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Abstract

The demand for alternative flame retardant materials such as phosphate flame retardants and plasticizers (PFRs) is increasing, although little is known of their possible effects on human health and development. To date, no information on the exposure of children or general Australian population to PFRs is available. The objective of this study was to characterize the average levels and age-related patterns of PFR metabolites in urine in the general Australian population and to identify novel hydroxylated PFR metabolites in urine. Surplus pathology urine samples from Queensland, Australia were stratified and pooled by age and sex (3224 individuals aged 0 to 75 years into 95 pools) according to two different pooling strategies at two different time periods. Samples were analyzed by solid phase extraction and liquid chromatography- tandem mass spectrometry following enzymatic treatment. Nine PFR metabolites were measured in the Australian population, including the first report of a hydroxylated metabolite of TCIPP (BCIPHIPP). Diphenyl phosphate (DPHP), BCIPHIPP and bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) were detected in >95% of samples. DPHP, a metabolite of aryl-PFRs, was found in several samples at levels which were one order of magnitude higher than previously reported (up to 730 ng/mL). Weighted linear regression revealed a significant negative association between log-normalized BDCIPP and DPHP levels and age $p < 0.001$). Significantly greater levels of BDCIPP and DPHP were found in children's urine compared with adults, suggesting higher exposure to PFRs in young children. BCIPHIPP was identified for inclusion in future PFR biomonitoring studies.

Key words:

Phosphate flame retardants; Children; Australia; Exposure; Urine; Triphenyl phosphate

Abbreviations

BBOEP: bis(2-butoxyethyl) phosphate

BBOEHEP: bis(2-butoxyethyl) 2-hydroxyethyl phosphate (or desbutyl-TBOEP)

BCEP: bis(2-chloroethyl) phosphate

BCIPP: bis(1-chloro-2-propyl) phosphate

BCIPHIPP: bis(1-chloro-2-propyl) 1-hydroxy-2-propyl phosphate

BDCIPP: bis(1,3-dichloro-2-propyl) phosphate

DPHP: diphenyl phosphate

HO-DPHP: 4-hydroxyphenyl diphenyl phosphate

HO-TBOEP: bis(2-butoxyethyl) 3´-hydroxy-2-butoxyethyl phosphate

HO-TPHP: 4-hydroxyphenyl diphenyl phosphate

TBOEP: tris(2-butoxyethyl) phosphate

TCEP: tris(2-chloroethyl) phosphate

TCIPP: tris(1-chloro-2-propyl) phosphate

TDCIPP: tris(1,3-dichloro-2-propyl) phosphate

TPHP: triphenyl phosphate

Short running title: Age related PFR exposure in Australian population

Conflict of interest: All authors declare they have no actual or potential competing financial interest.

Introduction

A number of household components including furniture, electronics, wall coverings, floor wax and paints may contain chemical additives including plasticizers and flame retardants (FRs). Following the addition of commercial polybrominated diphenyl ethers (PBDE) formulations to the Stockholm Convention (Stockholm Convention 2009), many regions across the globe have restricted or completely phased out the use of PBDEs, and consequently, the demand for alternatives, such as phosphate flame retardants and plasticizers (PFRs) has increased (van der Veen and de Boer, 2012).

Increased use of PFRs is reflected in increasing levels of PFRs in indoor dust from California in the United States (Dodson et al. 2012). Inhalation and ingestion of contaminated dust is assumed to be an important exposure pathway for FRs in humans (Harrad et al. 2010, Covaci et al. 2012). Due to their greater inhalation and metabolic rate, different breathing zone, and increased hand-to-mouth activity compared with adults (Tulve et al. 2002; WHO 2011), children are likely to experience greater indoor chemical exposures, including exposure to PFRs (Wilson et al. 2013, Harrad et al. 2010, Stapleton et al. 2011). The effects of chronic human PFR exposure have not yet been assessed, but there is some suggestion for decreased male fertility in vitro (Fang et al. 2003), and hepatotoxicity and carcinogenicity in animals (WHO 1991, 1998, 2000). Chemical exposures during critical windows of development, such as early childhood and adolescence are of particular concern as developing organ systems are susceptible to toxic influence; metabolic capacity may be diminished, particularly in the early post-natal period; and early-life exposure means more years of future life for the development of diseases with a long latency period (Scheuplein et al. 2002; WHO 2011).

There is little information available on human exposure to PFRs globally, and exposure of the Australian population remains uncharacterized. Biomonitoring is regarded as the gold standard in chemical exposure assessment since it is an aggregate measure and is not subject to the large uncertainties in estimating exposure based on dust or food ingestion rates (Sexton et al. 2004, Wilson et al. 2013). The target analytes of this study were the metabolites of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), tris(1-chloro-2-propyl) phosphate (TCIPP), tris(2-chloroethyl) phosphate (TCEP), triphenyl phosphate (TPHP), tris(2-butoxyethyl) phosphate (TBOEP), and tributyl phosphate (TNBP), as these parent compounds have been demonstrated to be the most abundant PFRs in indoor dust (Van den Eede et al. 2011; Dodson et al. 2012).

