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Impurities in technical mixtures of chlorinated paraffins show AhR agonist properties as determined by the DR-CALUX bioassay

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ABSTRACT

Chlorinated paraffins (CPs) are produced at more than one million tons per year. Technical CPs mixtures may contain impurities, which end up in consumer products. In the present study, 17 technical CPs mixtures were investigated for the potential occurrence of potential impurities. By applying the DR-CALUX bioassay, 3 out of 17 technical mixtures were shown to elicit responses at 4 h exposure time, but much lower at 48 h. Constitutional defined CPs materials did not show responses. Subsequently different groups of known AhR-agonists and compounds suspected to be present in technical CPs mixtures were investigated. Benzene, (poly)chlorobenzene, non-dioxin like polychlorinated naphthalenes (PCNs), and three-ringed polyaromatic hydrocarbons (PAHs) did not result in a significant response at 4 h or 48 h. TCDD, non-ortho PCBs, dioxin-like PCNs, four or five ringed PAHs and their chlorinated analogues resulted in a significant response. TCDD and the non-ortho PCBs showed the highest potency and stability, while dioxin-like PCNs, PAHs, and the chlorinated PAHs were clearly inactivated (metabolized) at longer incubation. Altogether, the present findings substantiate that AhR-mediated responses of CPs technical mixtures in the DR-CALUX bioassay are caused by impurities, most likely some intermediate stable AhR-agonists such as dioxin-like PCNs or (chlorinated) PAHs. The current study shows that impurities in CPs technical mixtures need to be investigated for assessing the safety of technical CPs mixtures.

1. Introduction

Chlorinated paraffins (CPs) are polychlorinated *n*-alkanes with varying chlorination degree. CPs are mainly used as lubricants, plastics additives, flame retardants, and metalworking fluids. Based on their carbon chain length, CPs are generally classified into three groups, short-chain (SCCPs) with 10–13 carbon atoms, medium-chain (MCCPs) with 14–17 carbon atoms, and long-chain (LCCPs) with more than 17 carbon atoms. CPs were also classified according to their chlorination degree in the Stockholm Convention nomination, in European Commission Regulations and US Environmental Protection Agency's actions (UNEP, 2017; ECHA, 2008; U.S.EPA, 2009).

During the last decades, CPs gained increasing attention, especially SCCPs. In 1990 the International Agency for Research on Cancer (IARC) assessed CPs with a carbon-chain length of 12 and average chlorination degree of 60% as possibly carcinogenic to humans (Group 2B) (IARC, 1990). In the late 1990s, the Joint Research Center of the European Commission concluded that SCCPs present a low risk for human health as human exposure to SCCPs was limited (European Commission, 1999). However, due to increasing exposure levels and its persistent bioaccumulative and toxic (PBT) properties, in 2008, the European Chemical Agency (ECHA) defined SCCPs as SVHC (Substance of Very High Concern) (ECHA, 2008). Since 2017, the Stockholm Convention listed SCCPs as persistent organic pollutants (POPs) in Annex A (UNEP, 2017), which required eliminating their use and production. Meanwhile, many countries all over the world, for instance, Australia (NICNAS, 2004), Canada (Canadian government, 2008), United States (U.S. EPA, 2009), Japan (Boer, 2010), and Denmark (Danish Ministry of the

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Environment, 2014), had made their risk assessments and subsequent regulations to ban or restrict the use and manufacturing of SCCPs. The European Food Safety Authority (EFSA) has recently published a draft-opinion (Schrenk et al., 2020) in which a risk assessment was performed and concluded that higher dietary exposure to the CPs due to some foods might be of concern.

Nowadays, it is considered that SCCPs have been replaced mainly by MCCPs (Schinkel et al., 2018). The worldwide production of CPs exceeds 1 million t/year, and the figure has been going up (Glüge et al., 2016). Due to increasing exposure levels of (MC)CPs to humans and the inadequate evaluation of their safety, researchers are mainly concerned about MCCPs (Zellmer et al., 2020). Human exposure to (MC)CPs is primarily caused by food and house dust (Schrenk et al., 2020). In recent years, the presence of CPs has been reported in food, food additives, the ecosystem, and biota (Du et al., 2019; He et al., 2019; Krätschmer et al., 2019; Sprengel et al., 2019; van Mourik et al., 2016; Vorkamp et al., 2019; Wang et al., 2018; Wu et al., 2020; Zhou et al., 2019; Zhuo et al., 2019) and Xia et al. revealed in their study that CP concentrations were increased in the human breast milk of Chinese population (Xia et al., 2017).

