

**The Measurement of the Dermal Bioavailability of Potentially Harmful Organic
Soil Contaminants.**

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Abstract

Currently only one value is used to predict the human health risk of the dermal absorption of polycyclic aromatic hydrocarbons (PAH) from soil for contaminated land risk assessments. Research has shown that soil PAH dermal bioavailability differs between soils, and additionally that differences in skin physiology results in variation in the quantity of PAH absorbed. The current PAH dermal bioavailability value used in risk assessments was generated using soil spiked with radiolabelled PAH in a solvent, overestimating PAH soil mobility and therefore creating uncertainty in the dermal bioavailability value.

Consequently, this study aims to develop a lab-based method to quantify the *in vitro* dermal absorption of native PAH from 'real-world' soils to produce soil-specific PAH dermal bioavailability values. PAH dermal bioavailability is defined here as: "The fraction of the PAH concentration that is absorbed into and/or through the skin".

Building on previous research, this study used a vertical diffusion cell experimental set-up, with an artificial skin membrane as a skin substitute, to reduce variability in PAH absorption data. PAH extraction and quantification methods were determined for: the soil, membrane (representative skin) and receptor solution (representative systemic circulation), in order to quantify the concentration of PAH dermally absorbed. The artificial membrane was validated by comparing the absorption rate of PAH from a solvent through the membrane, to published studies that had used excised human skin. The artificial membrane produced PAH absorption rates within the published values. The developed method was used to quantify the dermal absorption of PAH from contaminated soils. The results indicate that the soil-specific dermal absorption of native PAH is much lower than the current dermal bioavailability value (mean 97.5% reduction), and PAH dermal absorption varies between soils. Further research is needed to identify the key soil factors that impact soil PAH dermal bioavailability.

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“Take pride in how far you’ve come. Have faith in how far you can go. But don’t forget to enjoy the journey.” – Michael Josephson.

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1 Introduction

1.1 Context of this Research

This research developed an *in vitro* method for the quantification of the dermal bioavailability of polycyclic aromatic hydrocarbons (PAH) in soil for the use of deriving soil-specific dermal bioavailability values for human health risk assessment in the context of risk-based land management.

PAH are hydrocarbons composed of multiple aromatic rings (Hill and Holman, 1983), and are both persistent and ubiquitous in the environment (Boehm, 1964, Gökmen and Morales, 2014, Gupta and Pathak, 2020, Hamzai et al., 2021, Sultana et al., 2021). PAH are lipophilic and hydrophobic, with these characteristics increasing with molecular weight and the number of aromatic rings (Wittig et al., 2003, Lima et al., 2005, Larsson et al., 2018). The main process by which PAH are formed is pyrogenesis, which occurs by the incomplete combustion of organic matter (Lima et al., 2005, Delgado-Saborit et al., 2009, Zhu et al., 2009, Tobiszewski and Namiesnik, 2012, Kwon and Choi, 2014, Wei et al., 2014, Suman et al., 2016, Halfadji et al., 2017).

PAH bond strongly to soil organic matter (SOM) owing to their hydrophobic nature, reducing their mobility within soil (Semple et al., 2003, Semple et al., 2013, Larsson et al., 2018, Yu et al., 2018). The most important process affecting PAH soil mobility is the “ageing” process (Abdel-Rahman et al., 2002). The fast-ageing process is the rapid initial absorption of PAH to the soil surface, this is then followed by the slow aging process which involves the movement of PAH through the soil to less accessible sites in the soil matrix. These processes result in lower PAH soil mobility over time (Weissenfels et al., 1992, Abdel-Rahman et al., 2002, Bergknut, 2004).

Many PAH are known carcinogens, teratogens, and mutagens and have been shown to bioaccumulate (Angerer et al., 1997, Pessah et al., 2001, Wittig et al., 2003, Chen and Liao, 2006, Meudec et al., 2006, Unwin et al., 2006, IARC, 2010, Gomez-Eyles et al., 2012, IARC, 2012, Harris et al., 2013, Yang et al., 2014, Lemieux et al., 2015). Owing to their ubiquity in soils and potential to cause adverse health effects, there is a need to quantify the potential risk posed if PAH enters the body. There are three main pathways for substances to enter

the human body: inhalation, ingestion, and dermal absorption (Nathanail et al., 2009). Dermal absorption is a significant exposure route for PAH (Nathanail et al., 2009). For this study, the definition of dermal bioavailability is: *“The fraction of the concentration of a compound(s) or element(s) that is absorbed into the skin, that may remain in situ in the skin, and/or enter systemic circulation through the skin.”* Dermal bioavailability can be quantified *in vivo* (animal testing) or *in vitro* (labware based) (IPCS, 2006). Absorption of compounds through the skin have been reported to differ between species, individuals, and anatomical site (VanRooij, 1993, Monteiro-Riviere and Riviere, 1999, Brain and Chilcott, 2008), resulting in highly variable absorption data. *In vivo* testing is often more expensive and offers a lower sample throughput in comparison to *in vitro* methods (Collins et al., 2015).

In the UK, environmental regulators and other professionals carry out contaminated land assessment utilising a risk-based approach to the determination as to whether land presents a risk to human health. For a risk to exist, there needs to be at least one contaminant-pathway-receptor linkage, from which a relevant receptor might be affected by the contaminants present (DEFRA, 2012). Human health risk assessment (HHRA) involves deriving assessment criteria for soil contaminants that represent minimal or low risk to human health. At present, exposure to soil PAH via the dermal pathway is represented by one value derived from the *in vivo* study by Wester et al. (1990). This value was derived *in vivo* using soils dosed with radiolabelled PAH in a solvent. However, more recent studies have demonstrated that the bioavailability of soil PAH decreases with increasing time elapsed from when radiolabelled PAH are applied to the soil (Abdel-Rahman et al., 2002, Turkall et al., 2010, Peckham et al., 2017). Therefore, the chemical aging process was underrepresented for native PAH in contaminated soils, and it is likely that dermal bioavailability of soil PAH was overestimated. Furthermore, it has been demonstrated that soil properties also impact the proportion of soil PAH that are bioavailable (Abdel-Rahman et al., 2002, Semple et al., 2003, Xia et al., 2016). By using one value for assessing dermal exposure to soil PAH, the effects of soil properties are not represented, adding further uncertainty to the current value.

The ‘Housing White Paper’ was published by the UK government in 2017, aiming to prioritise the redevelopment of Brownfield land to provide thousands of new homes and help protect the countryside (Ministry of Housing, 2017b). Brownfield land is previously developed land that has real or perceived contamination issues (Oliver et al., 2005, CABERNET, 2006). An example of this, are former gasworks sites, which exhibit some of the highest PAH soil

concentrations (Law et al., 2002, Potin et al., 2004, Baylis and Allenby, 2006, Lundstedt et al., 2006, Thomas, 2014). Potin et al. (2004) found BaP soil concentrations of 133 mg/kg (Σ USEPA 16 PAH = 793.9 mg/kg) at a French former gasworks. In contrast, a typical urban soil normal background concentration (NBC) for BaP is approximately 17 mg/kg (Σ USEPA 16 PAH = 80 mg/kg) (Vane et al., 2021). This contamination is an issue in the modern day, as many gasworks are classed as brownfield land which the government wish to return to a habitable state. However, the uncertainties in current dermal bioavailability values pose an issue for the assessment and redevelopment of such sites, leading to remediation being required when not necessary (overprediction of dermal bioavailability), increasing costs and diverting funding from other sites (Alexander, 2000); or worse leading to sites not being remediated when it is needed to protect human health (underrepresentation of dermal bioavailability).

Consequently, a method needed to be developed to measure the dermal absorption of parent PAH from 'real-world' soils to produce soil-specific dermal bioavailability data to aid in the risk-based land management decision making process. With this in mind this research was developed with the following aims and objectives.

1.2 Aim

The aim of this research is to develop a laboratory-based method to quantify the *in vitro* dermal absorption of PAH from soils to produce soil-specific PAH dermal bioavailability values.

1.3 Objectives

To meet the aim of this research, the objectives are:

1. To develop laboratory-based methods to dose, extract and quantify pyrene (Pyr), benzo[a]pyrene (BaP) and dibenz[a,h]anthracene (DBA) (3-PAH) from the Strat-M™ membrane, receptor solution and contaminated soil for quantification by GC-MS.
2. Compare the absorption of BaP through the Strat-M™ artificial skin membrane to published *ex vivo* skin BaP absorption values.
3. To determine the diffusion cell time steps to be used for the *in vitro* quantification of soil 3-PAH dermal bioavailability.
4. To quantify the dermal absorption of 3-PAH from contaminated soils

5. To assess the developed *in vitro* soil PAH dermal bioavailability measurement method performance.
6. To compare the experimentally derived soil specific BaP dermal bioavailability values to published soil PAH dermal bioavailability studies.
7. To explore the possible implications of soil specific PAH dermal bioavailability data on contaminated land management.

1.4 Thesis Structure

Part One – Introduction and Literature Review

Chapter 1 - Introduction

This chapter provides research context along with the aims and objectives of this research.

Chapter 2 – Review of the Current Literature

This chapter is a literature review of PAH dermal bioavailability and subsequent human health risk assessment of PAH exposure. PAH occurrence and behaviour in the environment are explored to inform the potential for PAH dermal absorption from soils. *In vivo* and *In vitro* PAH dermal bioavailability studies methods, results and findings, dermal absorption theory and dermal physiology are considered for the basis and framework for the method development of this project. Current approaches to the assessment of contaminated land are established, to ascertain the output requirements of the developed PAH dermal absorption method. PAH toxicity, occurrence and characteristics are assessed for the selection of three focal PAH (3-PAH) for this research.

Part Two – Method Development

Chapter 3 - General Methods

This chapter outlines soil sampling site selection, along with soil sample collection, preparation, and storage. The methods used to extract and quantify PAH concentration in soils is also given.

Chapter 4 – The Development of an *In Vitro* Dermal Bioavailability Method

Section 4.1 - Methodology

This section outlines the approach to developing the *in vitro* dermal bioavailability method based on findings of the literature review. The experimental approach for the determination

of the optimum extraction and quantification methods for each sample media required for the developed PAH dermal bioavailability method is explained. The method for the quantification of PAH concentrations by GC-MS is outlined. Statistical approaches for determining the optimum extraction techniques are described, as well as a statistical decision-making flow diagram.

Section 4.2 - Selection and Optimisation of a Method to Extract Polycyclic Aromatic Hydrocarbons from the Strat-M™ Membrane

This section explores the efficiency and practicality of different PAH extraction methods for extracting and quantifying PAH from the Strat-M™ artificial membrane, by comparing extraction performance of methods encountered in the literature for PAH extraction from other sample media. The PAH extraction methods recoveries and precision are compared to select the best method for use in later dermal absorption experiments.

Section 4.3 - Receptor Solution PAH Extraction and Recipe Selection

This section determines a method that is capable of extracting PAH from the receptor solution, as well as which receptor solution recipe provides the best recovery and precision of PAH concentrations using said extraction method. The PAH concentration of the receptor solution represents systemic circulation for the developed PAH dermal bioavailability method.

Section 4.4 - Soil Loading and Unloading of the Strat-M™ Membrane

This section develops repeatable methods for the addition and removal of soil from the Strat-M™ membrane; and determines the contribution of soil remaining on the membrane to the PAH concentration of the membrane extract. Equations to correct for the contribution of soil remaining on the membrane to the membrane PAH concentration are proposed.

Part Three – Method Validation and Testing

Chapter 5 – The Dermal Bioavailability of PAH

Section 5.1 – The Dermal Absorption of PAH from a Solvent Vehicle

This section quantifies the absorption of 3-PAH from a solvent vehicle through the Strat-M™ membrane into the receptor solution over a period of 48h. Steady-state PAH flux (rate of diffusion) was calculated. The PAH absorption data was then compared to published values to determine if the Strat-M™ is representative of *ex vivo* skin. The data from this

experiment aided the experimental design (e.g., sampling time-steps) for the soil PAH dermal absorption experiment.

Section 5.2 – The Dermal Absorption of PAH from Contaminated Soils

This section reports the dermal absorption of 3-PAH compounds from three different soils over a period of 48h. Two of the tested soils were from former gasworks sites, the third soil was a certified reference material (CRM) from a former wood treatment plant. The 3-PAH concentrations were quantified in the soil, membrane, and receptor solution at each time-step over the 48h duration. PAH dermal absorption rates were calculated for each soil and compared between soils and to previous published works. The developed method performance was also compared to previous works for method assessment.

Part Four – Discussion and Conclusions

Chapter 6 – Implications of Experimental Dermal Bioavailability Data on the Contaminated Land Management Industry

The experimentally derived soil-specific PAH dermal absorption data is used to predict dermal exposure using the UK standard approach to HHRA for contaminated land assessment. The same model is then used to calculate dermal exposure for the same soils using the default PAH dermal bioavailability value. These outputs are then compared, to determine differences in the current approach and soil-specific PAH dermal bioavailability. The potential impacts of soil-specific dermal bioavailability values on UK risk-based land management are then explored.

Chapter 7 – Conclusions and Recommendations

This chapter presents the conclusions from the method development and discussion chapters, and also includes a section discussing recommendations for future research.

2 Review of the Current Literature

The aim of this literature review is to determine strengths and weaknesses in previous approaches for the measurement of the dermal bioavailability of PAH from soil. Furthermore, the toxicity of different PAH and the occurrence and behaviour of PAH are explored to determine the selection criteria for PAH and soil samples to be studied in this work. In addition, current approaches to human health risk assessment of potential PAH exposure are reviewed, so that the data outputs of the developed dermal bioavailability method are relevant to contaminated land risk assessment. This also informs on my decisions for which methodological approach to take when designing experiments in order that the dermal bioavailability results could be validated using previously published data.

2.1 PAH in the Environment

2.1.1 The Chemical Structure of PAH

PAH are hydrocarbons composed of multiple aromatic rings (C_6H_6) also known as benzene rings, with alternate C atoms having double H-bonds. PAH are known persistent organic pollutants (POPs) (Jones and De Voogt, 1999) owing to their stability in the environment, which is due to every C-C bond within their structure all being of equal length (Hill and Holman, 1983).

PAH are lipophilic and hydrophobic, with these characteristics increasing with molecular weight and the number of aromatic rings (Tobiszewski and Namiesnik, 2012). The K_{ow} (octanol-water partition coefficient) is the ratio at which a substance partitions into octanol and water, substances with low K_{ow} values are still considered relatively hydrophobic, but tend to have higher water solubility, low soil adsorption coefficients and small bioconcentration factors. Those with higher K_{ow} values are thought to be extremely hydrophobic, and they also show greater lipophilicity (Wittig et al., 2003, Lima et al., 2005, Larsson et al., 2018). Low molecular weight (LMW) PAH are defined as being composed of <4 aromatic rings, whereas high molecular weight (HMW) ≥ 4 aromatic rings Figure 1.

The USEPA list of priority PAH compounds (Figure 1) was introduced in the mid-1970s. These compounds are used frequently in research, as many believe they cover a wide variety of physico-chemical properties of PAH compounds and are commonly found in environmental samples (Zelinkova and Wenzl, 2015). Since the introduction of the list, it has allowed for a widespread understanding as scientists of different laboratories and fields of expertise are likely to be familiar with the compounds in the list. This has also allowed for good comparability amongst labs, as scientists are able to understand and analyse the same set of

analytes, and this results in effortless communication of results and findings. Also, limiting the list to 16 compounds results in a reduction in lab costs in comparison to measuring concentrations of hundreds of PAH compounds.

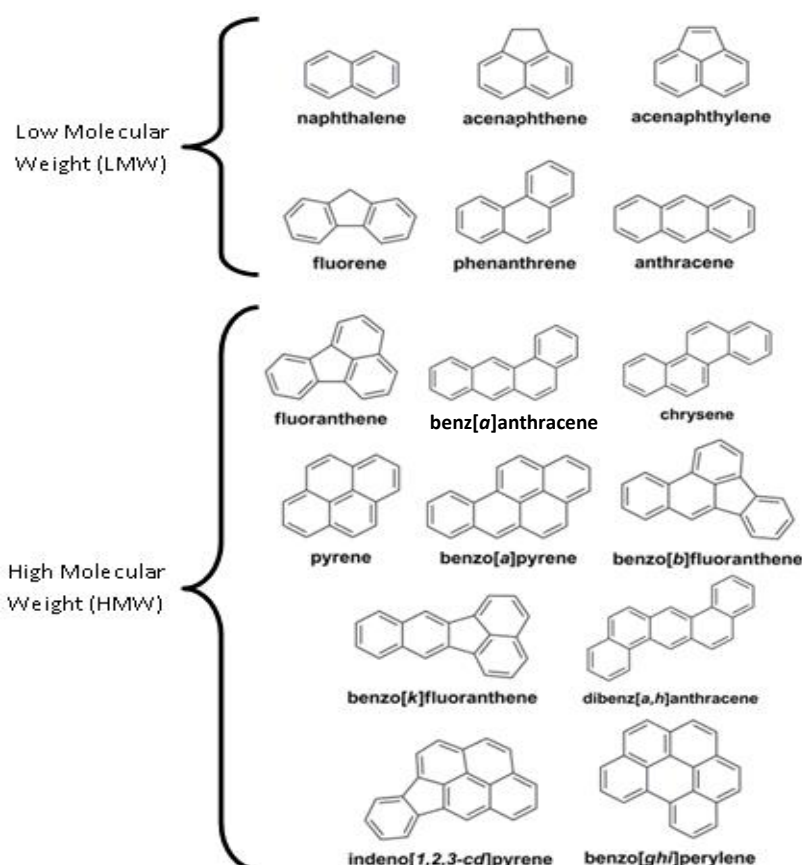


Figure 1: USEPA 16 PAH, adapted from: Nogueira et al. (2015). Low molecular weight (LMW) PAH are defined as being composed of <4 aromatic rings, whereas high molecular weight (HMW) ≥ 4 aromatic rings.

2.1.2 The Environmental Sources and Occurrence of PAH

PAH are ubiquitous in the environment (Boehm, 1964, Gökmen and Morales, 2014, Gupta and Pathak, 2020, Hamzai et al., 2021, Sultana et al., 2021). In general, there are four ways in which PAH are formed: diagenesis, petrogenesis, biosynthesis and pyrogenesis. A natural source of PAH is the diagenesis of organic matter, which is a rapid process which occurs at low temperatures (Morrison and Murphy, 2006). Another potential way in which PAH are formed is via biosynthesis. Stogiannidis and Laane (2015) report that there is evidence that some PAH are produced naturally in the environment by certain chemical/biological transformations of organic matter, or other biological processes. Petrogenic sources of PAH can be either natural or anthropogenic in occurrence. Natural sources for example would be

events such as oil seeps or the erosion of petroliferous shales. Anthropogenic petrogenic sources would include accidental oil/fuel spills, as well as storm water runoff and municipal waste treatment discharges (Morrison and Murphy, 2006).

The largest contributor to the occurrence of PAH in the environment is pyrogenesis (Lima et al., 2005, Delgado-Saborit et al., 2009, Zhu et al., 2009, Tobiszewski and Namiesnik, 2012, Kwon and Choi, 2014, Wei et al., 2014, Suman et al., 2016, Halfadji et al., 2017), which can occur naturally or anthropogenically. Pyrogenesis involves the formation of PAH through the incomplete combustion of organic matter, with natural sources including events such as forest fires and anthropogenic sources including fossil fuel combustion. A summary of potential sources of PAH can be found in Table 1.

Table 1: Various PAH sources and associated PAH-bearing materials. Table adapted from Ruby et al. (2016)

Type of Source	PAH Source	Primary PAH-bearing Materials
Natural	Forest/Grass Fires	soot, char
	Volcanic eruptions	soot, char
	Oil seeps	weathered crude oil
	Sedimentary Rock	kerogen
Industrial	Gasworks	coal tar, oil tar, pitch, coal, char, soot
	Coking	coal tar, coal, coke, soot
	Al production	coal tar or petroleum pitch
	Foundries	coal tar pitch, creosote, fuel oil, soot
	Wood treatment plants	creosote
	refineries	soot, crude oil, fuel oil, diesel
	C-black manufacture	soot, oil tar
Non-Industrial	Fuel spills and/or disposal	various NAPLs (crude oil, fuel oil, diesel)
	Skeet	coal tar pitch or bitumen
	Asphalt sealants	coal tar
	Landfill	creosote, soot, char
	Incinerators (industrial)	soot
	Open burning	soot, char
	Auto/truck emissions	soot

The formation of PAH pyrogenically involves the organic compounds present in the fuel/biomass being fragmented into smaller molecules/free radicals during combustion. These free radicals are much more unstable and reactive and through multiple different chemical pathways the first benzene ring is formed. Further reactions involving this ring and small carbon molecules (containing 2/3 carbon atoms) results in the formation of larger and more stable multiple aromatic ring systems.

A pioneering study by Wild and Jones (1995) attempted to quantify the PAH burden in the UK. This was achieved by calculating the surface area of the UK, and by assuming a bulk density of 1200 kg m^{-3} using the top 15cm of soil a total soil weight of 4.45×10^{10} tonnes for the UK was derived. It was then assumed that 10% of land cover is urban, another 10% is woodland and the remaining 80% is rural. They estimated a total PAH burden in soils of around 50,000 tonnes using these assumptions. Although this demonstrated the extent at which PAH are present in the UK environment, contaminated sites such as gas works were excluded from their estimations, and along with the other assumptions made, the UK PAH burden is likely to be underestimated. It has been estimated that there were between 2000 and 4000 gas works of sizes between 0.3ha and 200ha that were previously operated in the UK (Thomas, 2014), which would suggest that with just gasworks alone a significant burden would be added to their estimations. Figure 2 shows the extent at which gasworks soils PAH concentrations are higher than other soil types. This is due to the incomplete combustion of coal during the coal gasification products producing PAH and also many PAH-containing waste products such as coal tar being present at such sites (Tarr, 2014, Thomas, 2014).

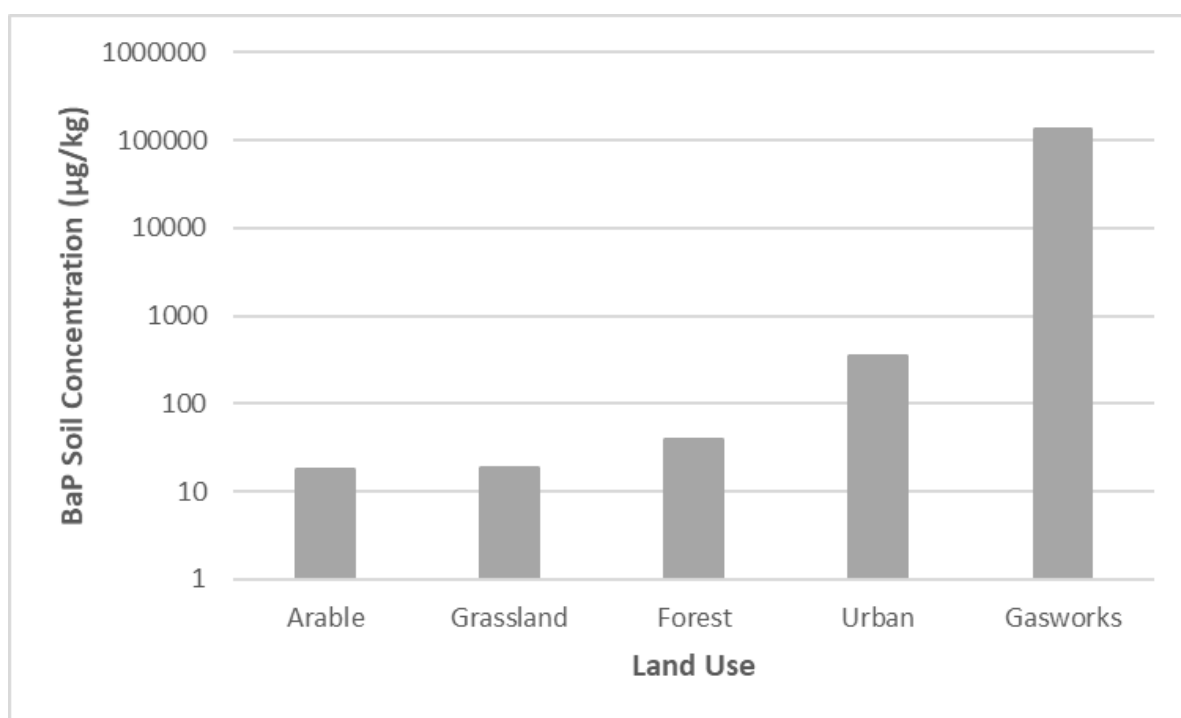


Figure 2: The mean BaP concentrations in soils of different land uses. Arable, grassland, forest and Urban soils data is from Wilcke (2000) and contaminated gas work soil from Potin et al. (2004).

The coal gasification process involves the heating of coal in the absence of air in retorts (an airtight vessel), with the resulting vapour being purified through tar traps, air- and water-cooled condensers, washers, scrubbers, and purifiers to remove substances such as: coal tar,

sulphur, cyanide and ammoniacal liquor (Figure 3). PAH were synthesised during the gasification process itself (Figure 3), as well as the presence of PAH in waste products such as coal tar, 17% of which is composed of US EPA 16 PAH, and often disposed of on site (Thomas, 2014). It is not surprising therefore, that these sites contain some of the highest PAH concentrations in their soils (Law et al., 2002, Potin et al., 2004, Baylis and Allenby, 2006, Lundstedt et al., 2006, Thomas, 2014). This contamination is an issue in the modern day, as many gasworks are now deemed brownfield land (Thomas, 2014) which the government wish to redevelop (CPRE, 2020).

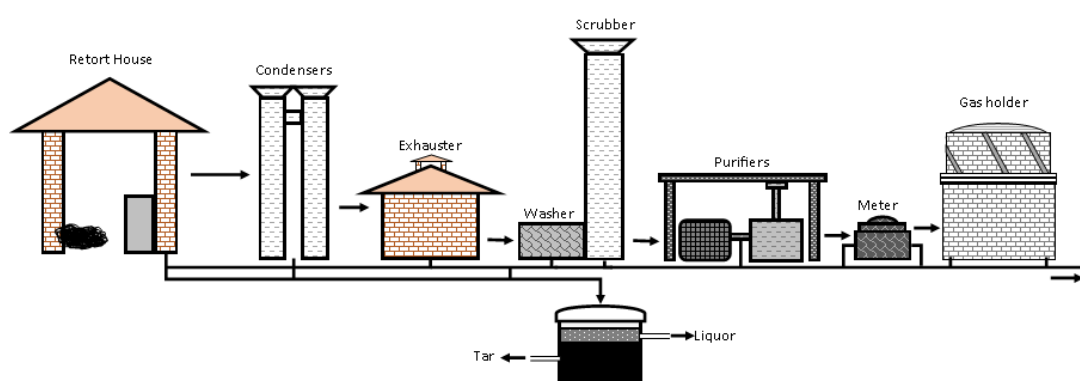


Figure 3: A schematic of a typical coal gasification process. Adapted from: Baylis and Allenby (2006).

2.1.3 Behaviour of PAH in Soil

PAH in soils can undergo chemical oxidation, photodecomposition, or volatilisation, but it is believed that they are mainly degraded by microorganisms (Weissenfels et al., 1992). In order to discuss PAH behaviour in soil, a broad definition of PAH bioavailability and bioaccessibility are applied. Bioavailability is the amount of contaminant that reaches systemic circulation; whereas bioaccessibility is the amount of contaminant that is free to be absorbed (Ng et al., 2015). PAH in soil tend to bond strongly to soil organic matter (SOM) owing to their hydrophobic nature, reducing their bioavailability and mobility (Semple et al., 2003, Semple et al., 2013, Larsson et al., 2018, Yu et al., 2018), with great reductions in PAH availability occurring if the soil organic carbon content is $>0.1\%$ (Bergknut, 2004). SOM can be divided into: fulvic acid, humic acid and humin, and it has been demonstrated that humic acid and humin are sources of site-specific non-linear sorption of hydrophobic organic compounds such as PAH, in soils (Chiou et al., 2000, Kang and Xing, 2005, Pan et al., 2006). It has also been shown that PAH bond very strongly to soot in soils from vegetation fires, and also that soot deposition from the atmosphere is a sink for PAH (Wilcke, 2000). It has been found from PAH sorption experiments that the sorption isotherms remain straight up to

around 50% of PAH sorption, where it starts to curve, showing that PAH sorption to soil is rapid, and an entropy driven process (Chiou et al., 1998, Wilcke, 2000).

The most important process affecting PAH bioaccessibility is the “ageing” process that occurs to PAH in soil over time (Abdel-Rahman et al., 2002). The fast-ageing process is the rapid initial absorption of PAH to the soil surface, this is then followed the slow aging process which involves the movement of PAH through the soil to less accessible sites in the soil matrix. The binding to the soil matrix occurs via processes such as π -bonding and hydrogen bonding (Bergknut, 2004). Hydrogen bonding is primarily an electrostatic force of attraction between a hydrogen atom which is covalently bound to a more electronegative atom or group, and another electronegative atom bearing a lone pair of electrons (Arunan et al., 2011). π bonds are covalent bonds, in each of which two lobes of an orbital (on one atom) laterally overlap with two lobes of an orbital on another atom (McNaught and Wilkinson, 1997) Mechanisms at play during these aging processes are not fully understood, but there are two main models which are generally accepted. The organic diffusion model suggests that PAH bind to the organic matter in soil (Brusseau et al., 1991, Nam et al., 1998). The alternative model, the intraparticle diffusion model, suggests that PAH migrate to nanopores within the soil and become trapped, with these pores being so small that even bacteria cannot reach the PAH therefore preventing biodegradation of the PAH (Weissenfels et al., 1992, Abdel-Rahman et al., 2002, Bergknut, 2004). This ageing process of PAH in soil is of utmost importance for dermal bioaccessibility studies, as the process effectively locks the PAH in the soil, reducing the ability for the compounds to move. In many studies, the use of artificially spiked soil does not consider this process, meaning that dermal absorption may be overestimated as PAH will not be bound to the soil such as in natural soils, so the use of “real life” soils is necessary for this project to better estimate and model the amount of PAH absorbed through the skin.

Another soil process that could potentially affect PAH bioaccessibility is the leaching process. As PAH have been found at greater depth from surface this indicates that they are subject to leaching (Wild and Jones, 1995). The enhanced solubility of PAH may be caused by their association with DOM (dissolved organic matter), which may act as a PAH carrier as DOM interacts with solid phases of the soil, DOM quality varies over time and space due to being altered by microbial action and either being produced or consumed. Soil horizons that contain high concentrations of Fe oxides, due to their scavenger like behaviour act as a barrier for PAH transport because DOM is retained. The association of PAH to DOM is

controlled by the polarity, molecular size, configuration, and chemical composition of the molecule the PAH sorbs to, and it has been shown that large molecules which contain non-polar compounds such as humic acids have a high capacity to bond to PAH (Wilcke, 2000). Overall, DOM could potentially have a large effect on PAH bioaccessibility, as DOM enhances PAH mobility within soils therefore allowing for greater movement from the soil to human skin resulting in a greater potential for skin absorption.

The soil moisture content is also likely to have an impact on PAH bioaccessibility. The bioaccessibility of phenanthrene was shown to be greater for a spiked moist soil (53.3% of applied dose) in comparison to a spiked dry soil (39.2% of applied dose) (Kottler et al., 2001). It has been shown that soil wet-dry cycles promotes PAH aging (White et al., 1997). This is thought to be due to structural changes occurring through the enlargement of soil pores during wetting coupled with the exposure of hydrophobic SOM areas to external surfaces during drying. (Schaumann et al., 2005, Wang et al., 2016). This same swelling of the soil pores may also be the mechanism by which PAH become more bioavailable when the soil has a higher water content.

2.2 Exposure to PAH

2.2.1 US EPA 16 Priority PAH

Many studies do not use the full US EPA priority list, but focus on one congener, benzo[a]pyrene (BaP) (e.g.) (Gelboin, 1980, Wester et al., 1990, Pessah et al., 2001, Moody and Joncas, 2007, Amodu et al., 2016). This is due to it being the most easily synthesised PAH known to be a carcinogen (Wester et al., 1990), and therefore, one of the most likely carcinogenic PAH to be encountered in the environment. BaP has long been used in toxicity equivalence factors (TEFs) which are used to estimate the combined potency of PAH mixtures such as coal tar, with relative potency in humans being derived from animal studies due to the lack of data of PAH potency in humans. Several of these studies have been carried out, such as Nisbet and LaGoy (1992), who created TEFs using BaP as the marker for 17 different PAH. Although these studies have their benefits, such as the reduction in the cost of analysis due to only BaP being analysed in complex mixtures, there are some alarming shortcomings. One of these is that other PAH may be misclassified in their carcinogenicity, resulting in either an over or worse underestimate of a mixtures potency. As the mixtures contain other compounds as well as PAH, the effect of other compounds on PAH toxicity is unknown, meaning that other compounds in the mixture may suppress or enhance PAH toxicity which

TEFs cannot at present account for. There may also be even more toxic PAH compounds other than BaP which are not currently measured as standard practice. For example, dibenzo[a,l]pyrene (DBaP) could be 10 to 100 times more potent than BaP (COC, 2003). This uncertainty is likely to contribute to the underestimation of carcinogenicity by the TEF method of PAH mixtures.

The surrogate marker approach assumes that the surrogate marker is present in all samples, and that the concentration of individual PAH relative to the marker remains similar in all samples (PAH profile). It is also assumed for the surrogate marker approach that the PAH profiles are similar to those used in toxicity studies used in risk assessment to derive critical toxicological parameters. As with the TEF approach, the presence of high potency PAH which are not commonly measured in soils such as DBaP also presents uncertainties as to if the surrogate is representative. On the other hand, the surrogate marker approach has the advantage of using dose response data from a *in vivo* carcinogenic study of a coal tar mixture, therefore assessing mixture potency rather than individual PAH (Culp et al., 1998, Public Health England, 2017). It has been argued that BaP is a suitable marker for other carcinogenic PAH, due to high correlation (r^2 between 0.72 and 0.98) between BaP and other carcinogenic PAH within soils of different origins (rural, urban, and industrial) (Bull and Collins, 2013). Public Health England (2017) also recommend the surrogate marker approach (BaP). Public Health England (2017) tested PAH concentration ratios variability at different UK sites, including 52 contaminated sites. The review concluded that BaP is a good surrogate marker, being ubiquitous in sites contaminated with PAH and providing a consistent indicator of the amount of PAH in contaminated soil. For the surrogate marker approach to be valid, the PAH profile at potentially contaminated sites should be similar to that seen in the oral carcinogenicity study in mice by Culp et al. (1998).

2.2.2 Health Effects of PAH Exposure

PAH are known carcinogens, teratogens, and mutagens and have been shown to bioaccumulate (Angerer et al., 1997, Pessah et al., 2001, Wittig et al., 2003, Chen and Liao, 2006, Meudec et al., 2006, Unwin et al., 2006, IARC, 2010, Gomez-Eyles et al., 2012, IARC, 2012, Harris et al., 2013, Yang et al., 2014, Lemieux et al., 2015). Billiard et al. (2007) found that the teratogenicity of PAH mixtures was not additive as previously presumed, which is likely due to the high variety in PAH structures and subsequent potential toxicity after being metabolised by CYP1 enzymes.

Meudec et al. (2006) found that PAH can bioaccumulate in plants, by measuring PAH concentrations in *Salicornia fragilis* (Yellow Glasswort) they found that PAH accumulate in shoots by a soil-to-plant transfer through the root system, and even found HMW PAH in the upper part of plants. Plants may also be exposed to PAH through the volatilisation of PAH from the soil in which the plant is growing with subsequent aerial deposition to the plant (Collins and Finnegan, 2010). Furthermore, D'adamo et al. (1997) found that BaP bioaccumulates in mussels, but to a lesser extent in fish, possibly due to different detoxification efficiencies. It has also been found that PAH are phytotoxic, as they disrupt mechanisms such as photosynthesis, respiration, transpiration. and nutrient absorption (Meudec et al., 2006). Wittig et al. (2003) also found that when PAH are present in soil, root development is inhibited, with growth inhibition most evident when exposed to fluoranthene.

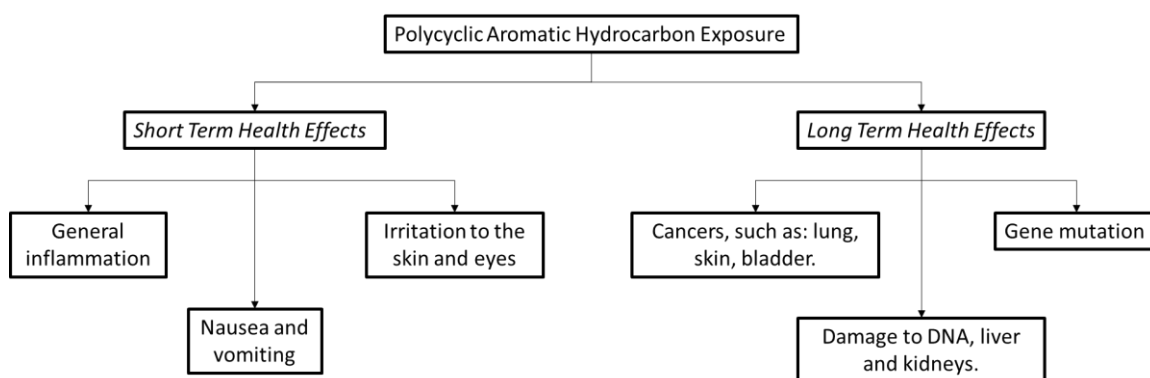


Figure 4: Human health effects caused by PAH exposure.

A summary of other human health effects is shown in Figure 4. The most concerning health impact of PAH is their ability to cause mutations and cancers. BaP is the only PAH at present to be classed as a known human carcinogen (IARC, 2010, ATSDR, 2017). The International Agency for Research on Cancer (IARC) classification of the USEPA 16 PAH is shown in Table 2 and the definitions of each classification in Table 3. BaP is the only USEPA 16 PAH that has been classified as a known human carcinogen, owing to the quantity and quality of human and animal toxicological data (IARC, 2010).

Table 2: IARC (International Agency for Research on Cancer) carcinogenicity classification of the USEPA16 PAH (IARC, 2010). AC = acenaphthene, ACL = acenaphthylene, AN = anthracene, BaA = benz[a]anthracene, BbFA = benzo[b]fluoranthene, BghiP = Benzo[g,h,i]perylene, BkFA = benzo[k]fluoranthene, CHR = chrysene, FA = fluoranthene, FL = fluorene, IP = indeno[1,2,3,c-d]pyrene, NA = naphthalene, PHE = phenanthrene, Pyr = pyrene. nd = no data.

IARC Classification of Carcinogenicity							
AC	ACL	AN	BaA	BaP	BbFA	BghiP	BkFA
3	nd	3	2B	1	2B	3	2B
CHR	DBA	FA	FL	IP	NA	PHE	Pyr
2B	2A	3	3	2B	2B	3	3

Table 3: IARC Group definitions (IARC, 2010).

IARC Group	Group Definition
1	Carcinogenic to humans.
2A	Probably carcinogenic to humans based on limited human data.
2B	Probably carcinogenic to humans based on animal studies
3	Not classifiable as to its carcinogenicity to humans.
4	Probably not carcinogenic to humans.

PAH are not carcinogenic until they are metabolised, and the bay region dihydrodiol epoxide pathway is the main mechanism for known strongly mutagenic and carcinogenic PAH. The formation of dihydrodiol epoxides is a result of three enzyme reactions involving P450's and epoxide hydrolases. The resulting metabolised congeners bind strongly to DNA adducts and are deemed to be a "strong pro-mutagenic event". This pathway produces many varieties of stereoisomeric metabolites of differing mutagenic potency (Gelboin, 1980, Pessah et al., 2001, European Food Safety Authority, 2008).

It should also be noted that there are PAH-containing mixtures that have been classified as known human carcinogens such as coal tar (IARC, 2010). Current human health risk assessment assumes potency increases with concentration addition, which may not be the case (Billiard et al., 2007). Previous industrial sites such as former gas works, or coke production sites will be heavily contaminated with PAH due to the production and improper disposal of waste products such as coal tar (Lemieux et al., 2015). This is of concern for regulators as many of these sites are to be assessed for redevelopment as many are now

brownfield sites, which is why pathways into the human body for these compounds and mixtures need to be better understood.

2.2.3 Dermal Bioavailability

There are three main pathways for substances to enter the human body: inhalation, ingestion, and dermal absorption. A common theme in the literature is that bioaccessibility and bioavailability are often defined and/or utilised incorrectly (Naidu et al., 2015). For this study, the definition of bioavailability is: *“The fraction of the concentration of a compound(s) or element(s) that is absorbed into the skin, that may remain in situ in the skin, and/or enter systemic circulation through the skin.”* Consequently, the definition of bioaccessible is: *“The proportion of the total concentration of a compound or element that is available for dermal absorption, and therefore can be used to estimate bioavailability.”*

The skin has many functions, one of which is its ability to act as an environmental barrier that also minimises insensible water loss (Monteiro-Riviere and Filon, 2017). The skin is also responsible for temperature regulation, it can act as an immunological effector axis by initiating inflammatory responses to foreign insults; contains neurosensory receptors for touch, pain, and heat; and can also metabolise compounds that do enter the skin to more readily remove substances that have been absorbed. The skin is a nonhomogeneous organ and is composed of two principal regions; the inner regions, the dermis which consists of connective tissues containing many cell types and specialised adnexal structures; and the outer region the epidermis, a stratified avascular cellular region. The epidermis is composed of cell layers with the outermost layer, the *stratum corneum*, functioning as a main barrier and consisting of keratinised dead cells which are constantly shed (Scheuplein and Blank, 1971). The viable epidermis can metabolise substances that are able to diffuse through the *stratum corneum*. The dermis offers physiological support for the avascular epidermis and contains blood vessels, sensory nerves, and lymphatics. The appendages, such as hair follicles, originate in the sub-papillary dermis (Monteiro-Riviere and Riviere, 1999, IPCS, 2006).

The epidermis consists of keratinised stratified squamous epithelium, and undergoes an organised pattern of proliferation, differentiation, and keratinisation. The cells of the basal layer are always undergoing mitosis, resulting in daughter cells migrating outwards resulting in the replenishment of the epidermis as the cells of the *stratum corneum* are shed/sloughed (Monteiro-Riviere and Riviere, 1999). The cells of the *stratum corneum* (corneocytes), are

stacked on top of one another to form an interlocking columnar structure, with this arrangement aiding in the facilitation of the skin's barrier function (Figure 5). Intercellular lipids originating in the *stratum granulosum* are located between the corneocytes, resulting in a "brick and mortar" type structure (the bricks being the corneocytes and mortar the intercellular lipids). This prevents penetration of substances through the skin, and also insensible water loss (Monteiro-Riviere and Riviere, 1999, Chilcott and Price, 2008, Monteiro-Riviere and Filon, 2017). The intercellular lipid composition is not homogeneous, and changes throughout the layers of the epidermis, along with anatomical and interspecies differences (Monteiro-Riviere and Riviere, 1999), but is mainly composed of ceramides, cholesterol, fatty acids along with small quantities of hydrolytic enzymes (Monteiro-Riviere and Filon, 2017). The lipid matrix presents a lipophilic environment and is therefore an influencing factor in the absorption of substances, although the underlying dermis is predominantly hydrophilic. Solubility of a substance can be determined by the measurement of the partitioning of a substance between two immiscible liquids at equilibrium. An example of this is the octanol-water partition coefficient ($\log P$), and as a general rule, substances with a $\log P$ of 1 to 3 are optimal for dermal absorption. For a substance to migrate through the epidermis to the dermis or other site, it cannot be highly lipophilic or hydrophilic for it will be sequestered in either layer and will be unable to pass to the other (Brain and Chilcott, 2008). The dermis consists of dense irregular connective tissues that extends to the hypodermis or subcutaneous tissues. Cells present in the dermis include macrophages, mast cells, fibroblasts, blood vessels, nerves and lymphatics, plasma cells, chromatophores, fat cells as well as extravasated white blood cells. Appendages such as sweat glands, sebaceous glands and hair follicles are also fixed within the dermis (Monteiro-Riviere and Riviere, 1999).

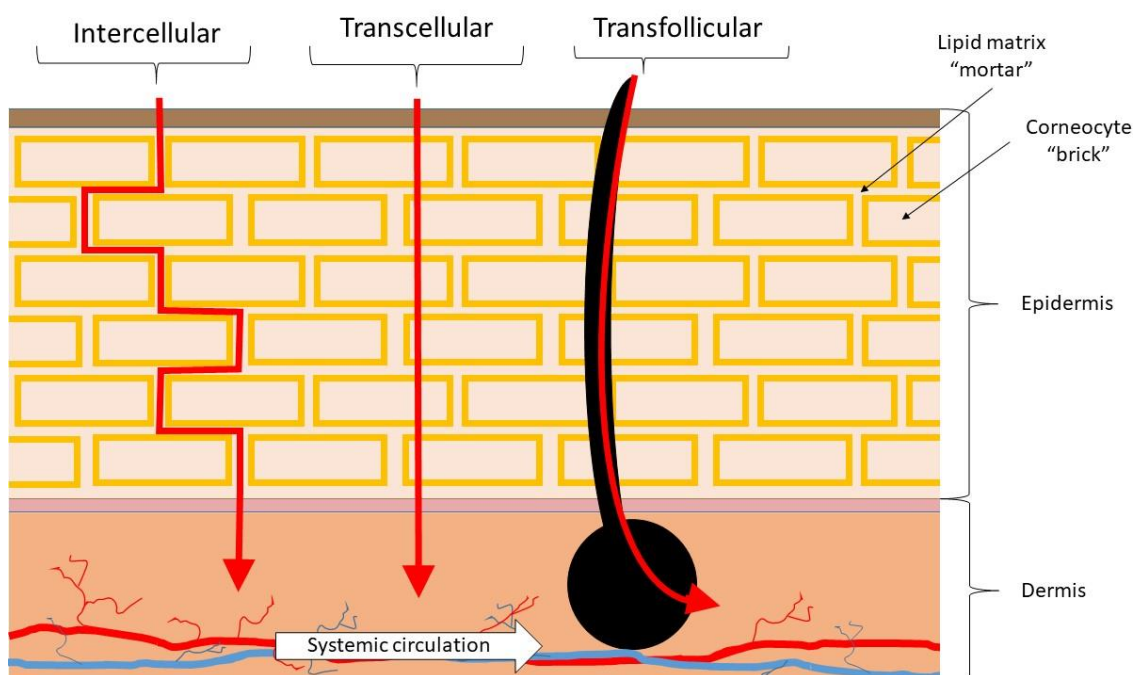


Figure 5: The three main dermal absorption pathways and skin structure.

Four pathways by which a substance can penetrate into the skin have been identified: intercellular, transcellular, as well as two transappendageal/transfollicular through sweat glands and hair follicles (Crosera et al., 2009) (Figure 5). The main route of dermal absorption for most substances is the intercellular pathway, which is heightened and enabled by lipids in the *stratum corneum*. Most chemicals are absorbed through the intercellular pathway, therefore the partitioning into and diffusion through the lipid matrix (Log P) can be predictive of the compound's absorption (Monteiro-Riviere and Filon, 2017). This pathway is long owing to the "brick and mortar" structure reducing the rate of absorption. Passage via the transcellular route occurs when substances are equally able to diffuse through the corneocytes and lipid matrix. The transappendageal route through epidermis into the lower dermis (e.g., through hair follicles) may enable the *stratum corneum* to be circumvented, but the overall contribution of this pathway to absorption is disputed, as although this route bypasses the epidermis, the entrance to the appendages still has corneocytes, sebum and enzymes present which will limit absorption (Chilcott and Price, 2008).

2.2.4 Dermal Absorption Theory

Dermal absorption is a thermodynamically driven process. There are three main phases by which a substance is dermally absorbed. The first of these, is the substance partitioning from a vehicle into the *stratum corneum*. Secondly, the diffusion of a substance through the

stratum corneum is driven by the thermodynamic concentration gradient, whereby the substance will diffuse from an area of high concentration to an area of lower concentration. The final stage involves the partitioning of a substance from the *stratum corneum* into deeper tissues in the epidermis and potentially reaching systemic circulation. The movement of a substance across a membrane at steady-state can be described by Fick's first law of diffusion (Equation 1) (Crank, 1975). The penetration rate (flux) is usually presented in the units of concentration per exposure area per time duration (e.g., ng/cm²/h).

$$J_{ss} = K_p \cdot C_o$$

Equation 1: Fick's First Law of Diffusion

Where J_{ss} is the steady state flux per unit area, K_p is the permeability coefficient expressed in distance and time (for soil studies K_p is known as the skin/soil partition coefficient (K_s) (Roy and Singh, 2001)), and C_o is the concentration of the substance in the vehicle. The time taken to achieve steady-state flux under an infinite dose scenario is known as lag time (τ).

After the application of a substance to a membrane, there will be a gradual equilibrium of the concentration of the substance in the vehicle (C_v) and the concentration of the substance in the *stratum corneum* (C_{sc}), which are related to the membrane partition coefficient (K_{sc}) shown in Equation 2 (Pugh and Chilcott, 2008).

$$K_{sc} = \frac{C_{sc}}{C_v}$$

Equation 2: Membrane Partition Coefficient

Partitioning is an important parameter for dermal absorption as it impacts the concentration of substance in the *stratum corneum* which consequently impacts the thermodynamic gradient through the *stratum corneum*, which is related to the permeability coefficient (K_p) as shown in Equation 3.

$$K_p = \frac{K_{sc} \cdot D}{x}$$

Equation 3: Permeability Coefficient

Where D is the diffusion coefficient, and x is the *stratum corneum* thickness. By inserting the terms of equation into Equation 3 gives Equation 4, which shows the association of the main factors that impact dermal absorption.

$$J_{ss} = \frac{K_{sc} \cdot C_v \cdot D}{x}$$

Equation 4: Relationship between the main factors that impact dermal absorption

Alternatively, the dermal bioavailability of a substance can be reported as the percent absorbed of the dose applied (PADA (%)). It is also often alternatively referred to in the literature as the bioavailable fraction (BAF (%)) (e.g. Wester et al. (1990)). The PADA (%) is calculated using Equation 5.

$$PADA (\%) = \frac{\text{Mass PAH dermally absorbed}}{\text{Mass PAH applied}} \times 100$$

Equation 5: Calculation of percent absorbed of the dose applied (PADA (%)). Alternatively referred to in the literature as the bioavailable fraction (BAF (%)).

Where: The mass PAH dermally absorbed represents the PAH mass that has entered the receptor solution and membrane *in vitro*, or alternatively the excreted metabolites *in vivo*. The Mass PAH applied is the dose applied to the membrane *in vitro*, or onto the skin of the animal *in vivo*, at the beginning of the experiment.

Another alternative expression of bioavailability, more often used for oral bioavailability, is Relative bioavailability (RBA). RBA refers to the comparative bioavailability of different forms of a substance or for different exposure media containing the substance (Ruby et al., 2016). For example, Juhasz et al. (2014) tested the oral bioavailability of BaP in creosote contaminated soil post microbial remediation *in vivo* using mice. Calculated the BaP RBA in the creosote contaminated soil by comparing oral uptake in mice from a BaP spiked sand (reference material), and the remediated soils using Equation 6.

$$\begin{aligned} \text{a) } PAH \text{ absolute bioavailability } (\%) &= \left(\frac{PAH \text{ applied} - \text{residual PAH in faeces}}{PAH \text{ applied}} \right) \times 100 \\ \text{b) } PAH \text{ relative bioavailability } (\%) &= \left(\frac{PAH \text{ absolute soil bioavailability}}{PAH \text{ absolute reference bioavailability}} \right) \times 100 \end{aligned}$$

Equation 6: Calculation of oral absolute and relative bioavailability. A) the calculation of absolute bioavailability, b) the calculation of relative bioavailability. Adapted from Juhasz et al. (2014).

Typically, bioavailability is calculated from the concentration of the chemical in blood over time, and reported as the area under the curve (AUC) from the blood concentration versus time profile curve (Ruby et al., 2016). RBA of a chemical can also be used in risk assessment, and is derived from the ratio of the AUC following an oral dose of the chemical in the soil versus the AUC from an equivalent oral dose of the chemical in the medium used

in the critical toxicity (Ruby et al., 2016). Although RBA can be a useful tool for determining differences in bioavailability of substances or exposure media, there are some shortcomings. Firstly, to measure an AUC with reasonable accuracy, blood concentrations at several time points are needed. The measurements at progressively lower PAH concentrations in soil and diet can result in blood concentrations decreasing below analytical detection limits, resulting in the error in estimating the AUC becomes unacceptably high (Ruby et al., 2016). Furthermore, for the ratio of AUCs of PAH from soil versus diet to reflect RBA, the rate of removal from the blood must be the same under both dosing conditions, which is difficult to achieve under several subsequent PAH doses. Furthermore, the faecal approach taken by (Juhasz et al., 2014), also suffers limitations as it cannot account for PAH losses via degradation in the intestine.

2.2.5 Biological and Chemical Factors that Impact the Dermal Absorption of a Substance

There are both biological and chemical factors that can result in different amounts of a substance being dermally absorbed. The ability for a substance to cause dermatotoxicity depends on its ability to enter the skin, and also the interactions of a substance with biological components of the skin that could produce a toxicological response (Monteiro-Riviere and Riviere, 1999). Molecular size can limit the ability of a substance to penetrate the skin because of the molecular packing of the brick-and-mortar structure of the *stratum corneum*. Consequently, it is generally accepted that substances with a molecular weight greater than 500 Da are unable to passively diffuse through the skin, this rule is coined the “rule of 500” (Chilcott and Price, 2008, Crosera et al., 2009). Owing to the molecular size of the USEPA 16 PAH all being less than 500 Da (DBA is the largest at 278 DA), molecular mass is unlikely to hinder their dermal absorption. The solubility of a compound is another physico-chemical characteristic that governs its ability to be dermally absorbed. The epidermis is a lipophilic environment, whereas the dermis is a hydrophilic environment, as the hydration of the skin increases with depth from the surface (outermost layer of the *stratum corneum* ~ 15%w/w compared to the viable epidermis ~70% w/w (Hou et al., 1991). As such, a substance cannot be strongly lipophilic, for it will sequester in the epidermis and not penetrate the dermis, or it cannot be too hydrophilic, for it will not penetrate the *stratum corneum*. As such, it is said that for a substance to be able to effectively traverse through the skin, a Log P of between 1 and 3 is required (Chilcott and Price, 2008). The *stratum corneum* also contains an array of lipids and proteins capable of hydrogen bonding with a penetrant,

the strength and number of this hydrogen bonding governs either the rate or ability of a substance to traverse through the *stratum corneum* (Brain and Chilcott, 2008). Consequently, as PAH are strongly lipophilic (US EPA 16 PAH Log K_{ow} between 3.37 and 7.1 (Tobiszewski and Namiesnik, 2012, Stogiannidis and Laane, 2015)), and are therefore likely to be sequestered in the lipid rich environment of the epidermis (Brain and Chilcott, 2008). PAH lipophilic and hydrophobic nature increases with molecular weight, meaning that those PAH that pose greater risk owing to their toxicity (Lerda, 2011), may in fact produce more localised health effects because of their inability to permeate the dermis because of its hydrated environment. These PAH that remain in the epidermis may be metabolised and produce a localised toxicological response, or be sloughed and subsequently removed as a result of the outward migration of corneocytes (Monteiro-Riviere and Riviere, 1999, IPCS, 2006). The charge of a substance impacts its capability of penetrating the skin. The *stratum corneum* contains proteins which contain both negatively and positively charged groups, which along with the lipophilic environment, results in an effective barrier against ions. As PAH are non-polar (Hill and Holman, 1983), this function of the *stratum corneum* may have negligible impact on PAH dermal absorption.

There are also biological factors that impact the amount of penetrant absorbed. It has been noted that regional differences in skin thickness, hair follicle density, blood flow, age and disease are likely to influence the barrier function of the skin (Chilcott and Price, 2008, Monteiro-Riviere and Filon, 2017). It has been shown that damaged skin can result in 4 to 100 times greater permeability than for healthy skin (Monteiro-Riviere and Filon, 2017). Anatomical site selection also has a marked impact on the amount dermally absorbed, it has been shown that for non-ionised toxicants the rate of penetration occurs in the order of: scrotal > forehead > axilla = scalp > back = abdomen > palm and plantar. The reduction in absorption is largely due to thickness of the *stratum corneum* (e.g., palm and plantar regions have a thickness of 100-400 times greater than other regions). This thickness results in an increase in lag time. Along with thickness, variations in hair follicle density. Another anatomical difference is cutaneous blood flow of difference sites, resulting in varying degrees of ability to remove penetrants (Monteiro-Riviere and Riviere, 1999). For PAH, VanRooij (1993) showed that there were inter-individual differences in PAH dermal absorption of 7%, and 69% variability depending on the anatomical site used.

Although the use of ex vivo human skin is the “gold standard” for in vitro dermal absorption experiments (Abd et al., 2016), owing to high cost, storage and ethical considerations, many studies have used animal skin in place of human, including: rat, pig, hairless guinea pig, and

monkey (Yang et al., 1989, Wester et al., 1990, Ng et al., 1991, Moody and Joncas, 2007, Xia et al., 2016). There are evident physiological differences between human and animal skin such as hair follicle density, thickness, and *stratum corneum* structure. For example, rodent skin has been shown to have different barrier properties to that of human skin, owing to the arrangement of the corneocytes in the *stratum corneum* (Chilcott and Price, 2008). In human skin, the corneocytes are offset in adjacent rows (like a brick wall) resulting in a long intercellular transport route, whereas in rodent skin the corneocytes are stacked in columns resulting in a much shorter intercellular absorption route. Out of the relevant animal models, pig skin is believed to be the most similar and therefore representative of human skin (Chilcott and Price, 2008), although pig skin can be difficult to handle and work with (Abd et al., 2016). Overall, the magnitude by which the dermal absorption of substances occurs is largely influenced by their physico-chemical properties, although biological factors such as anatomical site and species/individual differences are the major sources of variation in the quantity absorbed (Brain and Chilcott, 2008).

2.2.6 Ex Vivo Skin Alternatives for Dermal Absorption Studies

Previous *in vitro* dermal absorption studies have used human (Moody et al., 1995, Roy and Singh, 2001, Moody et al., 2011), rat (Yang et al., 1989, Moody et al., 1995), porcine (Moody et al., 1995, Abdel-Rahman et al., 2002) and guinea pig (Moody et al., 1995) skin. Although recently some researchers have been moving away from *ex vivo* animal and human skin, instead utilising reconstructed human epidermis (RHE) skin and artificial membrane substitutes (Schmook et al., 2001, Netzlaff et al., 2005, Schäfer-Korting et al., 2006, Schäfer-Korting et al., 2008, Ackermann et al., 2010, Abdallah et al., 2015, Uchida et al., 2015, Abd et al., 2016, Haq et al., 2018). The 3R's approach to ethical research (replacement, reduction and refinement) is being increasingly included in legislations, guidelines and practice of *in vivo* animal experiments in order to safeguard animal welfare (Williams, 2006, Törnqvist et al., 2014). This movement away from animal and human skin is due to the high cost and difficulty in acquiring the skin, restrictions for ethical reasoning, difficulty in storing tissue, poor correlation of absorption data with *in vivo* studies, and variability between individual extracts of skin. The issues presented by the variability of *ex vivo* skin could potentially be avoided using RHE or artificial membranes, as the structural and chemical variability would be minimised, reduce cost, not require ethical approval, and are more easily stored compared to *ex vivo* skin. Several *ex vivo* skin substitutes have been assessed over the past decade or so, including: EPISKIN, EpiDerm, SkinEthic, isopropyl myristate (IPM), certrimides, Strat-M and Graftskin™ LSE™ (Schmook et al., 2001, Netzlaff et al., 2005, Schreiber et al.,

2005, Schäfer-Korting et al., 2008, Karadzovska and Riviere, 2013, Uchida et al., 2015). The general structure, composition, and some biochemical aspects of EPISKIN, EpiDerm and SkinEthic closely resemble human skin, with these models being useful in toxicity testing and to a lesser extent transport studies, and vary less in permeability and responsiveness in comparison to human skin (Netzlaff et al., 2005). Schäfer-Korting et al. (2008), measured absorption of several substances: nicotine, flufenamic acid, caffeine, testosterone, clotrimazole, benzoic acid, mannitol, and digoxin in: EPISKIN, SkinEthic and Epiderm, as well as human epidermis sheets (HES). Strong correlation with HES absorption was reported between EPISKIN ($r=0.86$), SkinEthic ($r=0.92$) and Epiderm ($r=0.918$). Karadzovska and Riviere (2013) studied the feasibility of the *ex vivo* skin substitutes: isopropyl myristate (IPM), ceramides and Strat-M™, and compared the absorption of sodium diclofenac through these membranes to porcine skin. The absorption results showed a strong correlation between the skin substitutes and porcine skin, with r values of 0.82 (IPM), 0.82 (ceramides) and 0.85 (Strat-M™). The strong association between absorption data of the Strat-M™ membrane and porcine skin is compelling for the measurement of PAH because sodium diclofenac has a similar $\log K_{oc}$ (4.5) and MW (318) to PAH in that are present in soil samples. This suggests that the Strat-M™ membrane may be a suitable *ex vivo* skin substitute for the development of an *in vitro* soil dermal bioavailability method.

Table 4 compares commercially available RHE and artificial membrane substitutes for *ex vivo* skin. The cost shown in the table is only an estimate as currency conversion rates and supplier pricings are subject to change over time. Therefore, cost was shown relative to one another. Strat-M™ was the cheapest, and the RHE prices were between 3 and 5 times greater per membrane.

Table 4: Comparison of commercially available ex vivo skin substitutes. Note than cost estimates are only indicative, and it is recommended quotes are obtained from the supplier at the time of intended use.

