INTERACTIONS BETWEEN MICROBIAL ORGANISMS AND PAHS

INTERACTIONS BETWEEN MICROBIAL ORGANISMS AND POLYCYCLIC AROMATIC HYDROCARBONS, AS INVESTIGATED THROUGH THE USE OF PHOSPHOLIPID FATTY ACID ANALYSIS

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ABSTRACT

PAH contamination is a significant threat to human and ecosystem health worldwide. The development of novel, efficient bioremediation systems can serve to reduce this threat by more effectively removing PAHs from contaminated environments. An improved understanding of the interactions between microbes and PAHs is essential to developing better methods to monitor PAH biodegradation, which should in turn lead to improvements in actual PAH biodegradation. This thesis developed novel process-level and community-level understanding of the responses of microbes to PAH contamination, knowledge which could prove useful in the development of improved techniques for monitoring and enhancing PAH biodegradation.

The first paper (Chapter 2) examined the response of microbial communities to PAH contamination, through PLFA analysis of specific microbial communities in Hamilton Harbour, Ontario, Canada. Increased PAH contamination was found to have caused reductions in microbial biomass, and while community composition was different between sites exposed to high and low levels of PAH contamination, none of the differences in community composition could be definitively identified as having been caused by the presence of PAH. The PAH profile at the less contaminated study site was found to have changed relative to a previous study, suggesting either a change in PAH source occurred, or resuspended PAH-contaminated sediment from elsewhere in the harbour was deposited at that site.

In the second paper (Chapter 3), a novel approach using stable carbon and radiocarbon PLFA analyses was used to establish bacterial preference for PAH as a carbon source and identify microbial carbon cycling pathways in *P. frederiksbergensis* bacteria. Stable and radiocarbon isotopic analyses of *P. frederiksbergensis* PLFA suggested *P. frederiksbergensis* metabolism was heterotrophic, but it was found to primarily utilize dissolved inorganic carbon (DIC) as a carbon source for PLFA biosynthesis. Isotopic data suggested metabolism of organic carbon was minimal, and as such, *P. frederiksbergensis* is most likely an unsuitable candidate organism for use in *in situ* PAH biodegradation projects.

Increased knowledge of microbial carbon cycling pathways and microbial community responses to PAH contamination will lead to improvements in PAH remediation, in turnleading to improvements in ecosystem health and reduced exposure risks to humans.

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PREFACE

This thesis is the result of research carried out by the author under the supervision of Dr. Greg Slater in partial fulfilment of the degree of Master of Science (M.Sc.). While Dr. Slater provided guidance during the research process and editorial commentary during the writing process, the research was carried out solely by the author. All research in this thesis, including literature review, field sample collection, laboratory analysis and manuscript preparation was conducted by the author, with the exception of the total petroleum hydrocarbon, natural organic matter, and polycyclic aromatic hydrocarbon data in Chapter Two. For that data only, sample collection and data generation was carried out by Dr. Penny Morrill and Natalie Szponar, however, the author carried out all analysis of that data.

Chapter One of this thesis is an introduction to PAH biodegradation, microbial carbon cycling and isotope analysis. Chapter Two (Assessment of the impact of PAH and TPH contamination upon Hamilton Harbour surface sediment microbial communities via PLFA profiling) and Chapter Three (Modelling *P. frederiksbergensis* metabolism and carbon cycling behaviour using stable and radiocarbon isotopic analyses) represent manuscripts for submission to peer-reviewed journals. The final Chapter provides a summary of major findings and conclusions from the research conducted in this thesis.

CHAPTER 1

Introduction

Environmental contamination represents a significant danger to human and ecosystem health worldwide. Many contaminants, including organic compounds and heavy metals, are characterized by acute or chronic toxicity, or have carcinogenic, teratogenic or mutagenic properties. Commonly-employed techniques for the remediation of such compounds are typically expensive, manpower-intensive, and can degrade soil quality (eg. incineration), or pose dangers to remediation workers (eg. gas phase chemical reduction) (Tavlarides et al., 2000). More recently developed methods, such as phytoremediation and bioremediation, have the potential to resolve such issues by leveraging natural processes to detoxify large areas with limited expense or requirement for supervision. However, widespread application of these novel remediation techniques has yet to occur, as accurately monitoring remediation progress, and confirming the removal of toxic substances from contaminated sites can be challenging. An improved understanding of the mechanisms governing microbial interactions with contaminant compounds has the potential to reveal novel, more effective means by which to monitor the progress of microbial contaminant degradation, which in turn may contribute to the development of more effective remediation strategies.

This thesis expands current understanding of microbial interactions with polycyclic aromatic hydrocarbons (PAHs) at both the community level and process level,

through the examination of laboratory cultures and microbial communities in Hamilton Harbour, Ontario, Canada. The novel contributions to understanding of natural microbial communities and microbial carbon cycling presented in this thesis have the potential to contribute to improvements in the efficiency of bioremediation system design, enable improvements in monitoring and mitigating environmental contamination, and enhance understanding of microbial PAH metabolism.

Polycyclic aromatic hydrocarbons (PAHs) are among the most common of environmental contaminants, and can be found in air (Szidat et al., 2004), sediment and soil (van Herwijnen et al., 2003; Mandalakis et al., 2004b; Johnsen et al., 2007; Short et al., 2007a), groundwater and surface water (Martens et al., 1997; Holman et al., 1999), and in remote locations including deep-sea sediments (Ohkouchi et al., 1999) and arctic ice and snow (Kawamura and Suzuki, 1994; Mascalet et al., 2000). Most of the global PAH inventory is generated via natural and anthropogenic discharges of pyrogenic PAHs (PAHs formed from the incomplete combustion of carbon). While insignificant to the global PAH inventory, petrogenic PAHs (those found in unburned petroleum) also enter the environment though natural (eg. oil seeps) and anthropogenic activities (eg. oil spills, dumping of coal tar) (Lima et al., 2005). Certain PAHs can also be formed diagenetically, but this process again represents a minor contribution to the global PAH inventory (Wakeham et al., 1980). Most of the PAHs present in Hamilton Harbour are either pyrogenic or petrogenic in origin, and are produced mainly from the combustion of petroleum or petroleum by-products and subsequent atmospheric fallout (Sofowote et al., 2008), and direct releases of PAH into the harbour (Curran et al., 2000; Murphy, 2000).

While natural sources, particularly forest fires, contribute substantially to pyrogenic PAH emissions, anthropogenic emissions are of much greater concern, as they generally cause continuous contamination of a single, well-defined area, leading to accumulation of dangerous levels of PAHs (Environment Canada, 1994). As many PAHs are acutely toxic, or have carcinogenic, teratogenic or mutagenic properties (Wislocki and Lu, 1988; Mastrangelo et al., 1996; Boffetta et al., 1997; Samanta et al., 2002), their removal from contaminated sites is a high priority. At many sites, novel remediation techniques such as phytoremediation and bioremediation are being tested for their ability to remove target pollutants *in situ*. Often, however, these studies rely on only a superficial understanding of the biological mechanisms actually causing the degradation and removal of PAHs.

Bioremediation, the use of microbiological organisms in the degradation of environmental contaminants, has received substantial interest in the past few decades as a method for *in situ* remediation of contaminated sediments, primarily due to the reduced costs associated with this approach as compared to *ex situ* methods, particularly over large areas (Haritash and Kaushik, 2009). Naturally occurring (intrinsic) bioremediation is common in natural systems. Soil and sediment microbial communities are often sufficiently diverse to contain microorganisms capable of degrading most petroleum-derived contaminants, including PAHs (Gibson and Subramanian, 1984; Cerniglia and Heitkamp, 1989; Cerniglia, 1992), and intrinsic bioremediation, while a slow process, represents the primary method by which PAHs are removed from sediments (Cerniglia, 1992).

Despite the ubiquitous occurrence of PAH biodegradation in natural systems, the establishment of enhanced or engineered PAH biodegradation as a viable remediation technology has been hindered because better methods are needed for assessing the progress of microbial degradation. Most currently available methods have serious limitations in their ability to monitor microbial PAH degradation. While changes in substrate concentration or distribution can reflect active microbial degradation, they can also result from sorption to unmeasured components in a system, or other non-degradative processes of mass loss (Slater et al., 2006a). Traditional microbial culturing techniques, and the genetic analysis of microbial cultures, can identify the presence of specific organisms in a microbial community, but cannot directly establish if organisms are actively carrying out metabolic processes resulting in contaminant degradation, and cannot be used *in situ*. Labelled isotopic tracer compounds can be used to demonstrate the occurrence of metabolic processes of interest, through the *in situ* incorporation of ^{13}C- or ^{14}C-labelled carbon substrates into microbial cells and lipids (Roslev et al., 1998; Fang et al., 2004; Hesselsoe et al., 2005). However, the process is costly, requires long time intervals to achieve resolvable signal intensities, and in many cases the isotopically-labelled compound may behave in a substantially different manner to its non-labelled counterpart (Slater, 2003; Slater et al., 2005a; Slater et al., 2006a). Natural abundance stable isotope analysis, particularly if performed at a compound-specific level, can also be used *in situ*, and has been used to identify microbial carbon sources, and to establish the occurrence of specific types of microbial metabolism (eg. methanotrophy) (Boschker and Middelburg, 2002b). The primary challenge with this technique is the need to constrain

the variable effects of isotopic fractionation in biosynthetic pathways in order to properly interpret results (Hayes, 2001). As a further complication, there is often no distinction between the isotopic signatures of contaminants and other "natural" carbon sources that are present (Slater, 2003). The development of novel techniques which permit the flow of carbon in natural environments to be traced directly (eg. radiocarbon isotopic analysis of cell components), should provide the ability to directly establish the occurrence of microbial PAH degradation *in situ*. With the needed monitoring methods in place, more widespread use of biodegradation systems at PAH-contaminated sites should be possible.

The ability to directly monitor microbial carbon cycling could also provide the capability to determine if a specific organism is capable of PAH degradation *in situ*. To date, most studies which have clearly demonstrated the capability of specific species to degrade one or more PAHs have been carried out through the examination of microbial pure cultures of that species provided with the PAH as the sole available source of carbon. Clearly such conditions do not accurately reflect those of natural systems, and thus, while many organisms have been shown capable of degrading PAHs in laboratory studies (Kiyohara et al., 1982; Andersen et al., 2000; April et al., 2000; Kim and Lee, 2007), their ability to do so *in situ* is questionable, given possible interactions with other microbial species, and the potential for microbial incorporation of carbon from non-contaminant sources. Novel techniques permitting the ability to directly establish microbial carbon sources *in situ* will contribute tools to allow more effective assessment of such species' ability to degrade PAHs under natural conditions.

The overarching theme in this thesis was the utilization of phospholipid fatty acids (PLFAs) to investigate interactions between PAHs and microbes, in order to develop a better understanding of microbial responses to PAH contamination. In Chapter 2, this relationship was examined at a broad level, investigating the impact of a specific PAH (phenanthrene) on the size and composition of entire microbial communities *in situ*. Chapter 2 also provided specific insight into PAH contamination and microbial communities in Hamilton Harbour. This first study provided the foundation for the more detailed isotopic-tracer-based study of PAH metabolism carried out in Chapter 3. In Chapter 3, a novel approach using stable carbon and radiocarbon PLFA analyses was employed to establish bacterial preference for PAH as a carbon source, and to identify microbial carbon cycling pathways. As the study was performed under controlled laboratory conditions with several carbon sources provided to bacterial pure cultures, including a PAH, investigation into the likelihood that pure culture bacteria would be able to degrade PAH in natural systems which contain numerous other carbon sources was also possible.

1-1. PAHs in Hamilton Harbour

Hamilton Harbour, Ontario, Canada is a 22 km^2 embayment of western Lake Ontario located within a highly urbanized area with a population of roughly 750 000. The southern shore of the harbour is dominated by heavy industry, including the two largest

steel mills in Canada: ArcelorMittal Dofasco and US Steel (Stelco). Designated in 1985 as one of 43 Areas of Concern (AOC) in Canada and the United States by the International Joint Commission, it is one of the most highly impacted urban sites in Canada (Marvin et al., 2000c).

PAHs are a contaminant of major concern within Hamilton Harbour, and have been identified as such under the Hamilton Harbour Remedial Action Plan (HHRAP), a public-private partnership established to improve harbour conditions (Remedial Action Plan Office, 2009). PAHs enter the harbour system by a number of pathways, including atmospheric fallout from vehicle, ship and industrial emissions (Sofowote et al., 2008), industrial effluent discharges and petroleum spills (Murphy, 2000), and runoff from coal piles (Curran et al., 2000). Extremely high levels of PAHs, in some cases >1400μg/g, have been found in deep harbour sediments (Marvin et al., 2000c; Murphy, 2000). These contaminant concentrations are a legacy of fundamentally untreated waste discharges from the steel mills, related industries, and, to a lesser extent, municipal water treatment plants, prior to improvements in industrial and municipal water treatment in the late 1970s (Remedial Action Plan Staff, 1992). In deep sediments, the highest concentrations of PAHs are found in localized hotspots often associated with old effluent pipes from the steel industry (Murphy, 2000).

Over the past three decades, significant progress has been made in reducing PAH loadings into the harbour, but little to no progress has been made in containing or controlling releases from PAH hotspots. Records of PAH contamination are only available as far back as 1982, and indicate sediments of indeterminate depth at

unspecified locations along the industrial shoreline, or in the central harbour, had concentrations of 8 to 50μg/g. The higher PAH concentrations were observed closer to the steel mills, which were at that point still dumping effluent containing 20μg/L of PAHs into the harbour (Poulton, 1987). A similar range of PAH concentrations was observed in deep sediments (0-20cm) sampled in 1987-88 by Murphy (2000).

By the early 1990's, evidence suggested considerable progress had been made in reducing point source loadings to the harbour. Surface sediments (0-2cm), sampled from the open harbour in 1989-91 (Borgmann and Norwood, 1993) and 1992 (Murphy, 2000), showed up to 90% reductions in PAH concentrations relative to deep (0-20cm) sediments. These studies also showed smaller, but still substantial, reductions in PAH concentrations at PAH hotspots adjacent to industrial docks. However, recent data from the central harbour and Windermere Arm also showed PAH concentrations in suspended sediments were as high or higher than PAH concentrations in deep sediments at the same locations (Marvin et al., 2000c). These high suspended sediment PAH concentrations were likely caused by the re-suspension and transport of the highly toxic deeper bed sediments throughout the harbour. The extensive sampling of deep sediment conducted by Murphy (2000) in 1987-88 revealed a band of highly contaminated deep sediment running north to north-east from Randle Reef, suggesting PAH-laden effluents or sediments from two hotspots near the US Steel boatslip had been re-suspended and deposited across the central harbour in the recent past. Given that Rao et al. (2009) very recently established a model of Hamilton Harbour currents, which suggested the existence of a deep subsurface current flowing in the exact direction of the sediment redeposition suggested in Murphy

(2000), there is no reason to believe this erosion and subsequent redeposition of toxic sediment is not still occurring. Further support for harbour-wide PAH circulation can be found in the highly similar PAH ratios found in June, 2007 by Slater et al. (2008) at sites at opposite ends of Hamilton Harbour, which suggested PAH at both sites originated from the same source. Extensive uncontrolled and highly PAH-contaminated runoff from coal piles on industrial docks (Curran et al., 2000), and, in shallow areas, the turbulent passage of ship traffic (Irvine et al., 1997), presumably also contribute to current suspended sediment PAH loads.

The toxicity of deeper sediments to a variety of organisms, both in terms of mortality and growth inhibition, has been shown to be very high near PAH hotspots (Marvin et al., 2000b; Murphy, 2000; McCarthy et al., 2004), and PAH concentrations lower than those detected at hotspots are known to produce cancerous lesions and tumours in fish (Fabacher et al., 1988; Brand and Goyette, 1989; Swartz et al., 1989). As such, it is clear that remediation of PAH-contaminated deep sediments is essential to reducing human exposure to PAHs and associated health risks. Remediation is also essential to the recovery of a healthy fishery, a key objective of the Hamilton Harbour Remedial Action Plan.

1-2. Carbon Isotope Analysis

The relative abundance of carbon isotopes in organic compounds represents a highly useful source of information for interpreting environmental processes. Three carbon isotopes are present in natural systems: ^{12}C (98.9% abundance) and ^{13}C (1.1% abundance) are stable isotopes, while ^{14}C (1 part per trillion abundance) undergoes radioactive decay.

Throughout this thesis, isotope values are reported in delta notation, which compares isotope ratios or activities in a sample to those of an international standard. ^{13}C data is reported as $\delta^{13}C$, which represents the difference in parts per thousand or per mil (‰) between the ratio of $^{13}C/^{12}C$ in a sample and the $^{13}C/^{12}C$ ratio for the international standard reference material, NBS-19 (VPDB) (equation 1-1). ^{14}C data is reported as $\Delta^{14}C$, which represents the difference, in per mil (‰), between the ^{14}C activity (in counts per minute) measured for the sample (A_{sn}) and for NBS Oxalic Acid I (Standard Reference Material 4990B), the international standard for ^{14}C dating (A_{on}) (Equation 1-2) (McNichol et al., 1994). ^{14}C measurements are reported normalized to $\delta^{13}C$ values of -25‰, the postulated mean value of terrestrial wood. Normalization is standard practice and corrects for any ^{13}C isotopic fractionation during biosynthesis or other processes (Stuvier and Polach, 1977).

$$\delta^{13}C = \frac{^{13}C/^{12}C_{sample} - {}^{13}C/^{12}C_{std}}{^{13}C/^{12}C_{std}} * 1000 \qquad [1\text{-}1]$$

$$\Delta^{14}C = \frac{A_{sn} - A_{on}}{A_{on}} * 1000 \qquad [1\text{-}2]$$

The relative quantities of the stable carbon isotopes (^{12}C and ^{13}C) in organic compounds is variable, and depends upon the original source of the carbon atoms in the particular organic compound, and the biogeochemical reactions involved in the production of that compound. Ratios of $^{13}C/^{12}C$ can often be used to differentiate between multiple possible carbon sources in a system, much like an isotopic "fingerprint", which can be used to identify the specific carbon source or sources from which a specific compound was formed. Both abiotic and biotic processes can alter $^{13}C/^{12}C$ ratios, and thus for source identification to be possible, the isotopic composition of the product compound must remain unaltered by the biogeochemical processes involved in compound formation, or, alternatively, the effects of those processes on isotopic composition must be precisely known. Conversely, if the stable carbon isotope ratios are known for both the carbon source and the product compound, the nature and extent of differences between the two ratios has the potential to reveal which specific process or processes were involved in the production of the product compound.

Alterations in stable isotope ratios are termed isotopic fractionation effects, and can result from the slightly faster reaction rates of ^{12}C over ^{13}C and/or the mass differences between ^{12}C and ^{13}C. When the product of a reaction or series of reactions has a higher $^{13}C/^{12}C$ ratio than the reactant, the product is said to have been isotopically enriched relative to the reactant, or isotopically heavy. When the reaction(s) result in a lower $^{13}C/^{12}C$ ratio in the product, the product is said to be isotopically depleted relative to the reactant, or isotopically light. Abiotic processes can produce products which are either enriched or depleted. Processes carried out by biological organisms, such as

microbial incorporation of carbon, almost always cause the product to become enriched relative to the reactant, due to a biological preference for utilization of ^{12}C over ^{13}C.

Unlike ^{12}C or ^{13}C, radiocarbon (^{14}C) undergoes radioactive decay with a half life of 5730 years (Lima et al., 2005). This decay, along with the trace amounts of $^{14}CO_2$ present in natural systems (roughly 1 part per trillion carbon atoms), allows for a very precise estimate of the age of a substance to be established based upon the absolute quantity of ^{14}C present within the given substance, up to a limit of 60,000 years. Beyond 60,000 years, ^{14}C is present at levels undetectable by modern instrumentation, and, therefore, all substances older than 60,000 years are simply termed "ancient". Like ^{13}C, ^{14}C undergoes fractionation during environmental processing, however, fractionation effects are eliminated via corrections made during data processing, permitting direct comparison of the ^{14}C values (ages) of difference substances.

The ability to differentiate substances based on their age means that ^{14}C analysis can be used to compliment or replace ^{13}C analysis in establishing the identity of the carbon source or sources being used to form a specific compound, as long as the relevant carbon sources are of different ages (and thus have different ^{14}C compositions). In many cases, due to greater resolution and the correction for fractionation, ^{14}C analysis is able to resolve carbon sources when ^{13}C analysis is not.

Use of ^{14}C to differentiate between compounds generally works well in systems contaminated with petroleum hydrocarbons or their derivative products, which can include PAHs. Due to their geologic age, petroleum hydrocarbons contain no detectable ^{14}C, meaning they have a $\Delta^{14}C$ value of -1000‰. Conversely, modern carbon sources

such as natural organic matter (NOM), a common bacterial carbon source composed of a mixture of recently synthesized organic material from a variety of sources, still contain a great deal of ^{14}C ($\Delta^{14}C$=100±50‰, depending upon the time and place fixation occurred). This is because photosynthetic organisms actively fix $^{14}CO_2$ present in the atmosphere, and thus maintain biomass ^{14}C concentrations approximately equal to that of the contemporary atmosphere until their deaths (Lima et al., 2005).

Recently photosynthesized material generally has $\delta^{13}C$ values between -13‰ and -27‰. Most plants fix CO_2 via C3 photosynthesis, a process which causes a strong depletion in ^{13}C, yielding average $\delta^{13}C$ values in recently photosynthesized C3 plant material of -27‰ (Schidlowski, 2001). Fixation of CO_2 by C4 plants is associated with a smaller depletion, yielding average $\delta^{13}C$ values of -13‰ (Schidlowski, 2001).
Since they were originally derived largely from C3 plants, petroleum hydrocarbons generally retain the same $\delta^{13}C$ value of roughly -27‰ as the plants (Stahl, 1977). Due to the large differences in stable carbon and radiocarbon isotopic values between carbon sources such as those mentioned above, ^{13}C and ^{14}C analysis represent important techniques for identifying carbon sources and tracing the flow of carbon in natural systems.

1-3. Application of PLFA and DGGE Techniques in Microbial Ecology

Phospholipid fatty acid (PLFA) and denaturing gradient gel electrophoresis (DGGE) are established techniques for investigating microbial community composition, and community member identities, in soils and sediments. PLFAs are integral components of all bacterial and eukaryotic cell membranes, and as they degrade within days to weeks following cell death (Boschker and Middelburg, 2002a), their composition reflects that of the viable microbial community at the time of sampling. Many PLFA serve as biomarkers, their detection indicating the presence of particular types of microbes such as bacteria or fungi, or particular metabolic processes, such as methanotrophy or sulphate-reduction (Green and Scow, 2000; Boschker and Middelburg, 2002a). Reviews by Green and Scow (2000) and Kaur et al. (2005) have discussed the advantages and disadvantages associated with community characterization by PLFA analysis in detail. Key benefits associated with PLFA techniques include faster analysis times than molecular genetics-based approaches, the ability to sample the entire microbial population directly without bias (unlike culture-based approaches), and the ability to directly estimate total cellular biomass in soils and sediments. The primary weakness of PLFA analysis is the inability to identify the microbial species present in natural systems, as multiple different species present within microbial communities will produce the same PLFAs. For this reason, DGGE analysis naturally complements PLFA analysis, as it is an efficient means for establishing the identity of organisms present in natural systems. DGGE involves separation of microbial DNA sequences, allowing identification, often to

the species level, of any organisms present within a sample whose DNA comprises >1-2% of the total sample DNA.

The unique advantages associated with using PLFAs for microbial community analysis discussed above, as well as the ability to effectively extract and purify PLFA from soils or sediments, make stable isotope analysis of PLFA an ideal method for examining microbial carbon cycling and microbial metabolism in contaminated environments. A review of the theory, methods and applications of PLFA stable isotope analysis is available in Boschker and Middelburg (2002a). Modern gas-chromatography combustion isotope ratio mass spectrometry (GC-IRMS) equipment permits high precision measurements of $\delta^{13}C$ values of either bulk PLFA or individual PLFAs at concentrations of as little as 1 nmol (Boschker and Middelburg, 2002a). Measurements of naturally-occurring variations in $\delta^{13}C$ values resulting from isotopic fractionation during environmental (eg. sorption, volatilization) or microbial metabolic (eg. respiration, carbon fixation) processes can reveal the specific isotopic fractionation effect associated with a specific biogeochemical process. Just as with any other organic compound, natural-abundance measurements of PLFA can also be used to identify the source of carbon utilized by microorganisms for PLFA biosynthesis, if minimal fractionation occurred during the formation of PLFA, or if the exact fractionations associated with PLFA formation are known (Abraham et al., 1998).

Both natural abundance ^{13}C studies and ^{13}C-labelled tracer studies have been used successfully to constrain microbial metabolic processes in natural systems (Gibson and Subramanian, 1984; Boschker et al., 1999; Fang et al., 2004; Cowie et al., 2009), but

these approaches do have limitations. In many environmental systems, two or more carbon sources of interest have $\delta^{13}C$ values which are so similar that they are indistinguishable by natural abundance ^{13}C measurements. Labelled ^{13}C studies can be useful in investigating environmental and microbial processes and pathways, particularly with respect to rate measurements, but can be very expensive, are difficult to effectively implement and monitor *in situ* and the labelled compounds can have inhibitory effects on microbes (Fang et al., 2004), or may be preferentially incorporated relative to the non-labelled target compound (Steinberg et al., 1987).

Many of the difficulties associated with stable isotope PLFA analysis can be effectively overcome via the use of radioisotopic analysis of PLFA. As previously discussed, petroleum hydrocarbons and petroleum-derived compounds contain no detectable ^{14}C ($\Delta^{14}C = -1000‰$), while recently photosynthesized natural organic matter (NOM) generally contains modern levels of ^{14}C ($\Delta^{14}C = +100 \pm 50‰$) (Slater et al., 2006a). As ^{14}C isotopic signatures in bacteria have been shown to reflect the isotopic signatures of the carbon sources they assimilate (Petsch et al., 2001; Kramer and Gleixner, 2006), microbial uptake of petroleum carbon will usually result in lower ^{14}C content in microbial cellular structures than the uptake of NOM. As a result, ^{14}C analysis of microbial PLFAs can be used to differentiate between bacterial utilization of modern and ancient microbial carbon sources. Radiocarbon measurements can be performed on as little as 5-25μg C via accelerator mass spectrometry (AMS) (Shah and Pearson, 2007). Due in part to the higher precision of ^{14}C analysis relative to ^{13}C analysis, and the ability to sample an entire microbial community *in situ* without disturbance by tracer addition,

^{14}C analysis represents a powerful tool to investigate carbon cycling in contaminated systems, either alone or in conjunction with ^{13}C analysis. A dual ^{13}C and ^{14}C analysis approach was employed in Chapter 3 of this thesis to determine the source of microbial carbon in controlled laboratory cultures, providing new insight into the carbon cycling pathways involved in PAH degradation in *Pseudomonas frederiksbergensis*.

1-4. PLFA Biosynthesis

PLFA biosynthesis necessarily requires a cell to acquire carbon to use for that purpose, and to transfer it along the metabolic pathways which produce PLFAs as an end product. During microbial assimilation of carbon, and its transport within the cell, the potential exists for isotopic fractionation to occur at any branch point where carbon is transferred into two different metabolic pathways (eg. acetyl-CoA molecules can be transferred to the TCA cycle, or used to form acetogenic lipids, including PLFA). Such fractionations result from kinetic isotope effects caused by the slightly greater reaction rates of ^{12}C relative to ^{13}C. Fractionation resulting from flow of carbon through a specific series of branchings is consistent, and, therefore, it is often possible to differentiate between specific metabolic pathways based on the extent of fractionation observed between the starting and end products of those pathways. As an example, fixation of CO_2 in photoautotrophic bacteria is typically catalyzed by bacterial (Type II) Rubisco, resulting in an isotope effect of roughly 22‰, while CO_2 fixation by different species of

autotrophic bacteria which employ the enzyme PEP carboxylase is associated with an isotope effect of only 2‰ (Hayes, 2001).

Generally, the largest fractionations during PLFA biosynthesis are associated with CO_2 fixation (in autotrophs only) and fatty acid biosynthesis (both autotrophs and heterotrophs), with relatively minimal fractionation occurring at other branch points. As touched on previously, autotrophic fixation of CO_2 produces biomass carbon more depleted than the source CO_2, but the extent of that depletion is variable, depending upon which particular enzyme catalyzes fixation. Synthesis of n-alkyl lipids, including PLFA, generally produces lipids more depleted in $\delta^{13}C$ than the bulk cell biomass, a consequence of fractionation during the transfer of carbon along fatty acid biosynthetic pathways. In heterotrophic organisms this depletion is on the order of 2-3‰ (Blair et al., 1985; Teece et al., 1999; Hayes, 2001), while autotrophic organisms, including chemoautotrophs, exhibit depletions on the order of 5-12‰ (Sakata et al., 1997; Hayes, 2001; Londry et al., 2004; Sakata et al., 2008).

When the specific metabolic pathways employed by a given bacterium for PLFA biosynthesis are known, PLFA $\delta^{13}C$ values can be used to assist in the identification of microbial carbon sources, as the expected fractionation between PLFA and the carbon source is known. If the specific metabolic pathways are not known for an organism, fractionation between carbon source and PLFA can sometimes be used to constrain if an organism is autotrophic or heterotrophic and to establish which carbon fixation process is employed by autotrophs.

1-5. Research Objectives

The primary objective of Chapter 2 was to establish the impact of PAH contamination upon microbial communities, with specific focus upon communities within Hamilton Harbour. Microbial community PLFA profiles were examined for signals corresponding to increased exposure to PAHs. Despite the fundamental role microbial communities play within ecosystems, the impact of contamination upon those communities is often overlooked. Developing a better understanding of the nature and extent of changes in community composition and population size resulting from contaminant exposure can assist in development of guidelines for protection of ecosystems as a whole, and can also contribute to the development of better means for monitoring and evaluating remediation progress. A secondary objective in Chapter 2 was to establish the sources of PAH contamination influencing the study sites in Hamilton Harbour. For sites located far apart within Hamilton Harbour, such as those in this study, PAH profiling provides insight into the flow of PAHs throughout the harbour and/or changes in PAH source to specific sites. This understanding has implications not only for improving knowledge of PAH transport mechanisms, but can also provide insight into the potential exposure of microbial communities to PAH in areas of the harbour which have not been directly sampled.

The primary objective of the second study (Chapter 3) was to identify the source(s) of carbon utilized for PLFA biosynthesis by a specific bacterium, in order to develop a better understanding of the carbon source preferences and carbon cycling

behaviour of a very common bacterial genus (*Pseudomonas*), and the effectiveness of PLFA ^{14}C analysis in identifying the occurrence of microbial contaminant degradation. The initial goal of this study was to investigate if the bacterium would degrade a PAH contaminant (phenanthrene) when an alternative, potentially more bioavailable environmental carbon source (natural organic matter) was also readily available to bacteria. Chapter 3 also presented a mechanistic model of carbon metabolism in *P. frederiksbergensis*.

Enhanced understanding of microbial carbon cycling pathways and microbial community responses to PAH contamination can lead to improved methods for contaminant remediation, which in turn can lead to improvements in ecosystem health and reduced risk to humans. The continued development and application of novel methods for identifying physical and metabolic responses of bacteria to contaminants of concern, such as the PLFA isotopic analyses employed in this study, may permit the development of new techniques for the preservation and remediation of natural systems.

1-6. References

Abraham, W., Hesse, C., Pelz, O. (1998). Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. Applied and Environmental Microbiology 64, 4202.

Andersen, S.M., Johnsen, K., Sørensen, J., Nielsen, P., Jacobsen, C.S. (2000). *Pseudomonas frederiksbergensis* sp. nov., isolated from soil at a coal gasification site. International Journal of Systemic and Evolutionary Microbiology 50 1957-1964.

April, T., Foght, J., Currah, R. (2000). Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. Canadian Journal of Microbiology 46, 38-49.

Blair, N., Leu, A., Munoz, E., Olen, J., Kwong, E., Des Marais, D. (1985). Carbon isotopic fractionation in heterotrophic microbial metabolism. Applied and Environmental Microbiology 50, 996-1001.

Boffetta, P., Jourenkova, N., Gustavsson, P. (1997). Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. Cancer Causes and Control 8, 444-472.

Borgmann, U., Norwood, W.P. (1993). Spatial and temporal variability in toxicity of Hamilton Harbour sediments: Evaluation of the *Hyalella azteca* 4-week chronic toxicity test. Journal of Great Lakes Research 19, 72-82.

Boschker, H., de Brouwer, J.F.C., Cappenberg, T.E. (1999). The contribution of macrophyte-derived organic matter to microbial biomass in salt-marsh sediments: Stable carbon isotope analysis of microbial biomarkers. Limnology and Oceanography 44, 309-317.

