AN ABSTRACT OF THE DISSERTATION OF

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Title: Use of Targeted and Non-Targeted Analysis to Study Complex PAH Environmental Mixtures.

Abstract approved:

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Staci L. Massey Simonich

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants formed from the incomplete combustion of organic material. These contaminants are of concern because of their widespread presence in the environment and toxic properties. In addition, PAHs encompass a class of diverse compounds with varying physicochemical properties and exist in the environment as complex mixtures. Research has primarily focused on parent (or unsubstituted) PAHs, mainly the 16 U.S. Environmental Protection Agency priority PAHs. However, many other PAH derivatives, such as oxygen containing PAHs, co-occur in these complex mixtures and may be equally, if not more, toxic than parent PAHs. This dissertation investigates PAHs in complex soil and house dust environmental mixtures.

Previous studies have suggested that the partial degradation of PAHs during bioremediation may result in increased toxicity postbioremediation. However, there is limited

information on these transformation products in soil. An effects-directed analysis approach, incorporating targeted gas chromatography mass spectrometry (GC/MS), the *DT40* genotoxicity and zebrafish developmental toxicity assays demonstrated increased toxicity postbioremediation in four soil extract fractions (C, D, E and F). Non-targeted analysis, combing comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC/TOF-MS) and liquid chromatography quadrupole time-of-flight mass spectrometry (LC/QTOF-MS), was subsequently used to characterize the toxic soil extract fractions postbioremediation. Several of the tentatively identified compounds contained an oxygen and/or nitrogen in their elemental compositions, suggesting they would be relatively polar. Additionally, targeted GC/MS analysis of high molecular weight 302 PAHs (MW302-PAHs, PAHs with molecular weight $\geq$ 302 a.m.u), which are highly mutagenic, showed no degradation postbioremediation. The data suggest that monitoring the disappearance of a subset of parent PAH compounds may potentially overestimate the efficiency of bioremediation.

In the second part of the dissertation, a targeted approach, using GC×GC/TOF-MS, was used to investigate the long-term associations between parent PAHs, methylated PAHs, selected oxygenated PAHs, and thirdhand smoke (THS) in house dust. THS contains a complex mixture of toxic chemicals, and was recently shown to be a significant contributor of PAHs in settled house dust in smoker and non-smoker homes. However, less is known about how PAHs, more specifically other PAH derivatives, may associate with THS. In a pilot study, five homes were evaluated for the different PAH classes, tobacco-specific nitrosamines (TSNAs) and nicotine, up to six months after smoking had ended. Individual PAHs, 2+1 methylphenanthrene (2+1MPHE), 2-methylanthracene (2MANT), retene (RET), dibenz[a,h]anthracene (DBahA), 9,10-

anthraquinone (9,10AQ), 2-methylanthraquinone (2MAQ), benzanthrone (BZ), benzo[cd]pyrenone (BcdP), 5,12-naphthacenequinone (5,12NQ) and 9-fluorenone (9FLO) dust loadings were significantly correlated with nicotine and TSNA dust loadings over time (R^2: 0.47-0.72, $p < 0.05$). This suggests that these compounds may strongly associate with THS and should potentially be considered when assessing the long-term risks associated with THS exposure.

This dissertation demonstrates that PAHs exist in complex mixtures, and uses various tools (i.e. targeted and/or non-targeted methods, toxicity testing, complementary instrumental techniques) to investigate PAH compounds beyond those routinely monitored. Many of these compounds, such as transformation products, may be of ecological and toxicological significance.

Use of Targeted and Non-Targeted Analysis to Study Complex PAH Environmental Mixtures

by
Leah Chibwe

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Leah Chibwe, Author

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LIST OF ABBREVIATIONS

EDA	Effects-Directed Analysis
EI	Electron impact
ESI	Electrospray ionization
GC/MS	Gas chromatography mass spectrometry
GC×GC/TOF-MS	Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry
HRMS	High resolution mass spectrometry
IARC	International Agency for Research on Cancer
LC/QTOF-MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
m/z	Mass-to-charge ratio
MACs	Maximum allowable concentrations
MGP	Manufactured gas plant
MTBSTFA	*N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide
MW302-PAHs	High molecular weight 302 PAHs
NAT	N'-Nitrosoanatabine
NAB	N'-Nitrosoanabasine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone
NNN	*N′*-nitrosonornicotine
NPAH	Nitro PAHs
NSOBCs	Organo-bromine compounds
OHPAHs	Hydroxy PAHs
OPAH	Oxygenated PAHs
PAH	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis

LIST OF ABBREVIATIONS (Continued)

THS	Thirdhand smoke
TIE	Toxicity Identification Evaluation
TOF-MS/MS	Time-of-flight tandem mass spectrometry
TSNAs	Tobacco-specific nitrosamines
US EPA	United States Environmental Protection Agency

DEDICATION

I would love to dedicate this thesis to my baby brother, Mwansa Chibwe, who had the gentlest of souls and who will always remain in my heart and memory. Completing the last years of this dissertation without you was challenging, and if I could, I would move the earth and skies to share this accomplishment with you. Thank you for being my fiercest of supporters and may you always rest in peace.

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CHAPTER 1 – INTRODUCTION

1.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are pollutant products from the incomplete combustion of organic matter. Sources of PAHs include the burning of fossil fuels, vehicle exhausts, forest fires, coke production, cigarette smoking and charbroiled meats – among others.[1] PAHs are of particular health and environmental concern because they are ubiquitous and persistent. Several PAHs are classified as suspected or known carcinogens and/or mutagens, with 16 currently listed as priority pollutants by the United States Environmental Protection Agency (US EPA).[2]

PAHs encompass a class of diverse compounds composed of two or more fused benzene rings. These compounds are primarily characterized as parent PAHs (or unsubstituted PAHs), alkylated PAHs, high molecular weight 302 PAHs (MW302-PAHs, PAHs with molecular weight $\geq$ 302 a.m.u.), oxygenated PAHs (OPAHs, PAHs containing oxygen, e.g. quinones and ketones), hydroxy PAHs (OHPAHs, PAHs containing an OH group), nitro PAHs (NPAHs, PAHs containing a NO_2 group) and heterocyclic PAHs (HPAHs, PAHs containing an O, N, or S atom within a benzene ring) (Figure 1.1). As a result of this structural diversity, the physicochemical properties of this class of compounds varies widely. This diversity can also dictate the behavior and fate of these chemicals in the environment. For instance, the more hydrophobic higher molecular weight PAHs are more likely to sorb, accumulate and persist in soils and sediments.[3]

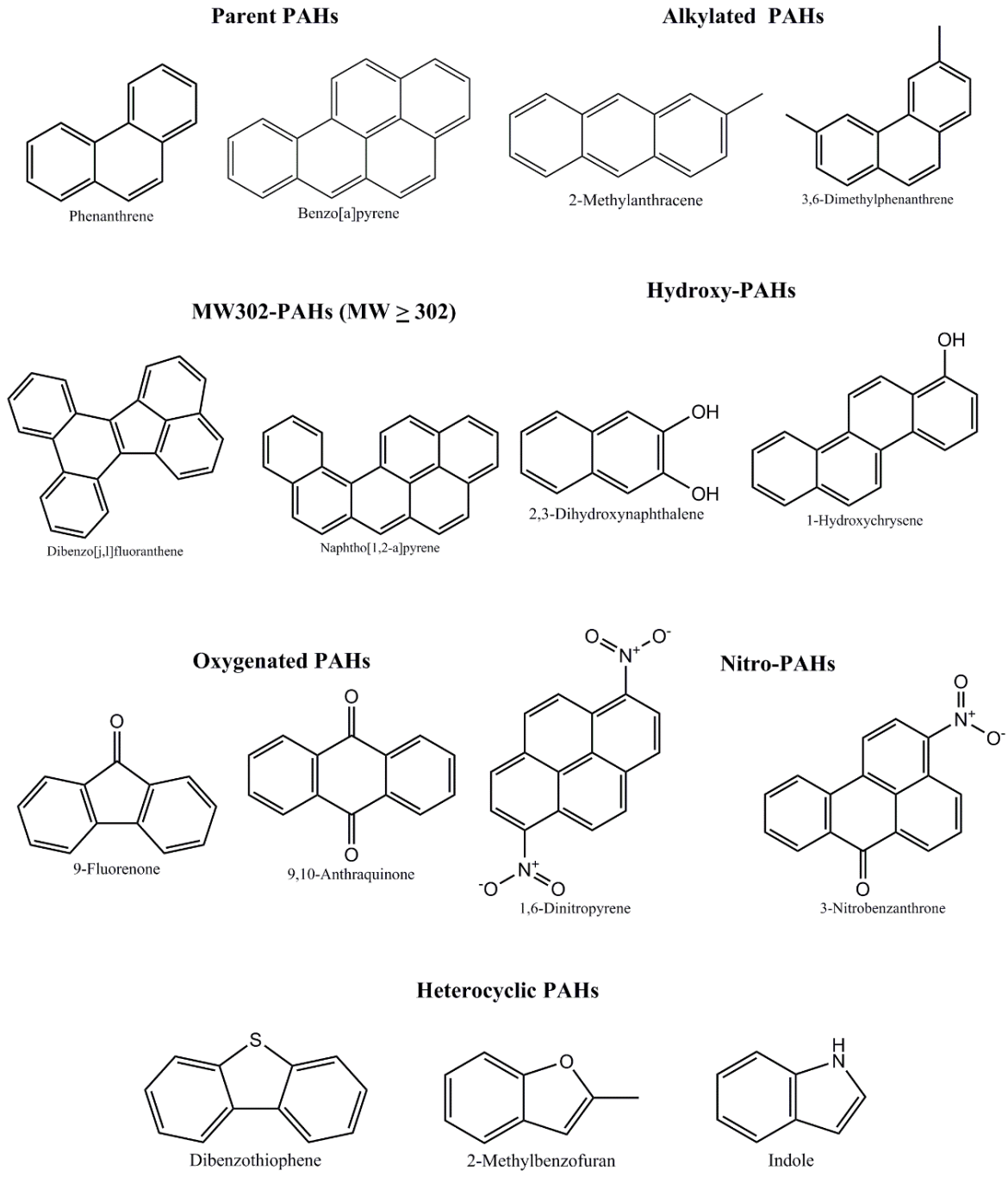

Figure 1.1. Examples of different classes of polycyclic aromatic hydrocarbons (PAHs).

Similar to many other pollutants, PAHs tend to exist in complex environmental mixtures. While, historically, the focus has been on the 16 EPA priority PAHs, there is concern regarding many non-routinely monitored PAH compounds. For example, MW302-PAHs were recently shown to contribute significantly to the mutagenic potential of asphalt and coal tar based seal coat.[4] Although, targeted methods offer sensitive and selective determination of pollutants, the use of non-targeted analysis to detect unknown chemicals with potential toxicological and ecological relevance, including derivatives of PAHs, is increasing.[5–10]

1.2 Non-targeted Analysis of Complex Environmental Mixtures

Non-targeted analysis offers a non-discriminatory approach of screening for compounds, potentially identifying unknown and unregulated chemicals. Some of these unknown compounds are expected to be bioaccumulative and persistent. For example, previously unknown biogenic and anthropogenic halogenated organic compounds, including compounds related to the pesticide chlordane, were detected in dolphin blubber in amounts comparable to target compounds.[7] In the mentioned study, more than half of the identified compounds were not routinely monitored, suggesting that targeted analysis would only encompass a minor proportion of the detected pollutants.[7] Similarly, another group of halogenated contaminants, natural and synthetic organo-bromine compounds (NSOBCs), were reported to be increasing in concentrations over time in the sediments of Lake Michigan.[9] This observation was concerning because some well-known NSOBCs, such as hydroxylated polybrominated diphenyl ethers, have been associated with adverse health effects.[11]

Transformation and degradation products also encompass a large proportion of unregulated chemicals, and may be equally, if not more, persistent and toxic than their precursors.[12–17] For example, previous studies have reported the formation of polar NPAHs from PAH photo-oxidation reactions in ambient air.[15,18,19] NPAHs are recognized as direct acting mutagens and are more toxic than their corresponding parent PAHs, because they do not require enzymatic activation to exhibit toxicity.[20,21] Additionally, several NPAHs are classified as probable carcinogens by the International Agency for Research on Cancer (IARC),[22] and have been recently shown to significantly promote tumor activity.[23] Other toxic transformation products include those from the use of consumer products, such as persistent methylated derivatives from triclosan,[16] and those linked with increased toxicity in bioremedial applications.[12, 13] Non-targeted analysis aims to bridge the knowledge gap stemming from the presence of significant unknown contaminants in the environment.

1.3 Instrumental Techniques for Non-Targeted Analysis of Complex Environmental Mixtures

The analytical techniques used in the analysis of organic compounds in environmental mixtures are mainly based on gas (GC) and liquid chromatography (LC) mass spectrometric methods (MS). GC is typically used for the analysis of semi-polar to non-polar compounds, while LC is widely used for the analysis of polar and thermally labile compounds. However, polar analytes can become GC amenable by increasing their volatility through derivatization reactions (e.g. OH groups in OHPAHs can be modified with a silyl-group using derivatizing agent *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide).[23, 24]

The reproducibility of electron impact (EI) ionization spectra and availability of mass spectral libraries, such as the National Institutes of Standards and Technology (NIST) database, make EI the most widely used ionization mode in GC for non-targeted analysis. However, EI is a hard ionization technique and can result in extensive fragmentation, with the molecular ion at low abundances or absent, making it difficult to determine elemental formula. On the other hand, LC methods use softer ionization techniques, such as electrospray ionization (ESI), in which information on the molecular ion is retained, but LC based mass spectral libraries are less extensive and can be a limitation in mass spectral matching.

The analysis of environmental samples presents a number of challenges, which are typically more pronounced in non-targeted analysis, including the presence of hundreds to thousands of biogenic and anthropogenic compounds with a wide range of physicochemical properties and concentrations in complex matrices. Recent advancements in analytical techniques have brought to the forefront multidimensional and hybrid applications that offer high throughput, high resolution and high sensitivity capabilities, such as comprehensive two-dimensional gas chromatography (GC×GC) and high resolution accurate mass spectrometry (HRMS).[26–28]

The increased chromatographic resolution and peak capacity offered by GC×GC make it a favorable technique for the non-targeted analysis of complex environmental samples.[29,26,30,7,31,32] Separation of compounds on two columns with independent separation mechanisms minimizes co-elution, which is beneficial for structurally similar isomers.[26,30] Chromatographic resolution can further be enhanced by increasing the orthogonality of column

combinations.[30] The increased peak capacity from the multidimensional separation space enables high throughput screening of complex samples containing thousands of compounds. The multidimensional space can also provide visual insight on structural or functional information, as similar compounds tend to cluster or align together. GC×GC has been applied extensively in non-targeted analysis, including studying petroleum degradation products in the Gulf of Mexico, [32,33] monitoring pesticide, pharmaceutical and personal care degradation products in wastewater and soil,[17,34] and profiling environmental contaminants in indoor house dust and air particulates, such as flame retardants and pesticides.[31]

Liquid chromatography (LC) coupled to high resolution accurate mass spectrometry (HRMS) has been used in non-targeted analyses to study polar contaminants, such as metabolites from pharmaceutical and pesticide use in wastewater and groundwater.[27,35–40] Accurate mass measurements can help narrow down potential candidates. Furthermore, fragment ions from tandem MS provide additional structural information on unknown compounds. Previous studies have reviewed the use of high resolution mass spectrometry in environmental applications.[27,41,42] Although HRMS is usually coupled to LC, there a few applications in GC.[6,43] For instance, GC-HRMS was used in the targeted and non-targeted analysis of compounds in water samples from Spain to identify significant toxicants such as benzophenone, which has been linked to endocrine disruption.[6]

Currently, it is difficult to envision a truly "universal" non-targeted instrumental approach. Compound class biases will exist depending on extraction method, instrumental method and ionization mode, and different classes of compounds are known to behave differently under different ionization modes. For example, the lack of a proton accepting or

donating moiety of a given compound can undermine the ionization efficiency necessary for detection.[44–46]

Few studies have used complementary instrumental techniques to cast a wider net to enable the detection of a wider range of compound classes in non-targeted analysis. Recently, Hernandez *et al*. used high resolution mass spectrometry coupled to both GC (atmospheric pressure chemical ionization) and LC (ESI) to investigate contaminants in wastewater, groundwater and surfacewater.[47] This method presented an elaborate universal screening method that allowed them to identify over 100 compounds, a feat that might not have been as successful with a single analytical technique. The use of complementary techniques for non-targeted analysis provides a greater opportunity for measuring the widest range of compound classes.

1.4 Toxicity Testing and Effects-Directed Analysis (EDA) in Environmental Applications

The integration of toxicity and chemical analyses is important in understanding the link between environmental pollutants and their associated effects. Several studies suggest that most targeted priority contaminants contribute only partially to observed toxicity.[13,14,48,49] For instance, in some bioremedial applications, a decrease in routinely monitored pollutant concentrations did not coincide with decreased toxicity.[12, 13, 48] Realistically, both known and unknown compounds may contribute to the overall toxicity in environmental samples. The combination of toxicity and chemical testing shifts the focus in complex mixtures to unknown compounds with relevant toxicity.

There are two main approaches used to identify toxins in environmental samples, Toxicity Identification Evaluation (TIE) and Effects-Directed Analysis (EDA). Detailed

overviews on these methods have been discussed previously.[49, 50] Though the objectives of both methods is similar, they differ in assumptions, procedures and endpoints. TIE generally focuses on the effect of toxins on the whole organism and, as a result, emphasis on bioavailability dictates method procedures (e.g. the non-use of organic solvents, etc.). Generally, the TIE approach is based on the following phases: (1) measure toxicity in the whole organism, (2) identify the toxicants if increased toxicity is observed, and (3) confirm the results. The EDA approach, on the other hand, consists of the following phases: (1) extract samples in organic solvents, (2) measure toxicity of the samples, (3) fractionate samples if increased toxicity is observed, (4) measure toxicity of fractions, and (5) confirm the results.

The sample fractionation in EDA results in simplification of the complex mixture, making it easier to tie the cause to the effect of toxicants. For this reason, it is beneficial in the non-targeted analysis of complex environmental samples. An EDA approach combining GC/MS and LC/HRMS with the AR CALUX® assay was successfully used to identify androgen-disrupting chemicals in soil.[53,54] A study by Marvin *et al*. observed that MW302-PAHs contributed significantly to toxicity in coal tar contaminated sediment using the *Salmonella typhimurium* bacterial strain (YG1025) and high pressure LC/MS.[48] EDA has also been previously used to monitor the formation of toxic transformation or degradation products,[14,55] such as oil degradation products in soil.[55] Comprehensive reviews of EDA in environmental and biological applications, including challenges and limitations have been previously reported.[56–58]

Given that toxins have different modes of action and affect organisms differently, there are multiple toxicity assays available and applied in EDA. The Ames test incorporates bacterial *Salmonella typhimurium* and modified strains, and is widely used to detect mutagenic

compounds in environmental samples due its availability and rapid screening capability.[59] However, bacterial metabolic pathways are not conserved in higher eukaryotic cell lines. This has led to the use of higher model based assays, such as the relatively recent *DT40* lymphocyte assay that uses a reverse genetic approach to detect genotoxicins,[14,60–62] and the zebrafish *(Danio rerio)* developmental toxicity model used to assess early life stage exposures to environmental toxicants.[63–67] Nielen *et al*. used a yeast-based receptor gene assay to identify estrogen disruptors in urine,[68] Chibwe *et al.* used the chicken *DT40* lymphocyte genotoxicity and zebrafish *(Danio rerio)* developmental assays to assess the formation of toxic degradation products during bioremediation of soil,[14] while Dorn *et al.* used an earthworm, Microtox®, and plant germination assays to evaluate crude oil acute toxicity in soils.[69] The information garnered from toxicity assays is invaluable, regardless of the type and number of assays used, as long as a case can be made for the translational relevance to human or ecological health. It is difficult to discern or anticipate the modes of action of toxins with compounds of unknown identity. Moreover, there should be the realization that, especially in non-targeted EDA analysis, depending on the assay(s) used, the response and performance of the bioassay will drive the identification of the unknown compounds towards a group of chemicals that exhibit particular toxic effects.

An important aspect of combined toxicity and chemical methods is the verification of toxicity of the identified unknown compounds. This can be impeded by the lack of availability of authentic

standards. While assumptions can be made with regard to the toxicity of the tentatively identified unknowns, based on functional similarity to known compounds, various tools are also available to predict the toxic activity of suspected structures, such as the online standalone predictive

program, VEGA-QSAR (quantitative structure-activity relationship).[70] These tools are dependent on the assumption that the toxicity observed is structurally related to the compound. While these models may not always be the perfect representation, they provide toxicological insight and are meant to bypass the limitations caused by the lack of authentic standards for many unknown compounds. Nevertheless, the integrated role of toxicity testing and chemical analysis is unequivocal in "directing" the focus to toxicologically relevant compounds.

1.5 Software and Computational Tools in Environmental Analysis

The role of software and computational tools in environmental analysis is invaluable and broad. Software algorithms are constantly evolving to meet the requirements and advances of analytical techniques. These include peak finding, peak alignment and mass spectral deconvolution software, and have been covered elaborately in previous reviews.[71–73] Due to the scope of software tools available, the objective of this section is not to cover all available software, but to highlight certain roles in simplifying and facilitating non-targeted analysis.

The data output produced from the analysis of complex samples includes comprehensive coverage of all ions amenable to the analytical technique. This results in hundreds to thousands of peaks originating from the sample, sample matrix and background noise. Some software tools incorporate statistical modeling features, meant to unbiasedly characterize compositional differences between groups of samples. These tools can be used to isolate relevant peaks from background or interfering peaks, and/or to identify metabolites or transformation products. In non-targeted analysis, this is beneficial in limiting the presence of false positives, reducing the time and effort spent on irrelevant peaks.

The LECO ChromaTOF® Reference and Statistical Compare features have been previously applied to facilitate the identification of compounds of interest in environmental samples in GC×GC applications.[8,17] Prebihalo *et al.* used the ChromaTOF® Reference feature to investigate emerging contaminants in wastewater and soil.[17] A sample was used to create a reference method, containing a comprehensive list of peaks with accompanying retention times and mass spectra. The reference sample serves as the basis of comparison for other samples, and the software computes pairwise comparisons to determine peaks present in both the sample and reference ("match"), only in the reference sample ("found"), and only in sample ("not found/unknown").[17] The sample selected for the reference method is arbitrary; a blank sample can be set as reference to eliminate peaks that match both the reference blank and sample, eliminating background peaks. LECO's Statistical Compare feature is useful to determine the most distinct compounds between samples, and has been applied successively to identify putative biomarkers.[74] Peaks are aligned in samples according to both retention times (1D, 2D) and mass spectral similarity, and are subsequently compared with samples in other groups (or classes). Prior to data processing, parameters to define peak tolerance (i.e. MS similarity threshold) and acceptable retention time shifts are set to account for variations and to limit false negatives. However, it should also be noted that peak alignment can be unsuccessful for poorly resolved peaks at low intensities or for highly saturated compounds.[74]

MarkerView™ software from SCIEX is similarly designed to identify structurally related and unrelated components between grouped samples for LC data. MarkerView™ has been used to assess data in metabolomics and in biological fluids,[75–77] such as urine and plasma. Environmental applications have included the recent identification of novel fluorochemicals in

firefighter foams,[39] and the discovery of quaternary triphenylphosphonium industrial contaminants in aquatic systems.[78] This software utilizes algorithms to align ions (according to retention times and mass spectral similarity, etc.) across multiple samples for comparison. Statistical tools, including principal component analysis (PCA) and principal component variable grouping (PCVG), can then be applied to the processed data to group samples and form chemical profiles. PCA arranges data into principal components, highlighting variables contributing to sample clustering while PCVG explores variables that share similar expression patterns from the PCA loadings. Pairwise comparisons and t-tests can additionally be conducted on grouped samples, with tabulated *p*-values suggesting the degree of variation of components between grouped samples. These tools can be effective at minimizing the presence of false peaks or at highlighting components of interest in grouped samples.

The use of computational methods to predict transformation and reaction pathways is another area of interest in the non-targeted analysis of environmental samples. Transformation pathway prediction software is especially useful in monitoring the formation of toxic products. Predicted products can be incorporated as "suspect" compounds and serve as a further screening tool, possibly leading to the rapid identification of unknown compounds. The University of Minnesota Pathway Prediction System (UM-PPS) is one of the most prominent tools used to predict products of bacterial metabolism.[79] The prediction is based on compound structure and transformation rules from data collected from known reactions (i.e. pathways, enzymes reactions etc.) UM-PPS has been reasonably successful at predicting transformation products in wastewater and natural waters from pollutants, such as pharmaceuticals and pesticides.[80,81] Related microbial pathway prediction systems include METEOR and META, built on rules and

knowledge-based reactions.[82,83] Additionally, predictive software tools such as MetaRouter have been designed to account for heterogeneity, specifically in biodegradation and bioremediation applications, where microorganisms exist in communities and can mutually influence how pollutants are degraded.[84]

Computational modelling tools to investigate formation of pollutants are not limited to microbial studies. Tools such as the Gaussian software incorporate theoretical rules to computationally predict the formation of most likely products from environmental chemical reactions.[85] Jariyasopit *et al.* applied the Gaussian software to successfully predict the formation of the most thermodynamically stable of NPAHs from PAH transformation reactions with atmospheric radicals.[18]

There is a variety of available software meant to predict the formation of reaction products; software that may be advantageous in investigating unknown compounds in non-targeted methods. Some of these tools, such as UM-PPS, are easily accessible to investigators, partly because the foundation of such databases depends on what has been reported in previous studies (i.e. observed reactions, etc.). It should be noted that the predictions from software and computational modeling tools are as effective as the rules and databases they are based upon, and that reactions pathways in the environment are complex. Multiple reactions (e.g. photolysis, hydrolysis etc.) occur at different rates and depend on a multitude of other factors, including the pollutant structure.

1.6 Challenges in Non-Targeted Analysis

Robust and sensitive instrumental methods, capable of resolving low concentration compounds and detecting a wide range of unknown compounds are necessary in non-targeted analysis. Generally, the workflow following data acquisition entails mass spectral library or database matching, followed by verification with authentic standards. However, libraries and databases may not contain the unknown compounds, making it difficult to match experimental spectral information, and authentic standards may not be readily available to confirm tentatively identified compounds.

Retention indices are helpful in refining mass spectral matches in GC,[86] and linear solvation energy relationships have been applied to predict the retention time behavior to identify unknown compounds in LC.[87] Myer *et al.* used mass defect analysis to measure biota-sediment accumulation factors in freshwater organisms exposed to halogenated pollutants in soil,[88] while Barzen-Hanson *et al.* used a similar approach to identify novel perfluoroalkyl sulfonates in aqueous film-forming foams and groundwater.[89] Peng *et al.* developed a data independent precursor isolation and characteristic fragment method and identified over 1500 unique natural and synthetic organo-bromine compounds (NSOBCs) in sediment.[9,90] Furthermore, *in silico* fragmentation tools such as MetFrag and MassFrontier, predict fragmentation patterns based on mass-to-charge ratio (m/z) intensity and bond dissociation energy, and have been used to identify compounds that are not available in library spectral databases.[89, 90] All of these methods represent tools to address limitations, and further demonstrate the potential for advancement in non-targeted analysis.

In order to retain information on unknown compounds detected in non-targeted environmental applications, various studies reported identifications based on hierarchal degrees of confidence.[7,10] For instance, compounds confirmed with authentic standards are reported with the highest confidence, while compounds only identifiable with mass spectral library or database matching are reported with lower confidence. Compounds for which structures cannot be elucidated are not eliminated, but reported with the lowest degree of confidence.[7,8,10] More researchers are calling for data sharing of results from non-targeted analysis. Hoh *et al.* created an open access database using the R platform to share mass spectral information of halogenated organic compounds detected in dolphin blubber by GC×GC/TOF-MS, identified to various degrees of confidence.[7] Sharing of information on mass spectral libraries does not only build databases, but also provides screening tools for other researchers conducting related non-targeted analysis.

1.7 Thesis Objectives

Although PAHs exist in the environment in complex mixtures, there are many other PAH related compounds of ecological and health relevance currently not monitored. Thus the integration of non-targeted and targeted methods is necessary to bridge the data gap presented by many of these unrecognized pollutants in the environment. Additionally, tools such as toxicity testing and computational modeling can guide and facilitate the identification of unknown compounds.

The objective of this thesis was to investigate PAHs and PAH derivatives in complex environmental mixtures, using targeted and non-targeted methods. In Chapter 2, an effects-

directed analysis approach was used to evaluate the effect of bioremediation on PAH contaminated soils. Targeted GC/MS analysis and the *DT40* lymphocyte and zebrafish developmental toxicity assays were used to assess toxicity changes pre- and postbioremediation.

