Assessment of human hair as an indicator of exposure to organophosphate flame retardants. Case study on a Norwegian mother-child cohort

Assessment of human hair as an indicator of exposure to organophosphate flame retardants. Case study on a Norwegian mother child cohort

Agnieszka Kucharska[1,2], Enrique Cequier[3], Cathrine Thomsen[3], Georg Becher[3], Adrian Covaci[2], Stefan Voorspoels[1]

[1] VITO – Flemish Institute for Technological Research, Boeretang 200, 2400 Mol, Belgium

[2] University of Antwerp, Toxicological Centre, Campus Drie Eiken, Universiteitsplein 1, 2610 Wilrijk, Belgium

[3] NIPH – Norwegian Institute of Public Health, P.O. Box 4404, Nydalen, 0403 Oslo, Norway

Corresponding author

Stefan Voorspoels

Address: Flemish Institute for Technological Research (VITO), Boeretang 200, 2400 Mol, Belgium.

Tel. +32 14335021; e-mail: stefan.voorspoels@vito.be

Abstract

A major challenge of non-invasive human biomonitoring using hair is to assess whether it can be used as an indicator of exposure to Flame Retardants, such as Organophosphate Flame Retardants (PFRs), since the contribution of atmospheric deposition (air and/or dust) cannot be neglected. Therefore, the aim of this study was to evaluate the suitability of using human hair more thoroughly by comparison of (i) levels of PFRs in human hair (from 48 mothers and 54 children), with levels measured in dust and air in their respective households; and (ii) levels of selected PFRs in hair with the levels of corresponding PFR metabolites in matching urine samples collected simultaneously. Most PFRs (tri-*n*-butyl phosphate (TNBP), 2-ethyl-hexyldiphenyl phosphate (EHDPHP), tri-phenyl phosphate (TPHP), tri-*iso*-butyl phosphate (TIBP), and tris(2-butoxyethyl) phosphate (TBOEP)) were detected in all human hair samples, tris(2-ethylhexyl) phosphate (TEHP) and tris(1,3-dichloro-*iso*-propyl) phosphate (TDCIPP) in 93%, tri-cresyl-phosphate (TCP) in 69% and tris(2-chloroethyl) phosphate (TCEP) in 21% of the samples. Levels of individual PFRs ranged between < 1 and 3744 ng/g hair and were lower than in indoor dust from the participants' homes. Several statistically significant associations between PFR levels in human hair and PFR levels in house dust and/or air were found, e.g. Spearman correlation (r_S = 0.561, $p < 0.05$) between TBOEP in children's hair and in indoor air. Also, associations were found between TDCIPP in hair and its metabolite bis(1,3-dichloro-*iso*-propyl) phosphate (BDCIPP) in urine; they were stronger for children (e.g. Pearson correlation r_P = 0.475; p = 0.001) than for mothers (r_P = 0.395, p = 0.01). Levels of diphenyl phosphate (DPHP) in mothers' and children's urine were slightly correlated (r_S = 0.409, p = 0.008), suggesting similar sources of exposure. To the best of our knowledge, this is the first study with such design and our findings might help to understand human exposure to and body burdens of PFRs.

Keywords: hair analysis, organophosphate flame retardants, urine, exposure assessment

1. Introduction

Flame retardants (FRs), such as polybrominated diphenyl ethers (PBDEs), have received much attention lately, as widespread human exposure has been documented and concerns for health risks have increased based on human and animal research (DiGangi et al., 2010, Shaw et al., 2010). This has led to worldwide restrictions, bans and voluntary phase-outs of PBDE mixtures from the global market (Stockholm Convention). However, these actions have resulted in increased production and use of alternative FRs. Organophosphate flame retardants (PFRs) are considered as appropriate replacements for brominated FRs and are used in a wide range of polymers with varying type of side chains of the phosphate ester.

PFRs used as additives in materials are not chemically bonded, thus they may easily be released into the environment by abrasion and volatilization (Van der Veen and de Boer, 2012). In consequence, they have been widely detected in indoor air (Bergh et al., 2011, Cequier et al., 2014a, Saito et al., 2007), house dust (Araki et al., 2014, Ali et al., 2012, Brommer et al., 2012, Cequier et al., 2014a, Dirtu et al., 2012, Dodson et al., 2012, Garcia et al., 2007, Kim et at., 2013, Stapleton et al., 2009, Tajima et al., 2014, Van den Eede et al., 201), drinking water (Stackelberg et al., 2007), sediment and biota (Brandsma et al., 2014, Van der Veen and de Boer, 2012). In general, PFRs are more abundant in indoor than in outdoor environments which may significantly increase the extent of human exposure. However, data on human exposure and potential risks associated with PFRs are limited. Also, little is known about the toxicity of PFRs and only a limited number of reports about potentially adverse effects have been published. The toxicity for different PFRs has largely been investigated using animal experiments (WHO 2000, 1998, 1991a, 1991b). Neurotoxic properties after chronic exposure were shown for tri-*n*-butyl phosphate (TNBP) and tris(2-chloroethyl) phosphate (TCEP) (WHO 1998, 1991b). Further, the chlorinated PFRs such as tris(1,3-dichloro-*iso*-propyl) phosphate (TDCIPP) are suspected carcinogens (WHO 1998). Recently, elevated levels of PFRs in house dust were related to altered hormone levels and decreased sperm quality in men (Meeker et al., 2013a, Meeker and Stapleton, 2010). Concerns about human exposure and possible environmental effects are still rising and biomonitoring of such compounds is therefore needed. So far, assessments of human exposure to PFRs are mainly based on dust analysis and estimation of dust ingestion using mathematical modelling tools or based on analysis of PFR metabolites in human urine (Cequier et al., 2014b, Cooper et al., 2011, Van den Eede et al., 2013b, Van den Eede et al., 2011).

Human biomonitoring (HBM) is a useful tool in quantifying human exposure to environmental pollutants for risk assessment (Smolders et al., 2009). Blood is considered as an ideal matrix, but requires an invasive sampling procedure and suffers from ethical and practical constraints especially when children are involved. Also, non-persistent contaminants are best measured in urine and not in blood and in most cases, they are measured as metabolites (Esteban and Castaño, 2009, Rocket et al., 2004, Smolders et al., 2009). Recent studies are therefore also focusing on method development for biomonitoring using non-invasive matrices, such as hair, nails, urine, saliva, etc. (Alves et al., 2014).

Hair seems to be a promising non-invasive matrix that can measure external (by deposition from air and dust) and internal exposure (through contact with blood at the hair follicle/root) (Covaci et al., 2002). Since distinction between external and internal exposure to PFRs is impossible, latest research suggests hair as a biomarker that provides information about retrospective and integral exposure (Kucharska et al., 2015).

