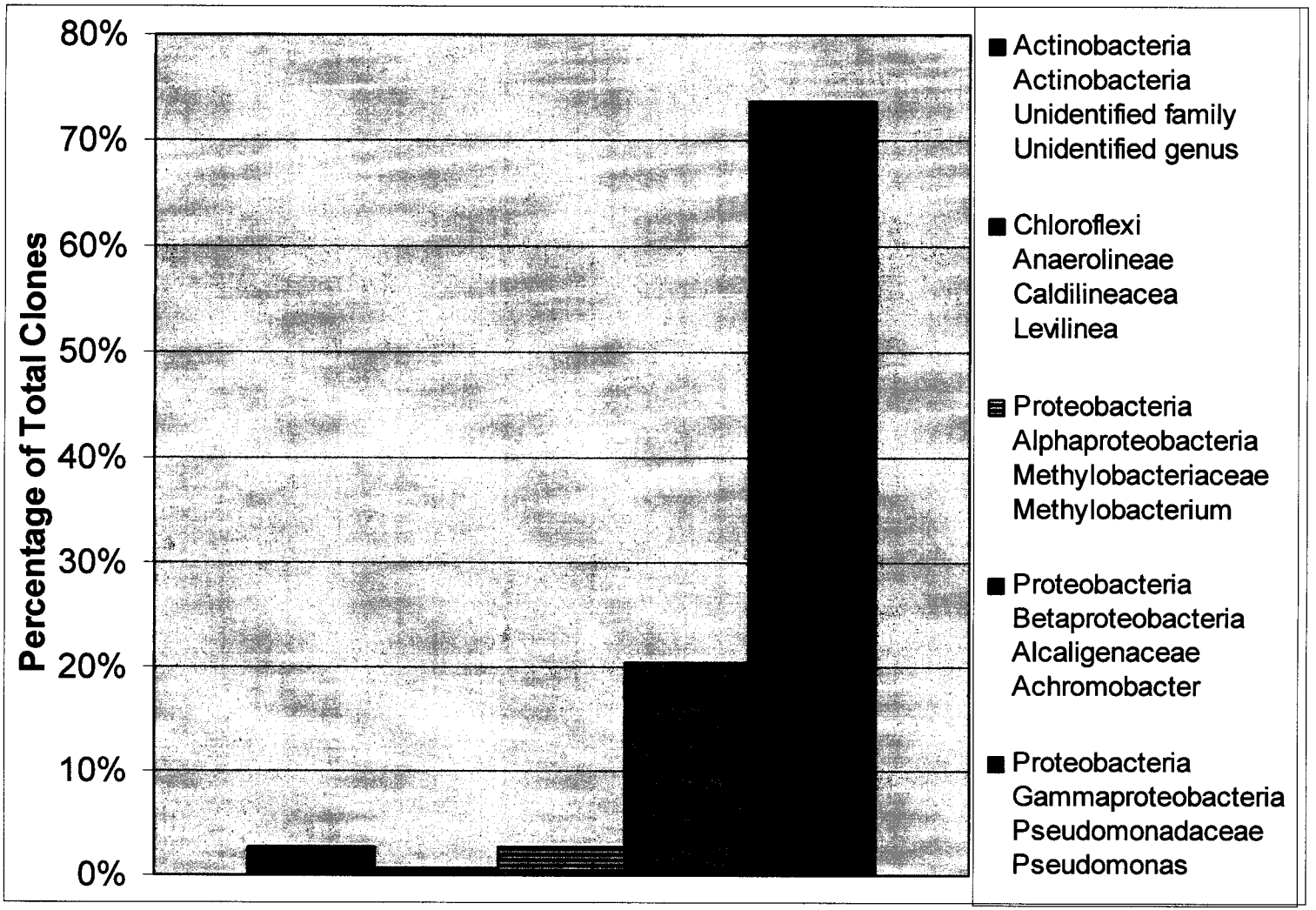
The starting microbial population of both reactors is fairly similar with *Pseudomonas* being the main genus present. It was observed that the *Pseudomonas* population decreased significantly to only a few clones in both reactors at days 45 and 90. Both reactors had a genus from the *Xanthomonadaceae* family as the dominating population when Treatment C was terminated at day 90.

3.2.4 EXOGENOUS MICROBIAL CONSORTIA

The dehydrated microbial seed (Medina) was rehydrated in nutrient broth (see Section 2.10) to determine its composition. The rehydrated seed was subjected to the same analyses as the Rock Bay samples discussed in Section 2.0. The results show that it contains high percentages of the genera *Pseudomonas* (73.7%) and *Achromobacter* (20.4%). The percentages of total clones of the microbial seed are illustrated below in Figure 3.13.

Selected samples from the exogenous microbial consortia (EMC) were analysed using RDP and determined to be primarily in the *Pseudomonas* or *Achromobacter*. These samples were compared using sequences alignments to samples given the same identity in Treatment C to investigate whether they were part of the microbial community involved in PAH degradation. Samples given the genus identity *Pseudomonas* or *Achromobacter* from the EMC were compared using BLAST2 [105] to several samples from days 45 and 90 of Treatment C that were also identified to be within the *Pseudomonas* or *Achromobacter* genera. These results show a high degree of similarity (>97%) between samples obtained from the EMC to those taken from Treatment C. These results show a high percentage of similarity between samples identified as *Pseudomonas* or *Achromobacter* in Treatment C to samples given the same identity in Treatment A and B.

Figure 3.13 **Percentage of total clones of the exogenous microbial culture (Medina) added to Treatment C.**



4.0 DISCUSSION

Three objectives of this thesis were outlined in Section 1.6.3.1. The first objective was to isolate the total metagenomic DNA from soil, and to amplify, clone and sequence the 16S rDNA. The second objective was to identify members of the soil microbial community by analysing rDNA sequences using the Ribosomal Database and Operational Taxonomic Unit (O.T.U.). The third and final objective was to examine temporal changes in bacterial community structure and PAH content for a PAH-contaminated soil during a 90-day bench-top bioslurry remediation exercise. It was hypothesized that the observed interplay between temporal changes in the soil microbial community and temporal changes in PAH concentration would yield insight into the elements of the microbial community that are determinants of PAH mineralization.

4.1 MICROBIAL COMMUNITY ANALYSIS

4.1.1 METAGENOMIC DNA RECOVERY: LIMITATIONS AND BIASES

Difficulties encountered during metagenomic DNA recovery could have been attributed to several methodological issues. The first difficulty encountered was primer bias. It has been observed that PCR amplification of the 16S gene region could be highly biased such that one out of four species can be preferentially amplified [96]. To control for this bias, multiple aliquots from each soil sub-sample were tested and observed to yield comparable community composition results. A second difficulty was related to the additional humic content in the slurry samples from treatments B and C. Consequently, it was necessary to optimize DNA

extraction and clean-up procedures. The optimized method involved clean-up of isolated DNA and PCR products to ensure effective ligation and cloning. In some instances, PCR products were re-tailed with *Taq* polymerase to ensure they would have a dA over-hang that would efficiently ligate with the dT over-hang of the TOPO vector. A third difficulty was related to the limitations caused by the identification of DNA sequences using the Ribosomal Database Project. Not all samples could be identified to the species or strain level with 100% certainty due to limitations in the database content. As of January 25th, 2010 the database contained 1,358,426 aligned and annotated 16S rRNA sequences [103].

