Secular trends of urinary phthalate metabolites in 7-year old children and association with building characteristics: Hokkaido study on environment and children's health

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Abstract

The widespread commercial production and use of phthalates as plasticizers in consumer products have led to significant human exposure. Some phthalates are known to disrupt the endocrine system and result in adverse health outcomes. As such, they have been regulated in materials used for children's items and food packages. In this study, we examined the secular trend of urinary phthalate metabolites in children and the association between metabolites and building characteristics. In total, 400 first-morning spot urine samples of 7 years old children collected from 2012 to 2017 from an ongoing birth cohort study were examined. Parents provided information on demographics and building questionnaires. We analyzed 10 urinary phthalate metabolites from five phthalate diesters using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS): MiBP, MnBP, MBzP, MEHP, MEOHP, MEHHP, MECPP, MiNP, OH-MiNP, and cx-MiNP. A multivariable regression model with creatinine-corrected metabolite levels was applied to assess secular trends during 2012–2017. The association between metabolite levels and building characteristics was investigated using a mutual-adjusted linear regression.

The metabolites MnBP, MEHP, MEOHP, MEHHP, MECPP, and OH-MiNP were detected in all samples. The highest median concentration was for MECPP 37.4 ng/mL, followed by MnBP and MEHHP at concentrations of 36.8 and 25.8 ng/mL, respectively. Overall, DBP, BBzP, and DINP metabolite concentrations in this study were comparable to or lower than those in previous studies from Japan and other countries in a similar study period. Higher concentrations of DEHP metabolites were observed in this study than in

children from the USA and Germany, as per previous reports. Despite updated phthalate regulations and reports of production volume change in Japan, all the measured metabolites showed a stable trend between 2012 and 2017. Higher phthalate metabolite levels were observed among children from households with low annual income, those who lived in old buildings, and those with window opening habits of ≥ 1 h than ≤ 1 h. In contrast, children in houses that vacuumed 4 or more days/week showed a lower level of MnBP than those in houses that vacuumed ≤ 3 days/week.

This study demonstrates that the internal exposure level of phthalates in Japanese children was stable from 2012 to 2017. Our findings suggest that phthalate exposure in children is consistent. Thus, improvements in the indoor environment, such as frequent vacuuming, may reduce exposure. Biomonitoring of phthalates is critical for elucidating their possible health effects and developing mitigation strategies.

Keywords

Urinary phthalate metabolites, Secular trend, Children, Human biomonitoring, Building characteristics

1. Introduction[1]

Phthalates or phthalic acid esters are a group of synthetic chemicals with the chemical structure of dialkyl or alkyl aryl esters of 1,2-benzene dicarboxylic acid (Cao et al., 2010). Phthalates are widely used as plasticizers, solvents, and additives in products such as polyvinyl chloride (PVC) materials, children's toys, food packaging, pharmaceuticals, and personal care products (Shinohara et al., 2020; Wang et al., 2019; Hauser and Calafat, 2005). The increased use of phthalates in several products results in its ubiquitous presence in the environment and exposure to the general population through inhalation, ingestion, or dermal contact (Anderson et al., 2018; Hauser and Calafat, 2005). Phthalate exposure has been reported to have endocrine-disrupting effects in humans (Hauser and Calafat, 2005; WHO, 2012) and experimental studies (Lyche et al., 2009).

Owing to the reproductive toxicity of phthalates, the United States and European government regulations were enacted to ban or restrict the use of phthalates, such as di(2-

[1] **Abbreviations:** ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBzP), Ministry of Health, Labor and Welfare (MHLW), di-iso-decyl phthalate (DiDP), di-isononyl-phthalate (DINP), di-octyl phthalate (DnOP), mono-n-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), mono-benzyl phthalate (MBzP), mono (2-ethylhexyl) phthalate (MEHP), mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono (2-ethyl-5-oxohexyl) phthalate (MEOHP), mono (2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-isononyl phthalate (MiNP), mono-hydroxy-isononyl phthalate (OH-MiNP), mono-carboxy-isononyl phthalate (cx-MiNP), UPLC charged surface hybrid (CSH), multiple-reaction monitoring (MRM), German external quality assessment scheme (G-EQUAS), limits of detection (LOD), limits of quantification (LOQ), standard deviation (SD), creatinine excretion (CE)

ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and butyl benzyl phthalate (BBzP), in the production of items associated with children and cosmetics (DIRECTIVE 2005/84/EC, 2005; Public Law 110-314 H.R. 4040, 2008). Such government regulations aim to change the production and use patterns of phthalates. Trend analysis studies in the general population have been used to monitor changes in exposure, such as in Denmark, Germany, Italy, and the US (Frederiksen et al., 2020; Koch et al., 2017; Tranfo et al., 2018; Zota et al., 2016). For instance, a study from the US using the National Health Nutrition and Examination Survey (NHANES) from 2001 to 2010 reported a decrease in DnBP, BBzP, and DEHP metabolite concentrations and increased levels of DINP in the general population of children and adults (CDC, 2019; Zota et al., 2014). Similar studies in Europe have also reported a decline in urinary metabolites of DBP, BBzP, and DEHP in adults (Frederiksen et al., 2020; Tranfo et al., 2018). Additionally, a biomonitoring study conducted in Germany reported a decline in DBP, DEHP, and DINP metabolites in samples collected between 1988 and 2005, mainly from students aged 20–29 years (Koch et al., 2017). Government regulations in the US and European countries have been considered effective, as decreased phthalate exposure has been attributed to them (Tranfo et al., 2018; Zota et al., 2014). In 2010, the Ministry of Health, Labor and Welfare (MHLW) in Japan updated restrictions on the use of DBP, BBzP, DEHP, di-iso-decyl phthalate (DiDP), di-isononyl-phthalate (DINP), and di-octyl phthalate (DnOP) in children's toys and food packaging materials (MHLW Notice No.370, 2010).

