Analysis of Oxygenated Polycyclic Aromatic Hydrocarbons in Contaminated Soil and Water Systems to Inform Remediation Strategy and Risk Assessment

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed:  ID No.: 15212974 Date: 16 June, 2020.

Table of Contents

List of Acronyms and Abbreviations

1-Naphacetic	—	1-Naphthylacetic acid
1-OHAcen/ 1-OHAcenaphthene	—	1-Hydroxyacenaphthene
1-OHNaph/ 1-OHNaphthalene	—	1-Hydroxynaphthalene
1-OHPyrene	—	1-Hydroxypyrene
1-OH-2-Naphthoic/ 1-OH-2Naph-acid	—	1-Hydroxy-2-naphthoic acid
1,2 *trans*-naphdiol	—	1,2 *Trans*-dihydroxy-1,2-dihydronaphthalene
2ME	—	2-Mercaptoethanol
4-OHBenzaldehyde	—	4-Hydroxybenzaldehyde
4-OHBenzoic	—	4-Hydroxybenzoic acid
9, 10-AnthQ	—	9, 10-Anthraquinone
9-OHFluo./ 9-OHFluorene	—	9-Hydroxyfluorene
9-OFluo	—	9-Fluorenone
ACE	—	Acetone
ACN	—	Acetonitrile
Acenaph	—	Acenapthene
Acenaphyl	—	Acenaphthylene
APCI	—	Atmospheric pressure chemical ionisation
APPI	—	Atmopsheric pressure photoionisation
ASAP-MS	—	Atmospheric-pressure solid-analysis probe mass spectrometry
a.u.	—	Arbitrary units
BCR	—	Community Bureau of Reference
BHT	—	Butylated hydroxytoluene
BSA	—	Bis(trimethylsilyl)acetamide
BS EN	—	British Standards in English
BSTFA	—	*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide
CA	—	Cellulose acetate
CAS	—	Chemical Abstracts Service, referring to a compound identifier number
Ch#	—	Refers to a chapter number
COOHPAH	—	Polycyclic aromatic hydrocarbons modified with carboxyl group

cq	—	Cautiously quantitative – while analytically stable in itself, some non-quantitative compounds, if present may transform into this compound during GC-MS analysis
CRM	—	Certified reference material
cycloHEX	—	Cyclohexane
DAD	—	Diode array detection
DCM	—	Dichloromethane
df	—	Refers to film thickness of gas chromatography column
DLLME	—	Dispersive liquid-liquid microextraction
DNA	—	Deoxyribonucleic acid
dSPE	—	Dispersive solid phase extraction
DTT	—	Dithiothreitol
EC_{50}	—	Half maximal effective concentration (i.e. the concentration required to produce a specified (adverse) effect 50% of the time)
ECD	—	Electrochemical detection
EDTA	—	Ethylenediaminetetracetic acid
EI	—	Electron impact ionisation
ERM	—	European reference material
EPA	—	United States Environmental Protection Agency
ESI	—	Electrospray ionisation
ET	—	Enhanced transformation - refers to group of samples described further Chapter 3
EtOH	—	Ethanol
EU	—	European Union
F#	—	F followed by a number refers to a specified solid phase extraction elution fraction
FID	—	Flame ionisation detection
FLD	—	Fluorescence detection
GC	—	Gas chromatography
GC-MS	—	Gas chromatography-mass spectrometry
GPC	—	Gel permeation chromatography
HA	—	Cellulose mixed ester membrane type
Hep	—	N-Heptane
HEX	—	Hexane
HLB	—	Hydrophilic-lipophilic-balanced sorbent

HMW	—	High molecular weight
HPAC	—	Polyaromatic compound with heteroatom incorporated into the fused ring structure
HPLC	—	High performance liquid chromatography
HRMS	—	High resolution mass spectrometry
i.d.	—	Internal diameter
IS	—	Internal standard
ISO	—	International Organization for Standardization
K_{OW}	—	Octanol/water partitioning constant
K_{TOC}	—	Total organic carbon/water partitioning coefficient
LC	—	Liquid chromatography
LC-MS	—	Liquid chromatography mass spectrometry check if LCMS is used anywhere checked intro-ch2 ok.
LDPE	—	Low density polyethylene
LE	—	Leachate fraction
LLE	—	Liquid-liquid extracction
LMW	—	Low molecular weight
LOD	—	Limit of detection
LOI	—	Loss on ignition
LOQ	—	Limit of quantitation
m	—	Slope of a calibration curve
m_x	—	Mass of analyte, with subscripts t= in test sample, u= in unspiked sample blank, r=in reference solution, s=of surrogate compound in sample,
MAE	—	Microwave assisted extraction
MeOH	—	Methanol
MRM	—	Multiple reaction monitoring
MS	—	Mass spectrometry
MSTFA	—	N-Methyl-N-trimethylsilyl-trifluoroacetamide
MTBSTFA	—	N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide
n	—	Indicates number of samples utilised in a test
nd	—	Not detected
NICI	—	Negative ion chemical ionisation
NIST	—	National Institute of Standards and Technology
NPAC	—	Polyaromatic compound with nitrogen incorporated into the fused ring structure
nq	—	Not quantitative

NR	—	Not representative
OHPAH	—	Polycyclic aromatic hydrocarbons modified with hydroxyl group
OPAC	—	Polyaromatic compound(s) with oxygen incorporated into the fused ring structure
OPAH	—	Polycyclic aromatic hydrocarbons modified with carbonyl group
p	—	Probability value (statistic)
PAC	—	Polyaromatic compound(s)
PAH	—	Polycyclic aromatic hydrocarbon(s)
PAHd	—	Indicates a mixture of deuterated polycyclic aromatic hydrocarbons
PAHM	—	Indicates a mixture containing target polyaromatic compounds
PCA	—	Principal components analysis
PDMS	—	Polydimethylsiloxane
PES	—	Polyethersulfone add to first time used (appendix A)
pH	—	Log_{10} of the molar proton concentration
pKa	—	Log_{10} of the acid dissociation constant
PLE	—	Pressurised liquid extraction
POM	—	Polyoxymethylene
PTFE	—	Polytetrafluoroethylene
PVDF	—	Polyvinylidene fluoride
P-WAX	—	Preparation-weak anion exchange
QTOF	—	Quadrupole-time of flight mass spectrometry
R	—	Correlation coefficient
R chart	—	Process control chart for measurement Range
R^2	—	Coefficient of determination
RA	—	Readily available
Rec_x	—	Recovery, with subscripts t=target compound, s=surrogate, SR=surrogate adjusted
ref	—	Refer to
RSD	—	Relative standard deviation
RT	—	Retention time
SA	—	Strong acid
SB	—	Strong base
SDME	—	Single drop microextraction

SIM	—	Single ion monitoring
SE_m	—	Standard error of slope of calibration curve
SEC	—	Size exclusion chromatography
SFE	—	Supercritical fluid extraction
SIM	—	Single ion monitoring
SPAC	—	Polyaromatic compound(s) with sulfur incorporated into the fused ring structure
SPE	—	Solid phase extraction
SPME	—	Solid phase microextraction
SR	—	Surrogate recovery standard; as a subscript indicates surrogate recovery adjustment has been applied
SRM	—	Standard reference material
SWE	—	Subcritical water extraction
TBDMS	—	*Tert*-butyldimethylsilyl
TC	—	Total carbon
TCE	—	Trichloroethylene
TE	—	Total extractable
TEA	—	Triethylamine
TFA	—	Trifluoroacetic acid
TLC	—	Thin layer chromatography
TMCS	—	Trimethylchlorosilane
TMS	—	Trimethylsilyl-
TMSI	—	Trimethylsilylimidazole
TN	—	Total nitrogen
TOC	—	Total organic carbon
TOF	—	Time of flight mass spectrometry
UPLC	—	Ultra-performance liquid chromatography
USE	—	Ultrasonic extraction
USEPA	—	United States Environmental Protection Agency
UV	—	Ultraviolet
$\bar{x}$	—	Mean value of sample

List of Figures

List of Tables

List of Publications

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Good intentions may do as much harm as malevolence, if they lack understanding **–Albert Camus**

Abstract

Analysis of Oxygenated Polycyclic Aromatic Hydrocarbons in Contaminated Soil and Water Systems to Inform Remediation Strategy and Risk Assessment

Coren E. Pulleyblank

Polycyclic aromatic hydrocarbons (PAH) have been regulated as priority pollutants since the 1970's. Since then, there has been increasing recognition that oxygenated PAH transformation products may present a greater risk than parent PAH. This is a concern for soil remediation sites where formation of oxygenated PAH can be accelerated. Currently, routine monitoring is challenged due to a lack of standard analytical protocols. This thesis applies new analytical methods to investigate the formation and distribution of oxygenated PAH in contaminated soil-water systems under different remediation scenarios. High performance liquid chromatography and gas chromatography - mass spectrometry were used to investigate the effect of lignin phenol amendments on PAH transformation processes in a simulated soil-water system. Samples with highest PAH attenuation were characterised by increased utilisation of lignin phenols and distinct patterns of oxygenated PAH removal/formation, suggesting a potential approach to enhance PAH biodegradation. Challenges for analysing soil-bound oxygenated PAH were addressed through the development of a novel aminopropylsilica solid phase extraction method. Strong recoveries of ketone- and hydroxyl-modified PAH were obtained, and the method also supported limited qualitative analysis for acid and aldehyde products. In addition, contamination level and clay content were shown to influence recovery of targeted compounds from different soils. Combining analytical methods for total extractable, leachate, and readily available soil fractions, the distribution of oxygenated PAH in gasworks soils undergoing remediation was monitored over a six-month period. Biochar, compost, and no amendment treatments were compared for effects on contaminant degradation/formation, and contaminant lability. It was shown that the biochar amendment was most likely to increase, and compost amendment most likely to decrease, risks associated with oxygenated PAH in these soils. Together, these studies show how new analytical techniques for the detection of oxygenated PAH can be used to enhance remediation science and support decision making at remediation sites.

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Chapter 1

Introduction

Sections of this chapter have previously been presented in an article in the journal Critical Reviews in Environmental Science and Technology

Pulleyblank, C., Cipullo, S., Campo, P., Kelleher, B., Coulon, F., 2019. Analytical progress and challenges for the detection of oxygenated polycyclic aromatic hydrocarbon transformation products in aqueous and soil environmental matrices: A review. Crit. Rev. Environ. Sci. Technol. 49, 357–409.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are some of the most widely reported persistent organic pollutants posing risk to soil and water systems at petroleum- and industrially-contaminated sites (Abdel-Shafy and Mansour, 2016). Comprised of two or more fused benzene rings (Figure 1.1), PAH are hydrophobic, highly stable in soil and water systems, and exhibit bioaccumulation potential (Kuppusamy et al., 2017). Due to their known toxic and carcinogenic effects, sixteen PAH have been listed as priority pollutants by the United States Environmental Protection Agency (EPA, USEPA) (Figure 1.1), with similar lists prepared for environmental and food safety legislation in the European Union (Lerda, 2011). Although the creation of these lists has been an important step for directing analytical developments, environmental research, and regulation of these pollutants, it has also limited the scope for considering additional risks associated with PAH-contaminated sites (Pulleyblank et al. 2019, Andersson and Achten, 2015). Other polyaromatic compounds (PAC) including larger PAH, hetero-PAC which incorporate N, S, or O into the aromatic ring system (N- S- O-PAC respectively), and alkyl-, and nitro-, and oxy- substituted PAC, are frequent co-contaminants at these sites and in many cases may present a higher risk than the 16 USEPA PAH (Andersson and Achten, 2015). Specifically, it has been understood for over 40 years that oxygenated PAH transformation products including PAH functionalised with ketone, hydroxyl, and/or carboxyl groups, (Figure 1.2 OPAH, OHPAH, and COOHPAH, respectively) may be more mobile, bioavailable, and more toxic than parent PAH (Arp et al., 2014; Boll et al., 2008; Cerniglia et al., 1983; Chibwe et al., 2015; Knecht et al., 2013; Lundstedt et al., 2014), but this class of compounds has remained understudied in the environment and at remediation sites (Arp et al., 2014).

This knowledge gap is particularly concerning as soil remediation often involves promoting PAH oxidation, which may lead to the unintended consequence of increasing site risk if resulting byproducts are not appropriately monitored and managed (Chibwe et al., 2015; Lundstedt et al., 2007). Already, several studies reporting PAH attenuation during soil treatment have observed *increased* rather than decreased soil toxicity (Brooks et al., 1998; Chibwe et al., 2015; Hu et al., 2014, 2012; Pradhan et al., 1998), and researchers have suggested this may be due to elevated levels of oxygenated PAH (Chibwe et al., 2015; Kang et al., 2019; Lundstedt et al., 2007). Since the ultimate aim of remediation should be to reduce health risk to humans and other ecological receptors,

it is imperative that we gain a better understanding of the dynamics of oxygenated PAH at contaminated sites in order to effectively inform regulation and risk assessment.

One of the primary barriers to including these compounds in regulation and risk assessment is a lack of knowledge of the overall extent and nature of oxygenated PAH contamination in real-world environmental samples from both unremediated and remediated sites. The paucity of available data is further conditioned by substantial challenges in detecting these compounds in soil and related water matrices (e.g. leachate, groundwater, runoff). While there have been some initial efforts to routinise analysis of OPAH (Lundstedt, 2014), there are currently no standard analytical methods that address the diversity of oxygenated transformation products in these matrices.

This thesis develops and applies novel analytical techniques for detecting oxygenated PAH alongside parent PAH in both unremediated and remediated soils and related water systems. The remainder of this chapter provides a general overview of PAH and oxygenated PAH in the environment and highlights specific research needs before outlining the rationale and objectives pursued throughout the rest of the work. Sections of this introduction (1-1.1, Figure 1.2, Table 1.1) have been adapted from our previously published review of analytical methods (Pulleyblank et al., 2019).

1.1 PAH and oxygenated PAH in the environment

1.1.1 Formation

PAH are formed through the incomplete combustion of organic materials. They may be produced naturally during wild fires, volcanic eruptions, petroleum formation, and through some microbial processes (Abdel-Shafy and Mansour, 2016), but most PAH pollution is anthropogenic in origin (Ghosal et al., 2016). Burning of wood and fossil fuels contributes atmospherically-deposited PAH to soils (Kuppusamy et al., 2017). However, fossil fuels, tars, and creosote are highly concentrated sources of PAH, and many of the most heavily contaminated soils are found at current or former industrial sites, with gasworks sites frequently carrying the highest PAH burden (Haritash and Kaushik, 2009; Kuppusamy et al., 2017). Oxygenated PAH are likewise produced during incomplete combustion and commonly co-occur with PAH in petroleum-based pollution (Arp et al., 2014; Avagyan et al., 2016; Obrist et al., 2015); but oxygenated PAH are also formed through later modification of PAH via photo-, chemical- or biological oxidation (Abdel-Shafy and Mansour, 2016; Gan et al., 2009; Marquès et al.,

2016). These processes occur naturally in the soil, water, or air, but may further be accelerated at remediation sites where oxidation is enhanced in order to promote PAH mineralisation (Chibwe et al., 2015). Under these conditions, oxygenated PAH are common intermediate or long term byproducts of PAH degradation (Lundstedt et al., 2007).

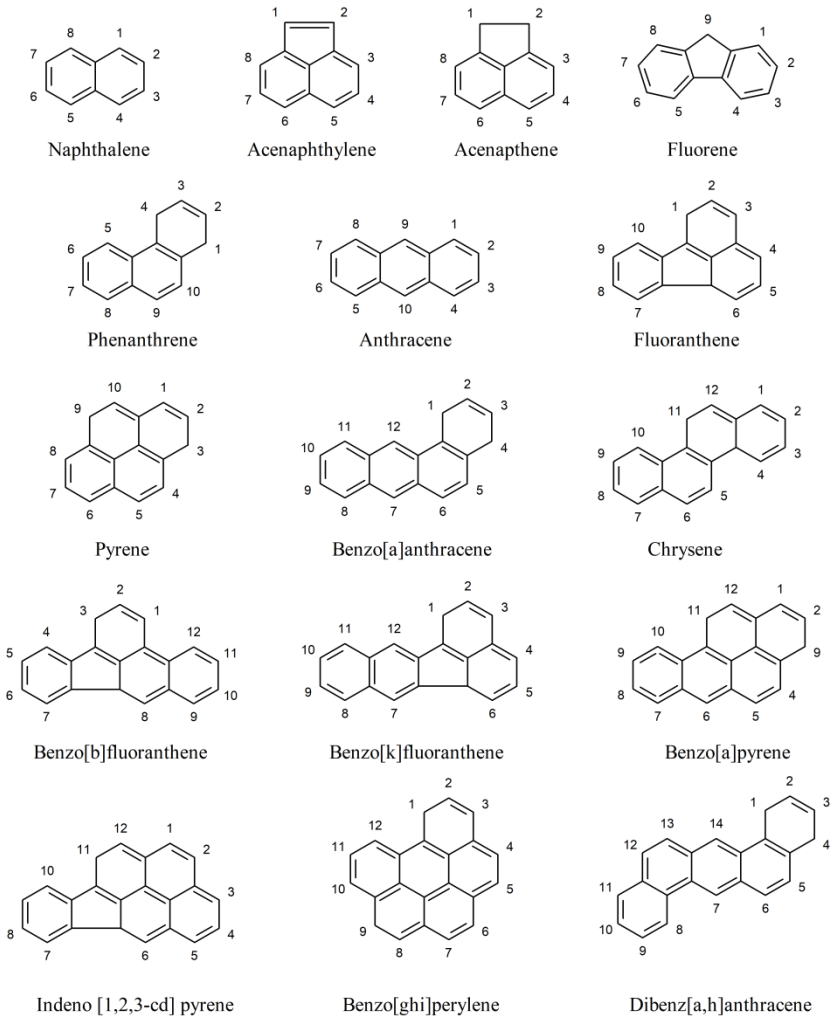

Figure 1.1 Sixteen USEPA PAH; numbered carbons indicate substitution positions.

In some cases, the formation of oxygenated PAH is a necessary aspect of PAH remediation. Specifically, bioremediation depends on stepwise transformation of PAH by bacteria, algae, fungi, and higher order animals. Mechanisms of PAH catabolism vary between organisms, with new pathways still being discovered (Cerniglia 1992; Ghosal et al., 2016; Habe and Omori, 2003; Haritash and Kaushik, 2009; Meckenstock et al., 2016, for more extended reviews). Although anaerobic pathways have been identified, most known PAH degrader species utilise aerobic mechanisms. Bacteria, fungi, and higher order animals typically initiate PAH degradation inside the cell through the action of specialised aryl dioxygenase or monooxygenase enzymes, which result in the formation of aryl epoxides and OHPAH. Lignolytic fungi, i.e. those involved in recycling lignins found in plant materials, also secrete extracellular enzymes - laccase, lignin peroxidase, and manganese peroxidase - which can initiate PAH oxidation, often resulting in the production of PAH quinones and acid transformation products (Ghosal et al., 2016). Through a cascade of enzymatic reactions, which may involve multiple organisms (Ghosal et al., 2016; Loick et al., 2009), initial daughter products are further oxidised and the aromatic rings eventually cleaved. At the later stages of PAH catabolism, some mono-aromatic compounds such as indanone, phthalate, catechol, gentisate, and salicylate are formed - these are considered collectively with oxygenated PAH species in the literature and in the current work. In addition, fungi and higher-order animals produce conjugated PAH metabolites which link glucoronide, glutathione, glycine, or sulfate at one or more of the hydroxyl or carboxyl groups (Figure 1.2). This facilitates excretion of the contaminant and increases its environmental mobility (Boll et al., 2008; Schmidt et al., 2010). Despite the wide array of catabolic pathways functioning in soils, it is important to note that in some cases oxygenated PAH resist further transformation and are considered dead-end products rather than intermediates of PAH mineralisation (Lundstedt et al., 2007).

The majority of research elucidating metabolic pathways of both bacteria and fungi has been performed using pure cultures of soil isolates under controlled laboratory conditions (Loick et al., 2009). Chemical- and photo-oxidation experiments have typically been similarly controlled and restricted. When viewed together, this ‘clean’ in vitro research has demonstrated an incredible variety of metabolites spanning a broad range of size, stability, solubility, polarity, acidity, and isomeric characteristics. In itself, the diversity of known oxygenated PAH can pose substantial analytical challenges, (ref. Chapter 2). In soils or other environmental matrices, even further complexity may be

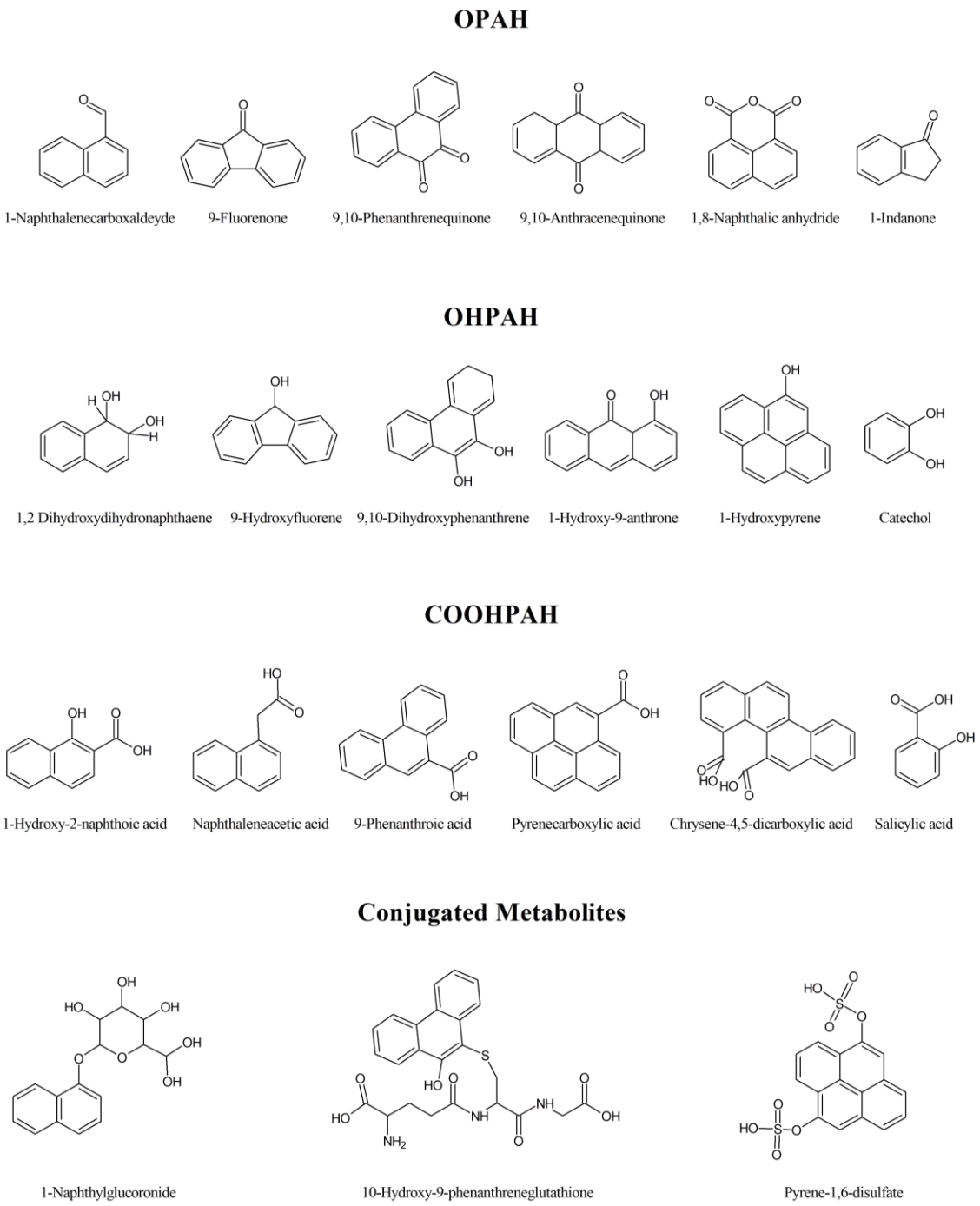

Figure 1.2 Selected oxygenated PAH transformation products and terminology used in this text. OPAH, OHPAH and COOHPAH modified by carbonyl, hydroxyl, and carboxyl groups, respectively, and conjugated forms. Late stage monoaromatic metabolites have been included. In cases of mixed functionality, the most polar group has been given priority. Dicarboxylic acid anhydrides are included with OPAH since they are typically analysed in the same fraction. In addition, OPAH species with an even number of carbonyl groups are classified as quinones. Dihydrodiols differ from other OHPAH as they are also characterised by loss of aromaticity in the substituted ring. These also exhibit stereospecificity with *cis*-forms associated with bacterial catabolism, and *trans*-forms with fungal and animal catabolism.

added through the interplay of large microbial consortia and the possibility for side reactions, abiotic rearrangements, or adsorption or binding of metabolites onto other soil components (Ghosal et al., 2016; Semple et al., 2001). For these reasons, it may not be possible to fully elucidate all transformation pathways, however, initial research has identified transformation products in natural and contaminated soils that are consistent with those known from soil-free studies (Meyer and Steinhart, 2001).

1.1.2 Environmental distribution

Since their regulation in 1976, the 16 USEPA PAH have been routinely monitored at contaminated sites and studied in diverse environments. Widely distributed through atmospheric processes, these compounds have been found in every environment, including remote mountain and arctic sites (Kuppusamy et al., 2017; Obrist et al., 2015; Wilcke et al., 2014a). In urban areas, background and roadside concentrations of USEPA PAH may range 0.1-50 mg/kg dry soil, while concentrations in industrially contaminated soils may reach upwards to the range of 10,000-20,000 mg/kg dry soil (Kuppusamy et al., 2017). As oxygenated PAH are not regulated for soil or water, they have, however, received considerably less attention, in turn making regulation difficult. In 2007, the Swedish Environmental Protection Agency reported that information regarding environmental concentrations of OPAH was lacking, and subsequently in 2008, included these compounds in their national soil screening programme which investigated a variety of environmental matrices including soil, sludge, aerosols, breast milk, and some urban fish (perch) (Brorström-Lundén et al., 2010). Over the past 15 years, continued research has provided data for soils obtained from both contaminated and uncontaminated sites across a number of countries, environments, and land use criteria (Table 1.1). In part due to challenges analysing these compounds, a majority of studies on oxygenated PAH in soils and sediments have only evaluated OPAH, though a few studies have also investigated OHPAH and COOHPAH. Aqueous environmental samples, e.g. surface waters, groundwater, and sewage effluent, have also shown detectable to high levels of these compounds. Even though soils remain the larger reservoir of oxygenated PAH, aqueous concentrations are considered the most mobile and available for interaction with ecological receptors, both positively in terms of potentially allowing further transformation to less harmful forms, and negatively in terms of increasing exposure to toxic elements.

Table 1.1 Concentrations of oxygenated PAH in various soil and aqueous environmental matrices

Location	Compounds	Land Use/ Matrix type	Concentration[1]	Reference
Soils, Sediments, Sludge			***ng/g***	
Sweden	Σ10 OPAH	background sediments	~195-410	Brorström-Lundén et al., 2010
		background soils	~110	
		urban sediments	~50-310	
		urban soils	~50-460	
		sewage treatment plant sludge	~800-2200	
		storm water treatment sludge	~250-700	
Germany, Mainz	Σ7 OPAH;	garden soil	6.6; *16.4*	Bandowe and Wilcke, 2010
Germany, Berlin	*Σ11OHPAH+COOHPAH*	former gasworks site soil	15,681; *518*	
Brazil, Manaus		forest soil	170.2; *36.3*	
Uzbekistan, Angren	Σ7 OPAH; *Σ5 OHPAH*	rural-industry gradient soils	29-1848; *7-63*	Bandowe et al., 2010
Slovakia, Bratislava	Σ9 OPAH; *Σ5 OHPAH*	urban soils, varying land use histories	33.8-2640; *nd-50.5*	Bandowe et al., 2011
Argentina	Σ15 OPAH	rural soils along a climatic gradient	0.1–124	Wilcke et al., 2014
Thailand, Bangkok	Σ15 OPAH	urban soils	6-234	Bandowe et al., 2014
Slovakia	Σ14 OPAH	forest soils near aluminium smelter	30-2900	Bandowe et al., 2018
Sweden, Karlstad	Σ11 OPAH	gasworks plant	6240-23570	Arp et al., 2014
Sweden, Riksten		tar factory	110-108080	
Sweden, Holmsund		wood impregnation site	219500-243710	
Belgium		gasworks plant	32820	
France		coking plant	10950-203900	
		coking and metallurgy site	9550-106070	
		gas factory	26860	
USA	Σ9 OPAH	remote forest sites	6-39	Obrist et al., 2015
China, Yangtze River	Σ4 OPAH	river delta topsoils	2.1-834.1	Cai et al., 2017
China	Σ4 OPAH	agricultural soils	1–42	Sun et al., 2017
Hong Kong	Σ5 OPAH *Σ8 OHPAH*	mangrove sediments	47.9-397; *36.0-180*	Wang et al., 2015

cont'd

Table 1.1 continued

Location	Compounds	Land Use/ Matrix type	Concentration[1]	Reference
Aqueous Samples			***µg/L***	
Former gasworks site	Σ7 COOHPAH [2]	groundwater - former gasworks site	2-98	Ohlenbusch et al.. 2002
Tokyo, Japan	Σ9 OPAH + O-OHPAH [3]	seawater (Tokyo and Suruga Bays)	0.0107-0.2116	Kurihara et al., 2005
Wastewater treatment plant	Σ3 OHPAH	municipal wastewater influent	0.110	Pojana and Marcomini, 2007
		industrial wastewater influent	1.417	
		wastewater treatment plant effluent	0.02	
Sweden, Belgium, France	Σ11 OPAH	porewater - industrial soils	0.00194-168	Arp et al., 2014
Germany	Σ17 OHPAH +OPAH[4]	river water (Elbe and Weser)	0.0128-0.0558	Siemers et al., 2015
North Sea		sea water	0.0027-0.0092	
Sweden, France	Σ11 OPAH	leachate - industrial soils	0.2518-160.64	Enell et al., 2016
Argentina	Σ3 OPAH	lake water	3.05	Guiñez et al., 2018
		drinking water	0.82	
Brazil	Σ2 OPAH	sea water	1605	Santos et al., 2017
		river water	1868	
		groundwater	nd	
China	Σ4 OPAH	river water,	0.13	Qiao et al., 2014
		untreated wastewater	0.17	
		treated wastewater	0.12	
USA	Σ22 OPAH	river water (Portland Superfund site)	0.279	Tidwell et al., 2017

Entries in italics indicate a second set of target analytes; *nd* - not detected (<limit of detection, LOD)
[1] mean concentrations, mean concentration ranges, or in some cases, approximate concentrations
[2] naphthalene derivatives
[3] anthracene derivatives
[4] primarily phenolics

Direct intercomparison of oxygenated PAH levels through available studies is challenging as different authors have included different compounds in their reports. Nevertheless, a few trends can be observed. Similar to PAH, highest concentrations of oxygenated PAH in soils are typically associated with industrial sites including former coking ovens, wood preservation sites, and especially gasworks sites, where oxygenated PAH may also be present in pore water, leachate, and groundwater (Arp et al., 2014; Enell et al., 2016; Ohlenbusch et al., 2002). Urban areas receive atmospheric inputs from traffic or other combustion sources and may be substantially impacted, along with areas such as river deltas and harbours which receive large loads of terrestrial and atmospheric materials. Rural and natural areas also reveal the presence of these compounds, likely sourced from local use of wood stoves (Avagyan et al., 2016) or agricultural equipment, natural or managed vegetation fires (Obrist et al., 2015; Wilcke et al., 2014a), or long range transport via atmospheric processes (Brorström-Lundén et al., 2010).

A substantial body of evidence now challenges the commonly-held assumption that due to their greater polarity, mobility, and bioavailability, oxygenated PAH are inherently more quickly degraded in the environment and are therefore less concerning than parent PAH (Lundstedt et al., 2007). Concentrations of OPAH that exceed parent PAH have been reported in several studies for both soil and water matrices (Bandowe et al., 2014; Brorström-Lundén et al., 2010; Kurihara et al., 2005; McKinney et al., 1999; Tidwell et al., 2017; Wilcke et al., 2014b). Studies have also demonstrated lower removal rates of some, though not all, of these compounds compared to the parent PAH (Andersson et al., 2003; Hu et al., 2014; Lundstedt et al., 2003), or have demonstrated similar stability to PAH under common environmental conditions (McKinney et al., 1999). Even the most polar constituents, conjugated metabolites, have been shown to be recalcitrant to mineralisation and to degrade at lower rates than parent PAH (Schmidt et al., 2010). The longevity of these highly mobile PAH metabolites could bring concomitant downstream risks that are not currently recognised (Boll et al., 2015; Schmidt et al., 2010). Moreover, even where further transformation proceeds at reasonable rates, the production of these compounds may still contribute to periods of elevated risk at contaminated sites, particularly in cases when these processes are accelerated by remediation initiatives (Chibwe et al., 2015; Lundstedt et al., 2006a).

1.2 Toxicity

Due in part to the high concentrations of PAH found in tobacco smoke, the toxicity of PAH has been well established in the medical literature (Armstrong et al., 2004). Because of their hydrophobic character, PAH associate strongly with lipids and exhibit moderate to substantial bioaccumulation potential (Abdel-Shafy and Mansour, 2016; Kuppusamy et al., 2017). Once incorporated into an organism, PAH interact deleteriously with nucleic acids, proteins, and other cellular processes resulting in acute or long term effects. Carcinogenicity and teratogenicity are perhaps the best known toxic effects of PAH exposure, and since high molecular weight (HMW) PAH (i.e. those comprised of 4+ aromatic rings) are more persistent and in biological tissues and more directly interfere with deoxyribonucleic acids (DNA), they are key compounds of environmental concern (Ghosal et al., 2016). Specifically, benzo[a]pyrene is considered the most genotoxic of the 16 USEPA PAH, and maximum allowable PAH levels at environmental sites may be assessed in terms of benzo[a]pyrene concentrations or benzo[a]pyrene equivalents (Andersson and Achten, 2015). However, PAH also interfere with other cellular systems, and low molecular weight (LMW) PAH (i.e. those comprised of 2-4 rings), though frequently deemed “less harmful” due to reduced genotoxicity and more rapid metabolism/excretion, are considered more toxic (i.e. causing acute stress or cell death), and are known to be involved in inflammation, endocrine disruption, and immunological disorders including sensitisation, development of allergies, autoimmune disease and cancer. Further, LMW PAH are more environmentally mobile and may exhibit higher bioaccumulation in soil organisms (Abdel-Shafy and Mansour, 2016; Kuppusamy et al., 2017; Wang et al., 2017).

Importantly, most PAH are not directly toxic, but require activation of the stable aromatic structure through initial metabolic oxidation (Abdel-Shafy and Mansour, 2016; Bolton et al., 2000). In humans, the most common pathway for PAH oxidation involves the cytochrome-P450 group of enzymes, also used by fungi during intracellular catabolism of PAH in soils (Ghosal et al., 2016). This activation results in the formation of OHPAH which may be further converted into more reactive arylepoxides and quinones (Bolton et al., 2000). It is these forms that cause direct oxidative damage to cells, form adducts with DNA or proteins, and mimic estrogenic effects (Bolton et al., 2000; Idowu et al., 2019; Schweigert et al., 2001). Inherently, OHPAH and OPAH occurring in the environment represent these activated forms and are therefore are

expected to be more directly toxic (Lundstedt et al., 2007). But despite recognition of the importance of oxygenated PAH in PAH toxicity, there has been little effort to establish ecological or human health exposure limits for these compounds in soil and water systems.

Integrating an overall view of the direct toxicity of oxygenated PAH is currently challenged by the diversity of potential chemical agonists, the role of further metabolic and system processes including excretion, the diversity of target organisms/measures used to evaluate toxicological response, and as described above, a lack of data regarding the environmental distribution of these compounds to inform exposure likelihood scenarios (Knecht et al., 2013; Lundstedt et al., 2007; USEPA, 2009; Wilson, 1996). It is known that the specific toxicity of oxygenated PAH depends on the size and arrangement of the aromatic centres as well as the type, degree, and position of oxygen-containing functional groups (Knecht et al., 2013; Wang et al., 2009). For example, one study found that 1-hydroxynaphthalene, 1,2-naphthoquinone, and 1,4-naphthoquinone, were more directly toxic to human mononuclear leukocytes than naphthalene (49-51% cell death vs. 19% cell death) but that 1,2 *trans*-dihydroxy-1,2-dihydronaphthalene and naphthalene 1,2-epoxide were not significantly cytotoxic or genotoxic under the experimental conditions (Wilson, 1996). Another study indicated that EC_{50} values (i.e. the concentration required to induce a specified adverse effect 50% of the time) for DNA adduct formation were 50% higher for 1-hydroxynaphthalene compared to 2-hydroxynaphthalene, and that nine isomers of hydroxybenzo[a]pyrene yielded EC_{50} values that ranged 329-~1443 mM (Wang et al., 2009). The dependency on test condition is also apparent. For example, it has been observed that when compared to the parent PAH anthracene, 9,10-anthraquinone causes greater oxidative stress for mammalian cells and greater toxicity toward the aquatic plant *Lemna gibba*, but exhibits reduced toxicity towards the invertebrate *Daphnia magna* (Lundstedt et al., 2007); further, several tests for the gentoxicity of 9,10-anthraquinone to *Salmonella typhimurium* (Ames assay) have presented conflicting results and have suggested toxicity may be strain-dependent (USEPA, 2009).

In view of this complexity, it is not difficult to understand that existing environmental policies have continued to target a simplified list of PAH rather than a diverse array of PAC including PAH transformation products. However, key environmental data emphasise the need to reevaluate the current approach. Specifically, measures of soil toxicity frequently do not correlate well with concentrations of 16

USEPA PAH or extended lists of parent PAH (Ahtiainen et al., 2002; Kang et al., 2019; Pradhan et al., 1998), and some studies have reported that using these metrics, soil toxicity may be underestimated by as much as 70 times (Andersson and Achten, 2015). In addition, several studies have revealed that more polar fractions of soil frequently exhibit equivalent or higher toxicity than the PAH-containing fraction (Chibwe et al., 2015; Lundstedt et al., 2007), a result also reported for aerosols (Walgraeve et al., 2010). In some cases, other semipolar PAC including nitro-substituted PAH may also be involved in the increased toxicity, however oxygenated PAH are more strongly associated with DNA adduct formation in aerosol extracts (Umbuzeiro et al., 2008). Further evidence also demonstrates that soil toxicity can increase as PAH are removed and oxygenated PAH are formed (Chibwe et al., 2015; Kang et al., 2019).

1.3 Remediation

Historically located at the urban periphery, former industrial sites including historic gasworks are now often situated in prime locations for urban expansion and redevelopment. It is estimated over 58,900 former gasworks sites and related coking plants exist worldwide, making strategies for their cleanup a global concern (Kuppusamy et al., 2017). Although specific regulations depend on jurisdiction and intended land use, land owners and developers are often required to ensure the safety of new projects, and this has created greater incentive to remediate PAH-contaminated sites. Until the late 1990's, the most common method for ameliorating site conditions involved the excavation and landfilling of hazardous materials, and this remains a common approach (Kuppusamy et al., 2017). But as costs associated with transport and landfilling of toxic materials have risen, there has been an increased interest in establishing remediation technologies that can be applied on site (Loick et al., 2009). Table 1.2 summarises PAH remediation studies discussed in comprehensive review articles. It should be noted that due to legal considerations, data for contaminated sites and commercial remediation projects are in most cases not accessible to the domain of public science. Scientists may rely on smaller scale studies and/or use spiked soils, which can be expected to influence observed/anticipated remediation outcomes compared to full scale operations in true contaminated soils (Ranc et al., 2016). In addition, there is considerable variation in experimental setup conditions including substantial differences in the original soil contamination level, compounds analysed,

specific treamtent conditions, and duration of the studies, to the extent that it has been stated "experimental conditions are as diverse as polluted soil parameters" (Ranc et al., 2016). Together, these challenges contribute to the substantial variations in evaluated remediation success (% PAH reduction), even within the same treatment type (Table 1.2). Many additional factors impact remediation outcomes, and Kupussamy et al. (2017) characterise key parameters under five categories:

1. Soil and Weather (organic matter, water content, temperature, texture, pH, nutrients, redox potential)
2. Microbial Community (diversity, population, symbiotic interactions, resistance, activity)
3. Contaminant and Co-Contaminant (concentration, toxicity, bioavailability, solubility, volatility, mass transfer)
4. Cost (including pre-treatment, treatment, and post-treatment costs, and consideration of site access)
5. Non-technical factors (regulation, intended land use, research funding, human resources, liability)

For these reasons, options appraisal for a remediation project is and must be a site-specific process, and it is not posisble to recommend a single remediation strategy. This said, there are some trends that can be identified in the literature, at least with respect to remediation of parent PAH. Physico-chemical approaches including onsite soil incineration, thermal desorption and venting, soil washing and solvent extraction, and chemical oxidation methods have all been applied with varying levels of success (Table 1.2; Gan et al., 2009; Kuppusamy et al., 2017). In some cases, these technologies represent the highest cost strategies, may be challenged by limitations in specific soil and site conditions, and/or may lead to deterioration of other soil properties, making them less desirable options (Kuppusamy et al., 2017). Chemical oxidation techniques are also in some cases effective for accelerating PAH removal; however a number of studies have highlighted concerns in relation to accelerated and lasting formation of oxygenated PAH during these processes (Lundstedt et al., 2007; Ranc et al., 2016). Monitoring these compounds has not yet become a priority for most workers using chemical oxidation for PAH attenuation, and this needs to be addressed (Ranc et al., 2016).

Increasingly popular are bioremediation strategies, which are widely considered safe, ecologically friendly, and economical (Table 1.2), and which may be applied in conjunction with other remediation technologies, often on site (Loick et al. 2009;

Table 1.2 PAH soil remediation strategies - summary records

Reference Type	Treatment type	no. recs[1]	PAH[2] mg/kg	duration days	% reduction [3]	Comments, Estimated costs USD/10,000 sq.m [4]
Kupussamy et al., 2017. Review	Thermal methods - incineration, thermal desorption	3	30-1000	35-480	90-99.9	incineration: 35 million thermal desorption: 8 million
	Physical methods - soil washing, surfactant- and solvent extractions	6	665-11,600	1-180	50-100	flushed materials require further treatment soil washing: 2 million surfactant flushing: 19 million
	Chemical oxidation	6	1089-4510	1-40	31-95	
	Bioremediation-					general bioremediation: 3 million landfarming: 9 million
	Landfarming	2	1140-13000	150-730	60-95	
	Bioaugmentation	4	16-3967	30-105	40-90	
	Slurry bioreactor	2	70.4-3700	30-35	60-70	
	Composting	5	3.6-6915	100-570	37-98	
	Phytoremediation	5	10.1-3000	75-540	30-90	
	Mixed methods[5]	11	10.2-35000	1-480	10-98	
Ranc et al., 2016. Review and metastudy	In-situ chemical oxidation	34	25-21400	--	0-99	
Zhou et al., 2019. Review	persulfate-based chemical oxidation	8	4.2-2800	0.17-35	26-100	
Loick et al., 2009. Review	Composting	66	1.3-24000	2 days-2 years, most 100-200 days	not significant-100	includes composting combined with other bioremediation approaches
Davie-Martin et al., 2017. Review and metastudy	No additions	19	150-3065	25-244	35 ±2	reduction of Σ PAH mean ± standard deviation.
	Biostimulation	28	330-7767	1-912	46±2	
	Bioaugmentation	39	96-6469	3-246	37±1	
	Surfactant	64	258-6746	10-60	55±1	
	Composting	25	150-3469	42-244	74±1	
Bianco et al., 2021. Review	Biochar application	10	0.1-1458	4-100	29-98	sediments

[1] number of records included in the summary
[2] initial concentration in soil; different studies include different target PAH
[3] percent reduction for individual or all PAH targeted in the studies
[4] cost estimates based on different dataset than otherwise summarised in Table. USD=United States Dollars sq. m=square meter
[5] mixed methods involve two or more methods which may be thermal, physical, chemical, and/or biological approaches, used simultaneously or consecutively

Kuppusamy et al., 2017). Bioremediation promotes the action of organisms, especially bacteria and fungi, to metabolically degrade PAH. This may be achieved through manipulating soil characteristics such as nutrient profile, pH, structure, moisture, aeration,and temperature, and/or through the introduction of PAH degrading organisms or symbionts (Gan et al., 2009). Specific strategies are diverse and include land farming (addition of simple nutrients, irrigation, etc. to the surface layers of soil using agricultural tilling equipment), biostimulation (addition of simple nutrients, usually fertilisers and simple carbon compounds), bioaugmentation (addition of PAH degrading bacteria or fungi), biochar application, and composting (addition of complex organic amendments to add structure, nutrients and support diverse microbiology), phytoremediation (using plants to stimulate PAH degraders, especially in the rooting zone - rhizoremediation), as well as the use of more controlled bioreactors. Each approach has specific advantages and limitations (Kuppusamy et al., 2017), however there has been increased interest in composting- and biochar- based approaches (Anyika et al., 2015; Bianco et al. 2021; Loick et al., 2009; Wu et al., 2014). In addition to providing nutrients and bulking material, addition of compost and biochar materials introduces diverse microbial habitats, changes water holding capacity, and provides unique effects on pollutant sorption and desorption which may help moderate contaminant availability and toxicity to PAH degraders (Anyika et al., 2015; Bao et al. 2020; Semple et al., 2001). Overall, a 2017 metastudy concluded that compared to other bioremediation approaches, composting was most effective at attenuating PAH, reducing median calculated PAH related cancer risk by 70%, compared to 28-53% for other approaches (Davie-Martin et al., 2017). However, due to a lack of available data, the study was not able to consider risk associated with oxygenated PAH. In addition, even in terms of PAH attenuation, composting and other bioremediation initiatives have not been considered equally successful, and at the end of these studies, calculated cancer risk still exceeded recommended levels (Davie-Martin et al., 2017). While somewhat less established than composting for PAH, biochars also exhibit high sorptive capacity, and have recently been trialed with respect to decreasing PAH bioavailability with potentially positive effects on biodegradation (Bianco et al. 2021). They may be especially considered for remediation of sites with concurrent heavy metal contamination, where the beneficial effects of biochar are more established (O'Connor et al. 2018). To date, the dynamics of oxygenated PAH in biochar amended soils have

not been substantially considered. Overall, further work is needed to understand how to enhance bioremediation, while at the same time addressing the concern that formation of oxygenated PAH intermediates during bioremediation treatments may play an adverse role in terms of site risk.

1.4 Developing analytical methods

In order to better understand oxygenated PAH within the context of improving remediation and reducing associated risks, the further development of methods for detecting and quantifying oxygenated PAH in environmental samples is essential. Six main stages of analytical method development are shown in Figure 1.3: 1) Initial Assessment, 2) Method Development, 3) Validation I - initial laboratory validation 4) Quality Control throughout sample analysis, 5) Reporting 6) Validation II - interlaboratory validation /standardisation. Stage 1 initial assessment involves the identification of target compounds of interest, choice of instrumentation, and selection of matrix type, as well as assessment of available methods. For this thesis, oxygenated PAH with a range functional groups (OPAH, OHPAH, and COOHPAH) and sizes were chosen (Table 3.1) in order to capture a broader picture of PAH transformation products than typically considered in whole-environmental samples. These compounds can be detected using high performance liquid chromatography (HPLC) with diode array detection, and by gas chromatography-mass spectrometry (GC-MS), which were the instrumental approaches used in this thesis. It was also decided that both aqueous and soil samples would be investigated, so the development of multiple sample preparation methods and consideration of matrix effects would be a key component of the research. Through conducting the literature review presented in Chapter 2, it became apparent that no standard methods are currently available for the analysis of oxygenated PAH in soil or water samples. This review, however, revealed select opportunities to trial, extend, simplify, clarify, and/or otherwise improve oxygenated PAH analysis that were ultimately pursued through Stages 2-5 as part of the experimental studies presented in this thesis. For aqueous samples, this included trialing of a biphenyl-modified stationary phase for HPLC separation of oxygenated PAH, and reducing solvent/sample requirements compared to traditional liquid-liquid extractions for subsequent GC-MS analysis, assessing effects of filter selection on sample analysis, and consideration of matrix effects. For the soil phase, this included addressing sample clean-up and

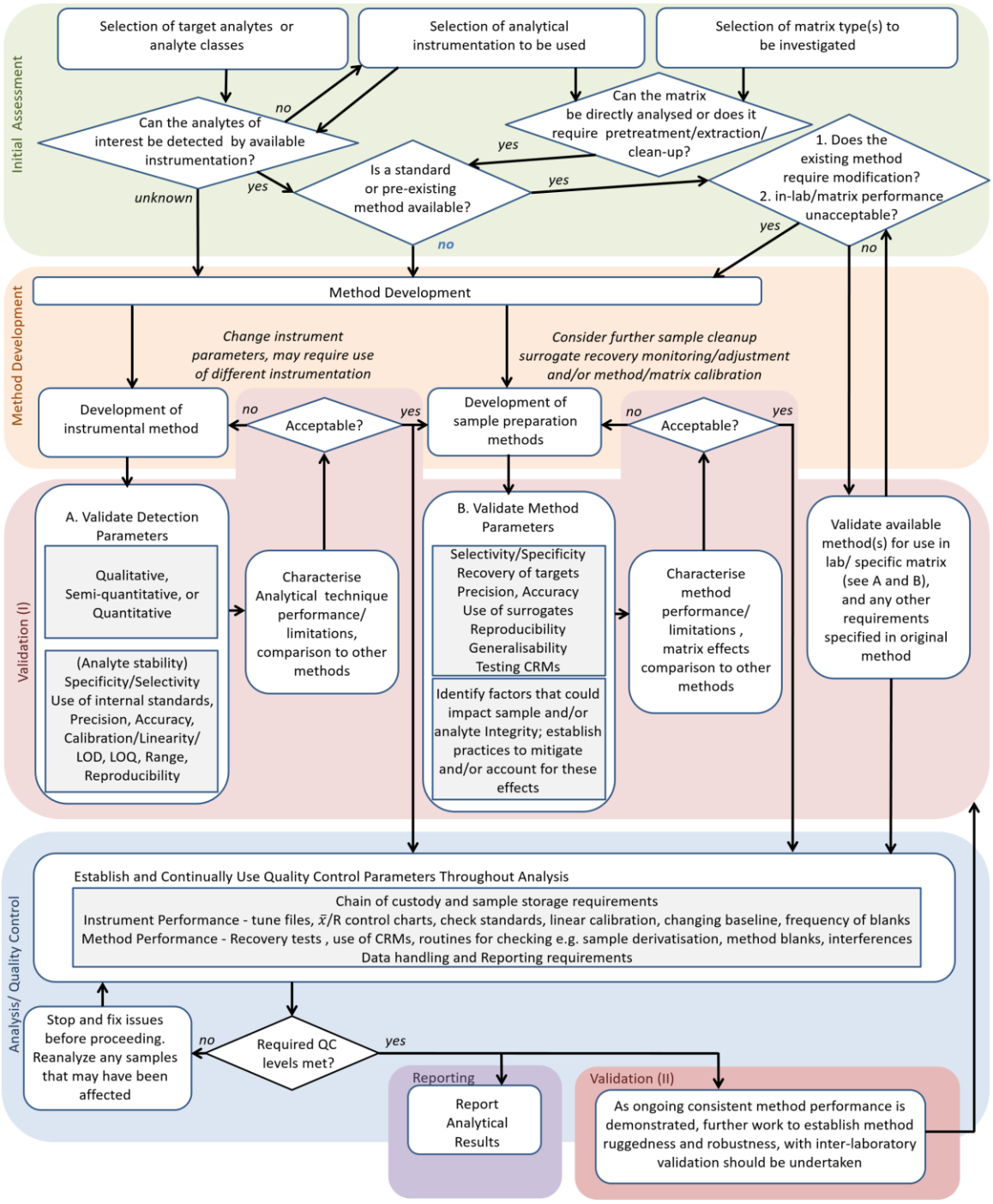

Figure 1.3 Analytical method development. LOD - limit of detection, LOQ - limit of quantitation, CRM - certified reference material, $\bar{x}$/R charts - process control charts for mean and range, respectively.

increasing the range of oxygenated PAH that can be analysed from a single soil extract, trialing aminopropyl silica as a solid phase extraction sorbent for this purpose, and addressing questions about sources of analyte loss, matrix effects, and appropriate use of deuterated PAH surrogates during sample preparation and analysis. Further general discussion of factors impacting method performance are presented in Chapter 2 (for quality control ref. 2.4), while specific approaches used to develop and validate the methods presented within this thesis are discussed within each chapter.