Boschker, H., Middelburg, J. (2002a). Stable isotopes and biomarkers in microbial ecology. FEMS Microbiology Ecology, 85-95.

Boschker, H.T.S., Middelburg, J.J. (2002b). Stable isotopes and biomarkers in microbial ecology. FEMS Microbiology Ecology.

Brand, D., Goyette, D. (1989). Further studies on the prevalence of idiopathic liver lesions in English Sole, *Parophrys vetulus*, from Vancouver Harbor, British Columbia, 1987. In: Protection, C.a. (Ed.). Environment Canada, Vancouver, p. 62.

Cerniglia, C. (1992). Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3, 351-368.

Cerniglia, C., Heitkamp, M.A. (1989). Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment. In: Varanasi, U. (Ed.). Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. CRC Press Inc., Boca Raton, Fla, pp 41-68.

Cowie, B., Slater, G., Bernier, L., Warren, L. (2009). Carbon isotope fractionation in phospholipid fatty acid biomarkers of bacteria and fungi native to an acid mine drainage lake. Organic Geochemistry 40, 956-962.

Curran, K., Irvine, K., Droppo, I., Murphy, T. (2000). Suspended solids, trace metal and PAH concentrations and loadings from coal pile runoff to Hamilton Harbour, Ontario. Journal of Great Lakes Research 26, 18-30.

Environment Canada (1994). Canadian Environmental Protection Act: Priority Substances List Assessment Report. Polycyclic Aromatic Hydrocarbons. Environment Canada,, Ottawa, p. 61.

Fabacher, D.L., Besser, J.M., Schmitt, C.J., Harshbarger, J.C., Peterman, P.H., Lebo, J.A. (1988). Contaminated sediments from tributaries of the Great Lakes: Chemical chacterization and carcinogenic effects in medaka (*Oryzias latipes*). Archives of Environmental Contamination and Toxicology 21, 17-34.

Fang, J., Lovanh, N., Alvarez, P.J. (2004). The use of isotopic and lipid analysis techniques linking toluene degradation to specific microorganisms: applications and limitations. Water Research 38, 2529-2536.

Gibson, D.T., Subramanian, V. (1984). Microbial degradation of aromatic hydrocarbons. In: Gibson, D.T. (Ed.). Microbial Degardation of Organic Compounds. Marcel Dekker, New York, pp 181-252.

Green, C.T., Scow, K.M. (2000). Analysis of phospholipid fatty acids (PLFA) to characterize microbial communities in aquifers. Hydrogeology Journal 8, 126-141.

Haritash, A.K., Kaushik, C.P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. Journal of Hazardous Materials 169, 1-15.

Hayes, J. (2001). Fractionation of the isotopes of carbon and hydrogen in biosynthetic processes. In: Valley, J.W., Cole, D.R. (Eds.). Stable Isotope Geochemistry. Mineralogical Society of America and the Geochemical Society, p. (to be published in this series) 31.

Hesselsoe, M., Nielsen, J., Roslev, P., Nielsen, P. (2005). Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of 14CO2. Applied and Environmental Microbiology 71, 646-655.

Holman, H.-Y.N., Tsang, Y.W., Holman, W.R. (1999). Mineralization of sparsely water-soluble polycyclic aromatic hydrocarbons in a water table fluctuation zone. Environmental Science & Technology 33, 1819-1824.

Hsueh, D., Krakauer, N., Randerson, J., Xu, X., Trumbore, S., Southon, J. (2007). Regional patterns of radiocarbon and fossil fuel-derived CO2 in surface air across North America. Geophysical Research Letters 34, 2816.

Irvine, K., Droppo, I., Murphy, T., Lawson, A. (1997). Sediment resuspension and dissolved oxygen levels associated with ship traffic: Implications for habitat remediation. Water Quality Research Journal of Canada. 32, 421-437.

Johnsen, A.R., Schmidt, S., Hybholt, T.K., Henriksen, S., Jacobsen, C.S., Andersen, O. (2007). Strong impact on the polycyclic aromatic hydrocarbon (PAH)-degrading community of a PAH-polluted soil but marginal effect on PAH degradation when priming with bioremediated soil dominated by mycobacteria. Applied and Environmental Microbiology 73, 1474-1480.

Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R., Kaushik, R. (2005). Phospholipid fatty acid - A bioindicator of environment monitoring and assessment in soil ecosystem. Current Science 89, 1102-1112.

Kawamura, K., Suzuki, I. (1994). Ice core record of polycyclic aromatic hydrocarbons over the past 400 years. Naturwissenschaften 81, 502-505.

Keeling, C.D., Bollenbacher, A.F., Whorf, T.P. (2005). Monthly atmospheric $^{13}C/^{12}C$ isotopic ratios for 10 SIO stations. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tenn, USA.

Kim, J., Lee, C. (2007). Microbial Degradation of Polycyclic Aromatic Hydrocarbons in Soil by Bacterium-Fungus Co-Cultures. Biotechnology and Bioprocess Engineering 12, 410-416.

Kiyohara, H., Nagao, K., Yana, K. (1982). Rapid Screen for Bacteria Degrading Water-Insoluble, Solid Hydrocarbons on Agar Plates. Applied and Environmental Microbiology 43, 454-457.

Kramer, C., Gleixner, G. (2006). Variable use of plant- and soil-derived carbon by microorganisms in agricultural soils. Soil Biology and Biochemistry 38, 3267-3278.

Lima, A., Farrington, J., Reddy, C. (2005). Combustion-derived polycyclic aromatic hydrocarbons in the environment - A review. Environmental Forensics 6, 109-131.

Londry, K., Jahnke, L., Des Marais, D. (2004). Stable carbon isotope ratios of lipid biomarkers of sulfate-reducing bacteria. Appl Environ Microbiol 70, 745-751.

Mandalakis, M., Gustafsson, O., Reddy, C.M., Xu, L. (2004). Radiocarbon Apportionment of Fossil versus Biofuel Combustion Sources of Polycyclic Aromatic .... Environmental Science & Technology.

Martens, D., Maguhn, J., Spitzauer, P., Kettrup, A. (1997). Occurrence and distribution of polycyclic aromatic hydrocarbons (PAHs) in an agricultural ecosystem. Fresenius Journal of Analytical Chemistry 359, 546-554.

Marvin, C., McCarry, B., Villella, J., Allan, L., Bryant, D. (2000a). Chemical and biological profiles of sediments as indicators of sources of contamination in Hamilton Harbour. Part II: Bioassay-directed fractionation using the Ames Salmonella/microsome assay. Chemosphere 41, 989-999.

Marvin, C., McCarry, B., Villella, J., Allan, L., Bryant, D. (2000b). Chemical and biological profiles of sediments as indicators of sources of genotoxic contamination in Hamilton Harbour. Part I: Analysis of polycyclic aromatic hydrocarbons and thia-arene compounds. Chemosphere 41, 979-988.

Mascalet, P., Hoyau, V., Jaffrezo, J.L., Cachier, H. (2000). Polycyclic aromatic hydrocarbon deposition on the ice sheet of Greenland. Part I: Superficial snow. Atmospheric Environment 34, 3195-3207.

Mastrangelo, G., Fadda, E., Marzia, V. (1996). Polycyclic aromatic hydrocarbons and cancer in man. Environmental Health Perspectives 104, 1166.

McCarthy, L.H., Thomas, R.L., Mayfield, C.I. (2004). Assessing the toxicity of chemically fractionated Hamilton Harbour (Lake Ontario) sediment using selected aquatic organisms. Lakes & Reservoirs: Research and Management 9, 89-103.

McNichol, A.P., Osborne, E.A., Gagnon, A.R., Fry, B., Jones, G.A. (1994). TIC, TOC, DIC, DOC, PIC, POC -- unique aspects in the preparation of oceanographic samples for 14C-AMS. Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms 92, 162-165.

Murphy, T. (2000). Coal tar in Hamilton Harbour - attempts at biological restoration guidelines. Environmental Toxicology 15, 484-495.

Ohkouchi, N., Kawamura, K., Kawahata, H. (1999). Distributions of three- to seven-ring polynuclear aromatic hydrocarbons on the deep sea floor in the Central Pacific. Environmental Science & Technology 33, 3086-3090.

Petsch, S.T., Eglington, T.I., Edwards, K.J. (2001). 14C-dead living biomass: evidence for microbial assimilation of ancient organic carbon during shale weathering. Science 292, 1127-1131.

Poulton, D.J. (1987). Trace contaminant status of Hamilton Harbour. Journal of Great Lakes Research 13, 193-201.

Remedial Action Plan Office (2009). Hamilton Harbour Stage 2 Update 2002 - Full Report - June 2003. Hamilton Harbour RAP Stakeholder Forum, p. 306.

Remedial Action Plan Writing Team (1992). Remedial Action Plan for Hamilton Harbour. Stage 1 Report: Environmental Conditions and Problem Definition. Hamilton Harbour Remedial Action Plan Office, Hamilton, Ontario, p. 247.

Roslev, P., Iversen, N., Henriksen, K. (1998). Direct fingerprinting of metabolically active bacteria in environmental samples by substrate specific radiolabelling and lipid analysis. Journal of Microbiological Methods 31, 99-111.

Sakata, S., Hayes, J., McTaggart, A., Evans, R., Leckrone, K., Togasaki, R. (1997). Carbon isotopic fractionation associated with lipid biosynthesis by a cyanobacterium: relevance for interpretation of biomarker records. Geochimica et Cosmochimica Acta 61, 5379-5389.

Sakata, S., Hayes, J., Rohmer, M., Hooper, A. (2008). Stable carbon-isotopic compositions of lipids isolated from the ammonia-oxidizing chemoautotroph *Nitrosomonas europaea*. Organic Geochemistry 39, 1725-1734.

Samanta, S., Singh, O., Jain, R. (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. TRENDS in Biotechnology 20, 243-248.

Schidlowski, M. (2001). Carbon isotopes as biogeochemical recorders of life over 3.8 Ga of Earth history: evolution of a concept. Precambrian Research 106, 117-134.

Shah, S., Pearson, A. (2007). Ultra-microscale (5–25 μg C) analysis of individual lipids by 14C AMS: assessment and correction for sample processing blanks. Radiocarbon 49, 69-82.

Short, J.W., Irvine, G.V., Mann, D.H., Maselko, J.M., al., e. (2007). Slightly Weathered Exxon Valdez Oil Persists in Gulf of Alaska Beach Sediments after 16 Years. Environmental Science and Technology.

Slater, G. (2003). Stable Isotope Forensics - When Isotopes Work. Environmental Forensics 4, 13-23.

Slater, G., Cowie, B., Harper, N., Droppo, I. (2008). Variation in PAH inputs and microbial community in surface sediments of Hamilton Harbour: Implications to remediation and monitoring. Environmental Pollution 153, 60-70.

Slater, G., Nelson, R., Kile, B., Reddy, C. (2006). Intrinsic bacterial biodegradation of petroleum contamination demonstrated *in situ* using natural abundance, molecular-level 14C analysis. Organic Geochemistry 37, 981-989.

Slater, G., White, H., Eglinton, T., Reddy, C. (2005). Determination of microbial carbon sources in petroleum contaminated sediments using molecular 14C analysis. Environmental Science & Technology 39, 2552-2558.

Sofowote, U., McCarry, B., Marvin, C. (2008). Source apportionment of PAH in Hamilton Harbour suspended sediments: comparison of two factor analysis methods. Environmental Science & Technology 42, 6007-6014.

Stahl, W.J. (1977). Carbon and nitrogen isotopes in hydrocarbon research and exploration. Chemical Geology 20, 121-149.

Steinberg, S.M., Pignatello, J.J., Sawhney, B.L. (1987). Persistence of 1,2-dibromoethane in soils - entrapment in intraparticle micropores. Environmental Science & Technology 21, 1201-1208.

Stuvier, M., Polach, H.A. (1977). Discussion: Reporting of 14C data. Radiocarbon 19, 355-363.

Swartz, R.C., Kemp, P.F., Schults, D.W., Ditsworth, G.R., Ozretich, R.J. (1989). Acute toxicity of sediment from Eagle Harbour, Washington, to the infaunal *Rhepoxynius abronius*. Environmental Toxicology and Chemistry 8, 215-222.

Szidat, S., Jenk, T.M., Gaggeler, H.W., Synal, H.A., Fisseha, R., Baltensperger, U., Kalberer, M., Samburova, V., Wacker, L., Saurer, M., Schiwikowski, M., Hajdas, I. (2004). Source apportionment of aerosols by radiocarbon (C-14) analysis of different carbonaceous particle fractions. Radiocarbon 46, 475-484.

Tavlarides, L.L., Zhou, W., Anitescu, G. (2000). Supercritical fluid technology for remediation of pcb/pah-contaminated soils / sediments. Proceedings of the 2000 Conference on Hazardous Waste Research, Denver, Colorado, pp. 239-255.

Teece, M., Fogel, M., Dollhopf, M., Nealson, K. (1999). Isotopic fractionation associated with biosynthesis of fatty acids by a marine bacterium under oxic and anoxic conditions. Organic Geochemistry 30, 1571-1579.

Turnbull, J., Lehman, S., Miller, J., Sparks, R., Southon, J., Tans, P. (2007). A new high precision 14CO2 time series for North American continental air. Journal of Geophysical Research 112, 1-10.

van Herwijnen, R., Wattiau, P., Bastiaens, L., Daal, L., Jonker, L., Springael, D., Govers, H.A.J., Parsons, J.R. (2003). Elucidation of the metabolic pathway of fluorene and cometabolic pathways of phenanthrene, fluoranthene, anthacene and dibenzothiophene by Sphingomonas sp. LB126. Research in Microbiology 154, 199-206.

Wacey, D. (2009). Early Life on Earth, A Practical Guide. Springer Science and Business Media B.V.

Wakeham, S., Schaffner, C., Giger, W. (1980). Polycyclic aromatic hydrocarbons in recent lake sediments - 2. Compounds derived from biogenic precursors during early diagenesis. Geochimica et Cosmochimica Acta 44, 415-429.

Wislocki, P.G., Lu, A.Y.H. (1988). Carcinogenicity and mutagenicity of proximate and ultimate carcinogens of polycyclic aromatic hydrocarbons. In: Yang, S.K., Silverman, B.D. (Eds.). Polycyclic aromatic hydrocarbon carcinogenesis: structure-activity relationships. CRC Press, Boca Raton, FL, pp 1-30.

CHAPTER 2

Assessment of the impact of PAH and TPH contamination upon Hamilton Harbour surface sediment microbial communities via PLFA profiling.

2-1. Introduction

Hamilton Harbour, Ontario, Canada is a 22 km^2 embayment of western Lake Ontario located within a highly urbanized area with a population of roughly 750 000. The southern shore of the harbour is dominated by heavy industry, including the two largest steel mills in Canada. Designated one of 43 Areas of Concern (AOC) in Canada and the United States by the International Joint Commission (Marvin et al., 2000a), it is one of the most highly impacted urban sites in Canada. High levels of suspended solids, heavy metals and organic compounds in surface waters and harbour sediments have lead to extensive degradation of water quality and reduced capacity for recreational water usage. In several areas within the harbour, concentrations of heavy metals and organic contaminants, which include polycyclic aromatic hydrocarbons (PAHs), organochlorine compounds and polychlorinated biphenyls (PCBs), exceed the "severe effect level" (SEL) for provincial sediment quality guidelines (PSQGs) in suspended and bed sediments (Zeman and Patterson, 2006). The SEL represents contaminant levels at which significant biological impairment is expected. Areas in which the SEL is exceeded are focused around contaminant point sources. These include steel mill effluent pipes, coal stockpiles on industrial docks (from which a total of >60kg/yr of highly toxic substances

enter the harbour from runoff) (Curran et al., 2000), and Randle Reef, a localized hotspot with total concentrations of 16 high priority PAHs exceeding 1400 ug/g dry sediment (Murphy, 2000). Extensive shipping traffic (Irvine et al., 1997), and subsurface harbour currents (Murphy, 2000; Rao et al., 2009), cause the disturbance and redeposition of surface and buried sediments near these contaminant point sources, resulting in the spread of contaminants throughout the harbour.

PAH-contaminated Hamilton Harbour sediments represent a serious danger to human and ecosystem health. Due to evidence of adverse impacts on the environment, PAHs are listed on the Priority Substances List under the Canadian Environmental Protection Act, with 5 specific PAHs listed as a possible danger to human life or health. The U.S. Environmental Protection Agency lists 16 specific PAHs as priority pollutants for remediation (Keith and Telliard, 1979), due in part to acutely toxic, carcinogenic, teratogenic or mutagenic properties (Wislocki and Lu, 1988; Mastrangelo et al., 1996; Boffetta et al., 1997; Samanta et al., 2002). PAHs are highly persistent in aquatic systems due to low aqueous solubility. This hydrophobicity leads to a rapid association between PAHs and particles in soil, air and water, and these contaminated particles are eventually transported into suspended and surface bed sediments where they accumulate (Means et al., 1980; Guha and Jaffe, 1996). In the U.S., this has resulted in the contamination of an estimated 10% of river, lake and coastal sediments (Boyd et al., 2005). Burial of contaminated surface sediments after deposition, generally caused by sedimentation, can further reduce the rate of PAH degradation and increase their environmental persistence. Burial reduces bioavailability, and those organisms still able to access the PAHs must rely

on anaerobic degradation processes, as dissolved oxygen levels typically decrease rapidly below the sediment-water interface in organic-rich sediments (Rothermich et al., 2002; Haritash and Kaushik, 2009). While recent evidence has suggested anaerobic degradation of a limited number of low molecular weight PAHs (2-3 benzene rings) can proceed at a relatively rapid pace, in general anaerobic degradation occurs much more slowly than aerobic degradation, particularly for high molecular weight PAHs (>3 benzene rings) (Rothermich et al., 2002; Haritash and Kaushik, 2009).

Under a defined remedial action plan (RAP), remediation efforts in Hamilton Harbour have been undertaken by local stakeholders, including steel manufacturers ArcelorMittal Dofasco and US Steel (Stelco), along with national, provincial and municipal environmental agencies (Remedial Action Plan Writing Team, 1992; Zeman and Patterson, 2006). Improved control over municipal and industrial effluent and air emissions has lead to substantial reductions in metal concentrations in Harbour waters as well as decreases in PAH and PCB concentrations in surface sediments (Murphy, 2000; O'Connor, 2002). Due to limited progress in treating or capping contaminant hotspots, however, bed sediments remain highly contaminated with PAHs, PCBs and trace metals, which very likely contributes to continued suspended sediment and water contamination (Murphy, 2000; Zeman and Patterson, 2006).

A primary goal of the RAP is the restoration of ecosystem function, leading to reestablishment of fish and wildlife (O'Connor, 2002). Assessing the impact of contamination upon ecosystem function is difficult, as different species can exhibit differing tolerances for the same contaminant. To date, multiple lines of evidence have

been examined, including studies of elevated contaminant concentrations on fish (Hayes et al., 1990; Karrow et al., 2003; Kavanagh et al., 2004), snapping turtles (de Solla et al., 2008), and insects and planktonic crustaceans (Murphy, 2000). These studies suggest the elevated levels of contaminants observed in Hamilton Harbour lead to greater organism mortality and higher incidence rates of cancer and birth defects.

Microbial communities are also a fundamental component of the Hamilton Harbour ecosystem, and as such have great potential to provide a useful measure of ecosystem health. Despite this, the effects of contamination upon the composition and metabolic activity of microbial communities in the harbour are largely unknown. Previous studies have investigated changes in microbial physiology caused by exposure to contaminated sediments, such as variations in light output of phospholuminescent bacteria due to contamination (Murphy, 2000), and contaminant-induced mutations in *Salmonella* strains (Marvin et al., 1993; Marvin et al., 2000a). Droppo et al. (2007) investigated the microbial community composition of Hamilton Harbour sediments using phospholipid fatty acid (PLFA) and denaturing gradient gel (DGGE) analyses, but the focus of that work was unrelated to contamination. Only one previous study, again utilizing PLFA analysis, has directly examined the impact of contamination upon microbial community composition in Hamilton Harbour. Slater et al. (2008) briefly examined PLFA profiles at sites characterized by high and low PAH concentrations, finding significant differences in total microbial biomass, but relatively limited differences in community composition.

For this study, PLFA and DGGE approaches were utilized to examine the microbial communities in Hamilton Harbour sediments. PLFA and DGGE analyses are ideal techniques for such an investigation as PLFA are integral components of cell membranes. As such, they degrade within days to weeks following cell death (Harvey et al., 1986; Boschker and Middelburg, 2002b), and thus, unlike many culture-dependent methods, they closely represent the viable microbial community at the time of sampling. Many PLFA also serve as biomarkers, being produced only by specific types of microbes (eg. bacteria, fungi, algae), or by microbial populations involved in particular metabolic processes (eg. methanotrophy, sulphate-reduction) (Green and Scow, 2000; Boschker and Middelburg, 2002b). PLFA concentrations can also permit direct estimation of microbial biomass, through the use of conversion factors (Green and Scow, 2000). PLFA analysis is limited in that specific organisms within the community cannot be identified. However, this can be resolved through the use of DGGE analysis, which permits the identification of dominant organisms within a microbial population (>1-2% of total population), often to the species level.

PLFA analysis has previously been employed to investigate changes in the microbial community composition of PAH-contaminated soils (Yang et al., 2007; Antizarladislao et al., 2008; Su and Yang, 2009) and sediments (Slater et al., 2008). Generally, increased PAH contamination has been observed to cause reductions in microbial biomass, and increases in the ratio of anaerobes to aerobes and bacteria to fungus. DGGE analysis has been used to investigate PAH-contaminated mangrove sediments, revealing decreases in microbial diversity with increased PAH contamination

and exposure times (Zhou et al., 2009). To the best of our knowledge, PLFA & DGGE analyses have not been previously combined to investigate the effects of PAH contamination on microbial communities.

In this study, we examined sediments at the same two sites in Hamilton Harbour previously examined by Slater et al. (2008), but PAH data was examined to greater depths, and a much more detailed microbial community analysis was conducted. Site 1 is located in Carole's Bay, near Cootes Paradise and the mouth of Grindstone Creek (Figure 2-1). The relative isolation of this area from the rest of the harbour, including the zone of heavy industry on the opposite shore, suggests that this site is located in one of the least contaminated zones of the harbour. Past studies have shown PAH concentrations in suspended solids (Marvin et al., 2000c) and bed sediments (Slater et al., 2008) at or near this site to be substantially lower than those near industrial activity on the opposite shore. No sources of PCBs to Site 1 are known. Site 2 is located in Windermere Arm, a narrow 50 ha channel commonly used as a shipping channel in the southeast of the harbour. The site is proximal to the boat slip for ArcelorMittal Dofasco, as well as other heavy industry. Bed sediments and suspended sediments in this area are contaminated with PAHs at much higher levels than at the western end of the harbour (Marvin et al., 2000c; Murphy, 2000; Slater et al., 2008). Site 2 surface sediments were previously measured and PCB concentrations did not exceed the GC-MS detection limit of 0.5ppm (unpublished data). Recent substantial reductions in metal loadings to the Harbour have reduced trace metal concentrations in surface sediments (0-10cm) near Site 2 below PSQG severe effect levels. These levels are still well above PSQG lowest effect levels, at

which the majority of sediment-dwelling organisms remain unaffected (Zeman and Patterson, 2003), and therefore metal contamination may still have an impact on microbial communities. Trace metal concentrations in sediments near Site 2 have traditionally been much higher than near Site 1 (OME, 1975, 1977; Remedial Action Plan Writing Team, 1992), but more recent data is not available. With evidence of significant improvements between 1975 and 1980 due to enhancements at the Dundas sewage treatment plant (OME, 1986), it seems a reasonable assumption that current metal loadings at Site 1 have been reduced at a rate commensurate to Site 2.

The primary goal of this study was to assess the effect of contaminant exposure upon microbial community composition, and to investigate possible signals in Hamilton Harbour microbial communities corresponding to contaminant exposure. To this end, PLFA and DGGE profiles for each site were compared to physical and geochemical site properties, as well as the concentrations of PAH, total petroleum hydrocarbons (TPH) and non-extractable organic residue (NEOR) associated with natural organic matter. Secondarily, PAH deposition data was used to investigate recent trends in the sources and concentrations of PAHs at each study site. Diagnostic ratios between specific PAHs were examined to assess PAH sources.

Beyond the immediate goals of this study, developing an understanding of current microbial population composition and size at key sites within the Harbour will help to establish a reference against which future changes in population size or composition can be compared. Establishment of such a reference would also allow monitoring of relevant

changes in population size or composition to be employed as an additional tool in assessing the effectiveness of future remediation efforts in Hamilton Harbour.

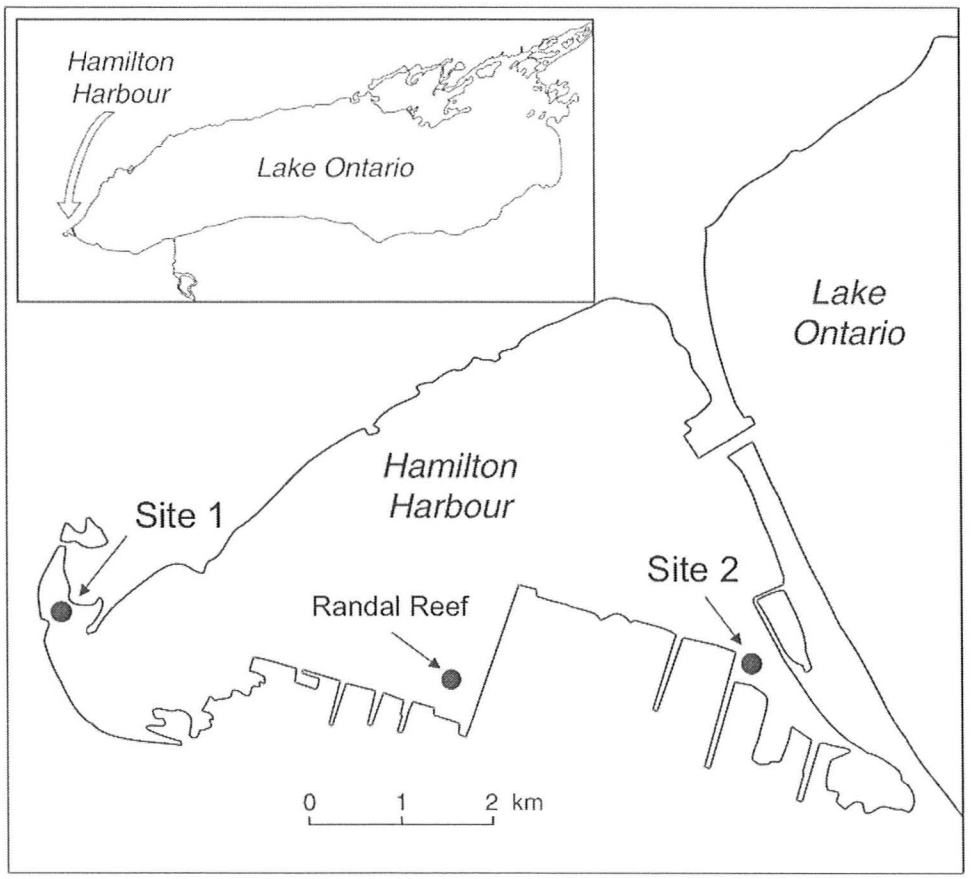

Figure 2-1: Location of study sites within Hamilton Harbour, Ontario, Canada

2-2. Methods

2-2.1 Field Sampling

2-2.1.1 Sediment Cores

In June, 2007, sediment was obtained from Site 1 (GPS coordinates: N 043° 16.943', W 079° 53.011') and Site 2 (N 43° 16.504', W 079° 47.596') using a large cubic box corer (roughly 120cm^3). Two box core samples were taken at each site, with the second box core sample taken within 15 feet of the first. From each box core sample, 4 push cores were taken with 10cm diameter plastic core barrels. These push core samples were plugged at the bottom with a rubber stopper within the box corer and then extracted. Cores were immediately extruded on the sampling barge, and the upper 12 cm of each core was sectioned into 3cm slices using clean, sterile metal implements. Core slices were immediately transferred to pre-combusted 500ml clear glass jars with solvent-rinsed PTFE lids. Care was taken to avoid inclusion of sediment at the edges of the core slice. Within hours of sampling, 0-3cm core slices from 3 push cores taken at each site (2 from the first box core sample, 1 from the second) were stored at 0°C in preparation for PLFA analysis, while remaining core slices were stored at -20°C. Samples were maintained at these temperatures until analysis.

2-2.1.2 Water Column Measurements

During the same sampling trip in which cores were obtained, temperature, pH, dissolved oxygen (DO), turbidity and chlorophyll concentration within the water column at Site 1 and Site 2 were measured at approximately 4mm intervals using a 6-Series Water Quality Sensor (YSI Inc., OH, USA).

2-2.2 Hydrocarbon/Organic Matter Analyses

2-2.2.1 Sediment Preparation

Sediment from Site 1 and Site 2 was analysed to establish bulk concentrations for total petroleum hydrocarbons (TPH) and individual PAH concentrations at 3cm intervals from a depth of 0 to 9cm below the sediment water interface (SWI). The bulk concentration of all organic residue not extracted during TPH analysis was also established at 3cm intervals by subtracting the TPH concentration from the total organic carbon (TOC) concentration of samples. We have termed the organic component represented by this subtraction non-extractable organic residue (NEOR), as it is composed of natural organic matter as well as other organic compounds recalcitrant to extraction.

20 g of sediment was taken for a single TPH/PAH analysis from each 3cm core slice in a single Site 1 and a single Site 2 sediment core. Replicability for these analyses had previously been established to be better than 10%. Samples were oven-dried at 50°C,

large organics such as twigs were removed, and samples were homogenized using a mortar and pestle. 3-5g of homogenized sediment was inserted in PTFE Greenchem microwave vessels (CEM Corp., Matthews, North Carolina, USA) with 20-30ml 1:1 hexane:acetone solution. 20ppm 5α-cholestane solution was added as an internal standard to all sediment samples and a sample blank in order to calculate recoveries. Samples were inserted into the MARS microwave-based solvent extraction unit (CEM Corp., Matthews, North Carolina, USA), which was programmed to increase sample temperatures to 115°C over 10min, hold at 115°C for 15min and then cool down to 40°C. Samples were filtered though solvent-cleaned glass fibre filter paper. Both the extraction vessel and the filtering apparatus were rinsed 3x with hexane. Resulting filtrate volume was reduced to 1mL under UHP N_2, activated copper was added, and samples allowed to sit overnight. Copper was subsequently removed by filtration exactly as described above. To remove excess organic analytes, the refiltered sample was fractionated in a glass silica gel column by elution with a solution of 1:1 hexane:DCM and methanol. The hexane:DCM fraction and methanol fractions were reduced to 400-500uL under UHP N_2 and then transferred to a 1mL vial. Prior to analysis on the GCMS, 25ul of 200ppm o-terphenyl solution was injected into the hexane:DCM vial as an internal standard and the total volume increased to exactly 1ml.

TOC concentrations were measured by the Stable Isotope Geochemistry Laboratory at McMaster University using an additional 20g of sediment from each core slice. NEOR concentrations were calculated by subtracting the TPH concentration from the TOC concentration.

2-2.2.2 PAH/TPH GC-MS Analysis

For PAH and TPH identification and quantification, a small subsample of the hydrocarbon mixture was analyzed by gas chromatography-mass spectrometry on an Agilent 6890 gas chromatograph with a J&W DB-5 column (30 m x 0.25 mm i.d.; 0.25 μm film thickness) coupled with an Agilent 5973 quadrupole mass spectrometer. The oven temperature was initially set at 80°C and ramped to 270°C at 10°C/min, with a final 15 minute hold at 270°C. PAHs monitored for this study were naphthalene, acenapthalene, fluorene, pheanthrene, anthracene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene & benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-c,d)pyrene and benzo(g,h,i)perylene. Reproducibility was better than 15% for all replicates and was greater for peaks which eluted earlier. TPH concentration was established by adding PAH concentrations to the concentration calculated for the unconsolidated mixture (UCM) hump detected on the GC-MS.