Our previous studies have shown higher urinary phthalate metabolite concentrations in children than in adolescents or adults (Ait Bamai et al., 2015). Moreover, urinary phthalate metabolite levels were positively correlated with phthalate concentrations in house dust

(AitBamai et al., 2016). Children are more vulnerable to the adverse effects of phthalate exposure on asthma and allergies than adults (Ait Bamai et al., 2014). Previous studies have indicated the importance of building characteristics and indoor environments on phthalate exposure (Ait Bamai et al., 2014; Hsu et al., 2017). Moreover, to the best of our knowledge, no prior study has been conducted on the biomonitoring of trends in consecutive years of phthalate exposure in the Japanese population. Therefore, we aimed to investigate the secular trend of phthalate exposure in Japanese children between 2012 and 2017 and examined the association between internal phthalate exposure levels and the building characteristics of their homes. Additionally, we estimated the daily intake of phthalates based on metabolite levels in the urine.

2. Materials and methods

2.1 Study population and data collection

This study is part of the ongoing birth cohort study, Hokkaido Study of Environment and Children's Health, Hokkaido Cohort. A detailed description of subject recruitment has been previously described (Kishi et al., 2017, 2011). A total of 20,926 participants were enrolled from February 2003 to March 2012. After accounting for the exclusion criteria of spontaneous abortion, stillbirth, loss to follow-up, and withdrawal, 10,655 singleton children aged 7 years until August 2017 were included. Since 2012, a follow-up questionnaire was sent to these 10,655 children in their birth months, and parents of the 6,218 children returned the questionnaire before September 2017 (response rate 58.4%). Among them, 2,451 children provided urine samples and submitted questionnaires on demographic and building

characteristics. The participants were chosen based on a case-cohort study of wheeze, eczema, and rhinoconjunctivitis. The sample size (n) was 100 for each symptom. A sub-cohort of 243 participants (11.1% of the original cohort) was randomly selected. Consequently, 83 cases and 160 controls were included in this study. To make up the required 100 cases for each symptom, participants with symptoms were randomly added from the original cohort with urine and questionnaire data. After selecting 100 participants for each symptom, 60 children had more than two symptoms. Finally, a total of 400 participants, 240 with symptoms and 160 without symptoms, were selected. Fourteen samples with insufficient urine volume were excluded, leaving only 386 participants for this study (Table 1). Between 2012 and 2017, the number of urine samples collected was randomly distributed as 62, 65, 54, 74, 86, and 45, respectively. The selection details of the 400 participants were described in a previous report (Ait Bamai et al., 2019).

The building questionnaire determined building age, annual household income, number of residents, housing type (single-family house/multi-family house), structure (wood/concrete), newly built or renovated within 1 year (yes/no), ventilation in living and/or child room(s) (yes/no), condensation (yes/no), mold odor (yes/no), visible mold (yes/no), water leakage (yes/no), humidity (yes/no), insecticide (yes/no), flooring (PVC/non-PVC), wall material (PVC/non-PVC), vacuum-cleaning/week, duration of the window opening, and whether the house was on the main road (yes/no).

Parents of children were asked to collect the first morning void urine samples of their children in a polypropylene cup, and these were sent to Hokkaido University, Center for

Environmental and Health Sciences, using a cool delivery service. When the shipped urine samples arrived at our center, the creatinine content was measured using an enzyme-linked immunosorbent assay at SRL, Inc. (Tokyo, Japan). On the same day, samples were transferred to glass test tubes with ground glass stoppers cleaned with acetone, sealed with fluoric tape, wrapped with aluminum foil, and kept at –20 °C until the day of analysis.

The research protocol regarding human sampling was reviewed and approved by the Institutional Review Board of the Hokkaido University Center for Environmental and Health Sciences before the study was conducted. The parents of all participants provided written informed consent to confirm their participation in this study.

2.2 Urinary chemical analysis

Ten phthalate metabolites were measured in the first morning void urine samples of children. The phthalate metabolites assessed included the DBP metabolites [mono-n-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP)], BBzP metabolite [mono-benzyl phthalate (MBzP)], DEHP metabolites [mono (2-ethylhexyl) phthalate (MEHP), mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono (2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono (2-ethyl-5-carboxypentyl) phthalate (MECPP)], and the DINP metabolites [mono-isononyl phthalate (MiNP), mono-hydroxy-isononyl phthalate (OH-MiNP), and mono-carboxy-isononyl phthalate (cx-MiNP)]. For sample preparation, 500 μL urine sample was spiked with 20 μL of a mixture of labeled internal standards and then buffered with 500 μL 100 mM ammonium acetate (pH 6.5), and deconjugated by 50 μL β-glucuronidase with incubation at 37 °C for 90 min. After incubation, 1 mL 0.5% ammonia water was added to

each sample. After this, sample extraction was performed using solid-phase extraction (SPE) that was conditioned with 1 mL 0.05% nitric acid in 90% methanol, 1 mL methanol, and then with 1 mL 0.5% ammonia water to activate the cartridge. Samples were loaded onto the conditioned SPE cartridge and sequentially washed with 0.5 mL ultra-pure water, 0.5 mL methanol, 0.5 mL ultra-pure water, and 0.5 mL 40% methanol containing 0.2% formic acid. The samples were eluted using a mixture of 90% methanol containing 0.2% formic acid. The eluted mixture (250 μL) was transferred to a vial and diluted with 750 μL ultrapure water. To quantify phthalate metabolites, 40 μL of the sample from the vial was injected into a UPLC-MS/MS (ACQUITY UPLC H-class) equipped with a Xevo TQ-S micro mass spectrometer (Waters Corporation, Milford, MA, USA). Detailed information including chemicals and reagents, urine sample collection, sample preparation, instrumental analysis, and chromatographic conditions can be found in the supporting information (Supplemental Figure 1 and Supplemental Tables 1–3).

2.3 Quality assurance

For each batch, two procedural blanks were analyzed to control for background contamination. For all target analytes, 12 calibration points ranging from 0 to 20 ng/mL were used to construct the calibration curves. A satisfactory correlation coefficient of calibration curves ≥ 0.998 was obtained for all measured metabolites. In each batch of 20 samples, replicated analysis of the calibration standard at a concentration of 5 ng/mL and the reference value of 63 G-EQUAS samples with known concentrations were conducted to determine both inter-and intra-day precision and were within acceptable limits with a coefficient of variation

174 < 10%. The limits of detection (LOD) and limits of quantification (LOQ) of individual
175 phthalate metabolites were determined based on 7 repeated analyses of spiked ultra-pure
176 water with 0.16 ng/mL for MEOHP, OH-MiNP, and cx-MiNP; 0.32 ng/mL for MEHP, MiNP,
177 MEHHP, and MECPP; 0.8 ng/mL for MBzP; and 1.6 ng/mL for MiBP and MnBP. The
178 standard deviation (SD) of these repeated analyses was calculated using the following
179 formula: LOD = 2 × t (n-1, 0.05) × SD and LOQ = 10 × SD. Here, t is the student's *t*-value
180 for the 95th percentile of n-1 degree of freedom, where n is the number of repeated samples.
181 The metabolites LOD and LOQ ranged 0.05–0.95 ng/mL and 0.13–2.5 ng/mL, respectively.
182 The recovery percentages of native and labeled internal standards spiked in pooled urine
183 samples ranged from 81% to 120%. The detailed quality assurance (QA)/quality control (QC)
184 results are shown in Supplemental Table 4.