1.5 Summary, aims, and outline of the work

Significant gaps remain in understanding how PAH transformation occurs in situ, what cofactors might help promote PAH bioremediation, and especially, how to monitor and assess the extent to which byproducts of incomplete degradation including oxygenated PAH might contribute to risks at both unremediated and remediated sites. These questions are timely, as there is both a need to develop effective and safe approaches to site remediation and a growing consensus that the traditional measures of parent PAH are no longer adequate measures of remediation success.

Several themes have emerged as important considerations for the development of this field, and these in turn will be further explored in the remainder of this thesis:

- Studies investigating the formation and distribution of oxygenated PAH in realistic environmental samples are limited in part due to a lack of standard analytical techniques. One of the major challenges facing this work is the development of methods for detecting and quantifying these compounds in soil and water samples.

- Soil may be a larger reservoir of PAH and oxygenated PAH, while related aqueous systems such as soil water and leachate likely represent the most available, most mobile, and most directly toxic fraction; therefore both warrant consideration.

- Although sometimes dismissed as less harmful, LMW PAH are more environmentally labile and may present significant risk; as they are also more rapidly transformed during bioremediation, the development intermediates may present a source of increased acute and immunotoxic risk. Similarly, more polar compounds OHPAH and COOHPAH are less well studied in environmental samples and warrant attention.

- In situ bioremediation strategies including composting currently represent some of the most economical and desirable options for site remediation; further understanding of how cofactors present during bioremediation might stimulate PAH transformation is desired.

Keeping these themes in mind, the remaining chapters develop and apply new analytical techniques to detect PAH, OPAH, OHPAH and COOHPAH in contaminated soils and related water systems. The use of these methods is further demonstrated through three studies which investigate the changing distribution of LMW oxygenated PAH transformation products for different soil types and under different bioremediation scenarios.

Chapter 2 provides an in-depth literature review of methods for the detection of oxygenated PAH transformation products in aqueous and soil environmental matrices.

Chapter 3 uses new high performance liquid chromatography and small sample liquid-liquid extraction techniques to investigate the attenuation of LMW PAH and formation of aqueous transformation products during a bench scale incubation study trialing lignin phenols as potential novel cofactor to promote soil remediation.

Chapter 4 develops a novel solid phase extraction method to improve the analysis of PAH transformation products in contaminated soils. The method is further tested for different soil types to investigate the role of matrix characteristics on method efficiency.

Chapter 5 applies techniques developed in Chapters 3 and 4 to investigate the formation and fractionation of oxygenated PAH products during the application of in situ remediation techniques, including compost and biochar application. In addition to tracking compounds of interest in soil and water, this study investigates the question of a ‘readily available’ fraction which is expected to become increasingly important for informing ecotoxicology and site risk assessments.

Chapter 6 discusses summary conclusions and final forward-looking remarks.

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Chapter 2

Analytical progress and challenges for the detection of oxygenated polycyclic aromatic hydrocarbon transformation products in aqueous and soil environmental matrices: A review

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Pulleyblank, C., Cipullo, S., Campo, P., Kelleher, B., Coulon, F., 2019. Analytical progress and challenges for the detection of oxygenated polycyclic aromatic hydrocarbon transformation products in aqueous and soil environmental matrices: A review. Crit. Rev. Environ. Sci. Technol. 49, 357–409.

Abstract

Despite increasing interest in the formation and fate of oxygenated PAH degradation products, significant challenges remain for detecting these compounds in environmental matrices. This review examines current approaches to analysing these compounds in soil, sludge, sediment and aqueous environmental samples. Opportunities for the consolidation, extension, or improvement of existing techniques are identified; novel approaches and other development needs are also discussed.

2. Introduction

It has been recognised for over 30 years that the inclusion of oxygenated PAH in environmental analyses could improve understanding of both site risks and in situ degradative processes at PAH contaminated sites. However, the lack of standardised analytical techniques has been a major challenge for the inclusion of these compounds alongside traditional hydrocarbons during site evaluation and monitoring (Lundstedt et al., 2014). In their recent paper 'Time to say goodbye to the 16 EPA PAH?", Andersson and Achten (2015) suggest that three conditions should be met for the inclusion of additional polyaromatic compounds into regulatory lists: (1) sufficient evidence of toxicity, mutagenicity, or carcinogenicity; (2) evidence of their common occurrence in the environment; and (3) sufficient and practical analytical separations are possible. Of 10 OPAH proposed as a starting point to form a list of oxygenated PAH for further monitoring in the environment, none were considered by the authors' estimation to yet have sufficiently validated analytical methods. Yet despite these challenges, progress made in analytical techniques over the last two decades has provided new information regarding the distribution, persistence, and toxicity of various oxygenated PAH, and has begun to shed light on the role that oxygenated PAH metabolites may have in regulating PAH biodegradation pathways in the environment (Knecht et al., 2013; Lundstedt et al., 2007; Vaidya et al., 2017). This chapter reviews current analytical techniques available for their analysis in solid matrices including soil, sediment, and sludge and aqueous freshwater matrices such as leachate, pore water, and groundwater. Emphasis is placed on sample preparation, including more traditional solvent extraction and solid phase extraction (SPE) techniques, as well as novel sorptive extraction, miniaturisation, and automation methods. Key insights into the use of hyphenated gas and liquid chromatography (GC and LC) - instrumentation coupled to various detection apparatus including mass spectrometry (MS) methods are discussed. Quality control tools including use of surrogate compounds, certified reference materials, and the importance of designing the overall sampling strategy for site assessment are also considered.

2.1 Analyte diversity and matrix complexity - A double challenge

The difficulty in establishing standard techniques for oxygenated PAH analysis is primarily due to two key challenges: (1) the large diversity of these compounds and their wide-ranging chemical characteristics and (2) the complexity and variability of the

matrices for which the analytical techniques must be validated. In addition, uncertainty in the best use of surrogates, internal standards, and reference materials, as well as their limited commercial availability, remains a major issue for the establishment of robust methods (Walgraeve et al., 2010, Lawal, 2017, Lundstedt et al., 2014).

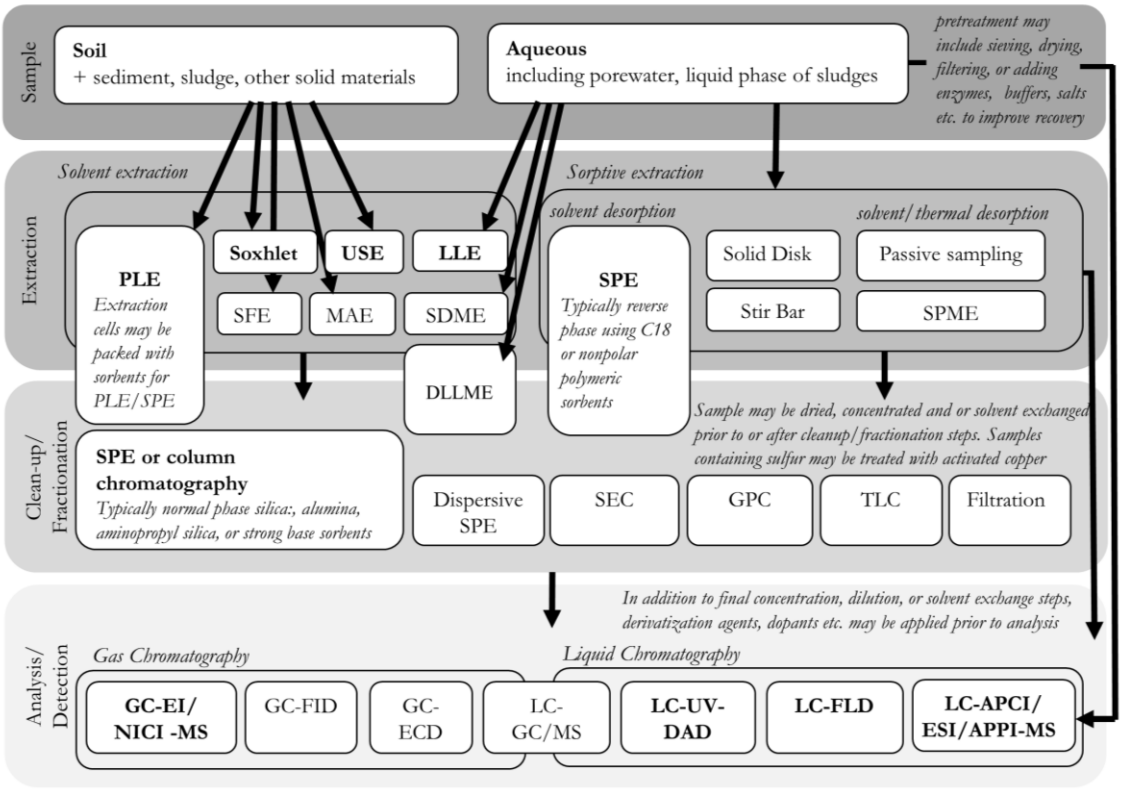
To date, an incredible variety of oxygenated PAH and PAH metabolites have been observed, though have not always been fully characterised (Letzel et al., 2001; Malmquist et al., 2013; Walgraeve et al., 2010). These span a broad wide range of size, stability, volatility, solubility, sorption, polarity, acidity, and isomeric characteristics (Arp et al., 2014; Boll et al., 2015; Hanna et al., 2012; Letzel et al., 2001; Walgraeve et al., 2010). This breadth of physicochemical characteristics of oxygenated PAH and PAH metabolites can pose substantial challenges to the development of comprehensive analytical approaches.

In general, researchers have addressed the challenge of analysing PAH metabolites by focusing on one class of compounds at a time (e.g. OPAH or OHPAH), most frequently the carbonyl-containing compounds in particulate matrices and the more soluble hydroxylated or conjugated forms in aqueous systems, though all compound groups may be found in either matrix type (ref. Table 1.1). A few studies have addressed a wider range of compounds through the use of multi-component instrumental approaches (Ahmed et al., 2015) or multistep fractionation protocols (Bandowe and Wilcke, 2010; Meyer et al., 1999). Fractionation can be particularly useful when target compounds require different processing (such as concentration and derivatisation steps) or are better suited to different instrumental analyses (ref. sect 2.3).

Subdivision by functional groups allows for some simplification in the analytical approach, as the grouped compounds tend to require similar treatments to render them detectable by GC- or LC-based techniques, but differences physicochemical characteristics within each group also remain substantial. For example, Walgraeve et al. (2010) provide a useful reference table providing estimated melting point, boiling point, vapor pressure, water solubility, and log octanol-water partitioning coefficient (K_{OW}) for 40 OPAH+OHPAH which shows estimated K_{OW} for four benzo[a]pyrene diones differ by over two orders of magnitude (log K_{OW} 3.05–5.24) and for 2–3 ring OPAH by three orders of magnitude (log K_{OW} 1.48 – 4.10) (Walgraeve et al. 2010), while two OHPAH isomers, 2-hydroxyfluorene and 9-hydroxyfluorene, are estimated to have water solubility of 71 and 4900 mg/L, respectively at 25 °C (Walgraeve et al., 2010). Further, in most cases, experimentally determined values for key chemical

characteristics are not available, and estimated values can only be used as a starting point. Recent research has demonstrated that current tools for estimating the partitioning of oxygenated PAH between soil and water phases, using (K_{OW}) and/or total organic carbon (TOC) measures, can be misleading. Boll et al. (2015) observed that some sulfate-conjugated metabolites of pyrene and phenanthrene without a carboxylic acid group showed much lower sorption than estimates obtained through the Estimation Programs Interface (aqueous distribution coefficients up to 150 times higher than estimates), though those metabolites with the carboxylic acid group were more accurately predicted. They also reported that for the three soils investigated with TOC ranging between 2.8 and 64% and pH ranging between 8.4 and 9.1, soil organic carbon content was less important than analyte functional groups for understanding sorption, though the impact of TOC characteristics may be more notable over a broader range of soils and pH conditions (Boll et al., 2015). Arp et al., (2014) also demonstrate the value of predicting organic carbon partitioning coefficients, $K_{TOC,}$ of PAH in heavily contaminated soils using Raoult's Law Coal Tar sorption model rather than K_{OW} alone, which tends to underestimate K_{TOC} for PAH in contaminated soils. Substantially improved agreement between experimentally determined and predicted K_{TOC} for OPAH was also obtained using the new approach. Although these insights may have greater impact for risk assessment, they are also important to understand for analytical technique development, particularly the development of passive sampling devices.

In addition to the complexity of the target compounds, matrix interferences in environmental samples can be substantial (e.g. O'Connell et al., 2013; Pojana and Marcomini, 2007). Solid and liquid environmental matrices contain additional macromolecules, humic substances, salts, and other interfering compounds. These may impact extraction efficiency (O'Connell et al., 2013, Sousa-Silva et al., 2015), preparative chromatography (Van de Wiele et al., 2004) and final analysis (Layshock et al. 2010). At the instrumental stage, interfering components may coelute with target analytes or contribute to overall increased baseline noise or to specific signal suppressions or enhancements, which may be particularly problematic where samples or extracts have been concentrated to improve detection of low-concentration analytes (Walgraeve et al. 2010). At the same time, analytical techniques used for aerosol, atmospheric, and urine analysis which frequently inform or adapt techniques developed for soil, sediment, and environmental aqueous samples, may not be directly applicable to these matrices.



PLE pressurized liquid extraction
USE ultrasonic-assisted extraction
LLE liquid liquid extraction
SFE supercritical fluid extraction
MAE microwave assisted extraction
SDME single drop micro extraction
SPE solid phase extraction
SPME solid phase microextraction
SEC size exclusion chromatography
GPC gel permeation chromatography
TLC thin layer chromatography
LC liquid chromatography
EI electron impact ionisation
NICI negative ion chemical ionisation
FID flame ionisation detection
ECD electrochemical detection
UV ultraviolet
DAD diode array detection
FLD fluorescence detection
APCI atmospheric pressure chemical ionisation
ESI electrospray ionisation
APPI atmospheric pressure photoionisation

Figure 2.1 Analytical techniques for the detection of PAH degradation products. Techniques in bold reflect most common approaches.

2.2 Extraction, fractionation, and storage

2.2.1 Preliminary sample processing

A variety of methods have been used for the preparation of soil and water samples prior to instrumental analysis (Figure 2.1). Extraction and clean-up methods are generally emphasised in the literature, but the impact of sample handling during pre-treatment and intermediate steps can also be substantial. Sample pre-treatment includes the separation of particulate and aqueous fractions, sieving and drying of sediments and the filtration of aqueous samples, as well as other steps including enzymatic deconjugation (Section 2.2.4) or pH adjustment.

For soils and similar matrices, the presence of water can adversely impact extraction, preparative chromatography, and instrumental detection. Excess water is therefore usually removed prior to extraction, and further drying steps may be included before final analysis. Very wet samples may be centrifuged (Hu et al., 2014), but typical preparations involve moderate air drying followed by chemical drying by sodium sulfate, which may also be performed after extraction or during sample clean-up (see Section 2.2.5). The sample is also ground and sieved to remove bulk materials and improve sample homogeneity. The <2 mm fraction is usually selected for extraction, though the finer fractions tend to have the highest levels of PAH and OPAH and therefore may be used. Since PAH and OPAH can sorb strongly to organic materials such as wood fibres, where these bulk materials are a substantial portion of the matrix, for example during composting remediation tests, it should be remembered that sorption may be an apparent source of loss from the final analysed soil matrix.

Filtration is sometimes performed prior to extraction of aqueous samples and may be applied to organic extracts prior to analysis. However, it has been generally recognised that hydrophobic organic compounds including PAH and many metabolites may adsorb on to filter membrane surfaces, leading to underestimates of analyte content (Enell et al., 2016). Despite this, filtration of samples or organic extracts is commonly overlooked as a source of loss during sample preparation. Workers report use of glass fibre (Boll et al., 2015; Niederer, 1998), cellulose acetate (Santos et al., 2017), mixed cellulose ester (Ohlenbusch et al., 2002), nylon (Avagyan et al., 2015; Hu et al., 2014), polytetrafluoroethylene (PTFE) (Lankova et al., 2016; Malmquist et al., 2013), polyamide (Lundstedt et al., 2006b), polyvinylidene fluoride (PVDF) (Hanna et al., 2012), or more ambiguously, ‘organic filter membrane’ (Liao et al., 2014), ‘filter paper’

and 'centrifugal filter' (Toriba et al., 2016), or just 'filtered' samples (Siemers et al., 2015), but most of these studies have not evaluated the potential impact of the filtration step. A few studies indicate that nylon may be appropriate for OHPAH prepared in methanol or methanol/dichloromethane (1:1) (Avagyan et al., 2015; Hu et al., 2014) and PVDF may be appropriate specifically for filtering aqueous samples containing naphthoic acids (Boll et al., 2015). Our own work (Appendix A) indicates PTFE is the most suitable filter material for a range of PAH and transformation products, though some losses were still observed. In general, centrifugation rather than filtration is the recommended approach for separating very wet soil samples or for removing particulate materials from aqueous samples (Enell et al., 2016). Where an aqueous sample has large colloid load, e.g. a simulated leachate (shaken aqueous extracts of soils), analysis of centrifuged samples may lead to overestimation of the analytes; sampling protocols may be adapted to avoid this error, for example, through the use of flow-through or recycled leachate collection systems (Enell et al., 2016).

2.2.2 Extraction from solid matrices

Soxhlet, ultrasonic-assisted extraction, and pressurised liquid extraction (PLE) are the most frequently used techniques for the extraction of oxygenated PAH in soils and sediments. Although less common, microwave assisted extraction (MAE) (Cai et al., 2017; McKinney et al., 1999; Sun et al., 2017) and supercritical fluid extraction (SFE) (Han et al., 2015) have also recently been used for analyses of OPAH and NPAC. With the exception of SFE which uses CO_2 as an extraction solvent (Han et al., 2015), typical solvent systems include dicholoromethane (DCM) or hexane mixed with DCM or acetone if PAH or OPAH are of interest, while acetonitrile (ACN), methanol (MeOH), ethyl acetate, acids, or occasionally water may be used to improve the extraction of more polar OHPAH and COOHPAH metabolites (Meyer et al., 1999; Bandowe and Wilcke, 2010; Wang et al., 2012; Blum 1997). For phenolic compounds specifically, citrate buffer may be added and is recommended over ethylenediaminetetracetic acid (EDTA) or water alone (Blum, 1997). Although sodium hydroxide is sometimes used for more exhaustive extraction at elevated pH, humic acids also become soluble and may cause matrix interference in subsequent chromatography (Vinken et al., 2005). Additional materials such as sand, diatomaceous earth, activated copper powder, sodium sulfate, or sorptive materials are sometimes added to the sample or extraction vessel in order to improve extractions, remove interferents, or facilitate fractionation.

In recent years PLE has become a dominant technique for the extraction of PAH, OPAH, and to a lesser extent OHPAH in soils. Many PLE methodologies for OPAH extraction have evolved from the work of Lundstedt et al. (2000) which initially focused on the recovery of PAH and was then extended to extract OPAH (Andersson et al., 2003; Lundstedt et al., 2003, 2006). The original work optimised PAH extraction and found best results were obtained using small sample mass (1 g), two short static extraction cycles and a large rinse volume (11 mL). Temperature was optimised at 150 °C to limit wear and tear on the instrument, while extraction pressures ranging from 6.9-19 MPa had limited influence on PAH recovery. Several solvents provided acceptable results but use of 1:1 hexane:acetone was preferred for minimising the use of chlorinated solvents and for its better suitability for subsequent silica clean-up. In order to capture OPAH in a subsequent study (Lundstedt et al., 2003), a second extraction cycle using 99:1 methanol:acetic acid was introduced as well as a solvent exchange step before silica clean-up, but PLE conditions were otherwise not re-optimised. Other authors have built on this core method with some modifications to improve clean-up and detection of OHPAH (ref. Table 2.2). A further development involved the addition of silica to the cell below the sample to perform extraction and fractionation/clean-up in an automated 2-cycle operation. This method provided comparable results to Soxhlet extraction followed by silica clean-up (Lundstedt et al., 2006a). More recently, PLE has been used as the basis for a laboratory inter-comparison study for the analysis of PAH, OPAH, and NPAC in spiked soils and unspiked reference soils (Lundstedt et al., 2014). During the inter-comparison study, 6 out of 8 laboratories used PLE methodologies without the integrated silica fractionation, though with some variations in instrument and solvent parameters; one other laboratory followed the integrated PLE-fractionation method; and the final laboratory used ultrasonication instead of PLE, with comparable results. Although none of the seven laboratories showed consistently higher or lower results, substantial inter-laboratory RSD was observed (21-97%), suggesting the need for further understanding of PLE parameters which specifically impact OPAH recovery (Lundstedt et al., 2014). Of particular concern for PLE extractions is the possible conversion of some PAH to PAH quinones, for example anthracene to anthraquinone (Lundstedt et al., 2014) and the rearrangement of PAH quinones and some ketones (Walgraeve, 2010). Bandowe and Wilcke (2010) reported low, highly variable, or unrealistic recoveries (e.g. 159+/-44%) for 9,10-phenanthrenequinone, 1,2-acenaphthenequinone, and 1,4-naphthoquinone, respectively which they attributed to

the lower stability of quinones during GC analysis, but also indicated that the elevated temperatures used in the PLE process may also be involved in the observed conversions. Rearrangement has also been observed by workers studying these compounds in aerosols, when PLE or elevated-temperature ultrasonic extraction was used (Kishikawa and Kuroda, 2014; Lintelmann et al., 2005). Although widely reported as a concern, best practices to address this issue have not yet been established.

Attempts to use PLE to investigate OHPAH and COOHPAH have yielded mixed results. Bandowe and Wilcke (2010) used a solvent mixture comprised of ethyl acetate/DCM/trifluoroacetic acid (TFA) (250:125:1 v/v/v), to extend their DCM-based PAH and OPAH extraction to include these compounds. Several OPAH and mono-hydroxylated OHPAH showed enhanced recovery, but the majority of OHPAH and COOHPAH had recoveries that were moderate to poor (0-7% for fourteen compounds). For the most polar compounds, only 1-3% improvement in recoveries were observed with the inclusion of this acidified system compared to DCM extraction alone, suggesting that insufficient acidity of the solvent was not the primary source of loss. The extent to which the subsequent silica fractionation protocol contributed to low recovery was not separately evaluated. Although further studies to extend PAH/OPAH PLE protocols should be undertaken, greater successes have generally been obtained when OHPAH have been investigated as a sole target class (Avagyan et al., 2015; Wang et al., 2012). By using methanol extractions performed at higher temperatures (i.e. 200°C) than usually used for OPAH, workers have obtained OHPAH recoveries of 70-102% for extracts of filters, with recoveries from soil deviating from these values up to 6% after clean-up (Avagyan et al., 2015). In a more unusual approach, Wang et al. (2012) explored a water/acetonitrile system for PLE extraction of 8 OHPAH from wetland sediments followed by dispersive liquid-liquid micro-extraction (DLLME) concentration procedure and obtained recoveries of 57-91% after both procedures.

2.2.3 Extraction from aqueous samples

Table 2.1 summarises the advantages and disadvantages of various techniques for the preparation of aqueous samples and references their use in primarily environmental contexts, but also in urine and culture media where much of the method development for the detection of mono-hydroxylated PAH metabolites in aqueous samples has been undertaken. In some cases, if the matrix is sufficiently free of interferents and metabolite concentrations are sufficiently high, extraction may be forgone entirely, and

the sample applied to an LC-based detection method. More commonly, however, solvent or sorbent based extraction is performed first. Liquid-liquid extraction (LLE), and reverse phase SPE are the most common approaches for the extraction of aqueous samples. Miniaturisation techniques such as DLLME and single drop micro-extraction (SDME), and other sorption techniques, including dispersive solid phase extraction (dSPE), solid phase micro-extraction (SPME), stir bar sorption, and passive sampling devices have also been utilised. These represent a growing field of sample preparation which aims to reduce sample preparation time, solvent use, and costs, and to facilitate the analysis of smaller sample volumes, or to investigate specific characteristics such as environmental partitioning or bioavailability. Although these have not yet widely been adopted for the analysis of oxygenated PAH more general reviews are available for factors affecting these techniques and their use in environmental analysis (Bizkarguenaga et al., 2013; U. Ghosh et al., 2014; Jain and Verma, 2011; Piri-Moghadam et al., 2016; Souza-Silva et al., 2015).

LLE, reverse phase SPE, and other aqueous sorptive extraction techniques take advantage of the relatively elevated K_{OW}s of target analytes and depend on facilitating the efficient fractionation of these compounds into a comparatively nonpolar liquid or solid phase. Ethyl acetate and DCM are most frequently used for the organic phase during LLE, though toluene and trichloroethylene (TCE) are also used, particularly in DLLME and SDME approaches; C18 and polymeric (polystyrene-divinyl benzene SDB) sorbents are most frequently used for solid phase extraction, while polydimethylsiloxane (PDMS) and polyacrylate have been investigated for SPME and stir-bar sorptive techniques respectively. Passive sampling devices, which utilise a longer sample exposure period, have been developed using low density polyethylene (LDPE), polyoxymethylene (POM), and silicone, as well as Tenax and hydrophilic-lipophilic-balanced (HLB) resins. For DLLME, an additional organic modifier which is miscible with water such as acetone, ACN, or ethanol is added as a disperser solvent in order to increase the interaction between the aqueous sample and the extraction solvent (Gupta et al., 2015; Wang et al., 2012). A similar strategy may be used during the conditioning of non-polar sorbents for aqueous samples.

During extractions, aqueous phase modification may also enhance transfer of more polar analytes, particularly those which dissociate in water. Acidic PAH metabolites and small phenols which may be ionised in aqueous solution must be converted into the unionised form in order to fractionate into the organic phase. This is

achieved by reducing sample pH below the pKa of target analytes, typically to pH <2. Basic compounds such as NPAC co-analytes are better transferred at higher pH (Siemers et al., 2015). Salts may also be added to promote aqueous exclusion of organic materials and facilitate analyte transfer to the organic phase (Letzel et al., 2001). Addition of HCl and NaCl to SDME protocols has improved recovery of OHPAH (Wang et al., 2017) with no significant effect for OPAH recovery (Santos et al., 2017).

Although C18 and polymeric sorbents are most often used for SPE work with oxygenated PAH, several studies have reported limitations of these sorbents (Table 2) and have applied mixed-mode or tandem devices. Newer sorbent materials and devices reviewed recently by Płotka-Wasylka et al., (2016) have yet to be applied to oxygenated PAH and may offer an area of continued analytical development. However, standardisation of SPE techniques is generally lacking (Andrade-Eiroa et al., 2016) and consolidation of existing techniques for oxygenated PAH may be more important for regularising the analysis of these compounds. This is particularly important because sub-optimal use of SPE may lead to poor precision, lead to sample loss, or provide incomplete method comparisons. For example, Wang et al., (2012) prepared subcritical water extracts (SWE) of soils with 20% ACN and DLLME and reported three times higher recovery than clean-up by C18 SPE; however the SPE method used had originally been developed for water samples without ACN, and its presence may have adversely impacted chromatography and contributed to premature elution of the target OHPAH. Van de Wiele et al., (2004) also reported loss of both low molecular and high molecular weight OHPAH in their C18 SPE methodology and attributed it to loss during a wash step, possibly enhanced in part by competition of matrix components for nonpolar interaction sites.

2.2.4 Conjugated metabolites

Although conjugated metabolites may be formed in soils and sediments, they are most often studied in aqueous matrices. For their analysis, enzymatic deconjugation is frequently, though not always, performed prior to extraction. Deconjugation improves interaction with the organic LLE solvent (e.g. (Cerniglia et al., 1982) or nonpolar sorbent system (Lankova et al., 2016) and may also be required in order to make use of established GC derivatisation and analysis techniques. As the availability of conjugated reference standards is limited (Ayala et al., 2015) deconjugation also facilitates detection through comparison to more readily available unconjugated standards.

Table 2.1 Methods for the extraction of oxygenated PAH from aqueous samples

Advantages	Disadvantages and Challenges	References and applications			
		Additional factors	Reference	Compounds identified	Matrix
1. Direct Analysis by LC-MS or LC-DAD - ***Best for relatively clean, concentrated samples, and the analysis of conjugated metabolites***					
• Minimal sample preparation • Polar compounds preserved in sample; are not lost during extraction • Matrix may better reflect real world sample matrix • Conjugated metabolites may be analysed directly • Conjugated and deconjugated metabolites can be considered alongside each other • Demonstrated utility in samples with expected higher concentrations (incubation studies)	• Limitations in the detectability of some analytes • Matrix interferences not removed prior to analysis, increased baseline noise or other instrumental problems; • Without fractionation, chromatograms may be overly complex • Pramauro et al. (1998) identified more analytes with LLE • Instrument LOD must be very low or samples must be concentrated prior to injection • Identification of compounds may require more analyst experience • Filtering prior to direct injection can lead to analyte loss • Aqueous samples may have reduced storage stability	*detector/ion source*			
		FLD-ESI-QTOF	Boll et al., 2015	sulfate-conjugated and COOHPAH	fungal mycelial microcosms
		FLD-ESI-QTOF	Schmidt et al., 2010	sulfate-conjugated and COOHPAH	fungal mycelial microcosms
		FLD-ESI-QTOF	Malmquist et al., 2013	conj. and unconj.metabolites	seawater/sediment microcosms
		ESI- MS/MS ESI-Q-TOF	Tang et al., 2016	conj. and unconj.metabolites	urine
		UV	Pramauro et al., 1998	OHPAH, OPAH	irradiated soil wash solutions

cont'd

2. LLE techniques - *Best for simplified method development, in reasonably concentrated samples, less polar compounds more easily extracted, or when a fractionation technique developed for organic extracts is desired*

- Utilises simple laboratory equipment
- As for soil extracts, further clean-up, fractionation, concentration, and derivatization techniques may be applied
- Sample volume range 10mL -1L
- Opportunities for scaling the technique
- May be performed sequentially to target different analytes
- May provide better recovery of large nonpolar compounds compared to direct SPE (Siemers et al., 2015)

- Solvent volumes are frequently larger than for direct SPE
- Extraction of large volumes of aqueous sample is not tenable
- pH adjustment and/or ionic adjustments required for recovering phenolic, acidic, and basic compounds
- Transfer of polar analytes may be incomplete after pH adjustment
- Conjugated metabolites require deconjugation before extraction
- Recovery for nonpolar compounds may be reduced compared to some SPE methods (Siemers et al., 2015)

solvent/disperser solvent pH and salts/subsequent sorbent shaking, temperature, number of extraction cycles

A. LLE + application to analytical instrument with or without derivatization

- Minimises further sample preparation and potential sources of analytes loss
- Reduces method development time
- Does not require chromatographic apparatus or consumables

- Matrix effects of raw extract may be substantial, particularly if extract must be concentrated prior to analysis
- No opportunity for further preparative fractionation and application of different techniques to different compound classes

DCM/pH <2	Pramauro et al., 1998	PAH, OPAH, OHPAH, COOHPAH	irradiated TiO2-treated soil washing solutions
hexane+toluene	Siemers et al., 2015	PAH, HPAC, OHPAH, OPAH	river water, sea water
ethyl acetate/ pH <2	Cajthaml et al., 2001	OPAH, OHPAH, COOHPAH	fungal mycelial microcosms
pentane+toluene	Li et al., 2014	OHPAH	urine (deconjugated)

cont'd

B. LLE + Normal phase SPE

• Allows for sample clean-up and removal of interferents • Allows for fractionation • Provided load solvent and volume is the same, fractionation technique developed for other organic extracts such as soils may be applied to LLE extract • Different fractions may be analysed by varying techniques	• Direct SPE may be preferred to reduce sample processing steps • More time- and materials than direct analysis of LLE extracts • Scaling method may be time consuming. • Some solvents used for LLE must be exchanged prior to load step • Additional steps over direct LLE may result in analyte loss	DCM/silica	Liao et al., 2014	PAH, OPAH	aq. phase soil slurry
		DCM/silica	Lundstedt et al., 2006b	PAH, OPAH	aq. phase soil slurry
		DCM/pH 5-8, 10-11 /KOH-silica	Enell et al., 2016	PAH, OPAH, NPAC	leachate - contaminated soils
		toluene/NaCl/ silica	Letzel et al., 2010	phenols, OPAH OHPAH	lignite pyrolysis wastewater

C. LLE + Dispersive SPE

• Simple purification protocol with simple lab equipment obtained better results than direct SPE (C18) for urine (C18) (Lankova et al., 2016)	• Methods typically intended for purification but not fractionation • Underdeveloped for aqueous environmental samples	ethyl Acetate/Z-Sep; also tested Z-Sep+, C18, PSA and ENVI-Carb	Lankova et al., 2016	OHPAH	deconjugated urine

D. Dispersive Liquid Liquid Microextraction (miniaturisation technique)

• Very low solvent volumes (<500µL); high enrichment factors • Low cost, high speed option • Utilises simple laboratory equipment • Yielded strong recoveries for 4:1 H_2O:ACN • Extremely small solvent volumes; high enrichment factors • Derivatization may be completed during extraction step, with good efficiency models for OHPAH (Wang et al. 2017) • Can be automated	• Although method optimization has been reported for urine, further optimization • Applicability to highly contaminated samples is unknown • Manual extraction may be challenging, specific automation apparatus recommended for some approaches • Matrix effects may be substantial and greatly increase LODs • Applicability to contaminated samples is unknown	toluene/ACN	Wang et al., 2012	OHPAH	sediment extracts H_2O:ACN
		toluene/ACN	Wang et al., 2015	OHPAH	sediment extracts H_2O:ACN
		TCE/ EtOH/ pH6,	Gupta et al., 2015	OHPAH	deconjugated,
		toluene: cyclohexane /HCl+NaCl	Wang et al., 2017	OHPAH	estuarine waters
		toluene; cyclohexane, isooctane/ HCl+NaCl also	Santos et al., 2017	OPAH, PAH , Nitro-PAH	river water, sea water, groundwater

cont'd

• Better recovery and LODs than C18 SPE or SPME have been obtained (Wang et al.; 2017 Santos et al., 2017)	• Analytes recalcitrant to derivatization may not be fully quantifiable if this step completed simultaneously	tested

3. Direct SPE Techniques - *Preferred for removal of matrix interferents and processing larger sample volumes*

• Requires less solvent than many LLE protocols • Larger sample volumes may be processed e.g up to 1L • Concentrates target analytes • Provides clean-up and fractionation opportunities • May increase recovery of small polar analytes over LLE • Storage on column possible for some applications	• May require increased method development time • Few methods cover a broad range of oxygenated PAH • Methods cannot be adapted from methods for organic extracts • Fractionation is largely untested for oxygenated PAH • Typically requires vacuum manifold and pump • Storage of oxygenated PAH on SPE devices not yet investigated	*specific sorbent* *maximum load* *sample pH* *eluent(s)* *compatibility with further processing*

A. C18- based sorbents

• Well known sorbents in environmental literature • Adequately retain OHPAH (Olmos-Espejel et al. 2012; Luan et al., 2007) • Well known in medical literature • Methods available for PAH and EPA phenols • May be preferred for OPAH analysis, as more polar compounds are less well retained (Qiao et al., 2014)	• Premature elution of polar compounds • pH stability lower than polymeric sorbents; may be particular issue with acidified or base-treated samples for analytes • Strongly hydrophobic constituents may be retained on the column • Insufficient selectivity including poor removal of humic acids (Ferrer and Barceló, 1999) and urinary interferents • Cleaner chromatograms obtained using stir bar sorption, LLE+ dispersive SPE for urine (Zhao et al., 2013; Lankova et al., 2016)	Strata-E ;DSC-18 Envi-18 Envi-Chrom P	Pojana and Marcomini, 2007	OHPAH	treated and untreated wastewater
		Sep-Pak	Luan et al., 2007	OHPAH	water
		Chromabond C18-PAH	Olmos-Espejel et al., 2012	PAH, OPAH	aqueous phase algal degradation study
		PrepSep C18	Van de Wiele et al., 2004	OHPAH	simulated human gastrointestinal matrix
		C18	Qiao et al., 2013, 2017	OPAH	river water, wastewater

cont'd

B. Polymeric Sorbents (Polystyrene Divinyl Benzene)					
• Relatively well known sorbents in environmental literature • Higher retention of polar compounds than C18 • May capture a broad range of compounds • Recovery of conjugated metabolites (Malmquist et al., 2013) • Environmental brands demonstrate utility in medical literature • Methods available for PAH and EPA phenols	• Generally non-specific sorbents • May show reduced recovery of PAH compared to some LLE applications (Siemers et al., 2015), or more variable response to OPAH (Qiao et al., 2013) • Recovery of PAH may be improved by the inclusion of C18 column in the protocol (Motorykin et al., 2015)	Lichrolut EN	Siemers et al., 2015	Phenols PAH, few O/OHPAH, NPAC	river water, sea water
		Oasis HLB	Malmquist et al., 2013	conjugated pyrene metabolites	seawater- spiked sea sediment microcosms
		Focus, Isolute 101, Bond Elut Plexa	Motorykin et al., 2015	OHPAH, PAH	deconjugated urine
C. Additional nonpolar sorbents : Cyclohexyl, Phenyl, C8					
• Strong recovery has been reported for naphthols and phenols with cyclohexyl and phenyl phases • Moderate to good recovery of PAH using these phases	• Few follow up studies using cyclohexyl phase • Phenyl phase also led to overestimation of compounds • C8 not recommended as tested	cyclohexyl, phenyl,C8, C18	Rostad et al., 1984	Phenols, Naphthols, PAH, HPACs	groundwater from contaminated site
D. Mixed mode sorbents (Polymeric-Weak Anion Exchange) (P-WAX)					
• May facilitate the retention of more than one class of analyte • P-WAX may facilitate the capture of acidic, and charged, and conjugated oxygenated PAH metabolites (Boll et al., 2015)	• Limited studies for oxygenated PAH in environmental samples	P-WAX	Boll et al., 2015	acidic and sulfate-conjugated metabolites	aqueous fungal microcosms
		Oasis Max	Kakimoto et al., 2008	OHPAH conjugates	urine
E. Tandem SPE					
• Found to be advantageous for some medical applications • Facilitates removal of specific interferents or target recovery of more than one class of compounds • Improved recovery of larger PAH compared to polymeric column alone (Motorykin et al., 2015)	• More materials intensive than single-phase SPE • Insufficiently tested for environmental matrices	Bond Elut Plexa/ C18	Motorykin et al,. 2015	OHPAH, PAH	deconjugated urine
		C18/silica; aminop-propyl silica, cyano- and diol	Chetiyanikornkul et al., 2006	OHPAH	deconjugated urine
		Immuno-sol gel / C18	Letzel et al., 2001	OPAH	deconjugated urine

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F. Solid Phase Disk Extraction

• Larger surface area for sample and solvent interaction • Longer dwell times for ample transfer on to stationary phase. • May be used in passive sampling applications • Vacuum manifold/other device not required	• Limited studies for oxygenated PAH in environmental samples • Directional fractionation not possible	Empore C18 and Empore SDB-XC	Kurihara et al., 2005	OPAH and OHPAH	Seawater Tokyo Bay
		Envi C-18 Disk	Lundstedt et al., 2003	OPAH	aqueous phase of Fenton agent slurry

4. Other sorptive extractions - *Key advantages are application specific - may be preferred for low solvent use, high concentration factors, and field sampling options*

• Minimal or no solvents used (0-5mL) • Reusable materials- reduces waste and cost • More selective sorbents available –reduces interfering compounds, baseline noise • Potential for developing field-extraction protocol • Options for thermal or solvent desorption • Automated or in-situ derivatization protocols may be applied • On-site applications may be feasible	• Fractionation not used • Only individual classes of oxygenated PAH have been studied • More selective membranes may also exclude compounds of interest • On-site methods are underdeveloped, • Calibration may be a challenge • Storage stability of analytes on devices not known	*sorbent material and format, thickness, sampling time, pH, ionic strength, matrix effects mixing desorption conditions*			

A. Solid Phase Micro Extraction (SPME)

• Minimal or no solvents; significant concentration factors • Sample preparation time can be reduced • Aqueous sampling and headspace applications possible • OHPAH have been successfully analysed • Substantially lower LODs achieved compared to C18 SPE (Luan et al., 2007) • Established methods for PAH, phenolics in aqueous media.	• Underdeveloped for enviro samples • Small sorptive surface area; better for cleaner samples • Competition for binding sites may be issue for dirty samples • Extraction efficiency matrix dependent • Calibration can be challenging • Lower upper calibration limits and poorer precision than traditional C18 SPE (Luan et al., 2007)	*Material*			
		polyacrylate	Luan et al., 2007	OHPAH	water, culture media, algal degradation experiments
		polyacrylate	Luan et al., 2006	OHPAH	aqueous phase of PAH degradation experiments
		polyacrylate	Smith et al., 2002	OHPAH	deconjugated urine

cont'd

• Adaptation for GC-MS or HPLC possible, though latter not yet studied for oxygenated PAH • Range of sorbents available • Automation is possible and recommended	• Manual injection finicky, automation recommended • OHPAH and COOHPAH require on-fibre derivatization • Investment in devices can be expensive; fibres can easily break	polyacrylate	Gmeiner et al., 2002	OHPAH	deconjugated urine
B. Stir Bar Sorptive Extraction					
• Larger surface area and more robust apparatus than SPME • More easily applied to large volumes than SPME or SPE • Minimal solvent use compared to LLE or SPE (200µL-5mL) • Improved clean-up over C18 SPE for OHPAH in urine, with strong recoveries (Zhao et al., 2013). • Can apply traditional solvent-based derivatization	• Recovery of OHPAH was somewhat low when acetylation- derivatization applied prior to sample extraction; • Recovery of higher molecular weight aromatics was reduced when compared to a direct-SPE method using Oasis HLB sorbent; (Poulain et al , 2016) • Automation is not possible	PDMS	Itoh et al., 2005	OHPAH, naphthoquinone	seawater, puddlie water
		PDMS	Zhao et al., 2013	OHPAH	deconjugated urine
<u>5. Passive sampling devices</u> - *Special applications, field deployment, estimation of mobile or bioavailable fractions*					
• Can be used to estimate mobile or bioavailable fraction in combined water/sediment systems • May be deployed in field	• Current techniques have not addressed OHPAH or COOHPAH • Applicability and QC required for field applications not established for oxygenated PAH • May require long periods to equilibrate - e.g. 7-28 days	*material and device format, conditioning, sampling duration, extraction, calibration*			

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A. Tenax and HLB in dialysis tubing					
• Used to investigate distribution of genotoxic elements • Use of dialysis tubing to suspend sorbent simplifies sample preparation compared to direct application of Tenax beads without adverse impacts on recovery	• Equilibration period of one week • Though it reflects the most mobile constituents, the bioavailable fraction of genotoxic elements in the whole slurry was not fully represented through this technique, • Larger solvent volumes, and longer extraction period (overnight) than other sorptive techniques	Tenax/HLB	Hu et al., 2014	PAH, OPAH	phosphate-buffered suspension of contaminated soil bioreactor slurry
B. Polyoxymethylene strips (POM)					
• OPAH, PAH, and NPAC can be analyzed at the same time • Extracts can be further processed as other organic extracts • Used to estimate porewater/KTOC parameters for OPAH	• Considered complimentary but not replacement technique for leachate sampling (Enell et al., 2016) • Long equilibration period (28 days for studies referenced here) • Larger solvent volumes used than for other sorptive techniques	POM	Enell et al., 2016	PAH, OPAH, NPAC	pore water/leachate from contaminated soils
		POM	Arp et al., 2014	PAH, OPAH, NPAC	simulated pore water from contaminated soils
		POM	Josefsson et al., 2015	PAH, OPAH	water
C. Silicone and Low Density Polyethylene (LDPE) passive sampling devices					
• Have been used to quantify OPAH in river water • Silicone provides greater sensitivity for individual OPAH than LDPE passive sampler, which may be preferred for PAH analysis	• Notable differences were observed for some OPAH between using standard addition and internal standard quantitation methods • Silicone sampling device contributed to matrix effects, extensive pre-cleaning required; selection of sorbent format important	Silicone, LDPE	O'Connell et al., 2013, 2014	OPAH	river water
		LDPE	Tidwell et al., 2017	OPAH	river water

Additional abbreviations used : QTOF - quadrupole time of flight mass spectrometry; HPLC - high performance liquid chromatography; PSA Primary-secondary amine; EtOH ethanol

The deconjugation process involves the application of phosphate or acetic acid buffer as well as hydrolysing enzymes arylsulfatase and beta-glucoronidase followed by incubation at 37 °C, typically overnight. Jacob et al., (2007) found that glucuronidase and arylsulfatase isolated from *E. coli* and *Aerobacter aerogenes* respectively gave cleaner extracts and better recovery than commonly-utilised enzyme mixtures obtained from *Helix pomatia*. The sample is then extracted and cleaned, although hydrolysed samples may have altered consistency causing clogging of SPE tubes (Lankova et al., 2016).

Since deconjugation inherently renders conjugated and unconjugated metabolites indistinguishable, sample preparation must be planned to differentiate the components when separate consideration of the two groups is important. In one approach, analysis can be completed with and without the deconjugation step (Jacob et al., 2007). Alternatively, stepwise extraction may be used to fractionate these metabolite groups: for example, fungal naphthalene degradation microcosms were first extracted with ethyl acetate to obtain unconjugated metabolites, then deconjugation conducted and a second extraction with ethyl acetate were performed to obtain the newly deconjugated products (Cerniglia et al., 1982). More recently, LC-MS (and LC-Fluorescence detection (FLD)-MS) methods have been used to identify both conjugated and unconjugated metabolites directly in a variety of aqueous matrices and extracts (Ayala et al., 2015; Boll et al., 2015; Malmquist et al., 2013; Schmidt et al., 2010a; Tang et al., 2016). Because of the consistent structure of the conjugate unit, its presence may also help with MS identification of metabolites.

2.2.5 Clean-up of organic extracts

Due to the wide range of compounds present in environmental matrices, the use of broad specificity solvent systems, and the concentration steps required to improve detection of trace metabolites, crude extracts tend to require additional clean-up or fractionation prior to analysis. The majority of these methods have been developed in the context of extracts from solid matrices, but some have also been applied to LLE extracts of aqueous samples. Most often, this involves preparative chromatography using open column (larger sorbent volumes), SPE (smaller sorbent volumes), or PLE-based methods (sorbent packed extraction cells) which allow for simultaneous extraction and fractionation (Lundstedt et al., 2006a). Table 2.2 presents a summary of extraction, clean-up, and fractionation methods that make use of preparative column

chromatographic techniques. In some cases, gel permeation chromatography or size exclusion chromatography (SEC) has been recommended to be used prior to SPE, and the latter has been reported to substantially improve baseline noise, although total recovery may be reduced with the inclusion of this step (Bandowe and Wilcke, 2010; Layshock et al., 2010). Activated copper powder and sodium sulfate are sometimes added to SPE protocols in order to remove sulphur and residual water respectively. As recovery values presented in Table 2.2 include different stages of the methodology, these values are not directly comparable in all cases, but give an indication of method performance

Similar issues regarding standardisation and optimal use of SPE protocols described above for aqueous applications are also of concern for soil extracts. Selecting appropriate sorbent and eluents is a key step, but determining appropriate conditioning steps, sample analyte load, load solvent type and volume, and flow rates is also essential. Insufficient separation may lead to direct analyte loss (e.g. nonpolar compounds lost during wash step), highly complex chromatograms (Letzel et al., 2001), the necessity of recombining separated fractions (Chibwe et al., 2015; Layshock et al., 2010), or non-ideal further processing (e.g. OHPAH being missed in an underivatised OPAH fraction). In general, fractionation need only be sufficient to the question at hand, and while some workers (Chibwe et al., 2015) have been able to demonstrate interesting trends in toxicity associated with smaller fractionation bands, the inclusion of 14 elution cycles recombined into 6 composite fractions, is overly complex and undesired for most investigations. Pre- or post- SPE concentration, dilution, or derivatisation, must also be carefully considered, as improvements here could help reduce analyte loss observed in some protocols.