2-2.3 PLFA Analysis

2-2.3.1 PLFA Extraction

PLFA sediment analysis was initiated within 2 weeks of sample collection. For each sampling site, ~15-20g of wet sediment from three separate 0-3cm core slices was

extracted following a modified Bligh and Dyer protocol (White and Ringelberg, 1998; Slater et al., 2006b). In brief, 3-5g of sediment from each bottle was dried at 110°C for 24 hours to determine sediment moisture content. The remaining 10-15g of sediment was sonicated in a 2:1:0.5 methanol:dichloromethane:phosphate (v/w) buffer solution for 2 minutes and then allowed to sit for 18-24 hours. Samples were gravity filtered and the organic phase was fractionated in a glass silica-gel column by elution with dichloromethane, acetone and methanol. The phospholipid fraction was evaporated to dryness under UHP N_2 and derivatized to fatty acid methyl esters (FAMEs) via the mild alkaline hydrolysis reaction. A second silica-gel column fractionation step with 4:1 hexane:DCM, DCM and MeOH ensured FAME purity prior to GCMS analysis.

2-2.3.2 GC-MS Analysis of PLFAs

For FAME identification and quantification, a small subsample of the FAMEs mixture was analyzed by gas chromatography-mass spectrometry on an Agilent 6890 gas chromatograph with a J&W DB-XLB column (30 m x 0.25 mm i.d.; 0.25 μm film thickness) coupled with an Agilent 5973 quadrupole mass spectrometer. The oven temperature was initially held at 40° C for 1 min, ramped to 130°C at 20°C/min, to 160°C at 4°C/min and finally to 300°C at 8°C/min. FAME identification was based upon mass-fragmentation patterns and retention times as compared to several bacterial reference standards (Bacterial Acid Methyl Esters CP Mix, Matreya Inc., 14-methylhexadecanoic acid, Supelco Inc., 17-methylstearic acid, Supelco Inc.). FAME double bond positions

were determined by analysis of dimethyl disulphide adducts as per Nichols et al. (1986), whose method was determined to produce identical results to the more recent methods employed by Shibahara et al (2008) and Yamamoto et al. (1991). FAME quantification was established relative to an external standard curve generated using C-12, C-14, C-16, C-18 and C-20 FAME standards at concentrations ranging from 2ppm to 30ppm.

FAMEs are identified according to the following nomenclature: total number of carbon atoms:number of double bonds, then an integer following a lowercase omega (ω) indicates the position of the double bond (if present) from the methyl end of the molecule (e.g., 16:1ω6). Cis and trans geometry is indicated by the suffixes c and t. The prefixes a and i refer to anteiso- and isobranching, 10me indicates a methyl group on the 10th carbon atom from the carboxyl end of the molecule, the positions of hydroxy (OH) groups, if present, are noted in the same manner, and the prefix Δ indicates cyclopropane fatty acids.

2-2.4 DGGE Analysis

Approximately 15g of wet sediment was taken from one of the 0-3cm core sections from each sample site. These were placed in 50mL glass jars with PTFE lids and shipped on ice to Microbial Insights, Inc. (Rockford, TN). While there, samples were subjected to analysis by denaturing gradient gel electrophoresis (DGGE) in order to identify dominant members of the sediment microbial community.

DGGE separates microbial DNA sequences, allowing identification of organisms, often to the species level. Separation is based upon the differing bond strengths of nucleotide base pairs in DNA. Adenine (A) forms 3 hydrogen bonds with Thymine (T) , while Cytosine (C) forms only 2 hydrogen bonds with guanine (G). DNA is isolated and specific sequences amplified by polymerase chain reaction. Amplification products are loaded in denaturing gradient gels, and exposed to steadily increasing quantities of denaturing chemicals as they are driven across the gel by an electric current. DNA sequences with greater numbers of A-T bonds move further down the gel, as they are more resistant to the chemicals. Identical DNA sequences migrate the same distance, grouping to form a visible band. This band can be excised and sequenced to determine microbial identity if the organism is present as >1-2% of the sample DNA. Sequencing results are compared to sequence databases to identify dominant organisms.

DNA in the sediment samples was isolated and amplified using both 16S (prokaryotes) and 18S (fungi) rRNA primers. Amplification products were run on DGGE gels and visible bands were excised, sequenced and identified by comparison with 16S and 18S sequence databases.

2-3. Results

2-3.1 Physical Properties of Study Sites

Study Site 1 had a depth of approximately 1.32 metres and Study Site 2 had a depth of approximately 7.80 metres. At comparable depths, Site 1 water temperature was marginally warmer than that at Site 2 (Figure 2-2), water column pH was lower than at Site 2 (Figure 2-2), water column dissolved oxygen (DO) and chlorophyll concentrations were slightly lower than at Site 2 (Figure 2-3) and turbidity was substantially higher than at Site 2 (Figure 2-4).

For Site 2 at increasing depths below 1.32m, the rate of decrease in temperature and pH steadily slowed until a plateau was effectively reached at the sediment-water interface (SWI), DO concentrations decreased at an increasingly rapid rate, until stabilizing at a depth of approximately 5.5 to 6.5m and then rapidly decreasing to a minimum value of 6.5 mg/L at the SWI. Site 1 was supersaturated with respect to DO throughout the water column and Site 2 was supersaturated from 0-5m, but dropped to a minimum percent saturation of roughly 68% at the sediment water interface. Changes in chlorophyll concentration with depth were similar to the profile for DO. From the surface they dropped slightly, reaching a local minimum around 2.5m depth, increased back to maximum observed levels at a depth of approximately 3.5m, and then dropped steadily until reaching a stable minimum value at roughly 6m depth. Turbidity levels decreased

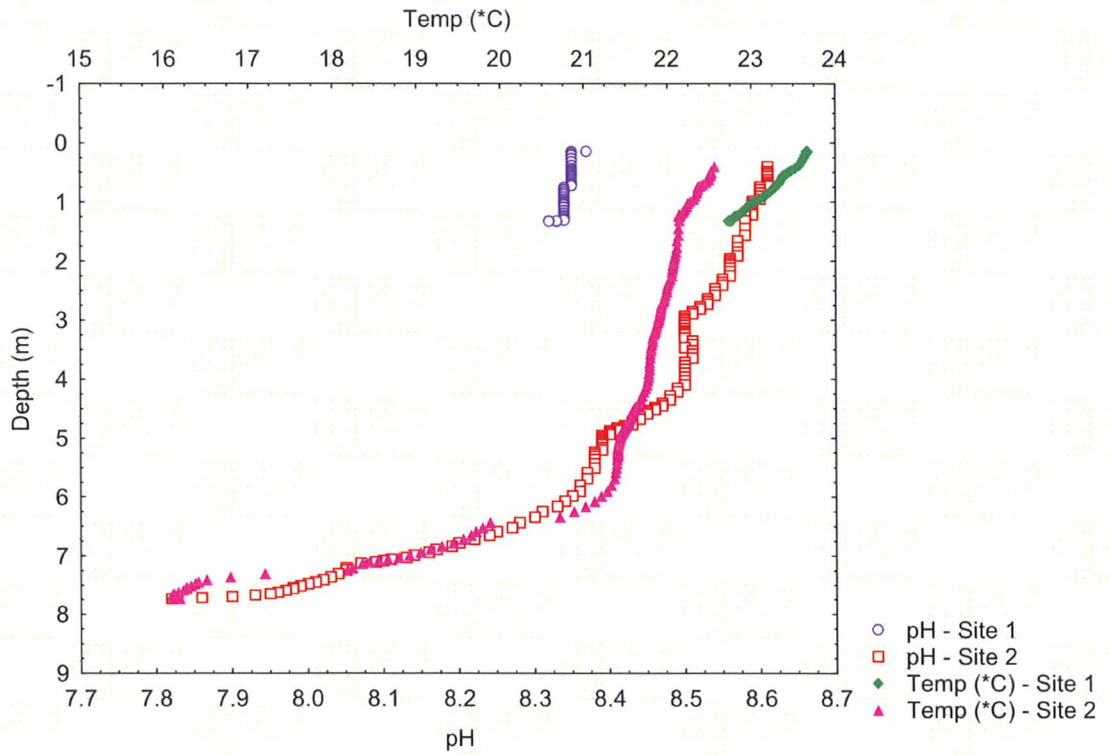

Figure 2-2: Temperature and pH of water column at Site 1 and 2 from lake surface (0m) to sediment-water interface.

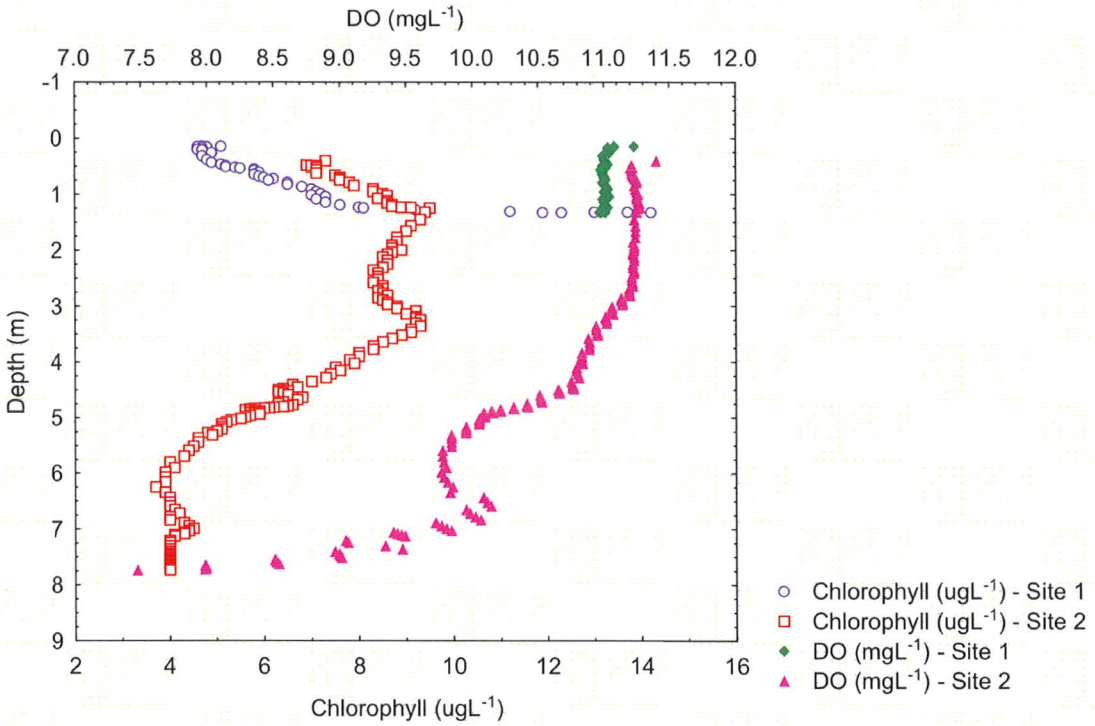

Figure 2-3: Concentrations of dissolved oxygen (DO) and chlorophyll in water column at Site 1 and 2 from lake surface (0m) to sediment-water interface.

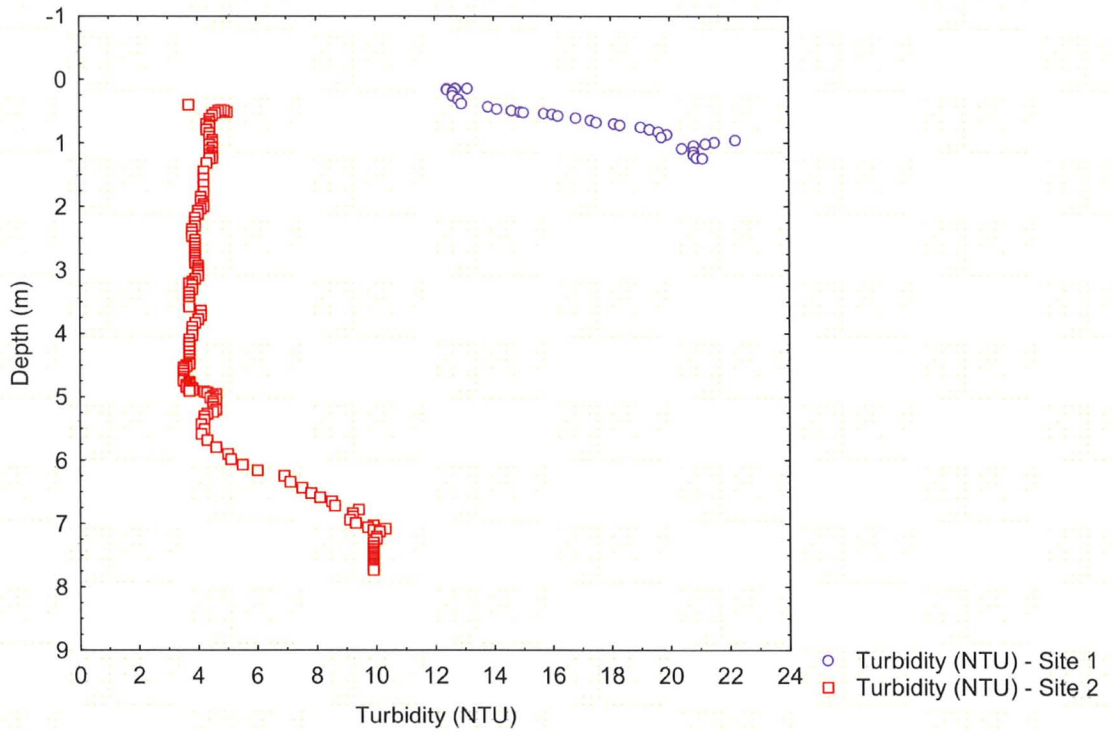

Figure 2-4: Turbidity within water column from lake surface (0m) to sediment water interface at Site 1 and Site 2. Units employed are nephelometric turbidity units (NTU).

slightly to a depth of approximately 5.5m, before increasing and reaching a stable maximum value at roughly 7.1m depth.

At comparable depths, temperature and pH at both sites decreased with depth at roughly the same rate, DO concentrations at both sites remained relatively stable, with a slight increase near the water surface, chlorophyll concentrations at both sites increased with depth at a similarly rapid rate and turbidity levels at Site 2 decreased slightly with depth, while at Site 1 they increased rapidly with depth.

Surface sediments at both sites should have been in approximate equilibrium with water directly overlying it. Thus, water conditions measured at the SWI provided important insight into conditions within surface sediments. Compared to the Site 2 SWI conditions, the Site 1 SWI temperature was approximately 6.5°C warmer, Site 1 pH was 0.7 higher, DO and chlorophyll concentrations at Site 1 were almost double those at Site 2, and turbidity at Site 1 was slightly more than double that at Site 2.

2-3.2 Hydrocarbon/Organic Matter Concentrations

Concentrations of all three types of organic matter measured were considerably higher at Site 2. At Site 2, polycyclic aromatic hydrocarbon (PAH) concentrations in 0-6cm sediments were approximately 3 times greater than at Site 1 and PAH concentrations in 6-9cm sediments were approximately 6 times greater than at Site 1 (Figure 2-5). The change in deeper sediment was largely driven by an approximately 5ug/g decrease in 6-

9cm sediment PAH concentrations at both sites, which had a small impact on the overall concentration at Site 2, but represented a 50% decrease at Site 1. Site 2 total petroleum hydrocarbon (TPH) concentrations at all depths were approximately 5 times greater than at Site 1, with concentrations remaining reasonably stable with increasing depths (Figure 2-5). Site 2 non-extractable organic residue (NEOR) concentrations at all depths were 1.5 to 1.8 times greater than at Site 1. NEOR concentrations at each site were relatively stable throughout the depth of the sediment cores, save for 0-3cm sediments at Site 2 which had significantly lower NEOR concentrations (closer to Site 1 values) than the rest of the Site 2 core.

At both sites, NEOR concentrations were on the scale of 2-3 orders of magnitude greater than PAH and TPH concentrations. At Site 1, TPH concentrations were roughly 5x greater than PAH concentrations, and, at Site 2, TPH concentrations were roughly 8x greater than PAH concentrations.

At each site, concentrations of individual PAHs were stable along the depth of the core, with differences exceeding measurement error in only 4 cases (Figure 2-6a,b). At Site 1, fluorine concentrations in 0-3cm sediments were lower than in 6-9cm sediments. At Site 2, naphthalene concentrations increased significantly with increasing depth, indeno(1,2,3-c,d)pyrene concentrations were significantly higher in 6-9cm sediments than 0-3cm sediments and benzo(g,h,i)perylene concentrations were significantly higher in 0-3cm sediments than 3-6 and 6-9cm sediments.

Between study sites, individual PAH concentrations varied substantially (Figure 2-6a,b). High molecular weight PAHs with 4-6 benzene rings (pyrene, chrysene,

benzo(b)fluoranthene & benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-c,d)pyrene and benzo(g,h,i)perylene) are most useful in comparing PAH signatures between sites, as their high environmental stability means degradation will not have affected them to a great extent. Acenapthylene, anthracene and phenanthrene were completely absent at Site 1, while at Site 2 they were present at an average of 115ug/kg, 375ug/kg and 1590ug/kg, respectively. On average, site 2 concentrations of fluorene, benzo(a)pyrene, indeno(1,2,3-c,d)pyrene and benzo(g,h,i) perylene were approximately 2-3.5 times greater than Site 1 concentrations, benzo(b)fluoranthene & benzo(k)fluoranthene and benz(a)anthracene concentrations averaged approximately 5 times greater at Site 2, fluoranthene, pyrene and chrysene concentrations averaged 11-20 times greater at Site 2 and naphthalene concentrations averaged 46x greater at Site 2. The overall PAH concentration profile at each site was nearly identical to the mole percentage profile.

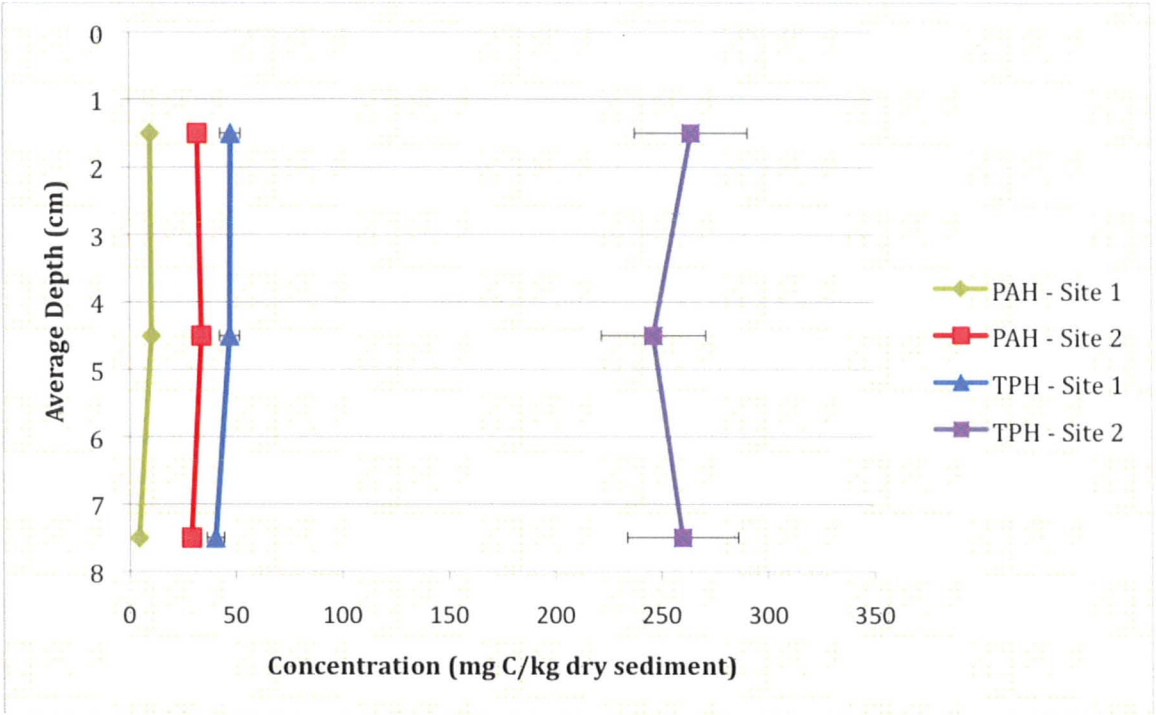

Figure 2-5: Total concentration of polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPHs) in sediments sampled in proximity to the Dofasco boat slip (Site 2) and within Carole's Bay (Site 1). Values are reported in milligrams of carbon per kilogram of dry sediment. Error bars represent 10% relative standard deviation resulting from GCMS machine error.

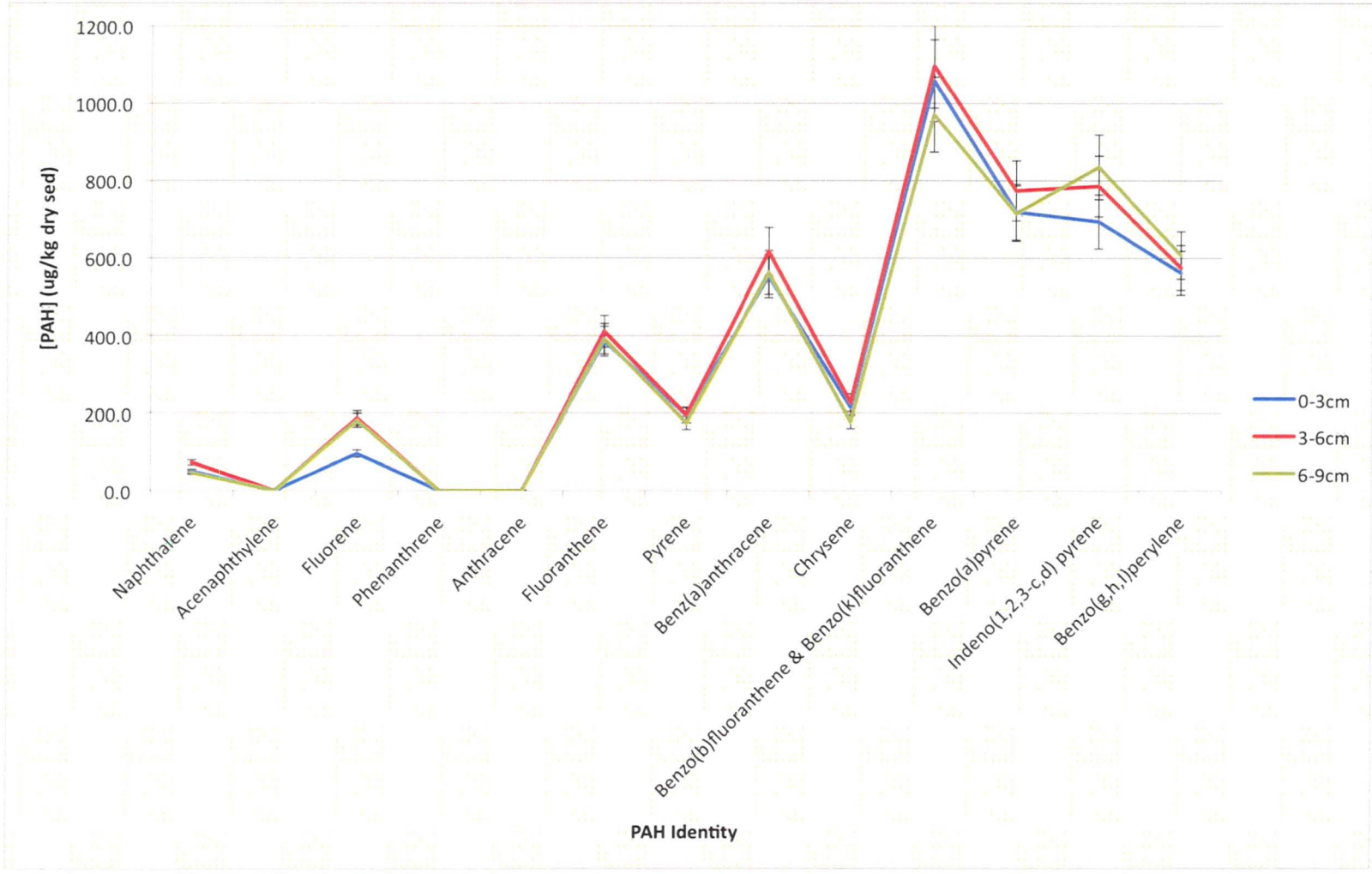

Figure 2-6a: Individual PAH concentrations in a sediment core obtained from Site 1 and sectioned into 3cm slices from 0-9cm depth below the sediment water interface. Error bars represent 10% relative standard deviation resulting from GCMS machine error. PAHs are arranged in order of increasing molecular weight (left - right).

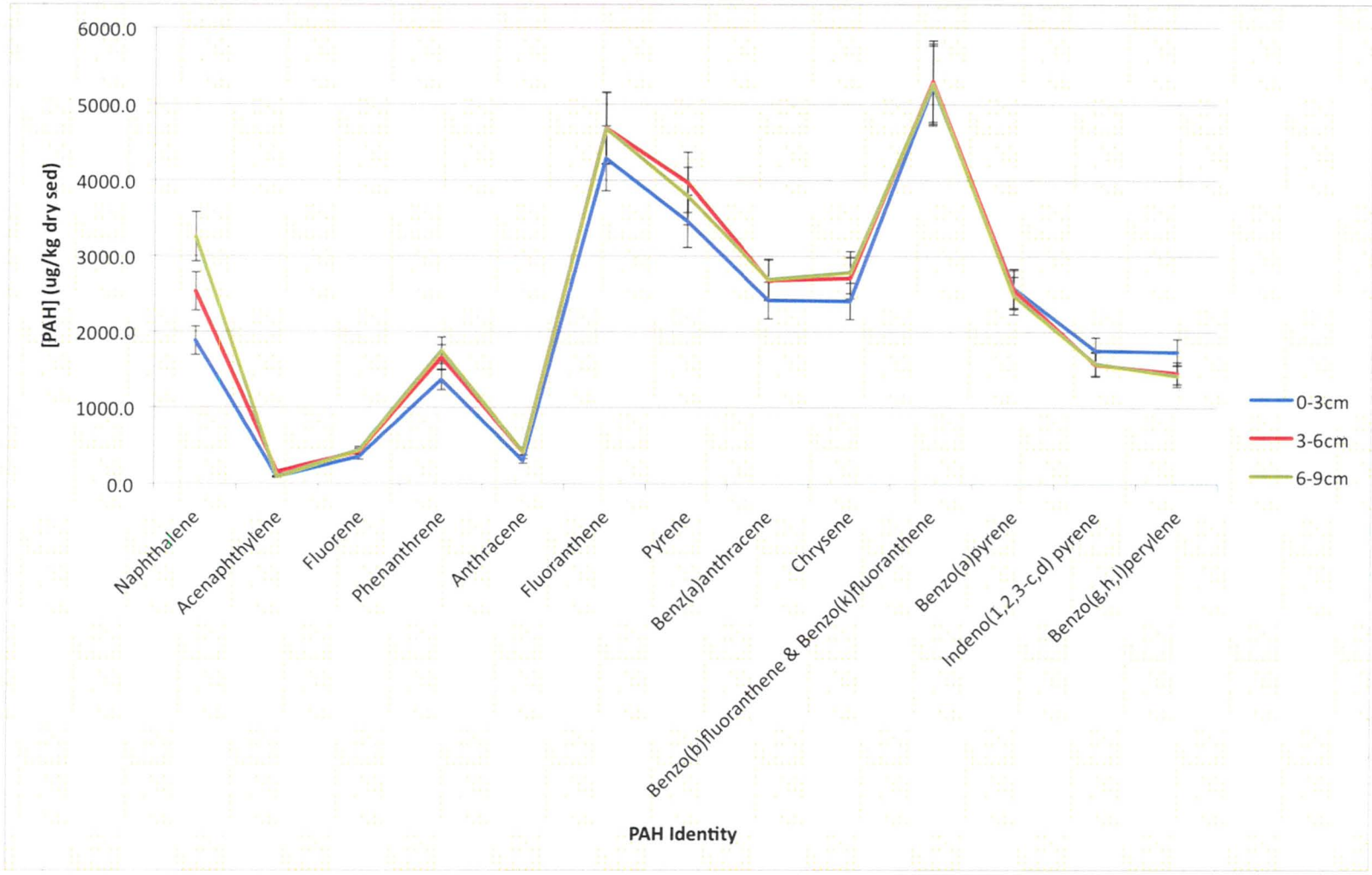

Figure 2-6b: Individual PAH concentrations in a sediment core obtained from Site 2 and sectioned into 3cm slices from 0-9cm depth below the sediment water interface. Error bars represent 10% relative standard deviation resulting from GCMS machine error. PAHs are arranged in order of increasing molecular weight (left - right).

2-3.3 Phospholipid Fatty Acids

2-3.3.1 PLFA Concentrations in Sediments

PLFA concentrations were measured in sediment cores obtained at the same time as sediment cores for PAH/TPH/NEOR analysis. PLFA concentrations and distributions were representative of the surface sediment microbial community at the time of sampling due to the rapid degradation of PLFAs following cell death (White et al., 1979).

Total PLFA concentrations in the highly contaminated sediment (Site 2) and relatively uncontaminated sediment (Site 1) are shown in Figure 2-7. The total concentration of PLFA at Site 1 was found to be slightly more than double the total concentration at Site 2 (Figure 2-7). Analysis of sediments at both sites showed percent water content was very similar between sites (11.4 ±0.11%), and therefore the comparison between total PLFA concentrations in Site 1 and Site 2 sediment should be accurate.

2-3.3.2 Microbial Community Composition

PLFA profiles for highly contaminated (Site 2) and relatively uncontaminated (Site 1) sediments were examined to determine if any significant changes in microbial community composition existed between study sites. Community PLFA profiles from both sites are presented in Figure 2-8. PLFAs ranging from C_{12} to C_{24} were identified,

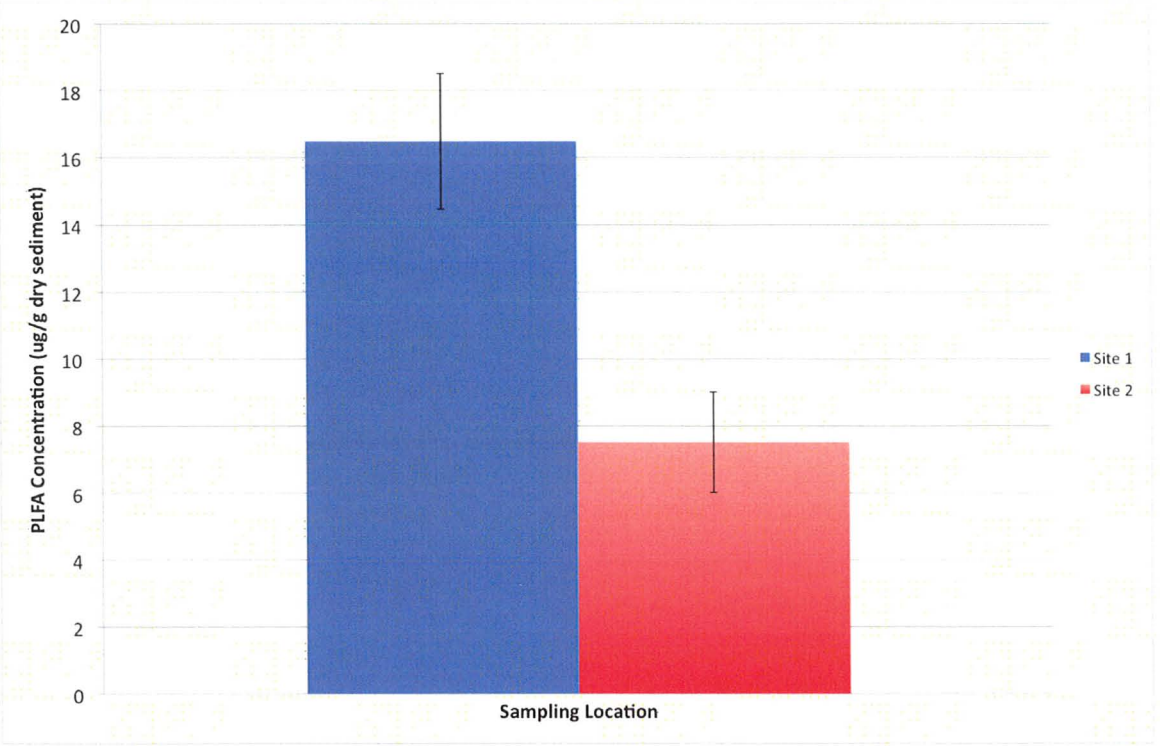

Figure 2-7: Mean total PLFA concentration in from three surface (0-3cm) sediment samples from separate cores obtained at Site 1 and Site 2. Error bars represent one standard deviation (1σ) from the mean.