4.1.2 INITIAL ANALYSES OF COMMUNITY STRUCTURE

The main phylum recovered from the soil for all treatments was the *Proteobacteria*. This was expected as both the *Beta-* and *Gammaproteobacteria* classes have been extensively reported to be ubiquitous in soil and aquatic environments [107]. Although the *Beta-* and *Gammaproteobacteria* are closely related through 16S rRNA analyses, their physiologies are so diverse that there is no obvious metabolic trend at this taxonomic level [107]. Within the *Beta-* and *Gammaproteobacteria* classes there was a great deal of diversity recovered from the Rock Bay soil.

Genera identified within the *Betaproteobacteria* class include *Alcaligenaceae '6'* (based on O.T.U. results), *Achromobacter, Burkholderia , Acidovorax, Variovorax, Methylibium, Methylophilus , Massilia, Rhodocyclaceae '35'*, and *Thiobacillus*. Table 4.1 summarizes the *Betaproteobacteria* genera present in the various reactors and

treatments examined, and provides a summary of those that represented at least 10.0% of the total clones for at least one of the sampling days. For detailed information about the percentage of total clones, the reader is referred to Tables 3.2 to 3.7.

Genera identified within the *Gammaproteobacteria* class include *Chromatiaceae '40', Legionella, Pseudomonas, Dyella, Lysobacter, Nevskia, Rhodanobacter,* and *Stenotrophomonas*. Table 4.2 summarizes the *Gammaproteobacteria* genera present in the various reactors and treatments, and provides a summary of those that were represented at least 10.0% of the total clones for at least one of the sampling days. Again, for detailed information about the percentage of total clones, the reader is referred to Tables 3.2 to 3.7.

Much of the metagenomic soil DNA results were not surprising. Organisms identified include either known common soil microbes and/or known PAH-degraders [4, 15, 37, 50, 56-63, 107]. The implications of the occurrence and temporal changes in the abundance of these organisms during the treatments are discussed below in Section 4.2.3.

Table 4.1 ***Betaproteobacteria*** **genera found in reactors 1 and 2 for all treatments.**

Genera	Treatment A		Treatment B		Treatment C	
	R1	R2	R1	R2	R1	R2
Alcaligenaceae '6'					✓	✓
Achromobacter	✓	✓	✓	✓	✓	✓
Burkholderia			✓			
Acidovorax	✓	✓	✓	✓	✓	✓
Variovorax	✓	✓	✓	✓	✓	✓
Methylibium	✓	✓				
Methylophilus		✓				
Massilia	✓				✓	
Rhodocyclaceae '35'	✓	✓	✓	✓		
Thiobacillus				✓	✓	✓

✓ denotes presence in reactor for any sampling day of a treatment.
Highlighted cells denote genera that represented at least 10.0% of the total clones for at least one of the sampling days.

Table 4.2 ***Gammaproteobacteria*** **genera found in reactors 1 and 2 for all treatments.**

Genera	Treatment A		Treatment B		Treatment C	
	R1	R2	R1	R2	R1	R2
Chromatiaceae '40'	✓	✓	✓			
Legionella			✓	✓		
Pseudomonas	✓	✓	✓	✓	✓	✓
Dyella					✓	
Lysobacter	✓	✓	✓	✓	✓	✓
Nevskia			✓	✓		
Rhodanobacter	✓	✓	✓	✓	✓	✓
Stenotrophomonas		✓	✓	✓	✓	✓

✓ denotes presence in reactor for any sampling day of a treatment.
Highlighted cells denote genera that represented at least 10.0% of the total clones for at least one of the sampling days.

4.2 BIOREMEDIATION OF PAH CONTAMINATED SOIL

As noted in Section 2.3, Whynot (2009) analyzed the temporal changes in the chemical composition of the Rock Bay soil slurry samples using HPLC [24]. The slurry was sampled at 15 day intervals for each reactor during each of the three treatments. The concentrations of 15 priority PAHs were determined in each sample for both the water and solid phases. The concentration of PAHs in the water phase was generally about three order of magnitude lower than in the corresponding soil phase [24].

HMW PAHs account for the majority of the total detected PAHs. LMW PAH concentrations were significantly lower, especially at later time points. In all treatments the level of priority PAHs was reduced over the course of the 90 day treatment. Reactor 1 of Treatment A was observed to have the most complete PAH removal (i.e., 90.4%) whereas reactor 2 of Treatment B was observed to be the least effective in terms of PAH removal (i.e., 65.6% removal of total PAHs). PAH levels were reduced at different rates during the different treatment scenarios. The rate of reduction also varied within each treatment.

4.2.1 TEMPORAL CHANGES IN PAH CONCENTRATION

Whynot (2009) reported that all treatments showed an initial rapid decrease in the total PAH concentration between days 0 and 15 [24]. The most remarkable reduction can be seen in reactor 2 of Treatment B (Figure 3.1 B). This initial reduction can be attributed to the rapid degradation of low molecular weight (LMW) PAHs that are more bioavailable. Whynot (2009) found that LMW PAHs were

generally degraded between days 30 to 45. Similar results were reported in a study by Viñas *et al.* (2005) that noted two- and three-ringed PAHs were greatly degraded during the first 45 days of treatment [49, 108]. In the Viñas *et al.* study all treatments were carried out in 1-litre sterile glass jars containing 600 g of sieved soil. Samples were aerated and moisture content corrected by mixing twice a week [49]. Several treatment parameters were tested including untreated, dry soil, basic, aerated soil, autoclaved soil, aerated soil with nutrients added, aerated soil with nutrients and either biosurfactant, inoculum or iron octoate added. Interestingly, they found that phenanthrene and anthracene were not completely degraded. The authors speculated that this may have been due to low bioavailability of the residual quantities of these compounds present after day 45. They also reported that the degree of degradation of certain PAHs (e.g., benzo[*a*]anthracene and chrysene) differed significantly when nutrients were added to the system. Both compounds were degraded more under biostimulation conditions without nutrient addition (i.e., the basic treatment, aerated with 40% water holding capacity) than in treatments with nutrient addition (i.e., treatments with nutrients and either biosurfactant, inoculum or iron octoate added) [49]. Moreover, the rate of biodegradation observed at later stages was significantly higher without the addition of nutrients [49]. A second study by Eriksson *et al.* (2000) showed that after 30 days, most substances (e.g., PAHs and coal tar creosote) were still present despite high microbial activity achieved through the addition of nutrients [108]. They reported that aromatics with four or more rings were unaffected, and remained at their initial

concentrations after 30 days. They hypothesized that this could be attributed to the physical-chemical properties of larger PAHs (i.e., K_{ow} and K_{oc}) that would contribute to strong sorption on particulate material and limited availability for microbial degradation [108, 109].

4.2.2 PAH CONCENTRATIONS AND MUTAGENIC HAZARD

The aforementioned Whynot (2009) study noted that as the concentration of PAHs decreased in the contaminated Rock Bay soil over the course of the bioremediation, there was a simultaneous increase in mutagenic activity assessed using the Salmonella reverse mutation test [24]. The net result was an increase in the mutagenic activity of the soil at the end of the 90-day treatment compared to the initial level at Day 0. This trend has been previously reported in the literature [65, 110], and several authors have speculated that the increase in mutagenic hazard can be attributed to the formation of polar PAH metabolites such as oxygenated PAHs (oxy-PAHs) [65, 110]. The production of mutagenic compounds, such as oxy-PAHs, aromatic amines, and nitro-PAHs is important to consider when attempting bioremediation of a contaminated soil for two reasons. First, these compounds can be responsible for a temporary increase in hazard. Second, the production of polar metabolites can yield a biocidal soil environment that detrimentally effects the indigenous microbial community [13].

