$^1$H NMR-based metabolomics for elucidating the mode of action of contaminants in the earthworm *Eisenia fetida* after sub-lethal exposure

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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There is a growing need to develop rapid and cost-effective ecotoxicological tools for risk assessment because traditional methods examine endpoints such as mortality, which do not provide any insight into the mode of action (MOA) of the chemical. Research presented within this thesis illustrates the potential of $^1$H NMR-based metabolomics as a rapid and routine ecotoxicological tool that can elucidate a chemical’s MOA and also aid in the identification of metabolites of exposure. Metabolomics involves measuring the fluctuations in the endogenous metabolites of an organism within a cell, tissue, bio-fluid or whole organism in response to an external stressor. We focused on the model polycyclic aromatic hydrocarbon (PAH) phenanthrene, and the perfluoroalkyl acids (PFAAs) perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), due to their recalcitrant nature and widespread prevalence in soil environments. $^1$H NMR-based metabolomics analysis of the exposure of *Eisenia fetida* earthworms to sub-lethal phenanthrene exposure via filter paper contact tests revealed a concentration-dependent two-phased MOA: a linear correlation between the metabolic response and exposure concentration at low concentrations followed by a plateau in the responses at high concentrations. Alanine, glutamate, maltose, cholesterol and phosphatidylcholine emerged as potential indicators of phenanthrene exposure. An increased energy demand and an interruption
in the conversion of succinate to fumarate in the Krebs cycle were observed due to phenanthrene exposure. Sub-lethal PFOA and PFOS exposure to *E. fetida* via contact tests for two days revealed heightened responses with higher PFOA and PFOS concentrations. Leucine, arginine, glutamate, maltose, and ATP were identified as potential indicators of PFOA or PFOS exposure. *E. fetida* responses were then investigated after exposure for two, seven and fourteen days to an artificial soil that was spiked with sub-lethal PFOS concentrations. An exposure time-dependent operation of two separate MOAs was identified. Both the contact tests and artificial soil exposure studies identified an elevation in fatty acid oxidation, a disruption in energy metabolism and biological membrane structure, and also an interruption of ATP synthesis following PFOA and PFOS exposure. This thesis illustrates the promise of NMR-based metabolomics as a routine tool for ecotoxicological assessment of contaminated sites.
ACKNOWLEDGMENTS

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I want to thank all the members of both Simpsons labs, past and present, for making this journey a memorable one. I wish you all the very best in your future endeavors and hope we stay in touch. I want to extend special thanks to Dr. Jimmy Yuk. We started grad school together, worked on similar projects, travelled to conferences (Australia, Washington and Edmonton), had our comprehensive exam on the same day and played sports together. I greatly miss our lunch and tea time discussions. I thank you for all the motivation and support you gave in encouraging me to finish. I don’t think I could have progressed as much as I did without us working together on many projects. Wish you all the very best in your future. I also extend special thanks to Hashim Farooq for being a great friend and source of support over the years. We started undergrad together and then are doing grad school together; can’t believe it has been almost nine
years!!! Thanks for teaching Jimmy and I the secrets of a proper workout in the gym. I wish you all the very best and hope you finish soon as well so that the three of us can celebrate together.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation spectroscopy</td>
</tr>
<tr>
<td>Cyt P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DSS</td>
<td>2,2-Dimethyl-2-silapentane-5-sulfonate sodium salt</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transform ion cyclotron resonance</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HEFS</td>
<td>2-Hexyl-5-ethyl-3-furansulfonate</td>
</tr>
<tr>
<td>HOC</td>
<td>Hydrophobic organic contaminant</td>
</tr>
<tr>
<td>HPV</td>
<td>High production volume</td>
</tr>
<tr>
<td>HSQC</td>
<td>$^1$H-$^{13}$C heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>JRES</td>
<td>J-resolved spectroscopy</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LC$_{50}$</td>
<td>Lethal concentration that causes 50% mortality of the population</td>
</tr>
<tr>
<td>LD$_{50}$</td>
<td>Lethal dose that causes 50% mortality of the population</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>MOA</td>
<td>Mode of action</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NHR</td>
<td>Nuclear hormone receptor</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NOEC</td>
<td>No observable effect concentration</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for economic cooperation and development</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCBs</td>
<td>Polychlorinated biphenyls</td>
</tr>
<tr>
<td>PFAA</td>
<td>Perfluorinated alkyl acids</td>
</tr>
<tr>
<td>PFOA</td>
<td>Perfluorooctanoic acid</td>
</tr>
<tr>
<td>PFOS</td>
<td>Perfluorooctane sulfonate</td>
</tr>
<tr>
<td>PLS-DA</td>
<td>Partial least squares discriminant analysis</td>
</tr>
<tr>
<td>PLS-regression</td>
<td>Partial least squares regression analysis</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator activated receptor alpha</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PURGE</td>
<td>Presaturation utilizing relaxation gradients and echos</td>
</tr>
<tr>
<td>QSARs</td>
<td>Quantitative structure activity relationships</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>QBARs</td>
<td>Quantitative biological activity relationships</td>
</tr>
<tr>
<td>QXI</td>
<td>Quadruple resonance inverse</td>
</tr>
<tr>
<td>REACH</td>
<td>Registration, evaluation, authorization, and restriction of chemicals</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>US EPA</td>
<td>United states environmental protection agency</td>
</tr>
</tbody>
</table>
**PREFACE**

This thesis is a combination of published works in peer-reviewed scientific journals (Chapters 2 to 5) and works submitted to peer-reviewed scientific journals (Chapter 6). There may be unavoidable repetition in the material presented. Contributions to the chapter and manuscripts are outlined in detail in the following section.

**CHAPTER ONE**

*Introduction:* $^1$H NMR-based metabolomics for elucidating the mode of action of contaminants in the earthworm *Eisenia fetida* after sub-lethal exposure

Contributions: Written by Brian P. Lankadurai with critical comments from Myrna J. Simpson

**CHAPTER TWO**

$^1$H NMR-based metabolomic observation of a two-phased toxic mode of action in *Eisenia fetida* after sub-lethal phenanthrene exposure


Contributions: The experiment was designed by Brian P. Lankadurai and Myrna J. Simpson. The experiments were conducted by Brian P. Lankadurai with assistance from David M. Wolfe. Data collection and analysis were performed by Brian P. Lankadurai with guidance from Myrna
J. Simpson. The manuscript was written by Brian P. Lankadurai with critical comments from André J. Simpson and Myrna J. Simpson.

CHAPTER THREE

$^1$H NMR-based metabolomics of time-dependent responses of Eisenia fetida to sub-lethal phenanthrene exposure


Contributions: The experiment was designed by Brian P. Lankadurai and Myrna J. Simpson. The experiments were conducted by Brian P. Lankadurai with assistance from David M. Wolfe. Data collection and analysis were performed by Brian P. Lankadurai with NMR guidance from André J. Simpson. The manuscript was written by Brian P. Lankadurai with critical comments from Myrna J. Simpson.

CHAPTER FOUR

$^1$H NMR-based metabolomic analysis of polar and non-polar earthworm metabolites after sub-lethal exposure to phenanthrene

Published online as: Lankadurai BP, Wolfe DM, Whitfield Åslund M, Simpson AJ, Simpson MJ (2012) $^1$H NMR-based metabolomic analysis of polar and non-polar earthworm metabolites after
sub-lethal exposure to phenanthrene. Metabolomics 1-13 DOI 10.1007/s11306-012-0427-3

Contributions: The experiment was designed by Brian P. Lankadurai and Myrna J. Simpson. The experiments were conducted by Brian P. Lankadurai and David M. Wolfe. Data collection and analysis were performed by Brian P. Lankadurai with NMR guidance from André J. Simpson and multivariate statistical help from Whitfield Åslund M. The manuscript was written by Brian P. Lankadurai with critical comments from Whitfield Åslund M. and Myrna J. Simpson.

CHAPTER FIVE

$^1$H NMR-based metabolomic analysis of the response of Eisenia fetida earthworms after sub-lethal exposure of perfluorooctanoic acid and perfluorooctane sulfonate


Contributions: The experiment was designed by Brian P. Lankadurai and Myrna J. Simpson. The experiments were conducted by Brian P. Lankadurai. Data collection and analysis were performed by Brian P. Lankadurai with NMR guidance from André J. Simpson. The manuscript was written by Brian P. Lankadurai with critical comments from Myrna J. Simpson.
CHAPTER SIX

$^1$H NMR-based metabolomic analysis of sub-lethal perfluorooctane sulfonate exposure to
the earthworm *Eisenia fetida* in soil

Submitted to: Metabolomics

Contributions: The experiment was designed by Brian P. Lankadurai and Myrna J. Simpson. The experiments were conducted by Brian P. Lankadurai. Data collection and analysis were performed by Brian P. Lankadurai with NMR guidance from André J. Simpson and MS guidance from Vasile Furdui. The manuscript was written by Brian P. Lankadurai with critical comments from Myrna J. Simpson.

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**Conclusions and Future Research**

Contributions: Written by Brian P. Lankadurai with critical comments from Myrna J. Simpson
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Figure 4-1. Mean principal component analysis (PCA) scores plot of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the (A) polar and (B) non-polar fractions of E. fetida tissue extracts. The mean scores for the controls are denoted in the figure by a ‘C’ and the corresponding exposure day (for example C1 identifies the control for day 1). The mean scores for the phenanthrene exposed earthworms are denoted by the exposure concentration followed by the corresponding exposure day (for example 0.4-2 identifies a phenanthrene exposure concentration of 0.4 mg/cm$^2$ for two days). The mean scores (with associated standard error) were obtained by averaging the scores of each earthworm concentration class for each day. The ellipses that group the exposed classes were constructed as visual aids. The concentration response trajectory is highlighted by a dashed line.

Figure 4-2. Loadings plot for (A) PC1, and (B) PC2 showing the metabolites that were major contributors to the separation observed in the average PCA scores plot for the polar fraction of the E. fetida tissue extracts. The abscissa refers to the $^1$H NMR chemical shifts (ppm).
Figure 4-3. Percent (%) change in selected metabolites of the polar fraction of phenanthrene exposed *E. fetida* tissue extracts compared with the control earthworms for each day. (A) Leucine, (B) Alanine, (C) Glutamate, (D) Arginine, (E) Lysine, (F) Phenylalanine, (G) Maltose, (H) Malate, (I) Fumarate, (J) Succinate, (K) Betaine, (L) *scyllo*-Inositol, (M) *myo*-inositol, (N) 2-hexyl-5-ethyl-3-furansulfonate (HEFS), and (O) Adenosine triphosphate (ATP). The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each day and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed for each day] are labeled with an asterisk (*; at α =0.05). The percent changes are shown with their associated standard error.

Figure 4-4. Percent (%) change in selected metabolites of the non-polar fraction of phenanthrene exposed *E. fetida* tissue extracts compared with the control earthworms for each day. (A) Fatty acid-CH$_3$, (B) Fatty acid-(CH$_2$)$_n$, (C) Cholesterol, and (D) Phosphatidylcholine. The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each day and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly different [based on a t-test (two tailed, equal variances) of control vs. exposed for each day] from the control are labeled with an asterisk (*; at α =0.05). The percent changes are shown with their associated standard error.
Figure 5-1. Average principal component analysis (PCA) scores plot of PC1 (first PCA component) versus PC2 (second PCA component) for the $^1$H NMR spectra of *Eisenia fetida* tissue extracts of (A) PFOA exposed, (B) PFOS exposed, and (C) both PFOA and PFOS exposed earthworms together. The mean scores for the PFAA exposed earthworms are denoted by the name of the exposed PFAA followed by the corresponding exposure concentration (for example, PFOA-50 identifies a PFOA exposure concentration of 50 µg/cm²). The mean scores (with associated standard error) were obtained by averaging the scores of each exposure concentration (n=10). The concentration response trajectory is highlighted by a dashed line. ................................. 122

Figure 5-2. Percent (%) change in selected metabolites of PFOA exposed *Eisenia fetida* tissue extracts (n=10) compared with the control earthworms (n=10). The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at $\alpha =0.05$) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding P-values. The percent changes are shown with their associated standard error. .......................................................... 125

Figure 5-3. Percent (%) change in selected metabolites of PFOS exposed *Eisenia fetida* tissue extracts (n=10) compared with the control earthworms (n=10). The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control
earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at \( \alpha = 0.05 \)) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding \( P \)-values. The percent changes are shown with their associated standard error.

Figure 6-1. Average principal component analysis (PCA) scores plot for the \(^1\text{H} \) NMR spectra of \textit{Eisenia fetida} tissue extracts after PFOS exposure of two days (A) PC1 (first PCA component) versus PC2 (second PCA component), (B) PC3 (third PCA component) versus PC4 (fourth PCA component), seven days (C) PC1 vs PC2, (D) PC3 vs PC4, and fourteen days (E) PC1 vs PC2, (F) PC3 vs PC4. The mean scores for the PFOS exposed earthworms are denoted by the exposure length followed by the corresponding exposure concentration (for example, 2-100 denotes a two-day exposure to 100 mg/kg of PFOS in organization for economic corporation and development soil). The mean scores (with associated standard error) were obtained by averaging the scores of each exposure concentration. The ellipses were constructed as visual aids.

Figure 6-2. Average principal component analysis (PCA) scores plot for the \(^1\text{H} \) NMR spectra of \textit{Eisenia fetida} tissue extracts after PFOS exposure of two, seven and fourteen days. (A) PC1 (first PCA component) versus PC2 (second PCA component), (B) PC3 (third PCA component) versus PC4 (fourth PCA component). The mean scores for the PFOS exposed earthworms are denoted by the exposure length followed by the corresponding exposure concentration (for example, 2-50 denotes a two-day exposure to 50 mg/kg of PFOS in organization for economic corporation and development soil). The mean scores
(with associated standard error) were obtained by averaging the scores of each exposure concentration for each day of exposure. The ellipses were constructed as visual aids.

**Figure 6-3.** Average predictions of PFOS concentrations ($\hat{y}_i$) given spectra $i$ by the PLS model derived from the leave-one-out cross-validation procedure with spectra $i$ omitted for PLS models constructed with the bucketed $^1$H NMR spectra as the X-table and the PFOS exposure concentrations as the Y variable. The solid line indicates a linear regression between the actual and predicted values. The PLS-regression models correspond to (A) two days of exposure, (B) seven days of exposure, and (C) fourteen days of exposure. The error bars represent the standard error of the mean.

**Figure 6-4.** Percent (%) change in selected metabolites of two-day PFOS exposed tissue extracts compared with the control earthworms. The % changes in the intensity of *Eisenia fetida* metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at $\alpha =0.05$) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding $P$-values. The percent changes are shown with their associated standard error.

**Figure 6-5.** Percent (%) change in selected metabolites of seven-day PFOS exposed *Eisenia fetida* tissue extracts compared with the control earthworms. The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control
earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at \( \alpha = 0.05 \)) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding \( P \)-values. The percent changes are shown with their associated standard error. ............................................................ 153

**Figure 6-6.** Percent (%) change in selected metabolites of fourteen-day PFOS exposed *Eisenia fetida* tissue extracts compared with the control earthworms. The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at \( \alpha = 0.05 \)) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding \( P \)-values. The percent changes are shown with their associated standard error. ............................................................ 156
Chapter One

Introduction: Environmental metabolomics as an ecotoxicological tool in assessing soil contamination
1.1 Ecotoxicology

The term ecotoxicology was originally defined by Truhaut [1] as the branch of toxicology that is concerned with the toxic responses initiated by exposure to natural and synthetic pollutants to the constituents of an ecosystem in an integrated context. Prior to World War II, the accepted practice in dealing with pollution was to dilute the chemicals, which was known as the dilution paradigm [2]. Since the end of World War II there was a slow shift from the dilution paradigm, to the boomerang paradigm, which suggested that what is released into the environment may end up returning to cause harm [2]. Two watershed events that were suggested to contribute to this paradigm shift were Minamata disease, a neurological disorder caused by the ingestion of methyl-mercury laden fish from Minamata Bay in Japan, and the accumulation of dichlorodiphenyltrichloroethane (DDT) in raptors and fish-eating birds and the resulting reproductive failure and death [2-4]. Rachel Carson’s Silent Spring also made the public aware of the dangers of the continued release of pesticides into the environment [5]. These events precipitated the need for scientific researchers to assess the production, fate, and toxicity of chemicals released into the environment. In addition, the rapid growth in technology has led to the production of countless classes of new chemicals in the past decade to fulfill the ever expanding needs of a growing global population. Brominated flame retardants, pharmaceuticals and personal care products, synthetic estrogens, hormones, fluorinated chemicals, chlorinated byproducts, engineered nanoparticles and manufactured antimicrobial products are examples of emerging classes of chemicals whose production and release into the environment has increased rapidly [2]. Each of these chemicals, have unique structures and have the potential to alter
biochemical mechanisms in organisms. Thus, the importance of ecotoxicological experts assessing the impact of these chemicals to the ecosystem has become imperative.

1.1.1 Current Approaches to Ecotoxicological Assessment and Their Limitations

To fulfill the growing need for toxicological data concerning new and existing chemicals and to alleviate the shortcomings of existing regulatory controls new legislation has been launched by the European Union in 2007 as the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH), and by the United States Environmental Protection Agency (US EPA) in 1998 as the High Production Volume (HPV) Challenge [6-8]. These legislations attempt to fulfill the gaps in ecotoxicological data by shifting the onus from the regulators to the manufacturers and importers of the chemicals to perform the necessary toxicological tests that will provide users with the safety information required to handle the chemicals [8-9]. Previous chemical safety programs focus on compounds manufactured or imported in quantities above 1000 t/annum [7]. REACH has decreased this limit to 1 t/annum and HPV to 500 t/annum, thereby increasing the number of chemicals for which risk assessment data is required [7, 9]. Also, toxicity testing is now being conducted for complex environmental matrices such as ambient waters, effluents and sediments, to monitor the level of pollution and to determine the appropriate treatment and remediation efforts [6]. In addition, the level of complexity of tests that are being incorporated, such as tests which capture the responses of organisms after exposure to chemicals that have the potential to disrupt specific endocrine pathways, have increased [6, 10]. These changes signal the need for the development of a high-throughput, more thorough and cost effective ecological testing methods [6].
Both REACH and HPV require a large number of toxicological data in order for manufacturers and importers to register high production volume chemicals [7-8, 11-12]. REACH and HPV also insist that vertebrate animal testing should be done only as a last resort [8, 13]. Quantitative structure activity relationships (QSARs) and in vitro testing have been suggested as possible alternatives to vertebrate animal testing [8, 14]. However, only a few in vitro assays have been accepted for some endpoints as acceptable alternatives [7]. Also, the data sets used to build the QSARs should also be reviewed to reduce the risk of obtaining unsatisfactory conclusions that fail to meet the high expectations of REACH and HPV. Therefore, most of the methods used to garner ecotoxicological data are based on whole animal exposures and the resulting adverse responses in survival, growth, and reproduction [6]. These types of studies are lengthy and require a lot of resources, especially when you switch from the short-term lethality tests to the longer full-life-cycle exposure tests [6]. Therefore, it may take decades before all the required testing has been conducted for the high production volume chemicals [7]. Hence, there is a bias to primarily obtain acute toxicity data and use that information to predict the responses to sub-lethal chronic exposures, although this extrapolation may not be accurate [15]. Also, these tests do not provide any information on how chemical toxicity occurs (i.e. the mode of action (MOA) of the chemical) [16]. Knowing the MOA of the chemicals will aid in more focused resource utilization and reduced uncertainty in regulatory decision making because the designing of the toxicity tests and the interpretation of the results will be less ambiguous [6]. Also, an understanding of the MOA will reduce the uncertainty in predicting synergistic and additive effects of chemical mixtures, because compounds with similar versus dissimilar MOAs can be grouped together, enabling better expectations of mixture toxicity [6]. Unraveling the toxicities of novel compounds will also benefit from an
understanding of the MOAs of common chemicals because the profiles from the novel compounds can be compared to the already known MOAs and potential toxic pathways can be identified [6, 17-18]. Therefore, developing rapid and reliable techniques that are capable of delineating the MOA of chemicals can have a great impact on ecotoxicological risk assessment.

Figure 1-1. A representation of the relationship between the detectability of an environmental distress signal and ecological relevance. Based on Fig. 1 of Moore et al. [19].

Biomarkers, broadly defined as a change in the response (biochemical, physiological or histological) that can be related to exposure of environmental chemicals, can provide insight into the MOA of chemicals [20-21]. Biomarkers can also be used as early “distress signals” that signify the exposure to environmental contaminants [19, 21]. For example, increased production of vitellogenin, a precursor protein to egg yolk, in adult and juvenile fish has been linked to the exposure to estrogenic compounds [22-24]. Examining contaminant exposure induced changes at the organism/population/ecosystem level is generally too complex and far removed from the
exposure events (Fig. 1-1) to enable the development of tools that help in early detection and prediction of consequences of exposure to environmental contaminants [19]. Due to the promise of an early warning system for chemical exposure and the potential to delineate the MOAs of chemicals has led to substantial research in developing and applying biomarkers in ecotoxicology and ecological risk assessment over the past two decades [20].

The global population has more than doubled in the past fifty years putting a tremendous strain on earth’s natural resources to meet the basic demands of the human population. This has led to the ever-increasing production of new synthetic chemicals as a means to compliment and enhance the use of earth’s natural resources. Legislations such as REACH and HPV have set very strict guidelines that need to be followed in order for chemicals with high production volume to be registered. As discussed before, obtaining the required ecotoxicological information for new and existing chemicals using existing protocols is going to take decades. Therefore, it is imperative to develop ecotoxicological tools and ecological risk assessment guidelines that are less time consuming, more efficient, and less resource intensive. Toxicological tests that delineate the MOA of the chemical and also provide biomarkers as early warning signals to chemical exposure has shown promise as a better alternative to acute toxicity tests and QSARs. Hence, it is essential to develop an analytical platform that is capable of detecting biomarkers of chemical exposure and is also able to elucidate the chemicals mechanism of toxicity.

1.2 Metabolomics

Metabolomics (also known as metabonomics or metabolic profiling) can be defined as the field of science that is interested in the analysis of endogenous and exogenous, low molecular
weight organic metabolites within a cell, tissue, or biofluid of an organism in response to some external stressor [25-26]. Just as genomics deals with gene expression, transcriptomics with gene transcripts and proteomics with expression of proteins, metabolomics deals with the metabolome. Any omics technology comes with the caveat that it is capable of generating a comprehensive data set of whatever is being measured [27]. Therefore, the metabolome constitutes all of the metabolites or small molecules within a cell, tissue, organ, biological fluid or the whole organism [28]. Metabolomic analyses can be categorized as either non-targeted or targeted analyses [29-30]. In non-targeted metabolomics a non-biased, quantitative analysis of all or large number of metabolites in a biological sample are analyzed [29]. In targeted metabolomics a specific group of metabolites are analyzed [29-30].

Figure 1-2. The relationship between the various omics technologies. (Adapted from Figure 1 of Fan et al. [31]). The deoxyribonucleic acid (DNA) structure is from http://www.csulb.edu/~cohlberg/storage/DNA.html. The transfer ribonucleic acid (tRNA) structure is from Fig. 1F of Bessho et al. [32]. The protein structure is that of a dehydrogenase enzyme from the bacteria *Colwellia psychrerythraea* obtained from the Argonne's Midwest Center for Structural Genomics. The diagram representing physiology is from http://www.refocus-workshop.com/pages/course_type.php?tp=9. The metabolite network diagram is from the Kegg database http://www.genome.jp/kegg/pathway/map/map01100.html.
Metabolomics, being the newest member of the omics platforms, has been traditionally viewed as a complementary technique to genomics, transcriptomics and proteomics [33-34]. However, metabolomics may actually provide a solution to many of the shortcomings that are encountered with the other omics methods [33, 35]. Exposure of an organism to an external stressor will result in changes to gene expression and protein production, which are both subjected to a variety of homeostatic controls and feedback mechanisms, and will lead to alterations of the metabolite profile (Fig. 1-2), resulting in a much more sensitive indicator of the external stressor with a higher-level of integration than other omics technologies [6, 26, 33]. Although methods have been developed to detect changes in genomic, transcriptomic and proteomic profiles, the basic information required to make meaningful interpretations based on these data is often not readily available [6]. However, knowledge concerning the structure and functioning of metabolites is much greater in comparison to genes or their corresponding proteins [33]. Also, the number of metabolites is often much lower than genes and proteins. For example, the human genome is composed of an estimated 30,000 genes and the proteome may have a 100,000 or more proteins, whilst the metabolome is expected to be about 2,000 to 20,000 compounds [6, 33, 36]. This allows metabolomics to be much more powerful statistically in detecting robust responses compared to other omics technologies because the number of sample replicates in a study is usually higher than the number of metabolites, whereas in proteomics or transcriptomics studies, the number of parameters being analyzed is usually higher than the number of replicates [37]. Therefore, metabolomics shows potential as a sensitive and rapid technique that in theory is capable of elucidating relationships between the metabolite levels and the external stressor (chemical, nutritional or disease) by using the information contained in the data set (i.e. the metabolome) [28].
The capacity to detect subtle changes and the comprehensive nature of metabolite measurements have resulted in the popularity of metabolomics to be a popular method in many fields [38]. The use of metabolomics is rampant in biology, clinical pharmacology and toxicology, nutrition and medicine [38-40]. For example, metabolic profiles of cancer cells are used to understand the development and progression of tumors [41-43]. In clinical applications, screening of readily accessible bodily fluids such as urine and blood have helped identify metabolite biomarkers that are capable of screening for cardiovascular diseases, hypertension, respiratory diseases, neonatal diseases and infectious diseases [44-50]. Metabolomics has also been used in preclinical toxicology to detect metabolite biomarkers of toxicity and to elucidate the MOA of newly developed drugs [27, 44, 51-52]. For example, Dieterle et al. [51] used metabolomics to select successful drug candidates from a group of drugs with equal pharmacological activities based on the least toxic side effects. They discovered that two of the five drugs tested resulted in an extreme excretion of choline into urine and hypothesized that this was probably due to a massive degradation of cell membranes or an inhibition of choline oxidation and therefore discarded those drugs from further testing. As mentioned previously (section 1.1.1), the field of ecotoxicology can greatly benefit from a method that is capable of identifying biomarkers of contaminant exposure and is also able to delineate the MOA of the chemicals. The potential illustrated by metabolomics to detect biomarkers of exposure and delineate MOAs of chemicals in other fields of science has contributed to the application of metabolomics techniques in the environmental sciences [25]. This emerging sub-class of metabolomics is referred to as environmental metabolomics. Fig. 1-3 compares the annual publications in metabolomics and the sub-class of environmental metabolomics between the years of 2001 to 2012. It is clearly evident that publications in both fields have increased
tremendously over the past decade.

1.2.1 Environmental Metabolomics

Environmental metabolomics was defined by the Metabolomics Standards Initiative-Environmental Context Working Sub-Group, a group that was established to set standards for reporting requirements of metabolomics data, as “the application of metabolomics to the investigation of both free-living organisms obtained directly from the natural environment (whether studied in that environment or transferred to a laboratory for further experimentation) and of organisms reared under laboratory conditions (whether studied in the laboratory or transferred to the environment for further experimentation), where any laboratory experiments specifically serve to mimic scenarios encountered in the natural environment” [25, 53]. Essentially, environmental metabolomics can be viewed as the application of metabolomics techniques to characterize the interactions of organisms with their environment [25]. The external stressors that organisms are exposed to in the environment can vary widely from abiotic stressors, such as temperature (natural) and pollution (anthropogenic), to biotic-biotic
interactions, such as infection and herbivory [25]. Organisms can also be exposed to a mixture of these stressors at the same time. Therefore, characterizing organism responses to environmental stressors can be complicated because multiple stressors can induce a variety of responses simultaneously.

Non-targeted metabolomics, due to its comprehensive and “open” nature of analysis allows for the detection of unexpected relationships and metabolic responses after exposure to various environmental stressors [25]. This enables hypothesis generation for hitherto unexplained responses to environmental stressors and thereby help unravel the underlying MOAs [25]. For example, metabolomics has been applied to understand the biochemical mechanisms responsible for thermotolerance in both terrestrial and aquatic organisms [25, 54-57]. The mechanisms underlying the phenotypic plasticity that is induced as an adaptive response to cold tolerance in the fruit fly *Drosophila melanogaster* (*D. melanogaster*) was poorly understood. Colinet et al. [54] investigated the cold tolerance of *D. melanogaster* using metabolomics. They illustrated that acclimation to cold stress resulted in an increase in the sugars sucrose, fructose and trehalose, an increase in the polyamines cadaverine and putrescine, and a decrease in the metabolic intermediates citrate, fumarate, malate and glycerate [54]. They also showed that the decrease in glycerate and the TCA cycle intermediates (citrate, fumarate and malate) signals a reduction in energy metabolism, which the fruit flies use as an energy-saving strategy after cold acclimation. Therefore, metabolomics revealed potential biomarkers of cold acclimation and also suggested a potential survival mechanism that involves energy metabolism. Metabolomics has also been used to understand the biochemistry of drought resistance in plants, since climate change, water availability and land management practices have exerted negative pressures on successful plant growth and reproduction [25, 58-59]. For example, Warren et al. [60] subjected
two closely related species of the *Eucalyptus* genus, the *Eucalyptus pauciflora* and the *Eucalyptus dumosa*, to severe water stress for two months. They reported that water stress resulted in significant responses of 30-40% of measured metabolites in *E. dumosa* and 10-15% in *E. pauciflora*. They also showed that some metabolites even had opposite responses in the two species. Overall, they illustrated that even species that are closely related can respond differently to water stress and identified species-specific metabolite biomarkers of water stress that can be used to assess the state of *E. dumosa* and *E. pauciflora* to drought conditions. Similarly, metabolomics has also been applied to analyze the responses of plants to emerging environmental concerns such as elevated atmospheric CO₂ levels [25, 61], increased exposure to Ultra-Violet-B radiation (UV-B; 280-320 nm) [25, 62] and also exposure to tropospheric ozone [25, 63-64]. In addition, to these natural abiotic stressors, metabolomics was also used to understand biotic-biotic interactions such as responses of plants to herbivores [25, 65] and symbiosis with fungi [25, 66]. These studies illustrate the power and versatility of metabolomics in elucidating the biochemical mechanisms and identifying potential biomarkers of exposure to a wide-array of environmental stressors in a variety of living organisms. These properties of metabolomics have been instrumental in propelling its development and application in the field of ecotoxicology, which is concerned with an ever increasing number of chemicals and the responses of both aquatic and terrestrial organisms to these chemicals [25, 67].

The capacity to detect biomarkers of exposure and elucidate the MOA of chemicals makes metabolomics a valuable tool for ecological risk assessment because it allows for early screening of chemicals which may result in adverse responses after chronic exposure [25]. In addition, metabolomics also aids environmental monitoring because by analyzing the biomarkers of exposure it is possible to identify which chemicals an organism is being exposed to, which
will greatly complement traditional chemical residue analysis, that only provide indirect inferences to the nature of the pollutants exposed to organisms [25, 68-69]. Metabolomics has been applied to investigate the toxicity of various environmental contaminants to many aquatic organisms [70-74]. For example, Jordan et al. [70] recently conducted a study in which gold fish were exposed to sub-lethal levels of three aquatic pollutants, 4,4’-isopropylindenediphenol (Bisphenol-A), di-(2-ethylhexy)-phthalate, and nonylphenol, both individually and as a mixture, and analyzed the liver and testis for changes in the metabolic profile. They showed that exposure to these chemicals resulted in a disruption of energy and lipid metabolism in the liver and an interruption of 5’ adenosine monophosphate-activated protein kinase and cyclic adenosine monophosphate signaling in the testis. They also revealed that contaminant mixture exposure resulted in a metabolic stress response that was not predicted by exposure to individual contaminants, even though any phenotypic differences between the two exposure methods were absent. This indicated the sensitivity of metabolomics in detecting responses to sub-lethal concentrations of chemicals and its potential in risk-assessment as an early indicator of chemical exposure.

Metabolomics has also been used to investigate the exposure of the endocrine disrupting chemical, 17α-ethinylestradiol (EE2), which has caused feminization in male fish, to several species of fish such as the juvenile rainbow trout (Oncorhynchus mykiss) [75], adult fathead minnow (Pimephales promelas) [76-77], the three-spined stickleback (Gasterosteus aculeatus) [78], and the roach (Rutilus rutilus) [79]. These studies identified various biomarkers of exposure to EE2, such as vitellogenin and the amino acid alanine [75] and also identified that sex steroid and glucocorticoid pathways are one of the primary targets of EE2 exposure in fish gonads [79]. In addition to fish, metabolomics has also been applied to aquatic invertebrates.
For example, Taylor et al. [80] exposed the water flea *Daphnia magna*, to cadmium, fenvalerate, dinitrophenol and propranolol and showed that metabolomics was able to distinguish *D. magna*'s responses between all four chemicals. Schock et al. [81] exposed the Atlantic blue crab (*Callinectes sapidus*) to the bacterium *Vibrio campbelli* and the chemical 2,4-dinitrophenol and revealed that glucose and lactate were reliable biomarkers of exposure and they also identified clear differences in the MOA of the two different stressors.

In addition to aquatic species, metabolomics has also been used to study terrestrial organisms as well. However, most of the metabolomics studies involving terrestrial vertebrates revolve around disease diagnosis and drug discovery with a very few that have direct environmental relevance [25]. Lu et al. [82] investigated the exposure of two endocrine disrupting chemicals, polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to rats. They illustrated that metabolomics was able to distinguish between the responses of PCB exposed and TCDD exposed rats. They reported several biomarkers of exposure such as lactate, glucose, creatine and 2-oxoglutarate and also identified mitochondrial dysfunction and perturbations in fatty acid metabolism in response to PCB and TCDD exposure.

The remainder of the environmental metabolomics studies on terrestrial organisms have focused on invertebrates [67, 83-84]. The nematode worm *Caenorhabditis elegans* [84-85] and various earthworm species (*Lumbricus rubellus* [86], *Lumbricus terrestris* [87], *Eisenia andrei* [88], *Eisenia fetida* [89], *Eisenia veneta* [90] and *Aporrectodea caliginosa* [91]) have been used in metabolomic analysis of various soil contaminants. For example, Mckelvie et al. [89] exposed *E. fetida* earthworms separately to, two pesticides (carbaryl and chlorpyrifos), three pharmaceuticals (carbamazephine, estrone and caffeine), two persistent organohalogenes (Aroclor 1254 and polybrominated diphenyl ether 209) and two industrial compounds (nonylphenol and
dimethyl phthalate) in order to identify contaminant-specific responses. They identified potential contaminant-specific metabolite biomarkers of exposure and were also able to delineate between the different toxic MOAs of the various contaminants. Therefore, these studies involving both aquatic and terrestrial species illustrate the potential of metabolomics in routine ecotoxicological assessment and environmental monitoring.

1.2.2 Experimental Design and Analytical Methods Used in Metabolomics

The basic steps that govern a typical environmental metabolomics study are illustrated in Figure 1-4. The experimental design will involve selection of the organism (e.g. fish, earthworms, plants or humans), type of external stressor (e.g. exposure to chemicals, heat/cold, starvation or disease), and the mode/route of exposure. Once the organism has been exposed to the external stressor, the biological medium to study will be selected (e.g. blood, urine, other biological fluids, and/or tissue/organ extraction) [92]. The extraction method to be used for tissues/organs also needs to be identified. No one extraction technique is capable of extracting all of the metabolites, therefore depending on the goal of the analysis the proper technique needs to be selected. For example a typical aqueous buffer extraction will be sufficient to obtain all of...
the polar metabolites, but a more rigorous technique involving a mixture of polar and non-polar solvents might be required to extract both polar and non-polar metabolites [93]. Once the extractions are completed the samples need to be prepared for the analytical platform of choice, which is typically either nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS). After the samples are analyzed by the analytical instrument, the data are processed and statistical analysis is performed using multivariate [principal-component analysis (PCA) and partial least-squares (PLS) regression analysis] and univariate (t-tests) analysis in conjunction with the quantification and identification of the metabolites. The final step would then involve biological interpretation of the data to make a connection between the external stressor and the metabolic response of the organism.