Several PFR metabolites may be of interest for assessing human exposure. Animal *in vivo* and human *in vitro* studies have suggested that PFRs are sensitive to two types of biotransformation reactions - hydrolysis and oxidative metabolism (Sasaki et al. 1984, WHO 1991, 1998). The hydrolysis pathway would form similar products, namely phosphate diesters as shown in Figure 1, compared to the biotransformation products of organophosphate pesticides (Sudakin and Stone 2011). Diesters of PFRs have already been considered as target metabolites in several biomonitoring studies (Schindler et al. 2009a,b; Cooper et al. 2011, Carignan et al. 2013, Meeker et al. 2013, Van den Eede et al. 2013a, Hoffman et al. 2014). A recent *in vitro* study on human liver fractions indicated significant formation of hydroxylated metabolites for TCIPP, TPHP, and TBOEP (Van den Eede et al. 2013b). The hydroxylated metabolite structures considered for monitoring in this study were BCIPHIPP, HO-TBOEP, BBOEHEP, HO-TPHP, and HO-DPHP (Figure 1). This selection was based on data on hydroxylated metabolites formed *in vitro* (Van den Eede et al. 2013b, Van den Eede et al. under review), and/or being tentatively detected in human urine (Dodson et al. under review). The suitability of these five metabolites as *in vivo* biomarkers of TCIPP, TBOEP, or TPHP exposure is unclear.

The aims of this study were to: (1) characterize exposure of the Australian population to PFRs using pooled samples of convenience, with a focus of children <5 years, through measurement of the major PFR metabolites identified previously *in vitro* or in vivo; (2) to identify novel urinary PFR metabolites; and (3) critically discuss the most suitable markers for future PFR monitoring efforts.

Materials and methods

Materials

Bis(2-chloroethyl) phosphate (BCEP), bis(1-chloro-2-propyl) phosphate (BCIPP), BCEP-D8, 4-hydroxyphenyl diphenyl phosphate (HO-TPHP), bis(2-butoxyethyl) 3´-hydroxy2-butoxyethyl phosphate (HO-TBOEP), bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), bis(2-butoxyethyl) phosphate (BBOEP), 4-hydroxyphenyl phenyl phosphate (HO-DPHP), 2-hydroxyethyl bis(2-butoxyethyl) phosphate (BBOEHEP or desbutyl-TBOEP), BBOEP-D4, BDCIPP-D10, and TBOEP-D6 were custom synthesized by Dr. Vladimir Belov (Max Planck Institute, Göttingen, Germany). Purity was more than 98% as measured by MS and NMR techniques. 1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP) was also custom synthesized, but consisted of 2 isomers which were both used for quantification.

TCEP was purchased from Chiron AS (Trondheim, Norway). Dibutyl phosphate (DBP), diphenyl phosphate (DPHP), DPHP-D10, and TPHP-D15 were purchased from Sigma–Aldrich (Bornem, Belgium). We used ultrapure water from a MilliQ system (Millipore), acetonitrile (analytical grade, Merck), methanol (analytical grade, Merck), triethyl amine (Sigma), β-glucuronidase (*Helix pommatia* H-1) from Sigma, and a RC-cellulose filter (Phenomenex).

Samples

De-identified urine specimens were obtained from a community-based pathology laboratory (Sullivan Nicolaides Pathology, Taringa, QLD, Australia) from surplus stored urine that had been collected and analysed as part of routine testing. Specimens were collected in sterile 50 mL polyethylene urine specimen containers, refrigerated immediately, and were sent to the pathology laboratory for testing within 12 h, where they were stored in monitored cold rooms (4 °C) for up to three days. Specimens were frozen immediately following collection by researchers (-20 °C).

Two separate sampling campaigns were undertaken from November 2010 to March 2011 (campaign 1) and November 2012 to November 2013 (campaign 2). This work was approved by the University of Queensland (UQ) Ethics Committee (approval number 2002000656).

Pooling protocol

Descriptive information about each specimen was limited to date of birth, date of sample collection and sex. Before pooling, samples were stratified according to age and sex. For sampling campaign 1 the age strata were: 0–0.5, 0.5–1, 1–1.5, 1.5–2, 2–2.5, 2.5–3, 3–3.5, 3.5–4, 4–4.5, 4.5–5 years, where 7 individuals contributed to a single pool (n=52 pools); 15–30, 30–45, 45–60, 60–75 years, where 28 individuals contributed to a single pool (n=20 pools). For sampling campaign 2, the age strata were 0–5, 5–15, 15–30, 30–45, 45–60 and ≥60 years, where 100 individuals contributed to a single pool (n=23 pools). The concentration measured in each pool is equivalent to the arithmetic mean of the concentration in each individual sample contributing to the pool (Mary-Huard 2007). During pooling, individual urine specimens were thawed, homogenised and aliquoted into amber glass bottles. The pooled sample was homogenised before being divided into smaller aliquots stored in 2 mL amber glass vials and frozen until analysis. Specimens were pooled based on volume, where each individual in the pool contributed the same volume to the pool. No measures of creatinine or specific gravity were available.