A variety of sorbents have been used for the clean-up and separation of PAH and OPAH in soil organic extracts. Silica is the most widely used, with partial deactivation through addition of 2-10% water to the silica often recommended. Since deactivation reduces analyte sorption, it can reduce the solvent intensity required for subsequent elution steps and limit the release of unwanted compounds (Lundstedt et al., 2006a). Considering additional common sorbents for a PLE-based methodology, Lundstedt et al., (2006) included Florisil and alumina (each deactivated 1.2 %) as a potential contenders for PAH/OPAH separation and clean-up. They found somewhat similar results between Florisil and silica during screening tests (see also Witter and Nguyen, 2016), but preferred the silica due to its reduced retention of PAH which led to

Table 2.2 Preparative chromatographic methods for the clean-up and fractionation of soil extracts for the analysis of oxygenated PAH

Reference and sample type	Sample Preparation and Extraction[1]	Column[2]	Procedure[3]	Analysis	Analytes Detected, Recovery %[4]	• *Comments* further applications and references
Lundstedt et al., 2003 solid phase of bioslurry treatment of former gasworks soil	**PLE** 1g soil sample mixed with 5g Na_2SO_4 Extract 1: HEX:ACE (1:1) Extract 2: MeOH:acetic acid (99:1) Combine extracts Evap. aliquot of 10% Reconstitute in 1mL HEX	**Open Column** 15mm 1g anhydrous Na_2SO_4 5g silica gel deact. 10%	Condition: HEX 20mL Load: Sample Wash: HEX 5mL F1: PAH, OPAC, SPAC: HEX:DCM (3:1) 15mL F2: OPAH, NPAC: DCM 30mL Evap. to dryness Reconstitute in toluene	GC-MS	69 PAH 64-102%*fa* for 9 tested 23 OPAHs + isomers 26 NPAC + isomers	• *PLE conditions optimised for PAH analysis* Andersson et al., 2003- Soil compost mixtures, sample pretreatment included acidification step, and only first extraction cycle was used. OHPAH also identified after BSTFA derivatisation. Lundstedt et al., 2006b and Liao et al., 2014 - Fractionation applied to LLE extracts (DCM) of slurry liquid phase Lundstedt et al., 2006a - Fractionation method applied to soxhlet extracts and compared to PLE-fractionation Lundstedt et al., 2014
Lundstedt et al., 2006a spiked uncontaminated and contaminated industrial soils	**PLE/fractionation** 1-5g soil homogenised with 20 g Na_2SO_4	**PLE Cell Column** 11mL Isolute sorbent cellulose filter soil homogenate (0.5-1g) cellulose filter 4g silica gel deact. 2%	F1: Extract/elute PAH : CycloHEX/DCM (9:1) F2: Extract/elute OPAH: CycloHEX/DCM (1:3)	GC-MS	33 PAH 55-91%*ma* for 33 tested; 13 OPAH 47-129%*ma* for 8 tested	• *Additional sorbent tested: florisil 1.2% deact., and alumina 1.2% deact., silica 5% deact. and activated* • *Solvent selection and extraction temperature discussed* • *Comparison to soxhlet and open column chromatography technique of Lundstedt et al 2003 with comparable results* Lundstedt et al., 2006b - slurry solids Lundstedt et al., 2014.. Liao et al., 2014 - slurry solids, extraction solvent modified

cont'd

Avagyan et al., 2015 soil samples from industrial area wood smoke particulates	**PLE** 1.5g soil or 40mg wood smoke particulates Extract: MeOH Evap to 0.5mL	**SPE** 100mg silica Biotage Isolute	Condition: 3mL HEX Load: 0.5mL prepared sample Wash: 0.5mL HEX Elute OHPAH: 3mL MeOH Concentrate to 0.5mL Filter 0.2µm nylon	HPLC/ APPI-MS	12 OHPAH and OPAH standard solutions: 70-102%*ea* 95-100%*fa* spiked soil extracts: 92-104%*fa*	• *PLE optimization explored, temperature and pressure positively related to extraction recovery; static time and number of cycles less important f* • *losses of 5-10% reported for evaporation step* • *Matrix effects ranged -15% to +20% without clean-up but were substantially reduced by SPE method*
Obrist et al. 2015 forest soils	**PLE** 1-5g soil Extract 1: DCM Extract 2: ACE Extracts dried by Na_2SO_4	**SPE-** silica Supelclean LC-Si	Elute PAH and OPAH: HEX/benzene (1:1)	Ion trap GC-MS	26 PAH and alkyl PAH 9 OPAH	
Wischman et al., 1996 soil/compost obtained from PAH-spiked microcosms	**Ultrasonication** 20g soil mixed with 1mL 4N HCl dried with 20g Na_2SO_4 Extract: 2x DCM (40mL) Evap. each extract to 5mL Combine extacts Evap. to 5mL	**Open Column** 8mL borosilicate glass PTFE frit 2g silica gel deact. 6% PTFE frit	Condition: 8mL HEX F1: PAH: 12mL HEX:DCM(9:1) F2: OPAH: 6mL DCM F3: OHPAH and COOHPAH:5mL 1% acetic acid in MeOH Evap. polar fractions Solvent exchange to 1mL MeOH 100µL aliquot dried Derivatize (BSTFA/TMCS)	GC-MS	6 OPAH 40-88%*ma* 3 OHPAH+ COOHPAH 49-89%*ma*	• *Higher recoveries obtained for most compounds when acidification was not used, but substantially lower recovery for COOHPAH and naphthalic anhydride*

cont'd

Bandowe and Wilcke, 2010 soils OPAH spiked and unspiked	**PLE** 20g soil mixed with diatomaceous earth Extract 1: DCM Extract 2: ACE/DCM /TFA (250:125:1 v/v/v). Combine extracts Dry with Na_2SO_4 Evap. and solvent exchange to 1 mL HEX	**Open Column** 8mL borosilicate glass glass wool 5g silica gel deact. 10% glass wool	Condition: 10mL HEX Load: 1mL sample preparation F1: PAH and alkylPAH: 9mL HEX/DCM (5:1) Add toluene, evap. to 0.5mL F2:OPAH, OHPAH, and COOHPAH: 5mL ACE Evap. to 1mL, add 10mL ACE, evap. to 0.5mL Derivatize F2 (BSTFA/TMCS)	GC-MS	7 PAH_d 36-84%*ma* 8 OPAHs 34-96%*mc*, most 78-96%*ma* 15 OHPAH+ COOHPAH 2 -70%*ma* for 7 targets; 8 others not recovered	• *Further elution with MeOH and acidified MeOH did not improve recovery of OHPAH and COOHPAH* Bandowe et al., 2010 Bandowe et al., 2011 Bandowe et al., 2014 (OPAH and azarenes) Wilke et al., 2014a (OPAH and azarenes) Wilke et al., 2014b Bandowe et al., 2018 (OPAH and azarenes)
Qiao et al., 2013river sediments river water suspended particulates	**PLE** Extract: 2x ACE: DCM(1:1) Solvent exchange Concentrate	**Open Column** 10mm alumina (6 cm) deact. 3% silica (12cm) deact. 3% Na_2SO_4 (1cm)	Condition: sorbents stored in HEX prior to use Elute: F1: 15mL HEX), F2: 75mL DCM:HEX (3:7), F3: 60mL DCM:HEX (1:1), F4: 60mL DCM:HEX (7:3) F5: 60mL DCM Evap. to 0.5mL	GC-MS	23 PAH 4 OPAH 101-148%*ma* 4 nitro-PAH	• *Target compounds recovered in F2, F3, and F4* Qiao et al., 2014 Qiao et al., 2017 wastewater particulates
Arp et al., 2014 contaminated and uncontaminated soils	**PLE** 1g soil mixed with solvent-washed sand to fill cell Extract: HEX/ACE(1:1) Evap half of extract	**Open Column** 16mm ID glass column 5g KOH-impregnated silica gel	Elute PAH, OPAH,NPAC: 30mL DCM Evap and solvent exchange to 1mL toluene for soils and 0.5mL toluene for worm tissue	GC-MS	16 PAH, 11 OPAH, 4 NPACs	• *Soil/water partitioning and bioaccumulation considered* • *Worm tissue also analyzed* Enell et al., 2016 - applied fractionation method to LLE extracts (DCM, KOH) of contaminated soil leachate

cont'd

Witter et al., 2016 urban stream sediments	**PLE fractionation** 10g lyophilised sediment	**PLE Cell Column + SPE** 1. PLE cell column 34mL diatomaceous earth florisil copper granules cellulose filter 2. SPE Silica Biotage	1. Extract sample with DCM. Evap. to 1mL 2. SPE Condition: 20mL DCM:HEX (1:4) Load: 1mL prepared sample Elute PAH, OPAH, NPAC: 2x20mL DCM:HEX (1:4)	GC-MS	10 OPAH 85-110%*ma* 4 HPAC 81-111%*ma*	
Meyer et al., 1999 spiked soil/compost mixtures and creosote and wood-impregnation site contaminated soils	**Soxhlet** 20g soil mixed and ground with 1mL 1M HCl and 20g Na_2SO_4 Soxhlet extraction: DCM (210 mL) + HEP (10mL) Rotary evap. to 5mL	**Open Column + SPE** 1: Open Column PTFE frit Chromabond SB 0.7g PTFE frit silica gel 2.0g (10% deact.) PTFE frit 2: SPE Chromabond SA cartridge	1: Open Column Condition: 12mL HEX Load: 5mL extract F1: PAH, SPAC, OPAC: 3mL HEX, 12mL HEX/ DCM (85:15; v/v), 2mL DCM F2: OPAH, OHPAH, NPAC: 1mL DCM, 6mL MeOH, 3mL 0.05N HCl in MeOH F3: COOHPAH : 6mL 0.05N HCl in MeOH 2. SPE (F2 only) Condition: 5mL MeOH F2a: Load and elute neutrals: F2 eluate + 5mL MeOH F2b::Elute Basics: 5mL 1N ammonia in MeOH Dilute fractions	F1,F2a GC-MS F2a, F2b, F3 HPLC-DAD	14 PAH 31-98%*ma* 6 OPAH 32-96%*ma* 2 OHPAH 87-94%*ma* 6 COOHPAH 93-100%*ma* 18 NPAC 60-101%*ma* 7 OPAC + SPAC 29-102%*ma*	• *Abbreviated method can be used without further separation of F2* Meyer and Steinhart, 2000 (PAH, OPAC, SPAC, NPAC) Meyer and Steinhart, 2001 (30 PAH metabolites identified. F2 used without further fractionation)

cont'd

Layshock et al., 2010 reference materials: sediment mussel tissue urban dust diesel particulate matter	**PLE** Solid samples mixed with approximately 30x Na_2SO_4 PLE extraction: DCM Some treated with SEC all samples: evap and solvent exchange to 1mL HEX prior to SPE	**SPE** Discovery 1000mg aminopropyl-silica and/or Discovery 1000mg bonded silica (used separately or in tandem)	F1: Elute nonpolar compounds: 3x2mL HEX:DCM 9:1 F2: Elute polar compounds: 3x2mL 4:1 HEX:DCM Evap. Recombine fractions	GC-MS	9 OPAH 34-97%*ma* for standards	• *Diesel extract also tested, applied directly to SPE.* • *Fractionation during preparative chromatography may not have been adequate since eluates were recombined prior to analysis* • *Losses of OPAH during fractionation were reported to be not attributable to the use of silica or aminopropyl silica* • *Authors report SEC improved chromatographic resolution of OPAH from sediment extracts compared their tandem SPE method, but also led to lower recovery for some OPAH compounds*

[1]HEX, ACE, MeOH, DCM, and HEP - hexane, acetone, methanol, dichloromethane, and n-heptane respectively;

[2] measurements in mm refer to column internal diameter; column materials are listed top to bottom, following the vertical setup; deact. denotes deactivation with water

[3]F# denotes a fraction number;

[4]where determined, reported recoveries reflect different stages of the process and are indicated in the brackets in italics: *m*=full method, spiked compounds added before extraction and all subsequent clean-up and evaporation steps included; *e*=extraction only- recovery evaluated before cleanup/fractionation *f*= fractionation, spiked compounds added after extraction and before cleanup/fractionation; *a*=absolute recoveries – i.e. not corrected for surrogate spike recovery; *c*= corrected for surrogate spike recovery.

greater PAH/OPAH separation efficiency. Alumina is not preferred for the fractionation of PAH and OPAH (Lundstedt et al., 2014) but has been used separately or in tandem alumina/silica sorbent systems for the removal of humic substances, macromolecules, and polar interferences in soil, water, and especially aerosol extracts. In these applications silica may also be used to separate aliphatic and aromatic compounds (Albinet et al., 2014, 2006; Cai et al., 2017; Qiao et al., 2013; Sun et al., 2017; Van Gestel et al., 2003; Walgraeve et al., 2010). The specific advantages of base-modified silica sorbents (KOH-impregnated silica gel, and aminopropyl silica) for PAH/OPAH analysis in soil has not been discussed in detail in the literature, though these likely improve the removal of acidic interferents, and may reduce RSDs for OPAH quantitation compared to deactivated silica (Lundstedt et al., 2014).

Less commonly, silica has been used to determine OHPAH and COOHPAH, with mixed results. Strong recoveries have been obtained for OHPAH when they were the sole class investigated and methanol was used as both extraction and elution solvent. Using these conditions, workers attributed only 5-10% losses in recovery of standards to the SPE methodology itself (Avagyan et al., 2015). However, when other workers have extended exisiting PAH/OPAH fractionation techniques for acidified DCM/acetone extracts to include OHPAH and COOHPAH, recovery of these compounds was either not achieved, or was low to moderate (Bandowe and Wilcke, 2010). In this case, the use of methanol and acidified methanol did not improve recovery in part because eluates exhibited turbidity, and were unworkable for subsequent derivatisation (Bandowe and Wilcke, 2010). It is possible that the use of acidified methanol could also lead to unintended methylation of carboxyl groups of COOHPAH (Antolovich et al., 2004).

The challenge of obtaining OH- and COOHPAH has been best addressed through the use of base-modified sorbents using the methodology of Meyer et al. (1999). This work used successive extraction on silica/strong base and strong acid sorbents to specifically isolate four classes of PAH-related compounds: (1) PAH and S- and O-PACs; (2) neutral metabolites and neutral NPACs; (3) acidic metabolites and (4) basic NPACs. An abbreviated version of their method allows for the separation of PAH, neutral metabolites, and acidic metabolites, without consideration of HPACs. This technique has been further validated in composting microcosm studies of spiked soils (Meyer and Steinhart, 2001), where 30 metabolites of 2-4 ring PAH and 4 additional metabolites of NPACs were identified, including metabolites with carbonyl, hydroxyl, diol, carboxyl, dicarboxyl anhydride, and mixed functionalities. Although this method

provides more extensive separation capacity for a wide variety of target analytes, it involves large solvent volumes and a lengthy Soxhlet extraction, and with the second column may be more involved than required for the analysis of PAH metabolites alone. Nevertheless, it is one of the few methods that has been evaluated for the range of PAH, OPAH, OHPAH, and COOHPAH which should be of interest in metabolite detection studies.

2.2.6 Undesired volatilisation, sorption, and leaching

Many workers report low recoveries and high variability for volatile and semi-volatile target analytes including naphthalene, indanone, and phenolic compounds, attributed to evaporative losses during sample preparation. Volatilisation may be minimised by avoiding harsh drying conditions including lyophilisation, excessively low vacuum, heating, or high drying gas flow rates and complete dry down of samples and extracts. Where possible, solvents should be selected to minimise the need for subsequent solvent exchange through dry down and reconstitution, a step sometimes implemented e.g. to allow for effective derivatisation or appropriate HPLC chromatography. The addition of keeper solvents, which are less volatile than the primary sample solvent, such as heptane (Meyer et al. 1999), dodecane (Fan et al., 2012; Li et al., 2014; Woudneh et al., 2016), and toluene (Bandowe and Wilcke, 2010; Siemers et al., 2015) may be helpful in preventing losses due to excessive drying, but these have not been specifically evaluated for oxygenated PAH and could lead to reduced solubilisation of more polar analytes. In many cases, keeper solvent use during PAH analysis has not substantially improved recovery of the smallest targets (Dabrowski, 2016). Non-extractive LC-MS or headspace techniques may be preferred for the recovery of the most volatile constituents.

Losses due to sorption on extraction equipment have also been reported and may impact recovery of both LMW and HMW components. Substantial losses of alklylated phenols have been attributed to free silanols in glassware, and recoveries of these compounds have been improved by >100% when rotary evaporation components were silanised prior to sample concentration (Berkner et al., 2004), a step that is also recommended for OHPAH analysis in atmospheric samples (Woudneh et al., 2016) and PAH in soil pore water (8272 - USEPA, 2007). Greater losses observed when PLE was used rather than ultrasonication have been attributed to losses in the PLE tubing (Berkner et al., 2004), and other workers have implemented a flushback mechanism

during PLE extraction (Walgraeve et al., 2010). Discussed above (Section 2.2.1), use of any filters should be carefully considered. PTFE vial caps and other implements are recommended. The use of plastics is not recommended, especially as phthalate contamination is common and can lead to interferences not only for the analysis of phthalate-related metabolites, but other analytes as well (3630C USEPA, 1996). Since not all losses are possible to control, depletion tests can be conducted to estimate losses due to sorption (Boll et al., 2015). Method calibration and the use of surrogate standards is also recommended though the latter approach may not be feasible (see Section 2.4.1).

2.2.7 Storage and stability

PAH and metabolites may be structurally altered or otherwise lost during extraction and storage. As discussed, quinones have been shown to be susceptible to rearrangement when heat is applied during extraction methods such as PLE or sonication and during GC-analysis (Bandowe et al., 2010; Lundstedt et al., 2014; O'Connell et al., 2013). The impact of elevated heat on other metabolites is less documented but studies suggest that stability under common storage conditions is less dependent on temperature and more dependent on solvent type and sample preparation. Loss of OHPAH occurred in water samples stored at temperatures from -80 ºC to +20ºC for 14 days (recovery ranging between 27 and 64%), and temperature itself did not substantially impact degradation of standards; however samples stored in toluene were well preserved for the 14 days within the same tested temperature range (Woudneh et al., 2016). OPAH stored in ethyl acetate at 4ºC were stable for 111 days (O'Connell et al., 2013).

Deconjugation tends to reduce while derivatisation tends to increase storage stability. When deconjugation was applied to urine samples prior to 14 days sample storage in clear vials at ambient temperatures, analyte loss was dramatic with less than 20% recovery of PAH metabolites; without deconjugation, recovery was 55-75% under the same conditions (Woudneh et al., 2016). Similar losses from deconjugated urine were observed by Motorykin et al. (2015), while undeconjugated urine samples containing OHPAH have also shown mean variation of only 15% of the original analytical results after 1 year of storage at -20ºC (Jacob et al., 2007). Derivatisation has been shown to improve subsequent recovery of several OHPAH compared to underivatised aqueous preparations. OHPAH derivatised with N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) to form trimethylsilyl (TMS) derivatives,

and prepared in a solution of 2-mercaptoethanol (2ME), acetonitrile, and toluene were stable for 55 days at -80ºC when stored in glass inserts (Woudneh et al., 2016). TMS derivatives in N,O-bis(trimethylsilyl)acetamide (BSA)-derivatised extracts prepared in hexane showed good stability for 24 hours at room temperature and -20ºC, but lost over 10% for some analytes after 1 week storage at room temperature (Toriba et al., 2016). Formation of TMS derivatives using N, O-Bistrifluoroacetamide (BSTFA) may also improve OHPAH stability (Schummer et al., 2009). Tert-butyldimethylsilyl (TBDMS) derivatives prepared using N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) in acetonitrile showed less than 10% loss when stored up to one week at -20ºC with the exception of 1,3 dihydroxynaphthalene and 9-hydroxyfluorene, and with some smaller quinone species also being lost by over 10% after two weeks (Motorykin et al., 2015). Pentafluorobenzyl ether derivatives showed good stability at room temperature for at least 24 hours, and at -20ºC for at least 5 days. Dansylated derivatives were also stable within 10% over 4-weeks storage at -20ºC.

In general, conditions that facilitate oxidation should be avoided during all sample handling and storage. Photodegradation is a well-documented issue for aromatic compounds and samples should be kept away from light by using amber or foil-wrapped extraction and storage vessels and darkened work conditions (Woudneh et al., 2016), with some workers recommending using ultraviolet (UV) light filters (Ahmed et al., 2015), or only red light (Lin et al., 2015; Ma et al., 2016). In addition, storage in toluene can minimise photodegradation compared to water (Woudneh et al., 2016). Processing samples under N_2 and with the addition of antioxidants has improved recovery of OHPAH in water and urine samples. In one study, 2-ME was preferred over butylated hydroxytoluene (BHT) for its solubility in both organic and aqueous mixtures and its improved characteristics for the subsequent HPLC methodology employed (Woudneh et al., 2016). However, the impact of antioxidants was of short duration and their addition did not improve analyte recovery over 14 days of storage (Woudneh et al., 2016). Antioxidants R-tocopherol, BHA, quercetin, gallic acid have also improved OHPAH recoveries during urine sample preparation (Jacob et al., 2007). For studies of anaerobic samples, even further care is required, as hydroxylation of the aromatic ring may also occur during sampling when reduced acceptors are present (Meckenstock et al., 2016). Microbial oxidation may be a concern for some samples. Filter-sterilised solutions containing phenolic acids and citrate showed little degradation of the acids at 9ºC over a 6 day period (Blum, 1997), but filter sterilisation should be avoided if the filtration step

has not been validated. Methanol and acetonitrile (5-20%) are sometimes added to aqueous solutions to inhibit microbes, and may have the benefit of maintaining target analytes in solution when the sample is stored at reduced temperatures (Boll et al., 2015; Jaitz et al., 2011; Ohlenbusch et al., 2002).

2.3 Hyphenated instrumental techniques

Both GC and ultra/ high performance liquid chromatography (UPLC/HPLC, inclusively, LC) have been used for the analysis of PAH, OPAH, OHPAH, COOHPAH, and conjugated PAH metabolites in environmental samples. MS analysis is preferred for metabolite work as it can offer full or partial identification of unknown compounds and help improve detection or quantitation of co-eluting compounds often present in complex environmental samples. Recent work has focused on developing LC-MS protocols and simplifying existing GC-MS techniques for the analysis of these compounds. Detection is ultimately performed using ion trap or quadrupole MS, MS/MS, or high resolution MS (HRMS) including or time of flight (TOF) or quadrupole-time of flight (QTOF) detectors, operating in scan, single ion monitoring (SIM), and, increasingly, multiple reaction monitoring (MRM) modes. Scan mode provides more information for compound identification and is best suited for untargeted analysis.

For the investigation of unknown compounds, Chibwe et al. (2017) suggested a hierarchical approach to compound identification of oxygenated PAH using LC-MS and GC-MS spectra, as follows: "(1) authentic standard (experimental mass spectral match and retention time match with an authentic standard), (2) isomer (experimental mass spectral match 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)" (Chibwe et al 2017). Using this approach, they found that most of the compounds which increased by at least 1.5x post bioremediation, and which therefore were of interest for potentially contributing to the increased toxicity observed after this treatment, were only able to be classified as level 5 compounds, though 11 compounds met criteria for levels 2-4, with most containing at least two phenyl rings and oxygen. At the same time, they

observed that 40 compounds identified by LC-MS, and 48 identified by GC/GC-MS showed little overlap based on mass spectral evidence. This highlights the importance of viewing LC-MS and GC-MS as complimentary approaches and also indicates the need for further development of compound libraries and increased diversity of authentic standards. SIM and MRM are preferred for targeted analysis as they offer reduced matrix interferences, lower detection limits, and especially in the case of MRM, greater confidence in compound identification. In order to take advantage of these different attributes, newer instruments which support the fast cycling of multiple modalities have also been used, though tradeoffs in sensitivity should be expected (Cochran et al., 2012)

Other hyphenated techniques, especially LC-FLD detection and LC-diode array detection (DAD) offer specific advantages for some analyses and are used separately or in tandem with either LC-MS or GC-MS. LC-GC-MS has also recently been used to analyse OPAH in aerosols in order to take advantage of the different separation capacities of the two chromatographic systems (Ahmed et al., 2015). Atmospheric-pressure solid-analysis probe mass spectrometry (ASAP-MS), which involves minimal sample preparation and does not use chromatographic separation, has also recently been investigated as a semi-quantitative screening tool for OPAH (Carrizo et al., 2015).

Despite their growing use, information on instrumental method development for oxygenated PAH for environmental soil and water analysis is still scarce. Instead, techniques have often been adopted from studies of aerosols, urine, or pure substances, or adapted from PAH methodologies (Hayakawa et al., 2017; Lundstedt et al., 2014; Walgraeve et al., 2010). These studies have provided useful insights into the factors impacting instrumental analysis, but greater attention within the community may be needed to understand particular matrix effects associated with soil and environmental aqueous samples, or to address particular research questions: e.g. the detection of bacterial or fungal metabolites in heavily contaminated samples.

2.3.1 Liquid chromatography and optical detection techniques

Liquid chromatography techniques take advantage of the same differences in size, polarity, and acidity that can make addressing the wide array of PAH transformation products difficult. Typically, samples are separated by reverse phase chromatography with C18 used most often as the stationary phase. Although C18 may provide inadequate resolution of the more polar PAH metabolites in complex samples, it has

been successfully applied for the separation of diverse oxygenated PAH with different functionalities during the same run, for example, both conjugated and unconjugated metabolites (Malmquist et al., 2013; Tang et al., 2016). Phenyl-modified silica has been preferred by some workers since the enhanced polarisability of the stationary phase can facilitate interaction with aromatic nuclei and improve separation of closely-eluting compounds; methods are available which address up to 81 PAH and oxygenated PAH of varying and mixed functionalities including low molecular weight phenolic acids in the same run (Letzel et al., 2001). Nevertheless, sample complexity and matrix interferences may present significant challenges to achieving adequate separation or identification of analytes, and most workers focus only on a subclass of these compounds after prior fractionation utilising SPE, online SPE (Olmos-Espejel et al., 2012) or a more selective extraction procedure (Wang et al., 2012).

UV absorbance detection, DAD, and FLD take advantage of highly conjugated π bond systems which absorb UV light. Compared to UV and FLD, DAD is more often used for the investigation of unknown compounds as not all oxygenated PAH fluoresce, and spectra may be used to support compound identification (Meyer and Steinhart, 2000; Pramauro et al., 1998; Wischmann and Steinhart, 1997). More selective and sensitive, FLD is widely used for monitoring PAH in water (Lerda, 2011), OPAH in aerosols (Hayakawa et al., 2017), OHPAH in urine (Fan et al., 2012; Onyemauwa et al., 2009) as well as metabolite production under pure culture conditions (Olmos-Espejel et al., 2012). Despite improved selectivity, interferences in complex matrices are common, and some OHPAH do not offer good sensitivity with this technique (Fan et al., 2012). Similarly, many quinones require derivatisation to enable fluorescence detection (Kishikawa and Kuroda, 2014). Rather than being used as singular technique, more often recently FLD, DAD, or DAD+FLD is used in-line with LC-MS to provide complementary evidence for identifying different PAH and oxygenated PAH in environmental samples (Boll et al., 2015; Hollosi and Wenzl, 2011; Letzel et al., 2001; Van de Wiele et al., 2004).

2.3.2 Liquid chromatography - mass spectrometry

The use of LC-MS is growing in popularity for investigations of oxygenated PAH in both medical and environmental samples. LC-MS shows some specific advantages over GC-MS including the potential for direct analysis of aqueous samples, the capacity to

analyse a greater number of compounds with carboxyl and hydroxyl groups without derivatisation, and lower operating temperatures which may limit the rearrangement and loss of quinones.

Ionisation of target analytes has been a key concern in the literature, particularly as small and nonpolar compounds may resist ionisation in common LC-MS sources. In general, LC-MS techniques are best applied to conjugated or acidic oxygenated PAH since these are either already ionised or may be easily ionised in the sample matrix. Grosse and Letzel (2007) compared the ionisation of 30 non-conjugated PAH metabolites using electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), and atmospheric pressure photoionisation (APPI), using both negative and positive ionisation modes. Nearly all tested metabolites yielded higher signals using APCI and APPI than ESI, which also failed to detect several compounds. Most compounds were preferentially ionised in the negative mode, except for lactones and ketones which were better ionised in the positive mode. Other workers have reported higher sensitivity of negative ESI for mono-hydroxylated PAH metabolites, but larger issues with ESI matrix interferences have also led these workers to prefer APCI for their specific applications (Jacob et al., 2007; Sakuma et al., 2011). ESI has been used in the quantitation of more polar components including COOHPAH in contaminated groundwater (Ohlenbusch et al., 2002), OHPAH in wastewater (Pojana and Marcomini, 2007), and OHPAH and conjugated metabolites in supernatants of fungal PAH degradation incubations as well as urine (Schmidt et al., 2010a; Tang et al., 2016). At the same time, the use of APCI is growing for these more polar compounds and is generally preferred for OPAH analysis, particularly in atmospheric samples where the technique has been more widely adopted (Boll et al., 2015; Cochran et al., 2016; Ghosh et al., 2014; Kishikawa and Kuroda, 2014; Nyiri et al., 2016; O'Connell et al., 2013; Walgraeve et al., 2010). APPI may be preferred for the analysis of parent PAH (Hollosi and Wenzl, 2011) and has been used more recently for analysis of OHPAH in soils (Avagyan et al., 2015)

Matrix effects can be substantial for all LC-MS applications, in particular as other constituents including salts, buffers, or the eluent solvents can cause residue buildup in instruments, compete with analytes, change charge-transfer characteristics, or lead to additional interactions such as ion-pairing. In addition to ensuring sufficient cleanup during sample preparation, the addition of dopants post-column can also improve ionisation of resistant PAH and breakdown products through matrix

modification. Hollosi and Wenzl (2011) compared dopants acetone, toluene, anisole, and xylene, separately and in combinations to improved detection of PAH by APPI and found that pure anisole yielded highest signal intensities for these compounds. Other researchers have found that a combination of methanol/toluene and toluene/anisole also support the ionisation of OPAH and OHPAH respectively by APPI (Avagyan et al., 2015; Ghislain et al., 2012), and that acetone improves signals of most classes of oxygenated PAH over methanol/water eluents alone (Grosse and Letzel, 2007). Another study using APCI demonstrated higher signals for individual OPAH when chloroform or hexane was used as eluents compared to methanol (Ghislain et al., 2012). O'Connell et al., (2013) found that the addition of DCM improved detection of OPAH by LC-APCI-MS up to five-fold while the addition of ammonium formate and formic acid did not improve OPAH detection. Nevertheless, detection of these more resistant compounds may be best assisted by the use of inline DAD or FLD devices as discussed above, or further application of the sample to GC-MS either offline (O'Connell et al., 2013), or online using LC-GC-MS (Ahmed et al., 2015).

2.3.3 Gas chromatography - mass spectrometry

GC-MS is the most widely used instrumentation for the analysis of PAH transformation products in environmental soil and water samples. GC-MS demonstrates advantages over LC for oxygenated PAH analysis in its higher resolution chromatography (Toriba et al., 2016), enhanced separation of isomers (Grova et al., 2013), lower instrumental detection limits (O'Connell et al., 2013), more normative ionisation parameters, and more established compound reference libraries. Method optimisation for oxygenated PAH has been conducted primarily for aerosol samples, and is reviewed elsewhere (Hayakawa et al., 2017; Kishikawa and Kuroda, 2014; Walgraeve et al., 2010), but several points warrant particular consideration or have not yet been reviewed.

Generally, separation of oxygenated PAH is conducted using DB-5, DB-5MS or HP5-MS-coated capillary columns, but these are not considered the most appropriate stationary phases for these analytes which may exhibit tailing or poor resolution of isomers. A comparison of DB17MS and DB-XLB columns for the analysis of OPAH demonstrated that the former offered improved separation, improved quantitation, and higher signal areas for most compounds. The study also presented concerns of the likelihood of overestimation of some compounds, particularly benzanthrone, in

reference materials, when columns with similar selectivity as DB-XLB including DB-5MS are used (Nocun and Schantz, 2013). In another study, it was shown that RTX®-2330 provided the best separation of OHPAH and specifically methylnaphthols when compared with column phases RTX®-440 and RTX®-50 and ZB-5MS but also led to peak broadening of late eluting compounds and required extended run times compared to the other columns; the authors therefore recommended using the ZB-5MS for applications where providing a summary area of all methyl-naphthol isomers was appropriate (Li et al., 2014). Use of GC×GC methods may improve resolution, but difficulties in integration may also contribute to deviating results (Lundstedt et al., 2014).

Electron impact ionisation (EI) at 70 eV is the most common ionisation technique used for oxygenated PAH. Negative ion chemical ionisation (NICI) utilising methane as the ionisation gas is used more widely in the study of OPAH in atmospheric materials and is preferred when nitro-PAH are included as target analytes (Cochran et al., 2012; Hayakawa et al., 2017). It may also offer improved stability of OPAH compared to EI (Albinet et al., 2006). However, higher limits of detection (LODs) (0.5-51x) using methane-NICI compared to EI have also been reported for OPAH (Cochran et al., 2012), and this method has only occasionally been used for soil extracts, where its specific utility remains undescribed (Niederer, 1998).

Due to their greater polarity, most COOHPAH and OHPAH are insufficiently volatile and require derivatisation prior to GC analysis. Derivatisation of OHPAH and COOHPAH involves the replacement of the -H, or -OH of hydroxyl or carboxyl groups with silyl, or less frequently, alkyl or acetyl groups (Itoh et al., 2005; Orata, 2012). Schummer et al., (2009) compared two of the most commonly applied derivatisation agents for the analysis of phenolic compounds and OHPAH: BSTFA and MTBSTFA. Both reagents were successfully utilised, but the use of MTBSTFA was recommended based on the increased signal strength, more consistent fragmentation patterns, and improved chromatographic resolution of OHPAH using an ULTRA-2 column. Further advantages of using MTBSTFA may also include improved stability of OHPAH derivatives when trace amounts of residual water are present (Motorykin et al., 2015). Nevertheless, it was also observed that for compounds such as 9-hydroxyfluorene which exhibit steric hindrance, MTBSTFA is less suitable; in these cases, the smaller BSTFA agent more readily accesses the hindered proton, and yields clearer signals (Schummer et al., 2009). In some GC-MS setups, it is possible to perform derivatisation online in

the auto sampling system or directly in the injection port. This can simplify sample preparation, reduce standard error measures, and reduce the loss of target analytes (Bizkarguenaga et al., 2013). MTBSTFA has been applied in in-port derivatisation techniques for acidic and polar organic pollutants (Bizkarguenaga et al., 2013), while BSTFA has been used specifically for automated derivatisation of OHPAH for in-port (Gupta et al., 2015) and SPME on-fiber applications (Luan et al., 2007).

PAH and OPAH do not require derivatisation for GC-MS analysis and are frequently analysed directly together in the same fraction. However, higher operational temperatures may make GC-MS unsuitable for analysis of thermally unstable OPAH, which are frequently reported to exhibit rearrangement, nonlinear or non-quantitative characteristics when this instrumentation is used (Cochran et al., 2012; Nocun and Schantz, 2013; O'Connell et al., 2013). Reducing initial oven temperatures (60 °C compared to 70 °C) has been shown to improve recovery of early eluting quinones such as 1,4 benzoquinone by ~400 fold (O'Connell et al., 2013), while the use of pulsed injection has been demonstrated to improve signal areas (Cochran et al., 2012). Signals may increase with higher injection port temperatures, but more moderate injection port temperatures (e.g. 140 °C) may also be preferred (Albinet et al., 2014). Quadratic fitting of calibration curves has been used to extend the calibration range of some compounds which would otherwise be considered non-quantitative or with calibration ranges limited to one order of magnitude (50-750 ng/mL) (O'Connell et al., 2013). Increased variability and reduced signal areas including non-detection of 1,2-naphthoquinone has been observed when glass wool was used as packing in the injection port liner compared to the use of CarboFrit™ filter liners or dimpled, unpacked glass liners (O'Connell et al., 2013). Another approach to improving the detection of quinones by GC-MS is to perform derivatisation after reduction of the ketone functional groups. The use of zinc and acetic anhydride has improved signals for 1,4 naphthoquinone by ~100 times (Kishikawa and Kuroda, 2014). A combination of zinc or dithiothreitol (DTT) with BSTFA effected the conversion of several PAH quinones to double TMS derivatives, while the use of the three reagents together allowed for the identification of all 37 PAH quinones studied as their TMS derivatives and doubled the signal intensities for orthoquinones compared to the use of zinc and BSTFA alone (Toriba et al., 2016). Further work demonstrated additional increases when a mix of BSA+ trimethylchlorosilane (TMCS) + trimethylsilylimidazole (TMSI) (3:2:3) was used as the silylation reagent (Toriba et al., 2016).

2.4 Assessing method performance

The adoption of analytical techniques depends on consistent performance of the proposed method. Regular instrument maintenance and use of quality control tools such as procedural blanks, internal standards, analyte check standards, precision tests, and recovery standards are essential. For ongoing analysis, the use of $\bar{x}$ (mean) and R (range) control charts offers a statistical and graphical approach to assessing whether a method process, such as instrument response or extraction recovery, falls within acceptable limits (NCSS Statistical Software, 2020). Beyond the scope of a single laboratory, interlaboratory validation and standardisation also depends on the establishment and testing of certified reference materials, as well as robustness and ruggedness studies (testing the effects of small deliberate procedural modifications, as well as non-procedural effects, e.g. different laboratories or specific instruments) (Dejaegher and Heyden, 2007).

2.4.1 Quantitation and use of internal and surrogate standards

Specific methods for conducting quantitation and assessing recovery can vary substantially, including differences in calibration strategy, selection and use of internal standards, and timing of inclusion and final use of surrogates (Table 2.2). Although these terms are sometimes interchanged, the USEPA designates that internal standards are used at the instrumental stage to monitor and normalise analyte signals for any shifts in instrument sensitivity, while surrogate standards are used to spike samples prior to sample preparation to monitor recovery through the specified procedure (USEPA Method 3500C, USEPA Method 8270). Most often, soil quantitation uses standard linear calibration curves constructed using the internal standard method, though quadratic calibration curves or single point calibration for select analytes may also be used and include the use of internal standards (USEPA Method 8270, Lundstedt et al. 2014, O'Connell et al., 2013). Matrix-matched calibration and standard addition are approaches which may be used to overcome additional uncertainty associated with matrix effects. These methods can be more time intensive, particularly in the case of standard addition, as individual samples need to be run multiple times. These approaches are not common for GC- based analyses of soil extracts of PAH or oxygenated PAH and have not been used in the any of the oxygenated PAH soil analyses reviewed throughout this paper. Instead, matrix effects for soil are most often

addressed by reporting/characterising changes in recovery of surrogate compounds (USEPA 3500), applying 'recovery-correction' factors (Lundstedt et al., 2014), and/or improving sample extraction and clean-up techniques (ref Sect. 2.2.5). Matrix matched calibration and standard addition methods are more often used for analyses of aqueous samples and LC- based analyses which may be due to the expected sensitivity of LC-techniques to matrix effects (Sect. 2.3.2) (Lankova et al., 2016; Sousa-Silva et al., 2015; O'Connell et al., 2013; Hollosi and Wenzl, 2011; Pojana and Marcomini, 2007; Ohlenbusch et al. 2002). For specific application using silicone passive sampling to analyse OPAH in river sediments by LC-MS and GC-MS, O'Connell et al. (2013) compared quantitation by the internal standard method and standard addition methods, and found that the internal standard method offered increased precision and similar quantitative accuracy compared to the standard addition method, while being less onerous. Within this thesis, matrix-matched method calibration was used for aqueous samples, while matrix effects were described for soil samples using comparative recovery testing and ongoing monitoring of surrogate recovery.

Due to the range of compounds considered and the number of steps involved in sample preparation, multiple isotopically labelled standards are needed to fully describe and/or correct for method recovery and variation in instrument sensitivity. Deuterated or ^{13}C labelled standards of oxygenated PAH have limited commercial availability, and this has been identified as a cause for the limited number of studies of oxygenated PAH in environmental samples (Nocun and Schantz, 2013). Since the use of large numbers of labelled reference compounds entails high expense or requires in-house production, researchers have often omitted their use or have relied on compounds with alternate chemistries to the target analytes, e.g. deuterated PAH (Obrist et al., 2015) or nitro-PAH (Niederer, 1998). Some labelled compounds are becoming more available (Walgraeve et al., 2010) and deuterated anthraquinones and fluorenones are increasingly used for OPAH recovery-correction in soil analyses (Enell et al., 2016; Layshock et al., 2010; Lundstedt et al., 2014; O'Connell et al., 2013). Nevertheless uncertainty in the selection of appropriate representative analogues remains. Bandowe and Wilcke, (2010) found that benzophenone-2,3,4,5,6-d_5 was suitable for representing OPAH, but 1-hydroxynaphthalene-d_7, transcinnamic acid-d_6, 1,4-naphthoquinone-d_6 exhibited substantial losses or unpredictable behavior which made them unsuitable to represent broader OHPAH, COOHPAH, and OPAH classes. Where labeled OPAH may also exhibit rearrangement during analysis, this might compensate for rearrangements of the

native unlabeled compounds, but could also lead to complications in interpreting the amount of deuterated standard and any labeled rearrangement products, as well as any additional unlabeled analytes they are intended to represent (Lundstedt et al., 2014).

2.4.2 Certified reference materials

Spiked test samples do not fully reflect real-world matrices in part because analyte-matrix interactions can change with time. Enhanced sorption through soil ageing tends to 'lock away' polyaromatic components, and spiked soils may be more easily extracted than heavily aged soils (Arp et al., 2014). For some matrices and instrumentation, it may also be very difficult to compensate matrix effects through the use of spiked surrogates (Lankova et al., 2016). More generally, spiking soils or water samples during individual laboratory studies does not provide an opportunity for ongoing detailed method comparisons, since variations in matrix type may confound comparison of analytical techniques.

In order to address these gaps, suitable Certified Reference Materials (CRMs) are needed. Currently no CRMs are available which provide reference analytical values for oxygenated PAH in soil or environmental water matrices (Lundstedt et al., 2014). This has presented challenges for the validation and intercomparison of analytical methods used in research and will continue to be a barrier to the adoption of a regulatory framework for these compounds (Lundstedt et al., 2014).

In the meantime, the use of CRMs for PAH analysis and/or alternate matrices provides a starting point for methodological comparisons in the literature. Workers have reported concentrations of OPAH obtained for diesel particulate matter and extracts (National Institute Science and Technology (NIST) Standard Reference Materials (SRMs) 1650, 1650b, 2975, 1975), urban dust and fine particulate material (NIST SRMs 1648a, 1648b, 2786, 1649a), and mussel tissue (NIST SRM 2977) with comparative information to support instrumental method development provided by several researchers (Albinet et al., 2006; Fushimi et al., 2012; Nocun and Schantz, 2013; O'Connell et al., 2013; Toriba et al., 2016). CRMs for select OHPAH are available for medical (urine NIST SRMs 3672 and 3673) (See Li et al., 2014) and marine studies applications (Community Bureau of Reference (BCR) fish bile BCR-720 and BCR-721), and these compounds are occasionally included in studies of the aerosol materials indicated above (Albinet et al., 2006). In soils specifically, limited data are available for OPAH superfund site soil 103-100 and NIST SRM 1941 (Lundstedt et al.,

2006a; Obrist et al., 2015). Initial analysis of NIST SRM 1944 NJ river sediment has also been undertaken and includes comparison of GC-MS and LC-APCI-MS analyses of these sample extracts, and further demonstrates that in some cases, particular matrix interferences may also lead to substantially different results when internal standards are used for quantitation compared to standard addition (Layshock et al., 2010; O'Connell et al., 2013). More extended data sets are also now available for reference soils European Reference Materials ERM CC013a and BCR-524 (Lundstedt et al., 2014).

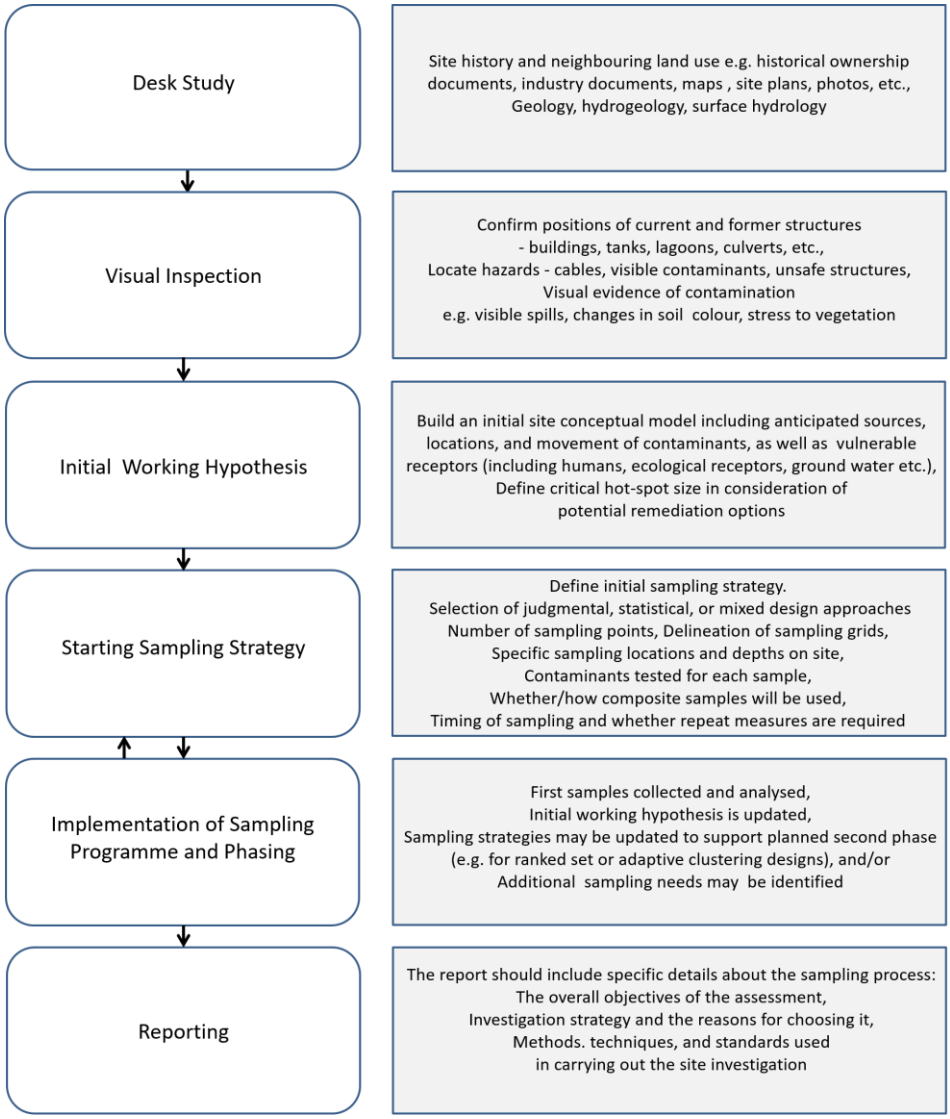

Figure 2.2 Development of a site sampling plan (based on Department of Environment 1994 and USEPA 2002)

2.5 Sampling design

While not all research questions will require overall site characterisation, for those that do it is important to consider the overall sampling plan in order ensure the final analysis is representative. For remediation projects, in addition to technical considerations such as site size, geology, hydrology, history, anticipated site homo/heterogeneity, contaminants of concern, and spatial statistics, requirements for site investigation and reporting frequently depend on non-scientific parameters such as jurisdiction, common practice, financial resources, intended site use, safety, risk aversion, and liability considerations (Department of Environment UK, 1994). While there have been standard grid-based approaches adopted for broad-scale soil mapping initiatives, such as the Tellus geochemical ground surveys in Ireland (RPS Group, 2019) and the land-use land cover topsoil survey of Europe (LUCAS-Toth et al., 2013), there is no one-size fits all sampling scheme for individual contaminated sites. Instead, a stepwise approach is often used to develop the sampling protocol (Figure 1.3). The purpose of this approach is to characterise the nature and distribution of contaminants on a site, if present; locate concentrations of contaminants which could lead to unacceptable risks to human health or the environment ('hot-spots'); establish the size and shape of such concentrations of contaminants (hot-spot/plume delineation); and to satisfy other project goals such as comparison of multiple sites, monitoring changes in a site over time, or to support further decision making about the site (USEPA 2002; Department of Environment UK, 1994). Importantly, throughout the sampling design process, "the efficient use of time, money, and human resources are critical considerations" (USEPA, 2002).

Initial investigations begin with a desk study and visual inspection of the site (Figure 1.3). The goal is to build a site model, or initial working hypothesis, of likely sources and types of contamination, the locations (including depth) of anticipated hotspots and other site hazards, geological conditions and any factors impacting or movement of contaminants, routes of exposure, vulnerable ecological receptors, etc. For statistical sampling approaches, the formulation of the initial working hypothesis also involves making decisions about accepted risk level, specifically the *critical hot-spot size* (i.e. the largest hot-spot that could be remediated if it were missed during sampling). This parameter is required for determining the number of sampling points required to obtain a strong statistical likelihood of locating a hot-spot, especially when little is known about the site (Department of Environment UK, 1994).

Depending on the amount of information available for the site, the expertise of the assessor, and the goals or requirements of the report, a judgmental, statistical, or mixed-mode approach may be considered for developing the sampling scheme (USEPA, 2002). Judgmental sampling depends on a high level of site knowledge and professional expertise to select the appropriate numbers, types, and locations of sampling points. While this approach does not allow for statistical inferences, it is easy to be implement, and can be more efficient and less expensive than statistical approaches. On the other hand, statistical (probability-based) sampling designs, allow statistical inference, such as estimations of uncertainty and error, but can be more expensive and difficult to implement on site (USEPA, 2002). It is important to note that the design of probabilistic approaches still requires the use of accurate site models and expert judgment in the choice of sampling setup and interpretation of any metrics arising. Common probabilistic sampling approaches include simple random, stratified random, and systematic grid sampling (square, herringbone, or triangular grids are common). Ranked set and adaptive cluster sampling procedures are mixed approaches which can involve the use of expert judgment on the likely location of hotspots (often based on data from a preliminary set of samples) in combination with statistical tools such as random sampling, grid sampling, and kriging or co-kriging (a group of statistical methods used to estimate the likely spatial variance of a measurement, informing sampling density plans) (USEPA 2002; Department of Environment UK, 1994). More extended discussion of the statistical bases for these approaches is given and referenced in the document EPA QA/G-5S-'Guidance on choosing a sampling design for environmental data collection for use in developing a quality assurance project plan'. Tools such as the Visual Sample Plan software package (United States Department of Energy, 2020), have also been developed to assist non-statisticians in the selection of optimal sampling strategies. In specific consideration of analysis of oxygenated PAH, the development of a cost-effective sampling plan could be supported through the initial use of proxy measures. For example, as OPAH presence tends to be highly correlated with PAH (Arp et al 2014), the initial localisation of OPAH could be estimated using more established methods for PAH analysis. It would, however, be important to follow up with quantifying oxygenated PAH concentrations in those hot-spot areas, and moreover, due to their anticipated higher mobility, determine oxygenated PAH concentrations outside and downstream from the delineated margins of the PAH-containing hot-spot or plume.

2.6 Conclusions

Significant gaps remain in understanding how PAH breakdown occurs in situ and the extent to which oxygenated PAH breakdown products contribute to risks associated with contaminated sites. These questions must continue to be supported by the development of robust analytical techniques that capture a sufficient range of PAH transformation products. Establishing methods for the extraction, identification, and quantification of oxygenated PAH from environmental matrices has excited increasing interest in the last 15 years. At the same time, the tandem concepts of cost (time) saving and green chemistry are driving a movement towards simplified analysis through reduced sample preparation, miniaturisation, simultaneous or online derivatisation, and the use of newer sorptive devices for extraction and passive sampling. This is an exciting area of development, but more work is still needed for method consolidation of oxygenated PAH analyses in soil and environmental water matrices. In soils, OPAH analyses may be the first to be standardised, but a concerted effort to formulate best practices to address OPAH rearrangement during extraction and analysis is still needed, as well as other parameters that may improve OPAH inter-laboratory data comparability and further push forward the certification of suitable CRMs. At the same time, more polar compounds which may more readily enter water systems should not be ignored, and continued extension of techniques should be undertaken. For oxygenated PAH in water, where these compounds may be most bioavailable, efforts to define initial target compound lists with acceptable LODs validated through toxicological assays would help establish analytical benchmark criteria and improve inter-study comparability. At the same time, it is expected that currently unidentified oxygenated PAH compounds will continue to emerge as relevant factors impacting site risk and management, and continued collaboration which supports sharing of compound mass spectral libraries and reference compounds will be needed.

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Chapter 3

Transformation of low molecular weight polycyclic aromatic hydrocarbons in the presence of lignin phenol amendments

Abstract

Bioremediation is a promising strategy for in situ remediation of PAH-contaminated soil and water systems. Further understanding of factors impacting bioremediation success is needed. We investigated the possibility of using two lignin phenols commonly present in composting-bioremediation mixtures, 4-hydroxybenzaldehyde and vanillin, as novel cofactors to modulate PAH degradation under bench scale conditions. Microcosms representing a combined contaminated soil/water system were supplemented with lignin phenols at concentrations of 0-120 µg/mL, and were monitored for LMW PAH, lignin phenols, and transformation products using new analytical methods for biphenyl-HPLC-DAD and small-sample LLE-GC-MS. After 18 days, most microcosms did not exhibit substantial removal of PAH, but a group of samples exhibited significant reductions for aqueous 2-3 ring PAH (30-70%) combined with distinct shifts in the profile of target and unknown transformation products. These characteristic shifts included significantly ($p<0.05$) increased concentrations of salicylic acid, 1-hydroxynaphthalene, 9-fluorenone, 9-hydroxyfluorene, 1-hydroxy-2-naphthoic acid, catechol, and vanillic acid, and reduced concentrations of amended lignin phenols, aqueous 1-hydroxyacenaphthene, 1,2 *trans*-dihydroxy-1,2-dihydronaphthalene, gentisic acid, 4-hydroxybenzoic acid, as well as alkylated phenols already present in the soil. This pattern of enhanced transformation (ET) was not directly associated with lignin phenol treatment level, however additional changes associated with lignin phenols amendment were also observed. Lignin phenol supplements directly supported the formation of 4-hydroxybenzoic acid, vanillic acid, gentisic acid, and three unknown compounds. Further, initially high levels of lignin phenols (120 µg/mL amendment) appeared to inhibit the formation of selected PAH transformation products, but at 18 days ultimately stimulated the formation of salicylic acid, 1-hydroxynaphthalene, and 9-hydroxyphenanthrene to highest levels, suggesting both independent and combinatorial effects with other ET processes. Results support the view that lignin phenols may modulate PAH transformation processes in situ.