In a recent study by Lunstedt *et al.* (2007) it was noted that oxy-PAHs can indeed be more toxic to both humans and the environment than their parent PAHs. They have relatively high persistence, and are generally considered to be 'dead-end

products' of many biological pathways [110]. In addition, oxy-PAHs tend to be more mobile in the environment due to increased polarity and water solubility, and thus have a tendency to move beyond the initial point of contamination via surface and groundwater. They are formed through the transformation of PAHs, and as a result, their concentrations can increase as sites are being remediated via methods that promote PAH degradation. Oxy-PAHs can also form in conjunction with PAH formation since both are the result of incomplete combustion, however, oxy-PAHs may also be formed through post-emission oxidation of parent PAH compounds in the environment [110]. One method of post-emission oxidation is via biological transformation of PAHs in the environment, and the reactions are generally catalyzed by microbial enzymatic systems either though intra- or extracellular methods [110]. Oxy-PAHs can also form through microbial cometabolism of parent PAHs by microorganisms utilizing another available substrate [110].

Due to the fact that some intermediate PAH breakdown products, such as oxy-PAHs, have been found to be considerably more toxic than their parent PAHs, it could be hypothesized that these compounds may have an effect on the indigenous soil microbial community. The microbial community in soil is a diverse consortium and the microbes present will interact with, and be affected by, PAHs and oxy-PAHs. For example, one organism may interact with a PAH to produce some form of intermediate (e.g., oxy-PAH) that could be harmful to other organisms in the consortium. Several studies have reported that oxy-PAHs can adversely affect a variety of organisms in the environment [110, 111], and some oxy-PAHs (such as

quinones) can form adducts with essential macromolecules such as proteins and DNA leading to both cytotoxic and genotoxic effects [110]. Some genera identified throughout the different treatments examined in this thesis did not behave as expected, particularly in light of the fact that they are known PAH-degraders. Although there would have been an abundance of nutrients in some instances, in addition to the PAHs (e.g., Treatments B and C), their relative abundance decreased during the treatment (e.g. *Stenotrophomonas*, see Section 4.2.3.7 below). This type of complex, and somewhat unpredictable, response pattern is presumably caused by a complex interplay of nutrient availability, substrate availability, physical-chemical conditions (e.g., oxygen availability, pH and redox potential), and local concentrations of toxic metabolites of PAH degradation.

4.2.3 TEMPORAL CHANGES OF PREVALENT GENERA AND INTERPLAY WITH TEMPORAL CHANGES IN PAH CONCENTRATION

Only genera that reached a relative abundance of more than 10.0% of total clones for at least one of the sampling days will be discussed in terms of noteworthy temporal changes, and interplay between temporal changes in community composition and PAH concentration. Table 4.3 provides a summary of temporal changes in the prevalent genera for Treatments A, B and C discussed below. It is important to note one universal limitation, previously discussed in Section 4.1.2, is the accuracy of rDNA analysis. Due to this limitation, closely related organisms may not have been accurately identified, and were either placed within the same genus or into different genera. Attempts to alleviate this limitation were done by aligning

questionable sequences using BLAST2. The results discussed below were analyzed so that they are as accurate as possible.

As discussed in Section 4.2.2 there was an observed increase in mutagenic activity throughout all treatments. Several studies have reported that this increase can be attributed to toxic intermediates, such as oxy-PAHs, forming during PAH metabolism. It has also been reported in the literature that these intermediates can be more toxic, and could therefore adversely affect growth of soil microbes. In this study, it was observed that several known PAH degraders did not behave as expected during treatment. It was expected these genera, which are outlined and discussed below, would thrive in a PAH-rich environment, however, this was not the case.

Table 4.3 **Summary of temporal changes in the prevalent genera for Treatments A, B and C. Values are percentages of the total number of observed clones.**

Phylum	Family	Genus	Day 0		Day 45		Day 90	
Treatment A			**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
β-proteobacteria	*Alcaligenaceae*	*Achromobacter*	14.0	13.6	28.1	28.7	35.5	12.1
β-proteobacteria	*Comamonadaceae*	*Acidovorax*	26.3	12.9	19.0	27.1	20.4	19.1
β-proteobacteria	*Comamonadaceae*	*Variovorax*	11.4	28.8	6.6	7.4	4.0	0.9
β-proteobacteria	*Rhodocyclaceae*	*'35'*	30.7	25.0	5.0	3.3	0.7	2.6
γ-proteobacteria	*Xanthomonadaceae*	*Lysobacter*	6.1	9.1	11.3	9.8	12.5	15.6
γ-proteobacteria	*Xanthomonadaceae*	*Rhodanobacter*	2.6[a]	0.0	0.8[a]	6.6	7.2[a]	19.1
γ-proteobacteria	*Xanthomonadaceae*	*Stenotrophomonas*	n/o	9.9	n/o	2.5	n/o	4.4
Treatment B			**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
α- proteobacteria	*Caulobacteraceae*	*Phenylobacterium*	0.0[a]	2.7	3.6[a]	8.7	2.5[a]	10.7
β-proteobacteria	*Alcaligenaceae*	*Achromobacter*	15.7	17.4	16.5	23.2	6.6	9.3
β-proteobacteria	*Comamonadacea*	*Acidovorax*	17.6	25.5	1.6	18.8	5.0	3.4
β- proteobacteria	*Hydrogenophilaceae*	*Thiobacillus*	n/o	0.0	n/o	2.2	n/o	17.0
β-proteobacteria	*Rhodocyclaceae*	*'35'*	24.1	19.0	13.4	10.9	4.1	4.2
γ-proteobacteria	*Pseudomonadaceae*	*Pseudomonas*	13.0	4.9[a]	0.8	0.0[a]	3.3	0.0[a]
γ-proteobacteria	*Xanthomonadaceae*	*Lysobacter*	22.2	19.0	0.0	5.1	5.0	11.0
γ-proteobacteria	*Xanthomonadaceae*	*Rhodanobacter*	0.9	3.3	26.0	12.3	36.4	20.3
Treatment C			**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
Gemmatimonadetes	*Gemmatimonadaceae*	*Gemmatimonas*	0.0	0.0[a]	0.0	0.0[a]	9.9	2.3[a]
β-proteobacteria	*Alcaligenaceae*	*Achromobacter*	0.0	3.8[a]	14.3	5.6[a]	7.6	3.8[a]
γ-proteobacteria	*Pseudomonadaceae*	*Pseudomonas*	98.1	87.0	2.4	0.0	2.3	0.0
γ-proteobacteria	*Xanthomonadaceae*	*Lysobacter*	0.0	0.5	9.5	9.3	40.2	9.9
γ-proteobacteria	*Xanthomonadaceae*	*Rhodanobacter*	0.0	0.5	11.1	54.2	3.8	62.9
γ-proteobacteria	*Xanthomonadaceae*	*Stenotrophomonas*	0.0	0.0[a]	15.9	2.8[a]	0.8	0.8[a]

[a]Representation of genus in one reactor was <10% of total clones for at least one sampling day.
n/o: not observed.