Analytical methods

A previously published method (Van den Eede et al. 2013a) was adapted for the analysis of hydroxylated PFR metabolites by inclusion of an enzymatic treatment and use of an alternate sorbent for solid phase extraction. All sample preparation and analysis was performed at UQ. Briefly, a volume of 2 mL urine was mixed with 0.7 mL sodium acetate buffer (pH 5, 1 M) and 100 µL of enzyme solution (1000 units per mL β-glucuronidase or 33 units per mL sulfatase activity in 0.2 M sodium acetate buffer pH 5) and 15 ng internal standard (Table S1). Samples were incubated overnight at 37°C. StrataX-AW cartridges were conditioned with 2 mL of acetonitrile and 2 mL of water. After loading the sample, cartridges were rinsed with 2 mL of water and the analytes were eluted with 2 mL of 5% triethyl amine in acetonitrile. The latter fraction was collected and evaporated until near dryness and solubilized in 250 µL 5% acetonitrile in water. Extracts were filtered through a 0.2 µm filter into a glass vial.

Analysis of the extracts was performed at UQ with a Shimadzu Prominence UFLC system coupled to an AB Sciex 5500 QTRAP in electrospray ionization mode. A volume of 2 µL was injected on a Kinetex C8 column (100 x 2.1 mm, 1.7 µm Phenomenex Inc.) using gradient conditions. Mobile phase composition was A: 5 mM ammonium acetate in acetonitrile-water (1:99, v/v) and B: 5 mM ammonium acetate in acetonitrile-water (98:2, v/v). Conditions were 5% B (0-0.5 min), 25% B (2-3 min), 50% B (5.5 min), 95% B (8.5-14 min), equilibration to 5% B during 6 min. Temperature was 55 °C, flow 0.25 mL/min. MS/MS parameters can be found in the Supplemental Material (Table S1). Extracts from campaign 1 and 2 (n=30 and n=23, respectively) were qualitatively screened at UA for HO-DPHP and BBOEHEP (LC-MS/MS parameters in Table S2).

Dust samples (n=4) from Australian households collected as part of a previous study (Toms et al. 2009) were analysed for DPHP. The analytical method was similar to the method for urine samples described above (Supplemental Material, page 4).

Method performance tests

Urine from five volunteers was used for quality control (QC) purposes. Aliquots were spiked at three concentrations and analyzed in triplicate on three separate days for validation purposes (Table S3). QC samples (n=14) consisting of urine spiked with 2 or 6 ng PFR

metabolite standards were included during sample analysis (Table S4). Procedural blanks (n=9) were analyzed and the average blank value for each analyte was subtracted from each sample concentration. Method limits of detection (MDL, Table S3) were calculated based on the average blank plus three times the standard deviation of the blanks. For metabolites not present in the blanks, MDL was calculated from S/N ratio of 10.

Statistical analysis

The datasets for each metabolite were examined for normality and checked for outliers. The distributions of PFR metabolite concentrations were generally lognormal without noticeable outliers, and data were ln-transformed before regression analyses were conducted. The influence of age (years) and sex (female vs. male), as well as sampling campaign (2 vs. 1) on ln-transformed urinary concentration was modelled using weighted linear regression, with each pool result weighted by the number of individuals included in the pool:

$$\ln(\text{concentration}) = I + \beta_1 * \text{Age} + \beta_2 * \text{Sex} + \beta_3 * \text{Campaign} \qquad \textbf{(Equation 1)}$$

Samples <MDL were substituted with MDL/√2.

Results

Method performance

Method validation results including recovery, precision, sensitivity, and procedural blanks can be found in Table S3. Accuracy of DPHP, BDCIPP, BBOEP, TCEP, BCIPHIPP, HO-TBOEP ranged from 80% (DPHP) to 116% (BDCIPP). Accuracy for DBP and HO-TPHP was 37 and 187%, respectively. Relative standard deviations were less than 16% except for HO-TPHP. BCEP and BCIPP were not included as their MDLs were >3 ng/mL, in contrast to the other target analytes for which MDLs were <0.5 ng/mL.

Urinary metabolites in sampling campaigns 1 and 2

The detection frequencies and range of detected concentrations for all six metabolites in each sampling campaign are summarized in Table 1. DPHP, BCIPHIPP, and BDCIPP were frequently detected in the pooled samples from both campaigns, with detection frequencies greater than 90%. The concentrations in sampling campaign 1 for DPHP, BCIPHIPP, and BDCIPP ranged from <0.15–727 ng/mL, 0.37–9.43, and <0.15–8.90 ng/mL, respectively. For sampling campaign 2, the ranges of detected concentrations for the frequently-detected

analytes were 10.2–225, 0.53–7.17 and < 0.15–3.41 ng/mL for DPHP, BCIPHIPP, and BDCIPP, respectively. The narrower range of concentrations observed in sampling campaign 2 may be a consequence of the greater number of individuals included in each pool in the second sampling campaign compared to the first campaign (100 vs. 7 or 28). With samples composited from a greater number of individuals, the influence of very high or very low concentrations from specific individuals would be reduced.

BCIPHIPP in urine was characterized by two peaks eluting on the same retention times as the two isomers of the standard solution, though in a different ratio relative to each other. MS fragmentation produced the same fragment ions but in a slightly different pattern as can be found in SM page 8 and figure S2. All reported concentrations are the sum of both isomers.

