Chemical and bioanalytical characterisation of PAH-contaminated soils
Always remember…
You’re Braver
than you believe,
Stronger than you seem
& Smarter than you think.

-Christopher Robin
Chemical and bioanalytical characterisation of PAH-contaminated soils - identification, availability and mixture toxicity of AhR agonists
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**Abstract**


Contaminated soils are a worldwide problem. Polycyclic aromatic hydrocarbons (PAHs) are common contaminants in soil at former industrial areas, especially at old gasworks sites, gas stations and former wood impregnation facilities. Risk assessments of PAHs in contaminated soils are usually based on chemical analysis of a small number of individual PAHs, which only constitute a small part of the complex cocktail of hundreds of PAHs and other related polycyclic aromatic compounds (PACs) in the soils. Generally, the mixture composition of PAH-contaminated soils is rarely known and the mechanisms of toxicity and interactions between the pollutants are far from fully understood.

The main objective of this thesis was to characterize remediated PAH-contaminated soils by use of a chemical and bioanalytical approach. Bioassay specific relative potency (REP) values for 38 PAHs and related PACs were developed in the sensitive H4IIE-luc bioassay and used in mass-balance analysis of remediated PAH contaminated soils, to assess the contribution of chemically quantified compounds to the overall aryl hydrocarbon receptor (AhR)-mediated activity observed in the H4IIE-luc bioassay. Mixtures studies showed additive AhR-mediated effects of PACs, including PAHs, oxy-PAHs, methylated PAHs and azaarenes, in the bioassay, which supports the use of REP values in risk assessment. The results from the chemical and bioassay analysis showed that PAH-contaminated soils contained a large fraction of AhR activating compounds whose effect could not be explained by chemical analysis of the 16 priority PAHs. Further chemical identification and biological studies are necessary to determine whether these unknown substances pose a risk to human health or the environment. Results presented in this thesis are an important step in the development of AhR-based bioassay analysis and risk assessment of complex PAH-contaminated samples.

*Keywords*: Polycyclic aromatic compounds; Soil; Risk assessment; Mixture studies; AhR-mediated activity; REPs; GC/MS; H4IIE-luc bioassay.

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List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals.


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>Bio-TEQ</td>
<td>Bioassay derived TCDD-equivalents</td>
</tr>
<tr>
<td>CA</td>
<td>Concentration addition</td>
</tr>
<tr>
<td>Chem-TEQ</td>
<td>Chemically derived TEQ</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOC</td>
<td>Dissolved organic carbon</td>
</tr>
<tr>
<td>DRE</td>
<td>Dioxin responsive element</td>
</tr>
<tr>
<td>d.w.</td>
<td>Dry weight</td>
</tr>
<tr>
<td>EC</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact ionisation</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental protection agency</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>MIF</td>
<td>Max induction factor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>Oxy-PAHs</td>
<td>Oxygenated PAHs</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>PLE</td>
<td>Pressurised liquid extraction</td>
</tr>
<tr>
<td>Priority PAHs</td>
<td>16 US-EPA priority PAHs</td>
</tr>
<tr>
<td>REP</td>
<td>Relative potency factor</td>
</tr>
<tr>
<td>RRF</td>
<td>Relative response factor</td>
</tr>
<tr>
<td>RS</td>
<td>Recovery standard</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>SIR</td>
<td>Selected ion recording</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TEQ</td>
<td>TCDD equivalents</td>
</tr>
<tr>
<td>TMI</td>
<td>TCDD maximum induction</td>
</tr>
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1 Introduction

According to the European Environment Agency (EEA, 2012), potentially polluting activities have occurred at nearly three million sites in the EEA member countries, and more than 8% of the sites need to be remediated. It is a challenging job, both technically and economically to clean-up these historically contaminated sites to background concentrations or concentrations suitable to use. Comprehensive risk assessment and a reliable classification of the sites is also a difficult task.

Only in Sweden, over 80,000 contaminated sites have been identified (S-EPA, 2012). Remediation of the most contaminated sites is a necessary step in order to achieve a non-toxic environment, which is one of the 16 national environmental quality objectives in Sweden. So far 2,543 sites, estimated to pose a very large risk to human health and the environment, have been remediated (S-EPA, 2012).

Polycyclic aromatic hydrocarbons (PAHs) are common contaminants in industrial areas, especially at old gasworks sites, gas stations and former wood impregnation facilities (Lundstedt et al., 2003; Nestler, 1974). Many PAHs are toxic and exposure can result in mutagenesis and carcinogenesis in humans and animals (Balch et al., 1995; Spink et al., 2008). Because of their toxicity, PAH-contaminated sites are highly prioritised for remediation.

Risk assessments of PAHs are complicated since these compounds mostly occur in the environment as complex mixtures of hundreds of PAHs and related compounds such as oxygenated PAHs (oxy-PAHs), azaarenes among others. Because of the similar source of origin or formation from parent PAHs during chemical or biological processes; alkylated-, oxygenated PAHs or heterocyclic compounds containing nitrogen are co-contaminants in the environment. Chemical compositions and concentrations in the soil are related to the contamination history, the availability and the degradability of the compounds. Generally, the mixture composition of PAH-contaminated soils is rarely known and the mechanisms of toxicity and interactions between the pollutants are far from fully understood.

There is a growing concern about polar polycyclic aromatic compounds (PACs) like oxy-PAHs and azaarenes. These compounds have been shown to be mutagenic and are more water soluble and thereby more mobile in the environment than their parent PAHs (Bleeker et al., 2002; Lemieux et al., 2008).

Today, generic guideline values for PAHs in contaminated soils are usually based on chemical analysis of the 16 priority PAHs listed by U.S. Envi-
The availability of the pollutants is an important factor in risk assessment of remediated soils. The availability influences to what extent organisms living in the soils are exposed and affects the potential transfer to ground or surface water and eventually the transfer to humans. Although it is well known that only a fraction of the total concentrations of contaminants may be available, most risk assessments are based upon measurements of the total concentrations of the contaminants in the soil (Alexander, 2000).

Many PAHs bind to and activate the aryl hydrocarbon (AhR) pathway, thus AhR-based bioassays have been used to screen PAH-contaminated samples (Denison et al., 2002). Mechanism specific bioassays are good complement to chemical analysis since they give an integrated response based on the overall mechanism specific effect of all chemicals present in a sample (Behnisch et al., 2001). Chemical- and bioanalytical studies of PAH-contaminated soils have shown that PAHs quantified by chemical analysis only can explain a small portion of the AhR-mediated activities observed in the soils (Andersson et al., 2009). Unexplained AhR-mediated activities in PAH-contaminated soils suggest mixture interactions and/or additional AhR agonists.

In the work underlying this thesis, analysis of PAH-contaminated soils with focus on chemical composition, availability, and mixture toxicity has been performed by use of both chemical- and bioassay analysis. The AhR-based H4IIE-luc bioassay has been used to estimate the AhR agonistic potencies of individual PAHs, including azaarenes, oxy-PAHs or methylated PAHs, and the combined effect of the compounds in artificial mixtures. Moreover, the AhR-mediated total toxic potential of PAH-contaminated soil samples has been studied. Bioassay derived data has been compared with chemical data to evaluate the risk of missing potentially toxic chemicals not targeted by the chemical analysis, and to identify possible AhR agonists.

The underlying hypothesis for this thesis is that remediated soils can still contain large numbers of PAHs and related compounds with significant toxic effects, which may pose a risk to the human health and the environment.
1.1 **Aim of this thesis**

The overall aim of this thesis is to refine and use an analytical methodology including both chemical and bioassay analysis to characterise remediated PAH-contaminated soils. Specific aims are:

- Develop H4IIE-luc assay specific relative potency factors (REPs) for PACs that can be used in mass balance analysis of PAH-contaminated samples.

- Study additive AhR-mediated effects of PACs in artificial mixtures by use of the H4IIE-luc bioassay. An additional aim is to investigate if the matrix, i.e. soil, or presence of non-AhR active PACs in a PAC mixture, affected the effect of PAC mixtures.

- Analysis of remediated PAH-contaminated soils to evaluate if the AhR-mediated toxic potential in remediated soils is reduced in proportion to the reduction in concentration of the 16 priority PAHs, by use of mass balance analysis. A secondary aim is to study the availability of PAHs and AhR agonists in the soils by use of different chemical extraction methods.
2 Polycyclic aromatic compounds

2.1 Sources
Polycyclic aromatic hydrocarbons (PAHs) are a group of widespread organic contaminants that are found in elevated levels in the environment, mainly as a consequence of human activities. PAHs are formed as a result of pyrolytic processes, especially from incomplete combustion of organic material during industrial processes, such as, wood treatment, combustion of fossil fuels and wood, coke production, coal tar production, metal smelting and asphalt production, and natural processes like forest fires and volcanic eruptions. They also exist naturally in crude oil and coal (Achten and Hofmann, 2009; Brandt et al., 2002; Nestler, 1974). PAHs are composed of two or more fused benzene rings, arranged in linear, angular or clustered formations (Figure 1).