4.2.3.1 *ACHROMOBACTER*

The *Achromobacter* genus is comprised of Gram-negative soil organisms that are strictly aerobic, and able to grow well at a pH of 6.5 to 8.5 [107, 112]. The genus has generally been associated with degradation of low molecular weight PAHs such as phenanthrene. Long *et al.* (2009) reported *Achromobacter xylosoxidans* isolate 2MN-2 as being capable of using phenanthrene as a sole carbon source as seen in an enrichment study [49, 56]. The *Achromobacter* are a prevalent genus in all three treatments (except for reactor 2 of Treatment C). In reactor 1 of Treatment A the population of *Achromobacter* was observed to increase continuously as the concentration of PAHs decreased. This trend would correspond to both the initial availability of phenanthrene in the soil as a carbon and energy source, as well as to the breakdown of larger PAHs. However, in reactor 2 of Treatment A, and in subsequent Treatments B and C, this is not the case. For the most part, the *Achromobacter* genus peaks at day 45, and then decreases towards the end of treatment. There are several possibilities for the observed pattern reported in the Results Section. The *Achromobacter* may have been outcompeted for metabolites by either the indigenous soil microbes or the exogenous microbial seed added in Treatment C. The addition of an alternative nutrient source may have caused a shift in the microbial metabolism of PAHs causing metabolites of choice to become unavailable. As previously stated, soil organisms interact intimately with one another [49, 54, 68], and the addition of an alternative food source, or other

microorganisms, may cause a shift in microbial interactions such that *Achromobacter* growth was not favoured.

4.2.3.2 *ACIDOVORAX*

The *Acidovorax* genus is compromised of aerobic [87], Gram-negative soil organisms [113]. The strain *Acidovorax delafieldii* P4-1 has been associated to phenanthrene degradation [50]. A variety of bacterial strains within the *Acidovorax* genus have been isolated and have been reported to have the ability to use phenanthrene as a sole carbon and energy source [50]. The *Acidovorax* are a prevalent genus in Treatments A and B, however, the observed temporal trends were not what was expected. This may in part be due to the inherent heterogeneity of soil and the fact that differences in community composition can occur over minute distances [44, 46]. In Treatment A, the initial *Acidovorax* abundance was markedly different between the two reactors. Reactor 1 has almost twice the relative abundance of reactor 2. Moreover, throughout the treatment, the two reactors behaved inversely to one another. The *Acidovorax* population of reactor 1 decreased between days 0 and 45, and then increased between days 45 and 90. The Reactor 2 showed the reverse. Similar to what was observed for Treatment A, the *Acidovorax* population of both Treatment B reactors did not show similar temporal trends. The *Acidovorax* population in reactor 1 decreased between days 0 and 45, and increased slightly towards days 90, whereas the population in reactor 2 continuously decreased throughout treatment. In Treatment C, the *Acidovorax* were hardly present at all, even in the initial microbial community. The behaviour of the

Acidovorax was not expected since low molecular weight PAHs, such as phenanthrene, should have been available in both reactors [28, 30]. It was expected that the *Acidovorax* would behave in a similar fashion to the *Achromobacter* in reactor 1 of Treatment A. Similar to what was observed for Treatment A, the *Acidovorax* population of both Treatment B reactors did not show similar temporal trends. In Treatment C, the *Acidovorax* were hardly present at all, even in the samples taken at the start of the treatment. This may be attributed to the anaerobic environment maintained in the soil storage container, or that they were not present in large enough numbers in the soil sub-sample that was added to the slurry system.

4.2.3.3 *LYSOBACTER*

The *Lysobacter* genus, which was prevalent in all treatments, is comprised of Gram-negative, aerobes that are known hydrocarbon degraders [107, 114]. Maeda *et al.* (2009) reported isolation of the novel carbazole-degrading *Lysobacter* sp strain OC7 from seawater that is also capable of utilizing naphthalene and phenanthrene as its sole carbon and energy sources [115]. Comparison between the temporal changes in growth trends in *Lysobacter* in all treatments, and temporal changes in PAH concentration, suggest their involvement in PAH mineralization. In both reactors of Treatment A, the *Lysobacter* genus grew at a steady rate throughout the treatment. At the start of Treatment B the *Lysobacter* genus showed a higher percentage of clones than Treatment A, however, it decreased towards day 45 before increasing at the end of treatment. At the start of Treatment C, the *Lysobacter* genus was not well represented in either reactor. However, by day 90 it

comprised a majority of the microbial community in both reactors. Temporal changes may be attributed to several possibilities including competition for PAHs or added nutrients.

4.2.3.4 PSEUDOMONAS

The *Pseudomonas* genus contains Gram-negative, chemoheterotrophic aerobes. The genus has been reported to be able to degrade hydrocarbons, and *Pseudomonas* are known to be involved the metabolism of naphthalene and phenanthrene [116, 117]. They are able to degrade a wide variety of organic molecules, and are important in the mineralization of noteworthy contaminants that are completely oxidized to carbon dioxide [107, 114]. Despite this, the observed pattern of relative abundance was unexpected. The *Pseudomonas* genus did not have a strong presence in Treatment A. They were initially present in reactor 1, but decreased throughout the treatment. They were not present in reactor 2 initially, but increased throughout treatment. Treatment B showed a decrease of the *Pseudomonas* genus throughout the duration of treatment despite favourable conditions and nutrient addition. Treatment C results showed a markedly different starting microbial community compared to the other treatments, with a high percentage of *Pseudomonas* present. The *Pseudomonas* genus is capable of utilizing LMW PAHs, but in Treatments A and C their relative abundance decreased over the course of treatment. This may be attributed to competition for preferred metabolites, or unfavourable growth conditions. Treatment C results showed a markedly different starting microbial community compared to the other treatments

due to the high percentage of *Pseudomonas* (NB. The microbial seed was added after sampling on day 0). The initial abundance of the *Pseudomonas* genus, followed by a general decrease in almost all instances, may be attributable to their ability to metabolize the smaller, more bioavailable PAHs. The decline in the smaller PAHs throughout the course of Treatment C, or competition for the breakdown products of the larger PAH, may have restricted the availability of suitable substrates for the *Pseudomonas* genus. Despite additional *Pseudomonas* being added to the system (exogenous microbial seed contained 73.7% *Pseudomonas*, see Figure 3.14 above) the relative abundance of *Pseudomonas* in the slurry decreased as treatment progressed.

4.2.3.5 *RHODANOBACTER*

The *Rhodanobacter* genus is comprised of Gram-negative, aerobic soil organisms The *Rhodanobacter* are known to be capable of hydrocarbon degradation and have been observed to make benzo[*a*]pyrene more bioavailable by solubilisation [62, 114, 118]. This is consistent with the results of Whynot (2009), which noted a decline in benzo[*a*]pyrene concentration in all reactors [24]. In reactor 1 of Treatment A the *Rhodanobacter* genus is not prevalent, and their behaviour is not consistent with the other treatments. In reactor 2 of Treatment A the *Rhodanobacter* genus increased continuously throughout the treatment as the concentration of PAHs decreased. The same trend was observed for both reactors of Treatment B. The results observed for Treatment C were not consistent across reactors. In reactor 2 of Treatment C, the *Rhodanobacter* genus increased significantly throughout treatment

representing more than half of the soil microbial community at the termination of treatment. It is possible that the *Rhodanobacter* are interacting with the benzo[*a*]pyrene throughout the course of treatment, and this interplay would correspond to the temporal changes in priority PAH concentrations, as well as to the increase in the relative abundance of *Rhodanobacter*. By making benzo[*a*]pyrene more bioavailable, it can be used by other organisms, and as such, overall PAH degradation is enhanced.