3.1 Introduction

Remediation strategies for PAH-contaminated sites may include a combination of physical, chemical, and biological approaches. Amongst these, in situ bioremediation has garnered increasing attention as a cost-effective option (Kotoky, 2018), and may be well suited to sites where more easily-degraded lower molecular weight (LMW) PAH, (typically 2-3 fused rings) such as naphthalene, fluorene, and phenanthrene are a primary concern (Loick et al., 2009). One in situ bioremediation strategy offering promising results is composting (Wu et al., 2014). Amending contaminated soils with fresh or partially degraded organic materials provides a complex mixture of nutrients and organic substrates that support PAH degraders and other soil microbes. This complex mixture may partially explain why composting tends to achieve higher transformation rates than addition of PAH degrading organisms, simple nutrient addition (N and P), or management of aeration and moisture alone (Davie-Martin et al., 2017). However, PAH degradation rates remain variable, and the underlying mechanisms that support PAH degradation are still in need of further study (Kuppusamy et al., 2017). Further understanding of the nature and role of these substrates could help elucidate how PAH catabolism is initiated and maintained in compost-based or other bioremediation scenarios.

One of the most abundant structural constituents of plant tissues is lignin, which contributes 15-20% of the dry mass of grasses and 20-40% of wood (Chen, 2014). As such, it is an important constituent of many composting mixtures. Lignin is a bulky polymer comprised of monomeric lignin phenols (Figure 3.1) linked by highly stable C-C, and ether bonds in apparently random arrangements. This structure makes lignin, like PAH, highly aromatic and stable in the environment (Ruiz-Dueñas and Martínez, 2009). Organisms that have evolved to degrade environmentally-familiar lignin compounds may also be involved in the degradation of xenobiotics including PAH; and, the co-occurrence of natural structural analogues such as lignin phenols may stimulate PAH-degrading activity (Olson et al., 2003). Lignolytic fungi represent a well-known example of this activity as they secrete extracellular enzymes including peroxidases and laccases that catalyse relatively non-specific oxidation of aromatic ring structures found in both lignin and PAH (Haritash and Kaushik, 2009). The presence of free lignin phenols may further enhance the activity of these enzymes (Chen et al., 2019; Librando and Pappalardo, 2013). Yet even when lignolytic fungi are present, it has been shown

that intracellular processes may be more important to PAH degradation (Kadri et al., 2017; Loick et al., 2009). Intracellular enzymes require smaller precursor molecules and exhibit greater substrate specificity. The phenolic subunits found in lignin are structurally similar to key PAH breakdown products and may provide a 'training ground' for PAH degrader species, promoting the expression of enzymes involved in PAH metabolism (Olson et al., 2003). Previous studies have demonstrated that enzymes specialised in aromatic ring-opening such as dioxygenases and hydrolases may be activated in the presence of phenolic compounds including those derived from lignin (Deveryshetty et al., 2007; Kim et al., 2006; Kotoky et al., 2018). In addition, plant phenols are expected to be involved in altering microbial community diversity and PAH degrader abundance in the rhizosphere (Qu and Wang, 2008; Técher et al., 2011). However, to date the possibility of using lignin phenol amendments to modulate PAH degradation has not been investigated.

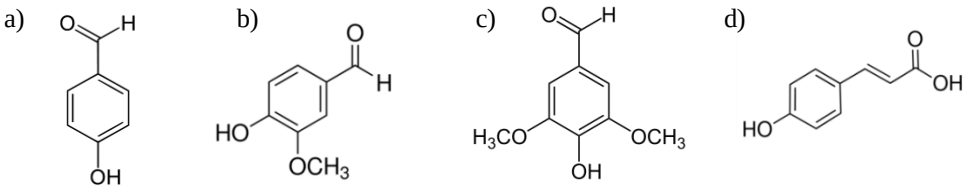


Figure 3.1 primary lignin phenols a) 4-hydroxybenzaldehyde, b) vanillin, c) syringealdehyde, d) p-coumaric acid

As improved remediation strategies are sought, there is growing interest in the formation and transport of intermediate byproducts resulting from PAH degradation. Oxygenated PAH transformation products are of interest both because they represent markers of specific degradation mechanisms, and because they may be more toxic and environmentally mobile than parent PAH (Lundstedt et al., 2007). Accelerated attenuation of PAH may result in the buildup of these intermediates and increased soil toxicity (Chibwe et al., 2017; Idowu et al., 2019), and this may especially be a concern where soils have a high leaching potential exposing connected groundwater systems

(Enell et al., 2016). These compounds should be monitored alongside parent PAH during the application of both traditional and novel remediation strategies.

Further understanding of substituents commonly found in composting materials, particularly lignin-derived compounds, may provide insights into bioremediation efficiency. Therefore, this study represents the first investigation into the attenuation of PAH and formation of PAH degradation products in a lignin phenol enrichment study. We investigated the possibility of using two lignin phenols, vanillin and 4-hydroxybenzaldehyde, to stimulate and/or alter the degradation pattern of LMW PAH in a contaminated soil/water system. Specifically, we considered whether differing lignin phenol amendment levels might: l) lead to a change in PAH prevalence, either by stimulating or inhibiting PAH removal; and 2) lead to changes in the distribution of (potentially toxic) PAH transformation products, especially in the more mobile aqueous phase. PAH, lignin phenols, and transformation products were monitored using novel biphenyl-HPLC-DAD and small-sample LLE + GC-MS as complementary analytical techniques. Although most PAH degradation studies focusing on simple organic amendments have utilised simplified matrices (no soil, or spiked, uncontaminated soils) and/or bacterial isolates (Pathak et al., 2009), here, the use of aged gasworks soil offered the possible presence of diverse indigenous PAH degrader species and the opportunity to examine transformation in a more realistic remediation-focused context.

3.2 Methods

3.2.1 Chemical reagents

Target analytes including PAH, lignin phenols, and oxygenated transformation products (Table 3.1) were obtained from Sigma Aldrich. The 18 individual target transformation products were selected to represent a range of size (1-4 aromatic rings) functional group (carbonyl, hydroxyl, and/or carboxyl-modified) and early/late stage PAH metabolites. Deuterated PAH surrogates used for soil analysis were obtained as a mixture from Thames Restek UK Ltd (semivolatile internal standard, 2000 µg/mL ea. in DCM). Organic solvents were obtained from Fisher Scientific and were LC-MS grade. Deionised water used for microcosm setup and HPLC elution was 15 MΩ-cm^{-1} grade. All other reagents were obtained from Sigma Aldrich, including mixture EPA-525 B, for PAH calibration; salicylic acid-d_4 (100 µg/mL in acetonitrile) and naphthalene-$d_{8,}$

used as surrogate standards for aqueous phase extractions; Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, $MgSO_4$, $CaCl_2$, for the preparation of M9 minimal salts medium; Na_2SO_4 and HCl, for preparation of soil and water extracts; and BSTFA with 1% TMCS, as a derivatisation agent. Fine sand and glassware were rinsed 3 times in deionised water and placed in a muffle furnace for 4 h at 450°C to remove organic residues. Volumetric glassware was sonicated with acetone instead of heating.

3.2.2 Soil physico-chemical characterisation

PAH contaminated soil was obtained from a former gasworks site located in southeastern UK. This soil had been associated with contamination from a former tar tank and had previously been excavated as part of site remediation (Ecologia Environmental Solutions Ltd.). The soil was air dried overnight then ground with a mortar and pestle and sieved to 2 mm. Particle size distribution was determined following International Organization for Standardization (ISO) method 11277 (2009) which involves hydrogen peroxide decomposition of soil organic matter and dispersal with buffered sodium hexametaphosphate, followed by sieving and sedimentation. Textural classification was assigned following Natural England Technical Information Note TIN037 (2008). Residual moisture content of the soil prior to setup and total organic content was determined by obtaining the mass lost on from triplicate 5 g soil samples after oven-drying 105°C for 24 hr and subsequent heating to 450°C in a muffle-furnace for 5 hr, respectively (British Standards Institution, BS-EN 13039, 2000). Total carbon, nitrogen, and phosphorus were determined following method BS-EN 13654-2 (2001) and ISO-11263, (1994) using a Vario EL III Elemental Analyser and Spectronic Helios Gamma spectrophotometer. Further details on nutrient analyses can be found in Cipullo et al. (2019) - results have been reproduced here with permission. Initial soil pH was determined in 0.01 M $CaCl_2$ (1:5 solid:liquid) using a Jenway 3540 pH meter after 1 hr shaking and 30 min equilibration period (ISO 10390, 2005). Further monitoring of pH during the experiment was conducted directly in the decanted aqueous phase of the sample using a handheld Hannah pH microprobe.

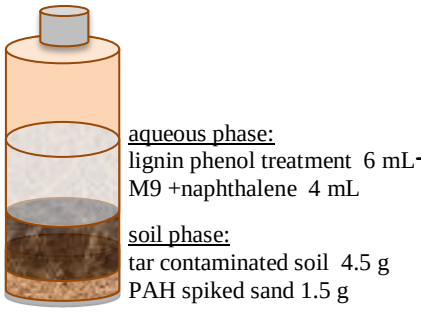
Table 3.1 Target analytes and instrumental analytical settings

Target Analyte	CAS #	mass g/ mol	HPLC[1] RT min	HPLC[1] λ **max,** monitored nm	GC-MS RT min	GC-MS SIM m/z **quant.,** reference	IS[5]	SR[6]
PAH								
Naphthalene	91-20-3	128	20.25	**220**, 220	7.79	1**28**, 64	IS1	SR1
Acenaphthylene	208-96-8	152			12.34	**152**, 126	IS1	SR2
Acenaphthene	83-32-9	154	22.27	**228**, 228	12.88	**154**, 76	IS1	SR2
Fluorene	86-73-7	166	22.54	**262**, 254	14.37	**166**, 83	IS1	SR2
Phenanthrene	85-01-8	178	23.20	**250**, 254	17.22	**178**, 89	IS1	SR3
Anthracene	120-12-7	178			17.38	**178**, 89	IS1	SR3
Pyrene	129-00-0	202	24.07	**240**, 228	22.90	**202**, 101	IS1	SR3
Amended Lignin Phenols								
4-Hydroxybenzaldehyde	123-08-0	122	9.92	**284**, 275	10.90	**179**, 151	IS1	
Vanillin	121-33-5	152	11.68	**230**, 275	13.52	**194**, 201	IS1	
OPAH								
1-Indanone	83-33-0	132	14.64	**206**, 220	9.431	**132**, 104	IS1	
9-Fluorenone	486-25-9	180	20.08	**256**, 254	16.60	**180**, 152[4]	IS1	
9,10-Anthraquinone	84-65-1	208			20.24	**208**, 180	IS1	
9,10-Phenanthrenequinone	84-11-7	208			24.21	208, 180[3]	IS1	
OHPAH								
Catechol	120-80-9	110	5.12	**194**, 220	9.98	**254**, 151	IS1	
1-Hydroxynaphthalene	1779-10-8	144	17.51	**210**, 220	13.65	**216**, 201	IS1	
1-Hydroxyacenaphthene	6306-07-6	170	16.60	**224**, 228	16.85	**242**, 152	IS1	
9-Hydroxyfluorene	1689-64-1	182			17.45	**254**, 165	IS1	
9-Hydroxyphenanthrene	484-17-3	194			22.45	**266**, 251	IS1	
1-Hydroxypyrene	5315-79-7	218	21.38	**242,** 275	30.41	**290**, 175	IS2	
1,2 *trans*-dihydroxy-1,2-dihydronaphthalene[1] (1,2-*trans*-naphdiol)[2]	771-16-4	162	10.67	**214**, 254	16.17	**191**, 203	IS1	
COOHPAH								
1-Naphthylacetic acid	86-87-3	186	16.32	**224**, 220	17.00	**258**, 168	IS1	
Fluorene-9-carboxylic acid	1989-33-9	210			19.58	282, 165[3]	IS1	
1-Hydrox-2-naphthoic acid	86-48-6	188	13.11	**218**, 220	20.45	**317**, 243	IS1	
Phenolic Acids								
Salicylic acid	69-72-7	138	3.21	202, 220	13.15	**267**, 209	IS1	SR4
4-Hydroxybenzoic acid	99-96-7	138	4.26	196, 254	14.83	**267**, 193	IS1	
Gentisic acid	490-79-9	154	1.64	206, 220	16.98	**355**, 297	IS1	
Ferulic acid	1135-24-6	194	2.11	266, 254	22.17	**338**, 323	IS1	
Deuterated surrogate standards								
SR1 Naphthalene-d_8	1146-35-2	136			7.75	**136**, 108	IS1	
SR2 Acenaphthene-d_{10}	15067-26-2	164			12.86	**164**, 162	IS1	
SR3 Phenanthrene-d_{10}	1517-22-2	188			17.22	**188**, 160	IS1	
SR4 Salicylic acid-d_4								
GC-MS internal standards								
IS1 Nonadecane-d_{40}	39756-36-0	308			18.12	**66**, 98		
IS2 Triacontane -d_{62}	93952-07-9	485			40.37	**66**, 98		

CAS#-Chemical Abstracts Service identifier; RT-retention time; [1]further details in Appendix A [2]product discontinued during project, semi-quantitative results reported; [3]non quantitative; [4]cautiously quantitative, as fluorene-9-carboxylic acid and 9,10-phenanthrenequinone, if present, may contribute to observed 9-fluorenone concentrations through rearrangement. [5] Selection of internal standard (IS) based on retention time, IS1 for all compounds before temperature-gradient associated baseline rise, and IS2 for all any compounds eluting after this rise. All quantitative compounds exhibited consistent linear behaviour when calibrated using these internal standards. For further discussion see. Sect 4.3.1. [6] Surrogate (SR) compounds used for estimating recovery of most polar and least polar analytes.

3.2.3 Microcosm setup

A schematic diagram of the microcosms, with five lignin phenol treatment levels and five sampling time points is shown in Figure 3.2. Since PAH may be tightly bound in aged soils, this soil was spiked to ensure the presence of a more available fraction of LMW PAH. Fine sand (1.25 mL - 1.5 g ± 0.1 g) was added to 20 mL amber headspace vials and spiked with 1 mL naphthalene, acenaphthene, fluorene, phenanthrene, and pyrene (225 µg/mL ea. in methanol / 50 µg ea. per gram of soil). After allowing the solvent to evaporate, 5 mL (4.5 g ± 0.2 g) contaminated soil was added. Microcosms were then amended with 4 mL (PTFE) filter-sterilised M9 minimal salts medium, prepared with additional naphthalene (appx. 32 µg/mL) as it was expected some may have been lost during evaporation of the original PAH spike. Microcosms were further supplemented with 6 mL vanillin + 4-hydroxybenzaldehyde prepared in autoclaved water at 0 (control), 10, 50, 100, or 200 µg/mL ea. (Treatments 1-5, prepared in duplicate, final additive concentrations 0-120 µg/mL ea. Figure 3.2). Individual lignin phenol concentrations of 10-50 µg/g are typical of the range to be found in UK natural and agricultural soils (Rimmer and Abbott, 2011). Microcosms were capped with PTFE- lined screw cap closures, hand shaken to combine constituents, and placed on an orbital shaker at ambient lab temperatures (40 revolutions per minute, rpm). Two replicate microcosms were sacrificially sampled from each treatment at the following intervals: 3 hr, 48 hr, 5, 9, and 18 d (T1 through T5, respectively) and stored at -80°C until further analysis.



Treatment #	Lignin Phenol Sup.		Sampling Time Points				
	µg/mL[1]	µg/g[2]	T1 3h	T2 48h	T3 5d	T4 9d	T5 18d
1(control)	0	0					
2	6	10					
3	30	50					
4	60	100					
5	120	200					

Figure 3.2 Soil/water microcosm setup. Initial incubation condition for individual samples shown on the left, with five treatment levels, and five sampling time points described on the right (50 samples total). Lignin phenol supplement reported [1]at final aqueous concentration after mixing with M9 + naphthalene, and [2] normalised to 6 g solids (soil+sand).

3.2.4 Preparation of aqueous and soil phases for extraction and analysis

Microcosms were thawed just before sampling. Initial work had demonstrated that several different filtration membranes removed analytes of interest from aqueous samples (Appendix A). Therefore, this step was removed from sample preparation protocols. A 1 mL luer lock syringe fitted with a 0.40 mm bore needle was inserted below the level of floating organic material (1 cm depth), and 0.9 mL of the aqueous phase was directly sampled into a 2 mL amber glass vial for HPLC analysis. This aliquot was spiked with 100 µL ACN to inhibit further transformation (Ohlenbusch et al., 2002) and to match the solvent mixture used for preparing calibration standards. An additional 2 mL was sampled into a foil-wrapped glass test tube with PTFE-lined cap for small-sample LLE + GC-MS analysis. The remaining aqueous phase was decanted and measured for pH. Solids were wrapped loosely in aluminium foil and air-dried overnight prior to extraction.

3.2.5 Liquid-liquid extraction of aqueous samples

Typically, LLE is performed on higher volume samples e.g. 100mL-1L for aqueous environmental matrices and micrcosms (Cajthaml et al., 2002; Enell et al., 2016; Letzel et al., 2001; Siemers et al., 2015). In order to monitor this study, we first tested the feasibility of applying traditional LLE techniques to small volume (2 mL) samples in order to detect PAH and oxygenated derivatives. Aqueous sample (2 mL) was spiked with 20 µL naphthalene-d_8 and 20 µL salicylic acid-d_6 (both 100 µg/mL) as surrogates, acidified with 200 µL HCl, and extracted with dichloromethane (DCM) in three cycles (3 × 2 mL DCM). For each cycle, the mixture was agitated on a rotary shaker (5 min) and the lower layer removed to a graduated conical tube (0.1 mL gradations). The full extract was reduced to 1 mL under a gentle stream of nitrogen. Recovery tests (1 µg/mL spike, n=3) and method calibration (0.01-10 µg/mL + 100, 200 µg/mL for amended phenols) were conducted in aqueous phase analogue (4.5 g experimental soil/10 mL deionised water shaken, sampled, and extracted as experimental samples). Relative standard deviation (RSD) of extraction was ≤5% with recovery ranging from 45%-134% for most analytes (Appendix B Table B.1 Soil 1 for recovery of individual analytes), and coefficient of determination, R^2, was ≥ 0.97 for all analytes. For every 10 experimental samples, two additional samples of 2 mL deionised water were also spiked, extracted, and analysed as method blanks.

3.2.6 Extraction of soils

For each microcosm, air dried soil (1 g) was weighed into a 40 mL amber glass vial with PTFE screw cap. Samples were spiked with 50 µL 160 µg/mL deuterated PAH as surrogates, and anhydrous sodium sulfate (4 g) was added to remove residual moisture. Vials were capped and stored overnight at 4° C to allow interaction of the spike and the soil matrix. Soils were extracted with 20 mL DCM, applying 20 min agitation and 10 min sonication. The sample was then centrifuged (90 min 900 g) and the supernatant decanted. For every 10 samples, two method blanks (sodium sulfate and surrogates but no soil) were also extracted. Additionally ~1.5 g each soil sample was used for the determination of residual moisture content, as described in Section 3.2.2. Final soil concentrations were calculated using the internal standard calibration method and adjusted to the dry soil mass equivalent. Surrogate PAH recovery for this method was 89-128% with RSD for individual surrogates 6-8%.

3.2.7 HPLC analysis

HPLC analysis was performed using an Agilent 1200 with DAD module. Separation of target compounds was achieved through a novel method we developed using a reverse phase biphenyl column (Restek Raptor 2.1 mm x 150 mm, 2.7 µm particle size), and gradient elution with water (Solvent A) and ACN (Solvent B). Further details of method development are given in Appendix A. The selected program was as follows: injection volume 7 µL, % B starting concentration 2%, hold for 2 min, increase to 80% over 25 min, hold for 3 min, decrease to 2% over 3 min and hold for 4 min. Peak development was monitored at 220, 228, 254, and 275 nm and identified peaks were compared for retention time (RT) and spectra based on reference standards. Replicate injections (2 µg/mL, n=3) indicated less than 1.7% RSD in signal area for all analytes except pyrene (2.3% RSD) and acenapthene (6.6% RSD). Blank injections of deionised water/ACN (9:1) were conducted every five samples to monitor for interferents or instrument carryover, and 2 µg/mL PAH standard was analysed every 10 samples to monitor for instrument drift. Presence of target analytes was not observed during blank runs, likely due to low concentrations of non-polar compounds in the aqueous samples, as well as the extended ramp and hold time at 80% B. Matrix calibration of PAH was conducted using 900 µL prepared matrix analogue (4.5 g soil/10 mL deionised water shaken and sampled as real samples) and 100 µL PAH calibration solution in ACN to achieve final additive concentrations of 0 and 0.010-20 µg/mL, with upper calibration limits selected

based on aqueous solubility and loss of linearity. Limits of detection and quantitation (LOQ) were based, respectively, on 3 and 10 times the standard deviation of seven replicate injections of a 0.01 μg/mL standard divided by the slope of the calibration curve. Discussion of challenges for full matrix-quantitation of targeted transformation products is discussed in Appendix A. Since GC-MS provided an alternate means for quantifying these compounds in matrix, HPLC analyses were primarily used for semi-quantitative and qualitative comparisons in this study.

3.2.8 GC-MS analysis

GC-MS analysis was performed using a Shimadzu TQ-8040 with AOC-6000 autosampler. The injection port was held at 200°C and operated in the splitless mode with 1 μL injection volume. Separation was performed using a 30 m Rtx 5 column (5% diphenyl / 95% dimethylpolysiloxane 0.25 mm ID, 0.25 μm df) and column flow was controlled for linear velocity at 36 cms^{-1}. The oven temperature program was as follows: initial 80°C, +5°C min^{-1} to 100°C, +8°C min^{-1} to 200°C, +3°C min^{-1} to 300°C, hold for 2 min. The transfer line and MS system were held at 300°C. The EI source was operated at 70 eV and the detector in the concurrent scan/SIM mode with event times of 0.3 s/ 0.060 s.

Sample extracts were derivatised prior to GC-MS analysis in order to facilitate detection of compounds containing OH and/or COOH groups. 100 μL extract, 20 μL internal standard mixture (IS-20 μg/mL deuterated nonadecane and triacontane in DCM) and 20 μL BSTFA+1% TMCS were added to a 200 μL glass insert in an amber GC vial, then shaken for 60 min. Target analytes were identified through comparison of retention time, quantitative and reference ion ratios (<30% difference between sample and standard), and general visual agreement of the spectra obtained from reference standards and experimental samples. LOD/ LOQ were based on 3/ 10 times the standard deviation of seven replicate injections of a 0.01 μg/mL standard divided by the slope of the IS-adjusted calibration curve (Ref Ch. 4 and Table 4.1 for instrument LOD/LOQ and discussion). For aqueous samples, the matrix-method-IS-calibration curve, as described in Section 3.2.5 was utilised in order to further account for characteristic losses/gains introduced by the LLE protocol and sample matrix (Section 3.2.5 Appendix B). For soil analysis, four-point calibration curves for standard solutions of target analytes were constructed using the internal standard IS method (0.01 μg/mL-10 μg/mL in DCM, with 0 μg/mL as blank) was conducted using the internal standard method

following the approach of Lundstedt et al. (2014), and USEPA method 3500C. Compound identification, integration, and chromatogram comparison were conducted using Lab Solutions Insight Postrun Analysis software (Shimadzu, 2015-2016). Samples were run in order of lowest lignin phenol addition to highest. Blank injections of DCM were conducted every 10 samples (for each time point) to monitor for instrument carryover. Due to the early elution times of target analytes and extended GC-temperature ramp at the end of each run, and based on the absence of interfering peaks in blank runs, instrument carryover was not observed to cause interference for these analyses, and the frequency of running instrument blanks was considered adequate. Any contribution of a targeted m/z value (mass to charge ratio), observed in method blanks was subtracted prior to final quantitation. The development of non-target peaks was also monitored in total ion chromatograms and where identification is suggested, spectral match with the NIST 14 reference compound library was >90%.

3.2.9 Note on the use of surrogate and internal standards used for GC-MS analysis

This text follows USEPA terminology to distinguish the use of isotopically labelled standards, 1) as Surrogate Standards, where the standard is spiked directly into the sample matrix in order to track recovery through stages of the extraction and sample preparation, and 2) as Internal Standards, where the standard is spiked into the fully prepared sample just prior to instrumental analysis; in this case, the internal standard accounts for small shifts in injection volume or instrument sensitivity. It is most common that the internal standard (IS) is then used to construct IS-normalised calibration curves, which is the approach used throughout this text (USEPA Method 3500C, USEPA Method 8270). Some researchers choose to further 'correct' quantitation of target compounds by normalising by the surrogate recovery (Lundstedt et al. 2014, further refs. Table 2.2, Sect. 2.4.1); however, the use of this approach in general is inconsistent in terms of which sample procedures are included in recovery assessment (Table 2.2), does not acknowledge likely differences in extractability of spiked and native compounds (Arp et al. 2014), and in some cases, could disguise poor recovery; in addition, as demonstrated in Chapter 4 of this thesis, this approach should only be considered when the labelled compound has been demonstrated to be a suitable analogue for the target compound and concentration range (see also, Bandowe and Wilcke 2010). In an ideal scenario, each target analyte could be both tracked for recovery and calibrated using its isotopically labelled counterparts, e.g. ^{13}C and

deuterium labelled compounds. In practice, this is not feasible due to the limited availability of suitable labelled compounds (Ref Ch. 2.4.1). The approach used in this thesis therefore follows the recommended approach for the use of surrogate compounds in standard USEPA Method 3500C-Organic Extraction and Sample Preparation, and deuterated aromatic compounds were prioritised for use as surrogate compounds to monitor recovery losses/gains throughout the sample preparation procedure for the most polar (salicylic acid) and least polar (PAH) constituents. It is noted that these compounds are also recommended for use as internal standards at the instrumental analysis stage in USEPA Method 8270 - Determination of Semi-Volatile Organic Compounds by Gas Chromatography-Mass Spectrometry; however this method also explicitly allows the use of other internal standards provided that the selected standards 1. are unambiguously identifiable (i.e. are chromatographically resolved and do not naturally occur in the sample), 2. elute in a smiliar timeframe as target analytes, and 3. that the IS-normalised calibration curve is consistent and linear. In this study, deuterated alkanes were used as the internal standards and the selected internal standard based on retention time, with IS1 (nonadecane-d_{40}) used for compounds eluting early, prior to the baseline rise associated with the temperature gradient, and IS2 (Triacontane-d_{62}) used for compounds eluting after this rise (Table 3.1). Calibration using these internal standards gave a consistent linear response (Table 4.1).

3.2.10 Data analysis

HPLC data were processed using Microsoft Excel 2010. GC-MS quantitation and all statistical tests were processed in MATLAB (2018b) Academic Use License, using custom scripts with built-in statistical functions for mean, standard deviation, Student's T-test assuming unequal variance, linear regression and correlation analysis (fitlm), and principal components analysis (PCA) using mean data centering and singular value decomposition. For all tests, significance was tested at $\alpha = 0.05$ level. Where a compound was detected but fell below the LOQ, the sample was assigned a concentration of ½ the LOQ prior to further statistical calculations, but has otherwise been reported as <LOQ. Subplot figures were prepared with customised versions of the Patrick Martineau code (2014).

3.3 Results

3.3.1 Soil characterisation

The soil exhibited a coarse sandy texture with a high proportion of organic matter, as expected due to the high level of tar contamination. An elevated C:N:P ratio suggested that the soil was limited in N and P content compared to USEPA guidelines which recommend a ratio of 100:10:1 to promote PAH degradation in composting scenarios (Cipullo et al. 2019). Elevated pH recorded under experimental compared to baseline soil characteristics was likely related to an increased soil: water ratio and differing ionic conditions.

Table 3.2 Basic characteristics microcosm test soil

Analysis	
textural classification[1]	coarse sand
sand %	88.3
silt %	8.1
clay %	3.6
residual moisture %	7.3
pH	8.4
organic matter (LOI)%	20.1
total C %	18.3
total N %	0.4
total P %	0.07
C:N:P	254:5:1

Table 3.3 Initial mean PAH concentrations in soil and aqueous phase. T1=3 hrs; n=10

Target PAH	aqueous [1] (μg/mL)	soil (μg/mL)	aq/soil
Spiked PAH			
naphthalene	5.6	1900	$2.95x10^{-3}$
acenaphthene	0.32	120	$2.67x10^{-3}$
fluorene	1.18	940	$1.26x10^{-3}$
phenanthrene	2.33	3500	$6.67x10^{-4}$
pyrene	0.61	1600	$3.81x10^{-4}$
Additional LMW PAH			
acenaphthylene	1.39	600	$2.32x10^{-3}$
anthracene	0.87	1500	$5.80x10^{-4}$

[1]Naphthalene quantified through HPLC analysis due to variable recovery of naphthalene-d_8 from LLE samples (65%±25%); other PAH quantified through GC-MS.

3.3.2 Transformation of PAH and oxygenated degradation by-products

PAH concentrations in the soil and aqueous phases at the onset of the experiment indicate high levels of initial contamination especially for 3-ring PAH and naphthalene (Table 3.3). Spike contribution to total soil PAH concentrations were moderate to minimal (~1-10%) for most PAH, with the exception of acenaphthene, where the spike contribution represented nearly half of the soil concentration. Aqueous concentrations increased with decreasing analyte mass as expected, and were 3-4 orders of magnitude lower than soil concentrations.

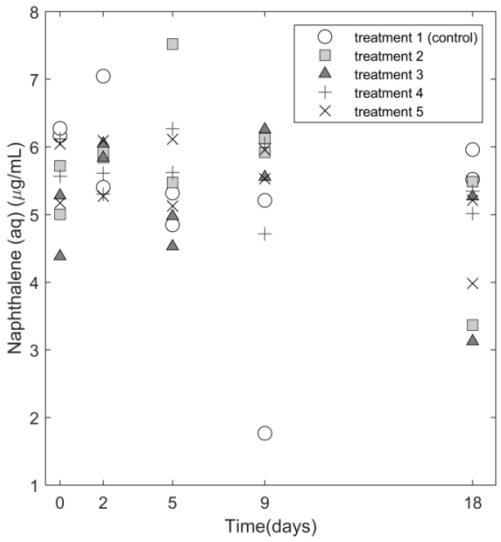


Figure 3.3 Aqueous phase naphthalene

Between 0 and 18 days, a majority of samples did not exhibit substantial changes in aqueous LMW PAH concentrations, and no clear differences in PAH attenuation rates were associated with lignin phenol treatment level (Figure 3.3). Low attenuation rates could be in part due to the high proportion of PAH and other compounds present in the soil (Table 3.3, Figures 3.4, 3.5, Ref. Section 3.4). In cases where soil PAH concentrations are very high, it may be difficult to detect PAH degradation through analysis of PAH alone, and consideration of PAH transformation products may more readily elucidate whether PAH transformation processes are active (Olsen et al. 2003). Examination of chromatographic profiles revealed a subset of samples at days 9 and 18 that exhibited characteristic shifts in both HPLC and GC-MS chromatograms indicative of enhanced transformation (ET) processes. Characteristic shifts observed in the ET sample group, corresponded to 1) enhanced removal of 2-3 ring PAH especially in the

aqueous phase, 2) formation and removal of known transformation products and unknown compounds, and 3) near complete removal of amended lignin phenols; each is discussed below. It is important to note that ET samples were obtained from all treatment levels: Treatment 2-4 each contributed one and Treatment 5 contributed two samples at 18 days, while one Treatment 1 control sample exhibited these patterns at 9 days; the observed decreases in aqueous PAH concentrations and chromatographic shifts therefore could not be directly attributed to lignin phenol amendment level. However, the clear removal of both amended and already-occurring phenols in combination with the removal and formation of other transformation products, suggested that ET samples were a group of interest and the potential relationship between phenols and PAH transformation warranted further consideration.

Comparison of ET samples with T1 revealed significant reductions (Student's T-test) of aqueous 2-3 ring PAH (mean reduction 30-70%) while other samples at 9 and 18 days (hereafter non-ET samples) samples showed no significant change for most of these compounds (Figure 3.6). Fluorene and acenapthene were exceptions, as declines in non-ET samples were also significant, though for acenapthene, ET samples still exhibited significantly increased removal (10% greater) compared to non-ET samples. Soil-bound naphthalene appeared to decline by ~27% in ET samples, however this change could not be confirmed significant at α=0.05; other soil-bound PAH also did not exhibit significant changes over time.

Examination of HPLC chromatograms for ET and T1 samples (Figure 3.4) at RT 9-16 min suggests that compounds were removed in succession, with more polar compounds including lignin phenols removed more completely, and less polar compounds including PAH exhibiting greater recalcitrance. Increases between RT 0-3 min indicate the most polar acidic compounds were also maintained at elevated concentrations due to limited continued degradation and/or higher replenishment rates. Although these increases were most substantial for ET samples, lesser increases were also observed in non-ET samples with greater peak areas generally associated with higher treatment level. Salicylic acid was suggested as a minor peak in ET samples in this window, however peak overlap excluded confirmation and quantitation, which was achieved through GC-MS analysis (Figure 3.6). Catechol on the other hand, was identified here but not observed during GC-MS analysis due to low recovery of this compound through the LLE method (Appendix B). Linear regression analysis revealed a highly significant ($p<0.001$) and well correlated ($R^2>0.80$) inverse relationship

between catechol and naphthalene areal signals for samples at day 9 and 18, suggesting catechol may have been present due to the transformation of naphthalene or was formed through related processes. Unidentified products h-k were present in ET samples both with and without added lignin phenols.

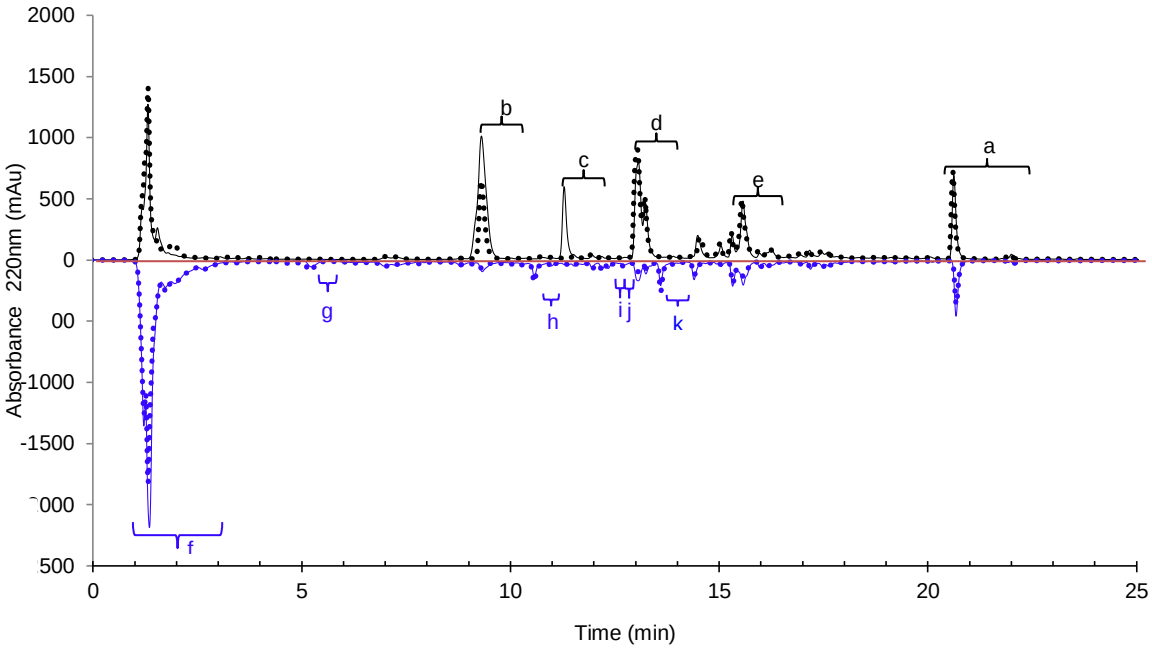


Figure 3.4 Mirrored representative HPLC chromatograms displaying characteristic shifts associated with enhanced transformation processes (ET) in the aqueous phase. Black lines represent samples at T1 = 3 hr, with lignin phenols amended at 0 µg/mL (Treatment 1- dotted) and 120 µg/mL (Treatment 5 - solid). Blue lines represent two ET samples at T5 = 18 d, both treated with lignin phenols (Treatment 3 - 30 µg/mL - dotted; Treatment 5- solid). Black/blue brackets indicate compound removal/formation respectively. a) attenuation of naphthalene and low molecular weight PAH; b) and c) complete or near complete removal of 4-hydroxybenzaldehyde and vanillin - the dotted black line at 9 min represents a co-eluting compound from the sample matrix identified by differing spectral properties, not 4-hydroxybenzaldehyde, also attenuated; d) reduction to near elimination of two compounds at 13.0 and 13.2 min; e) reduced peak areas at 15.0 and 15.6 min; f) increased concentrations of polar acidic compounds, also observed to a lesser extent in some non-ET samples; g) production of catechol; h) j) k) appearance of peaks at 10.6, 12.3 and 13.6 min, respectively; and i) increase of peak at 12.2 min also observed in some non-ET samples.

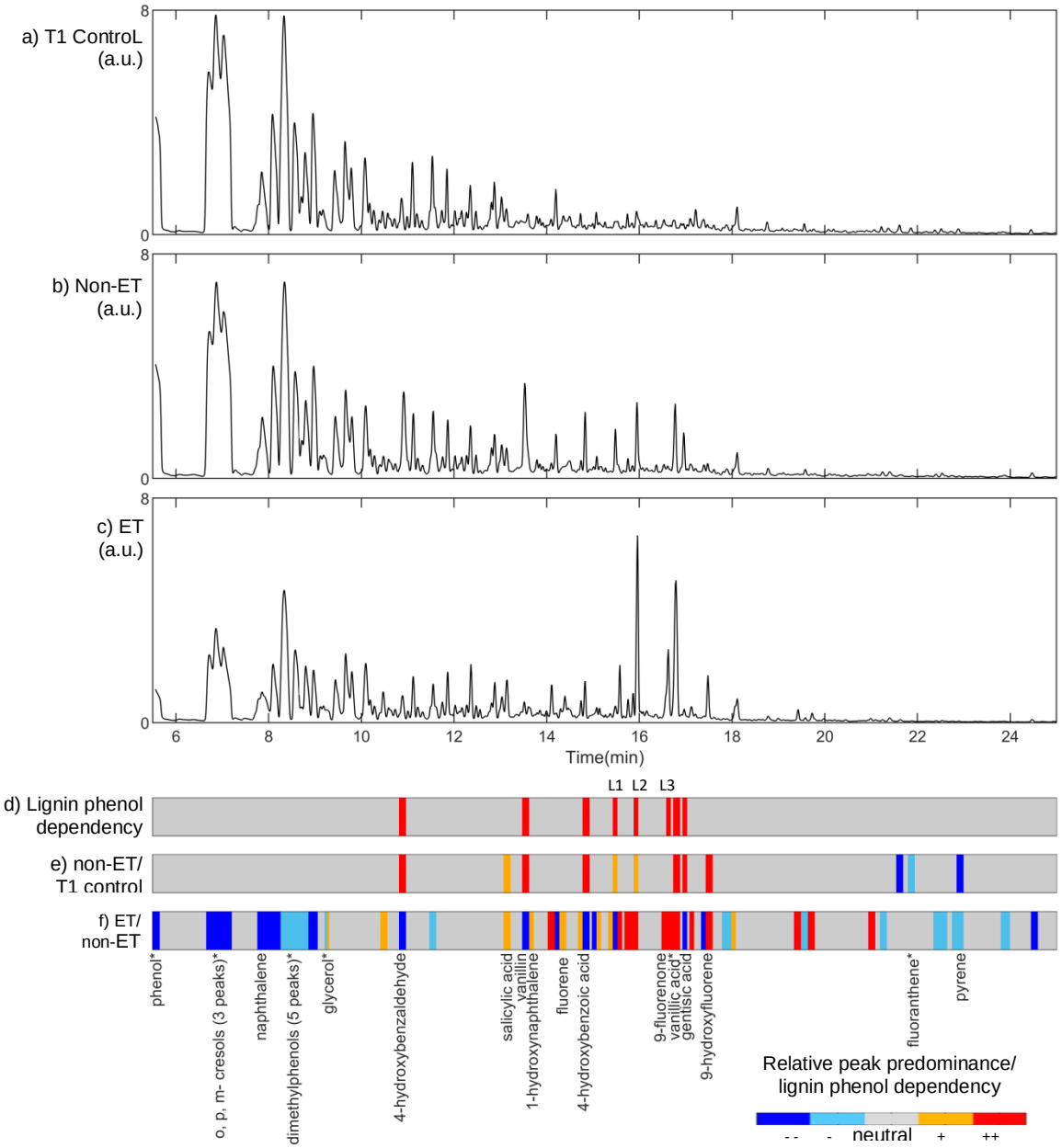
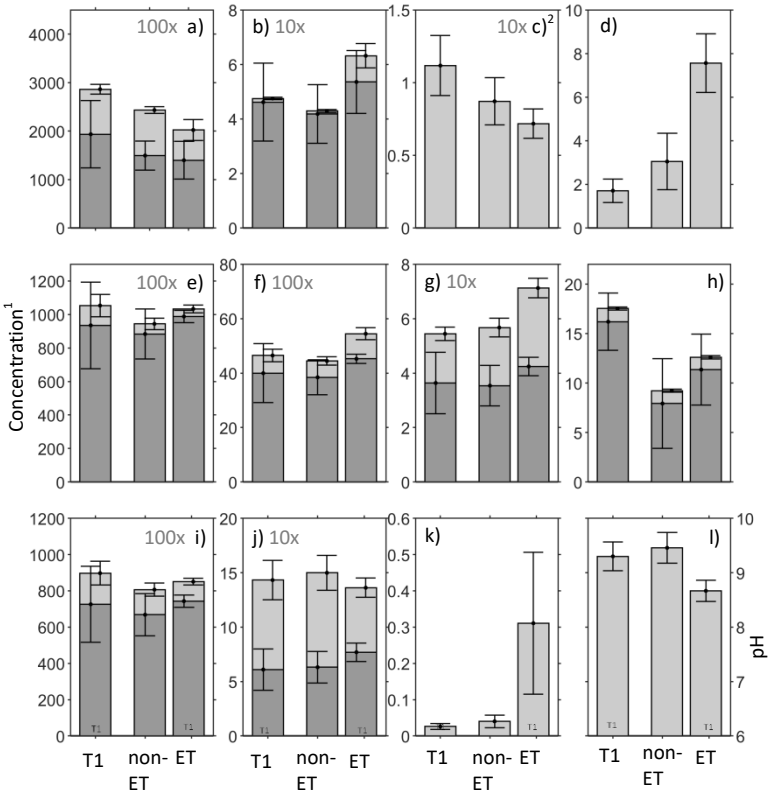


Figure 3.5 a-c) Average total ion chromatograms for LLE-GC-MS for T1 control, non-ET, and ET sample groups, respectively. All chromatograms displayed at same scale (arbitrary units - a.u.), which incorporates signal intensity of the total ion count, ion count for the internal standard, and averaging for the indicated group. d) lignin phenol dependency colourmap highlights peaks that demonstrated a clear and consistent relationship with lignin phenol treatment level, with positive relationships indicating peak areas increased with increasing treatment level. Lower colourmaps e-f) highlight chromatographic regions where the first-listed sample category showed moderate to substantial declines (light blue and dark blue respectively) and increases (orange and red) or neutral (no clear change) compared to the second-listed sample category. Named compounds have been identified through match to authentic reference standards, or where asterisked, exhibit >90% match with NIST library. Unknown compounds L1-L3 discussed further in text.

GC-MS analysis further demonstrated that ET samples exhibited substantial shifts in compound prevalence compared to non-ET samples, with 58 peaks showing moderate to substantial areal decreases or increases (Figure 3.5). Notable reductions were observed in early-eluting compounds (0-9 min) suggesting non-lignin phenolic compounds such as phenol, cresols, and larger alkyl phenols were also removed during ET processes. With the exception of glycerol, vanillic acid, fluoranthene, and targeted PAH analytes, remaining highlighted peaks, including 18 exhibiting increases, could not be identified using built-in NIST library searches. Excluding specific compounds associated with higher lignin phenol amendments (top colourmap), ET patterns obtained in the Treatment 1 sample showed good agreement with those observed in other ET samples.

SIM offered further resolution of target transformation products, and revealed ET-related shifts in 1-hydroxyacenaphthene, 1,2 *trans*-dihydroxy-1,2-dihydronaphthalene, and 1-indanone, not captured in GC-total ion chromatograms or HPLC analysis. Targeted PAH metabolites included in the ET/non-ET comparisons (Figure 3.6) did not exhibit direct dependency on lignin phenol treatment level, but exhibited a variety of trends associated with ET processes. Significant formation of 1-hydroxynaphthalene occurred in the aqueous phase of ET samples, and enhanced negative and positive response in ET samples were also observed for 1,2-*trans*-dihydroxy-1,2-dihydronaphthalene and salicylic acid respectively, though the latter compounds also exhibited general increases/declines over time. Fluorene transformation products 9-fluorenone and 9-hydroxyfluorene demonstrated significantly elevated levels in both aqueous and soil phases for ET compared to non-ET samples; but 1-indanone, a later stage fluorene metabolite, was removed to a greater extent in non-ET samples, possibly due to continued transformation of the larger fluorene metabolites in the ET samples. Unusually, 1-hydroxyacenaphthene, a byproduct of acenaphthene and acenaphthylene catabolism, demonstrated significant increases in the soil phase of ET samples, but significant decreases in the aqueous phase. Although this could reflect a change in sorption characteristics, it might also be explained by more rapid degradation occurring in the more available aqueous phase. Phenanthrene and its metabolite 9-hydroxyphenanthrene did not reveal a clear relationship with ET processes, but the later stage byproduct 1-hydroxy-2-naphthoic acid demonstrated substantial increases in ET samples. Other targeted products 1-hydroxypyrene and anthraquinone were detected but did not exhibit clear differences between ET/non-ET samples.



	Student's T p (aq, *soil*)		
	T1 vs nonET	T1 vs ET	nonET vs ET
a) naphthalene	0.782	0.016	0.014
	0.154	*0.113*	*0.604*
b) 1-OHnaph	0.199	0.006	0.005
	0.500	*0.323*	*0.064*
c) 1,2 *trans*-naphdiol	0.006	<0.001	0.022
d) salicylic acid	0.003	<0.001	<0.001
e) fluorene	0.028	0.009	0.267
	0.638	*0.611*	*0.031*
f) 9-fluo	0.558	0.039	0.014
	0.742	*0.248*	*0.003*
g) 9-OHfluo	0.013	<0.001	0.002
	0.841	*0.217*	*0.012*
h) 1-indanone	0.506	0.307	0.575
	<0.001	*0.025*	*0.101*
i) acenaph+ acenaphyl	0.201	0.012	0.015
	0.520	*0.836*	*0.049*
j) 1-OHacen.	0.533	0.004	<0.001
	0.799	*0.078*	*0.020*
k) 1-OH-2-naphthoic acid	0.015	0.016	0.019
l) pH	0.176	<0.001	<0.001

Figure 3.6 Soil (dark grey) and aqueous (light grey) concentrations of target PAH and transformation products for T1=3 h, and ET and non-ET samples. Analytes included in ET/non-ET distinction did not exhibit direct increasing/decreasing dependency on increasing lignin phenol treatment level. Plots with only aqueous concentrations indicate either that the compound was not detected in the soil phase, or that recovered concentrations were too low to permit quantitative comparisons. [1]concentrations in µg/g; aqueous phase values normalised to 1 g soil taking into account the full volume of the aqueous phase (10 mL). Error bars represent one standard deviation of the mean. Light grey 10x or 100x notation indicates scaling factor applied to aqueous phase for display purposes. [2]uncalibrated values - areas adjusted for the internal standard, and arbitrary calibration slope=1 applied; based on slopes obtained for other analytes, true values estimated to be 2-5 times greater. Results of Student's T-test at right. Non-italics indicate p value for aqueous samples, italics for soil measures.

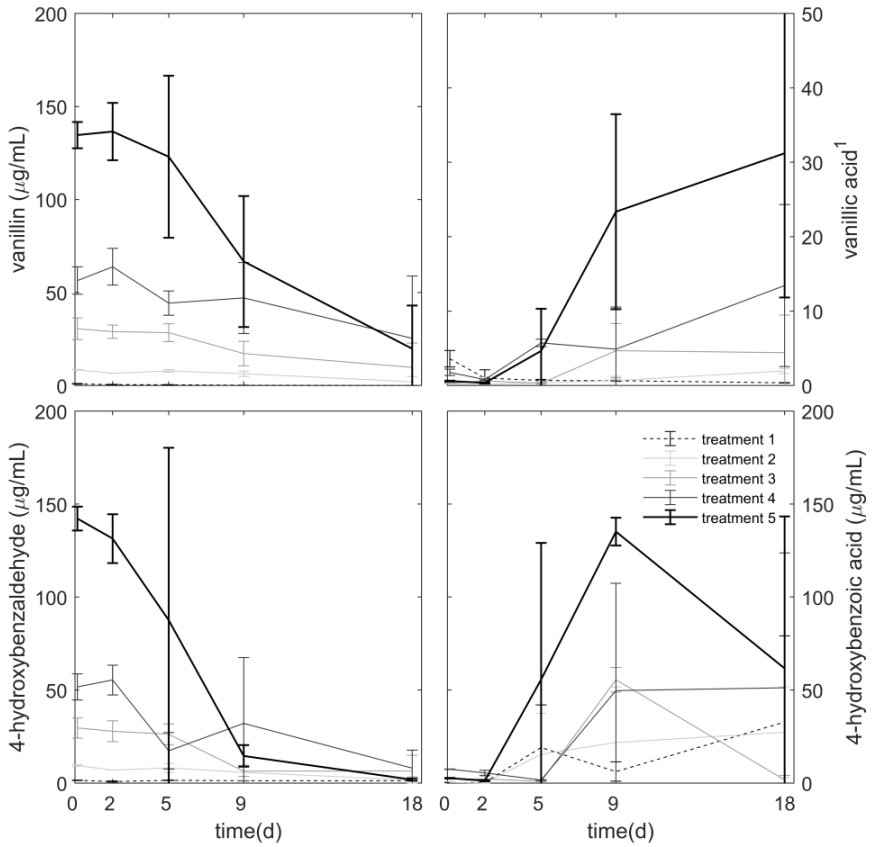


Figure 3.7 Transformation of lignin phenols amendments supplemented at 5 treatment levels. LLE-GC-MS results for aqueous phase analysis. [1]areas for vanillic acid were adjusted for the internal standard, and an arbitrary calibration slope of 1 applied; based on comparison of slopes obtained for other lignin phenols, true values estimated to be 2-5 times greater.