Membrane	Composition	Thickness	Surface Area (cm ²) (small, large)	Positives	Negatives	Cost ^a	Website
EPISKIN	Human keratinocytes cultured on a collagen matrix	Epidermis	0.38, 1.07	- Composed of human skin cells - Representative of Epidermis Structure - Biologically active (metabolism, irritation response etc.)	- Small surface area limits dose applied lowering method LOD. - Potential for batch to batch variability. - Short storage duration - Complex storage and preparation - Only representative of epidermis	£££	www.episkin.com/Episkin
EpiDerm	Normal, human-derived epidermal keratinocytes (NHEK)	Epidermis	0.6, 3.8	- Composed of human skin cells - Representative of Epidermis Structure - Biologically active (metabolism, irritation response etc.)	- Small surface area limits dose applied lowering method LOD. - Potential for batch to batch variability. - Short storage duration - Complex storage and preparation - Only representative of epidermis	£££	www.mattek.com/products/epiderm/
SkinEthic	Human keratinocytes cultured on an inert polycarbonate filter	Epidermis	0.5, 4	- Composed of human skin cells - Representative of Epidermis Structure - Biologically active (metabolism, irritation response etc.)	- Small surface area limits dose applied lowering method LOD. - Potential for batch to batch variability. - Short storage duration - Complex storage and preparation - Only representative of epidermis	£££	www.episkin.com/SkinEthic-RHE
Strat-M	Polyethersulfone, polyolefin, synthetic lipid post-treatment	Full-Thickness	4.9, 17.3	- Ease of storage. - Low batch-to-batch variability. - Inexpensive. - Ease of preparation (remove from sachet). - Low ethical consideration. - Structure/porosity is representative of epidermis and dermis - Large membrane surface area increases dosing potential and lowers method LOD	- Not composed of human skin cells. - Not "living" - no metabolism etc.	£	www.sigmaaldrich.com/GB/en/product/mm/skbm04760

^aCost £ = low, £££ = high. For true cost comparison users should request quotes at time of intended use as pricings are subject to change. Cost estimates presented in table will vary due to currency conversion rate changes over time

However, there are some limitations to RHE membranes. Due to the weak barrier function of EPISKIN, EpiDerm and SkinEthic, there is elevated permeability in relation to human skin consequently limiting the value of these RHEs for transport studies due to the *stratum corneum* not being appropriately represented. This could also potentially lead to some false positives in toxicity studies due to a greater rate of diffusion than what would occur in human skin (Netzlaff *et al*, 2005). Another major drawback of RHEs is that they do not consider the role of the dermis in the movement of compounds through the skin, to overcome this it has been suggested that full thickness methods be used, as this matches more closely to *in vivo* data; although this method involves human skin and the storage of which is only viable for 7 days, after which metabolic reactions reduce and autolysis results in the breakdown of cells (Nakamura *et al.*, 1990). The lack of replication of the dermis could lead to a greater rate of transport of lipophilic compounds such as PAH, as the dermis is an aqueous environment, it acts as a barrier to underlying blood vessels and consequently greatly influences the dermal absorption of PAH. The other issue is the lack of replication *in vitro* of the system that rapidly removes permeants from the epidermal-dermis interface, which is carried out by the permeation of the dermis by blood vessels under the skin (Netzlaff *et al*, 2005). Schmook *et al.* (2001) also studied the feasibility of using Graftskin™ LSE™, but found that this RHE had a greater permeability for hydrophobic compounds than human skin and therefore deeming it unsuitable for this study as PAH are hydrophobic. Also, RHEs do not contain the enzymes found in human skin, which metabolise PAH into polar compounds which are then much more mobile than the parent congeners and are also carcinogenic unlike the parent compounds. Consequently, these reactions have a substantial impact on the dermal absorption of PAH, as the parent congeners often are bound to the lipids in the *stratum corneum* (Karadzovska and Riviere, 2013), but once metabolised these polar PAH compounds can potentially reach systemic circulation.

Equation 4 shows the relationship between the main factors that impact dermal absorption. With increasing membrane/*stratum corneum* thickness, the substance will have a longer absorption pathway therefore reducing the rate by which it is absorbed (Figure 6).

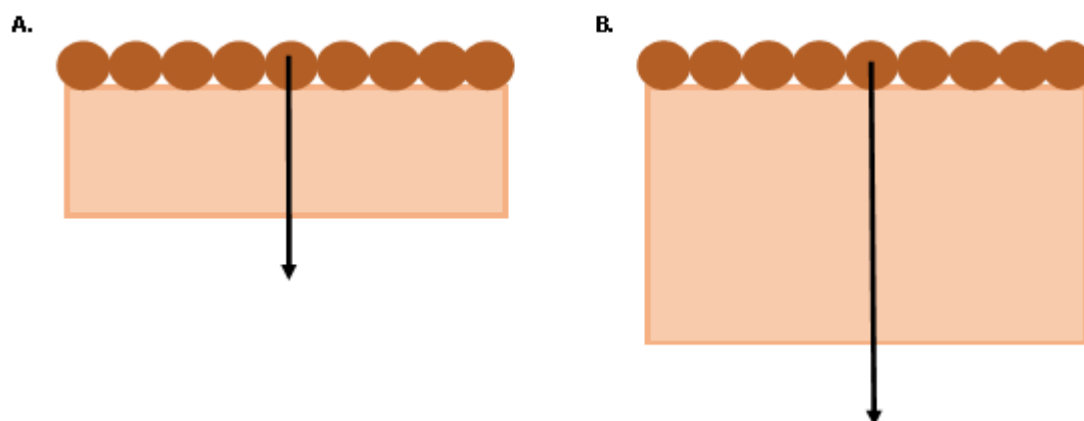


Figure 6: Differing length of absorption pathway with differing membrane thickness. B is double the thickness of A, resulting in a longer absorption pathway and consequently a reduction in the rate of chemical absorbed.

Wilkinson et al. (2006) showed that when measuring testosterone through dermatomed ($\approx 0.5\text{mm}$) and full-thickness ($\approx 1\text{mm}$) *ex vivo* human breast skin, that PADA(%) is significantly lower ($p < 0.01$) for full thickness skin (2.3 ± 0.4) compared to dermatomed (6.9 ± 1.0). Therefore, in order to make a true comparison of chemical flux between and within studies membrane thickness needs to be consistent. The Strat-M™ membrane is likely to have greater membrane thickness consistency compared to *ex vivo* skin and RHE membranes due to it being manufactured.

The Strat-M™ artificial skin membrane has been engineered to mimic the structure and chemistry of the multiple layers of full thickness skin, therefore representing a larger proportion of the skin in comparison to some RHEs. The membrane is composed of polymer layers, which increase in porosity with depth from the surface, reflective of human skin structure. The outermost layer is a tight top layer, resembling the *stratum corneum*, followed by two further layers of polyethersulfone (PES) which represents the dermis. The final layer consists of polyolefin, which represents the support structure beneath the skin, the subcutaneous tissues. The Strat-M™ membrane is also treated with a blend of lipids such as in human skin (such as ceramides, cholesterol, free fatty acids) (Haq et al., 2018). The most compelling study which utilised the Strat-M™ membrane was carried out by Uchida et al. (2015), who used 13 chemical compounds with molecular weights between 152-289 and $\log K_{OC}$ of 0.9-3.5 and compared the absorption of these compounds through Strat-M, human skin, and rat skin, in a Franz-type diffusion cell. The diffusion ($\log DL^{-2}$), permeability ($\log P$) and partition ($\log KL$) parameters of chemicals in Strat-M had very strong correlation with

human and rat skins (Table 4) showing that Strat-M could be a suitable substitution for animal/human skin for in vitro dermal bioavailability studies.

Table 5: correlation (r) of diffusion (DL-2), permeability (log P) and partition (KL) between Strat-M and human/rat skin. Pearson's Correlation Coefficient showed significance ($P < 0.05$). Data from Uchida et al, 2015.

	Human Skin (r)	Rat Skin (r)
Log P	0.93	0.97
Log KL	0.83	0.85
Log DL⁻²	0.77	0.71

For this study, an alternative for ex vivo skin was used to eliminate ethical considerations associated with using ex vivo skin and to reduce the hindrance to the development of an in vitro diffusion cell dermal absorption methodology as artificial membranes can be more readily acquired than human skin donors. The membrane of choice was Strat-MTM, due to high correlation of absorption parameters with human skin in previous studies, as well as requiring no specialist storage, being cost-effective in comparison to ex vivo skin for method development, as well as the probability of being less structurally and chemically variable in comparison to ex vivo skin resulting in less methodological variability.

2.3 PAH Dermal Bioavailability Studies

2.3.1 Introduction

The most reliable data of human chemical dermal bioavailability is generated through *in vivo* human volunteer studies performed under occupationally relevant test conditions (IPCS, 2006). However, owing to technical as well as ethical considerations, this approach was not valid for this study. Consequently, previous *in vivo* and *in vitro* PAH dermal absorption studies have been assessed to determine the methodological approach of this study and research gaps in the current literature. Thorough reviews on this have been carried out in recent years (Spalt et al., 2009, Beriro et al., 2016, Ruby et al., 2016).

In vitro methods tend to use diffusion cells, which are glassware consisting of a donor chamber and a receptor chamber (where a receptor fluid representative of systemic circulation is housed) between which the membrane/skin is positioned. The donor chamber is where the chemical dose is applied to the membrane in a study vehicle e.g., solvent. The receptor chamber houses a receptor solution, which is contact with the membrane. Any

chemical that diffuses into this solution is representative of the concentration that will be absorbed through the skin and reach systemic circulation.

There are two diffusion cell designs commonly encountered in the literature, static and flow-through. The main difference in the designs of static diffusion cells (e.g., Franz-type (Franz, 1975)) and flow-through (e.g. Bronaugh and Stewart (1984)) diffusion cells is that the receptor solution of a flow-through diffusion cell is constantly replenished throughout the experiment. Beriro et al. (2016) reviewed different types of diffusion cells, and in addition to Franz-type and flow-through designs, also discussed alternatives such as multi-plate systems and fibre arrays.

The Organisation for Economic Co-operation and Development (OECD) (2004) state that static or flow-through diffusion cells are acceptable for *in vitro* dermal absorption experiments. According to the IPCS (2006) (International Program of Chemical Safety), vertical diffusion cells are optimal for replicating *in vivo* conditions, and are excellent for the measurement of absorption of chemicals from semi-solids such as soil. The static (Franz) diffusion cell is the most used diffusion cell design for dermal absorption studies. The receptor fluid beneath the membrane is sampled manually and the receptor fluid is stirred by a magnetic stirrer. The receptor solution must not inhibit absorption so must have a high absorption capacity even for highly insoluble compounds. Flow through cells on the other hand have the receptor fluid continuously replaced, maximising sink conditions as fresh receptor solution is constantly supplied (IPCS, 2006).

2.3.2 Key PAH Dermal Bioavailability Studies

Recent reviews of PAH bioavailability methods by Spalt et al. (2009), Ruby et al. (2016), and Beriro et al. (2016) all demonstrate the strengths and limitations to current approaches for the measurement of dermal bioavailability of soil contaminants. Spalt et al. (2009) and Beriro et al. (2016) both present summaries of key studies, which provide critique and insight into each studies methodological approach. As such, a similar style to reviewing previous works was undertaken in order to assess previous methods, the findings of which will inform this studies methodical approach. This will enable this project to build upon previous methods in order to develop an *in vitro* method that will be able to quantify the dermal bioavailability of PAH of contaminated soils (Sections 1.2 and 1.3).

2.3.2.1 Yang et al, (1989)

Yang et al. (1989) used a standard reference material (SRM) of petroleum crude oil containing 1.1ppm BaP which was spiked with ^3H -BaP at 100 ppm. An air-dried loam soil was sieved, and the crude oil was added in 10 ml of DCM per gram of soil and the solvents then removed by rotatory evaporator. Rat dorsal skin was obtained, and sections cut to 350 μm and loaded into a 15 mm diameter Franz diffusion cell. For the *in vivo* part of the study female rats were used, areas of the dorsal were shaved and then a 7 cm^2 area had the crude mixture added to the skin. The amounts added to both the *in vitro* and *in vivo* experiments were equal. The rats were housed in metabolic cages with urine and faeces being collected once daily for four days.

They compared two amounts of soil loadings, 9 mg cm^{-2} and 56 mg cm^{-2} , and found that the amount absorbed was nearly identical at around 1.3 ng. This suggests that soil loading *in vitro* does not affect the total amount dermally absorbed at least in the period of this study. They also found that the *in vitro* and *in vivo* absorption data is strongly correlated, suggested the *in vitro* method was a fair representation of *in vivo*, but the strength of this correlation is not quoted in the paper. The study also showed that the absorption of BaP was reduced 4-5 times in the crude containing soil in comparison to just the crude on its own, showing that the soil is a major sink for PAH and consequently greatly reduces the dermal absorption of PAH.

This data shows good correlation between the two methods used, and although this correlation value is not quoted, this paper was one of the first to show that dermal absorption of contaminants from soil could be measured *in vitro*. It also demonstrated that the presence of soil has a large effect on the amount of PAH dermally absorbed. The main flaw of the study is the fact that they use rats, as rat skin is no longer thought to be representative of human skin due to overestimation owing to increased absorption through hair follicles in comparison to human skin (Williams, 2006), and also, the unethical practice of using animal skin and live animals during these experiments. This also would not be practical for the method developed in this project, as it is hoped that the developed method could be replicated in other laboratories. This is due to the cost and low availability of rat skins as well as gaining ethical approval to do so would make this replication in other labs much more difficult and hence reduce the potential impact of this project. Also, Yang et al. (1989) do not age the soils which contain crude, so the absorption data produced would not be representative of real-life conditions as the PAH would be more readily dermally absorbed from the crude mixed soil than in a real-life aged soil. The absorption data *in vitro* was derived

from the receptor solution only, which is acceptable for the comparison of absorption with and without the presence of soil within the study. However, absorption data from the *in vitro* study could not be used for human health risk assessment as absorption data is required for both the membrane and receptor solution to be deemed a suitable replacement for *in vivo* data (IPCS, 2006).

2.3.2.2 Wester et al, (1990)

The Wester et al. (1990) study is the most influential PAH dermal absorption study, as the generated 13% PAH dermal absorption value is currently used to derive human health assessment criteria in the UK. The study focused on BaP as this congener is believed to be the most carcinogenic. The study consisted of an *in vitro* and *in vivo* experiment. One test soil was used from California (26% sand, 26% clay, 48% silt) and spiked with ^{14}C -labelled BaP at 10ppm so that metabolites would be measurable in the urine. For the *in vitro* part of the study, two separate skin donor sources were used with six replicates per experiment. Diffusion cells of 1 cm² surface area were used with a flow rate of 3 mL/h with human plasma as the receptor fluid with an overall run time of 24 h. The membrane was loaded with 0.04g of soil and residual fluid was collected and analysed. The *in vivo* part of the study used four female rhesus monkeys, which were placed in metabolic chairs and dosed within a 12 cm² area of the abdomen and covered with a nonocclusive cover. Between the two patches used for the cover, a water vapour permeable membrane was placed to allow free passage of moisture. Urine was collected for 24 h in the metabolic chair. After the initial 24 h period, the skin of the monkeys was washed, and the monkeys were placed in metabolic cages for a further 6 days for urine collection.

The *in vivo* results showed that on average $51.0 \pm 22.0\%$ BaP was absorbed from the acetone vehicle (no soil) and a significantly lower ($p = 0.015$) $13.2 \pm 3.4\%$ was absorbed with the presence of soil. Consequently, demonstrating the significance the presence soil has in the reduction of the amount of BaP available to be dermally absorbed. This study has been accepted, with the 13% value currently used to derive human health assessment criteria as rhesus monkeys' skin is deemed to be similar to humans. However, there are multiple limitations to this study. One is that the dermal absorption estimates from urine had an extremely high correction factor (x15), with estimates being adjusted by correlating the urinal estimates to an excreted fraction from an intravenously administered dose. Also, it is highly likely that due to PAH being highly lipophilic that some were not metabolised and excreted over the course of this study and were still present in the bodies of the monkeys

when urinary measurement ceased to be taken. Due to these two factors, it may result in an under estimation of dermal absorption of BaP. Also, only four monkeys and one soil type were tested, which is not representative of a variety of dermal and soil conditions. The dermal absorption percentage may have also been over estimated, due to the soil being spiked with radiolabelled BaP a brief time before the experiment began, the natural aging process which binds PAH to soil matrix and consequently reducing the bioaccessibility would not have been considered. As this value is used to derive human health assessment criteria for natural soils the solvent spiking of soil is not necessarily reflective of soil PAH behaviour and therefore potential exposure.

2.3.2.3 Ng et al, (1991)

Ng et al. (1991) studied the dermal absorption of radiolabelled phenanthrene, using both *in vitro* and *in vivo* methods. Hairless guinea pigs were used for both experiments. Two dose levels were applied at 6.6 $\mu\text{g}/\text{cm}^2$ and 15.2 $\mu\text{g}/\text{cm}^2$ in acetone. The *in vitro* studies used a flow-through diffusion cell design with an exposed area of 0.64 cm^2 , with HEPES buffered Hank's Balanced Salt Solution (HBSS) 4% bovine serum albumin (BSA) receptor solution. Another receptor solution of distilled water and 4% BSA was also used for some higher dose applications. *In vivo* experiment applied the same dose of phenanthrene to the dorsal region of each guinea pig, which were kept in glass metabolic cages. The skin surface was washed at 24 h to remove any unabsorbed chemical. Urine radioactivity was quantified using liquid scintillation counting.

This study showed that the use of aqueous receptor solutions for the *in vitro* measurement of the dermal absorption of lipophilic compounds such as PAH hinders absorption. The water-based receptor solution resulted in lower absorbed dose than HBSS at each time step (6 h, 12 h, 24 h and 48 h (e.g., at 48 h water abs% = 57.4 whereas HBSS abs% = 71.3). This is due to PAH remaining in the membrane owing to the hydrophilic environment of the water-based receptor solution limiting the rate of diffusion. Therefore, it is recommended for this study to use a physiological buffer such as HBSS with the addition of a serum protein (either 10% fetal bovine serum or 4% bovine serum albumin) which has been shown to simulate *in vivo* absorption when the amount of chemical in the receptor and skin is totalled (Bronaugh et al., 1989a, Bronaugh et al., 1989b).

This study also demonstrated that *in vitro* studies could produce dermal absorption data representative of *in vivo* data. For example, 89.7% (6.6 $\mu\text{g}/\text{cm}^2$ applied) and 79.1% (15.2 $\mu\text{g}/\text{cm}^2$ applied) dose absorbed over 24 h *in vitro*, with 80% absorption of applied dose *in vivo* over same time at 15.2 $\mu\text{g}/\text{cm}^2$. However, hairless guinea pig is not accepted as

representative of human skin owing to different skin structure and hair follicle density (IPCS, 2006, Chilcott and Price, 2008). Furthermore, similar issues would be encountered with this *in vivo* study to previous works, with phenanthrene likely being retained in the skin meaning that the measured amount absorbed is unlikely to be representative. The authors did try to correct for this by applying intramuscular doses of phenanthrene to apply correction factors. However, it can be said that there is greater confidence in quantifying the fate of the PAH in the experimental system when using *in vitro* methods opposed to *in vivo*.

Another key finding of this study was that the penetration of phenanthrene through the skin is controlled by passive rate of diffusion rather than by metabolism. This means that the dermal absorption of PAH in soil is governed by the concentration gradient between the mobile PAH fraction of the soil and the PAH concentration of the skin.

2.3.2.4 Van Rooij, (1993)

VanRooij (1993), used 9 male volunteers between 22 and 37 years old to test for differences in dermal absorption rates of PAH between individuals and anatomical sites. 2.5 mg/cm² Coal tar ointment (containing 10% coal tar) was applied to 24 cm² of skin at six skin sites: forehead, shoulder, volar forearm, palm of the hand, groin, ankle. This ointment was then removed after forty-five minutes of exposure, and urinary 1-OH-pyrene was measured to calculate absorption. It was estimated that between 0.3-1.4% of pyrene was absorbed after 55 h. They found that the inter-individual differences in pyrene dermal absorption were low (7%) and whereas the anatomical differences were high (69%), with decreasing absorption of the order: shoulder > forehead, forearm, groin, > ankle, palm of the hand. This study used human volunteers, which although representative of real-life exposure, would be hard to come across every time dermal absorption of PAH from a soil is to be quantified. However, there were only 9 subjects tested, which would not be representative for a complete range of skin types. The differences between individuals and anatomical sites shows that there needs to be a standardised method to reduce inter-study variability. The use of an artificial membrane could reduce variability in absorption data by all tests being carried out on more uniform membranes. Also, although pyrene is deemed to be representative of other PAH, variations in K_{ow} will alter the absorption rates, which need to be quantified to a good degree of certainty for use in human health assessment criteria.

2.3.2.5 Moody et al, (1995)

Moody et al. (1995) performed cross-species *in vitro* dermal absorption tests using ¹⁴C-labelled benzo[a]pyrene in acetone for four different skin sources: rat, guinea pig, human, and tissue-cultured skin (Testskin). The study ran both *in vitro* and *in vivo* dermal absorption

tests for rat and guinea pig. For the *in vitro* study, 0.5 mm thickness skin was housed in a Bronaugh flow-through diffusion cell, with the radiolabelled BaP dose applied being between 8 and 12 $\mu\text{g}/\text{cm}^2$. For the *in vitro* tests, at 48h the radiolabelled BaP percent absorbed of dose applied (PADA) was: $95 \pm 9.6\%$ (rat), $51 \pm 3\%$ guinea pig), $43 \pm 8.7\%$ (human; 50-year-old), $34 \pm 12.4\%$ (Testskin), and $23 \pm 5.3\%$ (human; 32 years old). This further demonstrates that dermal absorption of PAH differs between species and within (VanRooij, 1993, Brain and Chilcott, 2008, Chilcott and Price, 2008), this study showing that in addition to other skin characteristics previously tested that the age of human skin is also a factor. The *in vivo* test measured radiolabelled BaP levels in urine and faeces at 48h, followed by necropsies at 14 days postexposure. The total % *in vivo* dermal absorption for rat was $70 \pm 7.6\%$ and for guinea pig $68 \pm 9.3\%$. The *in vitro* test produced $>97\%$ recovery. In comparison the *in vivo* recovery for rat was 69.4% (SD=7.59) and 67.8% (SD=9.33) for guinea pig.

The study demonstrated that radiolabelled BaP is well absorbed through the skin both *in vitro* and *in vivo*, although this was from an acetone vehicle, and therefore would overestimate BaP dermal absorption from soils. The study also demonstrated that as well as skin anatomical site and species, age of the skin source also has an impact on dermal absorption of PAH, with older skin sources resulting in greater absorption. This study did not show as strong of an agreement between *in vivo* and *in vitro* data as Ng et al. (1991), although this is due to different study protocols such as: skin thickness, sampling duration and intramuscular correction factors. This highlights the need for standardised testing protocols to improve inter-study comparability of PAH dermal absorption data, such as those given by OECD (2004).

2.3.2.6 Roy et al, (1998)

Roy et al. (1998) performed *in vitro* ^3H -benzo[a]pyrene dermal absorption tests with contaminated soils and organic solvent extracts of contaminated soils. ^3H -benzo[a]pyrene was applied to gasworks soils in hexane prior to dermal absorption testing. The organic solvent soil extracts were also spiked with ^3H -benzo[a]pyrene prior to application to the membrane. For the dermal absorption experiment, Franz static diffusion cells were used with human cadaver skin (age 31 to 88 years). Receptor fluid was sampled (200-400 μL) at 1, 3, 5, 7, 24, 48, 72, 96, 120, and 144 h post-dose. Upon termination, the remaining soil was removed from the skin surface by wiping with corn oil-wet and dry cotton applicators. Radioactivity was quantified using a Beckman liquid scintillation counter.

The use of PADA to display dermal absorption data in the literature is questionable, as the dose applied is impacted by the loading of soil applied during the experiment, resulting in

lower PADA values for the same dermally absorbed PAH concentration with increasing soil loading. Roy et al. (1998) acknowledge the inherent complication of the use of PADA by conducting *in vitro* dermal penetration experiments under infinite (nonlimiting) dose conditions. The loading applied of 25 mg/cm² of soil/soil extract dose used in this study is principally an unlimited supply of PAH which allows for the measurement of dermal flux (J) of the target PAH from soil or soil extract under steady-state conditions. The method performed well, with 83 – 106% mass balance, suggesting limited losses throughout the experimental and analytical process. Flux from soils ranged from 0.0064 ng/cm²/h to 2.2 ng/cm²/h in comparison to 210 ng/cm²/h to 750 ng/cm²/h from soil extracts. They found a 2-3 magnitude reduction in the dermal absorption of ³H-benzo[a]pyrene from spiked soils in comparison to the soil extracts, further demonstrating the significant impact the presence of soil has on reducing the mobility of PAH.

2.3.2.7 Abdel Rahman et al, (2002)

Abdel-Rahman et al. (2002) used two different soils in their *in vitro* dermal absorption experiments. They used a sandy and clay soil to which they added deionised water to achieve a moisture content of 11%. They autoclaved the soils, spiked the soils with ³H-BaP and aged for three months in Teflon-sealed glass in the dark to prevent microbial degradation. They used pig skin as the membrane in flow-through diffusion cells. Pig skin, although believed to be representative in structure and histology of human skin, is expensive and unethical to use and would also be impractical for storage and use for the regular testing of the dermal absorption of PAH. Freshly spiked sandy and clay soil showed 8.5% and 3.5% of the applied BaP had been absorbed into the skin respectively, and the aged soil 3.7% and 1.8%. This showed that there was an 89% reduction in absorption after a short time (the rapid aging process) and 95% reduction in absorption after long term aging (Abdel-Rahman et al., 2002, Bergknut, 2004, Weissenfels et al., 1992). This demonstrates the effect and importance of using aged soils in absorption studies to be representative of the real-life exposure as the use of freshly spiked soils results in PAH not being as extensively bound to the soil, resulting in increased PAH mobility and therefore an over estimation of dermal absorption.

2.3.2.8 Moody and Joncas, (2007)

Moody and Joncas (2007) applied ¹⁴C-BaP spiked soil to human breast skin (dermatomed 0.4-0.5 mm) in Bronaugh flow through diffusion cells (n =5). The soil tested was a commercial gardening soil, that was air dried, and sieved to <710µm (coarse sand (Wentworth, 1922)). The soil was dosed with ¹⁴C-BaP by application with acetone, and thoroughly mixed. The soil loading applied was 5 mg/cm². ¹⁴C-BaP was also applied in an acetone vehicle (n=6). The skin

was washed using Radiacwash and removed at 24 h and 42 h. Receptor solutions were subsampled every 6 h and replenished with fresh solution. The ^{14}C -BaP concentration was measured in the soil, skin and receptor solution using liquid scintillation counting. Both flux and PADA were reported, with this study also reporting a reduction in ^{14}C -BaP PADA and flux from soil in comparison to a solvent vehicle (Abdel-Rahman et al., 2002). The ^{14}C -BaP total flux with and without soil were both determined to be statistically different at 24h and 42h (Students t-test, $p < 0.05$).

This study produced excellent mass balances of 94.9% (SD=7.69) at 24h and 93.7% (SD=15.3) at 48h. The grain size applied ($<710\mu\text{m}$) by Moody and Joncas (2007) is coarser than the grain size accepted to adhere to hands ($<250\mu\text{m}$) (Duggan et al., 1985, Kissel et al., 1996, Abdel-Rahman et al., 2002, Choate et al., 2006, Cave et al., 2010, Beriro et al., 2020). This would result in a reduced soil grain surface contact with the membrane, resulting in a reduced potential for transfer of PAH from soil to skin leading to an underestimation of the soil dermal bioavailability of PAH. This study used ^{14}C -BaP spiked soil, which is likely to overestimate dermal bioavailability owing to the underrepresentation of the aging process of PAH in soils resulting in greater PAH mobility. Moody and Joncas (2007) also acknowledge that further research is needed to examine PAH dermal absorption from other soil types under a range of environmental conditions.

2.3.2.9 *Moody et al, (2011)*

Moody et al. (2011) used a “by difference” method to quantify the dermal bioavailability of real-world PAH using the same experimental set up as Moody and Joncas (2007). This required the analysis of the original contaminated soil and the same soil after 24 h of being loaded on a diffusion cell. The study used human breast skin surgical waste for the membrane. After 24 h, the soil was removed using a soap wash, with the difference between the concentrations of PAH in the original soil and soap washed soil producing a PADA value (percent absorbed of dose applied). Percentage dermal bioavailability data for only five of the 16 PAH originally analysed was reported: phenanthrene (60.8%); fluoranthene (49.4%); pyrene (48.7%); benz[a]anthracene (26.4%); and chrysene (33.5%). This study has the benefit of not using radiolabelled PAH congeners for the measurement of absorption like other studies as they used a naturally contaminated soil; this is important for risk assessment as when measuring real-life contaminated soils, the aging/weathering processes have been represented. They also point out that the use of flux data rather than PADA will allow for better comparison between labs. To give a greater chance of detection, the soil loading was increased from 5 mg/cm^2 (Moody and Joncas, 2007) to 50 mg/cm^2 .

A limitation of this method is that the system assumes that the skin is fully bioavailable, meaning that this method is extremely conservative. Although you need to be conservative for risk assessment, using this method may result in unnecessary remediation costs as a result. Another limitation is that the soap wash may not in fact remove all PAH, meaning that data may be underestimated. Also, the soap wash was found to produce concentrations for several PAH which were too low to be measured by HPLC. This was a result of only small amounts of sample being applied (32 mg) to the membrane at the start of the experiment therefore resulting in small amounts of soil being available for extraction and quantification. Consequently, as the soils tested was from a contaminated site with high PAH concentrations, this method is unlikely to produce data for soils containing lower concentrations of PAH unless higher soil masses were used.

2.3.2.10 Xia et al, (2016)

Xia et al. (2016) wanted to test the effect of PAH source material in soil and their impact on the concentration available for absorption. 30 contaminated soils were constructed with common PAH sources (fuel oil, soot, coal tar-based soot particles) and spiked with a solvent to different types and contents of SOM and minerals to achieve PAH concentrations in four orders of magnitude, between 0.1-100 mg/kg of BaP. The soils were sieved to $<150\mu\text{m}$, which simulated the fraction that most easily adheres to the skin. Soils were weathered for 8 weeks to attempt to replicate the natural aging processes which occur to PAH in soils. 100 mg of each soil was then loaded onto 5cm^2 of full thickness pig skin placed in an evaporating dish to achieve a loading of 20 mg cm^2 . The soil was spread evenly, and 10 drops of deionised water was added across the surface to provide moisture to better replicate real life conditions as wet/moist soil better adheres to the skin. The evaporating dish was then covered with parafilm and stored at 25°C for 16 h. After the 16 h, DI water was used to rinse the soil off the skin, and tissue paper used to mop up any residual soil. The skin was then cut up and placed in 25ml 0.5M KOH in methanol/water 1:3 in a vial and put in a water bath at 100°C for saponification over 4 h. Once cool, 20ml of hexane was added to the vial and extracted for 30 minutes by orbital shaker, then centrifuged for 2 minutes at 3000 rpm, and the hexane transferred to another vial. The solvent extraction was repeated twice, and the three aliquots combined for further clean-up and PAH analysis.

This method produced similar absorption fluxes to previous work, with $0.28\text{ ng/cm}^2/\text{h}$ for phenanthrene, which is within the range of values quoted by Abdel-Rahman et al. (2002) and $0.028\text{ ng/cm}^2/\text{h}$ for BaP which is within the range of Wester et al. (1990). It is common for *in vitro* studies to use diffusion cells to measure dermal absorption of substances (Wester et

al., 1990, Ng et al., 1991, Roy et al., 1998, Abdel-Rahman et al., 2002, Moody and Joncas, 2007, Peckham et al., 2017, Forsberg et al., 2021), but this method created similar dermal absorption data without the use of typical *in vitro* apparatus. Pig skin, although having comparable properties to human skin is unethical to use and presents logistical issues such as storage and availability. Pig skin cannot just be obtained from a butchers or abattoir as the skin is often scorched to remove the hair, with this practice creating PAH. Unlike many studies, an aging process was carried out on the soils, but this was only for 8 weeks so this may have skewed the isotherms reported owing to PAH being more mobile than in a real-world contaminated soil. This study demonstrated the impact of source material, soil composition (especially the presence of black carbon) on PAH mobility and subsequent dermal bioavailability. Dermal absorption of PAH is related to the freely dissolved aqueous PAH concentration in soil and not the total concentration in the soil.

2.3.2.11 Peckham et al, (2017)

Peckham et al. (2017) quantified the dermal absorption of ^{14}C -benzo[a]pyrene from four test soils of different organic (TOC) and black carbon (BC) contents. The soils were sieved to $<63\mu\text{m}$ (coarse silt (Wentworth, 1922)), spiked with ^{14}C -benzo[a]pyrene in toluene and artificially weathered for 8 weeks at two concentrations, 3 mg/kg and 10 mg/kg. Artificial weathering of soils was conducted by adding 0.5 ml of deionized (DI) water per gram of weathered soil once a week for 8 weeks to vials containing the eight test soils. The vials were then capped tightly for 3 days, after which the caps were removed and the samples were air dried for 2 days, followed by capping and mixing on a rotator for 2 more days. Soil homogenisation was tested by removing 5 mg aliquots of soil in triplicate during each experimental run (not significantly different). Unweathered test soils were also created by dosing the same concentration of ^{14}C -benzo[a]pyrene in toluene.

The *in vitro* dermal absorption experiments used flow-through diffusion cells, with an approximate 30 mg/cm^2 soil loading. Human Cadaver abdominal skin was used as the membrane (two females, one male: ages 59, 79 and 48 years). 20 mg of the test soils or 50 μL of the ^{14}C -benzo[a]pyrene in acetone were applied to the skin surface. Receptor solution was collected at specified time steps throughout the 8h (2h, 4h, 8h) or 24h (3h, 6h, 12h, 24h) exposure durations and mixed with scintillation cocktail prior to analysis by liquid scintillation counting. Soils were removed at the end of each experiment using dry applicator tips and extracted using scintillation cocktail for analysis. Skin samples were solubilized in 2 ml of Soluene 350 under sonication and mixed with 10mL of scintillation cocktail prior to analysis.

The soils used in the experiments had a 4-fold range of TOC and 13-fold range of BC. It was assumed by Peckham et al. (2017) that these properties would have influence sorbent capacity for BaP, and therefore the thermodynamic activity which drives the transfer of PAH from soil to and through the skin. However, no consistent effects were observed for the soils tested. This may be due to the soils being saturated with BaP, therefore exceeded sorbent capacity resulting in no observed effect of differing soil TOC and BC. Also, as the soil particle size was sieved to $<63\mu\text{m}$, the fine particles may have reduced the influence of TOC and BC BaP sorption. The study produced an excellent mass balance range of 89.2% to 103%. The saturation of soils in this study highlights the fact that contaminated soils often have much higher PAH concentrations than used in this study (Potin et al., 2004) where saturation was achieved. Therefore, suggesting that contaminated soils in the environment might exist at supersaturated conditions with PAH mobile for transfer from soil to skin.

Weathering for eight weeks produced fluxes 2.5 times lower than non-weathered soils, further demonstrating the importance of the ageing process being represented for dermal absorption studies. The two soil concentrations produced similar flux values into the receptor solution ($0.012\text{ ng/cm}^2/\text{h}$) at 24h whereas the membrane flux differed ($3\text{ mg/kg soil} = 0.033\text{ mg/cm}^2/\text{h}$; $10\text{ mg/kg soil} = 0.075\text{ mg/cm}^2/\text{h}$), suggesting that the membrane is the absorption rate limiting factor for PAH entering systemic circulation. This study suggested that the addition of sweat simulant to skin before addition of labelled soil or acetone should be avoided as it was deemed unrepresentative of chronic exposure. As BaP is a carcinogen chronic rather than acute toxicity is the concern for human health risk assessment.

2.3.2.12 Strandberg, (2018)

Strandberg et al. (2018) used a tape stripping method to sample the skin of firefighters and office workers (the control group) for an occupational exposure study. The tape stripping method assumes that any chemical uptake by the skin can be traced by repeatedly placing strips on an area of the skin and measuring the amount of chemical in each tape. But this is only true if 30 tapes are taken so that the skin is glistening post sampling, whereas in this study only 3-5 tapes were used. These tapes once collected were folded and placed in an airtight container and stored at -20°C prior to analysis. The tapes then went through a two-stage clean-up process. Firstly, they underwent a dialysis procedure, where semipermeable low-density polyethylene membranes (SPMs) were used. The SPMs were soaked and cleaned in hexane for 24 h and then air dried. Each sample was then transferred to an SPM by rinsing with 1.5mL of hexane three times. The film was then placed in a beaker, and 100mL of hexane (dialytic solvent) was added and the beaker covered with foil. The solvent was replaced with

another 100mL the next day. The combined dialysate was collected in a flask and reduced to a volume of 1mL. The second stage of clean-up involved the use of an aluminium oxide, silica gel and sodium sulphate packed column with samples eluted with 25mL of 1:1 DCM/hexane. Recoveries of the PAH measured were good, with most ranging between 60% and 90%, however naphthalene was lower at around 50% recovery, which is due to the volatility of the congener. Other factors to affect recovery include molecular size, mass, and shape of the molecule. One limitation of this study is that the blank tapes analysed also contained small concentrations of more volatile PAH such as naphthalene, but as PAH are ubiquitous in the environment this is to be expected. The tape stripping method will not be used in this project, due to it involving human subjects as well as the inability to analyse contaminated soils using this method.

2.3.2.13 Forsberg et al, (2021)

Forsberg et al. (2021) studied the *in vitro* dermal absorption of benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene and dibenz[a,h]anthracene. This was achieved by using weathered soils from two US Department of Defence sites, along with a BaP-spiked soil (grain size for all soils <250µm). A static diffusion cell system was used, with human breast and abdominal skin used as the membranes. 10 mg/cm² soil loading was applied for 24 h prior to removal, with receptor solution collected for a further 48 h post soil removal. Receptor solution was sampled at: 1, 2, 4, 8, 16, 24, 36, 48, 60, and 72 h post dose with replenishment. At 72 h post dose, the cells were dismantled. The skin was dried with tissue swabs, and the *stratum corneum* was partially removed using 5 successive tape strips (DSquame tapes), which were pooled as a single sample for analysis. The exposed skin was retained for analysis. Skin and tape strip samples were extracted with methanol (10 mL). Tape, skin, and receptor fluid samples were extracted and analysed using a validated high-performance liquid chromatography method with fluorescence detection.

This study showed an increase in the dermal absorption of PAH with increasing PAH soil concentration. However, the concentration of PAH in skin and receptor solution were not reported, so the partitioning of PAH in the dermal system cannot be examined. This study did produce site-specific ABS_d values, showing that when measuring native BaP from a soil that the dermal absorption is 6.5 times lower than when measuring BaP absorption from a spiked soil. It was also shown that PAH absorption into the receptor solution continues even after soil is removed, which further corroborates the guidance to measure PAH concentration in the membrane and receptor solution for *in vitro* studies (OECD, 2004, IPCS, 2006). However, Dibenz[a,h]anthracene dermal absorption data could not be produced,

which is needed for risk assessment as it has been shown that Dibenz[a,h]anthracene is more potent than BaP (Nisbet and LaGoy, 1992, Durant et al., 1999). The study showed high variability (e.g., BaP ABS_d for soil GS-1 was 1.3 with 95% confidence interval of -0.45 to 3.1), which could be due to the individual and anatomical variety of skin sources used in the dermal absorption experiments (VanRooij, 1993).

2.3.3 Soil Loading

The soil size fraction regularly encountered in the literature for soil dermal bioavailability testing is the <250 μm fraction, as it has been demonstrated that this fraction can adhere to the skin of children, who are often the critical receptor for human health risk assessments of potentially contaminated sites (Duggan et al., 1985, Kissel et al., 1996, Abdel-Rahman et al., 2002, Choate et al., 2006, Cave et al., 2010, Beriro et al., 2020). However, some studies have used finer or coarser grain sizes. Ruby and Lowney (2012) reviewed soil adherence to hands to assess the implications of soil grain sizes used in oral bioavailability experiments. They assessed studies that have used soil particle sizes ranging from <45 μm to <2000 μm . They state that it is not currently understood whether PAH bioavailability will be greater from large or small particles (as discussed above), and also because the data indicate that significant mass of five- and six-ring PAHs may be present in soil particles up to 250 μm . Therefore, it would be unwise to remove any particle within this soil particle size for bioavailability testing. The soil fraction applied (<710 μm) by Moody and Joncas (2007) is coarser than the fraction generally accepted to adhere to hands (<250 μm). This would result in a reduced soil grain surface contact with the membrane, resulting in a reduced potential for transfer of PAH from soil to skin leading to an underestimation of the soil dermal bioavailability of PAH.

Loading refers to the mass of soil applied per unit area of skin. Theoretically, monolayer coverage is the ideal loading scenario as this loading is assumed in human health risk assessment, which is the complete coverage of the study area of skin with a single layer of soil particles, although in practice this is highly unlikely to be achievable (Spalt et al., 2009). Soil loadings that produce broken skin coverage, with a single layer of soil particles are referred to as 'submonolayer', and those with more than one layer of soil particles covering the skin study area 'supermonolayer' (Spalt et al., 2009) (Figure 7).

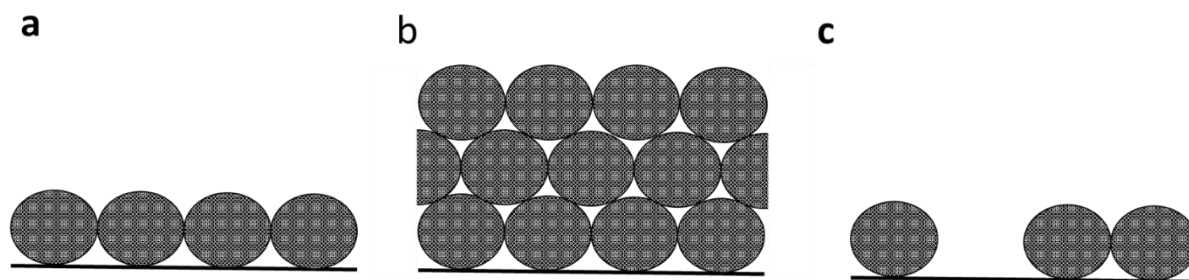


Figure 7: Soil Loadings. a) monolayer coverage. b) supermonolayer coverage. c) submonolayer coverage.

It has been suggested that dermal absorption data reported from submonolayer coverage and low soil loading is not a useful indicator of the dermal absorption capability of a substance due to broken coverage limiting substance transfer from soil to skin (Frasch et al., 2014). Soil loadings vary greatly in the literature, ranging from 1 mg/cm^2 (Roy and Singh, 2001) to 50 mg/cm^2 (Moody et al., 2011), with loading having a significant impact on results depending on which format the author presents their data. If the absorption data is presented as percentage absorbed of the dose applied (PADA%), the proportion absorbed will decrease with greater loading (Spalt et al., 2009). Presenting data in such a way also produces comparability issues, because for PADA% to be comparable between soils/studies, the loading must be the same to prevent effects of submonolayer or supermonolayer coverage on the PADA% value. Therefore, for this study, absorption data intending to be compared to previous works was presented as flux to remove the skewing of absorption data owing to soil loading.

In a real-world exposure scenario, it is likely the soil that the receptor is exposed to is not dry. The annual mean UK soil moisture content is approximately 40w/w% water (Stanley et al., 2019). It has been demonstrated that soil skin adherence increases with increasing soil moisture content. For example, Kissel et al. (1996) found in hand press trials that soil adherence of the same soil at <0.1% moisture was $0.22 - 0.54 \text{ mg/cm}^2$, compared to $1.64 - 14.8 \text{ mg/cm}^2$ at 21-27% soil moisture. Choate et al. (2006) also show an increase in soil adherence with increase in moisture content, with 0.64 mg/cm^2 at 1.85% moisture and 1.47 mg/cm^2 at 9.36% moisture. It has also been found that the adherence of larger soil grain size particles increased when the moisture content of soils was increased (14–19% moisture) (Kissel et al., 1996). Furthermore, a field study by Duff (1996), found that real world soil loadings on the skin of volunteers ranged from $<0.001 \text{ mg/cm}^2$ (submonolayer) to $>100 \text{ mg/cm}^2$ (supermonolayer). Therefore, with the high mean soil water content for the UK, and the critical receptor being a female child, it is likely that soil coverage will be

supermonolayer in real world exposure scenarios and will subsequently be replicated in soil PAH dermal bioavailability experiments.

Most previous *in vitro* soil PAH dermal bioavailability studies have only quantified experimental PAH soil concentrations at the end of the experiment rather than at each time-step at which the receptor solution was sampled (Abdel-Rahman et al., 2002, Moody and Joncas, 2007, Peckham et al., 2017, Forsberg et al., 2021). This final soil concentration was due to method performance/quality control, to ensure that there were no losses or gains of PAH to the experimental system throughout the study. This will also need to be achieved in this study so that dermal bioavailability of soil PAH and also method performance can be assessed in comparison to previous work.

Dermal bioavailability studies do not generally state how soils are removed from the skin surface and how soil concentrations are then quantified, with many referring to 'soap washes' (Ng et al., 1991, Moody et al., 1995). Previous studies (Moody et al., 1995, Moody and Joncas, 2007, Moody et al., 2011) have used soil removal methods that have involved washing with surfactants such as Radiacwash™ to remove soils from skin surfaces, owing to their use of radiolabelled PAH.

2.3.4 Summary of Findings of the Dermal Bioavailability Literature

In vivo methods have the benefit of representing the metabolic pathways of PAH which have been dermally absorbed. PAH are not harmful until they have been metabolised (Pessah et al., 2001, Zhang et al., 2012). Human volunteers for *in vivo* studies are deemed the gold standard for dermal absorption studies (IPCS, 2006), however, the aim of this work is to produce a laboratory-based test for the dermal absorption of PAH from soils, therefore the ethical implications and availability of humans to be tested every time a contaminated soil needs to be assessed for PAH dermal bioavailability are not practical and should not be considered a viable approach.

In vitro methods offer greater reproducibility and greater control over experimental parameters than *in vivo* approaches. This is due to significant differences in chemical dermal permeation depending on the anatomical site, the individual and species used for *in vivo* studies (VanRooy, 1993, Moody et al., 1995, Monteiro-Riviere and Riviere, 1999, Brain and Chilcott, 2008, Chilcott and Price, 2008). Furthermore, *in vivo* studies often produce lower chemical recovery in comparison to *in vitro* (Ng et al., 1991, Moody et al., 1995), meaning

that there is greater uncertainty of chemical fate within the system, resulting in greater uncertainty of the generated dermal bioavailability data.

Also, *in vivo* methods when testing soils pose the issue of the soil loading displacement on the animal skin over the course of the experiment which adds variability and uncertainty to subsequent absorption data. Additionally, *in vivo* testing is often more expensive and offers a lower sample throughput in comparison to *in vitro* methods (Collins et al., 2015). The OECD (2004) and IPCS (2006) state that *in vitro* dermal absorption data collected using diffusion cells can be representative of that from *in vivo* studies given that the chemical concentration is quantified in both the membrane and receptor solution. It has been shown in *in vivo* and *in vitro* method comparison studies that *in vitro* can produce PAH dermal absorption data representative of *in vivo* (Ng et al., 1991, Moody et al., 1995).

The improved cost and availability of artificial membranes such as the Strat-M™ in comparison to *ex vivo* skin also allows for PAH dermal absorption to be quantified in soil, membrane, and receptor solution at each time-step of a dermal absorption experiment. Previous studies (Abdel-Rahman et al., 2002, Moody and Joncas, 2007, Peckham et al., 2017, Forsberg et al., 2021) have only quantified PAH concentration of the receptor solution at each time step, only analysing the membrane and soil concentrations at the termination of the experiment. By quantifying the PAH concentration in all three media at each time step, this will allow for greater certainty of where PAH are in the dermal system over time, which is important for human health risk assessment. Furthermore, by quantifying PAH in all sample media at each time-step, any losses or gains of PAH can be located and rectified.

The use of PADA to display dermal absorption data in the literature is questionable, as the dose applied is impacted by the loading of soil applied during the experiment, resulting in lower PADA values for the same dermally absorbed PAH concentration with increasing soil loading. Roy et al. (1998) acknowledge the inherent complication of the use of PADA by conducting *in vitro* dermal penetration experiments under infinite (nonlimiting) dose conditions. To give a greater chance of detection, the soil loading was increased from 5 mg/cm² to 50 mg/cm² (Moody and Joncas, 2007). A similar approach was used in this study, a high loading ensures complete coverage of the exposed area for which dermal absorption is to be measured. A high soil loading also produces an infinite dose scenario, meaning that J_{ss} can be calculated for the soils tested in this study and compared to previous work to meet one of the objectives of this study (Chapter 1). A high soil loading will also increase peak flux, making it more representative of maximum flux by providing a large dose (Frasch et al.,

2014), which will increase the likelihood of the quantification of PAH in all three sample media and represent a conservative dermal exposure scenario (Moody et al., 2011). It was found that soil adherence to skin increases with soil moisture (Kissel et al., 1996, Choate et al., 2006), as well as high soil loadings ($>100 \text{ mg/cm}^2$) (Duff, 1996) on the skin of volunteers. Therefore, with the soils in the UK typically being moist (Stanley et al., 2019), it is likely that supermonolayer coverage would be the typical exposure scenario for critical receptors in the UK.

Another key point from the literature is that the presence of soil greatly reduces the dermal absorption of PAH (Roy et al., 1998, Abdel-Rahman et al., 2002), and even more so after the PAH have been exposed to the soil for several months (Abdel-Rahman et al., 2002, Moody et al., 2011, Xia et al., 2016). The aging process results in soil bound chemicals becoming increasingly resistant to desorption over time (Roy et al., 1998). Therefore, to be representative of real-life exposure conditions natural soils contaminated with PAH will be used, this is to prevent overly conservative dermal bioavailability values such as those derived in studies with artificially PAH spiked soil (e.g. Wester et al. (1990)). It is therefore likely that the current 13% dermal absorption value used in human health risk assessment is unrepresentative of the true dermal exposure to PAH from soil.

Two soil concentrations (3 mg/kg and 10 mg/kg) produced similar flux values into the receptor solution ($0.012 \text{ ng/cm}^2/\text{h}$) at 24h whereas the membrane flux differed (3mg/kg soil = $0.033 \text{ mg/cm}^2/\text{h}$; 10 mg/kg soil = $0.075 \text{ mg/cm}^2/\text{h}$), suggesting that the membrane is the absorption rate limiting factor for PAH entering systemic circulation (Peckham et al., 2017). Therefore, it was predicted that the membrane would contain the higher concentration of dermally absorbed PAH in comparison to the receptor solution. Peckham et al. (2017) also suggested that the addition of sweat simulant to skin before addition of labelled soil or acetone should be avoided as it was deemed unrepresentative of chronic exposure, as BaP is a carcinogen, and chronic rather than acute toxicity is the concern for human health risk assessment.

Differences in soil properties such as texture (Abdel-Rahman et al., 2002), TOC content (Xia et al., 2016, Peckham et al., 2017), and PAH source materials (Xia et al., 2016) also impact the availability of PAH from soils. Therefore, by using one PAH dermal absorption value of 13% to represent the dermal bioavailable fraction in human health risk assessment, the effects of different soil properties on the amount of PAH being dermally bioavailable is not considered. The *in vitro* soil PAH dermal bioavailability method developed in this study

therefore allowed for the generation of soil-specific parent PAH dermal bioavailability data from real-world soils. It should be noted that previous studies have only looked at one or two soil properties at a time, therefore reducing the power of the relationships found on PAH dermal bioavailability.

At present, there has been no use of a certified reference material (CRM) soil for dermal absorption studies. This reduces inter and intra lab and method comparability for soil PAH dermal bioavailability data. Yang et al. (1989) used an SRM of petroleum crude oil, but this was spiked with radiolabelled PAH. Therefore, solvent dosing volume, PAH mass and dosing vehicle as well as aging time would impact PAH bioavailability from the SRM. Consequently, this approach would not aid comparability of results inter and intra lab/study. This study is the first to measure the dermal absorption of parent PAH from a soil CRM. This will enable future inter and intra lab and study comparison of both methods and PAH dermal bioavailability data, acting as a quality control protocol (Bowen, 1975).

The developed method will be validated by comparing PAH absorption data from a solvent vehicle to previous *in vivo* and *ex vivo* studies. If absorption data from the developed *in vitro* method was within the range of values of those collected from *in vivo* and *ex vivo* studies, it could be assumed the developed *in vitro* method is representative.

Overall, the fact that the Wester et al. (1990) study is used to derive human health risk assessment criteria to assess brownfield sites is a potential issue, as the value of 13% BaP dermal bioavailability is disputable. By utilising artificial membranes, a naturally contaminated soil and solvent vehicle validation, a much more reproducible, accessible, cost-effective, validated *in vitro* method can be produced to generate soil-specific PAH dermal bioavailability values which can be used for human health risk assessment. The generation of such a method will then allow more comparative inter and intra lab studies, and the further assessment on the controls of soil PAH dermal bioavailability.

2.4 Brownfield Land

2.4.1 Definition

The Concerted Action on Brownfields and Economic Regeneration NETwork (CABERNET) define Brownfield land as sites that “*have been affected by the former uses of the site and the surrounding land; are derelict or underused; have real or perceived contamination problems; are mainly in developed urban areas; require intervention to bring them back to beneficial use*” (CABERNET, 2006).

In England, Brownfield land is a previously used land, other than for agriculture, which is currently underused or unused. The reduction of use could be due to the presence of one or more factors, which could include contamination (Longo and Campbell, 2017).

The generation of Brownfield land occurs during deindustrialisation, with the upheaval of industrial sectors resulting in potentially contaminated land left vacant in local communities (Tang and Nathanail, 2012). Brownfield sites tend to be located in urban areas, where the effective redevelopment of such sites present the opportunity to benefit environmental, economic, and social welfare of the surrounding communities (Lange and McNeil, 2004).

2.4.2 Brownfield Land in the UK

Typically, Brownfield sites in the UK are <1 ha in area, as are most Brownfield sites across Europe (Oliver et al., 2005). In England, the potential of Brownfield redevelopment is recognised, with an estimated 21,000 Brownfield sites, with the ability to provide 1.06 million homes covering 25,000 ha (CPRE, 2020). Using Brownfield land and other land with planning permission, there is currently land provision for over 1.5 million homes, providing enough land to meet the UK government's 300,000 homes a year target for the rest of this parliament (CPRE, 2020). The UK monitors the scale and nature of Brownfields in the UK, with the National Land Use Database and the Brownfield Land Registers. The purpose of the Brownfield Land Registers is to provide up-to-date consistent information on sites that local authorities deem appropriate for residential development in relation to criteria set out in regulation 4 of the Town and Country Planning (Brownfield Land Register) Regulations 2017. Registers are published locally as open data to allow transparency about the suitability and availability of such sites for development (Ministry of Housing, 2017a). Following reforms of the UK planning system, more than two thirds of all homes are built on brownfield land – with ambitions to go even further (Ministry of Housing et al., 2014).

Countries experiencing deindustrialisation and suburban migrations have used legislation to encourage developers to reuse Brownfield land (Tang and Nathanail, 2012). Alongside the 'Housing White Paper' published in 2017, the 'Home Builders Fund' provided £3 billion of support for the development of Brownfield sites, with an additional £1.2 billion to produce at least 30,000 starter homes on Brownfield land (Ministry of Housing, 2017b).

The main pieces of UK guidance regarding the procedure for the assessment of potentially contaminated land are the Town and Country Planning Act 1990, the National Planning Policy Framework (NPPF) and Environmental Protection Act 1990: Part 2A. The UK government

defines contaminated land as land where substances are or have the potential to: cause significant harm to people, properties, or protected species; cause significant pollution of ground or surface waters; or cause harm as a result of radioactivity (Part 2A of the Environmental Protection Act 1990, Section 78A). Land assessment under the Environmental Protection Act 1990: Part 2A involves a risk-based approach to determine if land is contaminated. The representation of the dermal pathway for the human health risk assessment of the dermal absorption of PAH from soil may not be accurate at present (Section 2.3.2, Section 2.3.2.2, and 2.3.4). This means that sites may be remediated when not necessary, or worse not remediated when there is a risk to human health. The unnecessary remediation of sites may lead to delaying and diverting funding away from the decontamination of sites for which it is required (Alexander, 2000).

Overall, the UK government wish to prioritise the redevelopment of brownfield sites, with the potential for 1.06 million homes to be developed on such sites (CPRE, 2020). But land assessment is currently based on dermal bioavailability data that could be inaccurate (Section 2.3.2, Section 2.3.2.2, and Section 2.3.4), which leads to remediation being required when not necessary, increasing costs and diverting funding from other sites, or worse leading to sites not being remediated when it is needed to protect human health. Therefore, a method needed to be developed to measure the dermal absorption of parent PAH from soils to produce dermal bioavailability data to aid in the risk-based land management decision making process.

2.4.3 Coal Gasification Works (Gasworks)

The gas industry flourished due to the need to develop an economical and efficient form of lighting in comparison to oil lighting methods that preceded (Palmer et al., 2012). The coal gasification process involved heating coal in the absence of oxygen in retorts. The manufactured gas industry was the first true utility industry (Palmer et al., 2012) and was a main sector of industry from the mid-19th century to mid-20th Century (Wehrer et al., 2011). This was not only due to gas supply, but also owing to the sale of valuable by-products from the coal gasification process (Wehrer et al., 2011). These included: coal tar which was sold as wood preservative or feedstock for the chemical industry (e.g., dyes); spent oxide for the manufacturing of sulphuric acid; coke for domestic heating; ammoniacal liquor for the

manufacturing of fertiliser (Wehrer et al., 2011, Thomas, 2014). A comprehensive history of the Gas Industry in the UK was produced by Professor Russel Thomas in collaboration with Historic England (Thomas, 2020a)

The coal gas industry left a significant legacy in the UK. By the late 19th and early 20th century, many of the smaller gasworks closed and were subsequently forgotten. These sites were often redeveloped during a time when the associated risks posed by the potentially contaminated land were not understood and safety and environmental standards were also less stringent. Most of these sites now have a residential land use, and pose the greatest concern to human health of the >4000 former gasworks that operated in Great Britain (Thomas, 2014). Larger gasworks are often owned by multiple parties, with many of these sites, but not all, having undergone remediation (Thomas, 2014). A comprehensive source of information on the locations, operations and history of former gasworks sites was produced by Professor Russel Thomas in collaboration with Historic England (Thomas, 2020b). It has been noted that pollution of gasworks sites was a result of everyday plant operations, accidents, and leaks and the deliberate disposal of polluting by-products into the environment (Tarr, 2014).

Coal tars are primarily dense non-aqueous liquids (DNAPLs) composed of thousands of organic and inorganic compounds, with the organic fraction being dominated by PAH (Gallacher et al., 2017). Coal tar was often used as part of a backfill mixture during plant demolition resulting in tar saturated soil layers (Baylis and Allenby, 2006). Coal tars along with many PAH have carcinogenic and mutagenic properties (IARC, 2010, IARC, 2012). Former gasworks soils can contain highly elevated PAH concentrations. For example, Lundstedt et al. (2003) found soil BaP concentrations of 120 mg/kg at a former Swedish gasworks, with USEPA 16 PAH soil concentrations ranging from 2.4 to 420 mg/kg. Similarly, Potin et al. (2004) found BaP soil concentrations of 133 mg/kg (Σ USEPA 16 PAH = 793.9 mg/kg) at a former gasworks in France. In comparison, a typical urban soil normal background concentration (NBC) for BaP is approximately 17 mg/kg (Σ USEPA 16 PAH = 80 mg/kg) (Vane et al., 2021).

Many of these former gasworks sites remain un-remediated (Thomas, 2014), and are located in urban areas where redevelopment would be beneficial for environmental, economic, and societal health. Therefore, these sites need to undergo human health risk assessment as part of the UK risk-based land management strategy before any redevelopment can occur. Consequently, the uncertainty surrounding the soil PAH dermal bioavailability value (Section

2.3.2, Section 2.3.2.2) could lead to added remediation costs when it is not required and/or discourage redevelopment (Alexander, 2000). Furthermore, it would be even more detrimental if the current value was an underestimate of potential human exposure, at this would put public health at risk. Therefore, a method for deriving soil-specific soil PAH dermal bioavailability data was required, to enable greater certainty of the human health risk-posed by these soils to aid in the redevelopment of former gasworks and brownfield sites.

2.5 UK Contaminated Land Management

In a 2016 report (Environment Agency, 2016) looking into the drivers of contaminated land assessment of 66 local councils between 2000 and 2013, it was found that unacceptable risk to human health was the main driver for land to be classed as contaminated. The presence of a wide range of substances can lead to land being considered contaminated. The Environment Agency (2016) reported, that during the years 2000-2013 that the most frequently occurring substance behind As (280 sites) and Pb (257 sites) was BaP (256 sites) (PAH were determined at 156 sites), as such BaP and PAH are the most frequent determining organic contaminants of the site data provided by the 66 councils.

The main objectives of the UK government on contaminated land, along with Part 2A are (DEFRA, 2012):

1. To identify and remove unacceptable risks to human health and the environment.
2. To guarantee that contaminated land is suitable for its current intended use.
3. To ensure that the burdens faced by individuals, companies and society as a whole are proportionate, manageable, and compatible with the principles of sustainable development.

The UK takes a phased approach to contaminated land risk assessment, where after each stage a decision is made regarding whether further action is required (Figure 8). For a risk to exist, there needs to be at least one contaminant-pathway-receptor linkage, from which a relevant receptor might be affected by the contaminants present (DEFRA, 2012). The critical receptor depends on the proposed land use, for example, for residential land use the critical receptor is a female child aged between 0 and 6 years old, whereas for a commercial land use, the critical receptor is a female worker aged between 16 – 65 (typical working lifetime) (Environment Agency, 2009, CL:AIRE, 2014a).