4.2.3.6 *RHODOCYCLACEAE '35'*

The *Rhodocyclaceae '35'* genus is compromised of Gram-negative soil organisms that grow photoheterotrophically preferably under anoxic conditions [119]. The *Rhodocyclaceae '35'* genus has not been reported to be involved in PAH mineralization or degradation; however, there is no information in the literature regarding any investigations on this topic. The observed pattern of the *Rhodocyclaceae '35'* genus in Treatments A and B (i.e., significant decrease between days 0 and 45) may be attributed to their preference to grow under anoxic conditions [119]. This trend may be a result of the aerobic environment in the reactors compared to the relatively anaerobic conditions in the soil storage container. It is interesting to note that in Treatment C this genus was not observed.

4.2.3.7 *STENOTROPHOMONAS*

The *Stenotrophomonas* genus is comprised of aerobic, Gram-negative organisms [120], and there is only one species within the genus, *Stenotrophomonas maltophilia* [107]. It is a ubiquitous organism that has been found in a variety of environments

including soil, sewage, and aquatic environments [120]. They are known to be capable of hydrocarbon degradation [114], including degradation of high molecular weight polycyclic aromatic hydrocarbons from three to seven rings [63]. The observed pattern of the *Stenotrophomonas* genus was not expected. The *Stenotrophomonas* genus was observed in all reactors and treatments except for reactor 1 of Treatment A; however, it was only a prevalent genus in reactor 2 of Treatment A and reactor 1 of Treatment C. In reactor 2 of Treatment A, as well as in Treatment B, the *Stenotrophomonas* genus decreased toward day 45 before it increased slightly toward the end of treatment. In Treatment C however, the trend is the reverse. The *Stenotrophomonas* genus was not detected at day 0, but was observed to increase at day 45 before decreasing toward the end of treatment. Whynot (2009) observed an overall decrease in pyrene, fluoranthene, benzo[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene [24], and it has been previously noted that *Stenotrophomonas* are capable of degrading these compounds. It is possible that intermediate breakdown products that are being formed are adversely affecting the growth of *Stenotrophomonas* [13, 110, 111], or competition for nutrients in the slurry system may have contributed to the unexpected pattern in the relative abundance of *Stenotrophomonas.*

4.2.3.8 *THIOBACILLUS*

The *Thiobacillus* genus is a Gram-negative, obligatory aerobe that is capable of oxidizing a variety of inorganic sulphur compounds. They are metabolically very flexible, ubiquitous in nature [107, 121, 122]. The *Thiobacillus* genus is able to

obtain carbon by fixing carbon-dioxide from the atmosphere, and is used industrially in metal leaching from mineral ores and in the microbial desulphurization of coal [121, 122]. To date, the *Thiobacillus* genus has not been directly associated with PAH degradation. They are only prevalent in reactor 2 of Treatment B, but were also present in smaller numbers in Treatment C.

4.2.3.9 *VARIOVORAX*

The *Variovorax* genus is compromised of Gram-negative soil organisms [123] that are known to be aerobic or faculatively anaerobic [124]. Currently, the *Variovorax* genus has not been reported to have a major role in PAH degradation, but they have been associated with an ability to degrade phenols and trichloroethylene [125]. The *Variovorax* genus was only prevalent in Treatment A and there was an overall decrease in the percentage of total clones of *Variovorax*. In Treatment B however, though they never achieve more than 10.0% of the total clones, both reactors showed an increase in the percentage of total clones between days 0 and 45 before decreasing towards the end of treatment. The *Variovorax* genus was only prevalent in Treatment A, and throughout Treatment A there was a decrease in the percentage of total clones of *Variovorax*. In Treatment B however, though they never achieve more than 10.0% of the total clones, both reactors showed an increase in the percentage of total clones between days 0 and 45 before decreasing towards the end of treatment. This may be due to the addition of micro and macro nutrients as described in Section 2.2.2.1, or the initial microbial community structure.

4.2.3.10 *GEMMATIMONAS AND PHENYLOBACTERIUM*

Two other genera were represented with more than 10.0% of total clones, but neither has been reported in the literature as having PAH-degradation ability. The *Gemmatimonas* genus consists of Gram-negative, aerobic organisms that grow well in a pH range of 6.5-9.5 [126]. The *Gemmatimonas* are widespread in nature, particularly in soil habitats [127]. Though the conditions within the reactors were favourable for their growth, the *Gemmatimonas* genus is only prevalent at day 90 in reactor 1 of Treatment C. Members of the *Phenylobacterium* genus are aerobic or facultative anaerobes, and are typical of the upper aerobic part of soil [128]. Different strains of *Phenylobacterium* have been isolated from various locations all over the world [128]. The *Phenylobacterium* genus is known to include xenobiotic compound degraders [129]. Compounds including chloridazon, antipyrin, and pyramidon have been reported as being sole carbon sources for *Phenylobacterium* [128, 130]. The Phenylobacterium genus is only prevalent at day 90 in reactor 2 of Treatment B. To date, neither genus has been reported to be involved with PAH catabolism but this may be due to no one investigating their PAH-degrading potential.

4.2.3.11 *PAENIBACILLUS*

The *Paenibacillus* showed a relatively high percentage of total clones (21.4%) in Treatment C. However, they were identified by uploading sequences to RDP, and the alignment results showed that O.T.U.s were not similar enough for the samples

to be considered to be in the same genus. They have been left as a genus group in the Results Section.

4.2.3.12 *ALCALIGENES AND SPHINGOMONAS*

Two genera that have been reported in the literature as having PAH-degrading ability but were not observed to be prevalent (≥10.0%) in any treatment, were the *Alcaligenes* and the *Sphingomonas* [4, 15, 37, 50, 58-61]. The *Alcaligenes* genus is within the *Alcaligenaceae* family along with the *Achromobacter* genus. Both the *Achromobacter* and an unidentified genus of the *Alcaligenaceae* family (called number '6' based on O.T.U. results) were found in some of the reactors during treatment however, there was no representation from the *Alcaligenes* genus. The *Alcaligenaceae '6'* was observed only in Treatment C, whereas the *Achromobacter* were observed throughout all treatments. The *Alcaligenes* genus has been reported to have the ability to degrade fluoranthene, phenanthrene and naphthalene (i.e., low molecular weight PAHs) [15, 50, 58-60].

The *Sphingomonas* genus is within the *Sphingomonadaceae* family along with the *Novosphingobium* and *Sphingopyxis* genera. The *Novosphingobium* genus is found in reactor 1 of Treatment B (see Table 3.4), and the *Sphingopyxis* genus is found in both reactors of Treatment C (see Table 3.6 and 3.7). The *Sphingomonas* genus has been reported to have the ability to degrade fluorene, phenanthrene, anthracene, fluoranthrene, naphthalene, benzo[*b*]fluorene, and pyrene [4, 37, 61].