NMR and MS usage in metabolomics is so widespread that in the past it was mistakenly assumed that ‘metabonomics’ deals with NMR-derived metabolic profiling studies, while ‘metabolomics’ deals with MS-derived metabolic profiling studies [27]. MS usage in metabolomics is usually coupled to liquid chromatography (LC) [85, 94-95], gas chromatography (GC) [60, 92, 96] or capillary electrophoresis (CE) [97-99]. These chromatographic techniques separate the complex sample mixtures such that they could be analyzed by MS, but this can make the overall analysis time-consuming [27, 100]. Also, GC methods usually entail elaborate derivatization steps that are very lengthy and thus inconvenient for high-throughput analysis [101]. Direct injection MS enables the injection of a crude extract directly into an electrospray mass spectrometer, resulting in one spectrum per sample, but this method is not particularly quantitative [101]. The great advantage of MS is its ultra sensitivity (typically pico gram level), which makes it very important for studies that are looking for novel biomarkers [27, 100]. Especially, Fourier Transform ion cyclotron resonance (FT-ICR) MS has
excessively high resolution and mass accuracy but is very expensive and is thus not widely used [100]. MS also has mass fragment databases that allow for easy identification of compounds [100, 102]. In addition to lengthy analysis times, some of the disadvantages of MS involve selectivity for certain analytes, destructive nature, matrix effects such as ion suppression that can cause widely varying signal intensities and lack of more robust methods for chromatographic separations [27, 100]. Due to the selective nature of MS, it has been mostly used in targeted metabolomics studies [29, 103-105]. NMR on the other hand is non-destructive, non-selective, possesses cross-laboratory reproducibility, and exhibits lacks of sample bias [27, 100]. Therefore, NMR has been used widely in non-targeted or ‘global’ analysis of all or most of all the metabolites [30, 83, 106-108]. The main disadvantage of NMR is its low sensitivity with limits of detection on the order of 10 \( \mu \text{M} \) [100]. This would especially be problematic for the analysis of novel biomarkers because these may be at too low of a concentration for NMR to detect. Steps to eradicate the problem of low sensitivity involve the usage of extremely high static magnetic field strength NMRs as well as usage of probes that are cryogenically cooled to 4.5 K in order to increase the signal to noise ratio (limits of detection on the order of few nmol). In addition, the use of microcoil probes has also decreased the limits of detection to tens of nanograms [100, 109]. The obvious problem with these methods is the cost to afford and use such high-end instrumentation. However, unlike MS the sensitivity of NMR is not dependent on the metabolite pKa or hydrophobicity, which makes NMR a very versatile choice for broad-based analyses [100]. Also, NMR is highly quantitative: Burton et al. [110] illustrated that using a 500 MHz NMR spectrometer in a quantitative \(^1\text{H}\) NMR analysis that utilized external standards, the precision and accuracy was calculated to be around 1%. In addition, most environmental metabolomics studies use NMR due to the comprehensive nature of non-targeted
metabolomics and the ability to generate hypotheses for complex environmental stressors for which there are no known MOAs [83, 111-115]. Therefore, NMR was the analytical instrumentation of choice for the metabolomic analyses presented within this thesis.

Most NMR-based metabolomics studies have utilized one dimensional (1D) \(^1\)H NMR experiments [75, 88-89, 116-118]. 1D \(^1\)H NMR experiments are advantageous for metabolomics studies, which usually have hundreds of samples, because of their short acquisition times (~15 mins), allowing for high-throughput analysis of samples [100, 119]. However, biological samples are complex due to the large number of molecules that they posses, resulting in a large number of peaks within the small chemical shift range (~ 10 ppm) of a \(^1\)H NMR spectrum. This leads to a greater a chance of overlap of peaks generated by different metabolites, which then leads to difficulty in identifying compounds that are present at low concentrations because their peaks are masked by larger peaks from compounds at high concentrations [100]. Several 1D NMR techniques have been developed to alleviate the spectral overlap and improve resolution between peaks. Carr-Purcell-Meliboom-Gill (CPMG) is one such technique, which removes broad resonances associated with molecules of high molecular weight or molecules whose motion is constrained and thereby provides better resolution of low molecular weight metabolites [120-121]. J-resolved spectroscopy (JRES) projections is another 1D technique that is used to improve spectral resolution. JRES spectroscopy is essentially a two-dimensional (2D) NMR technique, where the chemical shift information is on one axis and the spin-spin coupling information is on another axis. Projecting only the chemical shift axis, a 1D proton decoupled spectrum is obtained, which has less spectral overlap and enables better detection of specific metabolites [100, 119, 122]. In addition to 1D techniques, 2D NMR techniques have also been used to increase spectral resolution because they have an additional dimension into which the
signals can be dispersed. Some of the common 2D NMR techniques in metabolomics involve, \(^1\)H correlation spectroscopy (COSY), \(^1\)H-\(^{13}\)C heteronuclear single quantum coherence (HSQC) and \(^1\)H-JRES NMR spectroscopy [119, 123-125]. The additional benefit of using 2D NMR techniques such as HSQC, is that the \(^{13}\)C axis has a large chemical shift range (~ 200 ppm), which allows for greater spectral dispersion and enhanced resolution [123]. However, most of the 2D NMR techniques are less sensitive and require longer acquisition times (sometimes 3 to 4 times more than 1D experiments) [126]. For example, the NMR sensitivity of the \(^{13}\)C nucleus is 1.6% of the \(^1\)H nucleus and it also has very low natural abundance (1.1%) [127]. Therefore, 2D experiments such as HSQC require very long acquisition times for acceptable signal to noise (S/N) ratios. This has led to a limited use of 2D NMR techniques in metabolomics compared to 1D techniques. Nevertheless, 2D techniques can be a great complement in decreasing the ambiguity in compound identification from 1D \(^1\)H NMR experiments [119].

Analyzing aqueous samples using \(^1\)H NMR requires the application of water suppression techniques [77, 87, 91, 116, 122, 128]. Even though, D\(_2\)O (deuterium resonates at a different frequency than \(^1\)H in the NMR) solvents are mostly used there is always residual H\(_2\)O that is present. The concentration of water is much higher (~50 M) compared to millimolar metabolite concentrations, leading to a saturation of the NMR receiver by the H\(_2\)O signal and a suppression of signal intensities in the peaks of other compounds [129]. The three most common water suppression methods used in metabolomics are presaturation, Nuclear overhauser effect spectroscopy presaturation, and Presaturation utilizing relaxation gradients and echos (PURGE) [90, 118, 120, 122, 130-131]. Mckay [132] compared many water suppression techniques and concluded that PURGE provided the best suppression with the least amount of parameter optimization and the least of amount of spectral regions that need to be excluded due to
variations in the suppression of the solvent peak. Also, Yuk et al. [119] compared various 1D and 2D NMR techniques and concluded that PURGE $^1$H NMR to be the most rapid, informative and economical method for analyzing aqueous metabolomics samples, and will therefore be the method of choice in this thesis.

The choice of tissue/organ extraction method is also a critical step in metabolomics analysis [93, 133-134]. NMR-based metabolomics studies have mostly used D$_2$O-based phosphate buffer extractions [108, 112, 118-119, 128, 135-136]. The use of a buffer is advantageous because it maintains a constant pH across all of the samples, thereby decreasing the chances for changes in the chemical shift due to differences in the pH. However, aqueous buffer extraction methods only account for polar metabolites [137]. In order to obtain a holistic assessment of the metabolic profile of an organism both the polar and non-polar metabolites need to be analyzed. In addition, it is essential to quench metabolism as soon as possible after the exposure has been completed in order to decrease the influence of confounding variables into the analysis of the metabolic response [101]. This can be achieved by a rapid termination of enzyme activity, which is temporarily obtained by flash freezing the tissues with liquid nitrogen soon after the exposure period. However, unless the enzymes are precipitated from the extracts it is hard to limit their activity. Enzyme precipitation has been achieved by acid treatment or extraction with cold mixtures of organic solvents such as methanol, ethanol, acetone or acetonitrile [101]. Also, binding of the common NMR internal standard 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) to proteins in aqueous buffer extracts has led to large variations in the quantification of metabolite concentrations [138]. Therefore, it is essential to develop extraction methods that not only capture both polar and non-polar metabolites but also enable the precipitation of proteins. Lin et al. [101] examined various extraction methods
involving a mixture of organic solvents (methanol, chloroform, acetonitrile) and water in varying ratios and discovered that the bi-phasic methanol/chloroform/water (final solvent ratio of 2/2/1.8 respectively) extraction method, which was first described by Bligh and Dyer in 1959 [139], was the most reproducible and had the highest recovery of both polar and non-polar metabolites. Wu et al. [93] then went a step further, and examined three different strategies to add the methanol, chloroform and water to the tissue samples for extraction: 1. Stepwise addition - the original Bligh and Dyer [139] method of adding each solvent one by one, 2. Two-step addition - methanol and water are added in step one and chloroform and water are added in step two, 3. All-in-one addition – all three solvents are added together. They illustrated that the two-step addition was the best out of the three based on metabolite yield, extraction reproducibility, and sample throughput. Therefore, chapters 3 to 6 in this thesis utilize the two-step addition of methanol/chloroform/water described by Wu et al. [93].

1.2.3 Statistical Methods Used in Metabolomics

The advantage of metabolomics techniques is their ability to generate a large volume of data in a high-throughput manner. However, it is a challenge to visually analyze all of the collected data (i.e. NMR or MS spectra) and identify differences between samples in a timely manner [27]. Therefore, multivariate statistical methods are employed to ease the analysis of metabolomics data sets and obtain meaningful relationships between the external stressor and the metabolic response [140-141]. Before multivariate statistical analysis can be performed the spectra are divided into user-defined regions called buckets – this process is known as binning. For example, a typical $^1$H NMR spectrum that has a chemical shift range of 10 ppm can be divided into buckets that are 0.02 ppm wide resulting in close to 500 buckets or variables. Each bucket contains the areas of the integrated peaks within the bucket [27, 142].
advantage of pattern recognition methods is that they are able to reduce the dimensionality of metabolomics data from the hundreds variables into two or three components that are linearly orthogonally related to each other [141]. Therefore, analyzing two or three components then becomes a much easier task than analyzing hundreds of variables.

PCA is probably the most widely used multivariate statistical approach in metabolomics [83, 90, 120, 135, 141]. It is an unsupervised method, meaning the model is not provided with any prior information concerning the identity of the samples [142]. Therefore, the grouping of the samples in a PCA scores plot (Fig. 1-5) is based on the similarities of their metabolic profile. PCA explains the overall variability in the dataset, which is explained by a set of uncorrelated variables called principal components (PCs), which are linear combinations of the original variables [141]. The first PC (PC1) explains most of the variation in the data and PC2, which is orthogonal to PC1, explains the second most variation in the data and so on and so forth. As described in Fig. 1-5, PCA allows for dimensional reduction of the data into a low dimensional plane, such as PC1 vs PC2. The scores plot (e.g. PC1 vs PC2) allows for visual determination of the relationship between the samples based on their metabolic profile. As shown in Figure 1-5, the samples represented by the blue circles and the orange boxes have distinct metabolic profiles from each other as they are separated on the scores plot. The loadings plot illustrates the variables (or metabolites) that are responsible for the discrimination and/or clustering of the samples observed in the scores plots.

PLS regression analysis and partial least squares discriminant analysis (PLS-DA) are also used often as multivariate statistical tools in metabolomics [33, 77, 128, 143-144]. Both PLS-
regression and PLS-DA are supervised methods, which means that the classification of the samples as either control or experimental group is known and pre-defined variables are added to the model to maximize the separation between the sample classes and also to construct predictive models. The pre-defined variables for PLS-regression are usually measurable quantities such as the contaminant exposure concentration. For PLS-DA it is usually dummy variables such as zeros and ones [141]. PLS-regression and PLS-DA models are prone to over-fitting and therefore require proper validation methods to test the robustness of the models [145]. Therefore, cross-validation methods such as the leave-one-out cross validation (LOOCV) are used to validate the models [128, 145-146]. LOOCV is performed by randomly eliminating one of the samples from the data set (test set) and PLS-DA/PLS-regression is conducted on the remaining samples (the training set). This process is repeated until all of the samples have been left out of the model at least once. The models created with the training set are then used to
predict the test set. The ability of the model to predict the test set is represented by $Q^2_Y$, which is known as the goodness of prediction [145]. The $Q^2_Y$ value can be used to assess the robustness of a model: typically a $Q^2_Y > 0.4$ is considered a strong model [144-145]. In addition, response permutation testing is also conducted to assess the significance of PLS models [128, 147-148]. Permutation testing consists of keeping the data set constant, while randomly permuting the order of the pre-defined variables a set number of times. For each permutation a new PLS model is fitted and the $Q^2_Y$ is calculated providing a reference distribution of the $Q^2_Y$ statistic. The significance of the original PLS model and the confidence in its validity is increased if its $Q^2_Y$ value is higher than the values obtained for all of the PLS models built during the permutation tests [147].

Although, metabolomics mostly uses multivariate statistics, univariate statistical analyses are also conducted in a complementary manner to further enhance the amount of information gained from the study. T-tests can be used to assess the significance of the separation between the controls and exposed earthworms in PCA and PLS-DA scores plots. Also, t-tests can be utilized to determine which metabolites in the $^1$H NMR spectra of the treatment class increased or decreased significantly relative to the controls. Using this information a t-test filtered difference $^1$H NMR spectra can also be constructed by subtracting the buckets of the average controls from that of each average exposure class. The buckets representing metabolite peaks that were not statistically significant ($\alpha = 0.05$) from the controls can then be replaced with a zero resulting in a t-test filtered $^1$H NMR difference spectrum [76-77]. The t-test filtered difference $^1$H NMR spectra can be used together with the loadings plots to determine the metabolites that can be potential indicators or biomarkers of exposure to a particular contaminant.
1.3 Soil Contamination

Soil has undergone numerous changes especially within the past century. Intensive tilling of fields and clear cutting forests have vastly degraded the quality of soil [149]. Direct chemical contamination of soils due to wide-spread agricultural application of herbicides, insecticides, fungicides and other biocides over the years has led to high concentrations of these chemicals along with their degradation products in soils all over the world [150]. Numerous other synthetic chemicals such as flame retardants, fire fighting foams, pharmaceuticals and personal care products, along with contaminants such as polycyclic aromatic hydrocarbons (PAHs) that can be released through incomplete combustion due to anthropogenic and natural activities have also found their way to the soil environment. In addition to these organic chemicals, anthropogenic contamination of soil by heavy metals such as mercury, arsenic and lead have also increased in the past century and are causing serious health concerns due to their consumption either by plants that are grown in these soils or by leaching of these metals into groundwater which is used for drinking purposes [151]. These findings have warranted the need to perform experiments to identify the common contaminants in soil, understand their chemistry within the soil environment, find out their toxicities to organisms and design effective remediation methods to remove the problem contaminants from soil.

Earthworms have been used in ecotoxicological studies as indicators of soil toxicity [90, 137, 152-154]. A reliable indicator of soil toxicity requires an indicator that is naturally indigenous to soil. Earthworms are truly native to soil and their bodies are in direct contact with soil throughout their lives [155]. They contribute to the functionality of soil by aiding in the organic carbon turnover of soil by shredding of large pieces of organic matter thereby stimulating microbial activity. They also help in maintaining soil structure [90]. Earthworms
also serve as food for many animals and also facilitate in soil formation and thus are important in soils of many ecosystems [156]. Earthworms help in directly assessing the toxic effects of soil borne contaminants because they ingest large quantities of soil during their lifetime and also accumulate organic contaminants and metals from the surrounding soil environment via passive absorption [87, 155]. In addition, earthworms are cost effective and represent a non-controversial animal model and thus enable a convenient affordable method for research groups to pursue soil toxicity analysis [87]. Therefore, earthworms are used universally as ecotoxicological test organisms for soil contamination as they are viewed to be a good indicator species for environmental damage [90, 112, 116].

Ecotoxicological tests using earthworms have traditionally involved mortality tests that estimate LC$_{50}$ (concentration of a chemical that results in mortality to 50% of the population) values [157-158]. These tests that were developed and standardized by the organization for economic cooperation and development (OECD) involve exposure to contaminants using either contact filter paper tests or artificial soil tests [159]. *Eisenia fetida* is the earthworm species that has been recommended by the OECD for toxicological testing. *E. fetida* is found in soil rich in organic matter and possess several advantages as a good test species for ecotoxicological testing: its sensitivity to chemicals resembles that of true soil-inhabiting species, it has a short life cycle (hatching from cocoons in 3 to 4 weeks, and reaching maturity in 7 to 8 weeks), it is very prolific (each earthworm producing 2 to 5 cocoons per week) and is also easy to culture in the laboratory [159-160]. Therefore, *E. fetida* will be the earthworm species that is used in the studies presented within this thesis. The contact filter paper test is used as a quick initial screening method to determine the toxicity of chemicals to earthworms. The contaminant is applied to the filter paper as an aqueous solution or dissolved in a carrier solvent such as acetone, chloroform.
or hexane [159]. However, the contact filter paper test does not account for the differential sorption of chemicals to organic matter or clay particles found in soil [158]. Therefore, contact filter paper tests may not accurately represent the ecotoxicity of chemicals in soil. The artificial soil test is performed using a standardized soil medium that has the following composition: 10% finely ground sphagnum peat, 20% kaolinite clay, 70% industrialized quartz and a moisture content of 30 to 45% [158-159]. The exposure period is recommended to be either 7 or 14 days [159]. However, tests that estimate lethal concentrations do not provide any information regarding the exposure to the low-level chronic exposures that are usually experienced in the environment. Gibbs et al. [156] introduced tests that estimated the growth and reproduction of earthworms in addition to survival to try to understand the responses to sub-lethal exposure of chemicals. However, none of these methods allowed the elucidation of the MOA of the chemical or identified potential metabolite biomarkers of exposure.

1.3.1 NMR-based Earthworm Metabolomics

Nuclear magnetic resonance (NMR)-based metabolomics has been used to study earthworm responses to sub-lethal exposure of contaminants in both contact filter paper tests and soil exposure tests [25, 90, 92, 137, 152, 161-162]. For example, Baylay et al. [163] recently conducted a study, in which they exposed the earthworm species *L. rubellus* to two similarly acting (imidacloprid/thiacloprid) and two dissimilarly acting (chlorpyrifos/Nickel) chemicals to investigate the standard models of concentration addition and independent action using both metabolomics and classical toxicity tests that evaluate survival, weight change and cocoon production. They illustrated that even though reproductive responses indicated probable additivity for imidacloprid and thiacloprid, metabolomics detected distinct responses for each chemical, which at higher mixture concentrations was dominated by thiacloprid. Similarly, for
chlorpyrifos and nickel, even though phenotypic changes indicated independent action, metabolite changes were dominated by responses to nickel. This study revealed that NMR-based metabolomics is sensitive enough to elucidate the MOA of mixtures of chemicals. Soil exposure studies of the earthworm species *E. fetida* and *L. rubellus* to PAHs indicated that NMR-based metabolomics can identify concentration-dependent relationships between the metabolic profile and PAH exposure concentration [117, 144]. Bundy et al. [164] conducted an NMR-based metabolomic study in which they collected *L. rubellus* earthworms from seven geochemically contrasting sites in the United Kingdom with varying levels of metal contamination and wanted to determine if they can identify site specific metabolite biomarkers and also resolve which metals elicited significant responses in earthworms. They were able to distinguish both site- and contaminant-specific metabolic responses and identified zinc as the major contaminant eliciting a response in the earthworms. They also identified metabolites, which were correlated to zinc concentrations in all of the sites. This study illustrated the potential of using NMR-based metabolomics to assess polluted sites. These pioneering studies demonstrate the promise of NMR-based metabolomics as a novel tool to elucidate the MOA of chemicals and to assess the state of soil health. However, further studies are needed to test the ability of earthworm NMR-based metabolomic methods as a routine ecotoxicological tool.

This thesis focuses on NMR-based metabolomic analysis of two classes of contaminant exposure to *E. fetida*: PAHs (Chapters 2-4) and perfluorinated alkyl acids (PFAAs; Chapters 5 and 6). PAHs are ubiquitous in soil, are highly persistent due to their recalcitrant nature, and are also toxic to organisms with mutagenic and carcinogenic properties [165-170]. PFAAs, which have all of the C-H bonds in their hydrocarbon chain replaced by the highly stable C-F bonds, are highly persistent in the environment, are toxic to organisms, and are also found in soil [171-
Therefore, due to presence and persistence of PAHs and PFAAs in soil, and because of their toxicity to a wide variety of organisms including earthworms, we chose these two classes of contaminants as the focus of this thesis.

1.3.2 Polycyclic Aromatic Hydrocarbons

A group of organic chemicals that are of concern due to their wide-spread prevalence in the environment are PAHs [165-167]. PAHs are aromatic compounds that are distinguished by the number and arrangement of benzene rings they possess (Fig. 1-6). PAHs are ubiquitous in the environment, with soil and sediment considered as the major sinks [165-168]. PAHs are generally formed and released as a result of incomplete combustion of organic matter [168]. Natural sources of PAHs, although very minimal compared to anthropogenic sources, are due to

Figure 1-6. Structures of some common PAHs
volcanic eruptions, plant emissions and forest fires [177]. PAHs are found predominantly in petroleum products such as crude oils, motor oils, gasoline and heating fuels [166, 178]. Increase in the usage of petroleum products in the past few decades have greatly facilitated a massive release of PAHs to the environment [177]. Other pathways for entry of PAHs into the environment are through uncontrolled petroleum spills, marine transport, discharges from ships, and urban runoff [168]. PAHs released to the environment via incomplete combustion mainly enter soil and water bodies through wet or dry atmospheric deposition during rainfall [165, 168, 178]. PAHs are highly hydrophobic due to their structure composed of stable aromatic rings [166]. Their non-polar nature increases as the number of benzene rings increase [179]. Due to their highly hydrophobic nature they are practically insoluble in water, except for naphthalene [179]. They are slow to degrade and are extremely difficult to remove from soil due to their recalcitrant nature [169]. PAHs consisting of three or more rings have been observed to have negligible volatility [169, 180]. These qualities make PAHs highly persistent and immobile groups of contaminants in soil [180].

Due to the hydrophobicity of PAHs, they are also highly lipophilic [181] and are classified as ‘persistent toxic substances’. Persistent toxic substances are characterized by their exceptional toxicities towards many living organisms, reluctance in degradation and high lipophilicity [182]. They have also been designated by the US EPA as being toxic, partially mutagenic and carcinogenic ‘priority pollutants’ [170, 183]. The lipophilicity of PAHs is of major concern because they have the potential to cross lipid membranes and bioaccumulate in vital organs of organisms [184-185]. Since PAHs are potential mutagens and carcinogens the health of organisms can be severely hampered by the exposure to these toxic contaminants.
PAHs can exert their toxicity through several mechanisms, which include non-polar narcosis, adduct formation, the generation of reactive oxygen radicals and hormonal disturbance [186]. The general detoxification pathway in organisms exposed to PAHs involves two phases aimed at converting these hydrophobic compounds into more water soluble compounds, which can then be easily excreted [187]. In phase I, the cytochrome P450 (Cyt P450) enzyme system introduces a functional group, such as a hydroxyl or a sulphonyl, to the parent PAH molecule. In phase II, detoxification enzymes, such as glutathione S-transferase, attach a large water-soluble moiety to the product of phase I to further aid excretion [188]. Free radicals are formed as by-products of metabolism of PAHs by Cyt P450 in phase I. Organisms possess a set of antioxidant enzymes to counteract the production of free radicals. Catalase is a common antioxidant enzyme that breaks down the free-radical by-product hydrogen peroxide ($H_2O_2$), into water and molecular oxygen [187]. Ecotoxicological tests have monitored the activity of catalase and Cyt P450 in order to analyze the response of organisms including earthworms to PAH exposure. For example, Brown et al. [187] illustrated that exposure of the PAH pyrene to *L. rubellus* earthworms resulted in a decrease in catalase activity. Saint-Denis et al. [188] showed that exposure of *E. andrei* earthworms to benzo[a]pyrene resulted in a concentration-dependent increase in the activity of phase I enzymes but catalase activity was not altered. Achazi et al. [189] revealed that exposure of *E. fetida* earthworms to fluoranthene and benzo[a]pyrene did not result in an increase in the activity of Cyt P450 enzymes. Zhang et al. [190] observed a concentration-dependent relationship between total Cyt P450 levels in *E. fetida* and PAH exposure concentration. These previous studies that investigated the response of earthworms to PAH exposure using assays to monitor catalase and Cyt P450 enzyme activity revealed that
PAHs are toxic to earthworms but a reliable indicator to PAH exposure was not obtained using these traditional methods.

Several NMR-based earthworm metabolomic studies have been conducted to examine the response of earthworms to PAH exposure. For example, Brown et al.[161] exposed *E. fetida* earthworms via contact filter paper tests for two days to sub-lethal concentrations of naphthalene, phenanthrene and pyrene and suggested that earthworm metabolic responses may be correlated to contaminant concentration. They also identified the amino acids leucine, valine, alanine, lysine, and the sugar maltose as potential indicators of PAH exposure. However, this study only included 3 different exposure concentrations and did not clearly identify the MOA. Jones et al. [144] and Brown et al. [117] conducted sub-lethal soil exposure studies of pyrene to *L. rubellus* and phenanthrene to *E. fetida* respectively, and revealed that there is a positive linear correlation between earthworm metabolomic responses and PAH concentration. McKelvie et al. [118] exposed *E. fetida* to phenanthrene for thirty-days in soil and revealed that exposure time-dependent responses can also be analyzed using NMR-based earthworm metabolomics. These pioneering studies demonstrated the potential of NMR-based metabolomics as a novel soil testing tool to monitor sub-lethal PAH exposure. However, these studies did not assess whether or not the toxic MOA could be ascertained over a wide range of ultra-low sub-lethal concentrations and reliable metabolite indicators of exposure were also not identified. Therefore, further studies are needed to test the ability of earthworm NMR-based metabolomic methods as a routine tool in the ecotoxicological assessment of PAHs in the environment. In this thesis (Chapters 2-4) *E. fetida* earthworms are exposed to the model PAH phenanthrene [191-192] via contact filter paper tests to test both concentration and time-dependent responses and to identify potential indicators of exposure. The non-polar metabolites of *E. fetida* were also analyzed after
phenanthrene exposure for the first time to obtain a holistic assessment of the metabolic response (Chapters 3 and 4).

1.3.3 Perfluoroalkyl Acids

Perfluoroalkyl acids (PFAAs) are a class of organic fluorochemicals where all of the C-H bonds in the hydrocarbon chain have been replaced by C-F bonds [193]. They consist of a carbon backbone that is typically 4-14 carbons in length and a charged functional group such as a carboxylate, sulfonate, or phosphonate [174]. The stability of the C-F bonds in PFAAs renders them many unique physical and chemical properties such as hydrophobicity, lipophilicity, thermal stability, acid, base, and oxidizing agent resistance, and surfactant-like properties [171, 173-174]. These properties have led to a wide usage of PFAAs in many industrial and consumer-use applications such as fire-fighting foams, photographic emulsifiers, paints, adhesives, waxes, polishes, water-soil- and stain-resistant coatings for clothing fabrics, leather, upholstery, and carpets, oil resistant coatings for paper products approved for food contact, electroplating, electronic etching bath surfactants and aviation hydraulic fluids [171-175]. The chemical stability of PFAAs also results in environmental persistence [171-174]. PFAAs are distributed globally and have been detected in blood samples of many species of
wildlife and also in humans [171, 173, 194]. They have also been observed in arctic mammals and ocean going birds which are in remote locations that are far removed from PFAA sources [171, 173, 194-196]. PFAAs have also been detected in rivers, soils, sediments and oceans [197-199]. The two most common and prevalent PFAAs contain an eight-carbon backbone (Fig. 1-7): perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) [174]. The final breakdown product of many other PFAAs are also PFOA and PFOS [173-174]. Due to the widespread prevalence of PFOA and PFOS, their toxicity to a variety of organisms has been examined [173-174, 200]. Exposure studies involving rodents and monkeys have revealed decreased body weight and size, increased liver weight, tumors in the liver, pancreas and testicles, changes in lipid metabolism and even death at high doses [173, 200-201]. The structure of PFOA and PFOS resembles many common fatty acids and therefore, their main toxic MOA in mammals has been linked to an elevated level of fatty acid metabolism [173, 175, 200-202]. For example, PFOA and PFOS have been shown to cause peroxisome proliferation and thereafter increase the β-oxidation of fatty acids in rats and mice [173-174, 200, 202-205]. Peroxisome proliferator activated receptor alpha (PPARα) is a mammalian nuclear hormone receptor involved in lipid and lipoprotein metabolism [206-207]. Fatty acids binding to PPARα leads to the initiation of a cascade of events that eventually increase the production of peroxisomes in the liver. This leads to an enhanced oxidation of fatty acids by the peroxisomal enzymes [203, 207]. Since PFOA and PFOS mimic the structure of natural fatty acids they are able to bind to PPARα and initiate peroxisome proliferation leading to increased fatty acid oxidation that has resulted in the observed decreases in body weight and size, and the increases in liver weight [174, 203, 207].

Even though PFAAs are prevalent in soils [176, 199, 208-209], toxicity studies of PFAAs with terrestrial invertebrates and plants are limited. Joung et al.[176] investigated the exposure
of *E. fetida* earthworms to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) and observed a concentration-dependent increase in the mortality of the earthworms. This study illustrated that PFOA and PFOS were toxic to earthworms at high concentrations and may pose a threat to soil health and quality. Toxicity studies that examined the responses of earthworms to sub-lethal concentrations of PFOS are few. Stubberud [210] tested the fecundity of *E. fetida* after PFOS exposure and suggested that even at sub-lethal concentrations, adverse responses are being observed in the reproduction of *E. fetida*. However, the reproduction test does not provide any detail regarding the toxic MOA of PFOS in earthworms. The toxic MOA of the PFAAs has not been identified for earthworms. At the time of this thesis, there aren’t any published studies that utilized metabolomics techniques to investigate the response of organisms to PFAA exposure. Therefore, we attempted to bridge this gap in the literature and elucidate the MOA of PFAAs in earthworms by utilizing NMR-based metabolomics to analyze the response of *E. fetida* earthworms after exposure to sub-lethal concentrations of PFOA and PFOS via contact filter paper tests (Chapter 5) and PFOS via artificial soil exposure (Chapter 6).

### 1.4 Objectives

The overall objective of this thesis was to analyze the potential of $^1$H NMR-based metabolomics in elucidating the MOA of contaminants after sub-lethal exposure to *E. fetida* earthworms. Two classes of contaminants, PAHs and PFAAs, were chosen due to their prevalence and persistence in soil environments. Past metabolomic studies that investigated PAH exposure to earthworms revealed that NMR-based metabolomics is able to detect metabolic responses to PAH exposure and identified potential metabolite indicators of exposure [102, 103, 131, 197]. However, the MOA of PAHs wasn’t clearly identified. Also, the responses of non-
polar metabolites to PAH exposure was also not analyzed in *E. fetida* earthworms. Lipophilic contaminants such as PAHs bind to biological membranes and thereby have the potential to influence the non-polar metabolic profile as well [198, 199]. Therefore, a holistic evaluation of the metabolic responses of *E. fetida* to sub-lethal PAH exposure had not been performed at the start of this thesis. Metabolomics in general was not used to study the responses of organisms to PFAA exposure. Therefore, metabolite indicators that can be used as early indicators of PFAA exposure were also not identified. In addition, the MOA of PFAAs in earthworms has also not been identified. We attempted to contribute to fill the knowledge gaps in these areas through the following objectives:

**Objective 1:** To assess the potential of \(^1\text{H}\) NMR-based metabolomics in characterizing the metabolic response of *E. fetida* to a wide-range of ultra-low sub-lethal phenanthrene exposure concentrations in contact filter paper tests. We also aimed to determine if NMR-based metabolomics can be used to ascertain the MOA over a wide range of sub-lethal phenanthrene concentrations.

**Objective 2:** To investigate the potential of \(^1\text{H}\) NMR-based metabolomics in elucidating the toxic MOA of phenanthrene in *E. fetida* by exploring the time-dependent relationships between the metabolic response and exposure time in contact filter paper tests. Non-polar metabolites will also be analyzed in addition to the polar metabolites to obtain a holistic understanding of the biochemical changes in *E. fetida* to phenanthrene exposure.

**Objective 3:** To determine if NMR-based metabolomics was capable differentiating between the influences of exposure concentration and exposure time variables on the metabolic profile of *E. fetida* exposed to sub-lethal phenanthrene concentrations. We also tested whether
exposure time or phenanthrene concentration has a greater influence on the metabolic response of *E. fetida*.

**Objective 4:** To identify if NMR-based metabolomics is capable of detecting *E. fetida* metabolic responses to sub-lethal exposure concentrations of PFOS or PFOA via contact filter paper tests.

**Objective 5:** To determine if \(^1\)H NMR-based metabolomics was used to investigate the response of *E. fetida* after exposure to an artificial soil spiked with sub-lethal PFOS concentrations by exploring both the concentration-dependent and time-dependent relationships between the metabolic response and PFOS exposure.

These five objectives will be addressed in chapters 2, 3, 4, 5 and 6 respectively.

The hypotheses tested in this thesis include:

1.) We hypothesized that NMR-based metabolomics is capable of detecting concentration-dependent and time-dependent relationships between the metabolic profile of *E. fetida* and the exposure concentration and exposure time.

2.) Since, lipophilic contaminants such as PAHs bind to biological membranes, we hypothesized that phenanthrene exposure will elicit significant metabolic changes in the non-polar metabolic profile of *E. fetida* exposed to sub-lethal phenanthrene concentrations.
3.) The MOA of PFOA and PFOS in mammals involved an enhanced oxidation of fatty acids. We hypothesized that the changes in the metabolic profile of *E. fetida* in response to sub-lethal PFOA and PFOS exposure will also reflect an increased oxidation of fatty acids. We further, hypothesized that PFOA and PFOS will have similar MOAs in earthworms as was observed in other organisms.

1.5 Thesis Summary

**Chapter 1:** Environmental metabolomics as an ecotoxicological tool in assessing soil contamination

**Chapter 2:** $^1$H NMR-based metabolomic observation of a two-phased toxic mode of action in *Eisenia fetida* after sub-lethal phenanthrene exposure

This chapter has been published in *Environmental Chemistry* and addresses objective 1. $^1$H NMR-based metabolomics was used to analyze the response of *E. fetida*, which were exposed via contact tests to six sub-lethal concentrations of phenanthrene for 48 hours. A two-phased MOA was observed that was dependent on the exposure concentration of phenanthrene. Variations in the metabolites alanine, lysine, arginine, isoleucine, maltose, ATP and betaine were found to be proportional to the exposure concentration suggesting that NMR-based earthworm metabolomics is capable of elucidating concentration-dependent relationships in addition to elucidating the MOA of sub-lethal contaminant-exposure.
Chapter 3: $^1$H NMR-based metabolomics of time-dependent responses of *Eisenia fetida* to sub-lethal phenanthrene exposure

This chapter was published in *Environmental pollution* and addresses objective 2. $^1$H NMR-based metabolomics was used to examine the response of the earthworm *Eisenia fetida* after exposure to sub-lethal concentrations of phenanthrene over time via contact tests. Both polar and non-polar metabolic profiles were analyzed after one, two, three and four days of exposure. Heightened *E. fetida* responses were observed with longer phenanthrene exposure times. Polar metabolites were found to be more sensitive indicators of phenanthrene exposure at ultra-low concentrations than non-polar metabolites. Amino acids alanine and glutamate, the sugar maltose, the lipids cholesterol and phosphatidylcholine emerged as potential indicators of phenanthrene exposure. A heightened energy demand was observed signaled by significant decreases in sugars and fatty acids. The conversion of succinate to fumarate in the Krebs cycle was also interrupted by phenanthrene. Therefore, this study shows that NMR-based metabolomics is a powerful tool for elucidating time-dependent relationships in addition to the MOA of phenanthrene in earthworms.

