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A mixture of persistent organic pollutants relevant for human exposure inhibits the transactivation activity of the aryl hydrocarbon receptor *in vitro*

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Abstract: While humans are exposed to mixtures of persistent organic pollutants (POPs), their risk assessment is usually based on a chemical-by-chemical approach. To assess the health effects associated with mixed exposures, knowledge on mixture toxicity is required. Several POPs are potential ligands of the Aryl hydrocarbon receptor (AhR), which involves in xenobiotic metabolism and controls many biological pathways. This study assesses AhR agonistic and

antagonistic activities of 29 POPs individually and in mixtures by using Chemical-Activated LUciferase gene eXpression bioassays with 3 transgenic cell lines (rat hepatoma DR-H4IIE, human hepatoma DR-Hep G2 and human mammary gland carcinoma DR-T47-D). Among the 29 POPs, which were selected based on their abundance in Scandinavian human blood, only 4 exerted AhR agonistic activities, while 16 were AhR antagonists in DR-H4IIE, 5 in DR-Hep G2 and 7 in DR-T47-D when tested individually. The total POP mixture revealed to be AhR antagonistic. It antagonized EC_{50} TCDD inducing AhR transactivation at a concentration of 125 and 250 and 500 fold blood levels in DR-H4IIE, DR-T47-D and DR-Hep G2, respectively, although each compound was present at these concentrations lower than their LOEC values. Such values could occur in real-life in food contamination incidents or in exposed populations. In DR-H4IIE, the antagonism of the total POP mixture was due to chlorinated compounds and, in particular, to PCB-118 and PCB-138 which caused 90% of the antagonistic activity in the POP mixture. The 16 active AhR antagonists acted additively. Their mixed effect was predicted successfully by concentration addition or generalized concentration addition models, rather than independent action, with only two-fold IC_{50} underestimation. We also attained good predictions for the full dose-response curve of the antagonistic activity of the total POP mixture.

Keywords: Persistent Organic Pollutants; Aryl hydrocarbon Receptor; Antagonistic activity; Human relevant mixture; Generalized concentration addition model

Capsule:

A mixture of persistent organic pollutants relevant to human exposure antagonized the transcriptional activity of the Aryl hydrocarbon receptor *in vitro*, whose activity could be predicted.

1. Introduction

The aryl hydrocarbon receptor (AhR) was originally characterized as a xenobiotic mediator[1]. It is often called the "dioxin receptor" as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and several dioxin-like (dl) compounds are AhR agonists. Adverse health effects associated with exposure to these AhR agonists are widely studied, including abnormal reproduction and development, impaired immune system, liver toxicity and cancers[2].

AhR physiological roles have recently gained more attention since AhR is activated by a wide range of structurally diverse endogenous and exogenous compounds[3]. Also, AhR-deficient rodents suffer from various physiological defects in the immune system[4], liver[5], kidney[4,6], cardio-vascular system[7], urinary bladder[8], *etc*. Additionally, AhR homologs are preserved in animal evolution for 600 million years[9], while invertebrate AhR homologues cannot bind dioxin[10], indicating that dioxin detection was not the primary role of this protein. Increasing evidence supports important roles of AhR in normal development and homeostasis, while toxicity induced by AhR xenobiotic ligands could be due to perturbation of these normal processes[11].

Upon ligand binding, the AhR is translocated from cytosol into the nucleus where it joins its dimerization partner, aryl hydrocarbon receptor nuclear translocator (ARNT). This AhR/ARNT complex then binds to a DNA sequence called dioxin responsive element (DRE) to activate the expression of a battery of genes, including both phase I and phase II xenobiotic metabolism enzymes, of which *cyp1a1* is the best characterized. Hence, methods measuring *cyp1a1* gene expression are widely accepted for determining AhR activation[12], among which cell-based screening methods such as Chemical-Activated LUciferase gene eXpression (CALUX) are the most common[13–15].

Many potential AhR ligands are persistent organic pollutants (POPs). POPs are resistant to degradation and widely distributed in the environment. They can be detected in almost every human sample, including fetuses and embryos[16]. They tend to bioaccumulate and biomagnify in living organisms, resulting in toxic health effects to both humans and wildlife[17].

Presently, chemical risk assessment mainly relies on a chemical-by-chemical approach[18]. In real life, humans are exposed not to an individual POP, but to highly complex POP mixtures[19]. Understanding mixture toxicity is crucial to assess the potential adverse health effects associated with such real life exposure to mixtures of POPs[20]. The mixture effects may be additive, synergistic, or antagonistic depending on whether they are equal, stronger, or weaker than the sum of the effects of individual components, respectively[21]. The concepts called "something from nothing" and "a lot from a little" were introduced to describe the mixture effect[22] and proved in a study on fish when a significant effect was observed for a mixture combining individual compounds each at "no observed effect" concentrations[23].

Due to the various mixture forms and doses, models using the information of components to predict the combined effects are required. Three mathematical models have been developed for this purpose: i) the concentration addition (CA) model was designed for chemicals with similar mode of actions (MOAs)[24], but has also proven useful for mixtures with dissimilar compounds[23,25,26]; ii) independent action (IA)[27], successful in several applications[28,29], applies for chemicals which act independently and have different MOAs; and iii) the generalized concentration addition (GCA)[30], a CA modified model, was developed for predicting the effects of mixtures containing partial agonists[30–32].

In an effort to fill the gap in the knowledge of mixture toxicology, a defined mixture of 29 POPs (total POP mixture) was constructed based on their prominence in blood and/or food and

breastmilk with the concentration being average blood values from recent Scandinavian studies[33–35] published prior to 2012[36]. To mimic the exposure of cells (in a tissue) to chemicals that are in the blood stream, reporter gene assays involving cancer cell lines from different tissues (liver and mammary gland) and species (rat and human) were used in this study to determine the AhR transcriptional activities of the selected 29 POPs, individually and in mixtures (the total POP mixture and six sub-mixtures[36]). The aims were to (a) assess both AhR agonistic and antagonistic activities after exposing the cell lines to the 29 POPs and to the mixtures, (b) identify the main compound(s) responsible for the observed effects of the mixtures, and (c) predict the mixture activity by applying the three available models (IA, CA and GCA).

2. Materials and Methods

2.1. Chemicals and suppliers

The total POP and six sub-mixtures were designed and premade by the Norwegian University of Life Sciences, Oslo, Norway as described[36]. The former consists of 29 POPs, where most of them are listed as POPs under the Stockholm Convention on Persistent Organic Pollutants, belonging to three groups: six perfluorinated compounds (PFAAs), seven brominated compounds (BFRs), and 16 chlorinated compounds with seven polychlorinated biphenyls (PCBs) and nine organochlorine pesticides (OCPs). The latter consist of either one single class of compounds (PFAA, Br and Cl) or two combined classes (Cl+Br, Cl+PFAA, Br+PFAA). This way of mixture preparation was to enable the study of the effect of adding or removing one chemical group on different endpoints. As the design of the mixtures was focused on compounds occurring at high concentrations, most dl-PCBs (with the exception of PCB-118) and polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/PCDF) were deliberately excluded. These compounds were also omitted due to their high toxicity at low concentrations in several systems to allow the study of the effect of the non-dl and most prevalent compounds. The components included in the mixtures and their respective concentrations are given in Table S1.

Along with the mixture testing, 29 POPs from the total POP mixture were also examined individually. They were bought from Sigma Aldrich (Missouri, USA) except *o*-chlordane from Toronto Research Chemicals (North York, Canada) and PCB-118 from Dr Ehrenstorfer (Augsburg, Germany). All chemicals were dissolved in dimethylsulfoxide (DMSO) (Acros Organics, Molinons, France), except HCB in hexane (Merck, Massachusetts, USA).

The 29 individual POPs and the mixtures were stored as stock solutions at -20°C. Working solutions were prepared from the stock solutions to reach the concentrations mentioned in Table S1. The highest tested concentration was 50 µM for all PCBs and PFAAs and 20 µM for BFRs except BDE-100, BDE-153, BDE-154 and BDE-209 (1 µM) due to stocks available. OCPs were tested at the maximum concentration of 100 µM for *γ*-HCH and dieldrin, or 80 µM for all the others. The concentrations for mixture exposure are presented as "fold blood levels", relative to the average contaminant levels found in human blood of the Scandinavian population. The total POP mixture and sub-mixtures were tested at concentrations between the estimated concentrations in human blood and maximum 3000 fold blood levels.