There are several possible explanations regarding the low relative abundance of these two genera. First, limitations of the database for identification of the DNA

sequences (i.e., as discussed in Section 4.1.2). Second, the slurry may have provided unfavourable growth conditions for *Alcaligenes* and *Sphingomonas*. Finally, it is possible that the storage bucket simply did not contain a sufficient number of individuals from these genera to be able to observe their prevalence in the reactors.

4.2.4 EXOGENOUS MICROBIAL SEED

There have been several studies conducted both in the laboratory and *in-situ* to investigate the efficacy of additions of exogenous microbial cultures for the removal of pollutants from contaminated soil [37, 131]. These studies determined that the addition of these cultures did not enhance the rates of biodegradation compared to nutrient enrichment of the indigenous microbial community likely due to exogenous microbes not being sufficiently adapted [37, 132]. Examples of this are reported by van Hamme *et al.* (2003) [37]. In each instance they reported either indigenous microflora or 'well-acclimated' and prepared hydrocarbon cultures being employed to degrade refinery and petrochemical-like wastes. In this study, the results showed that the least effective PAH mineralization was observed in reactor 2 of Treatment B, and the most effective was reactor 1 of Treatment A. Although, Treatment C was, for the most part, more effective than Treatment B, it was unable to surpass the efficacy observed in Treatment A. Thus, with respect to this thesis, the addition of an exogenous microbial culture including organisms capable of hydrocarbon degradation did not enhance the removal of priority PAHs. Nevertheless, it may be possible to enhance the removal of PAHs during a bioslurry treatment via the addition of a highly concentrated bacterial inoculum derived from the same

contaminated site. Such treatments have been shown to be effective in increasing contaminant degradation and removal [108]. It is important to note that though the seed was formulated for the use in hydrocarbon degradation of oil field and refinery wastes several genera recovered and identified in the exogenous bacterial community are known PAH-degraders as well, such as the *Achromobacter* and *Pseudomonas*.

4.2.5 DIFFERENCES BETWEEN REACTORS AND TREATMENTS

Throughout each treatment (Treatments A-C) two reactors were set up in tandem and sampled over the course of 90 days. Multiple sub-samples were taken from each reactor, three were used in chemical and mutagenicity testing and a fourth was stored in 50% glycerol at -80°C for use in microbial community analyses.

Differences between the reactors of a single treatment can be attributed to several factors, the first being the heterogeneous nature of the soil sample, notwithstanding our efforts to homogenize the soil batch prior to treatment. Due to the intimate nature of interactions between soil microorganisms, minute spatial differences in soil chemistry and microbial content could easily have enormous repercussions for temporal changes in the composition of the microbial community [49, 68, 69]. This is apparent from the initial populations of each treatment. The two reactors of each treatment are never identical, though they are similar. For example, in reactor 1 of Treatment A the PAH concentration was almost three times greater than that reactor 2, and similar differences in soil microbial community composition are therefore, not unexpected.

Differences between treatments can be attributed to the differences in the treatment conditions (e.g., nutrient availability), as well as the way in which the soil was sampled before each treatment. The original soil batch was homogenized at the very start of the slurry treatments, and soil sub-samples were removed from the storage container at the commencement of each new treatment (i.e., stored soil not re-homogenized prior to each subsequent treatment). This likely caused differences in the initial composition of the microbial community. In addition, anaerobic conditions in the storage container may have contributed to temporal changes in the soil microbial community that would later be observed in the reactor vessels [37, 133]. This may be seen in the initial population of each treatment. The starting population for the two reactors of a treatment is generally similar, but tends to differ between treatments. Anaerobic metabolism has been observed as being a vital process with respect to petroleum hydrocarbon degradation and bioremediation [37]. It is likely that anaerobic metabolism is occurring within the storage container, and this may cause differences between treatments.

Differences in the temporal changes of common and/or prevalent soil organisms could be attributed to the intimate interactions that occur between members of the soil microbial community and their sensitivity to their environment [44, 47]. Changes in the treatment parameters could cause marked differences in the microbial community structure over time despite similarities in the initial microbial populations in reactors of the same treatment.

4.2.6 GENES INVOLVED IN PAH CATABOLISM

Genes involved in PAH degradation generally encode for enzymes such as dioxygenases (i.e., ring-hydroxylating, and naphthalene-2,3-dioxygenase), dihydrodiol dehydrogenase, hydroxylases, and meta-cleavage enzymes [15, 57]. Aerobic catabolism of PAHs by bacteria, such as the prevalent bacteria found in this study (i.e. *Achromobacter* and *Lysobacter*), generally involves the oxidation of a PAH to a dihyrodiol by a multicomponent enzyme system. The dihydrodiol intermediates may be broken down further by either *ortho* or *meta* cleavage pathways leading to intermediate products such as protocatechuates and catechols that can further be converted to tricarboxylic acid cycle intermediates [134]. Chadhain *et al.* (2006) notes that the initial step of aerobic bacterial degradation of PAHs involves the addition of both molecules of molecular oxygen to the aromatic ring by a dioxygenase system [135]. Aromatic dioxygenase systems are multicomponent enzymes that consist of an electron transport chain containing a ferredoxin, a reductase and a terminal dioxygenase [135]. The best studied PAH dioxygenase is naphthalene dioxygenase encoded by *nah* genes from *Pseudomonas* spp. [135]. These genes have been found in a variety of bacteria and geographic locations [135]. Table 4.4 below provides a summary of genes reported in the literature as being associated with the mineralization of PAHs and have been reported as being associated with the prevalent organisms from this study.

Genes involved in PAH-catabolism are induced by the presence of PAHs. Marlowe *et al.* (2002) reported that the presence of phenanthrene increased the

expression of the *nahAc* gene in *Pseudomonas putida* G7 [136], and Singleton *et al.* (2009) reported that nearly equivalent levels of increased expression for both the *phnAc* and *phnB* genes in *Acidovorax* sp. strain NA3 were observed following exposure to either phenanthrene or naphthalene. This suggests that the genes are co-transcribed [57].

Table 4.4 Genes in selected PAH-degraders that are know to be involved in PAH catabolism

Organism	Prevalent genera (Y/N)	PAH Substrates	Gene(s) involved	Reference(s)
Acidovorax	Y	Phenanthrene	*phnAc, phnB, phnC*	Singleton *et al.*, 2009 [57]
Alcaligenes	N	Fluoranthene, naphthalene, phenanthrene	*phn, nahA, nahH*	Weissenfels *et al.*, 1990 [137]; Habe and Omori, 2003 [6]; Ringelberg *et al.*, 2001 [15]
Pseudomonas	Y	Fluoranthene, fluorene, naphthalene, phenanthrene	*nah, nahAc, pah*	Chadhain *et al.*, 2006 [135]; Kasai & Harayama, 2004 [138]; Takizawa et al., 1994 [116]
Sphingomonas	N	Anthrancene, benzo[*b*]fluoranthene, benzo[*b*]fluorene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene	*cirA, flnA1-flnA2, flnE*	Schuler *et al.*, 2007 [4]

4.3 CONCLUSIONS AND FUTURE DIRECTIONS

4.3.1 CONCLUSIONS

The objectives outlined in Section 1.6.3.1 have been achieved. (1) Total metagenomic DNA was isolated from the PAH contaminated Rock Bay soil, amplified, cloned, and the 16S rDNA sequenced for analysis of community structure. (2) Members of the soil microbial community were identified using the Ribosomal Database and Operational Taxonomic Units. (3) Temporal changes in the bacterial community were compared to temporal changes in PAH concentration and mutagenic activity. Moreover, genera appear to play a role in PAH mineralization.

