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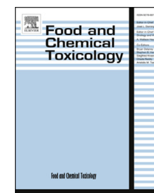

# Relative toxicological ranking of eight polybrominated diphenyl ether congeners using cytotoxicity, chemical properties and exposure data

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ABSTRACT

Polybrominated diphenyl ethers are widely used flame retardants which persist and diffuse in the environment thus entering the food chain. Eight congeners, most relevant for human exposure (BDE-28, 47, 99, 100, 153, 154, 183 and 209), were analyzed *in vitro* and *in silico* to derive a relative toxicological ranking.

Cytotoxicity was assessed on human liver (HepG2) and colon (DLD-1) cell lines, by three assays (MTS, ATP and DNA content) in a range of realistic concentration (1pM - 10 nM). Jejunum and Caco-2 passive absorptions were calculated *in silico*. Exposure estimates were calculated using EFSA database. By ToxPi we integrated the overall data.

No reduction of DNA content was observed, supporting absence of cytotoxicity. Otherwise, hormetic effects were exerted by all the congeners, except BDE-183. BDE-28, 47, 99, 100 differently affected the ATP content inducing a dose-related increase in HepG2 and depletion in DLD-1. Jejunum coefficients did not differ among congeners, whereas a higher Caco-2 coefficient indicates rapid absorption of BDE-28.

ToxPi relative rankings support the toxicological relevance of BDE-153 and 28 congeners for their potential hazard; the inclusion of exposure data in young and adult populations shifted BDE-209 and BDE-47 as top ranked due to their widespread occurrence in the diet.

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## 1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of chemicals mainly used as flame retardants in several household and commercial products such as textiles, furniture and electronic devices, or used as additives to plastics. PBDEs share structural similarities with polychlorinated biphenyls (PCBs) with 209 possible congeners differing in the number and position of bromine atoms (EFSA, 2011).

PBDEs are lipophilic and stable in the environment where they diffuse and persist. The consequent phenomenon of bio-accumulation and bio-magnification along the food chain raised concern on their potential adverse effects on wildlife and human health. Indeed, neurodevelopmental, endocrine and liver adverse effects have been documented for a number of PBDEs (Legler, 2008; Costa et al., 2014). Moreover, the increasing detection of several PBDE congeners in human compartments such as blood, cord blood, placenta, breast milk, liver and adipose tissue demonstrates the hazard these compounds may represent, especially for vulnerable population groups as fetuses and children (Fromme et al., 2016).

In Europe, diet represents the main route of exposure for humans with fish and meat providing the prevalent contribute (EFSA, 2011; U.S. Environmental Protection Agency, 2010). On the contrary, in U.S.A. the most relevant route of exposure for humans, accounting for the 90% of the total exposure estimate, is represented by dust ingestion in indoor environments like houses and offices, due to leaching from electronic devices (i.e. televisions, PC cabinets) or textiles, where PBDEs are added in variable amounts but not chemically bound (U.S. Environmental Protection Agency, 2010).

On the basis of prevalence data in food, the European Food Safety Authority (EFSA) recommended to collect toxicological data on eight most relevant congeners, i.e. BDE-28, 47, 99, 100, 153, 154, 183 and 209 (EFSA, 2011). To date, only some of these congeners have been investigated for their cytotoxic effects using human *in vitro* models (Hamers et al., 2006; Huang et al., 2010; Llabjani et al., 2011; Wang et al., 2012; Souza et al., 2013, 2016, Pereira

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et al., 2013) and no study compared the cytotoxicity of all the eight congeners in the same experimental system. Due to the lack of a comparison scale, such as the Toxicity Equivalent Factors established for dioxin-like compounds (Van den Berg et al., 2006), EFSA performed the risk assessment for separated congeners which also differ in the mode of action (EFSA, 2011).

The aim of the present study is to investigate potential toxicological differences among the eight dietary relevant PBDE congeners and to apply a method to classify their toxicological relevance by integrating results from *in vitro* cytotoxicity data, i.e. $EC_{50}/IC_{50}$ values or Benchmark doses, with chemical data on lipophilicity, gastrointestinal absorption and estimated half-lives, as well as with exposure intake data in different subpopulation groups. The cytotoxic potential of these compounds was investigated on two human cell lines representative of the digestive system, involved in metabolism and absorption of food nutrients and contaminants, namely the human hepatoma cell line (HepG2) and the adenocarcinoma cell line (DLD-1). This *in vitro* system previously proved to be useful to highlight different PCBs pleiotropic effects (Stecca et al., 2016). Hepatotoxicity is one of the main adverse effects ascribed to PBDEs (EFSA, 2011) and apoptotic effects induced by BDE-47, 99 and 209 have been observed on HepG2 (Hu et al., 2007, 2014; Souza et al., 2013; Wang et al., 2012). Otherwise, information about effects on colon cells is available only for BDE-209 *in vitro* (Curcic et al., 2014) and for BDE-47 in a fish model (Barja-Fernández et al., 2013).