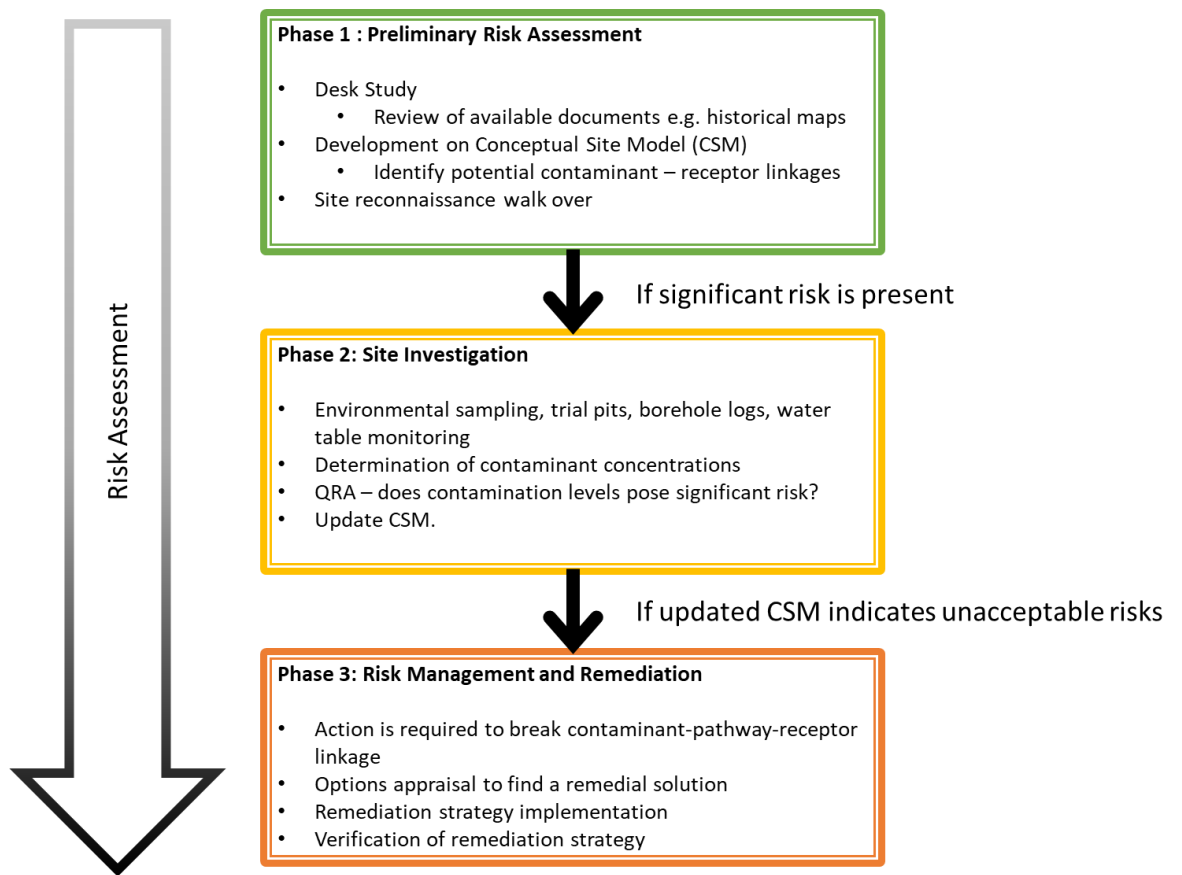


Figure 8: Overview of UK phased approach to contaminated land assessment.

Once land has been assessed as needed via the phased approach, the land can be categorised accordingly. Land in the UK can be placed into 4 categories (DEFRA, 2011, DEFRA, 2012, Ander et al., 2013) (Figure 9).

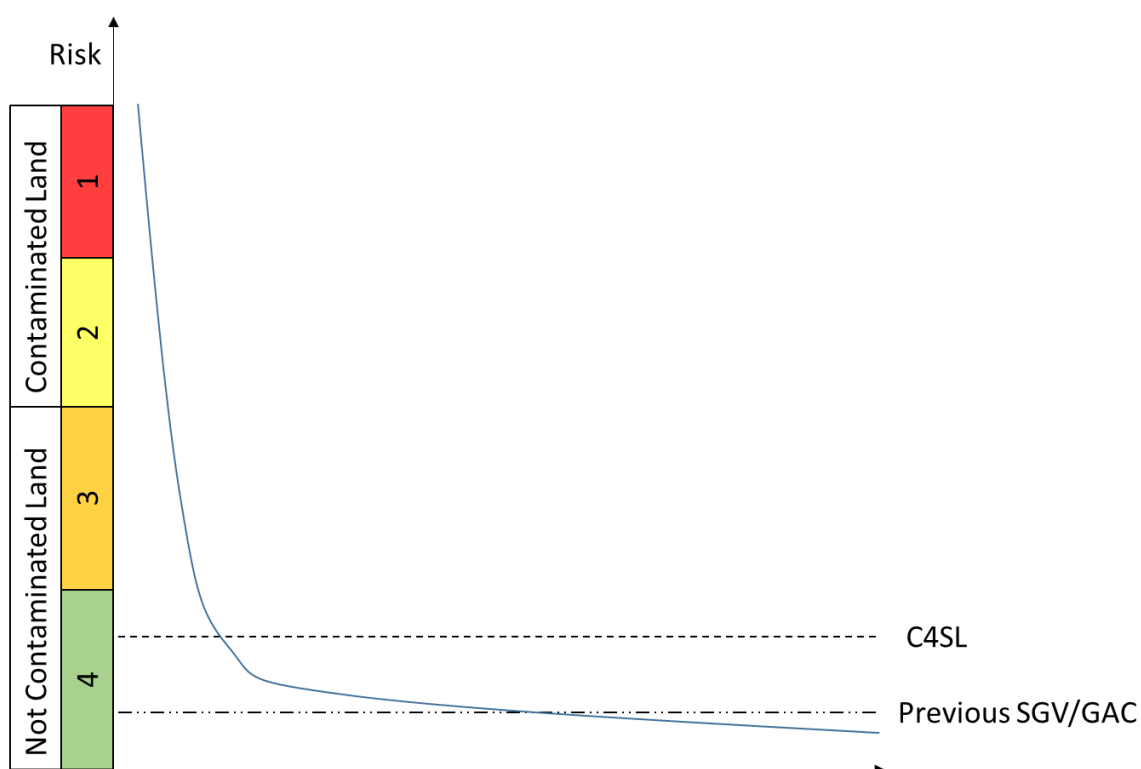


Figure 9: The 4 category land classification introduced by DEFRA, C4SL and previous GAC levels.

Categories 1 and 2 are classed as contaminated land, whereas categories 3 and 4 are classified as not contaminated land. For land to be placed in Category 1, there needs to be a high probability of significant harm, supported by strong science-based evidence that significant harm would occur if no mitigation was taken to prevent it.

GAC (Generic Assessment Criteria) represent tolerable/minimal risk levels (unless stated otherwise), and are guidelines on the chronic human exposure to individual chemicals in soil (Cole and Jeffries, 2009). There are GACs available for BaP as a surrogate marker for PAH. LQM/CIEH developed the Suitable 4 Use Levels (S4UL), which are a GAC that represent the soil concentration of a contaminant that poses minimal risk to human health (Nathanail et al., 2015). Also in 2013, the Category 4 Screening Levels (C4SL) were created by DEFRA. C4SL represent low risk, rather than minimal, but are still representative of land types in Category 4 (Figure 2). The C4SLs are intended to be used as a “relevant technical tool” to help determine whether to stop further assessment of a site on the basis that it is classed as Category 4 land (CL:AIRE, 2014b).

Currently, environmental practitioners in the UK derive GAC for soil contaminants using the CLEA model. The CLEA model utilises generic assumptions about the fate and transport of chemicals in the environment, as well as human behaviour to predict adult and child exposure levels to soil contaminants for people who may live, work and/or play on contaminated sites over long time periods (Nathanail et al., 2009). GAC are derived in accordance with UK legislation with the aim to have a database of toxicity and transport properties to inform HHRA. The current drawback of the CLEA model is that it currently uses the 13% dermal bioavailability value from Wester et al. (1990), which may not be representative of the dermal absorption of PAH from soil meaning that the assessment criteria produced by the CLEA model may not be reflective of a true exposure scenario via the dermal absorption pathway. This potential misrepresentation of the dermal absorption pathway is of concern, especially as risk to human health has been shown to be a main driver for land to be classed as contaminated (Environment Agency, 2016). Therefore, a further understanding and quantitation of the dermal absorption of PAH from soils is required, to better inform contaminated land assessments, environmental practitioners, local authorities, and regulatory bodies.

2.6 Selection of Three Focal PAH

It is common practice for PAH dermal bioavailability studies to focus on selected PAH from the USEPA 16 (e.g. (Wester et al., 1990, Ng et al., 1991, Moody et al., 1995, Stroo et al., 2005b)), and owing to the time and resources available during this project, the same approach was taken by choosing three of the USEPA 16 PAH to focus on. The selection was based on the dermal contribution to total exposure as calculated by the CLEA model, the IARC carcinogenicity classification (Section 2.2.2), PAH chemical characteristics, concentration in soils, and analytical stability.

BaP has been selected owing to its use in many dermal bioavailability studies (e.g. Yang et al. (1989), Wester et al. (1990), Abdel-Rahman et al. (2002), Moody et al. (2011), Xia et al. (2016), Strandberg et al. (2018)), due to it being defined as a “complete” carcinogen, and that it is classified as a IARC group 1 compound (known human carcinogen) (Section 2.2.2). BaP is the most easily synthesised parent PAH congener that will produce tumours (Wester et al., 1990, Pessah et al., 2001, Moody and Joncas, 2007, IARC, 2010). The use of BaP in many dermal studies will mean that flux values derived from this research can be compared to previous results (e.g., 0.024 ng/cm²/hr (Wester et al., 1990) and 0.028 ng/cm²/hr (Xia et al., 2016)). This will subsequently allow for validation of the method and also demonstrate the methods capability to measure the dermal bioavailability of a PAH that is a high-risk

driver in contaminated land assessment, therefore displaying its potential use as a screening method to assess potentially contaminated soils.

Table 6: Contribution to total exposure from the relevant pathways as calculated by CLEA 1.04 for the residential land use at 6% SOM. Adapted from: (Nathanail et al., 2009). AC = acenaphthene, ACL = acenaphthylene, AN = anthracene, BaA = benz[a]anthracene, BbFA = benzo[b]fluoranthene, BghiP = Benzo[g,h,i]perylene, BkFA = benzo[k]fluoranthene, CHR = chrysene, FA = fluoranthene, FL = fluorene, IP = indeno[1,2,3,c-d]pyrene, NA = naphthalene, PHE = phenanthrene, Pyr = pyrene.

	Contribution to total exposure for the residential land-use (%) ¹															
	AC	ACL	AN	BaA	BaP	BbFA	BghiP	BkFA	CHR	DBA	FA	FL	IP	NA	PHE	Pyr
Dermal contact (outdoor)	8	6.8	14.6	34.8	36.3	35.8	37.7	36.5	33.5	36.9	25.7	9.3	35.5	1.7	14.3	24.7
Inhalation of vapour (indoor)	4.9	4.4	1.4	<0.1	0	<0.1	0	0	<0.1	<0.1	0.4	3	<0.1	27	1.2	0.4
Consumption of homegrown produce and attached soil	74.2	78	60.6	9.5	5.9	7.1	2.2	5.2	4.2	4.2	32.6	72.9	8.4	45.1	60.7	35.3
Ingestion of soil and indoor dust	12.4	10.5	22.7	54.1	56.3	55.6	58.5	56.8	52	57.4	40	14.4	54.8	2.7	22.2	38.4

¹Not all pathways output from the CLEA model are included in this table, only the largest contributors to total exposure.

Table 6 shows the total exposure contributions for some of the pathways calculated by the CLEA model. The residential land-use was selected as the most sensitive receptor is a young female child as they are exposed to each pathway for a longer duration and more extensively than an adult. The table shows that naphthalene (NA), acenaphthene (AC), acenaphthylene (ACL), anthracene (AN), phenanthrene (PHE), and fluoranthene (FA) all have exposure contribution via the inhalation of vapour pathway. This suggests that these should not be selected to be focused on during this project, as these PAH are susceptible for evaporative losses, meaning that dermal contact may not necessarily be a main pathway of exposure (e.g., naphthalene) and losses will occur during analytical work. BghiP (37.7%), DBA (36.9%), BkFa (36.5%), BaP (36.3%), BbFa (35.8), IP (35.3), BaA (34.8), CHR (33.5%), FA (25.7) and Pyr (24.7) all had substantial dermal exposure contributions.

Several methods have been created with the hope of obtaining more accurate risk potentials for human exposure to PAH mixtures by using equivalency factors for BaP (Jung et al., 2010). TEF (Toxicity Equivalence Factor) relates to the carcinogenicity compared to BaP (TEF=1), and high and low dose values can be used (e.g., DBA high dose TEF = 5, Low dose TEF=1.1 (Nisbet and LaGoy, 1992). Another way is MEF (Mutagenic Equivalency Factor), which considers that

mutagenicity of a PAH relative to BaP. Mutagenic activity includes other non-cancerous health effects such as pulmonary diseases, and is not consistently related with cancer development (Jung et al., 2010). Durant et al. (1999) tested the mutagenicity of 11 PAH in an assay based on human h1A1v2 cells. DBA had a Minimum Mutagenic Concentration (MMC) of 9.7ng/ml and BaP had a MMC of 14.9ng/ml, which shows that DBA can be present at lower concentrations than BaP and can still cause adverse health effects. DBA has been found to have a higher cancer incidence in mice than BaP (Nisbet and LaGoy, 1992), but it is lower in abundance than BaP in most soils (Wilcke, 2000, Potin et al., 2004) (Figure 1). DBA also has a higher dermal pathway exposure contribution than BaP at 36.9% and is classed as a group 2A compound by the IARC (2010) (Section 2.2.2). Therefore, the toxicity, size (largest MW at 278) and lipophilicity (high log K_{ow}) (Section 2.2.1) of DBA means that it would be beneficial to understand the dermal bioavailability for human health risk assessment, as TEFs produced by Nisbet and LaGoy (1992) and MMC derived by Durant et al. (1999) suggest that DBA could have a higher potency than BaP for environmental exposures.

Table 7: Characteristics of the US EPA 16 PAH. MW = molecular weight (to 3 sig fig). Data from (Tobiszewski and Namiesnik, 2012, Stogiannidis and Laane, 2015)

Compound	Number of Rings	MW	Aqueous Solubility (mg/L)	Log K_{ow}
Napthalene	2	128	32	3.37
Acenaphthylene	3	152	3.9	4.1
Acenaphthene	3	152	3.9	3.9
Fluorene	3	166	1.9	4.18
Phenanthrene	3	178	1.1	4.57
Anthracene	3	178	0.05	4.54
Fluoranthene	4	202	0.26	5.22
Benzo[a]anthracene	4	228	0.009-0.014	5.6
Chrysene	4	228	0.002	5.86
Pyrene	4	202	0.13	5.18
Benzo[a]pyrene	5	252	0.003	6
Benzo[b]fluoranthene	5	252	0.0014	5.8
Benzo[k]fluoranthene	5	252	0.0007-0.008	6
Dibenz[a,h]anthracene	5	278	0.0005	6.5
indeno[1,2,3-cd]pyrene	6	276	0.00019	6.6
Benzo[ghi]perylene	6	276	0.00026	7.1

Table 7 shows various chemical characteristics for the US EPA 16 PAH. DBA and BaP have already been selected owing to their toxicity, contribution to exposure through the dermal pathway, and different MW. As both have 5 rings, it would be beneficial to have a 4 or 6 ring PAH included in the focal three to study a broader range of characteristics and their potential influence on dermal absorption. This leaves BghiP (6 ring), IP (6 ring), BaA (4 ring), CHR (4 ring), FA (4 ring) and Pyr (4 ring) (Table 7) to be considered owing to their dermal contribution

to exposure (Table 6). As two PAH have already been selected for toxicity, there needs to be a PAH selected which main purpose is quality control. Larger 6-ring PAH are more analytically variable producing broader chromatographic peaks than 4-ring PAH. Also, with the selection of a LMW 4-ring PAH a broader range of MW will be studied, this leaves FA and Pyr (MW 202 (Table 7)).

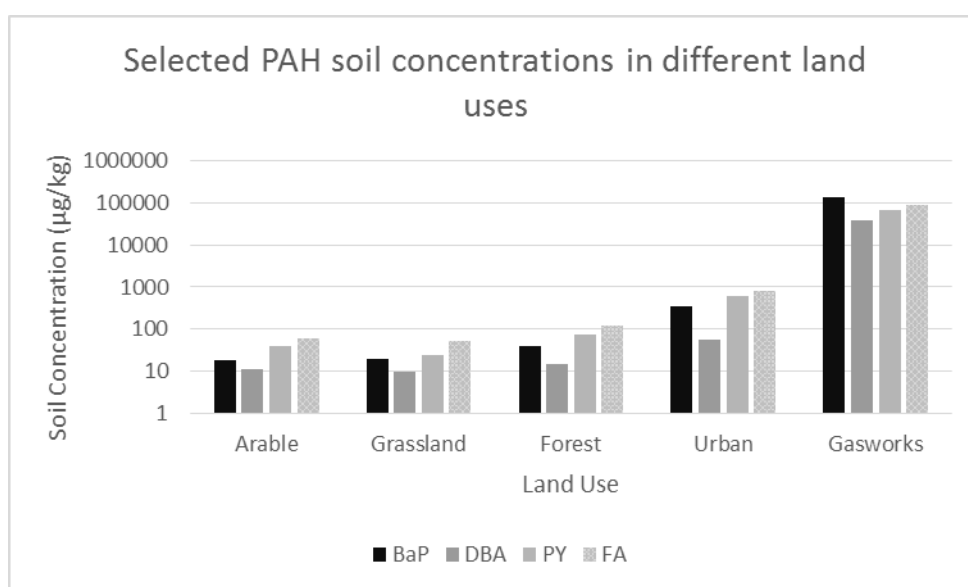


Figure 10: Soil concentrations of BaP, Pyr, DBA and FA at different land uses. Data for Arable, grassland, forest and urban soil are mean concentrations from Wilcke (2000); data for gasworks PAH concentrations from Potin et al. (2004).

Figure 10 shows mean soil concentrations for various land uses. FA and Pyr both have similar concentrations throughout. They are both Classified in IARC group 3 (Table 1 and 2), both produce good chromatographic peaks, and both have a MW of 202 (Table 4). Pyr has a lower K_{ow} at 5.18 compared to FA, giving a broader range of K_{ow} covered (also if BghiP was used the range would be smaller). No low molecular weight (LMW) PAH are to be prioritised in this study, as these compounds are present in lower concentrations than high molecular weight (HMW) in aged soils owing to the natural attenuation process and the preferential degradation and volatilisation of LMW PAH (Neff et al., 2005, Barnier et al., 2014). Although some LMW PAH such as naphthalene have been classified as group 2B compounds (possible human carcinogen) (IARC, 2010), the fact that they are often present in low concentrations and are prone to volatilisation, the soil-dermal pathway contributes a lower exposure for LMW PAH compared to the inhalation exposure pathway (Table 6) and this volatilisation would also result in analytical uncertainty. Pyr is the final congener to be focused on, as this congener is abundant in soils of different land use, it can be used to compare sensitivity and

also for quality control. Pyr is believed to be not as toxic as the other two congeners, and is placed in IARC group 3 (not classifiable as to its carcinogenicity to humans) due to lack of data (IARC, 2010), this PAH is ever present in soils (Figure 10) and is more analytically stable than 6-ring PAH, which is why a focus on this PAH would be beneficial to the study for quality control purposes.

With all the above considered, the three PAH (3-PAH) to be focused on during this research are: benzo[a]pyrene (BaP), dibenz[a,h]anthracene (DBA) and pyrene (Pyr).

2.7 Implications of Key Findings in the Literature

In summary, from the reviews of the literature above, the key points for consideration in this work are:

1. The current soil PAH dermal bioavailability value of 13% (Wester et al., 1990) used in HHRA (Section 2.5) holds many uncertainties and is likely to be unrepresentative of true exposure owing to being derived from a PAH spiked soil, not accounting for the effect a variety of soil properties has on PAH bioavailability (Sections 2.1.3, 2.3.2.2 and 2.3.4).
2. Owing to the advantages of the reproducibility, lower ethical considerations, and greater accuracy in determining the fate of PAH in the experimental system, an *in vitro* method was developed (Section 2.3.4).
3. By generating soil-specific dermal bioavailability values, the effects of soil properties and characteristics on PAH dermal bioavailability will be accounted for, allowing for greater certainty when performing human health risk assessments for contaminated land management (Sections 2.1.3, 2.3.2 and 2.5).
4. Former gasworks soils will be used in method development/testing owing to their high PAH concentrations (Section 2.4.3) because:
 - a. They are relevant for the intended purpose of the developed method (Section 2.5).
 - b. The high PAH concentrations provides a greater probability of dermally absorbed PAH being above a concentration that would allow quantification.
 - c. The soils have been contaminated with parent PAH and undergone weathering and aging (Section 2.1.3), therefore are more representative of potential real-world exposure than when using solvent spiked soils (Sections 2.3.2 and 2.3.4).

5. By using an artificial skin membrane such as the Strat-M™:
 - a. Clear differences in chemical absorption inter and intra species when using *ex vivo* skin will be avoided (Sections 2.2.5 and 2.3.2).
 - b. Owing to a more consistent structure and chemistry in comparison to *ex vivo* skin, the use of the Strat-M™ may reduce variability of absorption data (Sections 2.2.5, 2.2.6 and 2.3.4).
 - c. Ethical considerations associated with using *ex vivo* skin will be eliminated (Section 2.2.6).
 - d. Artificial membranes can be more readily acquired than human skin donors (Section 2.2.6).
 - e. The similarity and variability of PAH absorption through the Strat-M™ can be assessed by comparing results to previously published *ex vivo* PAH absorption data (Section 2.3.4).
6. It is common practice for PAH dermal bioavailability studies to focus on select PAH from the US EPA 16 (Section 2.3.2), owing to the time and resources available during the PhD, this project shall do the same by focusing on BaP, DBA and Pyr (3-PAH) (Sections 2.3.2 and 2.6).
7. Comparability between studies is also an issue in the literature (Section 2.3.4), which will be overcome in this study by:
 - a. Using a high soil loading for dermal bioavailability experiments as this produces an infinite dose scenario (Frasch et al., 2014), meaning that J_{ss} (steady-state flux) can be calculated for the soils tested in this study and compared to previous work (Sections 2.3.2, 2.3.3 and 2.3.4).
 - b. This study quantifying the dermal bioavailability of 3-PAH from a CRM to improve future inter and intra laboratory comparability of methods and quality control, if future works test the same CRM (Section 2.3.2 and 2.3.4).

3 General Methods

An overview of the methods for sample collection, preparation and PAH quantification (Figure 11) are described in this chapter.

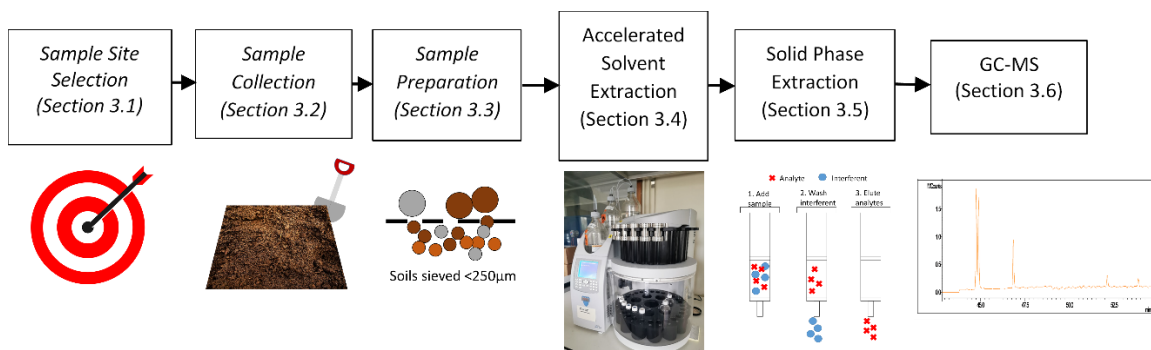


Figure 11: Diagram of soil sample PAH quantification process. *Italicised text represents stages of PAH quantification undertaken prior to author commencing his PhD project.*

3.1 Sample Site Selection

Sampling locations and sites were selected prior to the author beginning his PhD studies. UK former gasworks soils were collected owing to gasworks sites having soils with PAH concentrations elevated above background, often requiring further investigation and clean-up under Part 2A of the Environmental Protection Act 1990 (Sections 2.4.3 and 2.5). This project was fortunate to hold a CASE partnership with National Grid Property Holdings, who provided expert advice and site access. The site names and locations are confidential, consequently sample references are anonymised throughout this project.

The gasworks soils (Soil A and Soil B) used in this study were collected from the same former gasworks site in the UK. The former gasworks began operation in 1848, acting as a small-town gasworks. Coal gas was manufactured at the site until 1936 using low temperature stop ended horizontal retorts (Beriro et al., 2020, Thomas, 2020b). A summary of the two soils can be found in Table 8.

Table 8: Description of gasworks soils (Soil A and Soil B) used throughout this project. TOC(%) is Total Organic Carbon.

Soil	Industrial Activity	Soil Classification	TOC(%)
Soil A	Gasworks	Sandy Made Ground	11.72 ^a
Soil B	Gasworks	Sandy Made Ground	8.92 ^a

^aAs measured by Beriro et al (2020).

3.2 Sample Collection

Remediation contractors and National Grid Property Holdings provided the site access, personnel and equipment for fully supervised collection under their respective health and safety management systems.

The date, coordinates, sampling depth and description of soils were recorded for each sample. Window sampling as well as hand dug pit sampling approaches were used to collect soils. Each soil sample was placed in a 200 mL amber glass jar, stored in a cool box with a maintained temperature as close to 0°C for transport to the BGS. Once at the BGS the soil samples were stored in a chest freezer until sample preparation.

All work involving gasworks soils from collection to laboratory work followed the guidance and protocols of the Control of Asbestos Regulations 2012 (CL:AIRE, 2016).

3.3 Sample Preparation

Soil samples underwent sample preparation prior to the author beginning his PhD studies. Prior to comminution, debris such as stones, wood, metal and plastic were removed.

Soil samples were removed from the chest freezer, lids of the glass jars were removed, and samples transferred to a Christ Alpha 1-4 LD freeze dryer. Samples were then <0.5 mbar pressure at -50°C for 72 h. The sample container lids were then replaced, and the samples stored in a cool dry dark environment until disaggregation and sieving. Freeze drying was selected as it has previously been demonstrated that this approach provides greater data repeatability in comparison to other drying methods such as oven or air drying (Beriro et al., 2014).

Soils were disaggregated manually using a porcelain pestle and mortar. Soil samples were then passed through a stainless-steel mesh sieve with apertures of 250 µm. This size fraction was used owing to the <250µm soil fraction being the upper particle size limit that has been shown to adhere to children's hands, who are regularly the critical the receptor in the human health risk assessment of contaminated sites (Duggan et al., 1985, Kissel et al., 1996, Abdel-Rahman et al., 2002, Choate et al., 2006, Cave et al., 2010, Beriro et al., 2020). Following sieving, samples were stored in 150 mL airtight amber glass jars in dry and cool conditions away from sunlight until soil PAH extraction.

3.4 Accelerated Solvent Extraction (ASE)

Accelerated Solvent Extraction (ASE) is a method used to extract PAHs from solid sample media such as soils. It is performed at temperatures between 40-200°C to enhance elution speed and pressures between 1000-2500psi to keep the solvents in liquid form (Wang et al., 2007).

The ASE method used to extract PAH from soils was a BGS tried and tested standard method. The method is based on the Dionex (2011) application note 313, which meets the requirement of US EPA method 3545 (USEPA, 2007). To quantify the PAH concentration of each soil, 0.5g of each soil was weighed (Sartorius LP620S (± 0.001 g)) (5 replications) and spiked with 15 μ g of surrogate standards (pyrene-d10, benzo[b]fluoranthene-d12, benzo[a]pyrene-d12 and benzo[g,h,i]perylene). The spiked soil was then mixed with copper powder (≈ 2 g) to remove elemental sulphur. The soil and copper mixture was then mixed with granular anhydrous sodium sulphate to ensure thorough sample dispersal for effective ASE extraction. The soils were then extracted by ASE (Dionex™ ASE 350, Figure 12) using acetone:DCM (50:50). Full ASE operating parameters are shown in Table 7.



Figure 12: Dionex™ ASE 350 used for PAH extraction from soils.

Table 9: ASE operating parameters for the extraction of PAH from soils.

ASE Soil PAH Extraction Method	
Solvents	DCM:Acetone
Solvent Ratio	50:50
Flush (% vol)	50
Pressure (psi)	1500
Temp (°C)	100
Purge (secs)	60
Preheat (mins)	0
Heat (mins)	5
Static (mins)	5

The resultant extracts were reduced in volume to 2-3 mL using a TurboVap (Caliper Life Sciences), followed by a further reduction in volume to 1 mL volume under a steady stream of dry N₂ gas. Samples were then transferred into labelled 10 mL clear glass vials ready for SPE. Clear glassware was used throughout the extraction process, owing to extract colour being an indicator of the possible PAH concentration of the sample. Furthermore, clear glassware allowed for the author to check for the presence of emulsions in the samples indicating the presence of water. If moisture was visible in any sample, the sample was passed through a sodium sulphate packed Pasteur pipette until moisture was removed.

3.5 Solid Phase Extraction (SPE)

Solid Phase Extraction (SPE) is a common approach for the separation of compound groups post extraction in order to isolate the target compounds so that analytical interference can be reduced (Barranco et al., 2003, Dittmar et al., 2008, Purcaro et al., 2008). SPE involves passing liquid sample through a cartridge containing a solid phase sorbent. The chemical groups within the sample will have varying affinities to the sorbent, allowing the chemical groups to be separated by passing different solvents through the SPE cartridge.

The extract (Section 3.4) was quantitatively transferred to a conditioned polymeric sorbent SPE cartridge (Agilent, Bond Elute TPH w.500 mg Na₂SO₄, 1g sorbent, 3 ml reservoir volume) (Figure 13). SPE was performed using gravity. A full breakdown of the SPE method is shown in Table 8.



Figure 13: SPE of soil extracts.

Table 10: SPE protocol for the separation of PAH from soil extracts.

SPE Protocol	
Cartridge	Agilent Bond Elute TPH
Condition	6 mL hexane
Load	1mL sample
Wash (Aliphatics)	1.5 mL pentane
Wash (Aromatics)	6 mL hexane:isopropanol (97:3)
Wash (Other)	6 mL DCM

Briefly, the cartridge was conditioned prior to use with 6 mL of hexane, immediately before the introduction of the sample. The aliphatics (fraction A) were eluted using 1.5mL (0.5 ml + 1 mL) of pentane. The PAH containing fraction (fraction B) were eluted 6 mL hexane:isopropanol (97:3). This fraction was then reduced to a volume of 1mL and transferred to a GC-MS vial. Internal standards were then added (200pg/ μ L (fluoranthene-d10, perylene-d12 and indeno[1,2,3-cd]pyrene-d12) prior to analysis by GC-MS.

3.6 Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS was used for the quantification of PAH throughout this project. Chromatography involves the partitioning of a compound between two phases, one mobile (gas) and one stationary (solid). In a sample mixture, the compounds partition to a different degree

between the two phases depending upon their respective solubility in the mobile and stationary phase. As the compounds in the mixture are carried along by the mobile phase over a fixed bed of stationary phase, they will be slowed to varying amounts due to their different solubility and will become separated physically. In the GC the mobile phase is an inert carrier gas (e.g., H₂, He, N₂) and the stationary phase is a high molecular weight liquid which is deposited either on the surface of finely divided particles or on the walls of a long capillary tubing. The GC column introduces samples into carrier gas stream at a temperature high enough to vaporise all components, with the sample injected by microliter syringe and a detector (MS) attached directly to the column exit, monitors individual sample components as they are eluted from the column, with a recording of detector response with time forms a chromatogram (Karasek and Clement, 1988).

GC-MS has long been used for the analysis of PAHs due to it being a cheap, time efficient technique that also provides good sensitivity. To achieve good chromatographic separation, column oven temperature and the oven program (temperature ramp rate and temperature holding time) needs to be optimised (Britten and Naikwadi, 2009). There are also specialised PAH GC columns available (e.g., the “Select PAH” column by Agilent) to improve this separation.

GC-MS analysis of soil PAH was performed on a Thermo Scientific Trace 1300 gas chromatograph (GC) coupled to a Thermo Scientific TSQ9000 triple quadrupole mass spectrometer operating in single ion monitoring (SIM) mode (Figure 14).



Figure 14: Thermo Scientific Trace 1300 gas chromatograph (GC) coupled to a Thermo Scientific TSQ9000 mass spectrometer (MS).

Standard GC and MS operating conditions for PAH analysis are shown in Table 11.

Briefly, samples were introduced to the GC by programmable temperature vaporising (PTV) injection operated in splitless mode (1:10, 60°C to 300°C for 2 min at 14.5°C/s). The GC column used was a fused silica Agilent Select PAH column (Table 11). The GC oven-temperature programme began at 70°C (2 min isothermal) to 180°C at 85°C/min to 230°C at 2.7°C/min (7 min isothermal) to 280°C at 28°C/min. (10 min isothermal) to 350°C at 14°C/min (8 min isothermal). The carrier gas (helium) rate was 2 mL/min. All data processing was performed using Chromeleon software (version 7.2.10).

It should be noted that before the use of the Thermo Fisher GC-MS, a Varian CP-3800 GC coupled to a Varian 1200L triple quadrupole MS was used for method development. Ideally the same instrument would have been used throughout this project to minimise analytical variability. However, owing to maintenance issues with the Varian GC-MS owing to age and difficulty in locating required parts, the project analysis moved to the Thermo Fisher GC-MS after the dermal absorption of PAH from a solvent vehicle experiment. However, all soils and dermal absorption of PAH from soil measurements were carried out on the Thermo Fisher GC-MS, providing greater analytical sensitivity, and no change in analytical equipment within each individual experiment conducted throughout the project. The Varian GC-MS method is available in Appendix 8.1.

Table 11: Thermo Scientific TSQ 9000 GC-MS Standard operating conditions for PAH analysis.

GC operating conditions	
Description	Parameter
Instrument	Thermo Scientific Trace 1300 gas chromatograph
Injector	1 µL PTV injection operated in splitless mode (1:10, 60°C to 300°C for 2 min at 14.5°C/S)
Carrier gas	Helium (2 mL/min)
Column	Varian Select PAH column (30 m length × 0.25 mm i.d. × 0.1 µm film thickness)
Interface temperature	320°C
Oven temperature program	70°C (2 min isothermal) to 180°C at 85°C/min to 230°C at 2.7°C/min (7 min. isothermal) to 280°C at 28°C/min (10min. isothermal) to 350°C at 14°C/min (8 min. isothermal)
MS Operating Conditions	
Description	Parameter
Instrument	Thermo Scientific TSQ9000 triple quadrupole mass spectrometer
Mode of operation	Single Ion Monitoring (SIM)
Ionisation energy	70 eV
Source Temperature	250°C

The use of SIM quantification relies on the detection of selected ions as the chromatogram evolves. Internal standards are used as a reference during quantitative analysis, with the ratio of the peak area of the internal standard to analyte peak area used for quantification. Surrogate standards are used to determine extraction efficiency, which can then be corrected for during data processing (Karasek and Clement, 1988). A list of analytes, surrogates and internal standards and their respective ions monitored are shown in Table 12.

Table 12: Ions used for quantification of analytes, surrogate, and internal standards. I- number shows that analyte is an internal standard and for what analyte it was used. S- refers to a surrogate standard and for which analyte it was used.

Analyte	Reference to Internal Standard and Surrogate	Quantitation Ion	Confirmation Ion
Fluoranthene-d10 (I-1)	S-1	212	208
Pyrene-d10 (S-1)	I-1	212	208
Pyrene	I-1, S-1	202	200
Benzo[b]fluoranthene-d12 (S-2)	I-2	264	250
Benzo[a]pyrene-d12 (S-3)	I-2	264	260
Benzo[a]pyrene	S-2/S-3, I-2	252	250
Perylene-d12 (I-2)	S-2/S-3	264	260
Indeno[1,2,3-cd]pyrene-d12 (I-3)	S-4	288	284
dibenz[a,h]anthracene	S-4, I-3	278	276
benzo[g,h,i]perylene-d12 (S-4)	I-3	288	284

For BaP quantification, a choice of one of two surrogates were used (Table 12). This is due to the BaP-d12 (S-3) regularly failing QC, although other surrogates passed QC. It was assumed that the BaP-d12 standard was a bad batch and, in its place, BbF-d12 (S-2) was used.

3.7 Quality Assurance and Quality Control

Throughout the quantification of PAH concentrations in soils, different measures were put in place for quality assurance and control.

Quality control samples were used for the assessment of analytical precision and accuracy, following principles set out by the Environment Agency (2018). The guidelines were established using proven international standards for the chemical testing of soil to ensure

generated data is of high quality (Environment Agency, 2018). A summary of quality control samples used are shown in Table 13.

Table 13: Quality control measures and acceptance criteria for the laboratory extraction and analysis of soil PAH samples

Laboratory Method	Quality Control Samples	Assessment Criteria
ASE and SPE of soils	5 duplicate soil extracts per soil	±15% RSD
	1 blank extraction (no soil) per batch	<LOQ
GC-MS Analysis of Soils	Analytical Standard x2 beginning and end of sample batch (5-10 samples)	±15% RSD
	Certified Reference Material (1 per sample batch)	±15% RSD 80 - 100% recovery of certificate values
	Procedural blank (1 per sample batch)	<LOQ
	Analytical Blank (1 per sample batch)	<LOQ

Both procedural and analytical blanks were used, as this would aid in the determination of contamination sources if blanks failed their respective assessment criteria (Table 13). The limit of quantification (LOQ) for each compound were determined using the signal-to-noise (S/N) ratio of 10 (noise calculated for each peak on a blank chromatogram at the respective retention time) (Tennakoon et al., 2009, Canbay and Doğantürk, 2019, Aresta et al., 2020).

Quality assurance was achieved by following BGS laboratory good practice and quality monitoring system. The quality assurance measures are shown in Table 14.

Table 14: Quality assurance measures used throughout this project.

Quality Assurance Activity	Actions
Balance Checks	Checks performed and recorded using 0.001g, 0.002g, 0.005g, 0.01g, 0.02g, 0.05, 0.1g, 0.2g, 0.5g, 1g and 2g weights. If deviation $>\pm 1\%$ recalibration required.
Use of chromic acid cleaned glassware	Glassware submerged in chromic acid for 24 h prior to rinse (x5) with DI water.
ASE extraction vessels cleaning	ASE extraction cells disassembled and sonicated for 5 min in acetone:DCM (50:50). ASE cell components left to dry in fume cupboard overnight prior to reassembly.
ASE Rinse	ASE 350 underwent rinse cycle prior to each sample batch to ensure no residual analytes from previous extractions are in the system.
Use of personal protection equipment (PPE)	Nitrile gloves, safety glasses, laboratory coat. (Disposable coveralls, FFP3 mask, HEPA filtered laminar flow cabinet, HEPA vacuum cleaner used in addition for asbestos containing samples).
Storage of soil samples	Samples stored in amber jars in cool dry dark environment.
Storage of liquid samples	Stored in fridge at $<4^{\circ}\text{C}$. Fridge temperatures checked regularly to ensure correct storage temperature (refrigerator logs).
Use of DI water for equipment rinse	DI water produced by BGS.
Use of Milli-Q water for reagent preparation	Milli-Q water produced at BGS laboratories.
Syringe cleaning prior to/during use	μL syringes rinsed with full syringe volume of methanol, toluene, or DCM >7 times before use and between samples or changes in liquid.
Use of appropriate purity solvents (at least analytical standard)	Ordering and storage of appropriate solvents. Ensuring solvents and other consumables/reagents are in date.
Sample identification	Labelling of samples at all stages of laboratory work.
Laboratory Cleaning	Clean laboratory space with DI water and/or acetone.
Laboratory notebook for record of lab-based activities	Upkeep and storing securely.

4 Method Development

4.1 Methodology

This study, aims to develop a method to quantify the dermal absorption of parent PAH from contaminated soils so that PAH dermal bioavailability can be measured from soils that have undergone the aging process (Section 2.1.3 and 2.3). To achieve this, a different extraction and analytical approach to those in the literature are required to measure parent PAH in soils, membranes, and receptor solutions to allow for the quantification of PAH dermal absorption. Parent PAH will be quantified by GC-MS in all relevant sample media, owing to it being used for PAH quantification at a range of concentrations from a variety of sample media (Law et al., 2002, Bergknut et al., 2004, Afanasov et al., 2009, Lorenzi et al., 2010, Vane et al., 2020, Vane et al., 2021)

4.1.1 *In vitro* measurement of Dermal Bioavailability

The approach selected for the measurement of soil PAH dermal bioavailability was *in vitro* rather than *in vivo*. This is because *in vitro* methods offer greater reproducibility and enhanced control over experimental parameters than *in vivo* approaches. This is largely due to large differences in chemical dermal permeation depending on the anatomical site, the individual and species (Sections 2.2.5, 2.3.2 and 2.3.4). Also, *in vivo* studies often produce lower chemical recovery in comparison to *in vitro*, meaning that there is greater uncertainty of chemical fate within the system, resulting in greater uncertainty of dermal bioavailability data (Sections 2.3.2 and 2.3.4). Furthermore, there are ethical considerations to using an *in vivo* method (Sections 2.2.5, 2.2.6 and 2.3.4). Also, *in vivo* methods when using soils pose the issue of the soil loading on the animal skin being partially or completely removed over the course of the experiment, adding variability and uncertainty to subsequent absorption data. Additionally, *in vivo* testing is often more expensive and offers a lower sample throughput in comparison to *in vitro* methods (Collins et al., 2015). The OECD (2004) and (IPCS, 2006) state that *in vitro* dermal absorption data collected using diffusion cells can be representative of that from *in vivo* studies given that the chemical concentration is quantified in both the membrane and receptor solution. Therefore, methods needed to be derived for the quantification of PAH in the membrane and receptor solution by GC-MS.

There is still uncertainty as to whether *in vitro* methods are representative of *in vivo* dermal absorption. This is largely due to a limited number of directly comparable *in vitro* and *in vivo* studies (Williams, 2006). However, the developed method of this study was validated by comparing PAH absorption data from a solvent vehicle to previous *in vivo* studies. If

absorption data from the developed *in vitro* method was within the range of values of those collected from *in vivo* studies, it could be assumed the developed *in vitro* method is representative. Absorption data from a solvent dose was compared as any vehicle effect on the PAH availability for absorption was removed, meaning that PAH is 100% bioaccessible and has the potential to be absorbed. Therefore, any differences in dermal absorption could be attributed to experimental parameters and equipment.

The *in vitro* dermal absorption experiments were designed using the OECD guidelines (OECD, 2004). OECD (2004) state that static or flow-through diffusion cells are acceptable for *in vitro* dermal absorption experiments (Section 2.3.1). A Franz-type cell was selected because there was the possibility that owing to the receptor solution being replenished if using a flow-through diffusion cell design that any PAH dermally absorbed would be below the analytical LOQ. Consequently, the dermal absorption of PAH was measured *in vitro* using a glass Franz-type diffusion cell with an effective diffusion area of 11.3cm² (Figure 15), using the Strat-M™ artificial skin membrane (Section 2.2.6). The diffusion cells are composed of three compartments: The donor compartment (Figure 15, compartment A), which is where the dose is applied to the membrane (Figure 15, compartment B); which is in contact with the receptor solution housed in the receptor compartment (Figure 15, compartment C).

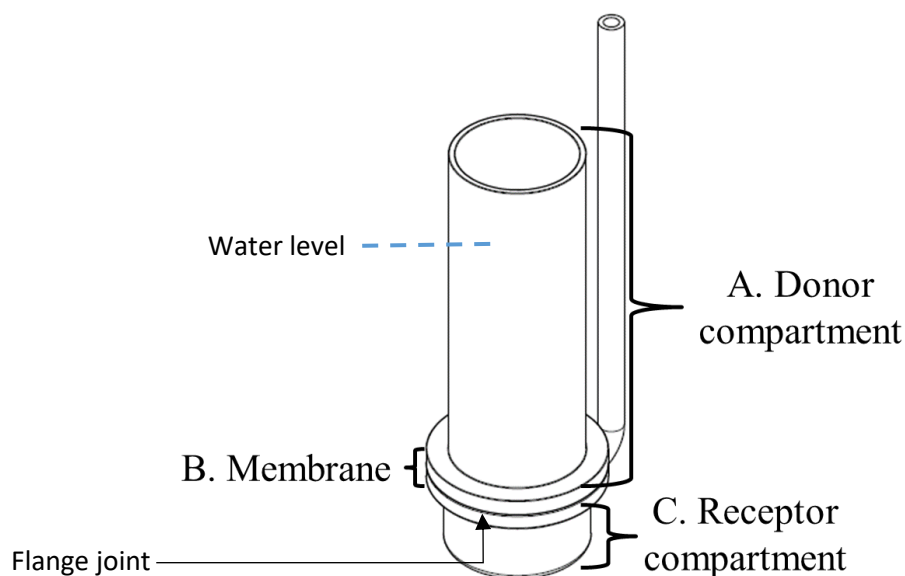


Figure 15: Schematic of the assembled Franz-type diffusion cell and its compartments: compartment A is the donor compartment; compartment B houses the membrane and compartment C houses the receptor solution. The blue dashed line indicates the level to which the diffusion cell was submerged in the water baths during the experiments. The flange joint is where the donor and receptor compartments are joined/separated during diffusion cell assembly/disassembly.

Typically, the receptor solution is sampled at different times throughout the duration of a dermal absorption experiment (known as time-steps) in order to produce a chemical dermal absorption curve (such as in Abdel-Rahman et al. (2002)) and also allows the calculation of flux (the rate of chemical diffusion over time) (Crank, 1975, Beriro et al., 2016). Owing to the potentially low concentrations of PAH that permeate the membrane into the receptor solution, a diffusion cell was removed at each time step. This enabled the whole ≈ 30 mL of receptor solution to be extracted and concentrated, therefore lowering the analytical PAH LOD in the receptor solution. The removal of individual diffusion cells at each time step may introduce some variation in comparison to subsampling the same diffusion cell over the experimental duration, but by lowering the LOD the time at which PAH reach the receptor solution will be better represented. The length of time taken for PAH to enter the receptor solution is of great interest to human health risk assessors, as this represents the time for which PAH take to diffuse from soil into and through the skin and enter systemic circulation (CL:AIRE, 2014b).

The diffusion cells were assembled, with degassed receptor solution along with a glass magnetic stir bar placed in the receptor compartment, ensuring that the receptor solution was in contact with the Strat-M membrane with no gas bubbles (OECD, 2004). The diffusion cells were held together using bulldog clips around the flange of the donor and receptor compartments (Figure 15). The assembled diffusion cells were submerged in a water bath (Figure 15) at 32°C (surface temperature of human skin (Sartorelli et al., 1999, OECD, 2004, IPCS, 2006)), with the magnetic stirrer set at 100rpm, and allowed to stabilise for 2 h to allow for the receptor solution to reach 32°C. The purpose of the magnetic stirrer in the receptor compartment is to promote the circulation of the receptor solution in the receptor compartment, so that “fresh” solution can be in contact with the membrane over time so that diffusion of PAH from the membrane into the receptor solution is not inhibited (OECD, 2004). Although, if the stirrer bar rpm is too high, there is the potential to increase flux by drawing down compounds in the membrane, resulting in an over estimation of the amount dermally absorbed. After stabilisation, each diffusion cell was removed from the water bath and checked by eye to ensure no gas bubbles had formed in the receptor solution prior to dosing.

4.1.2 Quantification of PAH in Diffusion Cell Compartments

PAH needed to be quantified by GC-MS in all three diffusion cell compartments in order to meet the main aim of the project: to develop an *in vitro* based method for measuring the dermal bioavailability of PAH in soil. This was achieved through developing methods to dose, extract and quantify Pyr, BaP and DBA (3-PAH) from the Strat-M™ membrane, receptor solution and contaminated soil for quantification by GC-MS. This section outlines how each experiment was designed (Figure 16) to be able to meet the aim and objectives of this project.

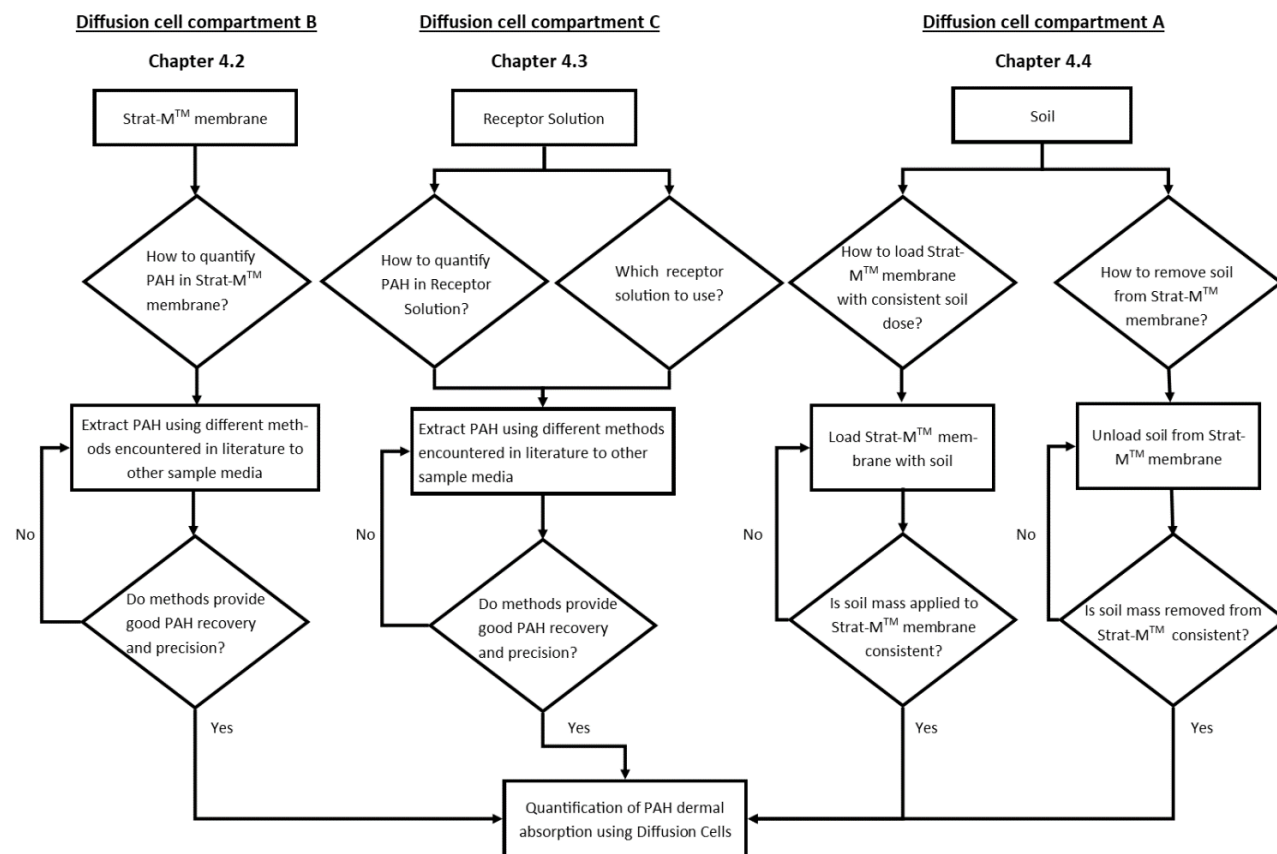


Figure 16: Method development chapter experimental flow chart. These experiments enabled the consistent PAH dosing and PAH quantification for the developed in vitro dermal bioavailability method.

4.1.2.1 Strat-M™ Artificial Skin Membrane

Figure 16 shows the experimental flow chart for the development of a method for the quantification of PAH in the Strat-M™ membrane. For this study, owing to the complications associated with attaining, storing and reproducibility of *ex vivo* skin an artificial skin membrane was used (Section 2.2.6). The Strat-M™ membrane has been designed to mimic the structure and chemistry of the multiple layers of full thickness skin. The membrane is composed of polymer layers (polyethersulfone), which increase in porosity with depth from the surface, reflective of human skin. It has been shown in previous work to produce similar dermal absorption data of chemicals to *ex vivo* skin (Uchida et al., 2015, Haq et al., 2018). Owing to the Strat-M™ membrane being engineered, it should be less variable in structure and composition in comparison to *ex vivo* skin. Consequently, by using an artificial membrane, the variability of absorption data due to the membrane should be reduced. There was some risk in selecting this membrane as there was uncertainty as to whether it would produce similar PAH dermal absorption data to previous *ex vivo* studies. The Strat-M™ membrane was validated by comparing BaP dermal absorption data from a solvent vehicle through the Strat-M™ membrane to previous studies which used *ex vivo* skin. The solvent vehicle dosing of PAH to the membrane ensured that BaP was 100% bioaccessible, meaning the rate limiting factor for absorption of BaP was the membrane itself. If BaP absorption through the Strat-M™ was within the range on literature *ex vivo* data, it was assumed that the Strat-M™ membrane was representative of *ex vivo* human skin.

The OECD (2004) and IPCS (2006) state that *in vitro* methods can only be used to produce dermal absorption data for the human health risk assessment if chemical concentrations of both the membrane and receptor solution are quantified. However, previous studies which have used the Strat-M™ membrane did not quantify PAH absorbed into the membrane itself (Uchida et al., 2015, Haq et al., 2018). Therefore, in order to develop a method that follows guidelines, a method needed to be established for the quantification of PAH in the Strat-M™ membrane (Figure 16). This was achieved by comparing the recovery and precision of PAH from the Strat-M™ membrane using methods found in the literature for extraction PAH from lipophilic sample media. PAH were applied to the membranes in solvent, to ensure no dosing vehicle effect on the extraction methods were observed. PAH extraction methods compared included: Accelerated Solvent Extraction (ASE) (Heemken et al., 1997, Zuloaga et al., 1998, Kim et al., 2018), Soxhlet (SOX) (De Castro and Garcia-Ayuso, 1998), Sonication (SON) (Guerin, 1999, Purcaro et al., 2009, El Hussein et al., 2018), and two Saponification Methods (SAP1 and SAP2) (Hyötyläinen et al., 2002, Anastasio et al., 2004, Xia et al., 2016).

4.1.2.2 Receptor Solution

The receptor solution (RS) is housed in the receptor chamber of the diffusion cell of choice, and in dermal in diffusion studies is often a simulated body fluid (SBF) with ion concentrations similar to that of human blood plasma (Oyane et al., 2003). IPCS (2006) and OECD (2004) suggest that the receptor solution should not limit the diffusion process by the compound(s) of interest being insoluble in the receptor solution. It is recommended that for lipophilic compounds (such as PAH), bovine serum albumin (BSA) or solvent mixtures such as ethanol:water should be incorporated into the receptor solution (Sartorelli et al., 2000, OECD, 2004). Human serum albumin (HSA) is the most abundant plasma protein, with one of its main functions being the transportation of metabolites around the body (Choi et al., 2004). So that the receptor solution is not rate limiting, BSA, HSA's bovine counterpart, was used in two of the three recipes tested. BSA is believed to bind the lipophilic solutes, consequently creating a sink that enables partitioning into the receptor solution from the membrane.

Figure 16 shows how PAH quantification of the optimal receptor solution was determined. Two methods have been encountered in the literature for the analysis of compounds in saltwater: solid phase extraction (SPE) (Dittmar et al., 2008, Trettin et al., 2014) and liquid-liquid extraction (LLE) (Reddy and Quinn, 1999, Keating et al., 2018). These extraction methods were assessed by dosing receptor solution with a known PAH concentration, and assessing the extraction recovery and precision, along with extraction method practicality.

To assess the optimal RS recipe to be used in the PAH dermal bioavailability method, three commonly used RS recipes from the literature were compared for their recovery (%) of PAH from the RS. The three RS recipes compared were: Hanks HEPES buffered receiver solution (HBSS) with 4% BSA (Moody et al., 1995, Moody and Joncas, 2007, Moody et al., 2011), phosphate buffered saline (PBS) with 4% BSA (Ackermann et al., 2010, Uchida et al., 2015, Peckham et al., 2017), and aqueous solution 6% polyethylene glycol 20 oleyl ether (Volpo-20TM/Brij-20TM) (Yang et al., 1989, Roy and Singh, 2001). It was assumed that owing to their use in previous studies, and that the recipes follow to OECD (2004) and IPCS (2006) recommendation for lipophilic compounds, that the receptor solutions selected do not limit the rate of PAH absorption in the dermal absorption experiments.

4.1.2.3 Soil loading and removal

The soil fraction used was <250µm throughout this study (Section 2.3.3). As monolayer coverage is highly unlikely achievable in practice (Spalt et al., 2009), supermonolayer was deemed a preferable loading scenario in comparison to submonolayer (Section 2.3.3). Furthermore, the use of submonolayer coverage would result in broken coverage of the diffusion area of the membrane, leading to a significant variation in soil-membrane contact and therefore PAH diffusion into and through the membrane.

Supermonolayer coverage will also have an impact on the dermal absorption data produced by an experiment. It can be widely seen in the literature that the fraction absorbed of the applied dose decreases with soil loading increase (Roy and Singh, 2001, Kissel, 2011, Frasch et al., 2014). Therefore, due to the range of loadings used in the literature (Chapter 2.3), fraction absorbed of dose applied/bioavailable fraction was not used for comparisons to previous studies. Furthermore, high loadings (such as supermonolayer) used in dermal bioavailability experiments are flux-limited, where there is ample load that cannot be significantly depleted over the duration of the experiment (infinite dose) (Frasch et al., 2014). This allowed for the calculation of BaP steady-state flux (Chapter 2.3), which can be compared to previous works to assess differences in soil PAH dermal bioavailability.

A tried and tested soil PAH extraction and quantification method was used to quantify soil PAH throughout this project (Sections 3.4, 3.5, and 3.6). However, there were no methods were found in the literature which adequately described how to add and remove soil from a membrane for the *in vitro* diffusion cell experiments. One soil removal method using Radiacwash™ was encountered in the literature (Moody et al., 1995, Moody and Joncas, 2007, Moody et al., 2011) But, owing to Radiacwash™ having a pH of 3.5, it is likely that due to a change in the ionic environment when the wash is applied that the polymers (polyethersulfone (PES)) of the Strat-M™ will denature. It was shown by Salgin et al. (2005), that PES interaction with low pH solutions enhances PES membrane absorption. Therefore, the use of Radiacwash™ for soil removal in this study could have resulted in the increased absorption of PAH from the soil into the membrane during the soil removal process, consequently skewing the dermal bioavailability results. It was consequently decided that an alternative method for removing soil from the Strat-M™ membrane would be needed. Therefore, methods needed to be devised to provide the Strat-M™ membrane with a consistent supermonolayer loading and soil removal (Figure 16). One soil was tested, with

soil loading consistency calculated by subtracting the membrane mass empty from the membrane mass with soil loaded. Soil removal efficiency was calculated by subtracting the membrane mass empty to the membrane mass post soil removal. One soil was used for this experiment, and it was assumed that the soil loading and unloading method would be as effective with other soils. Previous works had not factored the impact of soil remaining on the membrane on the PAH concentration of the membrane extract. Therefore, in order to produce accurate dermal absorption data, the PAH fraction of the soil remaining on the membrane needed to be removed from the membrane extract PAH concentration.

4.1.3 Data Analysis

4.1.3.1 GC-MS Data

GC-MS is the most widely used analytical method used to quantify non-polar organic chemicals. GC-MS has been used to quantify the PAH concentration of a variety of sample media, such as soils (Bergknut, 2004, Lorenzi et al., 2010, Vane et al., 2014, Wloka and Smol, 2014, Vane et al., 2021), sediments (Finardi et al., 2017, Kim et al., 2018, Vane et al., 2020), coal tar (Afanasov et al., 2009, Gallacher et al., 2017), even biological samples such as mussels (Law et al., 2002), earthworms (Jones et al., 2008) and plants (Dugay et al., 2002). Previous *in vitro* PAH dermal bioavailability studies have reported that the PAH concentration of each compartment of the diffusion cell decrease in the order soil > skin/membrane > receptor solution (Wester et al., 1990, Moody et al., 1995, Roy et al., 1998). GC-MS is capable of quantifying PAH on the micro, nano and pico scales, providing the analytical capability of quantifying PAH in all three diffusion cell compartments (Wloka and Smol, 2014).

GC-MS was run in selected ion monitoring mode (SIM) for PAH quantification. SIM involves using the instrument to record the ion current at selected masses that distinguish the compounds of interest within an anticipated retention time window. The instrument rapidly scans between the m/z values for the expected characteristic ions within a pre-determined time window (Kitson et al., 1996). This allows for high analytical sensitivity and high analytical specificity to be achieved (Kitson et al., 1996, Clench and Tetler, 2000). It has been established that gasworks soils and coal tar contain a wide variety of compounds (Gallacher et al., 2017), therefore, by running the GC-MS in SIM it allowed for the quantification of PAH in all three sample media even if they were present at low concentrations (Kitson et al., 1996). A full description of the GC-MS operating conditions is provided in in Section 3.6.

Quantification of PAH by GC-MS followed equations and direction given by McDonald et al. (2000) which are summarised below. Chromatographic peak integration was carried out using Chromeleon 7 software (Thermo Fisher). Peak area was used for PAH quantification calculations because peak height can lead to errors in quantitative calculations owing to peak distortion such as fronting or tailing.

A five-point relative response factor (RRF) calibration curve was established (Equation 7). Where: A_A is the peak area of the analyte measured, C_{IS} is the known concentration of the internal standard in the calibration solution ($\mu\text{g}/\text{mg}$), A_{IS} is the peak area of the internal standard, and C_A is the known concentration of the analyte in the calibration solution ($\mu\text{g}/\text{mL}$). Calibration standard concentrations varied depending on predicted PAH concentration of a sample. 50 $\text{pg}/\mu\text{L}$ internal standard five-point calibrations were carried out for quantification of low PAH concentration samples (analyte and surrogate range 10 – 700 $\text{pg}/\mu\text{L}$), and 200 $\text{pg}/\mu\text{L}$ internal standard calibrations (analyte and surrogate range 10 – 700 $\text{pg}/\mu\text{L}$) for samples with higher PAH concentrations.

$$RRF = \frac{A_A \times C_{IS}}{A_{IS} \times C_A}$$

Equation 7: Calculation of relative response factor for GC-MS calibration.

The response factors determined for each calibration level were averaged to produce a mean RRF for each analyte. The RSD% for the five response factors for each analyte must be $\leq 15\%$.

The mass of each analyte (ng) in analysed samples were then calculated using Equation 8.

$$M_A = \frac{A_A \times M_{IS}}{A_{IS} \times RRF}$$

Equation 8: Calculation of the mass of each target analyte (M_A) (ng).

Where: A_A is the peak area of analyte measured, A_{IS} is the peak area of the relevant internal standard, M_{IS} is the mass of internal standard added to the extract (ng), and RRF is the relative response factor for the analyte from the current calibration.

The PAH concentration (C) (ng/g) was then calculated using Equation 9.

$$C = \frac{M_A \times DF}{W}$$

Equation 9: Calculation of PAH concentration (C) (ng/g).

Where: DF is the dilution factor (Volume of extract (μL)/Volume of extract used to make dilution (μL)), W is the sample weight (g).

PAH analyte concentrations were reported as corrected for surrogate recoveries (3.6). Percent recovery for each surrogate was calculated using Equation 10.

$$SU_{Recovery} = \frac{C_{ESU}}{C_{SU}} \times 100$$

Equation 10: Calculation of surrogate recovery (%).