Hair has been used in several studies to assess exposure to POPs (Covaci et al., 2008, Tadeo et al., 2009, Zheng et al., 2011). A first positive and meaningful relationship between levels of PBDEs in hair and internal organs was reported by D'Havé et al. (2005). Also, Chen et al. (2015) observed positive correlations between Dechlorane Plus in human hair and serum. PFRs have been measured in human hair by Kucharska et al. (2014) giving first insights into analysis of these compounds from a small sample intake (200 mg). Although analysis of hair seems to be a good approach and alternative for invasive methods, there are still some concerns about data interpretation, especially for compounds such as PFRs and other FRs that are ubiquitous in indoor air and dust and thus potentially contribute to the PFR levels in hair by adsorption. Since this cannot be neglected, we suggested that untreated hair (i.e. no cleaning applied) might be a good indicator of retrospective and integrative exposure (Kucharska et al., 2015).

The present study aims to further evaluate the use of hair for exposure assessment to PFRs since this matrix is still underrated and not fully investigated. This research is also a continuation of our previous findings in which method development and validation for hair analysis of PFRs have been performed (Kucharska et al., 2014) and more insights into data interpretation were introduced (Kucharska et al., 2015). Consequently, we take this research one step further and explore human hair as a suitable matrix in biomonitoring of PFRs. To accomplish this, we have used several matrices relevant for indoor exposure and biomonitoring collected simultaneously in the frame of a mother child-cohort study conducted in the Greater Oslo area (Norway). These matrices are commonly considered as external contributors to the hair levels (dust/air) and internal indicators of exposure (urine). To this extent, we used methods that had been previously developed for the determination of the following PFRs: tris(2-ethylhexyl) phosphate (TEHP), tris(2-butoxyethyl) phosphate (TBOEP), triphenyl phosphate (TPHP), tris(1,3-dichloro-*iso*-propyl) phosphate (TDCIPP), 2-ethyl-hexyldiphenyl phosphate (EHDPHP), tri-*iso*-butyl phosphate (TIBP), tri-*n*-butyl phosphate (TNBP), tris(2-chloroethyl) phosphate (TCEP), tri-cresyl-phosphate (TCP) in human hair (Kucharska et al., 2014), dust and air (Cequier et al., 2014a). Also the following PFR metabolites: di-*n*-butyl phosphate (DNBP), bis(1,3-dichloro-*iso*-propyl) phosphate (BDCIPP) and diphenyl phosphate (DPHP), bis(2-butoxyethyl) phosphate (BBOEP) were measured in human urine (Cequier et al., 2014b). Using the outcome of these data, we attempted to assess the suitability of hair as an indicator of human exposure to PFRs.

2. Materials and methods

2.1 Chemicals and reagents

All solvents used for analysis were of analytical grade. *n*-Hexane (Hex), dichloromethane (DCM), toluene and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Ethyl acetate (EtAc) and formic acid were purchased from Biosolve (Valkenswaard, the Netherlands). All aqueous reagent dilutions were prepared with Milli Q water. Florisil, anhydrous sodium sulfate (Na_2SO_4), nitric acid (HNO_3, 65%), hydrochloric acid (HCl, 37%), and concentrated sulfuric acid (H_2SO_4, 98%) were purchased from Merck (Darmstadt, Germany). Silica gel was from J.T Baker Chemicals (Deventer, The Netherlands). All neat PFR standards except TIBP (99% purity, from Chiron) were purchased from Sigma-Aldrich (Munich, Germany): TEHP (purity 98%), TBOEP (94%), TPHP (99%), TDCIPP (96%), TCPP (68%), EHDPHP (92%), TNBP (98%), TCEP (97%), and TCP (90%). Deuterated PFRs were used as ISs: TPHP-d_{15} was purchased from Sigma-Aldrich, TNBP-d_{27} from Chiron, TBOEP-d_6, TDCIPP-d_{15} and TCEP-d_{12} were synthesized by Dr. Vladimir Belov (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). PFRs standard solutions were prepared in methanol. Empty polypropylene SPE cartridges (6 mL) Chromabond were purchased from Macherey-Nagel (Germany).

2.2 Study group

Detailed information about the cohort has been published elsewhere (Cequier et al. 2014a). In brief, samples of air and dust were collected from the living rooms and hair and urine were collected from mothers (n = 48) and their children (n = 54) between January and mid-May 2012. In six households, samples from two children were collected besides from the mother. The mothers' age ranged from 32 to 56 years (median 41 years) and the children's age was between 6 and 12 years (median 10 years). The participants answered questionnaires regarding diet and household characteristics for the assessment of factors potentially influencing the PFR exposure. Informed consent was received from all participants, and the study was approved by the Regional Committee for Medical Research Ethics.

2.3 Hair samples

Human hair samples from mothers (n = 48) and children (n = 54) were collected according to the procedure described by the DEMOCOPHES project (Becker et al., 2014). In brief, scalp hair was cut from the back of the head, wrapped in a paper envelope and a plastic bag, and stored at -20 °C until analysis. Before analysis, hair was cut into small pieces (1-2 mm) with stainless steel scissors and homogenized by manual shaking for 10 min.

2.3.1 Hair extraction and clean-up

Around 200 mg of dry and not washed hair was accurately weighed, spiked with ISs (TPHP-d_{15}, TCEP-d_{12}, TBOEP-d_6, TDCIPP-d_{15}, and TNBP-d_{27}) and ultrasonically extracted at 25 °C for 25 min

with 4 mL of 10% HNO_3 and 4 mL of Hex:DCM (4:1; *v/v*) and followed by two times solid-liquid extraction with 4 mL of Hex:DCM (4:1; *v/v*) using 1 min vortexing. After each extraction cycle, samples were centrifuged at 2500 g for 6 min. The supernatants were transferred to clean glass tubes. The combined fractions of organic solvent extracts were evaporated to near dryness under a gentle nitrogen stream and re-dissolved in 1 mL of *n*-Hex. Prior to fractionation, SPE cartridges (filled up from the bottom with 1 g of Florisil and 250 mg of anhydrous Na_2SO_4) were conditioned with 10 mL of EtAc and 6 mL of *n*-Hex. The extracts were quantitatively transferred and eluted with 10 mL of *n*-Hex and 6 mL of *n*-Hex:DCM (6:1; *v/v*) (Fraction FA) and 10 mL of EtAc (Fraction FB, containing PFRs). Fraction FB was evaporated to dryness and reconstituted in 500 µL of MeOH and measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This method allows measuring two groups of compounds, namely PFRs from fraction FB and PBDEs eluted in fraction FA (Kucharska et al., 2014).

2.4 Dust and air samples

Collection of samples of air and dust was done in the living room of the participants' houses. A detailed description about collection of air and dust and levels of PFRs has been published elsewhere (Cequier et al., 2014a). Briefly, pumps were deployed in the living rooms and worked for 24 hours at a flow rate of 12 L/min. Holders containing polyurethane foam (PUF) plugs trapped PFRs in the gas phase and the quartz filter used in front of the holders retained the airborne particles. Dust was collected vacuuming the entire floor of the living rooms. For air analysis, filter and PUFs were extracted together in an ultrasonic bath and PFRs were determined by gas chromatography-mass spectrometry (GC-MS; in electron ionization mode). For dust analysis, coarse particles were removed and approximately 75 mg of non-sieved dust was extracted in an ultrasonic bath and PFRs determined by the same GC-MS technique, respectively.