![Diagrams of PAH structures](image)

Figure 1. Structures of PAHs studied in this thesis. *indicates the 16 US-EPA priority PAHs.
Heterocyclic compounds containing nitrogen, sulphur or oxygen atoms, alkyl-substituted PAHs or oxygenated PAHs (oxy-PAHs) are often found together with PAHs in the environment (Brorström-Lundén E et al., 2008). Like PAHs they are formed during incomplete combustion of organic matter, or produced from chemical reactions of parent PAHs in the atmosphere or metabolic reactions in organisms (Lundstedt et al., 2007). The whole group of PAHs and related compounds are collectively referred to as polycyclic aromatic compounds (PACs). Oxy-PAHs, methylated PAHs and azaarenes studied in this thesis are presented in figure 2.

Figure 2. Structures of substituted PAHs; oxy-PAHs, methylated PAHs and azaarenes studied in this thesis.

2.2 Physicochemical properties
Physicochemical properties differ between individual PAHs. Generally, the lipophilicity (log Kow) and stability of the compounds increases with the number of aromatic rings in the PAH-molecule (Table 1). Transport and distribution of PAHs in the environment are mainly governed by their chemical and physical properties. Low molecular weight PAHs, that is, 2- or 3-ringed PAHs, are more soluble in water than heavier PAHs and are distributed in soil and groundwater more readily. They may occur in the
atmosphere mainly as vapours due to greater values of their Henry’s law constants. Consequently, the low molecular weight PAHs are more susceptible to degradation processes in the environment, such as microbial degradation, chemical oxidation and degradation by ultraviolet light than high molecular weight PAHs (Wild and Jones, 1995).

Table 1: Selected properties of 16 US-EPA priority PAHs (ATSDR, 1995). Swedish definition of low (L), intermediate (M) and high (H) molecular weight PAHs are also presented.

<table>
<thead>
<tr>
<th>PAH-L</th>
<th>Number of rings</th>
<th>Molecular weight</th>
<th>Henry’s law const. (Atm×m³/mol)</th>
<th>Aqueous solubility (mg/l)</th>
<th>Log Kow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>2</td>
<td>128</td>
<td>4.83×10⁻⁴</td>
<td>31</td>
<td>3.36</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>3</td>
<td>152</td>
<td>1.45×10⁻³</td>
<td>3.93</td>
<td>4.07</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>3</td>
<td>154</td>
<td>7.91×10⁻⁵</td>
<td>1.93</td>
<td>3.98</td>
</tr>
<tr>
<td>PAH-M*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorene</td>
<td>3</td>
<td>166</td>
<td>1.0×10⁻⁴</td>
<td>1.98</td>
<td>4.18</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3</td>
<td>178</td>
<td>2.56×10⁻⁵</td>
<td>1.20</td>
<td>4.45</td>
</tr>
<tr>
<td>Anthracene</td>
<td>3</td>
<td>178</td>
<td>1.77×10⁻⁵</td>
<td>0.076</td>
<td>4.45</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>4</td>
<td>202</td>
<td>6.5×10⁻⁶</td>
<td>0.26</td>
<td>4.9</td>
</tr>
<tr>
<td>Pyrene</td>
<td>4</td>
<td>202</td>
<td>1.14×10⁻⁵</td>
<td>0.077</td>
<td>4.88</td>
</tr>
<tr>
<td>PAH-H*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>4</td>
<td>228</td>
<td>1×10⁻⁶</td>
<td>0.010</td>
<td>5.61</td>
</tr>
<tr>
<td>Chrysene</td>
<td>4</td>
<td>228</td>
<td>1.05×10⁻⁶</td>
<td>2.8×10⁻³</td>
<td>5.16</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>5</td>
<td>252</td>
<td>3.87×10⁻⁵</td>
<td>7.6×10⁻⁴</td>
<td>6.06</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>5</td>
<td>252</td>
<td>1.22×10⁻⁵</td>
<td>0.0012</td>
<td>6.04</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>5</td>
<td>252</td>
<td>4.9×10⁻⁷</td>
<td>2.3×10⁻³</td>
<td>6.06</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>6</td>
<td>278</td>
<td>7.3×10⁻⁸</td>
<td>5×10⁻⁴</td>
<td>6.84</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>6</td>
<td>276</td>
<td>6.95×10⁻⁸</td>
<td>0.062</td>
<td>6.58</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>6</td>
<td>278</td>
<td>1.44×10⁻⁷</td>
<td>2.6×10⁻⁴</td>
<td>6.50</td>
</tr>
</tbody>
</table>

*PAH-M and PAH-H are carcinogenic according to the Swedish EPA.

High molecular weight PAHs (4 or more rings) are less water soluble, are highly lipophilic and exist mainly adsorbed to particles in environmental compartments, as air, water and soil. They are therefore less available for degradation processes and can be transported over long distances in the atmosphere. Oxy-PAHs have greater polarity compared to parent PAHs.
and are consequently more water soluble and less volatile. The lower volatility of these compounds leads to a higher tendency to adsorb to particles in the atmosphere. The distribution between gas and particle phase is very important for the distribution of PACs and their effects in the environment (Brorström-Lundén E et al., 2008; Wild and Jones, 1995).

2.3 Environmental fate and behaviour of PAHs in soil
Polycyclic aromatic hydrocarbons are found in all surface soils due to atmospheric deposition or urban runoff (Brorström-Lundén E et al., 2008; Johnsen et al., 2005; Wilcke and Amelung, 2000). Soil is a major sink for PAHs and other hydrophobic compounds. Concentrations of PAHs are generally greater in urban areas compared to background areas. Background concentrations of 16 US-EPA priority PAHs (PAH16) between 0.7 to 3.1 mg/kg have been detected in forest soils and between 0.1 to 0.7 mg/kg in arable soils (Šídlová et al., 2009). In urban soils, concentrations between 0.4 to 28 mg/kg (PAH16) have been detected with greatest concentrations observed in soil samples from roadsides from heavily trafficked roads (Jiang et al., 2009; S-EPA, 2008; Šídlová et al., 2009; Tang et al., 2005). Generally greater concentrations are found near industrial sources such as old coal coking and gas work sites (Eriksson et al., 2000; S-EPA, 2008; Wilcke et al., 1995). Concentrations of 300 mg/kg soil (PAH16) have been detected at an old Swedish gas work site and concentrations of 11 PAHs ranging between 10 to 32,000 mg/kg were detected at an old creosote production site (Ellis et al., 1991; Eriksson et al., 2000). In a screening study by Brorström-Lundén et al. (2008) PAHs and related compounds, such as azaarenes and oxy-PAHs were analysed; contaminants were frequently found in background and urban soils, with elevated concentrations in urban soils compared to background soils.

The composition of PAHs and related compounds in the environment can often be related to the source of contamination. In general, pyrogenic sources like wood-burning or vehicular emission are dominated by 3-, 4- and 5-ringed PAHs with generally lower concentrations of 2-ringed PAHs and methylated PAHs in contrast to petrogenic sources, including crude oil and refined products, where 2- and 3-ringed PAHs are dominating with abundance of alkylated PAHs (Jiang et al., 2009; O’Malley et al., 1996; Zakaria et al., 2002). Moreover the composition is depending on the age of contamination and the availability of the contaminants (Alexander, 2000).
2.3.1 Contaminated sites

In 1990, the Swedish EPA performed a nation-wide inventory of industrial branches for the purpose of identifying the sites in Sweden most urgently in need of remediation. To date over 80,000 contaminated sites have been identified in Sweden, and 2,543 of the most contaminated sites have been remediated. Mixed pollution situations with presence of both organic and inorganic pollutants simultaneously may occur at several sites, which complicate the risk assessment and remediation of these sites. Due to the toxicity of PAHs, sites contaminated with PAHs are highly prioritised for remediation. Soils from former gasworks sites, wood preservation sites and coke production facilities, have been shown to contain complex mixtures of hundreds of compounds (Bergknut et al., 2006). Figure 3 shows the distribution of main contaminants in contaminated soils in Sweden.

![Figure 3](image)

Figure 3. Distribution of main contaminants in contaminated soils in Sweden based on data from 2006. Adopted from EEA.