Previous studies have suggested that the incomplete degradation of PAHs could lead to the formation of polar and toxic PAH transformation products.[93–95] However, many of these transformation products are currently not monitored at bioremedial sites. Non-targeted analysis combining GC×GC/TOF-MS and LC/QTOF-MS was applied in Chapter 3, to identify toxic transformation products formed during bioremediation. An overview of the method is illustrated in Figure 1.2.

In Chapter 4, GC×GC/TOF-MS was used to investigate PAHs and their association with thirdhand smoke (THS) in house dust samples long-term. Although environmental tobacco smoke is known to be a significant contributor of PAHs in settled housedust,[96] the research focus has predominantly been on parent PAHs, namely the 16 US EPA PAHs. Less is known about the effects of PAH related compounds or how they associate with THS. Thus, not only parent PAHs but selected alkylated, oxygenated and nitrated PAHs were examined in homes, up to 6 months after active smoking had ended, to evaluate their contribution to THS.

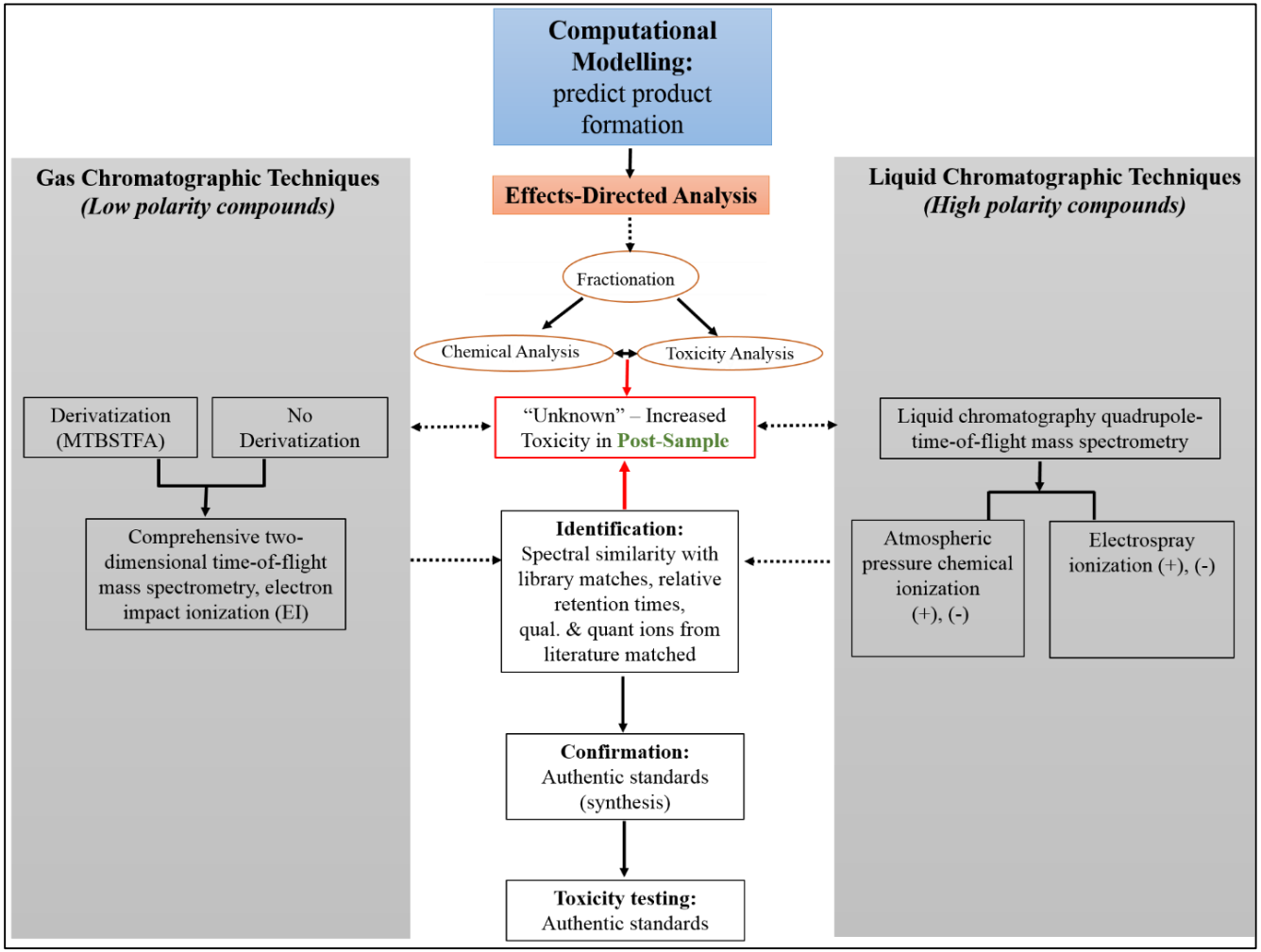

Figure 1.2. Complementary approach combing instrumental analysis, effect-directed analysis, and computational modelling tools

CHAPTER 2 – AEROBIC BIOREMEDIATION OF PAH CONTAMINATED SOIL RESULTS IN INCREASED GENOTOXICITY AND DEVELOPMENTAL TOXICITY

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2.1 Abstract

The formation of more polar and toxic polycyclic aromatic hydrocarbon (PAH) transformation products is one of the concerns associated with the bioremediation of PAH-contaminated soils. Soil contaminated with coal tar (prebioremediation) from a former manufactured gas plant (MGP) site was treated in a laboratory scale bioreactor (postbioremediation) and extracted using pressurized liquid extraction. The soil extracts were fractionated based on polarity, and analyzed for 88 PAHs (unsubstituted, oxygenated, nitrated, and heterocyclic PAHs). The PAH concentrations in the soil tested, postbioremediation, was lower than their regulatory maximum allowable concentrations (MACs), with the exception of the higher molecular weight PAHs (BaA, BkF, BbF, BaP, and IcdP), most of which did not undergo significant biodegradation. The soil extract fractions were tested for genotoxicity using the *DT40* chicken lymphocyte and developmental toxicity using the embryonic zebrafish (*Danio rerio*) bioassay. A statistically significant increase in genotoxicity was measured in the unfractionated soil extract, as well as in four polar soil extract fractions, postbioremediation ($p < 0.05$). In addition, a statistically significant increase in developmental toxicity was measured in one polar soil extract fraction, postbioremediation ($p < 0.05$). A series of morphological abnormalities, including peculiar caudal fin malformations and hyperpigmentation in the tail, were measured in several soil extract fractions in embryonic zebrafish, both pre- and postbioremediation. The increased toxicity measured postbioremediation is not likely to be due to the 88 PAHs measured in this study because most were not present in the toxic polar fractions and/or because their concentrations did not increase postbioremediation. However, the increased

toxicity measured postbioremediation is likely due to transformation products, including those of the 3- and 4-ring PAHs (PHE, 1MPHE, 2MPHE, PRY, BaA, and FLA) that were most degraded.

2.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of environmental contaminants formed through the incomplete combustion of organic matter. PAHs are of concern because some are toxic, suspected or known mutagens and/or carcinogens, and some tend to be persistent in the environment.[97–99] These pollutants are primary constituents in soils at manufactured gas plant (MGP) sites, where sources of PAHs often include coal tar.[100] Due to the relative stability and hydrophobic character of PAHs, soil ultimately acts as a major sink for these compounds.[101,102]

Bioremediation uses microorganisms to decrease PAH concentrations in soil, thus reducing their associated risks.[103] However, under certain conditions, reductions in PAH concentrations do not necessarily correspond with decreased soil toxicity.[13,104] Incomplete degradation, or oxidation, of PAHs may lead to the formation of more polar and mobile PAH transformation products, which may include PAH derivatives containing oxygen groups (OPAHs), and nitro groups (NPAHs). These more polar PAH compounds are not as well-studied in bioremediation systems, and could be present alongside PAHs, serving both as co-contaminants and/or remedial transformation products. Additionally, they may be more reactive and potentially more toxic due to the presence of electronegative atoms.[105–109] For instance, some OPAHs and NPAHs are known to exhibit greater toxicity than their corresponding unsubstituted PAH precursors and do not require enzymatic activation to express toxicity.[107–111] Heterocyclic

PAHs, HPAHs (PAH derivatives containing heteroatoms oxygen, nitrogen, or sulphur), have been shown to contribute significantly to toxicity at contaminated sites, and their metabolites have been linked to endocrine disruption.[112,113]

Beyond monitoring PAHs, chiefly those labeled as the 16 United States Environmental Protection Agency (U.S. EPA) PAH priority pollutants, the formation of PAH transformation products is not commonly measured at remediation sites. In complex and dynamic biological systems, it can be difficult to reliably predict the transformation products that will be formed. Additionally, environmental analysis of PAH transformation products, and more polar PAHs, is more challenging than that of the PAHs because they may be present in lower concentrations, are more reactive, and are strongly influenced by matrix interferences from soil organic matter and unresolved complex mixtures.[30] Compared with PAHs, there is also a lack of labeled standards and certified reference materials for these compounds.

Previous studies have used an effects-directed analysis (EDA) approach to assess toxicity changes during or after remediation. These previous studies have predominantly used bacterial and *in vitro* mammalian-cell assays,[94,95,114,115] which can be marred by high false positives and negatives, as well as limited sensitivities.[116,117] The *DT40* bioassay uses DNA damage repair-deficient mutants of the parental *DT40* cell line to measure genotoxicity, and the response to mutagenic chemicals in these repair-deficient mutants is marked by an increase in chromosomal aberrations relative to the parental *DT40* cell line.[118–120] The advantages of this assay include quick proliferation rates, a resemblance to higher eukaryotic cells, and high gene targeting efficiencies necessary in the production of deficient-repair mutants.[120] Another unique feature of *DT40* cells is their apparent lack of a functional p53 protein, which can induce apoptosis in the

presence of cell stress. The lack of a functioning p53 protein ensures that the cell death observed is due to failures in specific DNA-damage repair pathways rather than from apoptosis activated by the cell in response to DNA damage.[121] While many assays can determine whether a toxin is mutagenic or not, the *DT40* bioassay provides information on the mode of action, which can shed more light in understanding how certain chemicals are likely to behave in human exposure scenarios.[118]

The embryonic zebrafish assay (*Danio rerio*) is an effective *in vivo* model to assess the developmental toxicity of environmental toxicants.[64,122] Zebrafish share significant genetic and physiological homology with humans, and there is growing evidence that zebrafish can rival or exceed rodent models in predicting human disease outcomes.[123,124] To the best of our knowledge, no studies have used the embryonic zebrafish assay to study the effect of bioremediation on PAH contaminated soils. However, a recent study by Wincent *et al.* investigated the developmental toxicity in zebrafish in soil from multiple industrial sites, and found that in gas contaminated soil, there was greater developmental toxicity associated with the relatively more polar oxygenated fraction than with the PAH fraction.[125]

While some studies on the bioremediation of PAH contaminated soils measured a general decrease in soil toxicity following bioremediation,[126–128] other studies measured an increase, suggesting the formation of toxic transformation products and/or metabolites.[13,94,114,115,127] However, an in depth investigation into potentially toxic PAH transformation products has not been carried out. The objectives of this study were to (1) use an EDA approach to begin to identify potentially toxic PAH transformation products, as well as eliminate non-toxic PAH transformation products, in bioremediated soil; and (2) use changes in PAH, OPAH, NPAH, and

HPAH concentrations, pre- and postbioremediation, as a possible explanation for changes in soil toxicity. Soil contaminated with coal tar was extracted pre- and postbioremediation, the extract was fractionated based on polarity, and the fractions were evaluated for changes in PAH, OPAH, NPAH, and HPAH concentrations, as well as for genotoxicity and developmental toxicity using the *DT40* and zebrafish bioassays, respectively.

2.3 Materials and Methods

2.3.1 Chemicals

Standard solutions of PAHs and methyl PAHs were purchased from AccuStandard (New Haven, CT) and Chem Service (West Chester, PA), OPAHs from Sigma Aldrich (St. Louis, MO), HPAHs from AccuStandard (New Haven, CT) and Sigma Aldrich (St. Louis, MO), and NPAHs from AccuStandard (New Haven, CT). All 88 PAHs studied and their abbreviations are listed in Table 2.1. Isotopically labeled standards used as surrogates and internal standards for PAHs and methyl PAHs, OPAHs, HPAHs, and NPAHs were purchased from CDN Isotopes (Point-Claire, Quebec) and are listed in Appendix A.

2.3.2 Study Area and Soil Samples

Soil contaminated with coal tar was collected from a former MGP site in Salisbury, North Carolina.[13] The soil was treated in an aerobic laboratory-scale bioreactor under conditions previously described.[13,129] The contaminated soil before treatment was labeled as "prebioremediation" and after treatment as "postbioremediation."

2.3.3 Pressurized Liquid Extraction (PLE)

Approximately 0.5 g wet weight soil was extracted in 100 mL cells using an Accelerated Solvent Extractor (ASE) (Dionex ASE 350) in hexane:acetone (75:25, v/v) (1500 psi, 100 °C, 3 cycles, 240 s purge). ASE is an exhaustive extraction technique that is useful for extracting the majority of PAHs, OPAHs, NPAHs, and HPAHs from the soil samples.[130] However, it is a worst case scenario in terms of estimating bioavailable concentrations.[100,131] The extract was then split 75% for toxicity testing and 25% for chemical analysis and the portion undergoing chemical analysis was spiked with isotopically labeled surrogate standards. This was done so that the *DT40* cells and zebrafish embryos were not exposed to potentially toxic isotopically labeled PAHs and to ensure that the extracts being chemically analyzed were the same as the extracts undergoing toxicity testing. Dry weights of soil were obtained after drying at 120 °C for 24 h. All concentrations are reported on a dry weight basis.

Table 2.1. PAHs measured, their abbreviations, and the soil extracts that contained them. Where more than two fractions are listed, the first fraction was the primary fraction. Nitrated PAHs were not detected in study above LOD 0.3 ng g^{-1}.

Unsubstituted, methyl PAHs	Abbr.	Primary Fraction
Naphthalene	NAP	A
2-Methylnaphthalene	2MNAP	A
1-Methylnaphthalene	1MNAP	A
2,6-Dimethylnaphthalene	2,6MNAP	A
1,3-Dimethylnaphthalene	1,3MNAP	A
Acenaphthylene	ACEY	A
Acenaphthene	ACE	A
Fluorene	FLU	A
Phenanthrene	PHE	A
Anthracene	ANT	A
2-Methylphenanthrene	2MPHE	A
2-Methylanthracene	2MANT	A
1-Methylphenanthrene	1MPHE	A
3,6-Dimethylphenanthrene	3,6MPHE	A
Fluoranthene	FLA	A
Pyrene	PYR	A
Retene	RET	A
Benz[c]fluorene	BcF	A
1-Methylpyrene	1MPYR	A
Cyclopenta[cd]pyrene	CdeP	A
Benzo(a)anthracene	BaA	A
Chrysene + Triphenylene	CHR+TRI	A
6-Methylchrysene	6MCHR	A
Benzo(b)fluoranthene	BbF	A
Benzo(k)fluoranthene	BkF	A
Benz[j][e]aceanthrylene	BjeA	A
Benz(e)pyrene	BeP	A
Benzo(a)pyrene	BaP	A
Dibenz(a,c)anthracene	DacP/DahP	A
Indeno(1,2,3-cd)pyrene	IcdP	A
Benzo(ghi)perylene	BghiP	A
Anthranthrene	ANTH	A

OPAHs	Abbr.	Primary Fraction
9-Fluorenone	9FLO	C
1,4-Naphthoquinone	1,4NQ	C
Acenaphthenequinone	ACEN	B
Phenanthrene-1,4-dione	1,4PD	B
9,10-Anthraquinone	9,10AQ	B
1,4-Anthraquinone	1,4AQ	B
2-methyl-9,10-anthraquinone	2M9,10AQ	C
2-Ethyl-9,10-Anthraquinone	2E9,10AQ	B
9,10-Phenanthrenequinone	9,10PQ	C
Benzo[a]fluorenone	BaF	B
Benzanthrone	BZ	B
Aceanthrenequinone	ACEAN	C
Benzo[c]phenanthrene-[1,4]quinone	Bc1,4Q	B
7,12-Benzo[a]anthracene dione	7,12BaAD	B
Benzo[cd]pyrenone	BcdP	B
5,12-Napthacenequinone	5,12NQ	C
1,6-Benzo[a]pyrene quinone	1,6BaPQ	C

HPAHs	Abbr.	Primary Fraction
2-Methylbenzofuran	2MBZ	C
Thianapthene	THN	B
Quinoline	QUI	E, F
Indole	IND	E, F
8-Methylquinoline	8MQ	C
Dibenzofuran	DBF	A
Xanthene	XAN	B
5,6-Benzoquinoline	5,6BQ	A
Acridine	ACR	B
Carbazole	CAR	A, B
Dibenzothiophene	DBZ	A, B

NPAHs	Abbr.	Primary Fraction
1-Nitronaphthalene	1NNAP	B
2-Nitronaphthalene	2NNAP	B
2-Nitrobiphenyl	2NBP	B
3-Nitrobiphenyl	3NBP	B
4-Nitrobiphenyl	4NBP	B
3-Nitrodibenzofuran	3NBF	B
5-Nitroacenaphthene	5NACE	B
2-Nitrofluorene	2NF	B
9-Nitroanthracene	9NANT	B
9-Nitrophenanthrene	9NPHE	B
2-Nitrodibenzothiophene	2DBT	B
3-Nitrophenanthrene	3NPHE	B
2-Nitroanthracene	2NANT	B
2-Nitrofluoranthene	2NF	B
3-Nitrofluoranthene	3NF	B
1-Nitropyrene	1-NP	B
2-Nitropyrene	2NP	B
2,8-Dinitrodibenzothiophene	2-NP	B
7-Nitrobenz[a]anthracene	2NBaA	B
1-Nitrotriphenylene	1-NTRI	B
6-Nitrochyrsene	6NChr	B
3-Nitrobenzanthrone	3NBZ	B
2-Nitrotriphenylene	2NTRI	B
1,3-Dinitropyrene	1,3NP	B
1,6-Dinitropyrene	1,6NP	B
1,8-Dinitropyrene	1,8NP	B
6-Nitrobenzo(a)pyrene	6-NBaP	B

2.3.4 Fractionation

The toxicological and chemical portions of the extract were fractionated into fourteen 25 mL fractions using 20 g silica solid phase extraction (SPE) cartridges from Agilent (Santa Clara, CA) (Table 2.2). However, due to the intensive fractionation and to ensure there was enough soil residue to elicit a response in the *DT40* assay, these fractions were combined into six composite fractions A, B, C, D, E, and F, as shown in Table 2.2. Soil was also extracted, and not fractionated ("unfractionated"), and analyzed with the fractionated soil extracts. Lab blanks consisting of sodium sulfate were extracted and analyzed for target PAHs and toxicity alongside soil extracts. The extracts undergoing chemical analysis were evaporated down to a final volume of 300 µL. The extracts undergoing toxicological analysis were evaporated just to dryness under a flow of nitrogen in pre-weighed vials. The mass of the dry residue was measured using an analytical balance, and the residue was re-dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) to a concentration of approximately 10,000 µg soil residue per mL DMSO.

2.3.5 Chemical Analysis

Gas chromatographic/mass spectrometry (GC/MS) analysis was carried out using an Agilent 6890 GC system, equipped with a mass selective detector on a DB-5MS (30 m × 0.25 mm I.D. × 0.25 µm film thickness) capillary column. The soil extracts were spiked with isotopically labeled internal standards prior to GC/MS analysis. PAHs and methyl PAHs, and HPAHs were analyzed in electron impact ionization (EI) mode, while OPAHs and NPAHs were analyzed in electron capture negative ionization (ECNI) mode.[15,20,132] CHR and DahA were not

resolved from TRI and DacA, respectively, and were reported as a sum (i.e. CHR+TRI and Dah+acA).

Table 2.2 Silica solid phase extraction solvent elution composition for soil extract fractions A-F

Soil fraction	**Composite Solvent Elution [v/v]**
A (least polar)	100% Hexane 90:10 Hexane:Dichloromethane 80:20 Hexane:Dichloromethane 70:30 Hexane:Dichloromethane
B	60:40 Hexane:Dichloromethane 50:50 Hexane:Dichloromethane 40:60 Hexane:Dichloromethane
C	30:70 Hexane:Dichloromethane 20:80 Hexane:Dichloromethane
D	10:90 Hexane:Dichloromethane 100% Dichloromethane
E	100% Ethyl acetate
F (most polar)	100% Acetone (2 cycles)

2.3.6 DT40 Bioassay

The toxicological soil extracts were stored at -80 °C prior to exposure. They were serially diluted with phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY) and administered to the *DT40* cell line and the mutant *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ cells. A DMSO blank, diluted with PBS, was used as a negative control. The cells were incubated at 39.5 °C for at least

48 h, at 5% CO_2 and 95% relative humidity.[120] After incubation, the cells were treated with 2, 3-bis [2-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carbox-anilide salt (XTT dye) (Sigma, St. Louis, MO) and returned to the incubator to allow for dye metabolism. Once the dye was metabolized and the cells had developed sufficient color (approximately after 4 to 6 h), the absorbance was determined using a V_{max} kinetic microplate reader (Molecular Devices, Sunnyvale, CA) and related to percentage cell survival.[13] Details on the *DT40* bioassay cell culturing, exposure method, and maintenance are reported elsewhere.[120]

2.3.7 Embryonic Zebrafish Bioassay

The toxicological soil extracts were stored at -20 °C until 1 h prior to exposure. They were diluted in DMSO in a 96-well plate to 1171 µg residue per mL DMSO, then diluted further 8 times in a 5-fold serial dilution. Ten microliters were taken from the initial dilution to create a 10% DMSO in embryo media (EM) dilution row. Ten microliters were taken from the second dilution and added to the embryo-loaded 90 uL of EM. Ten microliters were added to each row of 4 exposure plates. The final DMSO concentration was 1% (v/v). A 1% DMSO vehicle control was used on every exposure plate. If mortality and morbidity, combined, were greater than 15% in the vehicle control, the exposures were re-run. Further details of the zebrafish method are reported elsewhere.[122,133]

2.3.8 Statistical Analysis

Median lethal concentrations (LC_{50}) were determined using Graphpad PRISM software, while statistical analyses were conducted using Microsoft® Excel 2013 and JMP (Statistical

Discovery™ from SAS) software. Student t-tests were used to identify statistically significant changes in PAH concentrations and toxicity, postbioremediation ($p < 0.05$).

2.4. Results and Discussion

2.4.1 Chemical Analysis

Unfractionated Soil Extracts. Prebioremediation, the total PAH (PAHs and methyl PAHs, OPAHs, and HPAHs) concentrations in the unfractionated soil extract ranged from 0.01 to 123 μg g^{-1}, while concentrations postbioremediation ranged from 0.03 to 60 μg g^{-1} (Figure 2.1, Appendix A1). No NPAHs were detected above the limit of detection (LOD) of 0.3 ng g^{-1}. The sum of PAH and methyl PAH concentrations accounted for about 97% of the total PAH, OPAH and HPAH concentration, with 3- and 4-ring PAHs (including PHE, 1MPHE, 2MPHE, PYR, BaA, and FLA), having the highest concentrations and showing the greatest reduction in concentration, postbioremediation (Figure 2.1A). The higher molecular weight 5- and 6-ring PAHs (ANTH, BghiP, IcdP, BaP, and BeP) were not biodegraded (Figure 2.1A).[134,135] Because higher molecular PAHs are more hydrophobic, they tend to sorb strongly to organic matter and may not be available to microorganisms for biodegradation.[102,134–136]

The sum of the 16 U.S. EPA PAH priority pollutants (excluding CHR and DahA) concentration was reduced 45% postbioremediation, and is comparable to previous studies, where removal percentages were between 40 and 77%.[13,94,115,134,136] Maximum allowable concentrations (MACs) for priority PAHs in industrial soils have been proposed by regulatory agencies and governments, including the U.S. EPA, the Canadian Council of Ministers of the Environment (CCME), and the German Federal Government (Table 2.3).[137–139] The PAH

concentrations in the soil, postbioremediation, were lower than their corresponding MACs, with the exception of the higher molecular weight PAHs (BaA, BkF, BbF, BaP, and IcdP) (Table 2.3). The higher molecular weight PAHs have the lowest regulated MACs (0.29 – 12 μg g^{-1}), likely because of their classification as B2 probable human carcinogens by the U.S. EPA.[140]

The sum of OPAHs accounted for about 2% of the total PAH, OPAH, and HPAH concentration, both pre- and postbioremediation (Figure 2.1B). The sum of OPAH concentration was reduced 58%, postbioremediation, with 9,10AQ, 2M9,10AQ, E9,10AQ, and BaF concentrations significantly reduced ($p < 0.05$). Though other studies have noted increases postbioremediation in certain OPAHs, including 9FLO,[95,141] we did not measure any significant increases in OPAH concentrations, postbioremediation.

The HPAHs were measured at the lowest concentrations, accounting for about 0.3% of the total PAH, OPAH, and HPAH concentration. Of the HPAHs, IND, 5,6BQUI, and ACR concentrations were significantly reduced postbioremediation ($p < 0.05$) (Figure 2.1C). Previous studies have shown that the presence of HPAHs can inhibit the degradation of PAHs.[142,143]

The formation of polar PAH transformation products during bioremediation may vary depending on a number of factors, including: degree of contamination, bioremediation conditions, microbial community composition, and soil properties.[144] In addition, compared to unsubstituted PAHs, less is known about the degradation pathways and microorganisms that can degrade these polar PAHs. For instance, Rodgers-Vieira et. al recently identified the first bacterial strain capable of degrading 9,10AQ, but noted that this strain differed from the ANT degrading strain, implying that while bacteria may be equipped to degrade the unsubstituted PAHs, they might not necessarily be equipped to degrade corresponding OPAHs.[145]

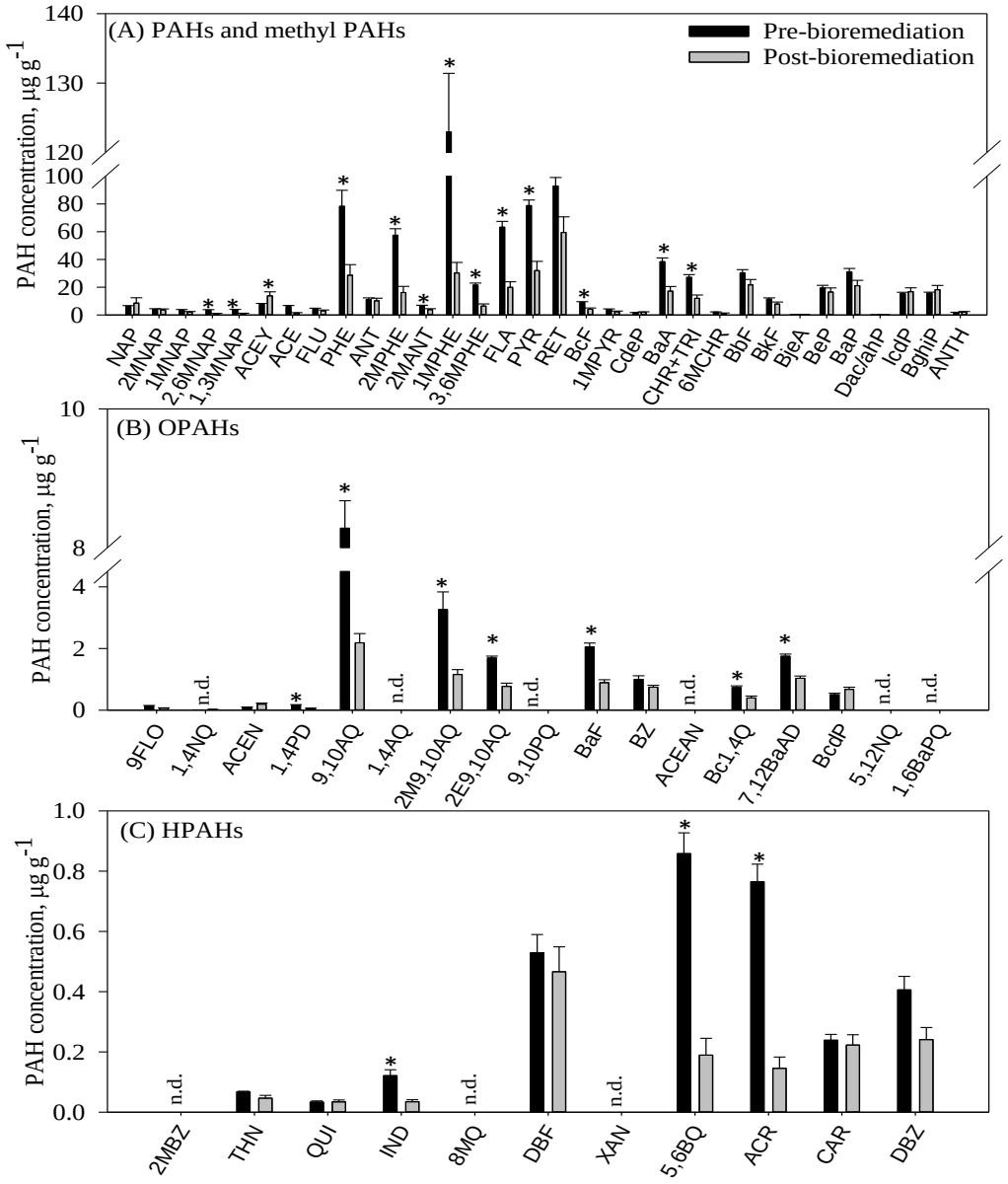

Figure 2.1. Mean concentrations in dry weight (with standard errors bars, $n = 3$) of investigated (A) PAHs and methyl PAHs, (B) OPAHs and, (C) HPAHs pre- and postbioremediation in the unfractionated soil extract. Compounds with asterisks (*) showed significant changes in concentration postbioremediation ($p < 0.05$). No NPAHs were detected above the limit of detection (0.3 ng g^{-1}). (n.d. = not detected).