2.2. Determination of aryl hydrocarbon receptor agonistic and antagonistic activities

2.2.1. Cell-based assays

Rat and human dioxin responsive (DR) cell lines were used. Rat hepatoma DR-H4IIE cells were from BioDetection System (Amsterdam, The Netherlands) while both human cell lines (hepatoma DR-Hep G2 and mammary gland carcinoma DR-T47-D) were previously home-made (Liege, Belgium)[37]. A vector containing an AhR-controlled luciferase reporter gene was stably integrated into these cells. The vector integrated into DR-H4IIE cells contained four native DREs (from the upstream promotor of the mouse *cyp1a1* gene), leading the MMTV (Mouse Mammary Tumour Virus) promoter[38], while both DR-T47-D and DR-Hep G2 cells were transfected with a vector

149 containing four synthetic DREs regulating the thymidine kinase promoter[37]. The cells were
150 routinely cultured in MEMα (Thermo Fisher Scientific, Massachusetts, USA) supplemented with
151 10% v/v fetal bovine serum (Greiner, Kremsmünster, Austria), 50 IU/mL penicillin and 50 μg/mL
152 streptomycin (Sigma Aldrich, Missouri, USA), and incubated in a H_20 saturated atmosphere
153 containing 5% CO_2, at 37°C.
154
155 The methodology for the DR-CALUX (Dioxin Responsive Chemical-Activated LUciferase gene
156 eXpression) bioassays was described in detail elsewhere[37,39]. Briefly, cells were first seeded in
157 white clear-bottomed 96 well microplates (Greiner, Kremsmünster, Austria) and incubated for 24h
158 to reach about 90% of confluence in the well. After 24-hour exposure, the cells were washed with
159 phosphate buffered saline (Sigma Aldrich, Missouri, USA) and treated with lysis solution
160 (containing Triton X100, Sigma Aldrich, Missouri, USA). Luciferin (Promega, Wisconsin, USA)
161 and ATP (Roche Diagnostics, Rotkreuz, Switzerland) were then added to the cell lysate to
162 produce luminescence, which was measured by using a luminometer (ORION II, Berthold
163 Detection System, Pforzheim, Germany). The cells were exposed, in triplicates, to a dilution series
164 of the tested compound/mixture in both agonistic and antagonistic tests. For the latter, the cells
165 were co-exposed with a constant concentration of 15 pM, 150 pM and 650 pM TCDD
166 corresponding to TCDD EC_{50} in DR-H4IIE, DR-T47-D and DR-Hep G2 cells, respectively.
167
168 In order to verify whether AhR antagonists compete for the same, single site on the AhR with the
169 agonist (TCDD), additional antagonistic tests were performed for selected compounds by co-
170 exposing DR-H4IIE cells to different concentrations of the tested compounds and a constant
171 saturating TCDD concentration (20 nM). Using the agonist (TCDD) at clearly saturating
172 concentrations would make it impossible for a lower affinity antagonist to affect transcriptional
173 activation at all.

All the exposure experiments were repeated at least three times independently. The final concentration of DMSO in the culture medium for the single POPs was 0.2% and 0.3% for agonistic and antagonistic tests, respectively, while they were 0.3% and 0.4% for the mixtures. For quality control, a TCDD reference curve was performed on each plate.

MTT cell viability and LDH cell cytotoxicity were performed along with visual inspection of cell morphology and attachment. The former was carried out in a replicate plate to the DR-CALUX assays. After 24-hour exposure, 25 μL MTT dye solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml, Sigma Aldrich, Missouri, USA) was added into each well, followed by a 4-hour incubation at 37°C to form insoluble purple formazan. Then, 100 μL isopropanol (Merck, Massachusetts, USA) was added into the plates to dissolve the formazan for two hours. The MTT formazan absorbance was read at 550/630 nm by a spectrophotometer (ELx800™ BioTek, Winooski, USA). Because the MTT data need to be interpreted with caution and are not necessarily related to cell death, we performed the LDH cell cytotoxicity as well. The Pierce™ LDH Cytotoxicity Assay Kit was purchased from Thermo Fisher Scientific (Massachusetts, USA) and operated according to the manufacturer's instructions (absorbance at 490/630 nm).

2.2.2. Calculations of EC_{50}, IC_{50} and efficacy (RPC_{Max})

Final results were presented as relative responses, *i.e.* percentages of the cell response to the tested compound/mixture compared to the maximum response of the cells to TCDD on the same plate for agonistic activities, or to spike-in TCDD EC_{50} for antagonistic activities. Dose-response curves were generated by Graphpad PRISM software, version 7 (San Diego, California, USA) by fitting a four-parameter non-linear regression for agonistic (Eq. 1) or antagonistic (Eq. 2) tests.

$$(1)\ Y_{agonistic} = B + \frac{x^H(T - B)}{x^H + {EC_{50}}^H}$$

202 $$(2)\ Y_{antagonistic} = B + \frac{T - B}{1 + \frac{x^H}{IC_{50}{}^H}}$$

199 where *x* is the concentration of a tested compound/mixture inducing the relative response $Y_{agonistic}$
200 or $Y_{antagonistic}$. EC_{50} and IC_{50} are the half maximal effective concentration for an agonist and
201 antagonist, respectively[40]. B = bottom, T = Top, H = Hillslope.
203
204 The lowest observed effect concentration (LOEC) is the lowest tested concentration at which a
205 significant effect ($p < 0.05$) was observed. The maximum observed effect concentration (MOEC)
206 is the lowest tested concentration causing the maximum effect ($p < 0.05$). ANOVA (Graphpad
207 PRISM) was used to determine statistical significance. Prior to ANOVA, tests for homogeneity of
208 variance and normal distribution (transformation if needed) were performed. When no full dose-
209 response curve was achieved, MOEC was considered as the highest concentration of the test
210 series. Efficacy was determined as RPC_{Max} (%), which is the maximum effect induced by the
211 tested compound[40]: AhR agonistic RPC_{Max} was the maximum relative response of the
212 compound/mixture compared to the maximum TCDD response, while AhR antagonistic RPC_{Max}
213 was the minimum relative response observed by the maximum inhibition of the test
214 compound/mixture to the spike-in TCDD EC_{50}. The compound/mixture was accepted as active
215 when its relative response was higher than the threshold level $RPC_{Max} \geq 10\%$ for AhR agonists
216 and lower than $RPC_{Max} \leq 70\%$ for AhR antagonists[40].
217
218 **2.2.3. Calculations of the predicted mixture antagonistic effects**
219 ***Concentration addition (CA).*** CA model is based on a dilution principle, all the chemicals behave
220 as they are simply the dilution of one another in the mixture. Hence, the effect contribution of one
221 compound to the mixture effect can be totally or partially replaced by the effect of the other. It
222 calculates the effect concentration ($IC_{mix,j}$) of the mixture inducing a specific antagonistic effect j

(from 1% to 100%) by considering the concentration partition (p_i) of compound i and its respective effect concentration (IC_{ij}) inducing the same effect j (Eq. 3). Previously published formulae were adapted[24,25,29]:

$$(3)\ IC_{mix,j} = \left(\sum_{i=1}^{n} \frac{p_i}{IC_{ij}}\right)^{-1}$$

The concentration partition p_i can either consider or not the non-active (NA) compounds. Because nonactive compounds do not give IC_{ij}, n is the number of the active compounds.

For each compound i, IC_{ij} inducing the effect j is calculated using its $IC_{i,50}$ and hillslope (H_i) from their fitted curves using Eq. 4 (Graphpad PRISM):

$$(4)\ IC_{ij} = IC_{i,50}\left(\frac{j}{100-j}\right)^{1/H_i}$$

Because the CA model allows only the calculation of $IC_{mix,j}$, to generate the full dose-response curves, we proposed several possible methods to calculate the hillslope and bottom (H_{mix} and B_{mix}) of the mixture response, while the top was set to 100% as no response.

- the Weighted Mean Hillslope and Bottom (WMHB) (Eq. 5a) considering p_i to weight the hillslope and bottom of the individual compounds:

$$(5a)\ H_{mix} = \left(\sum_{i=1}^{n} p_i H_i\right)\frac{100}{n}\ or\ B_{mix} = \left(\sum_{i=1}^{n} p_i B_i\right)\frac{100}{n}$$

- the Averaged Hillslope and Bottom (AvBH) considering the average of the hillslope and bottom of the individual compounds (Eq. 5b):

$$(5b)\ H_{mix} = \frac{\sum_{i=1}^{n} H_i}{n}\ or\ B_{mix} = \frac{\sum_{i=1}^{n} B_i}{n}$$

- the Formulated Hillslope and Bottom (FoBH) (Eq. 5c) using the formulae of the CA:

$$(5c)\ H_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{H_i}\right)^{-1}\ or\ B_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{B_i}\right)^{-1}$$

Independent action (IA). This method assumes that the effect of each component is an independent event[27]. Thus, the probability to exert a specific effect of the mixture is the joint probability of the effect of each compound applied independently. For calculating the relative response of the mixture, the data for individual compounds were converted into a probability.

An antagonistic effect induced by compound i is obtained by subtracting the measured relative response (R_{ik}) from 100% (100% being the relative response of TCDD EC_{50}) and then converted into a probability (scale 0-1, by dividing by 100). The relative response of the mixture (0%-100%) is calculated from the combination of individual probabilities of each compound using the adapted formula (Eq. 6)[28,29]:

$$(6)\ R_{mix,k} = 1 - \left(1 - \prod_{i=1}^{n}\left(1 - \frac{100 - R_{ik}}{100}\right)\right)$$

At a specific concentration k, $R_{mix,k}$ is the relative response of the mixture; R_{ik} is the relative response of compound i at that concentration k of the mixture, n is the number of the active components.

Generalized concentration addition (GCA). GCA (Eq. 7) assumes that the hillslope for each component is equal to 1 and considers also their RPC_{Max}[31]. It was adapted for AhR antagonistic activity similarly to the IA model by assuming 100% as no effect:

$$(7)\ R_{mix,k} = 100 - \frac{\sum_{i=1}^{n}\frac{RPC_{Max,i}C_{ik}}{IC_{50i}}}{1 + \sum_{i=1}^{n}\frac{C_{ik}}{IC_{50i}}}$$

where $R_{mix,k}$ is the relative response of the mixture at a specific concentration k, C_{ik} is the concentration of compound i in the mixture at that specific mixture concentration k. $RPC_{Max,i}$ is the maximum effect of compound i and IC_{50i} is the IC_{50} of compound i.

3. Results

In DR-H4IIE cells, while α-chlordane caused cytotoxicity at the highest tested concentration of 80 μM, *t*-nonachlor already did at 62.5 μM in three cytotoxicity tests (data not shown). They were also cytotoxic for the DR-T47-D at lower concentrations of 30 and 30.5 μM, respectively. These cytotoxic concentrations were excluded from the data analyses. None of the other compounds or mixtures induced cytotoxicity at any tested concentration.