The other metabolites analyzed were detected only infrequently. The detection frequency of the TCEP and DBP was greater in sampling campaign 1 (11 and 18%, respectively) compared with campaign 2 (4% for either analyte). BBOEP was found in 5.6% of samples from campaign 1, but not at all in samples from campaign 2. The maximum concentration of TCEP, DBP and BBOEP in samples from the first campaign was 24.5, 2.15 and 0.53 ng/mL, respectively. By visual inspection, the frequency of detection for TCEP, DBP and BBOEP was greatest in children aged 0–2.5 years. TCEP and DBP were only found in one sample of the second campaign, each at 0.37 and 0.94 ng/mL, respectively (Table 1). HO-TBOEP and HO-TPHP were not detected in any samples. BBOEHEP was detected in 96% of samples from campaign 2, and in 77% of 30 selected extracts of the first sampling campaign. HO-DPHP could not be positively identified in any extract.

The concentrations of the three frequently detected metabolites (DPHP, BDCIPP, and BCIPHIPP) showed significant associations with age, sex, and sampling campaign (Table 2). Concentrations were significantly negatively associated with age, with the strongest association for BDCIPP, followed by DPHP; the association with age was small for BCIPHIPP (Figure 2). Only BCIPHIPP showed a difference by sex, with females lower than males. DPHP concentrations were significantly higher in sampling campaign 2, which occurred 2 years later than campaign 1; the other two compounds were not significantly different between sampling campaigns. These three variables explained approximately 50% of the variance in pool concentrations for BDCIPP, while lower proportions (10 to 20%) were explained for the other two analytes.

Discussion

Method Performance

The analytical method proved to be reliable and provided adequate recovery and precision for DPHP, BDCIPP, BBOEP, TCEP, BCIPHIPP, and HO-TBOEP (Table S3). While BCEP and BCIPP were initially included, MDLs were high (>3 ng/mL) relative to the expected exposure levels, and made these metabolites unsuitable for quantitative analysis. Lower MDLs have been reported using GC-MS/MS methods (Van den Eede et al. 2013a). Recoveries of DBP were low but reproducible (Table S3), and independent of the urinary concentration. A correction factor based on the recovery of QC material was applied to reported results for DBP, yet these concentrations should be considered indicative. HO-DPHP and BBOEHEP were not included in the method optimization and validation, because the reference standards were not available at the time of method development. Enzymatic treatment proved necessary for the detection of BCIPHIPP in urine. The deconjugation reaction could not be quenched by addition of formic acid or organic solvent without affecting analyte recoveries during the extraction process. As such, incubation with glucuronidase/sulfatase was carried out overnight to decrease the variation due to small differences in incubation time.

Major PFR metabolites in urine

DPHP concentrations

The range of pool concentrations for DPHP was large across both sampling campaigns (<0.15–727 ng/ml) (see Table 1), and this study reports the highest ever levels of DPHP in urine, with the next highest report of DPHP at 37.7 ng/mL found in spot urine samples collected from pregnant women (Hoffman et al. 2014). This is particularly remarkable because of the pooled design used in this effort: a high pool concentration implies much higher individual concentrations for at least some of the individuals in that pool, since the pooling strategy that relies upon equal sample volumes from each individual in the pool effectively averages the individual concentrations. Similarly, the geometric mean DPHP level of both campaigns (24 and 37 ng/mL) suggests that TPHP exposure in the Australian population is one to two orders of magnitude higher than in Belgium, Germany and the United States (Schindler et al. 2009a, Cooper et al. 2011, Van den Eede et al. 2013a, Meeker et al. 2013). In addition to the quality control procedures (SM page 7), the quantification of low concentrations of DPHP in some samples (<10 ng/mL, n=23) confirms that systematic contamination was not responsible for the high concentrations reported.

Weighted linear regression shows a significant negative association between DPHP concentration and age in both sampling campaigns, with the highest concentrations observed in youngest age groups (Figure 2A). The association appears stronger in samples from campaign 1 compared with campaign 2 due to the pooling strategy employed, as the use of comparatively larger age ranges in campaign 2 partially obscures the age trend in the 0-5 year age range.

The high concentrations observed in the Australian population could be explained by high exposure to TPHP in dust, air, or from other sources, such as baby products, or by exposure to DPHP itself in dust (formed by spontaneous or microbial hydrolysis of TPHP). To explore the potential for high exposure from contaminated dust, samples from home, car and office environments in the Brisbane region were collected and analyzed for selected PFRs (personal communication, Brommer and Harrad). TPHP dust concentrations were in the low µg/g range and similar to the levels observed in other countries (Dodson et al. 2012, Van den Eede et al. 2011), and thus unlikely to be responsible for the high DPHP concentrations observed. Two other recent studies also found a lack of correlation between dust and urinary levels for this compound (Meeker et al. 2013, Dodson et al., in press). Information on other sources that might contribute to TPHP exposure, such as indoor air or diet, were not available, and may reveal information on alternate exposure pathways.

To assess the concentration of DPHP in dust, samples collected earlier from South East Queensland (Toms et al. 2009) were analyzed for DPHP (SM page 3). DPHP was detected in all samples (n=4) at concentrations ranging from 75 and 190 ng/g. While DPHP could be absorbed and excreted in urine unchanged (Sudakin and Stone, 2011), using a high dust ingestion rate of 140 mg/day for infants and toddlers (Wilson et al. 2013) and assuming complete absorption after intake and complete excretion via urine, the worst-case scenario DPHP excretion estimate is ~25-30 ng/day. Assuming a mean urinary output of 600 mL/day for children, this would result in a maximum concentration of 0.05 ng/mL, which is inadequate to explain the much higher DPHP concentrations observed in some samples (maximum concentrations of 727 and 225 ng/mL in campaigns 1 and 2, respectively).