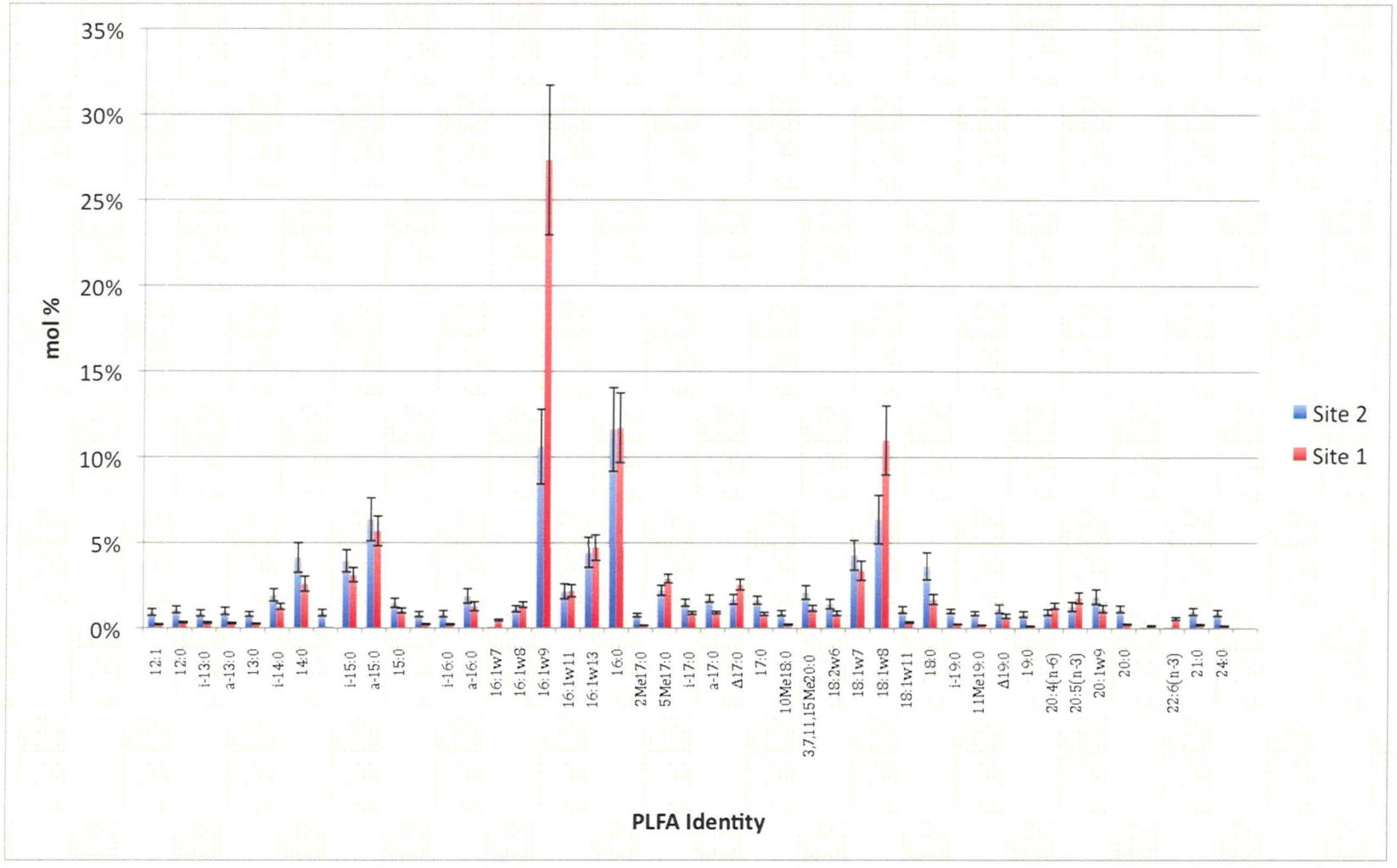

Figure 2-8: Average mole percentages of individual PLFAs identified in three 0-3cm sediment samples obtained from three separate cores taken at each of the study sites. Error bars represent one standard deviation from the mean

and included saturated, branched, monounsaturated, polyunsaturated and cyclopropyl fatty acid chains. Overall, community composition based on PLFAs was found to be very similar between the two sites. PLFAs identified at Site 1 were nearly identical to those identified at Site 2, with the exception that the PLFA 4,8,12Me16:0 was unique to Site 2, while 4,8,12Me21:0 and 22:6(n-3) were unique to Site 1.

Certain classes of PLFA are generally regarded to correspond to specific components within a microbial community. Table 2-1 shows a summary of the relative proportions of these PLFA classes at both sites. PLFA at both sites were predominantly composed of monounsaturates, which are generally indicative of Gram-negative bacteria (Ringelberg et al., 2008). A substantially greater proportion of monounsaturates was observed at Site 1 than at Site 2, (52% vs. 32%). At both sites, the next most dominant PLFAs were *n*-saturates (19% and 28%), which do not correspond to any specific community component due to their ubiquity. Terminal-branched saturated PLFAs, found in both Gram-positive and Gram-negative bacteria (Kaneda, 1991), comprised 14% of the PLFA at Site 1 and 21% of the PLFA at Site 2. Polyenoic PLFAs of carbon chain length <C19 are regarded as markers for microeukaryotes (White, 1988; Fang and Barcelona, 1998). The sole identified PLFA meeting those requirements, 18:2w9,12, comprised 0.9% of the PLFAs at Site 1 and 1.4% of the PLFAs at Site 2. Mid-branched saturated PLFAs, considered representative of Actinomycota (White, 1997), comprised 5% of the PLFA at Site 1, while making up 8.5% of the PLFA at Site 2. No branched monounsaturates were observed.

Ratios between certain classes of PLFA are also often useful indicators of differences in microbial community composition. The results of three commonly reported ratios, which represent the relative proportions of Gram positive to Gram negative bacteria, aerobic to anaerobic bacteria, and bacteria to microeukaryotes (fungi), are presented in Table 2-2. Saturated mid-branched PLFAs are indicative of gram-positive bacteria (O'Leary and Winkinson, 1988), while monoenoic, cyclopropyl unsaturated PLFAs, and saturated PLFAs containing at least one hydroxyl group are representative of gram-negative bacteria (Fang and Barcelona, 1998; Yang et al., 2007). The cyclopropyl fatty acids Δ17:0 and Δ19:0, as well as saturated or monoenoic PLFAs with fatty acid chains from C12 to C20, are characteristic of bacteria, while the sole observed <19C polyenoic PLFA (18:2ω9,12) is characteristic of fungi (Yang et al., 2007). Anaerobic bacteria commonly contain the cyclopropyl fatty acids Δ17:0 and Δ19:0 (Baird and White, 1985; White, 1988), while aerobic bacteria are characterized by C12 to C20 monounsaturated fatty acids (Findlay et al., 1990; Findlay and Dobbs, 1993). A much higher ratio of Gram positive to Gram negative bacteria was found at Site 2 than at Site 1. The ratio of fungi to bacteria was slightly higher at Site 2, but the difference in the ratio was driven by a nearly twofold increase in the fungal PLFA at Site 2, while the percentage of bacterial PLFAs remained similar at both sites. The ratio of anaerobic bacteria to aerobic bacteria was found to be greater at Site 2 than at Site 1, with errors that indicate the result was just barely significant. The difference in ratio was largely driven by the smaller percentage of the total microbial population made up by aerobic PLFAs at Site 2 compared to Site 1, while the relative presence of anaerobic PLFAs at both sites was similar.

Table 2-1: Microbial community composition in 0-3cm sediment at Site 1 and Site 2, as indicated by PLFA mole percentages for specific PLFA classes.

	Gram-negative bacteria (Monounsaturates)	Generic PLFA (n-saturates)	General Bacterial PLFA (Terminal-branched saturates)	Actinomycota (Mid-branched saturates)	Microeukaryotes (Polyenoic <C19)	Eukaryotes (Polyenoic)
Site 1	52.1 ±4.9%	19.2 ±2.1%	14.2 ±1.0%	5.0 ±0.3%	0.9 ±0.1%	4.5 ±0.4%
Site 2	32.8 ±3.0%	28.2 ±2.8%	21.0 ±1.6%	8.5 ±0.6%	1.4 ±0.3%	3.6 ±0.4%

Table 2-2: Key PLFA mole percentage ratios in 0-3cm sediment at Site 1 and Site 2.

	G+:G- bacteria	fungi:bacteria	anaerobic:aerobic
Site 1	0.09 ±0.02	0.012 ±0.002	0.06 ±0.01
Site 2	0.24 ±0.05	0.022 ±0.004	0.09 ±0.01

45 PLFAs were present at both sites. Between Site 1 and Site 2, 33 of those 45 PLFAs were present at significantly different percentages of the total PLFA at each site (Figure 2-8), revealing a distinct site-specific PLFA distribution pattern. 29 PLFAs made up a greater percentage of the total PLFA at Site 2, but for those PLFAs the difference between sites was fairly small. In contrast, only 4 PLFAs were present in greater quantities at Site 1, but two of them, 16:1ω9 and 18:1ω8, made up a greater percentage of the total PLFA than at Site 2.

2-3.4 DGGE Banding

Very few distinct bands were obtained from denaturing gradient gel electrophoresis (DGGE) analysis of surface sediments (Figure 2-9). At both sites only a single prokaryote was identified. At Site 1, a band was found for *Acidovorax spp.*, a nitrogen-fixing bacterium, while at Site 2, *Pseudomonas spp.* was identified. At both sites, fungal banding was limited to different uncultured members of phylum Zygomycota, with five bands found at Site 1 and two at Site 2.

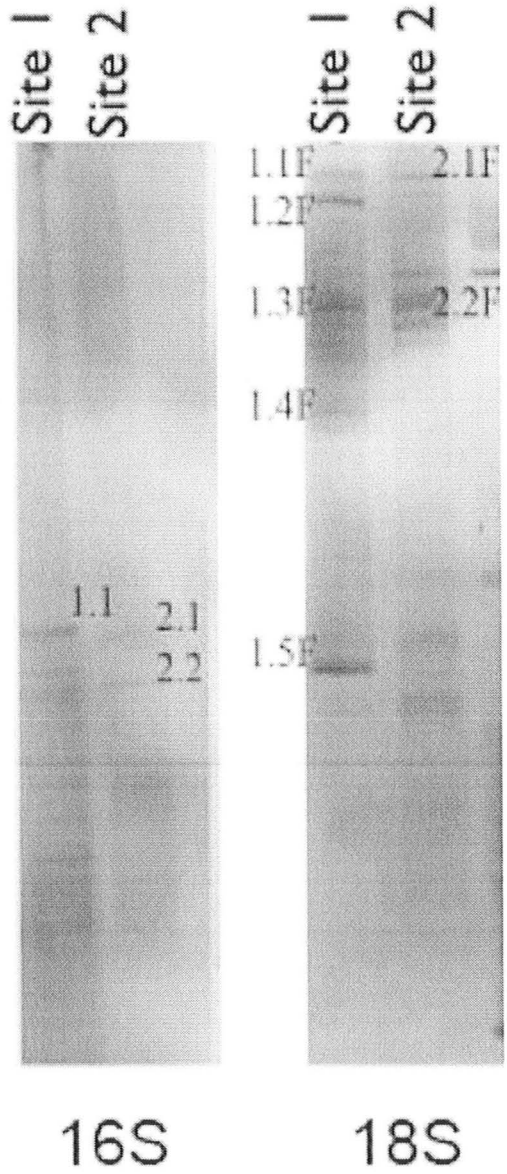

Figure 2-9: Denaturing gradient gel electrophoresis (DGGE) bands representing dominant microorganisms (>1-2% of total) in surface (0-3cm) sediment obtained from Site 1 and Site 2. 16S (prokaryotic) and 18S primers (fungus) were utilized. (1.1: *Acidovorax spp.*, 2.1: *Pseudomonas spp.*, 2.2: not identifiable, 1.1F-1.5F, 2.1F-2.2F: uncultured Zygomycete).

2-4. Discussion

2-4.1 PAH/TPH Loads at Study Sites

2-4.1.1 Recent trends in Hamilton Harbour PAH values

Extensive remediation efforts in Hamilton Harbour over the past 30-40 years have proven fairly successful in reducing PAH loads in harbour waters and surface sediments (see thesis introduction for details). Despite these successes, however, limited progress has been made in addressing PAH contamination in deeper sediments, which can exceed 1400ug/g in the most contaminated hotspots (Murphy, 2000). Strong evidence suggests resuspension of these hotspot sediments contributes to the high suspended sediment PAH concentrations currently observed in the harbour (Marvin et al., 2000c), and results in the redistribution of contaminated sediment throughout the harbour. As such, further harbour-wide PAH remediation efforts depend upon development of an effective method to contain or eliminate PAHs in hotspot sediments.

Surface sediment PAH concentrations have not shown any improvement over the past decade, presumably because hotspot sediments have not been contained or controlled. As shown by Slater et al. (2008), over the course of 3 months in the summer, total surface sediment (0-3cm) PAH concentrations in Windermere Arm (Site 1) and at the Western end of the harbour (Site 2) can vary by at least 15µg/g at Site 1 and 65µg/g at Site 2. All PAH concentrations reported at or near Site 1 and Site 2 between the years

2000-2007 fell within 15μg/g (Site 1) or 65μg/g (Site 2) of each other, indicating no resolvable decrease in total PAH concentrations occurred at either site over that period (Table 2-3). In this study (June, 2007), total surface sediment PAH concentrations of 4.5ug/g at Site 1 and 27.8ug/g at Site 2 were observed. At the same two study sites in May, June and July 2004, total PAH concentrations in surface sediments were found to range from approximately 5-13ug/g at Site 1 and 20-59ug/g at Site 2 (Slater et al., 2008). It should be noted that the upper sediment sampling resolution in the study conducted by Slater et al. (2008) was greater than in this study, with their measurements taken at 1cm intervals from 0-3cm below the sediment-water interface. In order to ensure valid comparisons between studies, the values from that study were averaged to produce a single PAH concentration value from 0-3cm. In October, 2000, Marvin et al. (2000a) found total PAH concentrations of 21-30ug/g in surface sediments and 34-47ug/g in suspended sediments from Windermere Arm and 7.6 ug/g in suspended sediments at the western end of the harbour, near Site 1.

The overall lack of change observed in sediment PAH concentrations at or near Site 1 and 2 could be the result of resuspension and redeposition of sediment from highly contaminated PAH hotspots present along the southern industrial shoreline. Such erosion and redeposition could potentially provide a steady flow of PAH to study sites, overriding and masking the effects of any biotic or abiotic PAH degradation processes that might be occurring in the harbour.

Table 2-3: Total PAH concentrations in ug/g observed at or in proximity to both study sites from Oct, 2000 through June, 2007. Note 2004 values are averages of 3 measurements from 0-3cm below the sediment water interface at 1cm intervals.

	PAH concentration (ug/g)	
	Site 1	Site 2
June, 2007	4.5	27.8
July, 2004[1]	13.1	59
June, 2004[1]	6.3	24.7
May, 2004[1]	5	20
Oct, 2000 (surface sed)[2]	n.d.	21-30
Oct, 2000 (suspended sed)[2]	7.6	34-47

[1] Slater et al. (2008)
[2] Marvin et al. (2000a)

Many PAH point sources, such as runoff from coal piles on industrial docks in Windermere Arm (Curran et al., 2000), or the heavily contaminated hotspot sediment near Randle Reef (Murphy, 2000), have yet to be adequately controlled, and strong evidence suggests that hotspot sediments near Randle Reef have been, and likely continue to be redistributed throughout the central harbour. Murphy (2000) demonstrated the existence of a band of sediment running north to north-east through the central harbour, connected to numerous PAH hotspots near Randle Reef and containing some of the most highly PAH-contaminated bed sediments in the harbour. As seen in Figure 2-10, when dominant harbour circulation patterns, based on modelling by Rao et al. (2009), are superimposed upon Figure 1 from Murphy (2000) (see Figure 2-10), there is a near-exact match between the shape of the central counter-clockwise circular current and the shape of the band of highly PAH contaminated sediment. Taken together, this data strongly suggests that the band of PAH-contaminated sediment was formed as the result of erosion of highly PAH-contaminated hotspot sediments along the southern shoreline, and subsequent transport and redeposition of those sediments by northerly harbour currents. The high concentrations of PAHs found in suspended sediments in the central harbour basin and Windermere Arm area by Marvin et al. (2000c) are also consistent with PAH-contaminated sediment resuspension and redeposition. Particularly in Windermere Arm, shipping traffic is likely partially responsible for initial hotspot sediment erosion and resuspension, as the passage of ships causes extensive perturbation of shallow sediments (Irvine et al., 1997).

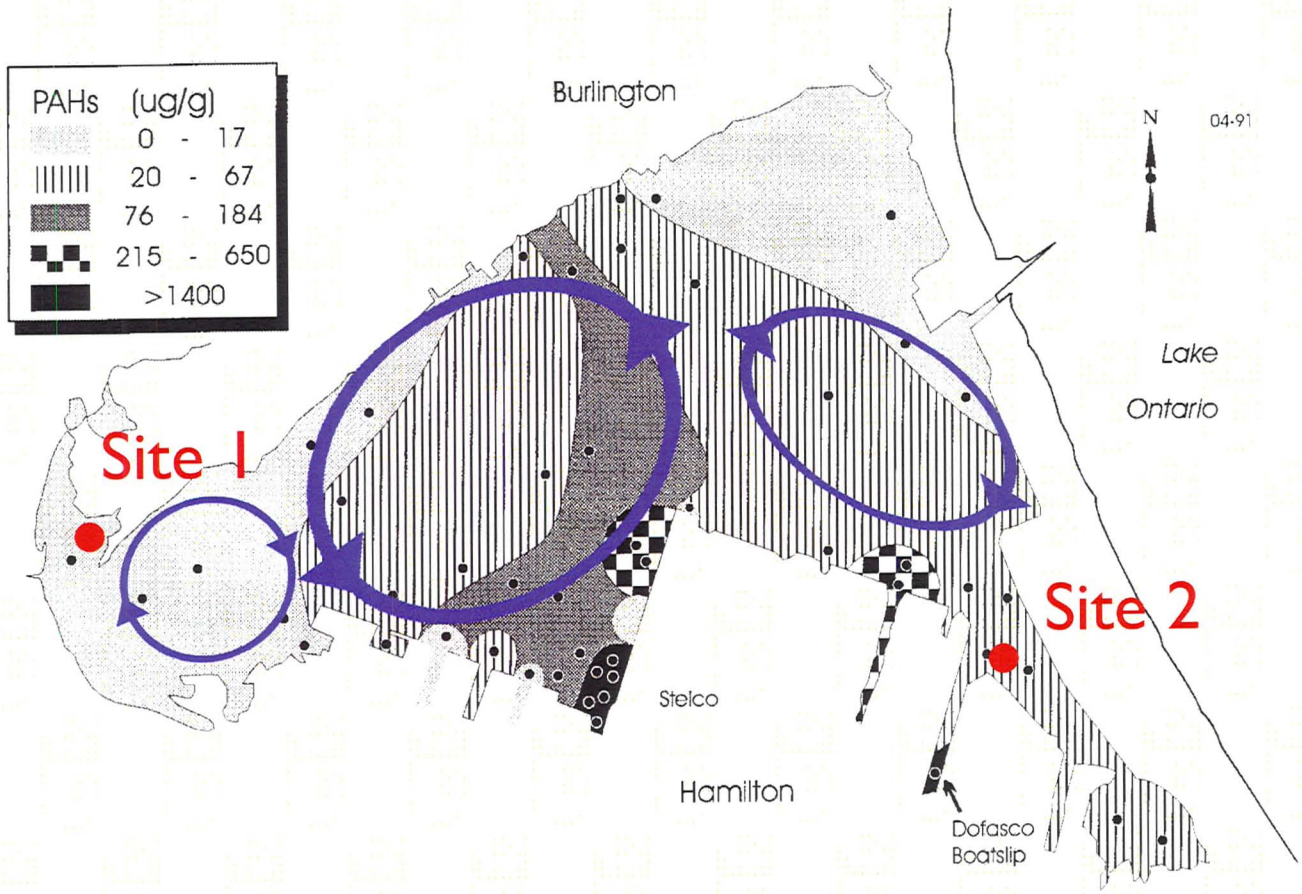

Figure 2-10: PAH concentrations in Hamilton Harbour sediment grab samples based on Murphy et al. (2000). Blue ellipses indicate primary current flows within Hamilton Harbour modified from Rao et al. (2009), with arrows indicating the direction of flow. This figure is modified from Figure 1 in Murphy et al. (2000). It should be noted that the clockwise direction of flow shown for the small circle to the left of this diagram is the reverse of what was reported in Rao et al. (2009), but the reported direction was likely a mistake (see Section 2-4.1.2 for details).

Given that there has been no progress in containing PAH-contaminated hotspot sediments in the harbour since the time of sampling conducted by Murphy (2000), there is a strong likelihood that the resuspension and redeposition of PAH-contaminated sediment continues to occur today. Based upon the discovery that a single PAH source influenced both Site 1 and Site 2 in June, 2004 (2008), it would also appear that this sediment redistribution can occur at a harbour-wide scale. Regular redistribution of PAH-contaminated sediment could potentially account for both the wide range in sediment PAH concentrations at or near Site 1 and Site 2 as reported in this study, Slater et al. (2008) and Marvin et al. (2000a), as well as the consistency of that range from 2000 to 2007. Sediment redistribution is also a possible explanation for the change in Site 1 PAH ratios between 2004 and 2007, as discussed in greater detail in Section 4.1.3.

A number of other factors could also contribute to the reasonably consistent PAH concentrations at Site 1 and Site 2, including steady PAH point loads from regular PAH sources influencing those sites (streams, effluent pipes, fallout of airborne PAHs etc.). More frequent and regular sampling at study sites, or higher resolution analysis of much deeper sediment cores would be an ideal first step in attempting to identify the cause(s) of the consistent PAH loads to both study sites, which could lead to the mitigation of whatever PAH-contributing mechanism was identified. The likelihood that regular redistribution of highly contaminated sediments is occurring in at least the central basin of Hamilton Harbour clearly indicates that remediation efforts should first focus upon controlling PAH point sources, prior to attempting to remediate other areas of the harbour.

2-4.1.2 Variability in PAH Ratios at Study Sites

Very different PAH distribution patterns were observed for Site 1 and Site 2, suggesting PAHs at each site must have originated from different sources, or PAH-contaminated sediments (Figures 2-6a,b). In this section we restrict our focus to source variability; the possible identities of these sources are discussed in Section 4.1.4. At Site 2, the source or sources appear to have been reasonably consistent over time, with PAH distributions closely matching those obtained at Site 2 in May-July 2004 (Slater et al., 2008). At Site 1, however, the distribution pattern differed significantly from any pattern observed in 2004. This indicates that the PAH source, or the relative contributions of multiple sources, must have changed at some point between the 2004 and 2007 sampling periods. As PAH distributions in this study were quite consistent at both sites over the depth of the cores (0-9cm), the change must have occurred prior to the record provided in the core. Based on the average 2004 sedimentation rates observed at Site 1 by Slater et al. (2008), the 9cm depth of the Site 1 core represents approximately 265 days. As the average sedimentation rate was calculated based upon sedimentation rates measured over 3 months in a single summer, this must be considered a very rough approximation. On this basis, the change in source is estimated to have occurred prior to November, 2006.

Differentiation between PAH sources can also be carried out by examining variations in the ratios of specific PAH concentrations (Furlong et al., 1988; Canton and Grimalt, 1992; Zhang et al., 1993; Benner Jr et al., 1995). In general, higher molecular

weight PAHs are employed in such ratios, as these compounds are more resistant to microbial degradation, and will therefore be present at concentrations more representative of the actual PAH source. Fluoranthene:fluoranthene+pyrene (or fluoranthene:pyrene) and indeno(1,2,3)pyrene:benzo(g,h,i)perylene ratios have been investigated previously to characterize the pyrogenic or petrogenic origins of PAHs (Canton and Grimalt, 1992; Yunker et al., 2002; Mahler et al., 2005), and were also previously used to investigate Site 1 and Site 2 PAH sources (Slater et al., 2008). Slater et al. (2008) also suggested the ratio dibenzo(a,h)anthracene:benzo(a)pyrene might be diagnostic of the PAH sources to Site 1 and Site 2.

The above ratios, along with a number of additional high molecular weight PAH ratios, were calculated for this study to investigate their utility for PAH source differentiation. Results supported the previous conclusion that the PAH profile at Site 1 changed substantially between 2004 and 2007. All ratios will be referred to henceforth in this paper by the letters A to G for simplification (see Table 2-4). The simultaneous use of multiple ratios can minimize the impact of sample variance in a specific PAH, common in complex natural systems, such as the variations observed in fluorine concentrations at Site 1 and naphthalene concentrations at Site 2 (Figure 6a,b) (Marvin et al., 2000c; Lima et al., 2003). All ratios for Site 1 and for Site 2 were quite consistent throughout the 9cm cores. Maximum percent errors between ratios from the 3 core samples taken at each site were less than 7% for Site 1 and less than 9% for Site 2 (Table 2-4). Between Site 1 and Site 2, however, ratios were clearly different throughout the

Table 2-4: Ratios of individual high molecular weight PAHs calculated from PAH concentrations measured at Site 1 and Site 2 in this study (2007) and by Slater et al. (2008) in 2004. 2007 values represent averages of measurements taken from 0-9cm depth, while 2004 measurements represent averages of measurements taken from 0-3cm depth.

		2007		2004	
Ratio ID	Ratio	Site 1	Site 2	Site 1 (May)	Site 2
A	Fluoranthene/pyrene	2.12 ±0.13	1.22 ±0.03	1.01 ±0.14	1.19 ±0.10
B	Indeno(1,2,3-c,d) pyrene/ Benzo(g,h,I)perylene	1.24 ±0.08	1.07 ±0.05	1.11 ±0.16	0.98 ±0.09
C	Dibenzo(a,h)anthracene/benzo(a)pyrene	n.m.[1]	n.m.[1]	0.85 ±0.12	0.08 ±0.01
D	Benzo(a)pyrene/pyrene	3.92 ±0.21	0.68 ±0.06	0.94 ±0.13	1.19 ±0.10
E	Chrysene/pyrene	1.10 ±0.07	0.70 ±0.03	0.89 ±0.13	1.11 ±0.10
F	Benzo(b)fluoranthene + Benzo(k)fluoranthene/pyrene	5.55 ±0.09	1.41 ±0.09	n.m.[2]	n.m.[2]
G	Benz(a)anthracene/pyrene	3.08 ±0.21	0.69 ±0.02	0.63 ±0.09	0.89 ±0.08

[1] dibenzo(a,h)anthracene was not measured in this study.

[2] Benzo(b)fluoranthene and Benzo(k)fluoranthene/pyrene were not measured in Slater et al. (2008)

cores (Table 2-4), strongly supporting the conclusion that the dominant source of PAHs to each site was very different throughout the period represented in the cores.

Ratios were also compared to those calculated in Slater et al. (2008) to determine whether values obtained in this study remained consistent with those obtained in 2004, or whether the PAH sources to the two sites had changed between studies. The results of these comparisons suggested the PAH source to Site 2 remained the same between studies, but the source to Site 1 changed between studies. At Site 2, the similarity of Ratio A, B and G between studies suggested the same PAH source influenced Site 2 in both studies (Table 2-4). Ratios A and B were also found to be very similar at sites proximal to Site 2 by Marvin et al. (2000c). This apparent consistency of the PAH source to Site 2 from 2000-2007 was expected, as the primary source(s) of PAHs to Site 2 are likely the industrial properties on the south shoreline. At Site 2, all other ratios measured in both this study and Slater et al. (2008) (Ratios D and E) had very different values between studies, suggesting these ratios might not be diagnostic at Site 2. At Site 1, much larger differences in Ratios A, D, and G, and significant differences in Ratio E, between studies suggested that the PAH source(s) at Site 1 changed between 2004 and 2007 (Table 2-4). In June and July of 2004, similar Site 1 and Site 2 PAH profiles had suggested Site 1 was being influenced by the same PAH source(s) affecting Site 2 (Slater et al., 2008). In this study, however, the highly dissimilar values for Ratios A, B, D, F and G between Site 1 and Site 2 suggest an entirely new and unknown PAH source influenced Site 1. The dissimilarity of the Site 1 fluoranthene/pyrene ratio calculated in this study from all fluoranthene/pyrene ratios found by Marvin et al. (2000c) in the

industrialized zone near Site 2 further supports the conclusion that the PAH source(s) influencing Site 1 had no association with that or those affecting Site 2. It should be noted (again) that due to the highly similar ratios found throughout the depth of the cores in this study, comparisons with ratios calculated by Slater et al. (2008) and Marvin et al. (2000c) were also made using averages of the three measurements taken for each entire core length (0-9cm), despite the fact Slater et al. (2008) only examined PAHs to a depth of 3cm.

2-4.1.3 Cause for Change in Site 1 PAH Ratios

A number of potential causes could have been responsible for the change in PAH source(s) at Site 1 which occurred during the 3 year period between sampling for this study and sampling conducted by Slater et al. (2008). Transport of PAH-contamined sediment from another area of the harbour is one possible cause. As discussed in Section 4.1.1, a mechanism for resuspension and redeposition of PAH-contaminated sediment clearly exists within the central harbour basin (see Figure 2-10). It is possible that the combined action of the central gyre seen in Figure 2-10, and the smaller gyre nearest Site 1 was capable of transporting PAH-contaminated sediments to Site 1 from elsewhere in the harbour, which could potentially have caused the change in PAH ratios observed at Site 1. It should be noted that Rao et al. (2009) reported that circulation in the smaller gyre was counter-clockwise. Given the stronger flow strength of the central counter-clockwise gyre, and it's proximity to the smaller gyre, counter-clockwise circulation is

illogical, however, and we must assume the flow direction reported was an error. If the smaller gyre did flow in the more logical clockwise direction, PAH-contaminated sediment could have been carried directly into the mouth of Carole's Bay where Site 1 is located.

As the frequency with which the source changed between this study and that conducted by Slater et al. (2008) is unknown, potential sediment transfers could have been continuous, or could have been driven by episodic events, during which greater amounts of sediment were transported. Such events (potentially caused by storm activity) were proposed by Slater et al. (2008) as an explanation for their discovery that a single PAH source affected both Site 1 and Site 2 in one, and only one, of the three months they conducted sampling in the harbour. To establish the frequency of PAH ratio changes and to correlate such events to potential causes, again more frequent and regular sampling at Site 1, or higher resolution analysis of much deeper sediment cores would be required.

Alternatively, it is also possible that there was an actual change in the PAH source or sources to Site 1 between studies. Site 1 was located very near the mouth of Grindstone Creek, and was also proximal to the channel connecting Cootes Paradise, a sheltered bay fed by Spencer Creek, to the rest of the harbour. As both creeks serve as primary connections between much of the Dundas Valley drainage basin and the harbour, all manner of potential developments upstream of Site 1 could have altered the natural composition and/or concentration of PAHs reaching Site 1 via creek-transported sediments. During the three year period in question, we know of only one specific change upstream of Site 1 which could have caused changes in PAH ratios at Site 1.

Over that 3 year period, efforts to reduce turbidity in Cootes Paradise were initiated through alterations to Spencer Creek. These changes were designed to reduce suspended sediment levels reaching Cootes Paradise, and thus it is possible PAHs sorbed to those suspended sediment particles were also prevented from reaching Cootes Paradise, and subsequently Site 1. Unfortunately, no information on PAH concentrations or compositions in Spencer Creek or within Cootes Paradise which could support this hypothesis is available in the literature. Further complicating matters, however, is that fact that even a change in the type of fertilizer used on a farm property abutting one of the creeks could have influenced the nature of the PAHs reaching Site 1.

2-4.1.4 Tracing Sources of PAH Inputs

Neither PAH ratios nor PAH distribution patterns could identify the exact PAH source(s) contributing to Site 1 and Site 2, as current PAH ratios and distribution patterns for potential sources were unavailable in the literature. Slater et al. (2008) were also unable to establish PAH sources to Site 1 and Site 2, for the same reason. Examination of ratios calculated from older reference samples yielded limited information, and even if clear relationships had been found, their validity would have been highly suspect given the constant state of flux in harbour conditions. Only 2 of the 4 diagnostic ratios used by Marvin et al. (2000c) to examine PAH reference samples employed PAHs which were measured in this study, and as such no clear relationship could be found between those reference samples and the study sites in this paper. Comparison of the ratios of all

measured PAHs to naphthalene (not shown) with the PAH to naphthalene ratios calculated by Curran et al. (2000) and Christensen et al. (1997) for coal pile runoff, coal and wood gasification, highway dust and air filter coke oven emissions revealed no clear relationship between reference sources and Site 1 or Site 2 ratios.. Canton and Grimalt (1992) suggested high pyrene/fluoranthene and benzo(g,h,i)perylene/ indeno(1,2,3-c,d)pyrene ratios were indicative of emissions from motor vehicles, but neither site exhibited particularly high ratios for these compounds.