Where: C_{ESU} is the calculated measured surrogate concentration in the extract, C_{SU} is the known concentration of surrogate standard added to the extract.

Finally, PAH analyte concentrations derived from Equation 9 were corrected for surrogate recovery using Equation 11.

$$C_{Corrected} = \frac{C}{SU_{Recovery}} \times 100$$

Equation 11: Calculation of surrogate recovery corrected analyte concentrations.

Quality control of GC-MS data included: analytical blanks, analysis of PAH standards and replicates. This allowed for analytical precision and accuracy to be assessed for each experiment and ensured that any variation in the data was due to differences in performance of the extraction methods being tested rather than analytical.

The limit of detection (LOD) and limit of quantification (LOQ) for each compound were determined using the signal-to-noise (S/N) ratio of 3 and 10 respectively (noise calculated for each peak on a blank chromatogram at the respective retention time) (Tennakoon et al., 2009, Canbay and Doğantürk, 2019, Aresta et al., 2020).

For the Strat-M™ and receptor solution experiments (Figure 16), PAH spiking solution was used to dose the sample media with a known PAH concentration (250ng PAH dose applied). The PAH spiking solution was created by diluting a PAH standard. The spiking solution PAH concentrations were quantified to provide more representative PAH recovery values rather than assuming a 10x dilution of the stock solution.

4.1.3.2 Calculation of Precision

The Relative Standard Deviation (RSD) is defined as the ratio of the standard deviation to the mean (Equation 12) (Gregory, 1978).

$$RSD(\%) = \frac{\sigma}{\mu} \times 100$$

Equation 12: Calculation of relative standard deviation (RSD) (%).

Where: σ is the standard deviation, and μ is the mean. The RSD is often used to assess precision of analytical techniques and shows the extent of variation in relation to the mean often expressed as a percentage. The lower the RSD% the less variability (i.e., greater precision) in the results of the extraction (Everitt, 1998).

The importance of variability for the development of an *in vitro* dermal absorption method is that there are three compartments that needed to have their PAH concentrations measured in order to understand the spread of PAH throughout the system and the amount of PAH dermally absorbed. Therefore, any variation in each compartment would add to the total cumulative variation of the overall method. Furthermore, the lower the variation in results generated in each compartment will result in a method that produces results with greater precision, which is critical for contaminated land human health risk assessment.

4.1.3.3 Calculation of Accuracy

Accuracy is defined as the degree of closeness to the true value (Palmer, 2014). Accuracy was calculated using Equation 13.

$$Accuracy (\%) = \frac{(C_A - C_o)}{C_A} \times 100$$

Equation 13: Calculation of accuracy (%).

Where: C_A is the accepted/certificate value of an analyte, and C_o is the observed/measured valued of an analyte.

The importance of accuracy throughout the development of the *in vitro* dermal absorption method is that the true concentration of PAH in each compartment need to be quantified in order to accurately quantify the dermal absorption of PAH.

4.1.3.4 Calculation and Comparison of PAH Extraction Recovery

Recovery is defined as: *'the proportion of the amount of analyte, present in or added to the analytical portion of the test material, which is extracted and presented for measurement.'* (Thompson et al., 1999).

The OECD (2004) state that all components of the diffusion cell system should be analysed, and chemical recovery determined. This approach allowed for any losses or gains of PAH in the experimental system to be located, and subsequently acknowledged and corrected.

Recovery of PAH from sample media throughout this project was calculated using

$$\text{Recovery (\%)} = \frac{\text{Measured PAH concentration}}{\text{PAH Dose Applied}} \times 100$$

Equation 14: Calculation of recovery (%).

Where measured PAH concentration is concentration determined by GC-MS analysis, and PAH dose applied is the known PAH concentration applied to/contained in the media.

A recovery near 100% (80-120%) gives confidence in the extraction and quantification method (Environment Agency, 2018). For method development, the importance of recoveries near 100% is that it is an indication of an almost complete mass balance for the extraction method being tested. This implies that complete extraction is being achieved, with minimal losses throughout the extraction and analytical procedure. For the quantification of PAH in the Strat-M™ membrane and receptor solution (Figure 16), the extraction method had to achieve a PAH recovery of 80-120% to give confidence that PAH would be able to be quantified in all diffusion cell compartments required for the measurement of the dermal absorption of PAH.

The recoveries of PAH were compared statistically (Figure 17), to determine differences in mean recoveries of different PAH extraction methods.

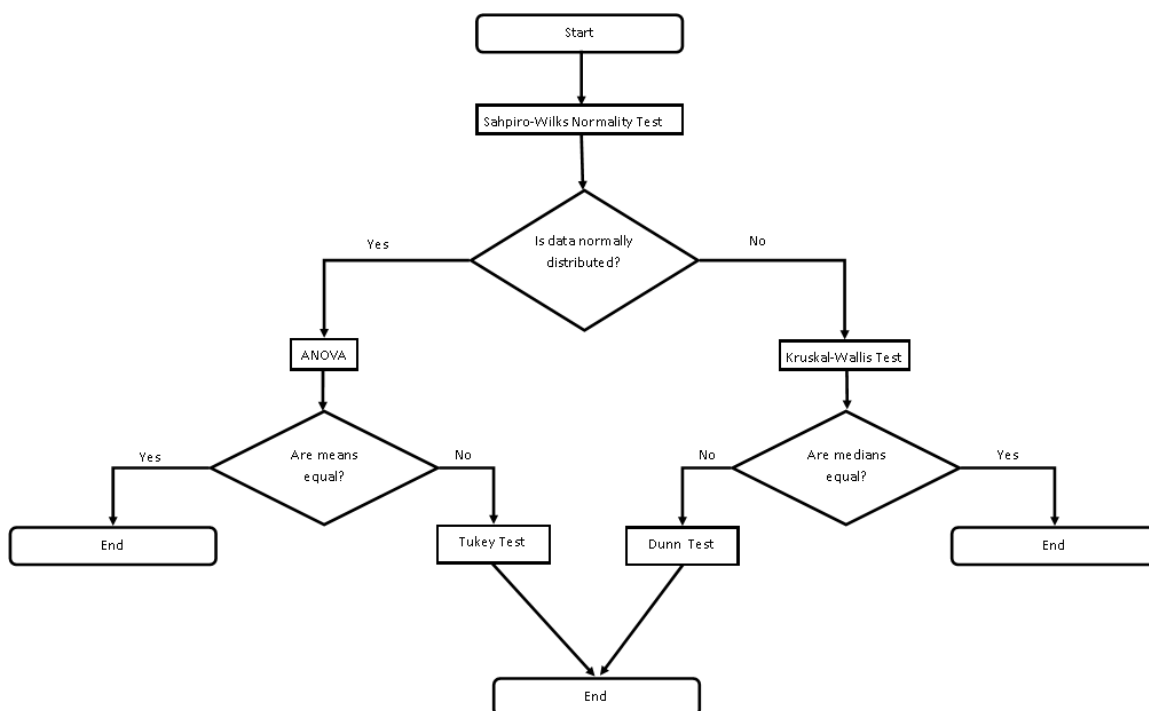


Figure 17: Statistical decision-making flow chart. All statistical tests carried out to 95% confidence.

Figure 17 shows the flow of statistical decision making when testing for differences in 3-PAH recovery (%) from the Strat-M™ membrane and receptor solution (Figure 16). All statistical tests were carried out using R statistical programming language, using packages: readxl, tidyr, ggplot2, plyr, Rmisc, psych, FSA. All statistical testing was carried out to a 95% confidence level.

A Shapiro-Wilks test for normality was carried out on each data set, for which it was concluded that data is significantly different to the normal distribution if $p < 0.05$. The Shapiro-Wilks test was selected because of greater sensitivity even with small samples sizes (required sample size = 3 to 50 (Shapiro and Wilk, 1965)) in comparison to other common normality tests e.g., Kolmogorov-Smirnov test (Ahad et al., 2011). If it was shown that data was significantly different from the normal distribution, a Kruskal-Wallis was used to test differences in median recoveries, followed by a Dunn test post-hoc to locate sources of differences of medians between groups. On the other hand, if the Shapiro-Wilks test found no evidence for data distribution to differ from the normal distribution, parametric tests for differences in means were executed. This was achieved firstly by ANOVA, and if there was evidence for differences in mean recoveries for different extraction methods, a post-hoc Tukey test was used to determine the source of differences in means.

4.2 Selection and Optimisation of a Method to Extract Polycyclic Aromatic Hydrocarbons from the Strat-M™ Membrane

4.2.1 Introduction

The aim of this section is to discuss the selection, and optimise a method for, the extraction of PAH from the Strat-M™ membrane. The Strat-M™ membrane is an artificial skin substitute used in place of *ex vivo* skin often used in diffusion cell dermal chemical absorption testing (Section 2.2.6). The PAH concentration needed to be quantified in the Strat-M™ membrane to determine the fate of PAH in the diffusion cell system (Section 2.7). This was essential for meeting the main aim of this project, quantifying soil PAH dermal bioavailability *in vitro*.

As no evidence was found in the current literature for a method to extract PAH from the Strat-M™ membrane (Section 2.2.6), common extraction methods used to remove PAH from various sample media were assessed. Five laboratory-based methods used in the literature for the extraction of PAH from lipophilic sample media were assessed for PAH recovery, repeatability, as well as practicality (time, cost etc.). These methods are: Accelerated Solvent Extraction (ASE) (Heemken et al., 1997, Zuloaga et al., 1998, Kim et al., 2018), Soxhlet (SOX) (De Castro and Garcia-Ayuso, 1998), Sonication (SON) (Guerin, 1999, Purcaro et al., 2009, El Hussein et al., 2018), and two Saponification Methods (SAP1 and SAP2) (Hyötyläinen et al., 2002, Anastasio et al., 2004, Xia et al., 2016).

Strat-M™ membranes were dosed with USEPA 16 PAH (Section 2.2.1) and were extracted using the aforementioned methods (Table 11 for details). These extracts were then quantified by GC-MS (Section 3.6). Once the best performing extraction method for the quantification of PAH in the Strat-M™ membrane was determined, further optimisation of that method was carried out through the adjustment of different parameters of the extraction method (e.g., temperature).

4.2.2 Methods

Table 15 shows all reagents and materials used in the experiment to derive and optimise a method for the extraction of PAH from the Strat-M™ membrane. The stock solution for the creation of the analytical standards and spiking solution was Qmx PAH-Mix 9 (Qmx Laboratories, UK), which contains 100ng/μL USEPA 16 PAH in cyclohexane. All solvents used were purchased from Fisher Scientific (Loughborough, UK) and of HPLC grade, and were used without further purification.

Table 15: Summary of the reagents and materials used in the experiment to derive and optimise a method for the extraction of PAH from the Strat-M™ membrane.

Reagent/Material	Description	Supplier
Strat-M™ Membrane	Synthetic, non-animal based human skin model.	Merck Millipore (Watford, UK)
PAH Mix-9	100ng/μL USEPA 16 PAH in cyclohexane	Qmx Laboratories (UK)
Deuterated PAH surrogate standards	naphthalene-d8, acenaphthene-d10, dibenzothiophene-d8, phenanthrene-d10, pyrene-d10, chrysene-d12, benzo[b]fluoranthene-d12, benzo[a]pyrene-d12, and benzo[g,h,i]perylene-d12 (1000ng/μL in cyclohexane)	Qmx Laboratories (UK)
Deuterated PAH Internal standards	1-methylnaphthalene-d10, fluorene-d10, fluoranthene-d10, benz[a]anthracene-d12, benzo[e]pyrene-d12, perylene-d12 and indeno[1,2,3-cd]pyrene-d12 (1000ng/μL in cyclohexane)	Qmx Laboratories (UK)
KOH Pellets	Potassium Hydroxide, extra pure, pellets	Fisher Scientific (Loughborough, UK)
Dichloromethane (DCM)	HPLC grade	Fisher Scientific (Loughborough, UK)
Acetone	HPLC grade	Fisher Scientific (Loughborough, UK)
Hexane	HPLC grade	Fisher Scientific (Loughborough, UK)
Isopropanol	HPLC grade	Fisher Scientific (Loughborough, UK)
Pentane	HPLC grade	Fisher Scientific (Loughborough, UK)
Methanol	HPLC grade	Fisher Scientific (Loughborough, UK)
TPH SPE cartridges	Agilent Bond Elute TPH SPE Cartridges	SLS (Scientific Laboratory Supplies Ltd.) (Nottingham, UK)

4.2.2.1 Extraction and Clean-up Methods

To reduce the number of Strat-M™ membranes used the membranes were quartered using a scalpel prior to spiking solution application. All membranes were weighed before spiking and also after each extraction, so that any decomposition of the polyethersulfone layers (Section 2.2.6) (Simon et al., 2016) due to the membrane's exposure to solvents and caustic solutions during the various extraction methods could be evaluated. The same solvents were used in all solvent based extractions to allow for a direct comparison of the methods. The

solvent mixture of choice was DCM:Acetone (1:1), as most methods, in order to recover as much low molecular weight (LMW) and high molecular weight (HMW) PAH as possible, use a mix of both polar and non-polar solvents (Purcaro et al., 2009).

Extractions were performed in triplicate, along with an additional blank, non-PAH-spiked, membrane put through each extraction method. The spiking solution was composed of the US EPA 16 PAHs, in a standard concentration (10 ng/ μ L) in acetone, which was applied to each quartered Strat-M™ membrane with a standard volume of 25 μ L, to achieve membrane dosing of 250 ng of each of the 16 PAH compounds. The acetone of the spiking solution was left to evaporate overnight so that the PAH compounds could bond with the membrane, and so resulting recoveries were not overestimated.

Alongside the PAH extraction from the membrane, the spiking solution was also extracted by each technique without the membrane present. This was so that any impact of the membrane on PAH extraction efficiency could be quantified.

4.2.2.2 Accelerated Solvent Extraction (ASE)

Accelerated Solvent Extraction (ASE) is a common method used to extract PAHs from solid sample media such as soils and sediments (Section 3.4).

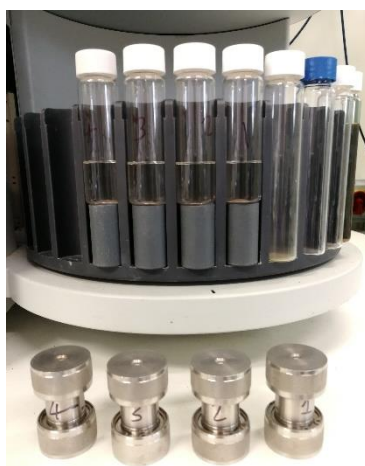


Figure 18: Accelerated Solvent Extraction (ASE). The glass sample collection vials can be seen in the carousel. The metal extraction vessels are on the workbench.

ASE extraction of PAH from sample media is achieved by first placing a known weight of sample media in an extraction vessel (Figure 18) with a dispersant such as NaSO₄. The purpose of the dispersant is to improve the contact of the solvent with the sample media to improve extraction efficiency. The extraction vessel is then placed on the carousel of the ASE (Figure 18), and the extraction vessel is filled with solvent under elevated temperature

and pressure to statically extract the sample. Compressed gas is then used to purge the solvent into sample collection vials (Figure 18) (Richter et al., 1996).

ASE is performed at temperatures between 40-200°C to enhance elution speed and pressures between 1000-2500 psi to keep the solvents in liquid form when heated (Wang et al., 2007). ASE has the benefit of being a temperature and pressure enhanced technique which reduces extraction time, as well as using a lower quantity of solvent compared to traditional solvent extraction techniques such as a Soxhlet extraction, subsequently reducing cost and environmental impact.

The ASE apparatus used was a Dionex™ ASE™ 350, with 10 mL extraction cells. An overview of key operating conditions is shown in Table 16. The method is based on the Dionex (2011) application note 313, which meets the requirement of US EPA method 3545 (USEPA, 2007).

Table 16: PAH Strat-M™ ASE Extraction conditions

ASE Extraction Conditions	
Solvents	DCM:Acetone (1:1)
Flush (%)	50
Pressure (psi)	1500
Temperature (°C)	100
Static Time (mins)	5
Purge Time (secs)	60
Cycles	1

The spiked membrane was added to a 10 mL ASE cell, using NaSO₄ as a dispersant. 250ng of surrogate PAH standards (Table 15) were then added to the cell and left overnight to allow for bonding of standards with the sample matrix. The assembled ASE cells were then loaded onto the ASE and extracted, with each sample taking approximately 20 min. The solvent of the sample collection vial was then emptied into a rotary evaporator flask. The samples were reduced to a final volume of 1mL, first by rotary evaporator, then by a steady stream of N₂ gas. 250 ng of internal standards (Table 15) were added prior to GC-MS analysis (Section 3.6). Extractions without membranes present followed the same steps, but with spiking solution applied to a cellulose filter instead of the Strat-M™ membrane.

4.2.2.3 Soxhlet

Soxhlet (SOX) is a traditional method which involves heating and condensing solvent which passes through the sample media consequently extracting compounds. Soxhlet has been the most widely used extraction method for decades owing to its extraction performance. This has subsequently resulted in Soxhlet often being used to compare the performance of different extraction techniques (De Castro and Garcia-Ayuso, 1998). However, it is more time consuming (24 h run time) and involves larger volumes of solvent than ASE.

Soxhlet extractions were carried out using 250mL round bottom flasks with DCM:Acetone (1:1). Anti-bumping granules (glass) were added to the solvent flasks to prevent the flash boiling of solvents as they were heated. Flasks were filled with 125 mL of DCM:Acetone. Both the solvent and bumping granules were topped up periodically over the course of the extraction to correct for evaporative loss of solvent and smoothing of the anti-bumping granules. The spiked membrane (along with surrogate standard (250 ng)) was introduced to the Soxhlet extractor above the solvent flask in a cellulose thimble and heated to >60°C by a heating mantle. The extraction ran for 24 hrs, after which the heat was turned off and the samples allowed to cool. Once cooled, the samples housed in a round bottom flask were reduced to 1 mL volume using a rotary evaporator, followed by a steady stream of dry N₂ gas. PAH internal standards (250 ng) were added prior to GC-MS analysis (Section 3.6). Extractions without membranes present followed the same steps, but with spiking solution applied to a cellulose filter instead of the Strat-M™ membrane.

4.2.2.4 Sonication

Sonication (SON) has been used to extract PAH from a range of media, such as: soils (Guerin, 1999), smoked meats (Purcaro et al., 2009) and grilled chicken (El Hussein et al., 2018). An ultrasonic bath (Elmasonic P60H, 220-240V, 50-60 Hz, 580W) was used to carry out this extraction method. Unlike ASE, Soxhlet or saponification, it is a non-thermal extraction technique. Sonication involves passing sound waves through a liquid which creates variations in pressure producing cavities which expand and contract in the extraction solvent. This converts sound waves into mechanical energy, which enhances solvent contact with target compounds (Garcia-Vaquero et al., 2017). Cavitation occurs by nucleation, growth and then collapse of bubbles in liquid, which is caused by the bulk pressure variation produced by the travelling ultrasound waves (Garcia-Vaquero et al., 2018).

The spiked membranes and surrogates (250ng) were added to a 10 mL vial containing 9mL of DCM:Acetone (1:1). The samples were sonicated at 40 °C for 3 minutes, with the solvent

pipetted into a 50 mL bottle. This was repeated three times with fresh solvent, and the solvent then combined. The final combined extract in a round bottomed flask was then reduced using a rotary evaporator followed by a steady stream of dry N₂ gas, to a final volume of 1mL.

Internal standards (250 ng) were added prior to GC-MS analysis (Section 3.6). Extractions without membranes present followed the same steps, but with spiking solution applied to a cellulose filter instead of the Strat-M™ membrane.

4.2.2.5 Saponification

Saponification is capable of extracting PAH from lipid matrices (Hyötyläinen et al., 2002, Anastasio et al., 2004, Xia et al., 2016). Owing to PAH being lipophilic, the lipids of the skin are a sink for PAH (Beriro et al., 2020), with this affinity of PAH needing to be disrupted for an effective extraction. This is achieved by using saponification, the alkaline hydrolysis of fatty esters, which involves the splitting of molecules by the addition of water (Killops and Killops, 2005).

Two saponification methods encountered in the literature were tested, KOH-MeOH:Water (1:3) (SAP1) (Xia et al., 2016) and KOH:MeOH (SAP2) (Anastasio et al., 2004) (Anastasio et al. used EtOH rather than MeOH)). The SAP2 method is similar to SAP1, with the only difference being that water is added to the mixture after heating, rather than before such as for SAP1. SAP1 involved placing a PAH spiked membrane, surrogate standards, and 25mL KOH-MeOH:Water (1:3) into a 50mL Pyrex bottle and heating for 4 h at 100°C. The samples were removed and allowed to cool prior to liquid-liquid extraction (LLE).

LLE was carried out using 3 mL of cyclohexane, the cyclohexane was added to the sample bottle and agitated by shaking vigorously for 1 minute. The immiscible layers of cyclohexane and KOH-MeOH:Water were then allowed to separate, with the cyclohexane pipetted off into a 10 mL vial. This was repeated 3 times, with each 3mL combined. The combined 9 mL of sample was then reduced to 1 mL volume using a rotary evaporator, followed by a steady stream of dry N₂ gas.

Internal standards (250 ng) were then added prior to GC-MS analysis (Section 3.6). Extractions without membranes present followed the same steps, but with spiking solution applied directly to the starting solution prior to introduction to the oven.

4.2.3 Results

4.2.3.1 Quality Control

Each extraction method was performed in triplicate with blanks. PAH recovery (%) was calculated using Equation 15. PAH recovery was compared to the respective measured PAH concentration of the spiking solution used to dose the membranes. This allowed for an improved representation of actual recovery of the extraction methods, whilst also considering analytical variation.

$$\text{Recovery (\%)} = \frac{\text{concentration of PAH compound measured}}{\text{concentration of PAH compound measured in spiking solution}} \times 100$$

Equation 15: PAH recovery calculation.

Analytical blanks (DCM) were analysed after samples for each method to ensure no retention of PAH in the GC-MS column, to prevent false positive results.

4.2.3.2 Quantification of PAH recovery from the Strat-M™ Membrane

The recoveries of the three PAH (3-PAH) selected to be the focus (Section 2.6) of this PhD project (Pyr, BaP and DBA) varied between extraction methods (Table 13), overall ASE had the highest mean recovery for Pyr (115.5%) and DBA (74.9%), and SON highest mean recovery of BaP (117.7%), although ASE also had very good mean recovery of BaP (100.5%). ASE produced the least variation for pyrene (3.74%), SON for BaP (0.91%), and SOX (2.04%) for DBA. Extraction recoveries >100% are due to analytical variability during GC-MS analysis.

Table 17: Mean recovery of Pyr, BaP and DBA from the Strat-M™ membrane. The number quoted in brackets is the RSD%

	Mean Pyr Recovery (%)		Mean BaP Recovery (%)		Mean DBA Recovery (%)	
ASE	115.5	(3.74)	100.5	(3.75)	74.9	(5.44)
SOX	112.1	(5.27)	79.3	(4.58)	66.3	(2.04)
SAP1	110.8	(4.72)	85.1	(6.78)	66.9	(5.25)
SAP2	82.7	(25.7)	25.0	(49.5)	15.3	(82.7)
SON	92.8	(6.21)	117.7	(0.91)	67.8	(5.91)

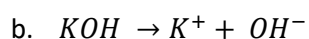
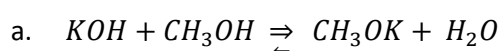
4.2.4 Discussion

4.2.4.1 Comparison of PAH Extraction Methods from the Strat-M™ Membrane

Three of the extraction methods, ASE SAP1 and SON, provided better recovery of 3-PAH than SOX. t-tests were used to determine if there were any differences in the mean recoveries of 3-PAH for ASE, SAP1 and SON. There was no significant difference between then mean

recoveries of 3-PAH using ASE and SON (p=0.3938), ASE and SAP1 (p=0.1905), or SAP1 and SON (p=0.239).

Table 17 also displays the large difference in the two saponification methods, with SAP1 resulting in higher recoveries than SAP2. This is thought to be due to the alkalinity of the two methods, with SAP1 being more alkaline than SAP2 due to the addition of water during different stages of each saponification method. This is due to KOH having a greater solubility in water than methanol resulting in more dissociation of KOH to K^+ and OH^- (Equation 1b). This therefore produces a more alkaline mixture than SAP2 as water is not present until later in the method.



Equation 16: KOH reactions with methanol (a) and dissociation in water (b)

The alkaline conditions create a mutual negative repulsion between the KOH^- and negatively charged functional groups in the polymer chain of the membrane, causing the membrane to warp and breakdown allowing for the hexane to have greater access to the PAH in the membrane. Also, the diphenol ether of the polymer chains can also be hydrolysed during saponification causing the breakdown of the membranes macro-molecular structure (Wang et al., 2015) resulting in greater extraction efficiency of PAH.

Table 18: Mean PAH recovery (%) from the Strat-M™ membrane and no membrane for ASE, SON, SOX, SAP1 and SAP2 extraction methods.

		Mean PAH Recovery (%)				
		ASE	SON	SOX	SAP1	SAP2
BaP	Membrane	101 ± 2.72	120 ± 2.51	79.3 ± 7.19	85.1 ± 17.8	25 ± 49.5
	No Membrane	97 ± 18.6	118 ± 12.1	95.2 ± 18.6	94.9 ± 49.9	94.9 ± 49.9
	Difference (%)	3.59	2.74	-15.89	-9.75	-69.9
Pyr	Membrane	116 ± 7.37	105 ± 16.4	112 ± 19	111 ± 15	82.7 ± 62.7
	No Membrane	112 ± 21.5	92.8 ± 0.87	102 ± 18	99 ± 48	98.7 ± 47.6
	Difference (%)	3.31	12.3	10.57	12.09	-16.0
DBA	Membrane	74.9 ± 3.04	67.8 ± 10.1	66.3 ± 2.90	66.9 ± 15.7	15.3 ± 56.7
	No Membrane	83.0 ± 12.1	66.8 ± 6.78	78 ± 10.1	75.5 ± 62	75.5 ± 61.8
	Difference (%)	-8.09	1.01	-11.82	-8.61	-60.2

Table 18 shows the mean recovery of the 3-PAH when the Strat-M™ membrane is/is not present for each tested extraction technique. This allows for the determination of the effect of the membrane on extraction performance. The lowest mean differences when extracting 3-PAH from the Strat-M™ membrane, and when the membrane was not present were provided by ASE (-0.39%) (Other methods: SON (5.35%), SOX (-5.71%), SAP1 (-2.09%), SAP2 (-48.69)). This suggests the presence of the Strat-M™ membrane does not impact the extraction method performance. There is little difference between the recoveries of 3-PAH from the membrane produced by ASE, SAP1 and SON (Table 17 and Table 18). With any sample preparation/extraction method, there are several challenges, such as: reduction in time, cost of materials and equipment, and error/uncertainty. It is well known in the analytical chemistry field that with the greater number of steps involved, the greater error likely to be introduced (El Hussein et al., 2018).

4.2.4.2 Strat-M™ Membrane Degradation Impact on PAH recovery

The Strat-M™ membrane was weighed before and after each extraction method to test the extent that the membranes are degraded by each extraction method, and whether the loss of membrane mass is connected to the extraction method efficiency.

Figure 19 shows the Mean US EPA 16 PAH Recovery compared to the %membrane loss for each extraction. There appears to be no correlation between membrane loss and recovery of PAH ($R^2 = 0.0009$), therefore it is unlikely that any variation in recovery of PAH from the Strat-M™ membrane could be connected to loss of membrane mass. It is known that the interactions between the polyethersulfone of the membrane and solvents can result in alterations to the membranes through expansion (intumescence), plastification or

dissolution (de Souza Araki et al., 2010). The dissolution of the polymers has been shown to occur through H-bonding between sites of the polymers and solvents such as dichloromethane (Rafiq et al., 2012). The solvents and alkaline mixtures used in the extractions altered the membrane polymers to differing extents resulting loss of membrane mass, but there is no evidence to suggest that this caused the difference in PAH extraction efficiency for each method. It is likely that owing to the lipophilic nature of PAH (Wittig et al., 2003, Lima et al., 2005, Larsson et al., 2018), that the varying success of the different extractions was largely down to the methods ability to dissociate PAH from the lipids of the Strat-M™ membrane (Haq et al., 2018).

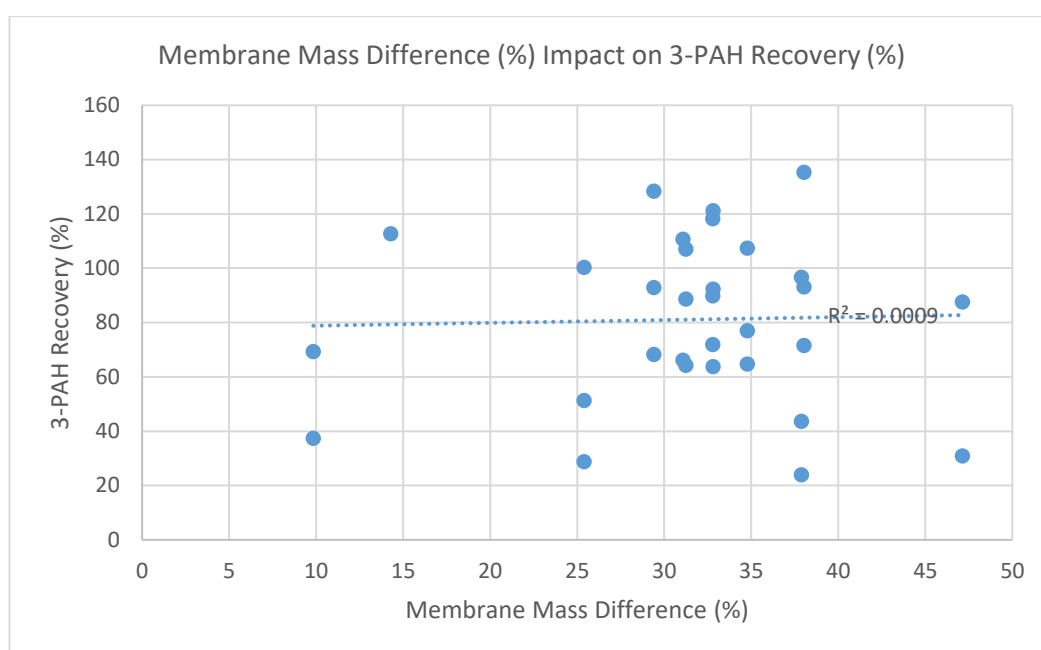


Figure 19: The difference in Strat-M™ mass pre and post extraction impact on mean Recovery% of 3-PAH. ASE data not included due to dispersant adhering to membrane post extraction resulting in a gain in membrane mass.

4.2.4.3 Evidence of Strat-M™ Lipid Content

When the extraction samples were analysed, a large peak of an unknown compound would appear on the chromatogram with a m/z 166, eluting at around 23 minutes into the run, fortunately not co-eluting or interfering with any of the analysed PAH or deuterated standards (Figure 20). As the samples were originally run in SIM mode, only selected ions were analysed so the composition of this compound was largely unknown (Section 3.6). To

overcome this, sample SAP2.1 was diluted by a factor of ten (owing to the relative size of the chromatographic peak) and the sample was analysed under the same GC conditions, but the MS-MS was run in full scan mode (mass range 50-400).

The compound in question appears to be isopropyl myristate, which is the ester of isopropyl alcohol and myristic acid. Myristic acid has been used as part of the fatty acid fraction in artificial sebum recipes (Lu et al., 2009). According to Stefaniak and Harvey (2006), 37% of the fatty acids in the *stratum corneum* are composed of “biologically valuable” fatty acids, with around 6.9% of these being myristic acid which contributes the second largest proportion of these biologically valuable fatty acids with the largest being palmitic acid (25.3%). On the basis of the evidence currently available in the literature (Haq et al., 2018), as well of the findings of this study, it seems fair to assume that the Strat-M™ membrane is treated with myristic acid as part of an artificial sebum recipe applied to the membrane in the manufacturing process in order to emulate the lipophilic nature of the outer layer of human skin.

Isopropyl myristate was present in all extractions. SAP1 and Soxhlet produced the highest responses SOX (85MCounts), with ASE SAP2 and SON producing lower responses (approximately 40MCounts). Although the peak does not interfere with any peaks required for the quantification of 3-PAH, and isopropyl myristate was not present in solvent blanks, it could reduce the lifespan of the GC column and may also have adverse effects on GC-MS operation. Therefore, the presence of isopropyl myristate should be reduced as much as possible by selecting the best performing 3-PAH extraction with the lowest isopropyl myristate detector response.

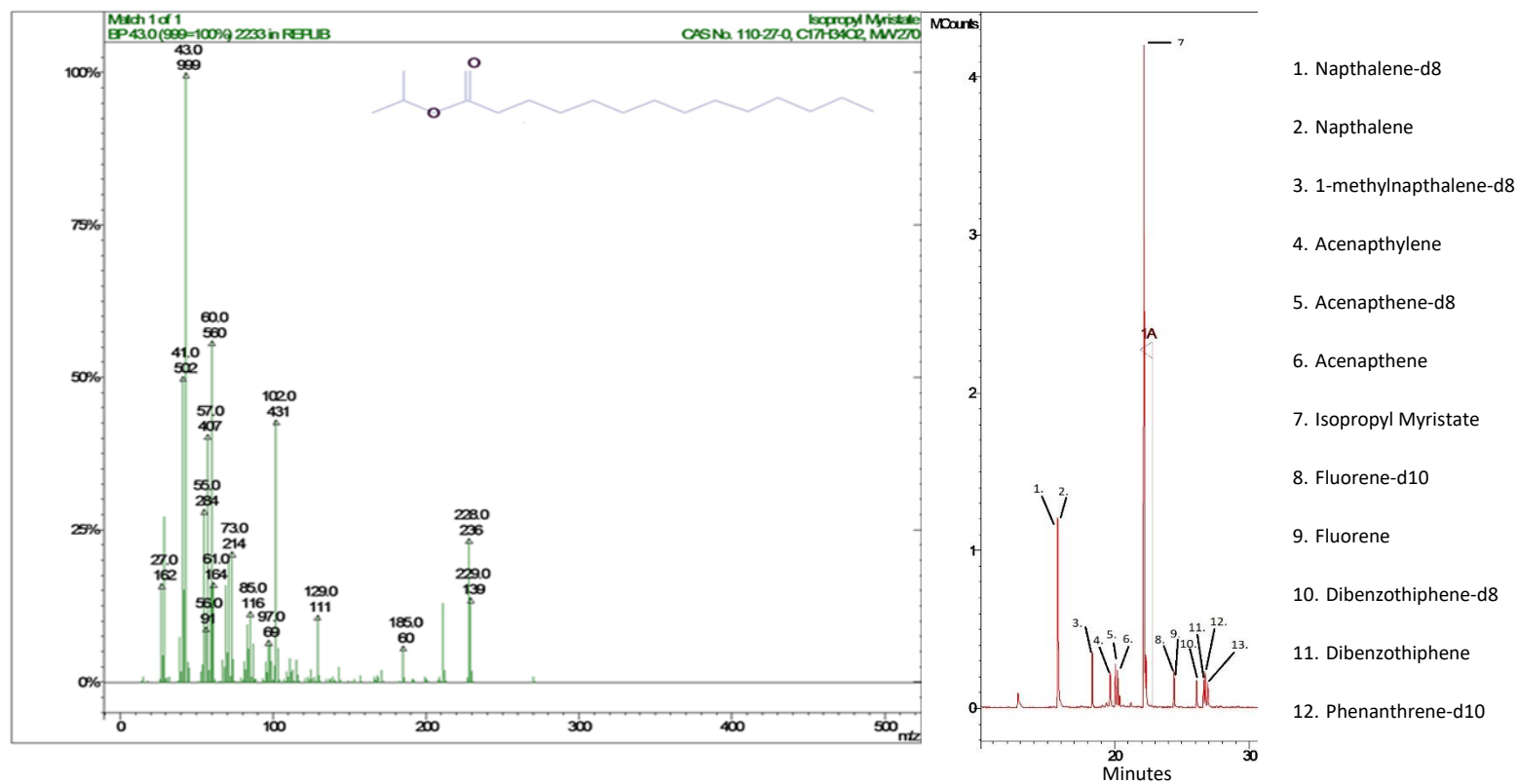


Figure 20: SAP2.1 Membrane sample GC-MS chromatogram and spectrum search for peak number 7 (isopropyl myristate).

The best performing extraction method in this study was ASE, as it has the smallest range of recoveries for 3-PAH (74.9%-116%) and low RSD% (Table 17) indicating good accuracy and precision, as well as the lowest difference in recoveries with the membrane present/not present (Table 18). Although isopropyl myristate was present in the ASE extracts, the detector counts were low compared to other methods (SAP1 and SOX). ASE is also favourable due to its rapid extraction time (approximately 20 minutes), low volume of solvents and few extraction steps, reducing the likelihood of analyte loss during the extraction process (Heemken et al., 1997).

4.2.5 Optimisation of Selected Strat-M™ Membrane PAH Extraction Method

ASE is the optimal extraction method (Section 4.2.4.1), providing high recovery of PAH with low variation. The ASE method used for extraction method comparison is a method used for PAH extraction from environmental sample media (Vane et al., 2009, Kim et al., 2018). Therefore, it is beneficial to determine the optimum ASE method for the extraction of PAH from the Strat-M™ membrane. Four principle components of the ASE method were tested for their impact on PAH recovery: ASE cell temperature, Solvent mixture, dispersant, and ASE flush (Appendix 8.2.)

4.2.5.1 *Methods*

To assess the impact of temperature, the cells were heated to 50°C, 75°C and 100°C. Three solvent mixtures were also tested: acetone:MeOH, DCM: acetone, and hexane:acetone (Guerin, 1999, Yamaguchi and Lee, 2010). The dispersants hold the samples inside the ASE cell, the three selected to be tested were: Cleaned sand, NaSO₄, and Hydromatrix (a high purity sorbent made out of diatoms (Agilent, 2019)). Levels of flush (volume of solvent that passes through the ASE cell and therefore the sample) were also compared: 30%, 50% and 70%. Only one criterion was changed for each sample group, with the original ASE PAH extraction method being altered to examine each condition (Appendix 8.2). Each condition was performed in triplicate along with a procedural blank. The membranes were spiked with 250ng of US EPA 16 PAH, and also 250ng surrogate standards (Table 15) prior to ASE extraction. The extractions were then reduced using a Turbovap, 250ng of internal standards (Table 15) added, and analysed by GC-MS (Section 3.6).

4.2.5.2 Results and Discussion

Figure 21, plot A, shows the variation of the recovery of 3-PAH at different temperatures. All statistics were carried out in R (V3.5.2) using FSA, dplyr, tidyr, ggplot2, dunn.test and psych packages (August 2019).

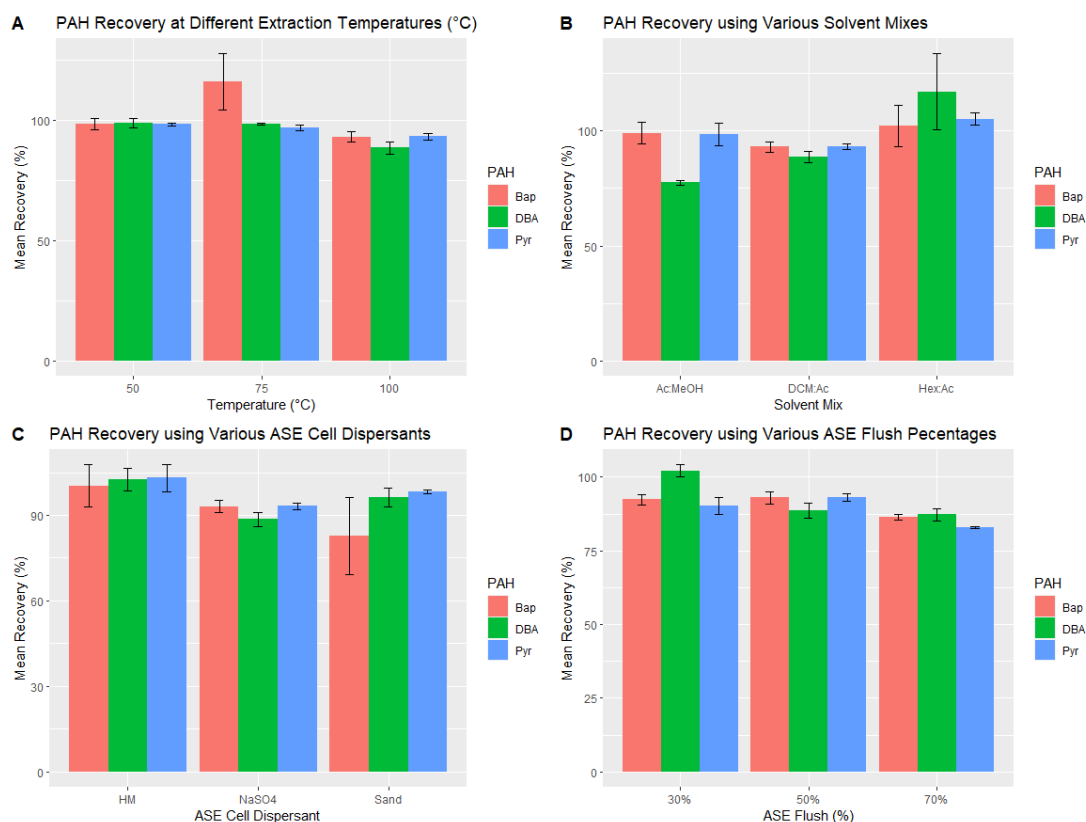


Figure 21: Results of the ASE optimisation experiment. Error bars represent the standard error. Plot A) The recovery of PAH (%) at varying ASE Cell temperatures (°C). Plot B) The PAH Recovery (%) by different solvent mixtures: acetone:MeOH (Ac:MeOH), DCM:acetone (DCM:Ac) and Hexane:Acetone (Hex:Ac). Plot C) The PAH recovery (%) using different ASE extraction vessel dispersant: HM (Hydromatrix), NaSO₄, and Sand. Plot D) %ASE Cell flush impact on PAH recovery (%): 30%, 50% and 70%.

A Shapiro-Wilks test for normality was carried out on each data set, for which all concluded that data is significantly different to the normal distribution ($p < 0.05$) (Section 4.1.3.4) (Table 19). Therefore, non-parametric tests for differences in median recoveries of 3-PAH under differing ASE parameters were carried out.

Table 19: Statistical testing of differences in 3-PAH recovery under different ASE operating conditions. All e=tests carried out to 95% significance level. Shapiro-Wilks No = not normally distributed.

ASE Parameter	Normality Test	Non-parametric Tests	
	Shapiro-Wilks	Kruskal Wallis Test	Dunn's Test
Temperature (°C)	No (p=0.000003)	Yes (p=0.0007)	Yes (50°C - 75°C (p=0.0014), 75°C-100°C (p=0.006))
Extraction Solvents	No (p=0.0007)	Yes (p=0.0116)	Yes (AC:MeOH - Hex:AC (p=0.024), DCM:Ac - Hex:Ac (p=0.004))
Dispersant	No (p=0.0008)	Yes (p=0.0187)	Yes (HM - NaSO ₄ (p=0.004))
Flush (%)	No (p=0.0154)	Yes (p=0.0157)	Yes (0%-70% (p=0.00079), 50%-70% (p=0.005))

All Kruskal-Wallis tests for comparison of median 3-PAH recoveries showed significant evidence for differences under different ASE operating parameters ($p < 0.05$) (Table 19). A Dunn Test was used to determine the source of the differences in 3-PAH recoveries between groups. The Dunn Test is a non-parametric test which can determine the specific means that significantly differ from others. The Dunn's test showed significant differences between the recoveries at 50°C and 100°C ($p = 0.0014$) and also between 75°C and 100°C ($p = 0.0006$). This is shown in Figure 21A, with recoveries dropping at 100°C. Figure 21A also shows greater variation in 3-PAH recoveries at 75°C. Polyethersulfone (PES) is often used in membranes owing to its thermal resistance and durability (Rahimpour and Madaeni, 2010). But it is probable that due to the high temperature (100°C) and pressure (1500psi), the membranes depolymerised to some extent, especially as the solvent mixture used for the temperature test contained DCM which is known to disrupt the polymeric structure (Rafiq et al., 2012, TPP, 2016). This may mean that polymer breakdown products were also extracted with the PAH, which could in turn form complexes resulting in lower PAH recovery and/or cause analytical interferences. Also, higher extraction temperature may result in greater extraction of lipids from the membranes, which could also lead to complications when the sample is injected into the GC (Sporring and Björklund, 2004) and also result in analytical interferences and matrix effects resulting in lower PAH recovery. Due to the lower recovery of PAH at higher extraction temperatures, as well as higher variation, a lower temperature of 50°C will be used for the extraction of PAH from the Strat-M™ membranes.

Figure 21B shows the impact of solvent mixture used in the ASE extraction on the recovery of PAH. To extract HMW and LMW PAH, a mix of polar/non-polar solvents is often used (Purcaro et al., 2013). The polar solvents such as acetone, have an affinity to water (e.g., acetone $\log K_{ow} = -0.24$), and are thus used in extractions to remove water from samples, allowing for less polar solvents such as DCM to dissolve PAH contained in the media. Also, the increased polarity of polar solvents allows for greater extraction efficiency of PAH with 4 or more rings (Brockmeyer et al., 2015). The dielectric constants at 20°C for each solvent are (in increasing polarity): hexane (1.89), DCM (9.08), acetone (20.7 at 25°C) and methanol (33.62) (Saim et al., 1998). DCM and hexane are both considered non-polar solvents (dielectric constant < 15). Statistical testing (Table 19) determined a difference in recoveries of Acetone:MeOH and Hexane:Acetone ($p=0.024$) and also DCM:Acetone and Hexane:Acetone ($p=0.004$). This implies that the source of the differences is Hexane:Acetone, which is due to the recoveries being slightly greater than the other two solvent mixtures (Figure 21B). All solvent mixes used for extraction of PAH from the Strat-M™ membranes produced excellent recovery. Although Hexane:Acetone produced highest recoveries, the solvent mixture to be used for extractions in future work will be DCM:Acetone, owing to good recovery, but largely due to low variability in recovery of PAH using this solvent mixture.

Figure 21C shows the effect of ASE cell dispersant on the extraction efficiency of PAH from the artificial membranes. A dispersant is usually mixed with samples to allow the solvent greater access to the sample media and also to prevent clogging of the ASE cell (Kettle, 2013). The statistical differences in recovery of 3-PAH from ASE cells containing differing dispersants was assessed (Table 19). These analyses concluded that there is significant evidence for a difference in recoveries when using Hydromatrix and NaSO₄ as a dispersant ($p=0.004$). Although there was no significant difference between the other groups (Hydromatrix and Sand ($p=0.172$) and NaSO₄ and Sand ($p=0.146$)). However, NaSO₄ is used to remove moisture from samples (Yu et al., 2008), complementing the polar solvents in the extraction process allowing for greater extraction efficiency of the PAH from the membranes by non-polar solvents, this could be why the NaSO₄ dispersant recoveries were less variable. Owing to producing low variability and good 3-PAH recovery (Figure 21C), NaSO₄ was used in future extractions.

Figure 21D shows the effect on ASE Flush % on the recovery of 3-PAH from the Strat-M™ membrane. Flush is the amount of solvent that passes through the ASE cell after the static time step and is expressed as a percentage of cell volume. The three flush percentages tested

were: 30%, 50% and 70%. The impact of different ASE Cell Flush (%) on 3-PAH recovery was statistically tested (Table 19). The analyses show differences between flushes of 30% and 70% ($p=0.0008$), and also 50% and 70% ($p=0.005$). This shows that the source of the difference is the recovery of PAH using 70% ASE cell flush, which as shown in Figure 21D is lower than the other two flush percentages. It appears that with lower flush, greater recovery is gained but at the expense of greater variability in the data, and although greater recovery is beneficial, this is outweighed by the addition of greater variability and therefore greater uncertainty. This may be due to the co-extraction of lipids from the artificial membranes, which in turn create analytical interferences with the PAH (Sporring and Björklund, 2004). For this reason, 50% flush was selected as it is a compromise of second-best recovery, and second lowest variability and will reduce the probability of lipids being co-extracted.

4.2.6 Conclusion

The preferred method for the extraction of 3-PAH from the Strat-M™ membrane was ASE. The ASE method was optimised further by adjusting four parameters: extraction temperature, solvent mixture, dispersant, and flush (%), and assessing their impact on 3-PAH recovery (Figure 21, Table 19). The optimised ASE method for the rapid extraction of 3-PAH from the Strat-M™ membrane is shown in the table below (Table 20).

Table 20: ASE parameters for the extraction of Pyr, BaP and DBA from the Strat-M™ artificial skin membrane. The conditions optimised in this work were: temperature, solvents, dispersant and flush (%).

Optimal ASE Conditions	
Temperature °C	50
Solvents	DCM:Acetone (1:1)
Pressure (psi)	1500
Dispersant	NaSO ₄
Flush (%)	50
Purge (seconds)	60
Heat up (mins)	5

This method will enable the quantification of 3-PAH that have diffused from a vehicle into the membrane, which will allow for mass balance to be achieved during dermal absorption experiments, and also enable the user to account for potential localised exposure effects of PAH in the skin.

4.3 Receptor Solution PAH Extraction and Recipe Selection

4.3.1 Introduction

The aims of this section are to give an overview of how an effective method for the extraction of PAH from receptor solutions (RS) was established and how which receptor solution recipe will produce the highest PAH recovery with lowest amounts of variation was determined. The receptor solution is housed in the receptor chamber of the diffusion cell of choice, and in dermal diffusion studies is often a simulated body fluid (SBF) with ion concentrations similar to that of human blood plasma (Oyane et al., 2003) (Section 4.1.2.2).

IPCS (2006) and OECD (2004) state that the receptor solution should not limit the diffusion process by the compound(s) of interest being insoluble in the receptor solution. Therefore, it is recommended that for lipophilic compounds (such as PAH), bovine serum albumin (BSA) or solvent mixtures such as ethanol:water should be incorporated into the receptor solution.

Two methods are widely described in the literature for the analysis of compounds in saltwater: solid phase extraction (SPE) (Dittmar et al., 2008, Trettin et al., 2014) and liquid-liquid extraction (LLE) (Reddy and Quinn, 1999, Keating et al., 2018).

Three receptor solution recipes are commonly used in the literature for the *in vitro* measurement of the dermal absorption of PAH. These three RS recipes are: Hanks HEPES buffered receiver solution (HBSS) with 4% BSA, phosphate buffered saline (PBS) with 4% BSA, and aqueous solution 6% polyethylene glycol 20 oleyl ether (Volpo-20™/Brij-20™) (Section 4.1.2.2). BSA is a serum albumin protein, and is the most abundant protein of the blood (Choi et al., 2004). HEPES is a zwitterionic compound often used a buffer, as it provides exceptional pH stability in the biological critical pH region of 7.4-7.8 (Duebener et al., 2002). The recovery of PAH from these three RS recipes will be compared, with the optimum RS recipe (PAH recovery 80 – 120%, lowest variability (Environment Agency, 2018)) then being used in this studies *in vitro* PAH dermal absorption experiments.

4.3.2 Method

4.3.2.1 Reagents and Materials

Table 21 shows a summary of the reagents and materials used in the experiments to derive a PAH extraction method from RS, and also RS recipe selection.

Table 21: Summary of reagents and materials used throughout the receptor solution extraction and recipe selection experiments

Reagent/Material	Description	Supplier
PAH Mix-9	100ng/μL USEPA 16 PAH	Qmx Laboratories (UK)
Deuterated PAH surrogate standards	naphthalene-d8, acenaphthene-d10, dibenzothiophene-d8, phenanthrene-d10, pyrene-d10, chrysene-d12, benzo[b]fluoranthene-d12, benzo[a]pyrene-d12, and benzo[g,h,i]perylene-d12 (1000ng/μL in cyclohexane)	Qmx Laboratories (UK)
Deuterated PAH Internal standards	1-methylnaphthalene-d10, fluorene-d10, fluoranthene-d10, benz[a]anthracene-d12, benzo[e]pyrene-d12, perylene-d12 and indeno[1,2,3-cd]pyrene-d12 (1000ng/μL in cyclohexane)	Qmx Laboratories (UK)
Dichloromethane (DCM)	HPLC grade	Fisher Scientific (Loughborough, UK)
Acetone	HPLC grade	Fisher Scientific (Loughborough, UK)
Hexane	HPLC grade	Fisher Scientific (Loughborough, UK)
Acetonitrile	HPLC grade	Fisher Scientific (Loughborough, UK)
Methanol	HPLC grade	Fisher Scientific (Loughborough, UK)
Strata PAH SPE cartridges	Strata PAH SPE cartridges (0.75g/6mL)	Phenomenex Ltd (Macclesfield, UK)
Phosphate-Buffered Saline (PBS)	Balanced salt solution, pH 7.2.	ThermoFisher Scientific (Loughborough, UK)
Hanks' Balanced Salt Solution (HBSS)	Balanced salt solution, pH 7.0 to 7.4.	ThermoFisher Scientific (Loughborough, UK)
BRIJ-20	Polyoxyethylene (20) oleyl ether	Merck Life Science UK Ltd. (Gillingham, UK)
Bovine Serum Albumin	pH 7, ≥98%	Merck Life Science UK Ltd. (Gillingham, UK)
HEPES	Fine white crystals. pH 5 to 6.5	Fisher Scientific (Loughborough, UK)

4.3.2.2 Receptor Solution Recipes

Table 22 shows the recipes of the three RS to be compared. Each RS was made in a 250mL Pyrex beaker. The liquid fraction of each recipe was added first, followed by the solid ingredients which were weighed using a Sartorius LP620S (± 0.001 g) balance. The solutions were then stirred until no visible solids were present, and allowed to equilibrate overnight stored in a fridge ($<4^{\circ}\text{C}$).

Table 22: Ingredients required to make 100mL of each receptor solution recipe.

HBSS	PBS	Brij-20
100 mL HBSS 4 g BSA 0.48 g HEPES	100 mL PBS 4g BSA	100mL Milli-Q water 6g Brij-20

4.3.2.3 Receptor Solution PAH Extraction Method

The first method tested was LLE. A 1mL volume of each receptor solution was spiked with 250ng of USEPA 16 PAH along with 250ng of surrogate standards (Table 21). 1mL of cyclohexane was introduced to each 10mL vial containing the 1mL of spiked RS and shaken, this process was repeated three times. However, an issue was encountered where the introduction and agitation of the RS with a solvent resulted in the formation of micelles within the samples, consequently there was poor separation of the two liquids making it difficult to determine where each layer began. It was intended to remove the top layer of cyclohexane similar to the LLE tested for PAH membrane extractions (Section 4.1.3.2) but owing to the poor separation of layers this was not possible to do accurately. As the LLE method did not come to fruition an alternative method for the extraction of PAH from the RS needed to be derived.

A Solid phase extraction (SPE) method was then tested (Dittmar et al., 2008, Trettin et al., 2014), using the Strata-PAH SPE cartridge made by Phenomenex (Phenomenex, UK), designed for the purpose of extracting PAH from water. As receptor solutions are often water based, it was theorised that these SPE cartridges would also be capable of PAH extraction from the receptor solutions. The main difference between the RS recipes and previous works using SPE for the extraction of PAH from water-based solutions, is the presence of BSA and Brij-20, which are known sinks for PAH (IPCS, 2006, Macii et al., 2019). It was unknown whether the SPE method would provide adequate separation of PAH from these components of the RS to produce the desired recovery (80-120%) (Environment Agency, 2018)).

Table 23: SPE protocol for PAH extraction from receptor solutions.

	RS PAH SPE Protocol
Cartridge:	Strata PAH (0.75 g/6 mL)
Condition:	10 mL DCM 10 mL MeOH 20 mL D.I. water
Load:	1 mL of receptor solution with acetonitrile (3:1).
Wash:	5 mL MeOH:water
Dry:	20 secs under 10" Hg vacuum
Elute:	6mL DCM

Table 23 shows the SPE protocol for the extraction of PAH from the three receptor solution recipes. The three receptor solution recipes were made up, with three 1mL aliquots of each receptor solution being spiked with 250ng PAH, and another one 1mL aliquot used as a procedural blank. 250ng of surrogate standards were applied to each sample prior to SPE. In order to activate the sites contained in the silica-based phase of the SPE cartridges, 10mL of DCM, 10mL of MeOH and 20mL of D.I. water was passed through the cartridge. The receptor solution samples were then mixed with acetonitrile (3:1) and loaded into the cartridge by Pasteur pipette, 5mL of MeOH:water (1:1) was passed through the cartridge, which elutes compound groups which are not of interest. The cartridge was then dried under a 10" Hg vacuum for 20 seconds. As this a reverse phase SPE method, the analytes of interest (PAH) are retained in the cartridge, with the final step being the addition of 6mL DCM to elute the PAH. To ensure no water remained in the samples post SPE, the samples were passed through a NaSO₄ packed Pasteur pipette. Samples were then reduced to 1mL using a steady stream of dry N₂ gas. Internal standards (250ng) were then added prior to GC-MS analysis.

4.3.3 Results

4.3.3.1 Quality Control

Each extraction was performed in triplicate, along with a procedural blank for each receptor solution. Recovery (%) was calculated using Equation 17. Such as with the previous Strat-M™ extractions, this allowed for the concentrations of each PAH in the spiking solution to be quantified to a greater certainty than by just assuming that the concentrations of the spiking solution were a factor of 10 dilution of the standard used to make it, therefore allowing for

an improved representation of actual recovery of the PAH from each receptor solution recipe, whilst also considering analytical variation.

$$\text{Recovery (\%)} = \frac{\text{concentration of PAH congener measured}}{\text{concentration of PAH congener measured in spiking solution}} \times 100$$

Equation 17: Formula used to calculate PAH recovery from each receptor solution recipe.

The spiking solution was analysed by GC-MS under the same conditions as the receptor solution extraction samples (Section 3.6). The mean mass of US EPA 16 PAH in the spiking solution was 214ng, with an RSD% of 1.37, suggesting low analytical variation between PAH congeners and good analytical performance. Therefore, any variation in results of receptor solution extraction recovery was likely to originate from the different receptor solution effects on PAH recovery.

DCM analytical blanks were also analysed after each receptor solution recipe during the GC-MS analytical run, all of which produced no data, ensuring that no carryover of PAH was occurring between samples and leading to false positive results.

4.3.3.2 Quantification of PAH recovery from Receptor Solutions

Recovery of PAH from each receptor solution recipe was calculated using Equation 17, with the results shown in Table 24.

Table 24: Mean recovery (%) of 3-PAH from each receptor solution recipe. Values reported in brackets are the RSD%.

	3-PAH Mean Recovery (%)					
	Pyr		BaP		DBA	
HBSS	97.6	(3.8)	96	(1.7)	100.6	(1.5)
PBS	94.9	(4.2)	99.7	(2.4)	113.6	(10.2)
Brij-20	79.6	(3.1)	84.6	(2.5)	99.2	(6.9)

Using the SPE method, HBSS and PBS both produced mean 3-PAH recoveries within the desired range of 80 – 120% (Environment Agency, 2018). However, Brij-20 produced lower 3-PAH recoveries in general, with mean Pyr recovery falling below the desired recovery range.

4.3.4 Discussion

4.3.4.1 Comparison of PAH Recovery from Receptor Solutions

The recovery of PAH from each receptor solution was excellent, and most recoveries fall within the desired range of recovery of 80% – 120% (Environment Agency, 2018). The

deciding factor for receptor solution selection therefore was likely to be the variability of recovery from each recipe, quantified by the RSD% (Section 4.1.3.2). The importance of variability for the development of an *in vitro* dermal absorption method is that there are three compartments that need to have their PAH concentrations measured in order to understand the spread of PAH throughout the system and ultimately the amount of PAH dermally absorbed (Section 4.1). Therefore, any variation in each compartment will add to the total cumulative variation of the overall method. Furthermore, the lower the variation in results generated in each compartment will result in a method that produces results with greater precision, which is critical when it comes to contaminated land HHRA.

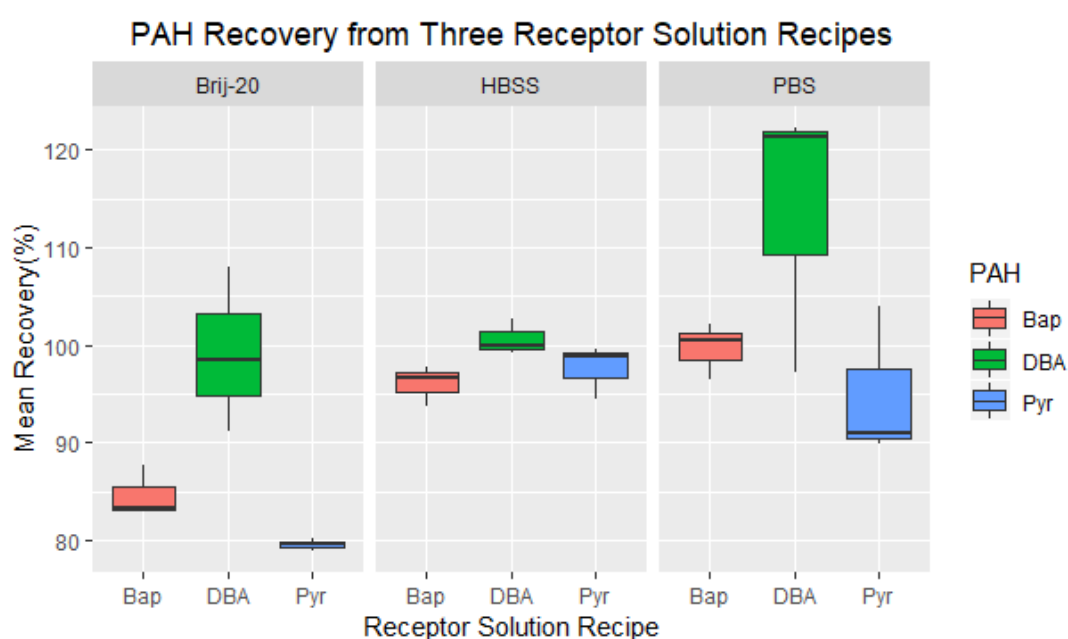


Figure 22: Mean Recovery (%) of Pyr, BaP and DBA from three receptor solution recipes; HBSS, PBS and Brij-20TM.

