



Detection of high PBDD/Fs levels and dioxin-like activity in toys using a combination of GC-HRMS, rat-based and human-based DR CALUX® reporter gene assays

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HIGHLIGHTS

- We determined DR CALUX and DR_{human} CALUX REP values for PBDD/Fs.
- In sampled plastic toys, we measured high levels of PBDD/Fs using GC-HRMS.
- GC-HRMS-based TEQ calculated using PCDD/F TEF were up to 3821 pg TEQ/g.
- Bioassay equivalents up to 2550 pg TEQ/g were measured by DR CALUX® bioassays.
- Mouthing of contaminated plastics may significantly contribute to dioxins TDI.

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ABSTRACT

Brominated dibenzo-*p*-dioxins and dibenzofurans (PBDD/Fs) are increasingly reported at significant levels in various matrices, including consumer goods that are manufactured from plastics containing certain brominated flame retardants. PBDD/Fs are known ligands for the aryl hydrocarbon receptor (AhR) but are not yet considered in the hazard assessment of dioxin mixtures. The aim of the present study was to determine if PBDD/Fs levels present in plastic constituents of toys could pose a threat to children's health. PBDD/Fs, unlike their chlorinated counterparts (PCDD/Fs), have not been officially assigned toxic equivalence factors (TEFs) by the WHO therefore, we determined their relative potency towards AhR activation in both human and rodent cell-based DR CALUX® bioassays. This allowed us to compare GC-HRMS PBDD/F congener levels, converted to total Toxic Equivalents (TEQ) by using the PCDD/F TEFs, to CALUX Bioanalytical Equivalents (BEQ) levels present in contaminated plastic constituents from children's toys. Finally, an estimate was made of the daily ingestion of TEQs from PBDD/Fs-contaminated plastic toys by child mouthing habits. It is observed that the daily ingestion of PBDD/Fs from contaminated plastic toys may significantly contribute to the total dioxin daily intake of young children.

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1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) are persistent organic pollutants that are highly toxic, particularly regarding developmental toxicity, following exposure at early stages of life (Birnbaum, 1995; Vreugdenhil Hestien et al., 2002). Most, if not all toxic effects of dioxins and dioxin-like compounds

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Abbreviations

AHR	: Aryl hydrocarbon receptor
BEQ	: Bioanalytical Toxicity Equivalent
BEQ _T	: Theoretical Bioanalytical Toxicity Equivalent
DecaBDE	: Decabromodiphenyl ether
EC50	: Half-maximal effective concentration
ESCHER	: European Scientific Committee on Health and Environmental Risks
GC-HRMS	: Capillary gas chromatography-high resolution mass spectrometry
LOEC	: Lowest observed effect concentration
PBDD/Fs	: Polybrominated dibenzo- <i>p</i> -dioxins and dibenzofurans
PBDEs	: Polybrominated diphenyl ethers
PCBs	: Polychlorinated biphenyls
PCDDs	: Polychlorinated dibenzo- <i>p</i> -dioxins
PCDFs	: Polychlorinated dibenzofurans
REP	: Relative Equivalent Potency
RIVM	: Dutch National Institute for Public Health and the Environment
TDI	: Tolerable daily intake
TEF	: Toxic Equivalent Factor
TEQ	: Toxic Equivalent
TWI	: Tolerable weekly intake
WHO	: World Health Organization

are mediated via their interaction with the Aryl hydrocarbon Receptor (AhR), which is a key transcription factor involved in numerous biological processes such as xenobiotic metabolism, immune response, and development (Larigot et al., 2018). Because of their detrimental effects on human health, PCDD/Fs are strictly regulated, and low limit values in e.g. food are in force in the EU and other parts of the world.

The standard analytical method for the quantification of dioxins and dioxin-like PCB congeners in various type of samples such as food, house dust and sediments, is capillary Gas Chromatography coupled to High-Resolution Mass Spectrometry (GC-HRMS). To translate quantitative GC-HRMS data into relevant toxicity information, the concentration of each individual toxic PCDD/F congener is multiplied by TEF-based toxic potency and added up to determine the total 2,3,7,8-TCDD related toxic equivalency (TEQ) of a sample (Van den Berg et al., 2006).

Since the turn of the century, innovative cell-based AhR-driven reporter gene assays, such as DR CALUX®, have been developed as an alternative high-capacity screening alternative to GC-HRMS for a wide variety of matrices (Murk et al., 1996; Besselink et al., 2004; Croes et al., 2013; Houtman et al., 2002; Vugt-Lussenburg et al., 2013). The quantification of the total toxic potency of PCDD/Fs, expressed in bioanalytical toxic equivalence (BEQ), using the DR CALUX bioassay is based on luminescence induction mediated by transactivation of the AhR through interaction of PCDD/Fs and related compounds. An additional advantage of DR CALUX, and related reporter assays, is that compounds similar to PCDD/Fs in their toxic mode of action, such as brominated dioxins and furans (PBDD/PBDFs) can also be detected and quantified. Combining the bioassay-based BEQ determination with GC-HRMS enables us to either confirm the results chemically, or find out which compounds are responsible for the additional bioactivity that may have been detected in a sample, and in this way identify the culprit chemicals, such as PBDD/Fs.

Recently, brominated dioxins (PBDD/Fs) have been generating

interest as their presence is increasingly reported in the outdoor and indoor environment (Bjurliid et al., 2018; Choi et al., 2003; Li et al., 2011; Pajurek et al., 2019). PBDD/Fs have been reported to be present at relatively high concentrations in e.g., in dust from indoor environment as described by several studies (Suzuki et al., 2017; Takigami et al., 2009, 2008; Tue et al., 2013). These studies strongly suggest that PBDD/Fs released via dust could represent a possible additional source of exposure for humans to dioxin-like compounds (van den Berg et al., 2013). As an example, PBDD/Fs can be released in an indoor environment from heat-resistant electronics, resulting in elevated PBDD/Fs levels in dust (Takigami et al., 2008). Brominated dioxins are originally present as impurities in some commercial brominated flame retardant mixtures, such as decabromodiphenyl ether (DecaBDE) or other PBDE mixtures which are used to flame-retard plastics and electronics (Altarawneh et al., 2019; Buser, 1986; Ren et al., 2017, 2011). Main sources of PBDD/F formation are during incomplete thermal degradation of flame-retarded plastics. Unsuitable temperature conditions used during recycling processes may also lead to the formation of PBDD/Fs from brominated flame-retardant precursors (Ebert and Bahadir, 2003; Hamm et al., 2001; Zhan et al., 2019).