Even when extensive PAH concentration data from potential sources is available, if more than 1-2 PAH sources contribute to the overall PAH load (very likely at Site 2), concentration ratios are generally limited in their ability to trace the exact source of the PAH due to the heterogeneity associated with natural systems (Sofowote et al., 2008). This could explain why Marvin et al. (2000c) were also unable to resolve exact PAH sources near Site 1 and Site 2, even with available and up-to-date PAH and thia-arene profiles from suspected possible sources. Using advanced statistical methods, Sofowote et al. (2008) proposed that PAH loads near both sites were likely derived from at least 3 major sources. The multiple linear regression and principle component analysis methods employed suggested a high likelihood that diesel and gasoline emissions and coal (coal tar or combustion) contributed to PAH loads at both study sites, with contributions from weathered PAHs and unburned fossils fuels also a possibility. They suggested diesel emissions were the dominant source of PAHs at Site 2, likely due to extensive shipping traffic. PAH sources at Site 1 were suggested to be more or less evenly distributed. Unfortunately, Sofowote et al. (2008) compiled their data from samples collected over a

span of 5 years (2002-2006), and major redeposition events in the harbour could well be occurring at much greater frequency. Therefore, it is questionable if their suggested distribution of sources at Site 1 correlates with either the PAH sources observed in 2007 or those contributing in 2004, or whether the distribution actually reflects the combination of multiple different sets of contributing sources over the 4 year study period. Regardless, their analysis represents the most reasonable assessment to date of PAH sources to the areas surrounding Site 1 and Site 2.

2-4.2 Microbial Community

2-4.2.1 Effect of Contamination on Microbial Biomass

PLFA analysis suggested PAH contamination had a substantial impact on microbial biomass, as total PLFA concentrations in 0-3cm sediments collected at Site 1 were more than twice as great as Site 2 PLFA concentrations. PLFA concentrations serve as proxies for microbial biomass, as they can be directly converted to biomass values through the use of a conversion factor (Green and Scow, 2000). The PAH to biomass relationship between Sites 1 and 2 has been very stable over a three year period, suggesting it is quite robust. In 2004, Slater et al. (2008) examined PLFA concentrations at Site 1 and Site 2, and again found total PLFA concentrations at Site 1 approximately double those at Site 2 at all depths measured in sediment samples taken in May, June and

July. Between the two studies, the difference in average total PAH concentrations in Site 1 and Site 2 surface sediments was also very similar, with Site 2 PAH concentrations 3-4.5x greater than Site 1 in Slater et al. (2008), and 3-4x greater in this study. The general trend described by this data, that of decreasing total PLFA concentrations (biomass) with corresponding increases in total PAH concentration, has also been observed in studies in which PAH-contaminated soil was examined. Relative to uncontaminated control sediment, Yang et al. (2007) found PLFA concentrations dropped by close to a factor of 10 in soil spiked with an average of 50μg/g of three 2- and 3-ring PAHs. Su and Yang (2009) found total PLFA concentrations were reduced 27% in soil spiked with an average of 25μg/g of the same three PAHs, with further decreases in PLFA concentration at greater PAH concentrations.

Increased environmental stress due to elevated PAH concentrations almost certainly contributed significantly to the decreased total PLFA concentration at Site 2, but a number of other factors are also likely to have played a role. Site 2 exhibited greater concentrations of TPHs than Site 1, and elevated levels of TPHs have also been shown to correlate with decreases in microbial biomass and diversity (Ringelberg et al., 2008). Additionally, water conditions nearest the sampled sediments at both sites suggested Site 2 sediments were colder and had lower dissolved oxygen (DO) concentrations. Lower temperatures are commonly associated with lower microbial activities and lower DO concentrations could certainly have resulted in reduced numbers of aerobic bacteria, although it would also likely have promoted an increase in the numbers of anaerobic bacteria. The relationship between DO concentrations and growth of aerobic and

anaerobic bacteria is examined further in the Section 4.2.2.3. Detailed surface sediment metal profiles could not be found for Site 1, making a direct comparison of metal contamination between sites impossible. However, surface sediment metal concentrations near Site 2 have been found at levels just above or below the lowest effect level of the Provincial Sediment Quality Guidelines, the level at which no biological impairment is expected to occur. Therefore, it is likely that even if Site 1 metal concentrations were lower than those at Site 2, the actual impact upon the microbial community at either site would have been minimal.

2-4.2.2 Effects of Contamination on Microbial Community Composition

2-4.2.2.1 PLFA Profiles

Comparison of PLFA profiles for Site 1 and Site 2 suggested microbial communities mainly produced the same PLFAs, but in different relative quantities (Figure 2-8), suggesting community composition was different at the two sites. This conclusion was supported by more rigorous PLFA class and PLFA ratio analyses (Sections 4.2.2.2 and 4.2.2.3). Three PLFAs were unique to Site 1, and one to Site 2, but none represented large differences in community composition, being present in quantities <1% of the total PLFA. The PLFA 22:6(n-3), unique to Site 1, was the only one of the site-specific PLFA with a known specific origin, being predominantly produced by planktonic algae (Sargent et al., 2002). The total absence of 22:6(n-3) at Site 2 is an important consideration, as this

compound, also known as docosahexaenoic acid (DHA), is an essential fatty acid for human health. It cannot be synthesized in the body and is most commonly ingested via consumption of fish which have fed upon such algae (Singh, 2005). The implications of the absence of DHA at Site 2 are discussed further in Section 4.3.

Most individual PLFAs made up similar relative percentages of the total PLFA at each study site. The exceptions were the much greater relative percentages 16:1w9 and 18:1w8 at Site 1 (Figure 2-8). These differences are more meaningfully discussed in subsequent sections of Section 4.2.2.

2-4.2.2.2 PLFA Classes

In order to more effectively assess changes in microbial community composition between study sites, the relative presence of specific classes of PLFAs were compared, and results indicated substantial differences in community composition between sites. Many classes of PLFA are commonly used indicators of change in community composition, as each class is known to be representative of a specific group of microorganisms. In order from greatest to smallest, the relative proportions at Site 1 and Site 2 of monounsaturates (52.1% and 32.8%), *n*-saturates (19.2% and 28.2%), terminal-branched saturates (14.2% and 21.0%), mid-branched saturates (5.0% and 8.5%) and polyenoic PLFA of <C19 chain length (0.9% and 1.4%) (Table 2-1), clearly suggested the microbial communities at both sites were very different. Relative to Site 2, Site 1 exhibited a significantly greater presence of Gram-negative bacteria (monounsaturates),

significantly decreased presences of nonspecific PLFAs (*n*-saturates), nonspecific bacterial markers (terminal-branched saturates) and Actinomycota (mid-branched saturates), as well as similar quantities of microeukaryotes (<C19 polyenoic PLFA) and total eukaryotes (all polyenoic PLFA).

Comparison with the PLFA class analyses conducted for the same sites in 2004 (Slater et al., 2008), revealed differences between the studies at Site 2. Slater et al. (2008) identified the bacterial groups corresponding to the PLFA classes differently, but those identities are not of concern to us as the same classes of PLFA were measured in both studies, and therefore direct comparison is possible. The relative percentages made up by PLFA classes at Site 1 were very similar to values obtained by Slater et al. (2008) at Site 1, while the results between studies at Site 2 were very different. Relative to 2004 values, the relative percentages of monounsaturates, *n*-saturates and terminal-branched saturates at Site 2 in 2007 were lower by approximately 16%, 10%, and 5%, respectively, while the 2007 value for mid-branched saturates was roughly 7% higher. Given that the PAH source is postulated to have changed at Site 1 between 2004 and 2007, and remained the same at Site 2, this was the opposite result to that expected.

At Site 1, if PAHs were serving as a microbial carbon source and a change in PAH source occurred between 2004 and 2007, as suggested in Section 4.1, there is a reasonable expectation that the microbial community composition also ought to change in response to the new carbon source. Natural microbial populations are very diverse. A change in carbon source, even if limited only to changes in the relative amounts of the individual PAHs making up the total PAH pool, would very likely favour the growth of

specific bacteria which had difficulty growing on the previous carbon source. PAH-degrading bacteria are known to often only be able to degrade only a limited number of carbon compounds (Cerniglia, 1992). The expected shift in community composition following a change in carbon source was observed by Su and Yang (2009), who saw decreases in monounsaturated PLFAs and cyclopropyl PLFAs when PAHs were introduced to uncontaminated soils, and by Yang et al. (2007) who saw changes in PLFA ratios representing Gram-positive to Gram-negative bacteria, bacteria to fungi and saturated to monounsaturated PLFAs with the addition of PAHs to uncontaminated soils. These studies investigated dramatic changes between conditions of contamination and non-contamination, and as such are not perfectly representative of the change experienced at Site 1. However, to our knowledge, no information on PLFA shifts resulting from changes in complex mixtures of PAHs is available, since Slater et al. (2008) sampled PLFAs during a period when both sites were influenced by the same PAH source.

Ultimately, the cause for the lack of change in community composition between studies at Site 1 is unknown, but one possible explanation can be proposed. Given that Carole's Bay and its primary tributary, Grindstone Creek, are highly rich in nutrients and organic material as a result of runoff from farms and erosion due to subdivision development, one explanation is that microbial metabolism of PAHs did not take place at Site 1 at all between 2004 and 2007. Instead, high levels of more labile and bioavailable organic compounds might have been used preferentially as a microbial carbon source. PAHs have low aqueous solubility and a high propensity to sorb to sediments, which has long been considered to mean PAHs have low bioavailability (Cerniglia, 1992; Johnsen,

2005). This would logically make PAH metabolism energetically unfavourable relative to a more labile carbon source. Slater et al. (2005b) may have observed this phenomenon in salt marsh sediments affected by an oil spill. In that study, the authors found no evidence of microbial petroleum carbon uptake and suggested it might result from a microbial preference for more labile natural organic matter within the salt marsh. An inconsistency with this theory arises with the individual PAH concentrations at Site 1, which show a general pattern in which PAHs with higher molecular weights are present at higher concentrations than PAHs of lower molecular weight (Figure 6a). Such a pattern is generally considered consistent with microbial PAH degradation as lower molecular weight PAHs can be more quickly metabolized due to their lower hydrophobicity and electrochemical stability (Means et al., 1980; Johnsen, 2005).

It is possible the greater presence of high molecular weight PAHs was not biologically-driven. If recirculation of contaminants throughout the harbour was frequent, microbial degradation of PAHs elsewhere could have resulted in delivery of predominantly higher-molecular-weight PAHs to Site 1. Another possible abiotic explanation for the pattern observed is photodegradation. However, photodegradation also produces characteristic PAH concentration patterns which were not observed, suggesting photodegradation did not occur. Certain PAHs are more environmentally unstable and thus susceptible to photodegradation. Benzo(a)pyrene, benz(a) anthracene, pyrene and anthracene are known to be rapidly degraded in near surface waters during direct exposure to sunlight at midday, with half lives for the their degradation of 0.54-0.75h. This was significantly shorter than the half lives calculated for chrysene (4.4h),

phenanthrene (8.4h), fluoranthene (21h) and naphthalene (71h). While the actual degradation rates may not be exact, as the impact of clouds, ozone and light scattering in water bodies were not considered, the relative sensitivity of these compounds to photodegradation is consistent in aqueous systems. Among the rapidly photodegraded PAHs, anthracene and pyrene concentrations were low to non-existent at Site 1 in this study, but benz(a)anthracene and benzo(a)pyrene concentrations were much higher than those of all four of the most recalcitrant PAHs (chrysene, phenanthrene, fluoranthene, naphthalene). Therefore, no clear indication of photodegradation was apparent in the data.

The observation of changes in the Site 2 community composition was also unexpected, given that the PAH source to Site 2 appeared to have remained consistent from 2000-2007 (see Section 4.1). Interestingly, with the exception of the Actinomycota, the relative percentages of PLFA classes present at Site 2 closely matched Site 2 sediments sampled 6cm below the sediment water interface in 2004 (Slater et al., 2008). As heavy shipping traffic occurs in proximity to Site 2 during in the summer, and Irvine et al (1997) have shown ship traffic can erode bed sediments in similarly shallow waters, we hypothesize that the passage of a ship or ships may have exposed deeper sediments in the sampling area, which were then sampled as surface sediments for the purposes of this study. Irvine et al (1997) conservatively estimated erosion would only resuspend sediment to a depth of 1mm. However, Site 2 was over a metre shallower than the shallowest water depth the estimate is based upon, and the repeated passage of multiple ships might have had a cumulative effect. Whether the lesser depth, conservative nature

of the estimate, and passage of multiple ships can account for an extra 5.9cm of erosion is unknown, but at this time we feel this is the best explanation without fingering factors which were not measured in both studies.

2-4.2.2.3 PLFA Ratios

Three key PLFA ratios, representing Gram-positive to Gram-negative bacteria, fungi to bacteria and aerobic to anaerobic bacteria were also calculated to examine changes in microbial community composition. These ratios suggested Site 1 and Site 2 microbial communities were different, but the differences in ratio could not be specifically correlated with differences in PAH concentration, meaning PLFA ratios do not appear to serve as a signal of PAH contamination in this system. Relative to Site 1, greater ratios of Gram-positive bacteria to Gram-negative bacteria, fungi to bacteria and anaerobes to aerobes were observed at Site 2 (Table 2-2). While the increases in the relative proportions of anaerobes and fungi at Site 2 were consistent with the effects of PAH exposure on soil microbial communities (Yang et al., 2007; Su and Yang, 2009), differences in aerobe:anaerobe ratios at Site 1 and Site 2 were likely not caused by differing PAH concentrations. Dissolved oxygen concentrations at Site 2 were much lower, and the difference in the ratio was largely due to site-specific differences in the quantity of aerobic bacteria. As such, the lower presence of aerobes at Site 2 most likely reflected the intolerance of aerobic bacteria for oxygen deprivation rather than an intolerance for higher PAH concentrations. The greater ratio of fungi to bacteria at Site 2

may also not reflect greater PAH contamination, as, compared to bacteria, fungal species have been shown to have a greater ability to degrade a broad range of PAHs, including high molecular weight PAHs, under low-oxygen conditions (Cerniglia, 1992; Silva et al., 2009). The observed changes in the ratio of Gram-positive to Gram-negative bacteria do not appear to serve as a marker for increased PAH contamination. Yang et al. (2007), Ringelberg et al. (2008) and Antizar-Ladislao et al. (2008) found increases in the relative proportion of Gram-positive bacteria with increasing PAH concentrations, while Su et al. (2009) found the opposite, under conditions very similar to Yang et al. (2007). PLFA ratios could not be compared with 2004 sediments, as ratios were not calculated by Slater et al (2008), and the exact PLFAs they identified were not provided.

2-4.2.2.4 Microbial Community Diversity

No clear differences in microbial community diversity could be detected based upon study results. The limited number of bands obtained from DGGE analysis was most likely due to such high microbial diversity that DNA smearing took place, rather than the development of distinct bands (Microbial Insights Inc., personal communication). PLFA results were inconclusive with respect to community diversity. Multiple species commonly produce the same PLFAs, and thus establishing the presence of particular species, or establishing the number of species present in a community is not possible.

2-5 Implications

2-5.1 Recovery of Hamilton Harbour fisheries

Reclamation of a healthy fishery in Hamilton Harbour is a major component of the Hamilton Harbour Remedial Action Plan (RAP) (O'Connor, 2002). Hamilton Harbour served as the nursery habitat for the largest fishery on Lake Ontario in the early 1900s (McCarthy et al., 2004), therefore recovery of the harbour fishery has even broader implications for fish recovery in Lake Ontario. The absence of the PLFA 22:6(n-3), also known as docosahexaenoic acid (DHA), at Site 2 is of significant concern to both fish habitat restoration and human health as it suggests DHA-rich photosynthetic and heterotrophic microalgae are absent at Site 2. Such microalgae represents a significant food source for fish and its consumption is the primary means by which fish accumulate and store DHA in their tissues and oils. The majority of DHA in human diets originates from ingestion of fish and fish oils (Singh, 2005), and therefore a diet containing fish harvested from the area surrounding Site 2 might not contain sufficient levels of DHA to support proper human development. No studies of DHA levels in Hamilton Harbour fish could be found to verify if DHA levels in their tissues were below normal.

DHA is an essential fatty acid in humans, contributing to growth and function of nervous tissue, particularly brain tissue, as well as helping to maintain proper cardiovascular function. As a key component of lipids in neuronal membranes, DHA modulates membrane fluidity and flexibility of the hydrophobic core of the membrane

bilayer as well as interacting directly with membrane proteins. Through this influence, DHA directly impacts the speed of signal transduction, neurotransmission and formation of lipid rafts (Innis, 2007). In the brain, DHA can comprise up to 35% of fatty acids in synaptic plasma membranes (Innis, 2007), and appears to assist in regulating gene expression and ion channel activity, while playing an important role in neurogenesis (Vreugdenhil et al., 1996; Kitajka et al., 2002; Kawakita et al., 2006). DHA is also believed to play an important protective role in the brain and retina, preventing inflammation and apoptosis caused by oxidative stress (Innis, 2007). With respect to cardiovascular function, DHA is known to reduce the risk of coronary heart disease (Leaf, 1992) and to prevent potentially fatal heart arrhythmias by serving to electrically stabilize myocytes (Kang and Leaf, 1994).

Current restrictions limit the number of fish from Hamilton Harbour which may be consumed in a given period, due to accumulated contaminants in fish tissues (Government of Ontario, 2009). Currently, these contaminants represent a far greater threat to human health than the absence of DHA. Most area residents are aware of these restrictions and consume fish from other sources, which presumably contain adequate DHA levels. However, with continued contaminant remediation, restrictions may be lifted in the future, allowing greater human consumption of harbour fish. Therefore, investigation into and promotion of conditions favouring a healthy community of microalgae is important not only to full recovery of fish habitat, but also to ensure that the dietary needs of area residents choosing to consume fish from the harbour would be properly met.

2-6. Conclusions

This study demonstrates there were clear differences in microbial biomass and community composition between Site 1 and Site 2. Higher PAH concentrations at Site 2 were correlated with clear reductions in microbial biomass relative to Site 1, indicating PAH contamination had deleterious effects on microbial communities. PLFA profile, ratio and class analyses demonstrated clear differences in community composition existed between Site 1 and Site 2, but given the potential influence of other environmental factors, no specific changes in PLFAs were found which could serve as a signal to definitively indicate that a microbial community was being affected by PAH contamination. Unexpected changes in Site 2 community composition between 2004 and 2007, despite the maintenance of a steady PAH source, and an unexpected lack of change in Site 1 community composition, despite a definitive change in PAH source, were observed. In both cases, factors not directly related to sediment PAH concentrations were most likely responsible.

The PAH fingerprint at Site 1 was found to be inconsistent with the PAH fingerprint at Site 2, indicating that the study sites were affected by different PAH sources. The PAH fingerprint at Site 1 was also found to be inconsistent with the fingerprint observed at Site 1 in 2004, indicating the PAH source at Site 1 is not temporally constant. The most likely cause for such source changes are harbour-wide sediment redeposition events, potentially resulting from storm activity. The variability in

PAH sources at Site 1 highlights the complex nature of PAH transport throughout the harbour, and suggests redistribution of PAH-contaminated sediment throughout Hamilton Harbour continues to be a problem. The exact sources of PAHs to Site 1 and Site 2 could not be identified using PAH ratios and fingerprints.

The increased understanding of PAH source variability, PAH transport throughout Hamilton Harbour, and the impact of PAH contamination upon sedimentary microbial communities in Hamilton Harbour developed through this study provides a framework upon which further studies of microbial communities in Hamilton Harbour can be based. Along with such future studies, baseline values for microbial biomass and community composition throughout the harbour can be established. Monitoring deviations from those baseline values would provide a greater ability to monitor the progress of remediation in Hamilton Harbour, and potentially develop more effective remediation methods. More frequent analyses at the current study sites, and/or analysis of greater numbers of sites could also aid in establishing the nature of and cause for changes in PAH ratios at Site 1, and in further constraining the effects of PAH contamination upon microbial communities.

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2-8. References

Antizarladislao, B., Spanova, K., Beck, A., Russell, N. (2008). Microbial community structure changes during bioremediation of PAHs in an aged coal-tar contaminated soil by in-vessel composting. International Biodeterioration & Biodegradation 61, 357-364.

Baird, B.H., White, D.C. (1985). Biomass and community structure of the abyssal microbiota determined from the ester-linked phospholipids recovered from Venezuela Basin and Puerto Rico Trench sediments. Marine Geology 68, 217-231.

Benner Jr, B., Wise, S., Currie, L., Klouda, G., Klinedinst, D., Zweidinger, R., Stevens, R., Lewis, C. (1995). Distinguishing the contributions of residential wood combustion and mobile source emissions using relative concentrations of dimethylphenanthrene isomers. Environmental Science & Technology 29, 2382-2389.

Boffetta, P., Jourenkova, N., Gustavsson, P. (1997). Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. Cancer Causes and Control 8, 444-472.

Boschker, H.T.S., Middelburg, J.J. (2002). Stable isotopes and biomarkers in microbial ecology. FEMS Microbiology Ecology.

Boyd, T., Montgomery, M., Steele, J., Pohlman, J., Reatherford, S., Spargo, B., Smith, D. (2005). Dissolved oxygen saturation controls PAH biodegradation in freshwater estuary sediments. Microbial Ecology 49, 226-235.

Canton, L., Grimalt, J.O. (1992). Gas chromatographic-mass spectrometric characterization of polycyclic aromatic hydrocarbon mixtures in polluted coastal sediments. Journal of Chromatography 607, 279-286.

Cerniglia, C. (1992). Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3, 351-368.

Christensen, E.R., Li, A., Ab Razak, I.A., Rachdawong, P., Karls, J.F. (1997). Sources of polycyclic aromatic hydrocarbons in sediments of the Kinnickinnic River, Wisconsin Journal of Great Lakes Research 24, 61-73.

Curran, K., Irvine, K., Droppo, I., Murphy, T. (2000). Suspended solids, trace metal and PAH concentrations and loadings from coal pile runoff to Hamilton Harbour, Ontario. Journal of Great Lakes Research 26, 18-30.

de Solla, S., Fernie, K., Ashpole, S. (2008). Snapping turtles (Chelydra serpentina) as bioindicators in Canadian areas of concern in the Great Lakes Basin. II. Changes in hatching success and hatchling deformities in relation to persistent organic pollutants. Environmental Pollution 153, 529-536.

Droppo, I.G., Ross, N., Skafel, M., Liss, S.N. (2007). Biostabilization of cohesive sediment beds in a freshwater wave-dominated environment. Limnology and Oceanography, pp. 577-589.

Fang, J., Barcelona, M. (1998). Biogeochemical evidence for microbial community change in a jet fuel hydrocarbons-contaminated aquifer. Organic Geochemistry 29, 899-907.

Findlay, R., Dobbs, F.C. (1993). Quantitative description of microbial communities using lipid analysis. In: Kemp, P.F., Sherr, B.F., Sherr, E.B., Cole, J.J. (Eds.). Handbook of Methods in Aquatic Microbial Ecology. Lewis Publishers Boca Raton, FL, pp 271-284.

Findlay, R., Trexler, M., White, D. (1990). Response of a benthic microbial community to biotic disturbance. Marine Ecology Progress Series. 62, 135-148.

Furlong, E.T., Carter, D.S., Hites, R.A. (1988). Organic contaminants in sediments from the Trenton Channel of the Detroit River, Michigan. Journal of Great Lakes Research 14, 489-501.

Government of Ontario (2009). Guide to Eating Ontario Sport Fish. In: Environment, M.o.t. (Ed.). Queen's Printer for Ontario, Toronto.

Green, C.T., Scow, K.M. (2000). Analysis of phospholipid fatty acids (PLFA) to characterize microbial communities in aquifers. Hydrogeology Journal 8, 126-141.

Guha, S., Jaffe, P. (1996). Biodegradation kinetics of phenanthrene partitioned into the micellar phase of nonionic surfactants. Environmental Science & Technology 30, 605-611.

Haritash, A.K., Kaushik, C.P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. Journal of Hazardous Materials 169, 1-15.

Harvey, H.R., Fallon, R.D., Patton, J.S. (1986). The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. Geochimica et Cosmochimica Acta 50, 795-804.

Hayes, M.A., Smith, I.R., Crane, T.L., Rushmore, T.H., Thorn, C., Kocal, T.E., Ferguson, H.W. (1990). Pathogenesis of skin and liver neoplasms in white suckers from industrially polluted areas in Lake Ontario. Science of the Total Environment 94.

Innis, S. (2007). Dietary (n-3) fatty acids and brain development. Journal of Nutrition 137, 855-859.

Irvine, K., Droppo, I., Murphy, T., Lawson, A. (1997). Sediment resuspension and dissolved oxygen levels associated with ship traffic: Implications for habitat remediation. Water Quality Research Journal of Canada. 32, 421-437.

Johnsen, A. (2005). Principles of microbial PAH-degradation in soil. Environmental Pollution 133, 71-84.

Kaneda, T. (1991). Iso-and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiology and Molecular Biology Reviews 55, 288.

Kang, J., Leaf, A. (1994). Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes. Proceedings of the National Academy of Sciences 91, 9886-9890.

Karrow, N.A., Bennie, D.T., Boermans, H.J., Bols ..., N.C. (2003). Effect of exposure to various sites within Hamilton Harbour on Oncorhynchus mykiss .... Journal of Great Lakes Research.

Kavanagh, R., Balch, G., Kiparissis, Y., Niimi, A., Sherry, J., Tinson, C., Metcalfe, C. (2004). Endocrine Disruption and Altered Gonadal Development in White Perch(Morone americana) from the Lower Great Lakes Region. Environmental Health Perspectives 112, 898-902.

Kawakita, E., Hashimoto, M., Shido, O. (2006). Docosahexaenoic acid promotes neurogenesis in vitro and in vivo. Neuroscience 139, 991-997.

Keith, L., Telliard, W. (1979). ES&T special report: priority pollutants: I - a perspective view. Environmental Science & Technology 13, 416-423.

Kitajka, K., Puskás, L., Zvara, Á., Hackler, L., Barceló-Coblijn, G., Yeo, Y., Farkas, T. (2002). The role of n-3 polyunsaturated fatty acids in brain: Modulation of rat brain gene expression by dietary n-3 fatty acids. Proceedings of the National Academy of Sciences 99, 2619.

Leaf, A. (1992). Health claims: omega-3 fatty acids and cardiovascular disease. Nutrition reviews 50, 150-154.

Lima, A.L.C., Eglinton, T.I., Reddy, C.M. (2003). High-Resolution Record of Pyrogenic Polycyclic Aromatic Hydrocarbon Deposition during the 20th Century. Environmental Science & Technology 37, 53-61.

Mahler, B.J., Van Metre, P.C., Bashara, T.J., Wilson, J.T., Johns, D.A. (2005). Parking Lot Sealcoat: An Unrecognized Source of Urban Polycyclic Aromatic Hydrocarbons. Environmental Science & Technology 39, 5560-5566.

Marvin, C., McCarry, B., Villella, J., Allan, L. (2000a). Chemical and biological profiles of sediments as indicators of sources of genotoxic contamination in Hamilton Harbour. Part II: Bioassay-directed fractionation using the Ames Salmonella/microsome assay. Chemosphere.

Marvin, C., McCarry, B., Villella, J., Allan, L., Bryant, D. (2000b). Chemical and biological profiles of sediments as indicators of sources of genotoxic contamination in Hamilton Harbour. Part I: Analysis of polycyclic aromatic hydrocarbons and thia-arene compounds. Chemosphere 41, 979-988.

Marvin, C.H., Allan, L., McCarry, B.E., Bryant, D.W. (1993). Chemico/biological investigation of contaminated sediment from the Hamilton Harbour area of Western Lake Ontario. Environmental and Molecular Mutagenesis 22, 61-70.

Mastrangelo, G., Fadda, E., Marzia, V. (1996). Polycyclic aromatic hydrocarbons and cancer in man. Environmental Health Perspectives 104, 1166.

McCarthy, L.H., Thomas, R.L., Mayfield, C.I. (2004). Assessing the toxicity of chemically fractionated Hamilton Harbour (Lake Ontario) sediment using selected aquatic organisms. Lakes & Reservoirs: Research and Management 9, 89-103.

Means, J., Wood, S., Hassett, J., Banwart, W. (1980). Sorption of polynuclear aromatic hydrocarbons by sediments and soils. Environmental Science & Technology 14, 1524-1528.

Murphy, T. (2000). Coal tar in Hamilton Harbour - attempts at biological restoration guidelines. Environmental Toxicology 15, 484-495.

Nichols, P., Guckert, J., White, D. (1986). Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. Journal of Microbiological Methods 5, 49-55.

O'Connor, K.M. (2002). Toxic Substances and Sediment Remediation Component. Remedial Action Plan for Hamilton Harbour: Stage 2 Update. Hamilton Harbour RAP Stakeholder Forum, Hamilton, Ontario, pp 81-102.

O'Leary, W., Winkinson, S. (1988). Gram-positive bacteria. In: Ratledge, C., Wilkinson, S.G. (Eds.). Microbial Lipids. Academic Press, London, pp 117-202.

OME (1975). Hamilton Harbour Study 1974. In: Branch, W.R. (Ed.). Ontario Ministry of the Environment, Toronto.

OME (1977). A water quality study of Cootes Paradise. In: Technical Support Section, W.C.R. (Ed.). Ontario Ministry of the Environment, Hamilton.

OME (1986). Cootes Paradise study. In: Technical Support Section, W.C.R. (Ed.). Ontario Ministry of the Environment, Hamilton.

Rao, Y.R., Marvin, C.H., Zhao, J. (2009). Application of a Numerical Model for Circulation, Temperature and Pollutant Distribution in .... Journal of Great Lakes Research.

Remedial Action Plan Writing Team (1992). Remedial Action Plan for Hamilton Harbour. Stage 1 Report: Environmental Conditions and Problem Definition. Hamilton Harbour Remedial Action Plan Office, Hamilton, Ontario, p. 247.

Ringelberg, D., Richmond, M., Foley, K., Reynolds, C. (2008). Utility of lipid biomarkers in support of bioremediation efforts at army sites. Journal of Microbiological Methods 74, 17-25.

Rothermich, M.M., Hayes, L.A., Lovley, D.R. (2002). Anaerobic, sulfate-dependent degradation of polycyclic aromatic hydrocarbons in petroleum-contaminated harbor sediment. Environmental Science & Technology 36, 4811-4817.

Samanta, S., Singh, O., Jain, R. (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. TRENDS in Biotechnology 20, 243-248.

Sargent, J.R., Tocher, D.R., Bell, J.G. (2002). The lipids. In: Halver, J.E., Hardy, R.W. (Eds.). Fish Nutrition. Academic Press, New York, pp 182-246.

Shibahara, A., Yamamoto, K., Kinoshita, A., Anderson, B.L. (2008). An Improved Method for Preparing Dimethyl Disulfide Adducts for GC/MS Analysis. Journal of the American Oil Chemists' Society 85, 93-94.

Silva, I., Grossman, M., Durrant, L. (2009). Degradation of polycyclic aromatic hydrocarbons (2–7 rings) under microaerobic and very-low-oxygen conditions by soil fungi. International Biodeterioration & Biodegradation 63, 224-229.

Singh, M. (2005). Essential Fatty Acids, DHA and the Human Brain. Indian Journal of Pediatrics 72, 239-242.

Slater, G., Cowie, B., Harper, N., Droppo, I. (2008). Variation in PAH inputs and microbial community in surface sediments of Hamilton Harbour: Implications to remediation and monitoring. Environmental Pollution 153, 60-70.

Slater, G.F., Nelson, R.K., Kile, B.M., Reddy, C.M. (2006). Intrinsic bacterial biodegradation of petroleum contamination demonstrated in situ using natural abundance, molecular-level 14C analysis. Organic Geochemistry 37, 981-989.

Slater, G.F., White, H.K., Eglinton, T.I., Reddy, C.M. (2005). Determination of microbial carbon sources in petroleum contaminated sediments using molecular 14C analysis. Environmental Science & Technology 39, 2552-2558.

Sofowote, U., McCarry, B., Marvin, C. (2008). Source apportionment of PAH in Hamilton Harbour suspended sediments: comparison of two factor analysis methods. Environmental Science & Technology 42, 6007-6014.

Su, Y., Yang, X. (2009). Interactions between selected PAHs and the microbial community in rhizosphere of a paddy soil. Science of the Total Environment 407, 1027-1034.

Vreugdenhil, M., Bruehl, C., Voskuyl, R., Kang, J., Leaf, A., Wadman, W. (1996). Polyunsaturated fatty acids modulate sodium and calcium currents in CA1 neurons. Proceedings of the National Academy of Sciences 93, 12559-12563.