185

186 ## 2.4 Daily intake estimation

187 Based on the measured concentrations of urinary phthalate metabolites, the daily
188 phthalate intake was estimated for each subject using the following equation (Wittassek et
189 al., 2007a).

190 $$\text{EDI} = \frac{UE_{sum} \times CE_{smoothed}}{F_{UE} \times BW} \times MW_P$$

191 where EDI (µg/kg bw/day) is the estimated daily intake of phthalate; UE_{sum} is the sum of
192 the creatine-adjusted molar concentration of phthalate metabolites (µmol/g Cr); $CE_{smoothed}$
193 (g/day) is the smoothed creatinine excretion (CE) rate; BW (kg) is the bodyweight; MWp

(g/mol) is the molecular weight of the respective parent phthalate; and F_{ue} is the urinary excretion factor of the parent phthalate, DnBP, DiBP, BBzP, DEHP, and DINP, which were set to 0.69, 0.69, 0.73, 0.62, and 0.39, respectively (Anderson et al., 2001; Koch et al., 2012; Wittassek et al., 2011). Gender-based values for urinary CE were determined using the following equations, where ht (cm) is the participant's height (Mage et al., 2008).

$$CE = ht \times \{6.265 + 0.0564 \times (ht - 168)\} \times 10^3 \text{ (male)}$$

$$CE = 2.045 \times ht \times \exp\{0.01552 \times (ht - 90)\} \times 10^3 \text{(female)}$$

2.5 Statistical analysis

To ensure that the study population (n = 386) was representative of the original cohort, we conducted sensitivity analyses with a sub-cohort that included 243 participants. The results showed similarity in the distribution of demographic characteristics, building characteristics, levels of phthalate metabolites, and their trends. Thus, we can anticipate minimal probable bias for our study population (n = 386) (Supplemental Table 5 and 6; and Supplemental Figure 2). For concentrations below the LOD, the LOD × detection frequency was assigned for statistical analysis (James et al., 2002). Additionally, the molar concentrations of DBP metabolites (MiBP and MnBP), DEHP metabolites (MEHP, MECPP, MEOHP, and MEHHP), and DINP metabolites (MiNP, OH-MiNP, and cx-MiNP) were combined to estimate the parent compound exposure. The distribution of phthalate metabolites is presented as minimum, percentiles (25, 50, 75, and 95), and maximum values. A regression model with creatinine-corrected metabolite concentrations was applied to

assess the secular trend from 2012 to 2017. Additionally, Dunnett's test, considering 2012 as a reference, was conducted to compare pairwise metabolite level mean differences by year. The *p*-values were adjusted using the Bonferroni correction. We first conducted a univariate analysis to analyze the distribution of urinary phthalate metabolite levels according to different building characteristics. We then investigated associations between phthalate metabolite concentrations and the building characteristics that showed a significant difference in univariate analysis using mutual-adjusted linear regression. We calculated the percent difference from the regression coefficient as $(e^{(\beta)} - 1) \times 100$ % with 95 % CIs estimated as $(e^{(\beta \pm \text{critical value} \times \text{SE})} - 1)$, where β and SE are the estimated regression coefficient and standard error, respectively. Yes indicators regarding dampness; condensation, mold odor, visible mold, water leakage, and humidity were assigned a value of 1 to compute the dampness index (1–5). Statistical analysis was performed using JMP Clinical 6.0, SAS.

3. Results and discussion

In this study, we presented the levels and secular trends of phthalate metabolites in Japanese children from Hokkaido between 2012 and 2017. Moreover, we investigated the association between building characteristics and levels of urinary phthalate metabolites and estimated daily phthalate exposure in children based on urinary metabolite levels.

3.1 Study population

Due to insufficient sample volume or sample preparation error, 14 samples were excluded, and 386 samples were included in this study. All children in this study were seven

years old; the gender participation was nearly balanced with males (52.6%) and girls (47.4%). The data represent the phthalate exposure trend of six consecutive years as children's urine samples were collected each year from 2012 to 2017. Nearly 60% of the participants lived in a mechanically ventilated house. Compared to our previous study, more participants in this study lived in houses with PVC flooring (16.9% vs. 7%) and slightly older buildings (median: 13 years vs. 10.5 years) (Kishi et al., 2018). The participants' demographic and building characteristics with urine collection years are presented in Table 1.

3.2 Concentration and secular trend of urinary phthalate metabolites

The distribution of urinary phthalate metabolite concentrations along with creatinine (Cr)-corrected levels in children is summarized in Table 2. Phthalate metabolites MnBP, MEHP, MEOHP, MEHHP, MECPP, and OH-MiNP were detected in all samples. The highest concentration was found among the DEHP metabolites MECPP and MEHHP, followed by MnBP. The creatinine-corrected concentrations showed a similar trend. All creatinine-corrected urinary phthalate metabolites in this study showed a significant positive Spearman's correlation. The highest correlation was found between DEHP and DINP metabolites (Supplemental Table 7). Although many studies have reported seasonal variations in phthalate exposure (Bi et al., 2018; Li et al., 2019), in this study, no association was observed between the sample collection seasons and phthalate metabolite levels (Supplemental Table 8). The secular trend of the evaluated urinary phthalate metabolite levels is shown in Figure 1. Regression analysis showed that all measured metabolites were stable throughout the study period. To the best of our knowledge, this is the first human