2.3.1.1 Regulations

Many PAHs are listed as priority substances by the European Commission (Regulation EC No166/2006) and concentrations of PAHs in environmental media, including soil, are regulated in most countries. In risk assessment of contaminated sites in Sweden generic guideline values are available for two different types of land use; sensitive land use (KM) and less sensitive land use (MKM). For PAH-contaminated sites guideline values have been derived for three groups of PAHs, defined as PAH-L, PAH-M and PAH-H
Guideline values for less sensitive land use are; sum of PAH-L < 15 mg/kg, sum of PAH-M < 20 mg/kg and sum PAH-H < 10 mg/kg (S-EPA, 2009). PAHs in group PAH-M and PAH-H are classified as carcinogenic by Swedish EPA. Guideline values are compared with measured concentrations on site, in order to assess the risk and the extent of remediation to be carried out. In special cases site specific guideline values can be determined, which take into account actual conditions at site. The guideline values can be used as threshold values in remediation of soils, however, in Sweden many soils are treated sufficiently and used in land filling.

Measured concentrations of a small number of PAHs, generally the EPA-priority PAHs, as a basis for classification and risk assessment of soils is an approach used in Sweden among other countries, for example, Canada, USA and the Netherlands (CCME, 2010; RIVM, 2012; US-EPA, 1996; US-EPA, 2007).

2.3.1.2 Soil remediation techniques

Many different remediation techniques have been developed as many countries have recognised the risk and problems associated with contaminated areas.

In the case of PAH-contaminated soils, bioremediation is a commonly used technique. Many commercial bioremediation methods exist, and they all involve the use of microorganisms stimulated to degrade the organic contaminants of concern, ideally to carbon dioxide and water. Nutrients, texture, moisture, oxygen and other additives can be added to enhance the biological degradation processes in the soil. To improve the water solubility of the contaminants and thereby the degradability, addition of surfactants can be done, which enhance the dissolution and desorption of the contaminants (Atagana et al., 2003; Bamforth and Singleton, 2005). In Sweden most remediation are performed ex situ, where the soil is excavated and treated elsewhere. Landfarming and composting are examples of above ground bioremediation approaches. Biological remediation has been shown to be effective for reducing concentrations of 2- and 3-ringed PAHs, however, more hydrophobic PAHs was less degraded (Ellis et al., 1991; Haritash and Kaushik, 2009).

Another remediation technique that is used in treatment of PAH-contaminated soils is soil washing. Soil washing is performed in a closed system and involves high-energy contact between the contaminated soil and an aqueous washing solution. During the soil washing process the soil masses are sorted in different fractions and the contaminants concentrated into the fine fraction. Addition of chemicals or surfactants enhances the aqueous solubility of the hydrophobic compounds and improves the re-
moval of the compounds (Chu and Kwan, 2003; Elgh-Dalgren et al., 2009; Yuan and Marshall, 2007). Separation techniques such as soil washing only separate the contaminants from the soil, no degradation takes place. Further treatment of the aqueous solution is necessary as well as disposal of the residual soil.

2.3.2 Availability

Availability is an important factor in risk assessment of contaminated soils. The availability of the contaminants affects the potential transfer of compounds to ground or surface water and the amount that organisms living in the soil are exposed to. Although it is well-known that only a fraction of the total content of contaminants may be available, most risk assessments are based upon total concentrations of contaminants in soils. Bioavailability of contaminants refers to the fraction that can be taken up by organisms. How much of a contaminant that is bioavailable depends on the distribution of the contaminant between different media, for example, between pore water and soil. The distribution depends on the characteristics of the soil and the contaminants (Alexander et al., 2002; Chung and Alexander, 2002; Totsche et al., 2006).

Low molecular weight PAHs are more readily degraded or leached out from soil while high molecular weight PAHs become strongly sorbed to organic matter in the soil, due to their lipophilic properties. The availability and degradability of PAHs commonly decreases with time, a phenomenon referred to as aging (Alexander, 2000; Chung and Alexander, 1998). Even though the concentrations of PAHs in ‘old’ contaminated soils still are relatively high, the risk of the PAHs may be reduced due to reduced availability of the compounds.

In case of polar PACs, they are likely more mobile in soil than parent PAHs due to their higher water solubility as indicated by their lower log $K_{ow}$ values (Blekker et al., 2002; Lundstedt et al., 2007). It has been shown in column leaching tests that oxy-PAHs are more mobile than parent PAHs. However, the mobility of oxy-PAHs studied in field experiments were only marginally higher compared to that of low molecular weight parent PAHs, which was suggested to be due to more complex interactions of oxy-PAHs than of PAHs with soil (Lundstedt et al., 2007; Musa Bandowe et al., 2010).

The fraction of a contaminant available for uptake by organisms is depending on the organism and the route of exposure. Humans and animals can be exposed to PAHs via oral intake (water, food, soil), skin contact, and via inhalation of air (dust, vapour). Most living organisms can transform PAHs and the degradation products formed may often be more toxic.
than the original compounds (Ramesh et al., 2004). Microorganisms, such as bacteria, may transform PAHs into carbon dioxide and water (mineralisation). However, oxy-PAHs are also often formed. Some oxy-PAHs have been reported to be even more toxic than the analogous PAHs (Haritash and Kaushik, 2009; Lundstedt et al., 2007). Humans predominantly transform PAHs into more polar products that will be readily excreted from the body but still a variety of reactive metabolites may be formed that can cause toxic effects (Shimada, 2006).

2.3.2.1 Bioavailability tests
A method for estimating bioavailability is to study the uptake of contaminants in earthworms. Earthworms are appropriate model organisms for bioavailability since they process large amounts of soil, have a thin permeable cuticle and play a major role in the transport of pollutants from the soil to organisms higher up the food chain. However, the use of earthworms in bioavailability studies is quite laborious and time-consuming and the worms do not survive exposure to certain substances or concentrations (Sun and Li, 2005). Consequently, several chemical and physical methods have been developed to estimate the bioavailable fraction of organic contaminants in soil (Bergknut et al., 2004; Bergknut et al., 2007; Cuypers et al., 2002; Liste and Alexander, 2002). Since there are many factors controlling the bioavailability, development of such methods is complicated and all compounds extracted by use of these methods may not be bioavailable or elicit biological effects. Moreover, it is problematic when using reference organisms to compare chemically and biologically derived data, for example, connecting the uptake of contaminants by the worms with the amount extracted by use of solvent extraction. The contaminant profiles in the test organisms and solvent extracts can be compared, but provide really no information about the available concentrations in the soils.

2.3.2.2 Leaching tests
Leaching tests estimate the proportion of contaminants in the soils, which are available for transport to the surrounding environment and groundwater and thereby also the portion available for uptake in plants and organisms. Development of leaching methods for organic substances in soil has been performed during the years (Bjuggren et al., 1999; Comans et al., 2001; Fortkamp et al., 2002) Today, criteria for leaching of inorganic substances from soil exist, and the physical and chemical properties of inorganic substances differ significantly from organic substances. In leaching of organic substances other parameters need to be considered, as the content
of dissolved organic carbon, the risk of adsorption to equipment and degradation of substances during the leaching.

Leaching tests are of importance for both economic and environmental reasons. As a complement to traditional total analysis of chemical concentrations, leaching test will provide a comprehensive basis for risk assessment. Leaching of PAHs from contaminated soils has shown low availability of the contaminants. Less than one percent of the initial amount of PAHs was leachable during experiments (Enell et al., 2004; Fortkamp et al., 2002). Use of leaching tests may lead to reduced costs due to less extensive remediation actions.

### 2.4 Toxicity

Polycyclic aromatic hydrocarbons have shown a wide range of toxicological effects, such as acute toxicity, developmental and reproductive toxicity, but the primary focus has been on their mutagenic and carcinogenic capacity. Even though PAHs are widespread, quite persistent pollutants that have been detected in various media, including air, water, food, soil, sediment, tissues of animals or humans, they are not classified as persistent organic pollutants (POPs) (Chen et al., 2004; Layshock et al., 2010; Söderström et al., 2005). In contrast to POPs, PAHs are readily metabolised in humans and most animals and consequently less bioaccumulative (Ramesh et al., 2004). The metabolic transformation of PAHs results in polar products and the increased water solubility facilitates their subsequent excretion from the body. Metabolism may also result in reactive metabolites that can form covalent adducts with DNA. The formation of reactive metabolites, like epoxides and dihydrodiols, which can bind to cellular proteins or DNA and cause cell mutations, is the underlying cause for the toxic effects of certain PAHs (Ramesh et al., 2004).

