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Characterization of polycyclic aromatic compounds in historically contaminated soil by targeted and non-targeted chemical analysis combined with *in vitro* bioassay☆

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ABSTRACT

Soil samples from a contaminated site in Sweden were analyzed to identify the presence of 78 polycyclic aromatic compounds (PACs) using gas chromatography coupled with mass spectrometry (GC-MS). The target analysis revealed large contributions not only from polycyclic aromatic hydrocarbons (PAHs), but also from alkylated- and oxygenated-PAHs (alkyl- and oxy-PAHs, respectively), and N-heterocyclics (NPACs). PAC profiles indicated primarily pyrogenic sources, although contribution of petrogenic sources was also observed in one sample as indicated by a high ratio of alkylated naphthalene compared to naphthalene. The aryl hydrocarbon receptor (AhR)-activity of the soil extracts was assessed using the H4IIe-pGudluc 1.1 cells bioassay. When compared with the calculated total AhR-activity of the PACs in the target list, 35–97% of the observed bioassay activity could be explained by 62 PACs with relative potency factors (REPs). The samples were further screened using GC coupled with Orbitrap™ high resolution MS (GC-HRMS) to investigate the presence of other PACs that could potentially contribute to the AhR-activity of the extracts. 114 unique candidate compounds were tentatively identified and divided into four groups based on their AhR-activity and environmental occurrence. Twelve substances satisfied all the criteria, and these compounds are suggested to be included in regular screening in future studies, although their identities were not confirmed by standards in this study. High unexplained bio-TEQ fractions in three of the samples may be explained by tentatively identified compounds (n = 35) with high potential of being toxic. This study demonstrates the benefit of combining targeted and non-targeted chemical analysis with bioassay analysis to assess the diversity and effects of PACs at contaminated sites. The applied prioritization strategy revealed a number of tentatively identified compounds, which likely contributed to the overall bioactivity of the soil extracts.

1. Introduction

Terrestrial ecosystems play an important role in supporting a healthy environment for soil microorganisms as well as for the environment and human safety in general, as highlighted in goal no. 15 of the United Nations Sustainable Development Goals (UN SDGs) (Rosa, 2017; "The 17 Goals | Department of Economic and Social Affairs," n.d.). Urban growth and industrial revolution led to the creation of manufacturing plants and industrial activities, which resulted in contamination of soil around the world (Arp et al., 2014; Hu et al., 2012; Trine et al., 2019). It has been estimated that around 80,000 contaminated land areas exist in Sweden ("Contaminated areas - SGI," 2020; Forslund and Barregård, n. d.). These sites must be assessed for toxicity and risk, and several of the sites must be remediated before they can be used in the future ("Specifications for A Non-Toxic Environment, 2020; Volchko et al., 2020). Efficient remediation requires detailed knowledge on the composition of

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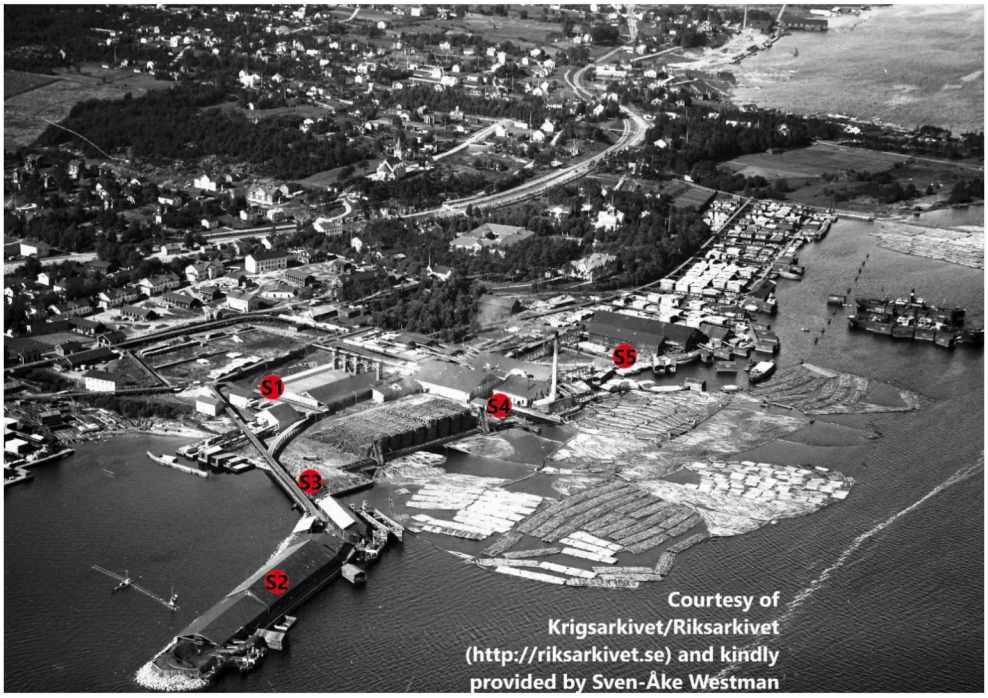


Fig. 1. Approximate sampling locations at Sundsvall municipality overlaid on the picture of the active industrial site taken in 1928.

the soil contaminants, which highlights the importance of profiling and quantifying pollutants found in contaminated soils.

One of the most commonly studied groups of soil contaminants are polycyclic aromatic compounds (PACs) due to their environmental and human health concerns (Lam et al., 2018b; Trine et al., 2019). Some PACs, such as polycyclic aromatic hydrocarbons (PAHs) and alkylated-PAHs (alkyl-PAHs), can activate the aryl hydrocarbon receptor (AhR) signaling pathway and, thus, their presence in soil extracts can be screened using AhR-based bioreporter assays (Andersson et al., 2009; Lam et al., 2018a; Larsson et al., 2013). Other PACs, including oxygenated-PAHs (oxy-PAHs), nitrogen-, oxygen- and sulfur-heterocyclics (NPACs, OPACs, and SPACs, respectively) are also known to be toxic and elicit AhR-mediated activities (Brinkmann et al., 2014; Machala et al., 2001; Swartz et al., 2009; Thomas et al., 2002). When the AhR-based bioreporter assay is used, the bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (bio-TEQs) of the soil extracts can be determined and compared with the chemically derived TCDD equivalents (chem-TEQs) (Larsson et al., 2013). The comparison of bio- and chem-TEQs are useful to illustrate the contributions of different PACs on the overall AhR-activity of the sample extract. It is of outmost importance to increase the chemical knowledge on reasons behind this gap in order to identify toxic substances posing a risk for soil health and potentially causing deteriorating quality in connected ground and surface water and secondary poisoning in food chains.