biomonitoring study to investigate internal phthalate exposure trends in the Japanese population. In 2010, the regulation of phthalates in children's toys and food packaging materials was revised in Japan. Consequently, changes in the production and exposure to phthalates have been reported in Japan, for example, from 2012 to 2017, the production of DEHP decreased by 13.3% (135,000 to 117,000 t). In contrast, the production of DINP increased by 43.2% (67,000 to 96,000 t) (IHS Markit, 2018; VEC, 2018). However, urinary phthalate metabolites showed a stable trend. This suggests that the reported changes in chemical production do not reflect children's exposure. A plausible explanation for the lack of a trend in the current study could be the limited scope of phthalate regulation. The regulation only concerns toys meant for children under 6 years of age and food containers containing fats and oils but excludes materials such as PVC flooring, wall and ceiling coverings (common in modern Japanese houses and apartments), and personal care products, which have been reported as potential phthalate exposure sources (Bornehag et al., 2005; Carlstedt et al., 2013; Husoy et al., 2020). Higher levels of DEHP in indoor dust in Japanese households than in other EU countries and the USA have been reported (Ait Bamai et al., 2014). Thus, the stable trend in exposure levels observed in this study can be attributed to phthalates emitted from non-regulated materials (Ait Bamai et al., 2014; Carlstedt et al., 2013; Husoy et al., 2020).

Comparing the results of our current and previous studies (Ait Bamai et al., 2015) in Japanese children, we observed a relatively higher median concentration for MiBP, MBzP, MEHP, MEOHP, and lower MnBP in the previous study than in the current study (Figure 2, Supplemental Table 9). However, this comparison should be interpreted with caution because

279 the method of analysis was different for the two studies. The previous study used
280 derivatization and was measured by GC-MS, while the current study used LC-MS/MS. The
281 concentrations of urinary phthalate metabolites in children among comparable age groups
282 show variations similar to those in the findings from other countries over a similar period
283 (Figure 2, Supplemental Table 9) (Ait Bamai et al., 2015; Becker et al., 2009; CDC, 2019;
284 Hartmann et al., 2015; Liao et al., 2018; Schwedler et al., 2020; Song et al., 2013; Wang et
285 al., 2015; Weng et al., 2017). For instance, a similar level of MiBP was observed in children
286 from the US (CDC, 2019), while a 2- to 3-fold higher median concentration was observed in
287 Germany (Schwedler et al., 2020) and China (Liao et al., 2018; Wang et al., 2015). A
288 noticeably higher level of DEHP metabolites was observed in our study participants than in
289 Germany (Schwedler et al., 2020) and the USA (CDC, 2019) (Figure 2). This indicates that
290 Japanese children still have high exposure to DEHP despite production regulations and
291 efforts to replace DEHP (Rowdhwal and Chen, 2018; VEC, 2018). We previously reported
292 that DEHP and BBzP concentrations in house dust are positively correlated with urinary
293 metabolite concentrations (Bamai, 2016). Additionally, a review revealed that house dust
294 ingestion is a significant exposure pathway for phthalates such as DEHP in Japan (Takagi
295 and Yoshinaga, 2009). Thus, the high levels of urinary DEHP metabolites in this study might
296 be due to non-regulated consumer products, such as PVC building materials, which can
297 release phthalates.

298 Considering the DINP in this study, consistent trends in its metabolites were observed.
299 This might be due to the wide use of DINP in PVC wallpapers, wire, and cable insulation
300 jacketing in Japan, which are less likely to be changed/installed frequently (IHS Markit,

2018). Moreover, most of the children in this study lived in houses with a mean age of 13 years (Table 1), which was built before the regulation and increased production of DINP, which could explain the stable trend observed in this study. Biomonitoring studies conducted in the early 2010s reported increased levels of DINP in Germany, Italy, and the USA (Tranfo et al., 2018; Wittassek et al., 2007; Zota et al., 2016). DINP has been subjected to regulation due to its various health risks and is substituted with alternatives such as DEHTP and DINCH, resulting in a decline in DINP exposure in recent years (CDC 2019, Frederiksen et al., 2020, Schwedler et al., 2020). Since the current study did not measure urinary DINP metabolites before the 2010 revised phthalate regulations, exposure levels of DINP in Japanese children before the regulation are uncertain. This warrants follow-up studies with a large population size to elucidate exposure changes over time.

3.3 Urinary phthalate metabolite levels and building characteristics

Despite the government regulations regarding phthalates in Japan, our trend analysis showed a stable level of phthalate metabolites. Additionally, the high detection frequency of urinary phthalate metabolites indicates that children are still widely exposed, probably from non-regulated products such as building materials. Thus, we evaluated potential phthalate exposure based on household characteristics and daily habits (Table 3). Our mutually adjusted regression model revealed a significant positive association between lower household income and OH-MiNP ($\beta = 0.138$) and $\sum$DINP ($\beta = 0.127$). This result is in agreement with those of previous studies that reported that lower socioeconomic status (SES) families tend to show a higher level of urinary phthalate metabolites, such as MBzP and

DEHP metabolites (Koo et al., 2002; Navaranjan et al., 2020). This may have several precipitating factors, such as the influence of SES on dwelling characteristics or fast-food consumption habits resulting from parental education levels, which have previously been reported as variables causing increased phthalate exposure (Ait Bamai et al., 2014; Zota et al., 2016). In the present study, MnBP levels showed a significant positive association with building age. Furthermore, our stratified analysis revealed an increased beta value of MnBP ($\beta > 0$) for older buildings, indicating an increased level of MnBP as the building age increased. Building age has been reported as a common predictor of indoor phthalate levels in the dust (Bornehag et al., 2005). There is evidence that the DnBP parent compound of MnBP was used as a plasticizer in PVC materials in the 1980s (Kavlock et al., 2002). Thus, the use of DnBP in interior materials for older buildings could explain our finding of a significant relationship between MnBP and building age. Metabolite MnBP was lower in children who lived in houses that were vacuumed 4–7 times/week than in those that were vacuumed ≤ 3 days/week. This result can be explained by the frequent vacuuming association with decreasing dust accumulation, resulting in lower phthalate levels (Wilson and VanSnick, 2017), as phthalates emitted from vinyl building materials can be absorbed into house dust (Liang and Xu, 2014).