Another important pathway concerning toxic effects of PAHs is the cytosolic aryl hydrocarbon receptor (AhR) signal transduction pathway. Halogenated aromatic hydrocarbons, such as polychlorinated dibenzo-p-dioxins and furans, polychlorinated biphenyls (PCB) together with a number of high molecular weight PAHs can bind to and activate the AhR, which starts the production of a battery of proteins, including the cytochrome P4501A (CYP1A) (Denison and Heath-Pagliuso, 1998; Marlowe and Puga, 2005). Induced proteins can alter cellular homeostasis, which may lead to toxic effects. Moreover, AhR-mediated induction of CYP1 enzymes can lead to genotoxicity, mutation, and tumour initiation due to metabolic activation of numerous PAHs (Bosetti et al., 2007; Nebert et al., 2000; Trombino et al., 2000). Carcinogenicity of PAHs in humans have primarily been indicated from occupational studies of workers who were
exposed to mixtures containing PAHs as a consequence of their involvement in processes as coke production, roofing, oil refining, or coal gasification (Bosetti et al., 2007).

Researchers disagree regarding the relationship between the AhR activation potency of PAHs and their ability to cause cancer. Some studies suggest that the relationship between affinity for the AhR and carcinogenic potency is unclear. For example, PAHs that strongly activate the AhR, such as benzo[k]fluoranthene have shown to be only weakly carcinogenic in animal studies (Bostrom et al., 2002; Machala et al., 2001b). Other studies have shown good correlations between AhR inducing capacity and carcinogenic potency (Sjogren et al., 1996; Trombino et al., 2000).

A relative potency factor approach for PAH mixtures to assess cancer risk from exposure to PAH mixtures is under development by US-EPA. Benzo[a]pyrene will be used as a reference chemical. Like the WHO-TEF system for dioxins and PCBs, this approach will be based on multiple studies, including in vitro assays, in vivo assays and occupational studies (US-EPA, 2010).

In contrast to the well monitored 16 priority PAHs, little information is available regarding toxicity of polar PACs. However, studies have shown that oxy-PAHs are acutely toxic and mutagenic (Lundstedt et al., 2007).

### 2.4.1 Bioassay monitoring and mixture effect studies

Bioassays are practical techniques to obtain estimates of the total toxic potential and risk of mixtures or single compounds. Mechanism specific bioassays are good screening tools for contaminants in different media because they enable an estimation of the total toxic potential of all compounds present in the sample with the same mechanism of action (Behnisch et al., 2001; Engwall and Hjelm, 2000; Machala et al., 2001a). Many PAHs are believed to elicit their toxicity via the AhR pathway, thus AhR-based bioassays, like the H4IIE-luc bioassay or the EROD assay, can be used in analysis of PAH-contaminated samples (Denison et al., 2002; Machala et al., 2001b).

H4IIE-luc bioassay studies of soils have reported bio-TEQs values of arable soils ranging between 96 to 478 pg/g soil. Greater bio-TEQ values have been observed in forest soils with values between 483 to 2095 pg/g soil. Analysis of traffic-affected soils has shown bio-TEQs between 225 to 27,700 pg/g soil (Šídlová et al., 2009). The greatest levels in the urban soils were almost as high as the bio-TEQ concentrations (50,000 pg/g) observed in soil samples from an old gas plant site (Andersson et al., 2009).

The advantage of mechanism-specific bioassays is that they measure the overall effect of all chemicals in a sample that act via the same mechanism,
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Characterisation of PAH-contaminated soils

which make bioassays excellent screening tools of contaminated environments. There is an inherent problem when using bioassays, namely the lack of absolute limit toxicity values for safe levels. Establishment of limit values is normally done for individual compounds, not on mixtures, and it is based on the toxicological risks of individual compounds. Bio-TEQ values in remote soils, like arable soils may be useable as safe levels for baseline toxicity in soils, since there is a noticeable difference between bio-TEQ values observed in arable soils compared to bio-TEQ values observed in highly contaminated urban soils.

2.4.1.1 H4IIE-luc bioassay

H4IIE-luc bioassay is a rat hepatoma cell line stably transfected with a luciferase reporter gene from the firefly, *Photinus pyralis* (Murk et al., 1996). The bioassay is mechanism-specific and detects all compounds that can bind to and activate the AhR. The AhR signal transduction pathway is illustrated in figure 4. The quantity of produced luciferase is an integrated result of the AhR ligands affinity to bind and activate the receptor and their concentration.

![Figure 4. The AhR signal transduction pathway in wild and recombinant cells (modified from Behnisch et al., 2001).](image-url)
2.4.2 Mixture toxicity

Both humans and organisms living in the environment are continuously exposed to complex mixtures of anthropogenic chemicals. However, for most mixtures, toxicity data is only available for a subset of all compounds present. Different approaches have been developed to assess the toxicity of mixtures, by use of toxic potencies of single compounds (Kortenkamp et al., 2009).

A commonly used concept is concentration-addition (CA) (Arrhenius et al., 2004; Fent and Bätscher, 2000). The CA concept assumes similar mechanisms of action and additive effects of mixture components. The relative potency factor (REP) approach is an application of the CA concept. Concentrations of mixture components are scaled relative to the concentration and toxic potency of a reference compound, and then summed up to give the total toxicity equivalent (TEQ) of a mixture (Machala et al., 2001b; Villeneuve et al., 2000). The REP approach is useful in studies combining instrumental chemical analysis and biological analysis (Hilscherova et al., 2000). Another application of the CA concept is the CA model (Altenburger et al., 2000; Berenbaum, 1985). Unlike the REP-concept, concentration-response curves of individual compounds do not have to be parallel. In the CA model, one chemical is supposed to behave as a dilution of the other, meaning that any compound can be substituted by an equally potent concentration of another compound without altering the overall effect. For example, 0.5 x EC50 of compound A can be replaced by 0.5 x EC50 of compound B in a mixture causing 50 % total effect. The CA model has shown to be an accurate reference model of mixtures of chemicals known to have similar modes of action (Faust et al., 2001; Zhang et al., 2008).

An alternative method is independent action (IA), also known as response addition (Bliss, 1939). IA is based on the assumption that the mixture components cause a common integrated effect through different mechanisms of action (Altenburger et al., 2000). Based on concentration-response curves of single compounds, the toxicities of mixtures can be predicted by the CA or IA model and compared with observed toxicities in the known mixtures.

Since the CA and IA models are based on additive behaviour of the compounds in the mixture, the models have been used to study combined effects of compounds in mixtures. Equitoxic mixtures have been widely used; generally the compounds are mixed in an equivalent-effect concentration, for example, an effective concentration for 50% (EC50) (Altenburger et al., 2000). The advantage of such mixtures is that no discrimination of low-potency compounds occurs since all compounds are expected to con-
Disagreement in predicted and observed toxicity data suggests non additive interactions, i.e. synergistic or antagonistic behaviour of the compounds in the mixture (Figure 5).

It has been shown that the predictive power of the models usually increases with numbers of compounds in the mixtures. The possible explanation is that both synergistic and antagonistic interactions might occur in multicomponent mixtures and thus cancelling each other out (Kortenkamp et al., 2009; Warne and Hawker, 1995).

Both concepts (CA and IA) are limited to mixtures of known chemical composition. Environmental samples contain complex mixtures of compounds with unknown identities and concentrations. However, both concepts can play a vital role when used in combination with advanced chemical-analytical techniques in order to identify important novel pollutants, and in risk assessment of environmental mixtures.
3 METHODOLOGY

Since both chemical and bioassay analysis are used in the characterisation of PAH-contaminated soils in this thesis, much focus have been on accurate comparisons of chemical and bioassay analysis. The strategy was to first develop H4IIE-luc bioassay specific relative potency factors that could be used in mass-balance analysis.

Earlier studies have reported relative potencies for a number of PAHs and related compounds (Sovadinová et al., 2006; Till et al., 1999; Villeneuve et al., 2002; Ziccardi et al., 2002), but they differ in methods, cell lines and time of exposure. REPs are species, assay and method specific and the use of nonspecific REPs can result in misleading mass-balance analysis. Since the REP-concept is based on additive behaviour of the compounds, combined interactions were studied in different mixtures of the compounds.

Use of mass-balance analysis, i.e. comparison of bioassay derived TEQs with chemical TEQs based on REPs and measured concentrations, gives an estimation of the contribution of instrumentally quantified compounds to the observed response in the bioassay.

Since the chemically identified PAHs only accounted for a fraction of the high AhR-mediated activities found in the remediated soils in paper II it was important to test and find possible explanations for the observed activities, both by testing additional compounds in the bioassay and to identify possible agonists in PAH-contaminated soils by chemical analysis.