Technical CP mixtures can contain other POPs as unintended contaminants, which are also regarded as a potential problem. The main contaminants or by-products in technical CPs reported are chlorinated benzenes (ClBz), polychlorinated biphenyls (PCBs) and polychlorinated naphthalenes (PCNs) (Takasuga et al., 2013). These may also be generated when CP mixtures undergo high temperatures (Xin et al., 2019). In a poisoning incident at the end of World War II, butter was mistakenly substituted by chlorinated paraffins, and PCNs were reported to be responsible for the observed intoxications (Herzberg, 1947).

Several POPs that have been detected in technical mixtures of chlorinated paraffins show structural similarities to dioxins (i.e., 2,3,7,8-polychlorinated dibenzo-p-dioxin (TCDD)) and related compounds. Dioxins, dioxin-like PCBs, and several PAHs are known agonists for the aromatic hydrocarbons receptor (AhR) and as such evoke a toxic response. The AhR can bind with AhR-agonists and subsequently be translocated into the nucleus where the ligand-bound AhR dimerizes with the AhR nuclear translocator (ARNT) and ultimately lead to gene transcription. AhR-agonists can be detected by in-vitro receptor-reporter transcriptional activation bioassays. One of these known screening methods for AhR-agonists is the DR-CALUX bioassay, which is based on a genetically engineered rat liver cell line (Baston and Denison, 2011; Behnisch et al., 2001; Bovee et al., 1996; Hoogenboom et al., 2006; Machala et al., 2001; Murk et al., 1997; Suzuki et al., 2017). This DR-CALUX bioassay has three essential features. The response is dose or concentration-dependent (1), i.e., AhR-agonists generate dose-response curves. After mathematical fitting of the curve, the half effective concentrations (EC50) can be calculated from full dose-response curves or 20% effective concentrations (EC20) from partial dose-response curves. By comparing the calculated EC50 value to that of 2,3,7,8-TCDD, the relative potency (REP) of a compound can be estimated. The dose-response and cognate EC50 or EC20 values in the DR-CALUX bioassay are structure-dependent (2). It has been demonstrated that planar hydrophobic compounds with halogenated positions in para positions display the highest potency (Behnisch et al., 2001; Denison and Heath-Pagliuso, 1998). The most potent and toxic dioxin congener is 2,3,7,8-TCDD, for which the REP is set on 1 and to which all other AhR-agonist are compared. The third feature of the DR-CALUX bioassay is, that the liver cells still have some metabolic activity and are, e.g., able to convert PAHs (3). This feature has been used to study PAHs' metabolic degradability for the risk assessment of environmental sample extracts (Larsson et al., 2012; Machala et al., 2001; Masunaga et al., 2004).

The aim of the present study was to investigate if 17 technical mixtures of chlorinated paraffins (CPs) contained AhR-agonists, either CPs themselves or as impurities resulting from the CP's manufacturing. This was investigated in the DR-CALUX bioassay. Exposure times of 4 and 48 h were used in order to determine whether responses are caused by stable, inert compounds like dioxins and dioxin-like-PCBs or due to less stable compounds like PAHs. The potency and metabolic stability of different known AhR-agonists, which are demonstrated to be present as unintended pollutants in technical CP mixtures, were investigated by using the DR-CALUX bioassay. These substances included TCDD, non-ortho PCBs, CP standards, benzene, (poly)chlorobenzene, dioxin-like, and non-dioxin-like PCNs, PAHs, and chlorinated PAHs.

