# UPTAKE AND SYSTEMIC RELATIVE EFFECT POTENCIES (REPs) OF 4-PeCDF, PCB-126, PCB-118, PCB-156 IN FEMALE C57/Bl6 MICE AFTER ACUTE EXPOSURE

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## Introduction

Risk assessment of mixtures of chlorinated dioxins (PCDDs), furans (PCDFs) and biphenyls (PCBs) is performed using the Toxic Equivalency (TEQ) concept, endorsed by the World Health Organization (WHO). TEQs are the sum of congener specific toxic equivalency factors (TEFs) multiplied by the concentration in a matrix, such as plasma. Current TEFs are derived from multiple relative effect potencies (REPs), obtained mainly from *in vivo* studies linking administered dose levels to toxic or biologic effects. Increasing evidence suggests that using administered dose levels (uptake) instead of systemic levels (e.g. liver or plasma concentrations), may lead to misinterpretation of risk[1-3].
In this study, REPs of 4-PeCDF, PCB-126, PCB-118 and PCB-156 were determined in female mice three days after a single oral dose. REPs were calculated based on cytochrome P450 1A1 (EROD) activity in liver samples and gene expression of CYP1A1, 1A2 and 1B1 in liver and peripheral blood lymphocytes (PBL) using administered dose, liver tissue levels and plasma levels.

## Materials and methods

*Chemicals*
2,3,7,8-tetrachlorodibenzodioxin (TCDD), 2,3,4,7,8,-pentachlorodibenzofuran (4-PeCDF), and 3,3',4,4',5-pentachlorobiphenyl (PCB-126) were purchased from Wellington Laboratories Inc. (Guelph, Ontario, Canada). 2,3',4,4',5-pentachlorobiphenyl (PCB118) and 2,3,3',4,4',5-hexachlorobiphenyl (PCB156) were purchased from Cerilliant Corp. (Round Rock, TX, USA). The chemicals were dissolved and diluted in corn oil (Sigma-Aldrich, Stockholm, Sweden). The compounds PCB-118 and PCB-156 were analysed for impurities by the department of Chemistry, Umeå University, Umeå, Sweden. Final TEQ contributions of impurities were 6.6 (118) and 36 (156) ng/g TEQ. These levels were considered to have no influence on the final outcome of our results.

*Animals*
Nine-week old female C57BL/6 mice (Harlan laboratories, Venray, The Netherlands) were randomly assigned to treatment groups (6 animals/group). The mice received a single dose of TCDD (0, 2.5, 10, 25 or 100µg/kg body weight), 4-PeCDF (0, 25, 100, 250 or 1000µg/kg body weight), PCB126 (0, 25, 100, 250 or 1000µg/kg body weight), PCB-118 or PCB-156 (0, 5, 15, 50, 150, 500mg/kg body weight). The chemicals were administered in corn oil by oral gavage at a dosing volume of 10ml/kg bw. Animals were sacrificed at day 3 by $CO_2/O_2$. Blood was obtained from the abdominal aorta directly after decease. Livers were removed, weighed, snap frozen and stored until use at -80°C. All animal treatments were performed with permission of the Animal Ethical Committee of the Utrecht University and according to the Dutch law on Animal Experiments.

*Chemical analysis*
Adipose and liver tissues samples were cleaned using a combined solid phase extraction using $Na_2SO_4$ and silica materials. Blood plasma samples were cleaned up using a solid phase extraction with Chem-Elut and NaCl. After clean up the samples were analysed by GC-MS. To retain unique individual results, liver tissue and plasma samples were not pooled within the same treatment group but between similar exposure levels. TCDD, 4-PeCDF

and PCB126 were pooled together in one group and PCB-118 and PCB-156 in a second group. The concentrations were calculated at lipid base.

*Plasma and lymphocyte isolation*
Blood from two mice was pooled after which plasma and peripheral lymphocytes were isolated by using Ficoll-Paque gradient according to manufacturer's instructions. Plasma samples were stored directly at -80°C until compound analysis. Isolated lymphocytes were lysed with RLT buffer (Qiagen, Venlo, the Netherlands) as described in the Qiagen RNAeasy kit protocol and stored until use at -80°C.

*EROD activity*
Hepatic CYP1A1 activity was determined by means of ethoxyresorufin-O-deethylase (EROD) activity in microsomal fractions of liver tissue.