White, D., Ringelberg, D. (1998). Signature lipid biomarker analysis. In: Burlage, R.S., Atlas, R., Stahl, D., Geesey, G., Sayler, G. (Eds.). Techniques in microbial ecology. Oxford University Press, New York, pp 255–272.

White, D.C. (1988). Validation of quantitative analysis for microbial biomass, community structure and metabolic activitty. Archiv fur Hydrobiologie - Beiheft Ergebnisse der Limnologie 1998, 1-18.

White, D.C. (1997). Biomass Measurements: Biochemical Approaches. In: Hurst, C.H., Knudsen, G., McInerney, M.J., Stetzenbach, L.D., Walter, M. (Eds.). Manual of Environment Microbiology. American Society for Microbiology Press, Washington, D.C., pp 91-101.

White, D.C., Bobbie, R.J., King, J.D., Nickels, J., Amoe, P. (1979). Lipid analysis of sediments for microbial biomass and community structure. In: Litchfield, C.D., Seyfried, P.L. (Eds.). Methodology for Biomass Determinations and Microbial Activites in Sediments. American Society for Testing and Materials, pp 87-103.

Wislocki, P.G., Lu, A.Y.H. (1988). Carcinogenicity and mutagenicity of proximate and ultimate carcinogens of polycyclic aromatic hydrocarbons. In: Yang, S.K., Silverman, B.D. (Eds.). Polycyclic aromatic hydrocarbon carcinogenesis: structure-activity relationships. CRC Press, Boca Raton, FL, pp 1-30.

Yamamoto, K., Shibahara, A., Nakayama, T., Kajimoto, G. (1991). Determination of double-bond positions in methylene-interrupted dienoic fatty acids by GC-MS as their dimethyl disulfide adducts Chemistry and Physics of Lipids 60, 39-50.

Yang, H., Su, Y., Zhu, Y., Chen, M., Chen, B., Liu, Y. (2007). Influences of polycyclic aromatic hydrocarbons (PAHs) on soil microbial community composition with or without vegetation. Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering 42, 65-72.

Yunker, M., Macdonald, R., Vingarzan, R., Mitchell, R., Goyette, D., Sylvestre, S. (2002). PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Organic Geochemistry 33, 489-515.

Zeman, A., Patterson, T. (2003). Characterization and Mapping of Contaminated Sediments, Windermere Arm, Hamilton Harbour, Ontario, Canada. Soil and Sediment Contamination (formerly Journal of Soil Contamination) 12, 619-629.

Zeman, A., Patterson, T.S. (2006). Characterization of contaminated sediments for remediation projects in hamilton harbour. In: Calabrese, E.J., Kostecki, P.T., Dragun, J. (Ed.). Contaminated Soils, Sediments and Water: Successes and Challenges. Springer Science+Business Media, Inc., New York, pp 401-422.

Zhang, X., Christensen, E.R., Yan, L. (1993). Fluxes of polycyclic aromatic hydrocarbons to Green Bay and Lake Michigan sediments. Journal of Great Lakes Research 19, 429-444.

Zhou, H., Wong, A., Yu, R., Park, Y., Wong, Y., Tam, N. (2009). Polycyclic Aromatic Hydrocarbon-Induced Structural Shift of Bacterial Communities in Mangrove Sediment. Microb Ecol 58, 153-160.

CHAPTER 3

Modelling *P. frederiksbergensis* metabolism and carbon cycling behaviour using stable and radiocarbon isotopic analyses.

3-1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are products of the incomplete combustion of organic material, are natural constituents of fossil fuels and can also be formed diagenetically and biosynthetically in globally-insignificant amounts (Kanaly and Harayama, 2000; Lima et al., 2005). They are pervasive, globally-dispersed environmental contaminants, being found in air (Szidat et al., 2004), sediment and soil (van Herwijnen et al., 2003; Mandalakis et al., 2004a; Johnsen et al., 2007; Short et al., 2007b), groundwater and surface water (Martens et al., 1997; Holman et al., 1999), and also in remote locations including deep-sea sediments (Ohkouchi et al., 1999), and arctic ice and snow (Kawamura and Suzuki, 1994; Mascalet et al., 2000). PAHs are emitted to the environment via natural processes including forest fires and volcanic eruptions as well as anthropogenic activities. Based on the most recently available data for Canada, atmospheric PAH emissions are dominated by forest fires, which release approximately 2010 tonnes of PAHs per year (~47% of total emissions). Industrial processes account for a further ~22% (roughly 960 tonnes), primarily the result of aluminium smelting. Residential, agricultural and industrial-related combustion of organic material account for

the majority of the remaining ~31%, with the largest contributions coming from wood heating, landfill incineration, agricultural burning and combustion of petroleum fuels (Environment Canada, 1994). Due to the temporal and spatial variability of their occurrence, forest fires do not cause continuous contamination of a specific area (Environment Canada, 1994), while most anthropogenic PAH sources repeatedly expose a specific area, and are, therefore, of greater concern. Besides incorporation of atmospheric PAHs, leaching from creosote-treated materials, petroleum spills, municipal effluents and burial of PAH-containing wastes also contribute substantially to PAH contamination in soils and sediments (Environment Canada, 1994).

The great environmental persistence exhibited by PAHs results primarily from their low aqueous solubility, which causes PAHs to rapidly become associated with particles in soil, air and water. These PAH-associated particles are eventually incorporated into suspended sediments and surface bed sediments in aqueous systems, where they accumulate (Means et al., 1980; Guha and Jaffe, 1996). In the U.S., this has resulted in the contamination of an estimated 10% of river, lake and coastal sediments (Boyd et al., 2005). The sorption of PAHs to particles reduces PAH bioavailability (Means et al., 1980; Guha and Jaffe, 1996), and burial of contaminated surface sediments after deposition can further reduce bioavailability. Following burial, organisms still able to access the PAHs must rely on anaerobic degradation processes as dissolved oxygen levels usually decrease rapidly below the sediment-water interface (Rothermich et al., 2002; Haritash and Kaushik, 2009). While certain low molecular weight PAHs (2-3 benzene rings) have been shown to be rapidly degraded under anaerobic conditions,

relative to aerobic PAH degradation, anaerobic degradation is usually a much slower process, particularly for high molecular weight PAHs (Rothermich et al., 2002; Haritash and Kaushik, 2009). Rates of photodegradation, another significant mechanism of PAH degradation in natural systems, are also reduced by sorption and burial, which reduce the extent to which solar radiation can reach PAHs. The susceptibility of a compound to photodegradation is determined primarily by its absorption spectrum and by the nature of the particle to which it is absorbed. PAHs absorb light of a wide range of wavelengths but typically, linear PAHs exhibit greater photosensitivity as they can absorb a wider and higher range of wavelengths than angular PAHs (Schwarzenbach et al., 2003). When all potential degradative factors are considered together, the overall residence time of PAHs in the environment tends to increase with increasing angularity and molecular weight of PAH molecules, due to greater hydrophobicity and electrochemical stability (Means et al., 1980).

Exposure to PAHs is regarded as a serious risk to ecosystems and to human health. Due to evidence of adverse impacts on the environment, PAHs are listed on the Priority Substances List under the Canadian Environmental Protection Act, with 5 specific PAHs listed as a possible danger to human life or ecosystem health. The U.S. Environmental Protection Agency lists 16 specific PAHs as priority pollutants for remediation (Keith and Telliard, 1979), due in part to the demonstration of acutely toxic, carcinogenic, teratogenic or mutagenic properties (Wislocki and Lu, 1988; Mastrangelo et al., 1996; Boffetta et al., 1997; Samanta et al., 2002). PAHs enter food webs directly at low trophic levels. A substantial amount of the PAH burden in the atmosphere is

removed by vegetation (Wagrowski and Hites, 1996), resulting in the contamination of plants consumed by humans and animals (Maggioni et al., 2009). Once present within the mammalian digestive system, the lipophilic nature of PAHs leads to their rapid absorption and distribution in a variety of body tissues, particularly body fat. While PAH metabolism appears to occur at a sufficient rate to prevent accumulation of PAHs in higher-trophic-level organisms (Nakata et al., 2003), many PAHs can be metabolized to produce highly reactive and carcinogenic bay- and K-region epoxides, which themselves may accumulate in mammalian tissues (Samanta et al., 2002). Organisms in lower trophic levels, such as lugworms, clams and crabs seem to lack the ability to metabolize PAHs and may be directly affected by the toxic effects of PAH accumulation (Nakata et al., 2003).

The worldwide distribution and deleterious impact of PAH exposure clearly highlight the need to develop viable PAH remediation schemes. Currently, commercial remediation of PAH-contaminated sediments is typically undertaken using physical or chemical approaches, such as sediment removal and incineration, gas phase chemical reduction and *in situ* thermal desorption. These methods can be extremely costly, slow, and can degrade soil quality, or pose a danger to remediation personnel (Tavlarides et al., 2000). In recent years, intrinsic and engineered bioremediation systems have received substantial interest as an alternative to traditional remediation methods, primarily due to their greater cost-effectiveness, especially in the treatment of large areas (Tavlarides et al., 2000).

A key challenge in establishing bioremediation as a viable remediation technology has been the need for better means of monitoring the extent and occurrence of microbial degradation. Most currently available monitoring techniques are very limited in their ability to monitor microbial activity. Changes in substrate concentration or distribution can reflect active microbial degradation, but can also result from sorption to unmeasured components in a system, or other non-degradative processes of mass loss (Slater et al., 2006a). Examination of laboratory cultures, or the genetic analysis thereof, can identify the presence of specific organisms in a microbial community, and can even (indirectly) reveal the presence of organisms able to degrade specific contaminants, if that ability has also been discovered as a result of other culture-based experimentation. However, this approach cannot directly establish if organisms are actively carrying out metabolic processes resulting in contaminant degradation, and cannot be used *in situ*. Isotopic tracers can be used to demonstrate the occurrence of metabolic processes of interest, through the *in situ* incorporation of ^{13}C- or ^{14}C-labelled substrates into microbial cells (Hesselsoe et al., 2005) and lipids (Roslev et al., 1998; Fang et al., 2004). However, the process is costly, requires long time intervals to achieve resolvable signal intensities, and in many cases the isotopically-labelled compound may behave in a substantially different manner to its non-labelled counterpart (Slater, 2003; Slater et al., 2005a; Slater et al., 2006a). Natural abundance stable isotope analysis, particularly if performed at a compound-specific level, also allows measurements to be taken *in situ*, and has been used to identify microbial carbon sources, and to establish the occurrence of specific types of microbial metabolism (eg. methanotrophy) (Boschker and Middelburg, 2002b). The

primary challenge with this technique is the need to constrain the variable effects of isotopic fractionation in biosynthetic pathways in order to properly interpret results (Hayes, 2001). As a further complication, there is often no distinction between the isotopic signatures of contaminants and other "natural" carbon sources that are present (Slater, 2003).

When organic contaminants of interest are derived from petroleum carbon, these difficulties can often be overcome through the use of natural abundance ^{14}C analysis. ^{14}C has a half life of 5730 years, meaning it is undetectable to modern instrumentation after approximately 60,000 years (Stuvier and Polach, 1977). Due to its geologic age, petroleum-derived carbon contains no detectable ^{14}C ($\Delta^{14}C = -1000‰$). In contrast, natural organic matter (NOM), defined here as the organic material not removed from sediments during the extraction of total petroleum hydrocarbons (TPH), is generally composed of photosynthesized organic material of geologically recent origin, and therefore usually contains modern levels of ^{14}C ($\Delta^{14}C = 100 \pm 50‰$ depending upon time and place of carbon fixation) (Slater et al., 2006a). As ^{14}C isotopic signatures of bacterial cell components have been shown to reflect those of the carbon sources they assimilate (Petsch et al., 2001; Kramer and Gleixner, 2006), microbial uptake of petroleum carbon will usually result in lower ^{14}C content in microbial cellular structures than the uptake of NOM. It must be noted that this works best for systems in which NOM is decidedly modern in age. In certain systems, a number of factors can cause NOM to become more aged, making differentiation of PLFA signatures more difficult. Limited contributions of new plant-derived carbon to NOM, such as might occur in deep soils or sediments, can

leave NOM dominated by older recalcitrant organic carbon. The presence of recalcitrant coal or petroleum as a component of NOM can also substantially reduce NOM $\Delta^{14}C$ values (Kramer and Gleixner, 2006). Petsch et al. (2001) showed NOM could also become more aged through the action of microorganisms which actively incorporated ^{14}C-free carbon in black shales, thus recycling ^{14}C-free carbon back into the NOM pool upon cellular death.

Assuming NOM and petroleum ^{14}C signatures are sufficiently distinct, it follows that the relative extent to which petroleum vs. modern carbon is incorporated into microbial cellular structures can be determined by measuring the $\Delta^{14}C$ levels of microbial cellular components. Microbial phospholipid fatty acids (PLFAs) are ideal cellular structures on which to perform such measurements. They are integral components of cell membranes, and, as they degrade within days to weeks following cell death (Harvey et al., 1986; Boschker and Middelburg, 2002b), their composition reflects that of the viable microbial community at the time of sampling. Many PLFAs serve as biomarkers, their presence indicating the presence of particular types of microbes such as bacteria or fungi, or microbial populations involved in particular metabolic processes, such as methanotrophy or sulphate-reduction (Green and Scow, 2000; Boschker and Middelburg, 2002b). PLFA concentrations can also be used to directly estimate cellular biomass through the use of conversion factors (Green and Scow, 2000).

Petsch et al. (2001) pioneered the differentiation of modern and ancient microbial carbon sources using PLFA ^{14}C analysis by demonstrating the assimilation of black shale organic matter (^{14}C-free) from organic-rich sedimentary rocks by a laboratory enrichment

culture of aerobic microbes. Slater et al. (2006a) expanded on this work, using PLFA ^{14}C analysis to directly demonstrate *in situ* microbial biodegradation of petroleum hydrocarbons. In that study, depletions in PLFA $\Delta^{14}C$ values resulting from microbial uptake of petroleum carbon were observed under constrained conditions (an oil spill on a rocky shoreline) in which petroleum carbon was a dominant carbon source and little soil or sediment (and associated organic matter) was present. In anaerobic sediments of more complex composition, in which petroleum carbon was less dominant, controversial results have been reported. In anaerobic salt-marsh sediments containing petroleum hydrocarbons from a spill which had persisted at depth for decades, Slater et al. (2005a) found no significant difference in the ^{14}C values of microbial PLFAs and NOM. This suggested no microbial uptake of petroleum carbon was occurring, despite extensive evidence that typical anaerobic microbial communities contain organisms capable of anaerobic degradation of the types of petroleum hydrocarbons that were present (Coates et al., 1997; Caldwell et al., 1998; Rothermich et al., 2002; So et al., 2003; Townsend et al., 2003). In contrast, Wakeham et al. (2006) found microbial PLFAs in different predominantly anaerobic salt-marsh sediments exhibited slightly lower ^{14}C values than the value for modern-aged NOM present in those sediments, which the authors suggested was indicative of microbial utilization of up to 10% petroleum carbon for PLFA biosynthesis. This level of petroleum carbon utilization was much lower than was indicated by ^{14}C measurements in Petsch et al. (2001) or Slater et al. (2006a). While such low values could have resulted from more limited utilization of petroleum carbon due to availability of a more complex mixture of available carbon sources, as suggested by the

authors, they could not definitively demonstrate the occurrence of any incorporation of petroleum carbon. The relatively depleted $\Delta^{14}C$ values could well have resulted from bacterial utilization of other carbon sources of intermediate ^{14}C content. Based on the results of these studies, it appeared possible that microbes might be preferentially metabolizing NOM over hydrocarbon compounds, meaning the presence of NOM was inhibiting petroleum hydrocarbon biodegradation. In Slater et al. (2005a), the authors suggested the total lack of microbial hydrocarbon metabolism in that study might have resulted from microbial preference for potentially more labile NOM, due to a greater energetic favorability for NOM metabolism versus metabolism of petroleum hydrocarbons. Most petroleum hydrocarbon compounds have traditionally been considered to have low bioavailability. However, the results in Wakeham et al. (2006) suggested bacterial metabolism of petroleum carbon might still be of sufficient energetic favorability to occur to some extent, despite the presence of large quantities of presumably more labile NOM. To further complicate matters, the recent finding that most hydrophobic organic contaminants (HOCs), including petroleum derivatives, did not strongly sorb to a variety of marine and freshwater sediments (White et al., 2008), implied HOCs actually remain highly bioavailable in the environment. Under such a scenario, differences in the energetic favorability of NOM and petroleum metabolism would be limited, suggesting any explanation based on energetic favorability is questionable. While it is possible the differences between studies could lie in site-specific parameters such as temperature, availability of electron receptors, or oxygen availability (Slater et al., 2005a; Wakeham et al., 2006), the question of bacterial carbon source

preference is certainly worthy of further investigation.

This study originated in an attempt to address the discrepancy in the Wakeham et al. (2006) and Slater et al. (2006a) salt-marsh studies. *In situ* studies, such as the above, pose difficulties for ^{14}C analysis, as the sheer number of possible carbon sources of different isotopic compositions make it difficult to establish which are used by microbes (and thus degraded). As such, a laboratory study was employed. The quantitative assessment of bacterial carbon source preferences in a controlled laboratory setting was expected to reveal bacterial preference for petroleum contaminants (represented by the PAH phenanthrene) as a carbon source, in the presence of modern-aged NOM. Phenanthrene is a low molecular weight PAH classified as a priority pollutant by the U.S. Environmental Protection Agency (Keith and Telliard, 1979). It was selected as a model PAH for this study for two key reasons: it is representative of the more easily biodegraded low molecular weight PAHs, and its biodegradation has been well documented and occurs on a timescale measured in weeks rather than years (Cerniglia and Heitkamp, 1989; Shuttleworth and Cerniglia, 1995; Kanaly and Harayama, 2000). *Pseudomonas frederiksbergensis*, a previously isolated bacterium known to be capable of at least co-metabolic degradation of phenanthrene under aerobic, terrestrial conditions (Johnsen et al., 1996; Andersen et al., 2000) was selected as a model PAH-degrading organism.

Laboratory pure cultures of *P. frederiksbergensis* were separately exposed to phenanthrene and to NOM in order to establish baseline microbial growth rates, PLFA concentrations and distributions, and bulk PLFA stable and radiocarbon isotopic

signatures with each organic carbon substrate serving as the sole source of carbon to the cultures. *P. frederiksbergensis* was also exposed to a mixture of equal quantities of phenanthrene and NOM. As phenanthrene is geologically ancient and therefore ^{14}C-free, and the NOM employed in this case exhibited a modern ^{14}C signature, exposure to both substrates was expected to result in a PLFA ^{14}C content between that observed for PLFA of *P. frederiksbergensis* cultures exposed to phenanthrene or NOM alone. This approach was designed to enable the first quantitative establishment of bacterial preference for one carbon source over the other via measurements of bacterial PLFA ^{14}C values. PLFA identities and concentrations, as well as ^{13}C isotopic analysis, were also used to evaluate carbon source utilization when *P. frederiksbergensis* was exposed to the different substrates. All initial expectations were proven incorrect, with experimental results suggesting a much more complex model of carbon source utilization in *P. frederiksbergensis* cells which involved dissolved inorganic carbon (DIC). While the involvement of DIC meant the question of bacterial preference for NOM over petroleum carbon could not be resolved, this study gives insight into *P. frederiksbergensis* carbon cycling pathways, as well as the potential for employing *P. frederiksbergensis* in future bioremediation efforts.

3-2. Methods

3-2.1 Microorganisms and culture conditions

Pure culture of *P. frederiksbergensis* was obtained from the American Type Culture Collection (Cedarlane Laboratories Ltd., Burlington, ON, ATCC #BAA-257). The initial cultures were initiated under sterile conditions in 3 250ml glass bottles with airtight Mininert ® PTFE caps, each containing 10ppm (w/v) powdered phenanthrene (Sigma-Aldrich, St Louis, MO), 200ml HCMM2 hydrocarbon minimal medium (Ridgway et al., 1990), and 250g fine grained silica sand combusted at 450° for 8 hours to remove all organic compounds. Media was vacuum filtered through a 0.2µm membrane to ensure sterility. Triplicate controls containing identical concentrations of phenanthrene, media and sand, but no cultures were also established. All culture bottles were grown in the dark and constantly agitated at 100rpm on an orbital shaker (VWR International). Initial culture sample bottle caps were partially removed under sterile conditions every 2 weeks to ensure cultures remained aerobic. Following a growth period of 200 days, 9 x 15ml of culture media was removed from each initial culture bottle and transferred to 3 sets of 9 bottles, all of which contained 250g silica sand and 200ml HCMM2 media. These new bottle sets were labelled as Set A, B, C (Table 3-1). Triplicate controls were established for each bottle set as described for the initial cultures. Each set of 9 bottles was made up of subsets of 3 bottles; all bottles in each subset received their 15ml of media from the same initial culture bottle. Set A bottles were supplemented with 10ppm of phenanthrene (Sigma Aldrich Inc.) and 10ppm of natural organic matter (NOM) from the Suwannee River, Georgia (International Humic Substances Society, St. Paul, MN), Set B bottles were supplemented with 10ppm of phenanthrene, and Set C bottles were supplemented

with 10ppm of NOM. Prior to its inclusion in bottles, NOM was wetted and incubated at 30°C for 48 hours to allow bacterial spores to germinate, then sterilized by heating at 200°C for 24 hours. NOM was then plated on LB agar to ensure sterilization was successful. This approach was chosen among those available as the least likely to physically or chemically alter the NOM (Trevors, 1996). Sets A, B and C were each allowed to grow approximately 1 month, prior to sampling. During this period, bottles were not vented. Previous analyses suggested that oxygen consumption over this period should not have been sufficiently rapid to affect culture growth.

Table 3-1: Culture Set nomenclature.

Bottle Set	Substrate
Initial culture	Phenanthrene (10ppm)
Set A	Phenanthrene (10ppm) + NOM (10ppm)
Set B	Phenanthrene (10ppm)
Set C	NOM (10ppm)

3-2.2 Sediment Extraction and Analysis

Wet sediment was extracted from all culture bottles (initial cultures, sets A, B, C) and their controls following a modified Bligh and Dyer protocol (White and Ringelberg, 1998; Slater et al., 2006a). In brief, 5-10g of sediment from each bottle was dried at 110°C for 24 hours to determine sediment moisture content. 200-225g of sediment from each bottle was sonicated in a 2:1:0.5 methanol:dichloromethane:phosphate (v/w) buffer solution for 2 minutes and then allowed to sit for 18-24 hours. Samples were gravity filtered and the organic phase was fractionated in a glass silica-gel column by elution with dichloromethane, acetone and methanol. The phospholipid fraction was evaporated to dryness under UHP N_2 and derivatized to fatty acid methyl esters (FAMEs) via the mild alkaline hydrolysis reaction. A second silica-gel column fractionation step with 4:1 hexane:DCM, DCM and MeOH ensured FAME purity prior to GCMS analysis.

Sampling of initial culture bottles and their controls took place when culture media was transferred to bottle Sets A, B and C. A total of 225g of sediment was extracted from each initial culture bottle. 10g from each bottle was used for PLFA analysis, and the

remaining sediment from all three bottles was combined for ^{14}C analysis to ensure a sufficient concentration of carbon was available for that analysis.

Sampling of Sets A, B and C and their controls took place roughly one month after they were established. 200g of sediment was extracted from each of the nine bottles in each set. Following gravity filtration, the organic phases of bottles from the same subset (ie. originating from the same initial culture bottle) were combined, effectively generating three 600g sediment samples for each set. This was again done to ensure sufficient carbon was available for ^{14}C analysis.

3-2.3 GC-MS Analysis

For FAME identification and quantification, a small subsample of the FAME mixture was analyzed by gas chromatography-mass spectrometry on an Agilent 6890 gas chromatograph with a J&W DB-XLB column (30 m x 0.25 mm i.d.; 0.25 μm film thickness) coupled with an Agilent 5973 quadrupole mass spectrometer. The oven temperature was initially held at 40° C for 1 min, ramped to 130°C at 20°C/min, to 160°C at 4°C/min and finally to 300°C at 8°C/min. FAME identification was based upon mass-fragmentation patterns and retention times as compared to bacterial reference standards (Bacterial Acid Methyl Esters CP Mix, Matreya Inc.). FAME double bond positions were determined by analysis of dimethyl disulphide adducts (DMDS) as per Nichols et al. (1986), whose method was found to produce identical results to the more recent methods

employed by Shibahara et al (2008) and Yamamoto et al. (1991). FAME quantification was established relative to an external standard curve generated using C-12,-14,-16,-18 and -20 FAME standards at concentrations ranging from 2ppm to 30ppm.

FAMEs are identified according to the following nomenclature: total number of carbon atoms:number of double bonds, then an integer following a lowercase omega (ω) indicates the position of the double bond (if present) from the methyl end of the molecule (e.g., 16:1ω6). Cis and trans geometry is indicated by the suffixes c and t. The prefixes a and i refer to anteiso- and isobranching, 10me indicates a methyl group on the 10th carbon atom from the carboxyl end of the molecule, the positions of hydroxy (OH) groups are noted, and Δ indicates cyclopropane fatty acids.

3-2.4 $\delta^{13}C$ and $\Delta^{14}C$ Analysis

FAME extracts not used for GC-MS analysis were sent for ^{13}C and ^{14}C analysis by AMS at the National Ocean Sciences Accelerator Mass Spectrometry (NOSAMS) facility at Woods Hole Oceanographic Institution, Woods Hole, MA. Due to time constraints, the single initial culture sample, containing phenanthrene as the sole carbon substrate, was analyzed in place of the Set B samples. All three Set A and Set C samples were analyzed. Pure samples of phenanthrene powder and sterilized natural organic matter were also submitted for analysis. Set A and Set C FAME samples, as well as the sample of pure NOM were processed according to NOSAMS standard small sample techniques, while

the initial culture sample and sample of phenanthrene were processed as large samples. At NOSAMS, NOM was prepared for analysis following methods for plant/wood samples, while all other samples were processed according to methods for sediments (total organics). ^{13}C data is reported in delta notation ($\delta^{13}C$) relative to the standard reference material, NBS-19 (VPDB) and expressed in units of per mil (‰). The term $\delta^{13}C$ represents the ratio of ^{13}C to ^{12}C in a sample relative to that in VPDB, as expressed by the following equation:

$$\delta^{13}C = \frac{^{13}C/^{12}C_{sample} - ^{13}C/^{12}C_{std}}{^{13}C/^{12}C_{std}} * 1000$$

^{14}C measurements are reported normalized to $\delta^{13}C$ values of -25‰, the postulated mean value of terrestrial wood, and expressed as $\Delta^{14}C$ values. Normalization is standard practice and takes into account any ^{13}C isotopic fractionation during biosynthesis or other processes (Stuvier and Polach, 1977). The term $\Delta^{14}C$ represents the deviation, in per mil (‰), from NBS Oxalic Acid I (Standard Reference Material 4990B), the international standard for ^{14}C dating (McNichol et al., 1994). After normalization, petroleum byproducts such as phenanthrene have a $\Delta^{14}C$ of -1000‰, while recently photosynthesized compounds are more enriched ($\Delta^{14}C$=100±50‰), depending upon the location and time at which their photosynthesis occurred.

$\delta^{13}C$ values for several PLFA samples sent to NOSAMS could not be established, and assumed values of -25‰ were reported instead. To establish the true $\delta^{13}C$ values for these samples, archived aliquots of the samples sent to NOSAMS were analyzed by

compound-specific GC-IRMS. Samples were concentrated to a volume of less than 10uL under UHP N_2 and then analyzed on an Agilent 6890 gas chromatograph with a DB-SMS column (30m x 0.32 mm i.d.; 0.25 μm film thickness) coupled to a Finnigan Delta Plus XP isotope-ratio mass spectrometer via a Finnigan GC Combustion 3 interface. Linearity of the CO_2 reference gas was sufficient to obtain reliable results from one or more of the largest PLFA peaks in each sample. Reported $\delta^{13}C$ values are an average of all peaks exceeding minimum acceptable linearity values in two IRMS analyses of each sample.

Contributions of methyl carbon derived from methanol to FAME $\delta^{13}C$ and $\Delta^{14}C$ values were removed via isotopic mass balance based on the equation below:

$$\delta^{13}C_{FAME} = [(N+1)*\delta^{13}C_{measured} - \delta^{13}C_{MeOH}]/N \text{ and } \Delta^{14}C_{FAME} = [(N+1)*\Delta^{14}C_{measured} - \Delta^{14}C_{MeOH}]/N$$

where N is the sum of the number of carbon atoms in the FAMEs present in each sample (Wakeham et al., 2006). $\delta^{13}C$ and $\Delta^{14}C$ values for methanol were established prior to its use in FAME preparation. Errors for $\delta^{13}C$ values are ±0.1‰ and cumulative errors for $\Delta^{14}C$ are given in Table 3-2.

3-2.5 DGGE Analysis

To establish sample purity, approximately 15g of wet sediment from each of the three initial culture bottles was placed in 50mL glass jars with PTFE lids and shipped on

ice to Microbial Insights, Inc. (Rockford, TN) for denaturing gradient gel electrophoresis (DGGE) analysis.

DGGE separates microbial DNA sequences, allowing identification of organisms present in sediments and other substrates, often to the species level. Separation is based upon the differing bond strengths of nucleotide base pairs in DNA. Adenine (A) forms 3 hydrogen bonds with Thymine (T), while Cytosine (C) and Guanine (G) are linked by only 2 hydrogen bonds. Therefore A-T bonding is stronger than C-G bonding. DGGE involves the isolation of DNA, specific sequences of which are amplified by polymerase chain reaction (PCR). Amplification products are then loaded in denaturing gradient gels and DNA migrates across the gel, driven by an electric current. Quantities of denaturing chemicals within the gel steadily increase along the path travelled by the DNA sequences, and therefore sequences with greater numbers of A-T bonds move further down the gel, as they are more resistant to the chemicals. Identical DNA sequences migrate the same distance, forming a band, which may be excised and sequenced to determine microbial identity if sufficient DNA is present. Generally, organisms must comprise >1-2% of a the total microbial population for their DNA to be sequenced and their identity revealed. Sequencing results are compared to sequence databases to identify organisms.

DNA from the sediment samples in this study was isolated and amplified using both 16S (prokaryotic) and 18S (fungal) rRNA primers. Amplification products were run on denaturing gradient gels and those forming defined bands were excised, sequenced and identified by comparison with 16S and 18S sequence databases.

3-3. Results

3-3.1 PLFA Isotopic Signatures

The stable carbon isotope content of bacterial PLFAs in all samples was clearly and consistently different from that of the organic carbon substrates, NOM and phenanthrene (Table 3-2). Suwannee River NOM was found to have a $\delta^{13}C$ value of -28‰, consistent with the expectation that it was largely composed of terrestrial plant/wood matter ($\delta^{13}C$ = -25‰) (Stuvier and Polach, 1977). Pure powdered phenanthrene was found to have a more enriched $\delta^{13}C$ value of -23.5‰. Measured bulk PLFA $\delta^{13}C$ values for all samples were very similar, regardless of the carbon substrate to which cultures were exposed (mean bulk PLFA $\delta^{13}C$ = -10.6 ±0.8‰), and all samples were substantially more enriched in ^{13}C than either of the organic carbon substrates. A $\delta^{13}C$ value for Set C Sample 3 could not be determined, but based on all other sample PLFA values, the value for Set C Sample 3 was assumed to also fall within the range of -10‰ to -11‰, and was assigned the average sample value of -10.6‰. A $\Delta^{14}C$ value of +30.8‰ was calculated based on that assumed $\delta^{13}C$ value.

Radiocarbon isotope results also showed distinctive differences between substrates and bacterial PLFA samples. Suwannee River NOM was found to have a $\Delta^{14}C$ value of +97.9‰, very consistent with expected values of +100±50‰ for recently photosynthesized organic matter (Slater et al., 2006a). The measured $\Delta^{14}C$ value of

-999.1‰ for powdered phenanthrene was similarly consistent with an expected value of -1000‰ for petroleum carbon, due to its age of >60,000 years. All bulk PLFA $\Delta^{14}C$ values were intermediate to the $\Delta^{14}C$ of the two substrates. Only two samples were significantly different from the other samples after error was taken into consideration, with the initial culture sample (phenanthrene only) and Set A Sample 3 (phenanthrene + NOM) both significantly more depleted than the other PLFA samples (Table 3-2). Errors associated with the precision of AMS analysis at NOSAMS were as high as ±16‰ (Table 3-2). The accuracy of $\Delta^{14}C$ values generated by small-sample techniques is generally considered to be in the range of ±10-20‰, while greater accuracy can be obtained with larger samples (Pearson et al., 1998). As accuracy cannot exceed precision (the error calculated from the actual analyses), it is a reasonable assumption that the actual total accuracy for the small sample $\Delta^{14}C$ measurements was ±16-20‰.