The main objective of this study was to characterize PAC profiles in samples from a contaminated site using targeted and non-targeted analysis combined with *in vitro* analysis. Soil samples from an industrial site in Sweden were extracted and analyzed by gas chromatography coupled with mass spectrometry (GC-MS) and by H4IIe-pGudluc 1.1 cells for targeted chemical and bioassay analyses, respectively. GC Orbitrap™-high-resolution MS (GC-HRMS) was used for non-targeted chemical analysis. Once unknown substances were tentatively identified, these compounds were prioritized to increase the identification confidence of AhR-active candidate compounds and to identify compounds that are potentially relevant for screening in future environmental monitoring and toxicity studies. The use of a prioritization strategy is virtually a necessity for successful non-targeted analysis (Blum et al., 2017; Lee et al., 2019; Rager et al., 2016). The strategy used in the current study relied on the unique combination of AhR-activity and environmental occurrence of the tentatively identified compounds. The results from this study will contribute to the risk assessment of contaminated soil sites, thus contributing to the advancement of the UN SDGs (Rosa, 2017), particularly SDG no. 15 concerning life on land.

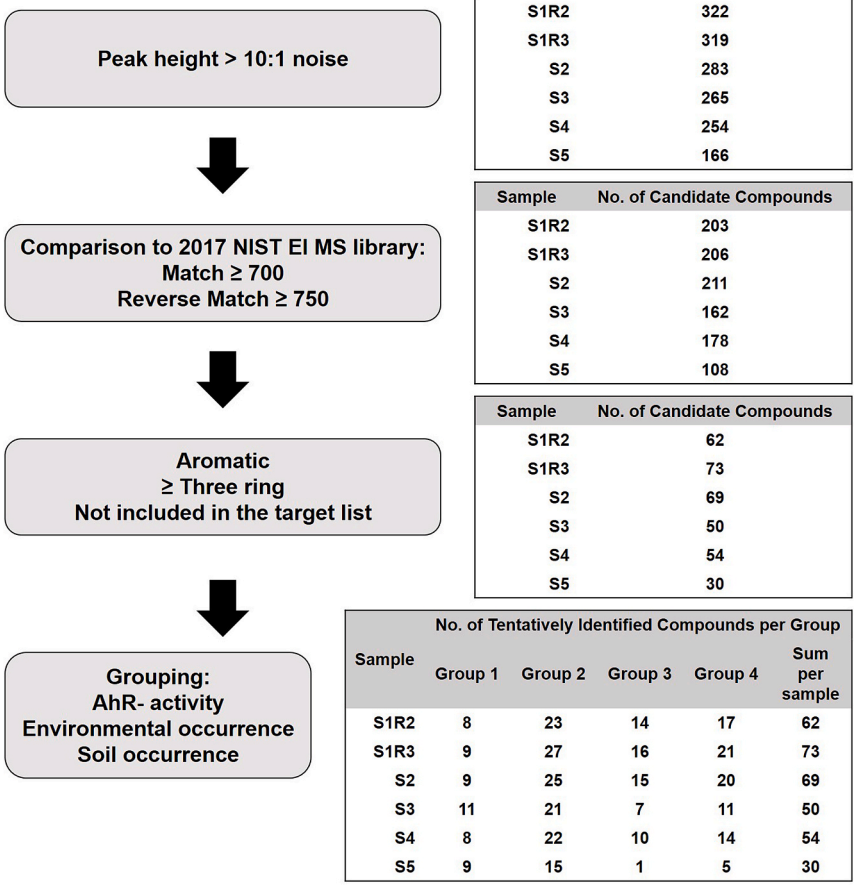
2. Materials and methods

2.1. Chemicals

Toluene (≥99.7%), *n*-hexane (≥98%), and anhydrous sodium sulfate (99%) were purchased from VWR (Stockholm, Sweden). Dichloromethane (99.8%), dimethyl sulfoxide (DMSO) (99.9%), and silica gel 60 were purchased from Sigma Aldrich (Stockholm, Sweden). List of 78 target PACs, isotopically labelled internal and recovery standards (IS and RS, respectively), their abbreviations, vendors, purities, and quantifier ions are listed in the Supplementary Materials (SM) (Table S1). The 2,3,7,8-TCDD standard (99.1%) for the bioassay analysis was purchased from AccuStandard (New Haven, CT, USA). Steadylite plus™ was purchased from PerkinElmer (Hägersten, Sweden).

2.2. Sample collection

Five soil samples (S1–S5) were collected at an industrial site in Sundsvall municipality, Sweden, at a sampling depth of 0–20 cm (Tiberg et al., 2019; Volchko et al., 2020) (Figs. 1 and S1). The samples analyzed in this study were a subset of the samples collected from a previous study (Tiberg et al., 2019) and were selected based on availability at the start of the current study. An aluminum production company, located south of the site has been identified as a source of PAH in the area, but elevated levels of PAHs found throughout the site indicated that PAHs most likely originated from past industrial activities such as charcoal production and sawmilling (Hifab, 2015). Elevated levels of metals, namely zinc, copper, and lead, has also been found throughout the site (Hifab, 2015; Tiberg et al., 2019). Samples S1, S2, S3, and S4 were all collected from areas where charcoal storage had taken place and consisted of loamy soil, a sandy soil with white fill, sandy loam soil with rocks, and sandy



Sample	No. of Candidate Compounds
S1R2	322
S1R3	319
S2	283
S3	265
S4	254
S5	166

Sample	No. of Candidate Compounds
S1R2	203
S1R3	206
S2	211
S3	162
S4	178
S5	108

Sample	No. of Candidate Compounds
S1R2	62
S1R3	73
S2	69
S3	50
S4	54
S5	30

Sample	No. of Tentatively Identified Compounds per Group				
	Group 1	Group 2	Group 3	Group 4	Sum per sample
S1R2	8	23	14	17	62
S1R3	9	27	16	21	73
S2	9	25	15	20	69
S3	11	21	7	11	50
S4	8	22	10	14	54
S5	9	15	1	5	30

Fig. 2. Workflow of non-targeted analysis based on full-scan screening with GC-HRMS. Grouping of tentatively identified compounds was based on three criteria (AhR-activity, other environmental occurrence and soil occurence). Tentatively identified compounds in Groups 1, 2, and 3 satisfied all three, two, or just one critera, respectively. Group 4 consisted of tentatively identified compounds that did not satisfy any criteria.

soil, respectively. Sample S5 was collected from the northern part of the site, which was historically used for sawmilling, wood impregnation with pentachlorophenol, and drying of logs. Within this area, two hotspots of polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, ethylene dichloride, and polychlorinated biphenyls (PCBs) have also been detected (Hifab, 2015). All soil samples were sieved in the field (≤2 mm), homogenized, and split into subsamples for subsequent analyses. The samples were stored at −18 °C prior to extraction. Water content was analyzed by heating the samples at 105 °C for 24 h. Information on water content and other soil characteristics of the samples (Tiberg et al., 2019) are provided in the SM (Table S2).