Chapter 4: $^1$H NMR-based metabolomic analysis of polar and non-polar earthworm metabolites after sub-lethal exposure to phenanthrene

This chapter was published in *Metabolomics* and addresses objective 3. $^1$H NMR-based metabolomics was utilized to elucidate the earthworm sub-lethal toxicity after exposure to phenanthrene via filter paper contact tests over one, two and three days of dermal contact. Polar and non-polar metabolic profiles of the earthworm tissue extracts revealed heightened *E. fetida* toxic responses with both longer exposure times and higher phenanthrene concentrations. PCA
scores plot of the polar fraction showed significant separation between control and exposed earthworms along PC1 for all phenanthrene concentrations on each day. The PCA scores plot of the non-polar fraction showed significant separation between the controls and exposed earthworms for only the first day of exposure. Alanine, glutamate, maltose, and fatty acids were identified as potential indicators of phenanthrene exposure. A heightened energy metabolism and possible deactivation of the succinate dehydrogenase enzyme in the Krebs cycle was also identified in exposed earthworms. PLS-regression models showed that the polar metabolic profile of *E. fetida* was weakly but significantly correlated to phenanthrene exposure concentrations after day one and day two of exposure. Overall, this study indicated that with longer exposures, contact time becomes more important than exposure concentration in discriminating between control and exposed earthworms. It was also suggested that because the non-polar metabolic profile appeared to be less sensitive to phenanthrene exposure even at the higher phenanthrene exposure concentrations used in this study future NMR-based metabolomic studies can focus only on the polar metabolites for a high-throughput analysis of the response of *E. fetida* to sub-lethal phenanthrene exposures.

**Chapter 5:** $^1$H NMR-based metabolomic analysis of the response of *Eisenia fetida* earthworms after sub-lethal exposure of perfluorooctanoic acid and perfluorooctane sulfonate

This chapter was published in *Environmental Chemistry* and addresses objective 4. $^1$H NMR-based metabolomics was used to elucidate the toxic MOA of PFOA and PFOS in *E. fetida* after exposure to sub-lethal concentrations for two days via contact filter paper tests. *E. fetida* metabolic responses intensified with higher PFOA or PFOS exposure concentrations. PCA exhibited significant separation between control and exposed earthworms along PC1 for all
PFOA and PFOS exposure concentrations. Leucine, arginine, glutamate, maltose, and ATP were recognized as potential indicators of PFOA and PFOS exposure as these metabolite concentrations fluctuated significantly with exposure. The metabolite responses suggested that PFOA and PFOS exposures may increase fatty acid oxidation, and also interrupt ATP synthesis. Significant decreases in the sugars glucose and maltose indicated a heightened energy demand in PFOA and PFOS exposed earthworms. This study revealed for the first time that NMR-based metabolomics is capable of detecting metabolic changes in response to PFOA and PFOS exposure and also provided possible insights into the MOA of these PFAAs in earthworms.

Chapter 6: $^1$H NMR-based metabolomic analysis of sub-lethal perfluorooctane sulfonate exposure to the earthworm Eisenia fetida in soil

This chapter has been submitted for publication in Metabolomics and addresses objective 5. $^1$H NMR-based metabolomics was utilized to determine the responses of E. fetida earthworms after exposure to an artificial soil that was spiked with sub-lethal concentrations of PFOS. Earthworms were exposed to a range of PFOS concentrations for two, seven and fourteen days. Multivariate statistical analysis indicated an exposure time-dependent operation of two separate MOAs: a non-polar narcosis type mechanism after two days of exposure and an MOA which suggested an increase in fatty acid oxidation following the seven and fourteen days of exposure. 2-hexyl-5-ethyl-3-furansulfonate (HEFS), betaine, leucine, arginine, glutamate, maltose, and ATP were identified as potential indicators of PFOS exposure as these metabolite concentrations fluctuated significantly with exposure. Elevation in fatty acid oxidation, disruption in energy metabolism and biological membrane structure, and also a possible interruption of ATP synthesis were also postulated based on the fluctuations of the metabolites to PFOS exposure. This
chapter indicates that NMR-based earthworm metabolomics has promise as a routine tool for ecotoxicological assessment of PFOS contaminated sites.

Chapter 7: Conclusions and future research
Chapter Two

\(^1\)H NMR-based metabolomic observation of a two-phased toxic mode of action in *Eisenia fetida* after sub-lethal phenanthrene exposure


2.1 Abstract

$^1$H NMR-based metabolomics was used to examine the response of the earthworm *Eisenia fetida* to sub-lethal phenanthrene exposure. *E. fetida* were exposed via contact tests to six sub-lethal (below the measured LC$_{50}$ of 1.6 mg/cm$^2$) concentrations of phenanthrene (0.8-0.025 mg/cm$^2$) for 48 hours. Multivariate statistical analysis of the $^1$H NMR spectra of earthworm tissue extracts revealed a two-phased mode of action (MOA). At exposures below $1/16$th of the LC$_{50}$ (the concentration that causes mortality to 50% of the population), the MOA was characterized by a linear correlation between the metabolic response and exposure concentration. At exposures $\geq 1/16$th of the LC$_{50}$, metabolic response to phenanthrene appeared to plateau, indicating a distinct change in the MOA. Further data analysis suggested that alanine, lysine, arginine, isoleucine, maltose, ATP and betaine may be potential indicators for sub-lethal phenanthrene exposure. Metabolite variation was also found to be proportional to the exposure concentration suggesting that NMR-based earthworm metabolomics is capable of elucidating concentration-dependent relationships in addition to elucidating the MOA of sub-lethal contaminant-exposure.
2.2 Introduction

Earthworms have been used in ecotoxicological studies as indicators of soil toxicity [90, 137, 152] and are considered to be excellent model organisms, because they are exposed to soil contaminants by both ingestion and via passive absorption [90, 211-212]. There are many studies that have examined the exposure of earthworms to lethal contaminant concentrations that result in mortality to 50% of the population; lethal dose (LD$_{50}$) or lethal concentration (LC$_{50}$) [90, 211-212]. However, these studies may not provide sufficient information concerning the toxic mode of action (MOA) of a given contaminant. Currently studies that explore changes in cellular metabolism, such as fluctuations in synthesis and breakdown of simple metabolites like amino acids and sugars due to exposure to very low or sub-lethal concentrations of contaminants are lacking [152, 213-214]. Sub-lethal contaminant exposure also results in adverse changes to the physiology of organisms [215-216]. Monitoring fluctuations in metabolite levels in response to sub-lethal contaminant exposure can potentially lead to an elucidation of the contaminant’s MOA [77, 217].

Nuclear magnetic resonance (NMR)-based metabolomics offers a reliable, reproducible and high-throughput platform that is currently being utilized to study earthworm responses to sub-lethal exposure of contaminants in both contact and soil exposure tests [25, 90, 92, 137, 152, 161-162]. Recent metabolomic studies with polycyclic aromatic hydrocarbons (PAHs) have suggested that the earthworm responses are concentration-dependent but these studies did not assess whether or not the toxic MOA could be ascertained over a wide range of ultra-low sub-lethal concentrations [117, 144, 161]. For example, Brown et al.[161] suggested that *Eisenia fetida* earthworm metabolic responses may be correlated to contaminant concentration but this study only included 3 different exposure concentrations and did not clearly identify the MOA.
Soil exposure studies have shown that there is a positive linear correlation between earthworm metabolomic responses and PAH concentration [117, 144]. These pioneering studies demonstrate the promise of NMR-based metabolomics as a novel soil testing tool however further studies are needed to test the ability of earthworm NMR-based metabolomic methods as a routine tool in the ecotoxicological assessment of PAHs in the environment. PAHs are prolific and persistent in soils [169-170, 218], thus developing better tools to monitor their risk to soil organisms is needed.

In this study, the metabolic response of the earthworm *Eisenia fetida* to ultra-low sub-lethal phenanthrene exposure in contact tests was examined using $^1$H NMR analysis of earthworm tissue extracts in combination with multivariate statistical methods. We specifically test if $^1$H-NMR metabolomics can be used over a wide range of sub-lethal concentrations (ie: fractions of the LC$_{50}$), which is important before metabolomics can be used as a widespread tool for elucidating sub-lethal toxic responses in soil environments [219-220]. Furthermore, by using six, sub-lethal concentrations, we hope to build upon previous studies [117, 161] and determine if NMR-based metabolomics can be used to ascertain the MOA over a wide range of sub-lethal phenanthrene concentrations. We report on the metabolomic responses of *Eisenia fetida* [the recommended earthworm species for use in toxicity tests by the Organization for Economic Cooperation and Development (OECD)] [137, 159, 161] after exposure to six sub-lethal concentrations of a model PAH [191-192], phenanthrene, ranging from 0.8 to 0.025 mg/cm$^2$ which are 1/2 to 1/64$^{th}$ of the LC$_{50}$ respectively. Metabolites that have the potential to be reliable indicators of phenanthrene exposure over a wide range of sub-lethal concentrations were also monitored to further build upon previous results [117, 161] with the main goal of further
determining the potential of NMR-based metabolomics as an ecotoxicity tool for soil contaminants.

2.3 Experimental Methods

2.3.1 Determination of phenanthrene LC$_{50}$

The 48-hour LC$_{50}$ of phenanthrene, using a concentration range from 0.016 mg/cm$^2$ to 10.4 mg/cm$^2$, was determined using the contact filter paper test as described by the OECD Guidelines [159]. The LC$_{50}$ for the OECD recommended reference substance, chloroacetamide, was also measured to assess the accuracy of the method. Standard 30 mL glass vials with Polytetrafluoroethylene (PTFE)-lined caps (Kimble Glass inc., Fisher Scientific), which are recommended by the US Environmental Protection Agency [221], were lined with Whatman no. 1 filter papers. Dichloromethane (DCM) was used as the carrier solvent. For all compounds, 1 mL of the carrier solution was added into each vial and evaporated under a slow stream of filtered, compressed nitrogen gas. In all cases, 1 mL of DCM was added to control vials (10 replicates) and vented. All vials were then vented for four hours.

For chloroacetamide, five concentrations between 0.05 μg/cm$^2$ to 31.25 μg/cm$^2$ were evaluated. A preliminary range-finding with 5 logarithmic concentrations of phenanthrene (between 0.001 mg/cm$^2$ to 10.0 mg/cm$^2$) was first conducted. This range was narrowed down to 0.016 to 10.4 mg/cm$^2$ (6 concentrations), with 10 worms per exposure concentration. Earthworms were depurated for three hours to void the intestinal tracts. A single, mature earthworm, weighing between 300 mg and 600 mg was added to each vial and 1 mL of deionised water was added to moisten the filter paper. All vials were stored on their side in the dark at
ambient room temperature. After 48 hours, mortality was determined. LC$_{50}$ values were calculated using the Trimmed Spearman-Karber Program Version 1.5 with automatic trim selected [221-222]. The LC$_{50}$ value for chloroacetamide was 3.9 μg/cm$^2$ (95% confidence interval of 2.6 μg/cm$^2$ to 5.8 μg/cm$^2$). This is in good agreement with the reported value of 2.7 μg/cm$^2$ for chloroacetamide [153].

2.3.2 Earthworm contact tests and tissue extraction

Mature earthworms (Accessory publication, Section A2-1) were depurated in the dark for 96 hours to empty their intestinal tracts prior to exposure tests [137]. Earthworms were exposed to six concentrations of phenanthrene: 0.8, 0.4, 0.2, 0.1, 0.05 and 0.025 mg/cm$^2$ (corresponding to: 1/2, 1/4$^{th}$, 1/8$^{th}$, 1/16$^{th}$, 1/32$^{th}$ and 1/64$^{th}$ of the measured 48-hour LC$_{50}$ respectively). Filter papers were placed in amber glass jars prior to the addition of phenanthrene solutions (1 mL in chloroform) [161]. For comparison to non-exposed earthworms (control set), only chloroform was added to filter papers (without phenanthrene) [161]. The chloroform in all jars (exposed and controls) was allowed to evaporate and then 1 mL of distilled water was added prior to addition of earthworms. Twelve earthworms per exposure concentration were used and twelve earthworms comprised the control set (without phenanthrene added to filter papers). The glass jars were kept in the dark for 48 hours [159]. Earthworms were then flash-frozen in liquid nitrogen, lyophilized and stored frozen until extraction [92, 137].

Lyophilized earthworms were homogenized in a 1.5 mL centrifuge tube using a 5 mm wide stainless steel spatula [92]. The homogenized earthworm tissue was then extracted using 1.20 mL of a 0.2 M monobasic sodium phosphate buffer solution (NaH$_2$PO$_4$·2H$_2$O; 99.3%; Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity; Sigma Aldrich) as a
preservative [137]. Buffer solution was made with D$_2$O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pH of 7.4 using NaOD (30% w/w in 99.5% D$_2$O, Cambridge Isotope Laboratories Inc). The buffer solution also contained 10 mg/L of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal standard [137, 161]. Samples were vortexed for 30 seconds and then sonicated for 15 minutes to aid with the extraction. Samples were then centrifuged at 14,000 rpm (~15,000 g) for 20 minutes and the supernatant was transferred into a new 1.5 mL centrifuge tube. The centrifugation procedure was repeated twice more to remove any additional particulates. Samples were then transferred into 5 mm High Throughput$^{\text{plus}}$ NMR tubes (Norell Inc.; NJ, USA) for $^1$H NMR analysis.

### 2.3.3 $^1$H NMR spectroscopy and data analysis

$^1$H NMR spectra of the earthworm extracts were acquired with a Bruker Avance 500 MHz spectrometer using a $^1$H-$^{19}$F-$^{15}$N-$^{13}$C 5 mm Quadruple Resonance Inverse (QXI) probe fitted with an actively shielded Z gradient. $^1$H NMR experiments were performed using Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression, 128 scans, a recycle delay of 3 s, and 16 K time domain points [130]. Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. All spectra were manually phased and calibrated to the DSS internal reference methyl singlet, set to a chemical shift ($\delta$) of 0.00 ppm.

Multivariate statistical analyses were performed on processed $^1$H NMR data using the AMIX 3.8.4 (Bruker BioSpin, Rheinstetten, Germany) statistics tool to identify differences in the metabolic profiles of *E. fetida* following phenanthrene exposure. The $^1$H NMR spectra were analyzed between $\delta$ of 0.5 and 10 ppm and divided into 0.02 ppm wide buckets, for a total of 475
The area between $\delta = 4.70$–$4.85$ ppm was excluded to eliminate the small residual H$_2$O/HOD signals. The integration mode was set to the sum of intensities and the spectra were scaled to total intensity [92, 161]. This created a matrix in which each row represents an earthworm sample and each column contains the integrated area of the original spectral intensities contained within each bucket region. Individual principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) scores plots were calculated to compare the metabolic response of the control and exposed worms for each exposure level. PCA is an unsupervised statistical method that identifies the maximum variation in data [147]. However, PLS-DA is a supervised statistical method that identifies variation between groups (for example, between controls and phenanthrene exposed worms) [147, 223]. The $R^2_X$ and $R^2_Y$ values, which denote the explained variance of X and Y respectively were obtained for PLS-DA [224-225]. The PLS-DA models were also cross-validated (internal cross-validation) using leave-one-out cross-validation (LOOCV) and the cross-validated $R^2_Y$ value (reported as $Q^2_Y$) was also obtained to determine the robustness of the model (Accessory Publication, Section A2-2) [144, 161, 225-226]. Levene’s test was used to test for variance homogeneity among the PCA and PLS-DA scores, which were found to have equal variances at $\alpha = 0.05$ [227]. A t-test (two-tailed, equal variances) was also performed on the first and second component PCA and PLS-DA scores to determine if there was a significant difference between the scores of the controls and the exposed worms at $\alpha = 0.05$ [228]. Corresponding PCA loadings plots, which show the relative weight for each bucket, were also acquired for each of the PCA scores plots to identify the metabolites that were contributing to the separation between the scores of the control and exposed earthworms. Both PCA and PLS-DA showed similar trends in discrimination (Fig. 2-1 and Accessory publication, Fig. A2-1). PLS-DA cross validation was also used to test the
robustness of the separation and the resulting $Q^2Y$ values for all exposure concentrations were greater than 0.5, suggesting that the models are robust (Accessory publication, Fig. A2-1) [144]. Since all of the replicates were used to make the PLS-DA models, external validation was not performed with an independent test set to determine the predictive ability of the model [223]. Thus, the resulting PLS-DA models provided no additional discrimination to that obtained with PCA; therefore further discussions will focus solely on the PCA models for brevity.

Difference class $^1$H NMR spectra were constructed to identify metabolites that had significantly increased or decreased relative to the control [76-77]. The buckets generated by AMIX 3.8.4 statistics tool, which represents the binned $^1$H NMR spectra of E. fetida extracts were then imported into Microsoft Excel. A Levene’s test performed on the buckets revealed that there was equal variance at $\alpha = 0.05$ [227]. A t-test (two-tailed, equal variances) was then performed comparing the buckets of the controls with that of the exposure class to identify the buckets that were statistically different at $\alpha = 0.05$. Average class $^1$H NMR spectra were obtained by averaging the buckets of each exposure class. Difference class $^1$H NMR spectra were then obtained by subtracting the buckets of the average controls from that of each average exposure class. The buckets representing metabolites peaks that were not statistically significant from the controls were then replaced with a zero resulting in a t-test filtered $^1$H NMR difference spectrum [76-77]. The buckets were then imported into ACD/1D NMR manager (Advanced Chemistry Development, version 12.0, Toronto, Canada) to acquire the difference spectra. The % changes in the intensity of metabolite peaks of exposed worms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms and then dividing again by the buckets in the control earthworms.
The metabolite peaks were identified using a database of the $^1$H NMR spectra of a series of standard metabolites that were previously identified in *E. fetida* [76, 92, 137].

A partial least squares (PLS) scores plot was also calculated using the AMIX 3.8.4 statistics tool, having the exposure concentration as the y-variable, to compare the control and exposed worms at all concentrations to deduce concentration-dependent relationships in the metabolic profile. A five-component model was found to be ideal using leave-one-out cross-validation (LOOCV). The scores from the PLS plot were then imported into Microsoft Excel (version 12.0.6504, Microsoft Corporation, Redmond, WA) and were averaged per class (concentration of phenanthrene exposure) and re-plotted with their associated standard errors.

### 2.4 Results and Discussion

PCA scores plots of $^1$H NMR spectra of *E. fetida* tissue extracts identified some statistically significant ($P < 0.05$) changes in the metabolic profiles of the phenanthrene exposed earthworms relative to the unexposed (control) earthworms (Fig. 2-1). Brown et al. [113] also showed that exposure of *E. fetida* to sub-lethal PAH concentrations via contact tests elicits changes in the metabolic profile. The t-test performed on the first and second component PCA scores showed that the higher exposure concentrations (0.80, 0.40, 0.20 and 0.10 mg/cm$^2$) resulted in a significant separation ($P < 0.05$) between the controls and the exposed worms along the x-axis (PC1; explains approximately 90% of the metabolic variation; Figs 2-1C-F). However, the lower concentrations (0.025 and 0.05 mg/cm$^2$) did not show significant separation ($P > 0.05$) from the controls along PC1 (Figs 2-1A and 2-1B). The 0.025 mg/cm$^2$ phenanthrene exposure had statistically significant separation ($P = 0.003$) from the controls along the y-axis (PC2; explains 4% of the metabolic variation), however this accounts for very little of the
Fig. 2-1. PCA scores plots of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of E. fetida aqueous buffer tissue extracts showing the separation of control worms (□) from exposed worms (○) at phenanthrene concentrations of (A) 0.025 mg/cm$^2$ (1/64$^{\text{th}}$ of LC$_{50}$), (B) 0.05 mg/cm$^2$ (1/32$^{\text{th}}$ of LC$_{50}$), (C) 0.10 mg/cm$^2$ (1/16$^{\text{th}}$ of LC$_{50}$), (D) 0.20 mg/cm$^2$ (1/8$^{\text{th}}$ of LC$_{50}$), (E) 0.40 mg/cm$^2$ (1/4$^{\text{th}}$ of LC$_{50}$), and (F) 0.80 mg/cm$^2$ (1/2 of LC$_{50}$). The $P$-values were obtained from t-tests that compared the scores of the control and exposed worms for each component.
variation in the metabolic profile (Fig. 2-1A). Therefore, higher phenanthrene exposure results in greater metabolic responses leading to greater differences in the overall metabolic profile. Brown et al. [117] also showed that high phenanthrene exposures in soil resulted in improved separation from the controls along PC1 (explains 74% of the metabolic variation), with lower concentrations separating from the controls only along PC4, which only explained approximately 2% of the metabolic variation.

PCA (PC1 and PC2) loadings plots were used to identify metabolites contributing to the separation (Fig. 2-2) [90, 117, 161, 229]. Leucine (δ 0.95 ppm), isoleucine (δ 1.25 ppm), alanine (δ 1.47 ppm), arginine (δ 1.91 ppm), lysine (δ 3.03 ppm), betaine (δ 3.25 ppm) and maltose (δ 5.41 ppm) were identified as the major metabolites contributing to the PCA separation (Fig. 2-2). The t-test filtered difference ¹H NMR spectra (Fig. 2-3) highlight these metabolites and the extent that the metabolic response varies with exposure to increasing phenanthrene concentration [76-77]. The percent change of the identified metabolites relative to the control (Fig. 2-4) suggests that metabolic changes are also related to exposure concentration over the range of concentrations studied. This confirms previous studies that suggest that NMR-based metabolomics is capable of detecting concentration-dependent responses in soil and contact exposure tests [117, 144, 161]. In our study, which included a wider range sub-lethal exposure concentrations, show that significant (at α = 0.05) metabolic responses can be detected consistently at exposure concentrations as low as 0.1 mg/cm² (1/16th of the LC₅₀) for select metabolites. For example, significant (at α = 0.05) increases in alanine, lysine and arginine, along with a significant decrease in maltose relative to the control were observed at exposures ≥ 0.1 mg/cm² (Figs 2-4A-C and 2-4F). The percent increase in isoleucine levels relative to the control was significant (α = 0.05) at all exposure levels (Fig. 2-4E). The magnitude of the
Figure 2-2. Loadings plot for PC1 and PC2 showing the metabolites that were major contributors to the separation observed in the PCA scores plot. The abscissa refers to the $^1$H NMR chemical shifts (ppm). (A) 0.025 mg/cm$^2$ (1/64th of LC$_{50}$), (B) 0.05 mg/cm$^2$ (1/32th of LC$_{50}$), (C) 0.10 mg/cm$^2$ (1/16th of LC$_{50}$), (D) 0.20 mg/cm$^2$ (1/8th of LC$_{50}$), (E) 0.40 mg/cm$^2$ (1/4th of LC$_{50}$), and (F) 0.80 mg/cm$^2$ (1/2 of LC$_{50}$).
Figure 2-3. T-test filtered \(^1\)H NMR difference spectra of *E. fetida* aqueous buffer tissue extracts obtained by subtracting the mean buckets of the control worms from the mean buckets for each phenanthrene exposure concentration and retaining the buckets that were statistically different from the controls at \(\alpha = 0.05\). (A) 0.025 mg/cm\(^2\) (1/64th of LC\(_{50}\)), (B) 0.05 mg/cm\(^2\) (1/32th of LC\(_{50}\)), (C) 0.10 mg/cm\(^2\) (1/16th of LC\(_{50}\)), (D) 0.20 mg/cm\(^2\) (1/8th of LC\(_{50}\)), (E) 0.40 mg/cm\(^2\) (1/4th of LC\(_{50}\)), and (F) 0.80 mg/cm\(^2\) (1/2 of LC\(_{50}\)).
percent changes in alanine, lysine, arginine, maltose and the energy molecule adenosine triphosphate (ATP), all increase with exposure, indicating concentration-dependent responses to phenanthrene exposure (Fig. 2-4) [117, 161]. However, the levels of the amino acids and maltose began to plateau at exposures ≥ 0.1 mg/cm². Brown et al. [117] also observed significant increases in amino acid levels and significant decreases in maltose relative to the controls at high phenanthrene exposures in soil. The consistency in the response of *E. fetida* to phenanthrene exposure in soil and in contact tests shows that contact tests are useful good for fast contaminant-exposure studies that may be representative of exposure responses observed in soil [117].

The observed amino acid increases may be attributed to an onset of protein catabolism that could have been triggered to meet the increased energy requirement in an attempt to counteract phenanthrene toxicity or a complete halt in amino acid breakdown [230]. Brown et al. [113] showed that maltose concentrations increased (but not statistically significant) in earthworms exposed to phenanthrene concentrations of 0.05 and 0.1 mg/cm² in contact tests. However, the variation in maltose concentration was high (i.e.: large standard deviations). The large variation in maltose was likely due to inherent natural variation in the earthworms and potential errors from using DSS as an internal standard [113]. In this study, the intensity of the maltose peak decreased significantly (at α = 0.05) for phenanthrene exposures ≥ 0.1 mg/cm² (Fig. 2-4F), which may be due to an increase in glycolysis to fulfill the energy needs of the cells [117, 230]. Increase in glycolysis also results in an increase in ATP production (Fig. 2-4G). However, a significant accumulation of ATP at exposures ≥ 0.05 mg/cm² also suggests that even though ATP is produced to meet the higher energy requirements, the mode that utilizes ATP may
Figure 2-4. Percent (%) change in selected metabolites of phenanthrene exposed *E. fetida* compared with the control worms. (A) Alanine, (B) Lysine, (C) Arginine, (D) Leucine, (E) Isoleucine, (F) Maltose, (G) ATP, (H) Betaine. The % changes in the intensity of metabolite peaks of exposed worms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly different from the control are labeled with an asterisk (*; at α =0.05). The percent changes are shown with their associated standard error.
be compromised due to phenanthrene exposure (Fig. 2-4G). The lack of efficiently utilizing ATP contributes to the plateau observed in the response, because energy is no longer available.

Chemical toxicity may either depress or stimulate metabolic activity [230-231]. Depression of metabolic activity has been correlated to a decrease in alanine, leucine and isoleucine levels in the marine mussel *Mytilus edulis*, which was exposed to lindane [230-231]. In this study, *E. fetida* exposure to phenanthrene showed a general increase in amino acids. Betaine, an osmolyte that is produced when organisms are exposed to conditions of drought, high salinity or temperature stress, also decreases as metabolism slows down [77, 230-231]. Betaine stabilizes cellular metabolic functions under varying conditions of stress by enhancing the water retention of cells and by replacing inorganic salts [232-234]. Betaine significantly (at α = 0.05) increased at the lowest exposure of 0.025 mg/cm² (Figs 2-3A and 2-4H). However, the increase in betaine at exposures > 0.025 mg/cm² was not significant (Fig. 2-4H). Therefore, the increases in amino acids and betaine levels suggests that there is stimulation in the metabolic activity of *E. fetida* on account of increasing phenanthrene exposure, however this plateaus as the exposure increases above 0.1 mg/cm² or 1/16 th of the LC₅₀.

PLS regression analysis was also used to further ascertain the relationships between metabolic responses and phenanthrene exposure (Fig. 2-5A) [147, 235-239]. The PLS scores plot shows distinct regions for high (0.8, 0.4 and 0.2 mg/cm²), medium (0.1 mg/cm²), and low (0.05 and 0.025 mg/cm²) exposure levels (Fig. 2-5A). The low phenanthrene exposure level clusters near the controls whereas the higher exposure levels are further away also suggesting a concentration-dependent response. A trajectory of responses to exposure of a chemical can signify its MOA, since exposure to chemicals with varying toxicities leads to trajectories that differ in their geometries or the overall shape of the curve [77, 217, 240-242]. The trajectory of
Figure 2-5. Multivariate statistical analysis showing (A) PLS scores plot of T1 (first PLS component) versus T2 (second PLS component) for $^1$H NMR spectra of *E. fetida* aqueous buffer tissue extracts. The mean scores (with associated standard error) were obtained by averaging the scores of each earthworm class. The ellipses that separate the high, middle and low exposure classes were constructed as visual aids. The exposure response trajectory is highlighted by a dashed line. (B) PLS scores plot of T1 versus U1. U1 represents the scores (first component) which describe the phenanthrene exposure concentrations. T1 represents the scores (first component) that describe the metabolic response of *E. fetida* obtained from the $^1$H NMR spectra. The mean T/U scores were obtained by taking the average of the scores for the worms belonging to each class. The mean T/U scores are shown with their associated standard error.
the metabolic profile of *E. fetida* is shown in Figure 2-5A. The metabolic response to phenanthrene exposure increases profoundly from 0.1 mg/cm$^2$ to 0.05 mg/cm$^2$ (large shift along T1 which explains 94% of the metabolic variation). However, increasing the exposure concentration beyond 0.1 mg/cm$^2$ alters the position only along T2 (explains only 4% of the metabolic variation). This suggests that at exposures $\geq 0.1$ mg/cm$^2$, there is little change in the intensity of the metabolic response. The PLS analysis also suggests a two-phased MOA for phenanthrene in *E. fetida* centered on the exposure of 0.1 mg/cm$^2$. This agrees with the results from the difference spectra and the percent change in metabolites, which also show that the metabolic response plateaus at exposures $\geq 0.1$ mg/cm$^2$ (Figs 2-3 and 2-4). The PLS T1/U1 scores plot, which is used to delineate any correlations between the metabolic response and exposure concentration [147, 235, 243], shows that there is a positive linear correlation ($r^2=0.99$) between exposure level and the metabolic profile that spans from the control to an exposure concentration of 0.1 mg/cm$^2$ (Fig. 2-5B). However, including exposure concentrations $>0.1$ mg/cm$^2$ results in an overall non-linear correlation ($r^2=0.41$) between exposure level and the metabolic profile. Therefore, the difference $^1$H NMR spectra, percent changes in metabolites and PLS analysis indicates that phenanthrene exposure of 0.1 mg/cm$^2$ is a critical concentration for *E. fetida* in contact tests. The MOA at the lower concentrations, which were 1/64$^{th}$ and 1/32$^{nd}$ fraction of the LC$_{50}$, may change to a more potent phase at exposures higher than the critical concentration of 0.1 mg/cm$^2$ and remains in that state as the exposure concentration increases towards the LC$_{50}$. Similar concentration-dependent behavior was also observed in *E. fetida* and other earthworm species exposed to PAHs where the concentration of cytochrome (Cyt) P450 was being monitored [190, 244-245]. Exposure to xenobiotics results in an increased expression of Cyt P450 enzymes, allowing the monitoring of total Cyt P450 content to be a biomarker for
exposure of xenobiotics to organisms [187, 190, 220]. PAHs have generally been shown to induce the Cyt P4501a isoenzyme in many species [187, 220, 246-247]. Zhang et al. [190] investigated the concentration-dependent behavior of *E. fetida* to pyrene and benzo[a]pyrene by monitoring the total Cyt P450 content. Total Cyt P450 content did not display any consistent correlation with PAH exposure and it was concluded that this observation was made because at certain concentrations, PAHs act as inducers of specific Cyt P450 isoenzymes while being an inhibitor for others. This may explain the observation of a MOA with two distinct phases in *E. fetida* to phenanthrene exposure. At concentrations ≥ 0.1 mg/cm² phenanthrene may act as an inhibitor for specific Cyt P450 isoenzymes. This would compromise the earthworm’s ability to combat the toxic MOA of phenanthrene and the chances for mortality are increased. This trend culminates with almost 50% mortality at the LC₅₀. The inconsistency in leucine levels, which contributed to the separation observed in the PCA scores plots, but did not increase significantly (at α=0.05) may also be partially attributed to the initiation in Cyt P450 expression (Figs 2-3 and 2-4D). Leucine, which comprises close to 15% of Cyt P450’s amino acid composition, is the major amino acid in its makeup [248]. Therefore, initiation in Cyt P450 production in exposed worms may have resulted in the observed fluctuations of free leucine levels in *E. fetida* after phenanthrene exposure. However, as we did not measure Cyt P450 levels directly, future studies are needed to confirm the observed MOA within the context of Cyt P450 activity.

2.5 Conclusion

This study further indicates that ¹H NMR-based metabolomics is able to detect earthworm responses to sub-lethal concentrations of phenanthrene and also suggests that earthworm responses are correlated to exposure concentration [117, 161]. Furthermore, in
addition to being able to detect earthworm responses over a wide range of ultra-low sub-lethal phenanthrene concentrations, metabolomics also has the potential to delineate the MOA of the contaminant in earthworms. Two phases of the toxic MOA were observed for *E. fetida* in response to phenanthrene exposure. At exposures below $1/16^{th}$ of the LC$_{50}$ phase I of the toxic MOA is in action, highlighted by a linear correlation between the metabolic response and the exposure concentration. At exposures $\geq 1/16^{th}$ of the LC$_{50}$, metabolic response to phenanthrene appeared to plateau, indicating the operation of a distinct phase II in the MOA. As in previous studies [117, 161], amino acids such as alanine, arginine, isoleucine and lysine and the sugar maltose emerged as potential response indicators of phenanthrene exposure. The consistency between the response of *E. fetida* to phenanthrene exposure in both contact and soil exposure studies suggests that even though contact test may not fully represent the soil environment, they provide a rapid and reliable screening method for potential measuring soil exposure responses. Therefore, the results of this study show that NMR-based earthworm metabolomics holds great potential for development as a routine tool in the ecotoxicological assessment of low and sub-lethal levels of contaminants in the environment.

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Chapter Three

$^1$H NMR-based metabolomics of time-dependent responses of *Eisenia fetida* to sub-lethal phenanthrene exposure


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3.1 Abstract

$^1$H NMR-based metabolomics was used to examine the response of the earthworm *Eisenia fetida* after exposure to sub-lethal concentrations of phenanthrene over time. Earthworms were exposed to 0.025 mg/cm$^2$ of phenanthrene ($1/64^{th}$ of the LC$_{50}$; the concentration that causes 50% mortality in the population) via contact tests over four days. Earthworm tissues were extracted using a mixture of chloroform, methanol and water, resulting in polar and non-polar fractions that were analyzed by $^1$H NMR after one, two, three and four days. NMR-based metabolomic analyses revealed heightened *E. fetida* responses with longer phenanthrene exposure times. Amino acids alanine and glutamate, the sugar maltose, the lipids cholesterol and phosphatidylcholine emerged as potential indicators of phenanthrene exposure. The conversion of succinate to fumarate in the Krebs cycle was also interrupted by phenanthrene. Therefore, this study shows that NMR-based metabolomics is a powerful tool for elucidating time-dependent relationships in addition to the mode of toxicity of phenanthrene in earthworm exposure studies.
3.2 Introduction

$^1$H nuclear magnetic resonance (NMR) -based metabolomics has gained in popularity over the past decade as a powerful tool for measuring organism responses to various environmental stressors, such as starvation, temperature and contaminant exposure, in both the aqueous and terrestrial environments [77, 152, 213, 229-230]. Non-targeted metabolomics involves measuring the fluctuations in the levels of low molecular weight endogenous metabolites such as sugars and amino acids in response to a defined external stressor [90, 118, 152]. In terrestrial systems, earthworms are commonly used as model organisms to monitor soil toxicity because they are directly exposed to soil contaminants through ingestion and passive absorption [155, 211-212, 219]. NMR-based earthworm metabolomics has shown great promise as a high throughput and reliable technique that can be used to analyze the biochemical responses after sub-lethal contaminant exposure [86, 92, 113, 144, 152, 229]. NMR-based metabolomics of organisms to sub-lethal or very low concentrations of contaminants has also allowed the elucidation of the contaminants mode of action (MOA) and also the identification of potential indicators (or biomarkers) of exposure [76-77, 113, 117, 217]. Increases or decreases in amino acids such as alanine, leucine and glutamate, the sugar maltose, the osmolyte betaine, and lipids such as fatty acids have been observed in organisms exposed to contaminants [76-77, 86, 90, 113, 117, 162]. NMR-based metabolomic studies have shown that exposure of earthworms to hydrophobic organic contaminants (HOCs) results in a heightened energy metabolism signaled by an increase in amino acids due to protein breakdown and a decrease in the sugar maltose [90, 92, 144]. Brown et al. [117] and Lankadurai et al. [249] showed that sub-lethal exposure of the earthworm Eisenia fetida (E. fetida) to the model polycyclic aromatic hydrocarbon (PAH), phenanthrene, resulted in concentration-dependent responses. Lankadurai
et al. [249] also showed that a two-phased MOA was observed due to phenanthrene exposure: phase I (at low exposure concentrations) showed a linear correlation between exposure concentration and the metabolic response, while phase II (at high exposure concentrations) showed that the metabolic responses reached a plateau. Saturation of the cytochrome (Cyt) P450 isoenzymes (the enzymes responsible for the metabolism of HOCs such as phenanthrene) at high phenanthrene concentrations was hypothesized as a possible explanation for the observed plateau in the metabolic responses in phase II of the MOA. Therefore, these prior studies have shown that NMR-based metabolomics is able to contribute to understanding the MOA of phenanthrene in *E. Fetida*. However, further studies are required to build on the existing information to obtain a better understanding of the MOA of phenanthrene.