An alternate hypothesis for the high urinary concentrations observed is the exposure to other aryl-PFRs which have the potential to form DPHP after being hydrolyzed. For example, *in*

vivo research has demonstrated formation of DPHP from 2-ethylhexyl diphenyl phosphate (EHDPHP) (Nishimaki-Mogami et al. 1988). Other aryl-containing PFRs, such as bisphenol-A bis(diphenyl phosphate), resorcinol bis(diphenyl phosphate), and isodecyl diphenyl phosphate could also contribute to urinary DPHP after uptake in the human body. Yet, little is known about their presence in indoor dust or air samples worldwide (Brandsma et al. 2013), and no information on their biotransformation pathways has been reported.

BDCIPP in urine

BDCIPP, a metabolite of TDCIPP, was detected with high frequency (> 90%) in both sampling campaigns, but at lower concentrations than DPHP and BCIPHIPP. BDCIPP levels in urine from adults (>15 y) were comparable to the levels reported in Boston and North Carolina (Carignan et al. 2013, Cooper et al. 2011, Meeker et al. 2013), but were 10 times lower than reported in pregnant women from North Carolina (Hoffman et al. 2014). This suggests that exposure of the Australian population is similar to that of the studied populations in the United States. BDCIPP also exhibited a strong negative association with age (Figure 2B). Stapleton et al. (2011) have previously reported the presence of TDCIPP in polyurethane foams collected from baby products, which may be an important exposure source for young children. In addition, correlations between BDCIPP in urine and TDCIPP in house or office dust have been previously reported (Carignan et al. 2013, Meeker et al. 2013). Thus, both indoor dust and the presence of TDCIPP in foams of baby products are possible exposure sources leading to BDCIPP in urine.

BCIPHIPP in urine

BCIPHIPP has not previously been reported *in vivo* and its formation *in vitro* was only recently discovered (Van den Eede et al. 2013b). The observation of 2 isomers being present in urine was similar to the two isomers which were found with liver fractions (Van den Eede et al. 2013b). These isomers could either be formed by hydroxylation on different carbons in the isopropyl group during oxidative dehalogenation or by degrading different isomers of TCIPP which are present in the commercial mixture (Collins et al. 2013). The magnitude of exposure to BCIPHIPP is significantly less than for DPHP (GM 1.74 and 24.4 ng/mL, respectively, for sampling campaign 1), yet in a comparable order of magnitude as BDCIPP (Table 1). There was a slight but significant negative association between BCIPHIPP urinary concentration and age, but the magnitude of the difference between the youngest and oldest age groups was not as pronounced as that seen with DPHP and BDCIPP. This could be due to

an adult-specific exposure source of approximately equal magnitude to the factors that typically result in higher exposures seen in children (see discussion below); for example, the prevalent use of TCIPP in insulation foams and sprays and indoor sealing used in office buildings is likely to be an important exposure source for office workers (EU 2008). Alternatively, immature or diminished expression of enzymes responsible for BCIPHIPP formation (as separate and distinct enzymes from those that catalyze organophosphate diester formation) in young children could result in lower formation of metabolic products (BCIPHIPP) in children compared with adults, even following the same high exposure to the parent compound.

TCEP in urine

A previous *in vitro* study has shown that TCEP is minimally transformed to BCEP or to hydroxylated metabolites by human liver enzymes (Van den Eede et al. 2013b), suggesting that TCEP rather than it's metabolites may be useful as a biomarker for human exposure to PFRs. To our knowledge no quantitative data on TCEP in human urine have been published to date, and this is the first study to target this parent PFR in urine. TCEP was detected in 35% of samples from children 0–3 years (Figure S1), but not in samples from older children or adults, suggesting that exposure may be higher in Australian children than in adults. Since the metabolite BCEP was not measured in any samples, it is not possible to compare TCEP exposure levels in Australia to levels reported in other populations.

Elevated PFR exposures in children

Urinary concentration was inversely associated with age for three of the frequently detected metabolites (Figure 2), with the highest concentrations reported in the youngest age groups. Children are disproportionately exposed to environmental chemicals including PFRs, due to differences in behavior, and consequently, differences in exposures. Due to their smaller size, children live in a different breathing zone than adults, and combined with their increased metabolic and ventilation rate, and larger surface area-to-body weight ratio, children have an increased potential for exposure via inhalation and dermal absorption compared with adults (Miller et al. 2002). Further, children have greater hand-to-mouth activity and more rapid and efficient absorption of nutrients than adults, resulting in greater exposure to environmental chemicals through non-nutritive ingestion, such as dust ingestion, than adults, which may be especially important for PFR exposures (Tulve et al. 2002; WHO 2011). For example, higher ingestion per kilogram bodyweight of aryl-PFR-containing dust by children relative to adults,

could explain the observed higher DPHP urinary concentrations in children. While this reasoning would be the same for child exposure in other regions, potentially higher concentrations of aryl-PFRs in Australia would cause the DPHP urine levels which were observed in this study.