2. Materials and methods

2.1. Standards and materials

2,3,7,8-Tetrachlorodibenzodioxin (2,3,7,8-TCDD, CAS 1746-01-6) and four non-ortho PCBs, being 3,3′,4,4′-tetrachlorobiphenyl (PCB 77, CAS 32598-13-3), 3,4,4′,5-tetrachlorobiphenyl (PCB 81, CAS 70362-50-4), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126, CAS 57465-28-8), and 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB 169, CAS 32774-16-6) were purchased from Cambridge Isotope Laboratories. Two constitutionally defined SCCPs, 1,2,13,14-tetrachlorotetradecanes (CAS 221155-23-3, purity 95.4%) and 1,1,3,9,11,11,11-octachloroundecanes (CAS 601523-25-5, purity 98.1%) and one constitutionally defined MCCP, 1,1,1,3,10,11-hexachloroundecanes (CAS 601523-28-8, purity 90.4%) were obtained from Chiron. Benzene (Bz, CAS 71-43-2, purity 99.9%), mono-chlorobenzene (ClBz, CAS 108-90-7, purity 99.96%) and 1,2,4,5-tetra-chlorobenzene (1,2,4,5-Cl_4Bz, CAS 95-94-3, purity 99.48%) were obtained from Dr. Ehrenstorfer.

Seven polyaromatic hydrocarbons (PAHs) and seven chlorinated polyaromatic hydrocarbons (Cl-PAHs) were used: anthracene (Ant, CAS 120-12-7, purity 99%), benzo[*j*,*k*]fluorene (fluoranthene) (Flu, CAS 206-44-0, purity 98%), pyrene (Pyr, CAS 129-00-0, purity 98%), fluorene (Fle, CAS 86-73-7, purity 98%), and phenanthrene (Phe, CAS 85-01-8, purity 98%) were purchased from Sigma-Aldrich. Benz[*a*]anthracene (BaA, CAS 56-55-3, purity 98.22%) and benzo[*a*]pyrene (BaP, CAS 50-32-8, purity 99.0%) were purchased from Dr. Ehrenstorfer. 6-chlorobenzo[*a*]pyrene (6-ClBaP, CAS 21248-01-1, purity 99%), 2-chloroanthracene (2-ClAnt, CAS 17135-78-3, purity 99.9%), 9-chlorofluorene (9-ClFle, CAS 6630-65-5, purity 99.0%), 3-chlorofluoranthene (3-ClFlu, CAS 25911-51-7, purity 89.5%), 1-chloropyrene (1-ClPyr, CAS 34244-14-9, purity 98%) and 9-chlorophenanthrene (9-ClPhe, CAS 947-72-8, purity 97.5%) were purchased from Chiron. 7-chlorobenz[*a*]anthracene (7-ClBaA, CAS 20268-52-4, purity 98%) from Cambridge Isotope Laboratories.

Nine polychloronaphthalenes (PCNs) were used: naphthalene (CAS 91-20-3, purity 99%) was obtained from Sigma-Aldrich, 2-chloronaphthalene (PCN 2, CAS 91-58-7, purity 99.9%), 1,2,3-trichloronaphthalene (PCN 13, CAS 50402-52-3, purity 98%), 1,2,3,5,8-pentachloronaphthalene (PCN 53, CAS 150224-24-1, purity 98%), 1,2,3,5,7,8-hexachloronaphthalene (PCN 69, CAS 103426-94-4, purity 98%) and 1,2,3,4,5,6,7-heptachloronaphthalene (PCN 73, CAS 58863-14-2, purity 98%) were obtained from Wellington Laboratories. 1,5-dichloronaphthalene (PCN 6, CAS 1825-30-5, purity 96.6%), 2,3,6,7-tetrachloronaphthalene (PCN 48, CAS 34588-40-4, purity 98%) and octachloronaphthalene (PCN 75, CAS 2234-13-1, purity 98.0%) were purchased from Dr. Ehrenstorfer, Cambridge Isotope and Laboratories Supelco, respectively.

The tested 17 technical chlorinated paraffin mixtures were obtained directly from CP manufacturers. The mixtures included two CP-42 mixtures (42% chlorination degree), eleven CP-52 mixtures (52% chlorination degree), and four CP-70 mixtures (70% chlorination degree). All the standards and technical mixtures were prepared in DMSO and stored in a dark place at room temperature.