Figure 22 shows the mean recovery (%) of the three focal PAH from the three receptor solutions tested. Figure 22 shows that there was greater variation and range in recovery (%) of the three focal PAH from Brij-20TM and PBS in comparison to HBSS. All statistics were carried out in R (V3.5.2). A Shapiro-Wilks test for normality was carried out on the data, generating a p-value >0.05 ($p = 0.0786$) therefore concluding that the data are not significantly different from a normal distribution and therefore normality can be assumed. Owing to the assumption of normality, an ANOVA was performed in order to determine if there are differences in mean recovery of the three focal PAH from the three receptor solution recipes. This generated a p-value of 0.0056, therefore H_0 was rejected, and we can

conclude that there was evidence for a significant difference in mean recovery (%) of the three focal PAH from the receptor solution recipes. To locate the source of the differences in means determined by ANOVA, a Tukey post-hoc test was carried out on the data. This test determined no significant difference in mean recovery between HBSS - Brij-20TM ($p = 0.058$), and PBS – HBSS ($p = 0.52$) at a 95% confidence level. However, there was evidence for a significant difference in mean recoveries from PBS – Brij- 20TM ($p = 0.0048$), showing a mean difference of 14.96%. HBSS – Brij-20TM showed a mean difference of 10.3% and PBS – HBSS a mean difference of 4.7%. This shows that PBS and HBSS produce similar levels of recovery of the three focal PAH via the SPE method. The deciding factor therefore was variation in recovery (%) values, and as shown in Figure 22, HBSS shows lower RSD% values in comparison to PBS, therefore producing less variable results.

HBSS may have produced the best recovery owing to the presence of HEPES in its recipe, whereas HEPES was not present in others. HEPES is a zwitterionic sulfonic acid buffer, developed by Good and colleagues and subsequently is part of a group of buffers known as Good's Buffers (Taha and Coutinho, 2016). HEPES shows excellent buffering in the critical pH region of 7.4–7.8. Physiologically this is important for the *in vitro* method as the pH of human blood is in the region of 7.4 (Duebener et al., 2002). Good's Buffers have high water solubility and low water solubility, and are insoluble in organic solvents (Taha and Coutinho, 2016). This pH stability will impact BSA structure, for example when pH is near to 7, albumin fatty acids will be present in compact N (normal) form, and will unravel under acidic conditions (<3.5) to its E (expanded) form (Michnik et al., 2005). Although pH was not highly acidic in any of the receptor solution recipes during this experiment, the presence of HEPES may create additional BSA stability, allowing for greater interaction between BSA and the stationary phase during SPE, and therefore greater extraction efficiency of PAH from the receptor solution. BSA has been shown to absorb to both hydrophobic and hydrophilic surfaces (Jeyachandran et al., 2009), but reaches higher saturation on hydrophilic surfaces (95% saturation) in comparison to hydrophobic surfaces (50% saturation). The silica-based stationary phase of the SPE cartridges, is most likely to be hydrophobic in nature, to promote PAH interaction and retention (as the SPE method is reverse phase). As different solvents are passed through the SPE cartridge, most BSA is likely to be removed owing to its greater affinity to hydrophilic surfaces, with PAH remaining in the cartridge to be later removed and recovered during the final wash phase of the SPE method.

Brij-20TM is a non-ionic surfactant, the use of non-ionic surfactants has previously been studied for the removal of hydrophobic organic compounds, such as PAH, from

contaminated soils. Surfactants are believed to aid in the solubilisation of sorbed hydrophobic contaminants, which is beneficial for soil-washing or similar remediation approaches, as well as perhaps influencing microbial remediation techniques by increasing the availability of soil PAH. The surfactant is amphiphilic, and therefore has two structural moieties. One moiety is polar and has an affinity to water and other polar compounds, whereas the other moiety is non-polar and is hydrophobic. Micelles can occur when the hydrophobic portion of each molecule is facing inward, creating a hydrophilic shell with a hydrophobic core (Edwards et al., 1991). It is likely, that PAH contained in the Brij-20™ receptor solution were in the centre of the hydrophobic core of these micelles. The interaction of these micelles with the stationary phase of the SPE cartridges and solvent mixes would influence recovery. The polar wash of MeOH:water (1:1) would likely degrade micelles due to the polar casing of the micelle being likely to have a strong affinity to the polar mixture as it passes through the SPE cartridge. The fate of the PAH may be that they are also removed along with the surfactant, or they remain on the stationary phase of the SPE cartridge depending on which substance the PAH has greater bonding capability with. This could be how PAH are lost during Brij-20™ extraction and also how recoveries vary with the extent of interaction of PAH with each phase.

4.3.5 Conclusion

The PAH recovery (%) of three receptor solution recipes encountered in the literature for measuring dermal absorption of PAH were tested to select the optimum recipe for the later diffusion cell method. These were: HBSS (Moody et al., 1995, Moody and Joncas, 2007, Moody et al., 2011), PBS (Ackermann et al., 2010, Uchida et al., 2015, Peckham et al., 2017), and Brij-20™ (Yang et al., 1989, Roy and Singh, 2001). LLE proved unsuccessful for the extraction of PAH from the receptor solution recipes owing to the formation of micelles distorting the separation of layers of extraction solvent and receptor solution sample. This was overcome by using the Strat-PAH SPE cartridge developed by Phenomenex for the extraction of PAH from water. The SPE method produced good recoveries of PAH from all three receptor solution recipes, with mean recoveries falling in the preferable range of 80% - 120% recovery. Variation in recoveries proved to be a deciding factor for which receptor solution to use, with the lowest variation desirable owing to its contribution to the overall diffusion cell method when multiple compartments require analysis for PAH content. HBSS produced the lowest variation for 3-PAH: Pyr (2.4), BaP (1.7) and DBA (1.5), and therefore was used in dermal bioavailability experiments throughout this research.

4.4 Soil Loading and Unloading of the Strat-M™ Membrane

4.4.1 Introduction

The aims of this section are to: develop methods for the addition and removal of soil from the Strat-M™ membrane; and to determine the contribution of soil remaining on the membrane to the PAH concentration of the membrane extract.

For the execution of soil PAH dermal bioavailability experiments, soils are needed to be added to the membrane consistently and removed effectively. A consistent soil loading ensures a constant PAH dose is applied to the Strat-M™ membrane when soil PAH dermal bioavailability testing. The removal of soil from the Strat-M™ membrane allowed for the soil PAH concentration to be quantified over the duration of soil PAH dermal bioavailability testing. This quantification is so that the fate of PAH in the diffusion cell system can be quantified accurately in order to produce soil PAH dermal bioavailability data (Sections 2.3.2, 2.3.4 and 2.7). A method is already known for the extraction and quantification of PAH from soil (Chapter 3), but a method needs to be determined for the repeatable application and removal of soil to/from the Strat-M™ membrane.

Strat-M™ membranes were loaded with supermonolayer coverage to reflect the increased soil adherence with increasing soil moisture, and provide an infinite dosing scenario in later soil dermal bioavailability experiments (Sections 2.1.3 and 2.3.3). Owing to the difficulty in producing monolayer coverage in practice (Spalt et al., 2009), supermonolayer coverage would ensure that broken submonolayer coverage (Section 2.3.3) would not be produced. Submonolayer coverage is undesirable as this could introduce variability into the dermal bioavailability data due to varying soil-membrane contact and subsequent PAH dermal absorption.

4.4.2 Methods

The soil loading/unloading method used for this research is as follows. A soil was weighed, and water added until the soil was moistened to 40w/w% water (Stanley et al., 2019). Soil was moistened by the gradual addition of Milli-Q water whilst mixing the soil until 40w/w% was achieved. Fresh membranes were weighed (Sartorius LP620S balance (± 0.002 g)), and then a target mass of 1.5 g of moistened soil was then loaded onto the membrane using a square brass template with a round aperture removed from the middle (Figure 23). This soil mass was selected as it would ensure supermonolayer soil loading to allow for the quantification of J_{ss} (Frasch et al., 2014). This amount of soil was selected so loading reflect the upper limits of soil adherence to volunteers (Duff, 1996). The template provided a

consistent surface area (8.55 cm²) at which soil was in contact with the surface of the membrane. The loaded membrane then had its mass weighed and recorded so that soil mass on each membrane could be calculated in order to assess variability in the soil loading method. The mass of soil applied to each membrane was calculated by subtracting the membrane starting mass from the loaded membrane mass.

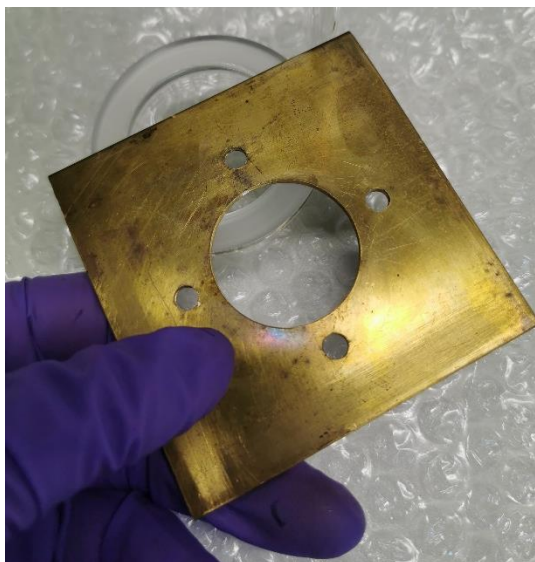


Figure 23: Brass template used for loading soils onto Strat-M™ membranes. The template has an internal diameter of 3.3cm.

To remove the soil from the membrane, the bulk of the mass was removed using a spatula into a 10mL vial, followed by a series of Milli-Q water rinses by squeegee into a 10 mL vial using a funnel until no visible soil was left on the Strat-M™ membrane. The membranes were allowed to dry, and then re-weighed. For PAH soil analysis, the soil was then freeze dried, ready for ASE extraction prior to GC-MS analysis (Sections 3.3, 3.4, 3.5 and 3.6). Freeze drying was selected owing to lower losses of analytes in comparison to oven or air drying (Beriro et al., 2014). Five replications of the soil loading and unloading of the Strat-M™ membrane was carried out in order to quantify method reproducibility.

4.4.3 Results

The results of the experiment are shown in Table 25. The method produced a mean soil removal from the Strat-M™ membrane of 99.8% (RSD% = 0.05), with a mean of 0.0032 g of soil remaining on the Strat-M™ membrane. The maximum soil removed from the membrane was 99.84% and the lowest was 99.76% (Table 25). The unloaded membranes had a mean weight of 0.278 g (RSD = 8.68%), a slight increase to their mean pre-weight of 0.275 g (RSD% = 3.08%) (Table 25). The mean soil mass (wet) applied was 1.38 g (RSD% = 8.6%), with a maximum of 1.6 g and minimum of 1.3 g (Table 25).

Table 25: Membrane soil loading and removal. It should be noted that soil weight (g) is wet (40% w/w).

Membrane	Membrane Pre-weight (g)	Loaded Membrane (g)	Unloaded Membrane (g)	Soil (g)	Soil removal (%)
1	0.272	1.51	0.274	1.24	99.84
2	0.278	1.88	0.281	1.61	99.81
3	0.289	1.65	0.293	1.36	99.71
4	0.263	1.62	0.266	1.35	99.78
5	0.274	1.65	0.278	1.37	99.71

4.4.4 Discussion

A paired t-test was carried out in R (version 1.4.1103, packages: readxl, dplyr, psych (February 2021)) to determine differences in the mass of the Strat- MTM membrane before and after soil loading (Section 4.1.3.4). A Shapiro-Wilk normality test was used to test whether the data is normally distributed. The Shapiro-Wilk test gave $p = 0.815$ ($p > 0.05$), so the distribution of the data are not significantly different from a normal distribution, therefore normality can be assumed. Consequently, a paired t-test was used to test for differences in mean membrane masses before loading and after unloading with soil. This test returned a p-value of 0.001 ($P < 0.05$). Therefore, it was concluded that there is a significant difference between the means. Furthermore, Figure 24 shows the Strat-MTM membrane masses before and after soil loading and removal. There was a difference in the means seen in Figure 24 (means are represented as red circles), with the membrane mean mass before loading being 0.275 g and after unloading 0.278 g. This difference in membrane masses could be from remaining soil on the membrane or variation in mass value produced by the balance (± 0.002 g) owing to the low difference in masses of the two groups of 0.003 g. Upon visual inspection of the membranes, small amounts of soil were present on the Strat-MTM membrane surface engrained in the small dips of the membrane surface.

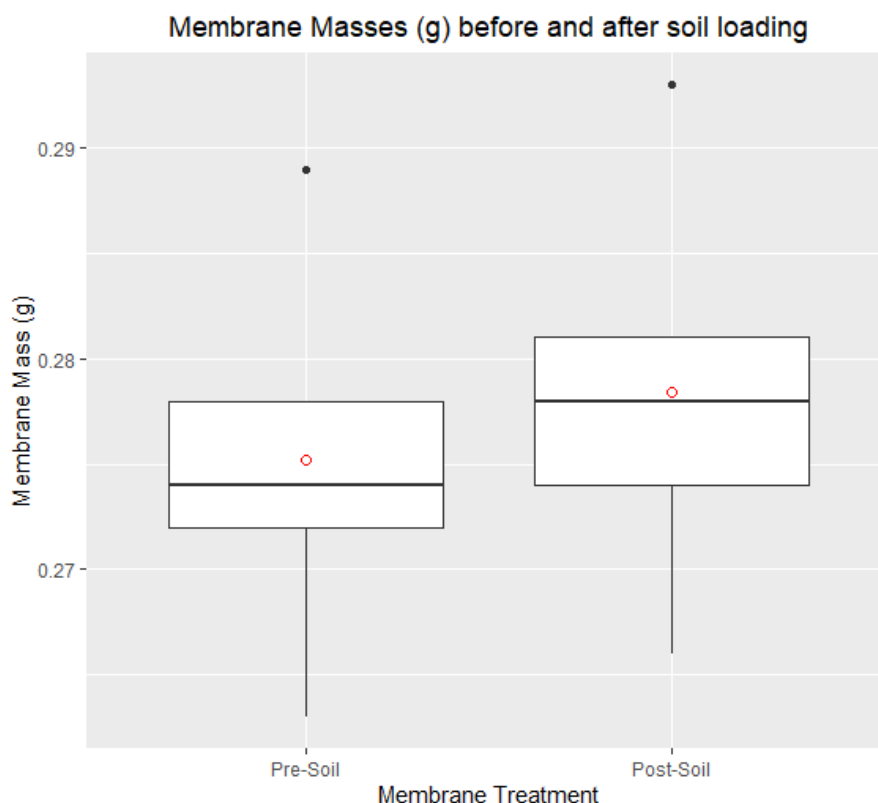


Figure 24: Membrane Masses (g) before loading with soil (Pre-Soil) and once soil was unloaded (Post-Soil). The red circle in each box represents the mean.

Consequently, in order to account for any additional PAH included in the soil remaining on the membrane skewing the measurement of PAH contained in the membrane a series of equations (Equation 18a and Equation 18b) were formulated.

$$a) \text{ Memb}_{\text{soilPAH}} = \text{Memb}_{\text{soil}} \times \text{Soil}_{\text{PAH}}$$

$$b) \text{ Memb}_{\text{PAH}} = \text{Memb}_{\text{ext}} - \text{Memb}_{\text{soilPAH}}$$

Equation 18: Soil remaining on membrane factoring equations. a) Calculate the PAH concentration of soil remaining on the membrane. b) Removal of remaining soil concentration on membrane from membrane extract PAH concentration to give PAH mass absorbed.

Where: $\text{Memb}_{\text{soilPAH}}$ is the mass of soil PAH remaining on the membrane after soil removal (g), $\text{Memb}_{\text{soil}}$ is the mass of soils remaining on the membrane after soil removal (g), Soil_{PAH} is the PAH soil concentration (mg/kg), Memb_{PAH} is the PAH mass contained in the membrane, and Memb_{ext} is the quantified PAH concentration of the membrane extract.

A series of tables were created using the formulae above (Equation 18) to estimate the potential contribution of the soil PAH remaining on the membrane may have on the measurement of PAH contained in the membrane for the soil PAH dermal bioavailability

experiment. The effect of: PAH bioaccessibility (1% to 25%), Mass of soil remaining on the membrane (0.001 g to 0.1 g), and PAH soil concentration (5 mg/kg to 100 mg/kg) were all assessed for their potential impact on the contribution of soil remaining on the membrane to the membrane PAH extract. If soil removal and soil PAH bioaccessibility remain constant, it was found that PAH soil concentration had no effect on soil contribution to the membrane extract concentration owing to proportionality (Figure 25). For example, as soil PAH concentration increased as would the PAH concentration absorbed into the membrane counteracting the impact of the increased soil PAH concentration remaining on the membrane surface.

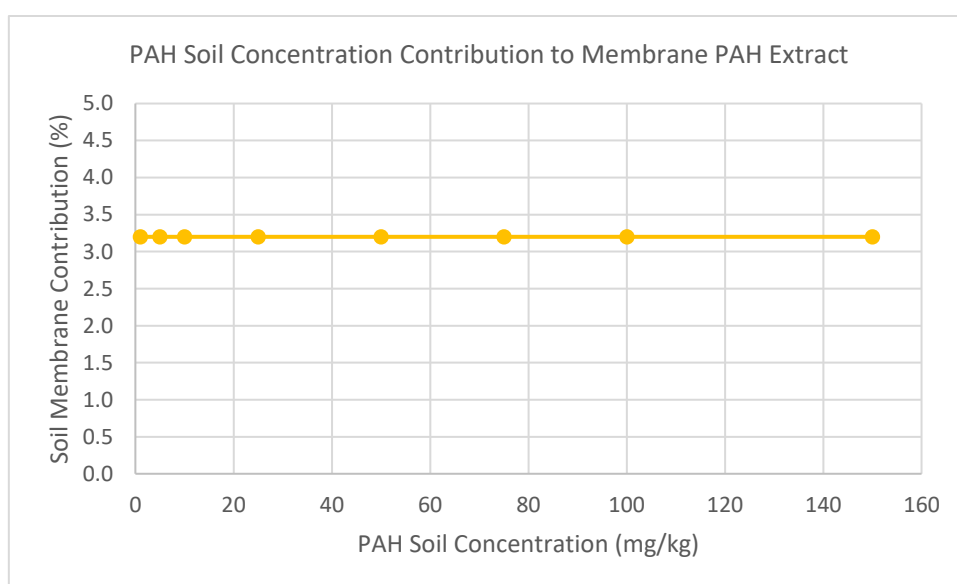


Figure 25: Soil PAH Concentration (mg/kg) impact on the contribution of soil remaining on the membrane to the membrane extract PAH concentration.

Figure 26 shows the potential contribution of different soil masses remaining on the membrane (g) for different PAH soil concentration (mg/kg) if soil PAH bioaccessibility is constant. Figure 26 shows that increasing soil mass remaining on the membrane (g) regardless of soil PAH concentration (mg/kg) leads to an increase in the contribution of soil remaining on the membrane to the PAH concentration of the membrane extract. However, soil removal during this experiment was effective and consistent, producing a mean removal of 99.8% with an RSD% of 0.05. Therefore, it can be assumed that soil remaining on the membrane during the soil PAH dermal bioavailability experiment will have a minimal impact on the membrane PAH concentration impact. Furthermore, any contribution will be corrected for using Equation 18.

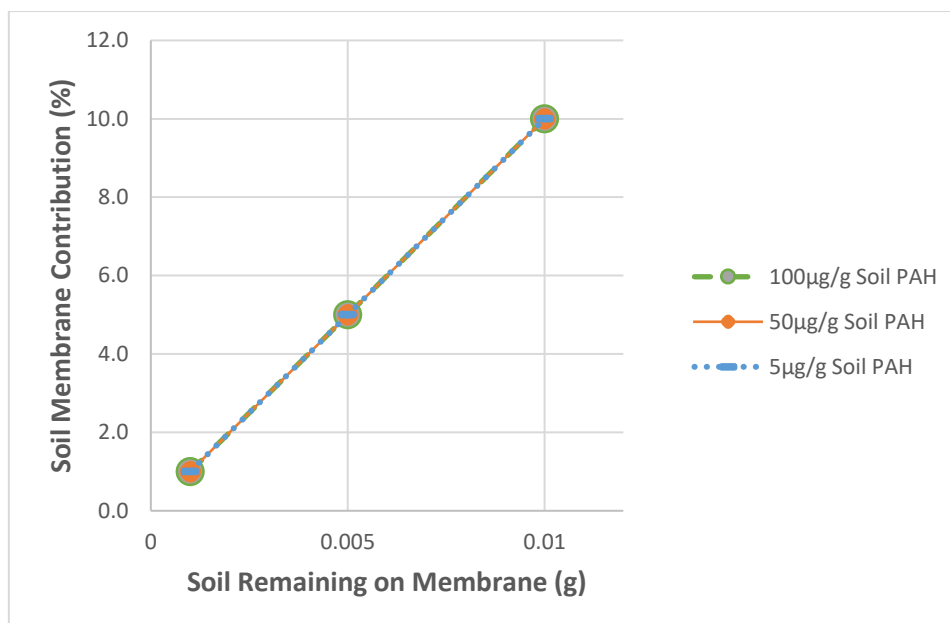


Figure 26: Soil remaining on the membrane (g) at different soil PAH concentrations (mg/kg) contribution to membrane PAH concentration (%).

In addition, Figure 27 shows that the higher the soil PAH bioaccessibility, and consequent higher PAH concentration absorbed into the membrane, results in a lower contribution of soil PAH remaining on the membrane extract PAH concentration.

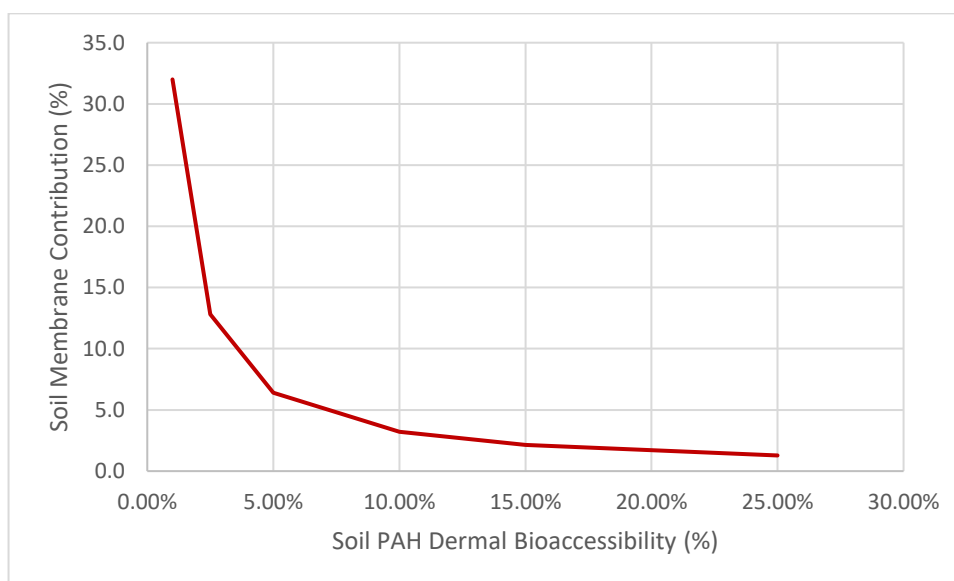


Figure 27: The impact of soil PAH dermal bioaccessibility on the contribution of soil PAH remaining on the membrane to the membrane PAH extract concentration.

For example, PAH bioaccessibility of 1% with 0.0032 g of soil remaining on the membrane would result in a soil contribution of 32%, whereas a PAH bioaccessibility of 10% with 0.0032 g of soil remaining on the membrane would result in a soil contribution of 3.2% regardless of soil concentration. It was found that for 0.0032 g remaining on the membrane (mean soil

mass remaining on the membrane from this experiment) would lead to an approximate membrane concentration contribution of 3%. The critical point was determined to be 0.1 g of soil remaining on the membrane, as this would result in 100% soil contribution, i.e., the same PAH concentration would be in both the membrane and the soil remaining on the membrane effectively counting each other out if the corrective equations were used (Equation 18).

Throughout the literature, methods for soil removal were encountered, such as washing skin surfaces with surfactants such as Radiacwash™ (Moody et al., 1995, Moody and Joncas, 2007, Moody et al., 2011). However, no data was found demonstrating the effectiveness of a soil removal method e.g. Moody and Joncas (2007). Some studies in addition to soil washes to remove soil from the skin surface, have used tape stripping (for example Forsberg et al. (2021)). This will have most likely removed any remaining soil on the membrane/skin during this tape stripping process. But the inclusion of PAH concentrations of these soil particles in the tape stripping compartment may have resulted in an overestimation of the dermally absorbed fraction if tape stripping was included. It should be noted that Forsberg et al. (2021) rightly did not include the tape stripping data in the dermally absorbed fraction calculations.

It has demonstrated by previous works that soil skin adherence increased with increasing water content (Kissel et al., 1996, Choate et al., 2006). Furthermore, real world soil loadings of $> 100 \text{ mg/cm}^2$ were reported in field studies involving volunteers (Duff, 1996). Therefore, it can be assumed that supermonolayer coverage is reflective of real-world exposure scenarios given the high mean water content of soils in the UK most likely increasing soil skin adherence. Another reason for the use of supermonolayer soil loading, is its implications on PAH flux. For exposure assessment, it is recommended that maximum flux is used, as this represents a worst-case scenario and therefore offers a conservative estimate of intake rate and associated human health risk (IPCS, 2006). Peak flux, is defined as the highest dose achieved from any given dose, with in vitro experiments presenting peak flux (Frasch et al., 2014). As flux is a concentration gradient driven process, peak flux will increase with dose as well as loading, with the greater increase in each of these resulting in a closer representation of maximum flux, and therefore presenting worst case scenario dermal absorption data in order to produce conservative human health risk assessment criteria.

Soil loading has an impact on the dermal absorption data produced by an experiment. It can be widely seen in the literature that the fraction absorbed of the applied dose decreases with soil loading increase (Roy and Singh, 2001, Kissel, 2011, Frasch et al., 2014). For example, if

a load of 5 units resulted in the absorption of 10% of the applied load, a load of 50 units would result in the absorption of 1% of the applied load (Frasch et al., 2014). Therefore, the percent absorbed of the dose applied is not a meaningful indicator for the potential dermal absorption of a chemical as it can significantly vary depending on loading (Frasch et al., 2014). High loadings used in dermal bioavailability experiments are flux-limited, where there is ample load that cannot be significantly depleted over the duration of the experiment (infinite dose) (Frasch et al., 2014). On the other hand, studies using low loadings are delivery-limited, owing to the depletion of load over the duration of the experiment (finite dose) (Frasch et al., 2014). However, as long as there is complete coverage of the membrane surface, initial flux into the skin from both loading scenarios will be the same (Kissel, 2011). J_{ss} is the steady-state dermal absorption rate for a chemical, which can be experimentally determined using infinite dose of a chemical. Owing to the supermonolayer coverage and high loading to be used in this experiment, the soil dermal bioavailability experiment is likely to be a flux-limited regime (infinite dose). Therefore, percent absorption cannot be used for comparison to previous studies. Most bioavailability studies assume infinite dose to calculate dermal absorption kinetic parameters such as flux (Beriro et al., 2016). However, chronic exposure to soil contaminants assumes a finite dose scenario (Jeffries and Martin, 2009). Nevertheless, this will allow for the calculation of steady-state flux, and peak flux, which can be compared to previous bioavailability studies to assess the developed *in vitro* dermal bioavailability method performance.

4.4.5 Conclusions

For soil PAH dermal bioavailability experiments, soil will be loaded onto the membrane by weight at 40% moisture content (Stanley et al., 2019) using a brass template with a 8.55 cm² aperture to produce supermonolayer coverage. The soil loading method produced a consistent mean wet soil loading of 1.38g (RSD% = 8.7%). This will allow for consistent membrane soil coverage for future soil PAH dermal bioavailability experiments. The soil removal method first scraped soil from the membrane using a spatula, followed by rinses of Milli-Q water. This soil removal method was effective and consistent, with mean soil mass removal equating to 99.8% (RSD% = 0.05). Therefore, it can be assumed for the soil PAH dermal bioavailability experiment that soil removal will be consistent owing to the extremely low RSD%.

Although the soil removal method was effective, some soil was still visible in the dips on the Strat-M™ membrane surface. The contribution of this soil to the PAH concentration of the membrane extract was assessed. PAH soil concentration was found to have no effect on soil

contribution to the membrane extract concentration. Mass of soil remaining on the membrane and soil PAH bioaccessibility were found to be the drivers for increased soil contribution to the membrane PAH concentration. The soil remaining on the membrane would only lead to a membrane PAH concentration contribution of 3%, which would lead to a minimal impact on the final PAH dermal bioavailability measurements and therefore it can be disregarded.

Overall, this experiment allowed for the assessment of the effectiveness of soil loading and removal methods. Both provide the consistency needed for future soil PAH dermal bioavailability methods. The potential contribution of remaining soil on the membrane on membrane PAH concentration will be corrected for using Equation 18. These methods allowed for the overall aim of the project to be achieved, by providing the membrane with a consistent soil PAH dose and also allowing for the fate of PAH in the experimental system to be determined, and consequently the quantitation of soil PAH dermal bioavailability.

5 The Dermal Bioavailability of PAH

5.1 The Dermal Absorption of PAH from a Solvent Vehicle

5.1.1 Introduction

The key aim of this chapter is to quantify BaP, DBA and Pyr (3-PAH) diffusion through the Strat-M™ membrane into the receptor solution from a solvent vehicle in order to optimise the time steps needed to quantify the dermal absorption of PAH from soil. This will enable the achievement of another aim, which is to calculate the steady-state flux of BaP through the Strat-M™ membrane, for comparison of BaP flux values from previous solvent vehicle *in vitro* studies which have used *ex vivo* skin, to test the Strat-M™ membranes suitability for the measurement of the dermal absorption of BaP from soil.

A common *in vitro* approach to the quantification of the dermal absorption of PAH is the use of diffusion cells (Yang et al., 1989, Wester et al., 1990, Abdel-Rahman et al., 2002, Moody and Joncas, 2007, Moody et al., 2011, Karadzovska, 2013, Duracher et al., 2015) (Sections 2.3 and 2.7). Some studies (Wester et al., 1990, Abdel-Rahman et al., 2002, Moody and Joncas, 2007, Xia et al., 2016) have utilised the application of PAH in a solvent vehicle to an *ex vivo* skin membrane *in vitro* to quantify the concentration of PAH that would partition into the receptor compartment if the applied PAH were 100% bioavailable. This allows for comparison of the concentration of PAH dermally absorbed from a spiked soil, for the purpose of quantifying the impact soil has on the reduction of PAH dermal absorption.

However, for this study, the data produced from previous *in vitro* PAH solvent vehicle dermal absorption studies can be compared to the data produced by the developed diffusion cell method for the purpose of method validation. There has been no evidence found in the current literature for the use of the Strat-M™ membrane for the *in vitro* measurement of the dermal absorption of PAH.

5.1.2 Method

5.1.2.1 Reagents and Materials

Table 26 shows the reagents and materials used for the measurement of the dermal absorption of 3-PAH from a solvent vehicle. The selected receptor solution to be used to measure the *in vitro* dermal absorption of PAH is HEPES Hank's Balanced Salt Solution with 4% bovine serum albumin (HBSS) (Section 4.2)).

Table 26: Summary of reagents and materials used for the measurement of the dermal absorption of 3-PAH from a solvent vehicle.

Reagent/Material	Description	Supplier
Strat-M™ Membrane	Synthetic, non-animal based human skin model.	Merck Millipore (Watford, UK)
PAH Mix-9	100ng/μL USEPA 16 PAH	Qmx Laboratories (UK)
Deuterated PAH surrogate standards	naphthalene-d8, acenaphthene-d10, dibenzothiophene-d8, phenanthrene-d10, pyrene-d10, chrysene-d12, benzo[b]fluoranthene-d12, benzo[a]pyrene-d12, and benzo[g,h,i]perylene-d12 (1000ng/μL in cyclohexane)	Qmx Laboratories (UK)
Deuterated PAH Internal standards	1-methylnaphthalene-d10, fluorene-d10, fluoranthene-d10, benz[a]anthracene-d12, benzo[e]pyrene-d12, perylene-d12 and indeno[1,2,3-cd]pyrene-d12 (1000ng/μL in cyclohexane)	Qmx Laboratories (UK)
Dichloromethane (DCM)	HPLC grade	Fisher Scientific (Loughborough, UK)
Acetone	HPLC grade	Fisher Scientific (Loughborough, UK)
Methanol (MeOH)	HPLC grade	Fisher Scientific (Loughborough, UK)
Strata PAH SPE cartridges	Strata PAH SPE cartridges (0.75g/6mL)	Phenomenex Ltd (Macclesfield, UK)
Hanks' Balanced Salt Solution (HBSS)	Balanced salt solution, pH 7.0 to 7.4.	ThermoFisher Scientific (Loughborough, UK)
Bovine Serum Albumin	pH 7, ≥98%	Merck Life Science UK Ltd. (Gillingham, UK)
HEPES	Fine white crystals. pH 5 to 6.5	Fisher Scientific (Loughborough, UK)

5.1.2.2 Selection of the spiking vehicle and dosage

MeOH was selected as the spiking vehicle owing to its chemical compatibility with the Strat-M™ membrane (Merck, 2018). PAH are also soluble in MeOH, and it is known to be a good medium in which to store PAH (Dąbrowska et al., 2008, Acree Jr, 2013). The concentration of PAH to be applied to the membrane (dosage) needs to produce PAH concentrations

absorbed into the receptor solution that are above the limit of quantification (LOQ (signal to noise ratio of 5)) of the GC-MS. BaP was used as the marker, as it is the most studied and also a driver for risk assessment owing to its toxicity (Pessah et al., 2001, Moody and Joncas, 2007). The LOQ of BaP was calculated to be 14.8pg/μL. Figure 28 shows the estimated concentration of BaP that would reach the receptor solution for different PADA% (percent absorbed of the dose applied) of various doses applied to the membrane. All doses produced estimated receptor solution BaP concentrations greater than the LOQ once the PADA% is greater than 0.03%, but the 1 μg dose applied produces receptor solution concentration that are above the LOQ with a PADA (%) of 0.005%. Larger applied doses were considered, but as a 0.005 % PADA (%) of a 1 μg dose would produce quantifiable concentrations of BaP in the receptor solution, which is much lower than the PADA (%) of 0.09% quoted by Wester et al (1990) for their *in vitro* study, the use of a higher dose was deemed unnecessary. As no previous study has used the Strat-M™ membrane to measure the dermal absorption of BaP before, the likely PADA (%) is unknown, and owing to this it was decided that the full volume of receptor solution from each diffusion cell would be extracted to increase the probability of recovering a mass of BaP greater than the LOQ. Consequently, a diffusion cell is to be removed at each time step.

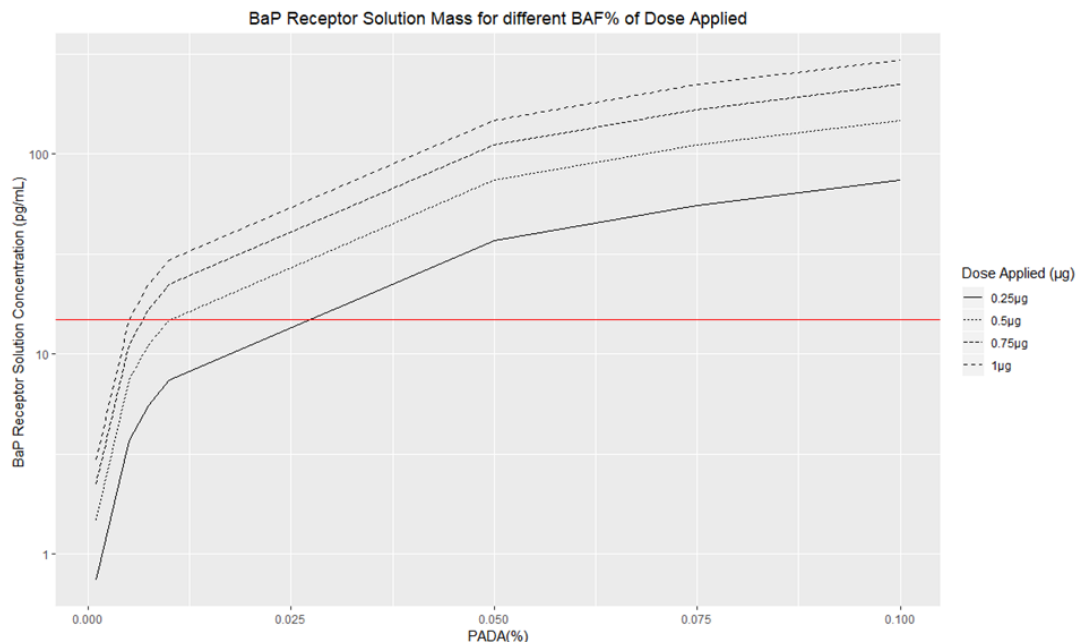


Figure 28: Estimation of receptor solution BaP concentration (pg/mL) for different PADA% (percent absorbed of the dose applied) for different applied dose masses (μg). The red line represented the LOQ for BaP of 14.8pg.

5.1.2.3 Dermal absorption experiment

The dermal absorption of PAH will be measured *in vitro* using a glass Franz-type diffusion cell with an effective diffusion area of 11.3cm² (Section 4.1.1), using the Strat-M™ membrane.

The selected time steps (n=12) for each experimental run (n=3) are shown in Table 27. These time-steps were selected based on those used by previous works (Section 2.3.2). Furthermore, a greater sampling density was used at the beginning of the experiment to determine how soon post-dosing 3-PAH could be quantified in the receptor solution. The experiment was carried out in three subsequent runs so that experimental variation could be quantified. A procedural blank diffusion cell was also run for the duration of the experiment (48 h) for each triplicate experimental run, to ensure that there was no addition of PAH from sources external to the experiment.

Table 27: Time steps for diffusion cell removal (n=12).

Time step	Start	1	2	3	4	5	6	7	8	9	10	11	12
Time (h)	0	0.25	0.5	0.75	1	2	4	6	8	12	24	36	48

Prior to the addition of the receptor solution to the diffusion cells, the receptor solution needs to be degassed to ensure that the receptor solution is in uninterrupted contact with the Strat-M membrane by preventing the formation of gas bubbles. It is vital to ensure that there is uninterrupted contact between the receptor solution and membrane so that the diffusion of compounds is not inhibited, as this would consequently lower the rate of diffusion of PAH through the Strat-M™ membrane into the receptor solution leading to an inaccurate measurement of the concentration of PAH that are dermally absorbed over time. To degas the receptor solution, a glass coated magnetic stirrer bar is placed in the base of a 500mL Büchner flask with 400mL of receptor solution then added. The Büchner flask is then connected to a pump and placed on a magnetic stirrer plate. The magnetic stirrer is set to 50rpm, and the pump switched on to create a vacuum under 10" Hg and left to degas for 12 h. The stirrer ensures the cycling of the solution, allowing any gas bubbles to rise under vacuum and be subsequently removed from the solution.

The spiking solution to be used was made up prior to the assembly of the diffusion cells by diluting the PAH-Mix 9 from Qmx by a factor of 10, resulting in a solution containing 10ng/μL USEPA 16 PAH in MeOH.

The diffusion cells were assembled, with degassed receptor solution (30mL of HEPES Hank's Balanced Salt Solution with 4% bovine serum albumin (HBSS) (Section 4.3)) along with a glass magnetic stir bar placed in the receptor compartment, ensuring that the receptor solution was in contact with the Strat-M membrane with no gas bubbles. The diffusion cells were held together using bulldog clips around the flange of the donor and receptor compartments (Section 4.1.1). The assembled diffusion cells were submerged in a water bath at 32°C (surface temperature of human skin (Sartorelli et al., 1999)), with the magnetic stirrer set at 100rpm, and allowed to stabilise for 2 h to allow for the receptor solution to reach 32°C. The purpose of the magnetic stirrer in the receptor compartment is to promote the circulation of the receptor solution in the receptor compartment, so that "fresh" solution can be in contact with the membrane over time so that diffusion of PAH from the membrane into the receptor solution is not inhibited. Although, if the stirrer bar rpm is too high, there is the potential to increase flux by drawing down compounds in the membrane, resulting in an over calculation of the amount dermally absorbed. After stabilisation, each diffusion cell was removed from the water bath and checked by eye to ensure no gas bubbles had formed in the receptor solution. 100µL of spiking solution (10ng/µL USEPA 16 PAH in MeOH) was added by 100µL gas tight syringe to the surface of the Strat-M™ membrane to create a mass loading of 1 µg of each USEPA 16 PAH.

At each time step (Table 27), the corresponding diffusion cell was removed from the water bath. The clips were removed allowing the donor and receptor compartments to be separated. The membrane was then removed using tweezers and placed in a 10mL screw cap vial and labelled with the sample name. The receptor solution was removed from the receptor compartment by Pasteur pipette into a Pyrex screw cap 50mL bottle and labelled with the sample name. The membrane was then extracted using the previously optimised ASE extraction (Section 4.2.6), and the receptor solution by SPE (Section 4.3.2.3), with both extracts then analysed by GC-MS (Section 3.6).

5.1.3 Results

5.1.3.1 Quality Control

During each GC-MS analytical run, a 30pg/µL standard containing USEPA 16 PAH, and was analysed in triplicate for quality control purposes. Table 28 shows the mean concentration, standard deviation, RSD% (relative standard deviation (%)) for 3-PAH. All three RSD% values are low, meaning that the analytical method is producing high precision for the standard.

Table 28: The mean 3-PAH concentration, standard deviation (S.D.), and RSD% of the 30pg/μL analytical standard.

	30pg/μL Std		
	Mean (pg/ μL)	S.D. (pg/ μL)	RSD%
BaP	34.7	0.57	1.6
DBA	36.2	1.41	3.9
Pyr	34.9	0.27	0.78

To assess the efficiency of the diffusion cell method and following extractions, the mass balance is calculated for each time step, the purpose of which is to calculate the proportion of each PAH accounted for in comparison to the concentration applied at the beginning of the experiment to subsequently account for any losses (Equation 19).

$$\text{Mass Balance (\%)} = \frac{\text{membrane PAH mass} + \text{receptor solution PAH mass}}{\text{applied PAH mass}} \times 100$$

Equation 19: Diffusion Cell PAH Mass Balance from a solvent vehicle (%)

The mass balance for 3-PAH is shown in Table 29. The mean mass balance for BaP is 92.7%, with a standard deviation of 36.4 and a subsequent RSD% of 39.3%. The mass balance is largely impacted by the extraction efficiency of the membrane as these hold the larger proportion of PAH mass. The high RSD% is largely due to the extremity of the PAH mass quantified in the membrane extractions; for example, at 32 h a mass of 364ng is reported compared to a mass of 1433ng at 12 h. The Mass balance of DBA produced a mean of 61.6%, with an RSD% of 46.8. This high RSD% is due to some of the membrane extractions producing DBA concentrations below the LOQ, meaning no data is available for some samples. Pyr produced a good mean mass balance of 95.8%, with an RSD% of 17.2. This PAH was included for quality control owing to the stability of Pyr analytically (Section 2.6), and therefore owing to the high mass balance, it is assumed that all extractions and the diffusion cell method did not result in significant PAH losses.

Table 29: The Mass Balance (%) of three focal PAH.

	Mean (%)	SD (%)	RSD%
BaP	92.7	36.4	39.3
DBA	61.6	39.8	64.6
Pyr	95.8	16.5	17.2

5.1.3.2 PAH concentration in the Receptor Solution

To produce PAH data as a concentration in the receptor solution, the mass of the PAH was divided by the volume of the receptor compartment of a diffusion cell (30 ± 0.54 mL).

Table 30: Concentration (pg/mL) of Bap, DBA and Pyr in the receptor solution (RS) over time. Number in brackets represents the RSD (%).

Time (h)	3-PAH Mean RS Concentration (pg/mL)					
	Pyr		BaP		DBA	
0.25	1081	(124)	676	(124)	1147	(141)
0.5	327	(57.7)	119	(100)	2952	(87.1)
0.75	223	(55)	144	(76.83)	357	(141)
1	237	(99.5)	242	(121)	1128	(76.7)
2	410	(99.6)	713	(136)	863	(137)
4	325	(70.3)	403	(105)	658	(125)
6	272	(13.7)	281	(72.9)	453	(95.9)
8	477	(43.1)	346	(48)	459	(89)
12	225	(38.8)	180	(129)	436	(123)
24	353	(11.1)	249	(77.3)	359	(99.7)
32	474	(19.1)	343	(97.3)	349	(134)
48	491	(15.5)	365	(87.8)	400	(106)

Table 30 shows the mean 3-PAH RS concentration (pg/mL) over time (h). The mean concentration of BaP in the receptor solution over three runs for each time step ranged from 119 – 713 pg/mL, with an overall mean concentration of 338 pg/mL. DBA is in the receptor solution in the highest concentration out of the three PAH, with the mean concentration of the three runs throughout the experiment ranging from 349 – 2952 pg/mL, with an overall

mean of 797 pg/mL. For Pyr, the concentration ranges from 223 – 1080 pg/mL, with an overall mean concentration of 408 pg/mL. The general trend is that the 3-PAH RS concentration increases with time, and also decreases in variability (RSD%) with time. The mean concentration of 3-PAH at 0.25 h is skewed heavily by one sample (0.25 h Run 1). For example, BaP mean concentration in the RS would be 81.4 pg/mL without sample 0.25 h run 1 and 676 pg/mL with this sample included. Dixon's Q-tests showed that this sample was statistically an outlier but owing to only carrying out three replications the statistical power of the test is low, and the sample included. Consequently, for future dermal bioavailability work, five replications will be carried out to allow for the statistical testing for outliers if necessary.

Some studies report data as percent absorbed of the dose applied (PADA) (Equation 20) which represents the proportion of the dose exposed to which has entered the skin and/or systemic circulation (For example: (Yang et al., 1989, Wester et al., 1990, Stroo et al., 2005b).

$$PADA (\%) = \frac{\text{Mass PAH in receptor solution}}{\text{Mass PAH applied}} \times 100$$

Equation 20: Formula for the calculation of Percent Absorbed of Dose Applied (PADA) (%)

PADA is calculated for BaP, DBA and Pyr for each run and timestep (Section 2.3.2). The mean PADA (%) of BaP in the receptor solution for each time step ranged from 0.036 - 0.21%, with an overall mean of 0.1% and an RSD% of 53.3. For DBA, the mean PADA (%) for each time step ranged from 0.1 – 0.88%, with an overall mean of 0.24% and an RSD (%) of 88.8%. Pyr mean PADA (%) for each time step ranged from 0.06 – 0.32%, and an RSD% of 54.8%. Figure 29 shows that the amount of each PAH absorbed into the receptor solution over the first 2 h is rapid. By looking at the amount of error/variability associated with each time step, it can be seen that the concentration in the receptor solution stabilises at 24 h for each PAH.

Table 31: Receptor solution Percent Absorbed of the Dose Applied (PADA) (%) for 3-PAH over time (h).

Time (h)	3-PAH Mean PADA (%)		
	Pyr	BaP	DBA
0.25	0.324 (124)	0.203 (124)	0.344 (141)
0.5	0.098 (57.7)	0.036 (100)	0.886 (87.1)
0.75	0.067 (55)	0.043 (76.83)	0.107 (141)
1	0.071 (99.5)	0.073 (121)	0.338 (76.7)
2	0.123 (99.6)	0.214 (136)	0.259 (137)
4	0.097 (70.3)	0.121 (105)	0.197 (125)
6	0.082 (13.7)	0.084 (72.9)	0.136 (95.9)
8	0.143 (43.1)	0.104 (48)	0.138 (89)
12	0.068 (38.8)	0.054 (129)	0.131 (123)
24	0.106 (11.1)	0.075 (77.3)	0.108 (99.7)
32	0.142 (19.1)	0.103 (97.3)	0.105 (134)
48	0.147 (15.5)	0.109 (87.8)	0.120 (106)



Figure 29: Mean PADA (%) for the three focal PAH in the receptor solution over time. Error bars represent standard error.

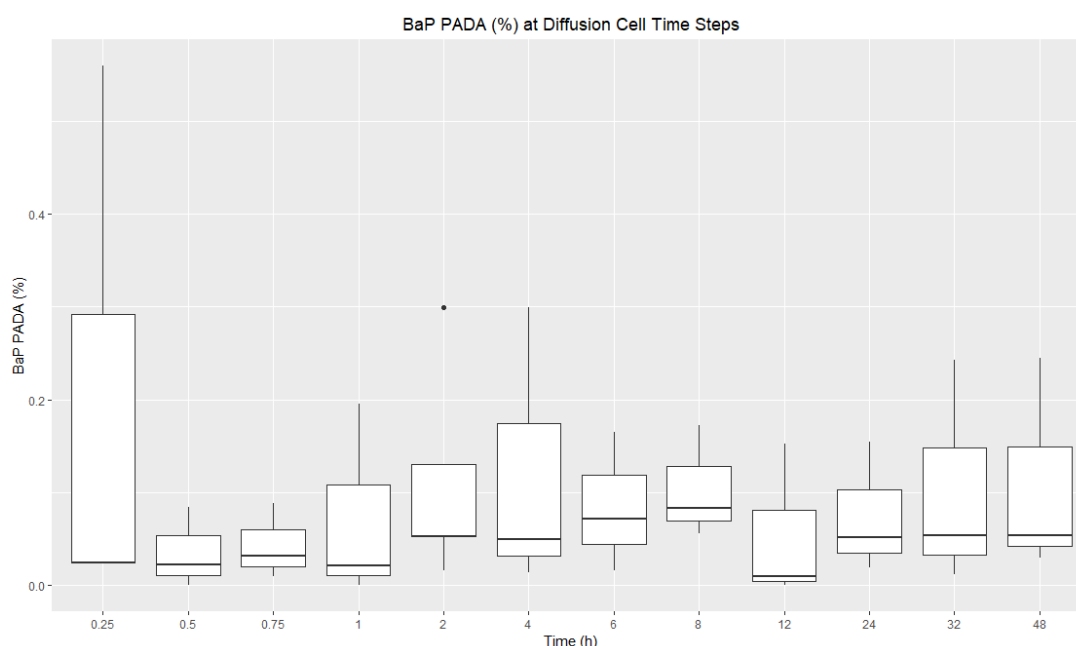


Figure 30: Boxplot of BaP PADA (%) in the receptor solution at each time step of diffusion cell experiment.

The data of Table 30 and Table 31 shows a decrease in variability between each run at 24 h. For example, the RSD (%) for the three runs for Pyr at 0.5h is 57.6%, whereas at 24 h it is 11.2%, staying at a similar level of variability thereafter. This suggests that when the dose is applied, the absorption of PAH is rapid and highly variable to begin with, and diffusion reaches steady state at around 24 h. Figure 30 also shows this, showing much more variable results at the first time step compared to $t=24$. The boxplot also shows that the mean concentrations are not significantly different including and after $t=24$.

5.1.3.3 Calculation of the Flux of BaP

Using the mass of BaP in the receptor solution at each time step, the rate of which its diffusion through the Strat-M™ membrane occurs (known as flux) can be calculated using Fick's first law of diffusion (Chilcott and Price, 2008, Pugh and Chilcott, 2008). The steady-state flux (J_{ss}) is reached after a lag phase in the flux has passed and occurs as the concentration in the receptor solution begins to stabilise (Beriro et al., 2016), which in this study occurs at the 24 h time step. J_{ss} is usually expressed as concentration per area per hour e.g., $\text{ng}/\text{cm}^2/\text{h}$. Equation 21 shows an alternative expression of Fick's first law of diffusion.

$$J = \frac{\left(\frac{\Delta C}{\Delta t}\right)}{A}$$

Equation 21: Alternative expression of Fick's First Law of diffusion. Where J is flux, ΔC is change in concentration (concentration gradient) and Δt is change in time. A is the surface area exposed to the compound of interest.

Using this equation, the mean J_{ss} for BaP was calculated to be 0.028 ng/cm²/h (with a standard deviation of 0.024).

5.1.4 Discussion

5.1.4.1 Data Quality

Overall, the mass balance (%) of BaP (Table 29) was excellent, with a mean of 92.7%. The mass balance (%) of BaP at 24 h is similar to that achieved by Moody and Joncas (2007) (This Study = 105% compared to 95% for Moody and Joncas (2007)). This suggests that the methods used to extract BaP from the Strat-M™ membrane and receptor solution and subsequent quantification by GC-MS is as efficient as the use of liquid scintillation counting (LSC) by Moody and Joncas (2007), which is a common approach to the quantification of radiolabelled compounds in dermal absorption studies (Wester et al., 1990, Moody et al., 1995, Moody and Joncas, 2007, Peckham et al., 2017).

Dixon's Q-test found several outliers (Run 1 time step 0.25 and Run 3 time step 2) but owing to the small number of replicates (n=3), the statistical power of the test is not great enough to justify the exclusion of these data points. Peckham et al. (2017) reported outliers which were also removed by Dixon's Q-test due to higher BaP concentrations in the receptor solution, which was found to be due to membrane failure. This could be a reason for much higher receptor solution PAH concentrations in some samples. Future experiments should be carried out with 5 replicate runs so outliers can be addressed with greater certainty.

Some variation in the data may be sourced from the fact that the triplicates were carried out consecutively rather than in parallel and therefore under different external environment conditions. Consequently, the soil diffusion cell experiment triplicates will be carried out in tandem to ensure that there is no impact from variations in the external environment which could contribute to variation in absorption.

The higher RSD% for DBA in comparison to BaP and Pyr throughout could be due to a number of factors. The first being that owing to it being the analyte with highest mass ($m/z = 278$) and secondly DBA having the lowest volatility (Henry's law Constant at 25°C of 0.00149 Pa m³ mol for DBA, 0.046 Pa m³ mol for BaP and 0.92 Pa m³ mol for Pyr (Nathanail et al., 2009))

it is the last analyte to elute from the column when the column is at its highest temperature, with the higher temperature creating a larger amount of column bleed and therefore greater background noise. This results in a larger signal response being required in comparison to earlier eluting compounds for the signal to noise ratio to be greater than 5 to pass the LOQ (Fialkov et al., 2007).

5.1.4.2 Flux of BaP

The flux of BaP from a solvent vehicle through the Strat-M™ membrane was calculated as 0.028 ng/cm²/h (standard deviation of 0.021). The calculated flux from this work is within the range of flux of BaP from a solvent vehicle reported in the literature (Table 32). As shown in Table 32, a wide range of BaP flux values from a solvent vehicle have been derived (0.019 – 22.4 ng/cm²/h) using *ex vivo* human skin. This further demonstrates the variability in the dermal absorption of compounds depending on skin condition and anatomical site as shown by VanRooij (1993). Table 32 also shows that previous works have used acetone as a vehicle for the application of BaP to the membranes, however, as acetone is incompatible with the polymers of the Strat-M™ membrane MeOH was used (Merck, 2018). It is known that vehicular effects can impact chemical absorption rates due to its interactions with the lipids in the *stratum corneum*, and also due to the influence of chemical solubility in the application vehicle (Davis et al., 2002, Karadzovska and Riviere, 2013). Consequently, It would be beneficial for other studies to use the Strat-M™ membrane to measure the dermal absorption of BaP from a range of solvent vehicles (e.g., acetonitrile, MeOH), as this will aid in the determination in the source of variation in flux values using the Strat-M™ as either method related, or membrane related. The higher flux of BaP through *ex vivo* human skin in studies such as Moody *et al* (2007) compared to the BaP flux through the Strat-M™ may also be due to the artificial membrane not representing the transappendageal dermal absorption pathway, which is present in human skin, which is the absorption pathway where rapid absorption occurs through sweat glands and hair follicles thereby avoiding the lipid matrix of the epidermis (Crosera et al., 2009) and increasing the rate of absorption of lipophilic compounds. Table 32 also shows the RSD% for BaP flux values derived by previous *in vitro* studies which have used *ex vivo* skin. The RSD (%) produced in this study using the Strat-M™ is (77.2%) is lower than those calculated for studies using *ex vivo* skin (RSD (%) 84.2% - 124.9%). This reduction in variation using the Strat-M™ is likely due to less variation in the membrane structure and lipid composition in comparison to *ex vivo* skin sources (Haq et al., 2018). The data suggests that the Strat-M™ has the capability to produce similar BaP flux

values to *ex vivo* human/animal skin with lower variability, suggesting it may be a suitable substitute for *ex vivo* skin, but further investigation inter and intra laboratory is required to confirm this as the data set for the Strat-M™ is much smaller than for *ex vivo* skin.

Table 32: Examples of measured flux of BaP from a solvent vehicle from previous studies. *a* is as reported by Peckham *et al* (2017). *b* was calculated using maximum error given in Peckham *et al* (2017).

Study	Apparatus	Membrane	Vehicle	n	BaP Flux (ng/cm ² /h)	Std Dev	RSD%
<i>This Study</i>	Franz-type Diffusion Cell	Strat-M™	Methanol	3	0.028	0.021	77.2
Moody <i>et al</i> (2007)	Bronaugh Flow-Through Diffusion Cell	Human breast skin	Acetone	6	22.4	20.01	89.3
Wester <i>et al</i> (1990)	Flow-Through Diffusion Cell	Human cadaver skin	Acetone	6	0.019 ^a	0.016 ^b	84.2
Peckham <i>et al</i> (2017)	Flow-Through Diffusion Cell	Human cadaver skin	Acetone	6	0.11	0.14 ^b	124.9
Sartorelli <i>et al</i> (1999)	Static Diffusion Cell	Monkey (<i>Cercopithecus aethiops</i>)	Acetone and artificial sweat	6	0.353	0.303	85.8

5.1.4.3 PAH receptor solution concentrations

Figure 30 displays the PADA (%) of BaP in the receptor solution for each time step. Figure 1 shows that the dermal absorption of BaP is a rapid process, as within 0.25h there is quantifiable concentrations of BaP that have reached the receptor solution. The receptor solution represents systemic circulation, and although BaP from a solvent vehicle application represents 100% bioavailability, it is possible that the same concentration of BaP will be available for uptake from soils (soil BaP will not be 100% bioavailable), and if this is the case, this could impact exposure scenarios used for human health risk assessments. If 0.1% of BaP for every 1 mg of BaP in soil is available for uptake, the same concentration of BaP would be available for dermal absorption as in this solvent vehicle experiment. Therefore, it is probable that BaP will reach the receptor solution from soils in quantifiable concentrations.

Figure 29 shows that 3-PAH display rapid diffusion through the membrane into the receptor solution within the first 2 h, which is a similar trend to the BaP solvent vehicle data of Abdel-Rahman et al. (2002). The receptor solution concentration appears to stabilise at around 6-8 h for BaP and DBA, and slightly later for Pyr at 24 h. This reduction in variation is clearly shown in the reduction in error after the aforementioned times for each congener. The drop in PADA (%) for each PAH at around 2 h may be due to an increase in analytical error owing to the matrix effect of the receptor solution samples. The drop in PADA (%) could also be caused by a back diffusion of PAH from the receptor solution into the membrane as the concentration gradient changes, with the curves flattening once the flux has equilibrated. The critical receptor for residential land for many exposure assessment approaches, such as the CLEA model, is a female child of up to 6 years old. This is due to a combination of higher childhood exposure to major pathways and lower bodyweight in comparison to adults. For the CLEA model it is assumed that the child will spend 1 hour per day in the garden (Jeffries and Martin, 2009). It has been shown previously that the presence of soil results in a decrease in the dermal bioavailability of PAH (Abdel-Rahman et al., 2002, Xia et al., 2016). However, if the same initial rapid rate of absorption shown in the solvent vehicle data is seen in later soil experiments it could suggest that a significant amount of the total PAH concentration that could be dermally absorbed will be absorbed in the 1 hour of exposure time of the critical receptor.

5.1.5 Conclusions

The aims of the experiment were to:

1. To quantify BaP, DBA and Pyr diffusion through the Strat-M™ membrane into the receptor solution from a solvent vehicle over a period of 0.25-48h to optimise the time steps needed to quantify the dermal absorption of PAH from soil.
2. To calculate the steady-state flux of BaP through the Strat-M™ membrane for comparison of BaP flux values from previous solvent vehicle *in vitro* studies which have used *ex vivo* skin to test its suitability for the measurement of the dermal absorption of BaP from soil.

Both aims have been achieved, with PAH being quantifiable in both the Strat-M™ membrane and the receptor solution over the 48h duration of the experiment, producing similar mass balances to previous studies which use the traditional LSC techniques for measuring the dermal absorption of radiolabelled PAH. The absorption of BaP through the Strat-M™ membrane reaches steady state flux (J_{ss}) at 24 h. The absorption of PAH into receptor solution is a rapid process, with quantifiable concentrations of the three focal PAH reaching the receptor solutions at the first time step (0.25 h). The BaP flux calculated from the collected data is within the range of fluxes previously reported in the literature, and also shows less variability in absorption values than *ex vivo* skin. Therefore, it is suggested that the Strat-M™ membrane may be an adequate replacement for *ex vivo* skin for the *in vitro* measurement of the dermal bioavailability of PAH.

5.2 The Dermal Absorption of PAH from Contaminated Soils

5.2.1 Introduction

The purpose of this chapter is to quantify the *in vitro* human dermal absorption of native PAH from contaminated soils over a period of 48 h. By doing so, the aim of this project was realised, which is to quantify the human dermal bioavailability of PAH from contaminated soils (Section 1.2). This was achieved by using the optimised diffusion cell method developed throughout this project (Chapter 4).

There is no evidence in the literature for the use of the Strat-M™ artificial skin membrane to quantify the *in vitro* dermal absorption of native PAH from contaminated soils. The Strat-M™ membrane was selected owing to its cost, accessibility, lack of need for specialist storage,

structural consistency, and ethical considerations (Section 2.2.6 and 4.1) The Strat-M™ has been shown to produce similar BaP flux values to studies that used *ex vivo* skin (Section 2.2.6, 5.1). Therefore, the Strat-M™ membrane was used in this study to measure the dermal absorption of PAH from real-world contaminated soils.

Previous *in vitro* studies have focused on the measurement of the dermal bioavailability of PAH from solvent spiked soils (Yang et al., 1989, Wester et al., 1990, Moody et al., 1995, Abdel-Rahman et al., 2002, Stroo et al., 2005b, Moody and Joncas, 2007, Moody et al., 2011, Peckham et al., 2017). Consequently, this likely underrepresents the aging process and the impact of other soil properties on PAH mobility (Sections 2.1.3 and 2.3.2). This leads to greater PAH mobility compared to real-world soils and subsequent potential for the overestimation of the soil PAH dermal bioavailability. As a result, the PAH dermal bioavailability values used in current methods for exposure assessment of PAH are unrepresentative of real-world exposure (Alexander, 2000, Abdel-Rahman et al., 2002). Therefore, this study quantified the dermal absorption of PAH from real-world contaminated soils (n = 2) from former gasworks using the optimised diffusion cell, and PAH extraction and quantification methods (Sections 3.4, 3.5, and 3.6). The quantification of native PAH dermal bioavailability will enable a more realistic representation of the real-world exposure scenario, which is of immense importance for the accuracy of HHRA and risk-based contaminated land management. The developed *in vitro* diffusion cell method performance was then assessed and compared to previous studies.