3.1. Aryl hydrocarbon receptor activities of the 29 POPs

AhR – mediated agonistic activities. Only four out of the 29 tested POPs presented AhR agonistic activities ($RPC_{Max} \geq 10\%$). BDE-153, PCB-138, and PCB-118 were active in DR-H4IIE, while BDE-99 was active in DR-T47-D. BDE-99 and BDE-154 were able to trigger a weak agonistic activity ($5\% < RPC_{Max} < 10\%$) in DR-H4IIE, as well as γ-HCH in DR-T47-D (Table S2). No agonistic response was recorded in DR-Hep G2 cells for any of the 29 POPs.

***AhR – mediated antagonistic activities*.** Sixteen out of the 29 individually tested POPs displayed AhR antagonisms. No antagonistic activities were observed for any of the PFAAs in all three cell lines. In contrast, in DR-H4IIE cells, AhR antagonistic responses were recorded for 16 POPs including all PCBs, most of the OCPs (except *p,p'*-DDE, α-HCH and β-HCH), and three out of the seven BRFs (BDE-47, BDE-99 and HCBD) (Table 1). PCB-118 and PCB-138 displayed a V-shaped dose-response curve, switching from antagonistic to agonist behavior at concentrations above 3.5 μM and 27.5 μM, respectively. Hence, their IC_{50} values were determined by only the antagonistic part of the curve. The dose-response curves obtained from DR-H4IIE cells co-exposed to TCDD EC_{50} and the 16 AhR antagonistic POPs are shown in Figure S1 (solid lines) with a detail in Table S3.

291 DR-Hep G2 cells were less responsive to the POPs than DR-H4IIE, with only five compounds
292 exerting antagonistic activities, namely PCB-28, PCB-118, PCB-138, HCB and BDE-47 (Table 1).
293 PCB-28 was the most potent compound, almost completely abolishing the activity of 650 pM
294 TCDD in DR-Hep G2, displaying an RPC_{Max} of 7.2 ± 3.6% and an IC_{50} of 6.1 ± 1.4 µM. In DR-
295 T47-D cells, seven out of the 29 POPs showed AhR antagonistic activities (PCB-28, PCB-118,
296 PCB-138, HCB, α-chlordane, *t*-nonachlor and γ-HCH). The highest potencies were found for α-
297 chlordane and *t*-nonachlor with RPC_{Max} of 7.4 ± 12.5% and 27.8 ± 3.5%, respectively.
298
299 ***Evaluating competitive inhibition of 16 antagonists in DR-H4IIE.*** The AhR antagonistic
300 activities were abolished for all compounds (except α-chlordane and *t*-nonachlor with RPC_{Max} of
301 56.6% and 56.8%, respectively) when co-exposing with excessive 20 nM TCDD, indicating they
302 are possible AhR competitive antagonists (Figure S1, dashed lines).
303
304 **3.2. Aryl hydrocarbon receptor activities of the POP mixtures**
305 ***AhR – mediated agonistic activities.*** Exposure to the total POP mixture or to the six sub-
306 mixtures described in Table S1 did not induce any significant ($RPC_{Max} \geq 10\%$) AhR agonistic
307 response in any of the cell lines (data not shown), as it could be expected with the exclusion of
308 dl-PCBs and PCDD/F.
309
310 ***AhR – mediated antagonistic activities.*** The total POP mixture triggered an AhR antagonistic
311 response in all cell lines (Table 2, Figure 1A). At a concentration in the culture medium
312 corresponding to the blood level, the total POP mixture did not interfere with the response of the
313 cells to EC_{50} TCDD. In contrast, significant and dose-dependent antagonistic responses were
314 already observed at concentrations of 125, 250 and 500 fold blood levels, respectively, for DR-
315 H4IIE, DR-T47-D and DR-Hep G2, although the concentrations of all 29 compounds were below

316 their respective LOEC at these levels or even at 1000 fold blood levels (Table S1). In DR-H4IIE,
317 the POP mixture displayed a significantly high AhR antagonistic efficacy of 52.5 ± 2.1% at 1000
318 fold blood levels and an IC_{50} = 374 ± 52 fold blood levels, while in both human cell lines, a
319 significant response was observed, but did not reach below 80%, making the calculation of an
320 IC_{50} not possible.
321
322 In parallel, six complementary sub-mixtures (PFAA, Br, Cl, Cl+Br, Cl+PFAA, Br+PFAA) were also
323 tested to study the possible interactions between these groups of compounds. Antagonism was
324 seen for all Cl containing mixtures (the total POP, Cl, Cl+Br and Cl+PFAA mixtures) in DR-H4IIE
325 and DR-Hep G2, while only the total POP and Cl+PFAA mixtures showed responses in DR-T47-
326 D (Table 2).
327
328 In DR-H4IIE cells, the three Cl containing sub-mixtures and the total POP mixture gave more or
329 less similar responses with $IC_{50} \cong$ 400 to 500 fold blood levels and $RPC_{Max} \cong$ 50%. This indicates
330 that the chlorinated compounds were responsible for the antagonism of all mixtures where they
331 are present. Also, the antagonistic response curve of Cl+PFAA mixture overlapped that of the
332 total POP mixture, which placed below those of the Cl and Cl+Br (Figure 1B). These observations
333 suggest that the effect of the Cl mixture was somehow enhanced in the Cl+PFAA mixture,
334 resulting in a dose-response curve overlapping that of the total POP mixture.
335
336 **3.3. Predictions of the rat aryl hydrocarbon receptor antagonistic activities of the total POP**
337 **mixture and Cl containing sub-mixtures**
338 We evaluated the capacity of the three different mathematical models (concentration addition
339 (CA), independent action (IA), and generalized concentration addition (GCA)) to predict the IC_{50}
340 and dose-response curves of the total POP mixture in the most sensitive cell line, the DR-H4IIE.

Calculation of bottom and hillslope values. Because the 16 rat AhR antagonists contributed only 4.3% for the mass of the total POP mixture, along with considering all of the 29 POPs to calculating p_i, we also considered only the active compounds, subtracting the weight of the non-active compounds (sNA) as mentioned in section 2.2.3. WMBH method was unable to predict both bottom and hillslope values. AvBH and FoBH showed reasonable predicted values, especially AvBH and sNA FoBH for the total POP mixture and the three Cl containing sub-mixtures (Table S4).

Prediction of mixture effects of the three models. The results obtained after running the three models are shown in Figure 2. While the IA predicted a really strong response even at the lowest concentrations of the mixture, far out of the range of the measured curve, both the CA (CA-AvBH and CA-sNA FoBH) and GCA predictions resulted in calculated curves comparable to the measured curve. This refers that the 16 active compounds in the total POP mixture acted additively rather than independently.

The measured IC_{50} of the total POP mixture (374 ± 52 fold blood levels) was lower than the predicted value (784 fold blood levels for both the GCA and CA models), while the IA model predicted IC_{50} = 2153 fold blood levels. Thus, both CA and GCA models underestimated IC_{50} of the total POP mixture by about two folds, much less than one order of magnitude.

Concerning the calculated dose-response curves, the two additive models appeared to diverge: GCA produced a somewhat better prediction in the low concentration range, both CA (CA-AvBH and CA-sNA FoBH) closely followed the experimental curve and only diverged at concentrations higher than around 1000 fold blood levels (Figure 2). Similar predictions were also shown for the three active Cl, Cl+Br, Cl+PFAA sub-mixtures (Table S4, Figure S2).

Toxic units. Derived from CA model, toxic units (*i.e*. the ratio of the concentration partition of a compound to its IC_{50} ($p_i/IC_{50,i}$)) scales the concentrations of the mixture components to its toxicity, represented by the transcriptional activity of the Cyp1a1 promotor. Thus, it has been applied to identify the main driver(s) for mixture effects in CA model[24,28,41]. Figure 3A clearly shows that PCB-138 and PCB-118 were the two main contributors for the AhR antagonism of the total POP mixture, constituting to 90% of the total combined activity. Since they were also partial agonists, it is likely that, in the presence of TCDD, they behaved mainly as antagonists especially at low concentrations. Following this prediction, a binary mixture of PCB-138 and PCB-118 was generated according to their concentration in the total POP mixture. The dose-response curve of this mixture followed that of the total POP mixture very closely, with an IC_{50} of 505 ± 67 fold blood levels (while IC_{50} = 374 ± 52 fold blood levels for the total POP mixture) (Figure 3B).

4. Discussion

AhR transactivation activities of the 29 POPs and the mixtures. This study shows that a majority of the chemicals composing the realistic total POP mixture are actually AhR antagonists (16 in DR-H4IIE, five in DR-Hep G2 and seven in DR-T47-D cells). As expected, the total POP mixture and the Cl containing mixtures were also shown to be antagonistic. These activities were AhR-dependent, and seemed to act through competition for the TCDD binding site, except for *t*-nonachlor and α-chlordane.

In our study, we tested the AhR transcriptional activity of POPs and POP mixtures using a transcriptional reporter assay, which basically reports the canonical AhR-driven pathways via AhR-ARNT-DRE interactions. However, we observed that two of the compounds (*α*-chlordane and *t*-nonachlor) do not seem to exert their antagonistic effect through competitive binding to AhR.