2.2. DR-CALUX assay

The AhR-mediated luciferase induction was measured in a recombinant rat hepatoma cell line (H4IIE-luc). Cell culture was performed

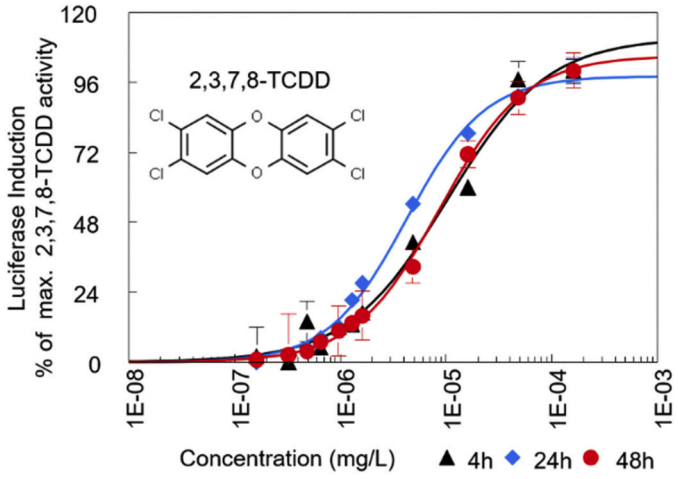

Fig. 1. Dose-response curves of 2,3,7,8-TCDD at 4, 24, and 48 h exposures as obtained in the DR-CALUX bioassay. The response is the mean of a triplicate ± standard deviations (SD).

under standard conditions at 37 °C in a 5% CO_2 atmosphere as described before (Bovee et al., 1998; Hoogenboom et al., 2006). In brief, Trypsin/EDTA(ethylenediaminetetraacetic acid) in PBS(phosphate-buffered saline) (EDTA)-(0.25%/0.02%, *v*/v) (Pan Biotech) was used for transferring cells. Cells were cultured in alpha-minimal essential medium (Gibco) with 10% fetal bovine serum (Gibco) and 0.5% penicillin/streptomycin solution (*v*/v, Sigma-Aldrich). For exposure, cells were seeded into 96-well plates (Corning) and incubated for 24 h to reach 80 to 90% confluency. Compounds dissolved in DMSO were added to the plates in triplicate (final DMSO concentration of 0.5%, v/v).

After 4 h and 48 h of exposure, the medium with test compounds was removed, and the cells were rinsed by PBS (Oxoid) and lysed by lysis reagent (Promega). After 30 min, the luciferase activities of the cell lysates were measured in a CLARIOstar microplate reader (BMG Labtech) that automatically added a substrate mixture mainly composed of luciferin and ATP followed by light measurement. DMSO (0.5%, v/v) was used as a blank sample control on each plate, and all responses for each plate were corrected with this control (blank subtraction).

2.3. Curve fitting and calculation

The dose-response curves were fitted by SlideWrite software, using the following equation:

$$y = \frac{a0}{1 + \left(\frac{a1}{x}\right)^{a2}}$$

Where y is the relative amount of light produced relative to the maximal response a0 by 2,3,7,8-TCDD (0.03 mg/L), and x is the compound's mass concentration. When the fitting coefficient of the curve is >0.9, the a1 represents the calculated EC50 (the half effective concentration), and a2 represents the Hill slope (Neubig et al., 2003).

Relative potency (REP) of each compound relative to 2,3,7,8-TCDD was calculated from the determined EC50, using the equation:

$$\text{REP for compound} = \frac{\text{EC50 for } 2,3,7,8-\text{TCDD}}{\text{EC50 for compound}}$$

In the present study, REP-values are expressed in mass concentrations. In some studies, REP values are expressed in molar concentrations. The difference between the mass concentration and mole concentration will differ by factors of 0.80 for Cl_8PN (the highest mass weight in the test) and 4.13 for benzene (the lowest mass weight tested). Therefore, the difference between mass and mole concentration-based REPs will be within one order of magnitude for the compounds in the present study.