2.5 Urine

Urine samples were collected from all participants over 24h. For children, minimum 2 urine samples were collected (1 spot morning urine and 1 to 2 urine samples in the afternoon/evening), and for mothers 2 to 8 urine samples were collected (always 1 spot morning urine). The total number of urine samples was 356 (244 samples from the mother and 112 from the children). All details about sample preparation, method validation, measurements and levels of PFR metabolites in urine are described elsewhere (Cequier et al., 2015, 2014b).

2.6 LC-MS/MS conditions

The details about the LC-MS/MS conditions are described elsewhere (Kucharska et al., 2014). Briefly, Fraction B containing PFRs was analyzed by Ultra Performance Liquid Chromatography (UPLC)-tandem mass spectrometry (MS/MS). The PFRs were separated on an Acquity UPLC BEH C18

column (2.1 mm × 100 mm, 1.7 µm) with a Van Guard Acquity UPLC BEH C18 precolumn (2.1 mm × 5 mm, 1.7 µm). Optimum separation was obtained with a binary mobile phase consisting of ultrapure water (solvent A) and MeOH (solvent B), both solvents acidified with 0.1% formic acid. Five µL of extract was injected into the LC system. The UPLC system was coupled to a Waters Quattro Premier XE Micromass tandem mass spectrometer that was operated in positive electrospray ionization mode (ESI+).

2.7 Quality control and quality assurance

Hair samples were analyzed according to a validated method (Kucharska et al, 2014). Procedural blank samples were run in parallel with each batch of samples (usually 3 procedural blanks per 10 human hair samples). As a procedural blank sample, 50 mg of extracted human hair was used, which allowed monitoring any potential contamination during sample preparation. However, no significant background contamination during sample preparation was observed. More details about the blank procedural issue are described elsewhere (Kucharska et al., 2014). Instrumental quality control also included injection of solvent blanks (methanol) every five samples and after standard solutions and in-house control samples (spiked human hair with native and ISs). The recoveries for the in-house control samples were always in agreement with the recoveries found in the method validation (Kucharska et al., 2014). The method limit of quantification (LOQ_m) was defined as 3 times the standard deviation (SD) of the mean of the blank measurements or, in case of absence of any peaks in the blank chromatograms, LOQ_m was based upon the system sensitivity using S/N = 10). Resulting LOQ_m were ranged between 1 and 9 ng/g, except for TCEP (33 ng/g) (Table 1). The results in Table 1 are presented as median, minimum and maximum concentrations (corrected for standard purity) with expanded uncertainty U (k = 2).

2.8 Statistical analysis

All statistical analyses were performed in SPSS (SPSS Statistics for Windows, Version 22.0, Armonk, NY). Descriptive statistics were calculated for PFRs in hair, dust and air and for PFR metabolites in urine (BDCIPP and DPHP) of mothers and children. These data indicated that the concentrations of PFR and their metabolites were highly skewed, kurtotic and not normally distributed. After log-transformation of the data and deletion of extreme outliers, only some of the data approached a normal distribution (Shapiro-Wilk test). In this study and for the purpose of the statistical analyses, the concentrations of urine metabolites were normalized to specific gravity (SG) which is considered to be less impacted by changes with age, body composition, physical activity, urine flow, time of day, diet, health conditions than other measures, such as creatinine normalization (Braun et al., 2011, Simerville et al., 2005). In order to increase the power of statistics, they were only performed for metabolites with the frequency of detection > 40%. All non-detects and values below LOQ_m were replaced by ½*LOQ_m and in case of metabolites, values below LOD_m were replaced by ½*LOD_m. Mann-Whitney U-test

(non-parametric test for not normally distributed data) was used to investigate the differences in the levels of parent compounds and metabolites between two groups (mothers and children). Including multiple children from the same family did not differ notably and did not change general outcome from the results that included only pairs, thus the data was not reported here. To investigate the strength of possible correlations between maternal and child levels of PFR metabolites, Pearson (for log_{10}-transformed values) and Spearman (for non-transformed values that did not meet normal distribution) correlations were assessed. Further, only Spearman correlations were assessed for the parent PFRs measured in hair, dust and air as a good and sufficient measure of potential associations (those sets of data were not normally distributed even after log transformation, thus this justifies our choice focused only on non-parametric tests) .

3. Results and discussion

3.1 Levels of PFRs in hair

All investigated PFRs were frequently detected (Figure 1). EHDPHP, TPHP, TIBP, TBOEP, TNBP were found in all hair samples from both mothers and children. TEHP and TDCIPP were found in about 93% of the analyzed hair samples. Only TCP and TCEP had a lower frequency of detection, 69% and 21%, respectively. Low detection frequency of TCEP is caused by high levels of this compound in procedural blank samples (included in the calculation of LOQ_m, Table 1). Levels of PFRs in mothers' and children's hair were in the range of < 1 to 3744 ng/g hair, and the relative distribution in the two groups was fairly the same, except for TBOEP which had higher concentrations in children's hair (Table 1). TBOEP, TNBP, and TPHP were the most abundant PFRs in the investigated population. TDCIPP, a suspected carcinogen, (Dodson et al., 2012, Van der Veen and de Boer, 2012) showed high detection frequency and relatively high concentrations in both groups. Further, in particular cases, we observed extreme levels of TNBP (Figure S1 in the supporting information) and TCP (Figure S2) in mothers' and their children's hair. Moreover, extreme values were found also in corresponding dust and air samples which might indicate a much greater individual exposure to these compounds in the particular households.

Differences between concentrations of PFRs in hair from mothers and their children were evaluated by the Mann-Whitney U test. Levels of EHDPHP and TDCIPP were usually higher in mothers' hair, but the difference was not statistically significant (p-value > 0.05). On the other hand, levels of TIBP, TNBP, and TEHP were significantly higher in mothers than in children, while levels of TBOEP were higher in children. This might suggest different exposures to these latter compounds. Both hair concentrations and detection frequencies of PFRs in this study are in line with our previous findings (Kucharska et al., 2014), and to our knowledge no other investigations of PFRs in hair have been reported.

3.1.1 Correlations between levels of PFRs in hair from mothers and children

Statistical analysis indicated very weak or no correlation of PFR levels in mothers' and children's hair. This lack of correlation indicates that the concentrations measured in hair of mothers and children are not driven by the (minor) amount of dust deposited onto the hair surface. The only PFR positively correlated between mothers' and children's hair was TBOEP (r_S = 0.410, p = 0.005) (Figure 2), suggesting households as a common source of exposure. TBOEP levels in human hair were also positively associated with TBOEP levels in house dust and air (see below). Furthermore, we found a number of statistically significant associations ($p < 0.01$) between concentrations of TCP and several PFRs in mother's and children's hair, such as TEHP, TDCIPP, TNBP, and EHDPHP with Spearman correlation coefficients of 0.640, 0.593 (for children), 0.588, and 0.521 (for mothers), respectively. These correlations could point out that these compounds might be used in the same type of products/applications.