Table 2.3. Maximum allowable concentrations (MACs) of the 16 priority PAHs in industrial soils regulated by the U.S. E.P.A. and Canadian Council of Ministers versus observed mean PAH concentrations and standard errors (SE) in unfractionated soil extracts in study.

Priority PAH	U.S. E.P.A[137] µg g^{-1}	Canada[138] µg g^{-1}	Germany [139] µg g^{-1}	Prebioremediation µg g^{-1}	Postbioremediation µg g^{-1}
NAP	17			6.26 ± 0.49	8.58 ± 3.82
ACE	45000			6.13 ± 0.71	1.31 ± 0.43
ACEY				8.03 ± 0.30	13.78 ± 2.94
FLU	30000			4.22 ± 0.68	3.02 ±0.58
PHE		50		78.32 ± 11.54	28.65 ±11.54
ANT	230000			11.17 ±1.21	10.21 ± 1.88
FLA	30000			63.32 ± 4.15	19.99 ± 3.98
PYR	23000	100		78.84 ± 3.99	32.03 ± 6.62
CHR	290				
BaA	2.9	10		38.38 ± 2.76	17.22 ± 3.30
BkF	29	10		11.40 ± 0.95	7.87 ± 1.53
BbF	2.9	10		30.42 ± 2.24	21.76 ± 3.87
BaP	0.29	0.7	12	31.00 ± 2.51	21.51 ± 3.84
IcdP	2.9	10		15.43 ± 0.95	16.77 ± 2.82
DahA	0.29	10			
BghiP					

Fractionated Soil Extracts. The soil extracts were fractionated into six fractions based on polarity, A to F (Table 2.2), and analyzed to identify which fractions contained the PAHs and methyl PAHs, OPAHs, HPAHs, and NPAHs (Table 2.1). The purpose of fractionating the soil extract was not to isolate the different PAH classes, but to simplify the complex mixture of PAHs in the soil extract and to better link the measured toxicity of a fraction to the chemistry of a fraction. The PAHs and methyl PAHs, the least polar of the PAH classes, were primarily contained in fraction A. The majority of the individual OPAHs, which are more polar than the PAHs and methyl PAHs, were primarily contained in fractions B and C. This includes the

potential degradation products of the 3- and 4-ring PAHs that biodegraded, such as 9FLO. The polarities of the HPAHs vary depending on the heteroatom and the number of rings. The least polar HPAHs were contained in fractions A and B, while the more polar HPAHs were contained in fractions E and F. Though NPAHs were not measured above the LOD in the soil, a spike and recovery experiment showed that they would be contained primarily in fraction B.

2.4.2 DT40 Bioassay

DNA damage repair-deficient mutants *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ were used to evaluate DNA damage in the soil extracts, pre- and postbioremediation. *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ are both sensitive to a wide range of DNA damaging agents and indicate whether the formation of DNA double-strand breaks (*Rad54*$^{-/-}$) or translesion synthesis (*Rev1*$^{-/-}$) DNA damage has occurred.[146,147]

In the unfractionated soil extracts, a significant decrease in median lethal concentration (LC_{50}), associated with increased toxicity, was measured postbioremediation for the parental *DT40* ($p < 0.001$) and mutants *Rad54*$^{-/-}$ ($p < 0.001$) and *Rev1*$^{-/-}$ ($p < 0.01$) (Figure 2.2, Appendix A2). The effect on both mutants suggests that compounds affecting the double-strand breaks and translesion DNA damage repair pathways likely contribute to the measured toxicity in the parental *DT40* cells, postbioremediation. These results are consistent with earlier work on this system by Hu et al.,[13] who noted an increase in (geno)toxicity in *DT40* cells and mutant *Rad54*$^{-/-}$ cell lines, postbioremediation.

In the fractionated soil extracts, a significant decrease in LC_{50} was measured postbioremediation in fraction E for *DT40* ($p < 0.05$), *Rad54*$^{-/-}$ ($p < 0.01$), and *Rev1*$^{-/-}$ ($p < 0.001$), and in fraction F for *Rev1*$^{-/-}$ ($p < 0.01$), suggesting that compounds in fractions E and F contribute

to the increased toxicity measured postbioremediation in the unfractionated soil extracts (Figure 2.2, Appendix A2). In fractions A, C, and D, we measured a significant increase in LC_{50} postbioremediation ($p < 0.05$), indicating a decrease in toxicity from compounds in these fractions after bioremediation.

While the LC_{50} provides information on general toxicity, the relative LC_{50} is a quantitative measure of how sensitive a DNA repair-deficient mutant is in relation to the parental *DT40* cell line (which has all functioning repair pathways). The relative LC_{50} was calculated by dividing the LC_{50} of the mutant (*Rad54*$^{-/-}$ or *Rev1*$^{-/-}$) by the LC_{50} of the parental *DT40*. A ratio less than 1 (and $p < 0.05$) signified the mutant was more sensitive to the soil extract than the parental *DT40*, and the soil extract could be considered genotoxic.[60,119] The smaller the LC_{50} of the mutant, the more toxic the soil extract is to the mutant, and the smaller the relative LC_{50}.

Rad54$^{-/-}$ was more sensitive than the parental *DT40* (relative $LC_{50} < 1$ and $p < 0.05$) to all soil extract fractions pre- and postbioremediation, except for fraction E prebioremediation. This suggests that these fractions contained genotoxic compounds that affected the DNA double-strand repair pathway (Figure 2.3A). The unfractionated extract was also genotoxic to *Rad54*$^{-/-}$, prebioremediation, with no significant change postbioremediation. However, we measured a significant decrease in relative LC_{50} for *Rad54*$^{-/-}$ in fraction D postbioremediation ($p < 0.05$), suggesting increased genotoxicity after bioremediation.

Rev1$^{-/-}$ was more sensitive than the parental *DT40* (relative $LC_{50} < 1$ and $p < 0.05$) to all soil extract fractions pre- and postbioremediation, except for fractions C and D prebioremediation, suggesting that these fractions contained genotoxic compounds that affected the DNA translesion repair pathway (Figure 2.3 B). It is important to note that fractions C and D

were not genotoxic prebioremediation, but were postbioremediation. This suggests that bioremediation resulted in the formation and/or increased concentration of genotoxic compounds in these fractions. We measured a significant decrease in relative LC_{50} for *Rev1*$^{-/-}$ in fractions C, D, E, and F postbioremediation ($p < 0.05$), suggesting increased genotoxicity after bioremediation. Since *Rev1*$^{-/-}$ is involved in error prone translesion DNA synthesis, the increased sensitivity to *Rev1*$^{-/-}$ compared to the parental *DT40* suggests that those soil extract fractions may include mutagenic chemicals.[148] However, *Rev1*$^{-/-}$ was not more sensitive than the parental *DT40* to the unfractionated soil extracts, pre- and postbioremediation. This may be due to antagonistic effects from the complex mixture of compounds in the unfractionated extracts that were not present in the fractions.

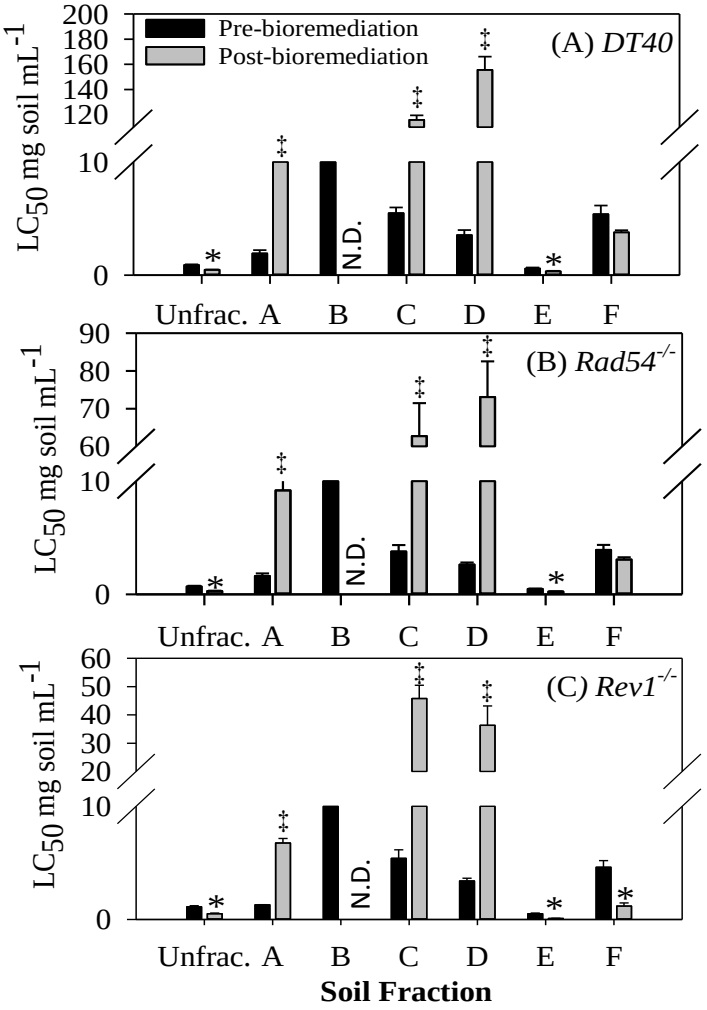

Figure 2.2 Mean of the median lethal concentrations (LC_{50}) (with standard errors bars, $n = 4$) of unfractionated soil extract (Unfrac.) and soil extract fractions (A – F) pre- and postbioremediation for (A) *DT40,* (B) *Rad54*$^{-/-,}$ and (C) *Rev1*$^{-/-}$ cells in mg soil residue per mL DMSO. LC_{50} values with asterisks (*) showed a significant decrease postbioremediation (increased toxicity), while (‡) showed a significant increase postbioremediation (decreased toxicity) ($p < 0.05$). The LC_{50} for soil extract fraction B postbioremediation could not be determined because the full dose-response curve could not be captured from the exposure concentrations (N.D. = not determined).

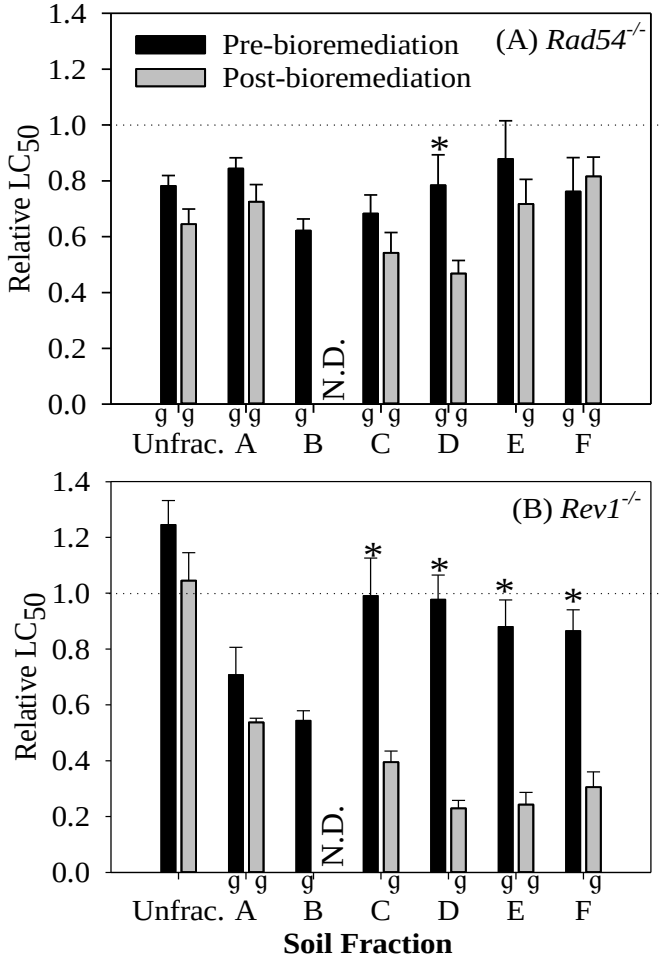

Figure 2.3 Mean of the relative LC_{50} values (with standard errors bars, $n = 4$) of unfractionated soil extract (Unfrac.) and soil extract fractions (A – F) pre- and postbioremediation for (A) *DT40*, (B) *Rad54*$^{-/-}$ and (C) *Rev1*$^{-/-}$ cells. "g" indicates the fraction was genotoxic (i.e. mean relative $LC_{50} < 1.0$ and $p < 0.05$). Relative LC_{50} values with asterisks (*) showed a significant decrease postbioremediation (increased toxicity), while (‡) showed a significant increase postbioremediation (decreased toxicity) ($p < 0.05$). The relative LC_{50} for soil extract fraction B postbioremediation could not be determined because the full dose-response curve could not be captured from the exposure concentrations (N.D. = not determined).

The vast majority of PAHs, OPAHs, HPAHs measured in this study, including those with known genotoxicity,[149–152] were contained in fractions A, B, and C (Table 2.1). Though these compounds may have accounted for the observed genotoxicity in fractions A, B, and C (Figure 2.3), the increased genotoxicity in fractions D, E, and F cannot be attributed to these compounds because they were not contained in these fractions and/or did not increase in concentration postbioremediation (Figure 2.1, Appendix A1). The degradation pathways of these PAHs have been studied and transformation products often include hydroxylated, carboxylated, and quinone PAH transformation products, such as 9-fluorenone (9FLO), 9-hydroxyfluorenone, 1-indanone, 1-hydroxynaphthoic acid, cis-4,5-dihydroxy-4,5-dihydropyrene, pyrene-4,5-dione, 2-carboxybenzaldehyde, 9-fluorenone-1-carboxylic acid, 9-carboxymethylene-9*H*-fluorene-1-carboxylic acid, and fluoranthene-2,3-dione etc.[106,153–156] Some potential transformation products of 3- and 4-ring PAHs(9FLO, 1,4PD, 9,10PQ, and 7,12BaAD) were measured in this study but their concentrations decreased or did not change postbioremediation (Figure 2.1, Appendix A1). This suggests the increased toxicity measured postbioremediation might likely be due to transformation products, including those of the 3- and 4-ring PAHs (PHE, 1MPHE, 2MPHE, PRY, BaA, and FLA) that were most degraded.

2.4.3 Embryonic Zebrafish Bioassay

The embryonic zebrafish bioassay was used to assess the soil extract fractions for developmental toxicity, both pre- and postbioremediation. Soil extract fractions A, B, and C had lower median effective concentrations (EC_{50}) (were more developmentally toxic) than fractions D, E, and F (Figure 2.4, Appendix A3). The EC_{50} for fractions E and F, postbioremediation, were

unable to be calculated because the concentrations tested were too low to capture the full concentration-response curve.

Fractions A, B, and C primarily contained the PAHs and methyl PAHs, OPAHs, and HPAHs in this study (Table 2.1). This suggests that the PAHs and methyl PAHs, OPAHs, and HPAHs measured in this study contributed significantly to the developmental toxicity of the zebrafish in these fractions. No significant change in EC_{50} was measured postbioremediation in fractions A and B, suggesting the developmental toxicity potential of these fractions did not change after remediation. A statistically significant decrease in EC_{50} postbioremediation was measured in fraction C ($p < 0.001$), indicating an increase in developmental toxicity after bioremediation. Fraction C contained 9FLO (Table 2.1), but 9FLO is unlikely to have caused the increase in developmental toxicity in this fraction because its concentration did not increase postbioremediation (Figure 2.1 and Appendix A1). It should be noted that though we measured increased genotoxicity in the *DT40* bioassay in fraction D (Figure 2.3), we measured a significant increase in EC_{50} postbioremediation ($p < 0.001$) in fraction D, suggesting that the compounds causing developmental toxicity in the embryonic zebrafish bioassay in this fraction were bio-transformed and/or decreased in concentration after bioremediation. Though genotoxicity increased postbioremediation in fraction D (Figure 2.3), and developmental toxicity decreased (Figure 2.4) in fraction D, it should be recognized that the two different assays provide information on different toxicological endpoints. While the *DT40* bioassay is a measure of DNA damage, the embryonic zebrafish is a comprehensive overview of any effect that can interfere with the normal development of the zebrafish.

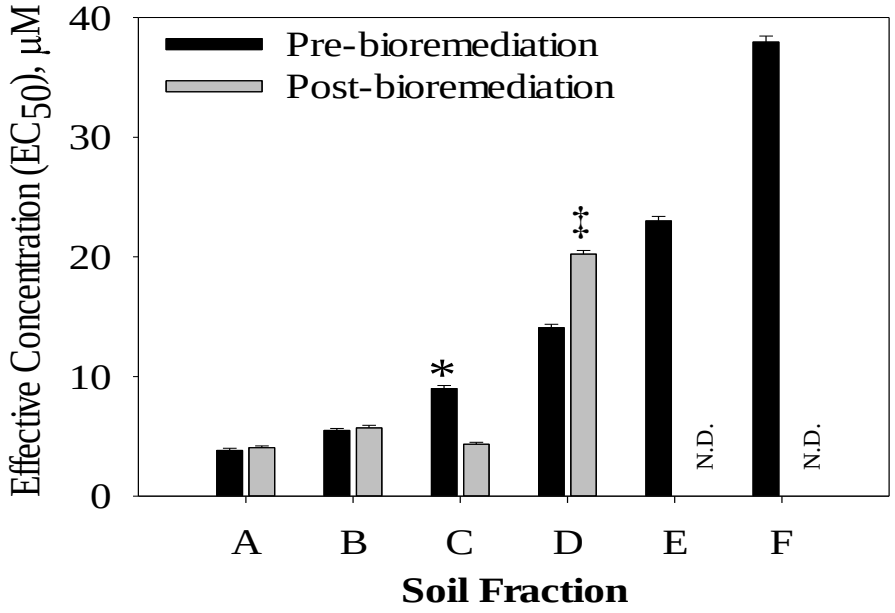

Figure 2.4. Mean of the median effective concentrations (EC_{50}) (with standard errors bars, n = 32) of fractionated soil extracts (A-F) pre- and postbioremediation in embryonic zebrafish. EC_{50} values with asterisks (*) showed a significant decrease postbioremediation (increased developmental toxicity), while (‡) showed a significant increase postbioremediation (decreased developmental toxicity) ($p < 0.05$). The EC_{50}s of fractions E and F postbioremediation were unable to be calculated because the concentrations tested were too low to capture the full concentration-response curve (N.D. = not determined).

In addition to EC_{50}, we evaluated 22 endpoints in the embryonic zebrafish, including swim bladder, pericardial edema, caudal and pectoral fin malformations. The malformations induced by each concentration level of the individual soil extract fractions, compared with the 1% DMSO vehicle control, are presented as a heat map of lowest effect levels (LELs) in Figure 2.5. Axis, jaw, caudal fin, and yolk sac edema malformations were measured prebioremediation in fraction A and were reduced postbioremediation. Fraction B had a similar malformation

profile to fraction A, except that the malformations were less pronounced. We measured a dominant swim bladder malformation in fraction C prebioremediation and this malformation was also reduced postbioremediation. Compared to all other fractions, fraction D had the lowest number of malformations, both pre- and postbioremediation. A swim bladder malformation was measured in fractions E and F and was reduced postbioremediation. We also measured mortality at 120 hours post fertilization (hpf) in fraction F postbioremediation, which was not present prebioremediation, suggesting that bioremediation produced larval mortality in the zebrafish (Figure 2.5).

Although we measured an increase in the LELs (decreased developmental toxicity) in individual malformations postbioremediation in fractions A and B (Figure 2.5), the EC_{50}'s for fractions A and B did not increase (developmental toxicity unchanged) postbioremediation (Figure 2.4). This suggests that the severity of the 22 malformations induced by the postbioremediation extracts for these fractions were reduced (i.e. while the number of fish with at least one of the 22 evaluated malformations were the same pre- and postbioremediation, the number of fish with more than one of the 22 evaluated malformations decreased postbioremediation). This may also be the case for fraction C where the EC_{50} decreased (increased developmental toxicity) postbioremediation (Figure 2.4) even though there was an increase in LELs (decreased developmental toxicity) overall in measured malformations in this fraction postbioremediation (Figure 2.5) (i.e. while the number of fish with at least one of the twenty-two evaluated malformations increased postbioremediation, the number of fish with more than 22 of the evaluated decreased postbioremediation)

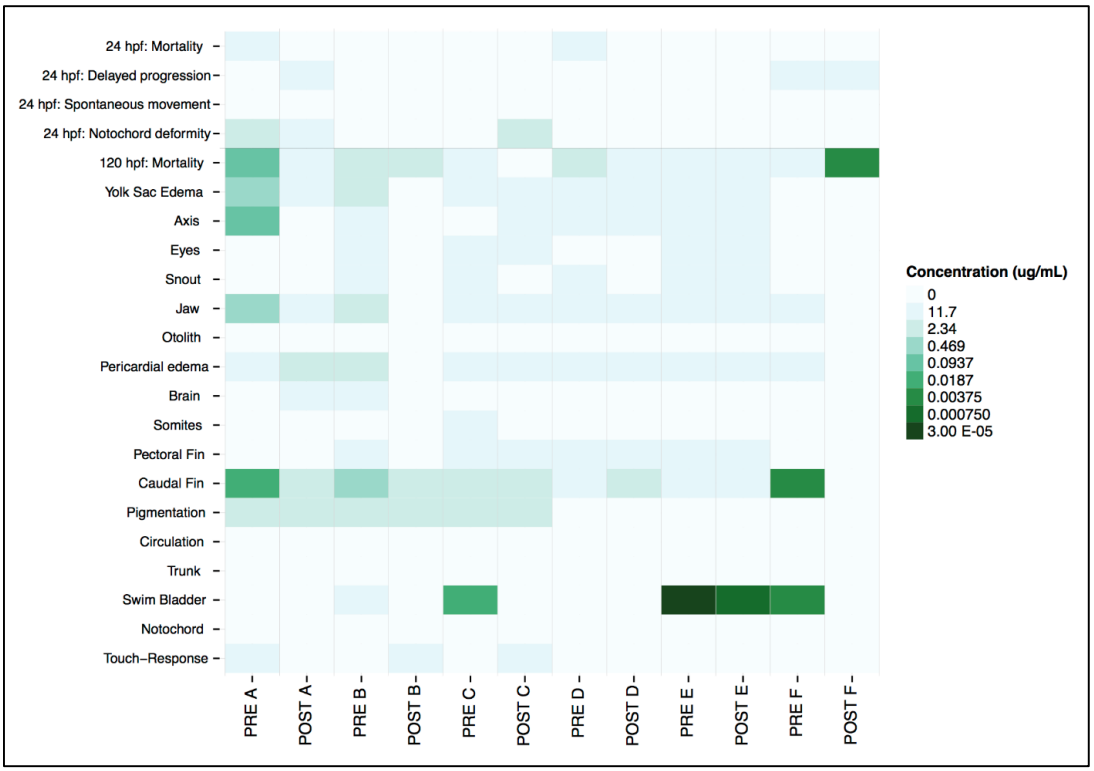

Figure 2.5 Heat map of Lowest Effect Levels (LELs) for each of the 22 evaluated endpoints in 24 hours post fertilization (hpf) and 120 hpf embryonic zebrafish. Darker color indicates lower LEL. (Pre = prebioremediation; post = postbioremediation, concentration "0" indicates no measured effect).

2.5 Implications

One of the implications of this research for sites contaminated with PAHs, including many Superfund sites, is that the higher molecular weight PAHs (including BaA, BkF, BbF, BaP, and IcdP) are not significantly decreased in concentration postbioremediation and may exceed regulatory MACs in the U.S., Germany, and Canada, even after bioremediation of the contaminated soil.[13,95,136] Another implication is that the genotoxicity and developmental toxicity of the soils may increase after bioremediation due to the formation of hydroxylated,

carboxylated, and quinone PAH transformation products,[154–158] that have not yet been positively identified. While the formation of polar transformation products merits attention due to their potential accumulation and toxicity,[106,141,145,159] their likely increased bioavailability needs to be accounted for as well.[106,160] Future work will focus on identifying, characterizing, and quantifying the potential 3- and 4-ring PAH transformation products responsible for the increased genotoxicity and developmental toxicity postbioremediation using non-targeted comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry (GC×GC/TOF-MS)[30,161] (with and without derivatization) and liquid chromatography-tandem mass spectrometry (LC/MS-MS).[162]

2.6 Acknowledgements

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CHAPTER 3 – POLAR TRANSFORMATION PRODUCTS INCREASE TOXICITY OF PAH CONTAMINATED SOIL FOLLOWING BIOREMEDIATION

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Contributions – Prepared by Leah Chibwe with editorial comments provided Michael D. Aitken, Eunha Hoh and Staci Simonich. Experiments were conducted by Leah Chibwe.

3.1 Abstract

Bioremediation is a commonly used technique to remove the toxicity associated with polycyclic aromatic hydrocarbons (PAHs) in contaminated soils. Risk assessment strategies frequently focus on the removal of a subset of parent (or unsubstituted) PAHs, failing to consider the potential formation of toxic transformation products and the presence of other more mutagenic. PAHs, such as MW302-PAHs (PAHs with molecular weight ≥ 302 a.m.u). In a prior study, an effects-directed analysis approach was conducted, whereby extracts from soil were fractionated into six fractions (A – F, in increasing polarity), analyzed for a target of 88 PAHs (parent PAHs and selected oxygen containing PAHs) and evaluated for genotoxicity and developmental toxicity using the *DT40* lymphocyte assay and zebrafish bioassay, respectively. Increased toxicity was observed in four of the relatively polar fractions (C, D, E and F). In the present study, an integrated non-targeted approach was used to characterize transformation products in soil extract fractions that had previously been established as toxic. This approach combined comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC/TOFMS) and liquid chromatography quadrupole time-of-flight mass spectrometry (LC/QTOF-MS). Additionally, targeted GC/MS analysis was used to evaluate degradation of MW302-PAHs. The non-targeted workflow resulted in the tentative identification of 10 peaks postbioremediation, isolated from more than 5,000 candidate peaks in the soil extract fractions, based on mass spectral similarity matching and/or fragment interpretation. Several of the tentatively identified compounds contained an oxygen and/or nitrogen in their elemental formulas, suggesting they would be relatively polar. Finally, targeted analysis showed that MW302-PAHs were not degraded postbioremediation, suggesting that the mutagenic potential

associated with these PAHs remained unchanged. The results of this study suggest that hazard assessment approaches focused on parent PAH removal, may understate the risks following bioremediation.

3.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are incomplete combustion products of organic matter. Sources include the burning of fossil fuels, forest fires, vehicle exhausts, tobacco smoke, coal coking and preparation processes.[96,163,164] In addition, PAHs are environmental contaminants of particular concern because of their widespread presence, persistence, and toxic properties.[1,97,98] Many PAHs are suspected or known carcinogens and mutagens, with 16 parent (or unsubstituted) PAHs classified as priority pollutants by the U.S. Environmental Protection Agency (EPA).[2,163,165,166]

The hydrophobic nature of many PAH compounds ultimately leads to their presence in soils, especially at industrial sites where direct contamination occurs.[167,168] Due to the environmental concerns associated with PAHs, remediation techniques, such as bioremediation, have been developed. Bioremediation uses microorganisms to break down PAHs in soils, with the anticipated outcome of reducing the toxicity of the soil.[168] However, several studies have reported increased toxicity in PAH contaminated soils following bioremediation, suggesting that the partial degradation of PAHs may result in the formation of more polar and toxic byproducts.[14,13,169,49,94,95] These products include oxygen containing PAHs (quinones, hydroxy PAHs, etc.) proposed in bacterial degradation pathways,[157,170,171] that may be more toxic because they are direct acting mutagens.[163] Previous studies that investigated the bioremediation of PAH

contaminated soils have reported the formation and accumulation of polar mutagenic aromatic ketones and azareenes,[95,94,172,150] such as 4-oxapyrene-5-one and indenopyridine, respectively.[94,95] Additionally, studies that applied an effects-directed analysis approach that combined biological and chemical analyses, observed greater toxicity associated with the semi-polar to polar fractions of the soil extract that could not be attributed to target mostly parent PAHs.[13,14,14,94,114,125,127]

In addition to concerns about transformation products, there is limited knowledge on the degradation of higher molecular weight PAHs (MW302-PAHs, PAHs with MW ≥ 302 a.m.u.) in soils. MW302-PAHs are significantly more mutagenic than parent PAHs and less likely to undergo bioremediation.[95,102,135] For example, MW302-PAH dibenzo[a,l]pyrene (D[a,l]Pyr) has an estimated relative potency factor that is 30 times higher than the commonly used reference compound for toxicity assessments, benzo[a]pyrene.[173] Recently, Titaley *et al.* observed that inclusion of the MW302-PAHs in the toxicity assessment increased the B[a]P carcinogenic equivalent concentrations of asphalt and coal tar based sealcoat by 4.1% to 38.7%.[4]

Risk assessment strategies for PAH contaminated soils at industrial sites frequently focus on the 16 US EPA PAHs and determine the efficiency of bioremediation based mainly on the reduction in concentrations of these compounds.[137] However, the potential formation of toxic transformation products and limited degradation of mutagenic MW302-PAHs in bioremediated soils, suggest that their contribution to risk in bioremediated soils may be overlooked when not incorporated in assessment strategies.

Comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC/TOF-MS) and liquid chromatography quadrupole time-of-flight tandem

mass spectrometry (LC/QTOF-MS) are both widely used for the targeted and non-targeted analysis of volatile and polar environmental contaminants, respectively.[7,9,27,31,35,39,55,89] GC×GC/TOF-MS offers increased chromatographic resolution, making it a high-throughput method for the separation of hundreds to thousands of chemicals in complex samples.[26,30] The application of high resolution MS in particular has presented major advances in the structural elucidation of unknown contaminants,[27,35] such as the recently discovered perfluoroalkyl sulfonates in aqueous film-forming foams and unrecognized degradation products in wastewater.[27,35,89] High mass accuracy measurements assist in narrowing down the pool of potential unknown compounds by providing a limited number of chemical formula that match a given exact mass. Additionally, high mass accuracy MS/MS product ions offer an additional level of identification, increasing the confidence in the designation of unknowns.[10]

In a prior study, an effects-directed analysis approach, incorporating targeted conventional GC/MS with the *DT40* lymphocyte and zebrafish *(Danio rerio)* assays, was used to evaluate the toxicity of a contaminated soil from a former coal tar contaminated manufactured gas plant site in North Carolina pre- and postbioremediation.[14] An increase in genotoxicity and developmental toxicity was observed postbioremediation in four soil extract fractions (fractions C, D, E and F). However, none of the 88 PAHs (parent PAHs and selected oxygen containing PAHs) could account for the observed increase in toxicity postbioremediation.

The objective of the present study was to use non-targeted and targeted analysis to identify transformation products in the toxic soil extract fractions postbioremediation, as well as to assess the efficiency of bioremediation for MW302-PAH degradation, respectively. A non-targeted approach, combining GC×GC/TOF-MS and LC/QTOF-MS techniques, was used to

profile the toxic transformation products in fractions C to F, and to ensure the widest coverage of potential unknown compounds (i.e. volatile to polar). Targeted GC/MS was used to evaluate the degradation of MW302-PAHs.

3.3 Experimental

3.3.1 Chemicals and reagents

Acetonitrile and formic acid were purchased from EMD Millipore (Gibbstown, NJ), purified water was purchased from E. Merck (Darmstadt, Germany), toluene (≥ 99%) and the derivatizing agent *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) were purchased from Sigma Aldrich (St. Louis, MO). All solvents used were HPLC grade. The purity of all available and tested commercial standards tested was at least 90%. MW302-PAHs standards were purchased from Chiron AS (Trondheim, Norway) and AccuStandard. Surrogate standards were purchased from CDN Isotopes (Quebec, Canada) and Cambridge Isotope Laboratories (Andover, MA). MW302-PAHs analyzed were: picene (PIC), naphtho[1,2-b]fluoranthene (N[1,2-b]Fla), naphtho[2,3-b]fluoranthene (N[2,3-b]Fla), dibenzo[a,e]+[b,k]fluoranthene (D[a,e]+[b,k]Fla), dibenzo[a,k]fluoranthene (D[a,k]Fla), dibenzo[j,l]fluoranthene (D[j,l]Fla), dibenzo[a,l]pyrene (D[a,l]Pyr), naphtho[2,3-k]fluoranthene (N[2,3-k]Fla), naphtho[2,3-e]pyrene (N[2,3-e]Pyr), dibenzo[a,e]pyrene (D[a,e]Pyr), coronene (COR) and dibenzo[a,i]pyrene (D[a,i]Pyr). Surrogate Standards used for analysis were d_{12}-benzo[ghi]perylene and d_{14}-dibenzo[a,i]pyrene.

3.3.2. Samples

Soil samples were collected from a former coal tar contaminated manufactured gas plant site in Salisbury, North Carolina, and treated in a laboratory-scale aerobic bioreactor representative of *ex-situ* bioremediation.[13] Methods of extraction, soil characterization, toxicity, and PAH profiles have been described previously (also see Chapter 2).[14] Briefly, soil was extracted in hexane:acetone (75:25, v/v) using pressurized liquid extraction, and the resulting soil extract was fractionated into six aliquots based on polarity (increasing from fraction A to F) using silica solid phase extraction. Soil extract fractions C, D, E, and F, identified as toxic postbioremediation in a prior study,[14] were analyzed using a non-targeted method, combining GC×GC/TOF-MS (derivatized and underivatized) and LC/QTOF-MS (underivatized) (see Figure 3.1 for the method overview).

3.3.3 Derivatization of Samples

Soil extract fractions were derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) to increase their detectability in GC×GC/TOF-MS. MTBSTFA is a common derivatization agent and is known to be more stable and less susceptible to the hydrolytic effects of moisture, when compared to other agents such as N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA).[25] Briefly, 100 µL of acetonitrile and 20 µL of toluene were added to 40 µL of the fractionated soil extract. The mixture was blown down to 20 µL under a steady flow of nitrogen and spiked with 30 µL of MTBSTFA. Samples were incubated at 65 °C for 25 mins and left to equilibrate to room temperature prior to GC×GC/TOF-MS analysis.[174]

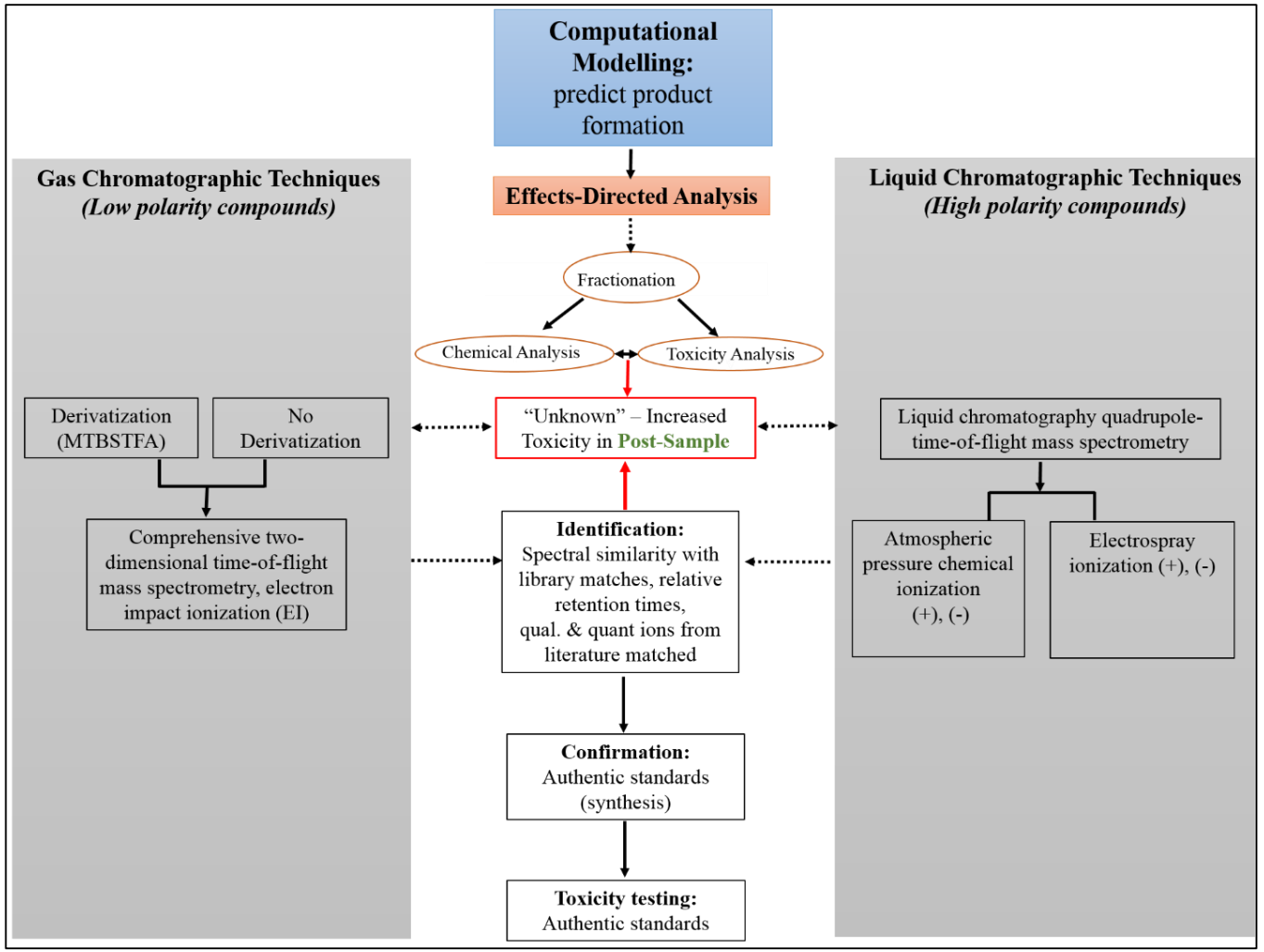


Figure 3.1. Complementary approach combing instrumental analysis, effect-directed analysis, and computational modelling tool.

3.3.4 Comprehensive Two-Dimensional Gas Chromatography Time-of-Flight Mass Spectrometry (GC×GC/TOF-MS) Analysis

Derivatized and underivatized soil extract fractions were analyzed using an Agilent 6890 gas chromatograph (Palo Alto, CA) featuring a non-moving quad-jet dual-stage modulator coupled to a Pegasus 4D time-of-flight mass spectrometer (Leco, St Joseph, MI). A Rtx-5 (35 m long × 0.25 mm internal diameter × 0.10 µm film thickness) and a Rxi-17 column (1.2 m × 0.10 mm × 0.10 µm) (both columns were from Restek, Bellefonte, PA) were used in the first and second dimension, respectively. The first dimension oven temperature program was: 60 °C (hold 1 min), ramp at 6 °C/min to 300 °C (hold 3 min), and then ramp at 20 °C/min to 320 °C (hold 15 min). The second dimension oven temperature program was: 85 °C (hold 1 min), ramp at 6 °C/min to 320 °C (hold 3 min), and then at 20 °C/min to 340 °C (hold 15 min). The modulator temperature was set to +35 °C relative to the first dimension, with a modulation period of 3.5 s. The transfer line and ion source temperatures were set at 100 °C and 230 °C, respectively.

Data processing was carried out using the LECO ChromaTOF® software version 4.33 (Leco, St Joseph, MI) and included baseline correction and deconvolution procedures. Peaks with signal-to-noise (S/N) ratios greater than 100 and 2D sub peaks with S/N > 50 were combined using a spectral match factor > 70%. The Statistical Compare feature of LECO ChromaTOF® (Leco, St Joseph, MI) was used to assign peaks into three groups (blank, prebioremediation and postbioremediation). Statistical Compare uses peak alignment algorithms to first match peaks across samples based on 1D and 2D retention times and mass spectral similarities. Pairwise comparisons are then conducted on peaks across samples, to determine the most common peaks between groups. After Statistical Compare processing, information on grouped peaks (1D, 2D retention times, peak areas, m/z, etc.) were exported to an Excel

spreadsheet, where peaks either only present in the postbioremediation fractions or with 1.5× increased responses postbioremediation were pre-selected. Finally, the pre-selected postbioremediation peaks were visually inspected to deselect chromatographically irrelevant peaks (i.e. poorly resolved, severe tailing etc.). Statistical Compare parameters were as follows: maximum modulation time shift of one 3.5 s period and the maximum retention time difference shift of 0.2 s, with a match spectral similarity > 60% between samples and groups.

Mass spectral matching with the National Institutes of Standards and Technology (NIST) EI library database and a *tert*-butyl silyl (TBS)-library, made available courtesy of Dr. Ute Roessner, president of the Metabolomics Society, were used to identify peaks in underivatized and derivatized samples, respectively.

3.3.5 Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC/QTOF-MS) Analysis

Liquid chromatographic analyses were performed on a Shimadzu Nexera ultra high-pressure liquid chromatography (UHPLC) system (Columbia, MD) coupled to a duospray source high-resolution hybrid quadrupole time-of-flight mass spectrometer (TripleTOF 5600, SCIEX, Framingham, MA). Separation was performed on an Agilent Extend-C_{18} column (2.1 mm × 150 mm, 5 µm particle size) (Palo Alto, CA). A binary gradient consisting of 0.1% (v/v) formic acid in water (A) and acetonitrile (B) was used for chromatographic separation with the following program: 0.0 - 0.01 mins, 2 - 2% B; 0.01 - 2.0 min, 2 - 20% B; 10 - 10.1 min, 20 - 20% B; 10.1 - 12 min, 20 - 70% B. The flow rate was 0.5 mL min^{-1}, injection volume was 5 µL, and the column temperature was 40 °C. Electrospray (ESI) and atmospheric pressure chemical (APCI) ionization in both positive and negative modes were used to monitor analyte ions.

Several authentic standards in acetonitrile from different classes of compounds were directly injected into the mass spectrometer to determine operational instrumental parameters, the purpose of which was to define initial parameters for a non-targeted method, rather than optimize the ionization conditions for all compounds in the soil extract fractions. Additional instrumental parameters were: collision energy, 30 eV; declustering potential, 80 V; temperature, 450 °C and ion spray voltage floating (ISVF), 5200 V. Conditions were similar between ESI and APCI (±), except that in APCI (±) the ISVF was 4500 V. Information-dependent acquisition was used to collect MS/MS scans every 250 ms in the range of 100 - 1200 Da and continuous internal recalibration was performed every 5 samples using the Calibrant Delivery System. The Analyst® software version 1.3.1 (Applied Biosystems, Foster City, CA) was used to collect raw data (TOF-MS and TOF-MS/MS) on acquired peaks.

MarkerView™ software (SCIEX, Framingham, MA) was used to pre-select peaks with increased response postbioremediation. Processing parameters included retention time (RT) tolerance, 0.50 min; minimum RT peak width, 4 scans; mass tolerance, 15.0 ppm and noise threshold, 4000 counts. Additionally, peak areas were normalized to the total peak area of a sample to account for any instrumental variations during analysis. The MarkerView™ software uses algorithms to align ions and retention times across multiple samples, in order to identify structurally related and unrelated components between groups. Following peak processing, pairwise t-tests were conducted and *p*-values calculated to examine the degree of variation in peak areas (of collected mass-to-charge ratios, m/z) between grouped samples (prebioremediation, postbioremediation and blank). Peaks of interest were selected if they had at least a 1.5-fold change increase in area postbioremediation, and if the *p*-value was less than 0.05.

Finally, profile plots were visually inspected to eliminate peaks that were also present in the blank fractions.

MasterView™ (SCIEX, Framingham, MA) was used to determine the elemental composition of the unknown compounds, pre-selected and exported from MarkerView™. The formula finder tool in MasterView™ proposes possible elemental formulas, based and ranked on how well the suggested formulas match the experimental isotope mass-to-charge ratio (m/z) and intensity. Peaks were prioritized for further identification if the determined elemental composition had a mass error < 5 ppm, a formula finder scores > 75%, and if both the TOF-MS and TOF-MS/MS were ranked in the top 2 for a proposed formula. Peaks with formula finder score < 75% and that did not produce a TOF-MS/MS were classified as unknown compounds, level 5, due to the degree of uncertainty associated with the elemental composition and the difficulty in elucidating the structure of an unknown compound without associated MS/MS product spectra.

MetFusion, a tool that combines *in silico* fragmentation prediction of MetFrag,[91] and mass spectral matching of compounds in the MassBank and Metlin MS/MS databases,[175,176] was used to assist in determining the structural formulas from determined elemental compositions (from MasterView™), experimental accurate masses and TOF-MS/MS fragment ions and intensities.[177]

3.3.6 Peak Prioritization and Characterization

Peaks were identified according to 5 levels adapted from Schymanski *et al.* and Hoh *et al* (Figure 3.2).[10,7] The workflow and criteria for peak selection and identification is shown in

Figure 3.2. The levels were: (1) authentic standard (experimental mass spectral match and retention time match with an authentic standard), (2) isomer (experimental mass spectral but retention time mismatch with an authentic standard), (3) library or database (mass spectral match with library, database or literature), (4) group (evidence for possible structures but insufficient for one exact structure allowing the definition of structural class or presence of certain functional groups), and (5) unknown (molecular formula or exact mass could only be assigned to structure or poor library matching).

3.3.4 GC/MS Targeted Analysis of MW302-PAHs in Unfractionated Soil Extracts

Gas chromatographic mass spectrometry (GC/MS) analysis of the MW302-PAHs was carried out using an Agilent 6890 GC system, equipped with a 5973N mass selective detector on an Agilent DB-17MS (60 m × 0.25 mm I.D. × 0.25 μm film thickness) capillary column.[178] The soil extracts were spiked with isotopically labeled internal standards prior to GC/MS analysis. MW302-PAHs were analyzed in electron impact ionization (EI) mode using a previously established method, with minor modifications in oven temperature program that included a 45 °C min ramp to 200 °C and 15 mins hold at 320 °C.[4,178]

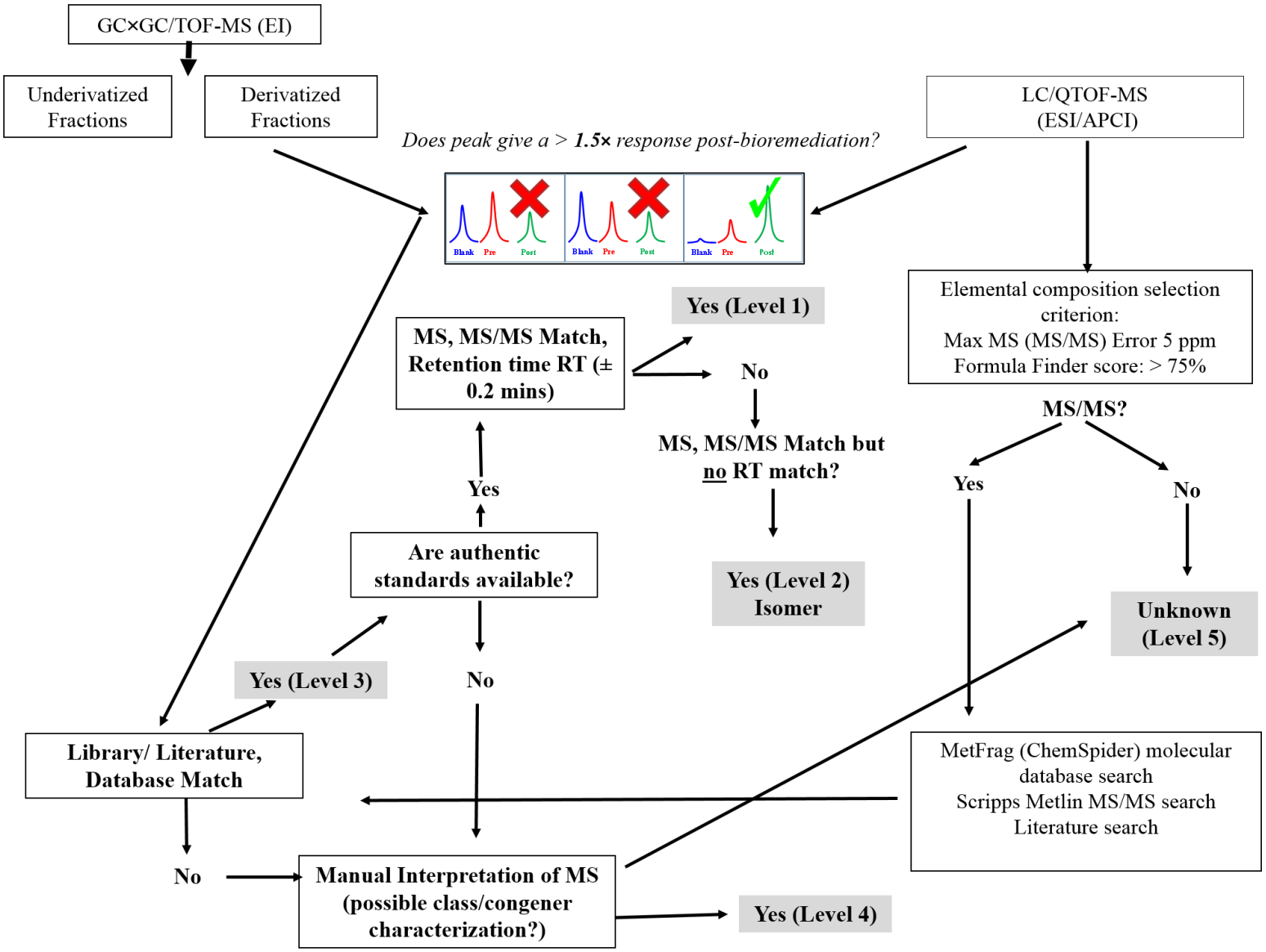


Figure 3.2 Peak identification and level characterization workflow.

3.4 Results and Discussion

3.4.1. Characterization of Polar Transformation Products in Toxic Fractions

LECO ChromaTOF® Statistical Compare (GC×GC/TOF-MS) and MarkerView™ (LC/QTOF-MS) were used to pre-select peaks with increased response in the soil extract fractions (C, D, E and F) postbioremediation. Peaks were selected for interpretation if they had at least a 1.5× increase in peak area postbioremediation and if they were absent in the blank fractions (to limit false positive identification). Prior to the use of Statistical Compare and MarkerView™, each soil extract fraction had at least 5,000 candidate peaks, indicating the complexity of the soil matrix. Peak pre-selection resulted in 48 and 40 unknown peaks in GC×GC/TOF-MS (derivatized and underivatized) and LC/QTOF-MS (underivatized), respectively. Of these 88 peaks, 10 peaks were tentatively identified in LC/QTOF-MS based on their accurate mass and TOF-MS/MS fragmentation patterns. The remaining 78 peaks were characterized as unknown compounds of interest (level 5) based on the following criteria: (1) poor NIST or TBS-library matching, (2) elemental composition formula finder score < 75%, or (3) no TOF-MS/MS spectra obtained at 30 eV. In general, soil extract fraction E contained the largest number of unknown peaks in LC/QTOF-MS and was the most toxic fraction in the *DT40* lymphocyte assay (Figure 2.2, 2.3).[14] Additionally, no substantial overlap in compounds was observed between GC×GC/TOF-MS and LC/QTOF-MS. Detection of a compound depended on whether the compound was GC or LC amenable and on the different instrumental conditions.

Information on the unknown peaks tentatively identified, including elemental compositions, elemental formula finder scores and mass errors detected in LC/QTOF-MS are given in Table 3.1. Structures are provided for unknown compounds classified as level 2

(potential isomer match) or level 3 (i.e. only one molecular structure predicted by the *in silico* fragmentation software for the determined elemental composition). Structures for the unknown compound where more than one molecular formula was possible are given in Appendix B, with accompanying ranked values, illustrating the similarity match between the predicted and experimental mass spectra.

An unknown compound with elemental composition $C_{13}H_8O$ was detected in soil extract fraction E in LC/QTOF-MS with a 3-fold change ($p < 0.001$) increase postbioremediation (Table 3.1). In a previous study, 9-fluorenone, which corresponds to the same elemental composition, has been observed to accumulate during bioremediation.[141] However, no significant change in 9-fluorenone concentration postbioremediation was observed in these soils previously (Figure 2.1).[14] Furthermore, comparison of TOF-MS/MS fragmentation patterns and retention times between authentic standards of 9-fluorenone and phenalenone, indicated that the unknown compound was more structurally related to phenalenone than to 9-fluorenone (Figure 3.3).

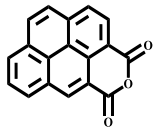
Tentatively identified compounds in LC/QTOF-MS were generally characterized elementally as CHO or CHNO compounds (Table 3.1). When several potential isomers were suggested for a given accurate mass and elemental composition, structures were predominantly composed of at least 2 phenyl rings, suggesting products were most likely PAH degradation products (Table 3.1, Appendix B). These degradation products may be from 3- to 4-ring parent PAHs that were observed to significantly decrease in concentration postbioremediation in the prior targeted study (i.e. phenanthrene, 2-methylphenantherene, 1-methylphenanthrene, fluoranthene and pyrene, etc.).[14] For many of the compounds it was difficult to assign a unique molecular structure due to the number of possible isomers for a given elemental composition

(Table 3.1, Appendix B). However, several of the proposed formulas contained an oxygen and/or nitrogen, suggesting they would be relatively polar. Oxygenated PAHs are known to be equally, if not more, toxic than parent PAHs and have been linked to severe malformations and developmental toxicity in zebrafish.[4,122,125] Additionally, nitrated and N-containing PAHs are known to exhibit ecotoxic effects comparable to parent PAHs.[150,179]

Though there are limited studies, an overview of transformation products tentatively identified in several previous bioremedial studies is given in Table 3.2. While transformation products with elemental compositions CHO, belonging largely to aromatic ketones, dominated the list,[93–95,141,180–182] Brooks *et al*. noted the formation of azaarene-related compounds (CHN) postbioremedition.[94] Transformation products identified in at least two of the previous studies, included 9-fluorenone, 1-indanone, anthracenedione and 9,10-phenanthrenedione (Table 3.2).[93,94,141,180,181] It should be noted that the PAH transformation products formed may vary because remedial applications are often site-specific and implemented based on various factors, such as soil properties, extent of pollution, etc. Brooks *et al*. additionally, evaluated four bioremedial applications using an effects-directed analysis approach on coal-tar contaminated soils and observed that biopile and bioslurry treatments resulted in increased mutagenicity postbioremediation, while land treatment and compost reduced mutagenicity.[94] Hu *et. al.* evaluated toxicity changes of PAH contaminated soils in a short-term 35-day residence time aerobic slurry treatment and a long-term 2-year column treatment in manufactured gas plant soils, and observed that the column treatment decreased toxicity, while the aerobic slurry treatment did not, postbioremediation.[13] Although, the decrease in overall toxicity

postbioremediation was observed in the column treatment, DNA damage was observed in the *DT40* bioassay, suggesting the formation of genotoxins.[13]

Table 3.1. Tentatively identified peaks in LC/QTOF-MS with retention times (RT), elemental composition and score, precursor mass-to-charge ratio (m/z), molecular m/z, mass error, fold-change and p-value of peak response postbioremediation.