3. Results

3.1. 2,3,7,8-TCDD

2,3,7,8-TCDD was tested at exposure times of 4 h, 24 h, and 48 h. These measurements were used as a reference for REP calculations. Fig. 1 shows that TCDD is a potent AhR-agonist, i.e., able to elicit a response in the low ng/L (1×10^{-6} mg/L) range, and the response is hardly affected by the exposure time, demonstrating its (metabolic) stability.

3.2. Technical mixtures of chlorinated paraffins

Seventeen technical mixtures of CPs from 9 different Chinese factories were tested in the DR-CALUX bioassay using exposure times of 4 and 48 h. At 4 h, three of them induced a response over 50% of the maximum response obtained by the reference compound TCDD. They are 8#CP, 12#CP, and 15#CP from different manufactures and with a chlorination degree of 52%, 42%, and 70%, respectively. The other 14 mixtures did not result in an apparent response (i.e., all well below 20% of the maximal response as obtained with TCDD). Fig. 2 shows the dose-response for the three positive technical mixtures as determined in the DR-CALUX bioassay. It is also demonstrated that at 48 h, the maximum response of these three mixtures dropped to lower dose-response levels and also that the dose-response curves shifted to the right, i.e., higher

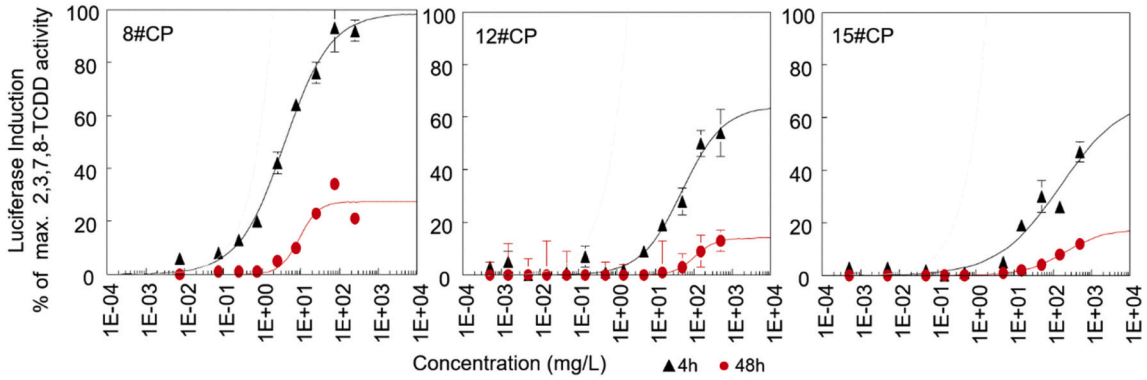

Fig. 2. Dose-response curves of three technical mixtures of chlorinated paraffins as obtained in the DR-CALUX bioassay at exposures of 4 and 48 h. The response is the mean of a triplicate ± standard deviations (SD).

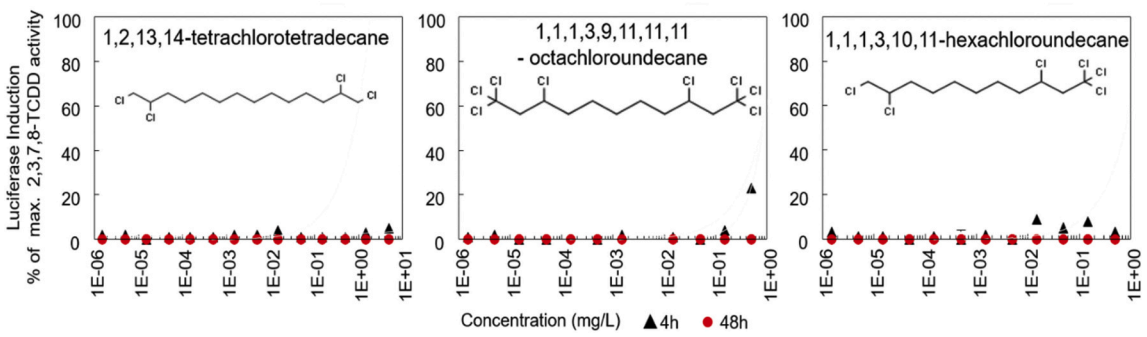

Fig. 3. Dose-response curves of two SCCP-materials and one MCCP-material with different chlorination degrees at 4 and 48 h exposures as obtained in the DR-CALUX bioassay. The response is the mean of a triplicate ± standard deviations (SD).