A problem arising with the potential presence of PBDD/Fs in plastic is linked to the fact that nowadays, a wide range of consumer products including toys are manufactured from recycled plastics instead of de novo synthesis. For instance, recycled black plastic often indicates plastic originating from e-waste, which is a type of plastics known to contain significant levels of polybrominated diphenyl ethers (PBDEs) and related flame-retardants (Digangi et al., 2017; Drage et al., 2018; Kuang et al., 2018; Samsonek and Puype, 2013; Strakova and Petriik, 2017). This suggests that consumer products manufactured using black recycled plastic, such as plastic toys, may represent another unsuspected route of exposure to PBDD/Fs via exposure to dust generated by the object or, particularly for young children, via normal mouthing behavior. Here the focus is on human hazards related to the possible exposure of, young children, to PBDD/PBDF-based contaminants in children's toys made of recycled plastic.

Human hazard assessment of dioxin congeners and mixtures is generally based on extrapolation from rodent models that are widely used because of their sensitivity towards dioxins. Indeed, the remarkable sensitivity of rodent-based models, such as DR CALUX, makes them very suitable for the determination of the total dioxin potency of e.g., mixtures of PCDD/Fs. However, in the objective of human hazard assessment from environmental contaminants it is essential to also consider human cell-based bioassays, as it is known that differences exist between humans and rodents in terms of sensitivity towards dioxin-related toxicity which may also be reflected in differences in sensitivity of AhR-mediated responses by dioxins and related compounds (Aarts et al., 1995; Brennan et al., 2015; Long et al., 2003; Larsson et al., 2015).

Therefore, we have created a human variant of the rat-based DR CALUX assay in the HepG2 cell line, the DR_{human} CALUX similar to the one described before by Aarts et al. (1995), the complement rat-based DR CALUX total dioxin-like activity measurement providing both sensitive quantification of dioxins and enhanced human-relevance of bioactivity determination.

In this study, we evaluated the presence of PBDD/Fs related dioxin-like toxicity in children's toys using a combination of GC-HRMS and cell-based AhR reporter gene assays (DR and DR_{human} CALUX) to determine toxicity equivalents in plastic toys. First, we determined the responsiveness of PBDD/Fs in both rat DR CALUX and its human variant in HepG2 cells, DR_{human} CALUX, we then quantified PBDD/Fs levels by GC-HRMS and calculated related TEQ levels by using the TEFs recommended by the WHO. Following

quantification and TEQ determination by GC-HRMS, we determined bioanalytical equivalents in the toy samples using DR and DR_{human} CALUX. Finally, we estimated the potential contribution of PBDD/Fs observed in the plastic toys to the total daily dioxin intake in young children.

2. Material and methods

2.1. Chemicals

For the analysis of individual congeners by means of the bioassay test, 2,3,7,8-TBDD, 2,3,7,8-TBDF, 2,3,4,7,8-PeBDF, 1,2,3,4,6,7,8-HpBDF and OBDF were purchased as analytical standards from Wellington Laboratories (Guelph, Canada). Congeners 2,3,7,8-TCDD and 1,2,3,4,7,8-HxBDF were purchased as analytical standards from Cambridge Isotope Laboratories (Andover, MA USA). Dimethyl Sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium) and the n-Hexane was purchased from Biosolve (Valkenswaard, The Netherlands). All ¹³C₁₂-labeled PBDD/Fs standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Samples and sample preparation

The toy subset selected for this study originates from a larger campaign of plastic consumer product sampling that took place between 2016 and 2018. All 6 selected toys contained a total PBDE content (>500 ppm) and Deca-BDE content (>250 ppm) in their black component (Strakova et al., 2018). All toys were made in China but purchased in different countries. Sample 1 was a puzzle cube from Argentina, Sample 2 was a hair clip from Czech Republic, Sample 3 a key fob puzzle cube, Sample 4 and 5 were puzzle cubes from India and Nigeria respectively, Sample 6 was a toy guitar from Portugal. The exact composition of the toys is not known. However, considering the potential e-waste origin of the plastic used to manufacture the toys, it is very likely that acrylonitrile butadiene styrene (ABS) is the major component of the plastic.

Before the extraction, we separated black plastic parts from the rest of the toy, since it is this type of plastic that is suspected to contain the polybrominated diphenyl ether (PBDEs) -type flame retardants and therefore are suspect of contamination with PBDD/Fs (Digangi and Strakova, 2016; Turner, 2018). After separation, the black plastic was cut in small pieces and between 5 g and 30 g of black plastic material were transferred into a glass bottle (Schott, Mainz, Germany) and 30 mL–60 mL of n-hexane was added, covering the plastic material completely. Each sample was then further broken down in small particles and homogenized with a homogenization apparatus (IKA Ultra-turrax, Staufen, Germany). Following 24 h of extraction in n-hexane, the organic solvent fraction containing the compounds of interest was collected and the sample extracted two times more with newly added n-hexane. Finally, all n-hexane extractions were pooled and evaporated to approximately 1 mL under a N₂ flow using a solvent evaporator (Dionex, Sunnyvale, CA USA). The remaining 1 mL solvent was cleaned-up twice by sulphuric acid silica gel columns. Finally, part of the cleaned extract dissolved in n-hexane was used for GC-HRMS analysis and the other part was evaporated and transferred to DMSO for CALUX analysis.

2.3. GC-HRMS analysis for PBDD/Fs

The GC-HRMS analysis for PBDD/Fs was performed by an ISO 17025:2005 accredited GC-HRMS laboratory (MAS GmbH, Münster, Germany) (Hamm et al., 2001; Imai et al., 2003). All PBDD/F analyses by MAS were performed by using the accredited test method

MAS_PA002:2013–10 “Determination of the mass concentration of PCDD/Fs, PBDD/Fs and dioxin-like PCBs in solid matter samples”. For 1,2,3,6,7,8-HxBDF, 2,3,4,6,7,8-HxBDF and 1,2,3,4,7,8,9-HpBDF congeners neither native nor isotope-labeled standards were commercially available, thus the assignment of the peak signals of these congeners was accomplished by relative retention time comparison with corresponding chlorinated dibenzofurans (Donnelly et al., 1991). As suggested by the WHO, toxic equivalent factors (TEF) for PCDD/Fs were used as a surrogate for calculation of WHO-TEQ levels of PBDD/Fs in samples (van den Berg et al., 2013).

