Accounting for interactions between PAHs in risk assessment of polluted soil 16-386

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Summary

Risk assessment of polluted soil in relation to levels of polycyclic aromatic hydrocarbons (PAHs) is today based on the measurement of 16 PAHs. It is however very well known that polluted soils contain a complex mixture of related compounds, including oxygenated (oxy-PAH), nitrated (N-PAC) and alkylated (me-PAHs) PAHs, which might be as potent toxicants as the PAHs, but are still not included in monitoring or risk assessment strategies. We show here that these compounds were found at relatively high levels in parks in Stockholm city and that pyrogenic processes were the main source of pollution. Moreover, a strong correlation was observed between the levels of these compounds and levels of PAHs. Assuming that no interaction occurs (so called mixture effects), this implies that the measurements of the 16 PAHs is enough to monitor the risk to human health. However, we show here for the first time, that oxy-PAHs and PAHs do interact, leading to unpredictable mixture effects in vitro and in vivo. This was shown for endpoints and markers for cancer potency/risk as well as for developmental effects on the cardiovascular system. We conclude that monitoring of only the PAHs in polluted soil is not enough to limit the risk of detrimental human health effects and due to the significant presence and interaction with related compounds such as oxy-PAHs, these should be included in strategies for environmental monitoring and human health risk assessment.

Background

The Swedish EPA (SEPA) has estimated that there are more than 80 000 contaminated sites in Sweden. Of these, more than 1200 may have a serious PAH contamination. The scientific basis for risk assessment of contaminated sites must be solid to permit sound and sustainable decisions. However, actual risk levels are often difficult to determine since contaminated areas generally are polluted by complex mixtures of contaminants, of which most never have been assessed. For PAHs and related compounds, only 16 of the large number of present compounds are routinely monitored. As it is impossible to test all combinations of PAHs in cancer bioassays, the risk estimation is based on the toxic equivalency factors (TEF)-model [1].

Results from our lab show that calculated TEF values do not correlate with the levels of DNA damage formed by complex PAH mixtures [2-4]. In some cases irreparable DNA damage was induced by samples containing low levels of toxic equivalents, indicating that the available TEF-scales are insufficient for predicting the cancer risk of complex PAH mixtures. This is in agreement with recent results from mammalian cell models [5, 6] and animals [7, 8]. Recent *in vivo* studies have also shown that the TEF concept cannot be used to predict the developmental effects of PAHs [9, 10]. The reasons behind the lack of correlation are numerous. PAHs are heterogeneous and their toxic mechanisms differ widely, and TEF-values do not account for interactions which might confer considerable effects. Tumor promoting and non-genotoxic PAHs lack TEF-values although they can act as potent co-carcinogens. Another factor is the toxic persistent transformation products of PAHs, oxy- and nitro-PAHs/N-PACs, which appear in significant amounts in soil but lack TEF values [11].

Our hypothesis is that interactions between different PAHs stand for a major toxic effect and that there is an urgent need to include these interactions in human risk assessment. The specific objective is:

To identify sensitive biological and chemical markers of toxicity for complex PAH mixtures that will improve the risk assessment of the mixtures.

This project was a continuation of a project that received support from ÅForsk 2014-2015 (Nr. 14-326).

PAH-analysis and identification of chemical markers

In this project 70 soil samples from 25 parks in urban Stockholm, collected for a project run by Stockholm's Miljöförvaltning in 2015, were analyzed. The PAH content in these soils was previously found to be above recommended guideline values for 8 of the 25 parks [12], but their complete contaminant profiles (PAHs and oxy- and nitro-PAHs) had not yet been determined. In this project we extended the analysis of the traditional PAHs to also include the highly potent dibenzopyrenes, methylated PAHs, oxy-PAHs and N-PACs (something that rarely has been done for urban soils) in order to obtain a comprehensive picture of the contaminant profiles. The type of PAHs found in the soil can reflect the type of activity taken place in the parks historically. Places where wood has been impregnated has proven to be dominated by pyrene and fluoranthene and in places where tar is produced, the proportion of oxy-PAHs and N-PACs are high. Similarly, pyrogenic and petrogenic sources also gives rise to specific profiles.