Instrument (Ionization)	Fraction	RT, mins	Elemental composition (Score)	Precursor, m/z	Elemental m/z	Mass error, ppm	Fold-change post bioremediation	P-value	Level of Identification
LC/QTOF-MS (ESI +)	E	4.85	$C_{13}H_8O$ (93.5)	181.06477	180.05749	0	3.00	1.62E-05	 2 (potential isomer match)
LC/QTOF-MS (ESI +)	F	4.75	$C_{14}H_7NO_2$ (85.9)	222.05478	221.0475	0.7	5.06	1.96E-05	4
LC/QTOF-MS (ESI +)	E	5.63	$C_{15}H_{10}O_2$ (90.1)	223.0754	222.06812	1.6	1.50	2.00E-02	4
LC/QTOF-MS (ESI +)	E	5.81	$C_{15}H_{13}NO$ (88.1)	224.107	223.09978	0.4	1.60	2.32E-03	4
LC/QTOF-MS (ESI +)	E	6.66	$C_{16}H_{10}O_2$ (87.8)	235.0754	234.06812	1.5	5.80	5.01E-06	4
LC/QTOF-MS (ESI +)	E	4.85	$C_{15}H_{12}O_3$ (85.8)	241.08587	240.07859	0.1	7.10	3.24E-05	4
LC/QTOF-MS (ESI +)	E	7.13	$C_{17}H_{12}O_2$ (91.9)	249.09088	248.09088	0.4	1.62	3.08E-03	4
LC/QTOF-MS (ESI +)	C	7.3	$C_{18}H_8O_3$ (83.3)	273.0541	272.04682	1.9	2.06	8.84E-03	 3 (database match) 3H,5H-Pyreno(1,10-cd)pyran-3,5-dione
LC/QTOF-MS (ESI +)	F	6.06	$C_{20}H_{26}O_4$ (94.8)	331.19041	330.1831	0	1.23	2.00E-04	5, unknown
LC/QTOF-MS (ESI +)	E	6.15	$C_{21}H_{18}O_5$ (91.7)	351.12242	350.11514	0.9	11.91	2.97E-03	4

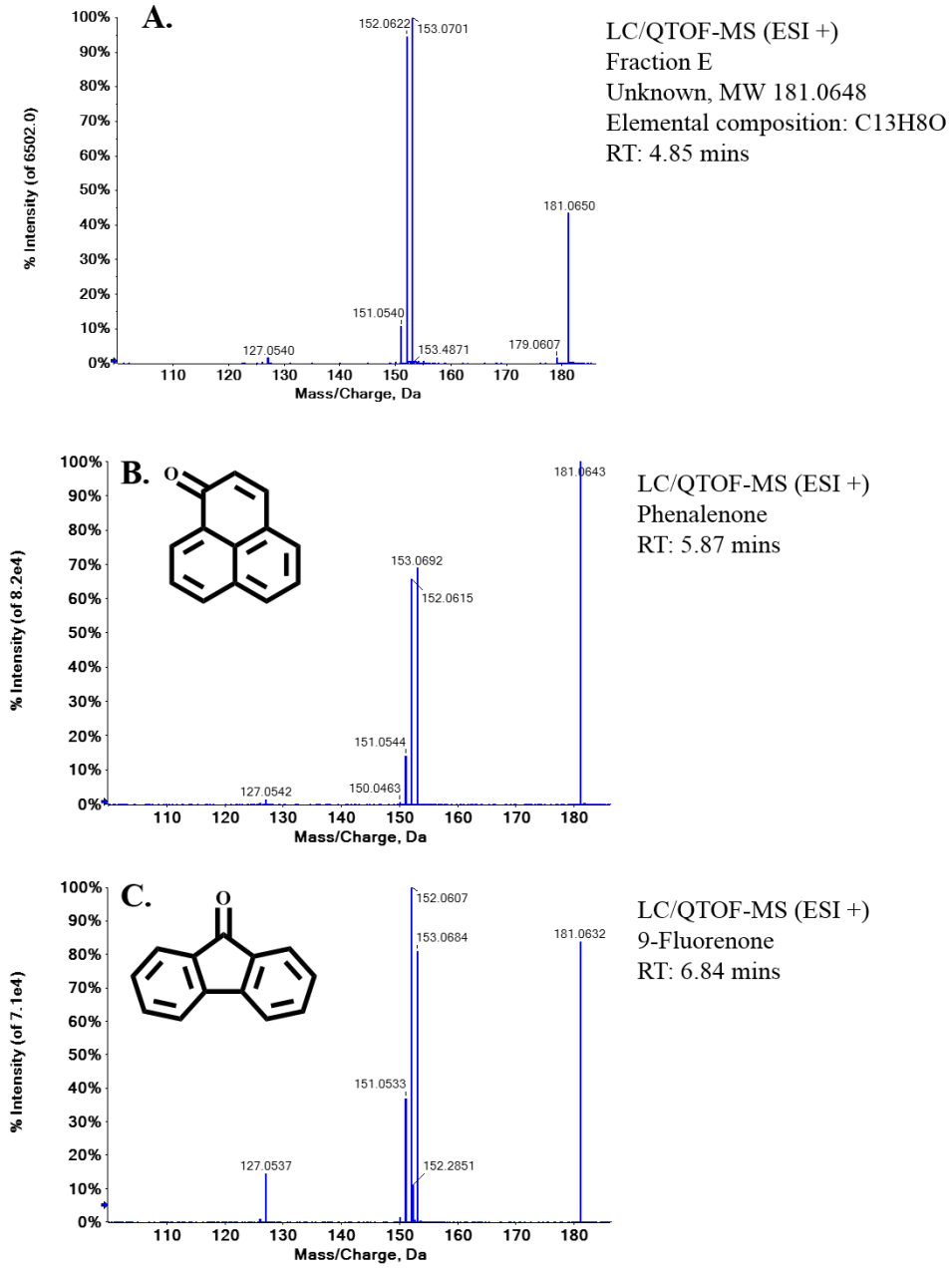


Figure 3.3 TOF-MS/MS mass spectra of (A) unknown compound 181, and (B) Phenalenone and (C) 9-fluorenone authentic standards analyzed in LC/QTOF-MS in fraction E

Table 3.2. Tentatively identified transformation products in previous bioremedial studies. **Bold** products indicate compounds identified in at least two studies. [a]Authentic standards confirmed.

Treatment (duration, weeks)	Contamination	Transformation products	Elemental compositions/functional group	Analysis	Source (year)
Bioslurry (6)	Creosote (soil)	methylbenzacridine, **benzanthracenone**, fluorenecarbonitrile, 1-azapyrene, tetramethylphenol, fluoroscein, **9,10-anthraquinone**, indacenedione, benzoquinoline, **cyclopentaphenanthrenone**, hydroxypyrene, **anthracenedione**, cyanopyrene, benzothiazolylphenol, diphenylpyrazol, 2-hexanol, methylcyclopentanone, tetrachloroethane, 3-methylpentanone, diaminotriazole, dihydrocyclobuta[b]naphthalene, phenanthrofuran, 2-pentanone, indenopyridine, acridine, methylacridine, methylazaphenanthrene, naphthopyrandione, anthracenecarbonitrile, palmitic acid	Aromatic ketones, hydroxy PAHs, alcohols (CHO); azareenes (CHN) etc.	Effects-directed analysis: *Salmonella* mutagenicity assay, HPLC (fractionation), GC/MS	94 (1998)
Biopile (20)	Creosote (soil)	benzanthracenone, fluorenecarbonitrile, phenethrol, indenoisoquinoline, hydroxy-thienyl-quinoline, anthracenecarbonitrile, 2-hexanol, tetrahydrophenanthrenone, therahydrobenzanthracene, phenanthrofuran, benzofuranol, **benzocoumarin**	Aromatic ketones, hydroxy PAHs, alcohols (CHO); azareenes (CHN) etc.	Effects-directed analysis: Salmonella mutagenicity assay, HPLC (fractionation), GC/MS	94 (1998)
Added nutrients to indegenous microorganisms (4)	Creosote (soil)	**9H-fluorenone**, **4-hydroxy-9H-fuorenone**, **4H-cyclopenta[def]phenanthrenone**, **9,10-phenanthrenedione**	Aromatic ketones and hydroxy PAHs (CHO)	GC/MS	179 (2000)
Bioslurry (4)	Gas works site, coal tar (soil)	**1-acenaphthenone**, 4-oxapyrene-5-one	Aromatic ketones, ester (CHO)	Accumulation of transformation products, GC/MS	95 (2002)
Soil/compost (29)	Tar oil	1-hydroxyacenaphthene, **1,2-acenaphthenedione**, ?-dihydroxyacenaphthylene, ? -hydroxydibenzofuran, 1H,3H-naphtho[cd]pyran-1-one,?-dihydroxyacenaphthene, 3-hydroxy-2-naphthoic acid,**?-benzocoumarin**,**?-hydroxy-9-fluorenone**, **1,8-naphthalic anhydride**, **9,10-phenanthrenedione**, **9,10-anthracenedione**, dibenzothiophenesulfoxide,?-dihydroxyphenanthrene, dihydroxyfluorenone,?-dihydroxydihydrophenanthrene, dibenzothiophenesulfone,?-hydroxyfluoranthene, **7,12-benz[a]anthracendione**	Aromatic ketones and hydroxy PAHs (CHO)	GC/MS and LC/MS	142 (2000)
Short-term incubation (19)	Fertile soil/Switzerland	**1-indanone**, **9-fluorenone**	Aromatic ketones (CHO)	GC/MS (accumulation of transformation products during treatment)	141 (2013)[a]
Bioslurry (3 - 13)	MGP soil	phthalic acids, fatty acid methyl-esters, **anthracenedione**, **benzanthracenone**	Aromatic ketones, phthalic acids, fatty acid methyl-esters (CHO)	GC/MS	181 (2001)
Soil/compost (15)	Artificially contaminated (Coal tar oil)	**1-indanone**, **acenaphthene-1,2-dione**, **1,8-naphthalic anhydride**, **anthracene-9,10-dione**, **benz[a]anthracene-7,12-dione**, **9-fluorenol**, 9(10H)-anthracenone, 2-methylanthracene-9,10-dione	Aromatic ketones, anhydrides (CHO)	HPLC, GC/MS	182 (1997)

Two anhydride related compounds, phthalic anhydride and 4-methylphthalic anhydride, were detected and confirmed with reference standards in GC×GC/TOF-MS (Figure 3.4, Appendix B). However, phthalic anhydride is known to be unstable in the presence of moisture.[183] Therefore, it is more likely that these compounds were from the in-source decomposition of larger unknown compounds as a result of high GC temperatures. For example, the phthalates are more likely to be from a tentatively identified compound, such as 3H,5H-Pyreno(1,10-cd)pyran-3,5-dione, which was tentatively identified in LC-QTOF-MS (Figure 3.4, Table 3.1) (at significantly lower oven temperatures of 40 °C). Brominated phthalic anhydrides were similarly observed to result from the thermal decomposition of *bis*-(2-ethylhexyl)-tetrabromophathalate and 2-ethylhexyl-2,3-4,5-tetrabromobenzaote by Hoh *et al*.[184] The possible in-source decomposition of unknown compounds presents challenges in elucidating the structures of unknowns in non-targeted methods, and should be considered because interpreted mass spectral fragments might only represent a part of an unknown molecule in both LC-QTOF-MS and GC×GC/TOF-MS.

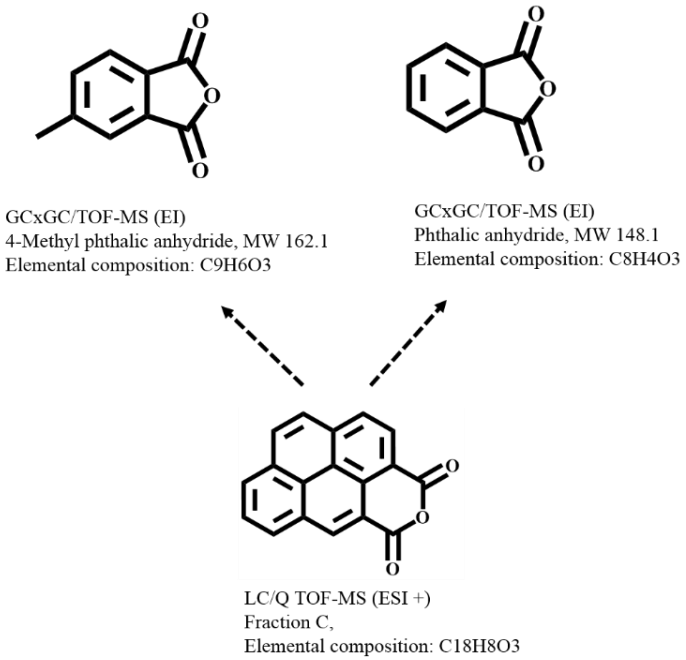


Figure 3.4 Phthalic anhydrides (top) as potential decomposition fragments for 3H,5H-Pyreno(1,10-cd)pyran-3,5-dione (bottom) tentatively identified in LC/QTOF-MS.

3.4.2 MW302-PAHs Characterization

In addition to non-targeted analysis of the transformation products in soil extract fractions C to F, MW302-PAHs were also measured in the unfractionated soil extracts, pre- and postbioremediation, to evaluate their extent of biodegradation (Figure 3.5). The total concentration of MW302-PAHs in the unfractionated soil extract was 155 ± 18.8 μg g^{-1} prebioremediation and 200 ± 18.0 μg g^{-1} postbioremediation ($p = 0.11$), indicating no significant biodegradation. For some of the MW302-PAHs (PIC, N[2,3-e] Pyr, and COR), a statistically significant increase in concentration was observed postbioremediation ($p < 0.05$). It is most likely that the continuous mechanical mixing of the bioslurry reactor enhanced the extractability

of some MW302-PAHs, causing the observed increased in concentrations, rather than it is likely that they were formed during bioremediation.[185] Moreover, due to the size and hydrophobicity of MW302-PAHs, they tend to sorb strongly to organic matter and, as a result, may be less accessible to microbes for degradation, than lower molecular weight PAHs.[102,134,135] Because of their non-polar properties, MW302-PAHs elute in the least polar fractions (A, B).[4,14]

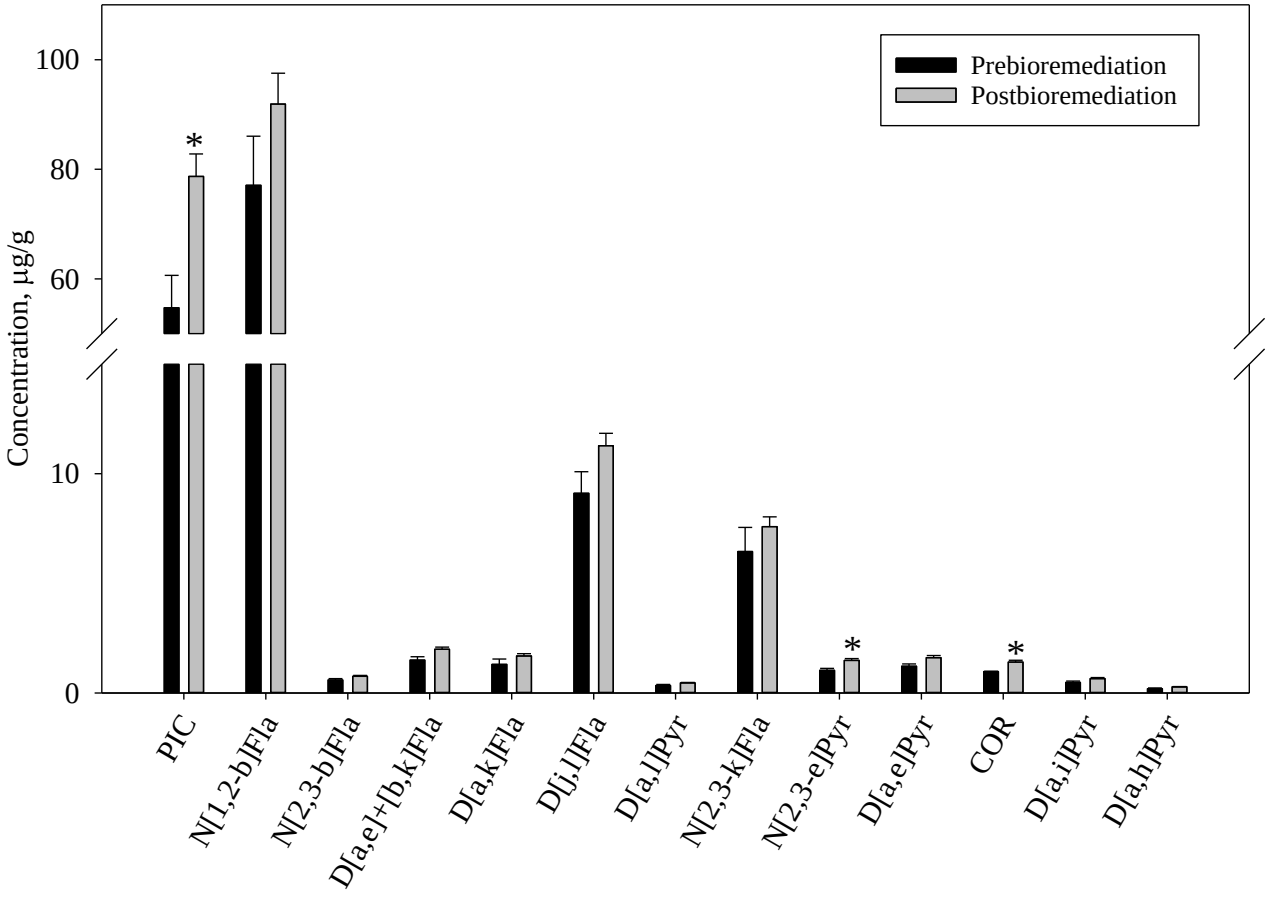


Figure 3.5. Mean concentrations in µg g^1 dry weight (with standard errors bars, n = 3) of investigated MW302-PAHs pre- and postbioremediation in the unfractionated soil extract. Compounds with asterisks (*) showed statistically significant changes in concentration postbioremediation ($p < 0.05$).

3.4.3 Implications and Future directions

In the present study, non-targeted and targeted analysis were combined to investigate polar transformation products and MW302-PAHs in a biodegradation study. There are relatively few studies that address the formation of toxic biodegradation products of PAHs,[93,95,141,180,181] or have studied the biodegradation of MW302-PAHs in soils. Additionally, hazard and risk assessment tends to focus on parent PAHs, suggesting that the calculated risk may be understated following bioremediation.[186]

It should be noted that many factors, including soil properties, contaminant profiles, and microbial populations, may influence the outcome of bioremediation. For example, Kazunga *et al.* observed that the formation of polar metabolites of pyrene resulted in decreased rates of degradation of phenanthrene and benzo[a]pyrene.[156] This suggested that the formation of certain metabolites could result in the persistence and limited degradation of certain compounds and further illustrate the heterogeneity of degradation reactions in natural systems.[156] Factors such as these make it challenging to predict the formation of toxic transformation products in natural systems, highlighting the need for combination of effects-directed and non-targeted analysis methods.

The mass spectral data collected in this study for all compounds that indicated increased responses postbioremediation (levels 2-5) has been incorporated into an in-house library (i.e. GC×GC/TOF-MS, LC/QTOF-MS). It will be used as a reference database for future investigation into PAH transformation products in bioremedial applications. Monitoring the frequency of formation of unknown compounds in related studies is one way of prioritizing the identification of potential toxic transformation products. This is especially vital given that for

many of these transformation products, including those in previous studies,[93,94,141,181] there is a severe lack of authentic standards and mass spectral databases. As a consequence, the toxicities of these transformation products are poorly understood even though they could be significant.

3.5 Acknowledgements

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CHAPTER 4 – POLYCYCLIC AROMATIC HYDROCARBONS AND RELATED TOXIC COMPOUNDS IN THIRDHAND SMOKE CONTAMINATED HOUSE DUST

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Contributions – Prepared by Leah Chibwe with editorial comments provided by Eunha Hoh and Staci Simonich. House dust collection and participation recruitment were conducted by Kayo Watanabe and Joy Zakarian. PAH and TSNA house dust extractions were done by Cher Vue and nicotine house dust extraction was done by Kathy Datuin. Nicotine and TSNA liquid chromatography mass spectrometry analyses were conducted by Kayo Watanabe. PAH analysis was conducted by Leah Chibwe. This study is part of the Healthy Home Project 3 & Co-PI: Georg Matt and Penelope (Jenny) Quintana), funded by the Californian State Tobacco-Related Disease Research Program (TRDRP).

4.1 Abstract

Indoor settled house dust is a potential reservoir and long term source for contaminants associated with thirdhand smoke (THS), including toxic and persistent polycyclic aromatic hydrocarbons (PAHs). Although tobacco-specific nitrosamines (TSNAs) and nicotine can remain in indoor environments for months after smoking has ended, there is limited knowledge on how PAHs associate with THS long-term. In this pilot study, PAHs, as well as TSNAs and nicotine were investigated in five homes up to six months after active smoking had ended. The total parent PAHs dust loadings were positively correlated with nicotine ($R^2 = 0.15$, $p = 0.10$) and total TSNAs ($R^2 = 0.28$, $p = 0.003$) dust loadings; and total oxygenated PAHs were correlated with nicotine ($R^2 = 0.24$) and total TSNAs ($R^2 = 0.44$) dust loadings ($p < 0.05$). Individual PAHs, 2+1 methylphenanthrene (2+1MPHE), 2-methylanthracene (2MANT), retene (RET), dibenz[a,h]anthracene (DBahA), 9,10-anthraquinone (9,10AQ), 2-methylanthraquinone (2MAQ), benzanthrone (BZ), benzo[cd]pyrenone (BcdP), 5,12-naphthacenequinone (5,12NQ) and 9-fluorenone (9FLO) were significantly correlated with nicotine and total TSNAs dust loadings (R^2: 0.47 - 0.72, $p < 0.05$), suggesting that these compounds may associate with THS and should possibly be considered when assessing the long-term risks associated with THS exposure.

4.2 Introduction

Thirdhand smoke (THS) is a complex, dynamic, persistent and toxic mixture of tobacco smoke pollutants generated from secondhand smoke.[187,188] Residual tobacco smoke deposits on indoor surfaces, sorbs to dust particles, or embeds in objects, and can gradually accumulate over

time.[188] Tobacco smoke pollutants can additionally be re-emitted back into the gaseous phase from surfaces, be re-suspended in particulate matter, or further react with each other and atmospheric oxidants to yield secondary and tertiary pollutants.[188,189] Toxic contaminants directly associated with tobacco smoke, include tobacco-specific nitrosamines (TSNAs).[190] Several TSNAs, such as *N*′-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK), have been detected in homes,[3, 4, 7, 10–12] shown to be highly active as carcinogens in animal studies,[197,198] and linked to lung and oral cancers.[197,199] Moreover, studies have shown that exposure to THS can lead to increased cancer risk,[191,192,200] and that young children are especially susceptible through increased exposure to contaminated surfaces and dust.[3, 5]

Recently, THS was shown to be a significant contributor of polycyclic aromatic hydrocarbons (PAHs) in settled house dust.[196] PAHs are ubiquitous contaminants of concern because of their persistence and toxicity.[1] Many of these compounds are recognized as known or suspected carcinogens and/or mutagens, with 16 PAHs listed as priority pollutants by the U.S. Environmental Protection Agency (US EPA).[186,202] Although PAHs have been detected in indoor dust and air particulates in both smoker and non-smoker homes, the research focus has predominantly been on parent (or unsubstituted) PAHs, namely the 16 U.S. EPA PAHs.[8, 12–14] Recently, Gao *et al.* detected over 50 PAHs including alkylated PAHs and heterocyclic PAHs in cigarette smoke,[203] and previous studies have reported detection of several alkylated PAHs in tobacco smoke.[204–206]

Even though TSNAs and nicotine may remain in indoor dust and surfaces at elevated levels months after smoking,[207] there is limited knowledge on the associations of PAHs with THS long-term. This is especially a concern for polar PAH derivatives, such as oxygen containing PAHs, which may be more potent than their parent PAHs because of their direct acting mutagenic potential.[9, 10] The objective of this pilot study was to characterize PAHs as a consequence of THS exposure in settled house dust over time. Specifically, concentrations and dust loadings of not only parent PAHs (PPAHs), but selected methylated PAHs (MPAHs), oxygenated PAHs (OPAHs) and nitro-PAHs (NPAHs) were examined in homes, up to 6 months after smoking had ended. Since PAHs have diverse sources including tobacco smoke, tobacco-specific nitrosamines (TSNAs) and nicotine, which are exclusively associated with tobacco smoke, were also investigated.

4.3 Experimental Methods

4.3.1 Materials

PPAH, MPAH and NPAH standards were purchased from AccuStandard (New Haven, CT) and Chem Service (West Chester, PA). OPAH standards were purchased from Sigma Aldrich (St. Louis, MO). Isotopically labeled PAHs (d_8-naphthalene, d_{10}-acenaphthene, d_{10}-phenanthrene, d_{12}-chrysene and d_{12}-perylene) were purchased from CDN Isotopes (Point-Claire, Quebec, Canada). Standard solutions and isotopically labelled standards of nicotine (d_4-nicotine) and TSNAs (d_4-NNK, d_4-NNN, d_4-NAT and d_4-NAB) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). The list of analytes (and their acronyms) studied are listed in Table 4.1.

Table 4.1 List of compounds and abbreviations investigated in this study.

	Analyte	Abbreviation		Analyte	Abbreviation
	Parent PAHs	PAHs		**Nitro-PAHs**[a]	**NPAHs**
1	1-Methylpyrene	1MPYR	32	2+3-Nitronaphthalene	2+3NN
2	1-Methylphenanthrene	2+1MPHE	33	2-Nitrobiphenyl	2NB
3	2-Methylanthracene	2MAnt	34	3+4-Nitrobiphenyl	3+4NB
4	6-Methylchrysene	6MChr	35	5-Nitroacenaphthene	5NACE
5	Anthracene	ANT	36	3-Nitrodibenzofuran	3NDBZ
6	Benzo[a]anthracene	BaA	37	9-Nitroanthracene	9NANT
7	Benzo[a]pyrene	BaP	38	9-Nitrophenanthrene	9NPHE
8	Benzo[b]fluoranthene	BbF	39	2-Nitrofluorene	2NFLU
9	Benzo[e]pyrene	BeP	40	2-Nitrodibenzothiophene	2NDBZT
10	Benzo[ghi]perylene	BghiP	41	3-Nitrophenanthrene	3NPHE
11	Benzo[k]fluoranthene	BkF	42	2-Nitroanthracene	2NANT
12	Chrysene	CHR	43	2-Nitrofluoranthene	2NFLA
13	Dibenz[a,h]anthracene	DBahA	44	3-Nitrofluoranthene	3NFLA
14	Fluoranthene	FLA	45	1-Nitropyrene	1NPYR
15	Indeno[1,2,3-cd]pyrene	IND	46	2-Nitropyrene	2NPYR
16	Phenanthrene	PHE	47	7-Nitrobenz[a]anthracene	7NBaA
17	Pyrene	PYR	48	6-Nitrochrysene	6NCHR
18	Retene	RET	49	2,8-Dinitrodibenzothiophene	2,8NDBZT
19	Triphenylene	TRI	50	3-Nitrobenzanthrone	3NBZ
	Oxygenated PAHs	**OPAHs**	51	1,3-Dinitropyrene	1,3NPYR
20	2-Methyl-9,10-anthraquinone	2M9,10AQ	52	6-Nitrobenzo[a]pyrene	6NBaP
21	5,12-Naphthacenequinone	5,12NQ			
22	9,10-Anthraquinone	9,10AQ			
23	9-Fluorenone	9FLO			
24	Benzo[cd]pyrenone	BcdP			
25	Benzo[a]fluorenone	BaF			
26	Benzanthrone	BZ			
	Tobacco-specific nitrosamines	**TSNAs**			
27	N'-Nitrosoanabasine	NAB			
28	N'-Nitrosoanatabine	NAT			
29	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	NNK			
30	N'-Nitrosonornicotine	NNN			
31	**Nicotine**	**NIC**			

[a] No nitro-PAHs were detected above limit of quantitation (LOQ) 0.2 ng g^{-1}.

4.3.2 Study Population and Sample Collection

Settled house dust samples were collected from five homes in the San Diego county area, and were part of a larger parent study that investigated nicotine, TSNAs and biomarkers (cotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol) in 90 homes six months after smoking had ended.[210] Detailed procedures outlining participant recruitment, eligibility, and sampling methods are outlined in the accompanying parent study.[210] Briefly, participants were recruited who had indicated intent to quit smoking and were either enrolled in a cessation program or were receiving assistance to quit from a medical professional. Participants had to have been the only active smokers in the homes, must have smoked at least seven months a minimum of seven tobacco products per week indoors, prior to the study. Homes of participants were sampled up to six months after active smoking had ended. Samples were collected at week 0 (smoking ends, baseline level) and weeks 1, 4, 12 and 24 after smoking. Dust samples were collected from a 1 m^2 taped off area in the living room using the High-Volume-Small Surface-Sampler (HVS4, CS3 Inc., Venice, FL). Samples were collected from a different area if the collected dust content did not exceed 1/4 inches of the dust collection bottles. House dust samples were pre-weighed, sieved through a 150 µm mesh sieve to remove artifacts such as large debris and pet hair, and weighed again. Samples were stored at -20° C until extraction.

4.3.3 Sample Extraction

For nicotine extraction, approximately 50 mg of sieved dust in an amber vial was spiked with internal standard and allowed to equilibrate for 15 minutes. Next, 1 mL of 0.1 M potassium hydroxide (KOH) was added and the mixture vortexed for 1-2 minutes. The extract was

transferred to a 15 mL centrifuge tube containing 1 g of magnesium sulphate ($MgSO_4$):sodium chloride (NaCl) (4:1, w/w) (1 UCT ENVIRO $MgSO_4$/NaCl myra pouch, UCT, Bristol, PA), vortexed for 1-2 minutes, and centrifuged for 5 minutes at 3000 rpm. Finally, 1 mL of the extract was filtered through a 0.22 micron PTFE filter (Restek, Bellefonte, PA).

For the extraction of PAHs and TSNAs, approximately 500 mg of sieved dust in a centrifuge tube was spiked with known amounts of deuterated TSNAs and deuterated PAH standards. Next, 5 mL of acetonitrile and 300 mg of $MgSO_4$ were added, the mixture vortexed, then centrifuged for 5 minutes at 3000 rpm. The extract was blown down to 1 mL in a glass tube using a Zymark TurboVap (Caliper Life Sciences, Hopkinton, MA), then mixed with d-SPE using Agilent Bond Elut (P/N 5982-5122 50 mg C18, 50 mg PSA and 150 mg $MgSO_4$). The extract was centrifuged for 1 minute at 10,000 rpm, then transferred to a liquid chromatography vial. The final extract was analyzed for TSNAs first, then further evaporated to 100 µL under nitrogen for PAH analysis.