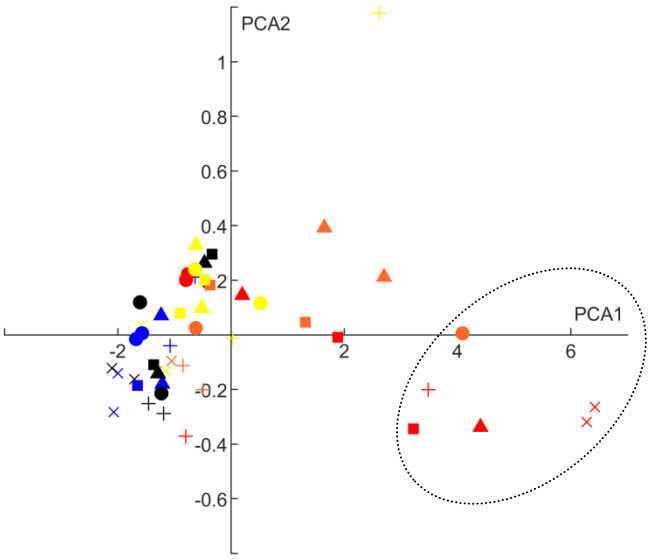
3.3.3. Influence of lignin phenols

Amended vanillin and 4-hydroxybenzaldehyde followed a general pattern of logistic decay over the 18-day period, which resulted primarily in the formation of vanillic acid and 4-hydroxybenzoic acid, respectively (Figure 3.7). Transformation of 4-hydroxybenzaldehyde was somewhat more rapid than vanillin, with the majority removed by day 9. Concentrations of 4-hydroxybenzoic acid also reached maximum or near-maximum average levels for treatments 2-5 at day 9. By day 18, a few individual samples showed continued increases, but the majority exhibited decline, demonstrating further transformation of this product. Increased levels of 4-hydroxybenzoic acid in control samples indicate that additional processes contributed to the formation (or leaching) of this compound besides the direct transformation of the amended phenols. Vanillin, 4-hydroxybenzaldehyde, and vanillic acid were not observed in control

samples throughout the experiment. All samples in the ET group exhibited increased removal of amended lignin phenols, with near elimination in ET samples (Figures 3.4 and 3.5), i.e. >97% removal in nearly all cases, with one case at 75% removal, compared to average removal of 35% vanillin and 63% 4-hydroxybenzaldehyde in the non-ET group. 4-hydroxybenzoic acid was also nearly eliminated in ET but not non-ET samples, while vanillic acid declined more rapidly in non-ET samples at 18 days, perhaps due to reduced transformation of vanillin.

In addition to 4-hydroxybenzoic and vanillic acid, four additional transformation products increased with lignin phenol treatment level: three unknown transformation products (L1-L3 Figure 3.5, top colourmap) and gentisic acid - a known transformation product of 4-hydroxybenzaldehyde, salicylic acid, p-cresol, and naphthalene (Lubbers et al., 2019). Gentisic acid (init. 0.003-0.60 μg/mL Treatments 1-5) and L1 were present as early as the 3 hr sampling time point and followed similar patterns to vanillin; L2 and L3 (RT 15.9, 16.6) followed trends observed for vanillic acid. Spectra for L1 and L3 exhibited strong similarity to 4-hydroxybenzoic and vanillic acid (i.e. dominance of m/z 297, 267, 223, 193, 190, 147, and 73) suggesting shared structural features; L2 exhibited a different pattern (m/z 281>237>207>73>296). None of these compounds corresponded to the most commonly reported transformation products for vanillin and 4-hydroxybenzaldehyde, i.e. vanillyl alcohol, 4-hydroxybenzyl alcohol, and 3,4-hydroxybenzoate (as TMS derivatives), and hydroquinone (Lubbers et al., 2019; Nishimura et al., 2018). It is possible that these compounds reflect the inducement of alternate transformation pathways of either amended phenols or other aromatic soil components.

Although no lignin phenol treatment level clearly outperformed any other in terms of aqueous or soil PAH attenuation, or in contributing ET samples, a PCA of the targeted PAH metabolites suggests lignin phenol amendments may have influenced the overall profile of targeted non-lignin phenol transformation products (Figure 3.8). PCA is a common tool used for exploratory analysis of multivariate chemometric datasets and involves the linear combination of variables to project the dataset into reduced dimensional space; by definition, the “new” first dimension or principal component 1 (PCA1) describes the greatest amount of variation in the data set, the second, (PCA2) the second greatest, etc. (Ballabio, 2015).



analyte	coefficients	
	PCA1	PCA2
1-indanone	0.0014	0.6328
salicylic acid	0.9912	0.0638
1-hydroxynaphthalene	0.1222	-0.4152
(1,2) *trans*-naphdiol	-0.0032	0.0485
9-fluorenone	0.0060	0.0434
1-hydroxyacenaphthene	-0.0200	0.6257
9-hydroxyfluorene	0.0189	0.1377
1-hydroxy-2-naphthoic acid	0.0410	-0.0824
9,10 anthraquinone	-0.0073	0.0306
9-hydroxyphenanthrene	0.0018	0.0219
1-hydroxypyrene	-0.00005	0.0004

Figure 3.8 Principal components analysis of PAH metabolite profile (PCA 1 and PCA2). Time: black - 3 h, blue - 48 hr, yellow - 5 d, orange - 9 d, red - 18 d; Lignin phenol treatment level ○ Treatment 1 (0 µg/mL), □ Treatment 2 (6 µg/mL), △ Treatment 3 (30 µg/mL), + Treatment 4 (60 µg/mL), × Treatment 5 (120 µg/mL); circled region identifies ET samples. PCA1 and PCA2 explained 97.5 and 2% of the total variability, respectively. Coefficients (right) indicate the extent to which individual analytes compounds influence each component, with absolute values approaching 1 indicating greater influence.

ET samples were associated with increasingly positive distribution along PCA1, which was most strongly influenced by changes in salicylic acid concentration and also suggested an overall trend with time (colour shift across PCA1). Linear regression analysis showed that PCA1 scores were well correlated with time ($p < 0.01$ $R = 0.66$) and with other treatment + time-dependent measures including removal of amended lignin phenols, formation of phenolic acids, and reductions in pH ($p < 0.01$ $|R| = 0.44$-0.60). Although this is not surprising, it is interesting that other samples at day 9 most resembling ET samples (i.e, positively distributed along PCA1) were found in Treatments 1-3, where levels of amended lignin phenols were very low. This could suggest that elevated levels of unconverted lignin phenols may have inhibited the initiation of ET processes. When samples from T1-9 days were further considered together, the production of select PAH transformation products appeared to be comparatively inhibited in Treatment 5 samples, and for some analytes also in Treatment 4 (Figure 3.9). However, it should be noted that at 18 days, Treatment 5 samples supported highest levels of salicylic acid, 1-hydroxynaphthalene, and 1-hydroxyphenanthrene (9.2, 1.48, 0.045 µg/mL, respectively), exceeding levels observed for other ET samples by 25-500% (those samples with concentrations ranging 6.1-7.3,

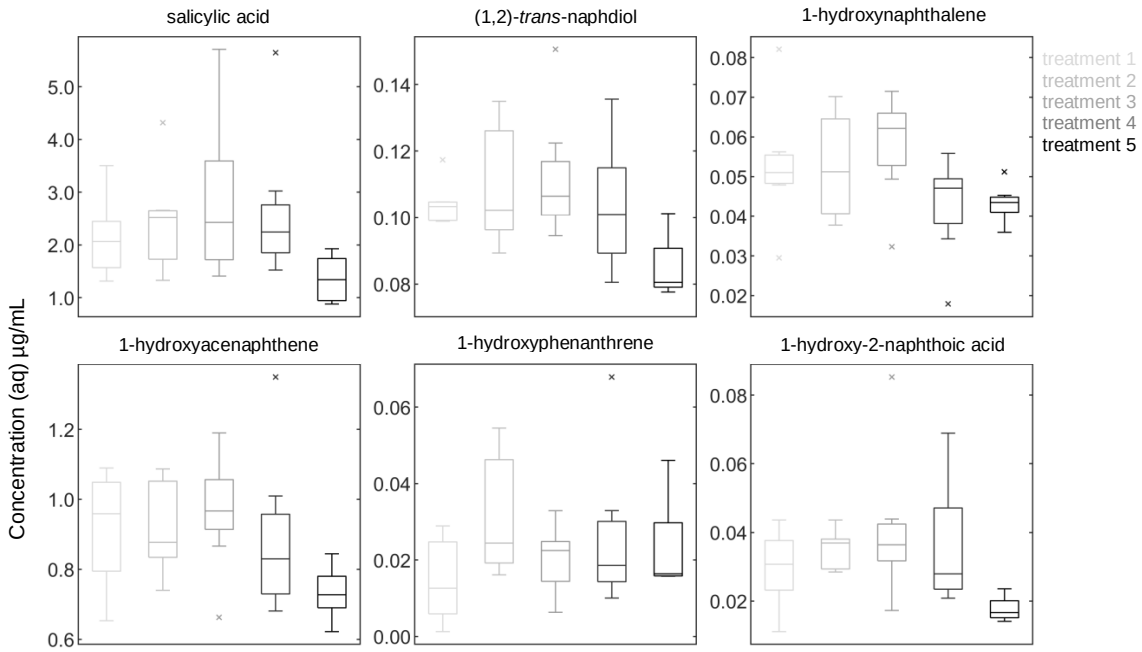


Figure 3.9 Aqueous PAH transformation products in microcosms from T1-9 days (non-ET samples). Whiskers display minimum and maximum values within ~2.7s, outliers (x) are plotted where values exceed the $75^{th}/25^{th}$ percentile ± the maximum whisker length times the interquartile range (MATLAB 2018).

0.3-0.97, and 0.01-0.02 µg/mL, respectively). This suggests that once the putative inhibitive effect was overcome, the highest lignin phenol amendment may have stimulated specific pathways associated with naphthalene and phenanthrene attenuation. To a lesser extent, trends in Figure 3.9 also suggest that prior to clear initiation of ET processes, moderate levels of lignin phenol amendment may have had a stimulatory effect to the production of some transformation products, particularly 1-hydroxynaphthalene and 9-hydroxyphenanthrene when compared to control samples. Although it described only a small part of the overall variability in PAH transformation products, PCA2 also appeared to exhibit a weak relationship with treatment level separate from time alone, as Treatments 4 and 5 were primarily distributed in the negative direction of PCA2, and Treatment 1 control samples primarily in the positive direction. Linear regression analyses confirmed PCA2 scores were not correlated with time or pH but were weakly correlated with vanillin removal ($p = 0.04$ $R = -0.30$), suggesting this process may have secondarily influenced the overall profile of PAH metabolites in both ET and other samples. Given the higher component loadings of 1-hydroxynaphthalene and 1-hydroxyacenaphthene, PCA2 may have in part captured the trends presented in Figure 3.9.

3.4 Discussion

Chromatographic analysis of lignin phenol-amended PAH microcosms revealed transformational processes that led to the attenuation of PAH, lignin phenols, and other aromatic compounds, and to the formation of new compounds. HPLC-Biphenyl-DAD and LLE-GC-MS were shown to be complementary techniques for observing qualitative and quantitative changes in the aqueous phase. Inter-laboratory proficiency studies should be undertaken to further validate the use of these methods over a range of samples. It is noted that some OHPAH and other polar transformation products detected in the aqueous phase were not detected in the soil extracts, which indicates further method development is needed to detect these compounds in contaminated soils – this project is undertaken in Chapter 4.

While this study did not specifically address abiotic vs. biotic transformation, identified transformation products and trends were consistent with known aerobic PAH and lignin phenol catabolic pathways, offering suggestive evidence that microbial transformation was the primary mechanism of PAH and phenolic degradation. Overall rates of PAH attenuation were low when compared to other aqueous incubation studies with or without soil and/or bacterial enrichments, which may exhibit removal rates of 40-90% (initial concentrations 10-250 μg/mL) within 7-28 days (Heitkamp et al., 1987; Pathak et al., 2009; Sharma et al., 2016). Low attenuate rates could be due to the comparatively high proportion of other chemicals present in the soil, which may cause competitive metabolism and slow the initiation of PAH catabolism (Johnsen et al., 2005). If this initial lag phase must be overcome, it could explain why only some samples exhibited ET processes in the 18-day period. Previous reports of biologically-treated creosote-contaminated soil and groundwater have demonstrated that the pattern of attenuation is phenolics > heterocyclics > LMW PAH > HMW PAH > pentachlorophenol, and importantly, that activity towards LMW PAH significantly increased after phenolic compounds were largely removed (Mueller et al., 1991; Wilson and Jones, 1993). This pattern seems to have held in the current study, as lignin phenols declined most rapidly, and phenol, cresols, C2-alkylphenols, and several lignin phenol byproducts were substantially removed only in the ET samples. Additionally, this pattern may help explain why Treatment 5 and Treatment 4 exhibited reduced abundance of specific PAH transformation products in non-ET samples.

In addition to the possibility that phenols represent a barrier to PAH degradation processes and must be utilised first, it is also possible that their initial presence ultimately helps stimulate degrader species through activation of metabolic pathways used during PAH catabolism, or influences the abundance of PAH degrading organisms. It has been demonstrated that for PAH degrader *P. putida* KT 2440, exposure to 4-hydroxybenzoate and vanillin induces activation of enzymes used for benzylic ring opening which may also be involved in the transformation of the condensed ring structure of PAH (Kim et al., 2006). Deveryshetty et al., (2007) showed that growth of phenanthrene degraders *Pseudomonas sp.* strain PPD and *Alcaligenes sp.* strain PPH was increased through culturing on 4-hydroxybenzoate and protocatechuate - common lignin phenol transformation compounds - and it is expected more broadly that lignin phenols may serve as an alternate energy source for PAH degraders (Olson et al., 2003). More generally, in agricultural soils, vanillic acid has been shown to stimulate growth and increase diversity of soil microbial consortia at application rates of 50-100 µg/g soil and to inhibit growth and show greater community selection at higher concentrations of 200 µg/g soil (Qu and Wang, 2008). In the present study, it is possible that the high levels of other phenolic compounds present provided sufficient 'training ground' for PAH degrader species, limiting the relative influence of the amended lignin phenols on ET processes. At the same time, elevated levels of specific PAH transformation products especially observed in Treatment 5 at 18 days may represent this type of stimulatory effect, and for soils with a smaller proportion of available phenolic compounds, the addition of natural aromatic compounds including lignin phenols could yet be revealed to modulate PAH degradation pathway or PAH degrader community. Additional microbiological studies of whole-soil systems would be valuable for better understanding the influence of lignin phenol abundance on the structure and activity of the PAH degrader community.

While the results presented in this study suggest that PAH transformation may be enhanced after the utilisation of high levels of amended lignin phenols, further work is required to establish causative rather than correlative relationships. Future studies should consider using soils that have been substantially depleted of phenolic compounds prior to treatment, and could be especially impactful if trialed using soils where PAH attenuation rates have plateaued during remediation. Ultimately, as it can be expected that different soils may behave differently, a large number of soil types will need to be investigated to further assess the generalisability of any conclusions. Future studies

could benefit from changing the experimental design to use continuous rather than sacrificial sampling. The sacrificial sampling approach used here is most useful to ensure sampling processes do not disrupt the experimental conditions (including loss of volatile components, changes in aeration, mixing etc.), and may be especially important for microbiological assessments. The sacrificial sampling approach is also beneficial when headspace analysis is undertaken. Initial exploration of headspace analysis (1 mL gas phase sampling/injection volume) suggested that select LMW PAH and higher-concentration OPAH (1-indanone, 9-fluorenone) might be detectable in the headspace, but other target compounds were not detected, which may be attributed to reduced volatility and detectability of the underivatised polar compounds (Schummer et al. 2009), Based on the comparative utility of LLE for this study, further development of headspace methods were not pursued. Despite potential benefits of sacrificial sampling, using continuous sampling of larger experimental units would offer other benefits: this approach would allow monitoring of single experimental units over time, even out the effects of small changes in individual samples (reduce issues of high variability within sample treatments as observed during this study), offer the potential for applying higher concentration factors during LLE, and allow the researcher to extend the experimental timeframe beyond the initial set up conditions e.g. if rates of attenuation are low, or further transformation processes are of interest.

3.5 Conclusions

Although a definitive relationship between lignin phenol amendment level and initiation of observed enhanced PAH transformative processes could not be established, lignin phenols participated in and may have modulated these processes. Lignin phenols directly influenced the distribution of specific transformation products including gentisic acid and 4-hydroxybenzoic acid, known from both PAH and lignin phenol transformation pathways, and especially when applied at highest concentrations of 120 µg/mL, lignin phenols appeared to induce an inhibitory phase followed by a stimulatory phase on naphthalene and phenanthrene byproduct formation. These results are consistent with the view that natural aromatic compounds are involved with PAH degradation, and that enhanced transformation of PAH and degradation byproducts may be stimulated after removal of more easily degraded phenolic compounds. Increasing free concentrations of lignin phenols could help support bioremediation efforts where

population or activity of PAH degraders is low, including late stages of compost-based bioremediation. We further support other findings which have demonstrated the formation of soluble toxic PAH intermediates during short-term incubation, including elevated concentrations of catechol, salicylic acid, 1-hydroxynaphthalene, 9-hydroxyfluorene, 9-fluorenone, and 9-hydroxyphenanthrene, as well as 18+ unknown compounds identified through the developed HPLC and LLE-GC-MS methodologies presented here. As our findings suggest variation in the overall pattern of PAH transformation products may be influenced by the utilisation of lignin phenols, we recommend monitoring both lignin phenols and PAH transformation products in compost-remediation scenarios.

Acknowledgements

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Chapter 4

Recovery of polycyclic aromatic hydrocarbons and their oxygenated derivatives in contaminated soils using aminopropyl silica solid phase extraction

This chapter has been previously presented as an article in the journal Chemosphere

Pulleyblank, C., Kelleher, B., Campo, P., Coulon, F., 2020. Recovery of polycyclic aromatic hydrocarbons and their oxygenated derivatives in contaminated soils using aminopropyl silica solid phase extraction. Chemosphere 258, 127314.

Abstract

In this study we developed the first method using aminopropylsilica solid phase extraction (SPE) for the analysis of OPAH, OHPAH, COOHPAH alongside PAH in soil extracts. We further investigated the efficacy of the developed method for three soils representing a range of contamination levels and soil textural characteristics and assessed the impact of different sample preparation steps on the recovery of targeted compounds. Average recovery of PAH, OPAH, and OHPAH standards for excluding the most volatile compounds 1-indanone and catechol were 99%, 84%, and 86%, respectively for the SPE method. In contrast, COOHPAH exhibited the lowest recovery (0-82%) and poor inter-batch reproducibility. Soil texture and contamination levels influenced full method efficiency. Specifically, the soils with higher proportion of clay contributed to the loss of the higher molecular weight OHPAH prior to SPE. Soils with the highest contamination showed enhanced recovery of some lower-concentration mid-weight PAH and OPAH, while the least contaminated soils exhibited greater sensitivity to evaporative losses during sample preparation. Recommendations for reducing and/or accounting for matrix effects as well as the practice of using deuterated PAH surrogate standards for OPAH analysis are further discussed. Quantitation of recovered PAH and oxygenated PAH across the three soils showed high reproducibility (<10% relative standard deviation for a majority of compounds), supporting the use of this method for monitoring PAH, OPAH, and OHPAH at contaminated sites.

4.1 Introduction

A greater understanding of oxygenated PAH transformation products with a range of chemistries is expected to improve decision making at contaminated sites; however, as discussed in Chapter 2, this research is currently hampered by a lack of standard protocols for identifying and quantifying these compounds in environmental matrices. Chapter 3 emphasised analysis of the aqueous phase, while this chapter directly addresses improving methods for soil analysis. Effort has been made towards routinising OPAH analysis (Lundstedt et al., 2014), with the most typical approach involving extraction of the soil with dichloromethane, hexane, or hexane/acetone, followed by a clean-up or fractionation step over silica or strong base sorbents using column chromatography or solid phase extraction (SPE). However, only a few studies have extended these methods to include OHPAH and COOHPAH (Bandowe and Wilcke, 2010; Chibwe et al., 2015; Meyer et al., 1999), and there has been little broader uptake in the use of these methods (Table 2.2). Further work is needed to address specific limitations of available methods such as uncertain quantitative performance (Chibwe et al., 2015; Meyer et al., 2013), uncertainty in the best application of surrogate recovery compounds, low recovery of OHPAH and COOHPAH (Bandowe and Wilcke, 2010), and/or the amount of material and complexity of the methods (Chibwe et al., 2015; Meyer et al., 1999). In addition, available methods have thus far reported recovery of oxygenated PAH from standard solutions or single spiked uncontaminated soils (Bandowe and Wilcke, 2010, Meyer et al., 1999). Yet even amongst soils, variations in soil texture and contamination level may also be expected to affect the identification and quantitative recovery of target analytes. As discussed in Chapter 2, a greater understanding of method performance across multiple preparative stages and soil types could reduce uncertainties in method applicability, help focus efforts to improve the most relevant steps of the protocols, and increase adoption and standardisation of methods.

To date, the utility of weak base aminopropyl silica for the fractionation of PAH, OPAH, OHPAH, and COOHPAH from soil extracts has not been investigated. Retention characteristics of aminopropyl silica are primarily influenced by the terminal primary amine group which increases hydrogen bonding capacity and offers basic functionality that can be modulated by adjusting eluent pH and solvent polarity. The capacity of aminopropyl silica to allow the separation of a wide range of neutral, polar,

and acidic lipids in crude sediment extracts has been demonstrated (Murphy et al., 2016; Pinkart et al., 1998), suggesting it could also be applied to enhance separation of oxygenated PAH of multiple functionalities. An SPE method using aminopropyl silica has been proposed for the separation of nitro-substituted and oxygenated PAH in aerosol samples (Cochran et al., 2012). However, limited data have been presented for the recovery of oxygenated PAH and the use of methanol for the elution of OHPAH requires full dry-down and solvent exchange step prior to derivatisation, which may contribute unnecessarily to analyte loss. At the same time, protocols developed for alternate sample types may not be suitable for soils due to the possibility of substantial matrix effects (Avagyan et al., 2015; Cochran et al., 2012).

Therefore, the aim of this study was to investigate the use of aminopropyl silica SPE for the fractionation of target PAH and their transformation products in crude soil extracts, specifically isolating three fractions for subsequent GC-MS analysis: (A) less polar PAH and OPAH which can be analysed together without requiring derivatisation; (B) moderately polar compounds, OHPAH and related natural non-acidic phenolic compounds requiring derivatisation, but avoiding solvent exchange via sample dry down; and (C) acidic transformation products, including COOHPAH and related acidic phenolic compounds, which requires removal of protic, acidic, or basic solvents prior to derivatisation via silylation (Schummer et al., 2009). We further investigated the application of the method to three soil matrix types, presenting the first study to consider the impacts of soil textural class and contamination level on the method recovery of this broad range of polar aromatic compounds. In order to elucidate factors affecting the selected methodology, this study separately examined recovery and the effect of matrix using a four-stage approach:

Stage 1: SPE method development using analytical standards: comparison of eluents, load volumes, linearity tests, consideration of post-SPE evaporative losses;

Stage 2: testing the SPE method with pre-prepared soil extracts spiked with target analytes (hereafter Matrix-SPE tests); comparison of three soils and standards. This helped elucidate whether other constituents in the soil extracts specifically influence recovery of the SPE method, e.g. through competition for sorption sites on the SPE column, and the extent to which whole-method recovery is affected at the SPE stage;

Stage 3: full method recovery testing - analytes spiked into soil prior to extraction; comparison of recovery for three soils. When combined with Stage 2 results, this helped elucidate the extent to which losses occur during the extraction step including specific matrix-associated effects; and,

Stage 4: Qualitative and quantitative characterisation of the original unspiked soils.

Finally, surrogate standards, i.e. compounds of known concentration and similar chemistry to target analytes, typically isotopically labelled, are frequently added prior to extraction or SPE. By tracking the amount of surrogate lost (or gained), the recovery of related target analytes can be estimated, and/or final target analyte concentrations may be adjusted or "corrected" for losses/ gains/ matrix effects introduced during sample processing. Although the availability of deuterated OPAH internal or surrogate standards is improving, these compounds are not widely available, and there is uncertainty in their best use during OPAH analysis (Ref Ch. 2.4.1). In the meantime, more readily available deuterated PAH are sometimes used as surrogates estimating OPAH recovery or correcting for losses during sample preparation (Obrist et al. 2015). We investigated the impact of using deuterated PAH to estimate recovery of OPAH during each stage of the study in order to evaluate this practice.

4.2 Methods

4.2.1 Chemical reagents and materials

Target PAH, transformation products, and internal standards were obtained from Sigma-Aldrich including 13 2.5-6 ring PAH in EPA semi volatile mix B (Table 4.1). Supelco Discovery aminopropyl silica SPE columns (500 mg/3 mL), derivatisation agent BSTFA with 1% TMCS, sodium sulfate, hydrochloric acid, and triethylamine (TEA) were also obtained from Sigma Aldrich. Deuterated PAH used for surrogate recovery were obtained as a mixture (2000 µg/mL each in acetone) from Thames Restek, UK. Solvents hexane (HEX), dichloromethane (DCM), acetone (ACE), acetonitrile (ACN) methanol (MeOH) were analytical reagent grade or higher and were obtained from Fischer Scientific. Amber vials used for extraction and SPE eluent collection were acid washed, rinsed with deionised water, then furnaced at 450°C for 4 h to remove residual organic materials. Volumetric glassware used during the preparation of standards, spike solutions, and samples, was rinsed 3x in acetone and 3x

Table 4.1 GC-MS method for target PAH and oxygenated transformation products

Target analyte	CAS#	Mass g/mol	SR grp [1]	RT min	SIM m/z **quant.,** ref	R^2	LOD[2] (µg/mL)	LOQ[2] (µg/mL)	Fig.4[3]
PAH									
Naphthalene	91-20-3	128.2	SR1	7.79	1**28**, 64	0.999	0.009	0.030	a
Acenaphthylene	208-96-8	152.2	SR2	12.34	**152**, 126	0.999	0.003	0.012	b
Fluorene	86-73-7	166.2	SR2	14.37	**166**, 83	0.999	0.004	0.013	c
Phenanthrene	85-01-8	178.2	SR3	17.22	**178**, 89	0.998	0.005	0.017	d
Anthracene	120-12-7	178.2	SR3	17.38	**178**, 89	0.998	0.004	0.013	e
Pyrene	129-00-0	202.3	SR3	22.90	**202**, 101	0.999	0.003	0.009	f
Benz[a]anthracene	56-55-3	228.3	SR4	29.80	**228**, 114	0.998	0.001	0.004	g
Chrysene	218-01-9	228.3	SR4	30.11	**228**, 114	0.998	0.001	0.004	h
Benzo[b]fluoranthene	205-99-2	252.3	SR5	36.78	**252**, 126	0.998	0.002	0.006	j
Benzo[k]fluoranthene	207-08-9	252.3	SR5	36.92	**252**, 126	0.997	0.003	0.009	k
Benzo[a]pyrene	218-01-9	252.3	SR5	38.69	**252**, 126	0.997	0.002	0.006	l
Indeno[1,2,3-cd]pyrene	193-39-5	276.3	SR5	45.21	**276**, 138	0.999	0.002	0.008	m
Dibenz[a,h]anthracene	53-70-3	276.3	SR5	45.48	**278**, 139	0.999	0.004	0.015	n
Benzo[ghi]perylene	191-24-2	276.3	SR5	46.52	**276**, 138	0.999	0.003	0.009	o
OPAH									
1-Indanone	83-33-0	132.2	SR1	9.431	**132**, 104	0.999	0.002	0.007	p
9-Fluorenone[4]	486-25-9	180.2	SR2	16.60	**180**, 152	0.999	0.002	0.006	q
9,10-Anthraquinone	84-65-1	208.2	SR3	20.24	**208**, 180	0.999	0.002	0.007	r
9,10-Phenanthrenequinone	84-11-7	208.2	--	24.21	**208**, 180	nq[5]	nq	nq	-
OHPAH									
Catechol	120-80-9	110.1		9.98	**254**, 151	0.999	0.003	0.011	nd[6]
1-Hydroxynaphthalene	1779-10-8	144.2		13.65	**216**, 201	0.999	0.003	0.011	s
1-Hydroxyacenaphthene	6306-07-6	170.1		16.85	**242**, 152	0.999	0.001	0.004	t
9-Hydroxyfluorene	1689-64-1	182.2		17.45	**254**, 165	0.999	0.001	0.004	u
9-Hydroxyphenanthrene	484-17-3	194.2		22.45	**266**, 251	0.999	0.002	0.008	w
1-Hydroxypyrene	5315-79-7	218.3		30.41	**290**, 175	0.998	0.003	0.010	x
Phenolic aldehydes									
4-Hydroxybenzaldehyde	123-08-0	122.1		10.90	**179**, 151	0.999	0.003	0.010	A
Vanillin	121-33-5	152.1		13.52	**194**, 201	0.999	0.003	0.009	B
COOHPAH									
1-Naphthylacetic acid	86-87-3	186.1		17.00	**258**, 168	0.999	0.059	0.197	C
Fluorene-9-carboxylic acid	1989-33-9	210.2		19.58	**282**, 165	nq	nq	nq	-
1-Hydrox-2-naphthoic acid	86-48-6	188.2		20.45	**317**, 243	0.996	0.001	0.004	D
Phenolic Acids									
Salicylic acid	69-72-7	138.1		13.15	**267**, 209	0.999	0.004	0.014	E
4-Hydroxybenzoic acid	99-96-7	138.1		14.83	**267**, 193	0.999	0.026	0.087	F
2,5-Dihydroxybenzoic acid	490-79-9	154.1		16.98	**355**, 297	0.999	0.001	0.004	G
Ferulic acid	1135-24-6	194.2		22.17	**338**, 323	0.998	0.010	0.036	H
Deuterated PAH for surrogate recovery (SR)									
SR1- Naphthalene-d_8	1146-35-2	136.2		7.75	**136**, 108	--	--	--	i
SR2- Acenaphthene-d_{10}	15067-26-2	164.3		12.86	**164**, 162	--	--	--	ii
SR3- Phenanthrene-d_{10}	1517-22-2	188.3		17.22	**188**, 160	--	--	--	iii
SR4- Chrysene-d_{12}	1719-03-5	240.4		30.00	**240**, 120	--	--	--	iv
SR5- Perylene-d_{12}	1520-96-3	264.4		39.08	**264**, 132	--	--	--	v
Internal standards									
Nonadecane-d_{40}	39756-36-0	308.8		18.12	**66**, 98	--	--	--	I
Triacontane-d_{62}	93952-07-9	485.2		40.37	**66**, 98	--	--	--	II

[1] SR group identifies surrogate standard used for recovery adjustment
[2] LOD and LOQ based on 1 µL injection.
[3] peak reference for Figure 4.4.
[4] cautiously quantitative, see text for further discussion.
[5] nq not quantitative, see further discussion in text.
[6] nd not detected in sample displayed in Figure 4.4

in the 'incoming' solvent and wrapped in foil below the fill line in order block excess light which can cause transformation of aromatic compounds (Woudneh et al., 2016). PTFE lined screwcaps were rinsed 3 times with acetone prior to use.

4.2.2 Soil characteristics

In order to consider the influence of soil matrix on SPE method performance, three soils with varying texture and contamination level were investigated. As the goal of this study was not full characterisation of the site, the sampling scheme focused on obtaining substantial amount of materials from a single location that could be used in this study as well as the larger incubation study presented in Chapter 5. Soils 1 and 2 were contaminated soils obtained from former gasworks sites in the southern UK. Both soils were associated with the location of former tar tanks. Soil 1 had been previously excavated and was received 'as is' (appx. 100 kg) (Ecologia Environmental Solutions Limited). Soil 2 (appx. 150 kg) was hand dug at a depth of 10-30 cm based on prior evidence of contamination and permission from the associated remediation company (Paddock Geo Engineering). Soil 3 was obtained from a residential garden area in Bedford UK (10-15 cm depth, appx 5 kg) to provide comparison to a relatively uncontaminated soil. Soils were loosely wrapped in foil and air dried overnight, then ground and sieved to 2 mm, and appx 1 kg sieved material was stored frozen at -80°C before further analysis. Subsamples of each soil were prepared using the coning and quartering method outlined in USEPA guidance document EPA/600/R-03/027 (Gerlach and Nocerino, 2003). Soil pH was measured in 0.01 M $CaCl_2$ (5:1 liquid:solid) following ISO procedure 10390 (2005). Total soil organic matter was determined

Table 4.2 Basic soil characteristics for three test soils

Soil	Location (UK)	Contamination level	Soil texture	Sand %	Silt %	Clay %	LOI %	pH $CaCl_2$
Soil 1	Kent former gasworks	very high strong tar odour	coarse sand	88.3	8.1	3.6	20.1	8.4
Soil 2	Northamptonshire former gasworks	moderately high	fine sandy loam	51.8	22.8	25.3	8.1	7.4
Soil 3	Bedford garden soil	very low	clay	31.6	28.8	39.6	11.0	7.2

as loss on ignition (LOI): oven dried soils (24 h 105°C) were heated to 450°C for 5 h to ash organic material, and the mass lost was calculated as a percentage of the total oven-dried mass (BS EN 13039, 2000). Soil texture was determined by sieving and sedimentation procedure (ISO 11277, 2009; Natural England TIN037 2008).

4.2.3 Developing SPE fractionation protocol with analytical standards (Stage 1)

The final SPE method is illustrated in Figure 4.1b. All SPE tests were performed using a vacuum manifold. For each cycle, gravity feed was used until only hold up volume remained, after which a gentle vacuum was applied to elute residual solvent. This provided more consistent flow rates in our setup than continual use of the vacuum pump. For all tests, SPE blanks were prepared using all materials but no 'load' solution.

During method development, several eluent systems were tested to optimise recovery of analytical standards. Each cartridge was conditioned with 6 mL of the first test eluent, then loaded with 100 µL of a test spike mixture containing 20 µg/mL each target analyte (PAHM) and 5 µg/mL each deuterated PAH (PAHd) in 1:1 DCM:ACN. Eluent selection was considered using a sequential process, where the preferred solvent system for fraction A was determined then used first during eluent selection for fraction B, and both systems applied prior to testing eluents for fraction C. For each fraction, two full column volumes (6 mL) were used. Protic solvents such as methanol, which have been used in previous methods for the elution of OHPAH, were avoided for fraction B in order to eliminate solvent exchange prior to derivatisation and reduce associated analyte loss. Eluents tested were as follows: for fraction A - HEX, DCM, and HEX:DCM (9:1); for fraction B - ACE, ACN, and 4 mL ACE followed by 2 mL ACN (ACE+ACN); for fraction C - 0.5% HCl in MeOH, and in ACE, ACN, and ACE:H_2O (9:1), as well as ACE:H_2O:TEA (90:10:0.05) and ACN:H_2O:TEA (90:10:0.05). Fraction C tests with HCl and no water were conducted in duplicate, while all other tests were conducted in triplicate. For eluent selection tests, post-SPE processing was conducted as in Figure 4.1c, with the exception that fraction A was also derivatised in order to detect early-eluting OHPAH, and fraction B was not subject to further concentration under N_2.

After final selection of the eluent system, increased load volumes of 1 mL and 500 µL were tested, but this led to early elution of OHPAH in fraction A, so 100 µL was maintained for all subsequent tests. The linearity of the method was evaluated

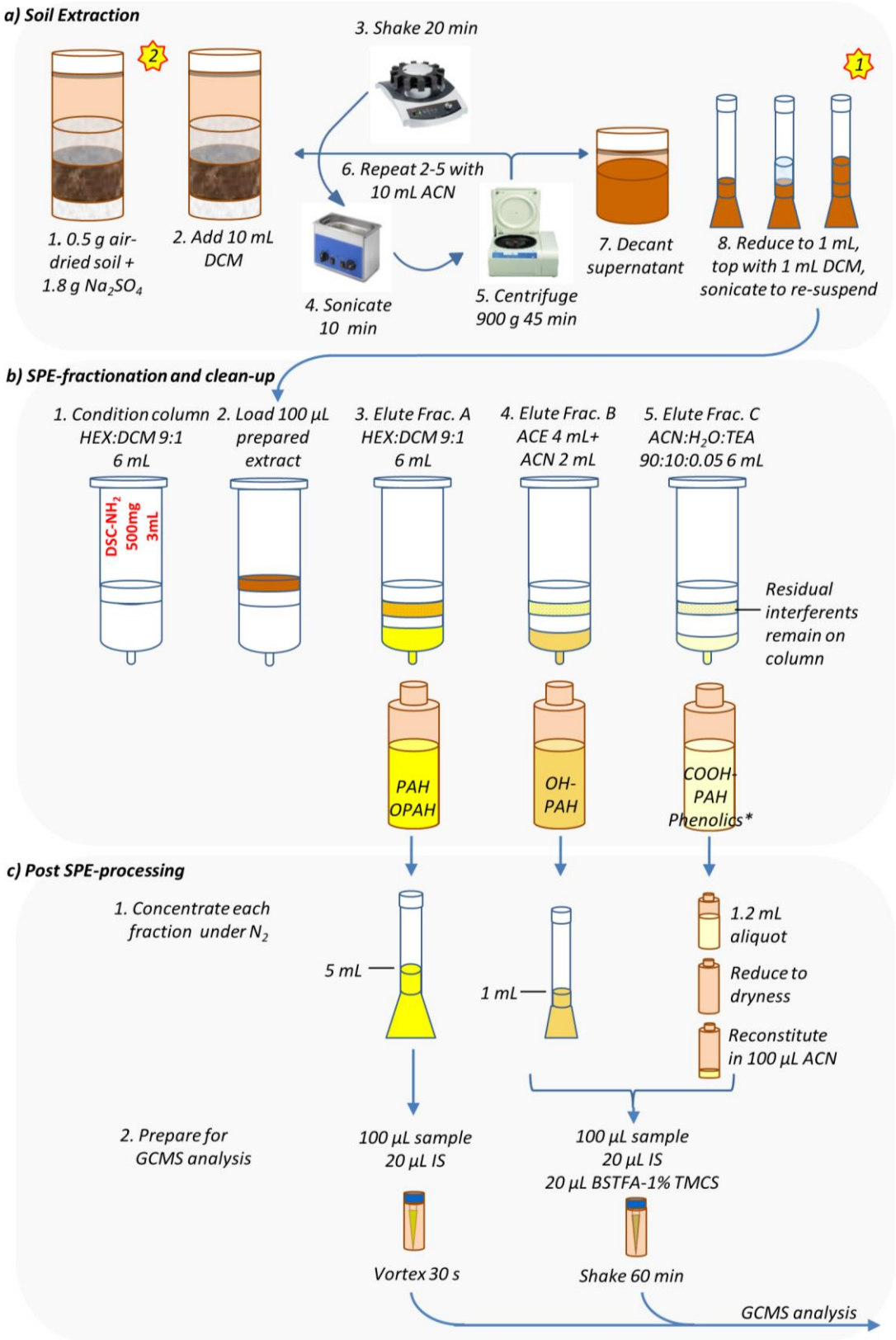

Figure 4.1 Method for the analysis of PAH and their oxygenated derivatives in crude soil extracts using aminopropyl silica SPE: a) extraction b) fractionation and clean-up, c) post-SPE processing. Gold stars 1 and 2 indicate points where spiked compounds were added to assess recovery from Matrix-SPE tests and full method-recovery respectively. *qualitative analysis.

through triplicate tests of PAHM at 10, 1, 0.1, and 0 μg/mL (the last as blanks), each with 5 μg/mL PAHd. As genuine samples would often require further concentration for the analysis of transformation products, fractions A and B were further reduced to 1 mL under N_2 prior to instrumental analysis.

4.2.4 Preparation of soil extracts for Stages 2 - 4:

The preparation of soil extracts using ultrasonic assisted extraction is shown in Figure 4.1a. Preliminary tests using 1 g soil demonstrated that Soil 1 extracts became very tarry and difficult to re-suspend after initial concentration. Therefore, a 0.5 g mass was selected and used for all soils to maintain consistency during these trials. Each sample was extracted in two cycles, first with 10 mL DCM, then with 10 mL ACN to target polar transformation products (Wang et al., 2012). The supernatants from each extraction cycle were combined and reduced to 1 mL then topped with an additional 1 mL DCM and sonicated briefly in order to re-suspend materials which had collected on vessel walls during the concentration steps and to maintain consistency of the 1:1 ACN:DCM load solvent system used for all SPE tests. All extractions, including blanks (all reagents except soil), were conducted in triplicate.

4.2.5 Matrix-SPE tests - testing the SPE-method with spiked soil extracts (Stage 2)

To investigate the effect of the soil matrices on target analyte recoveries through the SPE method alone, 1 mL of each previously-prepared concentrated soil extract as well as 1:1 ACN:DCM solvent standard were spiked to obtain added concentrations of 20 μg/mL PAHM and 5 μg/mL PAHd (Figure 4.1- starred point 1). An additional 1 mL each soil extract/solvent standard was spiked with 5 μg/mL PAHd only in order to account for any target analytes already present in the soil extract/blank. Triplicate 100 μL aliquots were then processed as in Figure 4.1b-c.

4.2.6 Full method recovery tests and quantitation in soils (Stage 3 and 4)

In order to investigate the recovery and the effect of the soil matrices on the full method (extraction + SPE), triplicate samples of each soil were spiked prior to extraction (Figure 4.1- starred point 2) to yield target added soil concentrations of 20 μg/g PAHM and 8 μg/g PAHd, respectively. PAHM spike levels reflect a mid-range concentration of OPAH previously reported in industrially contaminated soils (Arp et al., 2014). An

additional set of samples was spiked with the PAHd mixture only to quantify contaminants present in the original soils. Six samples were also prepared with sodium sulfate and the same spike solutions to assist characterisation of losses independent of the presence of soil matrix (n=3), and as extraction blanks (n=3). During sample preparation, an additional 3 samples for each soil of approximately 1 g each were used to obtain moisture content at the time of analysis (as percent mass difference after oven drying 105°C for 24 h). The average moisture content was used to calculate final dry-mass analyte concentrations.

4.2.7 GC-MS analysis

Target compounds were analysed by GC-EI-MS (Shimadzu TQ-8040) with AOC-6000 autosampler and Lab Insight Solutions software (Shimadzu, 2015-2016). Compound separation was achieved using a 30 m Rtx-5 column (5% diphenyl/95% dimethylpolysiloxane 0.25 mm i.d., 0.25 μm df). Instrument settings were as follows - injection port: 200°C, 1 μL injections, splitless mode; column flow, 36 cms^{-1}; oven program: initial temperature 80°C, increasing 5°C min^{-1} to 100°C, then 8°C min^{-1} to 200°C, and 3°C min^{-1} to 300°C, hold for 2 min; transfer line and MS system: 300°C; EI: 70 eV SCAN /SIM mode, event times of 0.3 s and 0.060 s respectively.

To prepare samples for analysis, 100 μL sample and 20 μL of internal standard (IS) mixture (20 μg/mL nonadecane-d_{40} and triacontane-d_{62}) were added to a 200 μL glass insert inside a 2 mL amber glass GC vial then capped (silicone/PTFE) and shaken to combine. For samples requiring derivatisation, 20 μL of BSTFA 1% TMCS was also added, and vials were shaken at room temperature for 60 min. BSTFA reacts with hydroxyl and carboxyl groups, to form trimethylsilyl (TMS) derivatives; this increases the volatility of polar compounds allowing for their analysis by GC-MS.

PAH and OPAH were identified as the $M^{\cdot+}$ ion, while OHPAH, COOHPAH, and phenolic compounds were identified as their trimethylsilyl (TMS) derivatives (SIM-monitored ions are given in Table 4.1). A compound was considered identified if the retention time deviated <0.1 min from the standard, the ratio of quantitative and reference ion differed less than 30% of the analytical standard, and other ions present in the overall mass pattern showed good visual agreement with the standard spectrum. Reference check mixtures containing all analytes were run with each experiment in

order to monitor for any shifts in retention time or substantive changes in instrument sensitivity. Derivatised samples were also monitored for the presence of residual underivatised target compounds. SPE and full method blanks were also checked for interferences, and an additional blank injection was included every 10 samples to monitor for instrument carry over (ref. Sect. 3.2.8). Any contribution of a target m/z value over the specified integration period in a blank sample was subtracted from experimental samples prior to the calculation of recovery.

Calibration was conducted using the internal standard method with four point calibration curves (0.01-10 μg/mL), with 0 μg/mL serving as a blank. PAH and OPAH were prepared in DCM, while all other analytes were prepared in ACN with derivatisation. By introducing an internal standard with known concentration, it is possible to monitor and normalise GC-MS signals to account for any changes in injection volume or instrument sensitivity for a given run. Ideally each analyte would be normalised to the signal of its isotopically labelled counterpart; however in practice this is not feasible, and it is most important that the concentration-dependent signal of the target analyte and the internal standard is consistent during instrument calibration (i.e. the IS-normalised calibration curve is consistent and linear - see also Sect 3.2.9). In this study, deuterated alkanes were used as the internal standards while deuterated aromatic compounds were prioritised for use as surrogate compounds to monitor losses/gains throughout the extraction and fractionation procedures. It should be noted that recovery of surrogate compounds also accounts for any differences in instrument response introduced by the matrix, and that when an analyte signal is also adjusted by the recovery of the surrogate, the signal - and chemistry - of the internal standard becomes mathematically irrelevant. Limits of detection (LOD) and quantitation (LOQ) were established through calculation of, respectively, 3 and 10 times the standard deviation of the IS-corrected signal from seven replicate injections of the 0.01 μg/mL calibration solution divided by the slope of the calibration curve.

4.2.8 Calculations

All post-processing was conducted in Microsoft Excel (2010) or in MATLAB (2018b Academic License) using custom scripts incorporating common functions (mean, standard deviation) and linear regression analysis using the fitlm function. Recovery of the target analyte and surrogate deuterated PAH was calculated as follows:

$$Rec_t = \frac{(m_t - m_u) * df}{m_r} \times 100\% \qquad Rec_s = \frac{(m_s) * df}{m_r} \times 100\%$$

where Rec_t and Rec_s are the recovery of the target and surrogate respectively; m_t is the mass of analyte in the test sample; m_u is the mass of the analyte or interference equivalent in the unspiked sample or blank - in soil extraction tests, m_t and m_u were normalised to the mass of soil extracted in each replicate; m_s is the mass of the surrogate in the sample, m_r is the mass of the analyte in the reference load solution; and df is the relative dilution factor between the test sample and the reference load solution.

Where application of a correction factor for surrogate recovery was used, it was calculated as follows:

$$Rec_{SR} = \frac{Rec_t}{Rec_s} \times 100\%$$

where Rec_{SR} is the recovery of the target adjusted for surrogate recovery. Surrogate-adjusted values are denoted by the subscript SR

4.3 Results and discussion

4.3.1 GC-MS method performance

The majority of the target analytes demonstrated good linearity over the calibration range (R^2>0.997; Table 4.1). Limits of detection were comparable to or lower than previously reported values for EI-SIM-MS for this range of compounds (Cochran et al., 2012). Two compounds were considered to give non-quantitative results: fluorene-9-carboxylic acid, which yielded the reduced transformation product 9-fluorenone, and phenanthrenequinone, which was only observed at the highest concentration levels, and which has been reported elsewhere to exhibit rearrangement including the formation of 9-fluorenone (Bandowe and Wilcke, 2010). Therefore, while 9-fluorenone showed strong quantitative behaviour throughout the recovery experiments, the positive identification and quantitation of 9-fluorenone is treated here with caution, as it may reflect the presence of multiple compounds in actual samples. Potential contributors to the quantitation of 9-fluorenone in this study would be chemically consistent with elution in fraction A, primarily PAH and OPAH.

4.3.2 Stage 1: Development of the SPE protocol

All solvent mixtures provided satisfactory recoveries with average recovery for total PAH ranging between 102 and 104% (Figure 4.2), and for individual PAH between 82% (naphthalene-DCM) and 126% (benzo[ghi]perylene-DCM). Average recovery of larger OPAH ranged between 82 and 90%, but was comparatively poor for 1-indanone, which was also somewhat adversely impacted when surrogate adjustment was applied (46-51% vs. 40-49%$_{SR}$). It should be noted that further tests discussed below demonstrated improved recovery of 1-indanone when BSTFA derivatisation was not used. DCM improved recovery of OPAH, but led to the premature elution of OHPAH when used as sole eluent. As HEX and HEX:DCM 9:1 offered comparable results in these tests, the latter was selected since the addition of DCM improved recovery of 1-indanone specifically, and the inclusion of a more polar solvent is often recommended for PAH and OPAH analysis (Lundstedt et al., 2014).

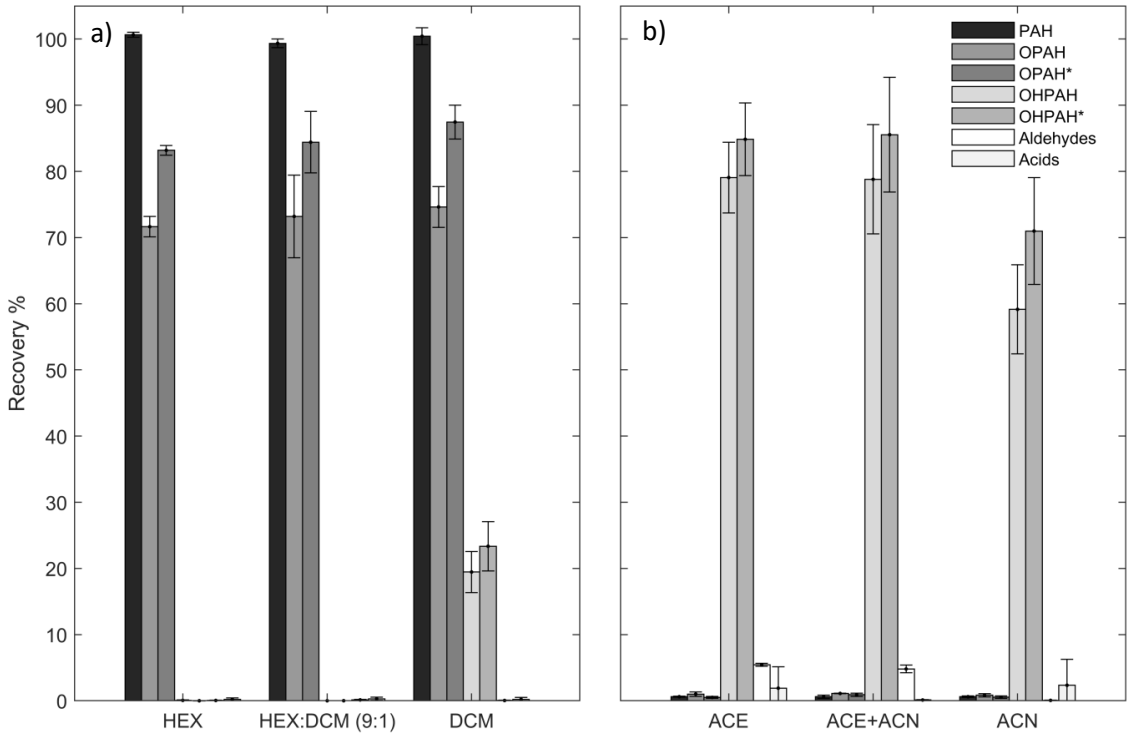

Figure 4.2 Average recovery of target PAH and derivative groups during solvent selection method development phase targeting a) Fraction A: PAH, and OPAH, and b) fraction B: OHPAH. Both fractions were derivatised with BSTFA in order to quantify polar compounds during this stage. Asterisked series omit the most volatile component: i.e. OPAH* omits 1-indanone, and OHPAH* omits catechol. Error bars indicate the standard deviation of the summary recovery of all analytes included in each group from triplicate SPE trials.