*RNA isolation and expression*
Total RNA extraction was performed using a Qiagen RNAeasy kit. RNA was reverse transcribed to complementary DNA (cDNA) using iScript cDNA sythesis Kit, according to the manufacturer's instructions (Bio-Rad, Veenendaal, the Netherlands). Quantitative real-time PCR (RT-PCR) analyses were performed using the iQ Real-Time PCR Detection System using SYBR green (Bio-rad, Veenendaal, the Netherlands). The PCR primers CYP1A1, and β-actine (reference gene) were designed with the Primer designing tool (NCBI), primers CYP1A2, CYP1B1 where designed by Flaveny *et al.*[4] and Berge *et al.*[5], respectively. Data were analysed using iCycler iQ Optical System Software (Bio-Rad, Veenendaal, the Netherlands).

## Results and discussion

The treatment with a single oral dose of various concentrations TCDD, 4-PeCDF, PCB-126, PCB-118 or PCB-156 did not affect body weight three days after exposure. Compared to the controls, all compounds tested provoked a dose-dependent decrease in thymus weight and a dose-dependent increase in liver weight (data not shown). Further, the lipid fraction in the livers (% lipid/g liver) dose-dependently increased to between 1.4- and 2.5-fold compared to the control at the highest dose tested. Lipid adjusted liver and plasma concentrations increased linear with the oral dose given. Although all congeners were present at higher concentrations in the liver than in plasma, clear differences were seen in liver:plasma concentration ratio among the compounds (Table 1). PCB-118 and PCB-156 showed the lowest liver:plasma concentration ratio of ~2, at all concentrations and 4-PeCDF the highest, with 485 at 100µg/kg bw, indicating high accumulation of 4-PeCDF in the liver. Compound analysis in adipose tissue was analyzed for dose groups 10µg/kg bw (TCDD), 100µg/kg bw (4-PeCDF, PCB-126) and 50 000µg/kg bw (PCB-118, PCB-156). Lipid adjusted concentrations in adipose tissues were lower compared to the lipid adjusted liver concentrations for all compounds, but were higher (4-PeCDF, PCB-118, PCB-156) or lower (TCDD, PCB-126) compared to lipid adjusted plasma concentrations. Based on wet weight (ng/g tissue) TCDD, 4-PeCDF and PCB-126, but not PCB-118 and PCB-156 were present at higher concentrations in liver than in adipose tissue.
Dose response relationships were determined for cytochrome P450 1A1 activity in liver samples and gene expression of CYP1A1, 1A2 and 1B1 in liver and peripheral blood lymphocytes. REPs for all endpoints were calculated relatively to the $EC_{20}$ of TCDD using the oral dose, liver or plasma concentration. In the TEF methodology, the potency of a compound is usually calculated relative to TCDD using $EC_{50}$ values. In this study, the $EC_{20}$ of TCDD was used as a benchmark effect since not all dose-response curves reached a maximum or similar $Y_{max}$ or parallel slopes for different congeners. Using the administered dose, REPs for the various endpoints ranged between 0.01 – 0.2 for 4-PeCDF (WHO-TEF 0.3), 0.005 – 0.04 for PCB-126 (WHO-TEF 0.1), 0.000005 – 0.00003 for PCB-118 (WHO-TEF 0.00003) and 0.00001 – 0.00003 for PCB-156 (WHO-TEF 0.00003), (see figure 1). If REPs were calculated using plasma levels a 4- to 9-fold increase in REPs compared to those based on oral dose were observed for 4-PeCDF. PCB-126 followed a similar increasing trend, but with 2-fold induction, less pronounced than 4-PeCDF. In contrast, plasma REPs for PCB-118 and PCB-156 where slightly lower than REPs based on administered dose. Compared to the REPs based on administered dose, recalculating the REPs using liver levels resulted in a decrease of the REPs for 4-PeCDF and PCB-126. On the

other hand, PCB-118 and PCB-156 showed a 10- to 14-fold and 10- to 20-fold increase in REPs, respectively, compared to the REPs based on the administered dose.