Children in houses with ≥ 1 h/day window opening habits showed significantly elevated levels of MiBP, MnBP, and DEHP metabolites compared to children in houses with < 1 h/day window opening habits. This result is unpredicted, as opening windows are expected to facilitate air exchange and decrease phthalate levels in the indoor environment (Śmiełowska et al., 2017). A possible reason for this contradictory result could be the

344 confounding effect of building age on the association between window opening and
345 metabolite levels. We observed higher metabolite levels in children living in older buildings
346 (Table 3). Hence, this finding raises the question: "Did the families with fewer open windows
347 live in newer houses with air-conditioning installed?". Unfortunately, we did not have data
348 on the use/installation of air-conditioning, but in this study area Hokkaido, it was uncommon
349 for households to have air-conditioning due to the climate being cool. Moreover, we did not
350 find a significant difference ($p > 0.786$) in building age between less window opening houses
351 (median 15.9 year) and more window opening houses (median 16.8 year). Therefore,
352 building age was not found to be a confounding factor in the relationship between window
353 opening and metabolites. Another study that examined the impact of open and closed
354 windows on indoor air composition reported that emission of semi-volatile compounds such
355 as phthalates was enhanced when windows were open rather than closed (Fortenberry et al.,
356 2019). Additionally, Xu et al. (2010) revealed that the emission rate of DEHP from vinyl
357 flooring increased at a high ventilation rate because of the higher air velocity near the surfaces
358 and consequently results in an increase in the mass-transfer coefficients that promote the
359 emission of DEHP. This suggests that window opening enhances ventilation and increases
360 emissions to the indoor environment. Subsequently, the internal exposure level of the DEHP
361 increased.

362 3.4 Estimated daily intake (EDI) of phthalates

363 Hereafter, we estimated the daily intake of phthalates in children, as shown in Figure 3. Daily
364 intake of DEHP had the highest median EDI value of 3.7 μg/kg/day. The EDIs of DnBP were

365 slightly higher in boys than girls, with a mean value of 1.8 and 1.4 μg/kg/day, respectively.
366 Based on the European Food Safety Authority (EFSA), tolerable daily intake (TDI) reference
367 values for individual phthalates DnBP and DEHP, one child in each phthalate exceeded the
368 reference values of 10 and 50 μg/kg/day, respectively (EFSA, 2005a,2005c). Considering the
369 updated EFSA risk assessment of combined exposure to DBP, BBzP, DEHP, and DINP at a
370 group-TDI level of 50 μg/kg/day, two children with one child at a marginal level were
371 observed (EFSA 2019). Considering the US reference dose (RfD) of 20 μg/kg/day for DEHP
372 (US. EPA., 1991), two children exceeded the RfD value and another 2 were on the reference
373 borderline, representing 1.03 % of the participants (Figure 3). Comparing this study's median
374 EDI values with those of other studies in children, DiBP, DnBP, BBzP, DEHP, and DINP
375 were lower or comparable (Ait Bamai et al., 2015; Kasper-Sonnenberg et al., 2014; Yoshida
376 et al., 2020). In contrast, the EDI value of DINP in this study was higher than that of
377 Taiwanese children with a median of 0.5 and 0.2 μg/kg/day, respectively (Chang et al., 2017).
378 However, caution should be taken when interpreting phthalate EDI comparisons, since
379 variations in participant characteristics or study methods may alter EDI among different
380 studies and countries.

381 In the future, the use of PVC gloves and rubbing alcohol during the COVID-19 pandemic is
382 likely to increase exposure risk among the general population, which further highlights the
383 need for phthalate biomonitoring.

384

4. Strengths and limitations

Selecting participants of the same age (7 years) in this study allowed for a better comparison by eliminating age as a confounding factor. Additionally, the accuracy of our phthalate metabolite measurement method was validated by the external quality assessment scheme G-EQUAS, which strengthens the reliability of our results (Supplemental Table 10). The building characteristics data in this study also strengthened our investigation by facilitating the identification of possible phthalate exposure sources. The primary limitation of this study is the small sample size. However, this data still provides valuable evidence on changes in phthalate exposure during 2012–2017 in Japanese school-aged children. A secondary limitation is the participants' selection bias of including children with allergies, which could limit the generalizability of this study. However, since the distribution of children with allergies in each year was similar, approximately 16%. Thus, we anticipated that the inclusion of children with allergies would have a minimal effect on any probable bias from our trend analysis. Another limitation is that the urine samples were collected only once, which may not represent variance in urinary phthalate metabolite excretion based on individual activity, diet, personal care product use, cleanliness, and seasonal dust accumulation with an open window. Thus, to reduce the variability of metabolite levels during the day, we used the first morning void urine samples.

5. Conclusions

This study is the first to document the consecutive stable trend of the internal exposure level of phthalates in Japanese children between 2012 and 2017, indicating that consistent phthalate exposure exists even after the regulation update of phthalates. Furthermore, we identified correlations between a high exposure level of MnBP among children in old buildings, DINP metabolites among those with lower household income, and MiBP, MnBP, and DEHP metabolites among those with long window opening habits. Frequent vacuum cleaning was associated with lower MnBP levels in children. Finally, other personal care and protective equipment that are known to increase phthalate exposure risk should be evaluated in future studies.

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Declaration of consent

The authors have no conflicts of interest to report.

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Table 1: Study population demographic and building characteristics

		N (%) or median (range)
Gender	Boys	203 (52.6)
	Girls	183 (47.4)
Height	cm	119.8 (102.0-150.0)
Weight	Kg	22.0 (14.8-42.3)
Urine sample collection year	2012	62 (16.1)
	2013	65 (16.8)
	2014	54 (14.0)
	2015	74 (19.1)
	2016	86 (22.2)
	2017	45 (11.6)
Annual household income (JPY)	< 3 Million	48 (12.4)
	≥ 3 Million	321 (83.2)
Number of residents	≤4	251 (65.0)
	≥5	135 (35.0)
Home type	Detached	269 (70.0)
	Apartment	116 (30.0)
House structure	Wooden	269 (69.5)
	Concrete	114 (29.5)
Renovation within the past 1 year	Yes	22 (5.7)
	No	364 (94.3)
Mechanical ventilation system in living and/or child room	Yes	229 (59.3)
	No	157 (40.7)
Use of insecticide	Yes	125 (32.4)
	No	261 (67.6)
PVC flooring	Yes	65 (16.9)
	No	321 (831)
PVC wall material	Yes	310 (80.3)
	No	76 (19.7)
Vacuum cleaning/week	≤3 times	199 (54.8)
	4-7 times	164 (45.2)
Duration of window opening/day	<1 hour	247 (64.0)
	≥1 hour	139 (36.0)
Main road	< 50 meters	75 (19.5)
	No or ≥ 50 meters	310 (80.5)
Building age (years)	Continuous	13 (<1- 50)
Dampness index (0-5)	Continuous	2 (1 - 5)

Table 2: Distribution of urinary phthalate metabolite concentrations in 7 years old children.