2.3. Analytical methods

2.3.1. Soil samples extraction

Prior to extraction, the samples were homogenized again and the S1 sample was analyzed in triplicates (S1R1, S1R2, and S1R3) to investigate measurement variability. Soil samples were extracted using pressurized liquid extraction (PLE™, Fluid Management Systems, Watertown, MA, USA) with in-cell clean-up (Larsson et al., 2018b). 44 mL extraction cells were packed with, from bottom, 4 g basic silica, thin layer of anhydrous sodium sulfate, soil mix (two to four g soil and sodium sulfate, 1:5 ratio), and a layer of anhydrous sodium sulfate. The extraction was performed in two static cycles (120 °C, 12 MPa, 10 min) with *n*-hexane:dichloromethane (9:1, v/v) as the extraction solvent (Larsson et al., 2018b). Soil extracts were evaporated to ~5 mL using rotary evaporator and further down to ~0.5 mL under a gentle stream of nitrogen and gravimetrically split into two halves for chemical and bioassay analyses. The fraction for bioassay analysis was solvent exchanged into 50 μL DMSO, while the fraction for chemical analysis was solvent exchanged into 500 μL toluene and spiked with 50–500 ng IS and 500 ng RS solutions. Recoveries for the method ranged from 40 to 130%. Details on the extraction step can be found elsewhere (Larsson et al., 2013, 2018b).

2.3.2. Targeted chemical analysis with GC-MS

Soil extracts were analyzed with an Agilent 7890 A GC coupled with a 5975C MS. The GC column was the Agilent Select PAH capillary column (30 m × 0.25 mm, 0.15 μm film thickness). 1 μL of extract was injected in splitless mode and the MS was operated in electron ionization (EI) mode at 70 eV using selected ion monitoring (SIM) mode. Further details on the chemical analysis can be found elsewhere (Larsson et al., 2018b).

2.3.3. AhR-activity analysis

AhR-activity analysis was performed using the DR CALUX® assay (H4IIe-pGudluc 1.1 cells) (Larsson et al., 2013; Murk et al., 1996), which measured the AhR-activity of the soil extracts. Details of the analysis can be found in previous studies (Larsson et al., 2018b; Schönlau et al., 2019b). Briefly, dilution series (1:4) of the extracts were prepared in culture medium and two extracts at six concentrations (three replicates per concentration) were added to the cells in triplicate wells. On each of the 96 well plate, six concentrations of 2,3,7,8-TCDD (0–300 pM) and a solvent control (DMSO) were also added in triplicate wells. Final DMSO concentration in all wells was 0.4%. Following 24 h exposure, the medium was removed from plates and the cells were washed with

phosphate buffered saline solution (PBS) twice. 25 μL PBS and 25 μL Steadylite plus™ mix were added to the cells and the plates were stored at room temperature in dark in 15–20 min in order for cell lysis and enzymatic reaction to occur. Cell lysates were transferred to white 96 well microtiter plates and the luciferase activity in each cell was measured by a luminometer (FLUOstar® Omega). Bio-TEQ of the soil extracts were calculated from the concentration-response curves by relating the luciferase induction potency of the extracts to that of the 2,3,7,8-TCDD standard (Larsson et al., 2013, 2018b). Chem-TEQ of the soil extracts were calculated as the sum of the measured concentration of each PAC multiplied by its assigned assay specific REP value (Lam et al., 2018a; Larsson et al., 2014, 2012) (Table S1). Chem-TEQs were then compared with the bio-TEQs to determine if targeted chemical analysis was able to fully explain the AhR-activity in the extract.

2.3.4. Full-scan chemical analysis with GC-HRMS

The samples analyzed for PACs using GC-MS were also injected to GC-HRMS (Q Exactive™ GC Orbitrap®, Thermo Scientific, Bremen, Germany). The S1R1 sample was lost prior to the non-targeted analysis and was excluded from the analysis. The GC column was Thermo Scientific TG-5SilMS capillary column (30 m × 0.25 mm, 0.25 μm film thickness). 1 μL of extract was injected in splitless mode. The Orbitrap® MS was operated in EI mode at 70 eV using full scan mode with m/z range of 53.4–800 at the 60,000 FWHM at m/z 200 resolution. Helium was used as the carrier gas. The GC oven followed the temperature program described elsewhere (Schönlau et al., 2019a). The injector, MS transfer line, and ion source temperatures were 250, 280, and 280 °C, respectively (see Fig. 1).

2.3.5. Workflow to tentatively identify unknown compounds

Peak-by-peak analysis of each sample extract following analysis with GC-HRMS was performed manually. The tentative identification workflow consisted of four steps (Fig. 2) and was based on a previous study (Cha et al., 2019) with some modifications:

- *First*, for a positive peak detection, peak to noise ratio of 10:1 was required. No peaks were observed in the injected toluene blank. Candidate compounds at this stage fell under the level 5 category in the Schymanski identification confidence level (Schymanski et al., 2014), hereafter referred as "confidence level".
- *Second*, after peak detection, mass fragments of the peaks were compared to the 2017 NIST EI library database. Tentatively identified candidate compounds with a matching threshold ≥700 and reverse matching threshold ≥750 to the NIST EI library were selected for the next step (confidence level 3).
- *Third*, only aromatic compounds with at least three rings and not included in the list of targeted chemicals were selected. The selection of aromatic compounds with at least three rings was based on criteria used in previous publications, where the criteria were justified by observed AhR-activities of PAHs with three or more rings (Cha et al., 2019; Louiz et al., 2008; Xiao et al., 2016). Tentatively identified compounds fell under confidence level 2a.
- *Fourth*, once a tentatively identified compound was identified, information about its bioassay activity was gathered from PubChem ("PubChemDocument, 2019) and from the literature. To further increase the identification, AhR-activity information of the tentatively identified compounds was supplemented with its environmental occurrence using the Google Scholar function in the U.S. Environmental Protection Agency's (U.S. EPA's) CompTox dashboard (Williams et al., 2017). Environmental occurrence was divided between soil and non-soil occurrences with the assumption that tentatively identified compounds previously found in soil had higher identification confidence than those that were identified not in soil. Tentatively identified compounds were then categorized into four groups in descending order of priority (Fig. 2). Group 1 consisted of tentatively identified compounds that satisfied all three criteria (confidence level 2b). Groups 2 and 3 consisted of tentatively identified compounds that satisfied any two and one criteria, respectively, and group 4 consisted of tentatively identified compounds that did not satisfy any criteria. Tentatively identified compounds in groups 2–4 fell under the confidence level 2a. Finally, peak area of the tentatively identified compounds were divided by the peak area of d_{10}-PHE to determine the normalized abundance of the candidate compounds in soil.