Aliquots of sample extracts sent by BDS to the MAS laboratory were fortified with ¹³C₁₂-labeled PBDD/Fs standards and further purified by several liquid chromatography clean-up steps. Prior to GC-HRMS analysis, additional ¹³C₁₂-labeled PBDD/Fs standards were added to the PBDD/F fractions as recovery standards (Supplementary data 1). For PBDD/Fs analysis, a capillary gas-chromatograph (GC) (Thermo Scientific GC-Ultra), coupled with a high-resolution mass spectrometer (HRMS) (Thermo Scientific MAT 95XP HRMS) was used. The GC was equipped with a Programmable Temperature Vaporizer injection port and a 30 m DB-5MS capillary column (Agilent J&W GC column, 0.25 mm inner diameter, and 0.1 μm film thickness). The MS was operated in Selected Ion Monitoring (SIM) mode to monitor selected masses of the molecular ion cluster. Additionally, masses of the molecular ions of PBDEs were monitored to check for potential co-elution of PBDEs with PBDFs, which can lead to false positives results. Native PBDD/F congeners were quantified via the internal ¹³C₁₂-labeled PBDD/F standards. Due to the large concentration gradients of the PBDFs in the samples, the higher brominated Dibenzofurans partly had to be determined by separate analyses of diluted extract aliquots. Chromatograms of such determinations are given, along with other congener's chromatograms, in Supplementary data 2 for Sample 4 as an example.

2.4. Cell lines and culturing

The DR CALUX cell line (Murk et al., 1996) and the DR_{human} CALUX cell line were used for the analysis of individual congeners and samples. The DR CALUX cell line represents a H4IIE-based (rat) cell line stably transfected with the pGudLuc1.1 AhR-controlled luciferase reporter plasmid. The DR_{human} CALUX cell line is a human variant of the DR CALUX and was created in a human HepG2 cell line after stably transfection with the same pGudLuc1.1 AhR reporter construct. DR CALUX and DR_{human} CALUX cells were cultured in α-MEM (Gibco) medium supplemented with 10% fetal calf serum (FCS) and DMEM:F-12 (Gibco) medium that were supplemented with 7.5% FCS, 10% non-essential amino acids (NEAA), respectively, and streptomycin (10 μg/mL) plus penicillin (10 U/mL) antibiotics. Cultures were maintained in a humidified atmosphere with 5% CO₂ at 37 °C.

2.5. CALUX analyses of pure compounds and extracts from toys and consumer products

In the case of pure compound analysis, the automated CALUX assays were carried out as described earlier (van der Burg et al., 2013). Briefly, DR and DR_{human} CALUX reporter cells were manually distributed over a white 384-wells plate and the plates placed in an incubator. After 24 h, reporter cells (70–95% confluence) were retrieved and the exposure medium was prepared. Using a liquid handling robot (Hamilton Starlet, Hamilton, USA), the dilution series in 0.5 log unit increments of each test compound (dissolved in DMSO) were prepared in the assay medium. Then, the liquid handling robot used the dilutions series to expose the cells. All compounds were tested in triplicate and the same exposure

medium was used for both DR and DR_{human} CALUX. Following 24 h of incubation, the cells were lysed using Triton lysis buffer. Ultimately, the luciferase activity was measured using a luminometer (Berthold, Bad Wildbad, Germany) after the addition of a luciferin-containing solution.

In the case of the toy samples, the DR CALUX analysis was performed in the same way but in a 96-wells format. In this set-up, the preparation of the compound dilution series, as well as the exposure of the DR CALUX cells, were performed manually by an operator instead of a robot.

2.6. Data analysis and quality control of CALUX analysis

2.6.1. Analysis of individual congeners

Each congener was analyzed in triplicate in two independent CALUX analysis performed using the method described above. On each plate, a complete 13-points 2,3,7,8-TCDD reference dilution series was analyzed in two duplicates ($n = 4$) and the rest of the plate was used to analyses a 13-points full-concentration range of a maximum of 6 compounds.

For analysis of results, the software GraphPad Prism was used for dose-response modelling employing a four parameters nonlinear regression model ($Y = Bottom + (Top - Bottom) / (1 + 10^{(\log EC_{50} - X) * HillSlope})$). Using raw luminescence data, the maximum response of the reference compound 2,3,7,8-TCDD was set at 100% and the response of individual congener was expressed relative to the maximum response of the 2,3,7,8-TCDD reference curve. When reached, the half-maximal effective concentration (EC_{50}) of the tested compounds was determined from the dose-response curve.

2.6.2. Determination of relative potency values

Relative potency values were determined from the fitted dose-response curve of the analyzed compounds. Relative potency values were calculated by dividing the $EC_{10\%}$ -TCDD or EC_{50} of 2,3,7,8-TCDD by the $EC_{10\%}$ or EC_{50} of the PBDD/F congener. The $EC_{10\%}$ value refers to an interpolation of the concentration of the analyzed congener needed to induce activity equal to 10% of that of 2,3,7,8-TCDD total dose-response. Relative potency values were determined for both DR and DR_{human} CALUX and are expressed on a molar basis.

2.6.3. Sample extracts analysis and BEQ determination

Each sample was analyzed in triplicate twice in two independent CALUX analyses. On each 96-well plate, a complete 10-points 2,3,7,8-TCDD concentration range was analyzed in triplicates and the rest of the plate, excluding the outer wells, was used to analyses a dilution range of the sample. For both DR and DR_{human} CALUX assays, several parameters were checked to verify the validity of the results: (i) R^2 of standard curve > 0.98 , (ii) z-factor of standard

curve > 0.6 , (iii) 2,3,7,8-TCDD EC_{50} between assay-specific pre-determined limit values and (iv) SD of analyzed triplicate $< 15\%$. Then, relative light units from the samples were interpolated from the 2,3,7,8-TCDD standard dose-response curve of the plate and the CALUX BEQ content quantified between the limit of quantification and the EC_{50} of 2,3,7,8-TCDD and, as close as possible to the EC_{10} (Besselink et al., 2004).