Analysis of the fluctuations in the metabolic response with variation in exposure time has provided valuable information regarding the MOA of contaminants in organisms [76-77, 240]. In addition, establishing time-dependent relationships between the metabolic response and contaminant exposure is vital in developing NMR-based earthworm metabolomics as a routine tool in ecotoxicological assessment of PAHs, such as phenanthrene, in the environment [219]. For example, McKelvie et al. [118] showed that NMR-based metabolomics revealed potential time-dependent relationships due to exposure of *E. fetida* to phenanthrene in soil but the precise nature of the relationship between exposure time and metabolic response could not be examined in detail due to the complexity of the observed responses. Therefore in this study, $^1$H NMR-based metabolomics is used in combination with principal component analysis (PCA) to further investigate the toxic MOA of phenanthrene in the earthworm *E. fetida* by exploring the time-dependent relationships between the metabolic response and phenanthrene exposure in the absence of soil. *E. fetida* earthworms were exposed to a sub-lethal phenanthrene concentration
of 0.025 mg/cm² or 1/64th of the LC₅₀ (the concentration that causes 50% mortality) via contact tests with exposure times of one, two, three and four days which represents phase I of the phenanthrene MOA after two days of exposure [249]. Our first objective was to determine if NMR-based metabolomics is able to detect the heightened energy metabolism even at this ultra-low exposure concentration, secondly determine which cycles of the energy metabolism (as reported by Brown et al. [117] and Lankadurai et al. [249]) are potentially influenced by the toxic MOA of phenanthrene and finally confirm that fluctuations in previously observed metabolites namely alanine, lysine, arginine and maltose, are consistently observed over time [113, 117-118, 249]. The heightened energy metabolism may also decrease fatty acid levels, as they are also an important source of energy [250]. However, previous NMR-based earthworm metabolomic studies focused mainly on the polar metabolites such as sugars and amino acids obtained by the aqueous buffer extraction of earthworm tissues [90, 113, 117, 144, 229]. Lipophilic contaminants such as polycyclic aromatic hydrocarbons (PAHs) have also been shown to bind to biological membranes and thereby have the potential to influence the non-polar metabolic profile as well [251-252]. Consequently, we also target non-polar metabolites in addition to the conventional approach of measuring polar metabolites to obtain a holistic understanding of the biochemical changes to earthworms after contaminant exposure. Therefore, both polar and non-polar metabolic profiles were monitored in this study to further build upon previous results and gain further insight into the MOA of phenanthrene in E. fetida earthworms [113, 117-118, 249].
3.3 Experimental Methods

3.3.1 Earthworm contact tests and tissue extractions

Mature earthworms were depurated in the dark for 96 hours to empty their intestinal tracts (Supplementary Material, Section S3-1) [137]. Then the earthworms were exposed to 0.025 mg/cm\(^2\) of phenanthrene, which was 1/64\(^{th}\) of the 48-hour phenanthrene LC\(_{50}\) for *E. fetida* in contact tests [249]. Filter papers were placed in amber glass jars and then phenanthrene solutions (1 ml in chloroform) were applied to filter papers. For unexposed earthworms (control set), only chloroform was added to filter papers (without phenanthrene) [113]. The chloroform was allowed to evaporate and 1 ml of distilled water was added prior to addition of earthworms. The glass jars were kept in the dark during the exposure period as recommended by the OECD contact test guidelines [159]. Ten earthworms were used for each treatment (phenanthrene exposure and control treatments) and were isolated after 1, 2, 3 and 4 days. However, only six earthworms were analyzed after four days of exposure in the phenanthrene exposed treatment because four of the earthworms died. At each time point, earthworms were flash-frozen in liquid nitrogen, lyophilized and stored frozen until extraction [92, 137].

The lyophilized earthworms were homogenized while on ice in 1.5 ml centrifuge tubes using a 5 mm wide stainless steel spatula [92]. The homogenized earthworm tissue was then extracted using the two step methanol:water:chloroform (2:2:1.8 final solvent ratio) tissue extraction protocol developed by Wu et al. [93]. Ice-cold methanol (4 ml/g) and water (0.85 ml/g) were added to the tissue and vortexed for 15 seconds using a VX 100 vortexer (Labnet, NJ, USA). Chloroform (4 ml/g) and water (2 ml/g) were then added and vortexed for 60 seconds. The samples were then kept in ice for 10 minutes to allow partitioning between the polar and non-polar layers. The tissue mixture was centrifuged for 10 minutes at 12, 000 rpm (~11, 000 g).
using an International Equipment Company 21000 centrifuge (Fisher Scientific). The upper polar layer and the bottom non-polar layer were removed carefully into a 1.5 ml centrifuge tube and a 1.8 ml glass vial respectively. The samples were then dried under a constant nitrogen flow. The non-polar fraction was then reconstituted in 750 μl of chloroform-d:methanol-d4 (2:1), which contained 0.02% (v/v) tetramethylsilane (TMS; Cambridge Isotope Laboratories Inc) as an internal standard. The polar fraction was reconstituted in 750 μl of a 0.2 M monobasic sodium phosphate buffer solution (NaH₂PO₄·2H₂O; 99.3%; Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity; Sigma Aldrich) as a preservative [137]. Buffer solution was made with D₂O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D2O; Cambridge Isotope Laboratories Inc). The buffer solution also contained 10 mg/l of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal standard [113, 117]. The non-polar fraction and polar fraction were vortexed for 30 seconds and then centrifuged at 12,000 rpm (~11,000 g) for 10 minutes and the supernatant was transferred into a new 1.5 ml centrifuge tube. Samples were then transferred into 5 mm High Throughput plus NMR tubes (Norell Inc.; NJ, USA) for ¹H-NMR analysis.

3.3.2 ¹H NMR spectroscopy

¹H NMR spectra of the earthworm extracts were acquired with a Bruker Avance 500 MHz spectrometer using a ¹H-¹⁹F-¹⁵N-¹³C 5 mm Quadruple Resonance Inverse (QXI) probe fitted with an actively shielded Z gradient. ¹H NMR experiments were performed on the polar fraction using Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression and 128 scans, a recycle delay of 3 s, and 16 K time domain points [130]. ¹H NMR experiments were performed on the non-polar fraction using a standard single pulse sequence and 64 scans, a
recycle delay of 2 s, and 128 K time domain points. Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. All spectra were manually phased and calibrated. The spectra of the polar fraction were calibrated to the DSS internal reference methyl singlet, set to a chemical shift (δ) of 0.00 ppm. The spectra of the non-polar fraction were calibrated to the TMS internal reference methyl singlet, set to a chemical shift (δ) of 0.00 ppm.

3.3.3 Data and Statistical Analysis

Principal Component analysis (PCA) was performed on processed ¹H NMR data using the AMIX 3.8.4 (Bruker BioSpin, Rheinstetten, Germany) statistics tool to identify differences in the metabolic profiles of *E. fetida* following phenanthrene exposure. The ¹H NMR spectra were analyzed between δ of 0.5 and 10 ppm and divided into buckets 0.02 ppm wide, for a total of 475 buckets [135, 249]. The area between δ of 4.70–4.85 ppm was excluded to eliminate the small residual H₂O/HOD signals for the polar fraction. The area between δ of 4.28–4.8, 3.30-3.40 and 7.38–7.59 ppm were excluded in the non-polar fraction to eliminate the small residual H₂O/HOD, MeOH/MeOD and CHCl₃/CDCl₃ signals respectively [77]. The integration mode was set at the sum of intensities and the spectra were scaled to total intensity. This created a matrix in which each row represents an earthworm sample and each column contains the integrated area of the original spectral intensities contained within each bucket region. Individual PCA plots were constructed to compare the metabolic response of the control and exposed earthworms for each exposure level. Levene’s test was used to test for variance homogeneity among the PCA scores, which were found to have equal variances at α = 0.05 [253]. A t-test (two-tailed, equal variances) was also performed on the first and second component PCA scores to determine if
there was a significant difference between the scores of the controls and the exposed earthworms at $\alpha = 0.05$ [234]. Corresponding PCA loadings plots, which show the relative weight for each bucket, were also acquired for each of the PCA scores plots to identify the metabolites that were contributing to the separation between the scores of the control and exposed earthworms [90, 113, 117]. Average PCA scores plots were also constructed for both the polar and non-polar fractions to compare the control and exposed earthworms from all four days of exposure to deduce time-dependent relationships in the metabolic profile. The scores from the PCA scores plots were then imported into Microsoft Excel (version 12.0.6504, Microsoft Corporation, Redmond, WA) and were averaged per class (exposure time) and re-plotted with their associated standard errors.

Difference class $^1$H NMR spectra were constructed to identify metabolites that had significantly increased or decreased relative to the control [76-77, 249]. The buckets generated by AMIX 3.8.4 statistics tool, which represents the binned $^1$H NMR spectra of E. fetida extracts were then imported into Microsoft Excel. Levene’s test was used to test for variance homogeneity among the buckets, which were found to have equal variances at $\alpha = 0.05$ [253]. A t-test (two-tailed, equal variances) was then performed comparing the buckets of the controls with that of the exposure class to identify the buckets that were statistically different at $\alpha = 0.05$. Average class $^1$H NMR spectra were obtained by averaging the buckets of each exposure class. Difference class $^1$H NMR spectra were then obtained by subtracting the buckets of the average controls from that of the average exposure class for each day. The buckets representing the peaks of metabolites that weren’t statistically significant from the controls were then replaced with a zero resulting in a t-test filtered $^1$H NMR difference spectra [76-77]. The buckets were then imported into Origin 7 (version 7.0383, OriginLab Corporation, Northampton, MA) to plot
the difference spectra. The percent changes in the intensity of metabolite peaks of exposed earthworms relative to the control were obtained by dividing the buckets that pertain to the metabolites in the exposed by the corresponding buckets in the control for each day. The metabolite peaks were identified using a database of the $^1$H NMR spectra of a series of standard metabolites that were previously identified in *E. fetida* [92, 137].

### 3.4 Results and Discussions

#### 3.4.1 Principal Component Analysis of $^1$H NMR spectra

Average and individual PCA scores plots were constructed using the $^1$H NMR spectra of the polar and non-polar fractions of the *E. fetida* tissue extracts to compare the metabolic response of phenanthrene exposed earthworms to the control earthworms over time (Fig. 3-1 and Supplementary material, Figs S3-1 and S3-2) [90, 113, 254-255]. Both average and individual PCA scores plots of the polar fraction show that there is a significant time-dependent separation along PC1 between the scores of the control and exposed earthworms ($P = 3 \times 10^{-4}$ on day 1 to $P = 1 \times 10^{-6}$ on day 4; Figs 3-1A and S3-1). The time-dependent response of *E. fetida* to phenanthrene is also observed in the survival patterns of the earthworms: 40% of the exposed earthworms for four days died implying that prolonged exposures even at ultra-low sub-lethal levels can result in adverse alterations to the metabolism in earthworms. However, neither the average nor the individual PCA scores plots of the non-polar fraction show any significant separation between the phenanthrene exposed and control (unexposed) earthworms (Figs 3-1B and S3-2). This indicates that the polar metabolites in general seem to be more sensitive indicators of phenanthrene exposure at ultra-low sub-lethal concentrations. Both the polar and
Figure 3-1. Principal component analysis (PCA) scores plot of PC1 (first PCA component) versus PC2 (second PCA component) for mean 1H NMR spectra of the (A) polar and (B) non-polar fractions of *E. fetida* tissue extracts. C1, C2, C3 and C4 represent the mean scores for the control (unexposed) earthworms after days 1, 2, 3 and 4 of exposure respectively. E1, E2, E3 and E4 represent the mean scores for the phenanthrene exposed earthworms after days 1, 2, 3 and 4 of exposure respectively. The mean scores (with associated standard error) were obtained by averaging the scores of each earthworm class. The ellipses that group the controls and exposed classes were constructed as visual aids. The time-response trajectory is highlighted by a dashed line.
non-polar metabolic profiles have a distinct time-dependent trajectory relative to the controls (Fig. 3-1). Metabolic trajectories have a distinct geometry or overall shape based on the type of chemical an organism is being exposed to because depending on the mode of toxicity of the chemical the organism will exhibit a different biochemical response [76-77, 240]. Therefore, the trajectories for the polar and non-polar metabolites of the phenanthrene exposed earthworms may be used to estimate exposure time in future studies.

3.4.2 Metabolic changes after phenanthrene exposure

PCA loadings plots were obtained to identify the metabolites that contributed to the separation observed between the exposed and control earthworms in the PCA scores plots (Figs S3-3 and S3-4) [86, 90, 113, 117]. T-test filtered $^1$H NMR difference spectra were also constructed to determine which metabolites increased or decreased significantly (at $\alpha$=0.05) relative to the control [76-77]. Percent changes in selected polar and non-polar metabolites in phenanthrene exposed earthworms relative to the controls were also obtained to determine their relationship to exposure time (Figs 3-2 and 3-3) [117, 249]. The sugars maltose ($\delta$ 5.41 ppm) and sucrose ($\delta$ 5.23 ppm), Krebs cycle intermediates succinate ($\delta$ 2.39 ppm), fumarate ($\delta$ 6.51 ppm) and malate ($\delta$ 2.37 ppm), amino acids alanine ($\delta$ 1.47 ppm) and glutamate ($\delta$ 2.35 ppm), messenger molecules scyllo-inositol ($\delta$ 3.35 ppm) and myo-inositol ($\delta$ 4.05 ppm), osmolyte betaine ($\delta$ 3.25 ppm), energy molecule ATP ($\delta$ 8.23 ppm) and short-chain fatty acids ($\delta$ 1.27 ppm for CH$_2$ and $\delta$ 0.83 ppm for CH$_3$) were identified as the polar metabolites whose concentrations significantly changed ( $\alpha$=0.05) relative to the control during the course of the exposure period (Figs S3-3 and S3-5). Phosphatidylcholine ($\delta$ 3.21 ppm), cholesterol ($\delta$ 0.69 ppm) and long-chain fatty acids ($\delta$ 0.87 ppm for CH$_3$, $\delta$ 1.63 ppm for CH$_2$-CH$_2$COOH and $\delta$ 2.33 ppm for CH$_3$-
Figure 3.2. Percent (%) change in selected metabolites of the polar fraction of phenanthrene exposed *E. fetida* tissue extracts compared with the control earthworms. (A) Alanine, (B) Glutamate, (C) Leucine, (D) Arginine, (E) Lysine, (F) Phenylalanine, (G) Maltose, (H) Malate, (I) Fumarate, (J) Succinate, (K) Betaine, (L) scyllo-Inositol, (M) myo-inositol, (N)–CH$_2$ of short chain fatty acids, and (O) Adenosine triphosphate (ATP). The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms and then dividing again by the buckets in the control earthworms [76–77]. The percent changes that were significantly different from the control (based on a t-test of control vs. exposed for each day) are labeled with an asterisk (*; at $\alpha$ =0.05). The percent changes are shown with their associated standard error.
COOH) were identified as the non-polar metabolites whose concentrations significantly changed ($\alpha=0.05$) relative to the control during the course of the exposure period (Figs S3-4 and S3-6).

Examining the fluctuations in the levels of metabolites in response to external stressors can provide insight into the mechanism of action (MOA) of the stressor as well as identify possible biomarkers for the stressor [77, 86, 217, 249]. Succinate, fumarate and malate are part of the Krebs cycle, which is an integral pathway in energy metabolism [250]. The time-dependent profiles of succinate, fumarate and malate are very similar to each other in unexposed (control) earthworms, which are only under the stress of starvation (Fig. S3-7). This is likely because succinate, fumarate and malate are produced in subsequent steps of the Krebs cycle (Fig. S3-8). However, in phenanthrene exposed earthworms the time-dependent profiles of fumarate and malate vary from succinate (Figs 3-2H-J). Succinate begins to increase after the third day of exposure (Fig. 3-2J), whereas malate and fumarate begin to decrease significantly after the second day of exposure (Figs 3-2H and 3-2I). This may be due to an inactivation of the succinate dehydrogenase enzyme complex in exposed earthworms (Fig. S3-8) [250]. The succinate dehydrogenase enzyme complex, which catalyzes the conversion of succinate to fumarate, is the only enzyme of the Krebs cycle that is membrane bound [250]. Phenanthrene has been shown to decrease membrane stability and alter membrane fluidity by binding to biological membranes [219, 252, 256]. Parrish et al. [257] showed that binding of aromatic hydrocarbons to mitochondrial membranes disrupted the regular functioning of succinate dehydrogenase. Therefore, phenanthrene binding to the inner mitochondrial membrane may have lead to an inactivation of the enzyme, resulting in a decrease in fumarate and malate because succinate dehydrogenase could not catalyze the conversion of succinate to fumarate (Fig. S3-8). The fluctuations in the levels of the sugars, fats and amino acids in this study
indicates an increase in the rate of energy metabolism in phenanthrene exposed earthworms relative to the controls as was observed previously in studies that exposed *E. fetida* to phenanthrene (Figs 3-2 and 3-3) [117, 249]. The significant decrease in maltose levels in phenanthrene exposed earthworms relative to controls can signify an increased need for glucose (Fig. 3-2G). Maltose is broken into two glucose molecules that is then used by glycolysis to produce energy [250]. The resulting depletion of glucose will result in an increase in gluconeogenesis, especially for glucose-dependent tissues [117, 144, 250]. Fatty acid oxidation also increases due to stress because it generates energy [250, 258]. The significant increase in the levels of the short-chain fatty acids (extracted in the polar fraction) relative to the controls after the first day of exposure (Fig. 3-2N) may be due to the breakdown of the longer chain fatty acids by β-oxidation [250]. As fatty acids get used up mobilization of more fatty acids from their storage in adipose tissue ensues. Due to their lipophilic nature, fatty acids are transported in the blood within special vesicles called lipoproteins [250, 259]. Cholesterol and phosphatidylcholine (a type of phospholipid), both of which are used in the construction of lipoprotein vesicles, show significant increases after the second day of exposure, suggesting increased lipoprotein vesicle formation (Figs. 3-3A and 3-3D). The long-chain fatty acids (extracted in the non-polar fraction), which being delivered to target cells by the lipoprotein vesicles, show significant decrease after the third and fourth day of exposure, suggesting increased oxidation (Figs. 3-3C). The sustained demand for more energy will lead to protein catabolism, which results in an increase in free amino acid levels [113, 144, 260]. However, the percent changes in the amino acids leucine, arginine, lysine and phenylalanine do not increase or decrease significantly (at α=0.05) relative to the controls, which is consistent with what Lankadurai et al. [249] observed with the same exposure concentration for *E. fetida* after two
Figure 3-3. Percent (%) change in selected metabolites of the non-polar fraction of phenanthrene exposed *E. fetida* tissue extracts compared with the control earthworms. (A) Cholesterol, (B) Fatty acid-(CH$_2$)$_n$, (C) Fatty acid-CH$_3$, and (D) Phosphatidylcholine. The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly different (based on a t-test of control vs. exposed for each day) from the control are labeled with an asterisk (*; at $\alpha =0.05$). The percent changes are shown with their associated standard error.
days of exposure (Fig. 3-2A and Figs 3-2D-F). The percent changes of all four amino acids have very similar profiles to each other as a function of time; they do not show any noticeable changes until after the third day of exposure when there is a spike in their levels. Whitfield Åslund et al. [108] showed that the levels of leucine, lysine and phenylalanine were highly correlated with each other in control *E. fetida* earthworms. This may explain the observation of similar time-dependent profiles in the fluctuation of these amino acids in exposed earthworms relative to the controls. Continuation of protein catabolism is usually followed by death. Interestingly, the spike in the amino acid levels after three days of exposure was followed by the death of forty percent of the exposed earthworms after four days of exposure [250]. However, the amino acid levels in the earthworms that survived after the four days of exposure did not show any significant (at $\alpha=0.05$) changes relative to the controls suggesting that at the current exposure level these amino acids may not be reliable indicators for phenanthrene exposure. However, it will be interesting to analyze their changes as a function of time at higher exposure levels because they have been shown to increase significantly at higher exposures [249].

The amino acid alanine is considered as the universal stress signal, because it increases significantly in response to many kinds of stress and may be of use as a potential metabolite biomarker of stress in *E. fetida* due to phenanthrene exposure [113, 260-262]. The exact mechanism responsible for the increase in alanine is currently unknown, however it has been linked to an increase in gluconeogenesis during times of stress because alanine is one of its major substrates [261, 263-264]. Alanine levels had a positive linear correlation with exposure time in phenanthrene exposed earthworms in this study ($R=0.70, P <0.0001$; Fig. S3-9). Alanine levels were also increased significantly ($\alpha = 0.05$) relative to the controls after all four days of exposure, suggesting that phenanthrene exposure was responsible for the observed spike in
alanine levels (Fig. 3-2B). The percent change in alanine in the exposed earthworms relative to controls increased from about 70% after the first day of exposure to almost 220% after the fourth day of exposure (Fig. 3-2B). Since alanine levels are positively correlated to the time-dependent stress induced by phenanthrene it can be argued that high alanine levels (> 220% increase over controls) can indicate stress levels that might potentially be lethal. Glutamate levels showed significant decreases relative to the controls after the second and third days of exposure, which was in contrast to the other amino acids, which showed a general increase due to phenanthrene exposure (Fig. 3-2C). This is consistent with Whitfield Åslund et al. [108]’s finding of no correlation in control *E. fetida*, between glutamate levels and the levels of alanine, leucine, lysine and phenylalanine. However, they showed glutamate to be positively correlated to maltose, which may explain the percent decrease in glutamate as maltose also decreased in exposed earthworms (Figs 3-2C and 3-2D).

Betaine, an osmolyte [232-233], significantly increased relative to the controls after the second and fourth days of exposure (Fig. 3-2K). Inositols also function as osmoregulators [265-266]. *Scyillo* and *myo* - inositol show significant increases in phenanthrene exposed earthworms after the first and second day of exposure relative to the controls (Figs 3-2L and 3-2M). As previously mentioned, binding of phenanthrene to biological membranes decreases membrane stability and also alters its fluidity leading to unregulated movement of molecules across the cell membranes [251-252, 257]. Increases in the production of organic osmolytes such as betaine and inositol help cells maintain their osmotic balance by offsetting any changes brought about by the unregulated movement of molecules either into or out of the cell [233, 267]. Inositols also form the structural basis for two important secondary messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol, which are involved in the transduction of extracellular signals [250,
The increase in the inositols can be also due to an increased production of the secondary messengers IP$_3$ and diacylglycerol in response to phenanthrene exposure. Therefore, the increase in inositol concentrations may also be as a result of the cell-signaling pathways.

3.5 Conclusion

Our study has shown that $^1$H NMR-based metabolomics is capable of delineating time-response relationships for the exposure of E. fetida to phenanthrene even at ultra-low sub-lethal concentrations. A heightened energy metabolism was observed as in previous studies [117, 249]. We applied the biphasic extraction technique developed by Wu et al. [93] for E. fetida and showed that it was a reliable and reproducible tissue extraction method of polar and non-polar metabolites for NMR-based metabolomics. The non-polar metabolic profile of E. fetida was analyzed for the first time to our knowledge by $^1$H NMR metabolomics in this study. The heightened energy demand was also shown to significantly influence the levels of some of the non-polar metabolites such as fatty acids. However, the overall non-polar metabolic profile did not change significantly with time, suggesting that polar metabolites are more sensitive indicators of phenanthrene exposure at ultra-low concentrations. Our study also provided evidence for the possible interruption of the Krebs cycle by the MOA of phenanthrene due to an inactivation of the succinate dehydrogenase enzyme complex. Metabolites such as alanine, arginine, lysine and maltose were also identified in this study as potential indicators of phenanthrene exposure and is consistent with previous studies [117, 144, 249]. Therefore, the results of this study indicate that NMR-based earthworm metabolomics has great potential to
emerge as a powerful tool for determining the MOA of chemicals in organisms and also in routine ecotoxicological assessment of contaminated soils.

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Chapter Four

$^1$H NMR-based metabolomic analysis of polar and non-polar earthworm metabolites after sub-lethal exposure to phenanthrene


4.1 Abstract

$^1$H NMR-based metabolomics was utilized to elucidate the earthworm sub-lethal toxicity after exposure to the persistent environmental contaminant phenanthrene. Earthworms were exposed to 0.05, 0.2 and 0.4 mg/cm$^2$ of phenanthrene [which correspond to 1/32$^{\text{nd}}$ to 1/4$^{\text{th}}$ of the 48-hour LC$^{50}$ (concentration that causes 50% mortality) respectively] via contact tests over one, two and three days of dermal contact. $^1$H NMR-based metabolomic analysis of the polar and non-polar fractions of the earthworm tissue extracts revealed heightened *Eisenia fetida* toxic responses with both longer exposure times and higher phenanthrene concentrations. Principal component analysis (PCA) of the polar fraction showed significant separation between control and exposed earthworms along PC1 for all phenanthrene concentrations on each day. The PCA of the non-polar fraction showed significant separation between the controls and exposed earthworms for only the first day of exposure. These results suggested that alanine, glutamate, maltose, and fatty acids were potential indicators of phenanthrene exposure. Interruption in energy production due to a deactivation of the succinate dehydrogenase enzyme in the Krebs cycle was also postulated in exposed earthworms. Cross-validated partial least squares (PLS)-regression models showed that the polar metabolic profile of *E. fetida* was weakly but significantly correlated to phenanthrene exposure concentrations after day one and day two of exposure. Overall, this study indicates that with longer exposures, contact time becomes more important than concentration in discriminating between control and exposed earthworms. This study also shows that NMR-based metabolomics has promise as a powerful ecotoxicological tool for elucidating the mode of toxicity of contaminants.
4.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants [166-168], are highly persistent in the environment, and are known carcinogens and mutagens [168, 268]. Many studies have been conducted to determine the adverse effects of exposure to lethal concentrations of PAHs [269-271]. However, these studies have not been able to elucidate the toxic mode of action (MOA) in detail. Chemical exposure in the environment occurs mostly at sub-lethal or very low levels, which can also have adverse physiological responses in organisms [216, 272-273]. Sub-lethal or very low levels of contaminant exposure enable the monitoring of an organism’s endogenous metabolite flux (i.e. changes in amino acids and sugars). This in turn allows for the identification of potential endogenous metabolites as early indicators of response to contaminant exposure and also facilitates the elucidation of the chemical’s MOA [76-77, 217, 240]. Therefore, it is essential to develop reliable, rapid and reproducible analytical methods that are capable of assessing organism response to sub-lethal contaminant exposure.

$^1$H nuclear magnetic resonance (NMR)-based metabolomics is being utilized increasingly over the past decade as a powerful tool for measuring organism responses to various environmental stressors, including contaminant exposure, in both aqueous and terrestrial environments [77, 152, 229-230, 274]. Metabolomics involves measuring the fluctuations of low molecular weight endogenous metabolite levels, such as sugars and amino acids, in response to a defined external stressor [83, 275]. In terrestrial systems, earthworms are commonly used as model organisms to monitor soil toxicity because they are directly exposed to soil contaminants through ingestion and passive absorption [211-212, 219]. NMR-based earthworm metabolomics has shown great promise as a high throughput and reliable technique that can be used to elucidate
a contaminant’s mode of action (MOA) and also identify potential metabolite indicators (or biomarkers) of exposure in response to sub-lethal contaminant exposure [83, 86, 136, 144, 229, 276]. Increases or decreases in amino acids such as alanine, arginine and glutamate, the sugar maltose, the osmolyte betaine, and lipids such as fatty acids have been observed in organisms exposed to contaminants [77, 162, 230]. NMR-based metabolomic studies have shown that exposure of earthworms to hydrophobic organic contaminants (HOCs) results in a heightened energy metabolism signaled by an increase in amino acids due to protein breakdown, a decrease in the sugar maltose and a decrease in lipids such as fatty acids [92, 144, 275].

NMR-based earthworm metabolomics has also been able to delineate concentration-dependent relationships between metabolic response and exposure concentration to the model PAH phenanthrene [117, 249]. Lankadurai et al. [249] observed a two-phased MOA after phenanthrene exposure: phase I (at low exposure concentrations) exhibited a linear correlation between exposure concentration and the metabolic response, while phase II (at high exposure concentrations) showed that the metabolic responses reached a plateau. Saturation of the cytochrome (Cyt) P450 isoenzymes (the enzymes responsible for the metabolism of HOCs such as phenanthrene) at high phenanthrene concentrations was hypothesized as a possible explanation for the observed plateau in the metabolic responses in phase II of the MOA. However, the past two studies focused only on the polar metabolites of *E. fetida*. Hydrophobic organic contaminants such as PAHs have been shown to bind to biological membranes and disrupt membrane function and thereby, potentially alter the non-polar metabolic profile as well [251-252, 257]. Lankadurai et al.[277] measured time-dependent responses to ultra-low phenanthrene exposure concentrations (0.025 mg/cm² or 1/64th of the concentration that causes 50% mortality) by examining both the polar and non-polar metabolites but did not detect
significant overall changes in the non-polar metabolic profile. However, analysis of the polar metabolites suggested a potential deactivation of the succinate dehydrogenase enzyme, which catalyzes reactions for the Krebs cycle. These previous studies have shown that NMR-based metabolomics was able to contribute to understanding the MOA of phenanthrene in *E. Fetida*. However, further NMR-based metabolomic studies are required to build on the existing information to obtain a better understanding of the MOA of phenanthrene and to examine phenanthrene exposure responses to both non-polar and polar metabolites at a range of sub-lethal concentrations.

In this study, $^1$H NMR-based metabolomics was used to further investigate the toxic MOA of phenanthrene in the earthworm *E. fetida* by exploring both the time-dependent and concentration-dependent relationships between the metabolic response and phenanthrene exposure in the absence of soil. Establishment of time and concentration-dependent relationships have been proposed as critical criteria in developing regulatory soil quality assessments [219]. Determining the nature of the concentration-dependent relationship potentially allows for an estimation of the level of contamination based on the metabolic profile of exposed earthworms. Similarly, the characterization of the time-dependent relationship allows for an estimation of the length of the exposure. Therefore, it was essential to analyze the capability of NMR-based metabolomics in describing time and concentration-dependent relationships for both the polar and non-polar metabolites. This information will allow for a better gauge of the potential of NMR-based metabolomics as a routine tool in assessment of soil health. Both polar and non-polar metabolic profiles were analyzed to obtain a holistic understanding of *E. fetida* response to a range of sub-lethal phenanthrene exposure [86, 91]. *E. fetida* earthworms were exposed, via contact tests, to sub-lethal phenanthrene concentrations of
0.05, 0.2 and 0.4 mg/cm², which correspond to 1/32⁰⁻, 1/8⁰⁻ and 1/4⁰⁻ of the 48-hour LC₅₀, over exposure times of one, two, and three days [249]. This represents a range of concentrations which initiated either phase I (0.05 mg/cm²) or phase II of the phenanthrene MOA (0.2 and 0.4 mg/cm²) after two days of exposure [249]. Responses of *E. fetida* to phenanthrene exposure were observed to be consistent between both contact tests and soil exposure studies [117, 249]. Contact tests are therefore used in rapid contaminant-exposure studies, prior to soil exposure studies and can provide insight into toxic responses that may be observed in soil [113, 136, 162, 275-276]. Our first objective was to determine if NMR-based metabolomics was able to differentiate between the influences of exposure concentration and exposure time variables on the metabolic profile. Phenanthrene concentration and exposure time have been examined separately in past NMR-metabolomic studies [117-118, 249, 277]. However, determination of metabolite indicators that are unique to each variable will be very valuable in developing NMR-metabolomics as an early warning screening technique for soil toxicity. In addition, it was necessary to determine whether exposure time or phenanthrene concentration has a greater influence on the metabolic response of *E. fetida*. Consequently, we aimed to test previous observations of the toxic MOA of phenanthrene, such as a heightened energy metabolism and the potential disruption of the Krebs cycle due to inactivation of the succinate dehydrogenase enzyme. The use of non-polar metabolite analysis is rare in earthworm metabolomics [86, 91, 277]. Previous observations of higher sensitivity of the polar metabolites to phenanthrene exposure relative to the non-polar metabolites at an ultra-low concentration was significant considering the lipophilicity of phenanthrene and the known mechanisms of biological membrane disruptions by PAHs [277]. Therefore we also tested this observation at higher phenanthrene exposure concentrations. Finally, we monitored the response of *E. fetida* to
phenanthrene exposure to build upon previous results and gain further insight into the MOA of phenanthrene in *E. fetida* earthworms [113, 117-118, 249]. This study will contribute to the continued progression of NMR-based metabolomics as a reliable, rapid and routine ecotoxicological tool in assessing contaminant toxicity.

4.3 Experimental Methods

4.3.1 Earthworm contact tests and tissue extractions

Mature earthworms were depurated in the dark for 96 hours to empty their intestinal tracts (See Section S4-1 in Supplementary Material). The earthworms were then exposed to 0.05, 0.2 and 0.4 mg of phenanthrene per cm² on filter paper (Whatman GF/A 4.25 cm diameter circles, Fisher Scientific; mg/cm²), which corresponds to 1/32nd, 1/8th and 1/4th of the 48-hour phenanthrene LC₅₀ for *E. fetida* in predetermined contact tests [249]. The LC₅₀ reported was for a 2-day exposure test; all of the earthworms exposed to 0.2 and 0.4 mg/cm² of phenanthrene died after three days of exposure and one of the earthworms exposed to 0.05 mg/cm² of phenanthrene died after three days of exposure. However, since the metabolomic analyses presented here were performed on only earthworms that survived, the results are consistent with observations for sub-lethal phenanthrene exposure. Filter papers (Whatman GF/A 4.25 cm diameter circles, Fisher Scientific) were placed in amber glass jars. Phenanthrene solutions (1 ml in chloroform) were applied to filter papers and only chloroform was applied to filter papers for control (unexposed earthworms). The chloroform was allowed to evaporate and 1 ml of distilled water was added prior to addition of earthworms. The glass jars were kept in the dark during the exposure period as recommended by the OECD contact test guidelines [159]. The earthworms were exposed to phenanthrene for up to three days. Ten earthworms were used for each treatment (phenanthrene exposure and control treatments) and were isolated after one, two, and
three days. However, only nine earthworms survived after three days of phenanthrene exposure at 0.05 mg/cm$^2$ and none of the ten earthworms survived after three days of exposure to 0.2 and 0.4 mg/cm$^2$ phenanthrene concentrations. At each time point, earthworms were flash-frozen in liquid nitrogen, lyophilized and stored frozen until extraction [92, 137].

The lyophilized earthworms were homogenized while in ice in a 1.5 ml centrifuge tube using a 5 mm wide stainless steel spatula [92]. The homogenized earthworm tissue was then extracted using the two step methanol, water, chloroform tissue extraction protocol [93]. Ice-cold methanol (4 ml/g of earthworm dry weight) and ice cold water (0.85 ml/g of earthworm dry weight) were added to the tissue and vortexed for 15 seconds using a VX 100 vortexer (Labnet, NJ, USA). Chloroform (4 ml/g of earthworm dry weight) and water (2 ml/g of earthworm dry weight) were then added and vortexed for 60 seconds. The samples were then kept in ice for 10 minutes to allow partitioning between the polar and non-polar layers. The tissue mixture was centrifuged for 10 minutes at 12,000 rpm (~11,000 g) using an International Equipment Company 21000 centrifuge (Fisher Scientific). The upper polar layer and the bottom non-polar layer were removed carefully into a 1.5 ml centrifuge tube and a 1.8 ml glass vial respectively. The samples were then dried under a constant nitrogen flow. The non-polar fraction was then reconstituted in 750 μl of chloroform-d:methanol-d$_4$ (2:1), which contained 0.02% (v/v) tetramethylsilane (TMS; Cambridge Isotope Laboratories Inc) as an internal standard. The polar fraction was reconstituted in 750 μl of a 0.2 M monobasic sodium phosphate buffer solution (NaH$_2$PO$_4$:2H$_2$O; 99.3%; Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity; Sigma Aldrich) as a preservative [137]. Buffer solution was made with D$_2$O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D$_2$O; Cambridge Isotope Laboratories Inc). The buffer solution also contained 10 mg/l of 2,2-
dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal standard [113, 117]. The non-polar fraction and polar fraction were vortexed for 30 seconds and then centrifuged at 12,000 rpm (~11,000 g) for 10 minutes and the supernatant was transferred into a new 1.5 ml centrifuge tube. Samples were then transferred into 5 mm High Throughput\textsuperscript{plus} NMR tubes (Norell Inc.; NJ, USA) for \textsuperscript{1}H-NMR analysis.