Phthalate metabolite (ng/mL)	LOD		Percentile					
	(ng/mL)	% > LOD	Min.	P25	P50	P75	P95	Max.
MiBP	0.95	99.7	<LOD	7.1	12.1	27.4	86	463.1
MnBP	0.78	100	2.5	20.7	35.1	58.8	117.1	1259.3
MBzP	0.10	98.9	<LOD	0.7	1.5	3.5	22.8	498.1
MEHP	0.15	100	0.4	2.4	4.1	7	16.1	31.6
MEOHP	0.05	100	1.3	12.3	20.5	33.2	65.1	158.7
MEHHP	0.15	100	1.8	16.4	26.7	43.8	84.8	219
MECPP	0.12	100	2.4	23.3	38.4	67.1	134.8	323.3
MiNP	0.09	96.9	<LOD	0.4	0.6	1.2	2.9	7.8
OH-MiNP	0.05	100	0.3	2.2	4.1	7.5	17	60
cx-MiNP	0.11	99.7	<LOD	1.3	2.4	4.6	10.8	35.3
∑DEHP [a] (µmol/L)	n.a	n.a	0.01	0.18	0.29	0.49	0.97	2.38
∑DINP [b] (µmol/L)	n.a	n.a	0.00	0.01	0.02	0.04	0.09	0.29
Creatinine corrected (µg/g Cr)								
MiBP			1.6	8.4	13.3	25.0	96.6	341.4
MnBP			4.7	26.3	39.1	59.2	108.6	1516.4
MBzP			<LOD	0.7	1.7	3.9	37.1	533.9
MEHP			0.7	2.9	4.5	7.4	16.2	61.8
MEOHP			2.4	14.9	22.4	32.5	69.7	228.7
MEHHP			4.2	19.3	28.7	43.7	94.8	415.7
MECPP			4.9	27.0	42.8	68.5	136.1	554.4
MiNP			<LOD	0.4	0.7	1.3	3.2	15.0
OH-MiNP			0.7	2.8	4.5	7.4	18.3	113.7
cx-MiNP			<LOD	1.6	2.7	4.9	12.1	45.7
∑DEHP [a] (µmol/L)			0.04	0.22	0.34	0.51	1.01	4.16
∑DINP [b] (µmol/L)			0.00	0.01	0.02	0.03	0.08	0.49
Urinary creatinine (g/l)			0.1	0.6	0.9	1.2	1.7	2.2

[a] ΣDEHP: sum of molar concentrations metabolites [MEHP + MEOHP + MEHHP + MECPP]

[b] ΣDINP: sum of molar concentrations metabolites [MiNP + OH-MiNP + cx-MiNP]

Abbreviations; LOD: Limit of detection; Max: maximum; Min: minimum; P: percentiles; n.a: not applicable; MiBP: mono-isobutyl phthalate, MnBP: mono-n-buty phthalate, MBzP: mono-benzyl phthalate, MEHP: mono (2-ethylhexyl) phthalate, MEOHP: mono (2-ethyl-5-oxohexyl) phthalate, MEHHP: mono (2-ethyl-5-hydroxyhexyl) phthalate, MECPP: mono (2-ethyl-5-carboxypentyl) phthalate, MiNP: mono-isononyl phthalate, OH-MiNP: mono-hydroxy-isononyl phthalate, cx-MiNP: mono(carboxy-isononyl phthalate).

Table 3: Percent difference (95% CI) in phthalate metabolites concentrations with demographic and building characteristics of children house (N=386)

Variables	Categories	MiBP	MnBP	MBzP	∑DEHP	∑DINP
Annual household income, (in JPY)	≥ 3 Million	Ref	Ref	Ref	Ref	Ref
	<3 Million	8.2 (-7.0,25.9)	3.6 (-7.0,15.3)	-3.6 (-22.0,20.0)	10.3 (-0.6,22.4)	13.9 (1.1,28.3) *
Building age (years)	Continuous	-0.6 (-1.6,0.4)	1.0 (0.3,1.7) **	0.8 (-0.7,2.3)	0.1 (-0.6,0.8)	-0.5 (-1.3,0.2)
Vacuum cleaning/week	≤3 times	Ref	Ref	Ref	Ref	Ref
	4-7 times	-8.5 (-17.2,1.0)	-7.2 (-13.5, -0.4) *	-7.3 (-19.7,7.1)	-5.0 (-11.3,1.7)	4.7 (-3.2,13.3)
Duration of window opening	<1 hour	Ref	Ref	Ref	Ref	Ref
	≥1 hour	11.6 (0.6,23.8) *	9.7 (1.9,18.1) *	-1.4 (-15.2,14.6)	12.3 (4.6,20.6) **	-1.0 (-8.8,7.4)
Ventilation in living or child room	Yes	Ref	Ref	Ref	Ref	Ref
	No	3.0 (-7.6, 14.7)	-1.0 (-8.3,6.9)	-6.5 (-22.7,13.2)	0.4 (-6.7,8.0)	-4.0 (-11.8,4.5)
Dampness index (0-5)	Continuous	1.6 (-6.8,10.9)	5.3 (-1.0,12.0)	-4.7 (-16.0,8.1)	0.1 (-5.7,6.3)	0.8 (-5.9,8.0)

Ref: reference, *P<0.05, ** P<0.01, Phthalate metabolites in urine were natural log transformed and corrected for creatinine level before analysis. General regression analyses conducted with phthalate metabolites concentration as dependent variable and the all building characteristics were mutually adjusted. DEHP metabolites MEHP, MEOHP, MEHHP and MECPP showed similar estimates thus in this table ∑ DEHP is presented. DINP metabolites MiNP, OH-MiNP, and cx-MiNP showed similar direction estimate thus in this table ∑ DINP is presented.