392 Several possible non-canonical AhR-driven pathways could contribute to the observed results,
393 such as crosstalk with other nuclear receptors, regulation of cell cycle and MAP kinase cascades,
394 or novel AhR DNA-binding partners[42,43]. Further studies of these mechanisms are required, but
395 were outside of the scope of this paper.
396
397 Our results concerning single compound testing are in general consistent with previously
398 published studies, where available. For agonistic activity, PCB-118 displayed highest RPC_{Max} =
399 61.3% at 50 μM, with EC_{50} = 25 ± 13 μM similar to previously finding (9.3 ± 2.5 μM)[44]. EC_{50} = 4 ±
400 0.8 μM of BDE-99 was lower than previous report (EC_{50} > 15 μM)[45]. For PCB-138, we observed
401 an agonistic effect with a high EC_{50} = 28 ± 6.4 μM, which was not reported before[44] (Table S2).
402
403 For antagonistic activities, in this study, IC_{50} of PCB-28 and PCB-138 were 6.8 ± 1.7 and 0.6 ±
404 0.07 μM (Table 1), close to previous estimates of 9.0 ± 2.9 μM and 1.4 ± 0.1 μM, respectively[44].
405 BDE-47 activity (IC_{50} = 3.1 ± 0.5 μM) was similar to those previously reported (2.7 ± 0.7 μM)[45] and
406 (3.7 ± 0.8 μM)[44]. However, the IC_{50} = 5.2 ± 1.9 μM for BDE-99 found in this study was lower than
407 that previously reported (13 ± 0 μM)[45]. This study reports for the first-time data for the AhR
408 transcriptional activity of the 29 POPs in both human mammary gland carcinoma DR-T47-D and
409 hepatoma DR-Hep G2 cells.
410
411 Differences between IC_{50} or EC_{50} values of this study and those from previous findings[44,45] may
412 result from differences in the experimental design and the regression methods (*i.e.* the number of
413 concentration points, availability of a maximum effect if a full curve is generated, extrapolation if
414 the maximum effect is not reached, and the regression function used with either four or three
415 parameters). Our study not only confirms and consolidates previous findings[44,45], but it also
416 contributes new data including full dose-response curves with four parameter fit (see Table S3)

417 which can be used for further data treatment or calculation of the joint effect of any mixture made
418 from these 29 POPs for the rat DR-H4IIE cells.
419 We observed species and tissue differences in the AhR transcriptional activities of the individual
420 POPs and of the mixtures. In general, the rat DR-H4IIE cells were more sensitive than the two
421 human cells towards the effects on AhR transactivation when exposed to individual POPs or POP
422 mixtures. Several considerations may explain this result. Interspecies differences in AhR structure
423 will obviously shape the sensitivity. Rats are 1000 folds more sensitive to TCDD than guinea
424 pigs[46]. Mouse AhR has a higher affinity than human AhR due to the different position of the
425 important amino acid Valine (V381 in humans corresponding to V375 in mice)[47]. Human AhR has
426 shown a higher relative affinity for certain structurally compounds *i.e.* endogenous ligands or
427 polyphenols[48]. Moreover, the difference in genetic modification (origins of the integrated promotor
428 and DREs) of the rat DR-H4IIE compared to the two human cell lines could also play a role for
429 the specific responses. Differences in regulatory processes downstream of AhR binding may be
430 responsible, such as differential binding to transcriptional coactivators[49]. Finally, in the
431 antagonistic tests, the POPs have to compete with increasing TCDD concentration (15 pM, 150
432 pM and 650 pM, respectively for DR-H4IIE, DR-T47-D and DR-Hep G2), which could lead to the
433 lower sensitivity to detect an antagonistic activity of the POPs in the two human cells compared
434 to the rat cells.
435
436 ***Mixtures relevant for human exposure antagonize AhR activation.*** The most striking result of
437 this study is that the total mixture of 29 POPs, derived from concentrations found in the blood of
438 a Scandinavian population, and sub-mixtures thereof were found to exert only antagonistic effects
439 on AhR. This observation is consistent with our results obtained from testing each compound
440 alone, revealing a majority of antagonistic compounds. AhR antagonism of POPs has been
441 observed in several screening studies[44,45] and mixture studies[50,51]. However, while the AhR
442 agonistic activity of POPs has been studied for decades, the antagonistic counterpart has not yet

443 received much attention, especially regarding its physiological consequences on an organism's
444 health.
445 This finding challenges the method of using toxic equivalency factors (TEFs) and toxic equivalent
446 quantities (TEQ) for risk assessments of mixtures of AhR ligands. The World Health Organization
447 assigned TEFs for PCDDs/PCDFs/dl-PCBs, expressed as relative effect potencies compared to
448 the most toxic form TCDD. Regarding their additive mechanism, TEFs are also used to estimate
449 TEQ for a mixture of compounds by adding up the TEF fraction and the concentration of each
450 compound within the mixture[52]. However, PCB mixtures alone or in combination with
451 PCDDs/PCDFs (usually TCDD) have shown additive but also non-additive responses[53,54]. Also,
452 several environmentally abundant biphenyls antagonize the *cyp1a1* induction by TCDD[12], while
453 some dl-mono-ortho-substituted PCBs revealed both agonistic and antagonistic properties[55,56]. In
454 many environmental samples, the ratio between PCBs and AhR agonists is above 1000,
455 indicating that antagonisms, resulting from interactions between AhR agonists and PCBs[57], are
456 not irrelevant. Therefore, the antagonistic effect of these compounds should be also considered
457 to calculate the effect of mixtures. Since they are more abundant in real-life mixtures, their
458 antagonisms may undermine or even abolish the overall dioxin potency of the environmental
459 mixtures[12].
460
461 Our finding also raises the issue of the biological significance of a predominantly AhR antagonistic
462 mixture in the blood of a human population. We found antagonistic activities at levels of 125 fold
463 blood levels in rat liver cells or 250 or 500 folds in human mammary gland and liver cells, levels
464 that may realistically be reached after an accident or in exposed populations.
465
466 It is important to note that in the total POP mixture used here[36], no dioxins and dl-compounds
467 were included. That allows us to study the antagonism of the human-exposure relevant POP
468 mixtures by isolating them from the dioxin and dl-compounds. But the roles of the dioxin and dl-

compounds in human exposure should have also been considered. Therefore, we attempted to estimate the effect of the total POP mixture in a real-life situation. According to Kvalem *et al*.[58] , the median of dl-compounds in Norwegian human blood was 33.1 pg TEQ/g lipid, which is equal to 0.6 pM in blood assuming that blood contains 0.6% fat and 1 ml blood = 1 g. Thus, the respective LOECs (for the POP mixture AhR antagonistic activity) in DR-H4IIE, DR-T47-D and DR-Hep G2 of 125, 250, and 500 fold human blood level correspond to 75 pM, 150 pM and 300 pM of dl-compounds, respectively. This concentration is close to the TCDD EC_{50} (15 pM TCDD in DR-H4IIE, 150 pM in DR-T47-D and 650 pM in DR-Hep G2 cells) used in our antagonistic assays. Therefore, it is likely that the total POP mixture would antagonize the activity of these dl-compounds in the Scandinavian population.

Furthermore, the question arises whether an overall AhR antagonistic exposure would actually cause health problems by interfering with the normal AhR function. Increasing evidence suggests that endogenous AhR ligands exist[59,60], complemented by dietary phytochemical-derived AhR agonists/antagonists[61,62]. AhR induced functions are essential for a variety of normal physiological functions. In mammary tissue, AhR likely plays a physiological role in coordinating development, differentiation, cell growth, and signaling of hormones[63–67]. Knock out mice or mice with low affinity AhR variants display impaired survival, growth, fertility, liver function and innate and adaptive immunity[68]. It is thus conceivable that the presence of highly stable POPs may interfere with the essential function of AhR controlled by mostly short-lived endogenous and dietary ligands, and thus impair cellular AhR mediated processes. The risk caused by AhR antagonism as the major effect of POPs could well exceed that due to their agonistic effect, however the health effect associated to AhR antagonism is unclear and deserves further investigation.

Predictions of mixture effects. Risk assessment for mixture exposure is crucial to protect the health of both humans and wildlife. The individual chemical approach underestimates the mixture

exposure and decreases the accuracy of risk assessment[22,23,69]. In addition, the risk of exposure to multiple chemicals at doses below their threshold, which is the most common case in real-life, should not be underestimated or assumed as no-effect.

In this study, the best prediction results were obtained using the CA (concentration addition) and GCA (generalized concentration addition) models. They performed well in predicting $IC_{mix,50}$ = 784 fold blood levels within two folds from the measured value (374 fold blood levels). This is considered as well accepted in predicting the combined effect of complex mixtures *i.e.* the total POP mixture with its components present at low concentration (lower than their LOECs at 1000 fold blood levels) and belonging to different compound groups.