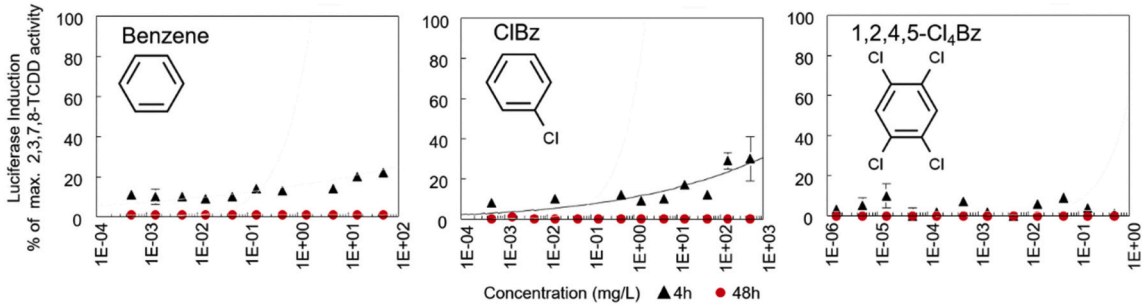

Fig. 4. Dose-response curves of benzene and two chlorinated benzenes as obtained in the DR-CALUX bioassay at exposures of 4 and 48 h. The response is the mean of a triplicate ± standard deviations (SD).