4.3.4 Chemical Analysis

The dust samples were analyzed for nicotine and TSNAs using an Agilent 1200 Series liquid chromatograph coupled with an Agilent 6460 triple quadrupole mass spectrometer detector (LC/MS) using electrospray ionization positive mode.

An Agilent HILIC plus column (2.1 mm x 50 mm, 1.8 µm particle size) was used for nicotine analysis. A binary gradient consisting of 0.1% (v/v) formic acid in ammonium acetate (A) and acetonitrile (B) was used for chromatographic separation. The flow rate was 0.3 mL min^{-1} and injection volume 1 µL. The column temperature was set at room temperature. Multiple

reaction mode (MRM) was used for identifying analytes: nicotine (163.1>130.1, 132.1, 117.1), and d_4-nicotine (167.3>136.1, 134.1, 121.1).

An Agilent Poroshell 120 SB-C18 column (2.1 mm x 50 mm, 2.7 µm particle size) was used for TSNA analysis. A binary gradient consisting of 0.1% (v/v) formic acid in ammonium acetate (A) and acetonitrile (B) was used for chromatographic separation, with the following program: initial composition was 10% B, changing over to 50% B at 10 min, changing to 90% at 10.1 min, maintaining for 9 min, changing back to 10% B at 11.1 min and maintained until 13 min. The flow rate was 0.2 mL min^{-1} and the injection volume was 1 µL. A multiple reaction mode (MRM) and MS/MS transitions were as follows: NNN (178.1>148.1, 120.1), d_4-NNN (182.1>152.2, 124.1); NAT (190.1>160.1, 79.1), d_4-NAT (194.1>164.1, 83.1), NAB (192.1>162.1, 133.1), d_4-NAB (196.1>166.1, 137.1), NNK (208.1>122.0, 79.1) and d_4-NNK (212.1>126.1, 195.1).

The dust samples were analyzed for PAHs using a comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC/TOF-MS) Pegasus 4D (Leco, St. Joseph, MI) composed of an Agilent 6890 gas chromatograph (Palo Alto, CA). The columns used were a LC-50 liquid crystal column (10 m × 0.15 mm × 0.10 µm) (J&K Scientific, Edwardsville, Nova Scotia, Canada) in the first dimension, and a NSP-35 nanostationary phase column (1.2 m × 0.10 mm × 0.10 µm) (J&K Scientific, Edwardsville, Nova Scotia, Canada) in the second dimension. Temperature and further operational parameters have been previously described in detail,[211] with minor modifications that included, a 7 s modulation period and 70 °C

initial 1D oven temperature. Six-point calibration curves ranging between 1 pg uL^{-1} and 2000 pg uL^{-1} (between 0.2 and 400 ng per gram of dust) were used for quantitation.

4.3.5 Quality Control

All glassware and materials were rinsed at least 3 times with hexane and methanol, or acetonitrile, prior to use and between samples where necessary, to minimize contamination. Analytical blanks consisting of hexane were analyzed at an approximate frequency of 30% to monitor background contamination and account for instrumentation variability. All dust concentrations and loadings were background subtracted.

4.3.6 Statistical Analysis

Statistical analyses were conducted using JMP (Statistical Discovery™ from SAS) software. For descriptive analysis, concentrations below the LOQ were reported as half the detection limit. Prior to statistical tests, the data was log transformed to normalize variance and *p*-values < 0.05 were considered statistically significant. Dust concentrations and loadings were reported as ng of analyte per gram of dust collected (ng g^{-1}; i.e. PAH concentrations) and in ng of analyte per square meter vacuumed (µg m^{-2}; i.e. dust loadings).

4.4 Results and Discussion

4.4.1 PAH Levels in Settled House Dust

Total parent and methylated PAHs (tPPAHs) concentrations measured in homes at any time were between 225 and 10,600 ng g^{-1} (dust loadings between 150 and14,200 ng m^{-2}) (Figure

4.1, 4.2 and Appendix C1, C2). The highest measured PPAH was pyrene (PYR) at 2,270 ng g^{-1} (dust loadings at 11,500 ng m^{-2}). Other PPAHs measured at generally high dust concentrations were fluoranthene (FLA), phenanthrene (PHE), chrysene (CHR), benzo[e]pyrene (BeP) and benzo[b]fluoranthene (BbF). These compounds have similarly been observed to be predominant in particulate matter associated with environmental tobacco smoke,[212] and in particulate and gaseous phases in non-smoking homes.[28, 29]

Total OPAHs (tOPAHs) were measured at lower dust concentrations than tPPAHs, between 13.7 and 1,220 ng g^{-1} (dust loadings between 6.20 and 6,160 ng m^{-2}). 9,10-anthraquinone (9,10AQ) was the most abundant OPAH with dust concentrations up to 825 ng g^{-1} (dust loadings up to 3,740 ng m^{-2}). 9,10AQ was measured at dust concentrations and loadings higher, or comparable to, some parent PAHs, highlighting the importance of looking beyond the routinely monitored PAHs in evaluating THS. No NPAHs were measured above the limit of quantitation of 0.2 ng g^{-1}. The reported formation of potentially more mutagenic NPAHs as a result of parent PAH oxidative reactions with atmospheric hydroxyl and nitro radicals,[10, 31] has raised some concerns that similar reactions could occur in homes. However, it should be noted that in homes similar reactions would likely occur at slower reaction rates because of limited indoor radical concentrations and sunlight. Nevertheless, NPAHs have been detected previously in indoor environments in smoker and non-smoker homes, though their presence was attributed to outdoor sources.[216]

Though PAH distributions varied between homes, PAH concentrations generally remained stable over time with the exception of one home (H33), where PAH concentrations

were higher than baseline levels (week 0) at the end of sampling. However, regression analysis to evaluate temporal trends in each home did not reveal any statistical correlations between contaminants and time, which might likely be due to the limited sample size.

Nicotine was measured at the highest dust concentrations between 1,010 and 51,300 ng g^{-1} (dust loadings between 1,630 and 259,000 ng m^{-2}), while total TSNAs (tTSNAs) were measured at the lowest dust concentrations (dust concentrations up to 129 ng g^{-1} and dust loadings up to 581 ng m^{-2}), with NNK being the most abundant (Figure 4.3 and Appendix C1, C2). Similar to the PAH distributions, the nicotine and TSNAs profiles remained relatively stable over time, with slight variation between homes. In the accompanying 90 home parent study, nicotine and NNK were observed to remain significantly elevated 6 months after smoking, concluding that quitters, as well as non-smokers continued to be exposed to THS toxicants well after active smoking had ceased.[210]

4.4.2 Associations between PAHS and THS

To determine the long-term associations of PAHs in THS, the relationships between PAHs, nicotine and TSNAs were evaluated. The tPPAHs dust concentrations were negatively correlated with nicotine (R^2 = -0.11) and tTSNAs (R^2 = -0.02) dust concentrations, but the correlations were not significant ($p > 0.05$) (Figure 4.4). The tOPAH concentrations were also poorly correlated with nicotine (R^2 = 0.07) and tTSNAs (R^2 = -0.006) dust concentrations ($p > 0.05$). However, stronger positive correlations were observed when dust loadings were considered (Figure 4.4B). A previous study that investigated associations between PAHs and nicotine in smoker and non-smoker homes,[196] suggested that dust loadings were potentially a

better indicator for contaminant exposure and accounted for greater variation in the data collected than dust concentrations, because they are directly related to the mass of dust per surface area.

The tPPAHs and tOPAHs dust loadings were positively correlated with nicotine and tTSNAs dust loadings (R^2 = 0.15 – 0.44), with the following individual PAHs statistically significant: 2+1 methylphenanthrene (2+1MPHE), 2-methylanthracene (2MANT), retene (RET), dibenz[a,h]anthracene (DBahA), 9,10-anthraquinone (9,10AQ), 2-methylanthraquinone (2MAQ), benzanthrone (BZ), benzo[cd]pyrenone (BcdP), 5,12-naphthacenequinone (5,12NQ) and 9-fluorenone (9FLO) (R^2: 0.47-0.72, $p < 0.05$) suggesting that these compounds could potentially associate with THS over time (Table 4.2). Several of the listed compounds are oxygenated PAHs, and include 9,10AQ, 9-FLO, BcdP and BZ, which have been linked to severe malformations and developmental toxicity in zebrafish.[122,125] Additionally, there is evidence that methylated PAHs, such as, RET, 2+1MPHE and 2MANT, may be as potent as their parent compounds.[217,218] For instance, methylated phenanthrene isomers, including 2MPHE, were observed to be at least 2 times more toxic than their corresponding parent PAH, phenanthrene, in the human aryl hydrocarbon receptor in a yeast bioassay.[217] These results suggest that these PAHs could contribute to risks associated with long-term THS exposure.

To further investigate the associations between the different individual PAH contaminants, principal component analysis (PCA) was conducted on the dust loadings (Figure 4.5). The first four principal components accounted for 98.1 % (82.2%, 10.1%, 3.96%, and 1.86%, respectively) of the total variance. PCA loadings were indicators for sources, in that

PAHs tended to cluster together in component 1, while compounds directly associated with tobacco smoke, TSNAs and nicotine, clustered together in component 2. Additionally, the separation between PAHs and nicotine clusters in loadings plot suggest the contribution of other indoor PAH sources, besides environmental tobacco smoke, such as indoor heating and cooking.[7, 34]

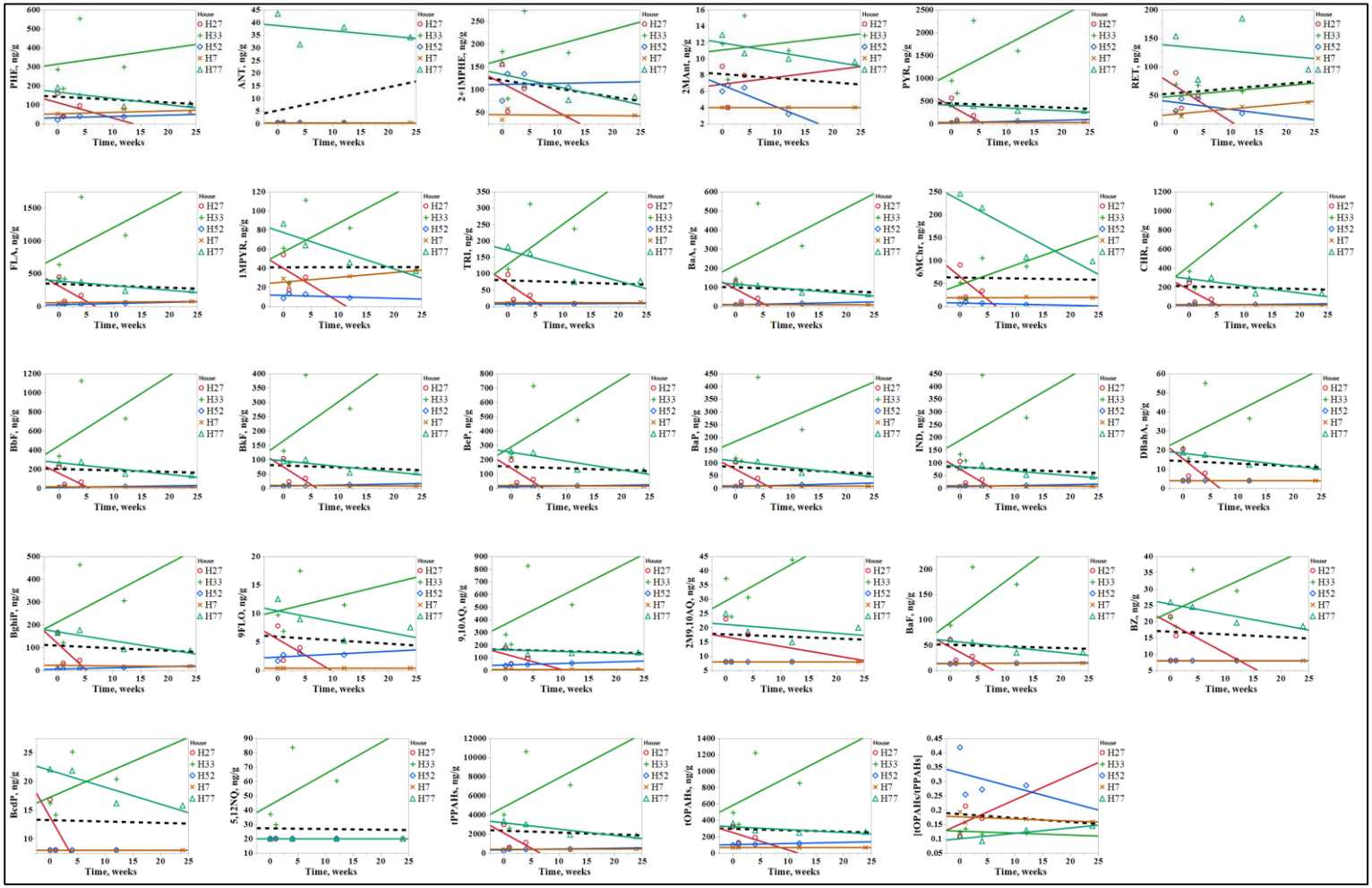
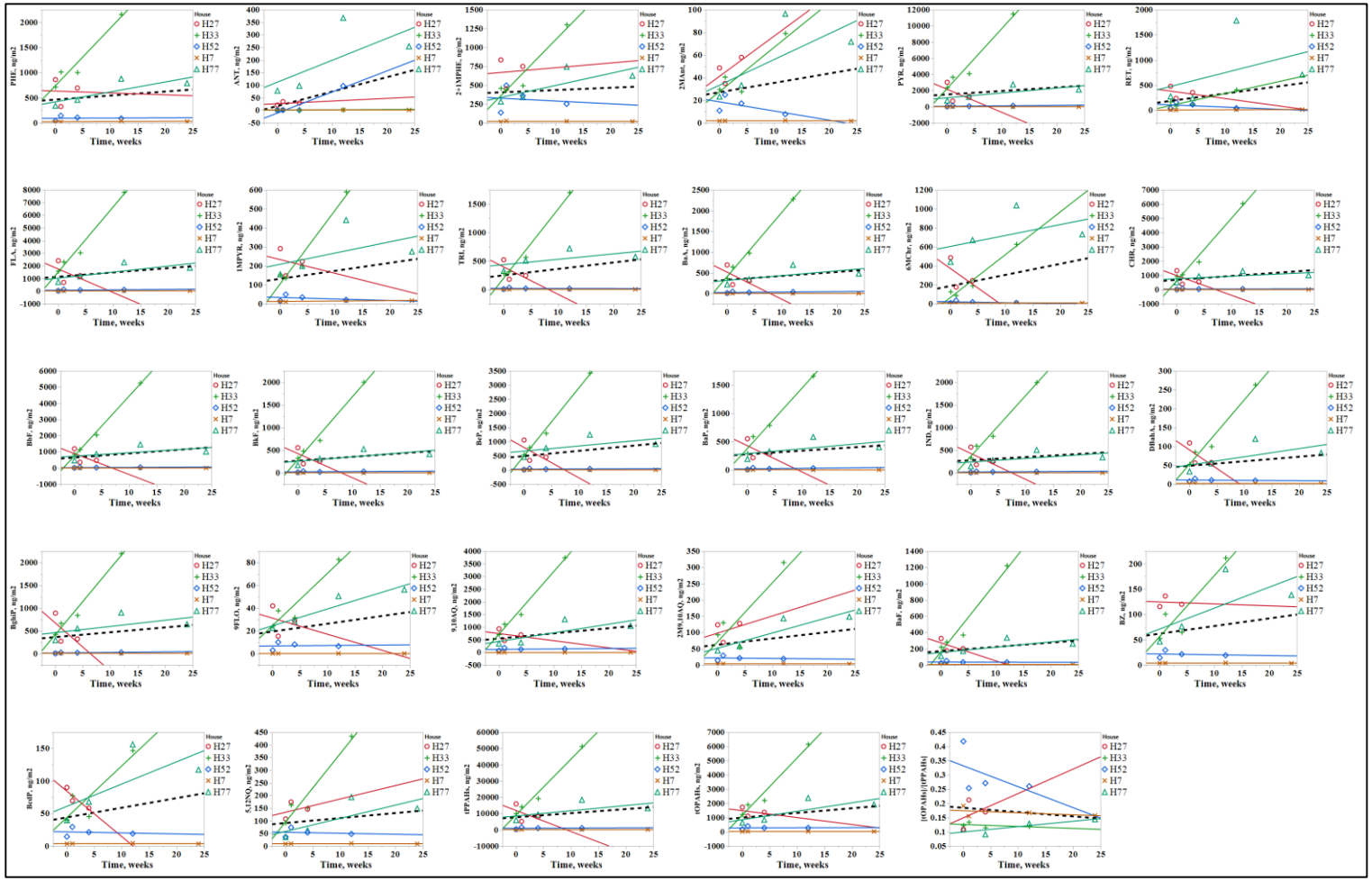


Figure 4.1 PAH concentration profiles (ng g^{-1}) in the five homes. Solid lines represent linear fit in respective houses, while dotted lines represent averaged linear fit regression across all homes.

Figure 4.2 PAH dust loading profiles (ng m^{-2}) in the five homes. Solid lines represent linear fit in respective houses, while dotted



lines represent averaged linear fit regression across all homes.

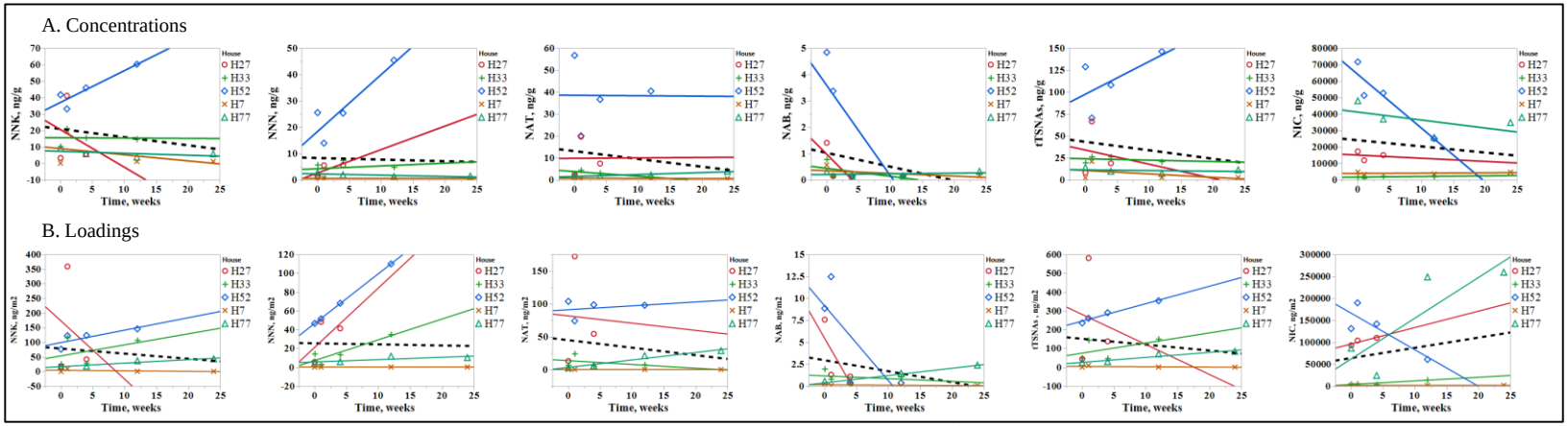


Figure 4.3 Nicotine and TSNA dust concentrations and loading profiles (ng m^{-2}) in the five homes. Solid lines represent linear fit in respective houses, while dotted lines represent averaged linear fit regression across all homes.

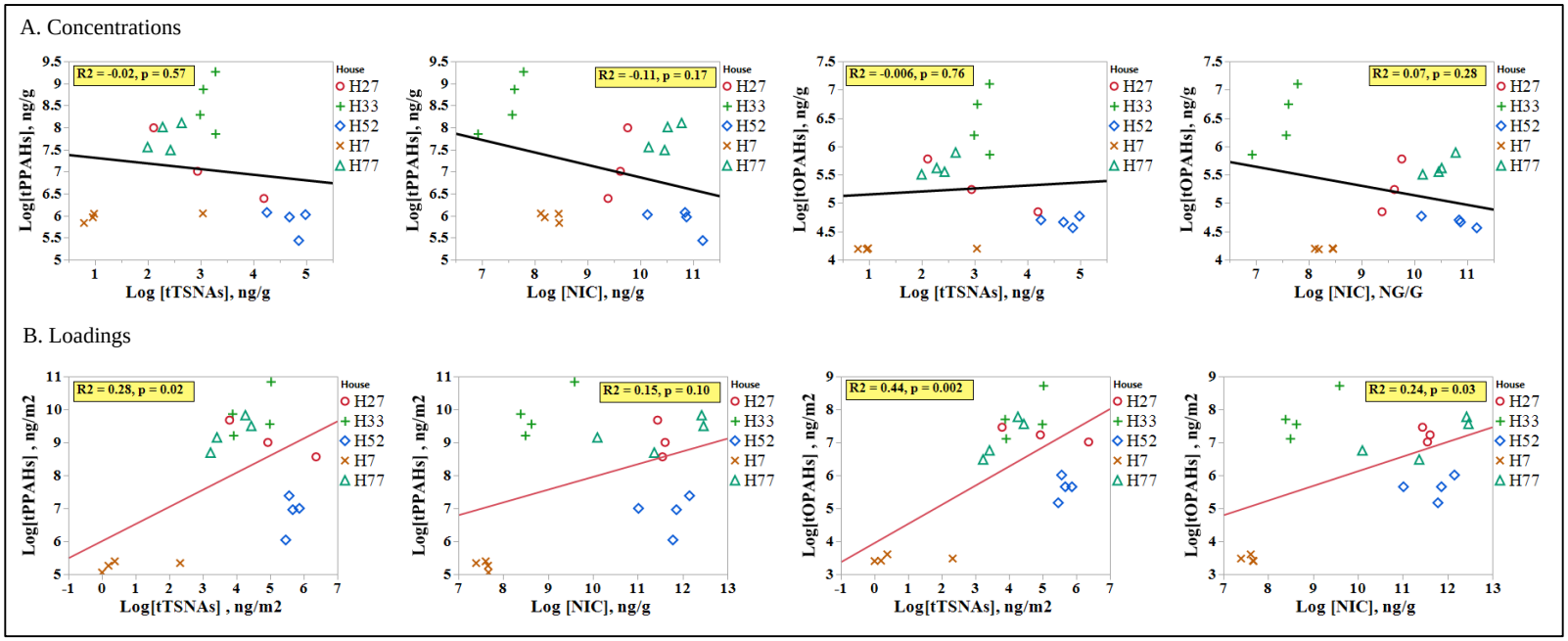


Figure 4.4 Correlation analysis between PAHs, and tTSNAs and nicotine dust (A) concentrations and (B) dust loadings

Table 4.2 Correlation coefficients in linear regression analysis of nicotine, TSNA, and PAH dust concentrations and loadings.

	tPPAHs	tOPAHs	NIC	tTSNAs	NNK	NNN	NAT	NAB	tPPAHs	tOPAHs	NIC	tTSNAs	NNK	NNN	NAT	NAB
PHE	**0.9522**	**0.8888**	**-0.5023**	-0.3094	-0.1427	-0.2454	**-0.4696**	-0.2679	**0.9925**	**0.9725**	0.3504	**0.4993**	**0.5407**	**0.4729**	0.4153	0.4384
ANT	0.3275	0.2346	**0.4595**	-0.2476	-0.1387	-0.2236	-0.1875	-0.155	0.4048	0.3801	**0.5654**	0.2117	0.2168	0.201	0.2651	0.138
2+1MPHE	**0.7126**	**0.8063**	0.0812	0.4143	**0.4812**	**0.5016**	0.2363	0.1242	**0.9049**	**0.9561**	**0.664**	**0.8022**	**0.804**	**0.7889**	**0.7391**	**0.6634**
2MAnt	**0.8702**	**0.8766**	0.0239	-0.0251	0.0887	0.0515	-0.1115	0.0677	**0.9347**	**0.9677**	**0.6524**	**0.7012**	**0.7059**	**0.6711**	**0.6623**	**0.6564**
PYR	**0.988**	**0.9666**	-0.3534	-0.0724	0.089	-0.0907	-0.244	-0.2209	**0.996**	**0.9739**	0.3317	**0.5172**	**0.5633**	**0.4727**	0.4104	0.4337
RET	**0.6148**	**0.5638**	0.3351	-0.2519	-0.1541	-0.0378	-0.1731	0.0124	**0.8726**	**0.889**	**0.7182**	**0.573**	**0.5773**	**0.5975**	**0.5775**	**0.6086**
FLA	**0.9842**	**0.9201**	-0.4483	-0.2581	-0.0763	-0.2475	-0.4307	-0.298	**0.9916**	**0.9554**	0.2886	0.4427	**0.4971**	0.4027	0.339	0.3801
1MPYR	**0.8483**	**0.717**	-0.3778	**-0.5722**	-0.4021	-0.4403	**-0.6921**	-0.2798	**0.9725**	**0.9399**	0.4098	0.4228	**0.4632**	0.4122	0.3556	0.4326
TRI	**0.9794**	**0.9166**	-0.2485	-0.2071	-0.0252	-0.1975	-0.3745	-0.233	**0.9896**	**0.9561**	0.3774	**0.4686**	**0.5219**	0.4307	0.3669	0.4241
BaA	**0.9924**	**0.975**	-0.3026	-0.06	0.0967	-0.0671	-0.23	-0.2081	**0.9984**	**0.9822**	0.3781	**0.5437**	**0.585**	**0.5034**	0.4425	**0.4574**
6MChr	**0.807**	**0.6701**	-0.0749	**-0.4908**	-0.3167	-0.4162	**-0.5607**	-0.2294	**0.9159**	**0.8638**	0.4323	0.3287	0.3781	0.3107	0.27	0.3931
CHR	**0.9948**	**0.9568**	-0.3119	-0.1206	0.0488	-0.1189	-0.2981	-0.2269	**0.9965**	**0.967**	0.3361	**0.4901**	**0.5404**	0.4536	0.38	0.4215
BbF	**0.9914**	**0.9562**	-0.3125	-0.1094	0.0595	-0.1242	-0.2858	-0.2368	**0.995**	**0.9653**	0.3295	**0.489**	**0.54**	0.4453	0.378	0.4125
BkF	**0.9924**	**0.9721**	-0.3188	-0.0765	0.0819	-0.0824	-0.253	-0.1959	**0.9977**	**0.9817**	0.374	**0.5417**	**0.5831**	**0.5008**	0.4392	**0.465**
BeP	**0.9898**	**0.937**	-0.3039	-0.1772	-0.0021	-0.1816	-0.3463	-0.246	**0.9943**	**0.9623**	0.3504	**0.4764**	**0.5271**	0.4336	0.3725	0.4172
BaP	**0.9907**	**0.9623**	-0.3206	-0.0837	0.0817	-0.1028	-0.2569	-0.2395	**0.9972**	**0.9778**	0.3696	**0.5359**	**0.5806**	**0.4888**	0.4339	0.4421
IND	**0.9917**	**0.962**	-0.3782	-0.0988	0.0678	-0.1217	-0.2874	-0.2357	**0.9963**	**0.9741**	0.3323	**0.52**	**0.5671**	**0.4707**	0.4099	0.4316
DBahA	**0.9897**	**0.9738**	-0.3179	-0.1025	0.0488	-0.0809	-0.2676	-0.1585	**0.9925**	**0.9934**	**0.4562**	**0.5986**	**0.6278**	**0.5617**	**0.5168**	**0.5283**
BghiP	**0.9673**	**0.8821**	-0.4053	-0.292	-0.1071	-0.3112	**-0.4662**	-0.3275	**0.9817**	**0.9352**	0.2809	0.4088	**0.4664**	0.354	0.3009	0.3488
9FLO	**0.8474**	**0.9106**	0.1031	0.2772	0.3687	0.2983	0.1914	0.0027	**0.9507**	**0.9791**	**0.608**	**0.7199**	**0.7304**	**0.7015**	**0.6621**	**0.6122**
9,10AQ	**0.8844**	**0.9612**	-0.0568	0.2729	0.3683	0.2847	0.1558	-0.0333	**0.9663**	**0.9947**	**0.5386**	**0.7106**	**0.7241**	**0.6901**	**0.6371**	**0.5888**
2M9,10AQ	**0.956**	**0.9402**	-0.3476	-0.1198	0.0216	-0.0918	-0.2827	-0.1038	**0.9694**	**0.9855**	**0.5208**	**0.6469**	**0.6634**	**0.606**	**0.5789**	**0.588**
BaF	**0.9842**	**0.9758**	-0.3761	-0.0856	0.0627	-0.0492	-0.2785	-0.1613	**0.9933**	**0.9934**	**0.4218**	**0.5944**	**0.6255**	**0.5644**	**0.5006**	**0.5145**