In addition, urinary flow rates normalized to body weight are higher in children than in adults. Thus exposure studies reporting urinary concentrations unadjusted for urine flow may underestimate the true exposure (Heffernan et al. 2013), and this effect will be most pronounced for the youngest participants. In the case of BDCIPP, DPHP and BCIPHIPP the use of flow-corrected values would strengthen the age-association (Figure 2). Elevated exposures are particularly important during windows of susceptibility, such as early childhood, as developing organ systems are particularly vulnerable to toxic influence, with the potential for adverse consequences for organ structure and function, and for cell growth and migration in later life (WHO 2011). Exposure to TPHP has been associated with adverse effects on the reproductive system both *in vitro* and *in vivo* (Fang et al. 2003, Meeker et al. 2010), but little information is known about the health impacts of other aryl-PFRs.

Suitability of biomarkers of exposure

Despite the relatively low MDL (0.15 ng/mL, see Table S3), and predictions from *in vitro* results (Van den Eede et al. 2013b), HO-TPHP and HO-TBOEP were not detected in any urine samples, and are thus less likely to be useful target compounds for future PFR exposure studies. BBOEP was detected in very few samples, and only in the first sampling campaign, and is also unlikely to be useful target compounds for future studies. The low detection frequency of BBOEP was in accordance with findings from a previous study (Van den Eede et al. 2013a).

A subset of samples (n=30 and n=23, campaigns 1 and 2, respectively) was investigated at UA for BBOEHEP and HO-DPHP, as reliable standards for quantification could not be sourced. In contrast to HO-TBOEP and BBOEP, BBOEHEP was detected in the majority of the extracts (77 and 96%, respectively), suggesting that BBOEHEP could be a more suitable biomarker of exposure for TBOEP than BBOEP or HO-TBOEP. However, more data on BBOEHEP in urine are needed to confirm this statement.

Mass spectrometric confirmation for HO-DPHP was unreliable, and since the extraction efficiency has not yet been tested, the results for HO-DPHP must be interpreted with caution.

As the usefulness of HO-TPHP and HO-DPHP for biomonitoring purposes remains uncertain, DPHP would still be the preferred target analyte in such studies. However, the low specificity of DPHP should be taken into account as it may be produced from more than one aryl-PFR.

BCIPHIPP was detected in all samples from both campaigns, including in infants less than 6 months old, indicating that BCIPHIPP might be an useful biomarker of exposure. Because of limited sample availability, we were unable to analyze BCIPP by GC-MS/MS, so we could not compare in the same set of samples. However, several studies used BCIPP to monitor TCIPP exposure with limited success, as detection frequencies were <30% (Schindler et al. 2009b, Van den Eede et al. 2013a). Considering the major importance of BCIPHIPP in *in vitro* metabolism studies (Van den Eede et al. 2013b) and the analytical difficulties encountered for BCIPP, namely low sensitivity with LC-MS/MS methods and a time and sample consuming procedure for GC-MS/MS analysis (Van den Eede et al. 2013a), we conclude that BCIPHIPP may be a more appropriate target for human biomonitoring.
The parent compound TCEP was tested as biomarker of exposure, but was only detected in urine from young children. While it may be useful to retain TCEP as a biomarker for exposure studies focusing on children and employing LC-MS/MS techniques, we recommend maintaining BCEP as a biomarker of TCEP exposure if samples can be analyzed by GC-MS/MS (Schindler et al. 2009a,b; Van den Eede et al. 2013a).

Limitations of the study

Study population and the use of pooled samples

The samples for this study were sourced from a community-based pathology laboratory and the health status of the individuals was unknown. However, we do not expect that significant medical intervention occurred or that PFR exposure would be systematically and significantly different from that of the general Australian population (Heffernan et al. 2013). As this was a retrospective study with convenience population, specific sampling protocols were employed. Pooled samples provide no information on population variance, but the measures of central tendency can be used to monitor population exposure with significantly reduced time and resource requirements compared with a traditional epidemiological approach. The utility and limitations of pooled samples for population monitoring has been discussed recently (Heffernan et al. 2014). Furthermore, we were not able to check the chemical stability of the analytes in frozen samples during long term storage. We assume however, that this would not

affect our results to a large extent, as was shown previously for other short half-life chemicals in urine (Calafat et al. 2005).

Spot samples and inter-individual variation

Assuming a short half-life of PFRs and metabolites in humans (WHO 1998, 1991), a single spot urine sample is likely to reflect relatively recent exposures (<24 h), and within-individual variation in urinary concentrations is to be expected. The within- and between-individual variability of PFRs is not well characterized and there is no information available on elimination kinetics for PFRs and their metabolites. Further, only limited information is available on PFR exposure sources and biomonitoring data for comparison. Two recent studies on short-term trends of PFR exposure in repeated urine samples from adults demonstrated greater between-individual variability than within-individual variability (Hoffman et al. 2014, Meeker et al. 2013), with calculated intraclass coefficients of 0.35–0.6 and 0.5–0.6 for DPHP and BDCIPP, respectively. This suggests that a single spot urine sample provides a moderately stable estimate of exposure over the time frame studied. The expected within-individual variability relative to exposure events will be somewhat mitigated by the averaging effect of a large number of individuals contributing to each pool, but significant variation may be expected in pooled samples with fewer individuals contributing to each pool (e.g. seven individuals per pool in sampling campaign 1).