The PAHs analyzed in this project were:

- 21 PAHs that are normally analyzed in environmental samples.
- 11 oxy-PAHs. These have a higher polarity and higher water solubility and lower volatility than the PAHs, making them interesting to study because they are more easily dissolved in water.
- 7 methylated PAHs (PAH with methyl groups attached to the molecular structure). Some of these have been shown to have a high biological activity.
- 4 N-PACs (PAH with nitrogen in the molecular structure). These also have a higher polarity than the PAHs.

Analysis

From each park, there were up to 5 subsamples and each subsample comprised themselves of up to five samples. Each subsample was pooled into one sample which was then analyzed for the PACs. A dry weight determination of each pooled sample and a LOI determination were performed. Before the extraction internal standards were added to quantify the amount of PACs found in the samples. The soil samples were extracted using PLE (pressurized liquid extraction) with a mixture of hexane and acetone (1: 1). The extract was concentrated and further purified on a basic silica gel column. The column as eluted with dichloromethane and the eluate was concentrated and a solvent exchange to toluene as

done. The last step is to add a re-inventive standard to determine how well the extraction and purification of the sample has passed. Analysis was performed by GC / HRMS.

Table 1. Non-polar and polar PACs that were analyzed.

PAHer	**MePAH**	**oxy-PAH**	**N-PAC**
Naphthalene	2-Methylnaftalen	1-Indanone	Quinoline
Acenaphthylene	1-Methylnaftalen	1-Acenaphthenone	Benzo[h]quinoline
Acenaphthene	2,6-Dimethylnaftalen	9-Fluorenone	Acridine
Fluorene	2,3,5-Trimethylnaftalen	Anthracene-9,10-dione	Carbazole
Phenanthrene	1-Metylfenantren	Cyclopentaphenanthrenone	
Anthracene	3,6-Dimetylfenantren	2-Methylanthracenedione	
Fluorantene	2,3-Dimetylantracen	Benzo[a]fluorenone	
Pyrene		7H-Benz[de]antracen-7-one	
Benzo[a]anthracene		Benz[a]antracen-7,12-dione	
Chrysene		Naftacen-5,12-dione	
Benzo[b]fluoranthen		6H-Benzo[cd]pyren-6-one	
Benzo[k]fluoranthen			
Benzo[e]pyrene			
Benzo[a]pyrene			
Perylen			
Indeno[cd]pyrene			
Dibenz[a,h]antracene			
Benzo[g,h,i]perylen			
Coronen			
Dibenzo[al]pyrene			
Dibenzo[ai]pyrene			

Results

The results are currently being summarized in a manuscript, *Polycyclic aromatic compounds in urban soils of Stockholm City: occurrence, source apportionment and human health risk assessment* which is expected to be submitted for publication during the first half of 2019 (See dissemination of results on page 10). The results showed a large variation in PAC content in the different parks. Highest PAH levels were found for fluoranthene, up to 10 mg/kg soil. For oxy-PAHs, N-PACs and me-PAHs, 6H-benzo[cd]pyren-6-one (up to 2.3 mg/kg soil), carbazole (up to 0.42 mg/kg) and 1-methylphenanthrene (up to 380 mg/kg soil) were found at highest levels. In Figure 1, the sum levels of PAHs, oxy-PAHs, N-PACs and me-PAHs are shown. As can be seen, 7 parks (numbered) had clearly much higher PAC levels compared to the other. These were also among the parks that were identified as having PAH levels above recommended guidelines. We show here that these parks are also highly contaminated by oxy-PAHs. Based on this we conclude that oxy-PAHs should be included as a chemical marker in national and international monitoring programs of contaminate soils.