3.1 H4IIE-luc bioassay analysis

3.1.1 Individual PACs

AhR-mediated potencies of 38 PACs were investigated by use of the H4IIE-luc assay. Stock solutions of the individual PAHs (paper I & III), oxy-PAHs and azaarenes (paper III) were made in dimethyl sulfoxide (DMSO). All PACs were tested in 10 concentrations, in triplicate wells. AhR-mediated potencies of the compounds were examined after 24, 48, or 72 h of exposure in the H4IIE-luc bioassay in order to investigate the metabolic persistency of the compounds. In each assay, a standard curve of TCDD (0.4 to 300 pM) and a solvent control (DMSO, 0.4%) were tested in triplicate wells. Concentration-response curves were constructed for all compounds by use of a sigmoidal concentration-response (variable slope) equation. The curve fitting was done with the GraphPad Prism® 5.0 software. Concentration-response curves of the compounds were used in calculations of relative potency factor (REPs) and in mixture predictions. The com-
pounds were tested in two to four independent assays per exposure duration.

In paper III, changes in concentrations of five chemicals (TCDD, benzo[a]pyrene, dibenz[a]anthracene, 9,10-dihydrobenzo[a]pyren-7(8H)-one and dibenz[ah]acridine) in the cell medium during the 24, 48, or 72 h of exposure in the H4IIE-luc assay were studied using low and high resolution GC/MS. Changes due to sorption or volatilization compared with the nominal medium concentrations were assessed by exposure of 96-well plates prepared the same way but without cells.

3.1.2 Mixtures

3.1.2.1 Soil extracts
Soil extracts aimed for bioassay analysis were solvent exchanged into DMSO (Paper II). Concentrations of stock solutions were selected to produce full concentration-response curves in the H4IIE-luc assay. Because of the diverse contamination history of the soils, different concentrations were chosen depending on the grade of contamination. In case of inaccurate concentration-response relationships, due to cytotoxicity, high background noise or a minimum response greater than 20% of TMI, the stock solutions were diluted with a factor of 10 with DMSO (Figure 7), and tested in the bioassay. Soil extracts from remediated soils (paper II) were tested by
24 h of exposure in the H4IIE-luc bioassay. A number of soil extracts were tested in three independent assays, as a measurement of the reproducibility of the assay.

![Graph](image)

*Figure 7. Concentration-response curves obtained from H4IIE-luc analysis of soil 1, before and after the stock solution had been diluted with a factor of 10 with DMSO.*

3.1.2.2 Synthetic PAH mixtures
Mixtures of PAHs, oxy-PAHs and azaarenes were made in DMSO. In paper I, additive interactions of PAHs were investigated after 24 h of exposure. Seven different mixtures of PAHs were obtained by mixing various numbers and combinations of individual PAHs in equal molar concentrations at a 1:1 ratio. To investigate the exposure time dependent effect on the mixture activity, three of the mixtures were also tested at 48 and 72 h of exposure.

In paper IV, additive interactions of PAHs, oxy-PAHs and azaarenes were investigated after 24 h of exposure. Eighteen mixtures of the compounds were composed in different combinations. Ten of the mixtures were, so called, equitoxic mixtures, where each component in the mixtures was combined in proportion to their EC5 or EC50 values. Since it is unlikely that the contaminants occur in the environment in a fixed ratio to their AhR-mediated potency concentrations, the other eight mixtures were prepared in non-equivalent effect concentrations. Various combinations of the contaminants were chosen in the mixtures to estimate the mixture toxicity and additivity in mixtures containing solely PAHs, oxy-PAHs or azaarenes and mixtures containing a combination of the contaminants. To investigate if the soil matrix might affect the AhR-mediated toxicity of PAC-mixtures, soil extracts in n-hexane from extraction of an agricultural soil were added to three of the PAC mixtures.
3.1.3 Calculations

3.1.3.1 Relative potency factors (REPs)
Relative potency factors (REPs) were obtained from the concentration-response curves by relating the luciferase induction potency of the PAH, oxy-PAH or azaarene in relation to that of the positive reference TCDD

\[ \text{REP}_i = \frac{\text{TCDD EC}_x}{\text{PAC EC}_x} \]

REPs based on EC50 and EC25 were calculated by dividing the ECx for TCDD by the ECx for the compound where x is 25 or 50% of TCDD max induction (TMI) (Figure 8). A mean value for each relative potency factor was calculated from two to four independent experiments. Moreover, REPs based on multipoint estimates (EC20 to EC80 range) were determined in paper III.

Figure 8. Example of concentration-response curves obtained with the H4IIE-luc assay. Lines indicate 25 or 50% of TCDD max.

3.1.3.2 Chemically derived chem-TEQ
Chem-TEQs were calculated by use of the H4IIE-luc assay specific relative potency factors (REP). Total chem-TEQ was calculated as the concentration of each compound in the samples multiplied by its specific REP (EC25 or EC50).
3.1.3.3 Bioassay derived bio-TEQ
Bio-TEQs were calculated from the concentration-response curves by relating the luciferase induction potency of the samples to that of the positive reference TCDD using the equation:

\[ \text{bio-TEQ (pg/g)} = \frac{\text{TCDD EC}_{25} \text{ (pg/ml)}}{\text{extract EC}_{25\text{TCDD}} \text{ (mg/ml)}} \]

where TCDD EC\(_{25}\) is the effect concentration of TCDD yielding 25% of the TCDD-induced maximum effect and extract EC\(_{25\text{TCDD}}\) is the effect concentration for the sample at 25% of the maximum effect of TCDD.

3.1.3.4 Prediction of mixture activities
The mixture activities were predicted by use of the REP concept (Paper I), CA model (Paper I & IV) or the IA model (Paper IV) on the basis of concentration-response curves of singly analysed PAHs, oxygenated PAHs and azaarenes. In order to make the response curves comparable, the mixture responses were normalised against the mean of the maximum response observed by the TCDD standard. The mean solvent control response was subtracted from both TCDD standard and mixture responses prior to conversion to get responses scaled from 0% to 100% of TCDD maximum induction (TMI). Calculation by use of the REP-concept is presented in paper I.

The CA model is based on the assumption that all mixture components have a common or similar mode and mechanism of action. When the composition of the mixtures is known, the concentrations of each compound can be expressed as a fraction of the total concentration. Concentration addition is expressed mathematically as

\[ EC_{x,\text{mix}} = \left( \sum_{i=1}^{n} \frac{p_i}{EC_{x,i}} \right)^{-1} \]

where \( n \) is the number of mixture constituents, \( EC_{x,\text{mix}} \) is the effect concentration of the mixture provoking x% effect, \( EC_{x,i} \) is the concentration of the ith mixture component provoking x% effect when applied singly, and \( p_i \) is the fraction of the ith component in the mixture (Berenbaum, 1985).

Concentrations giving 10-100% mixture effects were calculated in steps of 5% and the concentration/effect coordinates were plotted and analysed.
using the sigmoidal response (variable slope) curve fitting (GraphPad Prism®5 software) to give a predicted concentration-response curve.

The IA model, sometimes also termed response addition, is a common approach used for prediction of the mixture toxicity of compounds with diverse modes of action. Response addition is defined as

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i))$$

where $c_i$ denotes the concentrations of the $i$th mixture component, $E(c_i)$ its corresponding effect, and $E(c_{mix})$ the overall effect caused by the total concentration of the mixture ($c_{mix}$) (Bliss, 1939).

3.1.4 Quality of data

Only plates with a standard deviation of $\leq 16\%$ within triplicates and a TCDD maximum induction factor $> 6$ were used for quantification. For all plate measurements at 24 h of exposure, a criteria of a TCDD EC50 value between 8 to 18 pM, was used to include the measurement in the results. Limit of detection (LOD) was calculated as the mean luciferase activity of DMSO control triplicates + 3 times standard deviation (SD).

3.2 Analysis of PAH-contaminated soils

3.2.1 Soil sampling

Nine soil samples were collected during time period 2007/2008 from various Swedish remediation companies (Paper II). All soils had undergone remediation to levels below the Swedish limit for less sensitive land use with respect to PAHs. In the following sections these soils are referred to as soil 1 to 9. The contamination history of the soils is presented in paper II. Six of the soil samples had undergone biological treatment and the other three soil washing. Samples of agriculture soils were collected from different locations in Sweden. These soil samples are referred to as soil 10 to 12 in following sections.

Another set of soil samples from three biological treatment plants were collected during time period 2010/2012, under on-going remediation processes. These soils had a diverse pollution history; the first soil was composed of a mixture of PAH-contaminated soil, soil from old gas stations and residuals from treatment of oil contaminated soils, the second soil consisted of PAH-contaminated soil only and the third soil was composed of oil from the surrounding of a leaking oil boiler central. In the following
sections these soils are referred to as Soil-OP, Soil-P and Soil-O. These soil samples are included in an on-going study of PACs in soils from remote, urban and PAH contaminated areas and data are not published.

All soil samples were homogenised and passed through a 2-mm sieve and stored in a freezer at -18°C until extraction. The water content and organic matter (loss of ignition) were determined for all soils.