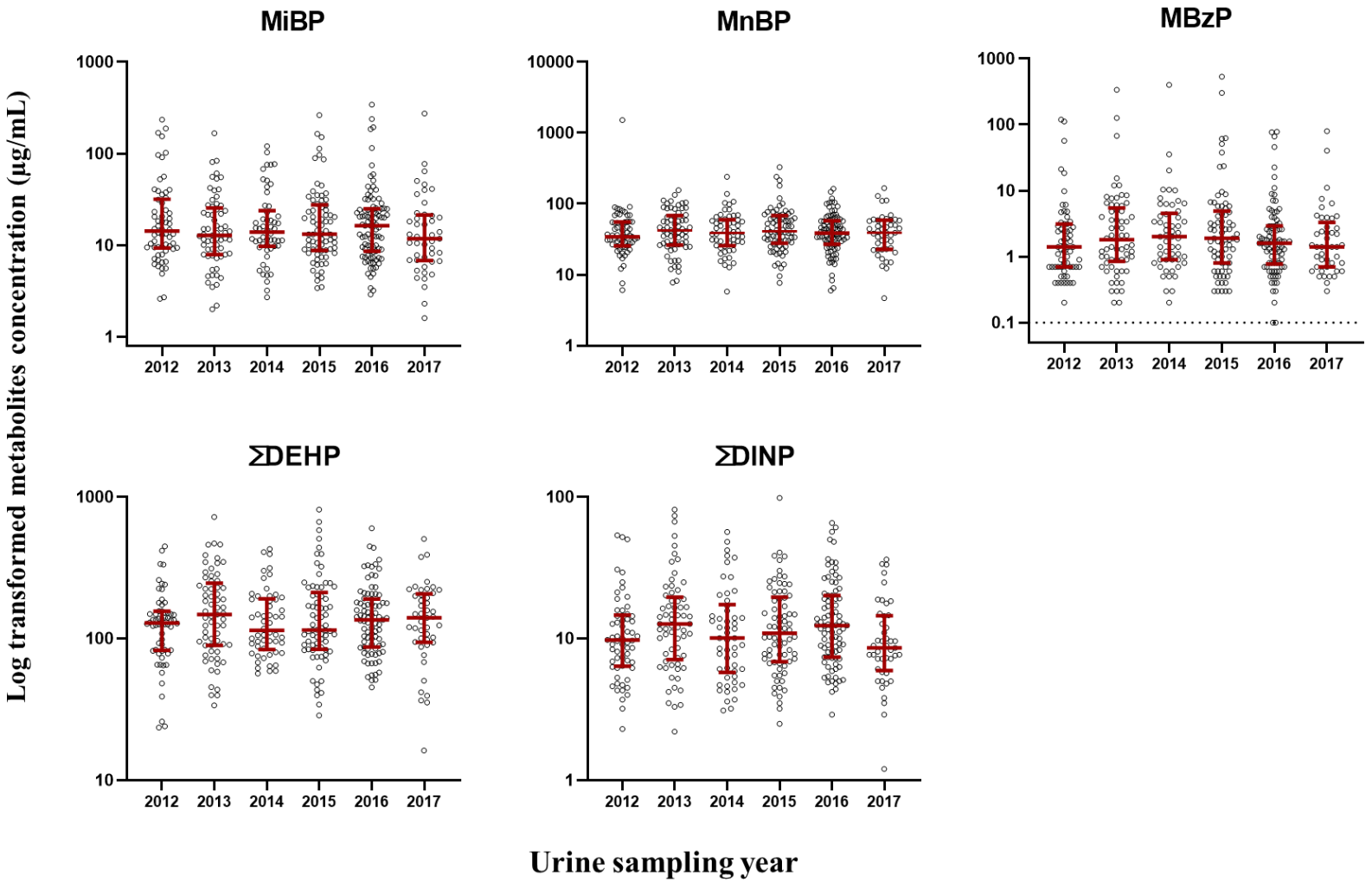

Figure 1: Natural log transformed creatinine corrected concentration level of urinary phthalate metabolites. Bars represent interquartile ranges and median. Points on dotted line indicates samples with concentration limit of detection (<LOD).