3. Results & discussion

3.1. Determination of relative potencies of individual PBDD/F and PCDD/F congeners in rat- and human-cell based CALUX assays

Unlike PCDD/Fs, PBDD/Fs are not assigned official WHO-TEF values. However, to allow the determination of PBDD/Fs-related toxicity equivalents, a WHO expert panel recently recommended using the TEF value normally assigned to the corresponding chlorinated congener for its brominated equivalent (van den Berg et al., 2013). Before determining the dioxin-like activity in the toys using CALUX bioassays, we decided to first investigate if PBDD/Fs TEF values were comparable to rat- and human-cell based CALUX Relative Potency (REP) values and consequently, if a comparison between analytical and bioassay TEQ would be meaningful. To assess the potential AhR-dependent luciferase expression by PBDD/Fs, we analyzed a selection of PBDD/Fs congeners using both the well-established DR CALUX and its human variant, the DR_{human} CALUX assay. We selected six PBDD/Fs congeners that were either representative of congener's prevalence in the toys (mainly PBDFS, as seen later in Table 2) (2,3,7,8-PeBDF, 1,2,3,4,7,9-HxBDF, 1,2,3,4,6,7,8-HpBDF and OBDF) or structurally similar to 2,3,7,8-TCDD (2,3,7,8-TBDD and 2,3,7,8-TBDF).

All compounds were active in a dose-dependent manner in both DR and DR_{human} CALUX assay. Therefore, it was possible to determine LOEC and $EC_{10\%}$ values for each compound (Table 1). In the case of compound displaying a full dose-response curve, we also determined the EC_{50} half-maximal concentration. With the DR CALUX bioassay, Suzuki et al. (2017) and Behnisch et al. (2003) obtained similar relative potency values for PBDD/Fs and a clear trend of potency decrease with bromine substitution increase can be seen. Contrastingly, this trend for potencies is less evident in the case of the DR_{human} CALUX where $REP_{EC_{50}}$ values, except for 1,2,3,4,5,7,8-HpBDF and OBDF, are relatively close to each other. Differences in potencies and sensitivity between DR CALUX and DR_{human} CALUX can clearly be seen in Fig. 1, indicating some species differences regarding PBDD/Fs bioactivity. The rat cell-based assay (DR CALUX) appeared to be more sensitive to PBDD/Fs than its human variant and we observed an overall 100-fold difference based on the EC_{50} value of 2,3,7,8-TCDD and between 10 and 100-fold difference the analyzed PBDD/Fs between the two cell-lines. This observation is in line with the notion, for chlorinated

Table 1
Lowest effective concentration (LOEC), $EC_{10\%}$, EC_{50} and Relative Potency (REP) values of several PBDD/Fs determined in the DR and DR_{human} CALUX bioassays.

Compound	DR CALUX					DR _{human} CALUX					WHO TEF (2005) ^a
	LOEC (M)	$EC_{10\%}$ (M)	REP $EC_{10\%}$	EC_{50} (M)	REP EC_{50}	LOEC (M)	$EC_{10\%}$ (M)	REP $EC_{10\%}$	EC_{50} (M)	REP EC_{50}	
2,3,7,8-TCDD	6.00E-13	5.75E-13	1	5.80E-12	1	1.50E-11	1.00E-10	1	4.80E-10	1	1
2,3,7,8-TBDD	2.60E-12	1.70E-12	0.23	2.50E-11	0.23	3.90E-12	6.00E-11	1.67	4.50E-10	1.1	1
2,3,7,8-TBDF	5.60E-13	8.98E-13	1.1	6.70E-12	0.86	6.10E-12	9.05E-11	1.10	1.29E-09	0.37	0.1
2,3,4,7,8-PeBDF	2.00E-12	7.00E-13	0.3	3.30E-11	0.18	7.10E-12	5.90E-11	1.69	4.90E-10	0.98	0.3
1,2,3,4,7,8-HxBDF	1.00E-11	2.10E-11	0.058	7.40E-10	0.0078	2.80E-11	4.15E-10	0.24	1.60E-09	0.30	0.1
1,2,3,4,6,7,8-HpBDF	2.20E-10	3.05E-10	0.0028	2.00E-09	0.0029	1.90E-09	1.35E-08	0.0074	2.80E-08	0.017	0.01
OBDF	6.70E-10	3.20E-10	0.00090	2.80E-09	0.0021	9.20E-09	4.80E-08	0.0021	nd	nd	0.0003

nd: EC_{50} could not be determined based on the fit of the curve (absence of plateau in the dose-response).

^a the TEF values presented are recommended TEF values for PBDD/Fs and derived from TEF values of the corresponding chlorinated congener (van den Berg et al., 2013).

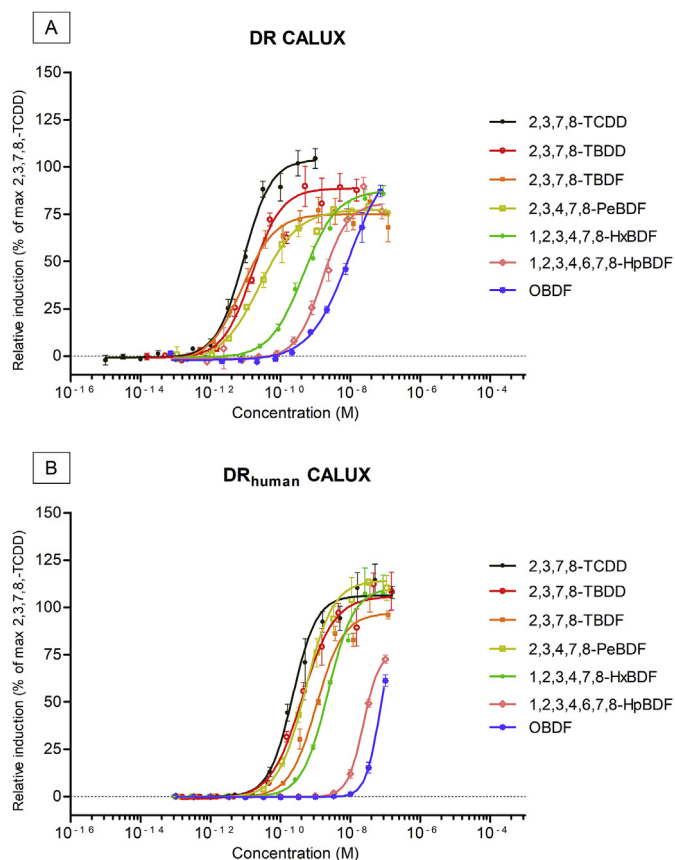


Fig. 1. Typical dose-response curve for several chlorinated and brominated dibenzo-p-dioxins and dibenzofuran in DR CALUX (A) and DR_{human} CALUX (B) ($n \geq 2$). Values represent the mean \pm SD.