4.3.2 \textsuperscript{1}H NMR spectroscopy

\textsuperscript{1}H NMR spectra of the earthworm extracts were acquired with a Bruker Avance 500 MHz spectrometer using a \textsuperscript{1}H-\textsuperscript{19}F-\textsuperscript{15}N-\textsuperscript{13}C 5 mm Quadruple Resonance Inverse (QXI) probe fitted with an actively shielded Z gradient. \textsuperscript{1}H NMR experiments were performed on the polar fraction using Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression and 128 scans, a recycle delay of 3 s, and 16 K time domain point [278]. \textsuperscript{1}H NMR experiments were performed on the non-polar fraction using a standard zg sequence and 64 scans, a recycle delay of 2 s, and 128 K time domain points. Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. All spectra were manually phased and calibrated consistently. The NMR spectra of the polar fraction were calibrated to the nine identical methyl protons of the trimethylsilyl group of the DSS internal reference (\(\delta=0.00\) ppm) and the NMR spectra of the organic fraction were calibrated to the twelve identical methyl protons of the tetramethylsilyl group of the TMS internal reference (\(\delta=0.00\) ppm).

4.3.3 Data and Statistical Analysis

The chemical range between 0.5 and 10 ppm represented all \textsuperscript{1}H NMR resonances in extracts and were divided into buckets that were 0.02 ppm in width using AMIX 3.8.4 statistics tool for a total of 475 buckets (Bruker BioSpin, Rheinstetten, Germany) [135]. The area between
4.70–4.85 ppm was excluded to eliminate the small residual H$_2$O/HOD signals for the polar fraction. The area between 4.28–4.8, 3.30–3.40 and 7.38–7.59 ppm were excluded in the non-polar fraction to eliminate the small residual H$_2$O/HOD, MeOH/MeOD and CHCl$_3$/CDCl$_3$ signals respectively [77]. The integration mode was set at the sum of intensities and the spectra were scaled to total intensity. This created a matrix in which each row represented an earthworm sample and each column contained the integrated area of the original spectral intensities contained within each bucket region. Individual principal component analysis (PCA) scores plots were constructed to compare the metabolic response of the control and exposed earthworms for each exposure treatment. Levene’s test was used to test for variance homogeneity among the PCA scores, which were found to have equal variances at $\alpha = 0.05$ [253]. A t-test (two-tailed, equal variances) was also performed on the PCA scores to determine if there was a significant difference between the scores of the controls and the exposed earthworms at $\alpha = 0.05$ [234]. Corresponding PCA loadings plots, which show the relative weight for each bucket, were also acquired for each of the PCA scores plots to identify the metabolites that were contributing to the separation between the scores of the control and exposed earthworms [113, 117, 275]. Average PCA scores plots were also constructed for both the polar and non-polar fractions to deduce time-dependent and concentration-dependent relationships in the metabolic profile. The scores from the PCA scores plots were then imported into Microsoft Excel (version 12.0.6504, Microsoft Corporation, Redmond, WA) and were averaged per class (exposure time) and re-plotted with their associated standard errors. PLS-regression analyses were also performed to determine the relationship between the metabolic profile and phenanthrene concentration after each day of exposure (see Section S4-2 in Supplementary Material).
Difference class $^1$H NMR spectra were constructed to identify metabolites that had significantly increased or decreased relative to the control [76-77, 249]. The buckets generated by AMIX 3.8.4 statistics tool, which represents the binned $^1$H NMR spectra of *E. fetida* extracts were then imported into Microsoft Excel. Levene’s test was used to test for variance homogeneity among the buckets, which were found to have equal variances at $\alpha = 0.05$ [253]. A t-test (two-tailed, equal variances) was then performed comparing the buckets of the controls with that of the exposure class to identify the buckets that were statistically different at $\alpha = 0.05$. Average $^1$H NMR spectra were obtained by averaging the buckets of each exposure concentration and control treatments separately for each day. Difference class $^1$H NMR spectra were then obtained by subtracting the buckets of the average controls from that of the average exposure treatments for each day. The buckets representing the peaks of metabolites that weren’t statistically significant from the controls were then replaced with a zero resulting in a t-test filtered $^1$H NMR difference spectra [76-77]. The buckets were then imported into Origin 7 (version 7.0383, OriginLab Corporation, Northampton, MA) to plot the difference $^1$H NMR spectra. The percent changes in the intensity of metabolite peaks of exposed earthworms relative to the control were obtained by dividing the buckets that pertain to the metabolites in the exposed by the corresponding buckets in the control for each day. The metabolite peaks in the $^1$H NMR spectra of the earthworm polar and non-polar extracts were identified by comparing to previously published assignments [76, 90-92, 137].
4.4 Results and Discussion

4.4.1 Multivariate statistical analysis

Individual and average PCA scores plots were constructed using the $^1$H NMR spectra of the polar and non-polar fractions of *E. fetida* tissue extracts to compare the metabolic response of phenanthrene exposed earthworms to the control earthworms (Fig. 4-1 and Supplementary Material, Figs S4-1 to S4-6) [113, 255, 275]. PCA scores plots were used to compare the time and concentration variables separately and also together (Fig. 4-1 and Supplementary Material, Figs. S4-3 to S4-6). Both the individual and average PCA scores plots for the polar fraction showed that there was significant separation (at $\alpha = 0.05$) between the scores of the control and phenanthrene exposed earthworms along PC1 even after one day of exposure for all three exposure concentrations (Fig. 4-1 and Supplementary Material, Fig. S4-1). This was consistent with Lankadurai et al.’s [277] findings with *E. fetida* polar extracts after one day of exposure to phenanthrene at a concentration of 0.025 mg/cm$^2$ ($1/64^{th}$ of the LC$_{50}$). The separation between the scores of the controls and exposed earthworms in the individual scores plots was significant (at $\alpha = 0.05$) only along PC1 after one day of exposure for all three exposure concentrations (Supplementary Material, Figs S4-1A, S4-1D and S4-1F). However, after the second day of exposure there were significant (at $\alpha = 0.05$) separations along PC1, and also increased in separation along PC2: the higher exposures of 0.2 and 0.4 mg/cm$^2$ both showed significant separations ($P = 0.0005$ and $P = 0.02$ respectively) along PC2 after the second day of exposure (Supplementary Material, Figs S4-1B, S4-1E and S4-1G). This suggests a change in the trajectory of the MOA after the second day of exposure. The average PCA scores plot summarizing the scores for all the study treatments also showed that there were time-dependent
Figure 4-1. Mean principal component analysis (PCA) scores plot of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the (A) polar and (B) non-polar fractions of *E. fetida* tissue extracts. The mean scores for the controls are denoted in the figure by a ‘C’ and the corresponding exposure day (for example C1 identifies the control for day 1). The mean scores for the phenanthrene exposed earthworms are denoted by the exposure concentration followed by the corresponding exposure day (for example 0.4-2 identifies a phenanthrene exposure concentration of 0.4 mg/cm² for two days). The mean scores (with associated standard error) were obtained by averaging the scores of each earthworm concentration class for each day. The ellipses that group the exposed classes were constructed as visual aids. The concentration response trajectory is highlighted by a dashed line.
increases in separation between control and exposed earthworms along PC2 (Fig. 4-1A). Therefore, PC2 seemed to account for the majority of the variation between control and exposed earthworms due to exposure time, whereas PC1 seemed to account for the majority of the variation due to exposure concentration. The average PCA scores plots depicting concentration-dependence showed that the metabolic profile of the earthworms exposed to 0.2 and 0.4 mg/cm$^2$ of phenanthrene were more similar to each other than the 0.05 mg/cm$^2$ exposure (Supplementary Material, Fig. S4-4). This was consistent with Lankadurai et al.’s [249] observation of a concentration-dependent two phased toxic MOA to phenanthrene centered around the exposure concentration of 0.1 mg/cm$^2$: phase I at phenanthrene concentrations <0.1 mg/cm$^2$ and phase II at phenanthrene concentrations ≥0.1 mg/cm$^2$. The average PCA scores plot revealed distinct regions for the scores of the exposed earthworms for each day of exposure, depicting time-dependence in the metabolic response (Fig. 4-1A). Within the region for each day, the scores of the exposed earthworms showed similar concentration-dependent trajectories that were separated along PC2 with the 0.4 mg/cm$^2$ exposure concentration having the highest PC2 value and the 0.05 mg/cm$^2$ exposure concentration having the lowest PC2 value. This suggests that the MOA of phenanthrene in *E. fetida* results in similar exposure concentration-dependent relationships with the metabolic profile regardless of the exposure time. However, when the scores of the exposed earthworms in the regions for the one and two days of exposure were compared, it was clearly evident that the scores were much more tightly grouped after two days of phenanthrene exposure (Fig. 4-1A). This suggests that with longer exposure the contaminant concentrations used in this study dictate the metabolic profile less. This is likely due to a gradual saturation of the mechanisms responsible for counteracting the toxicity either by an accumulation of phenanthrene overtime, or due to phenanthrene metabolites, which may be more toxic than the
parent compound, produced by enzymes such as Cyt P450 [279-281]. The time- and concentration-dependent behavior of *E. fetida* to phenanthrene exposure was also seen in the survival patterns of the earthworms: at the higher exposure concentrations of 0.2 and 0.4 mg/cm², none of the earthworms survived beyond two days of exposure, whereas at the 0.05 mg/cm² exposure concentration 90% of the earthworms survived after three days of exposure.

Unlike the polar fraction, the average and individual PCA scores plots of the non-polar fraction did not show clear time- and concentration-dependent responses (Fig. 4-1B and Supplementary Material, Figs S4-2, S4-5 and S4-6). There was insignificant (at $\alpha = 0.05$) separation between control and exposed treatment groups for all of the exposure concentrations after one, two and three days of exposure (Supplementary Material, Fig. S4-2). However, individual scores plots showed significant (at $\alpha = 0.1$) separation between exposed and control earthworms after one day of exposure along PC1 for the 0.05 and 0.4 mg/cm² exposures ($P = 0.06$ and $P = 0.05$; Supplementary Material, Figs S4-2A and S4-2D) and along PC2 and PC3 for the 0.2 mg/cm² exposure ($P = 0.08$ and $P = 0.03$; Supplementary Material, Fig. S4-2E). Lankadurai et al. [277] showed that at the lower exposure of 0.025 mg/cm² there was insignificant (at $\alpha = 0.1$) separation in the non-polar metabolic profile of exposed and control earthworms after one, two, three and four days of exposure. This suggests a concentration-dependence in the response of the non-polar metabolites because only exposure concentrations ≥0.05 mg/cm² showed a significant (at $\alpha = 0.1$) separation in the scores of the controls and exposed earthworms. The average PCA scores plots illustrated that there was clear separation between the scores of the control and exposed earthworms after one day of exposure for all three exposure concentrations (Supplementary Material, Figs S4-5 and S4-6). However, longer exposures did not result in a significant separation in the non-polar metabolic profile of the
control and exposed earthworms. This was in contrast to the polar metabolites, which revealed a
time-dependent increase in their response. Nevertheless, time appears to be an important
variable because one day of exposure seems to represent a significant response by the non-polar
metabolites.

PLS-regression models were also constructed (see Supplementary Material, Section S4-2
and Figs S4-11 and S4-12) to determine the strength and significance of the relationship between
the metabolic profile and exposure concentration for both polar and non-polar metabolites after
each day of exposure [128, 147]. The PLS-regression models revealed that the polar metabolic
profile of *E. fetida* had weak but significant linear correlation to phenanthrene exposure
centresations after one day of exposure (cross-validated PLS-regression with 2 components,
\( R^2_X=0.57, R^2_Y=0.64, Q^2_Y=0.48, P=0.00004 \)). The strength of the relationship was similar after
two days of exposure (cross-validated PLS-regression with 6 components, \( R^2_X=0.85, R^2_Y=
0.76, Q^2_Y=0.48, P=0.0001 \)), which suggests a stabilization in the metabolic response (relative to
the control) after both periods of time for the polar metabolites (Supplementary Material, Table
S4-1). The PLS-regression constructed using the non-polar metabolic profile after one day of
exposure was much weaker than either of the models using the polar metabolites (cross-validated
PLS-regression with 3 components, \( R^2_X=0.97, R^2_Y=0.48, Q^2_Y=0.25, P=0.008 \)) and after two
days of exposure this relationship had no apparent predictive power as reflected by a negative
\( Q^2_Y \) (cross-validated PLS-regression with 5 components, \( R^2_X=0.97, R^2_Y=0.54, Q^2_Y=-0.006,
P=0.05 \); Fig. S4-11). However, since this study only analyzed three exposure concentrations,
future studies with a larger range of phenanthrene exposure concentrations should be conducted
to obtain a much more definitive elucidation of the nature and strength of the relationship
between the metabolic profile and the exposure concentration. Nevertheless, since both PCA
and PLS-regression showed that the polar metabolites were more strongly correlated to phenanthrene exposure than the non-polar metabolites. Future earthworm metabolomic studies should focus mainly on the polar fraction.

4.4.2 Metabolic changes in response to phenanthrene exposure

PCA loadings plots were constructed to identify the metabolites that were responsible for the observed separation between the scores of the controls and phenanthrene exposed earthworms in the PCA scores plots (Fig. 4-2 and Supplementary Material, Figs S4-7 to S4-8). In addition to the loadings plots, t-test filtered \(^1\)H NMR difference spectra were also obtained in an exploratory capacity to identify any additional metabolites that may have increased or decreased significantly (at \(\alpha = 0.05\)) relative to the control (Supplementary Material, Figs S4-10 and S4-11) [76-77, 136]. The PCA loadings plots and the t-test filtered \(^1\)H NMR difference spectra identified the...
sugars maltose (δ= 5.41 ppm) and glucose/maltose (δ= 5.23 ppm), Krebs cycle intermediates succinate (δ= 2.39 ppm), fumarate (δ= 6.51 ppm) and malate (δ= 2.37 ppm), amino acids leucine (δ= 0.95 ppm), alanine (δ= 1.47 ppm), arginine (δ= 1.91 ppm), glutamate (δ= 2.35 ppm), lysine (δ= 3.01 ppm) and phenylalanine (δ= 7.31 ppm), messenger molecules scyllo-inositol (δ= 3.35 ppm) and myo-inositol (δ= 4.05 ppm), the osmolyte betaine (δ= 3.25 ppm), the energy molecule ATP (δ= 8.23 ppm), short-chain fatty acids (δ= 1.27 ppm for -CH₂ and δ= 0.83 ppm for -CH₃) and 2-hexyl-5-ethyl-3-furansulfonate (HEFS; δ= 1.27 ppm for -CH₂, δ= 0.83 and δ= 1.17 ppm for -CH₃, δ= 6.17 ppm for -CH from furan ring) as the polar metabolites that showed significant fluctuations in response to phenanthrene exposure (Fig. 4-2 and Supplementary Material, Figs S4-7, and S4-9). Both the loadings plots and the difference spectra illustrated that the δ= 3.40-4.00 ppm region of ¹H NMR spectra contain many overlapping peaks that can be assigned to both sugars and amino acids and which showed significant fluctuations in response to phenanthrene exposure. However, these peaks cannot be clearly assigned to individual metabolites due to overlapping resonances in this region. Phosphatidylcholine (δ= 3.21 ppm), cholesterol (δ= 0.69 ppm) and long-chain fatty acids (δ= 0.87 ppm for CH₃, δ= 1.25 ppm for (CH₂)ₙ, δ= 1.63 ppm for CH₂=CH₂COOH and δ= 2.33 ppm for CH₃-COOH) were identified as the non-polar metabolites that showed significant fluctuations in response to phenanthrene exposure (Supplementary Material, Figs S4-8 and S4-10). The PCA loadings plots (Fig. 4-2) pertaining to the average PCA scores plots of the polar fraction (Fig. 4-1A) summarizing all the treatments used in the study were also obtained to identify which metabolites contributed to the separation in the scores plots along PC1 and PC2. PC1, which was shown to explain the variation due to exposure concentration, was influenced more by sugars compared to PC2 (Fig.
PC2, which explained the majority of the variation due to exposure time, had a very large influence from alanine (Fig. 4-2B). This suggests that alanine is an important metabolite indicator for exposure time and is consistent with Lankadurai et al.’s [277] study which also showed that alanine had a positive linear correlation to exposure time in response to phenanthrene exposure at ultra-low sub-lethal levels.

The percent changes in discernible metabolite bucket intensities were examined to determine the fluctuations in the metabolites of phenanthrene exposed earthworms relative to the control earthworms and also to delineate the MOA of phenanthrene (Figs 4-3 and 4-4) [77, 86, 249]. Previous studies that examined contaminant exposures to organisms, including exposure of phenanthrene to earthworms, have shown that majority of the metabolite fluctuations can be ascribed to a heightened energy metabolism in the exposed organism relative to the controls [77, 113, 144, 249, 275]. The amino acids leucine, arginine, lysine and phenylalanine showed similar concentration and time-dependent profiles to each other (Figs 4-3A, and 4-3D-F). Lankadurai et al. [277] also illustrated that at a phenanthrene exposure of 0.025 mg/cm² these amino acids had similar time-dependent profiles. Whitfield Åslund et al. [108] observed a positive correlation between leucine, lysine and phenylalanine levels in control E. fetida and may explain the observed similarities in the responses of these amino acids in this study. It is important to note that Lankadurai et al.[277] also showed that these amino acids did not change significantly in response to phenanthrene exposure. However, at the higher exposure concentrations used in this study, significant (at α=0.05) changes were observed, suggesting concentration-dependence in the behavior of these amino acids (Fig. 4-3). Increased amino acid levels in response to contaminant exposure, including phenanthrene exposure, was observed by previous studies and was attributed to protein breakdown during times of stress to provide the starting materials for
gluconeogenesis [118, 144, 249, 282]. Interestingly, at the highest exposure concentration of 0.4 mg/cm², there wasn’t any significant increase in these amino acids even after two days of exposure (Figs 4-3A and 4-3D-F). This may be because at very high concentrations the toxic MOA of phenanthrene may disrupt the mechanisms responsible for initiating energy production through gluconeogenesis. Tintos et al. [283] also showed that there was a concentration-dependent decrease in the gluconeogenic capacity of rainbow trout exposed to the PAH naphthalene.

The amino acids alanine and glutamate showed different time and concentration-dependent profiles compared to leucine, arginine, lysine and phenylalanine (Figs 4-3B and 4-3C). Increased alanine levels during times of stress are well documented in the literature [117, 144, 249, 260, 262]. Alanine, considered as the universal stress signal in various organisms exposed to differing external stresses [262], also increased significantly (at α=0.05) in this study relative to the controls for all treatments (Fig. 4-3B). Lankadurai et al. [277] showed that even at the lower phenanthrene exposure concentration of 0.025 mg/cm², alanine increased significantly (at α=0.05) after just one day of exposure. The consistency in the response of alanine to phenanthrene exposure from ultra sub-lethal to higher exposure concentrations indicates that alanine may be a very sensitive indicator for phenanthrene exposure in earthworms. The reason for this increase in alanine during times of stress has not been exactly determined: it has been postulated to be due to the increase in gluconeogenesis because alanine is one of its major substrates [261, 263-264]. Alanine increases have been linked to a stimulation of stress protein production in mammalian kidneys as well [284]. In contrast to the other amino acids mentioned above, glutamate levels showed significant (at α=0.05) decrease in exposed earthworms relative to the controls (Fig. 4-3C). Furthermore, Lankadurai et al. [277] also observed a significant
Figure 4-3. Percent (%) change in selected metabolites of the polar fraction of phenanthrene exposed *E. fetida* tissue extracts compared with the control earthworms for each day. (A) Leucine, (B) Alanine, (C) Glutamate, (D) Arginine, (E) Lysine, (F) Phenylalanine, (G) Maltose, (H) Malate, (I) Fumarate, (J) Succinate, (K) Betaine, (L) *scylo*-Inositol, (M) *myo*-inositol, (N) 2-hexyl-5-ethyl-3-furansulfonate (HEFS), and (O) Adenosine triphosphate (ATP). The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each day and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed for each day] are labeled with an asterisk (*; at α=0.05). The percent changes are shown with their associated standard error.
decrease in glutamate levels due to phenanthrene exposure at a lower exposure concentration. Whitfield Åslund et al. [108] showed that glutamate was not correlated to the amino acids alanine, lysine, phenylalanine and leucine, which were positively correlated to each other in control *E. fetida*. However, glutamate was shown to be correlated to the sugar maltose in the control earthworms [108]. Both maltose and glutamate decreased significantly relative to controls in this study (Figs 4-3C and 4-3G). The decrease in glutamate may be due to its increased conversion to α-ketoglutarate (a Krebs cycle intermediate) in exposed earthworms to cope with increased energy demands. Glutamate levels showed an exposure concentration-dependent decrease after the first day of phenanthrene exposure for all three exposure concentrations (Fig. 4-3C). But, the 0.4 mg/cm$^2$ phenanthrene exposure did not show a significant decrease in glutamate levels after the second day of exposure (Fig. 4-3C). This may also be a result of the phenanthrene toxic MOA compromising the energy production mechanisms at higher exposure concentrations.

Maltose, a disaccharide, showed significant decreases (at $\alpha=0.05$) in the phenanthrene exposed earthworms relative to the controls (Fig. 4-3G). Previous studies that examined the response of *E. fetida* to sub-lethal phenanthrene exposure also observed a decrease in maltose concentrations in exposed earthworms [117, 249, 277]. Maltose breaks down to two glucose molecules, which are the substrates for glycolysis [250]. A decrease in maltose was also suggested to be as a result of the increase in energy requirements of the exposed earthworms, which are under the toxic MOA of phenanthrene [117, 135, 277]. At the 0.05 mg/cm$^2$ exposure concentration the percent decrease in maltose for all three days was nearly identical. However, for the two higher exposure concentrations the percent decrease in maltose was lower on day two
compared to day one (Fig. 4-3G). This observation may also be a result of the energy metabolism being compromised by the toxic MOA of phenanthrene at high exposure concentrations and longer exposure times. The Krebs cycle intermediates malate and fumarate significantly (at $\alpha=0.05$) decreased relative to the controls in phenanthrene exposed earthworms at all three exposure concentrations (Figs 4-3H and 4-3I). However, succinate generally showed a significant increase in the exposed earthworms (Fig. 4-3J). This pattern in the Krebs cycle intermediates was linked to a possible disruption in the functioning of the succinate dehydrogenase enzyme, which catalyzes the conversion of succinate to fumarate [257, 277]. Succinate dehydrogenase is the only enzyme of the Krebs cycle that is bound to the inner mitochondrial membrane [250]. Phenanthrene binding and the ensuing destabilization of the structure of the inner mitochondrial membrane may have contributed to the disruption in the functioning of the succinate dehydrogenase enzyme [252, 257, 277]. In addition to the destabilization of biological membranes, binding of HOCs such as phenanthrene to membranes can also alter their fluidity, which results in unregulated movement of molecules across the membranes and a disruption of the homeostatic osmotic pressure [251, 257]. During these times organic osmolytes such as betaine are produced to safely maintain the osmotic pressure within cells and organelles [233, 267]. Accordingly, betaine levels generally showed an increase in the exposed earthworms and were also significantly (at $\alpha=0.05$) increased after one day of exposure at the two higher exposure concentrations (Fig. 4-3K). Another potential metabolite indicator for destabilization of biological membranes in earthworms is the increased production of the amphiphilic molecule 2-hexyl-5-ethyl-3-furansulfonate (HEFS) [86, 275]. HEFS was identified as a compound that is unique to earthworms and was shown to be present in the earthworm species *Eisenia veneta, Lumbricus rubellus* and *Aporrectodea caliginosa* [86, 91, 275, 285].
Bundy et al. [275] mentioned that HEFS was also observed in *E. fetida* in unpublished observations. HEFS showed significant (at \( \alpha=0.05 \)) increase in exposed earthworms after one day of exposure to phenanthrene at all exposure concentrations (Fig. 4-3N). The higher phenanthrene exposure concentrations of 0.2 and 0.4 mg/cm\(^2\) showed greater increase in HEFS relative to the controls than the 0.05 mg/cm\(^2\) exposure concentration. The highest exposure concentration (0.4 mg/cm\(^2\)) shows significant increase in HEFS even after the second day of exposure. The exact function of HEFS in earthworms has still not been fully characterized. However, Bundy et al. [275] suggest that HEFS may play a role in membrane stabilization due to its amphiphilic nature. This may explain the increase in HEFS in exposed earthworms relative to the controls as a means to counteract the destabilization of the membranes caused by phenanthrene binding.

The long-chain fatty acids showed significant (at \( \alpha=0.05 \)) decrease for only the first day of exposure (Fig. 4-4B). As the sugar levels become depleted, the heightened energy demand would lead to an oxidation of the fatty acids to produce energy [250]. The breakdown of the long-chain fatty acids by \( \beta \)-oxidation, two carbon units at a time, will lead to a decrease in their levels [250]. However, longer exposures did not result in significant changes in the fatty acids relative to the controls. This may suggest an interruption in fatty acid breakdown mechanisms due to phenanthrene exposure because energy requirements remain high as shown by the significant decrease in maltose and significant increase in amino acids with longer exposure times. Perera et al. [286] also showed that PAH exposure results in a down regulation of fatty acid oxidation. Therefore, the toxic MOA of phenanthrene may be responsible for an interruption of fatty acid oxidation as well.
Figure 4-4. Percent (%) change in selected metabolites of the non-polar fraction of phenanthrene exposed *E. fetida* tissue extracts compared with the control earthworms for each day. (A) Fatty acid-CH$_3$, (B) Fatty acid-(CH$_2$)$_n$, (C) Cholesterol, and (D) Phosphatidylcholine. The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each day and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly different [based on a t-test (two tailed, equal variances) of control vs. exposed for each day] from the control are labeled with an asterisk (*; at $\alpha$ =0.05). The percent changes are shown with their associated standard error.
4.5 Conclusion

Our study demonstrated that $^1$H NMR-based metabolomics was capable of delineating both time-dependent and concentration–dependent relationships for the exposure of *E. fetida* to phenanthrene for a range of sub-lethal concentrations in both polar and non-polar endogenous metabolites. For the polar fraction PC1 explained most of the variation due to exposure concentration, whereas PC2 explained most of the variation due to exposure time. The PCA loadings showed that sugars were more influential in explaining the variation due to exposure concentration, whereas the amino acid alanine was highly significant in explaining the variation due to exposure time. With increasing exposure time, exposure concentration became less important in discriminating between controls and exposed earthworms, possibly due to a saturation of the enzymes involved in detoxification by an accumulation of phenanthrene and by production of its metabolites, which may be more toxic than the parent compound [281]. The non-polar metabolic profile appeared to be less sensitive to phenanthrene exposure even at the higher phenanthrene exposure concentrations used in this study. The only significant difference between control and exposed earthworms in the non-polar metabolic profile was after the first day of exposure due to the significant decrease in long-chain fatty acids. The polar metabolic profile was much more informative than the non-polar metabolic profile in enabling to delineate the MOA of phenanthrene in *E. fetida* and in also identifying possible metabolite indicators of phenanthrene exposure. Therefore, future NMR-based metabolomic studies can focus only on the polar metabolites for a high-throughput analysis of the response of *E. fetida* to sub-lethal phenanthrene exposures. A heightened energy metabolism was observed as in previous studies with significant decreases in the sugar maltose and long-chain fatty acids as well as significant increases in amino acids [117, 249, 277]. Our study also confirmed a previous report’s
observation of the interruption of the Krebs cycle by the MOA of phenanthrene due to an inactivation of the succinate dehydrogenase enzyme complex [277]. Metabolites such as alanine, arginine, lysine, phenylalanine, betaine, and maltose were also identified in this study as potential indicators of phenanthrene exposure [117, 144, 249, 277]. Therefore, the results of this study indicate that NMR-based earthworm metabolomics has great potential to emerge as a powerful tool for determining the MOA of chemicals in organisms and also in routine ecotoxicity assessments of contaminated soils.

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Chapter Five

$^1$H NMR-based metabolomics analysis of the response of *Eisenia fetida* after sub-lethal exposure of perfluorooctanoic acid and perfluorooctane sulfonate


5.1 Abstract

Metabolomics entails the analysis of endogenous metabolites within organisms exposed to an external stressor such as an environmental contaminant. We utilized $^1$H NMR-based metabolomics to elucidate sub-lethal toxic mechanisms of *Eisenia fetida* earthworms after exposure to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). Earthworms were exposed to a range of concentrations of PFOA (6.25 to 50 μg/cm$^2$) and PFOS (3.125 to 25 μg/cm$^2$) via contact tests for two days. Earthworm tissues were extracted using a mixture of chloroform, methanol and water, and the polar fraction was analyzed by $^1$H NMR. NMR-based metabolomic analysis revealed heightened *E. fetida* toxic responses with higher PFOA and PFOS exposure concentrations. Principal component analysis (PCA) exhibited significant separation between control and exposed earthworms along PC1 for all PFOA and PFOS exposure concentrations. Leucine, arginine, glutamate, maltose, and ATP are potential indicators of PFOA and PFOS exposure as these metabolite concentrations fluctuated with exposure. Our data also indicate that PFOA and PFOS exposure may increase fatty acid oxidation and interrupt ATP synthesis due to a disruption in the inner mitochondrial membrane structure. NMR-based metabolomics has promise as an insightful tool for elucidating the environmental toxicology of sub-lethal contaminant exposure.
5.2 Introduction

Perfluoroalkyl acids (PFAAs) are widely used in many industrial and consumer-use applications [172-174]. PFAAs have many unique physical and chemical properties such as hydrophobicity, lipophobicity, thermal stability, acid, base, and oxidizing agent resistance, and possess surfactant-like properties [171, 173-174]. The chemical stability of PFAAs also results in environmental persistence [171-173]. PFAAs have been distributed globally [171, 173, 194] and have been detected in blood samples of many species of wildlife and also in humans. They have been observed in arctic mammals, which are in remote locations from PFAA sources [171, 173, 194-196]. PFAAs have also been found in rivers, soils, sediments and oceans [197-199]. Due to the widespread prevalence of PFAAs in the environment, some studies have examined their toxicity to a variety of organisms [173-174, 200]. PFAA exposure studies involving rodents and monkeys have revealed decreased body weight and size, increased liver weight, tumors in the liver, pancreas and testicles, changes in lipid metabolism and even death at high doses [173, 200-201].

Toxicity studies of PFAAs with terrestrial invertebrates and plants are limited. Since, PFAAs are also found in soils [176, 199, 208-209] it is important to investigate their toxicity to terrestrial species such as earthworms, which are commonly used as model organisms to monitor soil toxicity because they are directly exposed to soil contaminants through ingestion and passive absorption [211, 219]. Joung et al.[176] investigated the exposure of *Eisenia fetida* earthworms to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) and observed a concentration-dependent increase in the mortality of the earthworms. This study illustrated that PFOA and PFOS, the two most common and persistent types of PFAAs in the environment, were toxic to earthworms at high concentrations and may pose a threat to soil health and quality.
However, exposure studies of earthworms to sub-lethal concentrations of PFOA and PFOS are currently lacking [176, 287]. Chemical exposure in the environment occurs mostly at sub-lethal or very low levels, which can also have adverse physiological responses in organisms [215-216]. The toxic mode of action (MOA) of the PFAAs has also not been identified for earthworms. Monitoring the fluctuations in endogenous metabolite levels (such as amino acids and sugars) in response to sub-lethal contaminant exposure may potentially elucidate the MOA of the chemical [217, 277]. Therefore, it is essential to develop reliable, rapid and reproducible analytical methods that are capable of assessing organism responses to sub-lethal contaminant exposure.

$^{1}$H nuclear magnetic resonance (NMR)-based metabolomics has gained in popularity over the past decade as a powerful tool for measuring organism responses to various environmental stressors, including contaminant exposure, in both aqueous and terrestrial environments [152, 213]. Metabolomics involves measuring fluctuations in low molecular weight endogenous metabolite levels, such as sugars and amino acids, in response to a defined external stressor [152, 213]. NMR-based earthworm metabolomics has shown great promise as a high throughput and reliable technique that can be used to elucidate the contaminant’s MOA and also identify potential metabolite indicators (or biomarkers) of exposure in response to sub-lethal exposure [76, 90, 144, 277]. NMR-based metabolomic studies have identified increases and decreases in amino acids such as alanine, arginine and glutamate, the sugar maltose, the osmolyte betaine, and lipids such as fatty acids in organisms exposed to various contaminants [76, 277].

In this study, $^{1}$H NMR-based metabolomics was used to investigate the toxic MOA of PFOA and PFOS in the earthworm *E. fetida* by exploring the concentration-dependent relationships between the metabolic response and PFAA exposure in the absence of soil. *E. fetida* earthworms were exposed to sub-lethal PFOA or PFOS concentrations via contact tests for
two days of dermal contact. To the best of our knowledge this is the first study to investigate sub-lethal PFOA and PFOS exposures to earthworms using NMR-based metabolomics. Therefore, our first objective was to determine if NMR-based metabolomics is able to differentiate between the responses of control (unexposed) earthworms and earthworms exposed to sub-lethal or very low concentrations of PFOA or PFOS. Toxicity tests in other organisms have demonstrated that PFOA and PFOS may have similar toxic MOAs [173-174]. Therefore, we aimed to compare the metabolic response of E. fetida to PFOA exposure to the metabolic response after PFOS exposure to test this hypothesis and to determine if changes in biochemical processes such as increased fatty acid oxidation and perturbations in energy metabolism that were observed in other organisms [173, 201] also occur in E. fetida. This study will help assess if NMR-based metabolomics is a reliable, rapid and routine ecotoxicological tool for assessing the toxicity of PFOA and PFOS to earthworms at sub-lethal concentrations.

5.3 Experimental Methods

5.3.1 Earthworm maintenance prior to contact tests

E. fetida earthworms were obtained from a healthy population of E. fetida cultured within our laboratory since 2006 [137]. The initial culture was purchased from The Worm Factory (ON, Canada) and raised under controlled nutrient and environmental conditions [137]. They were raised in earthworm bins containing sphagnum peat bedding (Magic Worm bedding; Magic Products; WI, USA) at an approximate temperature of 24°C and a moisture content of 67% water (by weight). The controlled conditions have been tested and implemented to ensure that differences within the 1H NMR profile from diet and other environmental factors are minimized [137].
5.3.2 Earthworm exposure via contact tests and tissue extractions

Contact tests are used in rapid contaminant-exposure studies prior to soil exposure studies and can provide insight into toxic responses that can be observed in soil [90, 113]. *E. fetida* was chosen because it is the recommended earthworm species for ecotoxicological tests by the organization for economic co-operation and development (OECD) [159]. Preliminary earthworm contact tests were conducted with *E. fetida* to confirm the range of sub-lethal concentrations for PFOA (pentadecafluoro-octanoic acid; 99%, Synquest Laboratories Inc.) and PFOS (heptadecafluorooctane sulfonic acid potassium salt; 98%, Sigma-Aldrich). PFOS was found to be more toxic than PFOA: PFOS exposure concentrations <50 μg per cm$^2$ on filter paper (Whatman GF/A 4.25 cm diameter circles, Fisher Scientific; μg/cm$^2$) and PFOA exposure concentrations <100 μg/cm$^2$ resulted in 100% survival of the earthworms after two days of exposure. Joung et al.[176] also demonstrated that PFOS was more toxic than PFOA to *E. fetida* in a soil exposure study. Therefore, exposure concentrations of 6.25, 12.5, 25 and 50 μg/cm$^2$ for PFOA, and 3.125, 6.25, 12.5 and 25 μg/cm$^2$ for PFOS were selected. Mature earthworms, indicated by a visible clitellum were chosen and depurated in groups of 10 in the dark for 96 hours in 500 ml glass jars containing moist Whatman 4 Qualitative filter papers with a diameter of 9 cm (Fisher Scientific) to empty their intestinal tracts [137]. The earthworms had an average mass of 0.55 g (±0.1 g; standard deviation) after depuration. Whatman GF/A 4.70 cm diameter glass filter papers (Fisher Scientific) were pre-treated by first rinsing once with dichloromethane (DCM), followed by evaporation of DCM in the fume hood and then baking in the oven for ~1hr at 80°C to remove residual DCM. The pre-treated filter papers were placed in 120 ml amber glass jars. PFOA and PFOS solutions (1 ml in acetone) were applied to filter papers and only
acetone was applied to filter papers for control (unexposed) earthworms [136-137, 277]. Acetone was allowed to evaporate and then 1 ml of distilled water was added prior to addition of the depurated earthworms (one per jar). The glass jars were kept in the dark during the exposure period as recommended by the OECD contact test guidelines [159]. The earthworms were exposed to PFAAs for two days. Ten earthworms were used for each treatment (PFAA exposed and control treatments). After the two days of exposure the earthworms were flash-frozen in liquid nitrogen, lyophilized and stored frozen until extraction [92, 137].