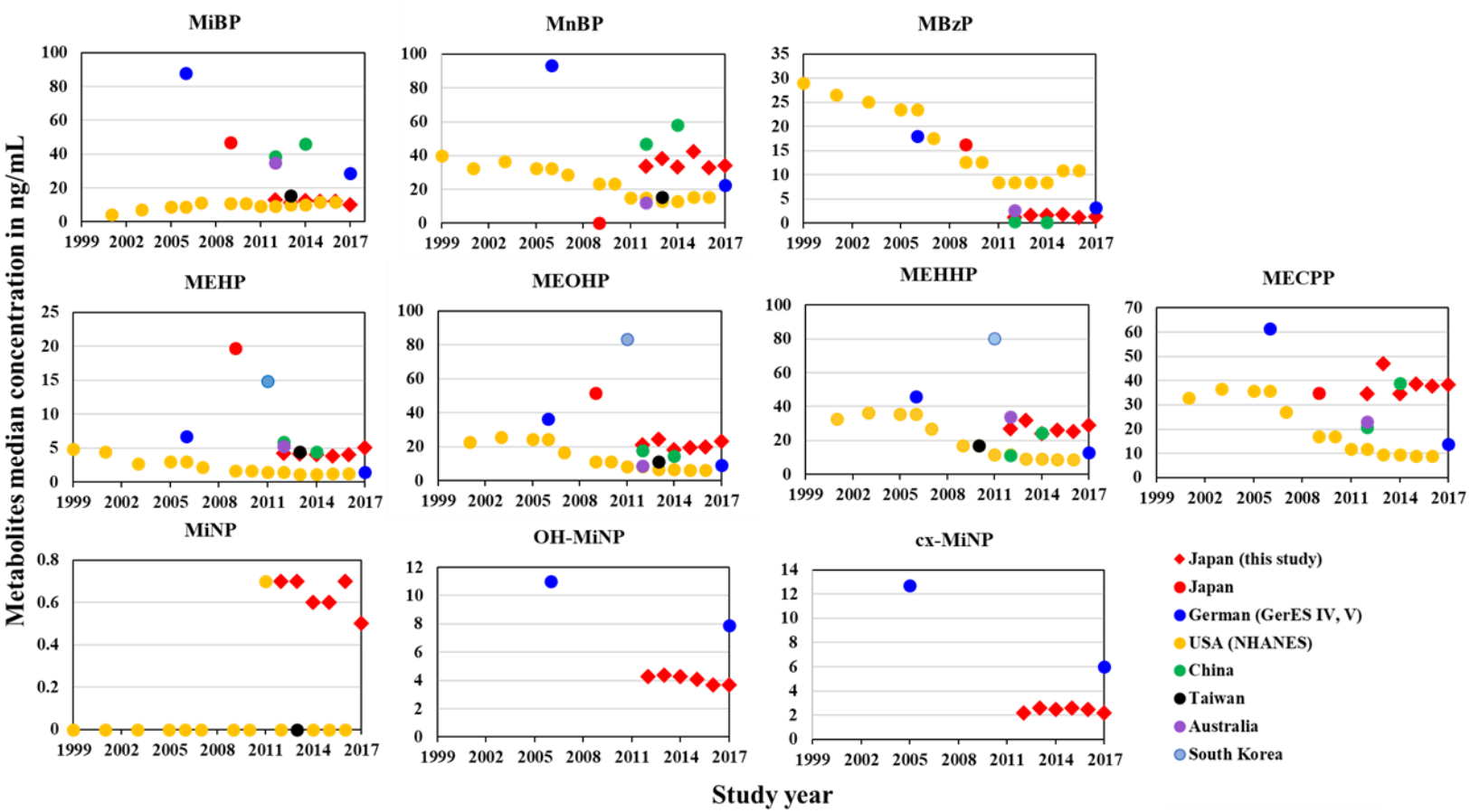

Figure 2: Secular trends and comparison of phthalate metabolites concentration (median ng/mL) in children from different countries. Data sources: Japan from present study and (Ait Bamai et al., 2015); German from (Becker et al., 2009; Schwedler et al., 2020) ; USA from (CDC, 2019) ; China from references (Liao et al., 2018; Wang et al., 2015); Taiwan from reference Weng et al.,2017; Australia from (Hartmann et al., 2015) reference; South Korea from (Song et al., 2013). Points on zero represent concentrations with less than limit of detection (<LOD).

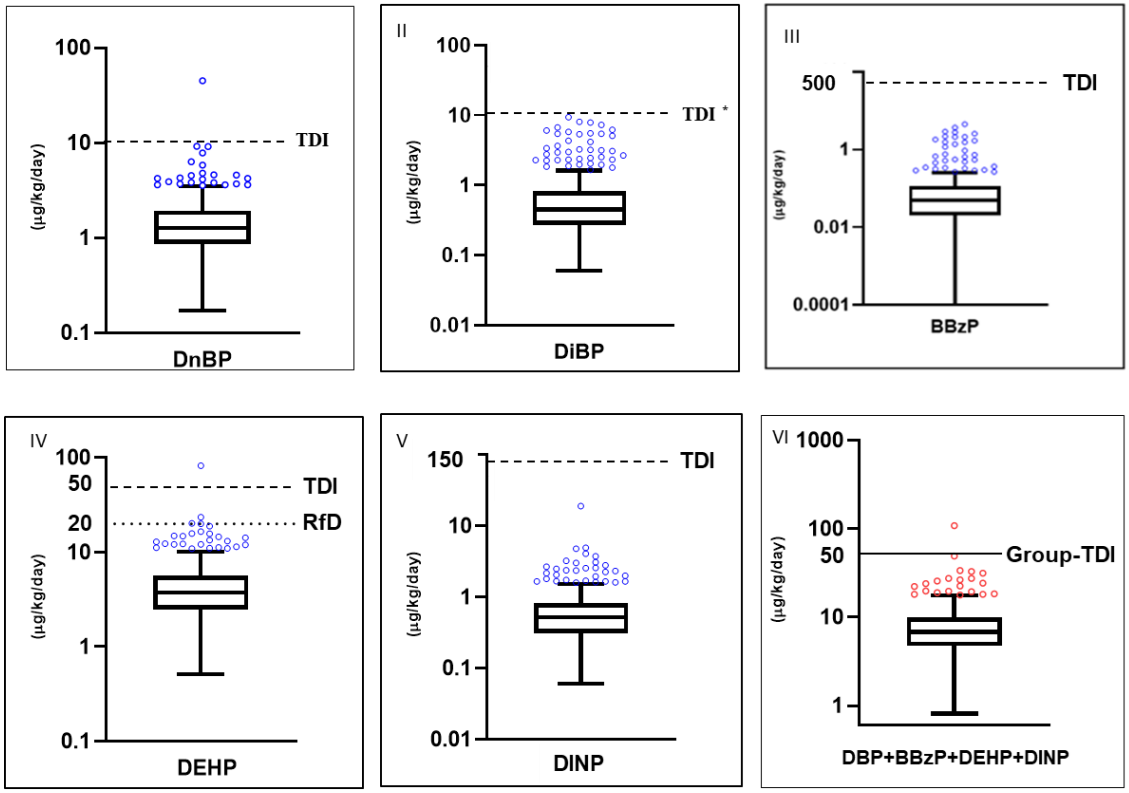

Figure 3: Estimated daily intake (µg/kg/day) of (I) di-n-butyl phthalate (DnBP) based on mono-n butyl phthalate, (II) di-isobutyl phthalate (DiBP) based on mono-iso butyl phthalate * TDI assumed by analogy to DnBP, (III) Butyl benzyl phthalate (BBzP) based on mono-benzyl phthalate MBzP, (IV) di(2-ethylhexyl) phthalate (DEHP) sum of molar concentrations metabolites mono(2-ethylhexyl) phthalate [(MEHP) + mono(2-ethyl-5-oxohexyl) phthalate (MEOHP)+ mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) + mono (2-ethyl-5-carboxypentyl) phthalate (MECPP)], (V) di-iso-nonyl-phthalate (DINP) sum of molar concentrations metabolites [mono-isononyl phthalate (MiNP) + mono (hydroxy-isononyl) phthalate +(OH-MiNP) mono (carboxy-isononyl) phthalate (cx-MiNP)]. (VI) grouped DBP, BBzP, DEHP and DINP. The horizontal dot-dashed lines represent EFSA (2005 and 2019) tolerable daily intake (TDI) and the U.S. EPA reference dose (RfD) and plots above these lines represent values that exceeded EFSA or RfD reference.