Figure 9. Study design for analysis of remediated PAH-contaminated soils.

3.2.2 Extraction
In chemical analysis, internal standards (IS) and recovery standards (RS) can be added and possible losses during sample preparation and analysis can be verified. In bioassays there is no way to compensate for losses during sample preparation and analysis. In comparison studies between chemical and bioassay analysis, there are two alternatives; parallel extractions or to split the extracts prior to analysis. Both alternatives have their uncertainties. In paper II, parallel extractions of the remediated soil samples were performed in all extraction methods. Arable soils and the soils from the remediation processes were split after extraction and clean-up. Certified reference material was extracted in parallel to the soil samples as a quality control sample.
3.2.2.1 Analysis of total concentrations

Total concentrations of PAHs in the remediated soil samples in Paper II were determined by use of pressurised liquid extraction (PLE\textsuperscript{TM}, Fluid Management Systems, Inc.). That is an extraction technique that involves high pressure and high temperature to enhance the effectiveness of the extraction (Lundstedt et al., 2000). In cell clean-up was used in paper II to minimise the work-up time and solvent volume (Ong et al., 2003). To optimize the clean-up of soil extracts and obtain good recoveries of the PAHs, different proportions of deactivated silica gel and certified reference material were tested in the PLE system. An amount of 4 gram of 10% deactivated silica gel generated the best results and was further used in paper II. This work up methodology reduces both time and solvent volume significantly compared to traditional extraction and clean-up methods.

Another extraction method was used for the arable soils and soils, Soil-O, Soil-P and Soil-OP, as the scope of the study had been widened to include additional PAHs, oxy-PAHs and azaearenes. Pressurised liquid extraction was used but with \textit{n}-hexane:acetone 1:1 (v/v) as an extraction solvent. Extracts were further cleaned up on open columns as described in table 3.

### Table 2. Different bioavailability mimicking extractions techniques tested by use of methanol or \textit{n}-butanol as an extraction solvent. The method used in the thesis, paper II, is highlighted.

<table>
<thead>
<tr>
<th>Mild shake</th>
<th>Pressurised liquid extractions, PLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ml solvent + 10 g soil</td>
<td>Cells packed with 5 g soil + Na\textsubscript{2}SO\textsubscript{4}</td>
</tr>
<tr>
<td>Vortex mixer, 5 s</td>
<td>Fill cells with solvent, 1 min</td>
</tr>
<tr>
<td>Settle for 15 min</td>
<td>Pressurise to 1 700 psi, 1.5 min</td>
</tr>
<tr>
<td>Centrifugation 7,000 g, 60 min</td>
<td>Heating to 25, 50, 75 or 100°C, 1.5 min</td>
</tr>
<tr>
<td></td>
<td>Cooling cells, 9 min</td>
</tr>
<tr>
<td></td>
<td>Flushing cells, 1.5 min</td>
</tr>
</tbody>
</table>

3.2.2.2 Analysis of available PAHs

A mild chemical extraction with methanol was used for the estimation of the bioavailable fraction of PAHs in the remediated soils (Paper II). The extraction method was based on a previously reported method (Liste and Alexander 2002) but further developed in our laboratory. A number of extraction methods were tested by use of different extraction solvents (\textit{n}-butanol or methanol), extraction temperatures and extraction times (Table 2).
An uptake study by earthworms was performed on the same soil. PAH-profiles in extracts were compared with the PAH-profile in earthworms, by summation of the relative deviation from the concentration of each PAH in the earthworms. The mild shake method developed by Liste and Alexander (2002), but by use of methanol as an extraction solvent, provided the best prediction of the priority 16 PAH uptake pattern in earthworms exposed for the same soil (Figure 10).

To obtain an indication of the leachable fraction of PAHs and AhR-agonists in the soil after remediation, a batch leaching test was performed on two of the soil samples according to ISO/DIS 21268-2, with minor modifications (paper II). All extracts were cleaned up on open columns packed with 10% deactivated silica gel, and contaminants were eluted with 15 ml \( n \)-hexane followed by 15 ml \( n \)-hexane:dichloromethane 3:1 (v/v).

![Figure 10. Comparison of the PAH pattern in worms and extracts from different extraction methods; PLE extraction by use of methanol or \( n \)-butanol at 25, 50, 75 or 100°C, and a shake method with methanol or \( n \)-butanol as solvent. The figure shows the individual PAHs AhR-mediated activity (pg/g TEQ) as a percentage of the overall expected AhR-mediated activity in the extracts, calculated from chemical data and REP values. The PAHs named in the figure accounted for approximately 98% of the toxicity by the chemically analysed PAHs. Note that these PAHs only account for a small part of the total observed effect of the extracts in the H4IIE-luc bioassay.](image-url)
3.3.1 Gas chromatography–mass spectrometry
Concentrations of 20 PAHs in remediated PAH-contaminated soils (Paper II) were quantified by use of a HP 6890 gas chromatograph coupled to a HP 5973 low resolution mass spectrometer using electron ionization (EI) at 70 eV. The gas chromatograph was equipped with a DB-5 capillary column (30m×0.25mm, 0.25μm film thickness; J&W Scientific). The GC oven temperature program was optimised to enhance the separation of benzo[b]fluoranthene and benzo[k]fluoranthene. The GC temperature program started with an initial oven temperature at 80°C which was held for 2 minutes, heating 15°C min⁻¹ to 180°C (held for 1 min), heating 8°C min⁻¹ to 250°C (held for 1 min), and finally heating 3°C min⁻¹ to 300°C (held for 6 min).

In paper III, concentrations of PACs in cell medium during exposure of H4IIE-luc cells were measured by use of an Agilent 7890A gas chromatograph coupled to a 5975C low-resolution mass spectrometer, and equipped with a ZB-SemiVolatiles column (30 m×0.25 mm, 0.25 μm film thickness; Phenomenex). Temperature program; initial 90°C for 2 minutes, ramped 8°C min⁻¹ to 300°C (held for 10 min). The same GC-parameters were used in quantification of the 43 PACs in arable and soil samples, Soil-O, Soil-P and Soil-OP.

Concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were quantified by use of a high-resolution GC/MS system (a Micromass Autospec Ultima) operating at 10,000 resolution using EI ionization at 35 eV (Paper III). All measurements were performed in the selective ion recording mode, monitoring the two most abundant ions of the molecular chlorine cluster. Quantification was performed using the internal standard method. Splitless injection was used to inject 1 μl of the extract on a 30 m (0.25 mm i.d, 25 μm film thickness) DB-5MS column (J&W Scientific; Folsom, CA, USA). The temperature program was as follows: initial temperature of 180°C (held for 2.0 minutes), then the oven temperature was increased by 3.5°C min⁻¹ till 230°C, then by 15°C min⁻¹ to 300°C with a final hold of 4.0 min. Chemicals included in each analysis are presented in table 3.

3.3.2 Quality of data
Quantification was performed using quality assurance/quality control procedures including the internal standard method using labelled standards. In lack of labelled standards, relative response factor (RRF) values for the compounds were calculated using the compound nearest in retention time. Target compounds were quantified by use of three to four point calibration curves. Relative standard deviation (RSD) of the RRFs was less than 15% for PAHs and 25% for oxy-PAHs and azaarenes. Samples which had con-
centrations exceeding the range of the calibration curve were diluted and reanalysed. Quantification standards were analysed after every tenth sample. Procedure blanks were included in all batches. The limit of detection (LOD) was defined as mean concentration in blanks + 3 times the standard deviations. External validation was achieved for PAHs, oxy-PAHs and azaarenes by participation in an intercomparison study (Lundstedt et al., unpublished data).