Comparisons of PAH dermal absorption data between studies is also difficult, due to variations of experimental parameters, such as soil loading and access to the same samples (Sections 2.3.3, 2.3.4 and 2.3.3). Therefore, in order to allow for a direct comparison between dermal bioavailability studies in future, a certified reference material (CRM) will also undergo the diffusion cell method.

5.2.2 Method

5.2.2.1 Reagents and Materials

Table 33 shows the reagents and materials used for the measurement of the dermal absorption of 3-PAH from soils. The selected receptor solution to be used to measure the *in vitro* dermal absorption of 3-PAH is HEPES Hank's Balanced Salt Solution with 4% bovine serum albumin (HBSS) (Chapter 4.3). The Strat-M™ (47mm diameter) membranes were purchased from Merck Millipore (Watford, UK). The stock solution for the creation of the analytical standards was Qmx PAH-Mix 9 (Qmx Laboratories, UK), which contains 100 ng/μL

USEPA 16 PAH in cyclohexane. All solvents used were purchased from Fisher Scientific (Loughborough, UK) and of HPLC grade, and were used without further purification.

Table 33: Summary of reagents and materials used for the measurement of the dermal absorption of 3-PAH from soils.

Reagent/Material	Description	Supplier
Strat-M™ Membrane	Synthetic, non-animal based human skin model. 47mm diameter.	Merck Millipore (Watford, UK)
PAH Mix-9	100 ng/μL USEPA 16 PAH in cyclohexane	Qmx Laboratories (UK)
Deuterated PAH surrogate standards	pyrene-d10, benzo[b]fluoranthene-d12, benzo[a]pyrene-d12, and benzo[g,h,i]perylene-d12 (1000 ng/μL in cyclohexane)	Qmx Laboratories (UK)
Deuterated PAH Internal standards	fluoranthene-d10, perylene-d12 and indeno[1,2,3-cd]pyrene-d12 (1000 ng/μL in cyclohexane)	Qmx Laboratories (UK)
Dichloromethane (DCM)	HPLC grade	Fisher Scientific (Loughborough, UK)
Acetone	HPLC grade	Fisher Scientific (Loughborough, UK)
Strata PAH SPE cartridges	Strata PAH SPE cartridges (0.75g/6mL)	Phenomenex Ltd (Macclesfield, UK)
Hanks' Balanced Salt Solution (HBSS)	Balanced salt solution, pH 7.0 to 7.4.	ThermoFisher Scientific (Loughborough, UK)
Bovine Serum Albumin	pH 7, ≥98%	Merck Life Science UK Ltd. (Gillingham, UK)
HEPES	Fine white crystals. pH 5 to 6.5	Fisher Scientific (Loughborough, UK)

5.2.2.2 Selection of Soils and CRM

Two former gasworks soils were chosen with BaP concentrations that exceeded the BaP Category 4 Screening Levels (C4SL) (Chapter 2.5) of 5 mg/kg for residential land with home grown produce (CL:AIRE, 2014b) (Table 34**Error! Reference source not found.**). Exceedance of the BaP C4SL warrants further investigation into potential human health risk under UK contaminated land guidance (Section 2.5)

Table 34: Mean measured Pyr, BaP and DBA concentration, soil classification, and Total Organic Carbon (TOC%) of the three soils to undergo dermal bioavailability testing.

Soil	Industrial Activity	Measured PAH Concentration (mg/kg)			Soil Classification	TOC(%)
		Pyr	BaP	DBA		
Soil A	Gasworks	115	179	26.7	Sandy Made Ground ^b	11.72 ^b
Soil B	Gasworks	86.8	104	11.8	Sandy Made Ground ^b	8.92 ^b
BCR-524	Wood Treatment	121	6.15	0.96	Contaminated Industrial Soil ^a	Nd ^a

^aNo soil classification given by JRC (De Voogt *et al*, 1996). Soil was homogenised and dried during CRM preparation. Soil described as "Contaminated Industrial Soil". Nd = No data

^bAs determined by Beriro *et al* (2020).

The selected CRM is "BCR-524 Industrial Soil (PAH's)" from the European Commission Joint Research Centre Catalogue of Certified Reference Materials. The CRM was originally collected from a former industrial wood treatment plant, with the PAH contamination source being from creosote oil (De Voogt *et al.*, 1996). This CRM was selected as it represents another former industrial activity that can result in soil PAH concentrations that would warrant further investigation into potential human health risks under UK contaminated land management regulations (Dahlgren *et al.*, 2003, Coronas *et al.*, 2013).

The two gasworks soils and CRM were extracted (five replications of each soil) using previously described methods (Sections 3.4 and 3.5) prior to GC-MS analysis (Section 3.6). These starting soil concentrations (Soil^{Total}) were used to calculate the applied PAH dose to the membranes for the soil PAH dermal bioavailability experiment.

5.2.2.3 Soil Dermal Absorption Experiment

Owing to the amount of PAH directly in contact with the membrane in the soil compared to a solvent spike, it was assumed that the lag time (The time taken to achieve steady-state flux under an infinite dose scenario (Pugh and Chilcott, 2008)) is greater when measuring soils in comparison to solvents (Peckham *et al.*, 2017). This is because the PAH delivered by the solvent vehicle will be on the skin surface and free to be absorbed. Whereas in comparison, only a fraction of soil PAH would be in contact with the membrane and mobile, with the majority of PAH in soil layers above the soil monolayer which is in contact with the membrane surface. Consequently, the first sample was taken at 0.5h rather than 0.25h with the remainder of the timesteps as detailed (Section 5.1) (Table 35).

Table 35: Time-steps at which diffusion cells were removed for PAH quantification (n=8).

Time Step	Start	1	2	3	4	5	6	7	8	8 (blank)
Time (h)	0	0.5	1	2	6	12	24	36	48	48

An overview of the experimental procedure of each diffusion cell experimental run is shown in Figure 31.

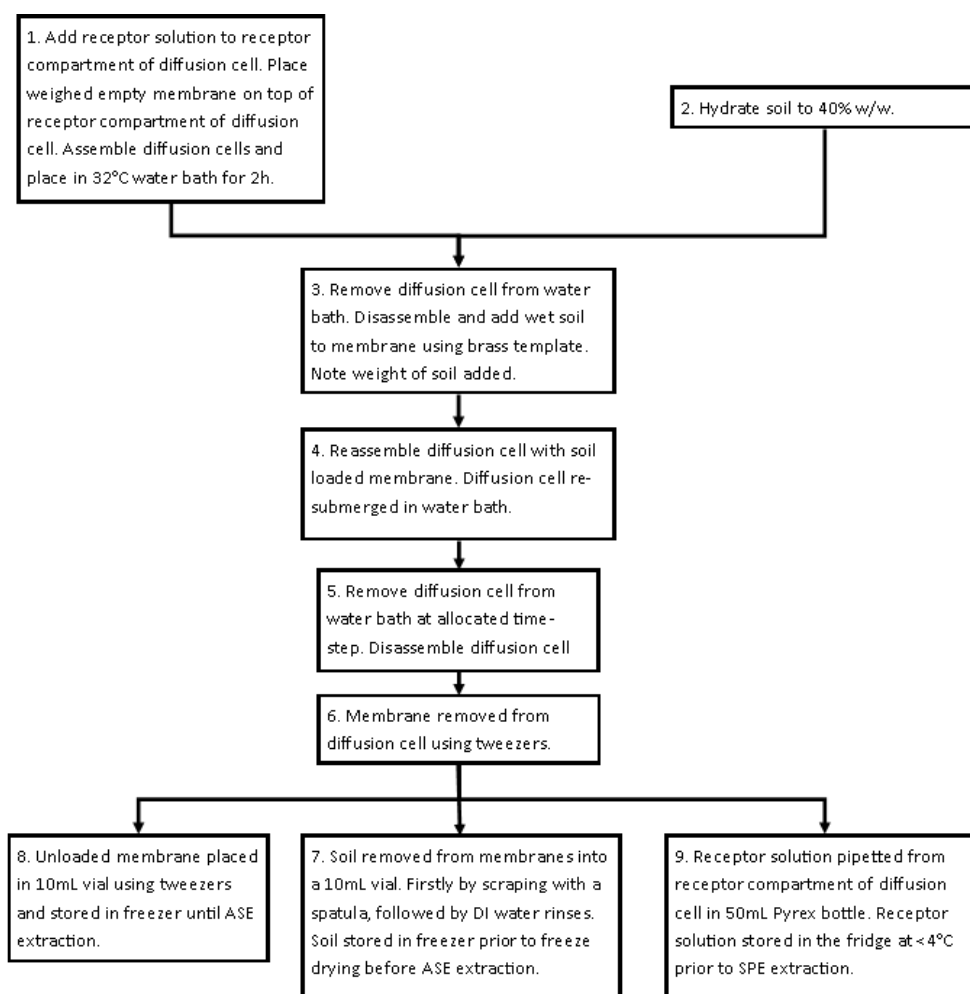


Figure 31: Overview of the experimental steps for an experiment run of the 48h soil PAH dermal bioavailability diffusion cell experiments.

The same experimental apparatus was used as for the dermal absorption of PAH from a solvent vehicle experiment (Chapter 5.1). The receptor solution was prepared and degassed as in the PAH dermal absorption from a solvent vehicle experiment (Chapter 5.1). The receptor solution volume of each diffusion cell was measured gravimetrically and noted prior to diffusion cell assembly. The assembled diffusion cells (Figure 32A) were stabilised in a water bath at 32°C for 2 h, prior to being loaded with soils (Chapter 4.4). The soils were

saturated to 40% w/w (Stanley et al., 2019), prior to being loaded onto the (pre-weighed) membranes using the brass template (Section 4.4.2) to achieve a mean dry weight loading of 80 mg/cm². The diffusion cells were then assembled with the now soil loaded membrane and re-submerged in the water bath with magnetic stirrers set at 100rpm. The diffusion cells remained submerged in the water bath (Figure 32B) under these conditions until each diffusion cell was then removed from the water bath at their allocated time step (Table 35).

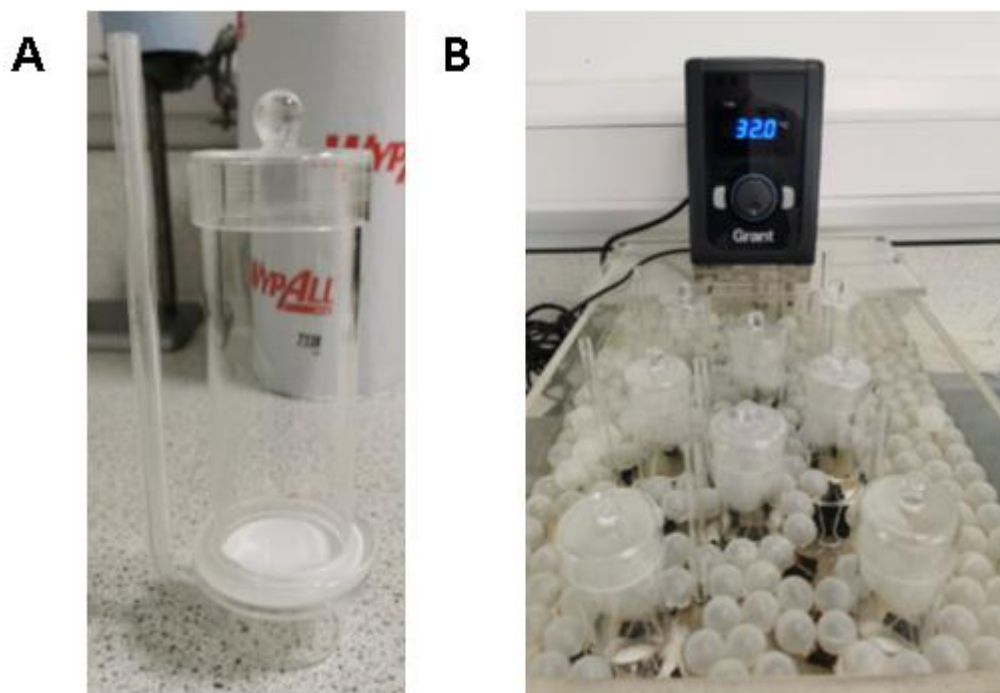


Figure 32: A) Empty Franz-type diffusion cell used for throughout this study. The flange joint was held together using bulldog clips. B) Loaded diffusion cells submerged in the water bath. An experimental run consisted of eight soil-loaded diffusion cells, and one blank unloaded diffusion cell.

Once removed, diffusion cells were disassembled, and each compartment removed and prepared for extraction so that the fate of PAH in the system could be determined. The membrane was removed using tweezers, and excess soil scraped off using a spatula followed by rinses of Milli-Q water (Section 4.4). The soils that underwent the diffusion cell experiment and removed from the membranes will be referred to as soil^{DERM} herein. The soil^{DERM} then underwent freeze drying prior to ASE extraction (Beriro et al., 2014) (Section 3.3). Once the soil^{DERM} had been removed from the membrane, the membrane weight was noted. The membrane was then placed in a 10mL vial and then stored in a freezer until extraction. The receptor solution was pipetted from the receptor compartment of the diffusion cell into 50mL Pyrex screwcap bottles until extraction (4.3)

Each diffusion cell experimental run consisted of eight soil loaded diffusion cells, with an additional experimental blank diffusion cell which was run for the full 48 h experimental duration. This was carried out in 5 replications for Site A and B soils, and with 4 replications for BCR-524. It was originally intended to carry out 5 replications for BCR-524 as well, but this was not possible due to BCR-524 supply issues brought about by the COVID-19 pandemic.

The soil^{DERM} samples underwent the same extraction and clean-up procedure as mentioned in Section 5.2.2.2. For analysis, each soil^{DERM} extract was dosed with 200pg/μL of internal standards and diluted so that the PAH concentrations would fall within the working linear range of the GC-MS calibration. This was achieved by diluting BCR-524 soil^{DERM} extracts by a factor of 10, and Site A and B soil^{DERM} extracts by a factor of 100. The dilution factors were devised by analysing a 200pg/μL PAH standard by GC-MS (Section 3.6), followed by diluted soil^{DERM} samples and comparing PAH chromatographic peak responses.

The membranes were extracted using the optimised ASE method discussed in Section 4.2.6. Different surrogate PAH concentrations were applied to membrane samples owing to their respective theoretical PAH dose from the soil applied: Site A – 50ng, Site B – 30ng, and BCR-524 – 10ng (pyrene-d10, benzo[b]fluoranthene-d12, benzo[a]pyrene-d12 and benzo[g,h,i]perylene). 200pg/μL internal standards (fluoranthene-d10, perylene-d12 and indeno[1,2,3-cd]pyrene-d12) were applied to each sample once extracted. Then time-step and PAH dose applied dependent dilution factors were applied (Table 36). This was carried out under the assumption that the membranes would have absorbed greater PAH masses with increased time in contact with the soils, as shown in previous work (Roy and Singh, 2001, Abdel-Rahman et al., 2002). The diluted samples were then analysed by GC-MS (Section 3.6)

Table 36: Dilution factors applied to membranes used to test different soils dependent on starting soil concentration and time in contact with exposed soil.

Time Step	Time (h)	Membrane Dilution Factor	
		Site A and B	BCR-524
1	0.5	2	2
2	1	2	2
3	2	2	2
4	6	4	2
5	12	4	2
6	24	10	4
7	36	10	4
8	48	10	4

Receptor solutions were extracted using the SPE method described in Chapter 4.3. Prior to extraction, the receptor solutions were spiked with different surrogate (pyrene-d10, benzo[b]fluoranthene-d12, benzo[a]pyrene-d12 and benzo[g,h,i]perylene) concentrations, again respective of the soil starting PAH concentration: Site A – 30 ng, Site B – 20 ng, and BCR-524 – 10 ng. After SPE extraction, the fraction containing PAH was reduced to 1mL. Following this, owing to the receptor solution being likely to contain the lowest PAH concentration, the samples were concentrated by a factor of 4 into 250µL GC-MS vial inserts (final volume 0.25mL). The equivalent concentration of internal standards (fluoranthene-d10, perylene-d12 and indeno[1,2,3-cd]pyrene-d12) of the corresponding surrogate standards were then added to each sample prior to GC-MS analysis (Section 3.6).

5.2.3 Results

5.2.3.1 Quality Control

Overall, a total of 530 samples were analysed by GC-MS. During each GC-MS analytical run for membrane and soil^{DERM} samples, a 200pg/µL standard consisting of USEPA 16 PAH was analysed in duplicate (n=44 total) and the beginning and end of a batch of samples from the same diffusion cell run. A total of 126 membrane samples (14 blanks), and 112 soil^{DERM} samples that underwent dermal bioavailability testing were analysed. Table 37 shows the mean measured concentration of Pyr, BaP and DBA in the 200 pg/µL standard over the course of the GC-MS analysis. The mean Pyr measured concentration of the 200 pg/µL PAH standard produced an accuracy of 105%, BaP an accuracy of 104%, and DBA produced an accuracy of 86.5%.

Table 37: The mean measured concentration of Pyr, BaP and DBA of a 200pg/µL analytical standard (n=44).

	200 pg/µL Standard		
	Pyr	BaP	DBA
Mean	212	207	173
Std Dev	11.1	8.62	13.2
RSD%	5.23	4.16	7.64

The same approach was taken for the receptor solution (RS) samples, but owing to their lower predicted concentrations, a USEPA 16 PAH 50pg/μL analytical standard (n=22 total) was used. A total of 126 RS samples including 14 blanks were analysed. Table 38 shows the mean measured concentration of Pyr, BaP and DBA over the course of the GC-MS analysis. The mean Pyr measured concentration of the 50pg/μL PAH analytical standard produced an accuracy of 84%, BaP an accuracy of 96.8%, and DBA an accuracy of 99.8%. All analytical standards passed QC, with <15% RSD (Section 3.7).

Table 38: The mean measured concentration of Pyr, BaP and DBA of a 50pg/μL analytical standard (n=22).

	50 pg/μL Standard		
	Pyr	BaP	DBA
Mean	42.0	48.4	49.9
Std Dev	1.03	0.98	3.55
RSD%	2.46	2.02	7.12

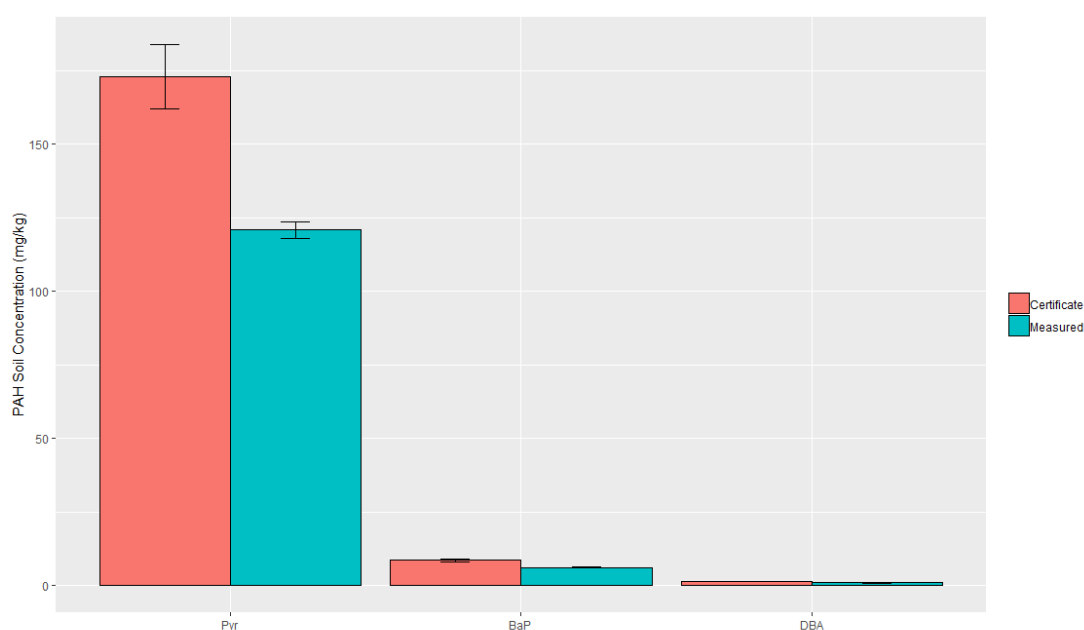


Figure 33: Certificate and Measured Pyr, BaP and DBA concentrations (mg/kg) of BCR-524 (CRM). Five replications were extracted and quantified. Error bars represent the 95% confidence interval.

Previous PAH dermal bioavailability studies have not used a CRM, mostly owing to that fact that previous works have spiked soils with radiolabelled-PAH compounds (e.g. (Wester et al.,

1990, Moody and Joncas, 2007, Peckham et al., 2017). Figure 33 shows the certificate and measured concentrations of Pyr, BaP and DBA. The Pyr certificate value was 173 ± 11 mg/kg, with the measured concentration being 121 mg/kg (RSD% = 2.74%). The BaP certificate value was 8.6 ± 0.5 mg/kg, with the measure value being 6.1 (RSD% = 4.07%). The DBA certificate value was 1.4 mg/kg, with the measured value being 1 mg/kg (RSD% = 15%).

In total, 112 Strat-M™ membranes were loaded and unloaded with soil. The mean dry soil loading was 81.5 mg/cm^2 (RSD% = 17.7%). Supermonolayer coverage (Section 2.3.3) was used to allow for an improved representation of peak flux, and also owing to field experiments showing that loadings can exceed 100 mg/cm^2 (Duff, 1996). Supermonolayer coverage also ensured that the entire exposure area was covered with soil, to remove the potential of another source of variation in PAH dermal absorption through incomplete coverage (Section 2.3.3).

The assessment of the developed *in vitro* diffusion cell PAH mass balance involves the comparison of the mass of substance applied ($\text{Soil}^{\text{Total}}$), to the measured mass of substance recovered from all compartments ($\text{Soil}^{\text{Derm}}$, membrane, receptor solution) of the experimental method. This is so that any gain or loss of 3-PAH can be located, and attributed to either the experimental, extraction or analytical method. The method PAH mass balance was calculated using Equation 22 and the aim is that both sides of are equal, where: SoilPAH_t is the measured soil PAH mass of that time step; MembPAH_t is the measured PAH mass contained in the membrane at that time step; RSPA_t is the measured PAH mass in the receptor solution at that time step.

$$\text{Applied PAH mass (ng)} = \text{SoilPAH}_t + \text{MembPAH}_t + \text{RSPA}_t$$

Equation 22: Diffusion cell method mass balance equation.

Table 39 shows the mass balance (%) of each PAH for the three soils tested. The mean Pyr mass balance for BCR-524 (CRM) over the 48 h was 103% (RSD%=80.3). BaP mean mass balance was 86.3% (RSD% = 42.1%) and DBA 65.3% (RSD% = 77.7). Site A produced a Pyr mass balance of 69.9% (RSD%=82.5%), BaP of 58.7% (RSD% =72.2) and DBA of 54.7% (RSD% = 83.8). Site B produced a mean Pyr mass balance of 82.1% (RSD% = 74.1), BaP of 61.2% (RSD% = 56.8) and DBA of 39.1% (RSD%=64.1).

Table 39: Mean method mass balance for Pyr, BaP and DBA for the three soils tested. Std Dev is the standard deviation. RSD (%) is the relative standard deviation. For BCR-524 $n = 32$. These were sampled over 4 diffusion cell experiment runs, with 8 time-steps in each run). For Site A and B soils, $n = 40$ for recovered. These were sampled over 5 diffusion cell experiment runs, with 8 time-steps in each run).

	BCR-524 (CRM)			Site A			Site B		
	Pyr	BaP	DBA	Pyr	BaP	DBA	Pyr	BaP	DBA
Mass Balance (%)	103	86.3	65.3	69.9	58.7	54.7	82.1	61.2	39.1
Std Dev (%)	82.5	36.3	50.8	57.7	42.4	45.9	60.9	34.8	25.0
RSD (%)	80.3	42.1	77.7	82.5	72.2	83.8	74.1	56.8	64.1

It was found that SPE 3-PAH soil^{DERM} SPE losses had a large impact on the method mass balance. However, the dermal absorption of PAH can still be quantified for both Sites A and B as the calculation of flux does not require the concentration of 3-PAH in the soil^{DERM} at each time-step. This is explored further in the discussion of data quality (Section 5.2.4.1.2)

5.2.3.2 PAH Concentration in each Diffusion Cell Compartment

Table 38 and Figure 34 show the mean 3-PAH soil^{DERM} concentration for the three soils at the various time steps throughout the diffusion cell experiment. For BCR-524 (CRM) (Figure 34A), the highest mean soil^{DERM} Pyr concentration was at 6 h (120 mg/kg), and lowest at 24 h (62.3 mg/kg). For BaP, the highest mean soil^{DERM} concentration was at 1 h (6.83 mg/kg), and lowest at 24 h (3.15 mg/kg). DBA soil^{DERM} concentration peaked at 1 h (0.741 mg/kg) and was at its lowest at 24 h (0.335mg/kg).

For Site A (Figure 34B), the mean soil^{DERM} concentration for Pyr was greatest at 6 h (102 mg/kg) and lowest at 24 h (33.8 mg/kg). The highest mean soil^{DERM} BaP concentration was found to be at 1 h (150 mg/kg), and lowest at 2 h (70.1 mg/kg). The 1 h time step had the highest DBA soil^{DERM} concentration at 26.2 mg/kg, and the lowest was found to be at 48 h (15 mg/kg).

The mean Site B soil^{DERM} (Figure 34C) Pyr concentration was highest at 36 h (91.3 mg/kg) and lowest at 24 h (45 mg/kg). For BaP, the mean soil^{DERM} concentration peaked at 24 h (79 mg/kg) and was lowest at 6 h (50.2 mg/kg). The highest DBA mean soil^{DERM} concentration occurred at 0.5 h (5.98 mg/kg), and the lowest occurred at 2 h (4.22 mg/kg). The overall trend for the PAH soil^{DERM} concentration is that with some variation, they decrease in concentration over time. The RSD% also is generally at its highest at later time steps in comparison to earlier times.

Table 40: Mean Pyr, BaP and DBA soil^{DERM} (mg/kg), membrane (ng/cm²) and receptor solution (ng/mL) concentration at each time step (h) for each soil tested. BCR-524 had 4 replications, Site A and B 5 replications. RSD% shown in brackets. nd = no data, owing to being below LOQ.

Time (h)	BCR-524 Mean Soil ^{DERM} Concentration (mg/kg)						Site A Mean Soil ^{DERM} Concentration (mg/kg)						Site B Mean Soil ^{DERM} Concentration (mg/kg)					
	Pyr		BaP		DBA		Pyr		BaP		DBA		Pyr		BaP		DBA	
0.5	83.2	(20.8)	3.60	(23.3)	0.336	(4.06)	72.4	(63.3)	90.9	(70.1)	19.9	(39.1)	48.6	(64.7)	68.8	(18.3)	5.98	(14.6)
1	111	(30.1)	6.83	(33.5)	0.741	(39.4)	96.1	(70.5)	150	(31.3)	26.2	(39.8)	79.2	(71.8)	59.4	(73.3)	5.17	(3.2)
2	83.7	(14.8)	5.52	(25.2)	0.555	(32.8)	65.0	(108)	70.1	(72.2)	16.0	(27.2)	76.1	(53.8)	67.1	(66.3)	4.22	(3.69)
6	120	(31.9)	5.34	(21.6)	0.552	(1.92)	102	(99)	127	(81.1)	17.8	(24.5)	73.5	(105)	50.2	(13.4)	5.77	(12.4)
12	67.5	(29.7)	5.20	(30)	0.545	(25.8)	89.1	(71.5)	97.9	(56.1)	17.8	(41.5)	60.4	(60.1)	76.2	(61.4)	4.35	(3.95)
24	62.3	(7.16)	3.15	(38.6)	0.335	(21.1)	33.8	(58.6)	80.7	(69.7)	18.9	(57.7)	45.0	(74.8)	79.0	(19.5)	4.95	(17.5)
36	86.5	(48.6)	4.58	(55.1)	0.598	(28.8)	83.7	(54.4)	134	(64.3)	25.4	(40.2)	91.3	(51.5)	68.1	(29.4)	5.56	(7.81)
48	85.5	(12.2)	6.48	(33.4)	0.551	(39.9)	87.5	(66.5)	86.3	(59)	15.0	(49.2)	68.6	(81.5)	69.5	(49.9)	5.38	(5.49)
Time (h)	BCR-524 Mean Membrane Concentration (ng/cm ²)						Site A Mean Membrane Concentration (ng/cm ²)						Site B Mean Membrane Concentration (ng/cm ²)					
	Pyr		BaP		DBA		Pyr		BaP		DBA		Pyr		BaP		DBA	
0.5	129	(30.9)	0.504	(30.8)	0.0972	(0)	55.2	(33.5)	43.0	(71.8)	nd	nd	25.8	(11.6)	5.25	(22.7)	nd	nd
1	241	(52.7)	1.19	(32.3)	nd	nd	35.5	(19.3)	21.3	(67.9)	nd	nd	27.6	(56.6)	15.3	(59.1)	nd	nd
2	69.3	(85.7)	0.668	(71.3)	0.0118	(0)	43.4	(19.3)	28.4	(14.4)	1.59	(0)	70.4	(81.9)	9.23	(28.3)	0.301	(0)
6	223	(41.2)	2.14	(115)	0.0898	(0)	146	(66.8)	37.6	(26.2)	2.08	(0)	65.2	(30.3)	13.4	(73.9)	nd	nd
12	1660	(57.1)	14.9	(113)	1.47	(0)	461	(118)	293	(110)	42.2	(38.8)	130	(43.6)	9.76	(12.2)	nd	nd
24	2709	(49.1)	10.5	(81)	0.535	(0)	179	(72.8)	175	(116)	nd	nd	159	(72.4)	34.5	(79.2)	nd	nd
36	2201	(55.8)	12.3	(96.9)	nd	nd	299	(82.4)	236	(22.7)	nd	nd	511	(37.9)	251	(50.6)	26.1	(93.4)
48	4902	(97.3)	19.1	(82.4)	1.72	(43.3)	216	(88.6)	112	(76.8)	27.6	(24.2)	536	(93.1)	513	(105)	22.6	(0)
Time (h)	BCR-524 Mean Receptor Solution Concentration (ng/mL)						Site A Mean Receptor Solution Concentration (ng/mL)						Site B Mean Receptor Solution Concentration (ng/mL)					
	Pyr		BaP		DBA		Pyr		BaP		DBA		Pyr		BaP		DBA	
0.5	3.69	(128)	0.178	(88.9)	0.021	(77.5)	0.175	(71.1)	0.131	(124)	0.084	(83.8)	1.86	(156)	1.78	(165)	0.745	(93.7)
1	9.12	(153)	0.432	(138)	0.128	(0)	0.117	(36.3)	0.064	(124)	0.054	(21.64)	0.359	(32.4)	0.071	(85.8)	nd	nd
2	6.61	(159)	0.293	(164)	0.119	(0)	0.193	(80.6)	0.168	(84)	0.098	(52.8)	1.52	(88.8)	2.15	(112.)	0.406	(40.7)
6	12.1	(114)	0.245	(166)	0.122	(0)	0.140	(26.3)	0.082	(84.3)	0.102	(0)	0.281	(35.9)	0.099	(90.9)	0.034	(47)
12	7.31	(51.6)	0.250	(22.3)	0.038	(0)	0.212	(44.4)	0.040	(42.5)	0.076	(0)	4.73	(115)	13.7	(136.6)	1.75	(60.4)
24	16.8	(43.2)	0.268	(72.4)	0.030	(50.9)	0.457	(38.9)	0.046	(85.8)	0.038	(71.3)	1.29	(80)	1.69	(126.8)	0.858	(29.4)
36	22.3	(35.3)	0.117	(0)	nd	nd	0.516	(83.2)	0.111	(140)	0.056	(101.4)	1.68	(89.2)	0.359	(134.6)	0.116	(94.6)
48	28.5	(41.3)	0.157	(108)	0.037	(68.8)	1.21	(56.8)	1.15	(105)	0.489	(93.7)	1.37	(57.2)	0.325	(102.9)	0.122	(64.9)

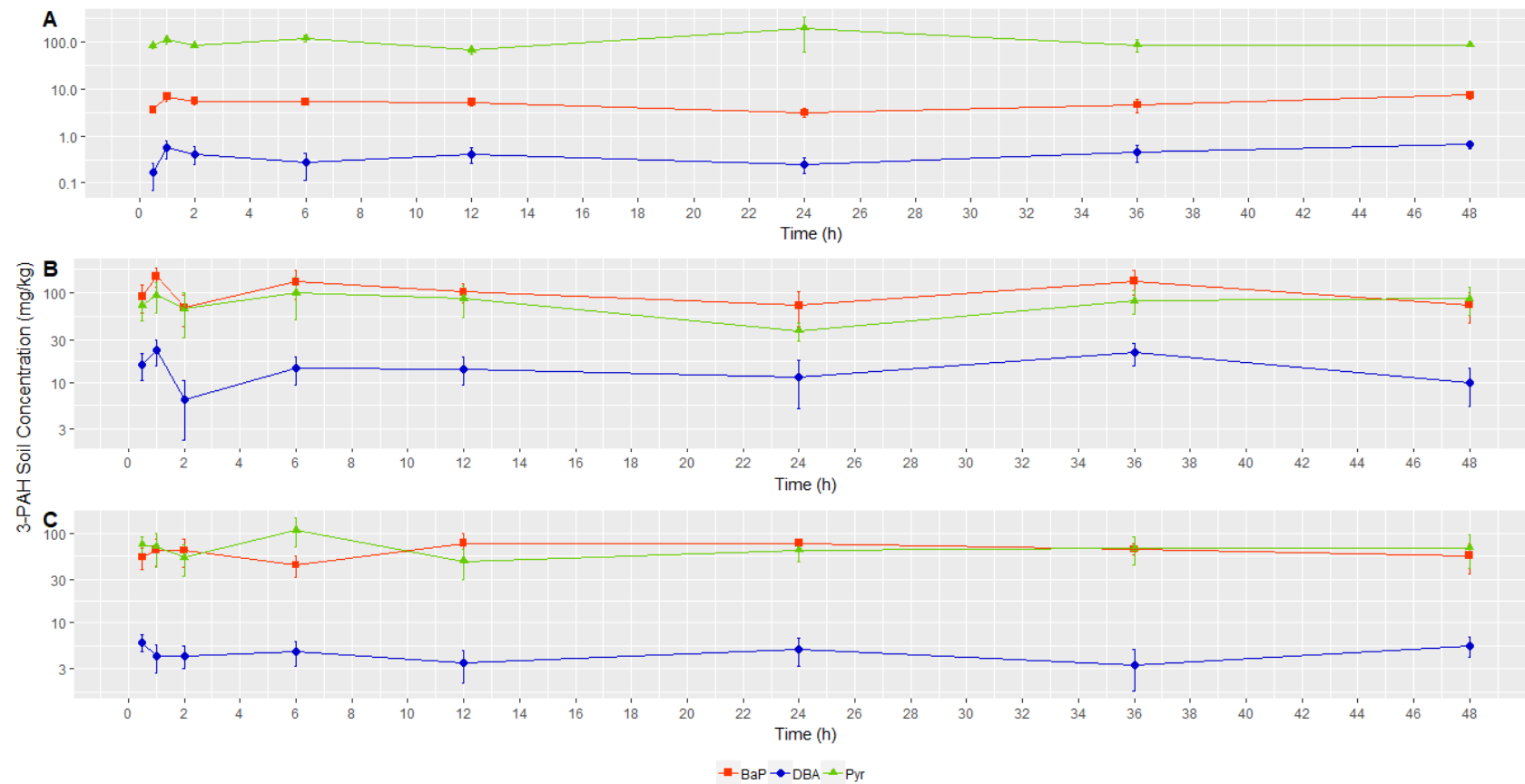


Figure 34: *Soil^{DERM}* 3-PAH concentrations (mg/kg) over time (h). A) BCR-524 (CRM), 4 replications B) Site A Soil, 5 replications C) Site B Soil, 5 replications. Error bars represent standard error

Table 38 and Figure 35 shows the mean Pyr, BaP and DBA concentrations in the membranes (Memb^{PAH}) throughout the course of the 48 h diffusion cell experiment. For BCR-524 (CRM), the mean Pyr membrane value is highest at 48 h (4902 ng/cm^2) and lowest at 2 h (69.3 ng/cm^2). BaP was present in the membrane highest at 48 h (19.1 ng/cm^2) and lowest at 0.5 h (0.504 ng/cm^2). The highest mass of DBA was present in the membrane at 48 h (1.72 ng/cm^2) and lowest at 2 h (0.0118 ng/cm^2). For the soil from Site A, the highest mean Pyr mass in the membrane occurred at 12 h (461 ng/cm^2), and the lowest at 1 h (35.5 ng/cm^2). The highest mean BaP mass in the membrane was found at 12 h (293 ng/cm^2) and lowest at 1 h (21.3 ng/cm^2). For DBA, the highest mean membrane mass was 42.2 ng/cm^2 at 12 h, and the lowest 1.59 ng/cm^2 at 2 h. For the soil of Site B, the highest mean Pyr membrane mass occurred at 48 h (535.5 ng/cm^2) and lowest at 0.5 h (25.8 ng/cm^2). The mean BaP mass in the membrane was highest at 48 h (513 ng/cm^2) and lowest at 0.5 h (5.25 ng/cm^2). At 36 h, the highest mean DBA mass was measured as 26.1 ng/cm^2 , and the lowest at 2 h was found to be 0.301 ng/cm^2 . Some analysis of the membranes produced no data (Table 40) (nd)), and this is largely due to the differences in starting soil concentrations of the three PAH analysed (Table 34) and the dilution factors required for the PAH to be in the working linear range of the GC-MS (Table 36). The general trend is that there is an increase in Pyr, BaP and DBA concentrations in the membrane over time, with most mean membrane PAH masses for each soil being highest at 48 h when the experiment was terminated.

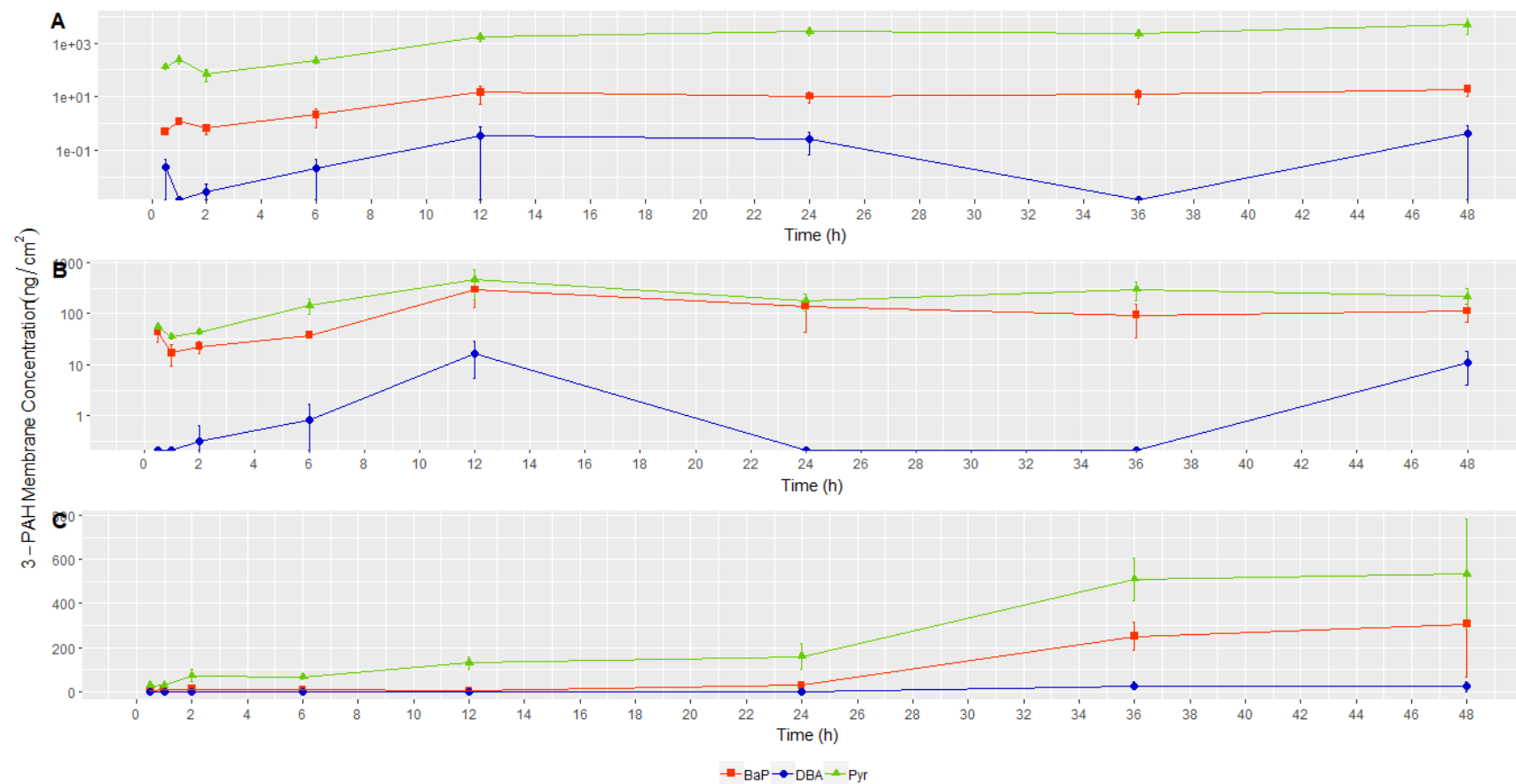


Figure 35: Strat-MTM membrane 3-PAH concentrations (ng/cm²) over time (h). A) BCR-524 (CRM), 4 replications B) Site A Soil, 5 replications C) Site B Soil, 5 replications. Error bars represent standard error.

The volume of the receptor compartment was measured prior to each diffusion cell assembly (Sections 4.1.1 and 4.1.2.2), with a mean receptor solution volume of 31.4mL (RSD% = 4.5). The recorded RS volume measurements of each diffusion cell were used to calculate the RS 3-PAH concentration. Figure 36 shows the 3-PAH receptor solution concentrations over time. For BCR-524 (CRM) the highest mean Pyr RS concentration occurred at 48 h (28.5 ng/mL), and lowest at 0.5 h (3.69 ng/mL). The uppermost mean BaP RS concentration occurred at 1 h (0.432 ng/mL) and lowest at 36 h (0.117 ng/mL). The mean DBA RS concentration peaked at 1 h (0.128 ng/mL) and was lowest at 0.5 h (0.021 ng/mL). For the Site A soil, mean Pyr RS concentration was highest at 48 h (1.21 ng/mL), and lowest at 1 h (0.177 ng/mL). At 48 h, the highest mean BaP RS concentration occurred (1.15 ng/mL), and the mean BaP RS concentration was at its lowest at 12 h (0.04 ng/mL). Maximum DBA mean RS concentrations were measured at 12 h (0.489 ng/mL), and minimum at 24 h (0.038 ng/mL). For the Site B soil, the uppermost mean Pyr RS concentration was at 12 h (4.73 ng/mL) and lowest at 6 h (0.281 ng/mL). The highest mean RS BaP concentration occurred at 12 h (13.7 ng/mL), and the lowest was found at 1 h (0.071 ng/mL). The maximum mean DBA RS concentration was found to occur at 12 h (1.75 ng/mL) and was lowest at 6 h (0.034 ng/mL). Overall, the mean PAH RS concentration increases with exposure time, with many of the higher PAH RS concentrations occurring in the latter time steps.

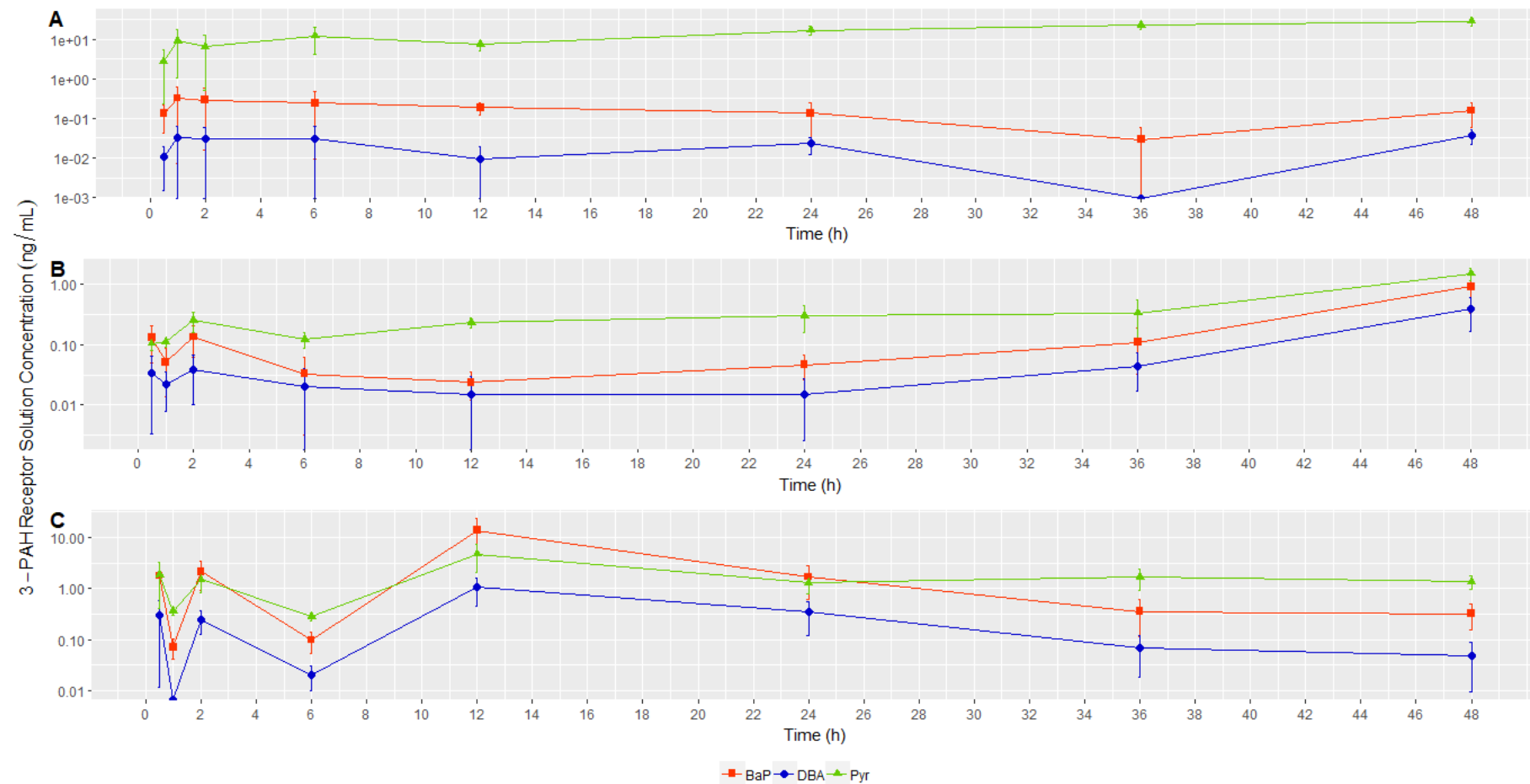


Figure 36: Receptor Solution 3-PAH concentrations (ng/mL) over time (h). A) BCR-524 (CRM), 4 replications B) Site A Soil, 5 replications C) Site B Soil, 5 replications. Error bars represent standard error.

5.2.3.3 Calculation of Flux

Previous studies have quantified the soil PAH bioavailable fraction (BAF%) using percent absorbed of the dose applied (PADA) (Sections 2.2.4, 2.3.2, and 2.3.3). However, the calculation of BAF% can be impacted by a variety of parameters, such as soil loading (Roy and Singh, 2001, Spalt et al., 2009, Frasch et al., 2014). As flux (rate of 3-PAH diffusion) calculation is not impacted by soil loading, flux can provide a better representation of the amount of BaP dermally absorbed for the prediction of human exposure and can also be compared to previous PAH dermal absorption studies. The soil loading applied in this study represents an infinite dosing scenario, allowing for the calculation of steady-state flux (J_{ss}) (Frasch et al., 2014).

Equation 23 was used to calculate the flux of PAH from soil into the membrane and RS, and also the flux of the entire dermal system (total). This is because the collective flux of the PAH into the membrane and RS represents the rate at which dermally bioavailable PAH are absorbed over time. This parameter is key for human health risk assessment, as this will allow for the estimation of daily intake rate of PAH via the dermal pathway.

$$J = \frac{\left(\frac{\Delta C}{\Delta t}\right)}{A}$$

Equation 23: Alternative expression of Fick's First Law of diffusion. Where J is flux, ΔC is change in concentration (concentration gradient) and Δt is change in time. A is the surface area exposed to the compound of interest.

Table 41 and Figure 37 show the mean flux (ng/cm²/h) of Pyr, BaP and DBA for BCR-524 (CRM) in the membrane and receptor solution (RS). The highest mean Pyr flux into the RS occurred at 1 h (33.5 ng/cm²/h) and lowest at 48 h (2.1 ng/cm²/h). The highest mean Pyr flux for the membrane also occurred at 0.5 h (257.2 ng/cm²/h), and lowest? at 2 h (ng/cm²/h). Total flux for Pyr from BCR-524 was highest at 0.5 h (277.3 ng/cm²/h), and lowest at 6 h (44 ng/cm²/h).

For the RS, the peak BaP flux occurred at 1 h (1.2 ng/cm²/h) and was lowest at 48 h (0.012 ng/cm²/h). Peak BaP membrane flux occurred at 12 h (1.24 ng/cm²/h) and was at its lowest at 36 h (0.34 ng/cm²/h). Total BaP flux was highest at 1 h (2.39 ng/cm²/h), and smallest at 36 h (0.35 ng/cm²/h).

The peak flux of DBA for the RS occurred at 1 h (0.0037 ng/cm²/h), and lowest between 12 and 48 h (0.0001 ng/cm²/h). Peak DBA flux for the membrane was measured at 0.5 h (0.0486

ng/cm²/h) and was at its lowest at 1 h (0.000 ng/cm²/h). Total DBA flux peaked at 0.5 h at 0.0511 ng/cm²/h and was lowest at 2 h (0.0032 ng/cm²/h).

Table 41: Mean Pyr, BaP and DBA Flux (ng/cm²/h) for receptor solution, membrane, and total for BCR-524. Number of replicates = 4. nd = no data as <LOQ.

	Pyr								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	20.1	31.7	158	257.2	79.6	31.0	277.3	88.5	31.9
1	33.5	51.5	153	240.9	126.8	52.7	274.4	140.0	51.0
2	12.8	20.4	160	34.7	29.7	85.7	47.4	27.0	57.0
6	6.9	7.8	111	37.1	15.3	41.2	44.0	21.6	49.0
12	2.3	1.2	53.0	138.3	78.9	57.0	140.6	80.1	57.0
24	2.6	1.1	43.2	112.9	55.4	49.1	115.5	54.3	47.1
36	2.3	0.8	35.5	61.1	34.1	55.8	63.4	34.3	54.1
48	2.1	0.9	40.3	102.1	99.4	97.3	104.2	98.8	94.8
	BaP								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	1.0	1.13	116	1.01	0.31	30.8	1.98	1.05	52.9
1	1.2	2.03	169	1.19	0.39	32.3	2.39	2.41	100.7
2	0.6	0.93	164	0.33	0.24	71.3	0.90	0.88	97.9
6	0.1	0.23	166	0.36	0.41	115.3	0.50	0.64	128.9
12	0.1	0.04	64.6	1.24	1.40	112.6	1.30	1.43	110.0
24	0.021	0.03	142	0.44	0.35	81.0	0.46	0.34	74.2
36	0.003	0.01	173	0.34	0.33	97.0	0.35	0.34	97.6
48	0.012	0.01	108	0.40	0.33	82.4	0.41	0.32	78.6
	DBA								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	0.0025	0.0037	148	0.0486	0.0842	173	0.0511	0.0834	163
1	0.0037	0.0065	173	0.0000	0.0000	0.0	0.0037	0.0065	173
2	0.0017	0.0030	173	0.0015	0.0026	173	0.0032	0.0032	100
6	0.0006	0.0010	173	0.0037	0.0065	173	0.0043	0.0075	173
12	0.0001	0.0002	173	0.0307	0.0531	173	0.0308	0.0533	173
24	0.0001	0.0001	82.5	0.0111	0.0144	129.4	0.0113	0.0144	127
36	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
48	0.0001	0.0001	68.8	0.0089	0.0155	173.2	0.0090	0.0155	171

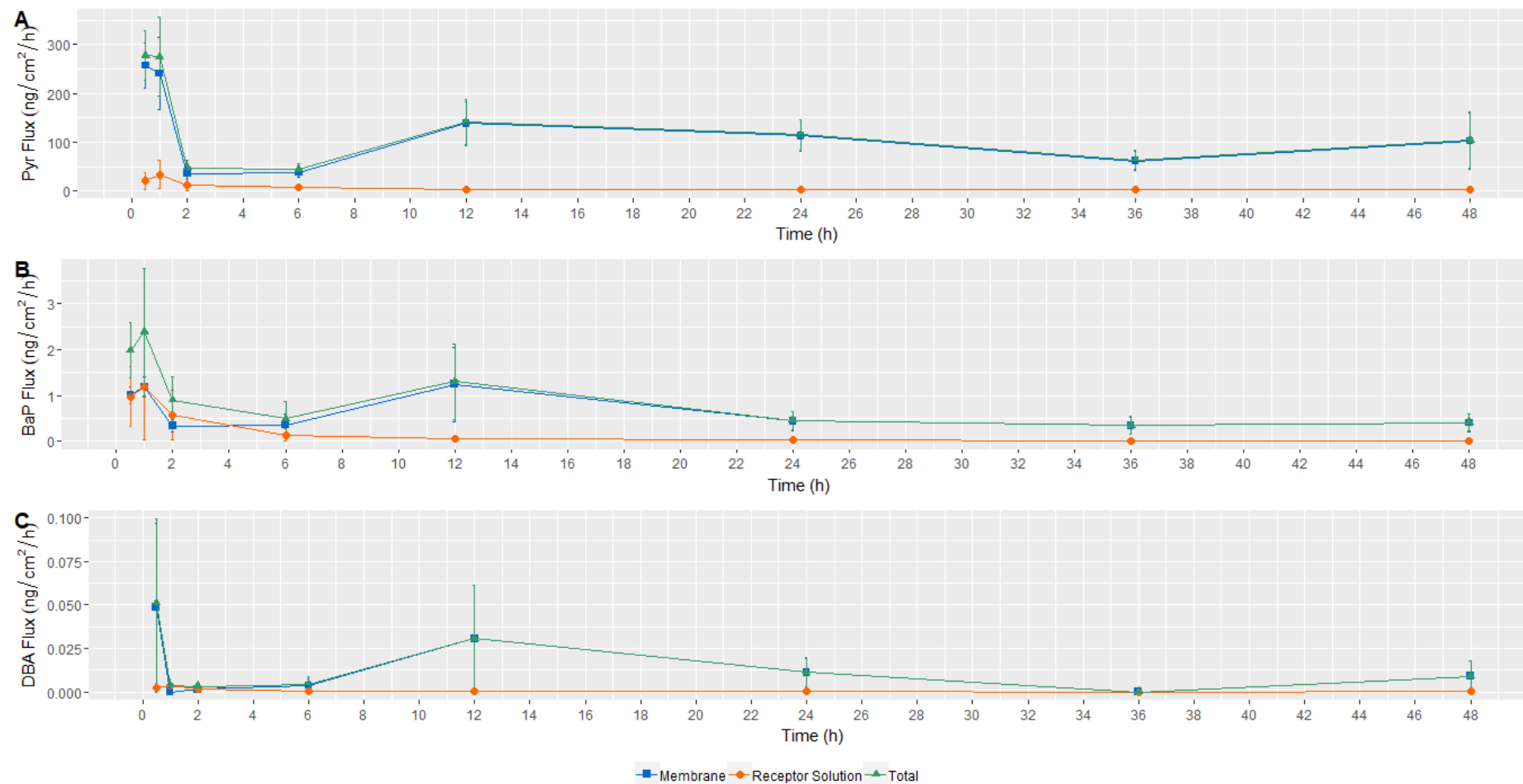


Figure 37: Flux of 3-PAH into the membrane, receptor solution (RS) and total over 48h from BCR-524 (CRM). Number of replicates = 4. Error bars represent standard error.

Overall, the flux of PAH from BCR-524 into the dermal system peaks at the earlier time-steps (0.5-1 h) and is lowest at the latter stages (24-48 h).

Table 42 and Figure 38 show the mean flux (ng/cm²/h) of Pyr, BaP and DBA for the soil of Site A in the various diffusion cell compartments. The peak RS flux of Pyr occurred at 0.5 h (1.3 ng/cm²/h), and lowest occurred at 36 h (0.04 ng/cm²/h). Peak flux for the membrane occurred at 0.5 h (110.47 ng/cm²/h) and was at its lowest at 48 h (4.5 ng/cm²/h). The peak total flux was 111.76 ng/cm²/h at 0.5 h, and lowest total flux was measured at 48 h (4.59 ng/cm²/h).

The highest mean BaP flux of the receptor solution occurred at 0.5h (0.96 ng/cm²/h) and was at its lowest between 12 and 36 h (0.01 ng/cm²/h). At 0.5h, the peak BaP membrane flux was measure (85.9 ng/cm²/h) and lowest at 48 h (2.3 ng/cm²/h). The mean total flux of BaP was highest at 86.9 ng/cm²/h at 0.5 h, and was at its lowest at 2.4 ng/cm²/h at 48 h.

The highest DBA flux of the RS occurred at 0.5 h (0.244 ng/cm²/h) and lowest at 24 h (0.002 ng/cm²/h). For the membrane, peak DBA flux occurred at 12 h (1.405 ng/cm²/h) and was at its lowest at 0.5-1 and 24-36 h (0.000 ng/cm²/h). DBA was present in the membrane samples (above LOD) but could not be quantified as the peaks did not pass quality control (below LOQ). Total DBA flux peaked at 12 h (1.41 ng/cm²/h) and was lowest at 24 h (0.002 ng/cm²/h).

Overall, the flux of PAH from the soil from Site A provides a similar trend to that of BCR-524 (Table 41), with the highest flux mostly occurring at early time steps (0.5-1 h) and lowest mostly occurring at later time steps (24-48 h).

Table 42: Mean Pyr, BaP and DBA Flux (ng/cm²/h) for receptor solution, membrane, and total for the soil from Site A. Number of replicates = 5. nd = no data as < LOQ.

	Pyr								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	1.30	0.91	70.2	110.47	36.99	33.5	111.76	37.69	33.7
1	0.44	0.16	37.4	35.49	6.86	19.3	35.93	6.91	19.2
2	0.34	0.27	77.6	21.72	4.19	19.3	22.06	4.39	19.9
6	0.07	0.04	57.0	24.27	16.22	66.8	24.34	16.24	66.7
12	0.07	0.03	44.5	38.45	45.68	118.8	38.51	45.71	118.7
24	0.06	0.04	65.5	7.48	5.45	72.8	7.53	5.45	72.4
36	0.04	0.04	103.3	8.30	6.84	82.4	8.34	6.87	82.3
48	0.09	0.05	54.7	4.50	3.99	88.6	4.59	3.97	86.5
	BaP								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	0.96	1.18	122.9	85.9	61.72	71.8	86.9	62.20	71.6
1	0.20	0.29	149.1	17.0	15.46	90.9	17.2	15.29	88.8
2	0.24	0.25	105.3	11.4	5.97	52.5	11.6	6.07	52.3
6	0.02	0.04	179.9	6.3	1.64	26.2	6.3	1.64	26.1
12	0.01	0.01	98.0	24.4	26.86	110.0	24.4	26.87	110.0
24	0.01	0.01	85.8	5.8	8.10	138.6	5.9	8.10	138.4
36	0.01	0.02	139.8	2.6	3.35	127.6	2.6	3.37	127.6
48	0.07	0.08	124.3	2.3	1.80	76.8	2.4	1.77	73.6
	DBA								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	0.244	0.441	180.5	nd	nd	nd	0.244	0.441	180.5
1	0.083	0.105	126.7	nd	nd	nd	0.083	0.105	126.7
2	0.069	0.102	146.8	0.159	0.318	200	0.228	0.299	130.9
6	0.012	0.024	200.0	0.139	0.277	200	0.151	0.272	180.7
12	0.004	0.009	200.0	1.405	1.925	137	1.410	1.922	136.3
24	0.002	0.004	165.3	nd	nd	nd	0.002	0.004	165.3
36	0.005	0.006	123.4	nd	nd	nd	0.005	0.006	123.4
48	0.028	0.033	116.4	0.230	0.295	128	0.258	0.298	115.3

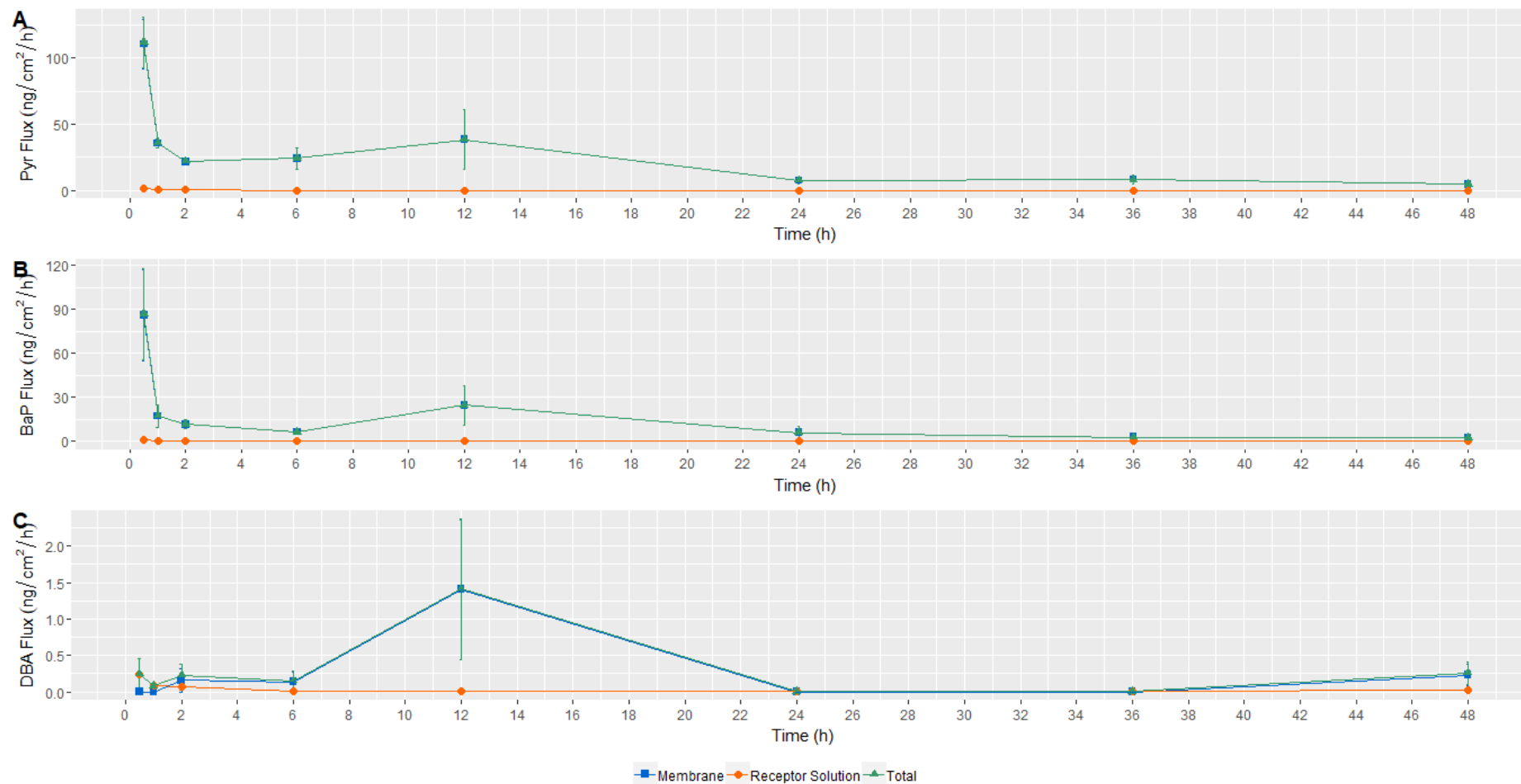


Figure 38: Flux of 3-PAH into the membrane, receptor solution (RS) and total over 48h from Site A. Number of replicates = 5. Error bars represent standard error.