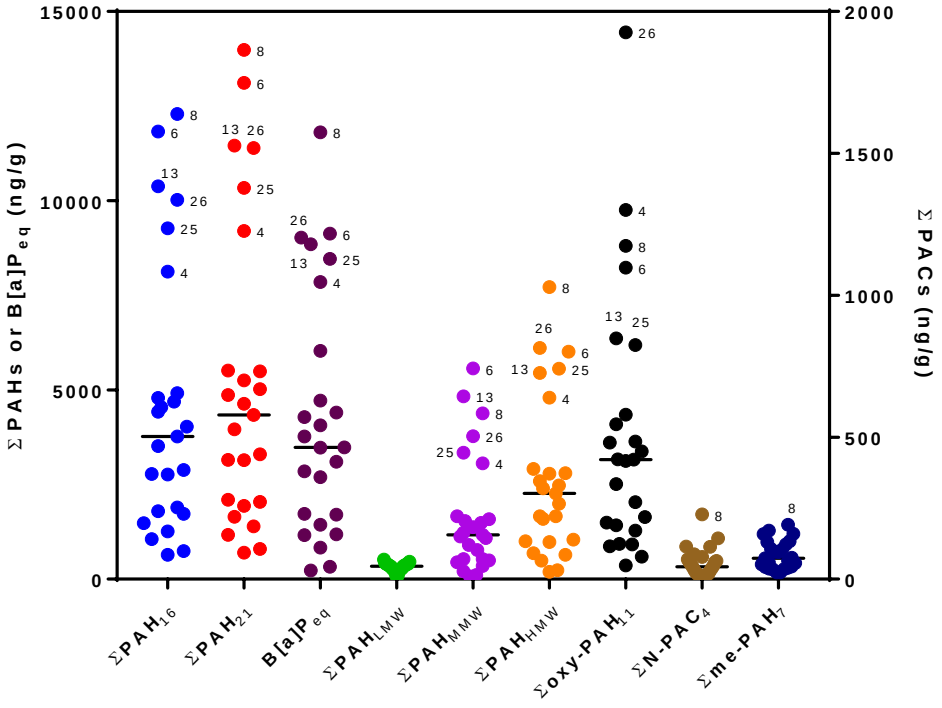

Figure 1. Distribution plots of sum values for PAHs, oxy-PAHs, N-PACs and me-PAHs. Each data point represents on park. Line indicates median value. The 7 most highly contaminated parks are indicated by their ID number.

Correlation analyses showed that sum levels of me-PAHs, oxy-PAHs and N-PACs strongly correlated with levels of PAHs, indicating similar sources. The same strong correlation was also observed for individual compounds, including the highly potent dibenzo[a,l]pyrene (DBP) with benzo[a]pyrene (BP) and the two oxy-PAHs 9-fluorenone (9-FLO) and benz[*a*]antracene-7,12-quinone (7,12-BAQ) with their parent PAH.

In an attempt to identify the major sources for the PAC contamination, we applied a set of established so called diagnostic ratios [13]. The results showed that the average diagnostic ratios in all parks were similar, with pyrogenic sources being dominant including petroleum emission, and biomass/coal combustion (Figure 2). The ∑pyrogenic PAHs/(∑pyrogenic PAHs + ∑petrogenic PAHs) ratios and the ∑LMW PAHs/∑HMW PAHs ratios did however suggest that petrogenic sources contributed in three parks (black rings). Biogenic sources, as assessed by levels of perylene, made a very smaller contribution, 1-3%, to the PAH load in the parks. In addition to these analyses, source apportionment and health risk assessments will be performed, taking the polar PACs into account.