<table>
<thead>
<tr>
<th></th>
<th>Paper II</th>
<th>Paper III</th>
<th>On-going study</th>
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</thead>
<tbody>
<tr>
<td><strong>Analytes</strong></td>
<td>20 PAHs</td>
<td>benzo[a]pyrene, dibenz[a,h]anthracene, 9,10-dihydrobenzo[a]pyren-7(8H)-one, dibenz[ah]acridine, TCDD</td>
<td>25 PAHs, 12 oxy-PAHs, 2-methylantracene, 5 azaarenes</td>
</tr>
<tr>
<td><strong>Sample matrix</strong></td>
<td>Remediated PAH-contaminated soils</td>
<td>Cell medium</td>
<td>PAH-contaminated soils; collected before or at the end of biological treatment, arable soils</td>
</tr>
<tr>
<td><strong>Extraction</strong></td>
<td>PLE</td>
<td>Liquid-liquid extraction</td>
<td>PLE</td>
</tr>
<tr>
<td><strong>Preparation</strong></td>
<td>In cell chromatography; 10% deactivated silica gel</td>
<td>Open column chromatography; 10% deactivated silica gel (PAC)</td>
<td>Open column chromatography; 5g silica gel 10% deactivated, 5 ml n-hexane + 15 ml n-hexane:dichloromethane 3:1 (v/v) + 30 ml dichloromethane</td>
</tr>
<tr>
<td><strong>Instrumental technique</strong></td>
<td>GC-LRMS</td>
<td>GC-LRMS (PACs)</td>
<td>GC-LRMS</td>
</tr>
</tbody>
</table>

*Table 3. Sample preparation techniques and instrumental analysis used in this thesis*
4 Results and discussion

4.1 AhR-mediated activity of PACs

4.1.2 Relative potencies of single PACs

Relative potencies of 38 PACs (including PAHs, oxy-PAHs, methylated PAHs and azaarenes) were investigated in papers I and III. Concentration-response curves of selected PAHs and their corresponding derivatives are presented in figure 11. Generally, PAHs were more potent inducers of AhR-mediated responses than their derivatives. Most PAHs achieved REP values (EC25) in the range of 2.2×10⁻³ to 1.3×10⁻⁵ after 24 h of exposure in the H4IIE-luc bioassay, compared to oxy-PAHs, which REP values ranged between of 7.4×10⁻⁶ to 1.7×10⁻⁷.

Oxidation of PAHs seems to reduce the AhR agonistic potency of the compounds, since all oxy-PAHs were less potent than their parent PAHs. In contrast, oxidation of methylated PAHs seems to increase the AhR-mediated potency of the compounds. The two methylated derivatives of anthracene elicited relatively weak agonist activity, but the response increased with the number of substitutions; anthracene < 2-methylanthracene < 2-methyleneanthracene-9,10-dione. A number of methylated chrysenes and methylated benzo[a]pyrenes have been reported to induce AhR-mediated activity in the H4IIE-luc assay, similar to, or even greater than their parent PAHs (Machala et al., 2008; Trilecova et al., 2011). Since methylated- and oxy-methylated PAHs are detected in PAH-contaminated environments, occasionally in relatively great concentrations they could contribute significantly to the overall hazard of PAHs (Lundstedt et al., 2003).

Dibenz[ah]acridine, an analogue to dibenz[ah]anthracene substituted with a nitrogen atom within one of the carbon rings (azaarene), was the most potent PAH derivative studied and it was more potent than dibenz[ah]anthracene at all durations of exposure. The other azaarenes tested had low molecular weights, and like low molecular weight PAHs they were weak AhR agonists.

Although many of the PACs had considerable luciferase inducing potencies, all PACs exhibited decreasing REP s as a function of duration of exposure. Concentration-response curves shifted towards the right on the x-axis with increasing exposure time and resulted in reduced REP values (Figure 11). This was likely due to metabolism of the compounds, as also supported by the GC/MS analysis (Paper III), showing decreasing concentrations of
PACs during exposure duration while the concentration of TCDD remained constant (Figure 12).

Figure 11. Concentration-response curves for induction of luciferase activity by PAHs and PAH derivatives at 24 h (squares) 48 h (triangles) and 72 h (diamonds) of exposure in the H4IIE-luc bioassay. Data represent the result of two to four independent experiments, three replicates each. Error bars represent standard deviations.
The results from the GC/MS analysis can also be used to evaluate the accuracy of the bioassay, since we were able to analyse and quantify the amount of the compounds stored in the cells, in the medium and thereby elucidate the amount adsorbed to the plastic wells in 96-well plates. In addition to metabolism, reduced potency of the compounds can be a consequence of vaporisation or adsorption to the plastic culture plates during exposure, or a function of cell crowding. Our results show that adsorption of compounds to the plastic wells during exposure may affect the results, and the potency of a compound relative to TCDD (or another chemical reference) can be both underestimated and overestimated depending on the adsorption of the compound or TCDD. The results in paper III provides new information regarding the degradation and distribution of compounds in wells during exposure.

Figure 12. Amounts of test chemicals in cell culture medium and cells after 24, 48 and 72 h of exposure in the H4IIE-luc assay in percent of the initial total amount of added chemical. Controls represent the total amount of chemicals in the cell medium in wells without seeded cells (grey bars), medium represents the amount in the cell medium in wells with seeded cells (black bars), and cells represent the amount in the cells (white bars). Bars represent the result of four replicates. Error bars represent the mean value and standard deviations. nd means no GC/MS data over limit of detection (LOD).
4.2 AhR-mediated activity of PACs in mixtures

Since the REP-concept is based on additive relative potencies of analysed compounds in a sample the additive behaviour of PACs in artificial mixtures was studied in papers I and IV. The results showed that it is possible to predict the mixture toxicity of PACs in the H4IIE-luc assay by use of concentration addition, like the CA-model and REP-concept. The predictive power was best for multicomponent mixtures, and independent of the mixture composition. Additive interactions were shown both in mixtures composed of PACs in equivalent- or non-equivalent effect concentrations. Example from the mixture predictions by the CA-model are presented in figure 13 (Papers I and IV).

![Figure 13](image-url)

Figure 13. Observed (solid line) and predicted (dashed line) AhR-mediated activity of; (A) a mixture of 18 PACs mixed in their equivalent effective concentrations (EC50) and (B) a mixture composed of 15 PAHs mixed in equal molar concentrations, at 24 h of exposure in the H4IIE-luc bioassay. The observed data is based on two experiments consisting of triplicates at each concentration. Predicted data calculated with concentration addition are based on two to four experiments for each of the individual compounds with triplicate exposures for each concentration. Error bars represent standard deviations of the observed curve.

These results suggest additive AhR-mediated potency of PACs and strongly support use of concentration addition, like REPs in risk assessment and mass balance studies of PAH-contaminated samples. Independent action was also evaluated in the same study, but seemed to be an inappropriate model for predictions of AhR-mediated activities in PAC mixtures. The predicted AhR-mediated activities by the IA-model overestimated the activity in most of the mixtures. Generally, single PACs have concentration-response curves with lesser steepness (slope=0.2 to 0.8), which will
lead to prediction of greater mixture activity by IA compared to the CA model (Backhaus et al. 2004; Brosche and Backhaus 2010). If all mixture components have curves with a slope of 1, predictions of both models are supposed to be similar.

The results in paper IV indicate that the AhR-mediated activity observed in soils contaminated with PAHs is not due to enhanced activity by the soil matrix, since similar concentration-response curves are observed for PAC mixtures with or without addition of soil extract.

### 4.3 Characterisation of remediated PAH contaminated soils

#### 4.3.1 Concentrations

Total concentrations of PAHs (PAH16) in all remediated soil samples (soil 1 to 9) studied in paper II, were well below the Swedish limit for less sensitive land use with respect to the priority PAHs.

![Figure 14. Distribution of PAHs, oxy-PAHs and azaarenes in soil samples collected at the start and in the end of remediation. Duration of remediation in days (n) is given in brackets. The asterisk (*) indicates the sum of additionally 9 PAHs not included among the 16 priority PAHs analysed in this study. Each bar represents the mean value of three soil samples collected from the remediation plant. Each soil sample consists of six to eight sub samples.](image-url)
Figure 15. Chromatograms of PACs in (A) a PAH-contaminated soil (Soil-P), (B) an oil contaminated soil (Soil-O) and (C) a soil composed of a mixture of oil- and PAH-contaminated soils (Soil-OP), showing their different PAC-profile.
Concentrations of PAHs in the soil samples Soil-P and Soil-O, collected in the end of remediation were also below the Swedish limit for less sensitive land use, in contrast to Soil-OP, which still contained too high concentrations of high molecular weight PAHs. The analysis of Soil-P, Soil-O and Soil-OP, showed that all three soils contained polar PACs, like oxy-PAHs and azaarenes, and the concentrations of the compounds are presented in figure 14. Moreover, the total concentrations of PACs had declined after remediation in contrast to Soil-OP and Soil-O, which concentrations of PACs were almost constant during remediation. Sample (Soil-P) contained the greatest initial concentrations of PAHs and the contaminant profile differed from the other two soils. The soil contained a greater fraction of PAH-M and a lower fraction of PAH-H compared to the other two soils. The different profiles are also illustrated by their chromatograms (Figure 15).

Similar to these soils, the soils studied in paper II most likely contained concentrations of azaarenes and oxy-PACs, since these soils also came from oil- and PAH contaminated areas.

The arable soils contained low concentrations of PAHs between 20 to 200 ng/g d.w. soil, which were in agreement with concentrations found in a study by Šídlová et al. (2009).