Table 1
Concentration of summed PAC groups in the soil samples (mg kg^{-1} d.m.) and their respective chem- and bio-TEQs in the soil extracts (pg g^{-1}). Chem-TEQs of the soil extracts were based on 62 PACs with assigned REP values. Bio-TEQs of the soil extracts were based on EC_{25} values. S1 data was provided as average ± standard deviation. Detailed information can be found in the SM.

PAC group	Concentration in sample (mg kg^{-1} d.m.) and chem- and bio-TEQs in extract (pg g^{-1})				
	S1	S2	S3	S4	S5
$\sum_{16\ PAHs}$	680 ± 80	190	550	73	20
$\sum_{Other\ 19\ PAHs}$	270 ± 28	49	250	31	8.1
$\sum_{Alkyl\text{-}PAHs}$	57 ± 10	27	25	4.6	1.5
$\sum_{Oxy\text{-}PAHs}$	56 ± 4.3	31	54	6.7	2.6
$\sum_{NPACs}$	91 ± 5.2	17	63	15	3.9
$\sum_{OPACs}$	6.1 ± 0.61	1.5	4.5	0.77	0.23
$\sum_{SPACs}$	4.9 ± 0.68	2.5	1.9	0.51	0.11
$\sum_{78\ PACs}$	1200 ± 120	320	950	130	35
Chem-TEQ	**S1**	**S2**	**S3**	**S4**	**S5**
$\sum_{62\ PACs}$	140,000 ± 15,000	38,000	200,000	20,000	5200
Bio-TEQ	**S1**	**S2**	**S3**	**S4**	**S5**
EC_{25}	360,000 ± 130,000	62,000	220,000	22,000	5400

2.3.6. Quality assurance/quality control (QA/QC)

For targeted chemical analysis, triplicates of certified reference material ERM®-CC013a (BAM, Berlin, Germany) soil containing 16 polycyclic aromatic hydrocarbons listed in the U.S. EPA priority pollutants (16 PAHs) were spiked with IS and extracted in the same way as the samples. RS was spiked prior to GC-MS. The average results fell within the certified values for the 16 U.S. EPA PAHs, with the exception of naphthalene (NAP), which was >30%. Relative standard deviations (RSD) of the triplicates were ≤15%. Procedural blanks were included in the targeted analysis.

Five to six point calibration curves were used to quantify the target PACs. The RSD of the relative response factor (RRF) values of the target PACs were <15% for PAHs and <25% for alkyl-PAHs, oxy-PAHs, NPACs, OPACs, and SPACs. Quantification standard was injected every tenth sample. Target PACs concentrations were calculated based on isotopic dilution method using the corresponding IS. RRF values for compounds with no matching IS were calculated using the compound nearest in retention time. Limit of detection (LOD) was calculated either based on the average response measured in six blanks plus three times standard deviation or based on the lowest point of the calibration curve, if there was no response detected in the blanks. Samples with concentrations exceeding the highest point of the calibration curve were diluted and reanalyzed. Levels of blank and <LOD analytes were substituted with LOD/ $\sqrt{2}$ in all data evaluation.

For bioassay analysis, bio-TEQ calculations were only applied if the plates had a standard deviation ≤14% within triplicates, a 2,3,7,8-TCDD EC_{50} between 8 and 18 pM, and a 2,3,7,8-TCDD maximal induction factor >6. LOD was calculated based on the mean luciferase activity of DMSO control triplicates plus three times the standard deviation (Larsson et al., 2018b).

QA/QC of the full-scan chemical analysis with GC-HRMS were already embedded in the identification workflow as described in section 2.3.5.

Table 2
Source tracing of PACs in the soil samples based on PAHs diagnostic ratios. Boundary values were obtained from previous studies (Budzinski et al., 1997; Hindersmann et al., 2020; Yunker et al., 2002). Specific differentiation on pyrogenic source (*i.e.,* liquid vs. solid) was not made. Pyrogenic source was observed for all samples, with the exception of S2 where two diagnostic ratios indicated potential contribution from petrogenic source.

Diagnostic Ratio	Boundary Values	S1R1	S1R2	S1R3	S2	S3	S4	S5
PHE/ANT	Pyrogenic <10 < Petrogenic	5.1	5.6	3.3	17	4.1	5.5	7.3
ANT/(PHE + ANT)	Petrogenic < 0.1 < Pyrogenic	0.16	0.15	0.23	0.055	0.20	0.15	0.12
FLT/PYR	Petrogenic < 1 < Pyrogenic	1.3	1.3	1.3	1.5	1.0	1.2	1.2
FLT/(FLT + PYR)	Petrogenic < 0.4 < Pyrogenic	0.57	0.57	0.56	0.60	0.50	0.54	0.55
BbjkF/(BbjkF + BeP)	Petrogenic < 0.5 < Pyrogenic	0.74	0.74	0.73	0.75	0.78	0.73	0.73
BaA./(BaA + CHR)	Petrogenic < 0.2 < Pyrogenic	0.59	0.60	0.60	0.52	0.63	0.59	0.58
IcdP/(IcdP + BghiP)	Petrogenic < 0.2 < Pyrogenic	0.56	0.57	0.57	0.59	0.59	0.55	0.52

3. Results and discussion

3.1. PAC profiles in soil samples

Total concentrations of PACs in soil samples ranged from 35 mg kg^{-1} dry matter (d.m.) (S5) to 1200 ± 120 mg kg^{-1} d.m. (S1) (Tables 1 and S3). Samples S1–S4 were collected in areas where charcoal storage used to exist (Fig. S1), which can explain the higher total concentrations of PACs in S1–S4 relative to sample S5 (Tables 1 and S3). Except for 6-ethylchrysene, 9,10-dihydrobenzo[*a*]pyren-7(8H)-one, and acridone, all target PACs were detected in at least one sample. Fluoranthene (FLT) was the predominant PAC in S1 and S5, while phenanthrene (PHE), indeno [1,2,3-*cd*]pyrene, and11H-benzo[*a*]carbazole (BaCARB) showed the highest concentrations in S2, S3, and S4, respectively. In every sample, the sum of the 16 PAHs ($\sum_{16\ PAHs}$) was the predominant PAC group relative to all measured PACs ($\sum_{78\ PACs}$), followed by the sum of non-16 parent-PAHs ($\sum_{Other\ 19\ PAHs}$) (Table 1). All 19 other PAHs were detected in all samples (Table S3). Compounds in this group included dibenzo-[*b*,*k*]fluoranthene, dibenzo[*a*,*e*]pyrene (DBaeP), and naphtho [2,3-*a*]pyrene, which have molecular weights of 302 a.m.u. and are known to be bioactive (Vondráček et al., 2020). These results showed the diversity of PACs found in soil samples collected at the site.