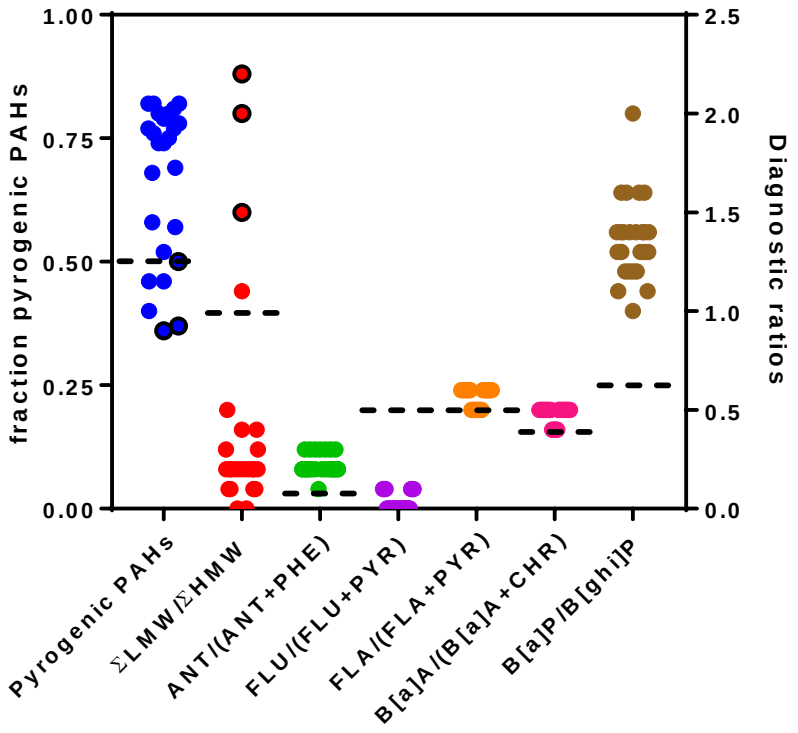

Figure 2. Plot of diagnostic ratios describing the major sources of contamination. For more details, see [13]. Dots marked with a black ring indicate soil samples with petrogenic sources. All other samples were dominated by pyrogenic sources.

Identification of biological effect markers

This part of the project aimed to identify biological effects or markers of effect that can be used to better study and understand the possible interaction effects of PACs in polluted soils and thereby improve the risk assessment. Our studies were performed in different mammalian in vitro test systems and in zebrafish embryos. The latter is an established alternative model to test for toxicity in the 3R efforts of Reducing and Replacing traditional animal testing. We have during the project published two articles, one manuscript is submitted and under review in the journal Environmental Pollution, and we are currently finalizing one manuscript (See dissemination of results on page 10).

Results

In the first paper, *Induction and inhibition of human cytochrome P4501 by oxygenated polycyclic aromatic hydrocarbons* [14], we determined that oxy-PAHs can be potent inhibitors of CYP450 and thereby affect the effects of PAHs and dioxins. CYP450 is the most important enzyme family for metabolizing PAHs, both in to less toxic and more toxic compounds. Our results also suggested that inhibition of CYP450 could be a biological marker for assessing interaction effects of PAHs in polluted soils. This is also supported by ours and other's earlier studies showing that inhibition of CYP450 often leads to synergistic (more than additive) mixtures effects, resulting in increased DNA damage and developmental effects.

In the second, *In vivo micronucleus screening in zebrafish by flow cytometry* [15], we for the first time applied a flow cytometry-based micronucleus assay in zebrafish. The results showed an as good sensitivity and specificity as the traditional microscopy-based assay in response to a number of established positive controls. We have now initiated to further use this method to screen for genotoxic *potencies* of oxy-PAHs and soil extracts in human cells and zebrafish.

In the submitted manuscript, *In vitro and in vivo genotoxicity of oxygenated polycyclic aromatic hydrocarbons*, we investigated the genotoxic potency of oxy-PAHs in human bronchial epithelial cells (HBEC). We found that all oxy-PAHs tested induced DNA strand breaks in a dose-dependent manner and some of the oxy-PAHs induced micronuclei formation, based on the methodology described in our above paper. Both of which are established markers for cancer potency. We further exposed zebrafish to single oxy-PAHs or a binary mixture with benzo[a]pyrene (B[a]P) and found both additive and non-additive mixture effects on the induction of DNA strand breaks. We concluded that oxy-PAHs elicit

genotoxic effects at similar or higher levels than B[a]P, which indicates that oxy-PAHs may contribute significantly to the total carcinogenic potency of environmental PAH mixtures.