3.1.2 Correlations between levels of PFRs in hair and dust/air

This study is the first that compares PFR levels in participants' hair with those found in dust and air from their respective households. Similar compound profiles were observed in hair and air/dust (Figure 1). The frequency of detection of EHDPHP, TPHP, and TBOEP was 100% in human hair and dust samples. TNBP, the most volatile among the investigated PFRs, was detected in only 58% of the dust samples. Further, volatilization from the dust and rapid distribution into the indoor air is proven by higher detection of TNBP in the air samples (80%) (Cequier et al., 2014a). On the other hand, TNBP was detected in all human hair samples, which demonstrates that the sole use of dust for exposure assessment of more volatile pollutants might lead to potential underestimation of exposure.

Statistically significant associations were found between the levels of PFRs in human hair and air/dust samples collected from the same households. In general, the correlations were stronger between hair and dust than between hair and air. However, this was not the case for TIBP, TNBP and partly TBOEP, for which correlations were stronger between hair and air, most probably due to their higher volatility. Further, we observed that the correlations between air and dust within the same PFRs were also moderate and the highest r_S was reported for TBOEP (r_S = 0.645, p = 0.01). The lack of strong correlations might be caused by various surface areas of the houses of the investigated population. Thus, the circulation and in consequence, the dilution of the compound's concentration in the air varied significantly. Another important reason might be a different source of exposure. Table S1 in the supporting information contains all relevant correlations between hair and dust/air. Stronger correlations were observed between levels of TBOEP in mothers' hair and dust (r_S = 0.526, $p < 0.05$) than in children (r_S = 0.414, $p < 0.05$). Although the differences between these two correlations are not statistically significant ($p > 0.05$), this might suggest again different sources of exposure as well as behavioral differences between adults and children. The strongest significant correlations were found between levels of TBOEP in children's hair and air (r_S = 0.561, $p < 0.05$). Lower correlations were

observed mainly between levels of EHDPHP and TDCIPP in children's hair and dust (r_S = 0.355, r_S = 0.347, respectively both at p = 0.05). TNBP and TEHP showed correlations between levels in mothers' hair and air of 0.457 and 0.413 (at $p < 0.05$), respectively (Table S1). The correlations showed above suggest that other sources, not investigated here, might significantly contribute to the exposure of the study population (school, work, cars, food, etc.). On the other hand, Cequier et al. (2015) reported that food does not seem to drive the exposure to these PFRs in their study. Furthermore, an interesting negative correlation between the living area in m^2 and TBOEP levels in children's hair was observed (r_S = -0.348, $p < 0.05$). This could suggest that a larger area gives a greater dilution of compounds in the air, or TBOEP would be mostly on particles and dust and consequently lower the levels found in human hair samples; however, that was not the case for the other compounds with higher detection frequency what might rather refuse our hypothesis.

Lack of strong correlations between human hair and dust/air confirm our assumptions that house dust might only partly contribute to the levels found in hair. Human hair samples might therefore be regarded as a passive sampler "attached" to the human body 24 h/day, thus integrating the exposure from all environments. Although dust might be a very important factor contributing to the human exposure to PFRs in general, we have to realize that it is not possible to arrange a perfect sampling campaign for human monitoring studies in which all types of dust from all microenvironments could be collected from participants (from schools, work places, cars, etc.)

Thus, we suggest using hair as an alternative or complementary to dust. It allows integrating exposure across multiple microenvironments which results in reliable and biologically more relevant data. In general, PFR levels in human hair are lower than in dust, but they might reflect the most relevant pathways of exposure to the investigated compounds. Finally, the scope of hair and dust analysis is different: hair sample analysis can provide information about the personal cloud of human exposure, while dust analysis yields more general information about exposure potential in the living area (e.g. house).

3.2 Correlations between PFR levels in hair and levels of PFR metabolites in urine

A detailed description of the results for urinary PFR metabolites can be found elsewhere (Cequier et al., 2015, 2014b). Overall, BDCIPP and DPHP were the most frequently detected metabolites in all spot urine samples from 48 mothers (n = 244) and 54 children (n = 112) (Figure 3 and Table S2 in the supporting information). We have observed both higher frequencies and higher median levels in children's than in mothers' urine for all metabolites, suggesting children are more exposed to the corresponding parent PFRs. The low detection frequency of BDCIPP in mother urine was surprising taking into account very high detection frequency of the corresponding native compound (TDCIPP) in dust and hair (Figure 1). Also, BDCIPP is considered as a major metabolite of TDCIPP (Van den Eede et al., 2013a) and thus, the breakdown in other metabolites is unlikely. In general, frequencies of detection and levels were similar to those found in Belgian samples by Van den Eede et al. (2013b),

but lower than those found in the samples from the US by Butt et al. (2014). However, levels of TDCIPP and TPHP in dust from several studies were higher in Japan (Araki et al., 2014) and the US (Meeker and Stapleton, 2010) than in the EU (Bergh at al., 2011, Van den Eede et al., 2011) what might significantly influence the levels of PFRs and their metabolites in human samples. Further, due to low detection frequency of BBOEP and DNBP (Figure 3), for our statistical analyses we have considered only DPHP and BDCIPP in urine as they have the highest detection frequencies in both groups. We also noticed that the levels were usually higher in morning urine than in other spot urine collected during 24 h by each participant. Nevertheless, knowing that the half-lives of the PFR metabolites in the body are likely to be in the order of several hours (Cooper et al., 2013, Meeker et al., 2013b), in our second approach we also used median values of the concentrations determined in all spot urine samples.

Figure 4 displays scatter plots for Pearson correlations between log-transformed concentrations of TDCIPP in children's hair and (A) BDCIPP in children's spot morning urine samples, and (B) BDCIPP in children's median urine samples. Spearman rank order correlations are depicted on Figure 5. In general, although the differences are not statistically significant, the correlations for children between TDCIPP in hair and BDCIPP in median urine samples were stronger ($r_S = 0.422$, $p = 0.003$) than the correlations for TDCIPP in hair and BDCIPP in morning spot urine samples ($r_S = 0.352$, $p = 0.02$), which suggests that the median values might be more relevant for calculations of correlations. In case of mothers, only one positive Pearson correlation was found ($r_P = 0.395$ at $p = 0.01$) for log-transformed TDCIPP in hair and log-transformed BDCIPP in morning spot urine samples. As pointed out above, the correlations are generally not strong, but they are statistically significant suggesting that there is a trend showing that elevated levels of TDCIPP in hair caused also elevated levels of corresponding metabolites in urine. Correlations for the children were stronger than for the mothers which might be caused by different exposure degree between these two groups different in age. No correlations were found for TPHP in hair and DPHP in urine, neither for mothers nor children. The lack of correlations between parent TPHP in hair and the metabolite DPHP is in line with findings of the study performed by Meeker et al. (2013b) and Butt et al. (2014) who were investigating correlations between TPHP in house dust and DPHP in human urine. Also Cequier et al. (2015) did not find positive correlation between TPHP in the house dust and DPHP in median urine. However, in the same study (Cequier et al., 2015) moderate correlation between TPHP in dust and DPHP in morning urine was observed (0.29 – 0.30, at $p < 0.05$). DPHP is only one of the most predictable metabolite on the metabolic pathway of TPHP but it is not a specific metabolite of it (Cooper et al., 2013, Cooper and Stapleton, 2011, Dodson et al., 2014, Van den Eede et al., 2013a), which might result in no or moderate correlations. Another reason include the potential of other chemicals and aromatic PFRs to be metabolized to DPHP (e.g. EHDPHP, resorcinol-bis (diphenyl) phosphate (RDP), bisphenol-A (diphenyl) phosphate (BDP), etc.) and also potential of individual differences in metabolism of the compound. Moreover, differences in toxicokinetics between two persons with the