dioxins, that humans are, compare to rodents, relatively insensitive to dioxins (He et al., 2011; Long et al., 2003; van den Berg et al., 2013; Wiebel et al., 1996)

We then compared the CALUX REP_{EC10%} and REP_{EC50%} values determined in both assays to the TEF values for PBDD/Fs hazard assessment which are, for the recall, derived from the TEF values for chlorinated dioxins. The TEF values for this set of PBDD/Fs congener appear to be roughly in the same order of magnitude than the REP_{EC10%} and REP_{EC50%} values determined with the DR and DR_{human} CALUX. This indicates that PBDD/Fs-related GC-HRMS estimated TEQ and DR/DR_{human} CALUX BEQ could potentially be compared. This, however, should be verified with a larger set of PBDD/Fs reference compounds.

3.2. Determination of PBDD/F levels (by GC-HRMS), and total dioxin-like activities in toy samples using DRs CALUX bioassays

As described earlier, black plastic parts from toys sampled in several countries selected by their PBDEs content (>500 ppm) and DecaBDE content (>250 ppm) (Strakova et al., 2018) were extracted and analyzed for PBDD/Fs. The samples were not analyzed for PCDD/Fs content. PBDD/Fs were found in all samples and high total levels of 17 PBDD/Fs congeners were measured, ranging from 5594 pg/g to 385856 pg/g (Table 2). The distribution pattern was comparable between the samples and PBDFs were the most abundant congeners, accounting for 98,8%–100% of the total profile. Particularly octa-, hepta-, and hexa-BDFs that were detected in every sample (Fig. 2, A). This predominance of PBDFs in plastics is in line with previous findings. Indeed, the PBDD/Fs pattern found in the toys does resemble the pattern of PBDD/Fs impurities found in some DecaBDE mixtures (Ren et al., 2011) used as a flame-retardant in plastics. This suggests that the recycling of DecaBDE containing plastics potentially also allows the recycling of significant amounts of PBDD/Fs into new products (Strakova et al., 2018). Additionally, the formation of PBDFs from DecaBDE also requires low amounts of energy and could, therefore, occur during plastic recycling process (Ebert and Bahadir, 2003; Hamm et al., 2001) leading to increasing PBDD/Fs levels in plastics.

From the PBDD/Fs concentrations determined by GC-HRMS analysis, we estimated the toxicity equivalent in the samples. The corresponding PBDD/Fs-based TEQ concentrations were estimated and ranged from 38 pg TEQ/g to 3384 pg TEQ/g (Table 3). As was the

Table 2

Concentrations of PBDD/F (pg/g) congeners as measured by GC-HRMS in plastic materials from toys; total sample concentration (pg/g) and WHO-2005 based TEQ values measured in plastic material from toys using GC-HRMS.

Congener	WHO TEF values (2005)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
2,3,7,8-TBDD ^b	1	3.69	< 4 ^a	< 8 ^a	2.23	< 2 ^a	< 5 ^a
1,2,3,7,8-PeBDD ^b	1	15.9	< 16 ^a	< 32 ^a	9.18	< 8 ^a	< 20 ^a
1,2,3,4,7,8/1,2,3,6,7,8-HxBDD ^{b,d}	0.1	24.2	< 32 ^a	< 64 ^a	14.4	< 16 ^a	54.4
1,2,3,7,8,9-HxBDD ^b	0.1	< 16 ^a	< 32 ^a	< 64 ^a	10.7	< 16 ^a	< 40 ^a
1,2,3,4,6,7,8-HpBDD	0.01	165	< 80 ^a	950	182	< 40 ^a	458
OBDD	0.0003	359	< 200 ^a	1861	226	< 100 ^a	612
2,3,7,8-TBDF ^b	0.1	42.8	< 4 ^a	230	29.7	5.84	227
1,2,3,7,8-PeBDF ^b	0.03	65.5	< 16 ^a	451	66.1	< 8 ^a	223
2,3,4,7,8-PeBDF ^b	0.3	95.4	< 16 ^a	856	69.9	15.4	596
1,2,3,4,7,8/1,2,3,6,7,8-HxBDF ^{b,c,d}	0.1	780	98	6759	1188	101	2187
1,2,3,7,8,9-HxBDF ^b	0.1	< 16 ^a	< 32 ^a	445	< 8 ^a	< 16 ^a	93.3
2,3,4,6,7,8-HxBDF ^{b,c}	0.1	358	32.5	4855	399	59.4	1714
1,2,3,4,6,7,8-HpBDF	0.01	51478	2751	226399	47937	2569	50476
1,2,3,4,7,8,9-HpBDF ^c	0.01	296	< 80 ^a	464	156	< 40 ^a	140
OBDF	0.0003	117070	2712	142586	37426	3943	41964
TOTAL (pg/g)		170753	5594	385856	87716	6694	98745
TOTAL (pg TEQ/g)^e		725	41	3821	693	48	1137

^a not detected above specified the limit of quantification.

^b data for these congeners represent maximum values since co-elution with other congeners of the same homologue group cannot be excluded.

^c due to uncertainty in the assignment of these congeners, results have to be considered as tentative for these congeners.

^d co-eluting congeners.

^e As no TEQ values have been officially established for PBDD/Fs, the TEQ values presented were calculated by using the WHO-2005 TEFs from their chlorinated analogues PCDD/Fs.