CA is often chosen as the default model[70] for predicting mixture activities, first for mixtures with similar compounds[24] then expended to dissimilar compounds[23,25,26]. Previous studies have shown the capability of the CA model to predict the mixture effect using the information of individual chemicals obtained *in vitro*[25,41,71,72], *ex vivo*[73] or *in vivo*[23,25]. *In vitro* research has mainly focused on an equimolar mixture with less than ten components and at high exposure concentrations. Birkhøj *et al*.[25] successfully applied the CA model to predict the antiandrogenic effect of a mixture of five commonly used pesticides at 10 μM each. In contrast, the CA model was unable to predict the effect on thyroid hormone function and AhR transactivation of another mixture of five different pesticides at the maximum concentration of 50 μM each, due to the presence of an inhibitory compound[71]. Other studies focused on more complex mixtures with multiple components at lower doses, typically below their threshold doses, or on human or environmentally relevant exposure scenarios. Two complex mixtures of 17 estrogenic chemicals were screened for estrogenic activities, reporter-gene (ERLUX) and cell proliferation (ESCREEN) endpoints[41]. This represents

519 one of the most comprehensive studies on the effects of mixtures where they were able to predict
520 the effects of the two mixtures.
521 GCA, on the other hand, has been recently developed and proven useful specifically for
522 calculating mixtures containing partial agonists[30–32], but has not been applied before to calculate
523 the activity of AhR antagonists. It allows to consider theoretically the fact that some agonists never
524 reach the full activity of TCDD, or that some antagonists present partial agonistic activities.
525
526 The difference between CA and GCA models resides in the predicted dose-response curve of the
527 mixture and in the maximum predicted activity of the total POP mixture (Figure 2). GCA predicting
528 the mixture response, allows to generate the full dose-response curve of the mixture using only
529 the data from testing the individual compounds (*i.e.* RPC_{Max}, concentration and IC_{50}). The reason
530 why this predicted curve diverged from the experimental curve at higher concentrations could
531 result from the assumption that the hillslopes of all components, and so of the POP mixture, are
532 equal to 1, which is clearly not the case (Table S3). However, GCA predicted the bottom (RPC_{Max}
533 = 52%) very close to the observed value for the total POP mixture (52.5%) thanks to its
534 consideration of the RPC_{Max}.
535
536 On the other hand, CA provides a prediction of $IC_{mix,j}$ without the full dose-response curve.
537 Therefore, we calculated the hillslope and the bottom values for the mixture response based on
538 its components by formulating several possibilities. The dose-response curve generated by CA
539 with averaged bottom hillslope (CA-AvBH) resulted in the best fit with reasonable hillslope and
540 bottom values (1.7 and 21%) compared to 1.3 and 52.5%, respectively of the measured POP
541 mixture curve. Subtracted non-activated compounds and formulated bottom hillslope (sNA FoBH)
542 predicted an overlapped curve with the observed up to 1000 fold blood levels because of its closer
543 H_{mix} = 1.5, but overestimated the extension of antagonism at higher concentration, leading to the
544 prediction of B_{mix} = 0% for the total POP mixture. B_{mix} is important when predicting the activity of

545 a mixture for risk assessment. Therefore, CA-AvBH rather than CA-sNA FoBH was chosen as a
546 more suitable prediction in our case. The CA model also provides a good prediction of $IC_{mix,50}$ for
547 the response of the DR-H4IIE cells to the active sub-mixtures as to the total POP mixture, and
548 reasonable predicted dose-response curves (Table S4, Figure S2).
549
550 Finally, IA (independent action) was designed specifically for mixtures of compounds with clearly
551 different MOAs to combine probabilities of action of individual compounds. Previous studies
552 showed that IA outperformed CA[23] or was comparable to CA with equal[29] or not more than five-
553 fold differences[28,74] in predicting the combined effects for chemicals having different MOAs. The
554 bad performance of IA to predict either IC_{mix} or the dose-response curve clearly results from the
555 mixture studied here, where we showed that most components act through the same MOAs. At
556 low doses, accumulation of the individual, low probabilities derived for a high number of individual
557 compounds presumably resulted in the dramatic overestimation of the antagonistic effect.
558
559 **5. Conclusions**
560 We tested the AhR agonistic and antagonistic activities of 29 POPs shown to contaminate human
561 blood, both in individual and mixture forms. AhR transactivation activities in three reporter cell
562 lines exposed to the 29 POPs and the mixtures were different due to the species and tissue-
563 specific responses. The predominant individual activities of the POPs were AhR antagonism, as
564 shown for 16 compounds out of 29 in rat DR-H4IIE cells, and for seven and five compounds in
565 human DR-T47-D and DR-Hep G2, respectively. The total POP mixture already induced a
566 significant AhR antagonistic activity at concentrations of 125, 250, and 500 fold human blood
567 levels, respectively in DR-H4IIE, DR-T47-D and DR-Hep G2, although each individual compound
568 was present at concentrations lower than its LOEC at these levels. Such blood levels of POPs
569 could realistically occur in food or environmental contamination incidents or in highly exposed
570 sub-populations. Chlorinated compounds, among which PCB-118 and PCB-138 contributed 90%

571 to the activity of the total POP mixture, were the drivers for AhR antagonism in DR-H4IIE cells.
572 Finally, CA and GCA proved to be good tools to predict the mixed effect of the total POP mixture
573 with only two-fold underestimated IC_{50} and acceptable does response curves. Hence, the
574 compounds acted additively in the mixtures. Although limitations remain to fully describe the
575 effects of realistic mixtures due to biological complexity, the predictions obtained using CA and
576 GCA seem suitable for establishing general regulatory guidelines for mixture toxicity
577 assessments. In addition, the data generated in this study for individual compounds will be useful
578 to predict the effect of other complex mixtures constituted by these compounds.
579
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584

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Figure Captions

Figure 1: (A) Dose-response curves obtained from DR-H4IIE, DR-T47-D and DR-HepG2 cells co-exposed to 15 pM, 150 pM and 650 pM TCDD, respectively, and the total POP mixture. (B) Dose-response curves obtained from DR-H4IIE cells co-exposed to 15 pM TCDD, and the total POP mixture, or the Cl, Cl+Br and Cl+PFAA sub-mixtures (Mean ± SD, n = 3, 0.4% DMSO).

Figure 2: Measured and predicted dose-response curves obtained from rat DR-H4IIE cells co-exposed to 15 pM TCDD and the total POP mixture, and from three prediction models. CA = Concentration addition, IA = Independent action, GCA = Generalized concentration addition, AvBH = averaged hillslope and bottom, and sNA FoBH = subtracted nonactive compounds, formulated hillslope and bottom. Green dashed lines represent the 95% confidence interval of the measured response.

Figure 3: (A) Distribution of toxic units of the 16 active AhR antagonists. (B) Dose-response curves obtained from rat DR-H4IIE cells co-exposed to 15 pM TCDD and the total POP mixture or a binary mixture consisting of PCB-138 and PCB-118 (Mean ± SD, n = 3, 0.4% DMSO).

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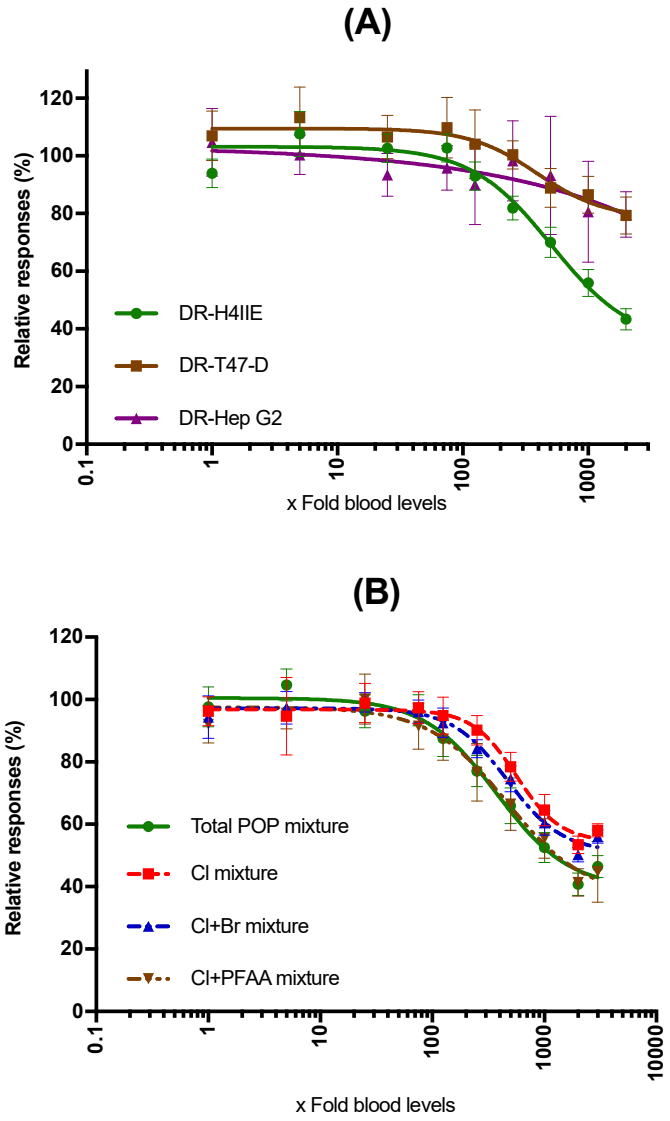

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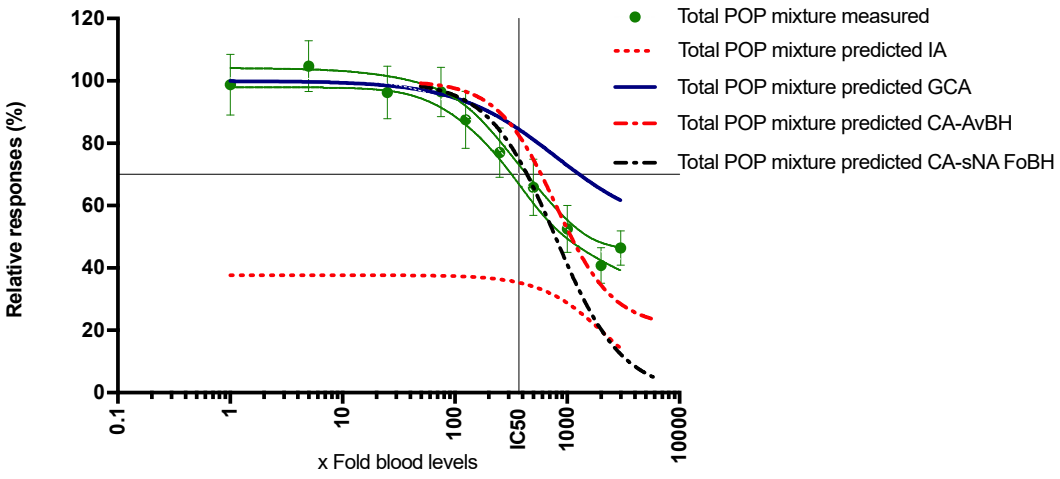