For OHPAH, the inclusion of ACE in the eluent system substantially improved recoveries over the use of ACN alone, with average recovery increasing by 15-20%, and recovery of catechol specifically increasing by 45-50%. In the ACE and ACE+ACN systems, average recoveries were respectively, for 1-hydroxynaphthalene 108 and 106%, mid weight OHPAH (1-hydroxyacenaphthene, 9-hydroxyfluorene, and 9-hydroxyphenanthrene) 82 and 85%, and heavier weight OHPAH (1-hydroxypyrene) 66 and 63%. As the two systems provided comparable results, the ACE-ACN system was selected because ACN is recommended for promoting the derivatisation of OHPAH (Schummer et al., 2009) and may be useful in reducing excessive volatilisation of analytes during concentration steps as it evaporates more slowly than acetone.

It was initially thought that all non-acidic phenolic compounds would elute in fraction B along with catechol, however 4-hydroxybenzaldehyde and vanillin did not follow this pattern. With typically less than 5% recovery of these compounds in the OHPAH fraction and no further elution in fraction C using initial HCl + solvent eluents, it was supposed that the aldehyde group might be bonding covalently with amino groups on the SPE sorbent to form Schiff bases, which is a reversible reaction in the presence of water and H^+ or OH^- (Nomura and Jones, 2013). As the use of acid-only-modified solvents initially tested for fraction C yielded poor recoveries of both target acids and phenolic aldehydes (<2%), three additional systems were tested, one incorporating water into the HCl-ACN eluent system and two incorporating water and TEA, ACN:H_2O:TEA (90:10:0.05) and ACE:H_2O:TEA (90:10:0.05). Both TEA-modified systems substantially improved the recovery of 4-hydroxybenzaldehyde, though total recovery was still low (15-20%), and marginally improved the recovery of vanillin (3-7%). The ACN:H_2O:TEA system also yielded the best recoveries for salicylic acid (58±11%), gentisic acid (38± 9.1%), and 1-hydroxy-2-naphthoic acid (82 ± 7.4%), offering apparent method improvements over a previous extraction+silica SPE protocol where recovery of these compounds was <10% (Bandowe and Wilcke, 2010). The presence of 9-fluorenone in the ACN:H_2O:TEA system, despite its strong recovery in fraction A and its absence in fraction B, also suggests elution of fluorene-9-carboxylic acid. Other acid analytes, 4-hydroxybenzoic acid, ferulic acid, and 1-naphthylacetic acid, were not recovered. As analyte loss might occur during the final evaporation and the solvent exchange step required for BSTFA derivatisation, the recovery for this procedure was also independently tested (n=3). Negligible losses were observed for 1-hydroxy-2-naphthoic acid and ferulic acid, but all other targets exhibited

losses between 27 and 45%, indicating that this solvent exchange step likely accounts for the lower recovery of some, if not all, phenolic aldehyde and acid analytes. Although the ACN:H_2O:TEA mixture provided the best results of the eluents tested, further tests of this system revealed poor inter-batch repeatability. This fraction is therefore not considered suitable for quantitative analysis by GC-MS, but it might be used qualitatively to suggest the presence of acid and phenolic aldehyde PAH transformation products of interest for further analysis, and has therefore been included here in further method investigations. The use of an alternative derivatisation technique or HPLC may extend the analytical utility of this SPE fraction (Meyer et al., 1999). However, further testing was beyond the scope of the present study.

Table 4.3 SPE linearity tests for 0.01-10 μg/mL target analyte. Lower limits of linearity are based on a reduction of the final extract to 1 mL as tested. All correlations are significant ($p<0.001$).

Target analyte	R^2	R^2_{SR}	SE_m/m[1]	$SE_{m\cdot SR}/m_{SR}$[1]
PAH				
Naphthalene	0.975	0.999	0.0438	0.0024
Acenaphthylene	0.973	0.999	0.0456	0.0027
Fluorene	0.970	0.999	0.0483	0.0079
Phenanthrene	0.967	0.999	0.0506	0.0022
Anthracene	0.970	0.999	0.0483	0.0039
Pyrene	0.965	0.999	0.0517	0.0067
Benz[a]anthracene	0.945	0.999	0.0655	0.0023
Chrysene	0.944	0.999	0.0661	0.0049
Benzo[b]fluoranthene	0.951	0.999	0.0618	0.0037
Benzo[k]fluoranthene	0.943	0.999	0.0668	0.0084
Benzo[a]pyrene	0.952	0.999	0.0609	0.0026
Indeno[1,2,3-cd]pyrene	0.948	0.998	0.0638	0.0158
Dibenz [a,h] anthracene	0.944	0.999	0.0662	0.0131
Benzo[ghi]perylene	0.946	0.998	0.0653	0.0177
OPAH				
1-Indanone	0.973	0.999	0.0456	0.0076
9-Fluorenone	0.965	0.998	0.0519	0.0152
9,10-Anthraquinone	0.955	0.998	0.0593	0.0186
OHPAH				
Catechol	0.947		0.0670	
1-Hydroxynaphthalene	0.992		0.0245	
1-Hydroxyacenaphthene	0.987		0.0312	
9-Hydroxyfluorene	0.994		0.0219	
9-Hydroxyphenanthrene	0.993		0.0226	
1-Hydroxypyrene	0.989		0.0288	

[1]standard error of the slope, SE_m, as a proportion of the slope, m

Results presented in Table 4.3 for PAH indicate that the method demonstrated strong linearity for concentrations of PAH, OPAH, and OHPAH in the load range 0.1-10 μg/mL. High R^2 and low error of the calibration slope was achieved for both absolute, and especially for surrogate-corrected values, including improvements for OPAH quantitation.

4.3.3 Stage 2: Matrix-SPE tests

Figure 4.3 displays recovery values for surrogates and OPAH obtained from Fraction A and B of matrix-SPE tests. Absolute recovery of all analytes tended to be lower for the standards and Soil 3 than for Soil 2 and especially Soil 1. This was notable for the 4-6 ring PAH, 9-fluorenone, 9,10- anthraquinone, and most OHPAH. It is possible that the presence of a more complex matrix improved the preservation of the targets in solution by offering competition to sorption sites on glassware, reducing the rate of solvent evaporation during concentration steps, and/or through the presence of natural antioxidant compounds (Woudneh et al., 2016).

Recoveries for Fraction A PAH surrogates and larger OPAH in Soils 1 and 2 were typically in an acceptable analytical range (<20% deviation from 100%) and RSD (1-10%). Exceptions were observed for Soil 1, where RSD for 9,10-anthraquinone was somewhat higher (15%), and elevated absolute recoveries near 150%, were obtained for both 9-fluorenone and acenaphthene-d_{10}. This indicated that matrix-associated baseline signal variation may be an issue for quantitation especially of 2.5-ring aromatic compounds in this soil (i.e. causing overestimation in spiked samples and/or underestimation in unspiked samples). In this case, it was demonstrated that matrix effects for PAH could be largely controlled by using surrogate recovery as an adjustment factor (e.g. absolute acenapthylene recovery was 147±20% but with adjustment was 95±5%$_{SR}$). Surrogate recovery adjustment also improved quantitation of PAH in Soil 3 and standards (recovery values closer to 100% and reduced RSD). Application of PAHd surrogate adjustment to OPAH recovery during matrix SPE tests tended to reduce RSD, suggesting that factors affecting surrogate recovery may also help explain partial variability in OPAH measurement. For 9-fluorenone and 9, 10-anthraquinone, surrogate recovery adjustment had marginal impacts on mean recovery observed in Soils 2, 3, and standards, but offered substantial improvements to interpretation of Soil 1. For 1-indanone, application of the surrogate adjustment factor

led to greater deviation from 100% in all cases, suggesting that additional or different factors inhibit the recovery of this compound in SPE-matrix tests compared to naphthalene-d_8 to an extent that the recovery of the latter does not reflect the recovery of the former.

In addition to impacts on calculated recovery of the spiked analytes, the complexity of the Soil 1 matrix also contributed to late elution of some high-concentration PAH in fraction B, typically less than 5% of the combined analyte signal of fractions A and B. Increasing the proportion of DCM by up to 50% in the first eluent, as suggested by Cochran et al. (2012) might reduce this effect for the most contaminated soils.

For fraction B, the average recovery of OHPAH excluding catechol, ranged between 63 and 113%, with most recovery values between 70 and 85%. RSD across matrix-SPE tests was generally <10%, except for Soil 1 where both apparent recovery and variability were greater. The somewhat lower recovery values obtained here when compared to original solvent selection experiment may be due to the addition of the evaporation-concentration step. Separate testing of the evaporation step (n=3) indicated this could alone account for target losses of up to 33, 30, 20, 19, 8, and 14% for catechol, 1-hydroxynaphthalene, 1-hydroxyacenaphthene, 9-hydroxyfluorene, 9-hydroxyphenanthrene, and 1-hydroxypyrene, respectively, and could explain 2-10% of the relative standard deviation in OHPAH recoveries. The consistency of the pattern observed across all OHPAH except catechol suggests that the use of a deuterated mid-weight hydroxylated PAH surrogate could be useful for the quantitation of these compounds at the SPE stage. With the exception of some catechol, carryover of OHPAH to fraction C was not observed.

4.3.4 Stage 3: Full method recovery tests

The recovery of target analytes after full method tests (Table 4.4) demonstrated greater variability than SPE-matrix tests, which was expected due to the inherent heterogeneity of soils and greater number of steps involved in sample preparation (Gerlach and Nocerino, 2003). For standards and Soil 3, the average recoveries of PAH and deuterated surrogates were generally very good (77-129%), with the exception of enhanced recovery of SR4 compounds, observed across all matrices, as well as lower recovery and greater variability for the more volatile compounds in SR groups 1 and 2.

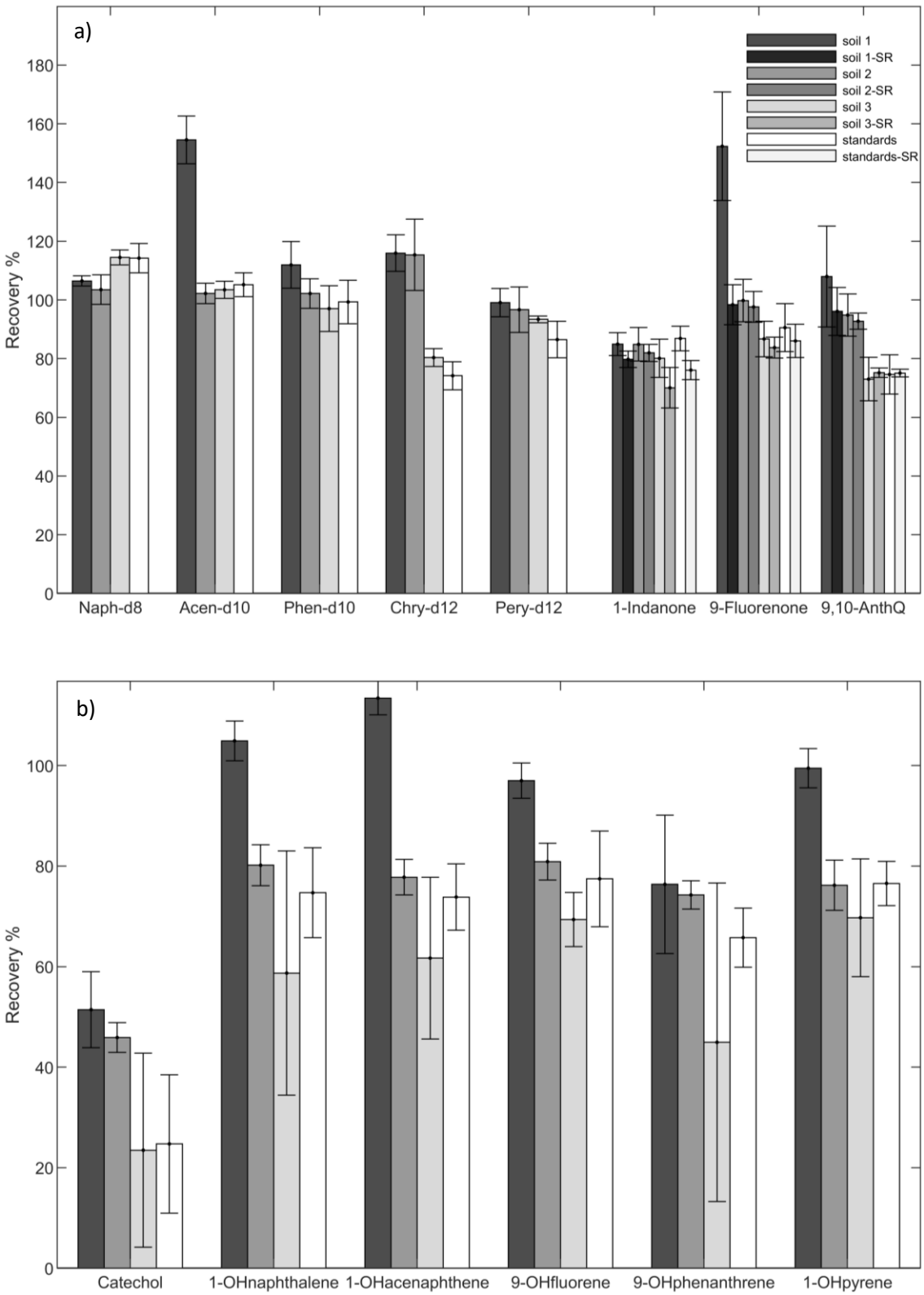

Figure 4.3 Average recovery of PAH surrogates, OPAH, OHPAH from Matrix-SPE tests in a) fraction A, with and without the application of surrogate recovery adjustment for OPAH (SR series); b) fraction B. Error bars indicate the standard deviation of recovery from triplicate Matrix-SPE trials.

For Soils 1 and 2, despite low RSD in concentrations (<10% for most PAH), at the high concentrations of specific PAH present in the original soils, this variability exceeded and thus masked the contribution of the spike used for recovery calculation, leading to calculated values not representative of true extraction recovery (e.g. >400% and/or negative values). The PAH calculated recoveries impacted specifically in this way have been marked not representative, 'NR', in Table 4.3. In most cases, the recovery of the associated deuterated surrogate indicated that the method had similar recovery to Soil 3. In some cases, very high recovery values obtained for the surrogate suggest the presence of residual matrix enhancement effects e.g. for mid-weight PAH (SR2-SR4) in Soil 1 and to a lesser extent in Soil 2. However, strong agreement between final quantitation for mid-weight PAH using this method and alternate analysis using simple DCM extraction (Ch. 3 method - agreement within 1-15% without surrogate adjustment) suggests matrix enhancements may have impacted lower-concentration analytes including surrogates, but did not substantially impact quantitation of high-concentration PAH (2-3 orders of magnitude higher) reported here.

Recovery of OPAH followed similar trends to PAH. Strong recoveries were obtained for standards and Soil 3, while Soil 1 yielded non-representative values due to elevated concentrations which masked the spike contribution. For Soil 2, the enhanced recovery of OPAH is likely attributed to the residual matrix effects also observed for lower-concentration PAH in groups SR2-SR4. Surrogate recovery adjustment increased RSD and had a negative effect on calculated recovery for both 1-indanone and 9-fluorenone in standards, Soil 2, and Soil 3, but tended to reduce RSD and improve calculated recovery for 9-fluorenone in Soil 1 and 9, 10-anthaquinone in all soils. In the latter cases, where surrogate adjustment offered improvements during recovery tests, the benefit of using this factor during final quantitation was ambiguous: adjustment either increased RSD, or did not substantially impact final quantitation. The inconsistencies and lack of clear benefit observed in these tests suggest use of the deuterated PAH as surrogates for tracking full-method recovery and quantitation of OPAH in soils is not suitable as a general approach.

The recovery of OHPAH for standards generally ranged between 68 and 89%, agreeing within ~10% with the matrix-SPE tests, with the exception of catechol, which was not recovered in any of the extraction-SPE tests. For the recovered OHPAH, deviations from the matrix-SPE recovery levels were greater when soil was present in the extraction (vs. standards in Na_2SO_4) and demonstrated different trends related to

specific analyte and soil type. It is possible that the presence of a more complex matrix improved the preservation of 1-hydroxyacenaphthene and 9-hydroxyfluorene in solution as suggested during the Matrix-SPE tests, with further enhancements for Soil 1 following trends for mid-weight PAH and OPAH in Fraction A. This does not appear to hold for the other OHPAH. In the case of 1-hydroxynaphthalene (recoveries 31-77%), the trend may reflect a trade-off between increased preservative effect of the more complex matrices and the more extended evaporation period required to concentrate highly contaminated sample extracts prior to SPE. It could also be related to differential sorption on soil particles. For 1-hydroxypyrene and especially 9-hydroxyphenanthrene, the range of recoveries across soil types was substantial (30-81% and 11-145%, respectively), and did not follow a clear trend associated with contamination level or preparative evaporation time. Instead, particularly low recovery of these compounds for Soil 2 and 3 can be best explained by the substantial and increasing proportion of clay in the soil mass (Biswas et al., 2015). The small size of clay particles as well as specific structure and charge characteristics of the minerals offers substantial sorptive capacity in soils even for non-polar PAH, and may also restrict the extractability of related aromatic compounds (Biswas et al., 2015). Low recovery of 9-hydroxyphenanthrene from clay soils has also been reported by Bandowe and Wilke (2010). As 9-hydroxyphenanthrene and 9-hydroxypyrene displayed similar trends in soil extractions, the use of deuterated hydroxypyrene as a surrogate could improve quantitation of both compounds without leading to overestimation of 9-hydroxyphenanthrene. Similarly, use of a 2.5-ring deuterated OHPAH could help track recovery could improve quantitation of 1-hydroxyacenaphthene and 9-hydroxyfluorene.

With the exception of lower recovery of 1-hydroxypyrene, this method offered a comparable or better range of OHPAH recoveries to those reported in previous methods targeting only OHPAH using methanol extraction followed by silica-SPE (Avagyan et al., 2015), or using water+ACN extraction followed by C18-SPE or dispersive liquid liquid microextraction and GC-MS (Wang et al., 2012). In comparison to methods targeting a range of functionalities, this method demonstrated elevated recovery of a greater number of target OHPAH when compared to PLE + silica clean-up and GC-MS analysis (Bandowe and Wilcke, 2010), especially when the contaminated soils presented here are considered. Improvements over the PLE+silica method were substantial for 9-hydroxyfluorene specifically (91% vs. ~60%), and were similar to those obtained using a Soxhlet extraction and silica/strong base clean up step followed

by HPLC analysis (93%) (Meyer et al., 1999). Recoveries of hydroxynaphthalenes for the current method were within range of and in some cases higher (by ~10-40%), than those presented in the PLE-silica method but were lower compared to the Soxhlet-silica/strong base/HPLC approach. Although the coarser texture of soil used in the development of the latter method may have improved recovery of these compounds through reduced sorption, our investigation also suggests that particle size shifts are not the primary controlling factor for recovery of 1-hydroxynaphthalene, and more likely, the evaporative step included prior to preparative chromatography is responsible for losses observed here.

4.3.5 Stage 4: Characterisation of unspiked tests soils

4.3.5.1 Qualitative identification of polyaromatic compounds

SPE offered substantial clean-up and fractionation capacities for the analysis of PAH, OPAH, and OHPAH in uncontaminated and contaminated soils (Figure 4.4). In the most contaminated soils, target breakdown products were difficult to detect in whole extracts even when these compounds were spiked directly into the extract, as in the load solutions for matrix-SPE tests. Interferences in the complex soil matrix led to poor chromatographic resolution (Figure 4.4a), obscurance of analyte mass spectral pattern, and possibly limited derivatisation or ionisation of these compounds. Fractionation assisted the identification of target compounds in these soils, which otherwise would not have passed detection criteria. In the unspiked sample presented in Figure 4.4, only 2 of 5 target OHPAH detected in fraction B, and only 2 of 6 (or of 7 if the presence of 9-fluorenone is indicative of fluorene-9-carboxylic acid) acid transformation products detected in fraction C were identified in the derivatised whole extract. The reduction of interferents in fraction A also improved signals for 9-fluorenone and 9,10-anthraquinone.

A search in the NIST14 library for Soil 1 extracts (70% match, following Chibwe et al., 2017) indicated the presence of additional polyaromatic compounds (PAC) which could suggest extended utility of this SPE method for a greater number of target compounds. Fraction A also revealed the presence of alkanes, additional PAH and alkyl PAH, S- and O- heterocyclic compounds, as well as additional carbonyl substituted PAC including 2-Butyl-10H-acridin-9-one and benzanthraquinone. Fraction B also contained a variety of semi-polar aromatic compounds, including cresols and other alkyl phenols, additional OH-phenanthrenes, a greater number of N- heterocyclic

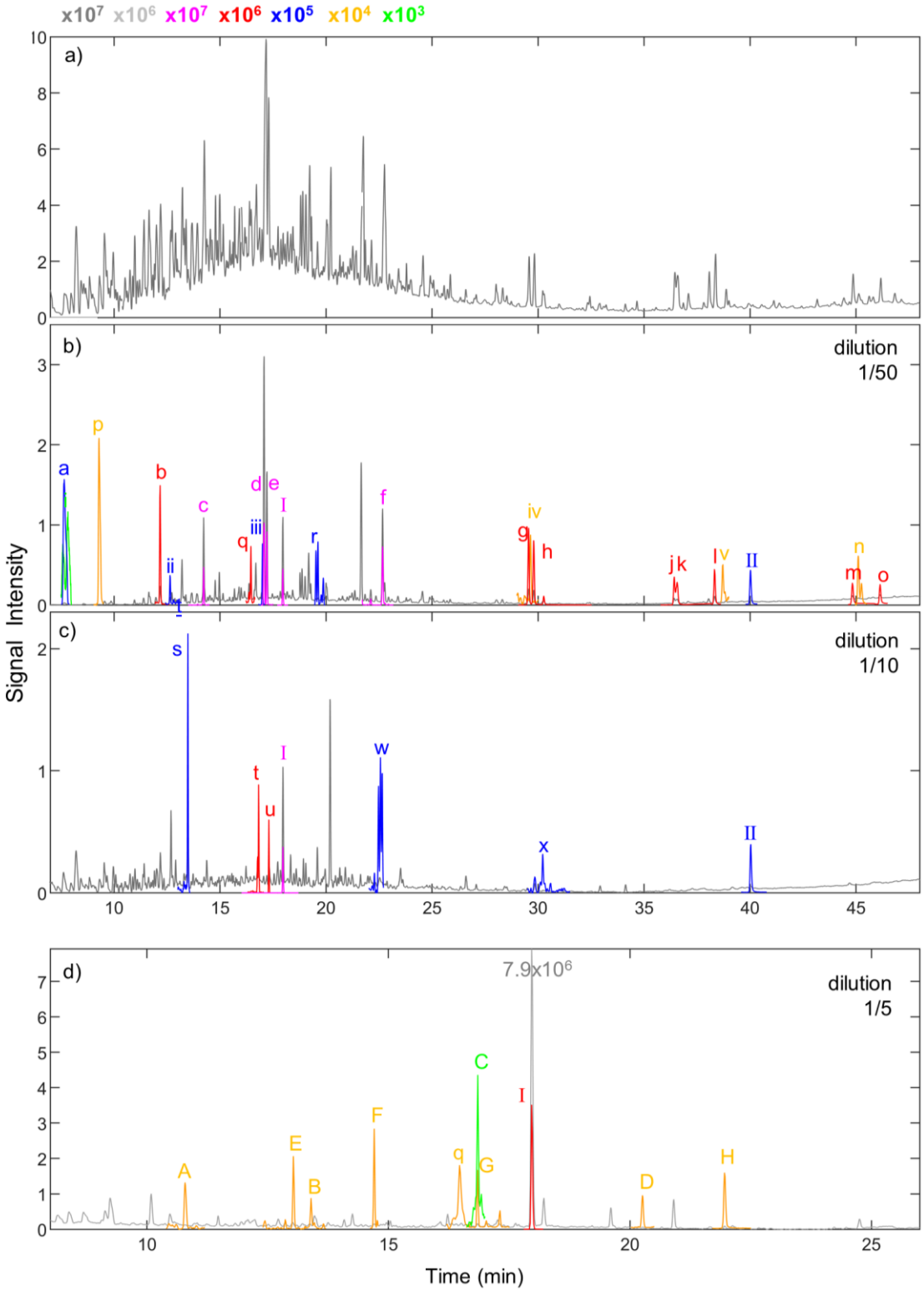

Figure 4.4 GC-MS chromatograms for SPE fractions of target PAH and transformation products in Soil 1 extract a) whole extract b) fraction A: PAH and OPAH c) fraction B: OHPAH d) fraction 3: COOHPAH, phenolic acids and aldehydes (note zoomed timescale). Black and grey traces refer to total ion chromatograms, while coloured represent specified analytical events from single ion monitoring traces. Specific shades relate to signal intensity order of magnitude, indicated at top left. Letters and Roman numerals refer to specific compounds identified in Table 4.1. Dilution levels given are relative to whole soil extract after SPE processing.

Table 4.4 Recovery and quantitation of PAH and their transformation products in three test soils

Compound/ compound group	Recovery extraction-SPE tests *with SR adjustment*			Average concentration in soil *with SR adjustment* µg/g			Method LOD[1]	Method LOQ[1]
	standards	Soil 1	Soil 2	Soil 3	Soil 1	Soil 2	Soil 3	µg/g	µg/g
OHPAH									
1-hydroxynaphthalene	77±2	42 ±6	51±3	31±2	8±6*	<LOD	<LOD	0.12	0.44
1-hydroxyacenapthene	89±3	141±10	118±6	95±9	23±2	0.50±0.05	<LOD	0.04	0.16
9-hydroxyfluorene	84±3	144±7	105±3	91±7	14±1	0.19±0.01	<LOD	0.04	0.16
9-hydroxyphenanthrene	145±7	72±3	25±1	11±1	7.1±7.0*	<LOD	<LOD	0.08	0.32
1-hydroxypyrene	68±4	81±2	50±2	30±3	2.5±1.0	<LOD	<LOD	0.12	0.40
OPAH									
1-indanone	86±3	NR[2]	82±2	65±6	35±10	0.57±0.03*	<LOD	0.4	1.4
	82±6	*NR*	*80±10*	*170±140*	*28±14*	*0.57±0.03**			
9-fluorenone	101±7	NR	135±7	90±1	140±10	3.9±0.3	<LOD	0.4	1.2
	123±14	*NR*	*140±15*	*140±30*	*141±14*	*3.7±0.4*			
9,10-anthraquinone	130±12	NR	200±30	115±2	90±7	5.8±0.5	<LOD	0.4	1.4
	133±9	*NR*	*160±20*	*107±1*	*90±7*	*5.8±0.6*			
PAH									
PAH-SR1	83±2	NR	125±25	46±42	1300±800	27±1	<LOD	1.8	6.0
	79±5	*NR*	*122±14*	*80±11*	*2100±140*	*28±3*			
(naphthalene-d_8)	(85±24)	(82±29)	(98±11)	(91±41)					
PAH-SR2	107±5	NR	141±22	77±11	300±400	28±3	<LOD	0.6-0.8	2.4-2.6
	130±10	*NR*	*146±31*	*118±7*	*980±160*	*33±4*			
(acenaphthene-d_{10})	(81±4)	(177±55)	(91±7)	(79±16)					
PAH-SR3	110±8	NR	NR	122±5	7000±500	370±50	<LOD	0.6-1.0	1.8-3.4
	120±11	*NR*	*NR*	*128±7*	*5690±90*	*330±40*			
(phenanthrene-d_{10})	(95±4)	(129±10)	(118±11)	(104±9)					
PAH-SR4	142±7	NR	NR	167±1	1000±100	150±5	<LOD	0.2-0.2	0.8-0.8
	107±4	*NR*	*NR*	*106±1*	*510±60*	*82±4*			
(chrysene-d_{12})	(129±10)	(185±25)	(197±17)	(142±17)					
PAH-SR5	108±3	NR	NR	129±5	2700±200	520±10	0.8±0.6*	0.4-0.8	1.2-3.0
	120±20	*NR*	*NR*	*104±9*	*2400±100*	*452±15*	*0.6±0.5**		
(perylene-d_{12})	(94±10)	(109±9)	(117±6)	(115±12)					

Recovery based on 20 µg/g analyte spike

[1]method LOD and LOQ based on instrument LOD/LOQ, method concentration/dilution factors and 0.5 g soil mass; range for individual PAH in group is given

*estimated value: asterisked compounds include estimated values for individual replicates >LOD, but <LOQ, where at least one replicate was >LOQ

[2] NR 'not representative' standard deviation of unspiked samples sufficient to mask spike contribution. For PAH, recovery of deuterated surrogate (8 µg/g, n=6),given in brackets, is an alternate indicator.

PAC and cyano, amino- and nitro-substituted PAH, as well as PAC with multiple polar functional groups. Fraction C indicated the presence of additional monoaromatic aldehydes such as 3-Methyl-p-anisaldehyde and 2,5-thiophene-dicarboxaldehyde, as well as additional phenolic acids including 4-methyl-2-hydroxybenzoic acid. It is worth noting that target compounds in calibration solutions typically exhibited match rates of 85-97% with the NIST14 library, but several OHPAH, i.e. 1-hydroxyacenaphthene, 9-hydroxyfluorene, 9-hydoxyphenanthrene, and 1-hydroxypyrene as well as 1-hydroxy-2-naphthoic acid did not yield NIST14 spectral matches. Further inclusion of TMS derivatives of OHPAH and COOHPAH to the NIST14 library could be beneficial for furthering oxygenated PAH research.

4.3.5.2 Quantitation of target analytes in soils

Concentrations of target analytes in soils are summarised in Table 4.4. Levels of PAH were highest in Soil 1, while PAH concentrations in Soil 2 were more moderate but at the mid-high range of for industrially contaminated soils presented in Arp et al. (2014). Minimal levels of high molecular weight PAH were detected in Soil 3, which could reflect the presence of combustion materials in the urban environment; more specifically, the relative abundance of benzo[a]pyrene as a proportion of benzo[a]pyrene + chrysene totals (i.e. ratio >0.35) may suggest the presence of coal combustion residues (Tobiszewski and Namieśnik, 2012). Concentrations of target oxygenated PAH were also highest in Soil 1 with lower levels detected in Soil 2. OPAH concentrations in Soil 1 and 2 were in line with those observed for other industrial soils with high-range and mid-range contamination levels, respectively (Arp et al., 2014). Comparison for OHPAH was somewhat more difficult due to the paucity of studies reporting concentrations of these compounds, but in general OHPAH concentrations here tended to be higher than those reported across soils of varying land-use histories (ref. Table 1.1). Soil 2 indicated levels that are similar to those evaluated for ERM-CC013a, obtained from a former gasworks sites in Berlin (Bandowe and Wilke, 2010), while Soil 1 indicated levels substantially higher levels than have previously been reported. This was likely related to the overall very high levels of contamination in this soil when compared to other industrial sites (Arp et al., 2014). It is acknowledged that matrix effects observed in recovery tests may have led to underestimation of select OHPAH in clay-dominated soils and moderate enhancement in select OPAH concentrations in the more highly contaminated soils. Although not commonly used for

anlaysis of oxygenated PAH in soils (Ref. Sect. 2.4.1), greater certainty might be obtained using standard addition calibration methods for low-concentration oxygenated PAH; however, as addressing matrix effects requires focus primarily on the extraction stage, the standard spikes would need to be introduced prior to extraction, substantially increasing the material and time cost of these analyses. These effects may be better addressed by the greater availability of a range of suitable surrogates. Overall, within the context of overall comparison of soil types and monitoring studies, based on the low RSD obtained from this method, it is suitable to report the quantitated values alongside recovery data.

4.4 Conclusions

The developed SPE method using aminopropyl silica offers good performance for the separation of PAH and oxygenated PAH into relevant groups for subsequent instrumental analysis by GC-MS, while improving recovery and/or reducing materials and simplifying preparation compared to previous methods. The combined extraction+ SPE method showed strong reproducibility for the quantitation of PAH, OPAH, and most OHPAH in individual soils, and offered further qualitative information for some COOHPAH and phenolic acids and aldehydes. It was demonstrated that the level of contamination and proportion of clay in the soil matrix may impact recovery at different stages of the protocol. In many cases, this may be adequately accounted for through the use of suitable deuterated surrogate compounds. The increased availability of a range of labelled oxygenated PAH would be especially useful when recovery is to be monitored throughout the full extraction method. The method presented here can be used to compare soils or to monitor changes in concentrations of these analytes in individual soils over time. In order to support the establishment of best practices for managing short- and long-term risk from oxygenated PAH at and downstream from contaminated sites, we recommend the continued development, adoption, and standardisation of analytical protocols which include these compounds alongside parent PAH.

Acknowledgements

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Chapter 5

Impact of compost and biochar on the distribution of oxygenated PAH in aged gasworks soils

Abstract

This study investigated the impact of compost, biochar and no amendment in situ remediation strategies on the distribution of target low molecular weight PAH, OPAH and OHPAH in two aged gasworks soils. In order to understand contaminant lability, we used analytical methods developed in Chapters 3 and 4 to investigate the total extractable and leachate fractions and further present the first study considering the readily available fraction of OHPAH transformation products using mild solvent extraction. After 180 days, attenuation of total extractable PAH for Soil1/Soil2 unamended samples was 24/29% and for biochar amended samples 20/21%. Compost amendment resulted in highest attenuation for Soil 1 (82%) but delayed PAH removal for Soil 2, putatively by supporting a sorption-and-release mechanism. Concentrations of individual OPAH ranged 13-410 µg/g (Soil 1), and 6-41 µg/g (Soil 2) and individual OHPAH 0-170 µg/g (Soil 1) and 0-20 µg/g (Soil 2). Transformation products exhibited a variety of temporal trends that differed between analyte, soil, treatment, and fraction considered. In in some cases, the biochar amendment supported sequestration of OPAH into less available fractions, but this treatment also supported highest concentrations, elevated lability, and increasing leachability over time for several individual PAH and transformation products, suggesting that in some cases biochar application may represent an additive risk factor due to potential adverse effects associated with degradation byproducts. On the other hand, the compost amendment most often led to reduced concentrations and/or reduced leachability of PAH and PAH transformation products. For all treatments, highest levels of individual transformation products were observed at intermediate timeframes, suggesting these compounds may be involved in increased toxicity previously reported for these soils, and supporting the view that monitoring of PAH oxidation byproducts should be ongoing at remediation sites as a part of site management.

5.1 Introduction

A variety of technologies that promote PAH degradation through chemical or biological oxidation have emerged as remediation strategies for heavily contaminated gasworks soils (Aparna et al., 2008; Gan et al., 2009; Kuppusamy et al., 2017). However, as attenuation of parent PAH has typically been used as a measure of remediation progress, potential risks associated with degradation byproducts produced during treatment have rarely been considered. There is growing evidence that although these technologies may reduce parent PAH concentrations, they can also lead to the accumulation of oxygenated PAH intermediates (Lundstedt et al., 2007). Oxidation byproducts including OPAH, OHPAH and COOHPAH are expected to exhibit increased toxicity (Chibwe et al., 2015; Xue and Warshawsky, 2005; Wilson, 1996), environmental mobility, and bioavailability (Enell et al., 2016; Schmidt et al., 2010) than parent PAH, but are only rarely monitored in genuine contaminated soils.

Examples of temporary or long-term build-up of intermediates in contaminated soils have been described for chemical oxidation with ethanol-Fenton (Lundstedt et al., 2006) and persulfate (Liao et al., 2014), bioslurry treatment (Hu et al. 2014, Lundstedt et al., 2003), incubation with (Andersson et al., 2003) and without (Wilcke et al., 2014b) wood-rotting fungi. In spiked soils, soil washing (Pramauro et al., 1998) and compost application (Meyer and Steinhart, 2001; Wischmann and Steinhart, 1997) have also resulted in the formation of oxygenated PAH transformation products. Less is known about oxygenated PAH in aged contaminated soils treated with compost or biochar. These technologies represent some of the lower cost strategies for in situ soil remediation (Kuppusamy et al., 2017). A recent metastudy of 180 treated soils demonstrated that the compost-based approach yielded highest reductions of parent PAH (mean reduction 70%) and associated calculated cancer risk in comparison to biostimulation, bioaugmentation, and surfactant application (mean reduction 28−53%) (Davie-Martin et al., 2017). Biochar amendments are known to increase sorption, nutrient availability, and microaggregate formation, and to support a diverse microbial community including PAH degrader species (Zhu et al., 2017), and it has recently been shown that biochar may substantially increase PAH degradation through increasing the population of specific PAH-degrading taxa Kong et al. (2018). However, because of their high sorptive capacity, biochars are more often considered most useful for contaminant immobilisation, and there is growing interest in their potential use to

reduce leaching and bioavailability of PAH (Bianco et al., 2021). Reduced mobility of PAH and some polar HPAC has been reported in sewage sludge (Oleszczuk et al., 2012), marine sediments (Bianco et al., 2021) and in combination compost+biochar soil remediation systems (Beesley et al., 2010; Sigmund et al., 2018). Importantly, biochars are considered effective for improving soil quality at sites with heavy metal contamination (O'Connor et al., 2018), and their application may therefore be desired at mixed-contamination sites. In addition, biochars are known sources of phenolic compounds (Zhu et al., 2017) and have been shown to ameliorate soils contaminated with organic pesticides with a range of polar functionalities (Varjani et al., 2019), suggesting the potential for biochar to be a source or sink of polar PAH metabolites, however to date this has been largely unstudied. For all these approaches, it is important to consider whether potential increases in risk/benefit of oxygenated PAH formation during treatment exceed those of no amendment interventions such as monitored natural attenuation (Davie-Martin et al., 2017).

While there is a need to recognise oxygenated PAH as potential risk drivers at contaminated sites, there is a concurrent interest to address potentially overly conservative estimates of risk by incorporating bioavailability into exposure-likelihood scenarios (Cipullo et al., 2018; Enell et al., 2016). Bioavailability has been variously defined in the literature, but in general, this concept aims to describe the proportion of a contaminant that is sufficiently labile in the whole soil and available to interact with the membrane of a receptor organism (Ortega-Calvo et al., 2015). In aged soils, PAH tend to become sequestered through strong interaction with clay particles, sediment micropores, humic materials, and black carbon residues to the extent that they are immobilised and become unavailable for interaction with soil organisms (Cipullo et al., 2018). Although there is uncertainty about the ultimate fate of strongly sorbed residues in aged soils, there is a normative view that these processes reduce the likelihood of adverse effects, and that the inclusion of this fraction in risk assessment is overly conservative (Cipullo et al., 2018). The bioavailable fractions, on the other hand, may include the freely-dissolved porewater fraction and the rapidly desorbing fraction, i.e. the larger pool of a contaminant which may be expected to interchange with the freely-dissolved fraction over time (Ortega-Calvo et al., 2015). These fractions are of interest for site assessment because they may provide greater insight into site risk as well as remediation potential (Ortega-Calvo et al., 2013).

Several methods have been proposed for the determination of bioavailable fraction of soil contaminants, and no single technique has been demonstrated to be best for describing all contaminant-matrix-organism relationships (Cui et al., 2013; Enell et al., 2016). Mild solvent extraction using methanol is a simple and convenient tool for estimating the bioavailable fraction of soil contaminants (Cui et al., 2013; Kelsey and Alexander, 1997), and methanol-extracted PAH content has been found to be correlated with earthworm acute toxicity and seed germination assays in an earlier report of the soils investigated here (Cipullo et al., 2019). Methanol-based extraction of soils has been shown to support strong recovery of polar PAH transformation products including OHPAH (Avagyan et al., 2015). In this study, we used the term 'readily available' since measures of bioavailability are typically defined in relation to specific receptor endpoints (Puglisi et al., 2007), which was beyond the scope of the present study. We also considered the leachable fraction, as it represents the highest risk to groundwater.

Therefore, this study investigated the formation and availability of target oxygenated PAH in two aged gasworks soils over a 180-day outdoor incubation study with compost amendment, biochar amendment, and no amendment. This study was undertaken in partnership with researchers at Cranfield University, and the division of roles and analyses is presented in Figure 5.1. A prior report discussing total petroleum contamination, heavy metal distribution, and toxicity indices has already been published (Cipullo et al., 2019). Since LMW PAH are both more bioavailable and more rapidly transformed (Davie-Martin et al., 2017), the study presented in this thesis focused on common transformation products of seven 2-4 ring USEPA PAH. Using a three-level analytical approach, this study quantified target degradation products in the total extractable, readily available, and leachate fractions, to demonstrate how consideration of all three fractions could help support decision making at contaminated sites. This study aimed to elucidate not only the impact of amendment and soil type on the (trans)formation of oxygenated PAH, but also on the relative lability of these contaminants. The datasets presented here provide additional insight into dynamics of PAH attenuation and toxicology presented in the earlier report, and the potential relationships between oxygenated PAH and previously-reported toxicology measures discussed in Section 5.3.6 of this thesis are my own analysis. To the best of our knowledge, this is the first study to investigate the formation and distribution of PAH transformation products during biochar application in hydrocarbon contaminated soils, and the first to investigate the readily available fraction of OHPAH in the soil matrix.

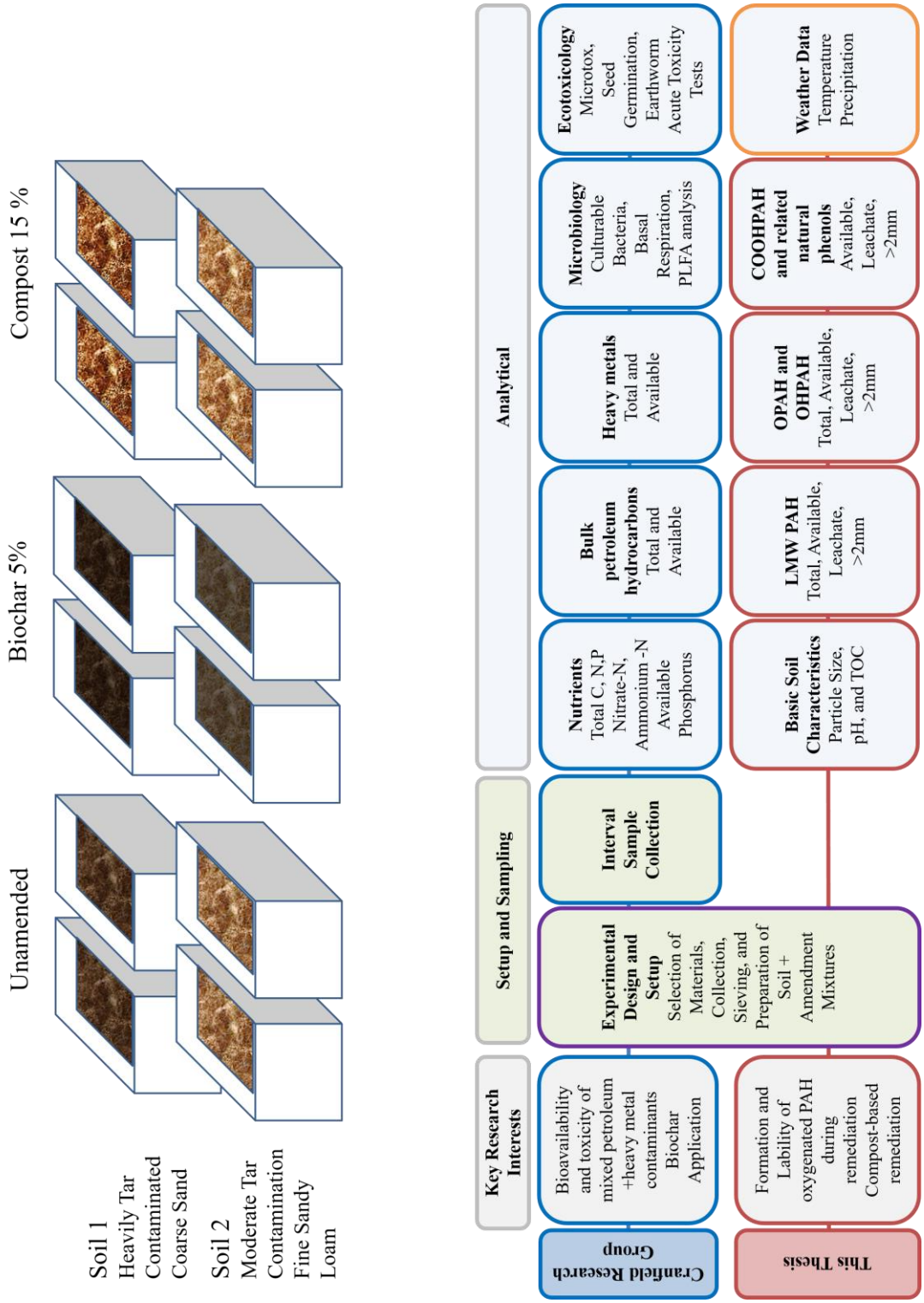

Figure 5.1 Experimental setup and division of shared experiment. Rationale and selection of application rates are discussed further in the text (ref. 5.1 and 5.2.2). Except where specifically indicated, the data presented in this thesis were fully prepared and analysed by myself - weather data was obtained by from the meteorological office, but aggregation and plots are my own; toxicity data referenced but not reprinted here were prepared by the Cranfield research team, but the interpretations included here are my own.

5.2 Methods

5.2.1 Chemicals and reagents

All organic solvents (analytical reagent grade) as well as PTFE syringe filters (0.2 μm) were purchased from Fisher Scientific UK. Deuterated semivolatile internal standards mix containing naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12} was purchased from Thames Restek UK. All other analytical standards were obtained from Sigma-Aldrich UK including PAH - EPA mix B and naphthalene; targeted transformation products - 1-hydroxynaphthalene, 1-hydroxyacenaphthene, 9-hydroxyfluorene, 9-hydroxyphenanthrene, 1-hydroxypyrene, 1-indanone, 9-fluorenone, 9,10-anthraquinone, catechol, salicylic acid, 4-hydroxybenzoic acid, 1-naphthylacetic acid, and 1-hydroxy-2-naphthoic acid; related phenolic compounds - 4-hydroxybenzaldehyde, vanillin, and vanillic acid; internal standards nonadecane-d_{40} and triacontane-d_{62}; and surrogate salicylic acid-d_4. Discovery solid phase extraction (SPE) columns (6 mL silica and 3 mL aminopropylsilica phases) and derivatisation agent BSTFA with 1% TMCS were also obtained from Sigma Aldrich UK.

5.2.2 Soils, amendments, and experimental setup

Two soils were obtained from UK former gasworks sites in Kent and Northamptonshire (Soil 1 and Soil 2 respectively, as presented in Ch. 4) and represent shifts in contamination level and soil textural classification (Table 5.1). Biochar and compost amendments were obtained commercially from Carbon Gold UK (enriched biochar) and Westland Horticulture Ltd. UK (multi-purpose peat-based compost with nutrients) (70%) with softwood shavings (30%) added as a fresh lignin supplement. Soil particle size analysis and textural classification were determined through a combination of sieving and sedimentation after oxidation of organic matter with hydrogen peroxide and dispersal with buffered sodium hexametaphosphate (ISO 11277, 2009). Amendment particle size was determined by sieving after oven drying (60°C 24 h) (Cipullo et al. 2019). Residual moisture content and total organic matter were determined, respectively, by mass lost after dehydration (sample oven dried 105°C for 24 h) and subsequent loss on ignition (LOI) (muffle furnace 450°C for 5 h). Soil pH was determined in 0.01 M $CaCl_2$ (1:5 solid:liquid v/v) using a Jenway 3540 pH meter after 1 hr shaking and 30 min equilibration period (ISO 10390, 2005). Aliphatic hydrocarbons (C_8-C_{40}) were determined by Cranfield University's Vincent Laboratories

through duplicate extraction of 2.5 g soil with dichloromethane:hexane (1:1 - 15 mL), filtration of the supernatants through 6 mL SPE DSC-Si silica tubes, and GC-MS analysis (ref. Cipullo et al 2019, for further details). Total carbon-TC and total nitrogen-TN were determined through combustion-elemental analysis using a Vario EL III Element Analyzer (Institution, 2001). Available phosphorous, NH_4^+-N, and NO_3^- -N, were determined using spectrometric methods (ISO 11263, 1994; Jackson et al., 1986). and nutrient analyses have been previously published (Cipullo et al., 2019), and results presented here (Table 5.1) are reproduced with permission of the authors.

For each soil, three common in-situ test treatment conditions were tested (Figure 5.1): (1) soil without amendment (unamended); (2) soil amended with 5% w/w biochar; and (3) soil amended with 15% w/w compost mixture. Amendment levels were selected based on previous studies which have shown effective reduction of toxicity, contaminant bioavailability, and/or total petroleum hydrocarbon content at these application rates (Novak et al., 2018; Sigmund et al., 2018; Taccari et al., 2012). Prior to mixing in amendments, soils were partially air dried then passed through a 2 mm sieve in order to remove pebbles and larger plant debris and to improve sample homogeneity. For each test condition, duplicate 10 L polypropylene buckets were filled with appx. 10 cm of gravel and topped with 5 kg of the unamended or amended test soil. The containers were then covered with a perforated lid to allow partial infiltration of rainwater while limiting exposure to light and wind. Test buckets were stored outdoors at Cranfield UK for the duration of the experiment, between May-November 2017. Local mean temperature for this period was 14.6°C, with maximum temperatures reaching up to 29.7°C in July; total rainfall over the study was 367 mm (Met Office, 2017, Appendix C for further details). After mixing the top 30 cm of each test bucket, composite samples of approximately 300 g obtained from 3 randomly selected areas of each bucket. Samples were collected at 0, 30, 90 and 180 days (T0-T180, respectively) and. stored immediately at -80°C to inhibit further chemical transformations.

5.2.3 PAH and metabolite extraction

Prior to extraction, samples were wrapped loosely in foil, thawed, and allowed to airdry overnight in a fume hood. Samples were then sieved through 2 mm screens to remove larger particles incorporated during the addition of the compost and biochar amendments. Subsamples of each soil were prepared using the coning and quartering method outlined in USEPA guidance document EPA/600/R-03/027 (Gerlach and

Nocerino, 2003). For all samples, three chemical fractions were considered as follows: (1) a 'total extractable' content determined using strong solvent and multiple extraction cycles (dichloromethane + acetonitrile); (2) a 'readily available' fraction obtained through weaker extraction with methanol; and (3) a 'leachate' analogue fraction from initial extraction with water followed by liquid-liquid extraction with dichloromethane. Together, the readily available and leachate fractions are termed 'labile fractions'. As the >2 mm fraction of composted samples included substantive proportions of wood shavings which may support unique ecological functions including increased sorption of contaminants (Zhang et al., 2014) and habitat for PAH-degrading organisms (Haritash and Kaushik, 2009), this fraction was reserved for separate analysis.

All samples were extracted in amber glass vials or foil-wrapped glassware fitted with PTFE lined caps to minimise exposure to light and reduce potential analyte sorption. Non-volumetric glassware was acid-washed, rinsed with deionised water, and furnaced at 450°C for 4 hr to remove any residual organic matter. Volumetric glassware and other implements were acid washed and rinsed in deionised water then sonicated in acetone prior to use. For all fractions, blank extractions (i.e. extractions containing all other reagents but no soil) were conducted for every 12 samples. Surrogate spikes containing deuterated PAH were used in all extractions to monitor consistency of the extractions. In addition, 2.5 g material was used to determine residual moisture content at the time of extraction and to normalise all final concentrations to dry soil mass content. Further details including method recoveries are presented in Appendix B.