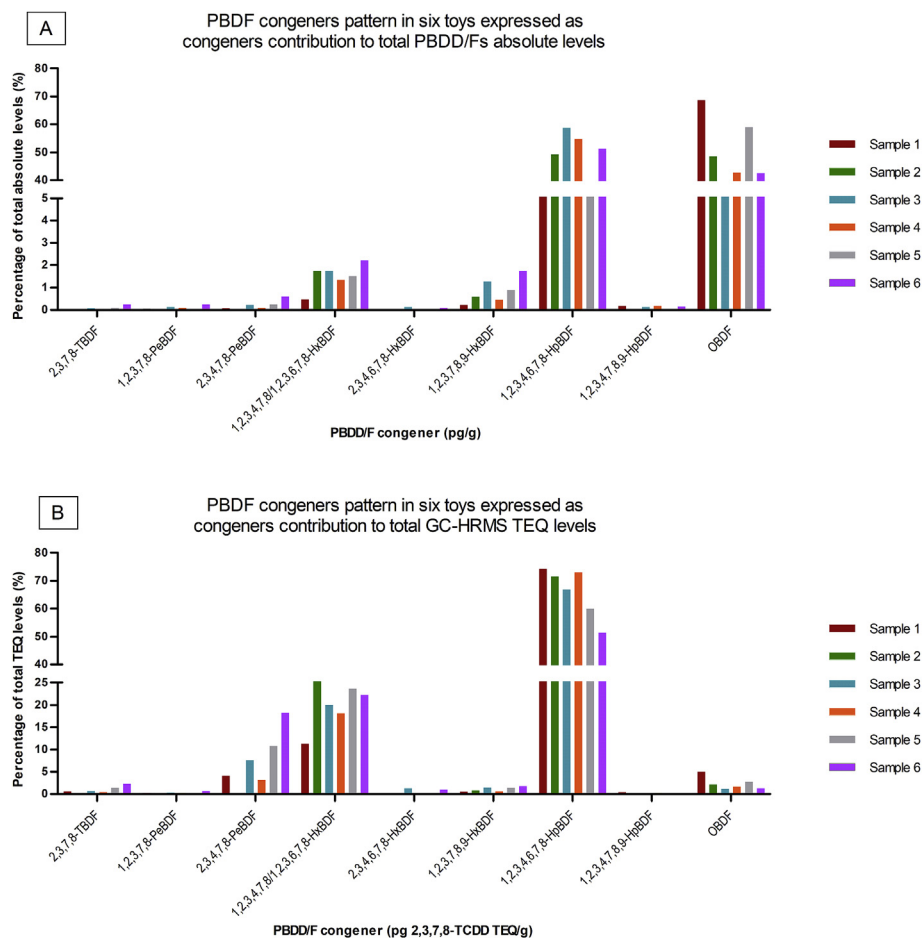


Fig. 2. PBDFs congeners pattern in six toys measured by HR-GCMS presented in percentage of contribution to the total concentration of PBDFs (pg/g) (A) and percentage of contribution to the total TEQ (B).

case for PBDD/Fs congener levels, the distribution pattern of PBDD/Fs relative to their contribution to the total Σ PBDD/Fs TEQ levels was similar between the analyzed samples and PBDF were accounting for 96,7–100% of the TEQ. Clearly, 1,2,3,4,6,7,8-HpBDF was found to significantly contribute to the overall TEQ of the mixture (Fig. 2, B). After determining PBDD/F levels with GC-HRMS and calculating of PBDD/Fs-related TEQ levels, we determined the total dioxin-like bioactivity in the samples using both DR CALUX and DR_{human} CALUX assays. CALUX activities were found in all samples tested and, the total dioxin-like activity ranged from 230 to 1520 pg

2,3,7,8-TCDD BEQ/g for the DR CALUX and from 640 to 2550 pg 2,3,7,8-TCDD BEQ/g for the DR_{human} CALUX (Table 3). Some differences are observed when comparing GC-HRMS-based PBDD/Fs TEQs and total bioanalytical equivalents.

To investigate these differences, we calculated theoretical BEQ (BEQ_T) values for the samples based on previously determined congener-specific REP_{EC10%} values, TEF values for non-analyzed congeners and GC-HRMS concentrations of PBDD/Fs (Table 2). Comparison between GC-HRMS-based TEQs and DR CALUX BEQ_T values shows comparable values and lower BEQ_{ST} values can

Table 3
Total equivalent activity (TEQ) values measured in analyzed toys and consumer goods using GC-HRMS (pg 2,3,7,8-TCDD TEQ/g), as well as DR CALUX and DR_{human} CALUX (pg 2,3,7,8-TCDD BEQ/g).

Sample ID	HR-GC/MS TEQ ^a (pg 2,3,7,8-TCDD TEQ/g)	DR CALUX BEQ (pg 2,3,7,8-TCDD BEQ/g)	Theoretical DR CALUX BEQ ^b (pg 2,3,7,8-TCDD BEQ/g)	DR _{human} CALUX BEQ (pg 2,3,7,8-TCDD BEQ/g)	Theoretical DR _{human} CALUX BEQ ^b (pg 2,3,7,8-TCDD BEQ/g)
Sample 1	725	1370	432	1230	1089
Sample 2	41	230	19	680	53
Sample 3	3821	1520	2222	2550	5855
Sample 4	693	550	348	890	930
Sample 5	48	370	34	650	90
Sample 6	1137	370	933	640	2442

^a as no TEF values have been officially established for PBDD/Fs, the TEQ values presented were calculated by using the WHO-2005 TEF values for the chlorinated analogues PCDD/Fs.

^b Theoretical CALUX BEQ values were determined based on the REP_{EC10%-TCDD} determined in this study and TEF values for PBDD/Fs.

potentially be explained by CALUX REP_{EC10%-TCDD} being generally lower than TEF values. In the case of DR_{human} CALUX, BEQ_{ST} are higher than TEQs, which can also be explained by differences between REP values and WHO-TEF as DR_{human} are overall higher than WHO-TEF values. Clearly, BEQ_{ST} values show that PBDD/Fs-related BEQ explained most of the total CALUX activities observed.

Differences between TEQs/BEQ_T and the total BEQ values may be explained by the presence of other, unknown compounds in the mixtures, acting either as agonists or antagonists of the AhR. In our case, it is expected that PBDEs present in the samples can partially explain the difference between TEQs/BEQ_T and BEQ. Unlike dioxins, PBDEs are not exclusively agonists of the AhR and some of them are capable to antagonize the AhR (Behnisch et al., 2003; Hamers et al., 2006; Peters et al., 2004). Therefore, since PBDEs have different potencies in human and rodent cell lines, their effect on the total mixture activity is expected to result in different BEQs determination in rodent versus human-based CALUX bioassays.

The suitability of the DR CALUX to accurately determine toxicity equivalence in complex mixtures of chlorinated dioxins and dioxin-like compounds has been demonstrated before (Besselink et al., 2004; Croes et al., 2013; Gizzi et al., 2005; Vugt-Lussenburg et al., 2013) however, it is equally important to consider the toxicological relevance of the total bioactivity determination. A bioassay-determined activity in a sample is the result of complex toxicological interactions (additive, non-additive, antagonism, synergism ...) which cannot easily be predicted using an analytical-chemical method. Because these interactions can differ between species, the use of the DR_{human} CALUX assay is relevant in terms of human hazard assessment based on the effect of complex mixtures on the human AhR. From that angle, the DR_{human} CALUX assay can be seen as a complementary tool to sensitive DR CALUX activity determination for increased human relevance.