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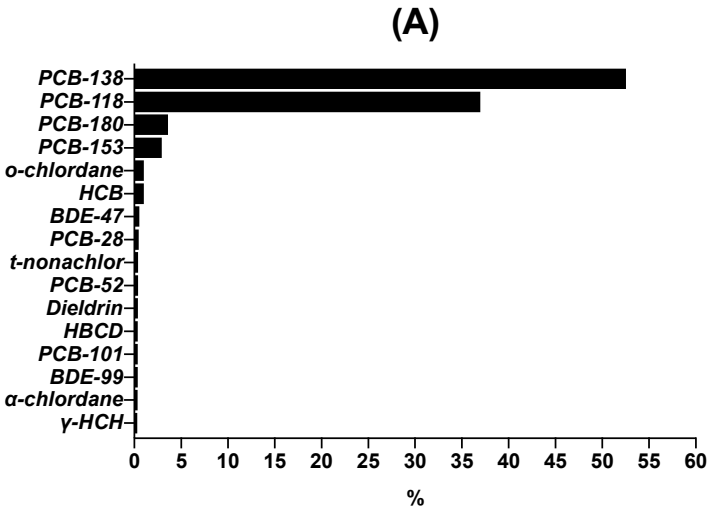

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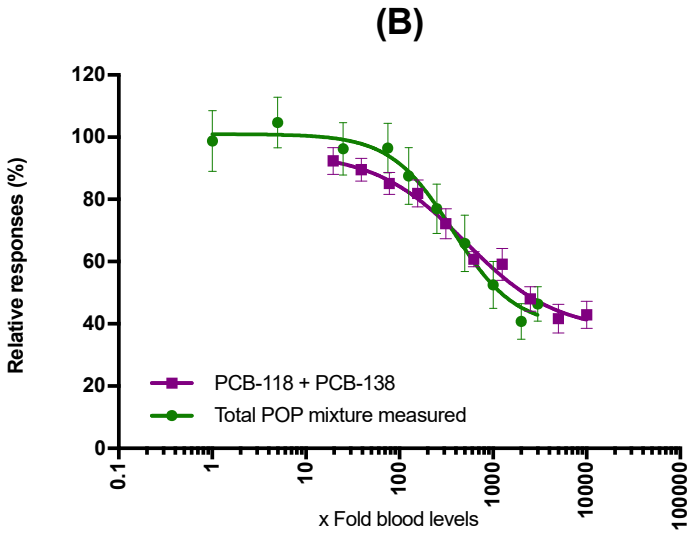

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839 Figure 3.

840 **Table Captions**

841 **Table 1:** AhR antagonistic responses (LOEC, MOEC, IC_{50} and RPC_{Max}) of 16 POPs in DR-H4IIE,
842 DR-Hep G2 and DR-T47-D cell lines (n = 3, 0.3% DMSO).

843

844 **Table 2:** AhR antagonistic responses (LOEC, MOEC, IC_{50} and RPC_{Max}) of the total POP mixture
845 and Cl containing sub-mixtures (Cl, Cl+Br, Cl+PFAA) in DR-H4IIE, DR-T47-D and DR-Hep G2
846 cells (n = 3, 0.4% DMSO).

847

Table 1.

Compounds	DR-H4IIE				DR-Hep G2				DR-T47-D			
	LOEC (µM)	MOEC (µM)	IC_{50} ± SE (µM)	RPC_{Max} (%)	LOEC (µM)	MOEC (µM)	IC_{50} ± SE (µM)	RPC_{Max} (%)	LOEC (µM)	MOEC (µM)	IC_{50} ± SE (µM)	RPC_{Max} (%)
PCB-28	2.5	25	6.8 ± 1.7	36.6 ± 4.3	2.5	25	6.1 ± 1.4	7.2 ± 3.6	3.5	25	11.4 ± 1.5	48.3 ± 5.9
PCB-52	2.5	50*	7.3 ± 1.2	17.1 ± 5.5	-	-	-	-	-	-	-	-
PCB-101	12.5	50*	17.9 ± 3.8	54.3 ± 3.6	-	-	-	-	-	-	-	-
PCB-118	0.5	2.5	0.3 ± 0.05	67 ± 4.7	12.5	25	9 ± 2.7	38.3 ± 16.5	12.5	27.5	13.6 ± 2.4	49.2 ± 4.9
PCB-138	0.5	2.5	0.6 ± 0.07	42.8 ± 2.8	3.5	25	ND	50.2 ± 9.5	12.5	50*	ND	42.6 ± 2.5
PCB-153	0.01	50*	18.5 ± 2.8	16.7 ± 4.6	-	-	-	-	-	-	-	-
PCB-180	1	50*	7.4 ± 3.3	16 ± 1.4	-	-	-	-	-	-	-	-
HCB	0.075	37.5	17.9 ± 11.6	42 ± 8.7	3.75	37.5	4.5 ± 2.3	39.4 ± 12.7	0.075	30	16.4 ± 2.1	51.7 ± 6.7
α-Chlordane	0.4	50*	28.3 ± 3.3	15 ± 1.1	-	-	-	-	10	30	20.2 ± 2.1	7.4 ± 12.5
o-chlordane	20	40*	26.5 ± 19.4	26.3 ± 1.5	-	-	-	-	-	-	-	-
t-nonachlor	25	50*	34.3 ± 1.8	34.2 ± 8.1	-	-	-	-	25	25	16.8 ± 2	27.8 ± 3.5
γ-HCH	0.5	50	27.5 ± 2.7	40.7 ± 3.5	-	-	-	-	50	75	61.2 ± 2.9	65.4 ± 13.7
Dieldrin	6.25	50	22.4 ± 11.4	59.6 ± 1.9	-	-	-	-	-	-	-	-
BDE-47	0.25	20*	3.1 ± 0.5	17.9 ± 2.7	1.25	12.5	ND	55.3 ± 7.2	-	-	-	-
BDE-99	0.25	10	5.2 ± 1.9	36.3 ± 1.5	-	-	-	-	-	-	-	-
HBCD	0.25	15	35.8 ± 63.9**	58.1 ± 3.1	-	-	-	-	-	-	-	-

LOEC: lowest observed effect concentration ($p < 0.05$); MOEC: maximum observed effect concentration ($p < 0.05$); IC_{50}: the concentration inducing half of the maximum inhibition response; SE: Standard Error; RPC_{Max} : observed efficacy expressed as a percentage of the cell response exposed to 15 pM, 650 pM and 150 pM TCDD, respectively for DR-H4IIE, DR-Hep G2 and DR-T47-D, corresponding to the MOEC; ND: Not Determined. * Corresponds to the highest tested concentration. ** IC_{50} estimated beyond tested concentrations. -: no response.

853 Table 2.

Mixtures	DR-H4IIE				DR-Hep G2				DR-T47-D			
	LOEC (x bl)	MOEC (x bl)	IC_{50} ± SE (x bl)	RPC_{Max} (%)	LOEC (x bl)	MOEC (x bl)	IC_{50} ± SE (x bl)	RPC_{Max} (%)	LOEC (x bl)	MOEC (x bl)	IC_{50} ± SE (x bl)	RPC_{Max} (%)
POP	125	1000	374 ± 52	52.5 ± 2.1	500	1000	ND	80.1 ± 5.8	250	1000	ND	86.6 ± 2.2
CI	250	1000	562 ± 54	53 ± 0.9	250	2000	ND	59 ± 1.6	-	-	-	-
CI+Br	125	2000	468 ± 38	64.6 ± 1.7	500	1000	534 ± 253	76.1 ± 3.9	-	-	-	-
CI+PFAA	75	2000	461 ± 78	41 ± 1.3	500	500	243 ± 104	77 ± 3.8	500	500	ND	77 ± 3.8

854 LOEC: lowest observed effect concentration ($p < 0.05$); MOEC: maximum observed effect concentration ($p < 0.05$); IC_{50}: the concentration inducing half of the
855 maximum inhibition response; SE: Standard Error; RPC_{Max} : relative response at MOEC expressed in % of the response of TCDD EC_{50} 15 pM, 650 pM and 150 pM
856 TCDD (respectively for DR-H4IIE, DR-Hep G2 and DR-T47-D) corresponding to the MOEC; x bl: fold blood levels; ND: Not Determined. -: no response.

Table S1: Composition of the total POP mixture and the six sub-mixtures (PFAA, Br, Cl, Cl+Br, Cl+PFAA, Br+PFAA). The minimum and maximum tested concentrations of each compound in individual compound testing and the concentrations of each compound corresponding to 1000 fold blood levels in the mixture testing are also given.