The observed differences in systemic REPs can partly be attributed to compound-specific tissue distribution. Liver:adipose concentration ratios greater than 0.3 suggest that distribution is not only based on lipid solubility, but is also sequestered in the liver, most likely due to CYP1A2 binding[6]. Differential disposition and hepatic sequestration of 4-PeCDF, PCB-126, PCB-118 and PCB-156 has previously been described by DeVito *et al.* in subchronically exposed female B6C3F1 mice[3]. 4-PeCDF is highly sequestrated in the liver due to high affinity binding to the CYP1A2 protein. It has been suggested that a higher CYP1A2 induction and subsequent sequestration may reduce the free cytosolic congener availability of AHR binding or serve as a continuous sink for AHR activation[7]. Our study shows that 4-PeCDF REPs based on plasma levels did not only increase the REPs determined in the liver but also in the peripheral blood lymphocytes. Considering that plasma levels might better reflect the free cytosolic concentration available (in the liver) for AHR binding. Using liver tissue levels to calculate REPs might underestimate the risk for congeners with a liver:adipose concentration ratio greater than 0.3.
Differential uptake *vs* systemic REP trends were seen for 4-PeCDF and PCB-126 compared to PCB-118 and PCB-156. Compared to the REPs based on administered dose, 4-PeCDF and PCB126 showed higher systemic plasma REPs, whereas PCB-118 and PCB-156 showed higher systemic liver REPs. As described above, this is most likely due to compound specific tissue distribution. The REPs calculated for PCB-126 based on either administered dose or systemic plasma / liver level were below the WHO-TEF of 0.1 for all endpoints. REPs could not be calculated for mRNA gene expression of CYP1A1 in the liver and CYP1B1 in the PBL since the induction of these enzymes by PCB-126 were below the $EC_{20TCDD}$ benchmark level. Calculated REPs ranged between 0.003 and 0.06 for all endpoints based on either administered or systemic levels. Similar REPs for PCB-126 have been found in other *in vivo* mice studies[8], as well as in some human *in vitro* systems using peripheral blood lymphocytes or keratinocytes[9,10]. Although, relative cancer potencies established from the NTP supported the TEF of 0.1 based on administered dose[11], recalculating the same study based on body burden lowered the relative potencies with 2- to 3-fold compared to the WHO-TEF[12]. This suggests that the current WHO-TEF can give an overestimation of the risk. For PCB-118, no CYP1A1 or CYP1B1 mRNA induction could be detected in PBLs. PCB-156 showed a minor CYP1A1 induction, but no CYP1B1 induction in PBLs. In the liver, PCB-156 induced CYP1A2 mRNA levels to a greater extent than TCDD. PCB-118 induced CYP1A2 as well as CYP1A1 mRNA levels to a lesser extent than PCB-156, but above the $EC_{20TCDD}$ benchmark. *Mono-ortho* PCBs are also known to cause phenobarbital-like effects in the liver, which are thought to be mediated by the pregnane X receptor (PXR). The greater induction of CYP1A2 mRNA levels may be due to a combination of AhR as well as PXR activation[9]. The lack of dioxin-like effects by these *mono-ortho* PCBs concurs with microarray analysis of these tissues, in which mainly induction of metabolizing enzymes was observed and very few common genes with TCDD were detected (data not shown).

Overall, the results of our study indicate that most REPs were below the WHO-TEFs, but deviation in REPs between administered and systemic levels was seen. However, if a 10-fold uncertainty is taken into account for the WHO-TEFs, the SYSTEQ-derived REPs fell largely within this range. Yet, the results presented here are only based on studies in the mouse. *In vivo* rat data and human *in vitro* SYSTEQ studies will also be used to further confirm and/or explore these findings.

**Table 1:** Lipid adjusted liver/plasma, liver/adipose and adipose/plasma ratio and wet weight liver/adipose ratio determined in female mice three days after a single oral dose of various dioxin-like compounds.

| Compound | Concentration µg/kg bw | Ratio liver/plasma Lipid adjusted | Ratio liver/adipose Lipid adjusted | Ratio adipose/plasma Lipid adjusted | Ratio liver/adipose Wet weight |
|---|---|---|---|---|---|
| TCDD | 10 | 32,59 | 48,66 | 0,67 | 4,51 |
| 4-PeCDF | 100 | 484,77 | 234,81 | 2,06 | 13,21 |
| PCB-126 | 100 | 158,20 | 183,18 | 0,86 | 9,98 |
| PCB-118 | 50000 | 2,22 | 1,66 | 1,34 | 0,07 |
| PCB-156 | 50000 | 2,07 | 1,84 | 1,13 | 0,13 |