3.3. Hazard assessment of plastic toys contaminant uptake by children through mouthing behavior

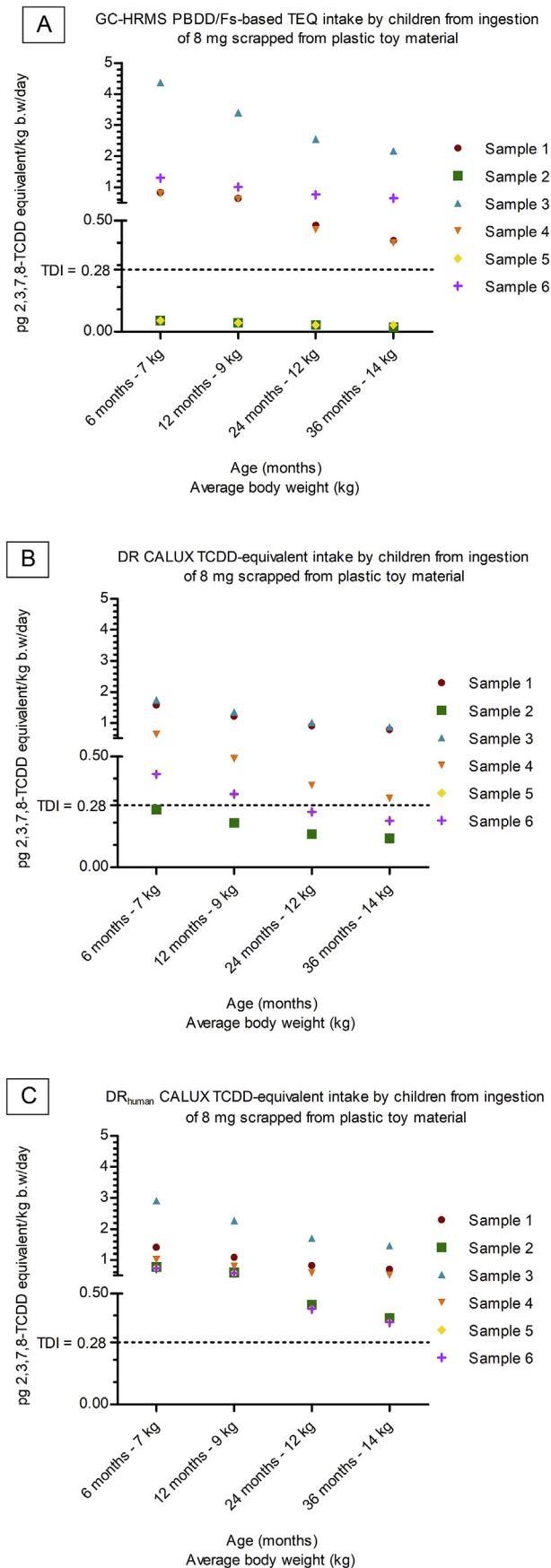
It is worthy to note that a value of 1000 pg 2,3,7,8-TCDD TEQ/g as can be found in plastic toys, is equivalent to the proposed hazardous waste limit for chlorinated dioxins in the Stockholm Convention for Persistent Organic Pollutants (Petriik, 2019). Considering this limit, toys 1, 3 and 6 were found to be as contaminated as hazardous waste. All other samples were also highly contaminated. Such high levels of PBDD/Fs contamination in consumer products indicate that they were most likely manufactured using flame retardant containing recycled plastics. The existence of this amount and type of contamination suggests a potential additional route of exposure of e.g., children to PBDD/Fs and other hazardous chemicals, such as PBDEs, which apparently is associated with the current way of recycling of plastics, and is very relevant with respect to the ambition of creating a global circular economy.

To estimate the potential for possible harm from plastic toys contaminated with relatively high PBDD/F levels, we have executed a first approximation of a hazard-assessment, bearing in mind the mouthing behavior as a possible route of exposure for children. It is known that for children under the age of 3, mouthing behavior plays an important role regarding the contact of children to toy materials (van Engelen JGM et al., 2007). The ingestion of plastic that is scraped off toys can then be an important route of exposure of young children to contaminants in plastics, including dioxins and dioxin-like compounds. In 2016, the European Scientific Committee on Health and Environmental Risks (ESCHER) reconfirmed the validity of the amounts ingested by children from toys by mouthing behavior as defined in a report from the Dutch National Institute for Public Health and the Environment (RIVM) (van Engelen JGM et al., 2009). In the ESCHER report, the daily ingestion of scraped-off

plastic toy material is estimated at 8 mg/day; 100 mg/day for dry, brittle, powder-like or pliable toy material, and 400 mg/day for liquid or sticky toy material. In this first approximation of hazard assessment, we used the lower value of 8 mg/day ingestion of scrapped-off plastic toy material. Because of the very limited data available on PBDD/Fs bioavailability, we assumed as a worst-case scenario that 100% of the PBDD/Fs would be bioavailable from the ingested plastic and absorbed over the intestinal tract. This is in line with the original RIVM report which states that in the scenario of direct ingestion and mouthing, 100% of the elements would migrate out of the plastic over the intestinal tract (van Engelen JGM et al., 2009).