Compounds	Individual compound testing: tested concentration (μM)		Mixture testing: individual concentrations in the mixture corresponding to 1000 fold blood levels (μM)						
	Minimum	Maximum	Total POP	PFAA	Br	Cl	Cl+Br	Cl+PFAA	Br+PFAA
PCB-28	0.01	50	0.031			0.031	0.031	0.031	
PCB-52	0.01	50	0.021			0.024	0.021	0.021	
PCB-101	0.01	50	0.025			0.025	0.021	0.025	
PCB-118	0.01	50	0.14			0.13	0.12	0.13	
PCB-138	0.01	50	0.43			0.45	0.39	0.42	
PCB-153	0.01	50	0.70			0.73	0.63	0.67	
PCB-180	0.01	50	0.34			0.38	0.33	0.35	
p,p'-DDE	0.5	50	1.066			1.088	0.95	1.006	
HCB	0.075	60	0.23			0.24	0.21	0.22	
α-chlordane	0.4	80	0.024			0.029	0.032	0.032	
o-chlordane	0.4	40	0.34			0.034	0.029	0.029	
t-nonachlor	0.5	50	0.099			0.099	0.101	0.101	
α-HCH	0.5	50	0.017			0.017	0.017	0.017	
β-HCH	0.5	50	0.076			0.076	0.069	0.069	
γ-HCH	0.5	100	0.017			0.021	0.017	0.017	
Dieldrin	0.5	100	0.055			0.063	0.060	0.058	
BDE-47	0.025	20	0.019		0.012		0.016		0.014
BDE-99	0.025	20	0.0071		0.0053		0.007		0.0071
BDE-100	0.0025	1	0.0035		0.0035		0.004		0.0035
BDE-153	0.0025	1	0.0016		0.0016		0.002		0.0016
BDE-154	0.0025	1	0.0031		0.0031		0.003		0.0016
BDE-209	0.0025	1	0.0094		0.0104		0.009		0.0094
HBCD	0.025	20	0.055		0.055		0.033		0.065
PFHxS	0.5	50	7.809	6.71				7.41	6.91
PFOS	0.5	50	41.52	18.19				40.83	16.37
PFOA	0.5	50	4.209	7.24				4.42	6.69
PFNA	0.5	50	1.092	1.76				1.036	1.74
PFDA	0.5	50	0.38	0.72				0.44	0.66
PFUnDA	0.5	50	0.34	0.32				0.17	0.28
Summary concentration (μM)			59.048	34.94	0.091	3.43	3.101	57.51	32.75

POPs: persistent organic pollutants. PFAA: perfluorinated compounds. Br: brominated compounds. Cl: chlorinated compounds. PCB: polychlorinated biphenyl. DDE: dichlorodiphenyldichloroethane. HCB: hexachlorobenzene. HCH: hexachlorocyclohexane. BDE: brominated diphenyl ether. HBCD: hexabromocyclododecane. PFHxS: perfluorohexanesulfonic acid. PFOS: perfluorooctanesulfonic acid. PFOA: perfluorooctanoic acid. PFNA: perfluorononanoic acid. PFDA: perfluorodecanoic acid. PFUnDA: perfluoroundecanoic acid.

Table S2: EC_{50}, RPC_{Max} and relative potency for AhR agonistic compounds in DR-H4IIE and DR-T47-D cells (n = 3, 0.2% DMSO).

Cell lines	DR-H4IIE					DR-T47-D	
Compounds	BDE-99	BDE-153	BDE-154	PCB-118	PCB-138	BDE-99	γ-HCH
EC_{50} ± SE (μM)	4 ± 0.8	ND	ND	25 ± 13	28 ± 6.4	1.9 ± 1.8	> 50
RPC_{Max} (%)	7.3	15.2	8.9	61.3	28.2	11	6
Relative potency	0.0000038	-	-	0.00006	0.000054	0.000013	0.000033

EC_{50}: concentration inducing half of the maximum response, extrapolated; RPC_{Max}: observed efficicacy of the maximum tested concentration expressed in % of the maximum response of TCDD; Relative potency: ratio between the EC_{50} of TCDD and the EC_{50} of the tested compound, with TCDD EC_{50} = 15 pM in DR-H4IIE and 150 pM in DR-T47D; SE: Standard Error; ND: not determined

Table S3: Four parameters obtained in the dose-response curves (Graphpad PRISM) of the 16 active AhR antagonists in the rat DR-H4IIE cells (n = 3, 0.2% DMSO, Mean ± SD).

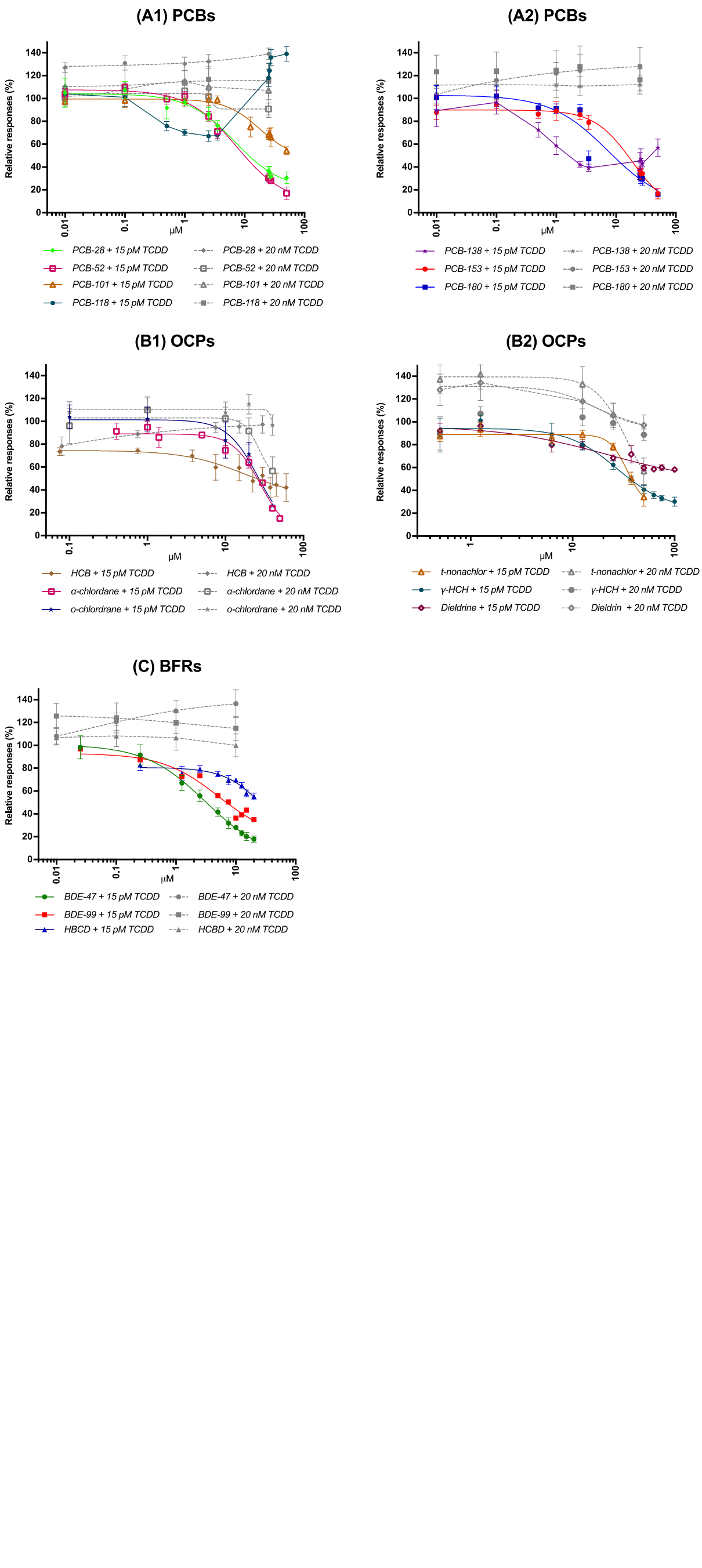
Parameters	BDE-47	BDE-99	HBCD	PCB-28	PCB-53	PCB-101	PCB-118	PCB-138
Bottom (%)	0.2 ± 7.05	16.9 ± 12.4	0	20.3 ± 7.4	8.2 ± 5.5	47.2 ± 7.4	67.2 ± 1.4	43.1 ± 1.5
Top (%)	100.7 ± 2.6	93.1 ± 3.3	80.7 ± 1.4	104.2 ± 1.8	107.6 ± 1.2	99.5 ± 1.3	104.2 ± 1.8	92.9 ± 2.1
HillSlope	0.9 ± 0.1	0.9 ± 0.2	1.3 ± 0.5	1.1 ± 0.2	1.1 ± 0.1	1.5 ± 0.3	2.2 ± 0.5	2.4 ± 0.6
IC_{50} (μM)	3.1 ± 0.5	5.2 ± 1.9	35.8 ± 63.9	6.8 ± 1.7	7.3 ± 1.2	17.9 ± 3.8	0.3 ± 0.05	0.6 ± 0.07
Parameters	PCB-153	PCB-180	HCB	*α*-Chlordane	*o*-chlordane	*t*-nonachlor	*γ*-HCH	Dieldrine
Bottom (%)	0	5.1 ± 14.7	33.01 ± 13.4	0	0	25.8 ± 5.8	23.2 ± 4.6	45.6 ± 11.5
Top (%)	89.9 ± 1.1	102.9 ± 3.2	74.6 ± 2.6	89.1 ± 1.2	101.4 ± 4.8	89 ± 1.0	94.4 ± 1.6	96.7 ± 3.3
HillSlope	1.4 ± 0.2	0.9 ± 0.2	1.1 ± 0.5	2.4 ± 0.4	2.2 ± 1.5	4.98 ± 0.9	1.8 ± 0.3	0.8 ± 0.3
IC_{50} (μM)	18.5 ± 2.8	7.4 ± 3.3	17.9 ± 11.6	28.3 ± 3.3	26.5 ± 19.4	34.3 ± 1.8	27.5 ± 2.7	22.4 ± 11.4

Table S4: Measured and predicted bottom, hillslope and IC_{50} of the CA models with several methods for the total POP mixture and Cl, Cl+Br, Cl+PFAA sub-mixtures.