same level of exposure could result in significant differences in the amount of a specific metabolite excreted in urine (Meeker et al., 2013b). Further, our study is the first that investigates correlations between PFR levels in human hair and PFR metabolites in urine, thus it is not possible to compare our results with other similar studies. Lack of or moderate correlations between metabolites in urine and parent compound in hair might be also explained by different nature of these two matrices – whereas PFRs in hair might be more stable and reflect long-term exposure, PFR metabolites in urine reflect short-term exposure (short half-lives of PFRs).

Further, since exposure sources of PFRs are mainly regarded as indoor environment such as furniture and flooring (in which PFRs are used), the levels of PFRs in dust/air might not change on daily basis, especially those in dust. Hence, it is expected that the correlations between hair and dust/air might be stronger than between hair and urine. However, our study has its limitations and not all correlations could be estimated due to low detection frequencies of some metabolites (e.g. for TBOEP in hair and BBOEP in urine). From a statistical point of view this kind of comparison would not be relevant and reliable.

3.2.1 Correlations of urinary PFR metabolites concentrations between mothers and children

Positive statistically significant associations (r_S = 0.409, p = 0.008) were found between DPHP in mothers' median urine and DPHP in children's median urine (Figure 6). Also, we found associations (r_S = 0.295, p = 0.05) for BDCIPP in mothers' and children's morning urine. This might suggest a common source of exposure to TPHP and TDCIPP, which are both used as additive flame retardants in household products (Van der Veen and de Boer, 2012).

Moreover, the Mann-Whitney U-test indicated that there was a significant difference ($p<0.05$) between levels of PFR metabolites (levels higher for children than for the mothers) for both morning and median concentrations in urine samples, which is in contrast to what we noticed for parent PFR compounds in hair, where most PFRs were at higher levels in mothers' than in children's hair. This again might suggest differences in the toxicokinetics and metabolic pathways in these two age groups with a much faster metabolism and excretion of metabolites by children than mothers what was the case in this study, and possibly different exposures outside the house.

Overall, the lack of strong correlations between mothers and children might be caused by the fact that these two groups are not spending their entire day together, adults being at work and children being at school, only sharing temporarily mutual environments and behavior (the same house, car, diet, genes, etc.).

4. Conclusions

Our results showed that hair might be a suitable matrix for measurement of PFRs and can be useful for future biomonitoring studies, since the knowledge on PFR levels in human blood is very limited. Hair is possibly a good indicator of human exposure in an integrative manner, combining both internal deposition and external adsorption. We also consider this matrix as a valuable alternative for and/or supplemental to dust and urine in which only several PFR metabolites could be measured and for which information about exposure to other compounds is not available. Gaining insights in such bioindicator may benefit in prevention and raise general awareness for potential exposure to PFRs.

Acknowledgements

We would like to acknowledge Dr. Vladimir Belov for the synthesis of the labeled PFR internal standards. The study was performed within the framework of a Marie Curie Initial Training Network – INFLAME (grant agreement n° 264600).

Supporting Information

Supporting information associated with this article can be found in the online version.

References:

Ali N, Dirtu A, Van den Eede N, Goosey E, Harrad S, Neels H, 't Mannetje A, Coakley J, Douwes J, Covaci A. Occurrence of alternative flame retardants in indoor dust from New Zealand: indoor sources and human exposure assessment. Chemosphere 2012; 88: 1276-82.

Alves A, Kucharska A, Erratico C, Xu F, Den Hond E, Koppen G, Vanermen G, Covaci A, Voorspoels S. Human biomonitoring of emerging pollutants through non-invasive matrices: state of the art and future potential. Anal Bioanal Chem 2014; 17: 4063-4088

Araki A, Saito I, Kanazawa A, Morimoto K, Nakayama K, Shibata E, Tanaka M, Takigawa T, Yoshimura T, Chikara H, Saijto Y, Kishi R. Phosphorus flame retardants in indoor dust and their relation to asthma and allergies of inhabitants. Indoor Air 2014; 24: 3–15

Becker K, Seiwert M, Casteleyn, Joas R, Joas A, Biot P, Aerts D, Castaño A, Esteban M, Angerer J, Koch H, Schoeters G, Den Hond E, Sepai O, Exley K, Knudsen L, Horvat M, Bloemen L, Kolossa-Gehring M, DEMOCOPHES consortium. A systematic approach for designing a HBM Pilot Study for Europe. Intern J of Hygiene Env Health 2014; 214: 312-322

Bergh C, Torgrip R, Emenius G, Östman C. Organophosphate and phthalate esters in air and settled dust - a multi-location indoor study. Indoor Air 2011; 21 (1): 67−76

Brandsma S, Leonards P, Leslie H, de Boer J. Tracing organophosphorus and brominated flame retardants and plasticizers in an estuarine food web. Sci Tot Env 2014; 505: 21-31

Braun J, Kalkbrenner A, Calafat A, Bernett J, Ye X, Silva M, et al. Variability and predictors of urinary phthalate metabolite and bisphenol A concentrations during pregnancy. Environ Health Perspective 2011; 119: 131-137

Brommer S, Harrad S, Van den Eede N, Covaci A. Concentrations of organophosphate esters and brominated flame retardants in German indoor dust samples. J Environ Monit 2012; 14: 2482–7

Butt C, Congleton J, Hoffman K, Fang M, Stapleton H. Metabolites of Organophosphate Flame Retardants and 2-ethylhexyl Tetrabromobenzoate in urine from paired Mothers and Todlers. Env Sci Technol 2014; 48: 10432-10438

Cequier E, Ionas A, Covaci A, Marcé R, Becher G, Thomsen C. Occurrence of a Broad Range of Legacy and Emerging Flame Retardants in Indoor Environments in Norway. *Environ Sci Technol* 2014a; 48: 6827-6835

Cequier E, Marcé R, Becher G, Thomsen C. A high-throughput method for determination of metabolites of organophosphate flame retardants in urine by ultra performance liquid chromatography-high resolution mass spectrometry. Analytica Chimica Acta 2014b; 845: 98-104