Conclusions

This study presents the first data on PFR exposures in children globally, and the first characterization of PFR exposure in the Australian population as a whole. DPHP, BCIPHIPP and BDCIPP were detected at a high frequency, with average Australian DPHP urinary concentrations exceeding previously reported maximum concentrations by one order of magnitude. Estimated intake following high dust exposure was insufficient to explain the elevated levels observed. It is possible that more than one PFR is contributing to DPHP formation and excretion, or that sources other than dust contribute to exposure in the Australian population. Weighted linear regression revealed a significant, negative association between DPHP, BDCIPP, and BCIPHIPP concentration and age, with the highest concentrations observed in the youngest age groups. In addition to DPHP, and BDCIPP, two new PFR metabolites have been positively evaluated for future biomonitoring studies:

BBOEHEP and BCIPHIPP, for assessing TBOEP and TCIPP exposure, respectively. The latter proved a more useful biomarker of TCIPP exposure than BCIPP.

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Table 1: Summary statistics for PFR metabolites in sampling campaign 1 (n = 28 pools of 28 individuals and n = 44 pools of 7 individuals) and sampling campaign 2 (n = 23 pools of 100 individuals). GM: geometric mean, N/A: not applicable.

	Sampling campaign 1			**Sampling campaign 2**		
	Detection frequency (%)	GM (ng/mL)	Range (ng/mL)	Detection frequency (%)	GM (ng/mL)	Range (ng/mL)
DPHP	97	24.4	<0.30–727	100	63.4	10.2–225
BDCIPP	92	1.00	<0.15–8.90	96	0.66	<0.15–3.41
BCIPHIPP	100	1.74	0.37–9.43	100	1.86	0.53–7.17
TCEP	13	<0.35	<0.35–24.5	4	<0.35	<0.35–0.37
DBP	18	<0.43	<0.43–2.15	4	<0.43	<0.43–0.94
BBOEP	6	<0.35	<0.35–0.53	0	N/A	N/A

631 **Table 2:** Weighted linear regression for urinary concentrations (ng/mL) and estimated
632 excretion rates for DPHP, BDCIPP, and BCIPHIPP vs. average age of pool donors, sex, and
633 sampling campaign. Each pool concentration was weighted by the number of donors
634 contributing to the pool.

635

	Concentration, ng/mL β (95% CI) *p*		
	ln(DPHP)	ln(BDCIPP)	ln(BCIPHIPP)
Pool avg. age (yrs)	-0.019 (-0.03, -0.007) ***0.002***	-0.026 (-0.031, -0.02) ***<0.001***	-0.005 (-0.010, 0.000) ***0.031***
Sex (female vs. male)	0.474 (-0.088, 1.035) *0.097*	0.224 (-0.047, 0.496) *0.104*	-0.271 (-0.494, -0.047) ***0.018***
Sampling campaign (2 vs. 1)	1.352 (0.724, 1.98) ***<0.001***	0.289 (-0.015, 0.592) *0.062*	0.159 (-0.091, 0.408) *0.210*
Intercept	3.157 (2.502, 3.812) ***<0.001***	0.007 (-0.309, 0.324) *0.964*	0.77 (0.509, 1.030) ***<0.001***
Model Adj. R^2	0.21	0.47	0.08