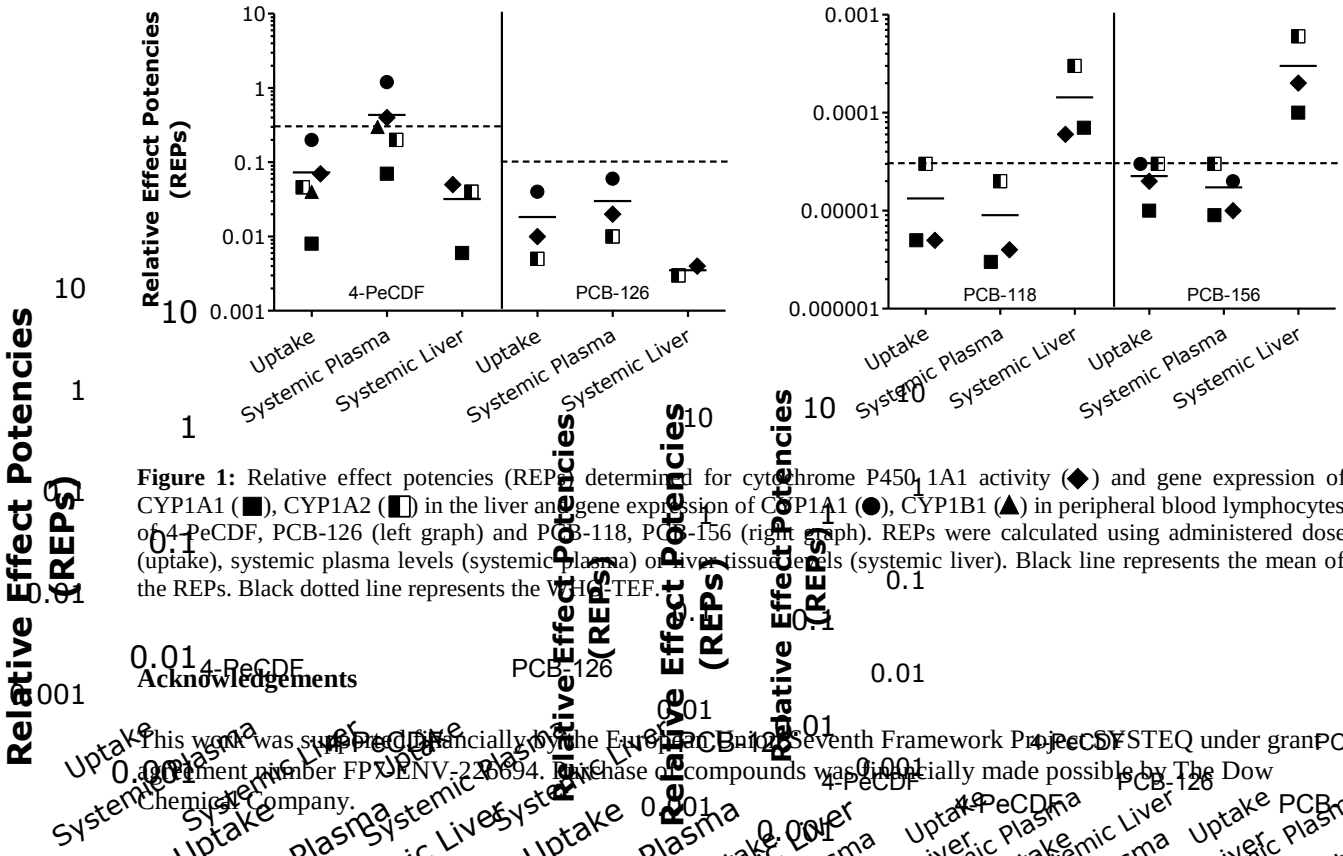


**Figure 1:** Relative effect potencies (REPs) determined for cytochrome P450 1A1 activity (◆) and gene expression of CYP1A1 (■), CYP1A2 (◧) in the liver and gene expression of CYP1A1 (●), CYP1B1 (▲) in peripheral blood lymphocytes of 4-PeCDF, PCB-126 (left graph) and PCB-118, PCB-156 (right graph). REPs were calculated using administered dose (uptake), systemic plasma levels (systemic plasma) or liver tissue levels (systemic liver). Black line represents the mean of the REPs. Black dotted line represents the WHO-TEF.

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