Cequier E, Marcé R, Becher G, Thomsen C. Human exposure pathways to organophosphate triesters — A biomonitoring study of mother–child pairs. Environ Int 2015; 75: 159-165
Cooper E, Getzinge G, Gooden D, Stapleton H. In vitro metabolism of the flame retardants tris (1,3-dichloro-2-propyl) phosphate (TDCPP) and triphenyl phosphate (TPP) by human liver. 2013, [under review]
Cooper E, Covaci A, van Nuijs A, Webster T, Stapleton H. Analysis of the flame retardant metabolites bis(1,3-dichloro-2-propyl) phosphate (BDCPP) and diphenyl phosphate (DPP) in urine using liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem 2011; 401(7): 2123–2132.
Cooper E, Stapleton H. Metabolism of the flame retardants by human liver microsomes and porcine esterase. SETAC North America 32nd Annual Meeting, 2011
Covaci A, Tutudaki M, Tsatsakis A M, Schepens P. Hair analysis: another approach for the assessment of human exposure to selected persistent organochlorine pollutants. Chemosphere 2002; 46: 413–418
Covaci A, Hura C, Gheorghe A, Neels H, Dirtu A. Organochlorine contaminants in hair of adolescents from Iassy, Romania. Chemosphere 2008; 72: 16-20
Chen K, Zheng J, Yan X, Yu L, Luo X, Peng X, Yu Y, Yang Z, Mai B., Dechlorane Plus in paired hair and serum samples from e-waste workers: Correlation and differences. Chemosphere 2015, 123: 43-47
Dirtu A, Ali N, Van den Eede N, Neels H, Adrian Covaci. Country specific comparison for profile of chlorinated, brominated and phosphate organic contaminants in indoor dust. Case study for Eastern Romania, 2010. Env Int 2012; 49: 1-8
D'Havé H, Covaci A, Scheirs J, Schepens P, Verhagen R, de Coen W. Hair as an indicator of endogenous tissue levels of brominated flame retardants in mammals. Environ Sci Technol 2005; 39: 6016-6020
DiGangi J, Blum A, Bergman A, deWit C, Lucas D, Mortimer D, Schecter A, Scheringer M, Shaw S, Webster T. San Antonio statement on brominated and chlorinated flame retardants. Environ Health Perspect 2010; 118: A516–A518
Dodson R, Perovich L, Covaci A, Van Den Eede N, Ionas A, Dirtu A, Brody J, Rudel R. After the PBDE Phase-Out: A broad suite of flame retardants in repeat house dust samples from California. Environ Sci Technol 2012; 46: 13056–13066
Dodson R, Van den Eede N, Covaci A, Perovich L, Brody J, Rudel R. Urinary Biomonitoring of Phosphate Flame Retardants: Levels in California Adults and Recommendations for Future Studies. Env Sci Technol 2014; dx.doi.org/10.1021/es503445c
Esteban M, Castaño A. Non-invasive matrices in human biomonitoring: a review. Environ Int 2009: 35: 438–449
García M, Rodríguez I, Cela R. Optimisation of a matrix solid-phase dispersion method for the determination of organophosphate compounds in dust samples. Anal Chim Acta 2007; 590: 17–25
Kim J, Isobe T, Sudaryanto A, Malarvannan G, Chang K, Muto M, Prudente M, Tanabe S. Organophosphorus flame retardants in house dust from the Philippines: occurrence and assessment of human exposure. Environ Sci Pollut 2013; 20: 812–822
Kucharska A, Covaci A, Vanermen G, Voorspoels S. Development of a broad spectrum method for measuring flame retardants - Overcoming the challenges of non-invasive human biomonitoring studies. Anal Bioanal Chem (2014); 406: 6665-6675
Kucharska A, Covaci A, Voorspoels S. Non-invasive biomonitoring for PFRs and PBDEs: new insights in analysis of human hair externally exposed to selected flame retardants.; Sci Total Environ 2015, 505: 1062-1071
Meeker J, Cooper E, Stapleton H, Hauser R. Exploratory analysis of urinary metabolites of phosphorus-containing flame retardants in relation to markers of male reproductive health. Endocr Disruptors 2013a; DOI: 10.4161/endo.26306
Meeker J, Cooper E, Stapleton H, Hauser R. Urinary metabolites of Organophosphate Flame Retardants: Temporal Variability and Correlations with House Dust Concentrations. Env Health Perspect 2013b; 121: 580-585
Meeker J, Stapleton M. House dust concentrations of organophosphate flame retardants in relation to hormone levels and semen quality parameters. Environ Health Perspect 2010. 118, 318

Rockett JC, Buck GM, Lynch CD, Perreault SD: The value of homebased collection of biospecimens in reproductive epidemiology. Environ Health Perspect 2004; 112: 94-104
Saito I, Onuki A, Seto H. Indoor organophosphate and polybrominated flame retardants in Tokyo. Indoor Air 2007; 17(1): 28-36
Shaw S, Blum A, Weber R, Kannan K, Rich D, Lucas D, Koshland C, Dobraca D, Hanson S, Birnbaum L. Halogenated flame retardants: do the fire safety benefits justify the risks? Environ Health 2010; 25(4): 261-305
Simerville J, Maxted X, Pahira J. Urinalysis: a comprehensive review. Am Fam Physician. 2005; 71: 1153-1162
Smolders R, Schramm K, Nickmilder M, Schoeters G. Applicability of non-invasively collected matrices for human biomonitoring. Env Health 2009; 8: 8
Stackelberg P, Gibs J, Furlong E, Meyer M, Zaugg S, Lippincott R. Efficiency of conventional drinking-water treatment processes in removal of pharmaceuticals and other organic compounds. Sci Total Environ 2007; 377: 255-272
Stapleton H, Klosterhaus, Eagle S, Fuh J, Meeker J, Blum A, Webster T. Detection of organophosphate flame retardants in furniture foam and US house dust. Environ Sci Technol 2009; 43: 7490-7495
Stockholm Convention - http://chm.pops.int/default.aspx
Tadeo J, Sanchez-Brunete C, Miguel E. Determination of polybrominated diphenyl ethers in human hair by gas chromatography-mass spectrometry. Talanta 2009; 78: 138-143
Tajima S, Araki A, Kawai T, Tsuboi T, Ait Bamai Y, Yoshioka E, Kanazawa A, Cong S, Kishi R. Detection and intake assessment of organophosphate flame retardants in house dust in Japanese dwellings. Sci Tot Env 2014 15; 478: 190-199
Van den Eede N, Maho W, Erratico C, Neels H, Covaci A. First insights in the metabolism of phosphate flame retardants and plasticizers using human liver fractions. Tox Letters 2013a; 223: 9-15
Van den Eede N, Neels H, Jorens P, Covaci A. Analysis of organophosphate flame retardant diester metabolites in human urine by liquid chromatography electrospray ionisation tandem mass spectrometry. J Chrom A 2013b; 1303: 48-53
Van den Eede N, Dirtu A, Neels H, Covaci A. Analytical developments and primary assessment of human exposure to organophosphate flame retardants from indoor dust. Environ Int 2011; 37: 454-461
Van der Veen I, de Boer J. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. Chemosphere 2012; 88: 1119-1153
World Health Organization. Environmental Health Criteria 218. Flame retardants: tris(2-butoxyethyl) phosphate, tris(2-ethylhexyl) phosphare and tetrakis(hydroxymethyl) phosphonium salts,. Geneva, Switzerland, 2000
World Health Organization. Environmental Health Criteria 209. Flame retardants: Tris(chloropropyl)phosphate and Tris(2-chloroethyl) phosphate. Geneva, Switzerland, 1998
World Health Organization. Environmental Health Criteria 111. Triphenyl phosphate. Geneva, Switzerland, 1991a
World Health Organization. Environmental Health Criteria 112. Tri-n-butyl-phosphate. Geneva, Switzerland, 1991b
Zheng J, Luo X-J, Yuan J-G, Wang J, Wang Y-T,Chen S-J, Mai B-X ,Yang Z-Y. Levels and sources of brominated flame retardants in human hair from urban, e-waste, and rural areas in South China. Environ Pollut 2011; 159: 3706-3713.