After lyophilization the earthworms were homogenized while on ice in a 1.5 ml centrifuge tube using a 5 mm wide stainless steel spatula [92]. The two step methanol, water, chloroform tissue extraction protocol [93] was then used to extract the homogenized earthworm tissue. Ice-cold methanol (4 ml/g of earthworm dry weight) and ice cold water (0.85 ml/g of earthworm dry weight) were added to the tissue and vortexed for 15 seconds using a VX 100 vortexer (Labnet, NJ, USA). Chloroform (4 ml/g of earthworm dry weight) and water (2 ml/g of earthworm dry weight) were then added and vortexed for 60 seconds. To allow partitioning between the polar and non-polar layers the samples were then kept on ice for 10 minutes. The tissue mixture was centrifuged for 10 minutes at 12,000 rpm (~11,000 g) using an International Equipment Company 21000 centrifuge (Fisher Scientific). The upper polar layer and the bottom non-polar layer were removed carefully into a 1.5 ml centrifuge tube and a 1.8 ml glass vial respectively. Previous studies have reported that the polar fraction provides the most information regarding the metabolic perturbations of E. fetida in response to phenanthrene exposure compared to the non-polar fraction using 1H NMR metabolomics [277, 288]. Also, preliminary analysis of the non-polar fraction using high-resolution mass spectrometry (MS) did not detect any fluctuations to lipid metabolites. Therefore, to obtain a high-throughput and consistent
analysis of the metabolic response of *E. fetida* to PFAA exposure only the polar fraction was analyzed. The polar fraction was dried under a constant nitrogen flow and was then reconstituted in 750 μl of a 0.2 M monobasic sodium phosphate buffer solution (NaH₂PO₄·2H₂O; 99.3%; Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity; Sigma Aldrich) as a preservative [137]. Buffer solution was made with D₂O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D₂O; Cambridge Isotope Laboratories Inc). The buffer solution also contained 10 mg/l of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal standard [113, 117]. The reconstituted extract was vortexed for 30 seconds and then centrifuged at 12,000 rpm (~11,000 g) for 10 minutes and the supernatant was transferred into a new 1.5 ml centrifuge tube. Samples were then transferred into 5 mm High Throughput NMR tubes (Norell Inc.; NJ, USA) for ¹H-NMR analysis.

5.3.3 ¹H NMR spectroscopy and data analysis

¹H NMR spectra of the earthworm extracts were acquired with a Bruker Avance III 500 MHz spectrometer using a ¹H-¹⁹F-¹⁵N-¹³C 5 mm Quadruple Resonance Inverse (QXI) probe fitted with an actively shielded Z gradient. ¹H NMR experiments were performed using Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression and 128 scans, a recycle delay of 3 s, and 16 K time domain points [130]. Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. All spectra were manually phased and
calibrated consistently. The $^1$H NMR spectra were calibrated to the nine identical methyl protons of the trimethylsilyl group of the DSS internal reference ($\delta=0.00$ ppm).

The chemical shift range between 0.5 and 10 ppm represented all $^1$H NMR resonances in extracts and were divided into buckets that were 0.02 ppm in width using AMIX 3.8.4 (Bruker BioSpin, Rheinstetten, Germany) statistics tool for a total of 475 buckets [135, 249, 277]. The area between 4.70–4.85 ppm was excluded in the $^1$H NMR spectra to eliminate the small residual H$_2$O/HOD signals. The integration mode was set at the sum of intensities and the spectra were scaled to total intensity. This created a matrix in which each row represents an earthworm sample and each column contains the integrated area of the original spectral intensities within each bucket region. Individual principal component analysis (PCA) scores plots were constructed to compare the metabolic response of the control (unexposed) earthworms to the earthworms exposed to PFOA or PFOS. A t-test (two-tailed, equal variances) was also performed on the PCA scores to determine if there was a significant separation between the scores of the controls and the PFAA exposed earthworms at $\alpha = 0.05$ [234]. To identify the metabolites that were contributing to the separation between the scores of the control and exposed earthworms PCA loadings plots, which represent the relative weight for each bucket, were also acquired for each of the PCA scores plots [90, 113, 117]. Average PCA scores plots were also constructed for PFOA and PFOS exposures separately and also together to deduce concentration-dependent relationships in the metabolic profile. The average PCA scores plots were constructed from a PCA model that described all of the data and then the scores belonging to each class were averaged to obtain the average PCA scores plot. The scores from the PCA scores plots were then imported into Microsoft Excel (version 12.0.6504, Microsoft Corporation,
Redmond, WA) and were averaged per class (exposure concentration) and re-plotted with their associated standard errors.

Difference class $^1$H NMR spectra were constructed to identify metabolites that had significantly increased or decreased due to PFAA exposure relative to the controls [76-77, 249]. The buckets generated by AMIX 3.8.4 statistics tool, which represents the binned $^1$H NMR spectra of *E. fetida* extracts were then imported into Microsoft Excel. Levene’s test was used to test for variance homogeneity among the buckets, which were found to have equal variances at $\alpha = 0.05$ [253]. A t-test (two-tailed, equal variances) was then performed to compare the buckets of the controls with that of the exposure class in order to identify the buckets that were statistically different at $\alpha = 0.05$. Average $^1$H NMR spectra were constructed by obtaining the average of the buckets of each exposure concentration and control treatments separately. Difference class $^1$H NMR spectra were then obtained by subtracting the buckets of the average controls from that of the average exposure treatments. The buckets that were statistically insignificant from the controls were then replaced with a zero resulting in a t-test filtered $^1$H NMR difference spectra [76-77]. The buckets were then imported into Origin 7 (version 7.0383, OriginLab Corporation, Northampton, MA) to plot the difference $^1$H NMR spectra. The percent changes in the intensity of metabolite peaks of exposed earthworms relative to the control were obtained by dividing the buckets that pertain to the metabolites in the exposed by the corresponding buckets in the control. Since discovery type/exploratory analyses were performed in the current study, which analyzed the metabolic response of *E. fetida* to PFOA and PFOS for the first time, false positive corrections was considered to be too limiting for formulating hypotheses and identifying potentially important metabolites as biomarkers. Therefore, false positive corrections were not made in this study [289-291]. The metabolite peaks in the $^1$H
NMR spectra of the earthworm tissue extracts were identified by comparison to previously published assignments for polar metabolites [76, 90-92, 137].

5.4 Results and discussion

5.4.1 Principal component analysis

Individual and average PCA scores plots were constructed using the $^1$H NMR spectra of *E. fetida* tissue extracts to compare the metabolic response of the PFOA and PFOS exposed earthworms to the control (unexposed) earthworms (Fig. 5-1 and Supplementary Material, Figs S5-1 and S5-2) [90, 113, 254]. Both the individual and average PCA scores plots exhibited that there were clear separations between controls and both the PFOA and PFOS exposed earthworms. The individual scores plots for both the PFOA and PFOS reveal significant separation along PC1 ($P<1 \times 10^{-3}$; Supplementary Material, Figs S5-1 and S5-2). Both PFOA and PFOS revealed increased separation from the controls with increasing exposure concentrations, except for the 25 $\mu$g/cm$^2$ PFOA exposure concentration which resulted in a greater separation ($P=8.3 \times 10^{-9}$) from the controls than the higher 50 $\mu$g/cm$^2$ exposure concentration ($P=1.1 \times 10^{-7}$; Supplementary Material, Figs S5-1 and S5-2). PFOS exposure resulted in a clear exposure concentration-dependent increase with respect to separation from the controls (Fig. 5-1B). At PFOS exposure concentrations $\geq$6.25 $\mu$g/cm$^2$ there was very little separation amongst the different PFOS exposure classes and any observed separation was along PC2 (PC2 explained only 15% of the variation in the metabolic response compared to 55% by PC1). This suggests that at PFOS exposure concentrations $\geq$6.25 $\mu$g/cm$^2$ the earthworm responses are less distinguishable from each other with varying exposure concentrations. This steep exposure concentration-response
observed in *E. fetida* was also observed in PFOS exposure to rats [173, 201]. The average PCA scores plots for PFOA were also differentiated from the controls with higher exposure concentrations (Fig. 5-1A). However, unlike with PFOS exposure there wasn’t a consistent trend at higher PFOA exposure concentrations because the 25 μg/cm² exposure class had a greater separation from the controls than the 50 μg/cm² exposure class (Fig. 5-1A). The PFOS exposed earthworms, which had lower exposure concentrations, were in general better separated from the controls than the PFOA exposed earthworms (except for the PFOS 25 μg/cm² exposure class), suggesting that PFOS exposure elicited a greater response at lower concentrations compared to PFOA (Fig. 5-1C). This is in agreement with the survival patterns of *E. fetida* to PFOS and PFOA exposures observed in the preliminary sub-lethal concentration range finding contact tests: PFOA exposure concentrations <100 μg/cm² resulted in 100% survival of the earthworms after two days of exposure, whereas only PFOS exposure concentrations <50 μg/cm² resulted in 100% survival of the earthworms. The PFOA and PFOS exposed earthworms were in close proximity to each other within the PCA scores plot, suggesting that there may be some
similarities in the metabolic response by *E. fetida* (Fig. 5-1C). This observation is consistent with the currently hypothesized toxic MOA based on studies with rats and mice [173, 193].

5.4.2 Metabolic changes in response to PFOA and PFOS exposure

PCA loadings plots were constructed to identify metabolites that were responsible for the observed separation between the scores of the controls and PFOA and PFOS exposed earthworms in the PCA scores plots (Supplementary Material, Figs S5-3 to S5-5). In addition to the loadings plots, t-test filtered $^1$H NMR difference spectra were also obtained in an exploratory capacity to identify any additional metabolites that may have increased or decreased significantly (at $\alpha = 0.05$) in response to PFAA exposure (Supplementary Material, Figs S5-6 and S5-7) [76-77, 136, 249, 277]. The PCA loadings plots and the t-test filtered $^1$H NMR difference spectra identified the sugars maltose ($\delta = 5.41$ ppm) and glucose/maltose ($\delta = 5.23$ ppm), Krebs cycle intermediates succinate ($\delta = 2.39$ ppm), and malate ($\delta = 2.37$ ppm), amino acids leucine ($\delta = 0.95$ ppm), alanine ($\delta = 1.47$ ppm), arginine ($\delta = 1.91$ ppm), glutamate ($\delta = 2.35$ ppm), lysine ($\delta = 3.01$ ppm) and phenylalanine ($\delta = 7.31$ ppm), messenger molecules scyllo-inositol ($\delta = 3.35$ ppm) and myo-inositol ($\delta = 4.05$ ppm), the osmolyte betaine ($\delta = 3.25$ ppm), the energy molecule ATP ($\delta = 8.23$ ppm), short-chain fatty acids ($\delta = 1.27$ ppm for -CH$_2$ and $\delta = 0.83$ ppm for -CH$_3$) and 2-hexyl-5-ethyl-3-furansulfonate (HEFS; $\delta = 1.27$ ppm for -CH$_2$, $\delta = 0.83$ and $\delta = 1.17$ ppm for -CH$_3$, $\delta = 6.17$ ppm for -CH from furan ring) as the metabolites that showed significant fluctuations in response to PFOA and PFOS exposure (Supplementary Material, Figs S5-3 and S5-7). The $\delta = 3.40-4.00$ ppm region of $^1$H NMR spectra contained many overlapping peaks that can be assigned to both sugars and amino acids and also revealed significant fluctuations in
response to PFOA and PFOS exposure. However, these peaks cannot be clearly assigned to individual metabolites due to overlapping resonances within the δ= 3.40-4.00 ppm region.

The percent changes in discernible metabolite intensities were examined to determine the fluctuations after PFOA and PFOS exposure and also to delineate the MOA of the two PFAAs (Figs 5-2 and 5-3) [77, 86, 217, 249]. In general, the percent changes of the metabolites that changed significantly (at α=0.05) relative to the controls in response to PFOA or PFOS exposure exhibited greater differences at high exposure concentrations as compared to low exposure concentrations (Figs 5-2 and 5-3). However, the metabolite changes did not reveal a consistent concentration-dependent trend for all metabolites. The amino acids leucine, valine, lysine, phenylalanine and arginine decreased significantly with both PFOA and PFOS exposure at all concentrations (Figs 5-2 and 5-3). PFOS exposure generally followed a concentration-dependent decrease in the aforementioned amino acids, however at exposure concentrations ≥6.25 μg/cm² the percent decrease appeared to plateau. These amino acids also exhibited similar concentration-dependent profiles to each other (Fig. 5-3). Previous studies have observed that the concentrations of the amino acids leucine, valine, lysine, phenylalanine and arginine are correlated to each other in control earthworms and in earthworms exposed to the contaminant phenanthrene [108, 277]. Earthworms exposed to PFOA displayed concentration-dependent decreases in leucine and valine relative to the controls (Fig. 5-2). Lysine, phenylalanine and arginine concentrations also decreased with increasing PFOA exposure concentration (Fig. 5-2). However, this trend is not consistent at the highest exposure concentration. The decrease in amino acids in response to PFOA and PFOS exposure may be due to the production of enzymes involved in fatty acid oxidation. Both PFOA and PFOS have been shown to cause peroxisome
Figure 5-2. Percent (%) change in selected metabolites of PFOA exposed Eisenia fetida tissue extracts (n=10) compared with the control earthworms (n=10). The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at α =0.05) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding P-values. The percent changes are shown with their associated standard error.
proliferation and there after increase the $\beta$-oxidation of fatty acids in rats and mice [173-174, 200, 202-205]. Peroxisome proliferator activated receptor alpha (PPAR$\alpha$) is a mammalian nuclear hormone receptor involved in lipid and lipoprotein metabolism [206-207]. Binding of fatty acids to PPAR$\alpha$ results in a cascade of events that eventually increase the production of peroxisomes and heighten the oxidation of fatty acids [203, 207]. Since PFOA and PFOS mimic the structure of natural fatty acids they are able to bind to proteins such as serum albumin and PPAR$\alpha$, which possess binding sites for fatty acids [174, 207, 292]. Binding of PFOA and PFOS to PPAR$\alpha$ initiates peroxisome proliferation [174, 203, 207]. The genome for E. fetida has not been sequenced and the nuclear hormone receptor involved in lipid metabolism has not been identified. However, for invertebrates such as the nematode worm Caenorhabditis elegans the nuclear hormone receptor-49 (nhr-49) was shown to be involved in regulating lipid metabolism much like PPAR$\alpha$ in mammals [293-294]. Although PFAA exposure studies on C. elegans have shown reduction in fecundity and longevity [295-296], these studies did not examine the MOA of these PFFAs in C. elegans. But because nhr-49 has binding sites for fatty acids, PFAAs may also bind to nhr-49 triggering fatty acid oxidation. Similarly, we hypothesize that E. fetida possesses nuclear hormone receptors like the nhr-49 that may be activated by the binding of PFAAs, leading to an initiation of fatty acid oxidation. Production of the peroxisomal enzymes involved in fatty acid oxidation requires the use of free amino acids. This might explain the decrease in the levels of free amino acids observed in the tissue extracts of PFOA and PFOS exposed E. fetida earthworms.

ATP, the energy currency of the cell, decreased significantly ($\alpha=0.05$) relative to the controls in response to PFOA and PFOS at all exposure concentrations (Figs 5-2 and 5-3). The
Figure 5-3. Percent (%) change in selected metabolites of PFOS exposed *Eisenia fetida* tissue extracts (n=10) compared with the control earthworms (n=10). The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at $\alpha =0.05$) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding $P$-values. The percent changes are shown with their associated standard error.
percent changes in ATP suggested concentration-dependence for both PFOA and PFOS exposure (Figs 5-2 and 5-3). PFOA and PFOS have been shown to hinder ATP synthesis by disrupting the structure of the inner mitochondrial membrane, thereby increasing its permeability and altering the proton (H\(^+\)) gradient required for the functioning of the ATP synthase enzyme [297-298]. This may explain the significant decrease in ATP that was observed in PFOA and PFOS exposed earthworms. Since ATP production by oxidative phosphorylation may be hindered the earthworm’s glycolysis may be increased to meet the required energy demands [250]. Maltose and the glucose/maltose resonances revealed that sugars decreased significantly (\(\alpha=0.05\)) relative to the controls in response to PFOA and PFOS exposures (Figs 5-2 and 5-3). Glucose is the major substrate for glycolysis [250] and maltose is a disaccharide composed of two glucose molecules. Therefore, the decrease in maltose in PFOA and PFOS exposed earthworms can be due to its breakdown into glucose to supply an increase in glycolysis [299]. Maltose concentrations in *E. fetida* have also been observed to decrease after sub-lethal exposure to phenanthrene [117, 249, 277].

The Krebs cycle intermediates succinate, fumarate and malate are observed at elevated levels in response to PFOA and PFOS exposure (Figs 5-2 and 5-3). Succinate exhibited significant (\(\alpha=0.05\)) increase relative to the controls in PFOA and PFOS exposed earthworms at all exposure concentrations (Figs 5-2 and 5-3). Fumarate did not reveal any significant (at \(\alpha=0.05\)) response to PFOA or PFOS exposure (Figs 5-2 and 5-3). Malate concentrations varied with different exposure concentrations and increased \((P=0.008)\) for the PFOA 25 \(\mu g/cm^2\) exposure concentration and the PFOS 3.125 \(\mu g/cm^2\) and 6.125 \(\mu g/cm^2\) exposure concentrations (Figs 5-2 and 5-3). The significant (at \(\alpha=0.05\)) increase in succinate and malate concentrations
suggests perturbation of the Krebs cycle by PFOA and PFOS. Previous studies that examined PFOA, perfluorododecanoic acid, and PFOS exposures to rats and humans also revealed that expression of genes that are involved in producing enzymes for the Krebs cycle were altered in response to PFAA exposure [300-302]. In addition, the increased oxidation of fatty acids due to the peroxisome proliferation caused by PFOA and PFOS results in elevated levels of acetyl-CoA, which is the starting substrate for the Krebs cycle [250, 303-304]. $\alpha$-Ketoglutarate (a Krebs cycle intermediate) and the amino acid glutamate are converted from one to another via the glutamate dehydrogenase enzyme [250]. Glutamate generally exhibited an increase in response to PFOA and PFOS exposures (Figs 5-2 and 5-3). The disruption in the normal functioning of the Krebs cycle in earthworms exposed to PFOA and PFOS may be responsible for an increased conversation of $\alpha$-ketoglutarate to glutamate resulting in the observed increases for glutamate.

The concentrations of osmolytes betaine, *scyllo*-inositol and *myo*-inositol generally increased relative to the controls after exposure to PFOA and PFOS (Figs 5-2 and 5-3). Similarly, HEFS a compound that is only found in earthworms and has been postulated to be involved in membrane stabilization [86, 90] also increased significantly (at $\alpha$=0.05) at all exposure concentrations in both PFOA and PFOS exposed earthworms (Figs 5-2 and 5-3). Both PFOA and PFOS possess surfactant-like properties and have caused disruption in membrane structure [298, 305-306]. Previous studies reported that PFOA and PFOS exposures altered the inner mitochondrial membrane fluidity and permeability which resulted in membrane leakage, disruption of the osmotic pressure and swelling of the mitochondria [297-298]. The increase in the osmolytes and HEFS in PFOA and PFOS exposed earthworms relative to the controls may be as a response to counteract the disruption in the membrane structure brought about by the exposure to the PFAAs.
5.5 Conclusion

Our study demonstrates that $^1$H NMR-based metabolomics is able to distinguish between the responses of *E. fetida* to PFOA and PFOS exposure from the control (unexposed) earthworms even at sub-lethal or very low exposure concentrations. We also show that PFOA and PFOS have similarities with respect to their toxic MOA in *E. fetida*, which is consistent with other studies [173, 193]. However, PFOA and PFOS elicited different concentration-dependent relationships between the metabolic response and exposure concentration, suggesting that even though the MOA is similar, the rate and extent to which each chemical elicits a response can vary [207]. This study only analyzed four exposure concentrations for each PFAA, thus future studies should include a wider range of sub-lethal concentrations to obtain a better understanding of the concentration-dependent relationships between the metabolic response of *E. fetida* and the exposure concentration of PFOA and PFOS. Evidence for increased fatty acid oxidation and disruption of biological membranes such as the inner mitochondrial membrane due to PFOA and PFOS exposure were observed and is in agreement with the hypothesized MOA of these chemicals [200, 202, 297-298]. Our study only analyzed the responses of the polar endogenous metabolites to PFOA and PFOS exposure. Since, PFOA and PFOS may alter the lipid metabolism of earthworms, future studies should also analyze the non-polar metabolites to obtain a holistic understanding of the MOA of the two PFAAs. In addition, sub-lethal PFOS and PFOA exposure studies should also be carried out in soils to better represent environmental exposures. Our study, which serves as the first attempt to utilize NMR-based metabolomics for delineating the MOA of PFOA and PFOS, highlights the potential for NMR-based metabolomics to elucidate the toxic mechanisms of these PFAAs at sub-lethal concentrations.
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Chapter Six

$^1$H NMR-based metabolomic analysis of sub-lethal perfluorooctane sulfonate exposure to the earthworm *Eisenia fetida* in soil

6.1 Abstract

$^1$H NMR-based metabolomics was used to measure the response of *Eisenia fetida* earthworms after exposure to sub-lethal concentrations of perfluorooctane sulfonate (PFOS) in soil. Earthworms were exposed to a range of PFOS concentrations (5, 10, 25, 50, 100 or 150 mg/kg) for two, seven and fourteen days. Earthworm tissues were extracted and analyzed by $^1$H NMR. Multivariate statistical analysis of the metabolic response of *E. fetida* to PFOS exposure identified time-dependent responses that comprised of two separate modes of action: a non-polar narcosis type mechanism after two days of exposure and increased fatty acid oxidation after seven and fourteen days of exposure. Univariate statistical analysis revealed that HEFS, betaine, leucine, arginine, glutamate, maltose, and ATP are potential indicators of PFOS exposure as the concentrations of these metabolites fluctuated significantly. Overall, NMR-based metabolomic analysis suggests elevated fatty acid oxidation, disruption in energy metabolism and biological membrane structure, and a possible interruption of ATP synthesis. These conclusions obtained from analysis of the metabolic profile in response to sub-lethal PFOS exposure indicates that NMR-based metabolomics is an excellent discovery tool when the MOA of contaminants is not clearly defined.
6.2 Introduction

Perfluoroalkyl acids (PFAAs) are a class of anthropogenic chemicals that have been distributed globally owing to their wide usage in many industrial and consumer-use applications [172-174]. PFAAs are lipophobic, hydrophobic, thermally stable, resistant to acids, bases, and oxidizing agents, and possess surfactant-like properties [171, 173-174]. As such, the high chemical stability of PFAAs also results in long-term environmental persistence [171-173]. Perfluorooctane sulfonate (PFOS) is reported to be the most prevalent type of PFAA in the environment [174, 307]. It is also the final breakdown product of many perfluorinated chemicals and has no known natural degradation pathway [173, 308]. PFOS has been detected in human breast milk and blood serum, wildlife blood serum and livers, and in fish [174, 309-310]. Neonatal mortality, decreased body weight and size, increased liver weight, tumors in the pancreas, liver and testicles, and changes to fatty acid metabolism were observed with PFOS exposure to rats, mice and monkeys [175, 200-201]. On account of its environmental persistence and toxicity to organisms PFOS has been recently added to Annex B of the Stockholm Convention on Persistent Organic Pollutants, which restricts the production of PFOS to a few specific applications [173, 311].

PFOS has been detected in various soil environments [176, 199, 208-209]. However, toxicity studies involving soil dwelling invertebrates and plants are rare. Earthworms are often used as model organisms to monitor soil toxicity because they are directly exposed to soil contaminants through ingestion and passive absorption [211, 219]. Previous studies have examined the toxicity of PFOS to the earthworm *Eisenia fetida* in artificial soil and reported LC50 (the concentration that causes 50% mortality) values of 405 mg/kg and 365 mg/kg after seven and fourteen days of exposure respectively [176, 312]. Joung et al.[176] reported a no
observable effect concentration (NOEC) in *E. fetida* based on mortality tests for PFOS soil concentrations of 160 mg/kg for both seven and fourteen days of exposure, whereas Sindermann et al.[287] observed a NOEC value of 77 mg/kg based on mortality tests in *E. fetida* after fourteen days of PFOS exposure. These studies illustrated that PFOS can be toxic to *E. fetida* and may pose a threat to soil quality and ecosystem health. In the environment exposure to chemicals mostly occurs at sub-lethal or very low concentrations, which may also exert adverse physiological responses in many organisms [215-216]. Stubberud [210] conducted reproduction tests with *E. fetida* after PFOS exposure and reported EC$_{50}$ (half maximal effective concentration) values of 103 mg/kg for number of cocoons, 80 mg/kg for number of juveniles, and 29 mg/kg for weight of juveniles. The NOEC-value from the reproduction tests was reported as 10 mg/kg, which suggested that the reproduction tests were more sensitive indicators of PFOS exposure than the mortality tests and that adverse responses are being observed in the reproduction of *E. fetida* with sub-lethal exposure [210]. Although valuable, reproduction tests do not provide any detail regarding the toxic mode of action (MOA) of PFOS in earthworms. Analyzing the fluctuations in endogenous metabolite levels (such as amino acids and sugars) in response to sub-lethal contaminant exposure may provide insight regarding the MOA of the chemical [217, 277]. Therefore, developing reproducible and high-throughput analytical methods that are capable of assessing organism responses to sub-lethal contaminant exposure may be indispensable.

$^1$H nuclear magnetic resonance (NMR)-based metabolomics has emerged as a powerful tool for measuring organism responses to various types of environmental stressors [152, 213]. Metabolomics involves measuring fluctuations in low molecular weight endogenous metabolite concentrations, such as sugars and amino acids, in response to a defined external stressor [152,
NMR-based earthworm metabolomics has shown promise as a rapid and reproducible technique that can elucidate the contaminant’s MOA and also identify potential metabolite indicators (or biomarkers) of exposure in response to sub-lethal contaminant exposure [76, 86, 240]. Our previous work has shown, for first time, that $^1$H NMR-based metabolomics is able to distinguish between the responses of PFOS and perfluorooctanoic acid (PFOA) exposed *E. fetida* earthworms in contact tests [313]. We were also able to elucidate the MOA of both PFOS and PFOA in *E. fetida* after short-term exposure (48 hours). However, we only examined four exposure concentrations and also conducted filter paper contact exposure tests, which may not represent all of the complexities that are involved in soil exposure (i.e.: bioavailability). Therefore, further research needs to be conducted to better understand the responses of *E. fetida* to PFAA exposure in the environment.

In this study, $^1$H NMR-based metabolomics was used to investigate the response of the earthworm *E. fetida* after exposure to an artificial soil spiked with sub-lethal PFOS concentrations by exploring both concentration-dependent and time-dependent relationships. Brown et al.[117] and Whitfield Åslund et al.[128] showed that two-day exposure to sub-lethal concentrations of phenanthrene and polychlorinated biphenyl (PCB) respectively, elicited significant metabolic responses in *E. fetida*. Joung et al.[176] reported similar LC$_{50}$ values for both the seven and fourteen-day PFOS exposures (405 mg/kg and 365 mg/kg respectively). Therefore, to determine an appropriate exposure period and also to test the exposure time-response of *E. fetida* to PFOS, we chose exposure time lengths of two days, seven days, and fourteen days of exposure (recommended by the OECD earthworm acute toxicity tests in artificial soil [159]). Our first objective was to compare the metabolic response of *E. fetida* to PFOS exposure in artificial soil to the metabolic response of *E. fetida* in contact tests reported in
our previous study and determine the appropriateness of contact tests in predicting soil exposure responses [313]. We also tested if NMR-based metabolomics was more sensitive than the traditional toxicity tests such as mortality tests and reproduction tests. Based on previous NOEC reports for PFOS, we chose six sub-lethal PFOS soil exposure concentrations ranging from 5 mg/kg to 150 mg/kg. The United States Environmental Protection Agency (US EPA) has set a residential soil screening level for PFOS of 6 mg/kg [314]. Therefore, we wanted to examine if there are significant metabolic responses at the lowest PFOS exposure concentration of 5 mg/kg. We also verified if changes in biochemical processes such as increased fatty acid oxidation and perturbations in energy metabolism that were observed in other organisms and were proposed in our previous study are observable after soil exposure as well [173, 201, 313]. This study will help assess if NMR-based metabolomics can be applied as a routine ecotoxicological tool for assessing the toxicity of PFOS in soil environments.

6.3 Experimental Methods

6.3.1 Soil spiking and total soil PFOS concentrations

An artificial soil medium was prepared by mixing sphagnum peat (Ward’s Natural Science), Kaolin clay (Ward’s Natural Science) and sand (Ward’s Natural Science) in a 1:2:7 ratio as described by the OECD Earthworm Acute Toxicity test protocol [159]. Initially, 125 g (dry weight) of the artificial soil was added to twenty-one 1 L clear glass jars. 10 ml of PFOS of 250, 500, 1250, 2500, 500 and 7500 mg/L (heptadecafluorooctane sulfonic acid potassium salt; 98%, Sigma-Aldrich) dissolved in acetone (HPLC grade, Fisher Scientific) was used to spike the soils of the six PFOS-exposed treatments for the two, seven and fourteen days exposure classes. The unexposed control treatment soil was treated with 10 ml of acetone only. The soils were
then left in the fume hood for 16 h to allow the acetone to evaporate [315]. An additional 375 g (dry weight) of soil was mixed thoroughly into each jar for a total of 500 g (dry weight) of soil per jar, resulting in total soil PFOS concentrations of 5, 10, 25, 50, 100 and 150 mg/kg (dry weight) for the PFOS-exposed treatments. Deionized water was used to wet the soils to a moisture content of 35% of soil dry weight and the soils were allowed to absorb the water for 24 h before introducing earthworms into the jars [159]. The PFOS concentrations in the spiked soils were confirmed by extraction and quantification via liquid chromatography/mass spectrometry (see Supplementary Information, Section S2 for the methods) following the two day, seven day and fourteen day exposure of the earthworms to the soils and found no evidence of degradation of PFOS during the experiment (data not shown).

6.3.2 Earthworm exposure and tissue extraction

Ten matured earthworms with a visible clitellum were added to each of the six PFOS-spiked soils and the control soil. Initial average mass of the earthworms was 400 ± 5 mg (standard error) wet weight. There was no significant difference in the initial earthworm mass between the different treatment groups (ANOVA, $F_{6,245} = 0.501, P = 0.8$). Earthworms were kept in closed jars for the duration of the exposure period in natural light. After which the earthworms were removed from the soils, and depurated for 96 h on damp filter paper. Earthworms were then flash-frozen in liquid nitrogen, lyophilized, reweighed and stored frozen until extraction.

The lyophilized earthworms were homogenized while in ice in a 1.5 ml centrifuge tube using a 5 mm wide stainless steel spatula [92]. The homogenized earthworm tissue was then
extracted using the two step methanol, water, chloroform tissue extraction protocol [93]. Ice-cold methanol (4 ml/g of earthworm dry weight) and ice cold water (0.85 ml/g of earthworm dry weight) were added to the tissue and vortexed for 15 seconds using a VX 100 vortexer (Labnet, NJ, USA). Chloroform (4 ml/g of earthworm dry weight) and water (2 ml/g of earthworm dry weight) were then added and vortexed for 60 seconds. The samples were then kept in ice for 10 minutes to allow partitioning between the polar and non-polar layers. The tissue mixture was centrifuged for 10 minutes at 12,000 rpm (~11,000 g) using an International Equipment Company 21000 centrifuge (Fisher Scientific). The upper polar layer and the bottom non-polar layer were removed carefully into a 1.5 ml centrifuge tube and a 1.8 ml glass vial respectively. Previous studies have reported that the polar fraction is more informative than the non-polar fraction regarding the metabolic perturbations of *E. fetida* in response to phenanthrene exposure using $^1$H NMR metabolomics [277, 288]. Preliminary analysis of the non-polar fraction using high-resolution mass spectrometry (MS) did not detect any significant fluctuations in the lipid metabolic profile. Therefore, for a rapid and consistent analysis of the metabolic response of *E. fetida* to PFAA exposure only the polar fraction was analyzed in this study. The polar fraction was dried under a constant nitrogen flow and was then reconstituted in 750 μl of a 0.2 M monobasic sodium phosphate buffer solution (NaH$_2$PO$_4$·2H$_2$O; 99.3%; Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity; Sigma Aldrich) as a preservative [137]. Buffer solution was made with D$_2$O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D2O; Cambridge Isotope Laboratories Inc). The buffer solution also contained 10 mg/l of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal standard [113, 117]. The extract was vortexed for 30 seconds and then centrifuged at 12,000 rpm (~11,000 g) for 10 minutes and the
supernatant was transferred into a new 1.5 ml centrifuge tube. Samples were then transferred into 5 mm High Throughput\textsuperscript{plus} NMR tubes (Norell Inc.; NJ, USA) for $^1$H-NMR analysis.

6.3.3 $^1$H NMR spectroscopy

$^1$H NMR spectra of the earthworm extracts were acquired with a Bruker Avance III 500 MHz spectrometer using a $^1$H-$^{19}$F-$^{15}$N-$^{13}$C 5 mm Quadruple Resonance Inverse (QXI) probe fitted with an actively shielded Z gradient. $^1$H NMR experiments were performed on the polar fraction using Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression and 128 scans, a recycle delay of 3 s, and 16 K time domain points [278]. Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. All spectra were manually phased and calibrated consistently. The $^1$H NMR spectra were calibrated to the nine identical methyl protons of the trimethylsilyl group of the DSS internal reference ($\delta=0.00$ ppm).

6.3.4 Data and Statistical Analysis

The chemical range between 0.5 and 10 ppm represented all $^1$H NMR resonances in extracts and were divided into buckets that were 0.02 ppm in width using AMIX 3.9.7 statistics tool for a total of 475 buckets (Bruker BioSpin, Rheinstetten, Germany) [135, 249, 277]. The area between 4.70–4.85 ppm was excluded to eliminate the small residual H$_2$O/HOD signals for the polar fraction. The integration mode was set at the sum of intensities and the spectra were scaled to total intensity. This created a matrix in which each row represents an earthworm sample and each column contains the integrated area of the original spectral intensities contained
within each bucket region. Individual principal component analysis (PCA) scores plots were constructed to compare the metabolic response of the control and exposed earthworms for each exposure treatment. A t-test (two-tailed, equal variances) was also performed on the PCA scores to determine if there was a significant difference between the scores of the controls and the exposed earthworms at $\alpha = 0.05$ [234]. Corresponding PCA loadings plots, which show the relative weight for each bucket, were also acquired for each of the PCA scores plots to identify the metabolites that were contributing to the separation between the scores of the control and exposed earthworms [113, 117, 275]. Average PCA scores plots were also constructed for PFOA and PFOS exposures separately and also together to deduce concentration-dependent relationships in the metabolic profile. The scores from the PCA scores plots were then imported into Microsoft Excel (version 12.0.6504, Microsoft Corporation, Redmond, WA) and were averaged per class (exposure concentration) and re-plotted with their associated standard errors.