Based on the results obtained, the thesis hypothesis statement is upheld. The analysis of 16S rDNA sequences in metagenomic samples of DNA from soil did permit the temporal analysis of community structure changes during a biolslurry treatment of a PAH-contaminated soil. Elements of the microbial community that may be determinants of PAH mineralization can be identified by examination of the interplay between temporal changes in community structure and previously recorded changes in PAH concentration.

The indigenous microbial community of the PAH-contaminated Rock Bay soil was found to be diverse and complex. For the most part the *Beta-* and *Gammaproteobacteria* classes dominated the community at the start of each treatment. However, given favourable conditions (i.e., temperature, pH, air and nutrients), in several instances, the soil microbial community increased in complexity over the course of the treatment. In total over the course of all three treatments, at

least 59 distinct genera were identified. Due to the alignment results of the RDP-identified *Paenibacillus* genera, there are probably several more genera to add to the total.

It is evident given the results obtained that interplay is occurring between the temporal changes in the soil microbial community and the previously observed temporal changes in PAH concentration. In each reactor the total PAH concentration was observed to decrease as the treatment progressed, and these changes corresponded with observed shifts in the soil microbial community. Some identified genera have been highlighted by other researchers, and their presence and changes in relative abundance are likely determinants of PAH mineralization. These genera may be responsible for the previously observed temporal alterations in mutagenic hazard. As previously discussed, the active players most likely include the *Achromobacter*, *Acidovorax*, *Lysobacter*, *Rhodanobacter* and *Stenotrophomonas* genera. These genera have been shown to be prevalent throughout this study.

Given the findings of this thesis, future bioremediation efforts should continue to focus on the innate PAH-degrading ability of the indigenous soil microbial community before attempting soil amendments (e.g., additions of nutrients or exogenous microbes) that may be costly and ineffective. However, the addition of a microbial inoculum isolated from the same contaminated soil may serve to enhance the abundance PAH-degrading microbes as previously reported by Eriksson *et al.* (2000) [108]. Longer periods of incubation would also be worth exploring as some studies indicated incubation times as long as eleven months [37]. Moreover, design

of an effective remediation system for PAHs must take into account potentially harmful intermediates that may form during the breakdown of PAHs. These can cause the soil environment to become biocidal, and may adversely affect the indigenous soil fauna. Further investigations regarding the metabolic capabilities of soil microorganisms (e.g. *Lysobacter* spp.) will be necessary to obtain an improved understanding of the complex interplay of microbial attributes that control the catabolism of PAHs in soil.

4.3.2 FUTURE DIRECTIONS

Promising areas for further research can be broken into three parts: additional remediation scenarios, increased optimization of laboratory protocols, and investigation of the PAH-degrading capacity of microorganisms discussed in this study. Assessment of additional remediation scenarios using would aid in determining the most effective means of PAH reduction and removal. Augmenting the soil with a microbial inoculum isolated from the same contaminated soil has proven to be an effective method of contaminant removal [108]. Organisms of interest to incorporate into this derived inoculum should include the *Achromobacter*, *Acidovorax*, *Lysobacter*, *Rhodanobacter*, and *Stenotrophomonas* since the relative abundance of these genera showed some correspondence with previously observed temporal changes in PAH concentration. Other remediation scenarios and experimental modifications that are worthy of additional effort include additional bioreactors running in tandem, longer incubation times, and more frequent nutrient supplementations [37, 132]. Running more bioreactor systems in

tandem and subjecting the soil to several different treatment scenarios simultaneous will aid in eliminating any bias due to settling out of the soil in storage, anaerobic reactions that may occur while the soil is in storage, and any variances that may occur between consecutive treatments. However, increasing the number of reactors would be tremendously expensive and would require a large amount of space beyond the capacities of this laboratory. Improvements to the experimental design outlined in thesis would include taking a large soil sub-sample from the storage bucket and adding it to water to make a large slurry that could then be dispensed into the replicate reactors.

In terms of increased optimization of laboratory protocols there are two areas that should be addressed: soil DNA isolation optimization, and the use of multiple 16S primer sets. Optimization of soil DNA isolation protocols serves to ensure that DNA from 'all' indigenous soil microbes is being isolated. Due to the nature of soil microorganisms there is a possibility that the isolation kit used in this project was not efficient in recovery of the total metagenomic DNA. Gram-positive organisms have a significantly more robust peptidoglycan layer compared to Gram-negative organisms making it more difficult to burst the cells. Moreover, there is a tendency for some microorganisms to sporulate when subjected to sub-optimal conditions making them resistant to rupture and DNA isolation [107]. Implementing the use of additional 16S primer sets could serve to eliminate potential primer biases [96], as well as to ensure that more indigenous soil microbes are being examined in case of slight variations in the priming region of the 16S rRNA gene.

One avenue that warrants additional investigation is the PAH-degrading ability of the *Lysobacter* genus. Currently, there is a paucity of information regarding the ability of the *Lysobacter* genus to play an active role in PAH mineralization, despite the fact it belongs to a family that includes known hydrocarbon degrading organisms. This thesis shows that changes in the abundance of the *Lysobacter* genus corresponded to temporal changes in PAH concentration. Moreover, that the *Lysobacter* genus was positively affected by the addition of nutrients. After the initial nutrient addition, the genus decreased in abundance, but then increased towards the end of treatment. The addition of the exogenous microbial seed in Treatment C seemed to enhance the relative growth of the *Lysobacter*, which increased continuously throughout treatment C. Another genus worthy of additional scrutiny is *Rhodanobacter*. When present in a reactor their relative abundance increased as the concentration of PAHs decreased. The *Rhodanobacter* genus has been reported as being involved in increasing the bioavailability of benzo[*a*]pyrene, but further research may prove useful in defining its role in PAH catabolism.

Another interesting endeavour would involve further investigation of the PAH-degrading potential of the *Gemmatimonas*, *Phenylobacterium*, and *Thiobacillus*. They have not been previously described in the literature as being involved in PAH catabolism, but their presence and changes in relative abundance in the slurry system warrants further attention. Investigation into their PAH-degrading potential could be as simple as attempting to grow pure cultures on select PAHs. Galvão *et al.*

(2005) describes several methods to explore and detect the presence of biodegradation genes in microorganisms [139]. The first method is a sequence-dependent method for the detection of biodegradation genes that relies on the conservation of the amino acid sequence in the active centres of catabolic enzymes. This provides a basis for identifying protein and gene variants that execute same or similar reactions. A second method to determine the presence and/or activation of catabolic genes involved in PAH metabolism is to use PCR-based techniques in order to survey enzymatic diversity. Genes coding enzyme variants from environmental samples can be isolated through primer or probe hybridization to the conserved regions of catabolic genes. A third method highlighted by Galvão *et al.* (2005) uses fluorescently labelled RNA probes that are hybridized to fixed cells in order to enable visualization of expressed transcripts. As previously noted genes involved in PAH metabolism are induced when in the presence of PAHs [57, 136]. A new technology that is still in the process of being optimized for use in environmental microbiology are DNA macro- and microarrays [139]. This technique can be sub-divided into functional gene arrays (FGAs), community gene arrays (CGAs), and phylogenetic genome arrays (PGAs). These will be discussed briefly: CGAs are made with genomic DNA isolated from environmental samples or pure culture whereas PGAs are made with oligonucleotides from rRNA genes. These two techniques give indirect information on the biodegradation gene landscape of an environmental sample. The FGA approach contains probes for genes encoding enzymes involved in

biodegradation and biotransformation and they probe for functional diversity in microbial communities.