636

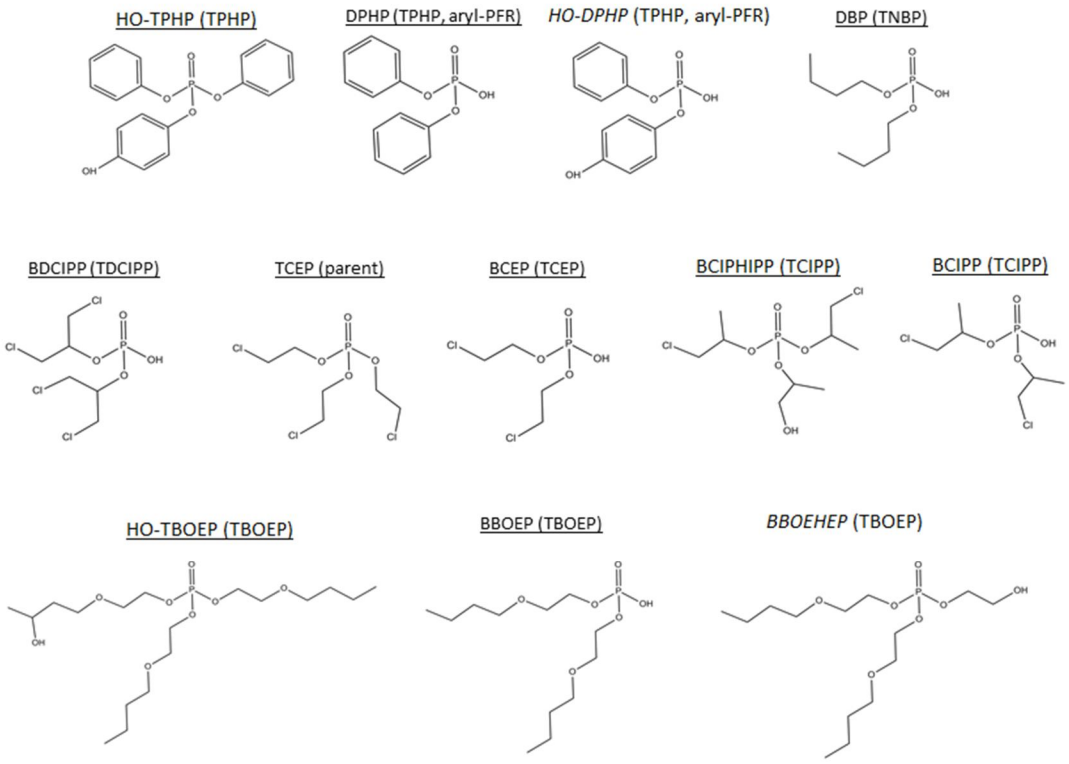
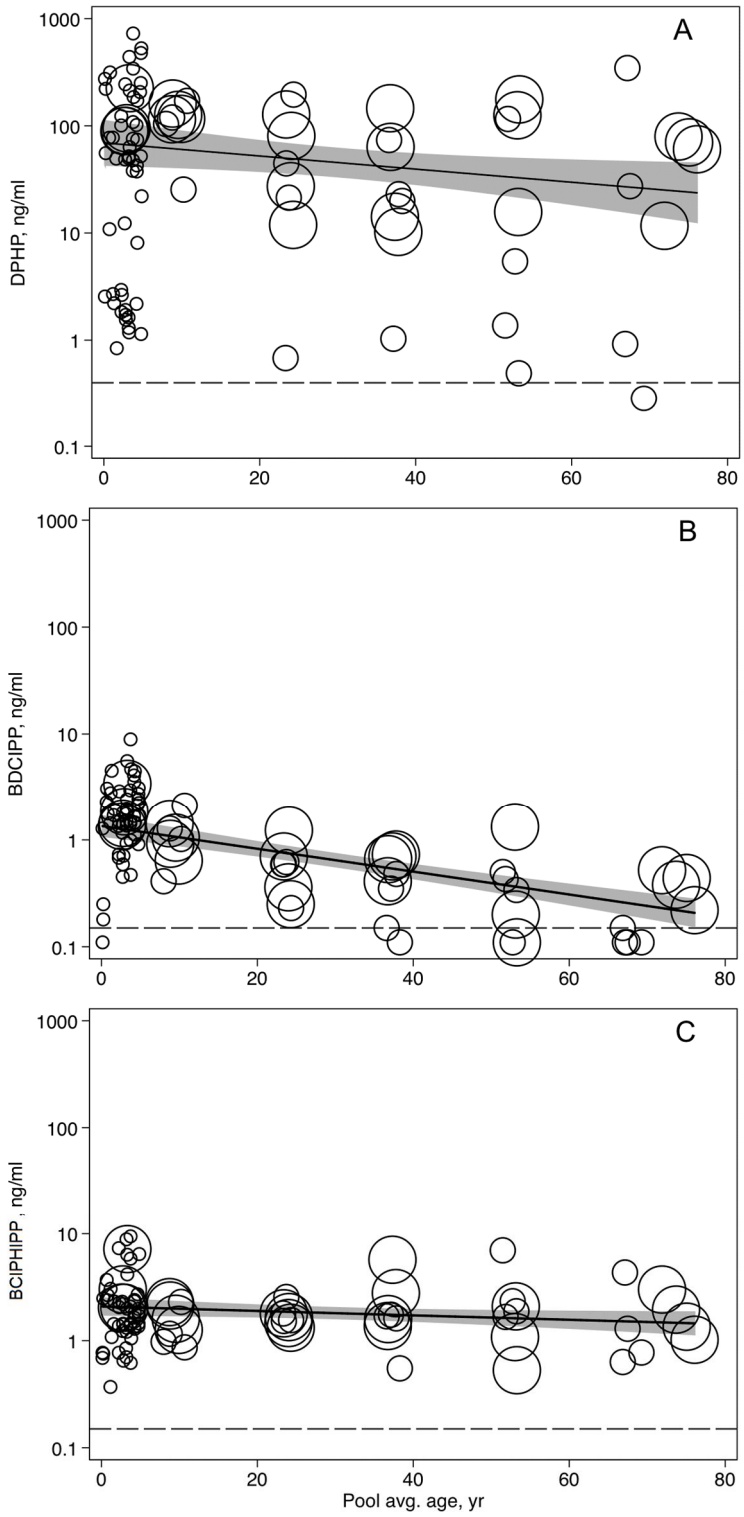


Figure 1: Structures of the target metabolites of phosphate flame retardants (PFRs). Abbreviations of the parent compound(s) are mentioned between brackets. Target PFR metabolites and one parent PFR used for quantitative monitoring are underlined; metabolites that were used for qualitative monitoring are in italics.



644

645 **Figure 2:** Urinary concentrations of DPHP (A), BDCIPP (B) and BCIPHIPP (C) in (ng/ml)
646 vs age. Lines with shaded areas represent simple weighted linear regression vs age and 95%
647 CI on the regression line; weights were assigned based on pool size. Symbol size corresponds
648 to pool size (n=7, 28, or 100). Horizontal dashed line represents the method detection limit
649 (DPHP: 0.30 ng/mL, BDCIPP and BCIPHIPP: 0.15 ng/mL).