Table 43 and Figure 39 show the mean flux (ng/cm²/h) of Pyr for the soil of Site B in various diffusion cell compartments. The RS mean Pyr flux peaks at 13.1 ng/cm²/h (0.5 h) and is lowest at 0.1 ng/cm²/h (48 h). Mean flux of Pyr in the membrane peaks at 0.5 h (42.2 ng/cm²/h) and is at its lowest at 24 h (4.68 ng/cm²/h). The total flux in the system peaks at 0.5 h (55.2 ng/cm²/h) and is at its lowest at 24 h (4.88 ng/cm²/h).

The mean flux (ng/cm²/h) of BaP for the soil of Site B in various diffusion cell compartments. The BaP mean flux peaks in the RS at 0.5 h (12.5 ng/cm²/h) and at its minimum at 48 h (0.02 ng/cm²/h). For the membrane, peak flux occurs at 1 h, at 7.11 ng/cm²/h, and is at its lowest at 12 h with a mean flux of 0.143 ng/cm²/h. The total mean flux of BaP is at its highest at 0.5 h, at 17.4 ng/cm²/h, and occurs at its lowest of 0.75 ng/cm²/h at 24 h.

The mean flux (ng/cm²/h) of DBA for Site B soil in the RS, membrane, and total flux of the diffusion cell system over time. The highest DBA mean flux occurs in the RS at 0.5h (2.06 ng/cm²/h) and is found at its lowest at 36 h (0.0076 ng/cm²/h). The mean measured flux of DBA in the membrane, was found to occur at 36 h (0.724 ng/cm²/h) and was lowest at 0.5, and 6-24 h (>0 ng/cm²/h). A zero flux value is reported due to DBA being above the LOD but below the LOQ, so it can be said that DBA is present in the samples, but the concentration cannot be quantified. The total mean flux for DBA was highest at 0.5 (2.06 ng/cm²/h) and lowest at 6 h (0.013 ng/cm²/h).

In summary, the flux of PAH into the various compartments as well as the entire represented dermal system (Total Flux) peaks between 0.5 and 1 h (Table 41, Table 42 and Table 43) and gets lower with time. Mean PAH flux is typically at its lowest between 24 and 48 h (Table 41, Table 42 and Table 43).

Table 43: Mean Pyr, BaP and DBA Flux (ng/cm²/h) for receptor solution, membrane, total for the soil from Site B. Number of replicates = 5. nd as <LOQ

	Pyr								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	13.1	19.9	152	42.2	21.8	51.8	55.2	7.26	13.1
1	1.33	0.47	35.5	17.5	13.8	78.9	18.8	13.9	73.9
2	2.88	2.50	86.8	31.4	31.9	101	34.3	31.9	92.9
6	0.17	0.06	35.2	8.44	5.32	63.0	8.61	5.35	62.1
12	1.32	1.47	111	8.66	6.39	73.9	9.98	6.06	60.7
24	0.20	0.16	80.7	4.68	5.11	109	4.88	5.16	106
36	0.18	0.17	92.1	11.4	7.84	68.9	11.6	7.77	67.2
48	0.10	0.06	60.4	9.42	11.34	120	9.52	11.4	120
	BaP								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	12.5	20.2	161	4.87	5.97	123	17.4	17.9	103
1	0.26	0.22	86.9	7.11	10.8	152	7.37	10.7	145
2	4.08	4.48	110	3.73	2.27	61.0	7.81	4.80	61.4
6	0.06	0.06	92.1	1.11	1.77	160	1.17	1.73	148
12	3.81	5.09	133	0.143	0.286	200	3.96	4.99	126
24	0.26	0.33	125	0.493	0.540	110	0.75	0.52	68.9
36	0.04	0.05	137	4.88	3.92	80.4	4.92	3.89	79.2
48	0.02	0.02	102	6.07	10.3	170	6.09	10.3	169
	DBA								
	Receptor Solution			Membrane			Total		
Time (h)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)	Mean Flux (ng/cm ² /h)	S.D. (ng/cm ² /h)	RSD(%)
0.5	2.06	3.95	191	nd	nd	nd	2.06	3.95	191
1	nd	nd	nd	nd	nd	nd	nd	nd	nd
2	0.474	0.467	98.4	0.151	0.301	200	0.625	0.407	65.2
6	0.0130	0.0133	102	nd	nd	nd	0.0130	0.0133	102
12	0.303	0.344	114	nd	nd	nd	0.303	0.344	114
24	0.0529	0.0683	129	nd	nd	nd	0.0529	0.0683	129
36	0.0076	0.0112	148	0.724	0.984	nd	0.731	0.982	134
48	0.0034	0.0052	155	0.472	0.943	200	0.475	0.942	198

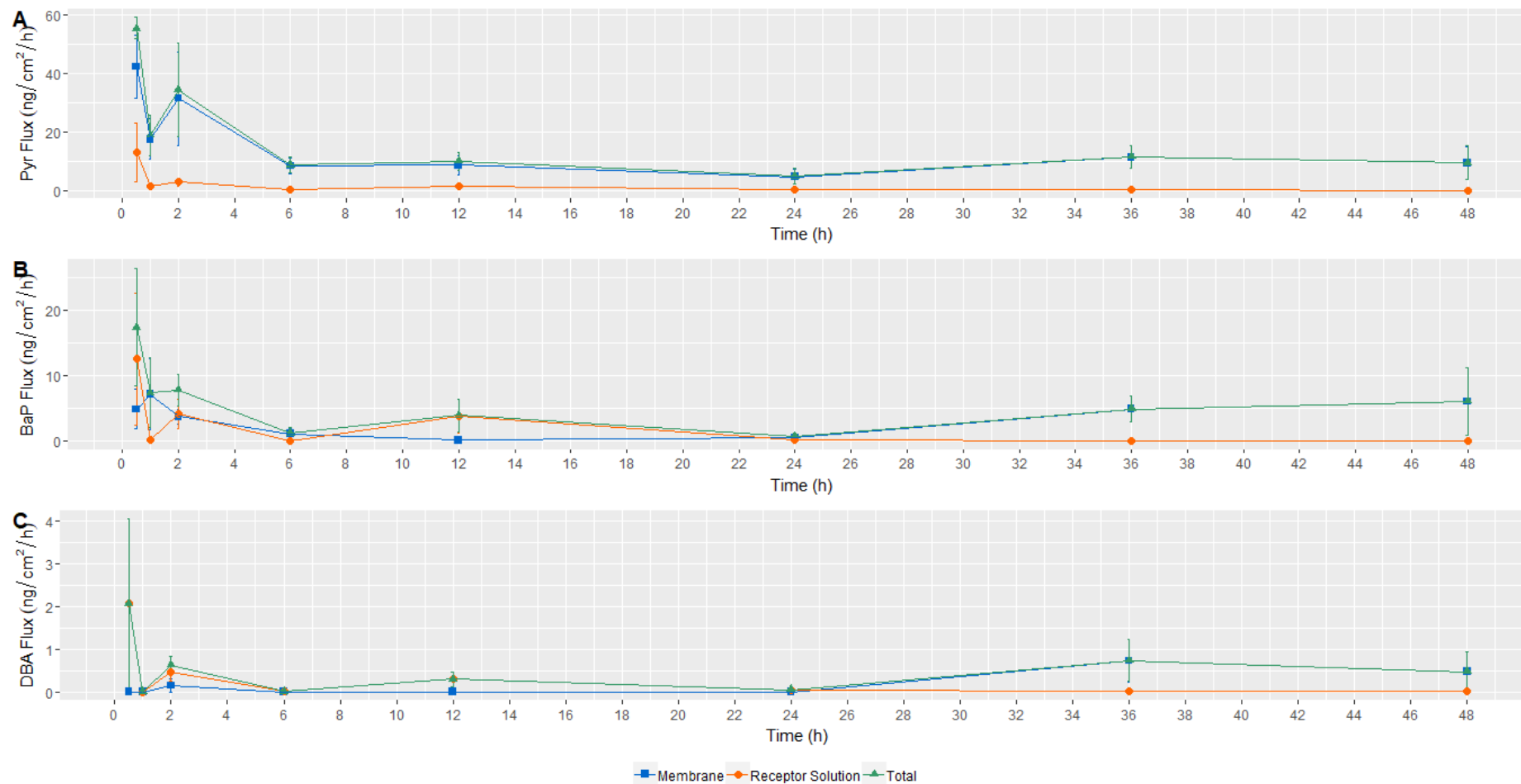


Figure 39: Flux of 3-PAH into the membrane, receptor solution (RS) and total over 48h from Site B. Number of replicates = 5. Error bars represent standard error.

5.2.4 Discussion

5.2.4.1 Data Quality

This section will explore the impact of different factors on the quality of PAH dermal bioavailability data produced by the developed diffusion cell method. These factors are summarised in Figure 40.

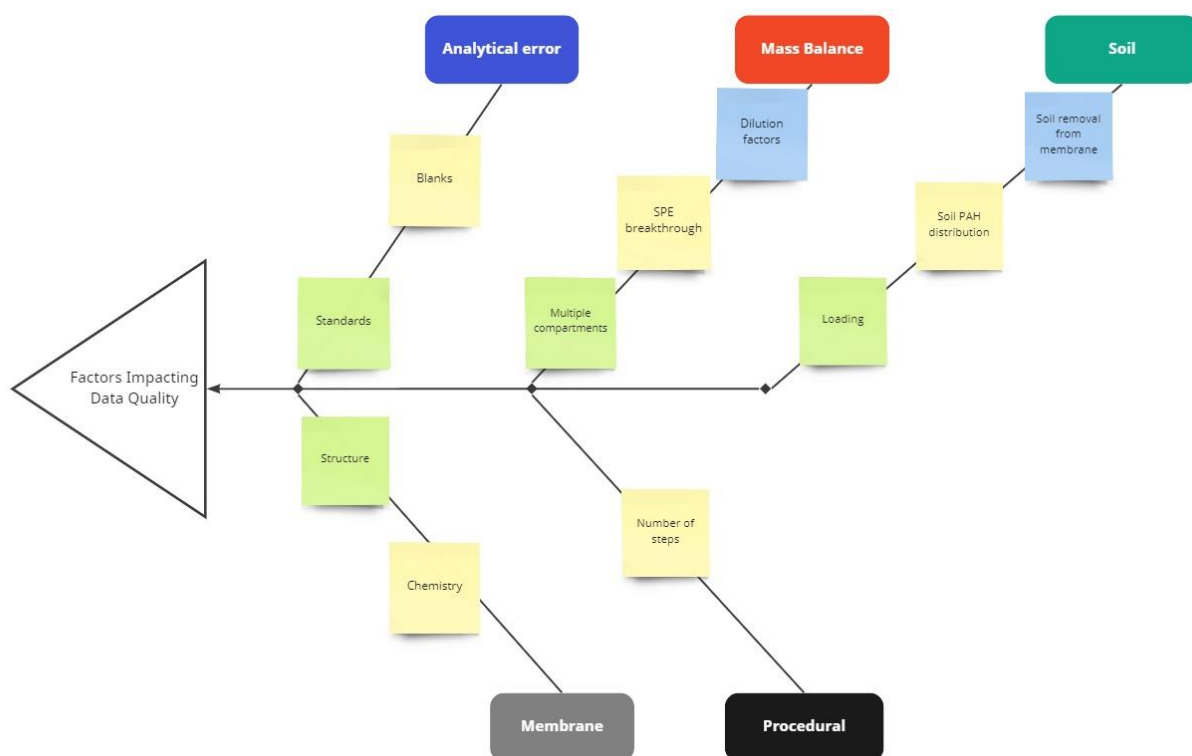


Figure 40: Factors impacting the data quality of the soil PAH dermal bioavailability data produced by the developed diffusion cell method.

5.2.4.1.1 Analytical Error

Two analytical standards were used throughout the GC-MS analysis of samples, a 200 pg/ μ L PAH standard ($n = 44$) for membrane and soil^{DERM} samples, and a 50 pg/ μ L PAH standard ($n = 22$) for receptor solution samples (Section 5.2.3.1). Accuracy of the standards ranged from 84% to 105%, with low RSD% (<8 %). Considering the duration of GC-MS analysis (> 1 month) and the number of samples analysed ($n = 530$), this accuracy and precision shows that the analytical system is stable and consistent even over prolonged periods of analysis. This, therefore, suggests that any large variation in dermal bioavailability data is not likely to be attributable to analytical variation. It should also be known that all experimental blanks fell below the LOQ (Sections 3.6 and 4.1.3.1) for 3-PAH.

5.2.4.1.2 Mass Balance

Table 39 shows the mass balance (Sections 4.1 and 5.2.3.1) of 3-PAH at 48 h for the *in vitro* soil dermal bioavailability diffusion cell experiment calculated using Equation 22. The purpose of mass balance is to ensure that there have been no significant losses or gains of PAH throughout the experimental and analytical procedure. Furthermore, if there are gains or losses, to locate at which point in the experimental or analytical procedure this occurs. BCR-524 produced excellent mass balance, for both Pyr (103%) and BaP (86.3) (Figure 41).

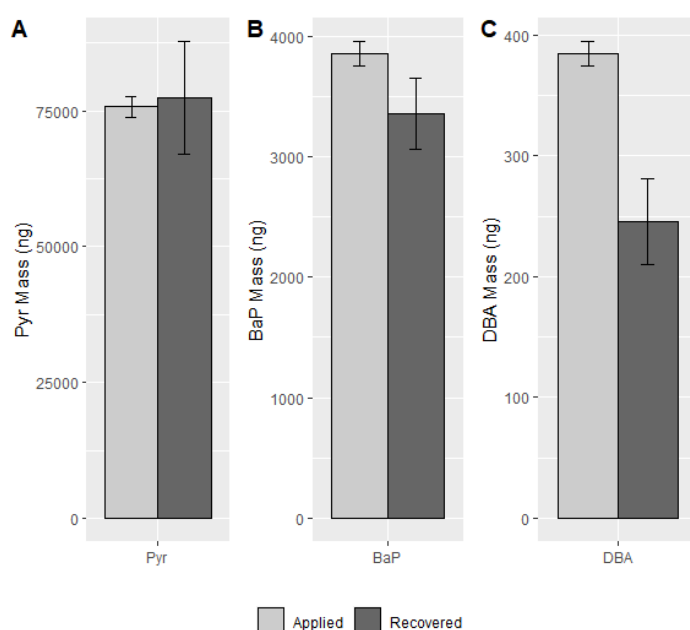


Figure 41: Experimental Mass Balance of 3-PAH the soil from BCR-524 (CRM) A. $n = 32$ for recovered (recovered is equal to the sum of 32 membrane extracts, 32 receptor solution extracts, and 32 soilDERM extracts. These were sampled over 4 diffusion cell experiment runs, with 8 time-steps in each run), $n = 5$ for applied ($Soil^{Total}$). Error bars represent standard error.

These values are similar to those in the literature that measured the dermal bioavailability of soil spiked radiolabelled-PAH *in vitro* using liquid scintillation counting. For example, Peckham et al. (2017) for radiolabelled BaP produced mass balance in the range of 89.2 – 103%. Roy et al. (1998) also produced a similar radiolabelled BaP mass balance of 83% - 106%. Moody and Joncas (2007) produced a mass balance of radiolabelled BaP at 48 h of 93.7% (RSD = 16.3%), again not too dissimilar to the Pyr and BaP mass balance of this study. Interestingly, Moody et al. (1995) reported lower *in vivo* mass balances of radiolabelled BaP of 69.4% (rat) and 67.8% (guinea pig), suggesting that *in vitro* dermal bioavailability studies

have a greater certainty of the fate of PAH in the experimental system in comparison to *in vivo* studies.

Figure 42 and Table 39 shows the mass balance of 3-PAH for the Site A soil. The 3-PAH mass balance (Pyr = 69.9%, BaP = 58.7%, DBA = 54.7%) was lower for Site A than BCR-524 (Table 39).

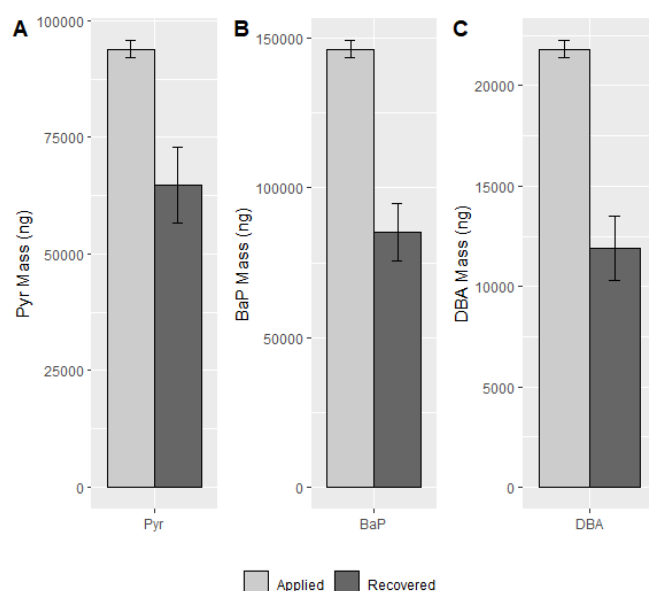


Figure 42: Experimental Mass Balance of 3-PAH the soil from Site A. $n = 40$ for recovered (recovered is equal to the sum of 40 membrane extracts, 40 receptor solution extracts, and 40 soilDERM extracts. These were sampled over 5 diffusion cell experiment runs, with 8 time-steps in each run), $n = 5$ for applied ($Soil^{Total}$). Error bars represent standard error.

Figure 43 and Table 39 shows the mass balance of 3-PAH for the Site A soil. The 3-PAH mass balance (Pyr = 82.1%, BaP = 61.2%, DBA = 39.1%) was lower than for BCR-524 (Table 39). The 3-PAH concentrations are highest in the soil^{DERM} compartment for all three tested soils (Table 40), with any 3-PAH losses from the soil during extraction and clean-up having a large impact on overall mass balance.

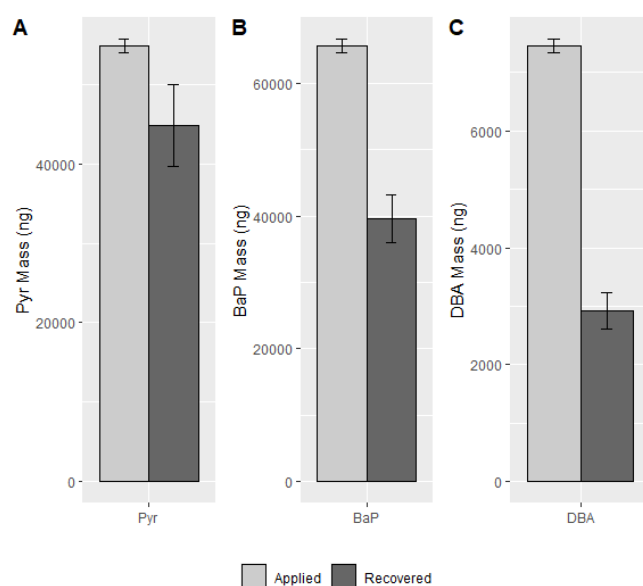


Figure 43: Experimental Mass Balance of 3-PAH for the soil from Site B. $n = 40$ for recovered (recovered is equal to the sum of 40 membrane extracts, 40 receptor solution extracts, and 40 soilDERM extracts. These were sampled over 5 diffusion cell experiment runs, with 8 time-steps in each run), $n = 5$ for applied (Soil^{Total}). Error bars represent standard error

The step at which 3-PAH losses for Site A and B soils occurs needed to be located in order to assess the potential impact on the credibility of the dermal bioavailability data. Due to the high 3-PAH concentration in the soils of Sites A and B (Table 34**Error! Reference source not found.**), it was speculated that the losses may have occurred during SPE clean-up of samples due to the SPE cartridges sorbent capacity being overloaded. This would result in the PAH breaking through into fraction A (aliphatics) rather than being retained and eluted as desired in fraction B (aromatics).

To test this, aliquots of SPE fraction A of soil^{DERM}, membrane and receptor solution samples were pipetted onto filter paper and checked under UV light (Figure 44). It is known that few aliphatic compounds show fluorescence under UV light (Díaz-García and Badía-Laiño, 2005), whereas PAH show fluorescence owing to their delocalised pi-electrons being readily excited (Howerton et al., 2002, Dartnell et al., 2012). Therefore, it can be assumed that any fluorescence is due to the presence of PAH.

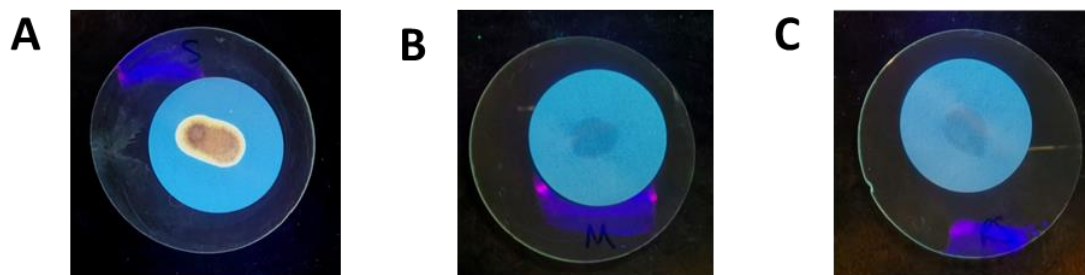


Figure 44: SPE Fraction A (aliphatics) under UV light. Image A is from a gasworks soil^{DERM} sample, B from a membrane, and C from a receptor solution.

Figure 44A shows the fluorescence of the SPE aromatic fraction of a gas work soil^{DERM} sample, Figure 44B and Figure 44C show no fluorescence. The 3-PAH soil^{Total} concentrations of Site A and B are much greater than that of BCR-524 (Table 34). Consequently, the extracts of Site A and B exceeded the sorption capacity of the SPE cartridge sorbent resulting in breakthrough and 3-PAH loss into SPE fraction A, which is not analysed for PAH. This meant that the measured 3-PAH soil^{DERM} concentrations of Site A and B were much lower due to losses during SPE. Consequently, as most of the 3-PAH is retained in the soil (Table 40), these losses had a large impact on the mass balance of the method for Site A and B. It is suggested that in future, this loss of PAH during SPE of soil extracts with high PAH can be reduced by either: extracting a lower soil mass (reducing the PAH concentration of the soil extract), use a different SPE method with a higher sorption capacity, or carry out SPE multiple times and combine fractions A and B prior to analysis. As BCR-524 produced good mass balance, and the Site A and B soils were treated the same, it is assumed that the diffusion cell method did not result in 3-PAH losses. The source of the loss of 3-PAH appears to be from the SPE of Site A and B soil^{DERM} samples, with no losses in the same manner for the membrane or receptor solution (Figure 44). This means that the data for the membrane and receptor solution still has a high level of confidence. Although the SPE 3-PAH soil^{DERM} SPE losses has a large impact on the method mass balance, the dermal absorption of PAH can still be quantified for both Sites A and B as the calculation of flux does not require the concentration of 3-PAH in the soil^{DERM} at each time-step.

DBA mass balance was lowest for all three tested soils (Table 39). DBA concentrations in the soil^{DERM} samples were lowest of the 3-PAH (Table 34). Consequently, the dilution factors applied to the soil^{DERM} and membrane samples (Table 36) required to ensure the much higher BaP and Pyr concentrations were within the working linear range of the GC-MS in turn resulted in some samples producing DBA concentrations below the LOQ. This coupled with DBA being the last analyte to elute off the GC column, the higher temperature creates a

larger amount of column bleed and therefore greater background noise. This results in a larger signal response being required in comparison to earlier eluting compounds for the signal to noise ratio to pass the LOQ (Fialkov et al., 2007). The dilution factors required for other PAH and higher column bleed not only lowered the mass balance, but also added a source of variation into the results. The soil^{DERM} and membrane samples contained the greatest DBA concentrations (Table 38), and the subsequent loss of quantification through dilutions resulted in a low mass balance of DBA in comparison to BaP and Pyr.

5.2.4.1.3 Soil

It is assumed that soil removal from the membrane is consistent throughout based on the low variability of soil removal (Chapter 4.4). To correct for the influence of remaining soil in the membrane 3-PAH concentration, 3% was deducted from the measured membrane concentration (Chapter 4.4). This could result in inaccuracies in the quantification of PAH absorption into the membrane. It is unlikely that any method would remove 100% of soil dose applied, and efficacy of soil removal methods cannot be found in the literature. In future work measuring dermal bioavailability using the Strat-MTM membrane, extra confidence in soil removal could be attained using tape stripping to remove any remaining soil particles, as well as remove any PAH in the outermost membrane layer that represents the *stratum corneum* (Forsberg et al., 2021). This will result in not only the influence of any remaining soil particles on the membrane PAH concentration, but also remove the addition of PAH included in the *stratum corneum* which could also be removed from skin over time through desquamation (Elias, 1983, Ingram and Phillips, 1993).

Soil loading is also another source of error, although this was fairly consistent in this study (RSD% = 17.7%) considering the number of membranes loaded with soil (n= 112). The PAH distribution in the soil is also another source of potential variability. It has been shown that PAH distribution in soils and sediments are highly variable at the micro-scale, due to different PAH affinity to different organic matter, grain size and source materials (Wilcke et al., 1996, Ghosh et al., 2000, Xia et al., 2016, Vane et al., 2020). Therefore, the soil fraction/component that is in contact with the membrane surface has a large influence on the quantity of PAH available for dermal absorption on a micro-scale, resulting in differences in PAH dermal absorption across the membrane surface. This is further demonstrated when comparing the RSD% of BaP flux through the Strat-MTM membrane from a solvent vehicle (5.1.4.2) in comparison to soils. The BaP flux from a solvent produced an RSD% of 77.2% (5.1.4.2), which is much lower than the largest RSD% of BaP flux from a soil (RSD% range = 26.1% to 169%).

Previous studies have potentially had less error introduced by soil PAH distribution, because after the PAH are applied to the soil in the solvent spike the soil is mixed before application. Therefore, the PAH partitioning into various soil compartments and PAH source materials is not represented such as when measuring native PAH from contaminated soils in this study.

5.2.4.1.4 Membrane

It has been shown in the literature that the use of different skin sources for dermal absorption experiments can result in variation in the chemical absorption data (Sections 2.2.5, 2.2.6 and 2.3.2). This is due to differences in skin structure and skin chemistry depending on the anatomical site and species from which the skin originates (Sections 2.2.5, 2.2.6 and 2.3.2). One of the reasons that the Strat-M™ membrane was selected, is that due to it being a manufactured artificial membrane, it was theorised that it would have lower chemical and structural variability in comparison to *ex vivo* skin. Consequently, it was thought that the flux of PAH through the Strat-M™ membrane would be less variable in comparison to previous studies that have used *ex vivo* skin. The variability between diffusion cell experimental runs resulted in high RSD (%) (Table 41, Table 42, Table 43), suggesting high variability in 3-PAH flux from the soils applied. This variability could be due to several factors. Firstly, it could be due to variations in PAH mobility in the soil (Section 2.1.3), resulting in variations in flux. Secondly it could be due to structural and chemical variations of the membranes impacting PAH flux (2.2.6). Generally, the RSD (%) decreases with time as the flux reaches steady-state (Table 41, Table 42, Table 43)

The membrane used in the *in vitro* measurement of dermal absorption controls the rate at which 3-PAH are dermally absorbed into the RS. The mean RS BaP flux values at 24h produced by this study for the three soils have a smaller range (0.021 ng/cm²/h – 0.26 ng/cm²/h) in comparison to those found in the literature (0.012 ng/cm²/h – 5 ng/cm²/h) (Wester et al., 1990, Stroo et al., 2005b, Moody and Joncas, 2007, Moody et al., 2011, Xia et al., 2016, Peckham et al., 2017). This lower range in flux values produced using the Strat-M™ membrane suggests that the structure and chemistry of the artificial membrane is more consistent than *ex vivo* skin (2.2.6). This provides greater confidence in the flux values generated than if an *ex vivo* skin membrane was used.

5.2.4.1.5 Procedural

The RSD% of BaP dermal bioavailability data produced by the developed diffusion cell method of this study is similar to previous *in vitro* PAH dermal bioavailability studies (e.g. Moody and Joncas (2007) RSD% up to 70%, Wester et al. (1990) RSD% = 75%, Peckham et al. (2017) RSD% up to 107%). Due to the method involving the PAH extraction and analysis of multiple compartments (soil, membrane, and RS), there are many steps at which variability can be introduced. It is well accepted in the analytical chemistry field that the greater number of steps involved, the greater error likely to be introduced (El Husseini et al., 2018). There is also the impact of analytical error, and although this was shown to be relatively low (Table 37 and Table 38), the cumulative error of measuring three sample media for each time-step will lead to larger overall analytical error. It should be noted however, that the certainty of the fate of PAH in the *in vitro* diffusion cell system is still greater than for *in vivo* studies. This is due to the lack of need for correction factors for urinary excretion of PAH, which in turn introduce error and uncertainty, such as the large x15 correction factor applied by Wester et al. (1990).

5.2.4.1.6 Data Quality Conclusions

Overall, this method produced 3-PAH mass balance (Table 39) in the same range as previous *in vitro* studies (Moody et al., 1995, Roy et al., 1998, Moody and Joncas, 2007, Peckham et al., 2017). Although the produced mass balance was lower for Site A and B soils, it was determined that 3-PAH losses occurred during SPE of the soil^{DERM} samples that had undergone the diffusion cell method (Figure 44). This had a large impact on mass balance for Site A and B owing to the PAH concentration being higher in the soil^{DERM} in comparison to membrane and RS samples (Table 40). But, due to the other sample media showing no signs of losses through SPE (Figure 44B and C), and also BCR-524 producing an excellent mass balance, it is assumed that no significant 3-PAH losses occurred due to the diffusion cell method. Due to the soil^{DERM} concentration not being required for the calculation of the bioavailable fraction or the flux of 3-PAH, the certainty of the calculation of the dermal bioavailability of 3-PAH from the soils is not hindered.

Due to the method involving the PAH extraction and analysis of multiple compartments (soil^{DERM}, membrane and RS), there are many steps at which variability can be introduced. Analytical error was low (Section 5.2.3.1), although the cumulative effect of analytical error from the quantification of each sample media of the diffusion cell experiment results in a

higher contribution to the variability of the diffusion cell method 3-PAH mass balance. The Strat-M™ artificial membrane results in a lower range of membrane 3-PAH flux values and 3-PAH flux RSD (%) in comparison to *ex vivo* skin (Section 2.3.2). This is most likely due to the artificial membrane having a more consistent chemistry and structure in comparison to *ex vivo* skin.

Soil contributes multiple sources of error to the diffusion cell method. Soil removal was consistent throughout, and membrane 3-PAH concentrations corrected for potential soil contribution. Soil loading applied to the membranes during the diffusion cell experiment was also consistent. The distribution of 3-PAH within the soils applied could have a large impact on the concentration of mobile PAH in contact with the membrane, and consequently, result in variations in the amount of 3-PAH absorbed.

Although there are multiple sources of error in the method, the developed methods still had a 3-PAH mass balance as good as previous *in vitro* methods and produced 3-PAH flux data with lower variability than previous works that had used radiolabelled PAH and *ex vivo* skin. This means that greater confidence can be given in the 3-PAH dermal absorption data for use in HHRA, than previous works.

5.2.4.2 Flux of PAH

Equation 23 was used to calculate the flux of PAH from soil into the membrane and RS, and the flux of the entire system (total). J_{ss} for 3-PAH were determined statistically (Chapter 4.1.3.4) for each soil by testing for differences in mean PAH flux at different time steps.

5.2.4.2.1 BCR-524 (CRM)

Figure 37 shows the flux of 3-PAH into the compartments over 24 h for BCR-524 (CRM). The flux of 3-PAH is rapid at the beginning of the experiment, with the highest flux of 3-PAH occurring over the first h from soil application. Table 41 and Figure 37 show that Pyr peak flux (J_{Peak}) occurs at 1 h for the RS and 0.5 h for the membrane and total. BaP J_{Peak} follows a similar trend, with the J_{Peak} occurring for the RS, membrane, and total at 1 h (Table 41 and Figure 37). DBA J_{Peak} occurs for the RS at 1 h, and the membrane and total at 0.5h. This shows that the highest rate of PAH dermal absorption from soils occurs within the first hour of soil dermal contact.

The 3-PAH flux in each compartment gradually decreases after 1h, which can be explained by Fick's Law of Diffusion. Fick's Law of diffusion states that the rate of diffusion is governed by the proportion between the surface area and the concentration gradient, and inversely related to the membrane thickness (Crank, 1975, Beriro et al., 2016). As the surface area and membrane thickness are constant throughout this study, any change in 3-PAH is most likely due to a change in concentration gradient. Therefore, the dermally bioavailable 3-PAH in the soil and 3-PAH in the membrane/RS concentrations are equalling out, resulting in a shallower concentration gradients and subsequent lower 3-PAH flux.

J_{ss} (steady-state flux) is expressed graphically as the gradient of the absorption profile prior to plateauing (Beriro et al., 2016). J_{ss} for 3-PAH were determined statistically (Chapter 4.1.3.4) by testing for difference in mean PAH flux at different time steps. It was determined that there was no significant difference between mean flux between time steps between 12 h and 48 h ($p=0.02$ to $p=0.04$). This suggests that there is no significant change in 3-PAH flux after 12 h, and therefore J_{ss} is reached at 12 h. The J_{peak} and J_{ss} for 3-PAH is shown in Table 44 .

Table 44: 3-PAH total J_{peak} and J_{ss} (ng/cm²/h) for BCR-524 (CRM).

	J_{peak}	Time (h)	J_{ss}	Time (h)
Pyr	277.3	0.5	105.9	12
BaP	2.39	0.5	0.63	12
DBA	0.051	0.5	0.017	12

5.2.4.2.2 Site A Soil

Figure 38 shows the flux of 3-PAH into the Strat-M™ membrane, RS, and total over 24 h from the Site A soil. The overall trend of the 3-PAH flux (Figure 38) is similar to that of BCR-524 (Figure 37), with highest of 3-PAH flux occurring within the first h, gradually decreasing and plateauing. Table 42 and Figure 38 show that Pyr peak flux (J_{peak}) occurs at 0.5 h for the RS, membrane and total. BaP J_{peak} follows the same trend (Table 42 and Figure 38). DBA J_{peak} occurs for the RS at 0.5 h, and the membrane and total at 12h. It should be noted that the 24h and 48h time-steps for DBA flux were heavily skewed owing to the lack of membrane data (Section 5.2.3.2).

J_{ss} for 3-PAH were determined statistically (Chapter 4.1.3.4) by testing for difference in mean PAH flux at different time steps. It was determined that there was no significant difference

between mean flux between time steps between 12 h and 48 h ($p=0.0002$ to $p=0.02$). Table 43 shows the total J_{Peak} and J_{SS} of the 3-PAH from the soil from Site A.

Table 45: 3-PAH total J_{Peak} and J_{SS} (ng/cm²/h) for Site A soil.

	J_{Peak}	Time (h)	J_{SS}	Time (h)
Pyr	111.8	0.5	14.7	12
BaP	86.9	0.5	8.83	12
DBA	1.41	12	0.335	12

5.2.4.2.3 Site B Soil

Figure 39 and Table 43 show the flux of 3-PAH into the Strat-MTM membrane, RS, and total over 24 h from the Site B soil. The overall trend of the PAH flux (Figure 39) is that the highest 3-PAH flux occurs within the first h, then gradually decreases and plateaus. However, unlike the other two soils, the 3-PAH flux of Site B reaches steady-state sooner (6h rather than 12h). J_{Peak} occurred for all 3-PAH in all media at 0.5h.

J_{SS} for 3-PAH were determined statistically by testing for difference in mean PAH flux at different time steps. It was determined that there was no significant difference between mean flux between time steps between 6h and 48 h ($p=0.0003$ to $p=0.01$) (Table 46).

Table 46: 3-PAH total J_{Peak} and J_{SS} (ng/cm²/h) for Site B soil.

	J_{Peak}	Time (h)	J_{SS}	Time (h)
Pyr	55.2	0.5	8.9	6
BaP	17.4	0.5	3.38	6
DBA	2.06	0.5	0.263	6

5.2.4.2.4 Comparison of 3-PAH Flux of the Three Soils

J_{Peak} for the 3-PAH occurred within 0.5-1h of applying the soil to the Strat-MTM membrane, suggesting that dermal absorption under the experimental conditions occurs shortly after application of the PAH contaminated soil. This is particularly important for HHRA, because the critical receptor for residential land is a female child aged between 0 and 6 years old, and it is assumed that a 1 h per day exposure period applies (Environment Agency, 2009, CL:AIRE, 2014a). The short time between soil application and quantifiable dermally absorbed 3-PAH (and J_{Peak}) may be due to the high soil moisture content (40 w/w%) resulting in greater mobility of the soil PAH as shown in previous work (Kottler et al., 2001). Also, Xia et al. (2016) found that the dermal bioavailability of PAH was directly linked to the

freely dissolved aqueous PAH concentration in the soil, and it is likely that with increasing wetting of the soil, that this freely dissolved phase increases.

The site B soil reached J_{ss} earlier than the other two soils tested using the developed diffusion cell method. This is likely due to the concentration gradient becoming shallower at a faster rate than the other soils due to there being a lower concentration of mobile PAH in the soil of Site B. The difference in J_{ss} time is not due to the PAH flux rates, as Site B (Table 46) has a lower 3-PAH J_{peak} in comparison to the other soils (Table 44 and Table 43) where J_{ss} is reached at 12 h. Flux variability reduces with time (Figure 37, Figure 38, Figure 39) for each soil. This is likely due to high variations in the amount of PAH in contact with the membrane at the beginning of the experiment until the system reaches steady-state, where variability reduces.

It has been shown in the literature that soil characteristics have a significant impact on soil PAH bioavailability (Sections 2.1.3 and 2.3.2). Soil organic matter (SOM) is one of the most important factors that controls PAH interactions with soils and their subsequent mobility (Semple et al., 2003, Semple et al., 2013, Yu et al., 2018). Small soil particles, such as in the <250 μm fraction for dermal bioavailability testing (Duggan et al., 1985, Kissel et al., 1996, Abdel-Rahman et al., 2002, Choate et al., 2006, Cave et al., 2010, Beriro et al., 2020), tend to have higher clay and organic matter contents. Consequently, this fraction tends to higher concentrations of adsorbable organic compounds such as PAH (Pernot et al., 2013). Xia et al. (2016) found that PAH source materials have a considerable influence on the partitioning of PAH. This is significant for this study because soils from site A and B are from former gas works, whereas BCR-524 (CRM) is from a former wood treatment plant. Therefore, it is likely that the soils not only have different SOM, particle size distributions, texture, but also contain different PAH source materials (e.g., coal tar for Site A and B, creosote oil for BCR-524 (Yu et al., 2018)), resulting in different 3-PAH fluxes regardless of the soil PAH concentration. The potential variation in soil properties may also contribute to some of the error shown (Figure 34, Figure 35, Figure 36), due to inhomogeneous soil PAH distribution and mobility owing to different SOM, soil texture, particle size and PAH source materials. This would result in varying mobile PAH concentrations coming into contact with the surface of the Strat-M™ membrane, resulting in varying concentrations of PAH being dermally absorbed at different rates.

The rate of 3-PAH flux from RS from the membrane remained relatively constant (once J_{ss} reached) throughout the 48h for all three soils tested (Figure 37, Figure 38, Figure 39). This

suggests that the 3-PAH at the soil-membrane interface was not depleted throughout the 48h exposure, and subsequently the 3-PAH soil to skin transfer was not rate limiting (Peckham et al., 2017). Some studies have used artificial sweat and/or sebum to measure the dermal bioaccessibility of chemicals (Schneider et al., 2016, Marin Villegas et al., 2019, Beriro et al., 2020). It was theorised that the addition of sebum and/or artificial sweat to the membrane surface would be required to mimic human skin physiology for any mobile soil PAH to enter the Strat-M™ artificial membrane. However, as the flux of PAH into and through the Strat-M™ membrane remained constant after J_{ss} was reached, the soil-membrane transfer of PAH was not inhibited. The Strat-M™ membrane is treated with artificial skin lipids (Haq et al., 2018), with these lipids enabling the penetration of lipophilic compounds (Elias, 1983). Schneider et al. (2016) tested the dermal absorption of toluene through *ex vivo* skin with and without the presence of artificial sebum and found no difference in percutaneous penetration under both treatments. Toluene, like PAH is lipophilic, suggesting that the lack of sebum used in this study is unlikely to influence the ability of the Strat-M™ membrane to dermally absorb PAH. However, dermal bioaccessibility studies/methods such as Beriro et al. (2020), would be useful for assessing the potential concentration of PAH in a soil that can be dermally absorbed.

Once flux is separated into membrane and RS, the membrane has the highest flux (Figure 37, Figure 38, Figure 39). As the PAH flux is high from soil to membrane in comparison to PAH flux from membrane to receptor solution, the membrane limits the amount of PAH that reaches systemic circulation (Kao et al., 1985, Abdel-Rahman et al., 2002, Peckham et al., 2017). This means that there is the potential for PAH, to either cause localised health effects, further diffuse into the skin and/or into systemic circulation over time. Forsberg et al. (2021) revealed that PAH skin permeation continues from the membrane to receptor solution even after the soil is removed.

The Strat-M™ membrane was shown to produce flux values representative of *ex vivo* skin (Section 5.1.4.2 and 5.2.4.2). But a limitation of the Strat-M™ is that unlike *in vivo* (Wester et al., 1990) and some *ex vivo* studies (Brinkmann et al., 2013), the membrane has no capability of metabolising PAH that have been dermally absorbed. PAH are not carcinogenic until metabolised (Gelboin, 1980, Pessah et al., 2001, Nathanail et al., 2009) (Sections 2.2.2 and 2.2.3), and have been shown to be metabolised by keratinocytes in the skin (Brinkmann et al., 2013). PAH metabolism is also a method by which the body removes PAH from the system (Sections 2.2.2 and 2.2.3). Previous works have demonstrated that the cutaneous and systemic fate of topically applied BaP was heavily influenced by the biochemical viability

and metabolic status of the skin (Kao et al., 1985). Therefore, without PAH metabolism being represented by the Strat-M™ membrane, greater PAH concentrations could remain in the *in vitro* system resulting in an overestimation of PAH concentration in the membrane in comparison to *in vivo* testing. On the other hand, the Strat-M™ membrane provides greater certainty of PAH fate in the dermal system than *in vivo* studies (Wester et al., 1990, Moody et al., 1995). This is due to better mass balance despite the errors in this research (Section 5.2.4.1), and the lack of need for large correction factors for PAH urinary metabolite excretion, such as used in Wester et al. (1990).

5.2.4.2.5 Comparison of this study's dermal bioavailability data to previous studies.

It has been shown in the literature that in general, higher soil PAH concentration applied results in higher PAH dermal flux (e.g. Peckham et al. (2017)). This was also seen in this study. When correlation was tested between mean 3-PAH soil^{Total} concentration (mg/kg) and 3-PAH dermal flux (total), a Pearson's Correlation Coefficient of 0.8571 ($p = 0.01071$). The p-value of the test is less than the significance level alpha of 0.05, and therefore it can be concluded that 3-PAH dermal flux is significantly correlated with 3-PAH mean soil^{Total} concentration (Figure 45).

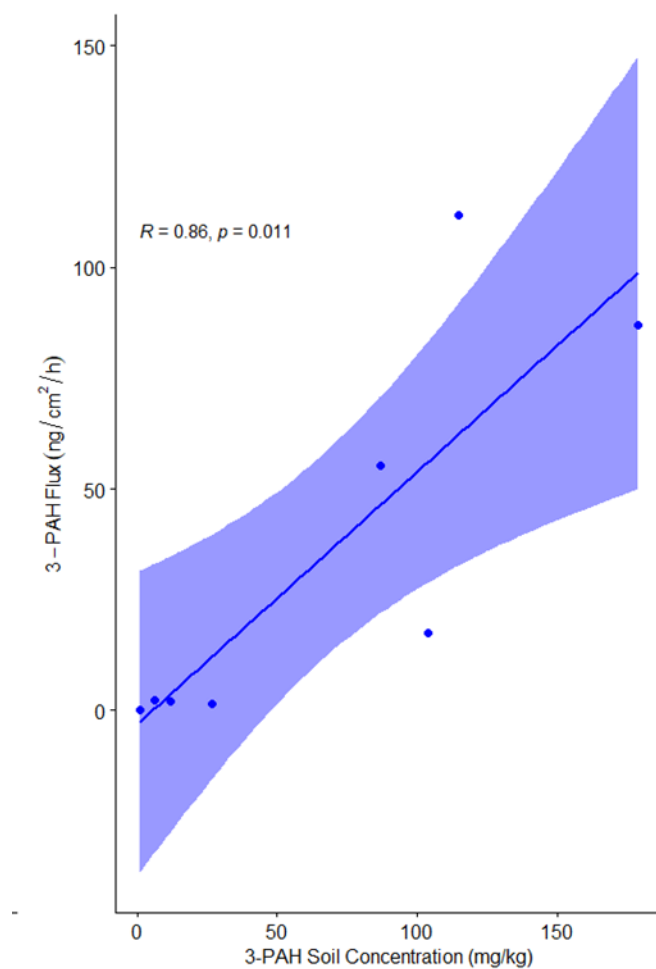


Figure 45: Pearson's correlation between 3-PAH SoilTotal Concentration (mg/kg) and 3-PAH Flux (ng/cm²/h). The shaded areas represent the confidence interval of the correlation coefficient at 95%.

Fick's Law of diffusion states that the rate of diffusion is governed by the proportion between the surface area and the concentration gradient, and inversely related to the membrane thickness (Crank, 1975, Beriro et al., 2016). Therefore, because the membrane thickness and surface area should remain relatively constant throughout the *in vitro* diffusion cell method, the changes in BaP flux are governed by changes in the concentration gradient. Therefore, this change in concentration gradient is governed by the concentration of soil BaP dermally bioavailable in comparison to the BaP concentration dermally absorbed.

Furthermore, Figure 46 shows a comparison of RS PAH flux at 24h between this work (n = 12) and previous work (n = 8) (Wester et al., 1990, Stroo et al., 2005b, Moody and Joncas,

2007, Moody et al., 2011, Xia et al., 2016, Peckham et al., 2017). A t-test found no evidence for a difference between means ($p=0.09$).

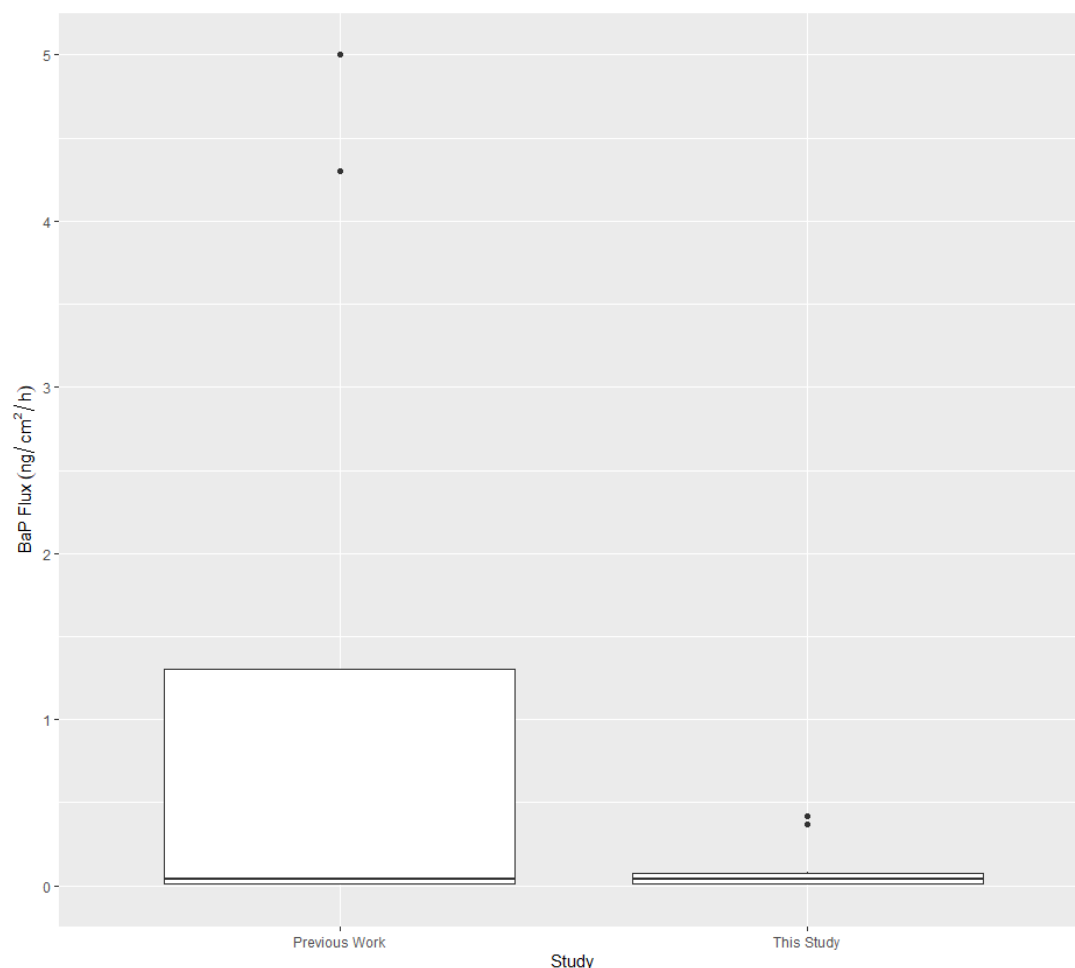


Figure 46: Comparison of PAH receptor solution flux at 24h of this study ($n=12$) and literature reported values ($n=8$).

However, Moody et al. (2011) spiked a soil with ^{14}C -BaP (10.1 mg/kg), which was a similar BaP concentration to that of BCR-524 (CRM) tested in this study (BaP = 6.15 mg/kg). The spiked soil of Moody et al. (2011) produced a BaP flux of 5 ng/cm²/h at 24h in the RS, in comparison to a mean RS BaP flux at 24h of 0.021 ng/cm²/h for BCR-524 (CRM) in this study. This has also been demonstrated in the literature, with Stroo et al. (2005b) reporting that BaP flux is 5-10 times lower in native than spiked soils. It has also been shown in the literature that aging decreases the dermal bioavailability of PAH from soil (e.g. (Roy and Singh, 2001, Abdel-Rahman et al., 2002), and that there is even a reduction in PAH dermal absorption just when soil is present due to the rapid adsorption of PAH to the soil matrix (Wester et al., 1990, Abdel-Rahman et al., 2002). Consequently, the use of solvent spiking PAH onto soil produces dermal bioavailability data with higher PAH dermal fluxes.

The RS flux values produced by this study are also less variable than in the literature (Figure 46), even though a wide range of PAH doses were applied (Table 34). The membrane used in the *in vitro* measurement of dermal absorption controls the rate at which analytes are dermally absorbed into the RS. The lower RS flux variation in this study could be due to the Strat-M™ membrane having a more consistent structure and chemistry in comparison to *ex vivo* skin (2.2.6), resulting in a more consistent barrier to the dermal penetration of PAH. It has been demonstrated in the literature that skin source (species and site) has a large impact on the quantity of compounds dermally absorbed (VanRooij, 1993, Moody et al., 1995). It is therefore recommended to use the Strat-M™ membrane for future work analysing the impact of soil properties and geochemistry on the dermal absorption of PAH. This will enable the variability of PAH flux from soil to be attributed to changes in soil properties with greater certainty than when using *ex vivo* skin.

Ideally, the use of the Strat-M™ membrane would be validated using an *in vivo* study of the same soils (OECD, 2004), but this would contradict the three R's (Replacement, Reduction and Refinement) approach to reduction in the use of animals for chemical hazard testing (Williams, 2006). Overall progress in assessing the suitability of *in vitro* methods to predict *in vivo* chemical exposure via the dermal pathway is due to the lack of direct *in vitro-in vivo* comparisons of the same dose and vehicle.

5.2.5 Conclusions

The aim of this project was to develop an *in vitro* method to quantify the human dermal bioavailability of PAH in soil. This has been achieved for the three soils tested, two soils from former gasworks (Site A and B) and BCR-524 (CRM). The method produced dermal absorption data over a 48 h exposure period for 3-PAH. The method mass balance for the Site A and B soils were impacted by the SPE method owing to the very high soil 3-PAH concentrations, and it was determined that the 3-PAH losses were unlikely due to the developed *in vitro* diffusion cell method (Section 5.2.4.1.2). However, this did not impact the calculation of 3-PAH flux. The developed *in vitro* diffusion cell method mass balance for BCR-524 (CRM) was in a similar range to previous *in vitro* methods and produced better mass balance than some *in vivo* studies (Section 5.2.4.1).

A larger proportion of dermally absorbed 3-PAH was retained in the membrane than in the RS over the duration of the experiment (Table 40). The dermal absorption of 3-PAH from soils is a rapid process, with the peak flux (J_{Peak}) occurring within the first 1 h for each PAH from each soil (5.2.4.2). 3-PAH J_{SS} was reached in all three soils between 6 and 12 h from when the

soil was applied (Section 5.2.4.2). J_{ss} in the RS at 24 h is lower and less variable than those from previous studies that spiked soil with radiolabelled PAH due to the aging process decreasing the dermal bioavailability of native PAH from soil (Roy and Singh, 2001, Abdel-Rahman et al., 2002). It is therefore recommended to use the Strat-M™ membrane for future work analysing the impact of soil properties and geochemistry on the dermal absorption of PAH, as this will enable the variability of PAH flux from soil to be attributed to changes in soil properties with greater certainty than when using *ex vivo* skin.

The developed *in vitro* dermal bioavailability method has allowed for the quantification of the dermal absorption of native PAH from ‘real world’ contaminated soils. The method has demonstrated that the dermal absorption of PAH does not hold a linear relationship with PAH soil concentration, and that soil properties have a key influence on the dermal bioavailability of PAH. Further understanding of soil property interactions with PAH and the impact of PAH soil desorption needs to be developed to better assess the potential soil PAH dermal bioavailability and the subsequent human health risk posed by the dermal absorption of PAH.

6 Implications to the Contaminated Land Management Industry Sector

6.1 Introduction

The aim of this chapter is to assess the potential implication of the soil^{DERM} PAH dermal flux data produced by the developed *in vitro method* of this study (Section 5.2) on contaminated land management. In the UK, land assessment under Part 2A involves a risk-based approach to determine if land is contaminated. For a risk to exist, there needs to be at least one contaminant-pathway-receptor linkage, from which a relevant receptor might be affected by the contaminants present (DEFRA, 2012) (Chapter 2.5). The National Planning Policy Framework (NPPF) also states that planning policies and decisions should ensure that a site is suitable for its intended use, considering any risks arising from land contamination (NPPF, 118c). In England, the current BaP dermal absorption fraction (ABS_d) of 0.13 (Environment Agency, 2009) is used in human health risk assessment (HHRA). This value originates from the Wester et al. (1990) *in vivo* study (Section 2.3.2.2). In this thesis soil-specific ABS_d values were derived using the same approach as Forsberg et al. (2021), using the measured soil^{DERM} BaP total J_{PEAK} data generated using the developed *in vitro* dermal absorption method

(Chapter 5.2.4.2). These ABS_d values are then compared to the 0.13 ABS_d default value. Furthermore, the soil-specific predicted BaP daily dermal intake, dermal pathway exposure contribution, and site-specific assessment criteria (SSAC) are compared to the default CLEA model outputs.

Ideally, J_{PEAK} would be input into the CLEA model directly to represent dermal exposure so that experimental soil loading would not impact the dermal bioavailability data (Sections 2.3.2 and 2.3.3). As this could not be achieved by the author, as this would require accessing and editing the formulae in the CLEA model, the impact of soil loading on the soil-specific ABS_d values and subsequent SSAC was assessed. It has previously been noted in the literature that soil loading has an impact on dermal bioavailability results if results are reported in the form of percent of the dose applied (i.e., BAF%, PADA, ABS_d). High loadings (81.5 mg/cm^2) were used in the *in vitro* soil dermal bioavailability diffusion cell experiment (Section 4.1.2.3) to achieve infinite dosing so that J_{ss} could be quantified and compared to previous work for method validation. Furthermore, it was hoped that applying a high 3-PAH dose would improve the method sensitivity allowing for 3-PAH to be quantified in all three diffusion cell compartments (Section 4.1 and 5.2). Spalt et al. (2009) state that if fractional absorptions are to be reported from experiments with supermonolayer loadings, that the data must be adjusted to monolayer equivalent values. Therefore, correction factors were applied to assume monolayer coverage (5 mg/cm^2) (USEPA, 1992). The effects of experimental soil loading on calculated ABS_d and subsequent SSAC are also explored.

The International Program on Chemical Safety (IPCS) state that results from *in vitro* methods can be considered adequate for risk assessment in the absence of representative *in vivo* data (IPCS, 2006). This is providing that the concentration of the substance absorbed into the skin is considered along with the receptor solution concentration. As this study has developed an *in vitro* based method that is able to quantify PAH in both the membrane (Section 4.2) and receptor solution (Chapter 4.3), it is possible for the absorption data from this study to be used to assess potential human exposure to PAH from soils via the dermal pathway.

6.2 Methods

The Contaminated Land Exposure Assessment Model (CLEA), was developed by the Environment Agency to develop Soil Guideline Values (SGVs) (Environment Agency, 2009). SGVs are generic assessment criteria (GAC) that may be used for the assessment of human

health risk of chronic exposure from soil contaminants (DEFRA and Environment Agency, 2004) (Section 2.5).

To calculate ABS_d values for the soils tested in this study, Equation 24a and b were used (Forsberg et al., 2021), using the parameters in Table 47. The Wester et al. (1990) study parameters were used to calculate an ABS_d for quality control of the Forsberg et al. (2021) ABS_d equations (Equation 24). This was achieved by comparing the ABS_d for generated input parameters from the *in vivo* Wester et al. (1990) study into Equation 24a and b, and comparing this to the CLEA standard 0.13 ABS_d value. The desired outcome was for the output of the Equation 24 using the Wester et al. (1990) parameters to equal the default 0.13 ABS_d value.

$$a) ABS_{exp} = (F_{rf} \times ET) + ABS_s$$

$$b) ABS_d = \frac{ABS_{exp}}{C \times ESLR}$$

Equation 24: Equations a and b, used to calculate ABS_d using experimental results and parameters. From Forsberg et al (2021).

Where: ABS_{exp} is the experimental PAH dose absorbed (ng), F_{rf} is the flux of a PAH into the receptor solution (ng/cm²/h), ET is the experimental time (h), ABS_s is the dose of a PAH in the membrane (ng/cm²), C is the concentration of PAH in the test soil applied (ng/mg), ESLR is the soil loading rate (mg/cm²).

Table 47: Parameters used to calculate experimentally derived soil-specific ABS_d .

	F_{rf} (ng/cm ² /h)	ET (h)	ABS_s (ng/cm ²)	ABS_{exp} (Dimensionless)	C (ng/mg)	ESLR (mg/cm ²)
Site A	0.96	1	21.3	22.26	179	81.5
Site B	12.5	1	15.3	27.8	104	81.5
BCR-524	1.2	1	1.19	2.39	6.15	81.5
Wester <i>et al</i> (1990)	2.2	24	nd ^a	48	10	40

nd^a due to *in vivo* experimental protocol not providing skin absorbed BaP concentration

It is recommended by the IPCS (2006) to use the maximum flux for human health risk assessment, either measured or estimated, in preference to K_p or percent chemical absorbed. Therefore, as this represents the highest potential rate of human BaP exposure through dermal absorption from soils, total BaP J_{PEAK} (Section 5.2) was used to calculate ABS_d . Also, the J_{PEAK} for all 3-PAH for all soils tested occurred within 0.5 – 1 h (Section 5.2) with 1 h per day being the predicted exposure duration for the critical receptor for residential sites. Therefore, the critical receptor would be exposed to the highest rate of PAH dermal

absorption from soils and this needs to be taken into consideration during the exposure assessment.