Contributions of alkyl- and oxy-PAHs to the total PAC concentrations were similar in all samples (3–6% and 5–7%, respectively), with the exception of S2, where the proportion of alkyl- and oxy-PAHs relative to the total PAC concentrations were higher than in other samples (8 and 10%, respectively). 2-methylphenanthrene was the most abundant alkyl-PAH in all samples, except for in S3, where 2-methylchrysene (2-MCHR) was the most predominant alkyl-PAH. For oxy-PAHs, 1,4-chrysenequinone + naphthacene-5,12-dione were the highest measured oxy-PAHs in S1 and S4, while anthracene-9,10-dione, 6H-benzo[*cd*] pyren-6-one, and 7H-benzo[*de*]anthracen-7-one were the predominant oxy-PAHs in S2, S3, and S5, respectively. Oxy-PAHs were commonly detected in contaminated soils (Arp et al., 2014; Bandowe et al., 2011; Lundstedt et al., 2014) and their presence may be due to occurrence in the original source and/or due to *in situ* formation by bacterial degradation of parent PAHs in soil (Lundstedt et al., 2007).

OPACs and SPACs contained six targeted compounds and only made up small percentage (up to 1.3%) of all PACs, with dinaphtho[2,1-*b*:1′,2’-]furan (DNF) and dibenzothiophene (DBT) as the predominant OPAC and SPAC in all samples, respectively. DBT is of concern because this compound is known to be active in the T47D*luc* cell-based estrogen response chemically activated luciferase expression (ER-CALUX) assay (Brinkmann et al., 2014).

Source tracing of PACs in the soil samples was performed based on seven diagnostic ratios of PAHs (Table 2) as well as on the distribution of NAP and DBT and their corresponding alkyl-compounds (Fig. S2). The ratio of some PAHs were calculated and was compared to boundary values as suggested in previous source tracing studies (Budzinski et al., 1997; Hindersmann et al., 2020; Yunker et al., 2002). Based on these analyses, PACs at this site likely originated from pyrogenic sources. However, diagnostic ratio of S2 also indicated potential contribution of PACs from petrogenic sources (Table 2), as indicated by a high ratio of mono (C1)-alkylated naphthalene compared to naphthalene. A previous study reported occurence of petroleum products in the area where sample S2 was collected (Hifab, 2015), which supports this source tracing result. Furthermore, the profile of NAP and alkyl-NAPs also indicated potential contribution from petrogenic source in S2 (Fig. S2). In this study, the source tracing assessment with alkyl-PAHs was performed only with a few isomers due to limited number of alkyl-PAHs in the target list of PACs; thus, future studies may consider incorporating the analysis of all possible isomers of alkyl-PAHs (Hindersmann et al., 2020) and using positive matrix factorization modelling (Norris et al., n. d.) to obtain more reliable information on the contamination origin of PACs in the soil samples.

Concentration data in this study were compared to PACs in soil samples collected from sites with similar land use as in our case study (Table 3). Levels of $\sum_{16\ PAHs}$ in this study were up to three orders of magnitude higher compared to concentrations found in topsoil next to an aluminum smelting factory in China (Hu et al., 2017), but were in the same range as concentrations found in soil from another Swedish site (Arp et al., 2014). Samples S1–S4 were collected from areas that were previously used for charcoal storage (Fig. S1). The $\sum_{16\ PAHs}$ concentrations in these samples were similar (S4) or up to two-fold higher (S1–S3) than what were measured at coal mine districts and a transportation facility in Asia (Huang et al., 2016; Liu et al., 2012; Mizwar and Trihadiningrum, 2015; Wang et al., 2010). Given that charcoal was produced through pyrolysis, we hypothesized that the PAHs from charcoal would be higher than PAHs from coal, which could explain the difference in PAH 16 concentrations between the different sites (Table 3). Sample S5 was collected from an area where a sawmill was located and contained lower concentration of $\sum_{16\ PAHs}$ than S1–S3, but

Table 3
Comparison of PAH 16 concentrations in this study to PAH 16 concentrations in soils from other sites in the world with similar usage to that at the Sundsvall municipality. NA refers to information not available.

Location	Site usage	Concentration range (ng g^{-1})	Sampling depth (cm)	References
Tapin District, Indonesia	Coal transporting facility	12,000–55,000	5–20	Mizwar and Trihadiningrum (2015)
Tiefa, China	Coal mine district	5–5600	0–150	Liu et al. (2012)
Anhui Province, China	Coal mine district	130–3500	0–25	Wang et al. (2010)
Guangxi, China	Coal mine district	80–4300	0–10	Huang et al. (2016)
China	Aluminum smelting	140–620	0–10	Hu et al. (2017)
Riksten, Sweden	Charcoal and wood tar production site	270–280,000	NA	Arp et al. (2014)
Sundsvall municipality, Sweden	Charcoal storage and sawmill	20,000–680,000	0–20	This study

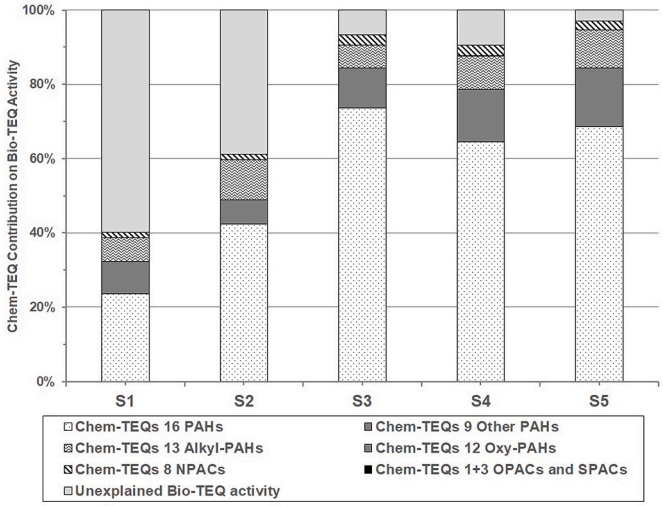


Fig. 3. Relative contribution (%) of chem-TEQs to bio-TEQ activities based on the concentrations and REP values of 62 PACs in the soil extracts (Tables 1, S1, and S4) and the EC_{25} of the bio-TEQs (Tables 1 and S5). Chem-TEQ for sample S1 was based on the average of the S1 replicates.

other samples collected within this area had concentrations in the same range as S1–S3 (i.e. >100,000 ng g^{-1}; data not shown in this study). High concentrations of PAHs in soil at sawmill sites often origin from wood impregnation with creosote; however, there is no record of using creosote at this site, and the main reason for the contamination at this area is still unknown.