Table 1. Levels of PFRs in mothers' and children's hair (ng/g)

		TBOEP	TPHP	EHDPHP	TNBP	TDCIPP	TCP[a]	TCEP	TIBP	TEHP
Mothers	% Detection	100	100	100	100	91	78	16	100	96
	Min	14	5	5	5	< 9	< 2	< 33	5	< 1
	Max	1253	1256	265	672	3744	134	163	82	53
	Median	65	52	27	22	30	8	72	22	12
Children	% Detection	100	100	100	100	92	62	26	100	90
	Min	34	6	2	3	< 9	< 2	< 33	5	< 1
	Max	2411	363	346	150	2698	74	118	82	114
	Median	318	63	21	11	31	8	59	14	8
	LOQ_m	5	4	2	2	9	2	33	4	1
	U (%)[b]	24	18	28	19	23	28	27	19	21

[a] Sum of TCP isomers; [b] U (Uncertainty was expressed as the expanded uncertainty U and calculated with the use of a coverage factor k = 2, corresponding to a confidence level of 95 %. U was calculated for each compound during the method validation (Kucharska et al., 2014).

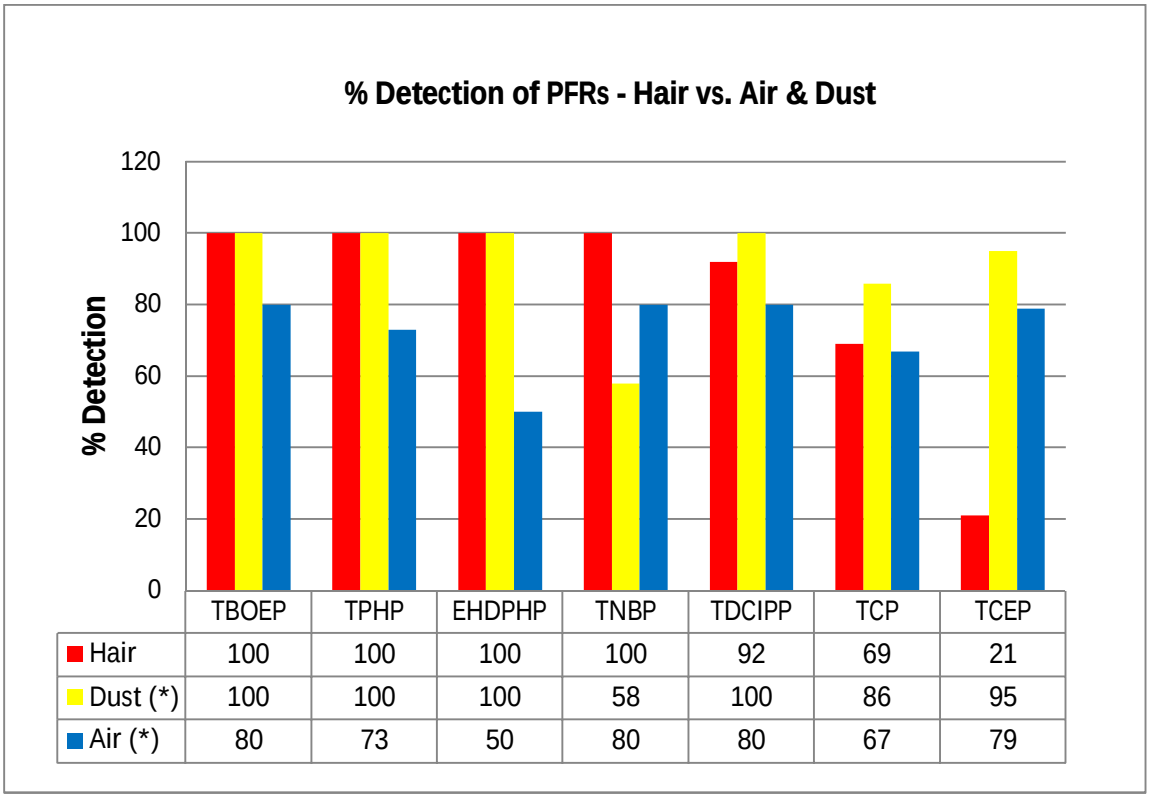

Figure 1. Detection frequency of PFRs in hair, dust and air from the same households; * - Cequier et al., 2014a

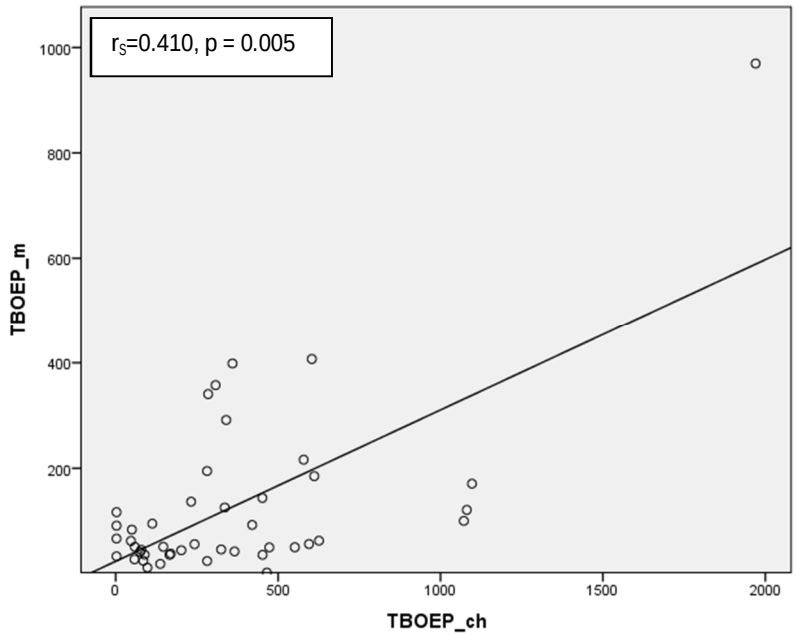

Figure 2. Relationship between concentrations of TBOEP in mothers' and children's hair (ng/g).