PLS-regression models were constructed to determine the relationship between the metabolic profile and PFOS exposure concentration after each day of exposure [128, 147]. PLS-regression analyses were performed in R [316] using the Chemometrics package [317] on the buckets generated by the AMIX 3.9.7 statistics tool, which represent the binned $^1$H NMR spectra. PLS-regression was performed via the NIPALS PLS algorithm using PFOS concentration as the Y (response) matrix and the normalized bucket intensities from the $^1$H NMR spectra of all the earthworms as the X matrix of multiple predictors [128, 288]. PLS models were cross validated using the leave-one-out cross validation [128, 145-146]. The single cross validation (1CV) strategy [145] was used to determine the optimal number of components for each final PLS model. The explained variation of X ($R^2_X$) and Y ($R^2_Y$) were obtained for each PLS model as a measure of how well the model fit the training data [147]. The cross validated
R²Y value (denoted as Q²Y) was used as a preliminary measure of the predictive ability of the PLS model [128, 146]. Response permutation testing was conducted to assess the significance of each PLS model [128, 147-148]. This method consisted of keeping the X matrix (normalized binned ¹H NMR spectra) constant, while randomly permuting the order of the PFOS exposure concentrations (Y matrix) 400 times. For each permutation a new PLS model was fitted and the Q²Y was calculated providing a reference distribution of the Q²Y statistic. The significance of the original PLS model and the confidence in its validity is increased if its Q²Y value is higher than the values obtained for all of the PLS models built during the permutation tests [147].

Difference class ¹H NMR spectra were constructed to identify metabolites that had significantly increased or decreased relative to the control [76-77, 249]. The buckets generated by AMIX 3.9.7 statistics tool, which represents the binned ¹H NMR spectra of E. fetida extracts were then imported into Microsoft Excel. A t-test (two-tailed, equal variances) was then performed comparing the buckets of the controls with that of the exposure class to identify the buckets that were statistically different at α = 0.05. Average ¹H NMR spectra were obtained by averaging the buckets of each exposure concentration and control treatments separately. Difference class ¹H NMR spectra were then obtained by subtracting the buckets of the average controls from that of the average exposure treatments. The buckets representing the peaks of metabolites that weren’t statistically significant from the controls were then replaced with a zero resulting in a t-test filtered ¹H NMR difference spectrum [76-77]. The buckets were then imported into Origin 7 (version 7.0383, OriginLab Corporation, Northampton, MA) to plot the difference ¹H NMR spectra. The percent changes in the intensity of metabolite peaks of exposed earthworms relative to the control were obtained by dividing the buckets that pertain to the metabolites in the exposed by the corresponding buckets in the control. The metabolite peaks in
the \textsuperscript{1}H NMR spectra of the earthworm tissue extracts were identified by comparing to previously published assignments [76-77, 90-92, 137].

6.4 Results and Discussion
6.4.1 Multivariate statistical analysis

Average PCA scores plots were constructed using the \textsuperscript{1}H NMR spectra of \textit{E. fetida} tissue extracts to compare the metabolic response of the PFOS exposed earthworms to the control (unexposed) earthworms (Fig. 6-1) [90, 113, 254]. The average PCA scores plot (PC1 vs PC2) for the two-day exposure period showed clear separation between controls and PFOS exposed earthworms (Fig. 6-1A). However, the separation of the PFOS exposed earthworms from the controls did not reveal a clear concentration-dependent trend. The PC3 vs PC4 scores plot also illustrated separation of PFOS exposed earthworms from the controls but the extent of the separation was not dependent on the exposure concentration. However, the separation in the PC3 vs PC4 scores plot was not as pronounced as was observed with PC1 vs PC2 (Fig. 6-1B). The average PCA scores plot (PC1 vs PC2) for the seven-day exposure period also revealed clear separations between controls and exposed earthworms that were not dependent on the exposure concentrations (Fig. 6-1C). However, the PC3 vs PC4 scores plot for the seven-day exposure illustrated that the higher exposure concentrations (50, 100 and 150 mg/kg) were more separated from the controls compared to the lower exposure concentrations (5, 10 and 25 mg/kg; Fig. 6-1D). The average PCA scores plot (PC1 vs PC2) for the fourteen-day exposure period did not illustrate a clear separation from the controls for all of the exposure concentrations (Fig. 6-1E). Nevertheless, the PC3 vs PC4 average scores plot revealed that the higher exposure concentrations (50, 100 and 150 mg/kg) were better separated from the controls compared to the
Figure 6-1. Average principal component analysis (PCA) scores plot for the $^1$H NMR spectra of *Eisenia fetida* tissue extracts after PFOS exposure of two days (A) PC1 (first PCA component) versus PC2 (second PCA component), (B) PC3 (third PCA component) versus PC4 (fourth PCA component), seven days (C) PC1 vs PC2, (D) PC3 vs PC4, and fourteen days (E) PC1 vs PC2, (F) PC3 vs PC4. The mean scores for the PFOS exposed earthworms are denoted by the exposure length followed by the corresponding exposure concentration (for example, 2-100 denotes a two-day exposure to 100 mg/kg of PFOS in organization for economic corporation and development soil). The mean scores (with associated standard error) were obtained by averaging the scores of each exposure concentration. The ellipses were constructed as visual aids.
low and mid exposure concentrations (5, 10 and 25 mg/kg; Fig. 6-1F). Overall, the PC1 vs PC2 (explained 63% of the variation in the metabolic response) scores plot showed better separation (not dependent on exposure concentration) from the controls for the two-day exposure period, whilst the PC3 vs PC4 (explained about 9% of the variation in the metabolic response) scores plots showed better (concentration-dependent) separations from the controls for the seven and fourteen-day exposures. This presents two interesting conclusions regarding the exposure time-dependent response of E. fetida to PFOS exposure: Firstly, exposure to PFOS for two days elicited a strong response by the earthworms that is independent of the exposure concentration and one that dominates the variation observed in the metabolic profile (based on PC1 vs PC2 scores plot). Secondly, longer exposures seem to demonstrate a different MOA that is exposure concentration-dependent and one that does not dominate the observed variation in the metabolic profile (based on PC3 vs PC4 scores plots). Longer exposure periods lead to prolonged starvation in both controls and exposed earthworms. Therefore, the overall variation in the metabolic profile of E. fetida may be dominated by starvation responses with longer exposure periods, which may explain the reduced separations from the controls at the seven and fourteen-day exposure periods in the PC1 and PC2 scores plots (Figs 6-1C and 6-1E). The average scores plot (both PC1 vs PC2 and PC3 vs PC4) summarizing all the exposure days and exposure concentrations (Fig. 6-2) reveals that the scores of the seven and fourteen-day exposures are clustered together, whereas the scores of the two-day exposure are separated from the seven and fourteen-day exposures. This also indicates that the seven and fourteen-day PFOS exposures activate a MOA that is different from the response of E. fetida after two days of exposure.

PLS-regression models were constructed to ascertain the strength and significance of the relationship between the metabolic profile and the PFOS exposure concentration (Fig. 6-3 and
Figure 6-2. Average principal component analysis (PCA) scores plot for the $^1$H NMR spectra of *Eisenia fetida* tissue extracts after PFOS exposure of two, seven and fourteen days. (A) PC1 (first PCA component) versus PC2 (second PCA component), (B) PC3 (third PCA component) versus PC4 (fourth PCA component). The mean scores for the PFOS exposed earthworms are denoted by the exposure length followed by the corresponding exposure concentration (for example, 2-50 denotes a two-day exposure to 50 mg/kg of PFOS in organization for economic corporation and development soil). The mean scores (with associated standard error) were obtained by averaging the scores of each exposure concentration for each day of exposure. The ellipses were constructed as visual aids.
Supplementary Material, Table S6-1 and Fig. S6-3) [128, 147, 288]. The PLS-regression model for the two-day exposure had no apparent predictive power as illustrated by a negative $Q^2_Y$ value (cross-validated PLS-regression with 2 components, $R^2_X=0.59$, $R^2_Y=0.15$, $Q^2_Y=0.10$, $P=0.3$; Fig. 6-3 and Supplementary Material, Table S6-1). However, the PLS-regression model for the seven-day exposure suggested a weak but significant linear correlation between the *E. fetida* metabolic profile and the PFOS exposure concentration (cross-validated PLS-regression with 7 components, $R^2_X=0.90$, $R^2_Y=0.74$, $Q^2_Y=0.34$, $P=7\times10^{-4}$). The fourteen-day exposure produced a PLS-regression model that had the best predictive power and strongest linear relationship between the metabolic profile and the PFOS exposure concentration (cross-validated PLS-regression with 6 components ($R^2_X=0.85$, $R^2_Y=0.74$, $Q^2_Y=0.42$, $P=2\times10^{-5}$). Similar to the PCA analysis, the PLS-regression models also suggested that two separate MOAs may be operational, one at the shorter exposure time of two days that is exposure concentration independent and the other at the longer exposure times of seven and fourteen- days that is concentration-dependent (Fig. 6-3). The clear separation observed between the controls and PFOS exposed earthworms even at the very low exposure concentration of 5 mg/kg in the PCA scores plots and the significant linear correlation between the metabolic profile and the PFOS exposure concentration observed in the PLS-regression analysis for the seven and fourteen-day exposures suggests that NMR-based metabolomics is a much more sensitive indicator of PFOS exposure than the traditional toxicity tests such as mortality (77 mg/kg) [287] and reproduction (10 mg/kg) [270] tests. In addition, we also observed clear separation from controls at the lower exposure of 5 mg/kg, which was below the residential soil screening level for PFOS (6 mg/kg) set by the US EPA [314].
Figure 6-3. Average predictions of PFOS concentrations ($\hat{y}$) given spectra $i$ by the PLS model derived from the leave-one-out cross-validation procedure with spectra $i$ omitted for PLS models constructed with the bucketed $^1$H NMR spectra as the X-table and the PFOS exposure concentrations as the Y variable. The solid line indicates a linear regression between the actual and predicted values. The PLS-regression models correspond to (A) two days of exposure, (B) seven days of exposure, and (C) fourteen days of exposure. The error bars represent the standard error of the mean.
6.4.2 Metabolic changes in response to PFOS exposure

PCA loadings plots were constructed to determine the metabolites that were responsible for the separation between the controls and PFOS exposed earthworms in the PCA scores plots (Supplementary Material, Figs S6-1 and S6-2). In addition, t-test filtered $^1$H NMR difference spectra were also constructed in an exploratory capacity to identify metabolites that increased or decreased significantly (at $\alpha = 0.05$) relative to the controls (Supplementary Material, Figs S6-4 to S6-6) [76-77, 136]. The PCA loadings plots and the t-test filtered $^1$H NMR difference spectra identified the sugars maltose ($\delta = 5.41$ ppm) and glucose/maltose ($\delta = 5.23$ ppm), Krebs cycle intermediates succinate ($\delta = 2.39$ ppm), fumarate ($\delta = 6.51$ ppm) and malate ($\delta = 2.37$ ppm), amino acids leucine ($\delta = 0.95$ ppm), valine ($\delta = 1.03$ ppm), alanine ($\delta = 1.47$ ppm), arginine ($\delta = 1.91$ ppm), glutamate ($\delta = 2.35$ ppm), lysine ($\delta = 3.01$ ppm), glycine ($\delta = 3.55$ ppm) and phenylalanine ($\delta = 7.31$ ppm), messenger molecules scyllo-inositol ($\delta = 3.35$ ppm) and myo-inositol ($\delta = 4.05$ ppm), the osmolyte betaine ($\delta = 3.25$ ppm), the energy molecule adenosine triphosphate (ATP; $\delta = 8.23$ ppm), short-chain fatty acids ($\delta = 1.27$ ppm for -CH$_2$ and $\delta = 0.83$ ppm for -CH$_3$) and 2-hexyl-5-ethyl-3-furansulfonate (HEFS; $\delta = 1.27$ ppm for -CH$_2$, $\delta = 0.83$ and $\delta = 1.17$ ppm for -CH$_3$, $\delta = 6.17$ ppm for -CH from furan ring) as the metabolites that significantly fluctuated in their concentrations in response to PFOS exposure (Supplementary Material, Figs S6-1, S6-2 and S6-4 to S6-6). Both the loadings plots and the difference spectra identified that the $\delta = 3.40$-4.00 ppm region of $^1$H NMR spectra, which contains overlapping resonances from sugars and amino acids, increased or decreased in response to PFOS exposure. However, these signals cannot be clearly assigned to individual metabolites due to overlapping resonances within
this region. The loadings plots for PC1 and PC2 (Supplementary Material, Figs S6-1A, S6-1C and S6-1E) for the two, seven and fourteen days of exposure illustrated that betaine and HEFS were the major contributors to the variation in the metabolic response, and had the greatest influence on the separation observed between the controls and the exposed earthworms in the PCA scores plots (Figs 6-1A, 6-1C and 6-1E). The PC1 vs PC2 scores plots didn’t reveal any concentration-dependent separations for any of the exposure lengths, suggesting that the fluctuations in betaine and HEFS are also exposure concentration independent. The PC3 and PC4 loadings plots for the two, seven and fourteen-day exposures showed that scyllo-inositol, alanine, glutamate and leucine also had substantial contributions to the metabolic variation in addition to betaine and HEFS (Supplementary Material, Figs S6-1B, S6-1D and S6-1F). It was also interesting to note that the decreased contributions of HEFS and betaine in the PC3 and PC4 loadings plots for the seven and fourteen day exposures were correlated with better concentration-dependent separations from the controls in the PC3 vs PC4 scores plots (Figs 6-1D and 6-1F) compared to the PC1 vs PC2 scores plots (Figs 6-1C and 6-1E). This illustrated that the responses of the metabolites other than betaine and HEFS to PFOS exposure is largely responsible for the observed concentration-dependent patterns in the PC3 vs PC4 scores plots for the seven and fourteen day exposures.

The percent changes in discernible metabolite bucket intensities were examined to determine the fluctuations after PFOS exposure and also to delineate the MOA (Figs 6-4 to 6-6) [77, 86, 217, 249]. In general, the percent changes in the metabolite concentrations relative to the controls did not reveal any consistent concentration-dependent patterns after two, seven or
Figure 6-4. Percent (%) change in selected metabolites of two-day PFOS exposed tissue extracts compared with the control earthworms. The % changes in the intensity of *Eisenia fetida* metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at $\alpha = 0.05$) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding $P$-values. The percent changes are shown with their associated standard error.
fourteen days of exposure. This was in contrast to what we observed in a previous study in which *E. fetida* were exposed to PFOS via filter paper contact test after two days of exposure [313]. This difference in the observed response of *E. fetida* between the contact and soil exposure studies reveals the complexity in the exposure routes of contaminants in soil as compared to a simple filter paper contact test. In the present study, leucine, valine, lysine, phenylalanine and arginine revealed significant (at $\alpha=0.05$) increases after two days of soil exposure to PFOS (Fig. 6-4). After seven and fourteen days of exposure the above-mentioned amino acids showed varying responses (Figs 6-5 and 6-6). In our previous study, leucine, valine, lysine, phenylalanine and arginine decreased significantly (at $\alpha=0.05$) at all exposure concentrations in the contact filter paper test [313]. This was attributed to the production of enzymes involved in fatty acid oxidation, which would have resulted in a decrease in these free amino acids [313]. Binding of PFOS to the peroxisome proliferator activated receptor alpha (PPAR$\alpha$), a mammalian nuclear hormone receptor involved in lipid and lipoprotein metabolism, results in increased peroxisome production and a heightened oxidation of fatty acids [203, 207]. The nuclear hormone receptor involved in lipid metabolism has not been identified in *E. fetida*. However, the nuclear hormone receptor-49 (*nhr*-49) is involved in regulating lipid metabolism in the nematode worm *Caenorhabditis elegans* much like PPAR$\alpha$ in mammals [293-294]. Therefore, our results led to the hypothesis that *E. fetida* also possesses nuclear hormone receptors similar to the *nhr*-49 that may be activated by the binding of PFOS, leading to an initiation of fatty acid oxidation and the subsequent decrease in amino acids due to the production of enzymes involved in $\beta$-oxidation [313]. Analyzing the percent changes in these amino acids over the various exposure lengths tested in this study revealed that as the exposure
Figure 6-5. Percent (%) change in selected metabolites of seven-day PFOS exposed *Eisenia fetida* tissue extracts compared with the control earthworms. The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at $\alpha = 0.05$) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding $P$-values. The percent changes are shown with their associated standard error.
time increased the amino acid concentrations generally tend to decrease in the exposed earthworms relative to the controls (Figs 6-4 to 6-6). The percent increase in these amino acids that was observed after two days of exposure is similar to what was observed in *E. fetida* after phenanthrene exposure [118, 277]. Exposure to phenanthrene elicits a non-polar narcosis type mechanism [118, 144, 256]. PFOS due to its surfactant-like properties disrupts biological membrane structure [298, 305-306]. The osmolyte betaine, and HEFS, a compound that is specific to earthworms and has been postulated to be involved in membrane stabilization [86, 90], also significantly decreased after two days of exposure (Fig. 6-4). In addition, betaine and HEFS dominated the PC1 and PC2 loadings plot for the two-day exposure (Supplementary Material, Fig. S6-1A) and therefore contributed the most to the clear separation observed between the controls and exposed earthworms in the PC1 vs PC2 scores plot (Fig. 6-1A). The significant fluctuations in betaine and HEFS after PFOS exposure may be as a response to counteract the disruption in the membrane structure brought about by PFOS. Therefore, we hypothesize that after two-days of PFOS exposure in soil there is a non-polar narcosis type MOA in *E. fetida*. The general decrease in these amino acids with exposure times > 2 days was similar to what was observed with our previous study involving contact filter paper tests [313]. This suggests that fatty acid oxidation may have been initiated by PFOS with longer exposures.

In our previous study, we observed that PFOS exposure resulted in a significant (at $\alpha=0.05$) concentration-dependent decrease of ATP (the energy currency of the cell) relative to the controls [313]. This was ascribed to the interruption of ATP synthesis by PFOS disrupting the structure of the inner mitochondrial membrane, thereby increasing its permeability and altering the proton ($H^+$) gradient required for the functioning of the ATP synthase enzyme [297-298]. However, ATP concentrations increased relative to the controls after phenanthrene exposure to
E. fetida via contact tests as was observed after the two-day and seven-day exposures in our current study [249, 277, 288]. As the exposure time increased to fourteen days ATP significantly decreased relative to the controls in the PFOS exposed earthworms (Fig. 6-6). Therefore, the disruption of the inner mitochondrial membrane structure by PFOS and the consequent altering of the proton (H\(^+\)) gradient and interruption of ATP synthesis may only take place after PFOS exposure that is longer than seven-days in soil. The sugars maltose and glucose/maltose generally increased significantly (at \(\alpha=0.05\)) relative to the controls at PFOS exposure concentrations \(\leq 50 \text{ mg/kg}\) for the two and seven-day exposures (Figs 6-4 and 6-5). After fourteen days of exposure maltose and glucose/maltose generally decreased relative to the controls, with significant (at \(\alpha=0.05\)) decreases at the 50 and 100 mg/kg PFOS exposure concentrations for glucose/maltose (Fig. 6-6). We also observed significant decreases in maltose and glucose/maltose after PFOS exposure in contact tests [313]. Maltose also decreased significantly (at \(\alpha=0.05\)) after phenanthrene exposure in both contact and soil exposure tests [117, 249, 277, 288]. The decrease in maltose and glucose was attributed to the increase in glycolysis due to an enhanced energy requirement brought about by the organisms attempt to counteract the toxicity of the xenobiotic. In this study, the significant increases in maltose and glucose/maltose were also correlated with a significant increase in ATP (Figs 6-4 to 6-6). This may be due to a feedback loop, which restricts glycolysis due to the accumulation of ATP [250]. As exposure time increased to fourteen days and ATP concentrations began to decrease significantly, probably due to a disruption of ATP synthase function, glycolysis is enhanced and maltose and glucose begin to decrease significantly.
Figure 6-6. Percent (%) change in selected metabolites of fourteen-day PFOS exposed *Eisenia fetida* tissue extracts compared with the control earthworms. The % changes in the intensity of metabolite peaks of exposed earthworms relative to the controls were obtained by first subtracting the buckets that pertain to the metabolites in the control earthworms from the exposed earthworms for each exposure concentration and then dividing again by the buckets in the control earthworms [76-77]. The percent changes that were significantly (at α =0.05) different from the control [based on a t-test (two tailed, equal variances) of control vs. exposed] are labeled with an asterisk (*) and also show the corresponding P-values. The percent changes are shown with their associated standard error.
Our previous contact test study identified significant (at $\alpha=0.05$) increases in both succinate and malate in response to PFOS exposure, but we did not observe any significant changes in fumarate concentrations [313]. Although, the percent changes in these Krebs cycle intermediates didn’t reveal any clear patterns in the present study (Figs 6-4 to 6-6), the significant (at $\alpha=0.05$) changes that were observed may reflect the altered expression of genes involved in producing enzymes for the Krebs cycle that were reported in previous studies of PFOS exposure to rats and humans [300-302]. Glutamate only decreased significantly at the 25 mg/kg exposure concentration after two days of exposure (Fig. 6-4). After seven days of exposure glutamate generally increased significantly (at $\alpha=0.05$) relative to the controls (Fig. 6-5), whilst fourteen days of exposure didn’t illustrate any significant changes in glutamate concentrations relative to controls (Fig. 6-6). In our previous study, glutamate was shown to increase significantly after PFOS exposure for two days via contact tests [313]. A disruption of the regular functioning of the Krebs cycle due to PFOS exposure was accredited as a possible reason for an increased conversion of $\alpha$-ketoglutarate (a Krebs cycle intermediate) to glutamate via the glutamate dehydrogenase enzyme [250]. Interestingly, the significant accumulation of fumarate after seven days of exposure was correlated with a significant increase in glutamate, suggesting a possible feedback mechanism resulting in an increased conversion of $\alpha$-ketoglutarate to glutamate (Fig. 6-6).

The concentrations of the inositol isomers (myo and scyllo-) didn’t reveal consistent trends in their fluctuations to PFOS exposure but did show significant (at $\alpha=0.05$) increases for some exposure concentrations after the seven and fourteen days of exposure (Figs 6-4 to 6-6). Significant (at $\alpha=0.05$) increases in glycine were only observed at the 150 mg/kg exposure
concentration after two days of exposure and at the 25 mg/kg exposure concentration after the fourteen days of exposure (Figs 6-4 to 6-6). The inositol isomers and glycine function as osmolytes and the significant increases observed may be as a means to cope with the changes in osmotic pressure brought about by a disruption in the membrane structure due to the surfactant-like properties of PFOS [265-266, 318].

6.5 Conclusion

Our study suggests that $^1$H NMR-based metabolomics was able to distinguish between the responses of PFOS exposed and control (unexposed) earthworms at sub-lethal or very low exposure concentrations. Multivariate statistical analysis identified that the longer exposures of seven and fourteen days of exposure revealed a concentration-dependent metabolic response. A comparison of E. fetida responses to PFOS exposure in soil in this study and to PFOS exposure in contact tests in our previous study [313] shows that contact tests elicited much more significant and consistent responses after two days of exposure. The MOA identified from soil exposure also appears to be more complex because we initially observed a non-polar narcosis type mechanism after two days of exposure and then observed an increase in fatty acid oxidation after seven and fourteen days. In contrast, we observed increased fatty acid oxidation in contact tests after only two days. This comparison illustrates that the modes of exposure of PFOS in soil and contact tests are clearly different. Sorption of PFOS to soil [319] may result in a decrease in its availability to the earthworms. Also, the movement of earthworms within the soil, compared to E. fetida being placed on a filter paper applied with PFOS in contact tests, may decrease the amount of exposure through limited bioavailability in soil. Therefore, these results suggest that although contact tests can be used as a rapid method for determining the responses of earthworms to contaminants, soil exposure tests are required for an accurate assessment of the
MOA. However, previous studies that exposed *E. fetida* to phenanthrene via contact and soil exposures observed similar MOAs by both methods after two days of exposure [113, 117]. Hence, response of *E. fetida* to contact and soil exposure routes seems to be contaminant specific. Increased fatty acid oxidation and disruption of biological membranes that were observed due to PFOS exposure conform to the hypothesized MOA [200, 202, 297-298]. 

¹H NMR-based metabolomics appears to be a more sensitive indicator of PFOS exposure than the traditional mortality tests and reproduction tests which identified 77 mg/kg and 10 mg/kg respectively as the no observable effect concentrations [210, 312]. We also observed significant responses at the lower exposure of 5 mg/kg, which was below the residential soil screening level for PFOS (6 mg/kg) set by the US EPA [314]. Our study highlights the potential for NMR-based metabolomics to be used as a routine tool in ecotoxicological assessment of contaminated sites.

### 6.6 Acknowledgements

Funding was provided by the Natural Sciences and Engineering Research Council (NSERC). BPL would like to thank the government of Ontario for a post-graduate scholarship. We would like to extend thanks to Jimmy Yuk, Edward Nagato and Chon Chio for technical assistance and valuable discussions.
Chapter Seven

Conclusions and Future Research
7.1 Conclusions

The continual production of new chemicals as a means to cope with the needs of a growing global population has raised the importance of the field ecotoxicology in assessing the risks associated with exposure to these chemicals [6-8]. Chemical legislations such as the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) and the High Production Volume (HPV) Challenge have set very strict guidelines that require a large number of toxicological data in order for manufacturers and importers to register high production volume chemicals [7-8, 11-12]. Most of the current methods used to gather ecotoxicological data are based on whole-animal exposures and the resulting adverse responses in survival, growth, and reproduction, which are lengthy and require a lot of resources [6]. Therefore, it is going to take decades for all the required testing to be conducted for the high production volume chemicals [7]. Hence, there is a need to develop ecotoxicological tools that are less time consuming, more efficient and less resource intensive. Toxicological tests that identify the mode of action (MOA) of chemicals will enable a more focused resource utilization and reduced uncertainty in regulatory decision making because the designing of the toxicity tests and the interpretation of the results will be less ambiguous [6]. Also, toxicological tests that can detect metabolite biomarkers of exposure are advantageous because they can serve as early warning signals for chemical exposure and have the potential to delineate the MOAs of chemicals as well [20]. Environmental metabolomics has shown promise as a technique that can be used to elucidate the MOA of chemicals and also identify metabolite biomarkers of exposure [25]. Research presented in this thesis illustrates the potential of $^1$H NMR-based metabolomics as a rapid and routine ecotoxicological tool in assessing the state of soil toxicity.
In this thesis we focused on the model polycyclic aromatic hydrocarbon (PAH), phenanthrene [191-192], and the perfluorinated alkyl acids (PFAAs), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) [173-174, 199-200], due to their recalcitrant nature and widespread prevalence in soil environments. *Eisenia fetida* earthworms were chosen, as they are the recommended species for soil toxicological testing by the organization for economic cooperation and development (OECD) [159]. We identified an exposure concentration-dependent two-phased MOA for sub-lethal phenanthrene exposure (Chapter 2): below 1/16\(^{th}\) of the LC\(_{50}\) (concentration that causes 50\% mortality in a population), a linear correlation between the metabolic response and exposure concentration was identified. At exposures \(\geq 1/16^{th}\) of the LC\(_{50}\), the metabolic response reached a plateau, indicating a distinct change in the MOA. We also observed a phenanthrene exposure time-dependent increase in the metabolic response of *E. fetida* to sub-lethal phenanthrene exposure (Chapter 3). Therefore, our first hypothesis that proton (\(^{1}\)H) nuclear magnetic resonance (NMR)-based metabolomics was capable delineating concentration and time-dependent relationships was confirmed. The response of the non-polar metabolites of *E. fetida* was analyzed for the first time (Chapters 3 and 4). The decrease in fatty acids in response to phenanthrene exposure suggested a heightened energy demand, which was also confirmed by the decrease in the sugar maltose and the increase in free amino acids. However, our second hypothesis that phenanthrene exposure will elicit significant fluctuations in the non-polar metabolites was not confirmed because even at exposure concentrations as high as 1/4\(^{th}\) of the LC\(_{50}\) a consistent significant response was not observed even with longer exposures (Chapter 3). Therefore, we suggested that since the polar metabolites were more sensitive to phenanthrene exposure future studies should focus only on the polar fraction for a high-throughput analysis (Chapter 3). Alanine, glutamate, maltose,
cholesterol and phosphatidylcholine emerged as potential indicators of phenanthrene exposure. In addition to an increase in energy demand, the MOA of phenanthrene also seems to interrupt the conversion of succinate to fumarate in the Krebs cycle possibly due to a disruption in the inner mitochondrial membrane structure (Chapters 3 and 4) [257]. Overall, we observed that exposure time became more important than exposure concentration in discriminating between control and exposed earthworms as exposure time increased. This was probably due to a saturation of the enzymes involved in detoxification by an accumulation of phenanthrene and its metabolites [320] (Chapter 4). Our results indicated for the first time that metabolomics is capable of detecting organism responses to PFOA and PFOS exposure (Chapters 5 and 6). Heightened \textit{E. fetida} responses were observed with higher PFOA and PFOS concentrations confirming again our first hypothesis that $^1$H NMR-based metabolomics can delineate concentration-dependent relationships. Leucine, arginine, glutamate, maltose, and ATP were identified as potential indicators of PFOA or PFOS exposure. The metabolic response of \textit{E. fetida} to PFOA and PFOS exposure was the same confirming part of our third hypothesis that these two PFAAs will have similar MOAs in \textit{E. fetida} (Chapter 5). In order to compare the responses of \textit{E. fetida} between filter paper contact tests and artificial soil exposure responses and to explore concentration and time-dependent responses we exposed \textit{E. fetida} for two, seven and fourteen days to an artificial soil that was spiked with sub-lethal PFOS concentrations. We identified an exposure time-dependent operation of two separate MOAs: a non-polar narcosis type mechanism after two days and an MOA which suggested increased fatty acid oxidation following the seven and fourteen days of exposure. 2-Hexyl-5-ethyl-3-furansulfonate (HEFS), betaine, leucine, arginine, glutamate, maltose, and adenosine-5’-triphosphate (ATP) were identified as potential indicators of PFOS exposure in soil. Both the contact tests and artificial
soil exposure studies identified an elevation in fatty acid oxidation, a disruption in energy metabolism and biological membrane structure, and also a possible interruption of ATP synthesis following PFOA and PFOS exposure (Chapters 5 and 6). This confirmed the first part of our third hypothesis that an increased oxidation of fatty acids will be observed in *E. fetida* as was seen in mammals. We have illustrated through this thesis that NMR-based metabolomics has great promise as a routine tool for ecotoxicological assessment of contaminated sites due to its capacity to elucidate the MOA of chemicals and also identify metabolite biomarkers of exposure.

This thesis was limited in that it focused only on the development and use of $^1$H NMR-based metabolomics to analyze responses of *E. fetida* earthworms after sub-lethal exposure to the model PAH, phenanthrene and the PFAAs, PFOS and PFOA. However, in addition to other PAHs and PFAAs, there are also a large number of emerging contaminants, such as brominated flame retardants, pharmaceuticals and personal care products, synthetic estrogens, hormones, other fluorinated chemicals, chlorinated byproducts, engineered nanoparticles and manufactured antimicrobial products [2], which were not analyzed in this thesis. Utilizing NMR-based metabolomics to detect the metabolic responses to the aforementioned classes of chemicals can greatly aid in determining the potential of NMR-based metabolomics in elucidating the MOA of these different chemicals and also identifying metabolite biomarkers that are unique to each class of chemicals. We were also limited in that we only used NMR and did not use mass spectrometry (MS) -based methodologies to further enhance the characterization of the responses of *E. fetida*. MS, with its greater sensitivity has the potential to identify a greater number of metabolites [27, 100]. Hence, future studies should investigate the advantageous of using MS, either coupled to gas chromatography (GC), liquid chromatography (LC), or capillary
electrophoresis (CE), in a complementary manner to NMR in analyzing the responses of *E. fetida* to phenanthrene, PFOS and PFOA. This thesis was also limited because the hypotheses generated for possible MOAs of the contaminants tested weren’t verified using either biochemical assays or other omics technologies. Future studies can greatly benefit by combining multiple omics technologies to verify the MOAs postulated by metabolomics. Finally, this thesis did not analyze the responses of earthworms exposed to contaminated field soils. Contaminated field soils may have confounding variables such as the presence of other chemicals, degradation products, and also decreased availability of contaminants due to their binding to soil organic matter to name a few. Therefore, to accurately assess the capability of metabolomics as a routine tool in ecotoxicological soil testing, exposure studies of earthworms to contaminated field soils are vital.

7.2 Future Research

7.2.1 Quantitative biological activity relationships (QBARs)

The guidelines set by REACH and HPV require a large number of toxicological data in order for manufacturers and importers to register chemicals with high production volumes [7-8, 11-12]. However, both these legislations insist that vertebrate animal testing should be done only as a last resort and that QSARs and *in vitro* testing should be used as possible alternatives [8, 13-14]. However, not much progress has been made in designing and implementing validated and regulatory acceptable alternative methods [7, 37, 321]. Therefore, animal testing will increase significantly to meet the demands of REACH and HPV [6, 37]. Some estimates of the number of animals that are needed to obtain the required toxicological data are well over 50 million [322]. Hence, there is an urgent need to develop alternative methods in an attempt to
decrease animal testing and also to decrease the time needed to finish the toxicological evaluation of the chemicals in a timely manner. One possible way to decrease the number of animal testing, whilst providing the required safety information is to group the chemicals based on a particular property and then read across from data rich chemicals belonging to the same group [37]. Initially, the grouping of chemicals was based on QSAR; however these groupings tend not to accurately predict the toxicity of chemicals in the same group consistently [37]. For example, van Ravenzwaay et al. [37] revealed that 2-acetylaminofluorene (2-AAF) and 4-acetylaminofluorene (4-AAF), which are categorized into the same group based on QSAR have very different toxic MOAs. They suggest an alternative to QSAR by introducing the concept of quantitative biological activity relationship (QBAR). Under QBAR the grouping of chemicals is based on the biological activity of the chemicals, which is related to their MOA. van Ravenzwaay et al. [37] illustrated the development of a metabolomics database based on the metabolic responses of rats exposed to several test substances which are known to induce toxicity to two target organs, the liver and thyroid, by sampling the blood after 7, 14 and 28 days of exposure. They established distinct metabolome patterns indicative of liver and thyroid toxicity. Afterwards, they compared the metabolic response of the rats exposed to various chemicals to the established database and were able to identify the MOA of those chemicals. This work demonstrates the huge potential of metabolomics in ecotoxicology because these metabolomics databases can be established based on in vitro tests as well, reducing the number of animal testing that is required, whilst enabling the elucidation of the MOA of chemicals. It also makes evaluation of the toxicities of novel compounds easier because the metabolic profiles from the novel compounds can be compared to the already known MOAs in the database enabling the identification of potential toxic pathways [6, 17-18]. This also reduces the
uncertainty in predicting synergistic and additive effects of chemical mixtures, because compounds with similar versus dissimilar MOAs can be grouped together, enabling better expectations of mixture toxicity [6]. Therefore, shifting from QSAR based groupings of chemicals to QBAR based groupings by utilizing metabolomics shows great potential in providing rapid and reliable safety information required by REACH and HPV, whilst decreasing the need for animal testing.