3.2. AhR-activity characterization of soil extracts

Chem-TEQs were calculated based on bioassay specific REP values at the 25% effective concentration (EC_{25}) and concentrations of 62 targeted PACs with known REP values (Tables 1 and S4), while bio-TEQs were calculated based on EC_{25} values of the bioassay analysis of the soil extracts (Tables 1 and S5). Chem-TEQs were able to explain between 40% and 97% of bio-TEQs in the soil extracts (Fig. 3). The bio-TEQ in S1 sample (360,000 ± 130,000 pg g^{-1} d.m.) was the highest among all soil samples, while the bio-TEQ in S5 sample (5400 pg g^{-1} d.m.) was the lowest (Tables 1 and S5). These observed bio-TEQs were higher than some of the previous measurements of bio-TEQs in contaminated soils in Sweden (Andersson et al., 2009; Larsson et al., 2013, 2018b). Chem-TEQs were able to explain 93%, 91%, and 97% of the bio-TEQs in S3, S4, and S5, respectively (Fig. 3). Meanwhile, chem-TEQs were only able to explain 40% and 61% of bio-TEQs in S1 and S2, respectively (Fig. 3). When comparing the S1 replicates, bio-TEQs from S1R1 and S1R3 were comparable to each other, but the bio-TEQ of S1R2 was less than half of the bio-TEQs from S1R1 and S1R3 (Tables 1 and S5). This variation can potentially be attributed to the heterogeneity of the soil samples.

Of the target compounds, chem-TEQs from 16 PAHs were the main contributor to total chem-TEQs in all soil extracts (Fig. 3). The only exception was the chem-TEQ contribution of alkyl-PAHs in S2, which was higher than the contribution from other 19 PAHs. The contributions from oxy-PAHs, OPACs, and SPACs were negligible in all samples (Fig. 3 and Tables 1 and S4).

While 16 PAHs were the highest contributors of the bio-TEQs (20–74%), this and other studies showed that 16 PAHs alone were not enough to explain the observed bio-TEQs of soil extracts (Fig. 3) (Andersson et al., 2009; Larsson et al., 2018b). Benzo[*b*]- and benzo[*k*]-fluoranthene (BbF and BkF, respectively) were two of the 16 PAHs that highly contributed to the chem-TEQs (Table S4). BjF, another isomer of BbF and BkF, also had high contribution toward the calculated

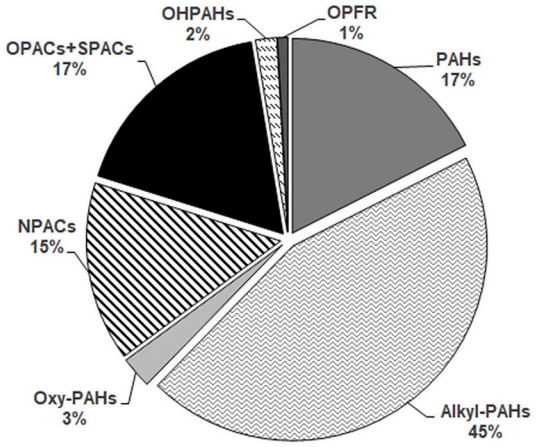


Fig. 4. Profile of tentatively identified compounds found in the soil samples based on full-scan screening with GC-HRMS. OHPAH and OPFR refer to hydroxylated-PAH and organophosphate flame retardant, respectively.

chem-TEQs (Table S4), indicating the AhR-activity of the benzofluoranthene isomers. For alkyl-PAHs and NPACs, 3- and 2-MCHR, and dibenzo[*a,h*]acridine (DBahACR), were compounds with significant chem-TEQ contributions toward bio-TEQs in all extracts.

The gaps between chem- and bio-TEQs in the extracts could be attributed to several factors. First, the chem-TEQs were only based on 62 of the 78 PACs included in the chemical analysis. The remaining 16 compounds did not have assigned REP values and could potentially induce AhR-activity in the extracts. Of these compounds, benzo[*b*]chrysene, DBaeP, and DNF have been shown to induce AhR-activity in previous studies (Larsson et al., 2018a; Machala et al., 2001; Vondráček et al., 2017). Second, mixture interactions, such as synergistic or antagonistic effects, could also contribute to the difference between chem- and bio-TEQs (Larsson et al., 2018b). Thirdly, metals have been known to induce AhR-activity (Elbekai and El-Kadi, 2004; Korashy and El-Kadi, 2005), and the presence of metals have previously been detected at this site (Tiberg et al., 2019). However, it is unknown whether the extraction method used in this study was able to extract metals. Alternatively, the presence of compounds not included in the list of targeted PACs could also contribute to the gap observed between chem- and bio-TEQs and was thus further explored using non-targeted analysis.

3.3. Full-scan analysis of soil extracts

A total of 114 unique compounds were tentatively identified following non-targeted analysis using the full-scan screening of GC-HRMS (Table S6). Of the 114 tentatively identified compounds, alkyl-PAHs made up the highest percentage of candidate compounds (45%), followed by PAHs (17%), and OPACs and SPACs (14%), which were counted together (Fig. 4 and Table S6). The presence of alkyl-PAHs highlighted the need for screening of more alkyl-PAHs in the environment, particularly because some alkyl-PAHs (e.g., 1-, 2-, 3-MCHR, 7-methylbenzo[*a*]anthracene, and 7-methylbenzo[*a*]pyrene) have been found to be more toxic than their corresponding parent compounds (Lam et al., 2018a). Inclusion of alkyl-PAHs may also help with source tracing efforts (Andersson and Achten, 2015; Hindersmann et al., 2020). The full-scan analysis also suggested the tentative presence of triphenylphosphine oxide, an organophosphate flame retardant, and 9H-fluoren-9-ol and 4,5-dihydro-3H-benzo[*cd*]pyren-5-ol, which are degradation products of PAHs. Higher number of candidate compounds were found in S1 and S2 samples (Table S6), indicating the greater presence of other compounds that could contribute to the bio-TEQs in these samples.