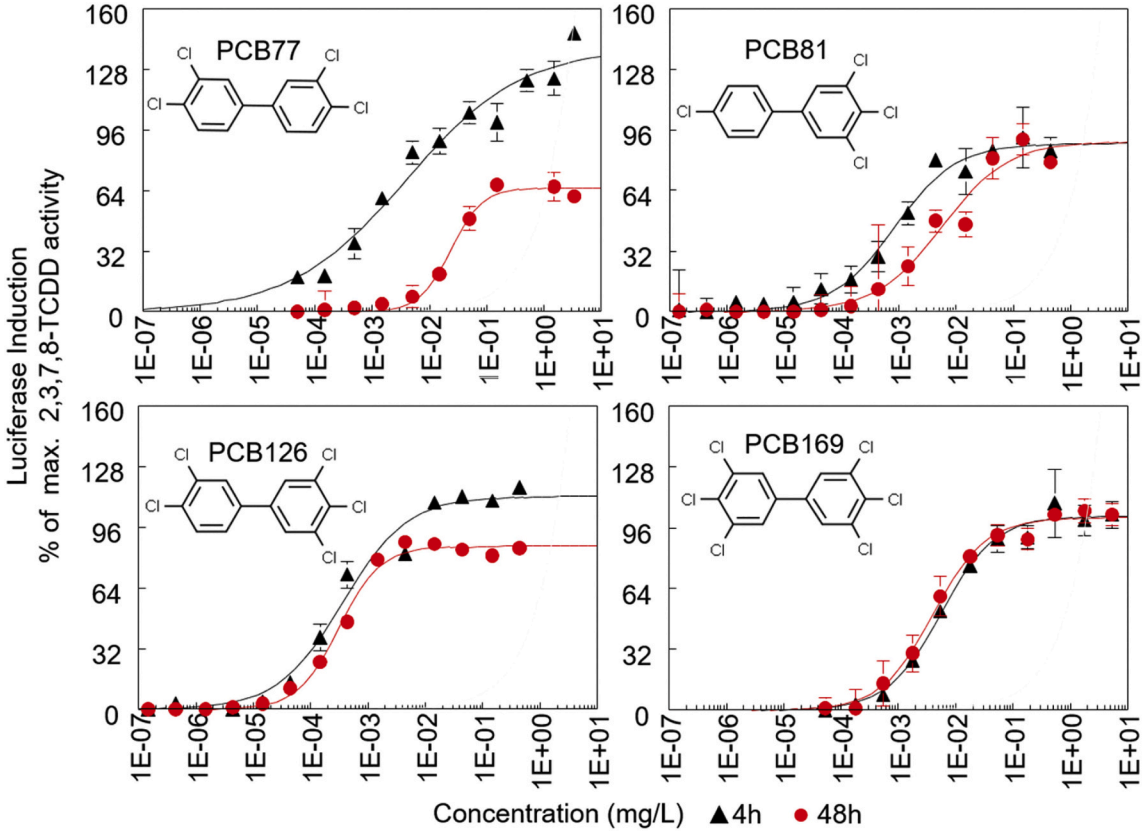

Fig. 5. Dose-response curves of four non-ortho PCBs as obtained in the DR-CALUX bioassay at exposures of 4 and 48 h. The response is the mean of a triplicate ± standard deviations (SD).

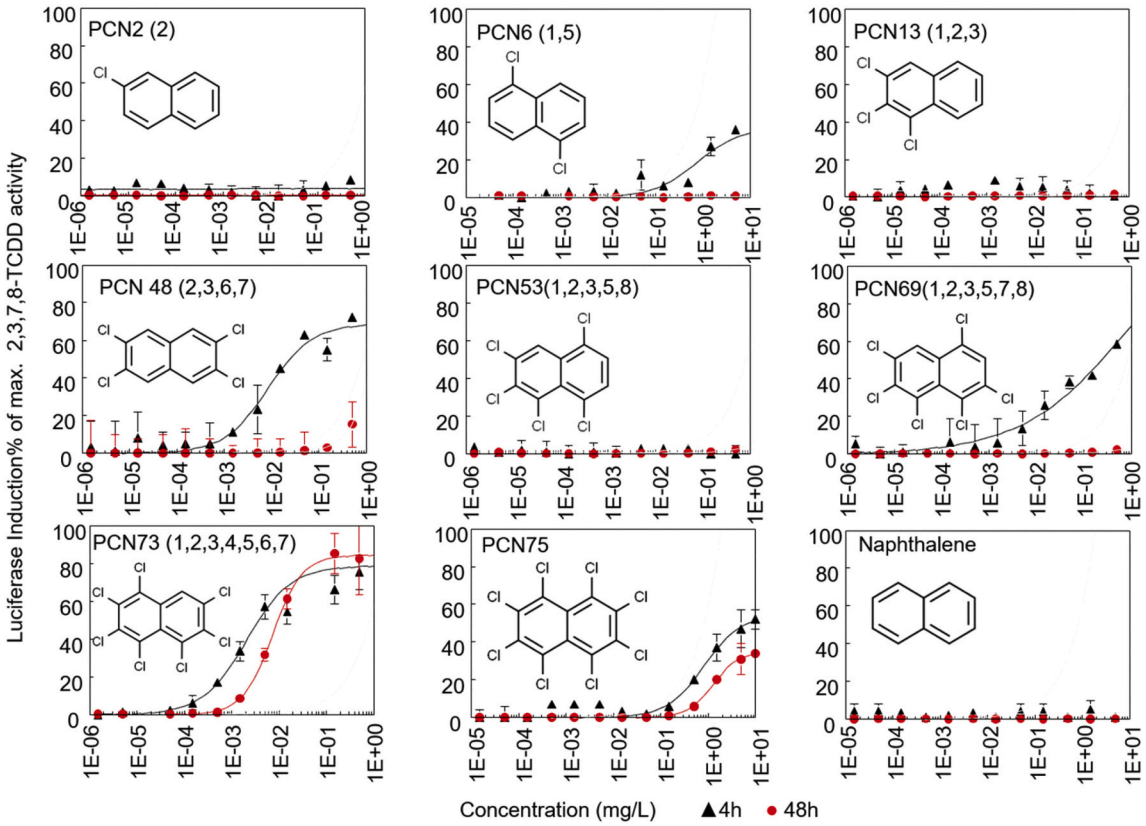

Fig. 6. Dose-response curves of naphthalene and chlorinated naphthalenes at 4 h and 48 h of exposures as obtained in the DR-CALUX bioassay. The response is the mean of a triplicate ± standard deviations (SD).