Targeting of the 16S rRNA is an established method to describe the phylogenetic diversity however, it provides few direct clues regarding the interactions and metabolic capacities of the microorganisms that the sequences in question represent [140]. In order to circumvent this issue stable-isotope probes (SIPs) have been employed to study microbial-mediated processes within complex environmental samples (such as the one investigate in this thesis project). Labelled substrates have been shown to reveal the metabolic function of microorganisms without the need to isolate them in culture [140]. The SIP method exploits physical properties of atoms that constitute all cellular components – isotopes of carbon. This method uses ^{13}C-labelled growth substrate to link microbial function with identity through selective recovery of ^{13}C-labelled DNA as well as using natural abundance levels of stable isotopes in cells or nucleic acids [140]. This technique allows the investigator to determine that a microbial population is involved in specific processes.

Another possibility for future research could address the catabolic pathways and genes involved in PAH degradation. Although there is detailed information about some pathways, genes and enzyme systems, the information is patchy. A promising strategy for identification of catabolic genes that is currently being explored is Substrate-Induced Gene Expression (SIGEX). The SIGEX method was developed to screen a metagenome library for catabolic genes whose expression is induced in

response to environmental stimuli (e.g., occurrence of a chemical substrate) [73]. SIGEX works by trapping the regulatory elements that respond to a specific substrate, and sorting a "promoter trap" library using fluorescence activated screening (FACS) [141]. Any substrate that is capable of entering the cytoplasm is effective for use in SIGEX screening.

5.0 REFERNCES

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6.0 APPENDICES

Appendix 1 Graphic Genera Colour Code

Phylum, Class, Family	Genus	Colour
Acidobacteria, Acidobacteria, Acidobacteriaceae	*Gp1*	Periwinkle
	Gp3	Aqua
	Gp4	Aqua and black hatched
Actinobacteria, Actinobacteria, Beutenbergiaceae	*Georgenia*	Grey 25%
Actinobacteria, Actinobacteria, Cellulomonadaceae	*Cellulomonas*	Ice blue and white hatched
Actinobacteria, Actinobacteria, Microbacteriaceae	*Microbacterium*	Coral and white hatched
Actinobacteria, Actinobacteria, Nocardiaceae	*Rhodococcus*	Dark blue
Bacteroidetes, Bacteroidetes, Porphyromonadaceae	*Proteiniphilum*	Indigo and bright green hatched
Bacteroidetes, Flavobacteria, Flavobacteriaceae	*Lutibacter*	Light turquoise
Bacteroidetes, Sphingobacteria, Flexibacteraceae	*Niastella*	Coral
Chloroflexi, Anaerolineae, Caldilineacea	*Levilinea*	Indigo
Firmicutes, Bacilli, Bacillaceae	*Bacillus a*	Yellow and red hatched
	Paenibacillus[2]	Ice blue
Firmicutes, Bacilli, Carnobacteriaceae	*Trichococcus*	Pink and green hatched
Firmicutes, Bacilli, Listeriaceae	*Aneurinibacillus*	Orange
Firmicutes, Bacilli, Paenibacillaceae	*Aneurinibacillus*	Orange and black hatched
	Bacillus	Dark Green
	Cohnella	Tan and blue hatched
	Paenibacillus[1]	Light blue
Firmicutes, Bacilli, Staphylococcaceae	*Staphylococcus*	White
Gemmatimonadetes, Gemmatimonadetes, Gemmatimonadaceae	*Gemmatimonas*	Tan
Planctomycetes , Planctomycetacia, Planctomycetaceae	*Gemmata*	Black and white
	Isosphaera	Light yellow
Proteobacteria, Alphaproteobacteria, Acetobacteraceae	*Paracraurococcus*	Teal and white hatched
	Roseomonas	Blue-grey
Proteobacteria, Alphaproteobacteria, Beijerinckiaceae	*Methylocella*	Orange and white hatched
Proteobacteria, Alphaproteobacteria, Bradyrhizobiaceae	*Afipia*	Grey 80%
	Bosea	Pink and white hatched

	Bradyrhizobium	Olive green
	Rhodopseudomonas	Pale blue
Proteobacteria, Alphaproteobacteria, Caulobacteraceae	*Caulobacter*	Dark yellow and white hatched
	Phenylobacterium	Dark yellow
Proteobacteria, Alphaproteobacteria, Hyphomicrobiaceae	*'11'*	Gold
	Hyphomicrobium	Light green and black hatched
Proteobacteria, Alphaproteobacteria, Methylobacteriaceae	*Methylobacterium*	Gold and black hatched
Proteobacteria, Alphaproteobacteria, Phyllobacteriaceae	*Mesorhizobium*	Black
Proteobacteria, Alphaproteobacteria, Rhizobiaceae	*Rhizobium*	Blue and white hatched
Proteobacteria, Alphaproteobacteria, Rhodospirillaceae	*'39'*	Light green
Proteobacteria, Alphaproteobacteria, Sphingomonadaceae	*Novosphingobium*	Violet and white hatched
	Sphingopyxis	Green
Proteobacteria, Betaproteobacteria, Alcaligenaceae	*'6'*	Rose and white hatched
	Achromobacter	Rose
Proteobacteria, Betaproteobacteria, Burkholderiaceae	*Burkholderia*	Green and white hatched
Proteobacteria, Betaproteobacteria, Comamonadaceae	*Acidovorax*	Yellow
	Variovorax	Sea green
Proteobacteria, Betaproteobacteria, Hydrogenophilaceae	*Thiobacillus*	Teal
Proteobacteria, Betaproteobacteria, Incertae sedis 5	*Methylibium*	Pink
Proteobacteria, Betaproteobacteria, Methylophilaceae	*Methylophilus*	Plum
Proteobacteria, Betaproteobacteria, Oxalobacteraceae	*Massilia*	Red
Proteobacteria, Betaproteobacteria, Rhodocyclaceae	*'35'*	Turquoise
Proteobacteria, Gammaproteobacteria, Chromatiaceae	*'40'*	Brown
Proteobacteria, Gammaproteobacteria, Legionellaceae	*Legionella*	Grey 25% and white hatched
Proteobacteria, Gammaproteobacteria, Pseudomonadaceae	*Pseudomonas*	Violet
Proteobacteria, Gammaproteobacteria, Xanthomonadaceae	*Dyella*	Blue and yellow hatched
	Lysobacter	Bright green
	Nevskia	Dark red
	Rhodanobacter	Light orange
	Stenotrophomonas	Lavender
Verrucomicrobia, Verrucomicrobiae, Opitutaceae	*Opitutus*	Grey 50%