Table 4.2 Continued

	tPPAHs	tOPAHs	NIC	tTSNAs	NNK	NNN	NAT	NAB	tPPAHs	tOPAHs	NIC	tTSNAs	NNK	NNN	NAT	NAB
BZ	**0.9639**	**0.9351**	-0.1995	-0.1052	0.0499	-0.0973	-0.2288	-0.2188	**0.9587**	**0.9782**	**0.5796**	**0.6693**	**0.6841**	**0.6219**	**0.6164**	**0.5753**
BcdP	**0.9401**	**0.8928**	-0.1376	-0.1793	-0.0128	-0.1505	-0.3228	-0.1306	**0.9561**	**0.9762**	**0.6081**	**0.6736**	**0.6908**	**0.6265**	**0.6216**	**0.6008**
5,12NQ	**0.7071**	**0.7758**	**-0.5967**	0.0812	0.1637	0.1391	-0.165	-0.1709	**0.9237**	**0.972**	**0.5247**	**0.748**	**0.7523**	**0.7102**	**0.6762**	**0.5819**

Bold values indicate $p < 0.05$

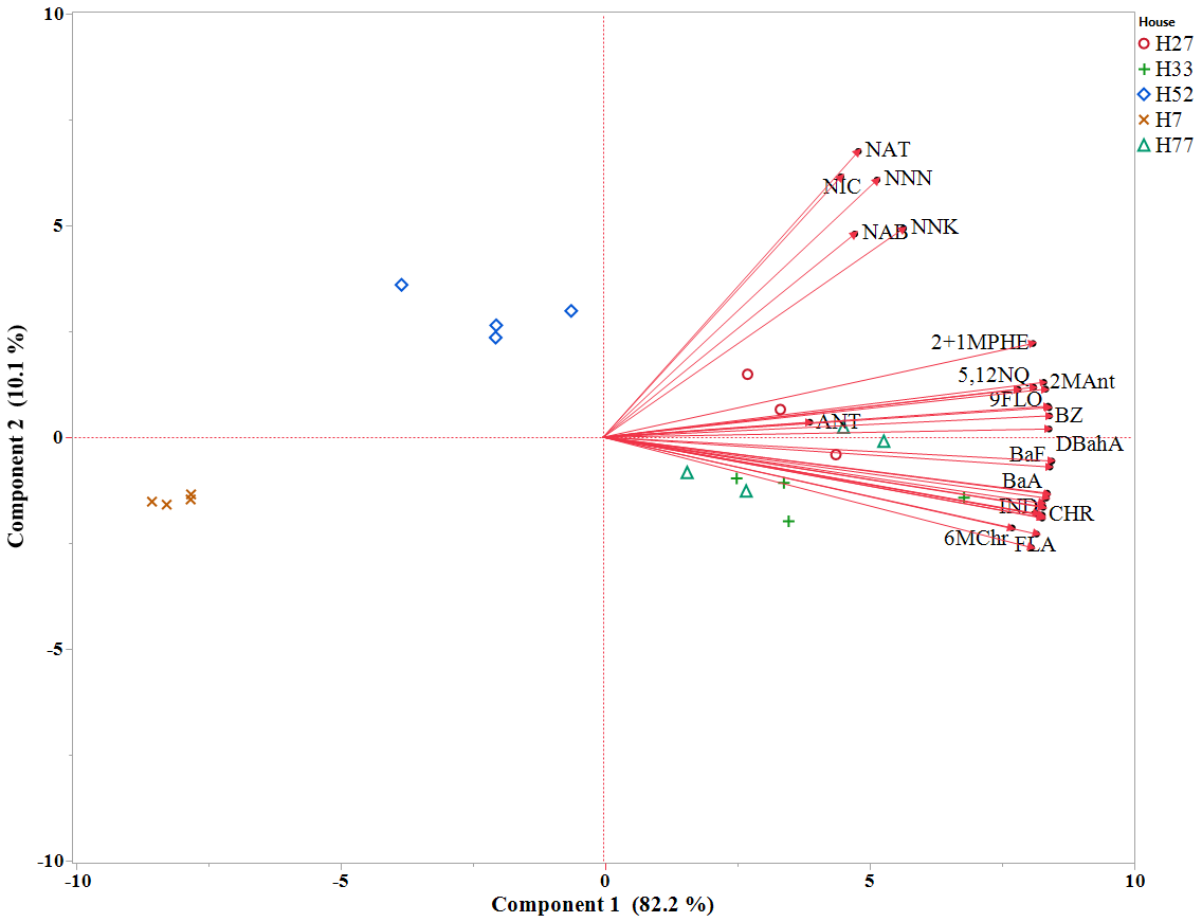


Figure 4.5 Principal component analysis plot of PAHs, nicotine and TSNAs dust loadings

4.5. Implications and Conclusions

Nicotine and TSNAs were recently shown to remain at elevated levels in indoor environments six months after active smoking had ended, suggesting that the risks associated with contaminants in THS may be long-term.[207,210] The results presented in this pilot study suggest that PAHs, ANT, 2+1MPHE, 2MANT, RET, DBahA, 9,10AQ, 2MAQ, BZ, BcdP, 5,12NQ and 9FLO (R^2: 0.52-0.72, $p < 0.05$), could additionally contribute to this risk. It should

be noted that, though the relatively small sample size and lack of repeated measurements limits our ability to make random inferences, the present study provides initial into insight in the role of PAHs in long-term THS exposure. This is important in understanding potential long term risk implications in indoor environments associated with these contaminants.

Notes

The authors declare no competing financial interest.

4.6 Acknowledgements

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CHAPTER 5 – CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation investigated PAHs and related PAH compounds in complex soil and house dust environmental mixtures, using targeted and non-targeted analysis. To evaluate the contribution of PAH transformation products in bioremediated soils, an effects-directed analysis approach with GC/MS targeted analysis was used in Chapter 2. Increased toxicity was observed postbioremediation in four soil extract fractions. However, the toxicity increase could not be attributed to targeted PAHs.

Non-targeted analysis was employed in Chapter 3, using GC×GC/TOF-MS and LC-MS/MS, to identify toxic transformation products in bioremediated soils. More than 50 peaks, with increased response postbioremediation, were isolated, with 10 unknown compounds tentatively identified based on accurate mass and TOF-MS/MS. Challenges in structural elucidation of unknown compounds included poor mass spectral library or database matching, and limited availability of commercial standards. Future work will include synthesis of standards and subsequent toxicity analysis of synthesized transformation products will be used to verify tentatively identified compounds. Finally, an in-house library containing MS, MS/MS and retention time information on all peaks with increased responses postbioremediation, was created and will be used to monitor these PAH transformation products in future remedial studies of PAH contaminated soils.

In Chapter 4 GC×GC/TOF-MS was used in a pilot study to evaluate the contribution of PAHs in THS long-term. Alkylated and oxygenated PAHs (2+1MPHE, 2MANT, RET, 9,10AQ, 2MAQ, BZ, BcdP, 5,12NQ and 9FLO), which may be more toxic than the routinely monitored

16 EPA priority PAHs, were observed to correlate with THS long-term. These data suggest that the long term risk implications associated with THS may be underestimated by the exclusion of these PAHs in analysis. Future work entails expanding the sample size with a focus on the aforementioned PAH compounds in indoor environments, after smoking has ended.

This dissertation demonstrates that PAH exist in complex mixtures, and that future studies need to incorporate and consider the contribution of other PAH derivatives, that may be more toxic in environmental samples, using complementary non-targeted analytical tools such as GC×GC/TOF-MS and LC-QTOF/MS.

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APPENDICES

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER TWO

Aerobic Bioremediation of PAH Contaminated Soil Results in Increased Genotoxicity and Developmental Toxicity

Appendix. Materials and methods. Chemicals

Appendix A1. Mean concentration in dry weight and standard errors (SE) of PAHs in unfractionated soil extracts pre- and postbioremediation

Appendix A2. Mean of median lethal concentrations (LC_{50}) and standard errors of unfractionated soil extract and soil extract fractions (A – F) pre- and postbioremediation for *DT40*, *Rad54*$^{-/-}$, and *Rev1*$^{-/-}$ cells in mg soil residue per mL DMSO

Appendix A3. Median of effective concentrations (EC_{50}) and standard errors of fractionated soil extracts (A-F) pre- and postbioremediation in embryonic zebrafish

Materials and Methods

Chemicals. Isotopically labeled standards used as surrogates for parent, methyl, and heterocyclic PAHs were d_{10}-fluorene, d_{10}-phenanthrene, d_{10}-pyrene, d_{12}-triphenylene, d_{12}-benzo[a]pyrene, d_{12}-benzo[ghi]perylene, for oxygenated PAHs were (d_6-1,4-naphthoquinone, d_8-anthraquinone), and for nitrated PAHs were (d_9-1-nitropyrene, d_7-1-nitronaphthalene, d_{11}-6-nitrochrysene, d_9-5-nitroacenaphthene, d_9-9-nitroanthracene and d_9-3-nitrofluoranthene). Isotopically labeled standards used as internal standards for parent, methyl and heterocyclic PAHs were d_{10}-acenaphthene, d_{10}-fluoranthene, d_{12}-benzo[k]fluoranthene, for oxygenated PAHs was d_8-9-fluorenone and for nitrated PAHs were d_9-nitrobiphenyl and d_9-2-nitrofluorene.

Appendix A1. Mean concentration in dry weight and standard errors (SE) of PAHs in unfractionated soil extracts pre- and postbioremediation. Compounds with asterisks (*) showed significant changes in concentration postbioremediation ($p < 0.05$, $n = 3$). No nitrated PAHs were detected above the limit of detection (1 pg μL^{-1}). (n.d. = not detected).

PAHs	Abbr.	Prebioremediation $\mu g\ g^{-1}$	Postbioremediation $\mu g\ g^{-1}$	*p* value
Naphthalene	NAP	6.26 ± 0.49	8.58 ± 3.82	0.58
2-Methylnaphthalene	2MNAP	3.90 ± 0.67	3.76 ± 0.67	0.89
1-Methylnaphthalene	1MNAP	3.35 ± 0.62	2.14 ± 0.41	0.18
2,6-Dimethylnaphthalene	2,6MNAP	3.40 ± 0.52	0.93 ± 0.22	0.01*
1,3-Dimethylnaphthalene	1,3MNAP	3.54 ± 0.50	1.08 ± 0.27	0.01*
Acenaphthylene	ACEY	8.03 ± 0.30	13.78 ± 2.94	0.12
Acenaphthene	ACE	6.13 ± 0.71	1.31 ± 0.43	<0.01*
Fluorene	FLU	4.22 ± 0.68	3.02 ± 0.58	0.25
Phenanthrene	PHE	78.32 ± 11.54	28.65 ± 7.60	0.02
Anthracene	ANT	11.17 ± 1.21	10.21 ± 1.88	0.69
2-Methylphenanthrene	2MPHE	57.48 ± 4.72	16.22 ± 4.38	<0.01*
2-Methylanthracene	2MANT	6.50 ± 0.56	3.85 ± 0.73	0.04*
1-Methylphenanthrene	1MPHE	122.95 ± 8.45	30.32 ± 7.62	<0.01*
3,6-Dimethylphenanthrene	3,6MPHE	21.57 ± 1.50	6.48 ± 1.55	<0.01*
Fluoranthene	FLA	63.32 ± 4.15	19.99 ± 3.98	<0.01*
Pyrene	PYR	78.84 ± 3.99	32.03 ± 6.62	<0.01*
Retene	RET	92.97 ± 6.15	59.57 ± 11.20	0.06
Benz[c]fluorine	BcF	9.02 ± 0.57	4.17 ± 0.89	0.01*
1-Methylpyrene	1MPYR	3.98 ± 0.38	2.35 ± 0.54	0.07
Cyclopenta[cd]pyrene	CdeP	1.65 ± 0.16	2.04 ± 0.35	0.36

Appendix A1 (Continued).

PAHs	Abbr.	Prebioremediation µg g^{-1}	Postbioremediation µg g^{-1}	*p* value
Benzo(a)anthracene	BaA	38.38 ± 2.76	17.22 ± 3.30	0.01*
Chrysene + Triphenylene	CHR+TRI	27.17 ± 1.90	11.99 ± 2.40	0.01*
6-Methylchrysene	6MCHR	2.09 ± 0.14	1.25 ± 0.28	0.05
Benzo(b)fluoranthene	BbF	30.42 ± 2.24	21.76 ± 3.87	0.12
Benzo(k)fluoranthene	BkF	11.40 ± 0.95	7.87 ± 1.53	0.12
Benz[j][e]aceanthrylene	BjeA	0.42 ± 0.04	0.43 ± 0.08	0.94
Benz(e)pyrene	BeP	19.47 ± 1.98	16.58 ± 2.99	0.46
Benzo(a)pyrene	BaP	31.00 ± 2.51	21.15 ± 3.84	0.10
Dibenz(a,c)anthracene	DacP/DahP	0.31 ± 0.02	0.26 ± 0.04	0.33
Indeno(1,2,3-cd)pyrene	IcdP	15.43 ± 0.95	16.77 ± 2.82	0.67
Benzo(ghi)perylene	BghiP	15.67 ± 0.80	18.25 ± 3.08	0.46
Anthranthrene	ANTH	1.88 ± 0.16	2.15 ± 0.37	0.53
OPAHs				
9-Fluorenone	9FLO	0.13 ± 0.02	0.07 ± 0.01	0.05
1,4-Naphthoquinone	1,4NQ	n.d.	0.00 ± 0.00	n.d.
Acenaphthenequinone	ACEN	0.08 ± 0.02	0.20 ± 0.03	0.05
Phenanthrene-1,4-dione	1,4PD	0.16 ± 0.02	0.05 ± 0.01	<0.01*
9,10-Anthraquinone	9,10AQ	8.28 ± 0.40	2.18 ± 0.30	<0.001*
1,4-Anthraquinone	1,4AQ	n.d.	n.d.	
2-methyl-9,10-anthraquinone	2M9,10AQ	3.26 ± 0.57	1.16 ± 0.16	0.02*
2-Ethyl-9,10-Anthraquinone	2E9,10AQ	1.69 ± 0.06	0.77 ± 0.10	<0.01*
9,10-Phenanthrenequinone	9,10PQ	n.d.	n.d.	

Appendix A1 (Continued).

PAHs	Abbr.	Prebioremediation µg g^{-1}	Postbioremediation µg g^{-1}	*p* value
Benzo[a]fluorenone	BaF	2.05 ± 0.13	0.89 ± 0.10	<0.01*
Benzanthrone	BZ	1.00 ± 0.12	0.74 ± 0.06	0.13*
Aceanthrenequinone	ACEAN	n.d.	n.d.	
Benzo[c]phenanthrene-[1,4]quinone	Bc1,4Q	0.73 ± 0.06	0.40 ± 0.06	0.01*
7,12-Benzo[a]anthracene dione	7,12BaAD	1.75 ± 0.07	1.03 ± 0.08	<0.01*
Benzo[cd]pyrenone	BcdP	0.50 ± 0.05	0.67 ± 0.07	0.11
5,12-Napthacenequinone	5,12NQ	n.d.	n.d.	
1,6-Benzo[a]pyrene quinone	1,6BaPQ	n.d.	n.d.	
HPAHs				
2-Methylbenzofuran	2MBZ	n.d.	n.d.	
Thianapthene	THN	0.07 ± 0.00	0.05 ± 0.01	0.09
Quinoline	QUI	0.03 ± 0.00	0.03 ± 0.01	0.92
Indole	IND	0.12 ± 0.02	0.03 ± 0.01	0.01*
8-Methylquinoline	8MQ	n.d.	n.d.	
Dibenzofuran	DBZ	0.53 ± 0.06	0.47 ± 0.08	0.57
Xanthene	XAN	n.d.	n.d.	
5,6-Benzoquinoline	5,6BQ	0.86 ± 0.07	0.19 ± 0.06	<0.01*
Acridine	ACR	0.76 ± 0.06	0.15 ± 0.04	<0.001*
Carbazole	CAR	0.24 ± 0.02	0.22 ± 0.03	0.70
Dibenzothiophene	DBZ	0.41 ± 0.04	0.24 ± 0.04	0.05

Appendix A2. Mean lethal concentration (LC_{50}) with standard errors bars of unfractionated soil extract and soil extract fractions (A – F) pre- and postbioremediation for *DT40, Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ cells in mg soil residue per mL DMSO. The LC_{50} values with asterisks (*) showed a significant decrease postbioremediation (increased toxicity), while (‡) showed a significant increase postbioremediation (decreased toxicity) ($p < 0.05$ $n = 4$). The LC_{50} for soil extract fraction B postbioremediation could not be determined because the full dose-response curve could not be captured from the exposure concentrations (N.D. = not determined).

Cell line	Soil/fraction	Prebioremediation mg mL-1	Postbioremediation mg mL-1	p value
DT40	Unfractionated	0.90 ± 0.06	0.48 ± 0.03	<0.001*
	A	1.94 ± 0.28	12.6 ± 0.65	<0.0001
	B	32.2 ± 2.81	N.D.	-
	C	5.48 ± 0.51	116 ± 3.59	<0.0001‡
	D	3.55 ± 0.44	156 ± 10.5	<0.0001‡
	E	0.59 ± 0.09	0.35 ± 0.04	<0.05*
	F	5.39 ± 0.78	3.80 ± 0.18	0.09
Rad54-/-	Unfractionated	0.71 ± 0.05	0.31 ± 0.02	<0.001*
	A	1.62 ± 0.23	9.20 ± 1.13	<0.001‡
	B	19.8 ± 1.22	N.D.	-
	C	3.80 ± 0.57	62.7 ± 8.77	<0.001‡
	D	2.64 ± 0.18	73.1 ± 9.45	<0.001‡
	E	0.49 ± 0.03	0.25 ± 0.03	<0.01*
	F	3.93 ± 0.45	3.08 ± 0.21	0.14
Rev1-/-	Unfractionated	1.12 ± 0.09	0.50 ± 0.06	<0.01*
	A	1.29 ± 0.01	6.75 ± 0.40	<0.0001‡
	B	17.4 ± 1.74	N.D.	-
	C	5.39 ± 0.75	45.8 ± 4.67	<0.001‡
	D	3.39 ± 0.25	36.3 ± 6.84	<0.01‡
	E	0.51 ± 0.05	0.09 ± 0.02	<0.001*
	F	4.60 ± 0.59	1.18 ± 0.26	<0.01*

Appendix A3. Median effective concentrations (EC_{50}) and standard errors of fractionated soil extracts (A-F) pre- and postbioremediation in embryonic zebrafish. Median EC_{50} values with asterisks (*) showed a significant decrease postbioremediation (increased developmental toxicity), while (‡) showed a significant increase postbioremediation (decreased developmental toxicity) ($p < 0.05$ $n = 32$). The median EC_{50}s of fractions E and F postbioremediation were unable to be calculated because the concentrations tested were too low to capture the full dose-response curve (N.D. = not determined).

Soil fraction	Prebioremediation µg mL-1	Postbioremediation µg mL-1	p value
A	0.89 ± 0.04	0.94 ± 0.03	0.36
B	1.29 ± 0.04	1.34 ± 0.05	0.43
C	2.11 ± 0.06	1.01 ± 0.04	<0.001*
D	3.30 ± 0.07	4.74 ± 0.07	<0.001‡
E	5.39 ± 0.09	N.D.	-
F	8.90 ± 0.12	N.D.	-

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 3

Polar Transformation Products Increase Toxicity of PAH Contaminated Soil Following Bioremediation

Appendix B1. GCxGC/TOF-MS (EI) for (top) phthalic anhydride authentic standard and (b) unknown compound in fraction F postbioremediation (underivatized.

Appendix B2. GCxGC/TOF-MS (EI) for (top) 4-methyly phthalic anhydride authentic standard and (b) unknown compound in fraction F postbioremediation (underivatized)

Appendix B3. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 222 detected in LC/QTOF-MS

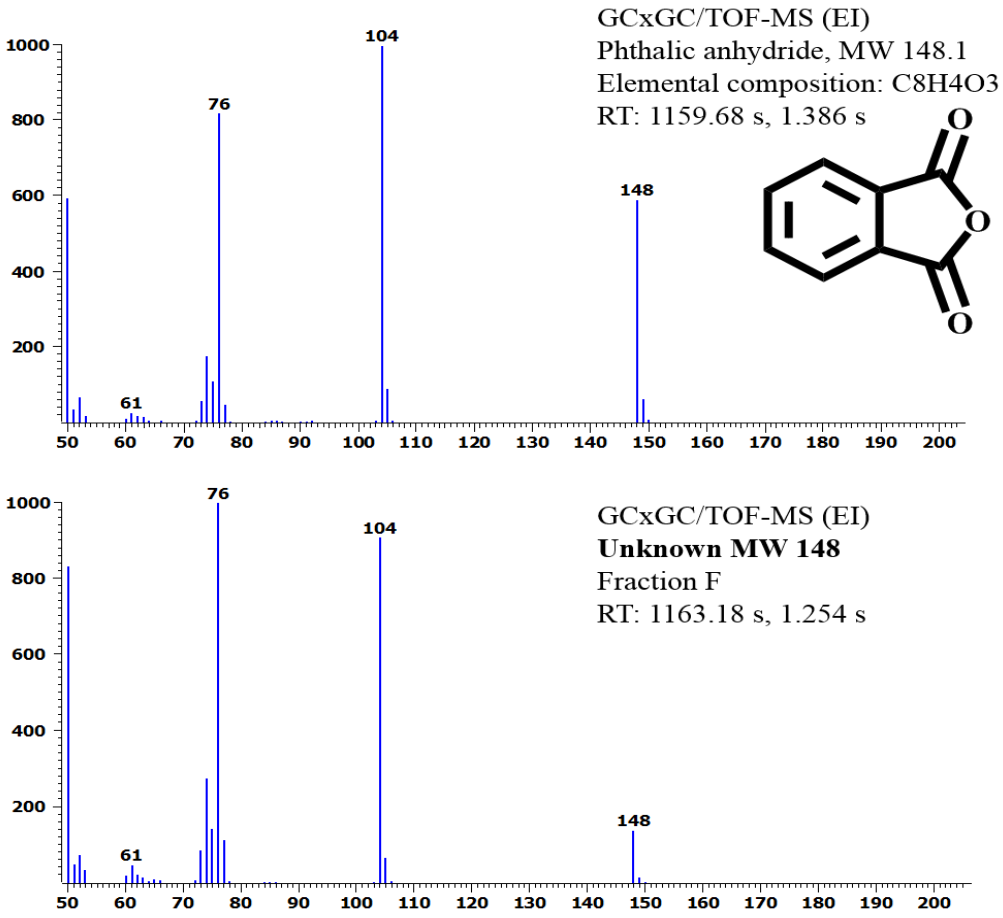
Appendix B4. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 223 detected in LC/QTOF-MS.

Appendix B5. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 235 detected in LC/QTOF-MS

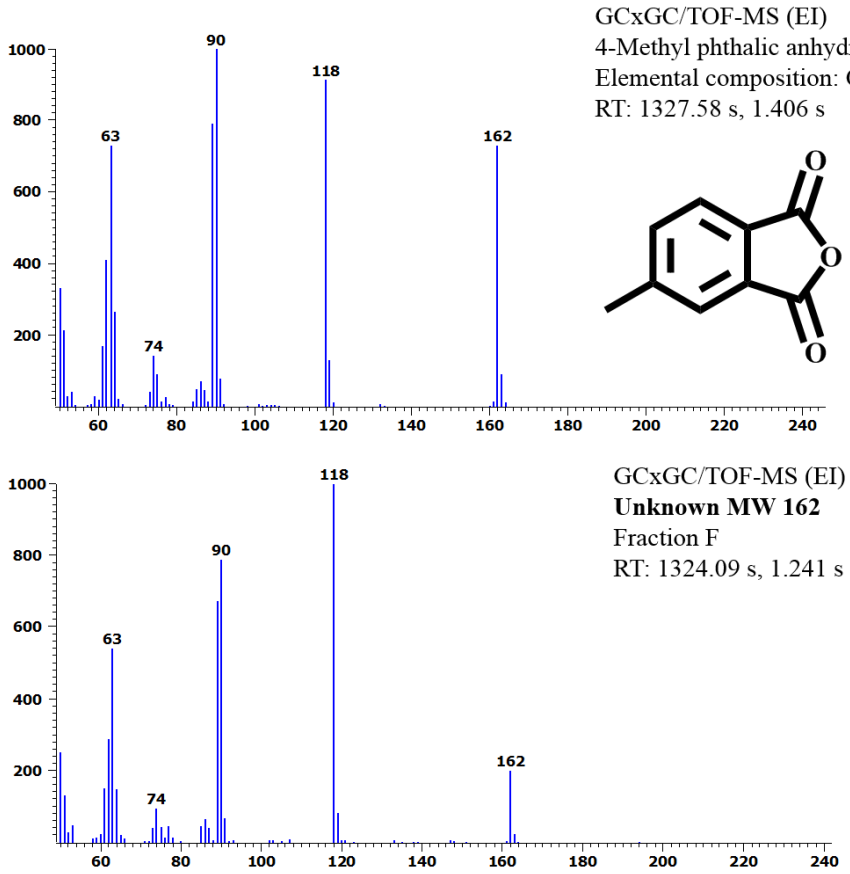
Appendix B6. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 240 detected in LC/QTOF-MS.

Appendix B7. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 249 detected in LC/QTOF-MS.

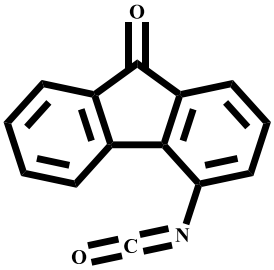
Appendix B1. GCxGC/TOF-MS (EI) for (top) phthalic anhydride authentic standard and (b) unknown compound in fraction F postbioremediation (underivatized)



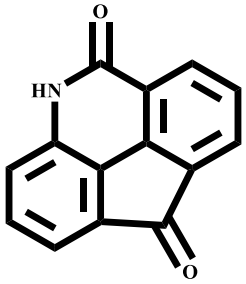
Appendix B2. GCxGC/TOF-MS (EI) for (top) 4-methyly phthalic anhydride authentic standard and (b) unknown compound in fraction F postbioremediation (underivatized)



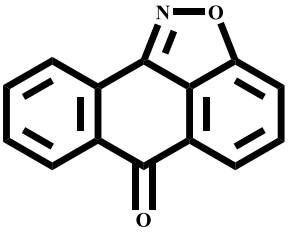
Appendix B3. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 222 detected in LC/QTOF-MS.