By using the values of previously determined GC-HRMS PBDD/Fs-based TEQs and DR/DR_{human} CALUX bioassay BEQs in toys and the average body weight values for children of 6, 12, 24 and 36 months, we estimated the possible daily intake of PBDD/Fs related to the ingestion of 8 mg scrapped contaminated plastic toy material expressed as pg TEQ/kg body weight/day (Fig. 3, Supplementary Table 3). Considering the recommended tolerable daily intake (TDI) for dioxins of 0.28 pg TEQ/kg body weight/day, our estimated contribution of the ingestion of contaminated black plastic to children's dioxin body burden is significant and often higher than the recommended TDI for PCDD/Fs and dioxin-like PCBs which we can use for comparison with exposure to PBDD/Fs. In the case of GC-HRMS PBDD/Fs-based TEQ and DR CALUX BEQ, two toys (Sample 2 and 5) were below the TDI at all ages (Fig. 3A and B). In the case of the DR_{human} CALUX BEQ, assay representative of the activity of the mixture on the human AhR, none of the toys were below the TDI at all age (Fig. 3C). As an example, the ingestion of 8 mg of black plastic scrapped off Sample 3 by a 12-month old toddler could potentially result in the ingestion of 2.27 pg BEQ/kg body weight/day based on DR_{human} CALUX BEQ activity. This represents an intake of 2,3,7,8-TCDD equivalents which are 9 times higher than the recommended TDI for dioxins of 0.28 pg TEQ/kg body weight/day (Knutsen et al., 2018). It is important to emphasize that this estimation only focuses on PBDD/Fs and do not include the dietary intake of chlorinated dioxins, therefore our finding supports the argument that PBDD/Fs can "contribute significantly to the total amount of TEQ" (van den Berg et al., 2013), and should therefore, in our opinion be included into overall dioxin-like toxicity estimations for a better hazard assessment of the total TEQ by chromatography based methods.

However, this hazard assessment study has potential limitations. As we could not pulverize the plastic into a fine powder, it is expected that PBDD/Fs extraction is incomplete. On the other hand, the dimension of the particles obtained was representative of what can be scrapped and swallowed by a child. Additionally, the fact that we only focused on the black plastic parts of the toys is a limitation, however, it should be noted that the toys analyzed were anyway essentially made of black plastic and that these parts were visible and accessible for a child for hand-to-mouth contact. Another potential limitation is that the type of toys analyzed may not be intended for children over 3 years old. Nevertheless, it is very likely that children under 3 years would have access to them as children's caretakers would probably not keep such toys out of reach from a child displaying mouthing behavior. In other cases, behavioral studies show that children mouth on a broad range of items not intended to be mouthed, and thus they could have access to other potentially contaminated objects (Juberg et al., 2001; Smith and Norris, 2003). Finally, congener-specific bioavailability was not considered and, based on PCDD/Fs studies, it is anticipated that the bioavailability of higher brominated congeners will be lower than for lower substitution congeners. Clearly, more studies investigating the bioavailability of PBDD/Fs from plastics are needed.



Overall, our findings suggest that the use of PBDD/Fs contaminated plastic could potentially represent a significant additional source and route of exposure for young children to hazardous compounds. Furthermore, since nowadays it is stimulated globally to recycle plastic for renewed product formation and use, the potential contamination of plastics by brominated flame retardants and associated PBDD/Fs, needs to be reconsidered. A solution could be the monitoring of PBDD/Fs levels in recycled plastics and removal of such contaminant-containing parts prior to their use for the manufacturing of new consumer products.

4. Conclusion

In plastic parts of selected sampled toys, we found high levels of PBDD/Fs and related TEQ levels as measured by GC-HRMS, and high dioxin-like activity as measured by the DR CALUX bioassay and its human variant DR_{human} CALUX. In three of the toys, GC-HRMS PBDD/F-related TEQ and CALUX activities are of similar or higher levels than the proposed limit value for toxic waste (>1000 TEQ pg/g). Because PBDD/Fs are mostly generated during the process of fabrication of brominated flame retardants mixtures that are later added to plastics (Ren et al., 2011) and to a lesser extent during the recycling of such plastic (Ebert and Bahadir, 2003; Zhan et al., 2019) it means that the sampled toys very likely were manufactured using flame retardant-containing recycled plastics containing high levels of PBDD/Fs. This is a worrying finding considering that PBDD/Fs are potentially toxic chemicals that, at present, do not fall under EU regulation nor are there limit values for these compounds in consumer products, or other goods. It is necessary also to underline that current limits for both trace contamination and definition of POPs waste set in EU POPs Regulation (EU Regulation, 2019/1021 of the European Parliament and of the Council of June 20, 2019 on persistent organic pollutants; Text with EEA relevance, 2019) for total content of PBDEs are weak (1000 ppm) and will allow a large number of such contaminated products to enter the market (Petrlík 2019).

Toxicity prediction and ranking of hazardous compounds are important issues in human hazard assessment and, because of the observed presence of high concentrations of PBDD/Fs in children's toys and observation that these compounds are being potent ligands for both the rodent and human AhR, more studies are needed towards the possible health-associated risks from exposure to PBDD/Fs from usage of recycled plastics. Moreover, since the use of recycled plastics is stimulated globally, it is essential to monitor the contamination level of batches of recycling material before it is converted into renewed plastic use.

Finally, the here applied DR CALUX in-vitro bioassays have identified an unknown risk of PBDD/Fs presence in PBDE pre-screened plastic products, which was confirmed later by analytical chemistry. Because of the challenges to be met by analytical chemistry, cost-efficient and high-capacity effect-based method such as bioassays are yet the only way forward to increase the globally needed capacities to test unknown, unexpected and various adverse chemicals contained in consumer products such as those made of recycled plastics.

Fig. 3. Estimation of the additional daily intake in young children of PBDD/Fs in plastic toys from mouthing behavior. Estimation of the pg TEQ/kg body weight/day is based on the ingestion of 8 mg of the black plastic parts of sampled toys and A) GC-HRMS congener concentrations converted to apparent TEQ values, using TEF values for analogous chlorinated congeners of dioxins and furans; B) using DR CALUX and C) DR_{human} BEQ values. Weight-for-age values are based on WHO child growth standards.

Declaration of competing interest

The authors, although several of them are associated with the BioDetection Systems company are not involved in plastic production, recycling of plastics, or any other activity related to the products, except for the analyses of contaminants. Therefore, we declare no conflict of interest.

CRedit authorship contribution statement

Clémence Budin: Conceptualization, Writing - original draft, Data curation, Formal analysis, Visualization. **Jindrich Petrlik:** Conceptualization, Resources, Visualization, Writing - review & editing. **Jitka Strakova:** Resources, Data curation. **Stephan Hamm:** Resources, Formal analysis, Writing - review & editing. **Bjorn Beeler:** Resources. **Peter Behnisch:** Conceptualization, Writing - review & editing, Investigation. **Harrie Besselink:** Validation, Visualization, Formal analysis. **Bart van der Burg:** Conceptualization, Visualization, Writing - review & editing, Investigation, Funding acquisition. **Abraham Brouwer:** Conceptualization, Visualization, Writing - review & editing, Investigation, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2020.126579>.

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