Significant differences existed between the $\Delta^{14}C$ values of the organic carbon substrates and of all PLFA samples, even if one were to assign the maximum estimated small-sample error in accuracy (±20‰) to all samples, even those processed by large sample techniques. It was particularly noteworthy that this significant difference held true for the PLFA samples exposed to only one of the two substrates; the PLFA values in those samples did not closely mirror the $\Delta^{14}C$ value of the sole provided organic carbon source, as would be expected if that source were being utilized for PLFA biosynthesis.

Table 3-2: Isotopic results from AMS and IRMS analyses. Samples of pure substrate are found below the break, bacterial PLFA samples above. $\delta^{13}C$ error was 0.1‰ for all samples. $\Delta^{14}C$ error shown is the total error associated with sample preparation and AMS analysis reported by NOSAMS[1].

Sample ID	Added Substrate(s)	$\Delta^{13}C$ (‰)	$\Delta^{14}C$ (‰)[1]
Initial Culture Sample	Phenanthrene	-10.9	-17.4 ±4.7
Set A Sample 1	Phenanthrene + NOM	-9.7	+24.8 ±11.2
Set A Sample 2	Phenanthrene + NOM	-11.3 [2]	+36.1 ±15.8 [2]
Set A Sample 3	Phenanthrene + NOM	-11.2	-28.8 ±9.9
Set C Sample 1	NOM	-10.7 [2]	+22.7 ±13.7 [2]
Set C Sample 2	NOM	-10.2 [2]	+5.4 ±15.6 [2]
Set C Sample 3	NOM	n.d.[3]	n.d.[3]
Suwannee R. NOM		-28.0	+97.9 ±15.9
Phenanthrene		-23.5	-999.1 ±0.4

[1] Error shown for ^{14}C analyses represents the precision of AMS analysis only. The total error (accuracy) for ^{14}C analysis for all samples was on the order of ±16-20‰.
[2] $\delta^{13}C$ and $\Delta^{14}C$ values represent values derived from re-analysis of $\delta^{13}C$ by GC-IRMS, and recalculation of $\Delta^{14}C$ based on the revised ^{13}C value
[3] $\delta^{13}C$ value could not be determined via either AMS or GC-IRMS analysis, but was assumed similar to other PLFA samples.

3-3.2 PLFA Identification and Quantification

A total of eight different PLFAs were detected across all sample bottles. 16:0, 16:1ω9c, 16:1ω9t, Δ17:0, 18:1ω11c and 18:1ω11t were detected in all samples. 18:0 was detected in all Set A samples, 2 of the 3 Set C samples, and was not detected in Set B. Δ19:0 was not detected in Set B, but was detected in all other samples.

Bulk PLFA concentrations were measured at the conclusion of the growth period for each sample. These concentrations are presented in Table 3-3.

Table 3-3: Bulk PLFA concentrations measured in sample bottles at the conclusion of the growth period for each Set.

Bottle ID	PLFA Concentration (ug/g dry weight)
Initial Culture Sample	1.24[1]
Set A Sample 1	0.16
Set A Sample 2	0.10
Set A Sample 3	0.19
Set B Sample 1	0.05
Set B Sample 2	0.03
Set B Sample 3	0.01
Set C Sample 1	0.07
Set C Sample 2	0.10
Set C Sample 3	0.05

[1] Initial Culture Sample PLFA concentration represents the combined total PLFA concentration from all three initial culture bottles.

3-3.3 Sample Purity

DGGE analysis results for 16S rRNA (prokaryotes) indicated the only prokaryotes present in the analysed samples were *Pseudomonas spp.*, the expected result for a pure culture of *Pseudomonas frederiksbergensis*. Results for 18S rRNA, however, indicated the presence of two types of fungus in the initial culture bottle used to generate Sample 1 in Sets A, B and C, one which could not be identified and one of the phylum Ascomycota. Fungus of the genus *Aspergillus and Cryptococcus* were present in the initial culture bottle used to generate Sample 2 in Sets A, B and C, and fungus of the genus *Malassezia* was present in the initial culture bottle used to generate Sample 3 in Sets A, B and C (Figure 3-1).

DGGE bands for *Pseudomonas spp.* were sharply defined, while fungal bands appeared more blurred. Bands for *Aspergillus* and Ascomycota appeared larger than those for *Pseudomonas spp.*, but of roughly the same intensity, while bands for the other fungal matches appeared to be of roughly equal size to *P. frederiksbergensis*, but were less intense.

Control bottles for all samples, sampled concurrently with the actual samples, were identical in composition to culture samples, save that *P. frederiksbergensis* cultures were not added to them. When subjected to GC-MS analysis, no PLFAs were detected in the controls, but they were not sent for DGGE analysis.

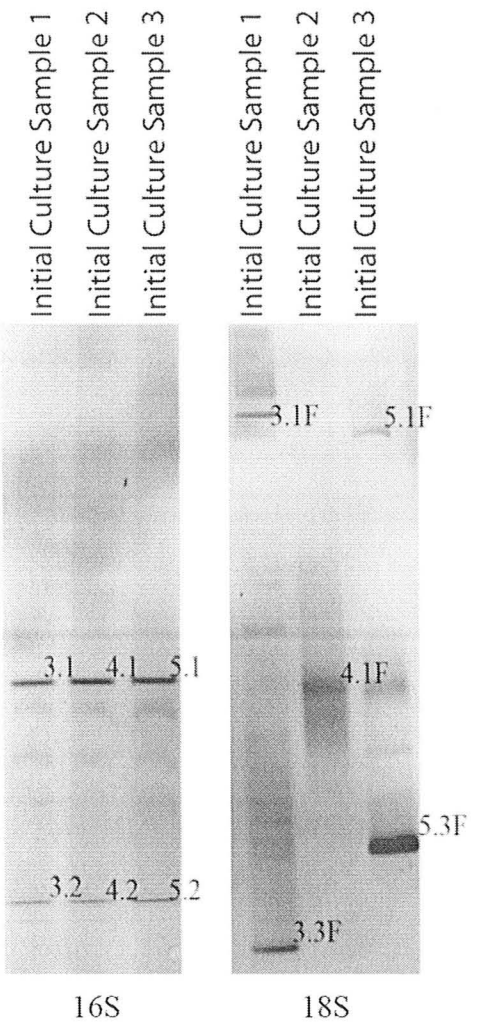

Figure 3-1: DGGE banding results for 16S and 18S rRNA primers. Numbered bands were excised and identified as follows 3.1, 3.2, 4.1, 4.2, 5.1, 5.2 – *Pseudomonas spp.*, 3.1F – unidentified, 3.3F – Ascomycota, 4.1F – uncultured *Malassezia*, 5.1F – *Cryptococcus*, 5.3F - *Aspergillus*

3-4. Discussion

3-4.1 Microbial Carbon Sources

Instead of the expected preferential uptake of one organic carbon source over the other (natural organic matter vs. phenanthrene), stable carbon and radiocarbon isotopic data suggested bacterial utilization of organic carbon for PLFA biosynthesis was minimal to nonexistent in all cultures, and dissolved inorganic carbon (DIC) was the dominant carbon source used for synthesis of PLFA.

3-4.1.1 Stable Isotopic Evidence for DIC as Primary Carbon Source

PLFA $\delta^{13}C$ values for all samples were obviously enriched relative to $\delta^{13}C$ values for either organic carbon source (Table 3-2). This suggested neither phenanthrene nor NOM could have been the dominant carbon source assimilated by *P. frederiksbergensis*. Biological organisms generally exhibit a greater preference for ^{12}C over ^{13}C during carbon assimilation. This results in an isotopic fractionation effect during carbon assimilation, producing biomass more depleted in ^{13}C than the source carbon. The extent of fractionation can vary widely depending upon the specific metabolic process by which carbon is assimilated (and in some cases no fractionation may occur). The formation of lipids (like PLFAs) from biomass carbon generally results in further fractionations, producing lipids more depleted than the biomass carbon. As a general rule, autotrophic organisms produce lipids substantially depleted in ^{13}C (-20‰ to -30‰) relative to the

source carbon (Hayes, 2001), while heterotrophs produce lipids with $\delta^{13}C$ values within ±4‰ of the source carbon (Petsch et al., 2001). The observed PLFA $\delta^{13}C$ values in all samples were more enriched in ^{13}C than could be explained by either heterotrophic or autotrophic utilization of either organic carbon source.

The only other carbon source that was present in sample bottles was DIC. DIC is composed of the carbonate species $CO_{2(aq)}$, H_2CO_3, HCO_3^- and CO_3^{2-}, although $CO_{2(aq)}$ is converted to H_2CO_3 so rapidly that the two species are often combined in practical use (Bunce, 1991). Since no carbon of any kind was added as an ingredient of the culture media, the DIC must have been derived from dissolution of atmospheric CO_2 into the sample media. Based on the most current published data, $\delta^{13}C$ levels of atmospheric CO_2 vary seasonally from roughly -7.7‰ to -8.5‰ in North America (Keeling et al., 2005), with a generally accepted value around -8‰ (Boschker and Middelburg, 2002a; Wacey, 2009). The $\delta^{13}C$ of CO_2 in the fourth floor laboratory in which cultures were grown was not measured during sample growth, but was previously measured to be between -11.5‰ and -11.9‰. Clearly, these estimates for $\delta^{13}C$ of atmospheric CO_2 represented a much closer match to the bulk PLFA $\delta^{13}C$ values for all samples (avg = -10.7‰±0.6) than the $\delta^{13}C$ values for pure phenanthrene (-23.5‰) or NOM (-28‰) (see Figure 3-2). The similarity between atmospheric DIC $\delta^{13}C$ values and PLFA $\delta^{13}C$ values provides strong support for the conclusion that DIC was the dominant carbon source for *P. frederiksbergensis* cultures

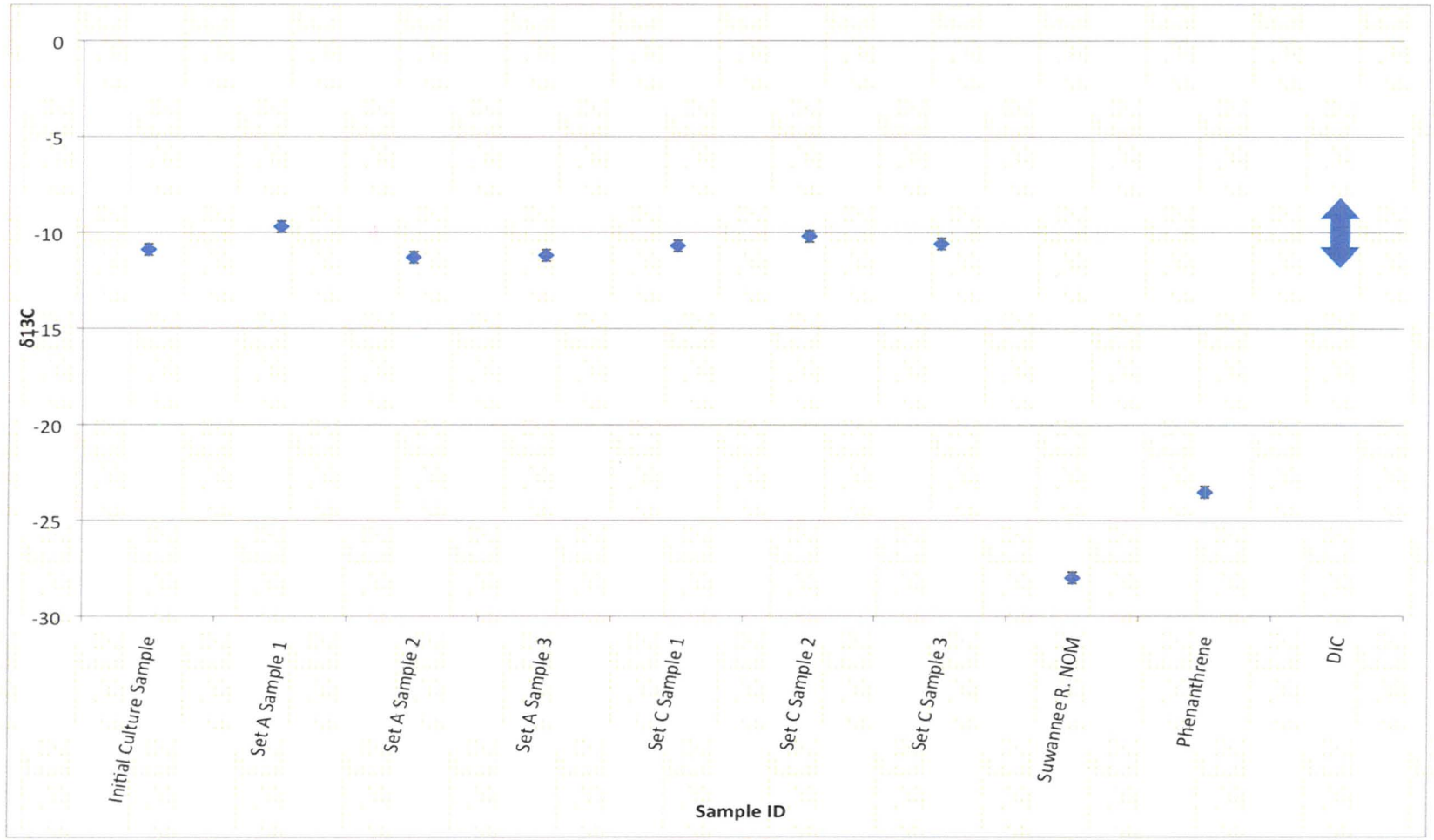

Figure 3-2: $\delta^{13}C$ values measured for PLFA samples and organic carbon substrates. The double arrow represents the range of values estimated to be the $\delta^{13}C$ value of DIC in this study. Error bars represent a total error in measurement accuracy of ±0.1‰ associated with AMS or GC-IRMS analysis.

3-4.1.2 Radioisotopic Evidence for DIC as Primary Carbon Source

Radiocarbon isotope results provided further evidence that DIC was the primary carbon source for *P. frederiksbergensis*. Bacterial assimilation of carbon from a single source can be expected to cause cellular components, including PLFAs, to take on ^{14}C signatures very similar to that of the carbon source. Both Petsch et al. (2001) and Slater et al. (2006a) observed such radioisotopic similarities between bacterial PLFAs and the carbon substrates utilized by the bacteria. From Table 3-2 and Figure 3-3, it can be clearly observed that such radioisotopic similarities did not exist between either NOM (+97.9±15.9‰) or phenanthrene (-999.1±0.4‰) and the bacterial PLFA samples in this study (-29‰ to +36‰), even when taking into consideration the total error for PLFA $\Delta^{14}C$ measurements could have been as much as ±20‰ (see Section 3.1). Instead, PLFAs from all bacterial cultures exhibited $\Delta^{14}C$ values intermediate to the $\Delta^{14}C$ values for the organic carbon sources.

The intermediate PLFA $\Delta^{14}C$ values implied DIC must have been the dominant carbon source utilized for PLFA biosynthesis by *P. frederiksbergensis* bacteria. DIC was the only other carbon source present in sample bottles, and none of the bulk PLFA isotopic values observed in this study could have been produced if the carbon utilized by bacteria for PLFA biosynthesis was primarily organic carbon. In sample Set C, the only form of organic carbon present was NOM. If the only source of carbon incorporated by Set C cultures had been NOM, PLFAs in those cultures ought to have had $\Delta^{14}C$ values close to the $\Delta^{14}C$ of NOM (+98‰). Actual PLFA $\Delta^{14}C$ values measured for Set C were

substantially more depleted than the value for NOM, meaning an additional carbon source with a $\Delta^{14}C$ signature more depleted than that of NOM must have been present and utilized by bacteria. The initial culture sample also contained only one form of organic carbon: phenanthrene. If phenanthrene had been utilized as the sole bacterial carbon source, the resulting PLFA $\Delta^{14}C$ value should have been approximately -1000‰, as was observed by Petsch et al. (2001) when bacteria utilized ^{14}C-free kerogen as a carbon source for PLFA biosynthesis. Actual PLFA $\Delta^{14}C$ values for that sample were much more enriched than phenanthrene, indicating that an additional carbon source substantially more enriched in ^{14}C than phenanthrene must have been present and utilized by bacteria. In Set A samples both NOM and phenanthrene were present, and therefore Set A PLFA $\Delta^{14}C$ values could theoretically have been produced by microbial utilization of a precise mixture of NOM and phenanthrene. The previously discussed stable isotope values for Set A, however, suggested neither NOM nor phenanthrene was a dominant carbon source in Set A.

The DIC in this study can also be shown to have had a $\Delta^{14}C$ signature intermediate to phenanthrene and NOM, consistent with the observed PLFA $\Delta^{14}C$ values (see Figure 3-3). The exact $\Delta^{14}C$ for the DIC (or atmospheric CO_2) was not measured as it was originally not considered likely to play a major role in the system, but a reasonable estimate for DIC $\Delta^{14}C$ can be established. A 2006 analysis of $\Delta^{14}C$ values in corn across the eastern United States and Canada suggested that atmospheric CO_2 in that region had an average $\Delta^{14}C$ of +58.5‰±3.9, with the most depleted values observed near highly urbanized areas similar to Hamilton (Hsueh et al., 2007). In the current "post-bomb"

period (post-1988), fluctuations in atmospheric ^{14}C are driven mainly by emissions of ^{14}C-free fossil fuel CO_2 and natural $^{14}CO_2$ production in the upper atmosphere. At current rates, fossil fuel emissions result in the depletion of atmospheric $\Delta^{14}C$ by 12-14‰ per year, while natural $^{14}CO_2$ production enriches the atmosphere by approximately 5‰ per year (Levin et al., 2009). Using the 2006 $\Delta^{14}C$ value, we can therefore estimate that average atmospheric $\Delta^{14}C$ levels when samples were collected in 2009 were in the range of +25‰ to +40‰. Even from this broad a range of values it is clear that DIC meets the requirements for the "additional" carbon source in bottles, being both more enriched than phenanthrene and more depleted than NOM.

Based upon the evidence presented in this Section and Section 4.1.1, the overall isotopic composition of the bacterial PLFAs in all samples could only be explained by utilization of DIC derived from atmospheric CO_2 as the source of carbon for PLFA biosynthesis.

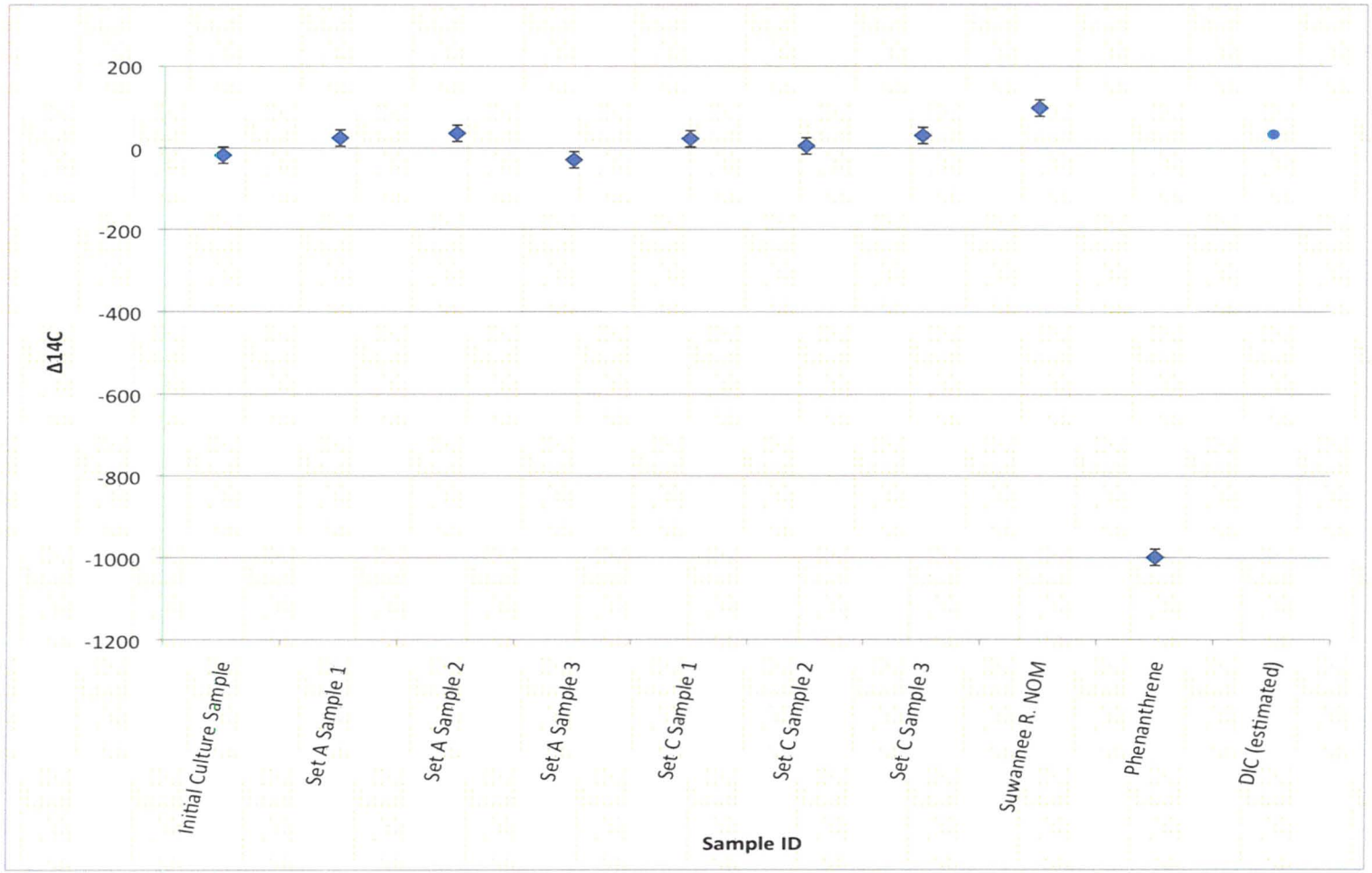

Figure 3-3: $\Delta^{14}C$ values measured for PLFA samples and organic carbon substrates. The blue circle represents the range of values estimated for $\Delta^{14}C$ of DIC in this study. Error bars represent a maximum total error in measurement accuracy of ±20‰ associated with AMS or GC-IRMS analysis. All but two PLFA samples fell within error of the range of values estimated for DIC.

3-4.1.3 Evidence of Organic Carbon Utilization in Samples

In all but two samples, PLFA isotope values supported the exclusive use of DIC as the carbon source for PLFA biosynthesis. In the initial culture sample and Set A Sample 3, however, PLFA $\Delta^{14}C$ values were more depleted than could be explained if DIC was the sole carbon source (see Table 3-2, Figure 3-3), which implies that phenanthrene must also have been used as a carbon source in those two samples. Given that PLFA $\delta^{13}C$ values for these samples were unchanged compared to other samples (see Figure 3-3), the extent of phenanthrene utilization in these samples must have been minimal. In the other samples, admittedly, organic carbon may also have been utilized, but if so, that isotopic signal could not be resolved. Overall, the PLFA isotopic evidence discussed throughout Section 4.1 suggests the bacterial carbon source utilized for PLFA biosynthesis was primarily DIC, and that metabolism of organic carbon did take place in at least some samples, but in limited amounts. The microbial metabolic pathways which could have lead to the observed PLFA results are considered in the next Section (4.2).

3-4.2 *P. frederiksbergensis* Cell Metabolism

We know living *P. frederiksbergensis* bacteria were present in all Set A, B and C sample bottles, based upon the substantial PLFA concentrations found in Set A, B and C culture bottles, the PLFA isotopic values, and the identification of *Pseudomonas* DNA via

DGGE analysis. DGGE results indicated *Pseudomonas* DNA was present in initial culture bottles just prior to transfer to Set A, B and C bottles. While the time required for DNA to degrade is somewhat variable, this suggests viable cells were present at the time of transfer. The PLFA concentrations observed for Sets A, B and C (Table 3-3) confirmed that viable cultures must have been transferred to those Sets from the initial culture bottles after the initial growth period of 200 days. If conditions had not been adequate for cell survival, that initial growth period would have been sufficient time for all cultures to have died and for all PLFAs to have degraded. Furthermore, PLFAs in all samples had $\delta^{13}C$ isotopic values indicating they were biosynthesized using carbon originally from atmospheric $CO_{2(g)}$. Prior to their involvement in this study, *P. frederiksbergensis* cultures were grown on tripticase soy agar, and would have utilized carbon from that agar for PLFA biosynthesis. Even in the unlikely scenario that the agar had the same stable carbon and radiocarbon isotopic signatures as the DIC, only minimal quantities of that agar carbon would have been present in Set A, B and C bottles, due to the long initial growth period and the small quantities of media transferred from the initial culture bottles to the Set A, B and C bottles. Therefore, the similarity between DIC and bulk PLFA isotopic values indicated that, in all samples, *P. frederiksbergensis* cells were actively biosynthesizing PLFAs using carbon from DIC, and were therefore alive.

Since viable cells were present in samples, those cells must have somehow obtained energy sufficient for survival, and possibly, but not necessarily, growth. The isotopic results strongly suggest *P. frederiksbergensis* cultures exhibited heterotrophic metabolism in this study in order to generate the required cellular energy.

3-4.2.1 Heterotrophic Metabolism

Based upon the PLFA isotopic results, *P. frederiksbergensis* cell metabolism in this study was most likely heterotrophic. This is consistent with the fact that nearly all studies of *Pseudomonas* species in the literature have indicated those species were heterotrophic. Only a single species of *Pseudomonas* capable of autotrophic metabolism under aerobic conditions can be found in the literature (Quayle and Keech, 1959; Dijkhuizen and Harder, 1984). The occurrence of autotrophy in this study is further contraindicated since, under the study conditions, an autotrophic organism could not have generated the cellular energy required for it to survive. Autotrophic organisms require a source of energy to fix inorganic carbon into the organic carbon. That organic carbon is subsequently oxidized during cellular respiration to generate cellular energy. In this system, no energy source for carbon fixation was available. Light energy could not be used for carbon fixation (photoautotrophy), as cultures were grown in total darkness. Chemical oxidation of inorganic electron donor species to provide energy for carbon fixation (chemoautotrophy) also could not have taken place, as no suitable electron donors were available in the culture media. Only a single inorganic species in the media, Fe^{2+} (from $Fe(NH_4)^2(SO_4)^2$), was present in a reduced form capable of serving as an electron donor, and at the experimental pH (roughly 5-7), any Fe^{2+} would have been rapidly oxidized to unsuitable Fe^{3+}. All other potential electron donor species were present already in their most oxidized states. Without the ability to fix carbon, an

autotrophic organism would not be able to survive, as no organic carbon would be generated and subsequently respired to generate the cellular energy required for cell maintenance and survival. Therefore, since *P. frederiksbergensis* cells were shown to be viable, we must assume they carried out heterotophic metabolism.

Proceeding on the basis that cell metabolism in this study was heterotrophic, cells in all samples must have metabolized organic carbon (NOM or phenanthrene) to obtain cellular energy, and possibly for use in PLFA biosynthesis. ^{13}C and ^{14}C isotopic results indicated organic matter could not have been the sole source of carbon utilized for PLFA biosynthesis (see Section 4.1). *P. frederiksbergensis* PLFAs were more enriched in ^{13}C than either organic carbon source, and all PLFA $\Delta^{14}C$ values were intermediate to the $\Delta^{14}C$ of the two organic carbon sources (see Figures 3-1, 3-2). In combination, these isotopic results could not have been obtained in any sample unless DIC was present and utilized by bacteria as the primary carbon source.

Metabolism of organic matter was demonstrated in two samples (see Section 4.1, Figure 3-3), but if *P. frederiksbergensis* exhibited heterotrophic metabolism as this model suggests, some organic carbon must have been metabolized in all samples to generate sufficient cellular energy for cell maintenance and survival. Presumably, isotopic signals indicative of bacterial utilization of organic carbon could not be resolved in the other five samples because too little organic matter was used for PLFA biosynthesis to significantly affect the PLFA isotopic results, with the organic carbon isotopic signal not able to be resolved from the isotopic signal generated by utilization of DIC.

As demonstrated graphically in Figure 3-4, extracellular DIC used for PLFA biosynthesis would have been formed from the dissolution of both atmospheric $CO_{2(g)}$ (when bottles were open to the atmosphere), and CO_2 formed during respiration, into the culture media. Depending upon whether phenanthrene or NOM was utilized by bacteria, the respired CO_2 would have been more depleted or enriched, respectively, in ^{14}C than the extracellular DIC pool it joined. Regardless of the type of organic carbon utilized, the respired CO_2 would have been more depleted in ^{13}C than the extracellular DIC pool. Again, however, the dominant utilization of DIC for PLFA biosynthesis suggests the organic carbon isotopic signal did not likely have a substantial effect on the isotopic values of the DIC pool, and the signal would not have been reflected in the PLFA isotopic values.

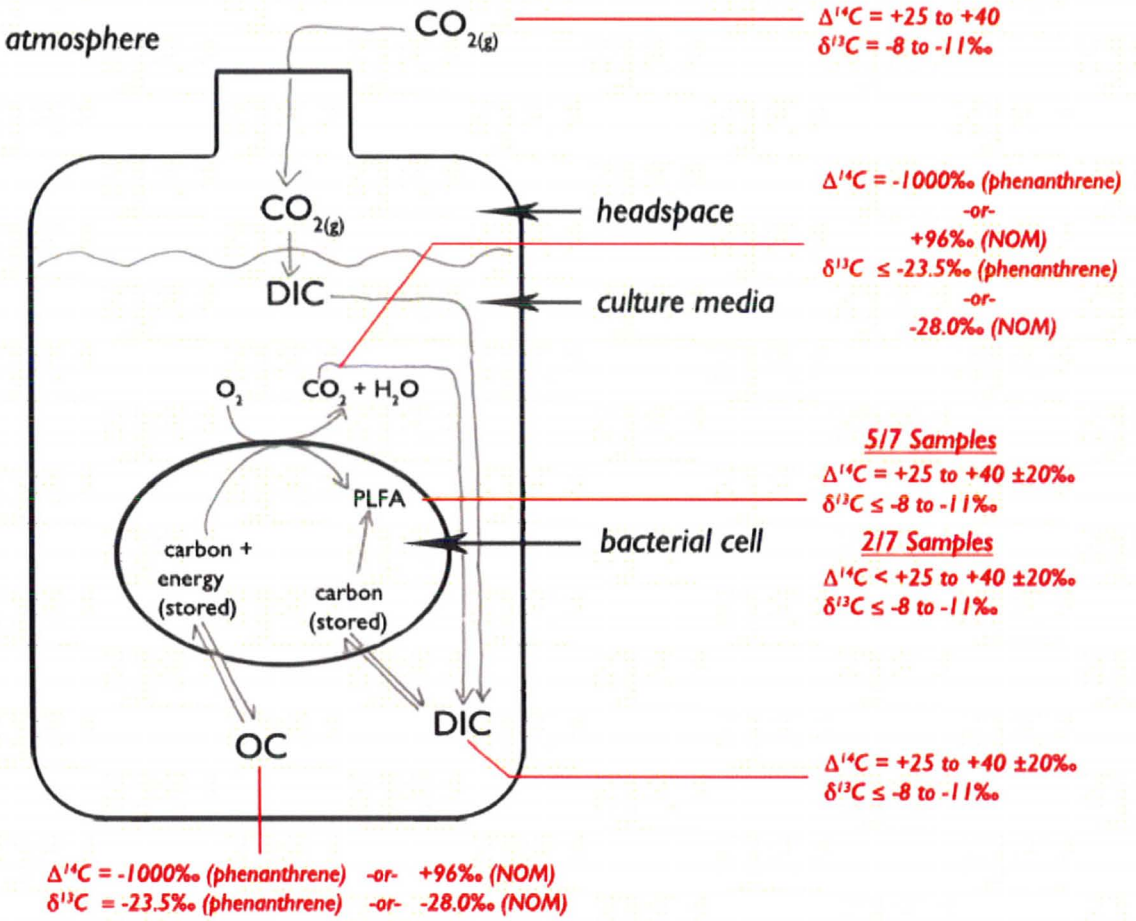

Figure 3-4: Diagrammatic model of heterotrophic *P. frederiksbergensis* cell metabolism in sample bottles. OC represents organic carbon (NOM or phenanthrene). Isotopic values or ranges of values for OC and PLFA represent measured values. Atmospheric $CO_{2(g)}$ isotopic values were estimated from previous measurements or literature values, and carry a high degree of certainty. Isotopic values for DIC and respired CO_2 are estimates based on the isotopic values of the other components of the system listed above and the overall interpretation of cell metabolism.