The part related to developmental effects in zebrafish embryos is also ongoing. We have purchased software and equipment that will allow us to monitor and measure effects on the heart (hear beat rate, blood flow etc) in response to PAHs, oxy-PAHs and soil samples. In our manuscript entitled *Interactions between oxy-PAHs and PAHs: cardiac toxicity in zebrafish,* we show that some oxy-PAHs very potently affect the development of the heart and that presence of B[a]P significantly increases the toxicity. One way of visualizing this is by looking at the structure of the heart. As can be seen in Figure 3, the heart (labelled in green) of the control zebrafish (blank) is nicely "rounded" and compact, while the hearts of zebrafish exposed to a single oxy-PAH (6H-BPO) or the combination of an oxy-PAH and a PAH (6H-BPO and BaP) is more elongated and tube formed. These morphological effects lead to reduced heart rate, blood flow and as a result other development effects. The cardiovascular development in zebrafish is very similar to mammalian and human development and is an established alternative model to study mechanisms and effects of cardiotoxicity. This manuscript is currently being finalized and is planned to be submitted during the spring of 2019.

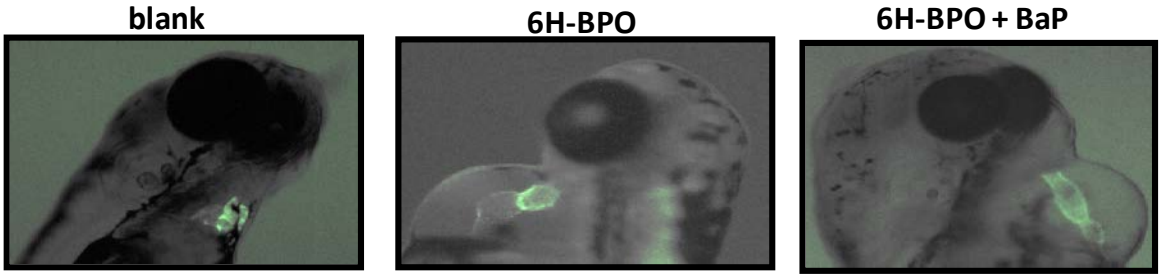

Figure 3. Heart morphology in zebrafish larvae (age 4 days) under control conditions (left panel), or in response to exposure to 6H-BPO (0.3 µM, middle panel) or 6H-BPO+BaP (0.3 + 1 µM, right panel) for 3 days.

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15. Le Bihanic, F., et al., *In vivo micronucleus screening in zebrafish by flow cytometry.* Mutagenesis, 2016. **31**(6): p. 643-653.

Dissemination of project results

Peer-reviewed research articles where the support from ÅForsk was acknowledged

Wincent E, Le Bihanic F and Dreij K. Induction and inhibition of human cytochrome P4501 by oxygenated polycyclic aromatic hydrocarbons. Toxicol Res 5, 788-799, 2016.

Le Bihanic F, Di Bucchianico S, Karlsson HL and Dreij K. *In vivo* micronucleus screening in zebrafish by flow cytometry. Mutagenesis 31, 643-653, 2016.

McCarrick S, Cunha V, Zapletal O, Vondráček J, Dreij K. In vitro and in vivo genotoxicity of oxygenated polycyclic aromatic hydrocarbons. Under review in Environmental Pollution.

Cunha V, Vogs C, Le Bihanic F, Dreij K. Interactions between oxy-PAHs and PAHs: cardiac toxicity in zebrafish. *Manuscript in preparation.*

Dreij K, Lundin L, Le Bihanic F, Lundstedt L. Polycyclic aromatic compounds in urban soils of Stockholm City: occurrence, source apportionment and human health risk assessment. *Manuscript in preparation.*

Presentations at international conferences where the support from ÅForsk was acknowledged

DEVELOPMENTAL AND CARDIOTOXICITY OF PAH/OXY-PAH MIXTURES ON ZEBRAFISH EMBRYOS. Virginia Cunha, PRIMO19, 30 June – 3 July, 2017, Matsuyama, Japan.

IMPACT OF PAH/OXY-PAH MIXTURES ON HEART DEVELOPMENT IN ZEBRAFISH. Virginia Cunha, SETAC 2018, 13-17 May, 2018, Rome, Italy.

ENVIRONMENTAL RISK OF CARCINOGENIC TOXICANTS AND EMERGING POLLUTANTS: TESTING THEIR INTERACTIONS IN VIVO AND IN VITRO. Carla Martins C, IMMR'18, 5-6 July, 2018, Peniche, Portugal.