4.3.2 AhR-mediated activity
A comparison of results from mass-balance analysis of all soil samples are presented in figure 16. Greater bio-TEQs than chem-TEQs were shown in all soil extracts. The proportion of unknown AhR agonists differed between the soils. Only 1 to 36 % of the high AhR-mediated activity in the remediated soil samples could be explained by the priority PAHs analysed. This indicates that all remediated soils contained a large fraction of AhR-active compounds that could not be explained by the priority PAHs present in the samples.

Although 43 compounds were included in the mass-balance calculations of Soil-P, Soil-OP and Soil-O samples, only 2 to 20 % of the high AhR-mediated activities in the samples could be explained by the compounds analysed. The contribution of the 16 priority PAHs to the total chem-TEQs of the 43 PACs analysed was 26 to 40 %, which indicates that the unexplained AhR-mediated activities in the soils most likely are due to unidentified PAHs, azaarenes among other substituted PAHs.

The sample (Soil-P) contained the lowest levels of AhR agonists, even though the initial measured concentrations of PAHs, oxy-PAHs and azaarenes in the start sample were greater than in the other two soils. Like
the concentrations of PACs in Soil-OP and Soil-O samples, AhR agonistic activities were almost constant during remediation.

Figure 16. Comparison of bioassay derived bio-TEQs (EC25) and chemically derived chem-TEQs (EC25) of the 16 priority PAHs in soil extracts from PLE extraction of nine remediated PAH-contaminated soil samples (soil 1 to 9), three arable soils (soil 10 to 12), and three soil samples collected at the start and the end of remediation (n days of remediation are given in brackets). Bio-TEQ bars show the mean value and SD of three independent determinations of bio-TEQ.

The bio-TEQ in the arable soils were low (50 to 190 pg/g d.w. soil), and comparable with bio-TEQs reported in an earlier study of arable soils (Šídlová et al., 2009). The proportions of unexplained AhR agonists in the arable soils were 80 to 95 % and probably a large part of those agonists exists naturally in many soils. However, if assuming that levels of natural occurring AhR agonists in the contaminated soils were similar as in the
arable soils, these gave a minimal contribution to the effects of remediated soils, whose absolute levels of unexplained AhR agonists were nine to 800 times higher. Previous studies have shown that PAH-contaminated soils consists of a variety of contaminants and naturally occurring compounds, which generated hundreds of peaks in MS spectrums when analysing these types of soils (Bergknut et al., 2006; Lundstedt et al., 2003). Most probably the complexity of the soils included in this study is similar, and the observed unexplained AhR-mediated activity is due to other AhR-active PACs in the soils.

### 4.3.3 Availability of the contaminants

Bioavailable fractions of PAHs in the remediated soils were assessed by use of a mild chemical extraction with methanol (Paper II). Comparison of bioavailable concentrations, defined by their extractability with methanol, and total concentrations in the soils is presented in figure 17.

![Figure 17. Concentrations of the 16 priority PAHs analysed by GC-MS in remediated PAH-contaminated soils extracted by use of two different extraction techniques. PAH-H sum of high molecular weight PAHs, PAH-M sum of intermediate molecular weight PAHs, and PAH-L sum of low molecular weight PAHs.](image)

There was no correlation observed between total PAH concentrations and concentrations of bioavailable PAHs, which indicate that other factors control the leachable fraction more than the absolute concentrations of
PAHs in the soil. Most PAHs are strongly sorbed to the organic matter in
the soil, making them less mobile and relatively unavailable for microbial
degradation. No significant correlation was found between the methanol
extractable fraction and the organic content in the soils, which indicates
that the available fraction of PAHs is influenced not only by soil properties
like organic content, but also on the contamination history and other fac-
tors. There was, however, a relationship between the proportion of metha-
nol leachable PAHs and the remediation technique used. The methanol
leachable portion of PAHs ranged from 36 to 44 % in the three samples (2,
7 and 9) that had undergone soil washing and from 10 to 21 % in the six
samples that had undergone biological treatments. The leaching test of
soils 2 and 7 showed that only 0.4-0.5 % of the total initial content of the
16 priority PAHs was leached out with water.

The results from the H4IIE-luc bioassay analysis of the soils suggest that
only a smaller portion of the AhR inducing compounds in the soils are
bioavailable. The methanol extracts accounted for 0.1 to 9 % of the bio-
TEQ obtained by the PLE total extraction of the soils, depending on sam-
ple. Soil 1, a bioremediated soil that consists of creosote contaminated soil
from a steel industry area, contained the greatest levels of unknown bioa-
vailable AhR ligands. It is not possible to comment on the risk of these
chemicals, because their identity are unknown, but there is a reason for
further investigation, since they seem to be leached out relatively easy from
the soil.
5 Concluding remarks and future perspectives

PAH-contaminated soils are a worldwide problem. Although extensive research has shown great concentrations of other polycyclic aromatic compounds in PAH-contaminated soils, risk assessment of PAH-contaminated soil is still based on a small number of PAHs, commonly the 16 priority PAHs. In this thesis it is shown that not only PAHs but also other polycyclic aromatic compounds, like oxy-PAHs, oxy-methylated PAHs and azaarenes should be considered in risk assessment and during remediation of PAH-contaminated soils. These compounds were detected in remediated soils, and PAHs, azaarenes and oxy-methylated PAHs were shown to be potent AhR agonists.

Results presented in this thesis are an important step in the development of AhR-based bioassay analysis of complex PAC-contaminated samples. The relative AhR-mediated potency of a number of PACs, included PAHs, oxy-PAHs, methylated PAHs and azaarenes, and their combined effect in different mixtures were investigated. Mixture studies showed additive effects of PACs in multi-component mixtures, which strongly support the use of concentration addition, like the REP-concept in risk assessment and mass-balance calculations of mixtures of PACs in environmental samples. Metabolic degradation of PACs in the H4IIE-luc bioassay was studied with both chemical and H4IIE-luc analysis, which showed that PACs are readily metabolised in H4IIE-luc cells.

Chemical and bioassay analysis were used in this thesis to characterise remediated PAH-contaminated soils. Bioassay specific REP-values were developed and used in mass-balance analysis of remediated PAH-contaminated soils. Chemical and bioassay analysis showed that PAH-contaminated soils contained a large fraction of AhR-active compounds that could not be explained by chemical analysis of the 16 priority PAHs. Mixtures studies indicated that the high AhR-mediated activity in contaminated soils was not due to AhR-active or facilitate effects of organic matter in the soil extract, or presence of non-AhR active PACs in the soils. These findings show that traditional methodology of using chemical analysis of the priority PAHs to determine the degree of PAH contamination in soils greatly overlooks toxicologically relevant PAHs or other AhR agonists still present in soils after remediation. That can be concluded that after most soil remediation actions there will remain non-analysed, biologically active compounds in the soil. These findings highlight the great need of bioassays in risk assessment of PAH-contaminated soils.
Introducing bioassay methodologies in risk assessment of remediated soil could reduce the risks contaminated soils may pose to humans and wildlife and provide a safer reuse of remediated soil.

Availability is an important issue to consider in risk assessment of contaminated soils. Results from this thesis indicate that the remediation techniques can affect the availability of the PAHs, since the soils that had undergone soil washing had a greater fraction of methanol leachable PAHs than the bioremediated soils. No conclusions can be drawn, since the availability studies only were performed on soils that had undergone remediation.

Questions remain regarding the identity of chemicals, their mechanisms of action, interactions, and whether the total AhR-mediated activating potency of complex PAC-mixtures corresponds to an adverse biological effect. Further chemical identification studies and biological studies and extensive soil characterisation methods are necessary to determine whether these unknown AhR agonists pose a risk to human health or the environment.

The following proposals for future studies have emerged during the studies underlying this thesis:

- **Effects studies of artificial mixtures in the H4IIE-luc bioassay:**
  Studies of different mixtures, like reference material or commercial mixtures will give an increased knowledge about the toxic potential of whole mixtures with a known chemical composition. These results can then be used in comparison with bioassay analysis of environmental samples. Moreover, the results will give an increased knowledge about the toxic potential of mixtures often found in contaminated areas. Component based mixtures need also to be tested, like mixtures of the PACs. Addition of PACs to reference material, like oil mixtures or creosote may facilitate the interpretation of bioassay data, since the results may give an enhanced understanding about the high AhR-mediated activity observed in PAH-contaminated soils.

- **Use multivariate analysis of full scan spectra to categorise PAH-contaminated soils; look for relationships between soil origin, compound composition and AhR-mediated activity i.e., markers for potentially toxic soils.** Analysis of these markers along with the 16
priority PAHs may provide a broader picture of the contaminants in the soils. Risk assessment of PAH contaminated soils would be easier and safer if adequate marker compounds were available.

- Study relationships between data generated from research of AhR-mediated toxicity of PACs \textit{in vivo} with the AhR-mediated potency of PACs observed \textit{in vitro}.
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