Table 4
List of tentatively identified compounds by GC-HRMS screening and included in Group 1. References on AhR- and other bioactivity, and soil and other environmental occurrence are provided in the SM (Table S6).

Tentatively identified compound not detected
Tentatively identified compound area/d_{10}-PHE area: 0 – 1
Tentatively identified compound area/d_{10}-PHE area: 1 – 10
Tentatively identified compound area/d_{10}-PHE area: 10 – 100
Tentatively identified compound area/d_{10}-PHE area: 100 – 1000

Compound	PAC group	CAS No.	MW (g/mol)	Sample S1R2	S1R3	S2	S3	S4	S5
Dibenzofuran	OPAC	132-64-9	168.19						
9H-xanthene	OPAC	92-83-1	182.22						
4-azapyrene	NPAC	194-03-6	203.24						
Benzo[*c*]acridine	NPAC	225-51-4	229.27						
Dibenzo[*a,c*]acridine	NPAC	215-62-3	279.3						
Benzo[*b*]naphtho[2,1-d]thiophene	SPAC	239-35-0	234.3						
1-methylphenanthrene	Alkyl-PAH	832-69-9	192.25						
3,6-dimethylphenanthrene	Alkyl-PAH	1576-67-6	206.28						
1-methylpyrene	Alkyl-PAH	2381-21-7	216.28						
Retene	Alkyl-PAH	483-65-8	234.3						
11H-benzo[*b*]fluorene	PAH	243-17-4	216.28						
Benzo[*b*]triphenylene	PAH	215-58-7	278.3						

3.3.1. Group 1

Twelve compounds were tentatively identified and included in Group 1 (confidence level 2 b), which constituted compounds that were known to be AhR-active, and have previously been detected in the environment, including in soil samples (Tables 4 and S6). Dibenzofuran, 9H-xanthene, benzo[*c*]acridine, 1-methylphenanthrene, 1-methylpyrene, and 11H-benzo[*b*]fluorene were tentatively identified in all samples. The AhR-activities of these compounds have previously been assessed (Barron et al., 2004; Dietrich and Kaina, 2010; Hawliczek et al., 2012; Hinger et al., 2011; Sun et al., 2014), but the assay specific REP-values of these compounds have not been assigned yet and hence, their chem-TEQ contributions are currently unknown. The remaining six compounds in Group 1 were tentatively identified in some samples, which could help explain the difference in unexplained bio-TEQ fractions between these samples. 3,6-dimethylphenanthrene was tentatively identified in five of the six samples, while retene was tentatively identified in three of the six samples. 4-azapyrene was only detected in S2 and dibenzo[*a,c*]acridine was only found in S3. The latter being an isomer of DBahACR, which has one of the highest REP-values, making dibenzo[*a,c*]acridine a potential AhR agonist too. Based on the profile of compounds in Group 1, there were no unique compounds that were found in S1 and S2 (which had 60% and 39% unexplained bio-TEQ fractions, respectively) that were not also found in S3, S4, and S5 (which had 7%, 9%, and 3% unexplained bio-TEQ fractions, respectively) (Fig. 3). Of note, the normalized abundance of 1-methylpyrene in S1R2, S1R3, and S2 were higher than in S3, S4, and S5 (Tables 4 and S6). The difference in 1-methylpyrene abundance among samples may explain some of the difference in the unexplained bio-TEQ fractions between these samples. The highest normalized abundance of the tentatively identified compounds in Group 1 was for retene in S3 (Tables 4 and S6).

3.3.2. Group 2

Tentatively identified compounds in Group 2 (confidence level 2a) (Table S6) have no known AhR-activity, but have been previously found in the environment and in soil, except for acenaphtho[1,2-b]pyridine (Barron et al., 2004). Five alkyl-PAHs and one PAH in this group were known to not be AhR-active (Table S6). Two compounds, 9H-fluoren-9-ol (tentatively identified in all samples) and triphenylphosphine-oxide (tentatively identified in S4 and S5), belong to groups of compounds that were not included in the targeted chemical analysis in this study. The most abundant tentatively identified compound of Group 2 was an OPAC, benzo[*kl*]xanthene (tentatively identified in all samples), followed by naphtho[2,1,8,7-klmn]xanthene (tentatively identified in S1R2, S1R3, S2, and S3) (Table S6).

3.3.3. Group 3

A total of 22 compounds were tentatively identified in Group 3 (confidence level 2a) and all have unknown AhR-activity and soil occurrence (Table S6). Therefore, their inclusion in this group was based either on their environmental occurrence alone. Similar to Group 2, alkyl-PAHs were the predominant tentatively identified compounds. Only one compound was tentatively identified in S5, indicating lower degree of contamination in this sample, which was in agreement with the low bio-TEQ of S5 (Table S5) and low amount of target PACs ($\sum_{78PACs}$) (Tables 1 and S3). Compounds such as iminostilbene, phenanthro[4,3-b]thiophene, and anthrone were only tentatively identified in S1 and S2, thus these likely contributed to the unexplained bio-TEQ fractions in these samples.

3.3.4. Group 4

Tentatively identified compounds in this group had no information on any of the prioritization criteria (Table S6), thus more research on their AhR-activity, and environmental and soil occurrences are needed. 4,5-dihydro-3H-benzo[*cd*]pyren-5-ol, and a few alkyl-PAHs and PAHs were only identified in S1 and S3 and thereby are potential candidates for AhR-activity screening. The normalized abundances of the compounds in this group were also low relative to other compounds in the other groups, with the exception of a few compounds in S1 and S3.

3.4. Implications

The results in this study highlighted both the success and failure of targeted chemical analysis to explain the bioactivity of soil extracts contaminated by PACs. For three samples (S3–S5), targeted chemical analysis was enough to explain the induced AhR-activity of the soil extracts. Such high degree of explanation could only occur because the target list of chemicals were expansive and included multiple compounds beyond the 16 PAHs, further underscoring the need to include other PACs beyond the 16 PAHs in future studies (Andersson and

Table 5
Tentatively identified compounds found in S1R2, S1R3, and/or S2, but not in S3, S4, or S5, and, thus, likely contributed to the unexplained bio-TEQ fractions in these samples. References on AhR-activity and soil and other environmental occurrence are provided in the SM (Table S6). Legend is provided in Table 4.