For a more realistic assessment of potential human health effects, we considered concentrations $\leq$10 nM according to maximum estimated environmentally relevant PBDEs levels to be used in *in vitro* studies (Wei et al., 2010) which are in the range of mean occurring PBDEs concentrations in food (EFSA, 2011). Therefore, HepG2 and DLD-1 cells were treated with PBDE congeners' concentrations ranging from 1 pM to 10 nM. We performed a cytotoxicity assessment of each congener on both cell lines by three methods, i.e. the metabolic MTS and ATP assays and the CyQuant® assay to determine the total amount of DNA. *In silico* calculation of Jejunum and Caco-2 passive absorption rates was also performed.

To estimate the relative toxicity of the eight PBDE congeners evaluated, we used the Toxicological Prioritization Index (ToxPi) tool (Reif et al., 2013) a powerful platform which allow the integration of different sources of information to derive a single weighed score for each chemical. In the model, we included obtained cytotoxicity data, chemical properties derived by our *in silico* calculation or publicly available, as well as exposure intake data from public repositories. As a result, we obtained provisional toxicological ranks of the eight dietary relevant PBDE congeners for young and adult subpopulation groups.

## 2. Materials & methods

### 2.1. Chemicals

PBDE congeners 28, 47, 99, 100, 153, 154, 183 and 209 were purchased by Wellington Laboratories (Ontario, Canada). Dr. Roberta Galarini and Dr. Arianna Piersanti from Istituto Zooprofilattico Abruzzo e Molise (in the frame of the Italian Ministry of Health funded project RF-2010-2311608) provided each congener as dry powder, following nitrogen flushing treatment to eliminate the organic solvent from the standard solutions. Upon arrival in our lab, each congener was dissolved in DMSO to obtain 50 μg/ml standard solutions which were stored at 4 °C.

### 2.2. Cell lines

HepG2 and DLD-1 cell lines were grown, respectively, in DMEM/F12 and RPMI1640 media (Life Technologies, Paisley, UK), both without phenol red and supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland), 2 mM L-glutamine (Lonza), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Cells were maintained in a humidified Steri-Cult 200 Incubator (Forma Scientific, Marietta, OH, USA) at 37 °C and 5% $CO_2$.

### 2.3. Cell lines treatment with PBDEs

Three different cytotoxicity assays were performed: 1) the colorimetric MTS assay to determine the amount of metabolically active cells able to reduce tetrazolium salts to formazan by dehydrogenases enzymes (Berridge et al., 2005); 2) the bioluminescent ATP assay to determine the intracellular amount of ATP as a parameter of proliferation/apoptosis (Crouch et al., 1993); 3) the fluorimetric CyQuant® assay to determine the total amount of DNA as directly proportional to cell number (Jones et al., 2001). According to the assay, 5000 (MTS and CyQuant®) or 2000 (ATP) cells/well were plated in transparent 96 flat-bottomed multiwells for MTS and CyQuant® Assays and in white 96 flat-bottomed multiwells for ATP Assay. Cells were incubated overnight in a humidified incubator at 37 °C to permit their adhesion. Medium was then replaced with fresh medium added with ten-fold serial dilutions of each PBDE congener in the range 1pM–10 nM, in triplicated wells, or with medium with vehicle alone (DMSO) as control, incubating cells for 72 h at 37 °C. Final DMSO concentration did not exceed 0.2%. Three independent experiments were performed for each assay. Values were normalized with respect to control cells set at 100%.

### 2.4. MTS assay

The Cell Titer 96® Aqueous One Solution reagent (Promega, Madison, WI, USA) was used to perform the MTS assay according to the provided protocol. Briefly, PBDEs treated cells in 96 flat-bottomed multiwells were added, after 72 h incubation, with 20 μl of MTS reagent to each well, incubating 60 min at 37 °C. Cell viability was determined by reading absorbance at 490 nm by a Victor 3 Multilabel Reader (PerkinElmer, Waltham, MA, USA).

### 2.5. ATP assay

The ApoSENSORtrade; Cell Viability Assay (Biovision, Milpitas, CA, USA) was used to assess intracellular ATP levels following manufacturer's protocol. Briefly, after 72 h incubation, PBDEs treatment medium or control medium were removed from 96 flat-bottomed white multiwells. Cells were added with 100 μl/well Nuclear Releasing Buffer incubating for 5 min at room temperature to lyse them. Next, 10 μl/well of ATP Monitoring Enzyme was added reading luminescence in a Victor 3 Multilabel Reader (PerkinElmer).

### 2.6. CyQuant® assay

The CyQuant® Direct Cell proliferation Assay (Life Technologies) was used to assess DNA content according to manufacturer's protocol. Briefly, PBDEs treated cells in 96 flat-bottomed multiwells were added, after 72 h incubation, with 100 μl (equal volume as culture media) of 2X Detection Reagent. Upon incubation for 60 min at 37 °C, fluorescence was red from bottom with a Victor 3 Multilabel Reader (PerkinElmer) using a standard green filter.

### 2.7. Calculation of $EC_{50}/IC_{50}$ values and benchmark doses

The GraphPad Prism v5.01 software (GraphPad Software Inc., La

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