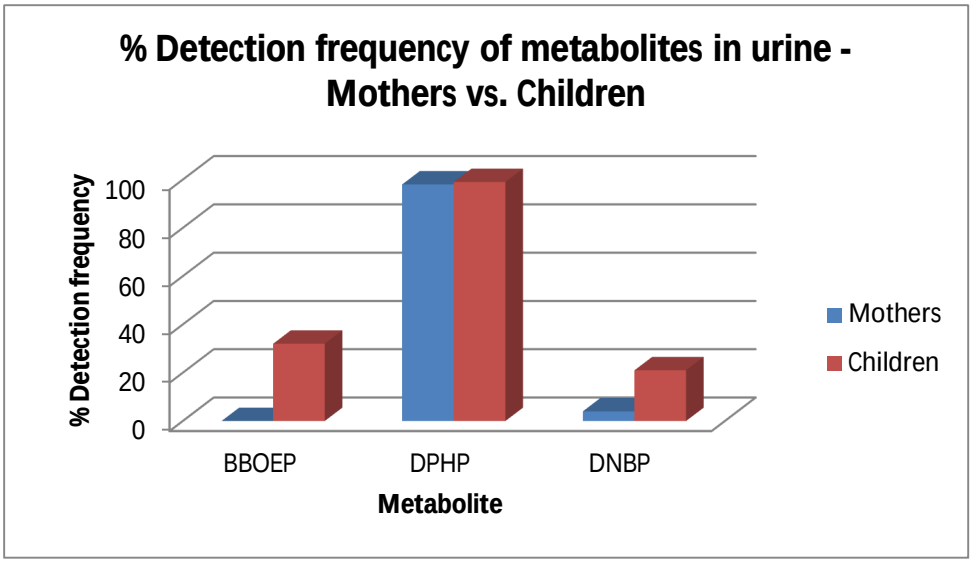

Figure 3. Detection frequencies of PFR metabolites; based on the publication of Cequier et al., 2015

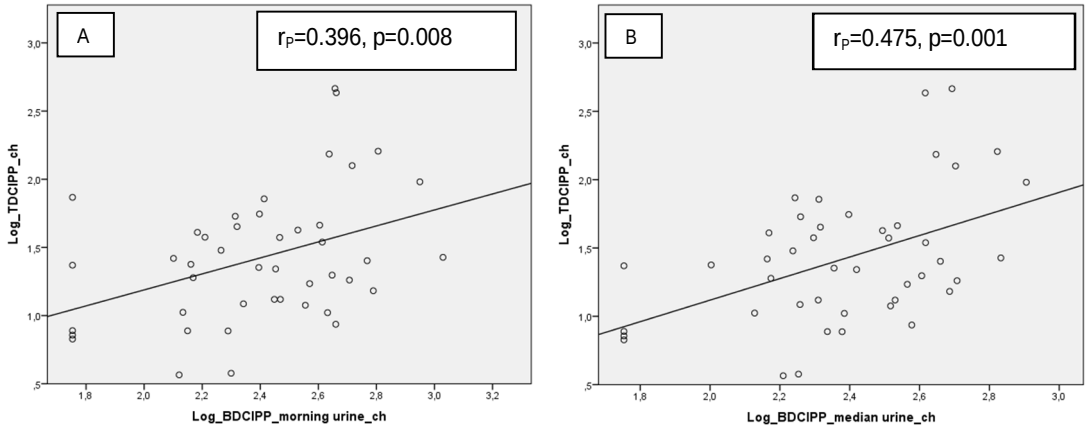

Figure 4. Correlations between log-transformed concentrations of TDCIPP in children's hair and BDCIPP in (A) children's morning urine, and (B) children's median urine.

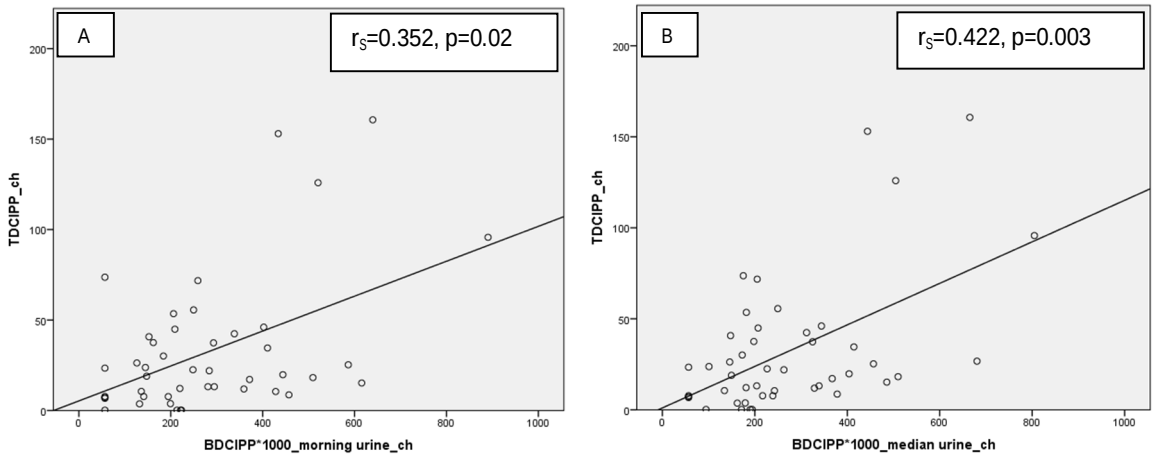

Figure 5. Correlations between concentrations of TDCIPP in children's hair and BDCIPP in (A) children's morning urine, and (B) in children's median urine; BDCIPP*1000 – concentrations of BDCIPP were multiplied by 1000 to have a better view on the scales.

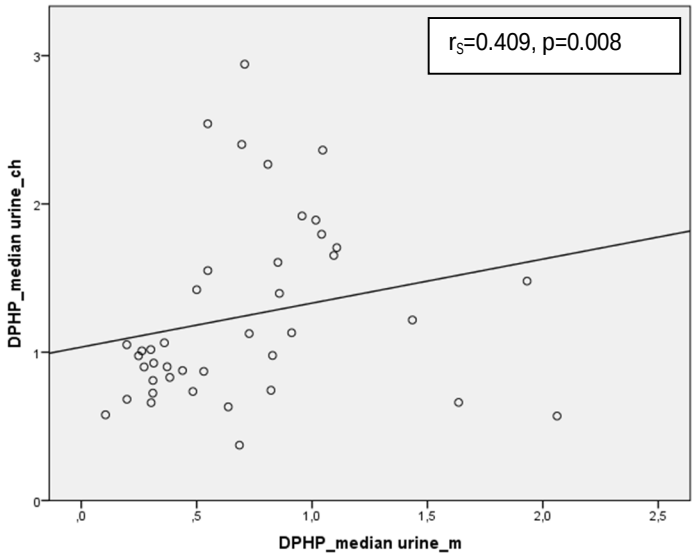

Figure 6. Correlation between log-transformed median concentrations of DPHP in urine for mothers and children.