Compound	PAC Group	CAS No.	MW (g/mol)	Sample S1R2	S1R3	S2
Phenanthridine	NPAC	229-87-8	179.22			
3-methylcarbazole	NPAC	4630-20-0	181.23			
9,10-dihydroacridine	NPAC	92-81-9	181.23			
Iminostilbene	NPAC	256-96-2	193.24			
3,6-dimethylcarbazole	NPAC	5599-50-8	195.26			
9-ethyl-9H-carbazole	NPAC	86-28-2	195.26			
1,7-dimethyldibenzothiophene	SPAC	89816-53-5	212.31			
Phenanthro[4,3-b]thiophene	SPAC	195-68-6	234.3			
8-methylbenzo[b]naphtho[2,3-d]thiophene	SPAC	24964-07-6	248.3			
4,5-dihydro-3H-benzo[*cd*]pyren-5-ol	OHPAH	NA	258.3			
Anthrone	Oxy-PAH	90-44-8	194.23			
6H-benz[*de*]anthracen-6-one	Oxy-PAH	NA	230.26			
9-methylfluorene	Alkyl-PAH	2523-37-7	180.24			
9H-fluorene-9-carbonitrile	Alkyl-PAH	1529-40-4	191.23			
1-methyl-9,10-dihydrophenanthrene	Alkyl-PAH	NA	194.27			
9,9-dimethylfluorene	Alkyl-PAH	4569-45-3	194.27			
9-ethylfluorene	Alkyl-PAH	2294-82-8	194.27			
9-anthracenecarbonitrile	Alkyl-PAH	1210-12-4	203.24			
9-(cyanomethylene)fluorene	Alkyl-PAH	4425-74-5	203.24			
9-ethenylanthracene	Alkyl-PAH	2444-68-0	204.27			
2,7-dimethylphenanthrene	Alkyl-PAH	1576-59-8	206.28			
2-ethylanthracene	Alkyl-PAH	52251-71-5	206.28			
9-ethyl-10-methylanthracene	Alkyl-PAH	19713-49-6	220.31			
9-isopropylanthracene	Alkyl-PAH	1498-80-2	220.31			
9-ethyl-7H-benzo[*de*]anthracene	Alkyl-PAH	NA	244.3			
4,12-dimethylbenz[*a*]anthracene	Alkyl-PAH	35187-19-0	256.3			
6,12-dimethylbenz[*a*]anthracene	Alkyl-PAH	568-81-0	256.3			
3-methylperylene	Alkyl-PAH	24471-47-4	266.3			
10-methylbenzo[*a*]pyrene	Alkyl-PAH	63104-32-5	266.3			
1-phenylpyrene	Alkyl-PAH	5101-27-9	278.3			
1,2,3,3a,4,5-hexahydropyrene	PAH	5385-37-5	208.3			
5,6-dihydrochrysene	PAH	2091-92-1	230.3			
8H-indeno[2,1-b]phenanthrene	PAH	241-28-1	266.3			
Pentacene	PAH	135-48-8	278.3			

Achten, 2015). For the remaining samples, targeted chemical analysis was only able to explain a fraction of the AhR-activity. This result demonstrated the need for high resolution mass spectrometer (HRMS) instruments to identify unknown compounds in soils that can induce high bioassay activities, thereby posing potential risks to the environment and human health.

Our study also highlighted the importance of a prioritization strategy to help reduce the data complexity as a result of HRMS analysis. The prioritization strategy also helped to tentatively identify a list of compounds that may be considered for inclusion in future regular screening studies (Table 4) and another list of tentatively identified compounds that could potentially explain the unexplained bio-TEQ fractions in S1 and S2 extracts (Table 5). However, confirmation of the tentatively identified compounds must be performed. This study also only considered aromatic compounds amenable by GC-MS, but more polar compounds amenable to liquid chromatography-MS (LC-MS) in soil can also cause AhR-activity (Cha et al., 2021; Lee et al., 2020).

Findings in this study not only demonstrated the diverse presence of organic pollutants in soil samples collected at different locations at a contaminated site, but they also showed variation within sampling spots. S1 samples were collected from a very low volume of soil (20 × 20 × 20 cm^3) and the results in this study showed some heterogeneity in terms of both the presence and concentrations of different PACs and the potential AhR-activity of the soil replicates (Tables S3, S5, and S6). It is also important to note that in order to further assess the risk posed by PACs in contaminated soils, leaching and bioavailability analysis (Enell

et al., 2016; Titaley et al., 2020) should also be considered.

4. Conclusion

Soil samples from a contaminated site in Sweden were screened for the presence of PACs using targeted chemical analysis. The PAHs, including 16 PAHs, were the predominant PACs in the soil samples. Source tracing analysis indicated pyrogenic sources as likely major contributor of PACs at this site, although petrogenic sources also contributed to the contamination in one sample as indicated from a high ratio of alkylated naphthalene compared to naphthalene. The AhR-activity of the soil extracts was measured using the H4IIe-pGudluc 1.1 cells assay. PAHs dominated the chem-TEQs contribution to the bio-TEQs, although considerable chem-TEQs contributions from alkyl-PAHs was also observed. Full-scan screening of the samples using GC-HRMS was used to tentatively identify other potentially or known toxic compounds that could contribute to the AhR-activity of the sample extracts. This analysis revealed the presence of 114 tentatively identified compounds that were divided into four groups of priority based on their AhR-activity and their environmental occurrence, which was divided between soil and non-soil occurrences. The study also demonstrated the benefit of combining chemical MS-analyses with bioassays to characterize PAC profiles in environmental samples. The results from this study contribute to a better understanding of the gap that is typically observed between bio- and chem-TEQs and provide evidence for the usefulness of non-targeted HRMS analysis of samples from the terrestrial environment.

While unknown compounds have been tentatively identified in our application, confirmation using reference standards is needed and represents a limitation of the study. Analysis using LC could be considered in future studies to determine the presence of polar AhR-active compounds in the soil. The number of samples in this study was relatively small; thus, future studies would benefit from increased number of examined samples.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117910.

Credit author contribution statement

Ivan A. Titaley: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization, Monika M. Lam: Methodology, Formal analysis, Investigation, Writing – Review & Editing, Rebecca Bülow: Formal analysis, Investigation, Anja Enell: Validation, Resources, Writing – Review & Editing, Project administration, Funding acquisition, Karin Wiberg: Validation, Resources, Writing – Review & Editing, Project administration, Funding acquisition, Maria Larsson: Conceptualization, Methodology, Validation, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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