3-4.3 Sample Purity

Fungal contamination of initial culture bottles was detected by DGGE analysis, but was unlikely to have had any substantial effect upon the results of this study. DGGE analysis is not a quantitative technique; certain organisms exhibit greater amplification efficiency than others, depending upon their DNA sequences (Microbial Insights Inc, personal communication). Therefore, DGGE analysis was able to provide only the identities of fungal contaminants, not their relative dominance in samples.

PLFA analysis represented a more effective means for assessing the extent of fungal contamination. Prokaryotic and eukaryotic PLFA biomarker concentrations can serve as proxies for prokaryotic and eukaryotic biomass (Green and Scow, 2000), and while Frostegard and Bååth (1996) caution against the use of fungal PLFA biomarkers for determination of precise fungal biomass estimates, such biomarkers can still be used to compare prokaryotic and eukaryotic biomass on a relative basis with reasonable accuracy.

3-4.3.1 Fungal PLFA Biomarkers

A number of specific PLFAs have been proposed as fungal biomarkers. 18:2ω6,9 , 18:1ω9 , 18:3ω6 , and 16:1ω5 have all been proven to be synthesized by fungal species (Olsson et al., 1995; Baath and Anderson, 2003; Klamer and Baath, 2004; Potthoff et al., 2006). Of these four PLFAs, none were detected in samples. 18:2ω6,9 is the most commonly reported specific fungal biomarker, and, therefore, we will use it as an

example to demonstrate the improbability of fungal contamination having impacted the results of this study.

18:2ω6,9 is common in fungal species, but has not been found in prokaryotes (Pinkart et al., 2002). Of the fungal strains detected by DGGE in this study, all but *Malassezia* have strong evidence to suggest they biosynthesize 18:2ω6,9. Six species of Ascomycota, including one of the genus *Aspergillus*, have been shown to have a dominant 18:2ω6,9 PLFA component (Klamer and Baath, 2004), and all species in an extensive list of Ascomycota compiled in Losel (Losel, 1988) had defined 18:2 PLFA components. Losel (Losel, 1988) did not directly reference *Cryptococcus*, however, every representative species in its class, Urediniomycetes, had defined 18:2ω6,9 PLFA components. No studies on the PLFA composition of *Malassezia* were available in the literature, likely because of the difficulties involved in growing *Malassezia* in culture (Kaneko et al., 2007).

3-4.3.2 Maximum Possible Fungal Contributions to Samples

Based on the non-detection of 18:2 PLFA biomarkers in all samples, we can demonstrate that fungal contaminants made minimal contributions to samples. The detection limit for PLFAs on the GC-MS equipment was found to be 0.5ppm (m/v). Any PLFA not detected during analysis would therefore have had a concentration of 0.5ppm or less. This concentration equates to a sediment concentration of 0.001μg of PLFA per gram of sediment. Therefore, if 18:2ω6,9 had been present in the samples in this study, it

would have had to be present at a concentration of less than 0.001μg of PLFA per gram of sediment. According to DGGE results, Sample 1's in sample Set A, B and C were contaminated with Ascomycota. As explained in detail below, a maximum of 6% of the total PLFA present in the samples could have originated from fungus, and if only 6% of biomass was fungus, the impact on isotopic results should have been minimal. Klamer and Baath (2004) found 18:2ω6,9 comprised 36% to 61% of the total PLFA detected in six species of Ascomycota. Considering the most extreme case (for the sake of argument), if only 36% of the PLFAs expressed by Ascomycota were in the form of 18:2ω6,9, and no other fungal PLFA biomarkers were detected, the remaining 64% must have been composed of PLFAs also synthesized by *P. frederiksbergensis* and therefore "hidden" in the PLFA profile. However, since 18:2ω6,9 was not detected, the 36% represented by that PLFA would have equated to no more than 0.001μg/g. As a result, counting the "hidden" fungal contribution, the total concentration of fungal PLFAs in Sample 1 of any culture Set could have been no more than 0.003ug/g. By dividing this concentration of fungal biomass by the total PLFA concentrations in Sample 1 of Set A, B, and C (0.05-0.15ug/g, see Table 3-3), it is clear that total fungal PLFA contributions to Sample 1 in any Set could have been at most 6% of the total PLFA.

In Sample 2 of each Set, DGGE indicated contamination by *Malassezia*. No estimate could be made of the percentage of the total PLFA comprised by 18:2ω6,9 in *Malassezia* as no published data could be found. Nonetheless, 18:2ω6,9 generally represents a substantial percentage of the total PLFA expressed by a given fungal species,

and therefore, it is likely fungal contributions to Sample 2 from each Set were as small as for Sample 1's and 3's.

In Sample 3 of each Set, DGGE indicated contamination by *Aspergillus* and *Cryptococcus*. As explained in detail below, a maximum of 4% of the total PLFA present in the samples subjected to isotopic analysis could have originated from fungus, and if only 4% of biomass was fungal, the impact on isotopic results should have been minimal. Klamer and Baath (2004) found 18:2ω6,9 represented 46% of the total PLFA in *Aspergillus* and an average of 9.1% of the total PLFA in Urediniomycetes, the class containing *Cryptococcus* (Losel, 1988). Since 18:2ω6,9 was not detected in any of the Sample 3's, the combined fungal percentages (55.1%) could have equated to an 18:2ω6,9 concentration no greater than 0.001ug/g, assuming the percentage values were not affected by interactions between the two fungal species. The remaining 44.9% of fungal PLFA must have been composed of PLFAs also synthesized by *P. frederiksbergensis* and therefore "hidden" in the PLFA profile. Counting the "hidden" fungal contribution, the total concentration of fungal PLFAs in Sample 3 of any culture Set could have been a maximum of 0.002ug/g. By dividing this concentration of fungal biomass by the total PLFA concentrations in Sample 3 of Set A, B, and C (0.01-0.19μg/g, see Table 3-3), it is clear that total fungal PLFA contributions to Sample 1 in any Set could have been at most 20% of the total PLFA. At 20% of the total PLFA, fungal impact upon study results would be a genuine concern. However, only Set B Sample 3 approached 20%, and Set B did not undergo isotopic analysis, meaning fungal contamination in Set B Sample 3 could

not have affected isotopic results. Sample 3 in Sets A and C had maximum total possible fungal PLFA contributions of less than 4%.

The initial culture sample was formed from the combined contents of the three initial culture bottles. Since these three bottles corresponded with Sample numbers in Set A, B and C (initial culture bottle 1 was used to propagate all Sample 1's, bottle 2 was used for all Sample 2's, and so forth), the initial culture sample can be considered to have been effectively formed from one Sample 1, one Sample 2 and one Sample 3 bottle. The initial culture sample would, therefore, have contained all the fungal contaminants discussed above. 18:2ω6,9 was not detected in the initial culture sample, again indicating the total 18:2ω6,9 concentration present could not have been greater than 0.001ug/g. Since the total PLFA concentration for the initial culture sample was 1.24 ug/g, the fungal contribution would have been less than 1% even if 18:2ω6,9 represented only 10% of the total fungal PLFAs, a percentage much smaller than suggested by the fungal contributions to the individual samples discussed above. Incidentally, since Sets A, B and C were generated from initial culture bottles, this also provides very strong evidence that the level of contamination in Sets A, B, and C would have been similarly minimal.

The lack of Sample-number-specific differences in results provides further evidence to suggest fungal contamination did not impact those results. Given that different species of fungus were found in different initial culture bottles, and cultures from those bottles were maintained separately as distinctly numbered samples (Sample 1,2,3), it would follow that any fungal influence upon results should produce Sample-number-specific patterns. From examination of study results, no such patterns were observed in isotopic

results. The lack of Sample-number-specific trends also suggests that the presence of *Malassezia* did not affect results in Sample 2 cultures.

3-4.3.3 Origin of Fungal Contamination

The origin of the fungal contamination was unknown, however, the ubiquity of fungal spores makes them a perpetual, although often overlooked, problem in cell culture studies. Fungus may have been present in the samples obtained from the ATCC, or originated from contamination during sample handling. The fungal origin is not of serious concern given the negligible impact of contamination to the results of this study.

3-5. Conclusions

This study demonstrated that pure cultures of *P. frederiksbergensis* bacteria primarily utilized dissolved inorganic carbon (DIC) for PLFA biosynthesis, despite the presence of large quantities of organic carbon (phenanthrene and NOM). Since *P. frederiksbergensis* cultures most likely exhibited heterotrophic metabolism in this study, some metabolism of organic carbon must have occurred in all samples to provide bacterial cells with sufficient energy for cell maintenance and survival. As isotopic signals indicative of the metabolism of organic carbon could only be resolved in two of the seven samples, and limited utilization of organic carbon for PLFA biosynthesis was

indicated even in those samples, use of organic carbon for PLFA biosynthesis must therefore have been minimal to nonexistent in all samples. This limited utilization of organic carbon implies that the organic carbon must not have been sufficiently accessible or energetically beneficial for *P. frederiksbergensis* cultures to use it effectively as a source of carbon and energy.

Since utilization of DIC would not have provided energy to a heterotrophic organism, the minimal metabolism of organic carbon would have meant very little cellular energy would have been produced in cells and bacterial growth would, therefore, have been very slow. Given that bacterial cells were clearly shown to be viable, however, cell maintenance would still have had to occur, and the results of this study clearly suggest the carbon required for that process was obtained primarily from DIC.

Fungal contamination was detected in sample bottles via DGGE analysis. However, as no fungal PLFA biomarkers were detected by PLFA analysis, fungus could only have been present in amounts below the detection limit of the GC-MS equipment. These amounts would not have been sufficient to affect experimental results.

This study was originally designed to investigate bacterial preference for PAHs in the presence of NOM, with the goal of determining whether modern-aged organic matter would be preferentially assimilated by bacteria. If modern organic matter had indeed been preferentially assimilated, this would have suggested the presence of NOM in natural systems could potentially inhibit *in situ* PAH degradation. While it was not possible to address that exact question, due to the utilization of DIC for PLFA biosynthesis in this study, the fact that DIC was utilized in large quantities demonstrated

that yet another carbon source available in natural systems can compete with organic carbon as a bacterial carbon source. This dominant utilization of DIC, and minimal utilization of organic carbon (including the PAH), strongly suggests that *P. frederiksbergensis* would be a poor candidate organism for use in bioremediation systems. Natural systems contain countless more carbon sources than present in this laboratory study, and if only minimal PAH metabolism (degradation) was observed in the presence of three carbon sources, it is likely that *P. frederiksbergensis* would metabolize PAHs to an even lesser extent in the environment.

The dominant utilization of DIC also implies that revisitation of studies in which microbial pure cultures have been shown to degrade petroleum hydrocarbons as the sole carbon source may be necessary. Simply demonstrating bacterial capability to degrade petroleum hydrocarbons has no relevance to bioremediation of natural systems if those systems contain other carbon sources preferred by the bacteria. The results of this study further suggest that ensuring the presence of microbes capable of degrading specific contaminants is likely insufficient for the design of effective bioremediation systems. As proposed previously by Slater et al. (2005a), carbon source limitation or increased competition for carbon sources might be necessary to ensure the degradation of contaminants less preferred as carbon sources. Future studies utilizing PLFA isotopic analyses to directly establish bacterial carbon cycling pathways are necessary to establish if carbon limitation or increased competition might be effective strategies in bioremediation system design.

3-6. Acknowledgements

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3-7. References

Andersen, S.M., Johnsen, K., Sørensen, J., Nielsen, P., Jacobsen, C.S. (2000). *Pseudomonas frederiksbergensis* sp. nov., isolated from soil at a coal gasification site. International Journal of Systemic and Evolutionary Microbiology 50 1957-1964.

Baath, E., Anderson, T.H. (2003). Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. Soil Biology and Biochemistry 35, 955-963.

Boffetta, P., Jourenkova, N., Gustavsson, P. (1997). Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. Cancer Causes and Control 8, 444-472.

Boschker, H., Middelburg, J. (2002a). Stable isotopes and biomarkers in microbial ecology. FEMS Microbiology Ecology, 85-95.

Boschker, H.T.S., Middelburg, J.J. (2002b). Stable isotopes and biomarkers in microbial ecology. FEMS Microbiology Ecology.

Boyd, T., Montgomery, M., Steele, J., Pohlman, J., Reatherford, S., Spargo, B., Smith, D. (2005). Dissolved oxygen saturation controls PAH biodegradation in freshwater estuary sediments. Microbial Ecology 49, 226-235.

Bunce, N. (1991). Environmental Chemistry. Wuerz Publishing Ltd., Winnipeg.

Caldwell, M.E., Garrett, R.M., Prince, R.C., Suflita, J.M. (1998). Anaerobic biodegradation of long-chain-alkanes under sulfate-reducing conditions. Environmental Science & Technology 32, 2191-2195.

Cerniglia, C., Heitkamp, M.A. (1989). Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment. In: Varanasi, U. (Ed.). Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. CRC Press Inc., Boca Raton, Fla, pp 41-68.

Coates, J., Woodward, J., Allen, J., Philp, P., Lovley, D. (1997). Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. Appl Environ Microbiol 63, 3589.

Dijkhuizen, L., Harder, W. (1984). Regulation of autotrophic and heterotrophic metabolism in *Pseudomonas oxalaticus* OX1. Growth on fructose and on mixtures of fructose and formate in batch and continuous cultures. Microbiology 130, 447.

Environment Canada (1994). Canadian Environmental Protection Act: Priority Substances List Assessment Report. Polycyclic Aromatic Hydrocarbons. Environment Canada,, Ottawa, p. 61.

Fang, J., Lovanh, N., Alvarez, P.J. (2004). The use of isotopic and lipid analysis techniques linking toluene degradation to specific microorganisms: applications and limitations. Water Research 38, 2529-2536.

Frostegård, A., Bååth, E. (1996). The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59-65.

Green, C.T., Scow, K.M. (2000). Analysis of phospholipid fatty acids (PLFA) to characterize microbial communities in aquifers. Hydrogeology Journal 8, 126-141.

Guha, S., Jaffe, P. (1996). Biodegradation kinetics of phenanthrene partitioned into the micellar phase of nonionic surfactants. Environmental Science & Technology 30, 605-611.

Haritash, A.K., Kaushik, C.P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. Journal of Hazardous Materials 169, 1-15.

Harvey, H.R., Fallon, R.D., Patton, J.S. (1986). The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. Geochimica et Cosmochimica Acta 50, 795-804.

Hayes, J. (2001). Fractionation of the isotopes of carbon and hydrogen in biosynthetic processes. In: Valley, J.W., Cole, D.R. (Eds.). Stable Isotope Geochemistry. Mineralogical Society of America and the Geochemical Society, p. (to be published in this series) 31.

Hesselsoe, M., Nielsen, J., Roslev, P., Nielsen, P. (2005). Isotope labeling and microautoradiography of active heterotrophic bacteria on the basis of assimilation of 14CO2. Applied and Environmental Microbiology 71, 646-655.

Holman, H.-Y.N., Tsang, Y.W., Holman, W.R. (1999). Mineralization of sparsely water-soluble polycyclic aromatic hydrocarbons in a water table fluctuation zone. Environmental Science & Technology 33, 1819-1824.

Hsueh, D., Krakauer, N., Randerson, J., Xu, X., Trumbore, S., Southon, J. (2007). Regional patterns of radiocarbon and fossil fuel-derived CO2 in surface air across North America. Geophysical Research Letters 34, 2816.

Johnsen, A.R., Schmidt, S., Hybholt, T.K., Henriksen, S., Jacobsen, C.S., Andersen, O. (2007). Strong impact on the polycyclic aromatic hydrocarbon (PAH)-degrading community of a PAH-polluted soil but marginal effect on PAH degradation when priming with bioremediated soil dominated by mycobacteria. Applied and Environmental Microbiology 73, 1474-1480.

Johnsen, K., Andersen, S., Jacobsen, C.S. (1996). Phenotypic and genotypic characterization of phenanthrene-degrading fluorescent *Pseudomonas* biovars. Applied and Environmental Microbiology 62, 3818-3825.

Kanaly, R., Harayama, S. (2000). Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. Journal of Bacteriology 182, 2059-2067.

Kaneko, T., Makimura, K., Abe, M., Shiota, R., Nakamura, Y., Kano, R., Hasegawa, A., Sugita, T., Shibuya, S., Watanabe, S., Yamaguchi, H., Abe, S., Okamura, N. (2007). Revised Culture-Based System for Identification of *Malassezia* Species. Journal of Clinical Microbiology 45, 3737-3742.

Kawamura, K., Suzuki, I. (1994). Ice core record of polycyclic aromatic hydrocarbons over the past 400 years. Naturwissenschaften 81, 502-505.

Keeling, C.D., Bollenbacher, A.F., Whorf, T.P. (2005). Monthly atmospheric $^{13}C/^{12}C$ isotopic ratios for 10 SIO stations. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tenn, USA.

Keith, L., Telliard, W. (1979). ES&T special report: priority pollutants: I - a perspective view. Environmental Science & Technology 13, 416-423.

Klamer, M., Baath, E. (2004). Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18:2ω6,9. Soil Biology and Biochemistry 36, 57-65.

Kramer, C., Gleixner, G. (2006). Variable use of plant- and soil-derived carbon by microorganisms in agricultural soils. Soil Biology and Biochemistry 38, 3267-3278.

Levin, I., Naegler, T., Heinz, R., Osusko, D., Cuevas, E., Engel, A., Ilmberger, J., Langenfelds, R.L., Neininger, B., Rohden, C.v., Steele, L.P., Weller, R., Worthy, D.E., Zimov, S.A. (2009). Atmospheric observation-based global SF 6 emissions–comparison of top-down and bottom-up estimates. Atmospheric Chemistry and Physics Discussions 9, 26653-26672.

Lima, A., Farrington, J., Reddy, C. (2005). Combustion-derived polycyclic aromatic hydrocarbons in the environment - A review. Environmental Forensics 6, 109-131.

Losel, D.M. (1988). Fungal lipids. In: Ratledge, C., Wilkinson, S.G. (Eds.). Microbial Lipids. Academic Press, London, p. 962.

Maggioni, S., Benfenati, E., Colosio, C., Moretto, A., Roots, O., Tasiopoulou, S., Visentin, S. (2009). Food contamination control in European new Member States and associated candidate countries: Data collected within the SAFEFOODNET project. Journal of Environmental Science and Health, Part B 44, 407-414.

Mandalakis, M., Gustafsson, O., Reddy, C., Xu, L. (2004). Radiocarbon Apportionment of Fossil versus Biofuel Combustion Sources of Polycyclic Aromatic Hydrocarbons in the Stockholm Metropolitan Area. Environmental Science & Technology 38, 5344-5349.

Martens, D., Maguhn, J., Spitzauer, P., Kettrup, A. (1997). Occurrence and distribution of polycyclic aromatic hydrocarbons (PAHs) in an agricultural ecosystem. Fresenius Journal of Analytical Chemistry 359, 546-554.

Mascalet, P., Hoyau, V., Jaffrezo, J.L., Cachier, H. (2000). Polycyclic aromatic hydrocarbon deposition on the ice sheet of Greenland. Part I: Superficial snow. Atmospheric Environment 34, 3195-3207.

Mastrangelo, G., Fadda, E., Marzia, V. (1996). Polycyclic aromatic hydrocarbons and cancer in man. Environmental Health Perspectives 104, 1166.

McNichol, A.P., Osborne, E.A., Gagnon, A.R., Fry, B., Jones, G.A. (1994). TIC, TOC, DIC, DOC, PIC, POC -- unique aspects in the preparation of oceanographic samples for 14C-AMS. Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms 92, 162-165.

Means, J., Wood, S., Hassett, J., Banwart, W. (1980). Sorption of polynuclear aromatic hydrocarbons by sediments and soils. Environmental Science & Technology 14, 1524-1528.

Nakata, H., Sakai, Y., Miyawaki, T., Takemura, A. (2003). Bioaccumulation and Toxic Potencies of Polychlorinated Biphenyls and Polycyclic Aromatic Hydrocarbons in Tidal Flat and Coastal Ecosystems of the Ariake Sea, Japan. Environmental Science & Technology 37, 3513-3521.

Nichols, P., Guckert, J., White, D. (1986). Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. Journal of Microbiological Methods 5, 49-55.

Ohkouchi, N., Kawamura, K., Kawahata, H. (1999). Distributions of three- to seven-ring polynuclear aromatic hydrocarbons on the deep sea floor in the Central Pacific. Environmental Science & Technology 33, 3086-3090.

Olsson, P.A., Baath, E., Jacobsen, I., Soderstrom, B. (1995). The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99, 623-629.

Pearson, A., McNichol, A., Schneider, R., Von Reden, K., Zheng, Y. (1998). Microscale AMS 14C measurement at NOSAMS. Radiocarbon 40, 61-75.

Petsch, S.T., Eglington, T.I., Edwards, K.J. (2001). 14C-dead living biomass: evidence for microbial assimilation of ancient organic carbon during shale weathering. Science 292, 1127-1131.

Pinkart, H.C., Ringelberg, D.B., Piceno, Y.M., Macnaughton, S.J., White, D.C. (2002). Biochemical approaches to biomass measurements and community structure analysis. In: Hurst, C.J., Crawford, R.L., Knudson, G.R., McInerney, M.J., Stetzenbach, L.D. (Eds.). Manual of Environmental Microbiology. ASM Press, Washington, D.C., p. 1138.

Potthoff, M., Steenwerth, K.L., Jackson, L.E., Drenovsky, R.E., Scow, K.M., Joergensen, R.G. (2006). Soil microbial community composition as affected by restoration practices in California grassland. Soil Biology and Biochemistry 38.

Quayle, J., Keech, D. (1959). Carbon assimilation by *Pseudomonas oxalaticus* (OX 1). 1. Formate and carbon dioxide utilization during growth on formate. Biochemical Journal 72, 623.

Ridgway, H.F., Safarik, J., Phipps, D., Carl, P., Clark, D. (1990). Identification and catabolic activity of well-derived gasoline-degrading bacteria from a contaminated aquifer. Applied and Environmental Microbiology 56, 3565-3575.

Roslev, P., Iversen, N., Henriksen, K. (1998). Direct fingerprinting of metabolically active bacteria in environmental samples by substrate specific radiolabelling and lipid analysis. Journal of Microbiological Methods 31, 99-111.

Rothermich, M.M., Hayes, L.A., Lovley, D.R. (2002). Anaerobic, sulfate-dependent degradation of polycyclic aromatic hydrocarbons in petroleum-contaminated harbor sediment. Environmental Science & Technology 36, 4811-4817.

Samanta, S., Singh, O., Jain, R. (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. TRENDS in Biotechnology 20, 243-248.

Schwarzenbach, R.P., Gschwend, P.M., Imboden, D.M. (2003). Environmental Organic Chemistry. Wiley-Interscience, Hoboken, New Jersey.

Shibahara, A., Yamamoto, K., Kinoshita, A., Anderson, B.L. (2008). An Improved Method for Preparing Dimethyl Disulfide Adducts for GC/MS Analysis. Journal of the American Oil Chemists' Society 85, 93-94.

Short, J.W., Irvine, G.V., Mann, D.H., Maselko, J.M., Pella, J., Lindeberg, M., Payne, J.R., Driskell, W.B., Rice, S.D. (2007). Slightly weathered Exxon Valdez oil

persists in Gulf of Alaska beach sediments after 16 years. Environmental Science and Technology 41, 1245-1250.

Shuttleworth, K., Cerniglia, C. (1995). Environmental aspects of PAH biodegradataion Applied Biochemistry and Biotechnology 54, 291-302.

Slater, G. (2003). Stable Isotope Forensics - When Isotopes Work. Environmental Forensics 4, 13-23.

Slater, G., Nelson, R., Kile, B., Reddy, C. (2006). Intrinsic bacterial biodegradation of petroleum contamination demonstrated *in situ* using natural abundance, molecular-level 14C analysis. Organic Geochemistry 37, 981-989.

Slater, G., White, H., Eglinton, T., Reddy, C. (2005). Determination of microbial carbon sources in petroleum contaminated sediments using molecular 14C analysis. Environmental Science & Technology 39, 2552-2558.

So, C., Phelps, C., Young, L. (2003). Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, strain Hxd3. Appl Environ Microbiol 69, 3892.

Stuvier, M., Polach, H.A. (1977). Discussion: Reporting of 14C data. Radiocarbon 19, 355-363.

Szidat, S., Jenk, T.M., Gaggeler, H.W., Synal, H.A., Fisseha, R., Baltensperger, U., Kalberer, M., Samburova, V., Wacker, L., Saurer, M., Schiwikowski, M., Hajdas, I. (2004). Source apportionment of aerosols by radiocarbon (C-14) analysis of different carbonaceous particle fractions. Radiocarbon 46, 475-484.

Tavlarides, L.L., Zhou, W., Anitescu, G. (2000). Supercritical fluid technology for remediation of pcb/pah-contaminated soils / sediments. Proceedings of the 2000 Conference on Hazardous Waste Research, Denver, Colorado, pp. 239-255.

Townsend, G.T., Prince, R.C., Suflita, J.M. (2003). Anaerobic Oxidation of Crude Oil Hydrocarbons by the Resident Microorganisms of a Contaminated Anoxic Aquifer. Environmental Science & Technology 37, 5213-5218.

Trevors, J. (1996). Sterilization and inhibition of microbial activity in soil. Journal of Microbiological Methods 26, 53-59.

van Herwijnen, R., Wattiau, P., Bastiaens, L., Daal, L., Jonker, L., Springael, D., Govers, H.A.J., Parsons, J.R. (2003). Elucidation of the metabolic pathway of fluorene and cometabolic pathways of phenanthrene, fluoranthene, anthacene and dibenzothiophene by Sphingomonas sp. LB126. Research in Microbiology 154, 199-206.

Wacey, D. (2009). Early Life on Earth, A Practical Guide. Springer Science and Business Media B.V.

Wagrowski, D., Hites, R. (1996). Polycyclic aromatic hydrocarbon accumulation in urban, suburban, and rural vegetation. Environmental Science & Technology 31, 279-282.

Wakeham, S.G., McNichol, A.P., Kostka, J.E., Pease, T.K. (2006). Natural-abundance radiocarbon as a tracer of assimilation of petroleum carbon by bacteria in salt marsh sediments. Geochimica et Cosmochimica Acta 70, 1761-1771.

White, D., Ringelberg, D. (1998). Signature lipid biomarker analysis. In: Burlage, R.S., Atlas, R., Stahl, D., Geesey, G., Sayler, G. (Eds.). Techniques in microbial ecology. Oxford University Press, New York, pp 255–272.

White, H., Reddy, C., Eglinton, T. (2008). Radiocarbon-based assessment of fossil fuel-derived contaminant associations in sediments. Environmental Science & Technology 42.

Wislocki, P.G., Lu, A.Y.H. (1988). Carcinogenicity and mutagenicity of proximate and ultimate carcinogens of polycyclic aromatic hydrocarbons. In: Yang, S.K., Silverman, B.D. (Eds.). Polycyclic aromatic hydrocarbon carcinogenesis: structure-activity relationships. CRC Press, Boca Raton, FL, pp 1-30.

Yamamoto, K., Shibahara, A., Nakayama, T., Kajimoto, G. (1991). Determination of double-bond positions in methylene-interrupted dienoic fatty acids by GC-MS as their dimethyl disulfide adducts Chemistry and Physics of Lipids 60, 39-50.

CHAPTER 4

Summary of Findings and Conclusions

This thesis contributed novel understanding of microbial responses to PAH contamination, both at a process level and at a community level, via laboratory and *in situ* investigation. This improved understanding of natural microbial communities and microbial carbon cycling has the potential to contribute to improvements in the efficiency of bioremediation system design, allow for improvements in monitoring and mitigating environmental contamination, and enhance understanding of microbial PAH metabolism.

The unifying theme in this thesis was the utilization of phospholipid fatty acids (PLFAs) to investigate interactions between PAHs and microbes, in order to develop a better understanding of microbial responses to PAH contamination. This was examined at a relatively broad level in Chapter 2, investigating the impact of a specific PAH (phenanthrene) on the size and composition of entire microbial communities *in situ*. The community-scale approach taken in Chapter 2 served as the foundation for the more detailed isotopic-tracer-based study of PAH metabolism carried out in Chapter 3. In Chapter 3, a novel approach using stable carbon and radiocarbon PLFA analyses was employed to establish bacterial preference for PAH as a carbon source and identify microbial carbon cycling pathways. While the study was performed under controlled laboratory conditions, the provision of several carbon sources, including a PAH (phenanthrene), permitted conditions to better reflect those of contaminated natural

systems than any previous study. This made for a more accurate investigation of how likely microbes are to degrade PAH in natural systems which contain countless other carbon sources.

Chapter 2 of this thesis addressed the impact of PAH contamination on microbial communities *in situ*, through the investigation of specific communities in Hamilton Harbour. GC-MS analysis of PLFA biomarkers indicated that microbial populations at the more contaminated site were substantially smaller. This biomass difference was directly correlated with increased PAH concentrations. PLFA analysis further indicated that substantial differences in community composition existed between the two sites, but due to the possible influence of other environmental factors, no specific signals could be found which clearly indicated PAH contamination was affecting bacterial cells.

The source of the PAHs affecting both study sites was also investigated in Chapter 2. While exact sources could not be determined at either site, PAH ratios at Site 1 and Site 2 indicated they were affected by different sources, as previously suggested in the literature. While the PAH source at Site 2 was found to be temporally steady, diagnostic PAHs ratios at Site 1 were unexpectedly found to have changed subsequent to a study at that site conducted three years earlier. This change may have been caused by current-driven resuspension and redeposition of PAHs from elsewhere within Hamilton Harbour, or it may have derived from a change in the PAH sources to Site 1.

Future research into the effect of PAH on microbial communities should involve analysis of sediment from many more locations within the Harbour to establish a more detailed model of community response to contamination. Such studies, or more frequent

investigation of the two current study sites could also provide insight into the exact nature and extent of the PAH re-suspension processes active within the harbour.

Chapter 3 of this thesis addressed the microbial carbon source preferences and carbon cycling pathways of the bacterium *P. frederiksbergensis,* a member of the common soil and sediment bacterial genus *Pseudomonas*. The initial goal of this study was to investigate if the bacterium would degrade a PAH contaminant (phenanthrene) when an alternative, potentially more bioavailable environmental carbon source (natural organic matter) was also readily available to bacteria. Stable carbon and radiocarbon analyses of the PLFA produced in *P. frederiksbergensis* bacteria indicated, however, that neither phenanthrene nor NOM served as the dominant carbon source used for PLFA biosynthesis. Instead, results indicated the bacterial cells mainly utilized a different non-PAH carbon source for PLFA biosynthesis: dissolved inorganic carbon (DIC) originating from the dissolution of atmospheric CO_2 in culture media. While metabolism of phenanthrene was shown to have definitely occurred in two samples, the PLFA isotopic results suggested the extent of phenanthrene metabolism was quite limited. As sample bottles were intended to more accurately reflect conditions in natural systems than samples in studies involving only a single carbon source, the results imply *P. frederiksbergensis* would also be unable to degrade substantial quantities of phenanthrene or other PAHs in natural systems, and would be a poor candidate organism for use in bioremediation systems.

Chapter 3 also presented a mechanistic model for carbon cycling mechanisms in *P. frederiksbergensis*. These pathways have not been previously elucidated. Isotopic

results clearly indicated both DIC and minimal amounts of phenanthrene were utilized by *P. frederiksbergensis* as a carbon source for biosynthesis of PLFAs in complete darkness. As bacterial cultures would have been unable to generate any cellular energy via autotrophic metabolism under the experimental conditions in sample bottles, *P. frederiksbergensis* cells most likely exhibited heterotrophic metabolism in this study. Based upon isotopic results, bacterial cells likely metabolized only sufficient organic carbon to meet energy requirements for cell survival and metabolism, and otherwise utilized DIC for PLFA biosynthesis.

Work expanding upon Chapter 3 should focus on establishing carbon source preferences for other organisms known to be capable of degradation of hydrocarbon contaminants as the sole carbon source. By exposing such organisms to different organic substances present in natural systems, as well as DIC, a knowledgebase could be developed to determine their relative preferences for contaminants versus other carbon sources. This knowledgebase would serve as a useful resource when selecting organisms for use in *in situ* bioremediation trials. Knowledge of isotopic values for various carbon sources would also further the ability to use isotopic methods to directly monitor *in situ* remediation progress.

Increased knowledge and understanding of microbial carbon cycling pathways and microbial community responses to PAH contamination will lead to improved methods for contaminant remediation, which in turn will lead to improvements in ecosystem health and reduced risk to humans. The continued development and application of novel methods for identifying physical and metabolic responses of bacteria

to contaminants of concern, such as the PLFA isotopic analyses employed in this study, may permit the development of new techniques for the preservation and remediation of natural systems. Such techniques will be required for future sustainable development.