Total Extractable Fraction

To determine total extractable PAH and metabolites, 0.5 g air dried soil was weighed into a 40 mL amber glass vial and mixed with ~1.8 g Na_2SO_4, then spiked with 50 µL 100 µg/mL deuterated PAH surrogate mixture. Each sample was extracted in two cycles - first with 10 mL dichloromethane (DCM), then with 10 mL acetonitrile (ACN). For each cycle, extraction vials were rotary-shaken for 20 min, placed in an ultrasonic bath for 10 min, then centrifuged (900 g, 90 min). The supernatants from each extraction cycle were combined and the whole extract reduced to 1 mL then topped with an additional 1 mL DCM. This extract was then cleaned and separated into three fractions using an aminopropylsilica-SPE method we developed in Chapter 4 (Pulleyblank et al. 2020). Briefly, 100 µL of the concentrated supernatant was applied to an aminopropylsilica column (Discovery 3 mL 500 mg) preconditioned with 6 mL 9:1

Hexane (HEX):DCM. PAH and OPAH were eluted with 6 mL 9:1 HEX:DCM, and the eluate reduced to 5 mL (eluate A); OHPAH were subsequently eluted with 4 mL acetone + 2 mL ACN (eluate B); finally COOHPAH and other phenolic acids/aldehydes were eluted with 6 mL ACN mixed with water and triethylamine (90:10:0.05) (eluate C – used for qualitative analysis only). For eluates B and C, a 1.2 mL aliquot was dried under a gentle stream of nitrogen and resuspended in 100 µL ACN prior to derivatisation. In addition to full extraction blanks, blank SPE runs (using all SPE reagents but no load) were also run for every 12 samples.

Readily Available Fraction –Methanol Extract

Following previously reported methods for determining readily available PAH (Cipullo et al., 2019), 2.5 g soil was mixed with 15 mL methanol, and samples were sonicated for 20 min at room temperature, shaken overnight at 150 rpm for 16 h, then sonicated again for 20 min, centrifuged (2000 g for 10 min), and passed through a 6 mL DSC-Si SPE cartridge. A 2 mL aliquot was then spiked with 50 µL of deuterated surrogate spike mix and evaporated under a gentle stream of N_2. The sample was resuspended in 2 mL 1:1 DCM:ACN, sonicated briefly, and passed through a 0.2 µm PTFE syringe filter unit prior to derivatisation and analysis.

Leachate Fraction- Aqueous Extract + Liquid Liquid Extraction

A leachate analogue was prepared by mixing 2.5 g soil samples with 10 mL 18 MΩ deionised water followed by rapid shaking on a vortex shaker for 20 mins. This ratio was selected to match the final soil/solvent extraction ratio for total extracts. Samples were allowed to sit overnight for 16 h at 4°C, then brought to room temperature, re-shaken for 10 mins, and centrifuged at 900 g for 45 mins. A 2 mL aliquot of supernatant was spiked with 20 µL deuterated PAH (10 µg/mL semivolatile internal standard mix, 100 µg/mL naphthalened-8) and salicylic acid-d_4 (100 µg/mL) as surrogates, pH adjusted to <2 with 200 µL HCl, and the sample extracted 3 times with 2 mL DCM. After each cycle, the DCM layer was removed to a graduated Pyrex test tube (0.1 mL increments), and the combined extract was reduced to 1 mL.

>2 mm Fraction (compost amended samples)

The >2 mm fraction of compost amended samples, primarily composed of larger softwood shavings, was also analysed to consider the possibility of apparent

contaminant removal via sorption onto this fraction and/or different transformation processes associated with these materials. 5 g material was mixed with 100 mL deionised water and shaken at 600 strokes per minute for 1 min to dislodge clinging particulates. The mixture was then drained over a 600 µm screen and quickly rinsed in three stages using a total of 100 mL deionised water. The materials were then transferred to foil and dried for 3 min under a stream of nitrogen. Samples were spiked with 50 µL deuterated PAH mixture (200 µg/mL each) and mixed with 5 g Na_2SO_4 to remove residual moisture. The sample was extracted in a similar manner to the <2 mm total extractable fraction, with two extraction cycles, first with 40 mL DCM, then with 40 mL ACN, each cycle including 20 min shaking followed by 10 min sonication. The combined supernatants were filtered through 0.2 µm PTFE membrane prior to analysis.

5.2.4 Derivatisation and GC-MS analysis

To prepare extracts for GC-MS analysis, 100 µL sample extract, 20 µL of internal standard mixture (20 µg/mL each nonadecane-d_{40} and deuterated triacontane-d_{62} in DCM), and 20 µL of BSTFA with 1% TMCS as a derivatisation agent were added to a 200 µL glass insert inside a 2 mL amber glass GC vial with silicone/PTFE screwcap. Samples were then vortex-shaken at room temperature for 60 minutes. As PAH and OPAH do not require derivatisation, the derivatisation agent was omitted for analysis of eluate A from the total extractable fraction.

Target compounds were analysed by GC-EI-MS (Shimadzu TQ-8040) with AOC-6000 autosampler and Lab Insight Solutions software (Shimadzu, 2015-2016). Compound separation was achieved using a 30 m Rtx-5 column (5% diphenyl/95% dimethylpolysiloxane 0.25 mm i.d., 0.25 µm df). Instrument settings were as follows: 1 µL injection at 200°C, splitless mode; initial oven temperature 80°C, raised at a rate of 5°C min^{-1} to 100°C, then 8°C min^{-1} to 200°C, and 3°C min^{-1} to 300°C and held for 2 min; column flow 36 cms^{-1}; transfer line and MS system 300°C; EI source, 70 eV; detector scan/SIM mode (0.3 s and 0.060 s event times respectively).

Calibration was conducted using the internal standard method at analyte concentrations of 10, 1, 0.1, and 0.01 µg/mL, with 0 µg/mL serving as a blank. Calibration solutions for each fraction were prepared in the same solvent mixture as samples, and in the case of leachate-LLE extractions, represent full method calibration. LOD and LOQ were established through calculation of, respectively, 3 and 10 times the

standard deviation of the IS-adjusted signal from seven replicate injections of the lowest calibration solution divided by the slope of the calibration curve.

Target analytes were identified as the molecular ion $M^{\cdot+}$ (PAH, OPAH) or as trimethylsilyl (TMS) derivatives (OHPAH, phenols, acids). A compound was considered identified if the retention time deviated <0.1 min from the standard retention time, the ratio of quantitative and reference ion differed less than 30% of the analytical standard, and other ions present in the overall mass pattern showed good visual agreement with major ions associated with the analytical standard. Derivatised samples were also monitored for the presence of residual underivatised target compounds. In addition, a blank injection of ACN was included every 6 samples to monitor for instrumental carry over between soils (ref. section 3.2.8). Any extraction blank contributing a targeted m/z signal at analyte retention time was subtracted prior to calculation of final concentrations.

5.2.5 Calculations

All post-processing was conducted in MATLAB (2018 Academic License) using custom scripts incorporating common statistical functions and visualisations (mean, standard deviation, boxplot) as well linear regression analysis using the fitlm function. Σ7 PAH included the following: naphthalene, acenapthene, acenaphthylene, fluorene, phenanthrene, anthracene, and pyrene. Figures were prepared using customised versions of Martineau subplot scripts for MATLAB (Martineau, 2014).

5.3 Results and discussion

5.3.1 Bulk soil characteristics

Soils 1 and 2 differed substantially in terms of soil texture, organic content, and nutrient profiles (Table 5.1). Soil 1 was coarse textured, alkaline, and contained a high proportion of tar, also evidenced by high levels of organic matter, carbon, aliphatic hydrocarbons, and PAH. Soil 2 was more finely-textured, with an elevated proportion of clay, lower proportion of organic matter, carbon, and PAH, and lower pH. Nitrogen availability was higher in Soil 2, but both soils fell short when C:N ratios were compared to USEPA recommended levels for the bioremediation of soils (10:1), suggesting remediation might be enhanced by increasing nutrient levels (USEPA 2002). Both compost and biochar contributed organic matter, carbon, nitrogen, and

phosphorus, but compost amendment supplied higher levels of the more readily available forms - available P and nitrate-N.

Table 5.1 Bulk characteristics of gasworks soils and amendments

Characteristic	Soil 1	Soil 2	Biochar	Compost mix
Source	Kent UK	Northamptonshire UK	Carbon Gold UK	Westland Horticulture Ltd. UK; Tesco UK
Material	gasworks contaminated soil associated with tar tank	gasworks contaminated soil	enriched biochar	peat-based compost with nutrients (70%); softwood shavings (30%)
<u>Particle Size Distribution</u>				
Amendments				
10-20 mm %	--	--	22.8	54.5
2-10 mm %	--	--	22.2	24.7
0.6-2 mm (coarse) %	--	--	15.5	7.5
0.2-0.6 mm (medium) %	--	--	37.7	9.0
<0.06 mm (fine) %	--	--	1.7	4.5
Mineral Fractions				
sand %	88.3	51.8	--	--
silt %	8.1	22.8	--	--
clay %	3.6	25.3	--	--
soil texture	coarse sand	fine sandy loam	--	--
Residual moisture at start	7.3	17.2	25.9	29.3
pH ($CaCl_2$)	8.4	7.4	10	6
Organic matter (LOI %)	19.1	8.8	76.0	78.7
Σ7 PAH mg/kg	8500±250	960±60	--	--
Σ Aliphatics	1112.4 ± 52.9	95.9 ± 6.3	--	--
<u>Nutrients</u>				
Total C%	18.3	5.8	59	40.2
Total N%	0.4	0.2	0.9	1.0
C:N	49.6	23.7	64.7	42.3
Available P mg/kg	35	33.7	74.1	328.1
NH_4^+-N mg/kg	9.5	71	n.d.	n.d.
NO_3^--N mg/kg	2	18	n.d.	310

n.d. not detected

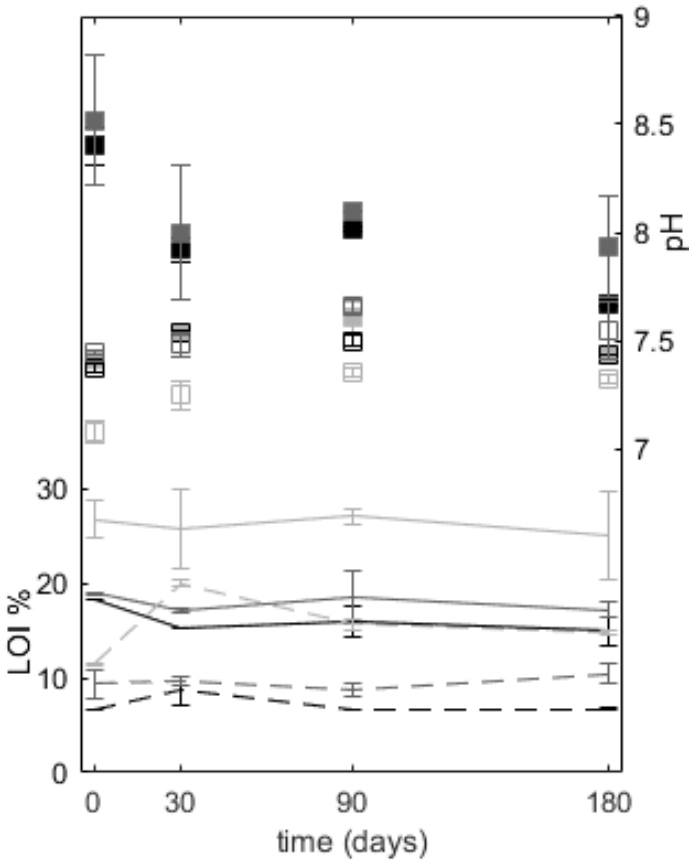

Figure 5.2 Total organic matter (LOI %) and pH: black - unamended, dark grey - biochar amended, light grey - compost amended; solid lines and filled boxes - Soil 1; dashed lines and empty boxes - Soil 2.

Organic matter content and pH were monitored in the <2 mm fraction for the course of the experiment (Figure 5.2). At T0, the >2 mm fraction comprised approximately 10% of the bulk mass of the compost-treated soils, but over time, the proportionate quantity and quality of this fraction changed as shavings and larger compost materials were also degraded and incorporated into the <2 mm fraction. This was reflected by increases in organic matter content in the <2 mm fraction for Soil 2 compost-amended samples, and by somewhat lesser reductions and higher variability for Soil 1 compost amended samples compared to other treatments. Soil pH decreased with the addition of compost and increased with the addition of biochar. Over time, pH of Soil 1 in biochar and unamended samples decreased, but in Soil 1 compost amended and in Soil 2 samples, pH increased moderately until T90, then declined.

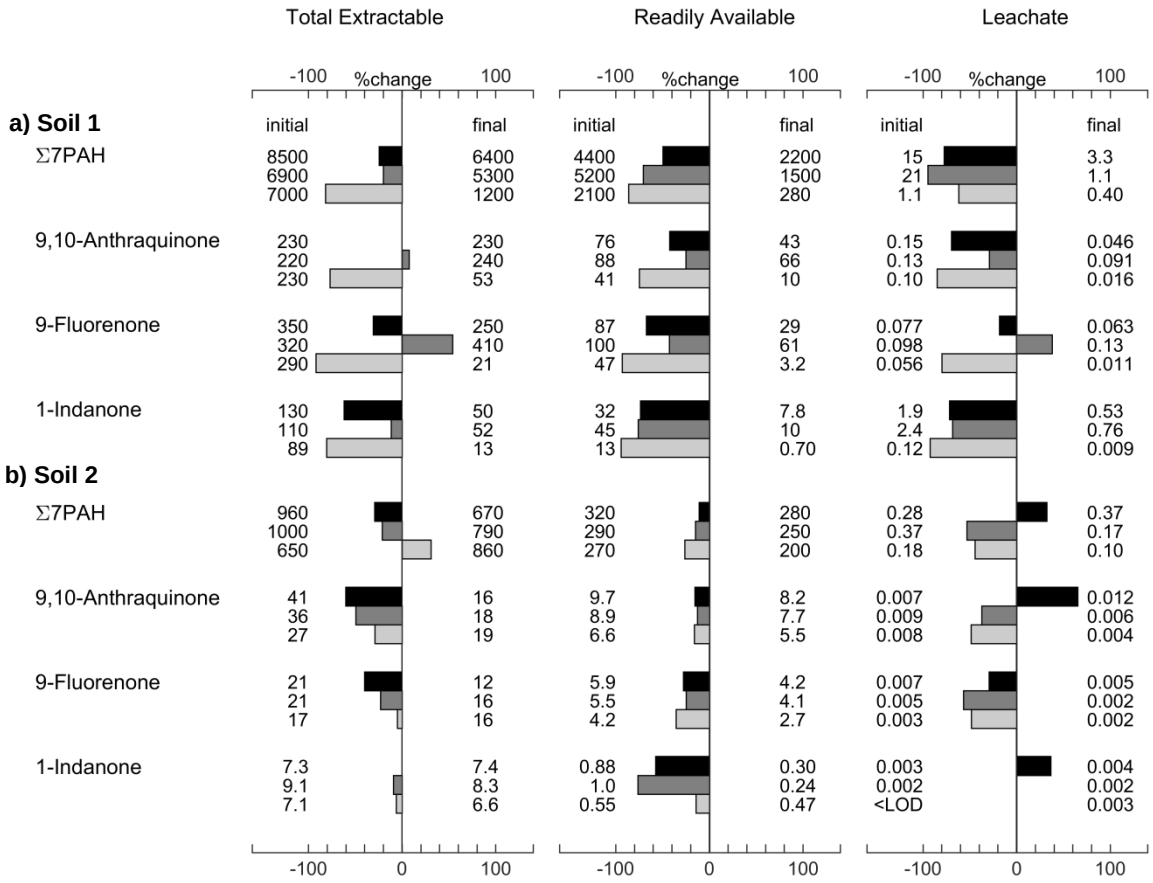

Figure 5.3 Mean concentrations of PAH and OPAH (µg/g dry soil) at initial (T0) and final (T180) sampling points in three fractions, a) Soil 1, b) Soil 2. Bars indicate percent change from T0, with negative values indicating contaminant removal. Black - unamended; dark grey - biochar amended; light grey - compost amended.

5.3.2 PAH

Summary trends for PAH are displayed in Figure 5.3. Individual 2-4 ring PAH in Soil 1 followed similar attenuation patterns (Figure 5.4), but lower molecular weight PAH exhibited increased removal rates and lesser differences between sample treatments. By T180, greatest reductions of Σ7 PAH in Soil 1 were observed in compost amended samples (82%), lesser total reductions in biochar amended samples (20%), with unamended samples indicating similar though slightly greater declines compared to biochar samples (24%) (Figure 5.3). Soil 2 exhibited a different trend: while the overall PAH attenuation rate in biochar amended and unamended samples (21% and 29%, respectively) were similar to Soil 1, compost amended samples exhibited substantially lower initial concentrations that increased by the end of the study (+30%). Both patterns of comparative attenuation have been previously reported: i.e. biochar addition may offer a comparatively limited or suppressive effect on PAH degradation (Bao et al.

2020; Han et al. 2016; Sigmund et al. 2018), but reduced PAH attenuation in compost treated soils compared to biochar treated soils has also been observed (Beesley et al. 2010). Results resented here indicate that soil type and contamination level are likely to impact outcomes and may partially explain these differing reports. In their study, Beesley et al. (2010) attributed reduced attenuation in compost-amended samples to differing mechanisms of contaminant removal (enhanced PAH sorption in biochar samples vs. and more gradual contaminant degradation in compost-amended samples). Here, reduced attenuation in Soil 2 compost amended samples may also be explained by initially stronger interaction between PAH and the organic amendment for the less contaminated soil. Individual PAH in Soil 2 compost amended samples exhibited temporary reductions in total extractable PAH at T30 that coincided with concentrations peaking in the >2 mm fraction (Figure 5.4). This suggests sorption on the organic amendment increased over the first stage of the study and was followed by partial release back into the extractable fraction, though it is noted that the extent of the observed 'dip' in the <2 mm fraction is likely in part also attributed to increased dilution by amended organic material at T30 (Figure 5.2). The sorption-and-release effect was also observed to a lesser extent for some PAH in Soil 2 unamended and biochar amended samples (Figure 5.5). Overall, these interactions delayed removal of LMW PAH from Soil 2 samples. Although enhanced sorption on the >2 mm fraction at T0 and T30 was also observed for Soil 1, greater PAH concentrations, higher attenuation rates, and lesser shifts in <2 mm total organic matter content meant this did not translate into a similar 'dip' or 'delay' effect (Figure 5.5).

In the labile fractions, compost amended samples exhibited lowest PAH concentrations for both soils throughout the study (Figure 5.3). In compost amended samples, attenuation rates in labile fractions samples were higher for Soil 1 (readily available 75%; leachate 62%) than Soil 2 (readily available 27%; leachate 44%), though it is interesting that Soil 2 supported these declines despite the increases observed in the total extractable fraction. This indicates that the sorption-and-release effect observed in the total extractable content was not sufficient to translate to the readily available and leachate fractions. Biochar amended samples exhibited highest initial levels of labile PAH for both soils, but final concentrations were lower than for unamended samples. For Soil 1 biochar amended and unamended samples, substantial reductions of labile PAH with lesser reductions in the total extractable fraction suggests the persistence or formation of recalcitrant residues by T180, as observed in other studies (Beesley et al

2010; Oleszczuk and Koltowski, 2018). On the other hand, Soil 2 unamended samples exhibited increasing levels of leachable PAH, contrasting the common contention that ageing processes tend to reduce PAH mobility (Riding et al., 2013). Here, the effect may have been influenced by the degradation of soil organic matter (Figure 5.2); this could have resulted in reduced bulk sorptive capacity or increased formation of humic materials/biosurfactants which can increase PAH solubility (Lipczynska-Kochany, 2018). If these processes also occurred in amended samples, it is possible that the amended sorptive materials limited the effect.

5.3.3 PAH transformation products

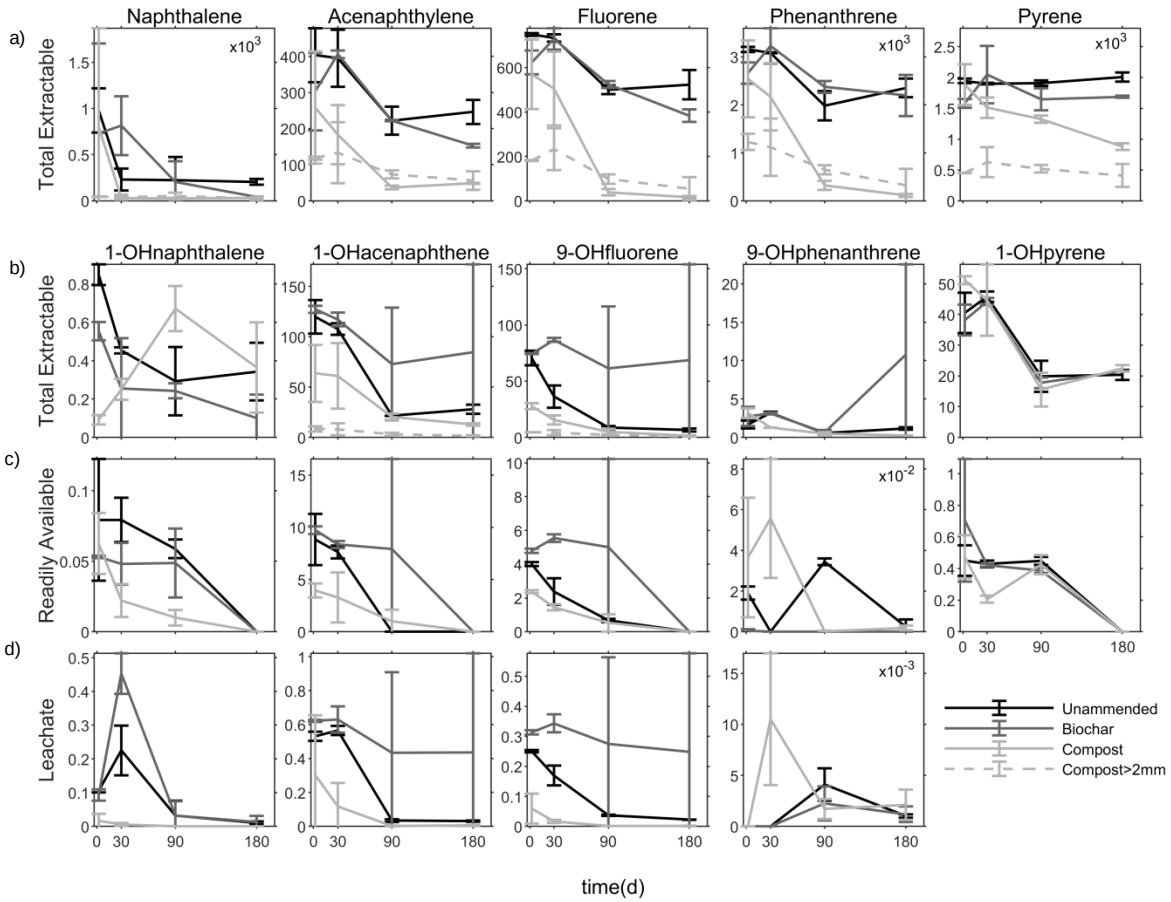

Figure 5.4 Soil 1: a) parent PAH in total extractable fraction; b-d) OHPAH in total extractable, readily available, and leachate fractions, respectively. All concentrations normalised to µg/g dry soil or in the case of >2 mm fraction, µg/g dry bulk material. Note scaling factors in top corners of selected subplots. 1-hydroxypyrene fell below LOD in leachate fraction. Less abundant, acenaphthene (init. 78 µg/g) and anthracene (init. 1180 µg/g) (not displayed), exhibited similar behaviour to acenaphthylene and pyrene, respectively.

5.3.3.1 OHPAH

PAH modified by hydroxyl groups often represent the first stages of PAH catabolism and may be considered positive indicators of PAH biodegradation (Haritash and Kaushik, 2009). Initial OHPAH concentrations in total extracts of unamended samples ranged from 0.9 μg/g (1-hydroxynaphthalene) to 145 μg/g (1-hydroxyacenapthene) for Soil 1 (Figure 5.4) and 1.0 μg/g (1-hydroxyfluorene) to 9.3 μg/g (9-hydroxyphenanthrene) for Soil 2 (Figure 5.5). Both soils, especially Soil 1, exhibited higher levels of OHPAH than previously reported (ref. Table 1.1), suggesting OHPAH may be more relevant in some soil systems than previously thought.

In most cases, Soil 1 total extractable concentrations of individual OHPAH declined over the course of the study, with lowest OHPAH levels most often observed in compost amended samples (Figure 5.4). One exception was the notable increase in 1-hydroxynaphthalene in compost amended samples by T90, suggesting this treatment supported more active naphthalene transformation than biochar, which is consistent with other studies (Bao et al., 2020; Sigmund et al., 2018). A second exception was the notable split observed in biochar samples, with one test system supporting substantial declines and the other substantial increases in 1-hydroxyacenapthene and 9-hydroxyfluorene concentrations at T90 and T180. This difference persisted after repeating all airdrying, extraction, and analysis steps, and occurred in all fractions, indicating this was not an analytical artefact. Instead, this suggests the outcome of biochar treatment may be highly variable even in the same soil and weather conditions. The different response showed no clear relationship to shifts in parent PAH concentrations, total organic matter, pH, or microbial respiration previously reported by Cipullo et al. (2019). However, an additional split in biochar amended samples (with inverse high/low concentrations) observed for 9-hydroxyphenanthrene at T180 associated elevated concentrations with increased removal of phenanthrene and reduced pH. The application of biochars are known to impact the microbial community in ways that are difficult to predict (Zhu et al., 2017), and it is possible that over time different communities of degrader species became established in the two biochar amended systems, leading to preferential removal/formation of specific PAH/OHPAH. It was previously observed that the microbial community was less stable in biochar amended samples compared to compost and unamended samples based on analysis of phospholipid fatty acid profiles (Cipullo et al., 2019); however, further microbiological analyses were beyond the scope of the current study. 1-hydroxypyrene exhibited similar

trends across all treatments, indicating that amendment was not a significant factor impacting the presence of this compound in Soil 1.

In Soil 1 labile fractions, OHPAH trends varied substantially between treatment, analyte, and fraction. Concentrations of labile 1-hydroxyacenaphthene and 9-hydroxyfluorene generally tracked trends observed in the total extractable fraction, with the primary difference that by T180, the readily available fraction was reduced in both biochar amended test systems. The greater persistence of these compounds in the leachate fraction despite their removal from the readily available fraction seems surprising, but might be explained by the presence of small amounts of colloidal materials in the prepared leachate (Enell et al., 2016). Labile fractions of other targeted OHPAH did not follow trends observed for the total extractable fraction. Importantly, increased concentrations of total extractable 1-hydroxynaphthalene in compost-amended samples did not translate into increases in the labile fractions, where concentrations remained lower than the other treatments and declined steadily throughout the study. For 9-hydroxyphenanthrene, highest labile concentrations were offset either later (T30 compost, T90 unamended) or earlier (T90 biochar) than maxima for total extractable contents (T0, T30, and T180, respectively). Readily available 1-hydroxypyrene declined across all samples and showed little difference between treatments, but showed persistence or increases between T30 and T90, which may have contributed to the more substantial declines observed in the total extractable fraction.

Comparing T0 with T180, OHPAH in Soil 2 total extracts exhibited either little net change (most OHPAH in compost amended samples; 1-hydroxyacenaphthene in unamended samples) or net declines (1-hydroxynaphthalene all treatments; most OHPAH in biochar and unamended samples) (Figure 5.5). Interestingly, final concentrations of OHPAH were very similar between all treatments. In some cases, however, OHPAH concentrations at T30 and T90 ranged generally from 20-300% and up to 10 times higher than initial levels. Highest concentrations of lowest, mid-size, and largest targeted OHPAH were observed in compost, unamended, and biochar samples respectively, indicating no one treatment led to highest or lowest levels of all OHPAH over the duration of the experiment, but that risk profiles of the treatments in this soil may be characterised differently with increasing contaminant size. Acknowledging a trade-off of increased concentrations of 1-hydroxynaphthalene (Wang et al., 2017), there may have been an overall advantage to compost application in terms of OHPAH speciation since larger OHPAH are expected to elicit increased genotoxic

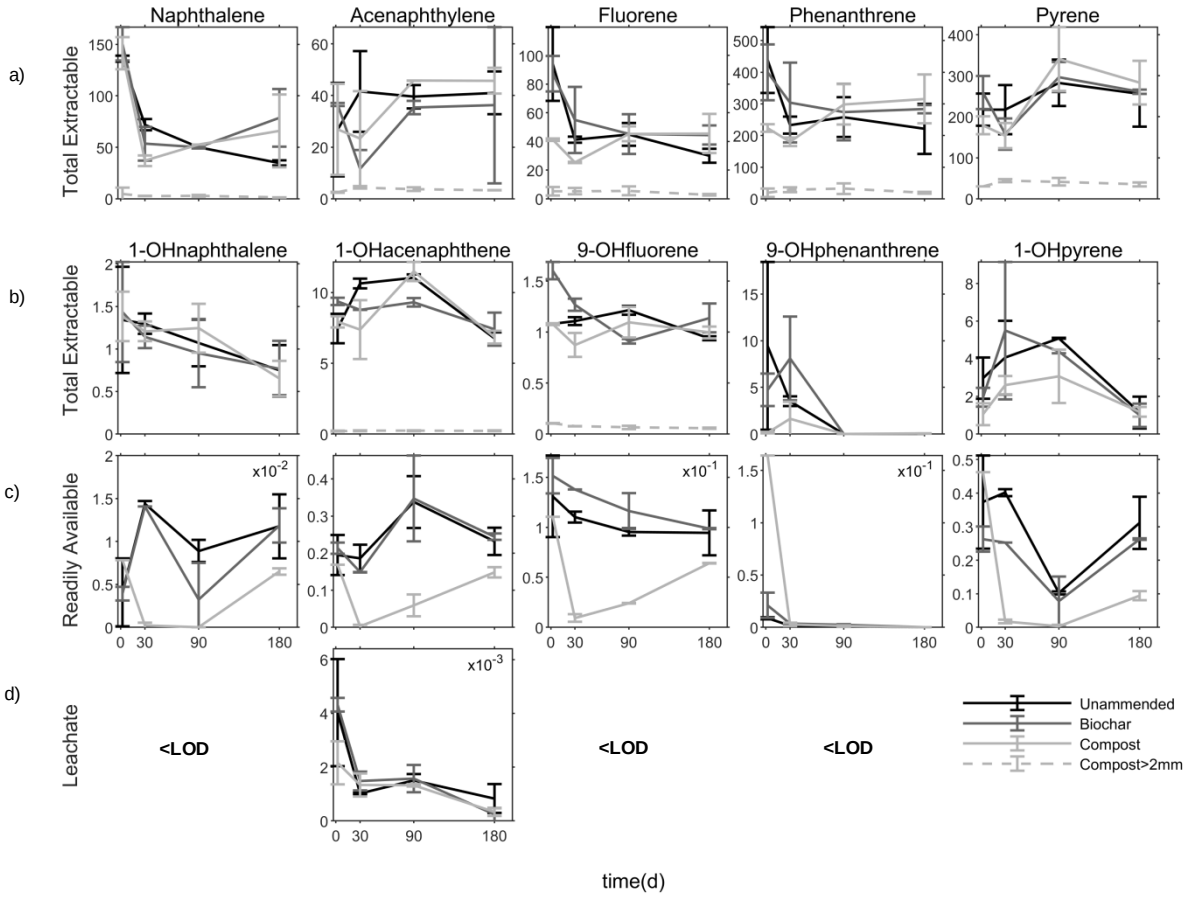

Figure 5.5 Soil 2: a) parent PAH in total extractable fraction; b)-d) OHPAH in total extractable, readily available, and leachate fractions, respectively. All concentrations normalised to µg/g dry soil or in the case of >2 mm fraction, µg/g dry bulk material. Note scaling factors in top corners of selected subplots. 1-hydroxypyrene also fell below LOD in leachate fraction. Less abundant, acenaphthene (init. 27 µg/g) and anthracene (init. 4 µg/g) (not displayed), exhibited similar behaviour to acenaphthylene and pyrene, respectively.

effects due to their association with larger, more toxic PAH (Ortega-Calvo et al., 2013), with related disadvantages for the biochar application.

Treatment-related trends in Soil 2 readily available fraction were more consistent than for Soil 1: most often, unamended and biochar amended samples exhibited highest and similar levels, while lowest concentrations were observed in compost amended samples. Compost amended samples were characterised by rapid initial attenuation of readily available OHPAH followed by gradual increases, possibly due to sorption-and-release from the organic amendments over time (or reduced sorption of newly-formed OHPAH). Although the final upward trend in the readily available fraction for OHPAH in compost samples and specific OHPAH in other treatments may be concerning, when paired with reductions in the leachate fraction as

observed for 1-hydroxyacenaphthene, could represent ideal conditions for promoting degradation in situ without increasing downstream risk. Together, results from the two soils support the view that temporal trends in OHPAH in the labile fractions in general cannot easily be directly predicted by total extractable levels, and that periods of highest contaminant mobility and availability may differ from periods of highest total concentrations.

5.3.3.2 OPAH

OPAH contamination may co-occur with original PAH pollution, and/or develop through the modification of PAH and OHPAH by soil flora or other abiotic processes. When formed in soil, OPAH often represent side reactions of primary PAH/OHPAH catabolism, with reduced rates of subsequent transformation (Lundstedt et al., 2007). Both of these processes may help explain observed OPAH concentrations that were generally half to 2 orders of magnitude greater than OHPAH concentrations in all soils and treatments (Figure 5.3).

For Soil 1, compost amended samples demonstrated lowest initial concentrations and largest reductions of OPAH between T0 and T180 across all fractions (Figure 5.3). Biochar amended samples supported highest mean final concentrations of OPAH across all fractions. OPAH in this soil tended to follow patterns associated with parent PAH, except that concentrations of 9-fluorenone tended to be lower in unamended samples than in biochar amended samples, as observed for 9-hydroxyfluorene. 9-fluorenone, 1-indanone, and to a lesser extent 9,10-anthraquinone, were also impacted by the 'split' in biochar treatment systems as observed for other OHPAH, described above (section 5.3.3.1 ref. Figure 5.4). This split also contributed to reduced attenuation and/or increases in mean concentrations of OPAH when compared to parent PAH and to other treatments (Figure 5.3). Importantly, total extractable concentrations of 9-10-anthraquinone in unamended and biochar samples were not substantially changed by the end of the study, supporting the observations of other researchers that this compound exhibits significant longevity in some soil systems, and that over the course of time, some OPAH may become more dominant in the soil profile than the parent PAH (Lundstedt et al., 2007; Wilcke et al., 2014a).

Soil 2 compost amended samples also exhibited lowest initial OPAH concentrations across total, readily available, and leachate fractions (Figure 5.3). However, in comparison to Soil 1, Soil 2 compost amended samples had substantially

lower OPAH removal rates, and in most cases supported final concentrations that were the same as, or higher than for other treatments. Reduced attenuation of 9-fluorenone in these amended samples was partially related to the 'dip' effect at observed for parent PAH and 9-hydroxyfluorene putatively attributed to sorption-and-release and changes in bulk dilution, described above (section 5.3.2 ref. Figure 5.4). This effect was less apparent for 9-10 anthraquinone and 1-indanone, which showed initial declines followed by increases at T180 and T90 respectively, but which exhibited similar fluctuations for all treatments. Biochar samples supported net declines in OPAH concentrations in total extracts as well as labile fractions. Unamended samples generally supported higher OPAH removal rates from total extracts suggesting that for this soil, the addition of amendments either did not enhance, or indeed inhibited removal of OPAH as well as the parent PAH. At the same time, increased levels of leachate PAH and OPAH represent a concern for the unamended soil, and attenuation may have been driven primarily by leaching. In contrast to Soil 1, removal of 9,10-anthraquinone in Soil 2 matched or exceeded removal of anthracene in nearly all samples, but 9-fluorenone exhibited attenuation rates that were 40-90% lower compared to fluorene. While it is possible this was a temporary effect due to increased fluorene turnover, it is in line with the view that the proportion and importance of OPAH compared to parent PAH may increase over time, and highlights that these trends are likely to be compound and soil-specific (Hu et al., 2014; Lundstedt et al., 2007).

5.3.4 Fractionation of PAH, OPAH and OHPAH

In addition to considering total concentrations of PAH and their transformation products in individual fractions of the two soils, we also compared the effect of soil type and treatment on the proportionate fractionation of these compounds over the duration of the experiment. This more readily captures the overall impact of soil type, treatment, and time, on the mobility of contaminants separate from considerations of specific analyte concentrations. Ratios describing the proportion of readily available/total extractable (RA/TE), leachate/total extractable (LE/TE) and leachate/readily available (LE/RA) PAH+OPAH, and OHPAH, are displayed in Figures 5.5 and 5.6, respectively.

Readily available Σ7PAH comprised ~40-60% and ~30-50% of the total extractable amount for unamended Soils 1 and 2 respectively (Figure 5.6). Surprisingly, RA/TE ratios for OPAH were frequently lower than those observed for individual parent PAH, most notably in Soil 1 (Soil 1 ~10-35% and Soil 2 ~2-65%). One

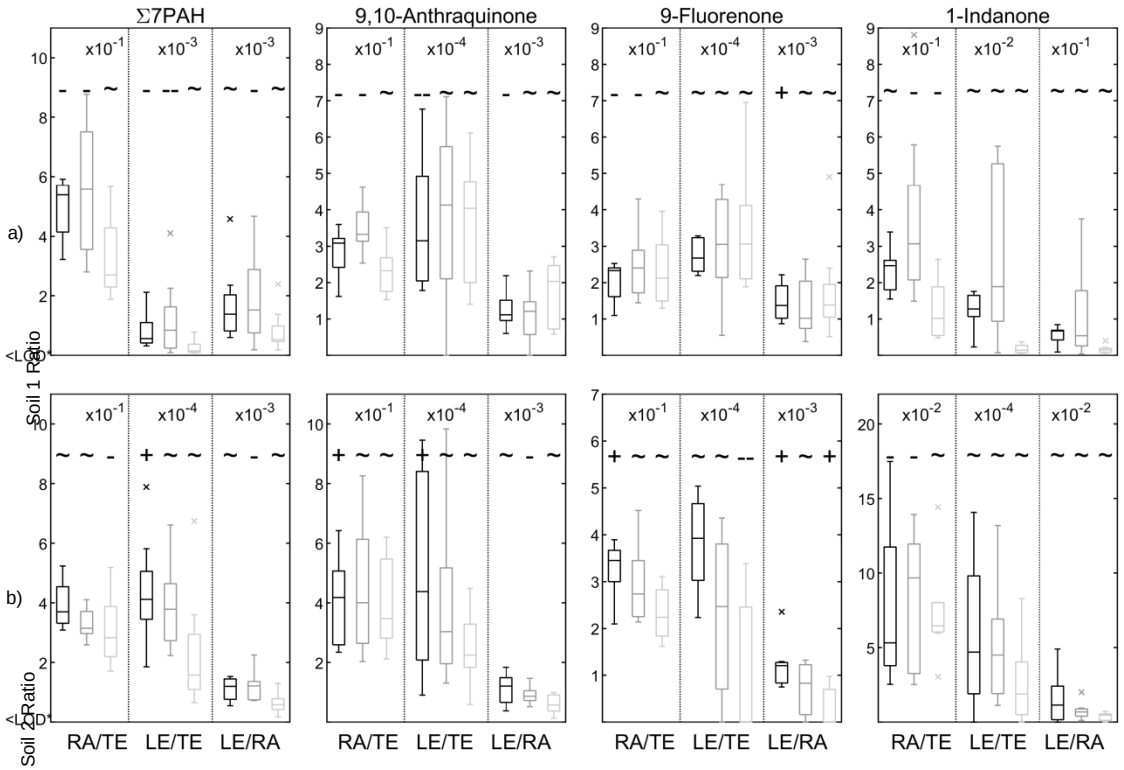

Figure 5.6 Fractionation ratios of PAH and OPAH between total extractable (TE) readily available (RA), and leachable (LE) fractions, comparisons specified at bottom. Note scaling factors indicated at the top of each panel. a) Soil 1, b) Soil 2; black-unamended, dark grey- biochar amended, light grey-compost amended. Higher ratios indicate reduced sorption/higher contaminant lability; lower ratios indicate higher sorption/reduced contaminant lability. Boxplots display the median (central line) and interquartile range (box). Whiskers show minimum and maximum values within ~2.7σ, and outliers indicated by an 'x' symbol are plotted where values exceed the $75^{th}/25^{th}$ percentile ± the maximum whisker length times the interquartile range (MATLAB 2018). * where a value extends to the bottom of the plot, this indicates that the measure contributing to the nominator value of the specified ratio fell below the LOD. Additional notation at the above each box indicates how the ratio changed over time: ++/-- ratio consistently increased/decreased; +/- generally increased/decreased, but exhibited greater fluctuation or variability; ~ fluctuated without clear positive or negative trend, exhibited high variability, or did not substantially change between T0 and T180.

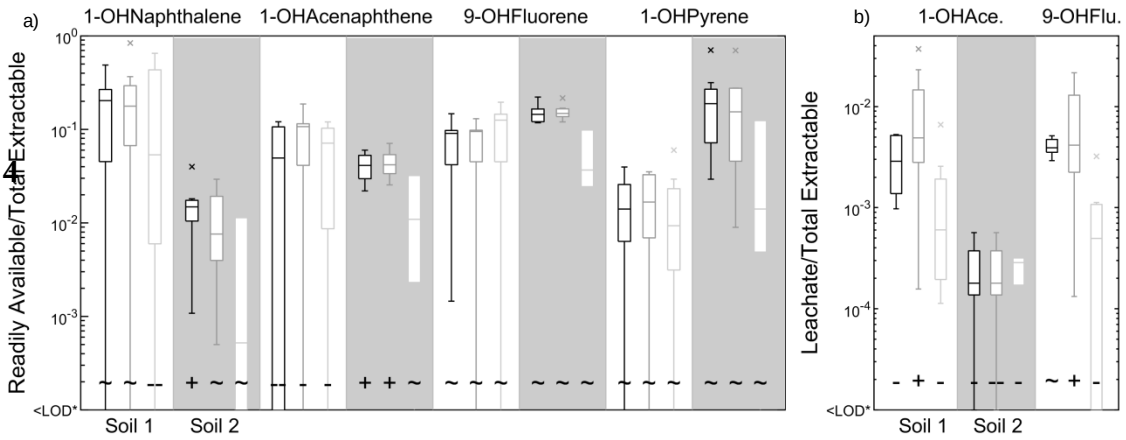

Figure 5.7 OHPAH fractionation ratios a) Readily Available/Total Extractable, b) Leachate/Total Extractable. White panels - Soil 1, grey panels - Soil 2; Box colour: black - unamended; dark grey - biochar amended; light grey - compost amended. Note semilog scale. Further boxplot symbology, including indications of temporal trends +/~/- described in Figure 5.6 caption. Cases where a majority of measures fell below the LOD were excluded.

explanation could be that the very high levels of PAH especially in Soil 1 might have led to a competition effect during non-exhaustive methanol extraction. Higher leachability of OPAH compared to parent PAH (median LE/TE 10-170% higher) was observed for both soils As expected. LE/TE ratios for 9,10-anthraquinone and 9-fluorenone (0.02-0.08%) agreed between two soils within 25%, but LE/TE proportions of Σ7PAH (~0.03-0.1%) and 1-indanone (~0.01-2%) were 1 and 2 orders of magnitude higher for Soil 1 than for Soil 2. Higher values could be due to overall reduced sorptive capacity of Soil 1 due to lower proportion of silt and clay (Table 1) (Biswas et al., 2015), and/or increased saturation of the soil with competing compounds. On the other hand, unamended Soil 1 PAH and OPAH lability declined somewhat over time, perhaps because these compounds interact more strongly with petroleum and black carbon fractions (Arp et al., 2014). The importance of organic matter on contaminant sequestration was also apparent in Soil 2, as unamended samples supported somewhat increased lability of PAH, OPAH, and some OHPAH over time despite a higher proportion of clay, while this effect was similar or reduced in amended samples.

Fractionation ratios for OHPAH (Figure 5.7) tended to extend over broader ranges, generally varying over half to 2 orders of magnitude within each soil, suggesting a greater diversity of factors impacted fractionation of OHPAH compared to PAH and

OPAH. Specific trends observed across OHPAH also support the view that contaminant lability was primarily controlled by different factors for the two soils. For Soil 1, the proportion of readily available OHPAH decreased with increasing analyte mass and increasing non-polar character, with maximum RA/TE proportions of 1-hydroxynaphthalene and 1-hydroxypyrene at 49 and 4% respectively, suggesting analyte availability was characterised primarily weaker or stronger interaction with the bulk petroleum fraction. Conversely, for Soil 2, the proportion of readily available OHPAH decreased with decreasing analyte mass, (3.9% 1-hydroxynaphthalene vs. ~30% 1-hydroxypyrene), which may be explained by increasing interaction of the smaller more polar compounds with the fine mineral fractions in this soil, and reduced sorption of larger compounds. Reduced leachability of 1-hydroxyacenaphthene was also observed for Soil 2 compared to Soil 1.

The effect of amendments on contaminant mobility depended on soil type and contaminant. Biochar amendment reduced contaminant lability of less polar compounds in Soil 2 - primarily PAH and larger OPAH (~15-20% lower), but in most cases either exhibited similar fractionation or increased contaminant lability compared to unamended samples. This was particularly notable for Soil 1 PAH, OPAH, 1-hydroxyacenaphthene, and 1-hydroxyfluorene, which exhibited increased median and/or maximum LE/TE ratios, and in many cases, RA/TE ratios as well. Especially when paired with increasing contaminant concentrations, as observed here for 9-fluorenone, 1-hydroxyacenaphthene, and 1-hydroxyfluorene, the elevated lability in biochar amended samples is potentially concerning, particularly as the leachability of the latter two compounds also tended to increase over time, and because the anticipated benefits of biochar application at contaminated sites are frequently associated with increased contaminant sorption/immobilisation (Beesley et al. 2010; Sigmund et al., 2018; Zhu et al., 2017). In contrast, the addition of compost most often reduced median proportions of readily available and leachable target compounds compared to unamended samples by 20-97% and 36-88%, respectively (median values; Figures 5.5 and 5.6) and also appeared to offer potential benefit of comparatively low LE/RA ratios for most PAH and OPAH, which could support continued biodegradation without increasing risks associated with leaching. Compost amendment did increase RA/TE fractionation of 1-hydroxyacenaphthene, 9-hydroxyfluorene in Soil 1, and 1-indanone in Soil 2 (median +44, 39, 21% respectively, compared to unamended samples); however both total concentrations and LE/TE fractionation ratios remained lower in compost amended

samples than for unamended and biochar samples, suggesting limited risk increase for downstream receptors. Increased leachability of 9,10-anthraquinone and 9-fluorenone in compost amended samples may be more concerning, but concentrations in this fraction exhibited substantial declines and remained lower than for other treatments.

5.3.5 Acid and phenolic transformation products

In addition to OHPAH and OPAH, several acidic transformation products and related natural phenolic compounds were observed in total extracts and quantified in readily available, labile, and >2 mm compost fractions. Identified compounds include 1-hydroxy-2-naphthoic acid, 1-naphthaleneacetic acid, catechol, salicylic acid, gentisic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, and vanillic acid. Results for individual compounds are displayed in Appendix C. Not all of these compounds are expected to be very toxic, and along with 1-indanone, in some cases may be of greater interest as biomarkers of PAH degradation. However, 1-naphthaleneacetic acid, also used as a pesticide and plant growth regulator, is regulated in the food industry as toxic compound (Guan et al., 2011), and salicylic acid and catechol are both known to be toxic to both ecological and human receptors (Nunes et al., 2015; Schweigert et al., 2001). In most cases, concentrations decreased over time or exhibited little change, but in some cases primary or secondary maxima were observed at T30 and/or T90 or persisted at the end of the experiment. Importantly, Soil 1 unamended samples supported the accumulation of leachate 1-hydroxy-2-naphthoic acid and catechol, Soil 2 unamended samples supported increased leachate concentrations of 1-naphthaleneacetic acid and catechol, and all Soil 2 treatments supported the late release of 1-hydroxy-2-naphthoic acid. Overall, in consideration of these specific COOHPAH and phenolic compounds, compost amendment appeared to reduce or maintain a similar risk profile to biochar and unamended treatments.

Late stage metabolites and natural phenolic compounds are also of interest as they may influence degrader proliferation and enzyme activities (Chen et al., 2019; Deveryshetty et al., 2007; Lin et al., 2010; Técher et al., 2011). It is interesting that in the Soil 1 compost amended samples early utilisation of readily available lignin phenols vanillin and 4-hydroxybenzaldehyde as well as other alkyl phenols observed in total ion chromatograms coincided with elevated production of 1-hydroxynaphthalene, as observed during the lignin phenol incubation study in Chapter 3. Acknowledging the complexity of these mixtures and the presence of additional interactive effects including

the enhanced transformation (ET) processes described in Chapter 3, this suggests that in some soils, removal of phenolic compounds may stimulate specific PAH degradation pathways. Soil 2 compost amended samples supported increasing release of lignin phenols 4-hydroxybenzaldehyde and vanillin over time, which could improve degrader efficiency in this system over time, however longer term studies for a diversity of soils are needed.

5.3.6 Relationship to toxicology

A previous paper describing toxicology of this study reported that for Soil 1 compost-amended samples, measures of acute toxicity to earthworms, seed germination assays (*B. alba, L. perenne, P. sativum*), and luminescent bacterium *V. fischerii* (Microtox® assay) were significantly reduced compared to other treatments, and decreased over the 180-day period (Cipullo et al., 2019). This trend is in keeping with overall highest reductions in both parent PAH as well as their transformation products, making it difficult to elucidate connections between transformation products and soil toxicity, though it does suggest the elevated total extractable levels of 1-hydroxynaphthalene did not substantially impact the overall toxicology in these samples. More interestingly, for Soil 1 unamended and biochar amended samples, seed germination improved over time and amelioration was higher in unamended samples. This contrasts trends for parent PAH, as Soil 1 biochar amended samples exhibited lower final concentrations than unamended samples across all fractions, but it is in keeping with results for OPAH and the most abundant OHPAH, 1-hydroxyacenapthene and 1-hydroxyfluorene. The earlier report also demonstrated that for Soil 2, measures of toxicity were less consistent across different assays, but no assay represented a consistent response to changes in parent PAH concentrations across all treatments. Interestingly, T30 and T90 represented periods of increased toxicity for seed germination and earthworm health (sublethal effects) in Soil 2 biochar amended and unamended samples, suggesting that toxicity to these ecological receptors may have been driven by the presence of intermediate compounds formed during treatment. Although further investigations could benefit from an untargeted approach to analysis of transformation products, based on the temporal trends for individual target compounds investigated here, 1-hydroxyacenapthene and 1-hydroxypyrene may have been involved in the observed increased toxicity and may be interesting analytes for further toxicological study.