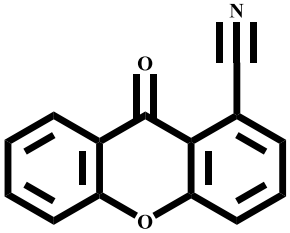
4-Isocyanato-9H-fluoren-9-one
Score: 0.874



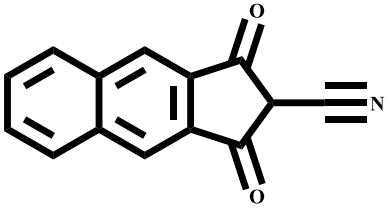
4H-Cyclopenta[lmn] phenanthridine 5,9-dione
Score: 0.818



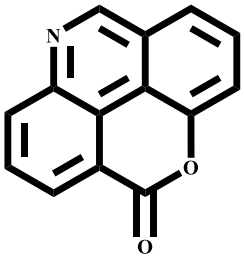
6H-Anthra[9,1-cd] [1,2]oxazol-6-one
Score: 0.818



9-Oxo-9H-xanthene -1-carbonitrile
Score: 0.818

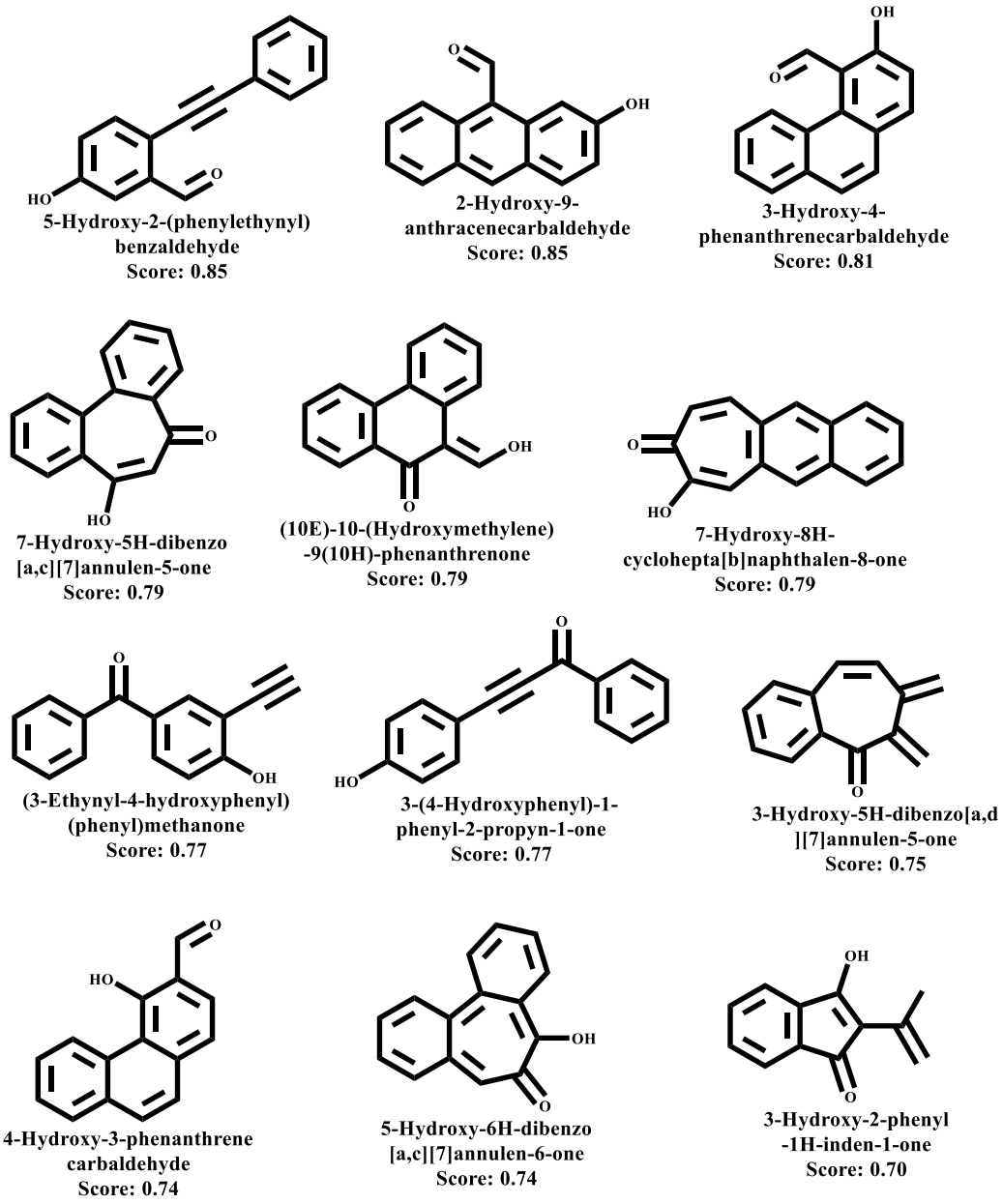


1,3-Dioxo-2,3-dihydro-1H-cyclopenta [b]naphthalene-2-carbonitrile
Score: 0.818

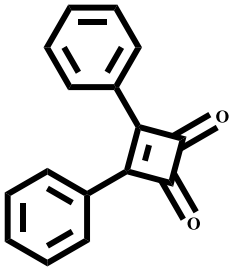


5H-Pyrano[2,3,4,5-lmn] phenanthridin-5-one
Score: 0.80

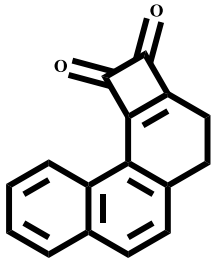
Appendix B4. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 223 detected in LC/QTOF-MS.



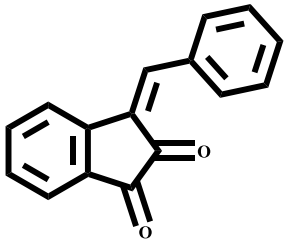
Appendix B5. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 235 detected in LC/QTOF-MS.



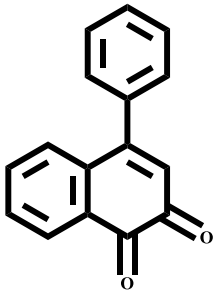
Diphenylcyclobutenedione
Score: 0.81



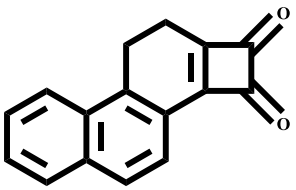
3,4-Dihydrocyclobuta[c] phenanthrene-1,2-dione
Score: 0.81



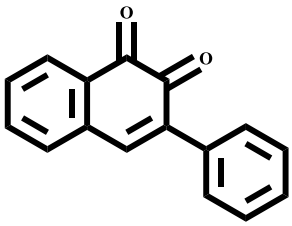
3-Benzylidene-1H-indene-1,2(3H)-dione
Score: 0.81



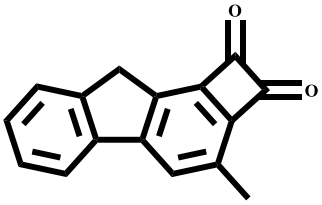
4-Phenyl-1,2-naphthalenedione
Score: 0.81



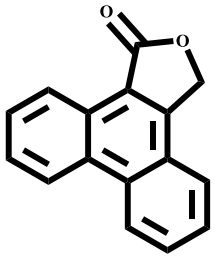
9,10-Dihydrocyclobuta[a] phenanthrene-1,2-dione
Score: 0.81



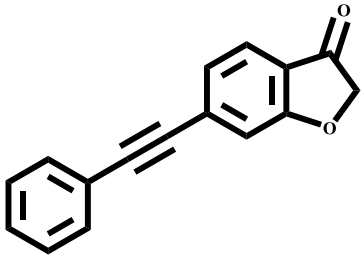
3-Phenyl-1,2-naphthalenedione
Score: 0.81



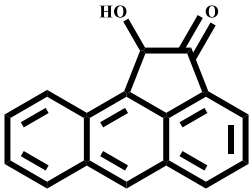
3-Methyl-1H-cyclobuta[a] fluorene-1,2(9H)-dione
Score: 0.81



Phenanthro[9,10-c]furan-1(3H)-one
Score: 0.69

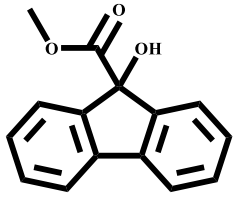


6-(Phenylethynyl)-1-benzofuran-3(2H)-one
Score: 0.69

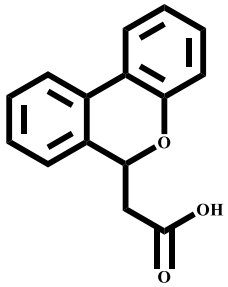


1-Hydroxy-2(1H)-aceanthrylenone
Score: 0.63

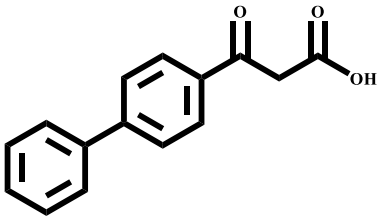
Appendix B6. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 240 detected in LC/QTOF-MS.



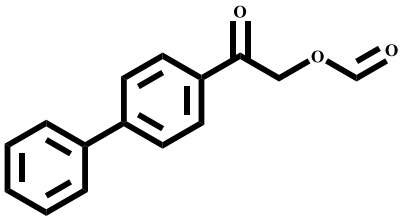
Methyl 9-hydroxy-9H-fluorene-9-carboxylate
Score: 0.90



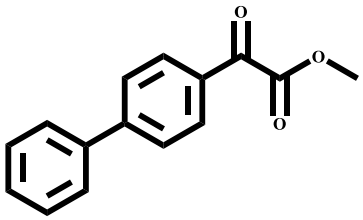
6H-Benzo[c]chromen-6-ylacetic acid
Score: 0.89



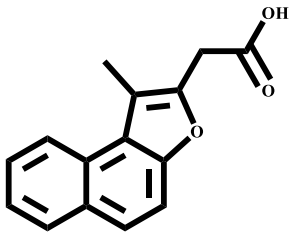
3-(4-Biphenylyl)-3-oxopropanoic acid
Score: 0.87



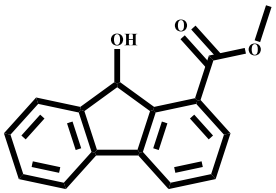
2-(4-Biphenylyl)-2-oxoethyl formate
Score: 0.87



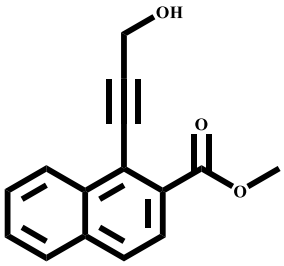
Methyl 4-biphenylyl (oxo)acetate
Score: 0.87



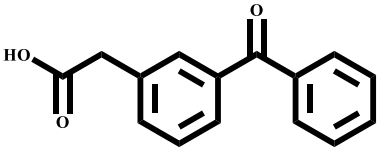
(1-Methylnaphtho[2,1-b]furan-2-yl)acetic acid
Score: 0.82



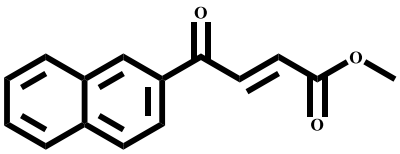
Methyl 9-hydroxy-9H-fluorene-1-carboxylate
Score: 0.81



Methyl 1-(3-hydroxy-1-propyn-1-yl)-2-naphthoate
Score: 0.81

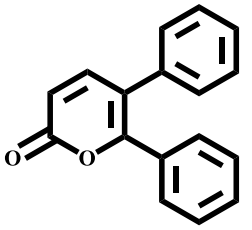


(3-Benzoylphenyl)acetic acid
Score: 0.71

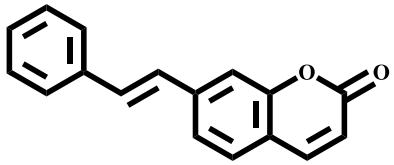


methyl 4-(2-naphthyl)-4-oxo-but-2-enoate
Score: 0.71

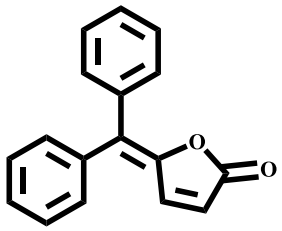
Appendix B7. Top ranked proposed molecular structures from *in silico* fragmentation prediction for unknown compound 249 detected in LC/QTOF-MS.



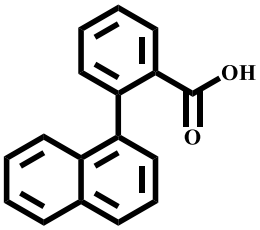
5,6-Diphenyl-2H-pyran-2-one
Score: 0.79



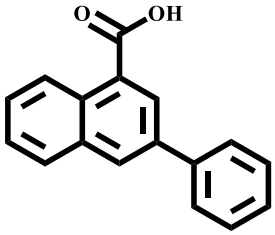
7-[(E)-2-Phenylvinyl]-2H-chromen-2-one
Score: 0.79



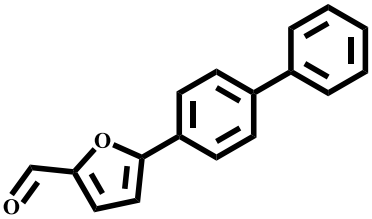
5-(Diphenylmethylene)-2(5H)-furanone
Score: 0.79



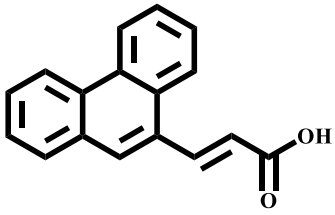
2-(1-Naphthyl)benzoic acid
Score: 0.53



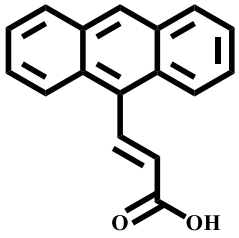
3-Phenyl-1-naphthoic acid
Score: 0.53



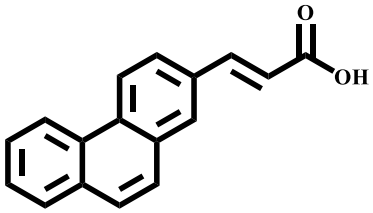
5-(4-Biphenylyl)-2-furaldehyde
Score: 0.53



3-(9-Phenanthryl)acrylic acid
Score: 0.51



3-(9-Anthryl)acrylic acid
Score: 0.51



3-(2-Phenanthryl)acrylic acid
Score: 0.51

APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 4

Polycyclic aromatic hydrocarbons and related toxic compounds in third hand smoke contaminated house dust

Appendix C1. Percentage frequency of detection, median and range of concentrations, nicotine, PAHs, and TSNAs in settled house dust in ng g^{-1} in five nonsmoker homes up to six months after smoking

Appendix C2. Percentage frequency of detection, median and range of concentrations, nicotine, PAHs, and TSNAs in settled house dust in ng m^{-2} in five nonsmoker homes up to six months after smoking.

Appendix C3 Principal Component loadings from compound dust loadings

Appendix C1. Percentage frequency of detection, median and range of concentrations, nicotine, PAHs, and TSNAs in settled house dust in ng g^{-1} in five nonsmoker homes up to six months after smoking.

	Week 0 (N=5)		Week 1 (N=4)		Week 4 (N=4)		Week 12 (N=4)		Week 24 (N=2)	
Analyte	**% Freq**	**Median (Range)**	**% Freq**	**Median (Range)**	**% Freq**	**Median (Range)**	**% Freq**	**Median (Range)**	**% Freq**	**Median (Range)**
1MPYR	100	54.2 (8.50-86.5)	100	19.6 (13.4-24.8)	100	47.2 (12.8-111)	100	38.5 (8.86-82.1)	100	37.2 (36.9-37.6)
2+1MPHE	100	155 (33.8-184)	100	69.1 (51.9-134)	100	122 (103-273)	100	90.8 (40.9-181)	100	63.5 (43.3-83.8)
2MAnt	80	9.05 (0-12.9)	75	3.36 (0-7.43)	100	9.29 (6.44-15.3)	50	4.98 (0-11.0)	50	4.81(0-9.62)
6MChr	100	51.6 (4.58-246)	100	17.2 (9.60-20.3)	100	69.6 (6.81-215)	100	54.2 (3.81-107)	100	58.4 (18.7-98.2)
ANT	20	0 (0-43.4)	0	n.d.	25	0 (0 -31.2)	25	0 (0-38.0)	25	17.1 (0-34.1)
BaA	100	121 (6.59-146)	100	18.4 (11.6-118)	100	75.8 (9.63-538)	100	43.6 (7.82-317)	100	36.5 (8.70-64.3)
BaP	100	103 (5.37-118)	100	18.4 (10.3-108)	100	71.8 (7.75-436)	100	37.1 (7.77-231)	100	31.3 (8.47-54.1)
BbF	100	222 (6.69-338)	100	30.5 (13.7-211)	100	171 (9.88-1126)	100	84.9 (10.3-730)	100	73.7 (11.5-136)
BeP	100	197 (7.73-256)	100	31.4 (15.1-146)	100	155 (10.8-714)	100	73.1 (14.4-476)	100	69.9 (16.5-123)
BghiP	100	164 (4.04-171)	100	29.5 (7.19-122)	100	110 (7.78-464)	100	53.9 (12.2-306)	100	52.5 (18.5-86.5)
BkF	100	94.4 (7.14-131)	100	17.3 (10.2-88.8)	100	67.5 (8.01-396)	100	33.4 (7.94-278)	100	31.8 (8.09-55.6)
CHR	100	248 (6.94-369)	100	32.7 (20.9-194)	100	188 (13.8-1072)	100	77.1 (14.2-841)	100	75.0 (14.6-135)
DBahA	60	18.7 (0-21.2)	0.6	3.23 (0-15.6)	75	12.7 (0-55.0)	50	6.20 (0-36.6)	50	5.54 (0-11.1
FLA	100	406 (16.9-638)	100	72.6 (36.2-424)	100	272 (32.0-1671)	100	149 (46.5-1087)	100	162 (75.9-248)
IND	100	81.0 (4.82-134)	100	16.9 (8.99-110)	100	62.2 (7.44-445)	100	31.7 (7.10-277)	100	27.4 (8.68-46.1)
PHE	100	160 (20.8-286)	100	42.2 (37.5-186)	100	121 (40.0-553)	100	84.6 (37.4-300)	100	84.1 (62.3-106)
PYR	100	426 (15.6-947)	100	60.4 (37.8-673)	100	284 (28.5-2266)	100	171 (24.6-1597)	100	157 (29.8-284)
RET	100	69.3 (20.8-153)	100	21.7 (13.1-44.0)	100	58.3 (43.7-77.5)	100	43.4 (18.3-184)	100	66.4 (37.7-95.1)
TRI	100	97.5 (5.49-182)	100	18.1 (9.39-58.2)	100	98.2 (7.37-313)	100	41.5 (8.02-237)	100	44.8 (12.5-77.2)
tPAHs		2,963 (225-4,013)		511 (416-2,600)		2,056 (387-10,622)		1,156 (381-7,134)		1,099 (413-1,785)
2M9,10AQ	60	23.0 (0-37.2)	25	0 (0-23.9)	75	18.0 (0-30.6)	50	7.43 (0-43.8)	50	9.97 (0-19.9)
5,12NQ	20	0 (n.d.-37.1)	25	0 (0-29.8)	25	0 (0-83.6)	25	0 (0-60.4)	n.d.	n.d
9,10AQ	80	173 (0-283)	75	51.4 (0-205)	100	110 (45.9-825)	75	96.2 (0-520)	50	70.3 (0-141)
9FLO	80	7.80 (0-12.5)	75	2.25 (0-6.91)	100	6.48 (3.03-17.5)	75	3.98 (0-11.5)	50	3.77 (0-7.54)
BcdP	60	16.2 (0-22.1)	25	0 (0-14.2)	25	10.9 (0-25.1)	50	8.08 (0-20.4)	50	7.88 (0-15.8)
BaF	100	60.9 (12.8-89.2)	100	17.3 (14.1-52.1)	100	41.6 (13.2-204)	100	24.5 (13.3-170)	100	24.8 (14.5-35.1)
BZ	60	21.1 (0-25.9)	50	7.81 (0-18.5)	75	20.4 (0-35.9)	50	9.80 (0-29.4)	50	9.28 (0-18.6)

Appendix C1 (Continued).

Analyte	Week 0 (N=5) % Freq	Week 0 (N=5) Median (Range)	Week 1 (N=4) % Freq	Week 1 (N=4) Median (Range)	Week 4 (N=4) % Freq	Week 4 (N=4) Median (Range)	Week 12 (N=4) % Freq	Week 12 (N=4) Median (Range)	Week 24 (N=2) % Freq	Week 24 (N=2) Median (Range)
tOPAHs		303 (13.7-494)		78.7 (14.1-351)		207 (62.1-1222)		150 (13.3-856)		126 (14.5-238)
NAB	100	0.79 (0.30-4.84)	25	n.d. (n.d.-3.37)	0	n.d.	0	n.d.	50	0.23 (n.d.-0.32)
NAT	100	2.44 (0.87-56.7)	75	12.1 (n.d.-20.0)	100	5.33 (1.91-36.6)	75	1.64 (n.d.-40.4)	50	2.25 (n.d.-3.88)
NNK	80	9.25 (n.d.-41.7)	100	27.2 (19.6-41.1)	100	10.7 (5.68-45.1)	100	9.35 (1.21-60.2)	100	3.53 (1.27-5.78)
NNN	80	2.70 (n.d.-25.5)	50	3.08 (n.d.-14.0)	100	6.56 (1.87-25.3)	75	3.04 (0.63-45.4)	50	1.00 (n.d.-1.38)
TSNAs		14.0 (2.21-129)		46.5 (21.0-70.5)		22.7 (9.78-108)		21.0 (2.61-146)		7.36 (2.67-11.36)
NIC	100	17,305 (1,932-71,651)	100	7,625 (1,011-51,289)	100	22,609 (2,391-52,727)	100	14,366 (2,014-25,741)	100	19,741 (4,679-34,804)

n.d.: NNN and NAB not detected above 0.15 ng g^{-1}; NAT and NNN not detected above 0.623 ng g^{-1}
% Freq: Frequency at which compounds were detected above LOQ

Appendix C2. Percentage frequency of detection, median and range of loadings, nicotine, PAHs, and TSNAs in settled housedust in ng m^{-2} in five nonsmoker homes up to six months after smoking.

		Week 0 (N=5)		Week 1 (N=4)		Week 4 (N=4)		Week 12 (N=4)		Week 24 (N=2)
Analyte	**% Freq**	**Median (Range)**	**% Freq**	**Median (Range)**	**% Freq**	**Median (Range)**	**% Freq**	**Median (Range)**	**% Freq**	**Median (Range)**
1MPYR	100	153 (13.2-291)	100	92.8 (10.7-148)	100	201 (34.4-223)	100	231 (17.5-591)	100	146 (17.0-275)
2+1MPHE	100	282 (15.3-835)	100	447 (28.1-497)	100	429 (342-750)	100	498 (22.9-1304)	100	322 (19.7-624)
2MAnt	80	23.2 (0-148.6)	75	12.4 (0-40.7)	100	30.5 (17.3-57.9)	50	39.6 (0-96.3)	50	35.8 (0-71.6
6MChr	100	130 (8.37-486)	100	62.5 (8.79-177)	100	219 (18.3-671)	100	321 (9.21-1036)	100	370 (8.48-731)
ANT	20	0 (0-78.0)	0	n.d.	25	0 (0 -97.6)	25	0 (0-367)	25	127 (0-254)
BaA	100	218 (3.94-690)	100	130 (5.66-644)	100	326 (25.9-980)	100	364 (4.38-2284)	100	241 (3.94-479)
BaP	100	195 (3.66-553)	100	127 (5.82-595)	100	305 (20.8-793)	100	308 (4.35-1663)	100	203 (3.84-403)
BbF	100	468 (5.38-1195)	100	205 (9.68-1157)	100	670 (26.6-2049)	100	751 (5.78-5257)	100	509 (5.21-1013)
BeP	100	461 (7.23-1061)	100	199 (11.4-799)	100	615 (29.1-1300)	100	645 (8.09-3431)	100	463 (7.48-918)
BghiP	100	296 (7.39-850)	100	157 (13.4-670)	100	437 (20.9-844)	100	465 (8.17-2201)	100	326 (8.39-644)
BkF	100	170 (3.88-557)	100	119 (5.65-486)	100	284 (21.5-720)	100	277 (4.44-2005)	100	209 (3.67-414)
CHR	100	514 (6.50-1331)	100	231 (10.4-1063)	100	742 (37.1-1951)	100	674 (7.96-6056)	100	508 (6.61-1009)
DBahA	60	33.6 (0-109)	0.6	28.2 (0-85.5)	75	56.3 (0-100)	50	59.9 (0-263)	50	41.2 (0-82.5)
FLA	100	731 (23.1-2410)	100	416 (31.8-2326)	100	1201 (86.1-3041)	100	1196 (34.8-7826)	100	940 (34.4-1846)
IND	100	146 (3.63-570)	100	108 (6.26-601)	100	265 (20.0-810)	100	265 (3.98-1998)	100	174 (3.93-344)
PHE	100	342 (23.5-862)	100	237 (21.8-1017)	100	577 (107-1007)	100	484 (43.8-2160)	100	408 (28.2-789)
PYR	100	766 (11.6-3034)	100	426 (19.2-3687)	100	1264 (76.7-4124)	100	1445 (13.8-11496)	100	1064 (13.5-2116)
RET	100	174 (9.42-480)	100	125 (6.36-239)	100	183 (117-356)	100	228 (16.6-1782)	100	363 (17.1-709)
TRI	100	286 (4.27-524)	100	109 (7.44-319)	100	377 (19.8-569)	100	370 (4.73-1708)	100	290 (5.67-575)
tPAHs		5929 (151-15931)		3379 (203-14244)		8728 (1040-19331)		9703 (213-51362)		6742 (187-13297)
2M9,10AQ	60	44.8 (0-124)	25	0 (0-131)	75	56.9 (0-128)	50	71.8 (0-315)	50	74.3 (0-149)
5,12NQ	20	0 (0-93.1)	25	0 (0-163)	25	0 (0-152)	25	0 (0-435)	n.d.	n.d

Appendix C2 (Continued)>

Analyte	Week 0 (N=5) % Freq	Week 0 (N=5) Median (Range)	Week 1 (N=4) % Freq	Week 1 (N=4) Median (Range)	Week 4 (N=4) % Freq	Week 4 (N=4) Median (Range)	Week 12 (N=4) % Freq	Week 12 (N=4) Median (Range)	Week 24 (N=2) % Freq	Week 24 (N=2) Median (Range)
9,10AQ	80	351 (0-932)	75	325 (0-1125)	100	543 (123-1502)	75	723 (0-3744)	50	524 (0-1048)
9FLO	80	22.5 (0-41.9	75	12.8 (0-37.8)	100	28.5 (8.16-31.8)	75	28.5 (0-82.8)	50	28.1 (0-56.2)
BcdP	60	39.7 (0-90.2)	25	0 (0-77.7)	25	22.9 (0-68.1)	50	73.5 (0-156)	50	58.7 (0-117)
BaF	100	110 (6.21-327)	100	116 (6.87-285)	100	188 (35.6-371)	100	185 (7.43-1225)	100	134 (6.56-262)
BZ	60	46.6 (0-116)	50	50.6 (0-136)	75	70.8 (0-120)	50	94.6 (0-212)	50	69 (0-138)
OPAH		615 (6.21-1,631)		521 (6.87-1,921)		985 (167-2224)		1181 (7.43-6161)		888 (6.56-1770)
NAB	100	1.97 (0.26-8.86)	25	1.06 (n.d.-12.5)	0	n.d.	0	n.d.	50	1.22 (n.d.-2.38)
NAT	100	7.05 (0.39-104)	75	49.3 (n.d.-172)	100	30.2 (5.84-98.5)	75	14.5 (n.d.-97.9)	50	14.6 (n.d.-28.9)
NNK	80	17.8 (n.d.-76.3)	100	120 (9.52-359)	100	34.9 (18.2-123)	100	71.9 (0.68-146)	100	21.8 (0.58-43.1)
NNN	80	5.83 (n.d.-46.7)	50	25.9 (n.d.-51.6)	100	27.5 (5.83-68.1)	75	23.4 (n.d.-110)	50	5.29 (n.d.-10.3)
TSNAs		44.3 (1.00-236)		203 (10.2-581)		93.2 (30.5-291)		151 (1.46-354)		71.1 (1.21-84.6)
NIC	100	86,433 (2,142-131,122)	100	54,716 (1,627-189,770)	100	102,076 (4,353-141,837)	100	37,659 (2,016-248,659)	100	130,706 (2,121-259,292)

n.d.: NNN and NAB not detected above 0.15 ng g^{-1}; NAT and NNN not detected above 0.623 ng g^{-1}

% Freq: Frequency at which compounds were detected above LOD

Appendix C3. Principal component loadings from compound dust loadings.

Analyte	Prin1	Prin2	Prin3	Prin4	Prin5
PHE	0.97287	-0.183	-0.07537	-0.01144	-0.0476
ANT	0.45802	0.0418	0.82816	0.25458	0.16011
2+1MPHE	0.95259	0.25966	-0.07968	0.01361	-0.05577
2MAnt	0.9765	0.1522	0.04102	-0.07796	-0.05499
PYR	0.97415	-0.19232	-0.08868	0.0245	0.04002
RET	0.91973	0.1322	0.27988	-0.11973	-0.17696
FLA	0.96043	-0.26799	-0.05115	0.01424	0.00465
1MPYR	0.96044	-0.21126	0.10587	-0.07122	-0.10244
TRI	0.97159	-0.21591	0.03936	-0.00596	0.00689
BaA	0.98384	-0.15475	-0.06575	0.02217	0.02564
6MChr	0.90633	-0.2509	0.28689	-0.09283	-0.05947
CHR	0.97325	-0.21573	-0.04887	0.01167	0.01264
BbF	0.97124	-0.2222	-0.04262	0.02714	0.04001
BkF	0.98368	-0.15698	-0.06853	0.01012	0.03362
BeP	0.97299	-0.22371	-0.0002	0.011	0.02973
BaP	0.9818	-0.16821	-0.05238	0.03673	0.03948
IND	0.97504	-0.19414	-0.09097	0.02488	0.03719
DBahA	0.99444	-0.0658	-0.05335	-0.01621	0.00303
BghiP	0.94911	-0.30555	0.01578	0.02906	0.04631
9FLO	0.98063	0.1333	-0.02185	0.01253	-0.01233
9,10AQ	0.98592	0.08597	-0.10678	0.02107	-0.01091
2M9,10AQ	0.9881	0.02282	-0.02925	-0.03903	0.01515
BaF	0.99003	-0.08233	-0.09946	-0.00959	-0.00892
BZ	0.98903	0.05924	0.0542	0.01079	0.01286
BcdP	0.98792	0.082	0.07692	-0.01047	0.02547
5,12NQ	0.95409	0.13828	-0.16496	0.045	-0.03677
NNK	0.66521	0.57756	-0.23084	0.30468	0.13907
NNN	0.60691	0.71248	-0.17555	0.17147	-0.13298
NAT	0.56555	0.79155	-0.07899	0.11409	0.03799
NAB	0.5575	0.56301	-0.03998	-0.57013	0.20156
NIC	0.52679	0.72121	0.4108	-0.08551	-0.09007