7.2.2 Using multiple analytical platforms

As mentioned previously in this chapter, this thesis was focused on the utilization of $^1$H NMR-metabolomics to analyze the responses of *E. fetida* to phenanthrene, PFOA and PFOS. Most studies in metabolomics have also focused on a single analytical platform [38, 90, 133, 136, 164, 323]. However, future studies should combine MS methodologies along with NMR to better characterize the metabolic response of *E. fetida*. Although MS requires lengthy analysis times, is destructive, is selective towards certain analytes, and is subject to matrix effects, its high sensitivity enables the potential to identify a greater number of metabolites [27, 100]. Especially, MS seems to be much better at characterizing the responses in the non-polar metabolites than NMR [80, 120, 131, 144]. For example, Jones et al. [144] investigated the response of *L. rubellus* earthworms to pyrene exposure and analyzed the polar fraction from the earthworm tissue extraction using both $^1$H NMR and GC-MS, whilst the non-polar fraction was analyzed using only GC-MS. The $^1$H NMR identified a greater number of polar metabolites compared to GC-MS. However, GC-MS was able to detect many non-polar metabolites such as various fatty acids and sterols that was more comprehensive than the detection of non-polar metabolites by $^1$H NMR illustrated in Chapters 3 [277] and 4 [288], and also by Ekman et al.
and Rochfort et al. [91]. Similarly, Taylor et al. [80] and Poynton et al. [131] also showed using fourier transform ion cyclotron resonance (FT-ICR) MS to analyze D. magna responses to various contaminants that non-polar metabolites were characterized well by MS. Beltran et al. [133] recently released a paper in which they advocate the use of both NMR and LC-MS methods in a complementary manner to analyze organism responses to external stressors. They argue that liquid chromatography LC-MS with its higher sensitivity can detect metabolites that are at concentrations too low to be detected by NMR and on the other hand NMR can easily detect polar metabolites such as glucose or lactic acid which are not readily ionized by electrospray ionization or not retained using reverse-phase chromatography. They also propose a tissue extraction protocol using a combination of methanol, chloroform and water that is compatible with NMR and LC-MS. Therefore, in order to progress metabolomics as a comprehensive analysis of the metabolite responses of organisms subjected to an external stressor, an inclusion of both NMR and MS based methods in a complementary fashion can be very beneficial. However, the potential cons of using multiple analytical platforms will be due to higher costs from simply using two expensive analytical instruments and also by hiring experts in both NMR and MS, and also a decrease in the high-throughput nature of the analysis due to the time required for sample prep, instrumental analysis and data mining for both instruments [133]. Since, as mentioned above NMR is good for detecting polar metabolic responses whilst MS is better for characterizing the responses of non-polar metabolites, one way to decrease the cost and analysis time associated with using multiple analytical platforms will be to use $^1$H NMR strictly for the polar metabolites and the MS based methods for the non-polar metabolites. Also, as mentioned in section 1.2.2, MS is ideally suited for targeted metabolomics and NMR is very good for non-targeted metabolomics. Therefore, another possible way to use NMR and MS in a
complementary manner whilst cutting costs and analysis time can be to use NMR to conduct non-targeted analysis of the metabolic response to generate hypotheses of possible MOAs and identify potential biomarkers of exposure. Then MS can be used to target only those metabolites that were identified as biomarkers and monitor their fluctuations to determine their responses to the exposure of contaminants.

7.2.3 Systems Toxicology approach

One of the biggest assets of metabolomics is its comprehensive and “open” nature of analysis, which allows for the detection of unexpected relationships between the metabolic response and the external stressor [25]. This allows metabolomics to be a hypothesis generating machine, which can potentially help unravel the underlying MOAs for previously unexplained responses to environmental stressors [25, 54, 83, 91, 324]. However, once potential MOAs have been identified they also need to be verified. Therefore, further tests can be conducted that utilize the other omics technologies to confirm the hypotheses generated by metabolomics. These studies that integrate multiple omics platforms have been tagged as ‘systems toxicology’ and are widely used in studies that investigate drug toxicity [325-327], but hasn’t been used much in ecotoxicology. Bundy et al. [328] conducted a study in which the response of the earthworm _L. rubellus_ to copper exposure was analyzed using both $^1$H NMR-based metabolomics and cDNA transcript microarrays. The study illustrated that both metabolomics and microarray analysis illustrated a disruption in energy metabolism: an increase in the transcripts of carbohydrate metabolizing enzymes was correlated to decreases in sugars such as glucose and mannose. Therefore, they were able to confirm that copper exposure to _L. rubellus_ disrupts energy production. Similarly, future studies that combine metabolomics with other
omics methodologies can greatly aid in elucidating and confirming unknown MOAs of a wide variety of environmental stressors. The great benefit of using a comprehensive technique such as metabolomics as a tool to delineate the MOA of novel external stressors is that it is able to direct the researcher to a single or a set of potential MOAs so that future tests to verify the toxic mechanisms can be designed to focus on those pathways saving both time and money. However, the potential hurdle to implementing a systems toxicology approach is the fact that costs will be higher due to the need for experts in multiple omics platforms and also special equipment requirements for each methodology.

In summary, the proposed future studies describe methods that can propel metabolomics into a routine tool for MOA elucidation and biomarker discovery of new and existing chemicals. Combining the non-selectivity and analytical resolving power of NMR with the sensitivity of MS can greatly enhance the characterization of the metabolic responses of organisms. This information can benefit the development of metabolic response databases for QBAR-based grouping of chemicals as well. Developing QBARs by using information from metabolomics studies will not only decrease the amount of animal testing but also lessen the time required to obtain the safety information required by legislations such as REACH and HPV to register high production volume chemicals. Once potential MOAs have been identified they can be verified using a systems toxicology approach that utilizes multiple omics platforms.
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Appendix A

Supplementary Material for Chapter Two


A2-1 Earthworm Maintenance Prior to Contact Tests

*E. fetida* were purchased from The Worm Factory (ON, Canada). They were raised in earthworm bins containing sphagnum peat bedding (Magic Worm bedding; Magic Products; WI, USA) at an approximate temperature of 24°C. The moisture content of the bedding was maintained at 67% (by weight). The earthworms were acclimated for several months to decrease variations in the $^1$H NMR profile due to differences in diet and other environmental factors [1]. The worms were fed Magic Worm Food (Magic Products; WI, USA). Mature earthworms, indicated by a visible clitellum, were selected and depurated in groups of 10 in the dark for 96 hours in 500 ml glass jars containing moist Whatman 4 Qualitative filter papers with a diameter of 9 cm (Fisher Scientific) to empty their intestinal tracts [1]. The earthworms had an average mass of 0.62 g (±0.15 g; standard deviation) after depuration. The earthworms were then transferred to individual 120 ml amber glass jars containing pre-treated Whatman GF/A 4.70 cm diameter glass filter paper (Fisher Scientific).

A2-2 Calculation of $Q^2_Y$ of Partial Least Squares Regression Models

The PLS model was fully cross-validated using leave-one-out cross-validation (LOOCV) [2,3]. In each iteration of LOOCV one sample (validation data) from the set of samples (the training set) that were used to build the PLS model is left out of the model, forming a new model. The $x$-value of the validation data is then used to predict the $y$-value ($\hat{y}$). The predicted value is then subtracted from the known value ($y$) and a residual is obtained. This procedure is repeated until all of the samples in the training set are left out once. The $Q^2_Y$ is then calculated according to the following equation [2]:

\[ Q^2_Y = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum y_i^2} \]
\[ Q^2_Y = 1 - \frac{\sum (y_i - \hat{y}_i)^2}{\sum (y_i - \bar{y})^2} \]

Where, \( \hat{y}_i \) is the predicted value of \( y_i \) of given sample \( i \) by the PLS model derived during LOOCV in which sample \( i \) was omitted from the model as the validation data. \( \bar{y} \) is the mean value of \( y \) for all of the samples.

**A2-3 References**


Figure A2-1. PLS-DA scores plots of T1 (first PLS component) versus T2 (second PLS component) for $^1$H NMR spectra of *E. fetida* aqueous buffer tissue extracts showing the separation of control worms (□) from exposed worms (○) at phenanthrene concentrations of (A) 0.025 mg/cm$^2$ (1/64$^{th}$ of LC$_{50}$), (B) 0.05 mg/cm$^2$ (1/32$^{th}$ of LC$_{50}$), (C) 0.10 mg/cm$^2$ (1/16$^{th}$ of LC$_{50}$), (D) 0.20 mg/cm$^2$ (1/8$^{th}$ of LC$_{50}$), (E) 0.40 mg/cm$^2$ (1/4$^{th}$ of LC$_{50}$), and (F) 0.80 mg/cm$^2$ (1/2 of LC$_{50}$). The *P*-values were obtained from t-tests that compared the scores of the control and exposed worms for each component. *Three phenanthrene exposed earthworms were identified as outliers in the DModY plot and removed from the model.
Appendix B

Supplementary Material for Chapter Three


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S3-1 Earthworm Maintenance Prior to Contact Tests.

*E. fetida* were purchased from The Worm Factory (ON, Canada). They were raised in earthworm bins containing sphagnum peat bedding (Magic Worm bedding; Magic Products; WI, USA) at an approximate temperature of 24°C. The moisture content of the bedding is 67% water by weight. The earthworms were acclimated for several months to decrease variations in the $^1$H NMR profile due to differences in diet and other environmental factors [1]. The worms were fed Magic Worm Food (Magic Products; WI, USA). Mature earthworms, indicated by a visible clitellum were chosen and depurated in groups of 10 in the dark for 96 hours in 500 ml glass jars containing moist Whatman 4 Qualitative filter papers with a diameter of 9 cm (Fisher Scientific) to empty their intestinal tracts [1]. The earthworms had an average mass of 0.47 g ($\pm$0.08 g; standard deviation) after depuration. The earthworms were then transferred to individual 120 ml amber glass jars containing pre-treated Whatman GF/A 4.70 cm diameter glass filter paper (Fisher Scientific).

S3-2 References

Figure S3-1. PCA scores plots of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the polar fraction of the *E. fetida* tissue extracts showing the separation of control worms (□) from exposed worms (○) after exposure to phenanthrene for (A) one day, (B) two days, (C) three days, and (D) four days. The *P*-values were obtained from t-tests that compared the scores of the control and exposed worms for each component.
Figure S3-2. PCA scores plots of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the non-polar fraction of the *E. fetida* tissue extracts showing the separation of control worms (□) from exposed worms (○) after exposure to phenanthrene for (A) one day, (B) two days, (C) three days, and (D) four days. The *P*-values were obtained from t-tests that compared the scores of the control and exposed worms for each component.
Figure S3-3. Loadings plot for PC1 showing the metabolites that were major contributors to the separation observed in the PCA scores plot for polar fraction of the *E. fetida* tissue extracts. The abscissa refers to the $^1$H NMR chemical shifts (ppm). (A) one day, (B) two days, (C) three days, and (D) four days of exposure.
Figure S3-4. Loadings plot for PC1 and PC2 showing the metabolites that were major contributors to the separation observed in the PCA scores plot for the non-polar fraction of the *E. fetida* tissue extracts. The abscissa refers to the $^1$H NMR chemical shifts (ppm). (A) one day, (B) two days (PC1), (B2) two days (PC2), (C) three days, (D1) four days (PC1) and (D2) four days (PC2) of exposure. For days two and four of exposure both the PC1 and PC2 loadings are shown because the PCA scores plot showed better separation ($P=0.01$ and $P=0.06$ respectively) from the controls along PC2.
Figure S3-5. T-test filtered $^1$H NMR difference spectra of the polar fraction of the *E. fetida* tissue extracts obtained by subtracting the mean buckets of the control worms from the mean buckets for the phenanthrene exposed worms for each day and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. (A) one day, (B) two days, (C) three days, and (D) four days of exposure.
Figure S3-6. T-test filtered $^1$H NMR difference spectra of the non-polar fraction of the *E. fetida* tissue extracts obtained by subtracting the mean buckets of the control worms from the mean buckets for the phenanthrene exposed worms for each day and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. (A) one day, (B) two days, (C) three days, and (D) four days of exposure.
Figure S3-7. Relative intensity of the Krebs cycle intermediates succinate, fumarate and malate as a function of time in the polar fraction of the unexposed (control) *E. fetida* tissue extracts. The relative intensities are shown with their associated standard error.
Figure S3-8. An illustration showing the possible mechanism by which the succinate dehydrogenase enzyme complex was inactivated due to binding of phenanthrene to the inner mitochondrial membrane. The % changes in the Krebs cycle intermediates succinate, fumarate and malate along with the energy molecule ATP relative to the control are shown with their associated standard errors.
Figure S3-9. The change in the relative intensity of alanine as a function of the length of exposure to phenanthrene in the polar fraction of the *E. fetida* tissue extracts. The correlation coefficient (R) and the *P*-value denoting the significance of the correlation between the intensity of alanine and exposure time were obtained by linear-regression.
Appendix C

Supplementary Material for Chapter Four


**S4-1 Earthworm Maintenance Prior to Contact Tests.**

*Eisenia fetida* were purchased from The Worm Factory (ON, Canada). They were raised in earthworm bins containing sphagnum peat bedding (Magic Worm bedding; Magic Products; WI, USA) at an approximate temperature of 24°C. The moisture content of the bedding is 67% water by weight. The earthworms were acclimated for several months to decrease variations in the $^1$H NMR profile due to differences in diet and other environmental factors [1]. The worms were fed Magic Worm Food (Magic Products; WI, USA). Mature earthworms, indicated by a visible clitellum were chosen and depurated in groups of 10 in the dark for 96 hours in 500 ml glass jars containing moist Whatman 4 Qualitative filter papers with a diameter of 9 cm (Fisher Scientific) to empty their intestinal tracts [1]. The earthworms had an average mass of 0.46 g (±0.09 g; standard deviation) after depuration. The earthworms were then transferred to individual 120 ml amber glass jars containing pre-treated Whatman GF/A 4.70 cm diameter glass filter paper (Fisher Scientific).

**S4-2 Partial Least Squares (PLS) –regression analysis**

A PLS-regression model was constructed to determine the relationship between the metabolic profile and phenanthrene concentration after each day of exposure [2, 3]. PLS –regression analyses were performed in R [4] using the Chemometrics package [5] on the binned $^1$H NMR spectra generated by the AMIX 3.8.4 statistics tool. PLS-regression was performed via the NIPALS PLS algorithm using phenanthrene concentration as the Y (response) matrix and the normalized bucket intensities from the $^1$H NMR spectra of all the earthworms as the X matrix of multiple predictors [2]. PLS models were cross validated using the leave-one-out cross validation [2, 6, 7] and the optimal number of components for each final PLS model was selected using the single cross validation (1CV) strategy described by
Westerhuis et al. [7]. The explained variation of X (R²X) and Y (R²Y) were obtained for each PLS model as a measure of how well the model fit the training data [3]. The cross validated R²Y value (denoted as Q²Y) was used as a preliminary measure of the predictive ability of the PLS model [2, 6]. Response permutation testing was also performed to estimate the significance of each PLS model [2, 3, 8]. This method consisted of keeping the X matrix (normalized binned ¹H NMR spectra) constant, while randomly permuting the order of the phenanthrene exposure concentrations (Y matrix) 400 times. A new PLS model was fitted and Q²Y calculated for each permutation. This provided a reference distribution of the Q²Y statistic. The significance of the PLS model and the confidence in its validity is increased if the PLS models built during the permutation tests consistently give lower Q²Y values than the original model [3].

Table S4-1 PLS-regression model parameters and output

<table>
<thead>
<tr>
<th>Day of Exposure</th>
<th>PLS-components</th>
<th>R²X</th>
<th>R²Y</th>
<th>Q²Y</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 – polar fraction</td>
<td>2</td>
<td>0.57</td>
<td>0.64</td>
<td>0.48</td>
<td>0.00004</td>
</tr>
<tr>
<td>Day 2 – polar fraction</td>
<td>6</td>
<td>0.85</td>
<td>0.76</td>
<td>0.48</td>
<td>0.0001</td>
</tr>
<tr>
<td>Day 1 – non-polar fraction</td>
<td>3</td>
<td>0.97</td>
<td>0.48</td>
<td>0.25</td>
<td>0.008</td>
</tr>
<tr>
<td>Day 2 – non-polar fraction</td>
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<td>0.97</td>
<td>0.54</td>
<td>-0.006</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* P-values were obtained from the normal distribution of the permuted Q²Y values obtained from a
S4-3 References


**Figure S4-1.** PCA scores plots of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the polar fraction of the *E. fetida* tissue extracts showing the separation of control worms (□) from exposed worms (○) after phenanthrene exposures of (A) 0.05 mg/cm$^2$ for one day, (B) 0.05 mg/cm$^2$ for two days, (C) 0.05 mg/cm$^2$ for three days, (D) 0.2 mg/cm$^2$ for one day, (E) 0.2 mg/cm$^2$ for two days, (F) 0.4 mg/cm$^2$ for one day, and (G) 0.4 mg/cm$^2$ for two days. The $P$-values were obtained from t-tests (two tailed, equal variances) that compared the scores of the control and exposed worms for each component.
**Figure S4-2.** PCA scores plots for the $^1$H NMR spectra of the non-polar fraction of the *E. fetida* tissue extracts showing the separation of control worms (□) from exposed worms (○) after exposure to phenanthrene. (A) PC1 vs PC2 for 0.05 mg/cm$^2$ after one day of exposure, (B) PC1 vs PC2 for 0.05 mg/cm$^2$ after two days of exposure, (C) PC1 vs PC2 for 0.05 mg/cm$^2$ after three days of exposure, (D) PC1 vs PC2 for 0.2 mg/cm$^2$ after one day of exposure, (E) PC2 vs PC3 for 0.2 mg/cm$^2$ after one day of exposure, (F) PC1 vs PC2 for 0.2 mg/cm$^2$ after two days of exposure, (G) PC1 vs PC2 for 0.4 mg/cm$^2$ after one day of exposure, (H) PC1 vs PC3 for 0.4 mg/cm$^2$ after one day of exposure, and (I) PC1 vs PC2 for 0.4 mg/cm$^2$ after two days of exposure. The $P$-values were obtained from t-tests (two tailed, equal variances) that compared the scores of the control and exposed worms for each component. The PC2 vs PC3 and PC1 vs PC3 scores plots for the 0.2 mg/cm$^2$ and 0.4 mg/cm$^2$ exposure concentrations respectively, are shown because there was significant separation between the exposed and control earthworms at these higher principal components.
Figure S4-3. Mean principal component analysis (PCA) scores plot of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the polar *E. fetida* tissue extracts comparing control (unexposed) earthworms and earthworms exposed to phenanthrene at concentrations of (A) 0.05 mg/cm$^2$ (B) 0.2 mg/cm$^2$ and (C) 0.4 mg/cm$^2$ after one, two and three days of exposure. The mean scores for the controls are denoted on the figure by a ‘C’ and the corresponding exposure day. The mean scores for the phenanthrene exposed earthworms are denoted by the exposure concentration followed by the corresponding exposure day. The ellipses shown in the figure were constructed as visual aids.
Figure S4-4. Mean principal component analysis (PCA) scores plot of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the polar *E. fetida* tissue extracts comparing control (unexposed) earthworms and earthworms exposed to 0.05 mg/cm$^2$, 0.2 mg/cm$^2$ and 0.4 mg/cm$^2$ of phenanthrene after (A) one and (B) two days of exposure. The mean scores for the controls are denoted in the figure by a ‘C’ and the corresponding exposure day. The mean scores for the phenanthrene exposed earthworms are denoted by the exposure concentration followed by the corresponding exposure day.
Figure S4-5. Mean principal component analysis (PCA) scores plot of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the non-polar *E. fetida* tissue extracts comparing control (unexposed) earthworms and earthworms exposed to phenanthrene at concentrations of (A) 0.05 mg/cm$^2$ (B) 0.2 mg/cm$^2$ and (C) 0.4 mg/cm$^2$ after one, two and three days of exposure. The mean scores for the controls are denoted on the figure by a ‘C’ and the corresponding exposure day. The mean scores for the phenanthrene exposed earthworms are denoted by the exposure concentration followed by the corresponding exposure day. The ellipse shown in the figure was constructed as a visual aid.
Figure S4-6. Mean principal component analysis (PCA) scores plot of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the non-polar *E. fetida* tissue extracts comparing control (unexposed) earthworms and earthworms exposed to 0.05 mg/cm$^2$, 0.2 mg/cm$^2$ and 0.4 mg/cm$^2$ of phenanthrene after (A) one and (B) two days of exposure. The mean scores for the controls are denoted on the figure by a ‘C’ and the corresponding exposure day. The mean scores for the phenanthrene exposed earthworms are denoted by the exposure concentration followed by the corresponding exposure day. The ellipse shown in the figure was constructed as a visual aid.
Figure S4-7. Loadings plot for PC1 and PC2 showing the metabolites that were major contributors to the separation observed in the PCA scores plot for the polar fraction of the *E. fetida* tissue extracts. The abscissa refers to the $^1$H NMR chemical shifts (ppm). (A) 0.05 mg/cm$^2$ after one day of exposure, (B) 0.05 mg/cm$^2$ after two days of exposure, (C) 0.05 mg/cm$^2$ after three days of exposure, (D) 0.2 mg/cm$^2$ after one day of exposure, (E) 0.2 mg/cm$^2$ after two days of exposure, (F) 0.4 mg/cm$^2$ after one day of exposure, (G) 0.4 mg/cm$^2$ after two days of exposure. PC2 loadings are shown only for treatments that showed significant separation between the exposed and control earthworms in the PCA scores plots at this higher principal component.
Figure S4-8. Loadings plot showing the metabolites that were major contributors to the separation observed in the PCA scores plot for the non-polar fraction of the *E. fetida* tissue extracts for the principal components indicated. The abscissa refers to the $^1$H NMR chemical shifts (ppm). (A) 0.05 mg/cm$^2$ after one day of exposure, (B) 0.05 mg/cm$^2$ after two days of exposure, (C) 0.05 mg/cm$^2$ after three days of exposure, (D) 0.2 mg/cm$^2$ after one day of exposure, (E) 0.2 mg/cm$^2$ after two days of exposure, (F) 0.4 mg/cm$^2$ after one day of exposure, (G) 0.4 mg/cm$^2$ after two days of exposure. PC3 loadings are shown only for treatments that showed significant separation between the exposed and control earthworms in the PCA scores plots at this higher principal component.
Figure S4-9. T-test filtered $^1$H NMR difference spectra of the polar fraction of the *E. fetida* tissue extracts obtained by subtracting the mean buckets of the control worms for each day from the mean buckets for the phenanthrene exposed worms and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. The t-test filtered $^1$H NMR difference spectra are shown after phenanthrene exposure of one day (A) 0.05 mg/cm$^2$, (B) 0.2 mg/cm$^2$, (C) 0.4 mg/cm$^2$, two days (D) 0.05 mg/cm$^2$, (E) 0.2 mg/cm$^2$, (F) 0.4 mg/cm$^2$, and three days (G) 0.05 mg/cm$^2$. 
Figure S4-10. T-test filtered $^1$H NMR difference spectra of the non-polar fraction of the E. fetida tissue extracts obtained by subtracting the mean buckets of the control worms for each day from the mean buckets for the phenanthrene exposed worms and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. The t-test filtered $^1$H NMR difference spectra are shown after phenanthrene exposure of one day (A) 0.05 mg/cm$^2$, (B) 0.2 mg/cm$^2$, (C) 0.4 mg/cm$^2$, two days (D) 0.05 mg/cm$^2$, (E) 0.2 mg/cm$^2$, (F) 0.4 mg/cm$^2$, and three days (G) 0.05 mg/cm$^2$. 
Figure S4-11. Average predictions of phenanthrene concentrations ($\hat{y}_i$) given spectra $i$ by the PLS model derived from the leave-one-out cross-validation procedure with spectra $i$ omitted for PLS models constructed with the bucketed $^1$H NMR spectra as the X-table and the phenanthrene exposure concentrations as the Y variable. The solid line indicates a linear regression between the actual and predicted values. The PLS-regression models correspond to both the polar fraction (A) one day of exposure, (B) two days of exposure and the non-polar fraction (C) one day of exposure, (D) two days of exposure. The error bars represent the standard error of the mean.
Figure S4-12. Histograms of PLS-regression $Q^2_Y$ values for cross-validated PLS models using the bucketed $^1$H NMR spectra as the X-table and random permutations of the phenanthrene exposure concentrations as the Y variable. Distributions were constructed using 400 permutations of the Y table. The histograms correspond to PLS models constructed for both the polar fraction (A) one day of exposure, (B) two days of exposure and the non-polar fraction (C) one day of exposure, (D) two days of exposure.
Appendix D

Supplementary Material for Chapter Five


**Figure S5-1.** PCA scores plots of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the *E. fetida* tissue extracts showing the separation of control earthworms (□) from exposed earthworms (○) after PFOA exposures of (A) 6.25 µg/cm$^2$, (B) 12.5 µg/cm$^2$, (C) 25 µg/cm$^2$, and (D) 50 µg/cm$^2$. The P-values were obtained from t-tests (two tailed, equal variances) that compared the scores of the control and exposed earthworms for each component.
Figure S5-2. PCA scores plots of PC1 (first PCA component) versus PC2 (second PCA component) for $^1$H NMR spectra of the *E. fetida* tissue extracts showing the separation of control earthworms (□) from exposed earthworms (○) after PFOS exposures of (A) 3.125 µg/cm², (B) 6.25 µg/cm², (C) 12.5 µg/cm², and (D) 25 µg/cm². The *P*-values were obtained from t-tests (two tailed, equal variances) that compared the scores of the control and exposed earthworms for each component.
Figure S5-3. Loadings plot for PC1 and PC2 showing the metabolites that were major contributors to the separation observed in the PCA scores plot of the *E. fetida* tissue extracts comparing the controls and PFOA exposed earthworms. The abscissa refers to the $^1$H NMR chemical shifts (ppm). The loadings plots refer to PFOA exposures of (A) 6.25 µg/cm$^2$, (B) 12.5 µg/cm$^2$, (C) 25 µg/cm$^2$, and (D) 50 µg/cm$^2$. 
Figure S5-4. Loadings plot for PC1 and PC2 showing the metabolites that were major contributors to the separation observed in the PCA scores plot of the E. fetida tissue extracts comparing the controls and PFOS exposed earthworms. The abscissa refers to the $^1$H NMR chemical shifts (ppm). The loadings plots refer to PFOS exposures of (A) 3.125 µg/cm$^2$, (B) 6.25 µg/cm$^2$, (C) 12.5 µg/cm$^2$, and (D) 25 µg/cm$^2$. 
**Figure S5-5.** Loadings plot for PC1 and PC2 showing the metabolites that were major contributors to the separation observed in the average PCA scores plots of the *E. fetida* tissue extracts comparing the controls and PFOA and PFOS exposed earthworms. The abscissa refers to the $^1$H NMR chemical shifts (ppm). The loadings plots refer to PFOS exposures of (A) PFOA exposure, (B) PFOS exposure, and (C) PFOA and PFOS exposure.
**Figure S5-6.** T-test filtered $^1$H NMR difference spectra of the *E. fetida* tissue extracts obtained by subtracting the mean buckets of the control earthworms from the mean buckets for the PFOA exposed earthworms and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. The t-test filtered $^1$H NMR difference spectra are shown after PFOA exposure of (A) 6.25 $\mu$g/cm$^2$, (B) 12.5 $\mu$g/cm$^2$, (C) 25 $\mu$g/cm$^2$, and (D) 50 $\mu$g/cm$^2$. 
Figure S5-7. T-test filtered $^1$H NMR difference spectra of the *E. fetida* tissue extracts obtained by subtracting the mean buckets of the control earthworms from the mean buckets for the PFOS exposed earthworms and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. The t-test filtered $^1$H NMR difference spectra are shown after PFOS exposure of (A) 3.125 µg/cm$^2$, (B) 6.25 µg/cm$^2$, (C) 12.5 µg/cm$^2$, and (D) 25 µg/cm$^2$. 
Appendix E

Supplementary Material for Chapter Six

Section S6-1. Earthworm Maintenance Prior to Contact Tests.

_Eisenia fetida_ were purchased from The Worm Factory (ON, Canada). They were raised in earthworm bins containing sphagnum peat bedding (Magic Worm bedding; Magic Products; WI, USA) at an approximate temperature of 24°C. The moisture content of the bedding is 67% water by weight. The earthworms were acclimated for several months to decrease variations in the \(^1\)H NMR profile due to differences in diet and other environmental factors [1]. The worms were fed Magic Worm Food (Magic Products; WI, USA). Mature earthworms, indicated by a visible clitellum were chosen and depurated in groups of 10 in the dark for 96 hours in 500 ml glass jars containing moist Whatman 4 Qualitative filter papers with a diameter of 9 cm (Fisher Scientific) to empty their intestinal tracts [1]. The earthworms had an average mass of 0.55 g (±0.1 g; standard deviation) after depuration. The earthworms were then transferred to individual 120 ml amber glass jars containing pre-treated Whatman GF/A 4.70 cm diameter glass filter paper (Fisher Scientific).

Section S6-2. Analysis of soil PFOS concentrations

S6-2.1 Soil Extraction

PFOS present in soil after two, seven and fourteen days of exposure were extracted in triplicate using the procedure based on Higgins et al. [2]. Homogenized and air-dried OECD soil (1 g) was transferred to a 40-ml polypropylene (PP) vial, to which 10 ml of an acetic acid solution (1%) was added. Each vial was then vortexed, sonicated for 15 min in a preheated bath (60 °C) and centrifuged at 4500 rpm (~1500 g) for 2 min using an International Equipment Company 21000 centrifuge (Fisher Scientific). The acetic acid solution was then decanted into another 40-ml PP vial. A 2.5 ml aliquot of a solvent mixture composed of 90:10 (v/v) methanol
and 1% acetic acid in Milli-Q (Millipore Synergy UV, Billerica, MA) water was then added to the original PP vial, which was followed by vortexing, sonication for 15 min at 60 °C and centrifugation at 4500 rpm (~1500 g) for 2 min. The supernatant was again transferred to the second PP vial. The acetic acid wash followed by the methanol/acetic acid extraction was repeated and a final 10 ml wash with acetic acid was performed. All washes and extracts were combined for each sample. The total volume of the extracts and washes was approximately 35 ml.

S6-2.2 Sample Cleanup

Solid phase extraction (SPE) was performed to concentrate the extracts and to remove the acetic acid, salts, and potential matrix interferences. A 500-mg SUPELCLEAN LC-18 cartridge (SUPELCO, PA, USA) was first conditioned with 10 ml of methanol followed by 10 ml of 1% acetic acid. The extracts were then loaded on to the SPE cartridges that were mounted on a vacuum manifold. The SPE cartridges were then rinsed with 10 ml of Milli-Q water prior to being allowed to dry under vacuum for 2 h prior to elution. PFOS was eluted from the SPE cartridge with 4 ml of methanol and was collected in a 1:1 (v/v) methanol/acetone washed 20 ml glass vials. The eluent was then concentrated to 2 ml under nitrogen and transferred to fresh 20 ml glass vials. The original 20 ml glass vials were then rinsed with 800 µL of methanol. The rinse was combined with the eluent and an additional 1200 µL of 0.01% aqueous ammonium hydroxide solution was added. The extracts were stored at 4 °C until analysis. Prior to analysis the extracts were diluted (10,000x) into 990 µL of 1:1 methanol/water to which 10 µL of 13C₄-PFOS aqueous internal standard (750 pg/ml) was added in a 2-ml autosampler glass vial.

S6-2.3 HPLC-MS/MS Analysis
Analysis of PFOS extracted from the spiked OECD soil was performed using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). An Agilent 1200 series HPLC was coupled with a 4000 QTRAP triple quadrupole mass spectrometer (Applied Biosystems-MDS Sciex, Concord, ON, Canada). Water and methanol (20 mM Ammonium acetate) were the solvents, which were delivered at a flow rate of 0.5 ml/min. The sample injection volume was 20 µl. The chromatographic separation was obtained using a Kinetex 2.6 µm Phenyl-Hexyl column (4.6 mm i.d. x 100 mm, 2.6 µm; Phenomenex, Torrance, CA). PFOS separation was obtained in 10 mins under gradient conditions, with 65:35 methanol:water initial mobile phase, followed by a 0.5 min ramp to 80:20 methanol:water, then a 2 min ramp to 95:5 methanol:water, which was held for 4 mins, followed by a 0.5 min ramp back to 65:35 methanol:water which was held for 3.50 mins.

The mass spectrometer was operated in negative electrospray ionization multiple reaction monitoring (MRM) mode using previously published methods [3, 4]. MRM transition related parameters were optimized for PFOS\(^-\) (m/z = 499) and SO\(_3\)F\(^-\) (m/z = 99): Ion transfer voltage = -4500 V, collision energy = -75 V, declustering potential = -103 V, collision exit potential = -10 V, and the dwell time = 200 msec. The \(^{14}\)C\(_3\)-PFOS (Wellington Laboratories, Guelph, ON) internal standard used the same MRM transition related parameters as PFOS for \(^{14}\)C\(_3\)PFOS\(^-\) (m/z=503) and SO\(_3\)F\(^-\) (m/z = 99). Quantification was performed using the internal standard method with a multi-concentration external calibration curve.

**S6-3 References**


Table S6-1. PLS-regression model parameters and outputs

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<th>Day of Exposure</th>
<th>PLS-components used</th>
<th>R²X</th>
<th>R²Y</th>
<th>Q²Y</th>
<th>P-value*</th>
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<tbody>
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<td>2 day exposure</td>
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<td>0.15</td>
<td>-0.10</td>
<td>0.3</td>
</tr>
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<td>7 day exposure</td>
<td>7</td>
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<td>0.74</td>
<td>0.34</td>
<td>7 x 10⁻⁴</td>
</tr>
<tr>
<td>14-day exposure</td>
<td>6</td>
<td>0.85</td>
<td>0.74</td>
<td>0.42</td>
<td>2 x 10⁻⁵</td>
</tr>
</tbody>
</table>

* P-values were obtained from the normal distribution of the permuted Q²Y values obtained from a permutation test that involved 400 permutations.
Figure S6-1. PCA loadings plots showing the metabolites that were major contributors to the separation observed in the average PCA scores plot of the *E. fetida* tissue extracts comparing the controls and PFOS exposed earthworms. The abscissa refers to the $^1$H NMR chemical shifts (ppm). The loadings plots refer to, two-day exposure (A) PC1 and PC2, (B) PC3 and PC4, seven-day exposure (C) PC1 and PC2, (D) PC3 and PC4, and fourteen-day exposure (E) PC1 and PC2, (F) PC3 and PC4.
Figure S6-2. PCA loadings plots showing the metabolites that were major contributors to the separation observed in the average PCA scores plot of the *E. fetida* tissue extracts comparing the controls and earthworms exposed to PFOS for two, seven and fourteen days. The abscissa refers to the $^1$H NMR chemical shifts (ppm). The loadings plots refer to, (A) PC1 and PC2, and (B) PC3 and PC4.
**Figure S6-3.** Histograms of $Q^2_Y$ values for cross-validated PLS models using the binned $^1$H NMR spectra as the X-table and random permutations of the PFOS exposure concentrations as the Y variable. Distributions were constructed using 400 permutations of the Y table. The histograms correspond to PLS models constructed for (A) two days of exposure, (B) seven days of exposure and (C) fourteen days of exposure.
Figure S6-4. T-test filtered $^1$H NMR difference spectra of the *E. fetida* tissue extracts obtained by subtracting the mean buckets of the control earthworms from the mean buckets for the PFOS exposed earthworms after two days of exposure and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. The t-test filtered $^1$H NMR difference spectra are shown after PFOS exposure of (A) 5 mg/kg, (B) 10 mg/kg, (C) 25 mg/kg, (D) 50 mg/kg, (E) 100 mg/kg, and (F) 150 mg/kg.
Figure S6-5. T-test filtered $^1$H NMR difference spectra of the *E. fetida* tissue extracts obtained by subtracting the mean buckets of the control earthworms from the mean buckets for the PFOS exposed earthworms after seven days of exposure and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. The t-test filtered $^1$H NMR difference spectra are shown after PFOS exposure of (A) 5 mg/kg, (B) 10 mg/kg, (C) 25 mg/kg, (D) 50 mg/kg, (E) 100 mg/kg, and (F) 150 mg/kg.
Figure S6-6. T-test filtered $^1$H NMR difference spectra of the *E. fetida* tissue extracts obtained by subtracting the mean buckets of the control earthworms from the mean buckets for the PFOS exposed earthworms after fourteen days of exposure and retaining the buckets that were statistically different from the controls at $\alpha = 0.05$. The t-test filtered $^1$H NMR difference spectra are shown after PFOS exposure of (A) 5 mg/kg, (B) 10 mg/kg, (C) 25 mg/kg, (D) 50 mg/kg, (E) 100 mg/kg, and (F) 150 mg/kg.