5.3.7 Bioavailability - future research needs

In this study we used mild solvent extraction with methanol to represent the readily available fraction. Although recognised as a simple and convenient metric to explore more available PAH (Ortega-Calvo et al., 2015), this method has sometimes been criticised for being too specific to individual soils (Cui et al., 2013) and because strong correlation with total extractable PAH suggests there may be only marginal benefit to the additional analyses (Bergknut et al., 2007). However, in this study we suggest that analysis of transformation products in methanol-extracted soils provides substantively different information than total extracts since trends related to treatment and time were frequently different than those observed for total extracts, especially for OHPAH. Methanol extractions (as well as leachate analyses) also allowed monitoring of a wide range of target compounds, including acidic transformation products which are more difficult to determine in soils extracted with stronger solvents due to increased matrix interference and the challenge of developing suitable clean-up methods (ref Ch. 2, Ch. 4). This suggests analysis of PAH transformation products in milder solvent extracts may provide useful insights into degradation efficiency and the management of site risk. It is acknowledged that further method development would be required to establish the relationship between PAH transformation products in the readily available fraction and toxicological endpoints. Given the increased interest in other methods of characterising the available fraction of PAH, e.g. cyclodextrin extraction, Tenax infinite sink methods (Ortega-Calvo et al., 2015), porewater sampling, flow-through leachate analysis, and passive sampling devices (Enell et al., 2016), further studies evaluating these methods as bioavailability proxies should incorporate a range of more polar PAH transformation products including OHPAH. In general, further development of toxicology profiles for a range of PAH transformation products is still needed and would also help establish appropriate analytical benchmarks including target detection limits.

5.4 Conclusions

This study demonstrates that even remediation technologies broadly considered safe and less intensive may support the formation, buildup, or leaching of toxic PAH transformation byproducts. While the specific impacts on oxygenated PAH development and distribution are likely to vary between soil type and local conditions, we demonstrate how a multi-fraction analytical approach could help inform decision making at contaminated sites, and specifically suggest that by using this approach it

may be possible to support enhanced biodegradation and the production of intermediates as long as risks associated with the readily available and leachate fraction can be managed. For the soils and conditions tested here, we report that compost amendment delayed PAH attenuation in soil with the lowest level of contamination, but on the other hand enhanced PAH attenuation in the soil with the highest contamination level and most often led to reduced concentrations and reduced leachability of PAH and PAH transformation products. In contrast, the biochar amendment in some cases supported sequestration of PAH and OPAH into less available fractions, but did not offer a substantial advantage compared to no amendment in terms of PAH attenuation for either soil type. Further to this, the biochar amendment supported highest concentrations, elevated lability, and increasing leachability over time for several individual PAH and transformation products. We therefore suggest that the application of biochar may represent an additive risk factor due to potential adverse effects associated with degradation byproducts. Although unamended samples exhibited higher PAH attenuation rates than either treatment in the less contaminated soil, we suggest this may have been primarily driven by leaching and does not represent a preferred treatment method. Increased levels of PAH transformation products observed at intermediate timeframes and in some cases persisting at the end of the study support the view that monitoring of PAH transformation products should be ongoing during site management, especially where leaching is a concern.

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Chapter 6

Concluding Remarks

6. Concluding Remarks

Over the past two decades it has been increasingly recognised that traditional measures of parent EPA PAH are no longer adequate for site risk assessment or evaluation of remediation success. At the same time, the formation and fate of oxygenated PAH degradation products has garnered increasing attention. These compounds may be viewed positively as markers of PAH degradation; however, the likelihood that these compounds exhibit greater toxicity, mobility, and bioavailability compared to parent PAH is concerning, especially when their formation is accelerated during remediation treatment. Despite these concerns, the number of studies directly addressing oxygenated PAH in environmental samples has been limited, particularly those considering the more polar OHPAH and COOHPAH. Progress in this field has been hampered by the substantial amount of time it takes to develop methods for the detection of these chemically diverse compounds in environmental samples. With a high environmental burden of gasworks and other industrially contaminated sites worldwide, it is imperative this hurdle be overcome in order to (1) promote the remediation of PAH-contaminated sites, and (2) ensure that remediation initiatives do not inadvertently increase harm.

The studies presented in this thesis developed and demonstrated the application of novel methods for the analysis of oxygenated PAH in contaminated soils and leachates, giving researchers new tools to investigate the role of oxygenated PAH in remediation and site risk. In Chapter 1, Figure 1.3 depicted an overall picture of method development. Here, in Figure 6.1, the general schematic has been revised to highlight key processes and outcomes of the work presented in this thesis. Analytical method development begins with an initial assessment, which includes an overall decision about target compounds and matrices, as well as appraisal of the available knowledge, resources, and methods for sample preparation and instrumental analysis. Chapter 2 provided an in-depth review of recent progress in the analysis of oxygenated PAH in soil and aqueous environmental matrices. The wide diversity of potential target compounds as well as the lack of standardised methods, suitable reference standards, and certified reference materials remain overarching challenges for oxygenated PAH research. However, as standard methods have not yet been established for oxygenated PAH research, method development and validation stages can be considered an iterative process through which individual or multiple laboratories seek to improve, extend, or simplify analysis prior to interlaboratory validation and standardisation.

Specific knowledge gaps in sample preparation and analysis identified through the literature review were addressed directly during the experimental studies presented in this thesis. Chapters 3 and 4 presented new methods for analysis of aqueous and soil bound oxygenated PAH respectively using HPLC-Biphenyl-DAD and small sample LLE-GC-EI-MS, and whole soil extraction + aminoproyl silica SPE and GC-EI-MS, while Chapter 5 integrated these methods and further considered non-exhaustive methanol extraction in order to understand the relative lability of oxygenated PAH in different soils and remediation treatments. Each method was validated and tested on realistic contaminated samples with varying contamination levels and/or soil characteristics. The HPLC method allowed direct analysis of the aqueous samples and was found to be most effective for detecting and characterising changes in the profile of PAH and lignin phenol transformation products. It also allowed the detection of catechol, which was difficult to detect using the other sample preparation techniques. The LLE-GC-MS provided lower detection limits for aqueous samples and allowed quantitation of a greater number of specific target compounds, while allowing the positive or partial identification of unknowns based on mass spectral evidence. In addition, this method used substantially less solvent and material cost when compared to traditional LLE and sorbent-based methods. The aminopropyl silica SPE method developed and tested in Chapter 4 reduced matrix effects and separated PAH, OPAH, and OHPAH in soil, while offering qualitative data about select COOHPAH and phenolic aldehydes. Additional observations and benefits of this approach are further summarised below, but the most important outcome is that this technique extends the utility of whole-soil extraction methods for oxygenated PAH analysis. This means that oxygenated PAH, especially OHPAH, can be studied alongside parent PAH with minimal additional sample preparation. Further work to undertake inter-laboratory testing and standardise methods is needed and is ultimately essential for the adoption of regulatory frameworks for oxygenated PAH in the EU and elsewhere. In the meantime, simplifying techniques increases the likelihood of their adoption, and as shown through the studies undertaken in Chapters 3-5, the techniques presented in this thesis can now be used in experimental and monitoring studies to inform remediation and risk assessment.

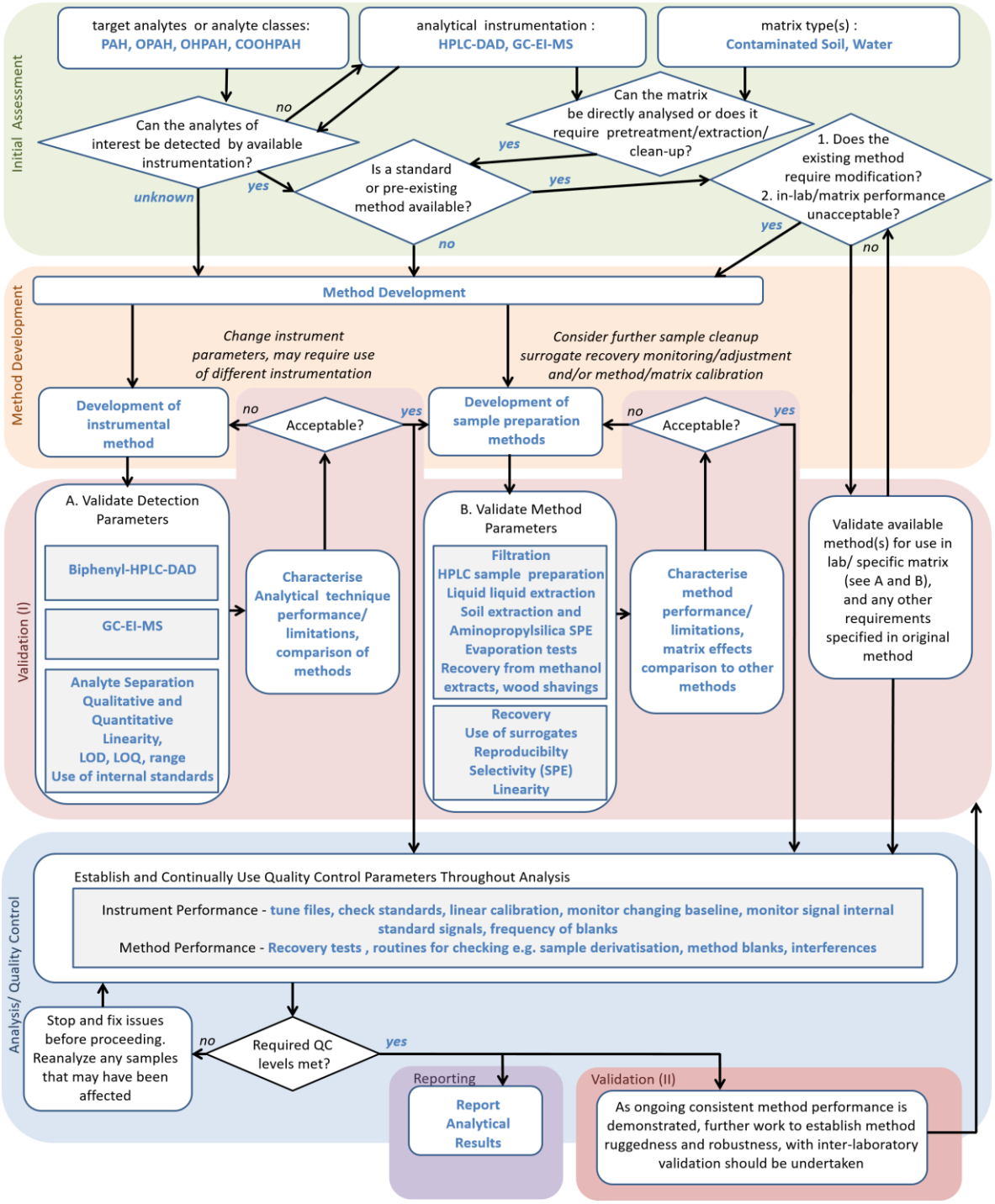


Figure 6.1 Analytical method development. Processes and outcomes of this thesis project highlighted in blue. Ref. Figure 1.3

One of the primary questions for the contaminated land industry has been how to increase rates of PAH removal. In-situ bioremediation, and especially compost-based approaches, have shown promise for attenuating parent PAH. However, as removal rates are highly variable and tend to slow over time, greater understanding of factors impacting remediation efficiency is still needed. One possibility investigated in this thesis is that the presence of natural structural analogues found in soil and compost mixtures may modulate PAH biodegradation pathways. Chapter 3 investigated the effect of applying two environmentally common lignin phenols, vanillin and 4-hydroxybenzaldehyde, on the (trans)formation of PAH and soluble oxygenated PAH intermediates. HPLC-DAD using biphenyl stationary phase and small sample (2 mL) LLE-GCMS – were shown to be complimentary techniques for monitoring PAH, lignin phenols, and degradation by-products in contaminated soil leachates. The targeted/non-targeted approach allowed for the quantitation of selected products while also identifying additional analytes of interest based on their association with observed enhanced transformation processes. Through these analyses, it was demonstrated that dominant processes affecting lignin phenol transformation also affected transformation of oxygenated PAH. It was also shown that high initial concentrations of lignin phenols may inhibit initiation of PAH degradation, but ultimately promote the formation of by-products associated with naphthalene and phenanthrene catabolism. Additional results presented for compost-amended samples in Chapter 5 are consistent with the view that utilisation of phenolic compounds may stimulate specific PAH degradation pathways. Further studies of the microbiology and functional gene expression would help elucidate these processes, and could lead to the development of strategies to overcome current limitations in composting based approaches. It is suggested here that where concentrations of natural phenolic compounds have become depleted and other (xenobiotic) phenolic compounds are low, application of lignin phenols could be used to help (re)initiate PAH biodegradation, provided that potential additive risks associated with degradation products can be managed.

While aqueous samples represent the most mobile fraction of contaminants, soil ultimately remains the larger reservoir of oxygenated PAH. Methods for analysing more polar oxygenated PAH including OHPAH and COOHPAH alongside traditional PAH are limited, and where available, have not seen broader use. Chapter 4 investigated the possibility of using aminopropylsilica SPE to separate multiple classes of PAH derivatives from whole soil extracts. The novel method offers improved recovery of

several oxygenated PAH while simplifying sample preparation and reducing material inputs compared to previous methods. It was further demonstrated that this method can be applied to several soil types, and that soil type and contamination level influence recovery of target compounds particularly during the soil extraction stage, prior to SPE. Soil with the highest contamination exhibited enhanced recovery of some lower-concentration midweight PAH and OPAH, while the least contaminated soil showed greater sensitivity to evaporative losses. Soils with higher proportion of clay also limited the extraction of the higher molecular weight OHPAH, though the ecotoxicological relevance of tightly bound contaminants is unknown. These matrix effects are likely to be soil specific and may be best addressed as a greater number of suitable deuterated surrogate compounds become commercially available. Nevertheless, this method allowed the identification of OHPAH and COOHPAH not observed in crude soil extracts, while low RSD for PAH, OPAH, and OHPAH across all soil types supports the use of the method for monitoring changes in these compounds during soil remediation.

The challenge in anticipating the formation and distribution of oxygenated PAH under realistic remediation scenarios was highlighted in the final outdoor incubation study presented in Chapter 5. This study investigated the impact of compost, biochar, and no amendment in situ remediation strategies on the distribution of target low molecular weight PAH, OPAH and OHPAH in two aged gasworks soils. In addition to using methods developed in Chapters 3 and 4, for the first time, mild solvent extraction was used to consider the readily available fraction of specific transformation products in order to further understand contaminant lability. By integrating these analyses, it was shown that the prevalence and lability of PAH, OPAH, OHPAH, and acid degradation products depended on soil type, contamination level, amendment characteristics, and changes over time. It should be acknowledged that in cases where the lability and toxicity of the compounds is low, and accumulation is minimal, increasing formation of one or more products might be viewed as an indicator of advantageous PAH degradation. Yet at the same time, in the absence of clear threshold levels set through ecotoxicology assays, it is reasonable and necessary to be cautious about the formation of these compounds, especially if they are present in the most available soil fractions or exhibit substantial longevity. While compost amendment delayed PAH attenuation in the less contaminated soil, it most often led to reduced concentrations and/or reduced leachability of PAH and PAH transformation products. In in some cases, the biochar

amendment supported sequestration of OPAH into less available fractions, but this treatment did not substantially improve rates of PAH attenuation compared to unamended samples. Moreover, biochar also supported highest concentrations, elevated lability, and increasing leachability over time for several individual PAH and transformation products. For the less contaminated soil, the no amendment treatment outperformed the amended soils in terms of PAH attenuation; however, this may have been primarily driven by leaching and does not necessarily represent an ideal treatment strategy. While no single treatment provided an unequivocal 'best' option for both soils, based on consideration of by-product levels and trends in contaminant lability, it was concluded that that the biochar amendment may increase risks associated with PAH by-products while compost amendment most often reduced these risks. Concerns raised about biochar application are especially timely as there is growing interest in applying these materials in situ at mixed-contamination sites and without proper monitoring, these initiatives could increase harm. Longer term study (> 6 months) of the formation and fractionation of oxygenated PAH in compost-amended soils is also still needed, especially as continued breakdown of organic materials can be expected, which could substantially alter contaminant-soil sorption characteristics. Differing temporal trends in total extractable, readily available, and leachate fractions also highlight the importance of monitoring each of these fractions in order to best inform remediation strategy and risk management. It is anticipated that by further integrating ecotoxicology and analytical chemistry, bioavailability science will be refined and ultimately incorporated into best practices for site management.

The studies presented here demonstrate that oxygenated PAH, and especially OHPAH may be more highly concentrated in aged gasworks soils and leachates than previously reported in the scientific literature. Results from the two incubation studies in Chapters 3 and 5 also support findings that some oxygenated PAH may build up over time in contaminated soils and/or leachates. While the short duration of these studies must be taken into consideration, these results stand in contrast to the common assumption that due to their greater polarity and availability, oxygenated PAH are quickly removed and therefore represent a lower risk than parent PAH. The increased toxicity observed for some soils during treatment, as reported for the biochar amended samples in Chapter 5, suggests that PAH degradation by-products are chemicals of concern that should be included into site risk assessment and monitoring.

This thesis joins a growing body of research that demonstrates that the application of amendments to promote remediation can modulate the distribution of oxygenated PAH in contaminated soils. Specifically, the studies presented demonstrate that even treatments which may be considered 'low intensity' or 'safe', such as the application of lignin phenols, biochar, and compost, can be expected to lead to unknown effects through the promotion of specific PAH degradation pathways and alteration of soil sorption characteristics. Beneficial site remediation will ultimately depend on finding the balance of promoting PAH degradation, but only to the extent that the risks associated with additional degradation by-products can be managed. By developing novel analytical methods and applying them to different remediation scenarios, the work presented in this thesis increases understanding of the prevalence of these compounds in gasworks soils and associated water systems. Further, new methods and approaches presented here are now part of the repertoire available to researchers to investigate these processes for specific soil remediation initiatives. The continued development of this work is essential in order to ensure remediation initiatives improve rather than deteriorate safety at contaminated sites.

Appendices

Appendix A: HPLC method development

A.1 Instrumentation

Method development was performed using an Agilent 1200 HPLC with binary pump and DAD module, with a reverse phase biphenyl column (Restek Raptor 2.1 mm × 150 mm, 2.7 μm particle size). Phenyl groups enhance the polarisability of the stationary phase, which is expected to improve separation of aromatic compounds (Letzel et al., 2001), however biphenyl has not previously been tested for a range of PAH, PAH transformation products and natural phenolic compounds. The small column diameter was initially selected for low load flows preferred for LC-MS applications, however a larger column and load volume could improve detection limits for DAD, which were elevated here (Table A1.2) compared to previous methods for PAH (Titato and Lancas, 2006). For all tests, the autosampler compartment was maintained at 4°C and column at 30°C. Target PAH, phenolic compounds, and PAH transformation products were monitored at 220, 228, 254, and 275 nm with a reference wavelength of 360 nm (Table A.1). Injection volumes 5 μL, 7 μL and 10 μL were tested at standard concentration of 2 μg/mL, and since 10 μL led to column saturation, 7 μL was selected.

A.2 Chromatographic separation

Chromatographic methods were developed using ultrapure water (Solvent A) and LC-MS grade acetonitrile (Solvent B). Although methanol is recommended for improving separation with biphenyl-modified columns (Restek), and has been used for comparable phenyl-LC-MS applications (Letzel et al., 2001), the UV absorbance cutoff is higher compared to acetonitrile (205 nm vs. 190 nm), occluding λ_{max} for several target analytes. Moreover, when tested as an alternate Solvent B in our setup, methanol did not substantially improve separation but contributed to larger baseline shifts associated with gradient elution and increased instances of backpressure limit exceedance (>400 bar). Following Dolan (2013), we also tested the addition of 0.1% phosphoric acid to the aqueous phase to improve issues of peak fronting observed for some acid analytes (Figure A.1), but this did not offer substantial improvement and caused further pressure profile issues requiring full column restoration to resolve. Acetonitrile and water were maintained for further method development.

Two chromatographic methods were developed: Method 1 was developed for 20 compounds, and provides good separation of LMW PAH and lignin phenols, as well as key naphthalene metabolites; Method 2 was developed after two larger hydroxylated

PAH, 9-hydroxyfluorene and 9-hydroxyphenanthrene, were added as reference compounds. Representative chromatograms and pump conditions are given in Figure A.1, with compound identification details in Table A.1. Though 9-hydroxyfluorene and 9-hydroxyphenanthrene could be partially differentiated through examination of spectral shifts across the coeluting peaks 1-hydroxynaphthalene and naphthalene using Method 1, by decreasing the ramp rate for the addition of acetonitrile, full separation of 22 compounds as well as a putative vanillin isomer was effected in Method 2. Although Method 2 may be valuable for analysis of complex chromatograms, peak splitting was observed for salicylic acid, and the slow ramp rate led to peak broadening of early eluting compounds causing substantially reduced resolution at low concentrations. For these reasons Method 1 was selected for monitoring shifts in the lignin phenol microcosm experiment (Ch.3).

Low RSD of triplicate injections (2 μg/mL), i.e. <2.3% for all compounds except acenapthene (6.6%) supported the use of single injections for further analyses.

A.3 Quantitation and matrix effects

We compared the chromatographic performance of Method 1 for target analytes in three sample matrices: 100% acetonitrile, 9:1 water:acetonitrile, and contaminated soil leachate analogue with acetonitrile (9:1)(hereafter 'acetonitrile, 'water' and 'leachate' matrices respectively, though all contain 10% acetonitrile in order to allow suitable calibration and match experimental sample preparation). Soil leachate analogue was prepared to represent the lignin phenol microcosm experimental conditions (Ch.3): deionised water was shaken with the tar contaminated soil in a ratio of 10 mL/4.5 g soil, heavy particulates were allowed to settle, and the aqueous phase was decanted and passed through a syringe fitted with a 0.40 mm bore needle.

Standards of naphthalene, acenaphthene, fluorene, phenanthrene, and pyrene were prepared in acetonitrile at 200, 50.0, 10.0, 5.00, 1.00, 0.50, 0.10, 0 μg/mL. Calibration standards were prepared by adding 100μL of the appropriate standard to 900 μL of each prepared matrix, i.e. acetonitrile, water, and leachate analogue. This yielded final concentrations of each species between 20.0 and 0 μg/mL, or in the case of the leachate analogue, final additions of the same value (Table A.2).

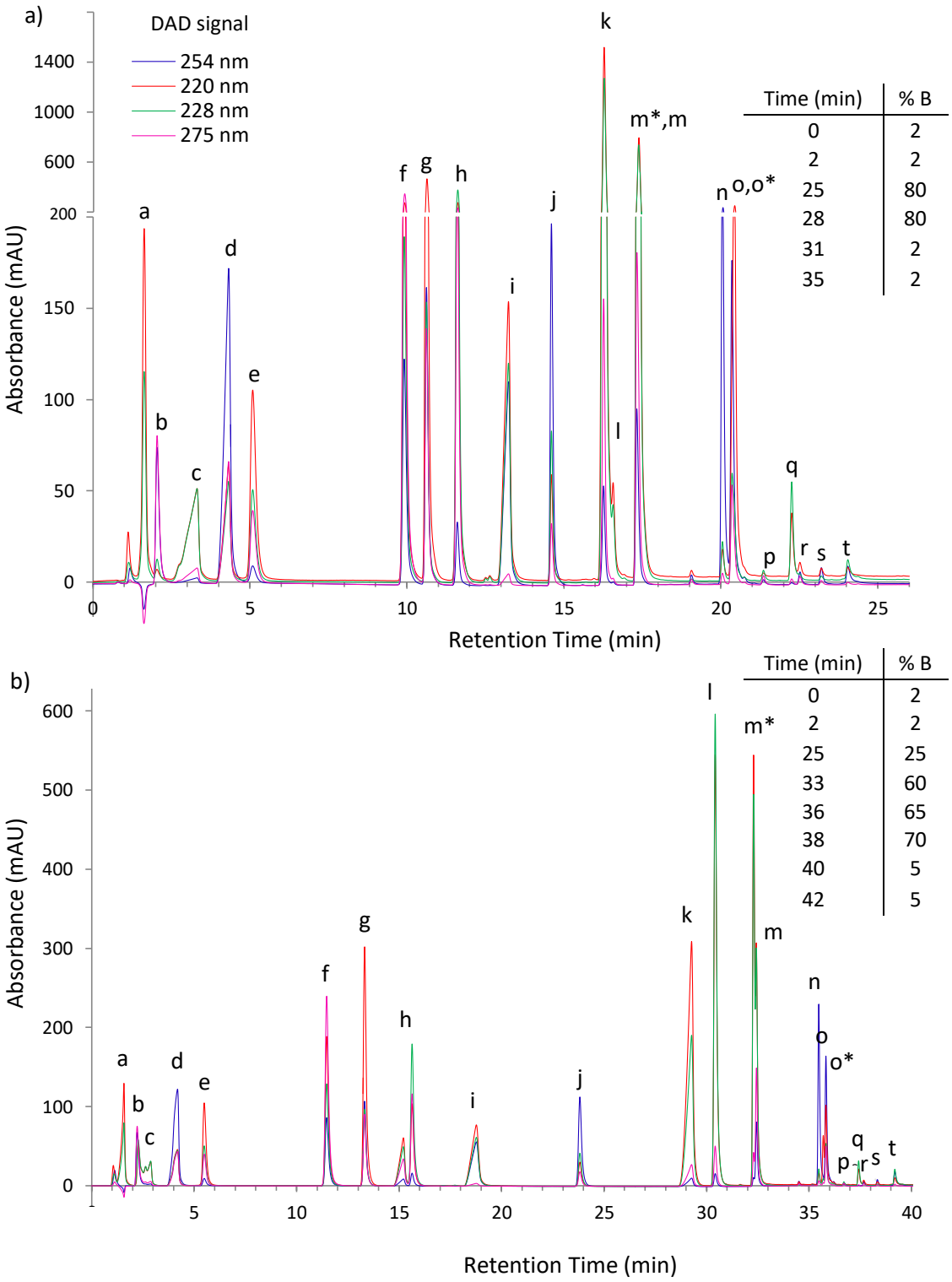
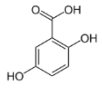
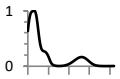
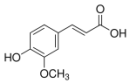
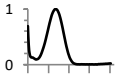
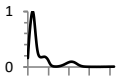
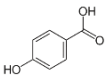
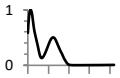
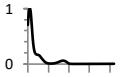
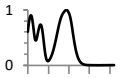
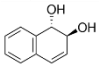
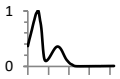
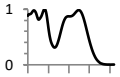
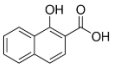
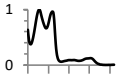
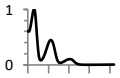
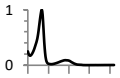


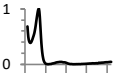
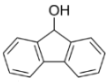
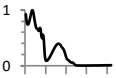
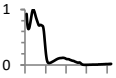
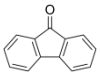
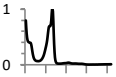
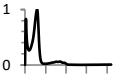
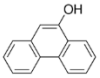
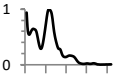
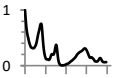
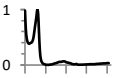
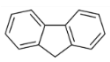
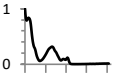
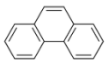
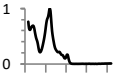
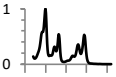
Figure A.1 HPLC-Biphenyl-DAD method for separating aqueous PAH, lignin phenols, targeted transformation products a) Method 1 for 20 compounds, and b) Method 2 for 22 compounds. Peak designations given in Table A.1. Solvent A-water, Solvent B-acetonitrile. Analytes at varying concentrations

Table A.1 HPLC-DAD identification of 22 PAHs, lignin phenols, and transformation products using two methods; Peaks designations refer to Figure A.1. RT1 = retention time Method 1, RT2 = retention time Method 2

Peak	RT1	RT2	Analyte	Structure	UV-abs[1] 190-400nm	λ_{max}[2] nm	λ_{monit} nm
a	1.64	1.57	gentisic acid			206	220
b	2.11	2.28	ferulic acid			266	254
c	3.21	2.63	salicylic acid			202	220
d	4.26	4.19	4-hydroxybenzoic acid			196	254
e	5.12	5.49	catechol			194	220
f	9.92	11.47	4-hydroxybenzaldehyde			284	275
g	10.67	13.32	1,2 *trans*-dihydroxy-dihydronaphthalene			214	254
h	11.68	15.21 15.63	vanillin			230	275
i	13.11	18.77	1-hydroxy-2-naphthoic acid			218	220
j	14.64	23.82	1-indanone			206	254
k	16.32	29.30	1-naphthylacetic acid			224	220

…cont'd

Table A.1 continued

Peak	RT1	RT2	Analyte	Structure	UV-abs[1] 190-400nm	λ_{max}[2] nm	λ_{monit} nm
l	16.60	30.42	1-hydroxyacenaphthene			224	228
m*	17.28	32.29	9-hydroxyfluorene			208	228
m	17.51	32.43	1-hydroxynaphthalene			210	220
n	20.08	35.48	9-fluorenone			256	254
o	20.25	35.69	naphthalene			220	220
o*	20.32	35.83	9-hydroxyphenanthrene			248	254
p	21.38	36.71	1-hydroxypyrene			242	275
q	22.27	37.42	acenaphthene			228	228
r	22.54	37.67	fluorene			262	254
s	23.20	38.34	phenanthrene			250	254
t	24.07	39.19	pyrene			240	228

[*] Analytes marked with an asterisk may coelute with peak of same letter designation if present in sufficient quantities (Method 1), but may be qualitatively monitored based on their differing spectral properties.

[1] UV absorbance spectra normalised to λ_{max} and to remove negative absorbance caused by gradient shifts in refractive index of mobile phase. Horizontal axis extends from 190-400 nm left to right.

[2] Where 190 nm provides highest absorbance, a second λ_{max} is indicated

Table A.2 Matrix effects of three solvent systems on PAH calibration
W=9:1 water:acetonitrile, L=9:1 leachate:acetonitrile, A=100% acetonitrile

Analyte	Calibration range [1]	Aqueous solubility 25°C[2]	LOD	LOQ	R^2			Response ratio	
	µg/mL	µg/mL	µg/mL	µg/mL	W	L	A	L/W	A/W
Naphthalene	0.010-20.0	31	0.01	0.032	0.997	0.986	0.999	1.03	1.41
Acenaphthene	0.010-1.00	3.57	0.003	0.010	0.996	0.991	0.998	1.26	1.01
Fluorene	0.010-1.00	1.69	0.005	0.016	0.999	0.983	0.994	1.24	1.04
Phenanthrene	0.010-0.50	1.15	0.003	0.011	0.999	0.994	0.999	1.37	1.18
Pyrene	0.010-0.10	0.135	0.003	0.010	0.978	0.930	0.976	1.55	0.94

[1] Upper calibration limits based on aqueous solubility and loss of linearity in water samples; acetonitrile-only series provided showed excellent linearity for all PAH over the range tested
[2] Solubility values obtained from PubChem database.
[3] LOD and LOQ calculated based on 3x and 10x the standard deviation of 7 replicate 0.01 µg/mL standards prepared in water divided by the slope of the calibration curve. LOD and LOQ were not determined separately for the leachate analogue since leachate contained all target PAH

Results presented in Table A.2 demonstrate the importance of using matrix-matched calibrations for soil leachate analysis. Leachate standards exhibited more deviation (lower R^2), and linearity for pyrene was unsatisfactory. Responses for samples prepared in 100% acetonitrile were similar to responses in the water matrix for acenaphthene, fluorene, and pyrene, but were more substantially increased for phenanthrene and naphthalene, at 18% and 41% increases respectively. Leachate samples exhibited a different pattern with increasing matrix effects related to increasing PAH size/retention time. Retention times for PAH in acetonitrile-only standards were slightly reduced compared to other matrices

Matrix effects for PAH transformation products and lignin phenols were investigated in the same way as PAH, but could not be fully quantified. Acetonitrile-only standards negatively impacted chromatography through substantial peak broadening of compounds eluting prior to 15 min, making them unsuitable for quantitation. Introducing the calibration solution to the leachate analogue led to the formation of an unknown precipitate. Precipitate formation did not occur in leachate after the addition of the 100 µL acetonitrile during matrix tests or analysis of the experimental samples in the lignin phenol microcosm study. Due to the possibility of multiple interactions between mixed target analytes and soil leachate matrix, it may be more suitable to calibrate for individual transformation products after their presence has been identified in samples.

A.4 Sample preparation - Filtration

Several common syringe-filter membranes were tested for filtration losses (Table A.3). A 1 mL syringe was used to prewet the filter assembly, then slowly filter 0.9 mL of aqueous target analytes directly into a 2 mL amber HPLC vial. The filtrate was supplemented with 100 µL acetonitrile in order to maintain consistent preparation with other tests. Since the addition of an organic solvent prior to filtration is recommended for PTFE filters (Restek, personal comm.) and is compatible with nylon filters, 10% acetonitrile was also added to the sample prior to filtration for these filters (referred to here as 'premixed samples'). Reference 'syringe-only' samples were prepared for filtrate samples by taking up and releasing the test mixture through a 1 mL syringe fitted with a 0.40 mm bore needle. Average recovery was calculated by comparing average signal area of three replicate filtrations to the average area obtained from triplicate 'syringe-only' preparations.

Filter types exhibited strong differences in analyte recovery related to the overall hydrophilicity and in some cases the presence of particular functional groups (Table A.3, following page). Cellulose acetate (CA), polyethersulfone (PES), and nylon are unsuitable for many or most of the compounds considered here. Cellulose mixed ester (HA) and PVDF membranes may have limited utility for studies considering degradation products of the smallest PAH, naphthalene and acenaphthene as well as lignin phenols, but PTFE is the only membrane that provides good recoveries of all compounds considered. The 0.45 µm pore size membrane is preferred over 0.2 µm for applications where sterilisation is not required, and pretreatment with 10% acetonitrile can further improve recovery. As filtration with PTFE still presents a source of loss compared to syringe-only tests, it should only be applied where necessary.

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Letzel, T., Pöschl, U., Wissiack, R., Rosenberg, E., Grasserbauer, M., Niessner, R., 2001. Phenyl-modified reversed-phase liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry: A universal method for the analysis of partially oxidized aromatic hydrocarbons. Anal. Chem. 73, 1634-1645.

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Table A.3 Filter performance for the recovery of 22 targeted PAH, transformation products and lignin phenols.

Filter membrane material and brand	pore size µm	unit diameter mm	Comments[1]
CA-cellulose acetate Sartorius Minisart sterile	0.2	28	Not recommended for any of the target compounds. Recovery less than 40% for nearly all compounds. No recovery of late eluting compounds.
Nylon VWR	0.45	25	Nylon performed only marginally better than CA, with an extended range of compounds recovered and with 80-99% recovery on early eluting neutral compounds (up to indanone). Compounds with carboxylic acid groups eluting in this time range had comparatively low recovery (0-68%).
premixed sample			Premixing with acetonitrile improved Nylon performance, through extension of range of compounds recovered, but not sufficiently to recommend the filter for this purpose.
HA-mixed cellulose esters Millex sterile	0.45	33	Not preferred. With generally >88% recovery for early eluting analytes (up to 1-hydroxynaphthalene), could prove suitable in experiments where degradation of PAH larger than naphthalene not considered.
PES-polyethersulfone Millex sterile	0.45	33	Not recommended. Similar to HA for early eluting compounds, but1-hydroxy-2-naphthoic acid and 1-hydroxynaphthalene not well recovered (<53%).
PVDF- Polyvinylidene fluoride Restek	0.45	13	May be suitable for studies monitoring naphthalene and acenaphthene degradation products as well as some fluorene-related products. (Recoveries greater than 96%). However, recovery of parent PAH and 1-hydroxy pyrene unsatisfactory (0-57%).
PVDF- Polyvinylidene fluoride Restek	0.22	13	Comparable to 0.45 µm PVDF, however recovery of parent PAH and 9-fluorenone further reduced (0-38%)
PTFE-Polytetrafluoroethylene Restek	0.45	13	PTFE performs very well with all compounds recovered in excess of 90% with the exception of fluorene (80%) and phenanthrene (66%).
premixed sample			premixing with acetonitrile improves PAH recovery to 87-98%
PTFE-Polytetrafluoroethylene Restek	0.22	13	Similar to PTFE 0.45 membrane. Recovery of PAH from acenapthene to pyrene somewhat lower (63-86%).
premixed sample			premixing with acetonitrile improves PAH recovery to 81-95%

[1] Recoveries have been compared to 'syringe only' samples. Since not all filters were the same dimensions, quantitative comparison between filters membrane types should be treated with caution.

Appendix B: Summary of extraction recoveries

B Summary of extraction recoveries

Table B.1 Recovery of targeted PAH and oxygenated transformation products for extracts of selected soil fractions

Target analyte	Leachate-LLE [1]			Readily Available – Methanol Extract[2]				>2 mm Frac.[3]
	Stands. 1 µg/mL %	Soil 1 leach. %	Soil 2 leach. %	Stands. 10 µg/mL %	Stands. 1 µg/mL %	Soil 1 RSD %	Soil 2 RSD %	2 µg/g spike %
PAH								
Naphthalene	85±5	54±13	44±4			19	5	92±8
Acenaphthylene	93±4	98±4	81±3	74±17	6±1	1	4	
Acenapthene						6	3	101±6
Fluorene	95±4	103±4	87±2	104±6	55±7	1	6	
Phenanthrene	98±7	119±2	98±3	107±7	68±8	7	6	113±4
Anthracene	105±9	119±2	94±1	106±9	89±3	3	10	
Pyrene	106±12	134±1	101±4	115 ±2	94±3	1	3	
Benz[a]anthracene	95±10	140±7	124±4	126±10	102±12	2	4	
Chrysene	96±8	128±1	154±7	124±8	99±13	2	4	123±8
Benzo[b]fluoranthene	93±6	135±6	102±1	119±3	101±3	5	4	
Benzo[k]fluoranthene	94±7	85±12	71±10	107±3	103±9	8	12	
Benzo[a]pyrene	91±8	67±8	74±9	117±4	101±7	8	6	111±3[4]
Indeno[1,2,3-cd]pyrene	51±10	31±10	37±11	106±11	70±5	9	7	
Dibenz[a,h]anthracene	49±10	31±12	33±10	128±15	101±10	7	3	
Benzo[ghi]perylene	46±10	29±12	36±11	150±24	73±6	7	6	
OPAH								
1-Indanone	90±5	56±1	90±5	95±8	22±9	3	7	108±7
9-Fluorenone *cq*	94±8	133±4	90±2	106±2	88±3	1	3	147±34
9,10-Anthraquinone	97±8	147±4	87±3	99±1	89±1	1	2	167±61
9,10-Phenanthrenequinone *nq*								
OHPAH								
Catechol	33±2	45±1	9±1	106±5	83±6	4	25	108±7
1-Hydroxynaphthalene	110±8	99±3	17±2	110±2	80±9	3	17	80±3
1-Hydroxyacenaphthene	105±6	131±4	90±5	107±2	94±4	7	3	120±15
9-Hydroxyfluorene	120±8	119±3	107±4	106±1	97±4	1	3	119±8
9-Hydroxyphenanthrene	119±11	133±5	<5	103±3	95±3	2	28	64±5
1-Hydroxypyrene	91±8	96±2	<5	111±8	94±8	3	23	91±10
Phenolic aldehydes								
4-Hydroxybenzaldehyde	90±4	95±3	75±2	112±3	97±6	3	6	75±5
Vanillin	110±6	108±5	84±1	109±2	91±5	3	3	84±10
COOHPAH								
1-Naphthylacetic acid	109±8	134±4	94±3	110±2	51±3	7	5	32±14
Fluorene-9-carboxylic acid *nq*								
1-Hydrox-2-naphthoic acid	49±4	108±9	39±5	102±1	74±5	6	50	<5
Phenolic Acids								
Salicylic acid	101±7	86±4	91±4	109±4	77±8	3	18	<5
4-Hydroxybenzoic acid	6±1	11±4	9±1	111±3	12±5	2	17	<5
2,5-Dihydroxybenzoic acid	6±1	14±2	<5	104±1	70±5	9	66	<5

Notes for Table B.1: All tests conducted in triplicate. *nq*= not quantitative by GC-MS analysis; *cq*=cautiously quantitative as this compound observed in GC-MS chromatograms may also reflect presence of *nq* compounds. Recovery was calculated after subtracting mean response of method blanks or unspiked samples. [1]Leachate-LLE recovery based on extraction of spiked (1 µg/mL) replicates prepared in water (standards) or soil leachate analogue. Soil 1 values correspond to Ch 1. microcosm test soil and Ch. 5 test Soil 1 [2]Recovery from prepared methanol extracts considered the final evaporation and reconstitution steps. Target analytes spiked (1 µg/mL) into methanol extracts of each soil to assess reproducibility (RSD=relative standard deviation) as soil extracts tended to require longer drying times than methanol standards. [3]Test material for >2 mm fraction of compost-amended samples obtained after sieving test materials (5 g each extraction); recovery calculated after subtracting mean response of three unspiked samples. Deuterated PAH surrogates were used to estimate PAH recovery for this fraction, including [4]perylene-d_{12}. Low recovery for some analytes in this case suggests strong sorption onto bulk organic material. Recovery and matrix effects for <2 mm Total Extractable Fraction is discussed in Ch. 4, with recovery of the SPE method for PAH, OPAH and OHPAH in soil extracts ranging 100-150, 90-120, and 80-120 %, respectively (ref Table 4.4).

Appendix C: Supplementary data for Chapter 5 compost and biochar remediation study

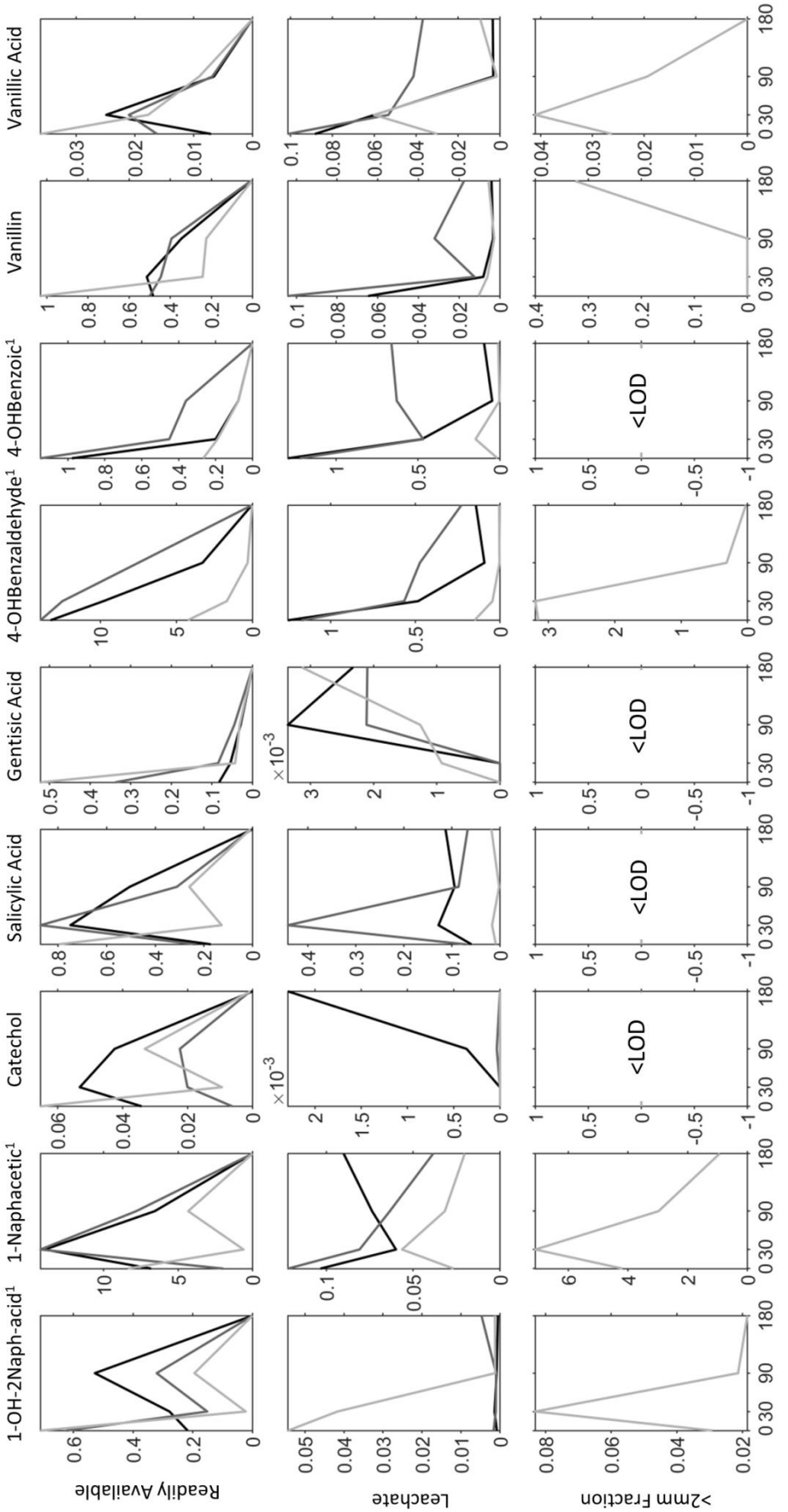

Figure C.1 Mean acid transformation products and related phenolic compounds in Soil 1 readily available, leachate, and >2mm fraction of compost amended samples. Black-unamended, dark grey-bochar amended, light grey-compost amended. All concentrations normalised to µg/g dry soil or in the case of >2 mm fraction, µg/g dry bulk material via extractant volume. [1]abbreviated compounds in order left to right: 1-hydroxy-2-naphthoic acid, 1-naphthaleneacetic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid.

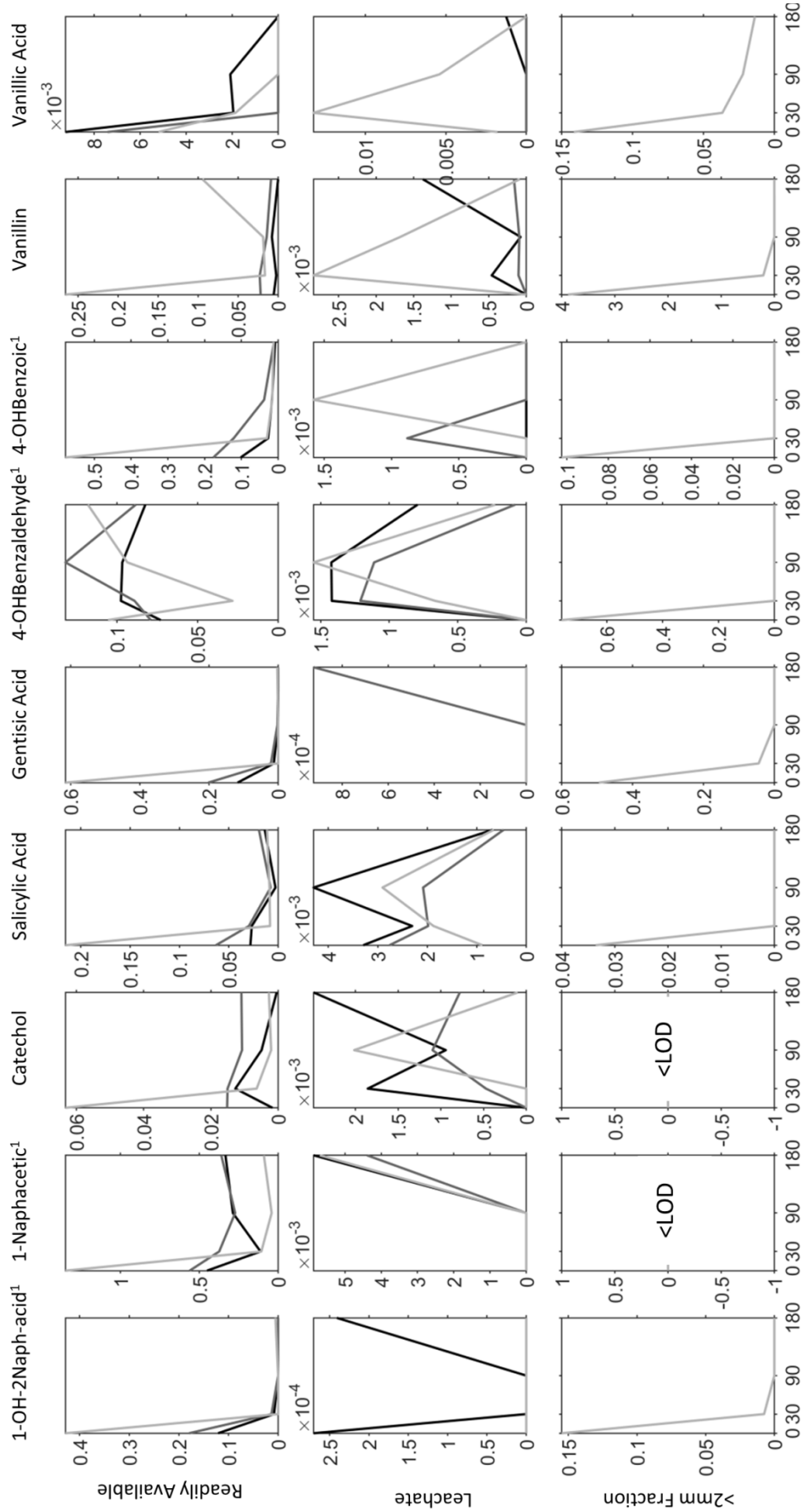

Figure C.2 Mean acid transformation products and related phenolic compounds in Soil 2 readily available, leachate, and >2mm fraction (compost amended). Black-unamended, dark grey-bochar amended, light grey-compost amended. Figure C.1 caption for further details.

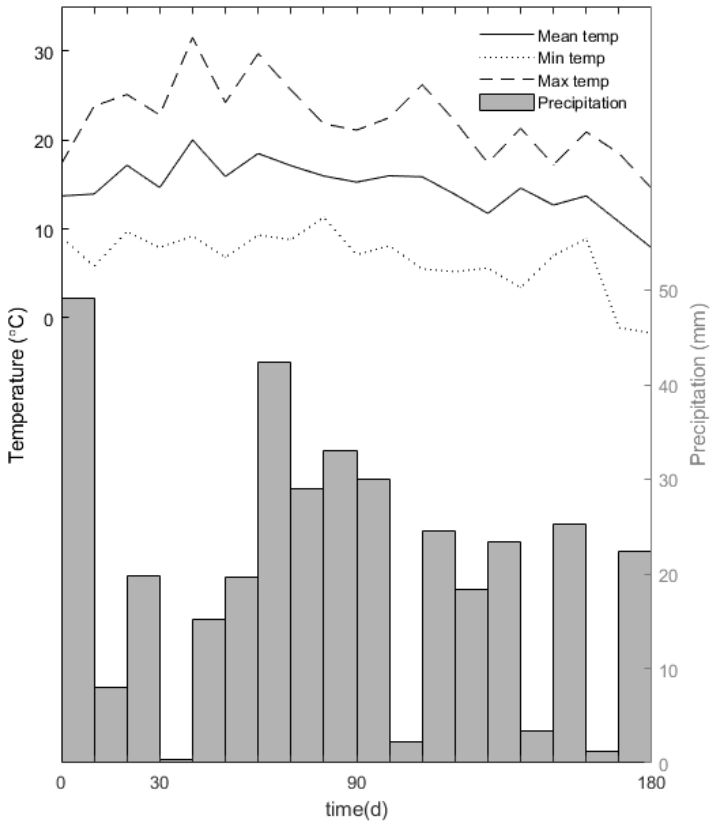

Figure C.3 Local weather conditions at Woburn, UK over duration of incubation study May-November 2017 (Data Source:Met Office, 2017). Hourly records aggregated over 10-day intervals, with sampling periods indicated along lower axis.