Once the soil specific ABS_d values were derived, the values were input into the daily dermal chemical uptake rate formula (Equation 25) from the CLEA model (Environment Agency, 2009). The critical receptor for residential land is a female child of up to 6 years old. This is due to a combination of higher childhood exposure to major pathways and lower bodyweight in comparison to adults. For the CLEA model it is assumed that the child will spend 1 hour per day in the garden (Environment Agency, 2009). Consequently, the parameters selected were that of a female child for the residential land exposure scenario (Table 48).

$$IR = C_s A_F ABS_d A_{skin} \times \frac{1}{1000} g\ mg^{-1} \times 10000 cm^2 m^{-2}$$

Equation 25: CLEA model daily dermal chemical uptake rate (mg/day)

Where: IR is the chemical uptake rate from outdoor dermal contact with soil (mg/day), C_s is the total chemical concentration in soil (mg/g), A_F is the soil-to-skin adherence factor (mg/cm²), ABS_d the dermal absorption factor (dimensionless), A_{skin} is the exposed skin area (m²), n is the number of daily soil contact events (day).

Table 48: Female Child (<6 years old) CLEA model exposure parameters for residential land. Data from: (Environment Agency, 2009).

Female Child Exposure Parameters		
n (day)	AF (mg/cm ²)	A _{skin} (m ²)
1	1	0.068

SSAC were then calculated using the CLEA model (v1.071) using default ABS_d and the soil-specific ABS_d calculated using Equation 24. The CLEA model calculates SSAC for the oral and inhalation pathways or combined pathways depending on whether exposure to a substance results in a systemic or localised critical toxicological effect (CL:AIRE, 2014b). The systemic exposure from oral, inhalation and dermal pathways should be considered as additive due to PAH being multi-site systemic carcinogens (Section 2.2.2). Therefore, oral, inhalation and dermal exposure were combined to assess systemic risk (CL:AIRE, 2014b).

The effect of experimental soil loading on ABS_d and subsequent SSAC and exposure pathway contribution (%) was assessed by changing the ESLR in Equation 24 with a range of soil loadings encountered in the literature (between 1 mg/cm² (Roy and Singh, 2001) and 100 mg/cm² (Duff, 1996)). All other variables in Table 47 remained unchanged when calculating

ABS_d for different soil loadings for each soil tested. These ABS_d for each soil loading were then input into the CLEA model under the same parameters as previously mentioned to calculate and consequently allow for the comparison of SSAC and exposure pathway contributions (%) under different theoretical experimental soil loadings. The ABS_d and SSAC could then be compared under different soil loadings.

Spalt et al. (2009) state that the data must be adjusted to monolayer equivalent values if fractional absorptions are to be reported from experiments with supermonolayer loadings. In order to achieve this for the values derived in this project a monolayer correction factor was applied to the soil-specific ABS_d values to achieve a theoretical soil loading of 5 mg/cm² using Equation 26 (USEPA, 1992).

Equation 26: ABS_d experimental loading correction to monolayer coverage (USEPA, 1992).

$$ABS_{scaled} = ABS_{exp} \times \frac{ESLR}{5mg/cm^2}$$

Where: ABS_{scaled} is the loading corrected dermally absorbed fraction (dimensionless), ABS_{exp} is the experimental dermally absorbed fraction (dimensionless), ESLR is the experimental applied soil loading (mg/cm²), and 5 mg/cm² represents an estimate of the soil load that would provide nominal monolayer coverage (USEPA, 1992).

6.3 Results

6.3.1 Soil-specific ABS_d and SSAC

Table 47 shows the calculated BaP ABS_d values using Equation 24 for the three soils using experimental BaP J_{PEAK} (Section 5.2). Table 47 also shows the calculated BaP ABS_d values using Equation 24 for the *in vivo* Wester et al. (1990) study.

Table 49: Calculated soil specific ABS_d values using Equation 24, and subsequent BaP SSAC (mg/kg) calculated using the CLEA model. Number in brackets represent 95% confidence interval.

Soil	BaP ABS _d	BaP SSAC (mg/kg)
Site A	0.00153 (0.00153)	4.31
Site B	0.00328 (0.0038)	4.28
BCR-524	0.00477 (0.006)	4.25
Wester <i>et al</i> , 1990	0.132 ^a	2.87

^a was calculated using parameters from Wester et al (1990) *in vivo* study.

The calculated soil-specific ABS_d of all three tested soils using the developed *in vitro* diffusion cell method were lower than the standard CLEA model ABS_d value of 0.13 (Table 47). The Wester et al. (1990) *in vivo* study parameters were input into Equation 24 for quality control. This produced an ABS_d value of 0.132. Furthermore, the BaP SSAC (mg/kg) derived using the CLEA model, were all higher than the BaP SSAC (mg/kg) using the default 0.13 value (Table 47).

6.3.2 BaP Calculated Dermal Intake Rate

Table 50 shows the predicted BaP IR dermal intake rate for a female child for residential land use. The BaP IR was calculated using Equation 25, the ABS_d value of Table 47 and CLEA model parameters of Table 48.

Table 50: BaP Intake Rate (IR) (mg/day) for female child (<6 yrs) for residential land use. Numbers in brackets represent 95% confidence interval.

BaP ABS_d Used	Soil	BaP IR (mg/day)
Default CLEA	Site A	0.0112
	Site B	0.00628
	BCR_524	0.00076
<i>In Vitro</i> Peak Flux	Site A	0.000132 (0.0001)
	Site B	0.000232 (0.00027)
	BCR_524	0.0000199 (0.000026)

Table 48 shows that there is a decrease in predicted BaP IR (mg/day) when using the soil specific ABS_d in comparison to the default CLEA model ABS_d of 0.13. Site A produced a 98.8% reduction in BaP IR, Site B a 96.3% reduction, and BCR-524 a 97.4% reduction.

6.3.3 Impact of Soil Loading on ABS_d and SSAC

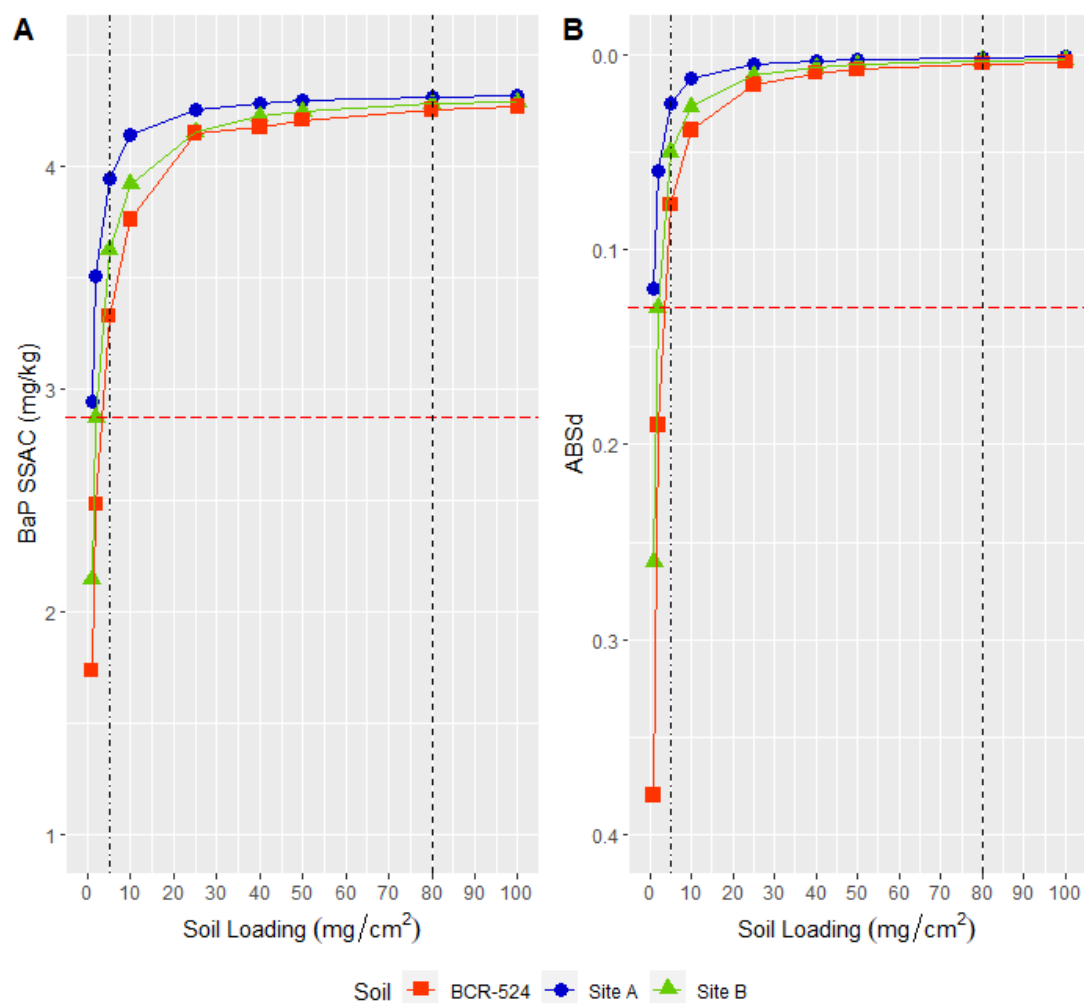


Figure 47: The impact of experimental soil loading (mg/cm²) on calculated BaP SSAC (mg/kg) (A) and ABS_d (B) of the three soils tested in this study. The red horizontal line represents the CLEA default output for SSAC in figure A, and default ABS_d in figure B. The vertical dot-dash line at 5mg/cm² loading represents the soil-specific ABS_d and SSAC (mg/kg) with experimental loading correction applied. The vertical dashed line at 81.5 mg/cm² represents the ABS_d and SSAC (mg/kg) with the experimental loading of this study.

Figure 47 shows the impact of soil loading (mg/cm²) on the calculated BaP SSAC (mg/kg) (Figure 47A) using soil-specific ABS_d (Figure 47B) calculated using Equation 24. Figure 47B shows that ABS_d increases as experimental soil loading (mg/cm²) decreases, consequently

resulting in the reduction in the BaP SSAC (mg/kg) as shown in Figure 47A. Furthermore, there is an increase in ABSd, and decrease in SSAC (mg/kg) when comparing the supermonolayer soil loading of this study (81.5 mg/cm²) when adjusted to an assumed monolayer coverage of 5 mg/cm².

6.3.4 Impact of Soil Loading on Exposure Pathway Contribution

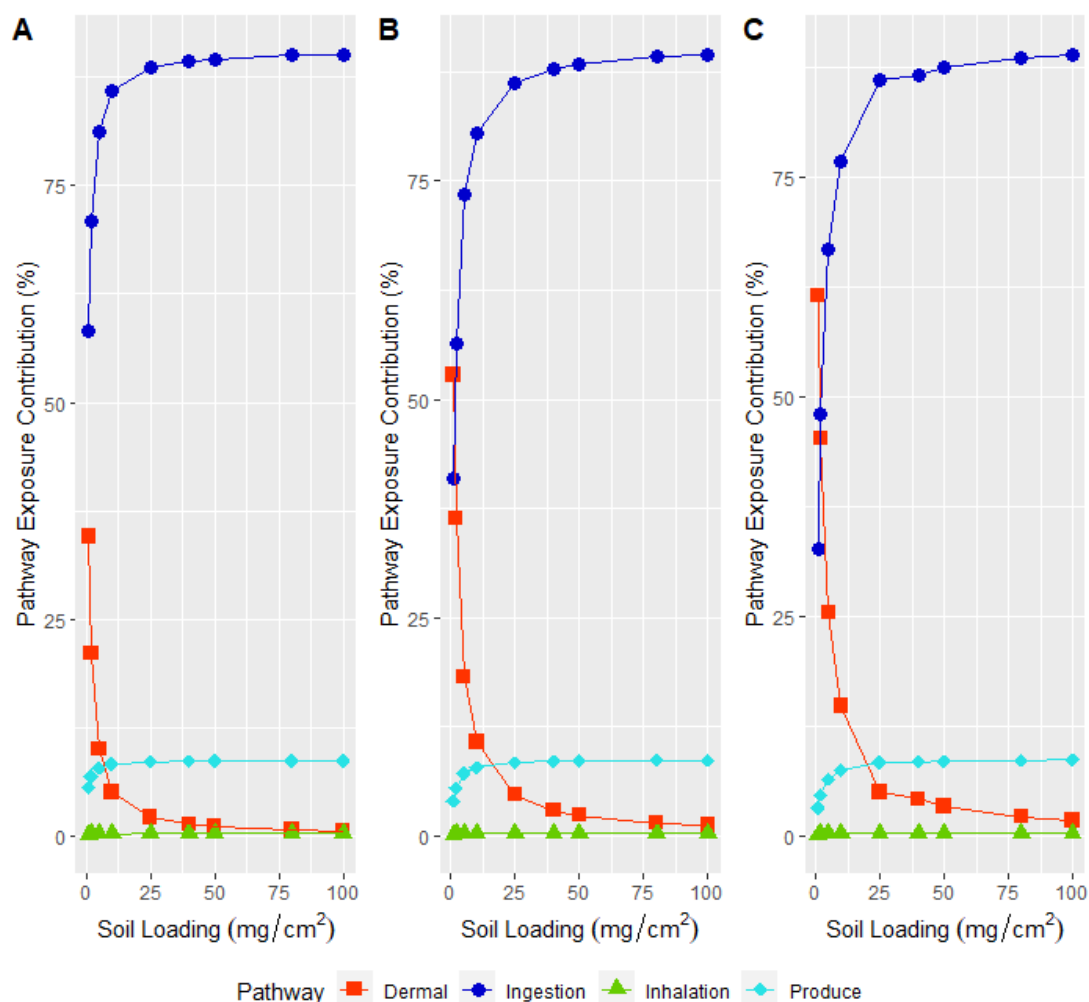


Figure 48: Dermal, ingestion, inhalation, and consumption of home-grown produce pathway contribution (%) to total BaP exposure at different experimental soil loadings. A) Soil A B) Soil B C) BCR-524.

Figure 48 shows the pathway exposure contribution to BaP total exposure by the dermal, inhalation, ingestion and consumption of home-grown produce as calculated by the CLEA model. Figure 48 shows that for each soil, dermal exposure contribution (%) increases as soil loading (mg/cm²) decreases. The default CLEA pathway soil BaP exposure contributions are in the order: soil ingestion (56.5%), dermal contact (36.3%), consumption of home-grown produce (5.46%), and dust inhalation (0.18%) (Section 2.6). Figure 48 shows that at higher soil loadings, the dermal exposure contribution (%) becomes lower than the consumption of

home-grown produce exposure contribution (%). Additionally, Figure 48B and C show that the dermal exposure contribution (%) becomes higher than the soil ingestion exposure contribution (%) for both Soil B and BCR-524 at lower soil loadings (approximately 1 mg/cm²).

6.4 Discussion

6.4.1 PAH *in vitro* dermal bioavailability impact on HHRA

Similar approaches to assess the impact of site-specific bioavailability values have been encountered in the literature (Stroo et al., 2005a, Haney Jr et al., 2020, Forsberg et al., 2021). Table 47 and Table 48 show the calculated ABS_d and BaP daily intake rate (mg/day) using BaP J_{PEAK} from the soils of this study. There was a mean reduction in soil-specific BaP ABS_d of 97.5% in comparison to the CLEA model standard ABS_d value of 0.13 for the three soils tested by using J_{PEAK} in Equation 24. Consequently, this led to a mean reduction in predicted daily BaP intake rate of 97.5% from the three soils when inputting the derived ABS_d value in comparison to the default 0.13 ABS_d value. There is an increasing body of evidence in the literature that dermal absorption of BaP from aged soil may be lower than freshly PAH spiked soil (Abdel-Rahman et al., 2002, Stroo et al., 2005b, Moody and Joncas, 2007, Turkall et al., 2010).

Abdel-Rahman et al. (2002) showed that aging of a spiked BaP dose onto soil led to a 95% to 98% reduction in the dermal bioavailability when compared to dosing BaP in a solvent vehicle, and this aging was only over a short period of 3 months. In comparison, the soils tested in this study have contained PAH contamination for decades. Consequently, the aging process has likely resulted in the PAH being less available for dermal absorption, with PAH being retained in soil to a greater extent with time. This aging process is not currently represented in the default dermal exposure parameter of ABS_d 0.13, owing to the aging process not being represented in the original study from which it is derived (Wester et al., 1990, Alexander, 2000). As a result, it is possible that Wester et al. (1990) provide an overestimation of human exposure, and subsequently the human health risk, from the dermal absorption of PAH from soils.

It should also be noted that a greater proportion of BaP is retained in the membrane rather than the receptor solution (Section 5.2) with the same trend being shown in previous *in vitro* dermal bioavailability studies (Wester et al., 1990, Moody et al., 1995, Abdel-Rahman et al., 2002, Moody and Joncas, 2007, Peckham et al., 2017). It has been shown that highly lipophilic

PAH such as BaP can form a reservoir in the *stratum corneum* owing to its lipophilic environment (Williams, 2006, Alalaiwe et al., 2020), reducing the immediate concentration that reaches systemic circulation (in this case the receptor solution). There is evidence to suggest that BaP is also a point of contact carcinogen, resulting in both local and systemic health impacts (Knafla et al., 2011). BaP contained in the membrane could have multiple pathways in a real world exposure scenario. The first is that BaP contained in the *stratum corneum* is removed from the skin during desquamation, resulting in BaP removal from the skin (Elias, 1983). The second is the inward diffusion of BaP from the stratum corneum into the dermis, and third through the dermis and into systemic circulation. A recent study by Forsberg et al. (2021) revealed that BaP continued to permeate into systemic circulation after the soil had been removed. Consequently, if the receptor was exposed to PAH contaminated soil for 1 hour, the peak flux will be encountered, and BaP will continue to enter systemic circulation post soil removal. Therefore, further demonstrating the importance of the rapid flux of BaP into the membrane shown in the *in vitro* soil dermal bioavailability diffusion cell experiment for exposure assessment (Section 5.2).

The BaP bioavailability dermal pathway contribution using CLEA model default values is 36.3%, the second largest contribution behind ingestion (56.3%) (Nathanail et al, 2009) (Section 2.6). Figure 48 shows the impact of soil loading on the dermal contribution to total exposure for the three soils tested in this study. For each soil, decreasing the soil loading applied results in greater dermal exposure contribution (%). This is due to soil loading effects on the calculation of ABS_d (Section 2.3.3). The individual soil dermal pathway exposure is in fact the same for all loadings as the same respective soil-specific BaP J_{PEAK} was used to calculate ABS_d therefore the same actual PAH concentration absorbed. As ABS_d represents the fraction dermally absorbed of the dose applied, as the dose applied reduces with lower soil loading, the fraction absorbed increases. Consequently, if soil-specific ABS_d is to be used in future to derive BaP SSAC, it is recommended that the CLEA model is adapted so that J_{PEAK} is used to assess dermal exposure to avoid the uncertainties introduced by soil loading (mg/cm^2).

Furthermore, the three soils show different dermal exposure contribution (%) (Figure 48). Dermal contribution to exposure increased in the order: Site A < Site B < BCR-524, which is the reverse order of their respective BaP soil concentrations. Additionally, the respective ABS_d values differ (Table 47), and increase in the order: Site A < Site B < BCR-524. These findings suggest that one value cannot be representative of BaP dermal exposure for all soils.

This is likely due to the impacts of soil properties, soil geochemistry and PAH source materials on PAH mobility (Sections 2.1.3, 2.3.2 and 5.2.4.2.4). It has been previously demonstrated by Xia et al. (2016) that PAH source material, and soil properties such as black carbon content, impact PAH soil mobility. BCR-524 originates from a former creosote works and may exhibit higher BaP dermal exposure owing to difference in PAH source material to former gasworks soils A and B (creosote oil vs coal tar). This relationship could be determined further in future work by testing the dermal absorption of PAH from soils with a range of soil concentrations, soil properties and PAH source materials. Furthermore, the findings of this work may be able to determine the key soil and PAH characteristics that govern soil PAH dermal absorption, enabling the creation of a predictive model for soil PAH dermal exposure.

Even though there is uncertainty in the ABS_d , SSAC and dermal exposure contribution (%) derived using the $soil^{DERM} J_{PEAK}$ owing to the impact of soil loading (mg/cm^2) (Figure 47 and Figure 48), this study still provides evidence to suggest that the current 0.13 ABS_d value used in CLEA over predicts soil BaP dermal exposure. Firstly, at the soil loading applied in the Wester et al. (1990) study ($40 mg/cm^2$), the calculated ABS_d for each soil (Site A = 0.0031, Site B = 0.0067, BCR-524 = 0.0097) are all lower than the ABS_d of 0.13. Consequently, these lower ABS_d values result in higher BaP SSAC at this loading than when using the default values (Figure 47). Furthermore, when the ABS_d for each soil tested is corrected to an assumed monolayer coverage of $5 mg/cm^2$, the soil-specific ABS_d are still lower than the Wester et al. (1990) 0.13 ABS_d value (Site A = 0.0249, Site B = 0.0535 and BCR-524 = 0.0777) (Figure 47B). These findings suggest that it is likely the current 0.13 ABS_d value over predicts dermal exposure to soil BaP.

Furthermore, as BaP was the third most common determinant of Contaminated Land between 2000 and 2013 (Environment Agency, 2016), the current representation of the dermal pathway may have resulted in sites requiring clean-up when in fact the potential risk posed is lower (Table 47, Figure 47) (Alexander, 2000). A recent study by Haney Jr et al. (2020) in the USA, found that by using soil-specific dermal and oral bioavailability data, that the soil screening values increased by between on average 4.4 and 6-fold in comparison to using the default ABS_d of 0.13. Furthermore, whilst also ensuring that the significant resources required for remediation of contaminated sites for residential land are used appropriately to reach contaminant levels which pose low or minimal risk of harm to human health. It should be noted that the method by which Haney Jr et al. (2020) attained the soil-specific dermal and oral bioavailability data is not known, and the author states in their paper that the methods will be explained in another related publication yet to be published.

The ingestion and dermal pathways represent the highest soil BaP exposure contribution out of the pathways determined by the CLEA model (Figure 48). Therefore, it could also be beneficial to include soil-specific ingestion/oral (e.g., unified BARGE method (Wragg et al., 2011) (ISO: 17924:2018(en)) bioavailability, to compare the effect of experimentally derived values and CLEA model default exposure values on human health risk assessment output. Nathanail and Ogden (2013) showed an increase in the BaP site-specific assessment criteria compared to CLEA default parameter GAC when using site-specific oral bioavailability values quantified using a colon-enhanced physiological extraction test (CEPBET). Although soil-specific oral and dermal bioavailability testing would add expense to the initial site investigation phase of contaminated land assessment, by performing DQRA with site-specific bioavailability data, expense could be lowered for the remediation phase by potentially demonstrating that the site poses less of a risk to human health in comparison to when using default values.

6.4.2 Differences in 3-PAH Dermal Bioavailability

At present, there are two ways by which the potential health risk of PAH exposure can be assessed, by using TEFs or the surrogate marker approach (Section 2.2.2). The experimental flux of 3-PAH were compared to assess whether BaP is an appropriate marker from which exposure and toxicological assessment can be made for the dermal pathway. The 3-PAH experimental flux varies, with flux being different between soils as well as between 3-PAH compounds (Section 5.2).

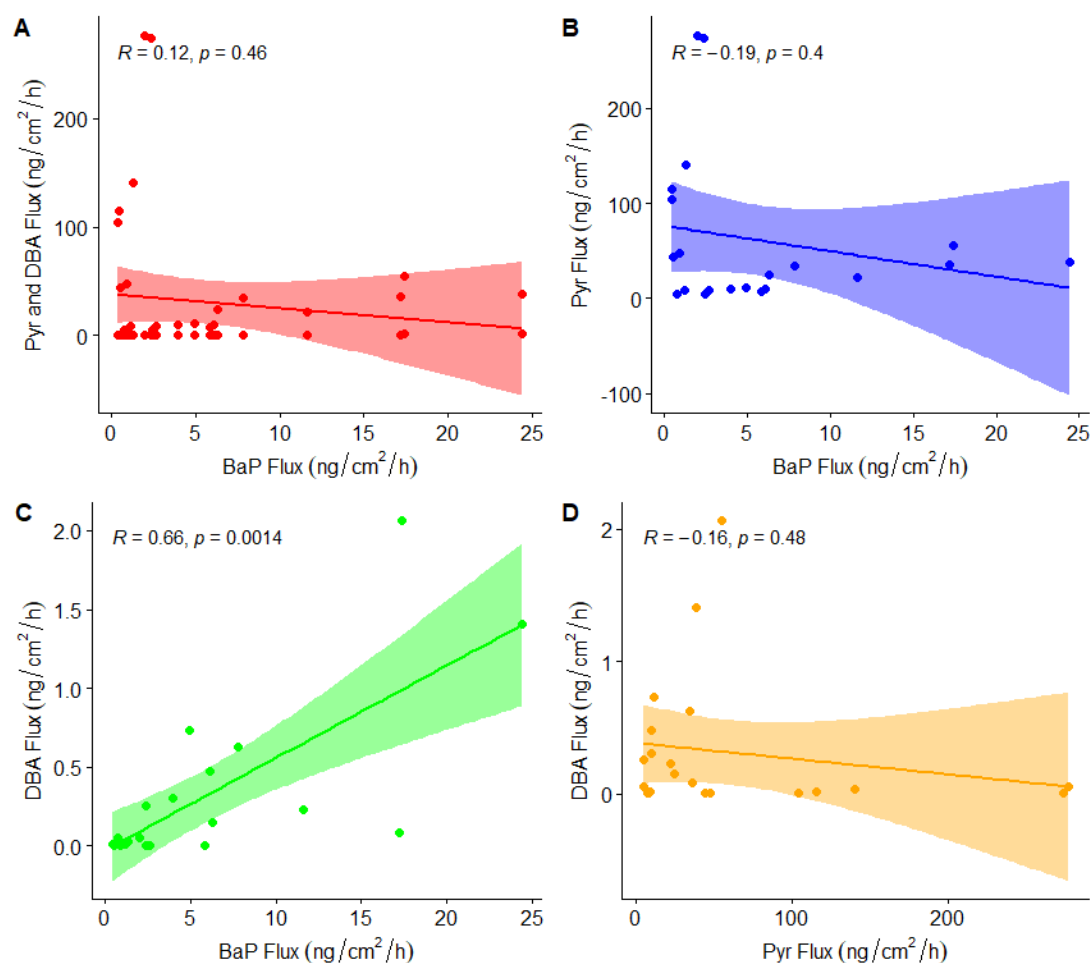


Figure 49: Spearman's correlation between fluxes (ng/cm²/h) of 3-PAH. A) Correlation between BaP and Pyr and DBA flux (ng/cm²/h). B) Correlation between BaP and Pyr flux (ng/cm²/h). C) Correlation between BaP and DBA flux (ng/cm²/h). D) Correlation between Pyr and DBA flux (ng/cm²/h).

A Shapiro-Wilks normality test of 3-PAH flux found that flux was not normally distributed ($p=0.0000009$), therefore a non-parametric Spearman's Correlation was used to test for linear relationships between 3-PAH flux (Figure 49). A Spearman's Correlation Coefficient was calculated between each 3-PAH (R version 1.4.1717, packages: ggpubr). The only comparison that showed significant correlation was between BaP and DBA flux (ng/cm²/h) (Figure 49C), with a Spearman's correlation coefficient of 0.6623 ($p=0.0014$). The relationship between BaP and Pyr flux provided a Spearman's correlation coefficient of -0.1922 ($p=0.4022$). BaP flux correlation between Pyr + DBA flux ($R = 0.1174$, $p=0.4589$) and also correlation between Pyr and DBA flux was also not significant ($R=-0.1636$, $p = 0.4768$).

The differences in flux relationships suggest that PAH characteristics have an impact on the rate at which PAH are dermally absorbed. Pyr has the smallest molecular weight (MW= 202), and lowest K_{ow} (5.18) (Tobiszewski and Namiesnik, 2012, Stogiannidis and Laane, 2015)

(Section 2.2.1), and the highest overall flux values and shows no significant correlation to BaP flux (Figure 49). However, BaP ($k_{ow} = 6$, MW= 252 (Tobiszewski and Namiesnik, 2012, Stogiannidis and Laane, 2015)) and DBA ($k_{ow} = 6.5$, MW = 278) do show a significant correlation (Figure 49). Potentially owing to their higher lipophilicity and molecular weight, BaP and DBA behave similarly within the soil and during transit into and through the skin resulting in a significant correlation of fluxes. There are seven PAH classified by the IARC as carcinogenic, or probably carcinogenic (Lerda, 2011). Of these, two are 4-ring PAH (chrysene and benzo[a]anthracene) and five are 5 or 6-ring (benzo[a]pyrene, dibenz[a,h]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene). It would be beneficial to compare BaP flux to the other six carcinogenic HMW PAH as these are the main known risk drivers for human health risk assessment, to see if BaP is a suitable surrogate for PAH exposure through the dermal pathway. As no relationship was found between BaP and Pyr in this study, further study should test the relationship between BaP and other 4-ring PAH to determine if BaP is a suitable surrogate. Further study should also investigate whether a significant relationship and strong correlation between Pyr and other 4-ring PAH exist. Since BaP flux shows no relationship, Pyr could be a viable alternative marker for 4-ring PAH owing to its abundance in soils (Wilcke, 2000, Public Health England, 2017) and analytical stability.

It should be noted that LMW-PAH are semi-volatile and this could result in losses during the dermal bioavailability experiment resulting in poorer method recovery and therefore greater uncertainty than for the heavier 3-PAH measured in this study. In order to assess LMW recovery, a deuterated surrogate standard (different to ones used to assess extraction method recovery) could be applied to the soil at the beginning of the experiment, quantified in all three sample media, and concentration corrected dependant on total surrogate standard recovery from all three media. Alternatively, Tenax could be housed in the donor chamber above the soil/dosing vehicle (not in contact), which will absorb any volatile PAH that would otherwise be lost to atmosphere. These have been previously used to measure the firefighter personal exposure to volatile/semi-volatile PAH (Kirk and Logan, 2015).

Although BaP and DBA flux show significant correlation, it is evident from the experimental flux data that the rate of dermal absorption of PAH differs between different PAH and soils (Section 5.2), resulting in different PAH dermal intake rates (Table 50). Owing to the varying dermal absorption of each PAH from different soils, it is recommended that the dermal bioavailability of each potentially harmful PAH is quantified for DQRA. The Dutch Institute of Public Health and the Environment (RIVM) considered that it was not possible to set a level

for total PAH exposure, and that each PAH should be evaluated separately (RIVM, 2001). Further study is also required on PAH mixture toxicity as many mixture potencies are not fully understood, as many believe that PAH toxicity is additive driven, with current management assuming potency increases with concentration addition, which may not be the case (Billiard et al., 2007).

6.5 Conclusions

The objective to assess the implications of the soil-specific measurement of PAH dermal absorption to contaminated land management has been met. This was achieved using Equation 24 (Forsberg et al., 2021) to calculate soil-specific BaP ABS_d and inputting these values into the CLEA model daily dermal intake rate equation (Equation 25) (Environment Agency, 2009). The Forsberg et al. (2021) ABS_d equation (Equation 24) was applied using the parameters and results from Wester et al, producing the same ABS_d value of 0.13 (Table 47). This resulted in a mean reduction in predicted BaP daily intake (mg/day) of 97.5%, from 0.006 mg/day to 0.0001 mg/day, for the three soils tested when using the soil-specific ABS_d values in comparison to the CLEA default ABS_d of 0.13 (Table 50).

Furthermore, the soil-specific ABS_d values were used in the CLEA model to calculate SSAC and pathway exposure contribution (%) and compared to default outputs. Due to ABS_d representing a percent absorbed of the dose applied, lower loadings result in higher ABS_d for the same concentration of PAH dermally absorbed (Section 2.3.3) (Figure 47). The high experimental soil loading of 81.5 mg/cm² therefore needed to be adjusted so comparisons of soil-specific ABS_d to the CLEA default ABS_d could be made. At the same experimental loading of the Wester et al. (1990) study (40 mg/cm²) the calculated site-specific ABS_d were 14 to 43 times lower than the default CLEA value of 0.13 (generated from the Wester et al. (1990) study). Furthermore, when corrected for assumed monolayer coverage (5mg/cm²) the calculated site-specific ABS_d were 1.7 to 6.5 times lower than the CLEA default ABS_d of 0.13. These findings suggest that the current 0.13 ABS_d value over predicts dermal exposure to soil BaP.

The dermal pathway exposure contribution (%) increases as soil loading decreases (Figure 48), again, due to ABS_d being influenced by soil loading (mg/cm²). Furthermore, the three soils show different dermal exposure contribution (%) (Figure 48). Dermal contribution to exposure (%) and ABS_d increased in the order: Site A < Site B < BCR-524, which is the reverse

order of their respective BaP soil concentrations. These findings suggest that one value cannot be representative of BaP dermal exposure for all soils. The ABS_d , SSAC, dermal pathway exposure contribution (%) and predicted daily dermal intake values were calculated to demonstrate the potential impact of soil specific dermal bioavailability measurements on the risk-based land management decision process and are not intended to suggest regulatory concurrence at present. It is recommended that inter and intra lab method comparisons should be carried out, to further assess the developed *in vitro* method validity and reproducibility. In future, Inter-laboratory trials on the same test soils could be used to bring this method into regulatory concurrence, taking a similar approach to the unified BARGE method (Wragg et al., 2011) (ISO: 17924:2018(en)).

Measurement of sample-specific dermal flux values is a useful tool in calculating the dermal risk at PAH-impacted sites (Stroo et al., 2005a, Stroo et al., 2005b). The current default ABS_d value of 0.13 used in HHRA may have resulted in sites requiring clean-up when in fact the risk posed is much lower. This in turn may have delayed and diverted funding away from the decontamination of sites for which it is required (Alexander, 2000). Studies so far have assessed the effect on soil-specific bioavailability data on one or two pathways (Nathanail and Ogden, 2013, Haney Jr et al., 2020, Forsberg et al., 2021). It was demonstrated in this study that dermal exposure contribution and ingestion are two of largest contributors to exposure to BaP at lower dermal soil loadings (mg/cm^2) (Figure 48). Furthermore, for all dermal soil loadings, the ingestion and dermal pathways contributed between 90.7% and 94.3% of total BaP exposure. Dermal exposure to BaP varies between soils, therefore it is likely the same is true for the ingestion pathway. Therefore, soil-specific values for these two pathways could provide more accurate exposure values for the main contributors to total exposure, reducing risk uncertainty. Consequently, it would be beneficial for future study to assess the difference in human health risk assessment criteria between default CLEA model parameters and soil-specific PAH dermal, and ingestion bioavailability.

If PAH follow the same profile as that in the Culp et al. (1998) study, then it is standard practice to use BaP as a surrogate marker for HHRA and assume that the toxicity of the soil is governed by BaP. It was shown that BaP flux showed significant correlation with DBA, but not Pyr, suggesting that BaP may be a suitable surrogate to predict the dermal absorption 5 and 6-ring PAH. However, further investigation is required into this relationship. Further investigation should also assess which PAH would be a suitable surrogate marker for the dermal absorption of 4-ring PAH. Ideally, it is suggested that as individual PAH dermal absorption varies between soils, that each PAH of concern to health is assessed individually.

It should be noted that the developed method of this study would need to be adapted for the measurement of LMW PAH owing to their volatility. In order for method recovery to be assessed, a surrogate standard (different to standards used for extraction efficiency corrections) could be applied to the dosing vehicle and quantified in each sample media; or Tenax beads could be housed in the donor chamber above the dosing vehicle which will absorb any volatiles that would otherwise be lost to atmosphere.

7 Conclusions and Recommendations

7.1 Introduction

Prior to this study, radiolabelled PAH soil dermal absorption studies were used to generate PAH dermal bioavailability data for human health risk assessment (HHRA) (Section 2.3). Upon reviewing the literature (Section 2.3), it was determined that this approach produced likely overestimates of dermal bioavailability, owing to a lack of representation of the chemical aging process (Section 2.1.5). Furthermore, as only one soil PAH dermal bioavailability value is currently used for HHRA (13% PAH dermal bioavailability (Wester et al., 1990)) the current approach does not consider the effect of the range of soil properties encountered across the UK on PAH dermal bioavailability (Section 2.1.5). Additionally, the use of a variety of skin sources in previous studies contributed to variability in the generated dermal absorption data, as well as the fate of PAH not being determined throughout dermal absorption experimental systems, created uncertainty in HHRA (Sections 2.2 and 2.3).

Throughout this study, an *in vitro* method to measure the dermal absorption of 3-PAH from contaminated soils was developed (Chapter 4). Methods were established to quantify 3-PAH in all three compartments of the diffusion cell method, to allow for PAH mass balance assessment and greater certainty of the fate of PAH in the experimental system in comparison to previous studies (Section 2.3, Chapter 4). The method was validated by comparing solvent vehicle BaP flux values to published literature BaP flux values which were generated using *ex vivo* skin (Section 5.1). As flux produced by the developed method of this study was within the range of published values, it was determined that the developed method and the Strat-M™ membrane produced representative PAH dermal bioavailability data.

The method was used to quantify 3-PAH soil dermal bioavailability from three soils: two contaminated soils from former gasworks sites; and one CRM from a former creosote wood

treatment plant (Section 5.2). The results showed that the dermal absorption of native PAH from contaminated soils is significantly lower (a mean reduction in BaP ABS_d of 97.5%) in comparison to the 13% ABS_d value from the *in vivo* Wester et al. (1990) study (Section 6.4.3). Furthermore, BaP dermal absorption differed between soils, demonstrating the impact of soil properties on PAH mobility. The dermal absorption of the 3-PAH from soils also differed, in the order Pyr > BaP > DBA, suggesting that PAH characteristics also impact dermal absorption.

The following sections expand on these findings and determine how the aim and each objective (Sections 1.2 and 1.3) have been met for this research.

7.2 Development of an *in vitro* PAH Dermal Bioavailability Method

To develop the *in vitro* PAH dermal absorption method, procedures needed to be derived to dose, extract, and quantify Pyr, BaP and DBA (3-PAH) from the Strat-M™ membrane (Section 4.2), receptor solution (Section 4.3) and contaminated soil for quantification by GC-MS (Sections 3.4, 3.5, 3.6 and 4.4). This would allow for method performance to be evaluated and know the location of PAH in the experimental system throughout the duration of the experiment. My findings showed that accelerated solvent extraction (ASE) provided the highest 3-PAH recovery with the lowest variability, and therefore was the method to be used for quantifying PAH absorbed into the Strat-M™ membrane (Section 4.2).

A novel solid phase extraction (SPE) method was used to extract PAH from the receptor solution (Section 4.3). Three commonly used receptor solution recipes were compared for PAH recovery using the SPE method, and it was found that Hank's Balanced Salt Solution (HBSS) with 4% BSA produced the highest 3-PAH recovery with highest precision (Section 4.3). My findings demonstrated that the recovery and quantification of 3-PAH using this SPE method was as effective as previous studies that had used liquid scintillation counting (LSC) for the quantification of radiolabelled-PAH in receptor solutions (Section 4.3).

Established soil PAH extraction and quantification methods were used throughout this project (Sections 3.4, 3.5 and 3.6). However, methods for applying soil to and removing soil from the Strat-M™ membrane were still required. The soil loading method produced a consistent supermonolayer soil loading (RSD% = 8.7%), and the soil removal process was also consistent (RSD% = 0.05) with 99.8% of soil removed (Section 4.4). The effectiveness of the soil removal method in relation to previous work is unknown as there is no reported data in

the literature. As this is not mentioned in literature, it is likely that previous works have assumed that soil removal techniques are 100% effective, which is improbable. It is likely that some soil remaining on the skin contributes to the absorbed fraction when the skin/membrane is extracted and quantified, subsequently overestimating the PAH concentration absorbed. I propose that the effect of this was minimised throughout this research by applying equations (Equation 1) to correct for soil contributions to the PAH concentration of the membrane (Section 4.4.4).

Soils of this study were applied to the membrane at 40% w/w (Sections 4.4.2 and 5.2.2). It has been demonstrated in previous works that PAH soil bioavailability increases with increasing soil moisture (Section 2.1.3). Therefore, the moisture content of soils that undergo the developed dermal bioavailability method should be consistent (unless the intention is to study the impact of soil moisture on PAH dermal bioavailability. If soils are found to have a moisture content <40%, a similar approach to wetting could be applied as used in this study (Sections 4.4.2 and 5.2.2). If soils are found to have a moisture content >40%, different drying methods could be applied such as freeze drying (Section 5.2.2) or chemical drying (e.g., by mixing soils with NaSO₄).

7.3 PAH Dermal Bioavailability Method performance

The developed *in vitro* PAH dermal bioavailability method used the Strat-M™ membrane, and this study was the first to extract and quantify PAH absorbed into the Strat-M™ (Section 5.1.5 and 5.2.3.2). The flux of BaP through the Strat-M™ membrane from a solvent vehicle was compared to published *ex vivo* skin BaP flux values. The BaP flux through the Strat-M™ membrane was within the reported range of fluxes in the literature and showed less variability in comparison to absorption values quantified using *ex vivo* skin (Sections 5.1 and 5.1.4). Consequently, the findings of this research suggest that the Strat-M™ membrane is an adequate replacement for *ex vivo* skin for the *in vitro* measurement of the dermal absorption of PAH.

The measurement of 3-PAH dermal absorption from a solvent vehicle also showed that steady-state flux J_{ss} was reached at 24 h (Section 5.1.3 and 5.1.4). Furthermore, the data showed that the absorption of 3-PAH from a solvent vehicle is a rapid process, with all 3-PAH being quantifiable in the receptor solution within 0.25 h of the dose being applied to the membrane (Section 5.1.3 and 5.1.4). This was used to determine sampling time steps during the soil PAH dermal absorption experiment, with greater sampling density at the beginning of the experiment (Section 5.1.5).

The developed method was used to measure the dermal absorption of 3-PAH from real-world contaminated soils (Section 5.2), resulting in the completion of the aim of this research (Sections 1.2 and 1.3). Previous studies had focused on measuring the dermal absorption of radiolabelled PAH applied to soil in a solvent, underrepresenting the chemical aging process in soil, consequently overestimating dermal bioavailability. However, this study allowed for the quantification of the dermal absorption of native PAH from real-world contaminated soils over a period of 48 h (Section 5.2.3.2). PAH concentrations in the sample media of the soil PAH dermal bioavailability experiments were in the order of soil > membrane > receptor solution (Section 5.2.3.2), consistent with previous work (Sections 2.3.2 and 2.3.4), further validating the data generated by the developed method.

The method mass balance was in a similar range to previous *in vitro* methods, and produced better mass balance than some *in vivo* studies (Section 5.2.4.1). The mass balance for both contaminated soils (Site A and B) were lower than for the CRM (BCR-524), this was determined to be associated with the soil clean-up SPE method and did not result in adding uncertainty to the derived dermal bioavailability values (Section 5.2.4.1). There was a reduction in variability in absorption data compared to previous studies (Sections 5.1.4 and 5.2.4.1). I propose that the lower variability in absorption data in this study could be due to greater structural and chemical consistency of the Strat-M™ membrane in comparison to *ex vivo* skin used in previous works (Section 2.2.6).

The removal of soil from the Strat-M™ membrane was found to be effective at 99.8% soil removal (RSD% = 0.05) (Section 4.4). However, it was found that the soil mass remaining on the membrane could contribute only 3% to the PAH concentration when the membrane was extracted. Previous studies have not reported soil removal effectiveness or have shown that they considered the possible addition of PAH concentration from soil remaining on the membrane.

Soil loading provided supermonolayer coverage to allow for infinite dosing for comparison to previous work (Section 4.4). This enabled the BaP flux from this study to be calculated and compared to previous studies so that the comparison of percent absorbed of dose applied (PADA) (which is affected by soil loading) was not required during method validation. Although variable, this method produced lower variability in absorption values compared to previous *in vitro* PAH dermal bioavailability studies (Sections 5.1.4 and 5.2.4.1). There was an increase in variability of dermal bioavailability data when measuring absorption from soil

in comparison to a solvent vehicle. This is due to micro-spatial variation in PAH concentration in soils resulting in different PAH concentrations being in contact with the membrane surface and therefore available for absorption (Section 5.2.4).

7.4 Impact of the Developed *in vitro* PAH Dermal Bioavailability Method

Prior to this study, the same value of 13% (Wester et al., 1990) was used for assessing the dermal bioavailability of PAH from soils for HHRA (Section 2.5). Conversely, this study measured native PAH dermal bioavailability from real-world contaminated soils from former gas sites, and generated PAH dermal bioavailability (ABS_d) values 97.5% lower than the 13% value currently used for HHRA when assessing contaminated land (Section 5.2 and 6.4.3). This is due to native PAH in 'real-world' contaminated soils being more strongly bound to the soil matrix owing to the having undergone extended periods of aging and weathering (Section 2.1.5). SSAC were calculated using the soil-specific ABS_d values in the CLEA model. These SSAC were higher than those calculated using the CLEA default ABS_d value.

The calculation of ABS_d is impacted by soil loading (Sections 2.3.3, 4.4 and Chapter 6). Therefore, the soil-specific ABS_d and SSAC were adjusted to account for this effect. At the same experimental loading of the Wester et al. (1990) study (40 mg/cm^2), the calculated site-specific ABS_d (Site A = 0.0031, Site B = 0.0067, BCR-524 = 0.0097) were all lower than the CLEA default ABS_d of 0.13. These lower soil-specific ABS_d values result in higher BaP SSAC at this loading than when using the default values (Chapter 6). Furthermore, when the soil-specific ABS_d was corrected to an assumed monolayer coverage of 5 mg/cm^2 , the soil-specific ABS_d are still lower than the Wester et al. (1990) 0.13 ABS_d value (Site A = 0.0249, Site B = 0.0535 and BCR-524 = 0.0777) (Figure 47B). My findings imply that the current soil PAH dermal bioavailability value of 13% (Wester et al., 1990) overestimates the exposure to soil PAH via the dermal exposure pathway in current HHRA approaches.

Furthermore, there were differences in 3-PAH dermal absorption between the three soils tested. Site B soil had a lower BaP concentration than Site A soil (Section 5.2.2.2), however site B produced a higher ABS_d value than Site A (Section 6.3). This demonstrated further that a blanket ABS_d value of 13% is not appropriate, as higher soil concentrations does not necessarily mean greater soil PAH dermal bioavailability owing to the effects of soil properties, soil conditions, PAH characteristics and PAH source materials on PAH soil mobility (Section 5.2 and Chapter 6).

Measurement of soil-specific dermal flux values is a useful tool in calculating the dermal risk at PAH-impacted sites (Chapter 6). As BaP was the third most common determinant of

contaminated land between 2000 and 2013 for local authorities (Environment Agency, 2016), and the dermal pathway is a major contributor to overall exposure (Nathanail et al., 2009), the current representation of the dermal pathway may have resulted in sites requiring remediation when in fact the risk posed is much lower, diverting funding from sites that require remediation to be fit for intended use (Section 6.4). The Strat-M™ membranes (47mm diameter) are sold in packs of 60, with a pack costing around £1200 (price subject to change). It is likely that the Strat-M™ membrane is single use because the PAH extraction method for the membrane is destructive, as shown in Chapter 4.2 (Section 4.2.4.3) the lipids of the membrane are co-extracted with PAH. Therefore, the chemical composition of the membrane would be altered post-extraction, resulting in a different PAH-membrane interaction if the membrane was reused and subsequently different PAH absorption data. Furthermore, although the extraction method provided an excellent mean 3-PAH recovery of 97% (Section 4.2.3), there may be PAH remaining in the membrane which would skew absorption data if the membrane is reused. The cost of sample analysis in a commercial laboratory can vary depending on the sample matrix, but analysis typically costs between £80 and £250 per sample. There are three different sample media (soil, membrane and receptor solution) would require PAH quantification. If the same number of time steps were tested as in this study ($n = 8$), with three replications, this would lead to 72 sub-samples per soil which underwent dermal bioavailability testing. Including dermal bioavailability consumables such as receptor solution, the cost of quantifying the soil-specific dermal bioavailability of a soil could be in the region of £3000 - £7000 per soil depending on laboratory specific pricing and cost of consumables. Soil-specific PAH dermal bioavailability testing may increase costs during the site investigation phase of contaminated land management, but would save costs overall by preventing unnecessary remediation, helping the delivery of the UK government's 300,000 homes a year target for the rest of this parliament (CPRE, 2020).

The IPCS (2006) states that *in vitro* dermal absorption data can be used for HHRA providing that the concentration of the substance absorbed into the skin is considered along with the receptor solution concentration. Furthermore, the IPCS (2006) recommends using the maximum dermal flux value of a substance for human health risk assessment. To generate the soil-specific ABS_d values, J_{PEAK} was used resulting in a conservative approach to risk assessment, as this flux will reduce with time when the concentration gradient lessens as more PAH diffuse into and through the skin (Chapter 6). This study showed that the absorption of 3-PAH from soils is a rapid process, with the peak flux (J_{Peak}) occurring within

the first hour for each PAH from each soil (Section 5.2.4.3). This has implications to HHRA, as the maximum rate of human exposure to PAH in soil via the dermal pathway occurs a short time after initial exposure (Chapter 6)

Like previous radiolabelled PAH dermal absorption studies, this study found that PAH concentrations were in the order: Soil > membrane > receptor solution (Section 5.2.3.2 and 6.4.3). This suggests that of the absorbed PAH, a higher concentration is sequestered in the skin, with three potential outcomes: localised health effects, continued inward diffusion post soil removal (Forsberg et al., 2021), or removal from skin by sloughing (Section 6.4.3). Furthermore, the rate of 3-PAH flux into the RS from the membrane remained constant (once J_{ss} reached) throughout the 48h for all three soils tested (Figure 37, Figure 38, and Figure 39). This suggests that the 3-PAH at the skin-membrane interface was not depleted throughout the 48h exposure, and subsequently the 3-PAH soil to skin transfer was not rate limiting (Peckham et al., 2017). Additionally, this demonstrates that the membrane limits the amount of PAH that reached systemic circulation.

The use of the Strat-M™ artificial skin membrane resulted in lower variability in absorption values through the membrane in comparison to previous works that use *ex vivo* skin (Sections 5.1.4.2 and 5.2.4.3). Therefore, when using the Strat-M™ membrane, any differences in PAH dermal bioavailability between soils can be attributed to the different soil properties and conditions with greater certainty in comparison to *ex vivo* skin. Furthermore, the Strat-M™ is easier to store, use, and cheaper and more easily acquired than *ex vivo* skin. This allows for higher sample throughput during either dermal bioavailability experiments for scientific exploration or for generating soil-specific dermal bioavailability values for contaminated land HHRA. Additionally, the use of the Strat-M™ as an artificial *ex vivo* skin substitute contributes to the EU three Rs aim of reducing the use of animals in research (Williams, 2006).

If PAH follow the same profile as that in the Culp et al. (1998) study, then it is standard practice to use BaP as a surrogate marker for HHRA and assume that the toxicity of the soil is governed by BaP. It was shown that BaP flux showed significant correlation with DBA, but not Pyr, suggesting that BaP may be a suitable surrogate to predict the dermal absorption 5 and 6-ring PAH but not 4-ring PAH (Section 6.4.4).

At present, it is challenging to compare literature PAH dermal bioavailability values and methods owing to different experimental soils/vehicles used (Sections 2.3.2 and 2.7). This is down to previous works focussing on measuring the dermal absorption of radiolabelled

PAH and a lack of access to the same samples (Section 2.3.2). This study was the first to measure the dermal bioavailability of native PAH from a CRM (BCR-524) soil (Sections 5.2.2.2 and 5.2.3.3). Therefore, I propose that if future studies use the same CRM, this will allow for a direct comparison between dermal bioavailability studies in future. This will enable much improved future inter and intra lab comparisons of PAH dermal absorption methods and results.

7.5 Recommendations for Further Research

This study found that the Strat-M™ membrane generates similar BaP flux rates to *ex vivo* skin, suggesting that it may be a suitable substitute for chemical dermal absorption testing (Sections 5.1.4.2 and 5.2.4.3). However, the previous *ex vivo* and *in vivo* absorption data used to compare were carried out in different labs under different experimental conditions (Sections 2.3, 5.1.4.2 and 5.2.4.3). Therefore, further comparison of Strat-M to *ex vivo* and *in vivo* data is needed to strengthen the validation of the Strat-M™ as a suitable *ex vivo* skin substitute shown in this project. The ideal comparison would be to run a series of *in vitro* dermal absorption experiments using the same dosing vehicle and PAH concentration using Strat-M™ and *ex vivo* skin under the same experimental conditions. The dermal absorption experiments should form a part of the same study, to reduce inter-lab and operator variability.

Throughout this study, PAH absorption data suggested that the Strat-M™ membrane also produced less variable RS flux values at 24 h in comparison to previous works that have used *ex vivo* skin (Section 5.2.4.3). This is due to a more consistent structure and chemistry in comparison to *ex vivo* skin (Section 2.2.6). Membrane thickness is also a source of variation in dermal absorption values of previous works (Section 2.2.6) and thickness can vary depending on the anatomical and/or species origin of *ex vivo* skin (Section 2.2.5). The Strat-M™ membrane represents full-thickness, and owing to its structural consistency would reduce variability in absorption data owing to skin thickness. It is therefore recommended to use the Strat-M™ membrane for future work analysing the impact of soil properties and geochemistry on the dermal absorption of PAH, as this will enable the variability of PAH flux from soil to be attributed to changes in soil properties with greater certainty than when using *ex vivo* skin.

It was demonstrated in this study that different soils with different PAH source materials yield different rates of PAH dermal absorption (Section 5.2.4.3, Table 51).

Table 51: Summary of the industrial activity, likely PAH source material, calculated ABS_d and site-specific assessment criteria (SSAC) for each soil of this study. CLEA default values from the Wester *et al* (1990) study are included for comparison.

Soil	Industrial Activity	PAH Source Material	BaP ABS _d	BaP SSAC (mg/kg)
Soil A	Gasworks	Coal Tar	0.00153	4.31
Soil B	Gasworks	Coal Tar	0.00328	4.28
BCR-524	Wood Treatment	Creosote	0.00477	4.25
CLEA Default	No Data	Solvent spiking ^a	0.13	2.87

^aWester *et al* (1990) spiked the Yolo County 65-California-57-8 soil sample with radiolabelled BaP in acetone

Therefore, a wide variety of soil types need to be tested for their respective PAH dermal bioavailability. This will enable the determination of key soil factors that impact soil PAH bioavailability, subsequently allowing for the creation of a robust statistical model that can be used to predict PAH exposure via the dermal pathway under different scenarios. Further study needs to be carried out on the controls of PAH dermal bioavailability, such as: PAH characteristics, PAH interaction with soil properties, and PAH soil concentration. This will allow contaminated land risk assessors to have a better understanding of PAH behaviours in soil, soil PAH bioavailability and subsequent risk posed to human health via the dermal pathway (Section 5.2.5).

Although there are guidelines for the *in vitro* measurement of the dermal bioavailability of substances (e.g., OECD (2004)), there is a need for a standard operating procedure if a method is to be used for producing data for HHRA. It has been made clear to the author throughout this project via literary evidence or experimental observations that small alterations in experimental parameters, equipment and dosing mechanism have a significant impact on the dermal absorption rate of chemicals (Sections 2.1.5, 2.2.5, 2.2.6, 2.3.2, 2.3.3 and 2.6. Chapters 4, 5 and 6). Consistency in experimental approach will therefore provide uniformity in how dermal bioavailability values are derived, and therefore facilitate genuine comparisons in chemical dermal absorption data. This is important for the generation of soil-specific HHRA values, the approach needs to be homogeneous to minimise bias.

Furthermore, this project was the first PAH dermal bioavailability study to quantify the dermal absorption of native PAH from a CRM (Section 5.2). It is recommended that in future research, the same CRM also undergoes any dermal absorption experiments. The use of the

same CRM will improve inter and intra lab comparability of PAH dermal absorption data and methods, and quality control.

It has been shown in previous studies that soil-specific bioavailability values can differ from the standard bioavailability values used in the CLEA model for HHRA (Section 6.4.3). This study provided evidence that soil-specific PAH dermal bioavailability value result in a mean reduction in ABS_d values of 97.5% compared to the CLEA model default value (Sections 6.3 and 6.4.3). Furthermore, it was demonstrated that the exposure pathways with the largest BaP exposure contribution under a variety of dermal soil loadings were ingestion and dermal absorption. These two pathways accounted for between 90.7% and 94.3% of total BaP exposure (Chapter 6). The dermal exposure to BaP varies between the soils tested in this study (Section 5.2 and Chapter 6), consequently it is likely oral bioavailability also varies for the ingestion pathway. Therefore, it would be beneficial for future study to assess the difference in human health risk assessment criteria between default CLEA model parameters and values; and soil-specific PAH dermal and ingestion bioavailability.

The ABS_d and predicted daily dermal intake values (Chapter 6) were calculated to demonstrate the potential impact of soil specific dermal bioavailability measurements on the risk-based land management decision process and are not intended to suggest regulatory concurrence at present. It is recommended that inter and intra lab method comparisons should be carried out, to further assess the developed *in vitro* method validity and reproducibility. In future, Inter-laboratory trials on the same test soils could be used to bring this method into regulatory concurrence, taking a similar approach to the unified BARGE method (Wragg et al., 2011) (ISO: 17924:2018(en)).

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8 Appendix

The data from any of the experiments in this thesis can be provided in Excel format upon request by emailing the author (jalort@bgs.ac.uk or lgxjl3@nottinham.ac.uk).

8.1 Varian GC-MS/MS operating conditions

Name	Parent Ion	Product Ion		Confirmation Ion		Internal standard used	Figures of Merit			
	m/z	m/z	CID voltage (eV)	m/z	CID voltage (eV)		Retention time (minutes)	R ²	LoD (pg)	LoQ (pg)
fluoranthene-d10	212	212	30	208	40	fluoranthene-d10 fluoranthene-d10 benzo[a]pyrene-d12 or benzo[b]fluoranthene-d12 benzo[a]pyrene-d12 or benzo[b]fluoranthene-d12 benzo[a]pyrene-d12 or benzo[b]fluoranthene-d12 benzo[a]pyrene-d12 or benzo[b]fluoranthene-d12 indeno[1,2,3-cd]pyrene-d12 indeno[1,2,3-cd]pyrene-d12	30.378			
pyrene-d10	212	212	30	208	40		31.525	0.999	1.9	6.2
pyrene	202	202	30	200	40		31.618	1	1.6	5.3
benzo[b]fluoranthene-d12	264	264	35	260	40		40.907	0.999	3.4	11.4
benzo[a]pyrene-d12	264	264	35	260	40		42.417	0.999	4.7	15.6
benzo[a]pyrene	252	252	35	250	40		42.524	0.999	4.4	14.8
perylene-d12	264	264	35	260	40		42.836			
indeno[1,2,3-cd]pyrene-d12	288	288	35	284	40		46.36			
dibenz[a,h]anthracene	278	278	35	276	40		46.453	0.995	5.9	19.7
benzo[ghi]perylene	276	276	35	274	40		47.944	0.995	5.9	19.6

GC Operating Conditions	
Description	Parameter
Instrument	Varian CP-3800 gas chromatograph
Injector	1µL split-splitless injection, 280°C, splitless 0-0.7min, split ratio 1:20 thereafter
Carrier gas	Helium at 1mL/min
Column	Agilent, PAH Select, 30m length x 0.25mm i.d. x 0.15µm film thickness
Oven temperature programme	60°C(1 minute isothermal), ramp 6°C/min to 320°C (10 minutes isothermal)
Interface temperature	320°C
MS-MS Operating Conditions	
Description	Parameter
Instrument	Varian 1200L triple quadrupole mass spectrometer
Mode of operation	Electron impact (EI), selected reaction monitoring (SRM), pseudo MS-MS mode
Ionisation energy	70eV
Source temperature	250°C
Scan rate	0.5 scans / second
Detector voltage	1500 V
Collision cell gas	Argon. analyser pressure with no argon = 2.9E-7 Torr, analyser pressure with argon = 1.2E-6 Torr, Q3 pressure with no argon = 0.962 mTorr, Q3 pressure with argon = 1.14 mTorr.

8.2 ASE parameters for each sample group

Sample ID	Sample Type	ASE Conditions	Testing
T1	Analysis	100°C, DCM:Acetone (1:1), Heat up time = 5 minutes, 1500psi, Dispersant = NaSO ₄ , Flush = 50%, purge= 60 seconds	Temperature
T2	Analysis		
T3	Analysis		
T4	Blank		
T5	Analysis	50°C, DCM:Acetone (1:1), Heat up time = 5 minutes, 1500psi, Dispersant = NaSO ₄ , Flush = 50%, purge= 60 seconds	
T6	Analysis		
T7	Analysis		
T8	Blank		
T9	Analysis	75°C, DCM:Acetone (1:1), Heat up time = 5 minutes, 1500psi, Dispersant = NaSO ₄ , Flush = 50%, purge= 60 seconds	
T10	Analysis		
T11	Analysis		
T12	Blank		
S1	Analysis	100°C, Hexane:Acetone (1:1), Heat up time = 5 minutes, 1500psi, Dispersant = NaSO ₄ , Flush = 50%, purge= 60 seconds	Extraction solvent mixture
S2	Analysis		
S3	Analysis		
S4	Blank		
S5	Analysis	100°C, Acetone:Methanol (1:1), Heat up time = 5 minutes, 1500psi, Dispersant = NaSO ₄ , Flush = 50%, purge= 60 seconds	
S6	Analysis		
S7	Analysis		
S8	Blank		
M1	Analysis	100°C, DCM:Acetone (1:1), Heat up time = 5 minutes, 1500psi, Dispersant = Sand, Flush = 50%, purge= 60 seconds	ASE cell dispersant
M2	Analysis		
M3	Analysis		
M4	Blank		
M5	Analysis	100°C, DCM:Acetone (1:1), Heat up time = 5 minutes, 1500psi, Dispersant = Hydromatrix, Flush = 50%, purge= 60 seconds	
M6	Analysis		
M7	Analysis		
M8	Blank		

F1	Analysis	100°C, DCM:Acetone (1:1), Heat up time	ASE cell flush (%)
F2	Analysis	= 5 minutes, 1500psi, Dispersant =	
F3	Analysis	Hydromatrix,Flush = 70%, purge= 60	
F4	Blank	seconds	
F5	Analysis	100°C, DCM:Acetone (1:1), Heat up time	
F6	Analysis	= 5 minutes, 1500psi, Dispersant =	
F7	Analysis	Hydromatrix,Flush = 30%, purge= 60	
F8	Blank	seconds	