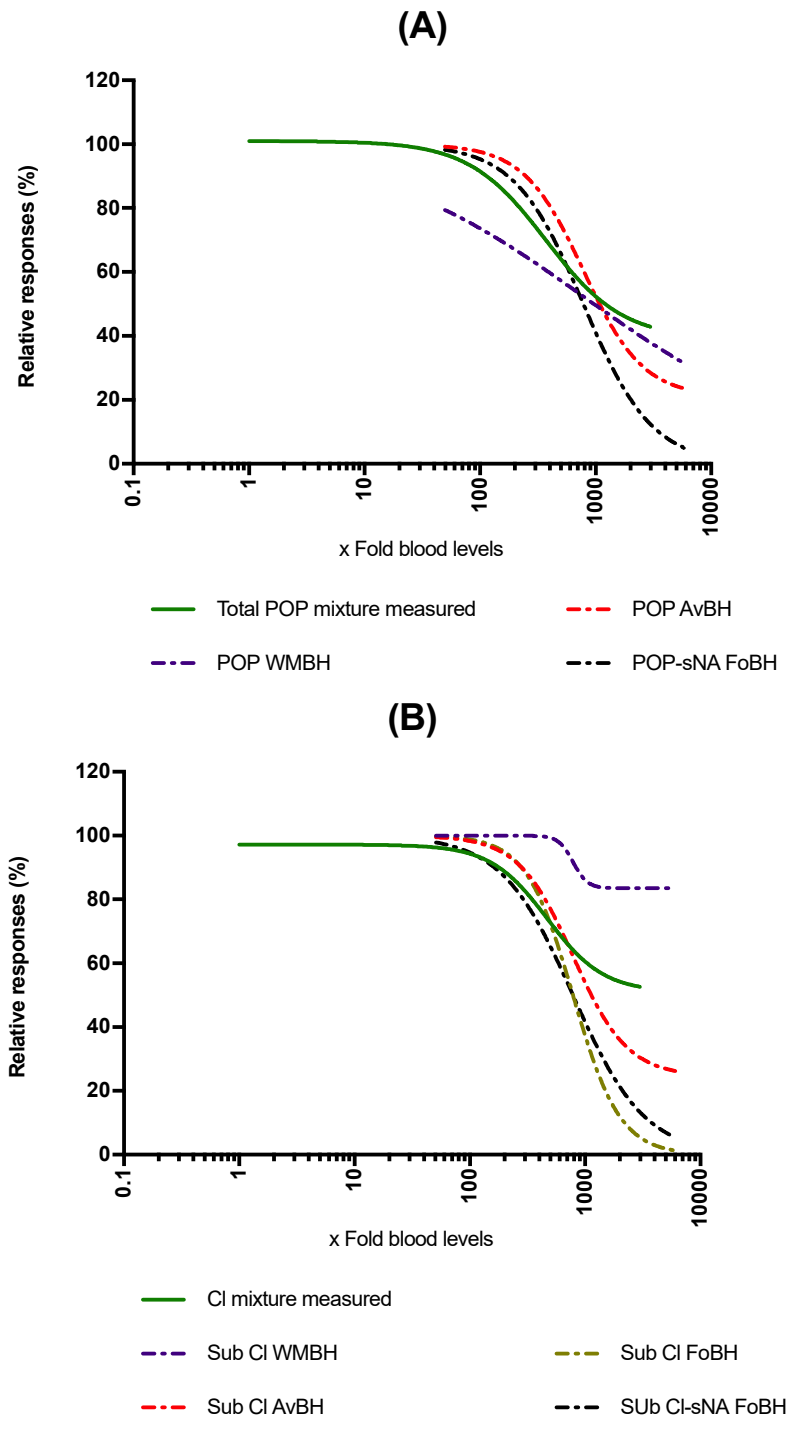
Methods	Measured	WMBH	AvBH	FoBH	sNA WMBH	sNA FoBH
Total POP mixture						
Bottom (%)	52.5	5	21	0	100	0
Hillslope	1.3	0.5	1.7	34.2	-10.9	1.5
IC_{50} (x bl)	374	784	784	784	784	784
Cl sub-mixture						
Bottom (%)	53	83	25	0	100	0
Hillslope	2.1	6.9	1.8	2.1	-10.6	1.4
IC_{50} (x bl)	500	786	786	786	786	786
Cl+Br mixture						
Bottom (%)	64.6	83	21	0	100	0
Hillslope	2.1	7	1.7	2.1	-10.6	1.4
IC_{50} (x bl)	500	875	875	875	875	875
Cl+PFAA mixture						
Bottom (%)	41	5	25	0	100	0
Hillslope	1.4	0.4	1.8	38.6	-10.7	1.4
IC_{50} (x bl)	576	840	840	840	840	840

WMBH = weighted mean, AvBH = averaged, formulated hillslope and bottom (FoBH), sNA = substracted nonactive compounds. x bl: fold blood levels.

Figure S1: Dose-response curves obtained from DR-H4IIE cells co-exposed to 15 pM TCDD (solid lines) or 20 nM TCDD (dashed lines) and PCBs (A1 and A2), OCPs (B1 and B2) and BRFs (C) (Mean ± SD, n = 3, 0.3% DMSO).



Figures S2: Predicted full dose-response curves of the concentration addition models with several methods to calculate the bottom and hill-slope for the total POP mixture (A) and Cl (B), Cl+Br (C), Cl+PFAA (D) sub-mixtures. WMBH = weighted mean, AvBH = averaged, formulated hillslope and bottom, sNA = subtracted nonactive compounds.



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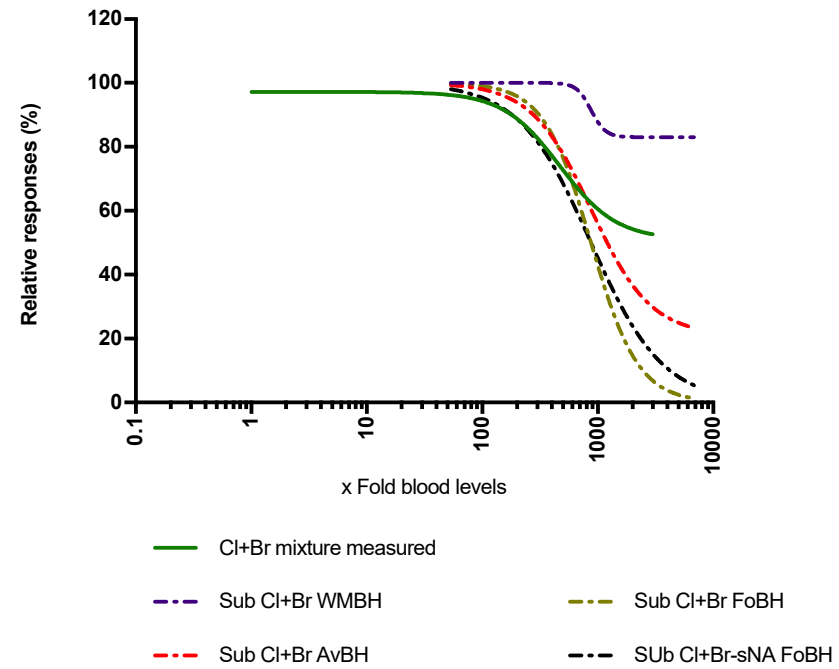
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0
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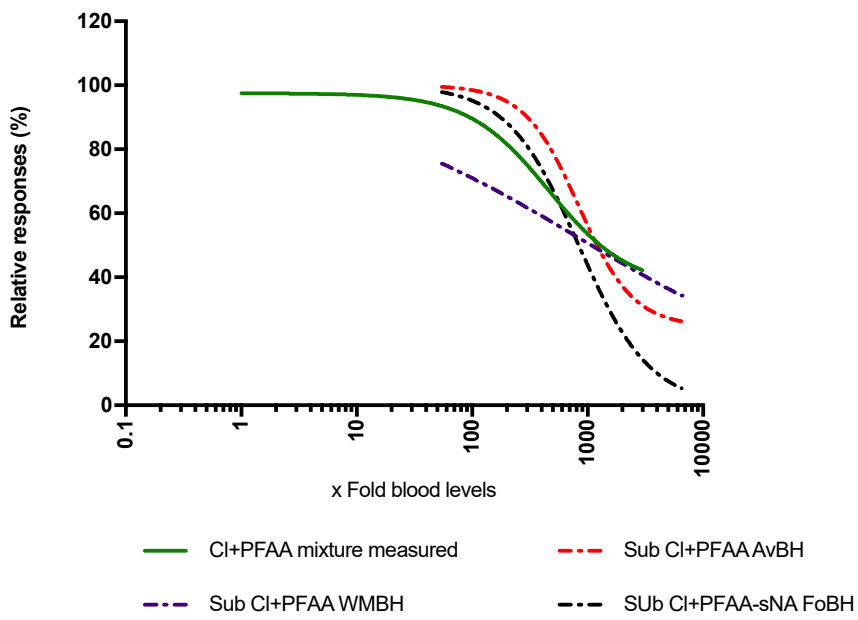
0.1
1
10
100
1000
10000
x Fold blood levels
Cl+Br mixture measured
Sub Cl+Br WMBH
Sub Cl+Br AvBH
Sub Cl+Br FoBH
SUb Cl+Br-sNA FoBH

(D)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0.1
1
10
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x Fold blood levels
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Sub Cl+PFAA WMBH
Sub Cl+PFAA AvBH
SUb Cl+PFAA-sNA FoBH