EC50 and EC20 values, which means that the responsible AhR-agonist(s) present as (an) unintended pollutant(s), are less stable than TCDD.

Because 14 out of 17 mixtures did not result in substantial responses, it can be assumed that CPs are not likely AhR-agonists themselves. It is expected that only aromatic hydrocarbons fit into the binding pocket of the AhR. Therefore, CPs will probably not fit in the AhR binding pocket. To test this hypothesis, we evaluated three constitutional defined CPs materials with the DR-CALUX bioassay, i.e., two constitutionally defined SCCPs and one MCCP.

3.3. SCCP and MCCP standards

Two constitutionally defined SCCP materials with C11 chain length with 6 and 8 chlorines, 1,1,1,3,10,11-hexachloroundecanes and 1,1,1,3,9,11,11,11-octachloroundecanes respectively and one constitutionally defined C_{14} MCCP with four chlorine atoms, 1,2,13,14-tetrachlorotetradecanes, were tested. Fig. 3 shows that these CP standards did not elicit a clear (dose-)response in the DR-CALUX bioassay at either 4 or 48 h. Only the highest concentration of 1,1,1,3,9,11,11,11-octachloroundecane and only at 4 h, resulted in a response above 20% of the maximal response as obtained with TCDD.

3.4. Benzene and chlorinated benzenes

Benzene, mono-chlorobenzene (ClBz), and the more dioxin-like 1,2,4,5-tetrachlorobenzene (1,2,4,5-Cl_4Bz) were tested in the DR-CALUX bioassay. Fig. 4 shows that these benzene standards did not elicit a clear (dose-)response in the DR-CALUX bioassay at either 4 or 48 h. Surprisingly, the highest response, above 20% of the maximal response relative to 2,3,7,8-TCDD, was obtained with the highest concentration of ClBz at 4 h, but no responses were observed after 48 h of exposure.

3.5. Non-ortho PCBs

Four non-ortho PCBs, i.e. PCB 169, PCB 126, PCB 81, and PCB 77, were tested. Fig. 5 shows that all four tested non-ortho PCBs are AhR-agonists, that PCB 126 is the most potent non-ortho PCB, and that PCB 126 and PCB 169 are more stable than PCB 81 and PCB 77.

3.6. Naphthalene and polychlorinated naphthalene (PCNs)

Naphthalene and PCNs with one to eight chlorines were tested. Fig. 6 shows that at 4 h of exposure, 1,5-dichloronaphthalene (PCN 6), 2,3,6,7-tetrachloronaphthalene (PCN 48), 1,2,3,5,7,8-hexachloronaphthalene (PCN 69), 1,2,3,4,5,6,7-heptachloronaphthalene (PCN 73) and octachloronaphthalene (PCN 75) resulted in clear dose-response curves and that PCN 73 and PCN 75 even resulted in dose-response curves after 48 h of exposure, the two most stable PCNs. PCN 73 was the most potent PCN tested, and the obtained maximal responses at 4 and 48 h were close to those obtained with the reference compound TCDD. PCN 48 induced relatively high effects at lower exposure times, indicating that this compound is metabolized. Naphthalene, 2-monochloronaphthalene (PCN 2), 1,2,3-trichloronaphthalene (PCN 13) and 1,2,3,5,8-pentachloronaphthalene (PCN 53) did not result in a clear response, neither at 4 h nor at 48 h of exposure.

3.7. PAHs and Cl-PAHs

Seven PAHs with 3 to 5 rings and their corresponding monochlorinated PAHs (Cl-PAHs) were tested. Results are shown in Fig. 7. All, except Pyr and Ant, were able to induce activity in the bioassay. PAH-structures with chlorine, the Cl-PAHs, generally induced a higher response than the analogs without Cl atoms. Responses of active PAHs and Cl-PAHs relative to 2,3,7,8 TCDD strongly declined at longer

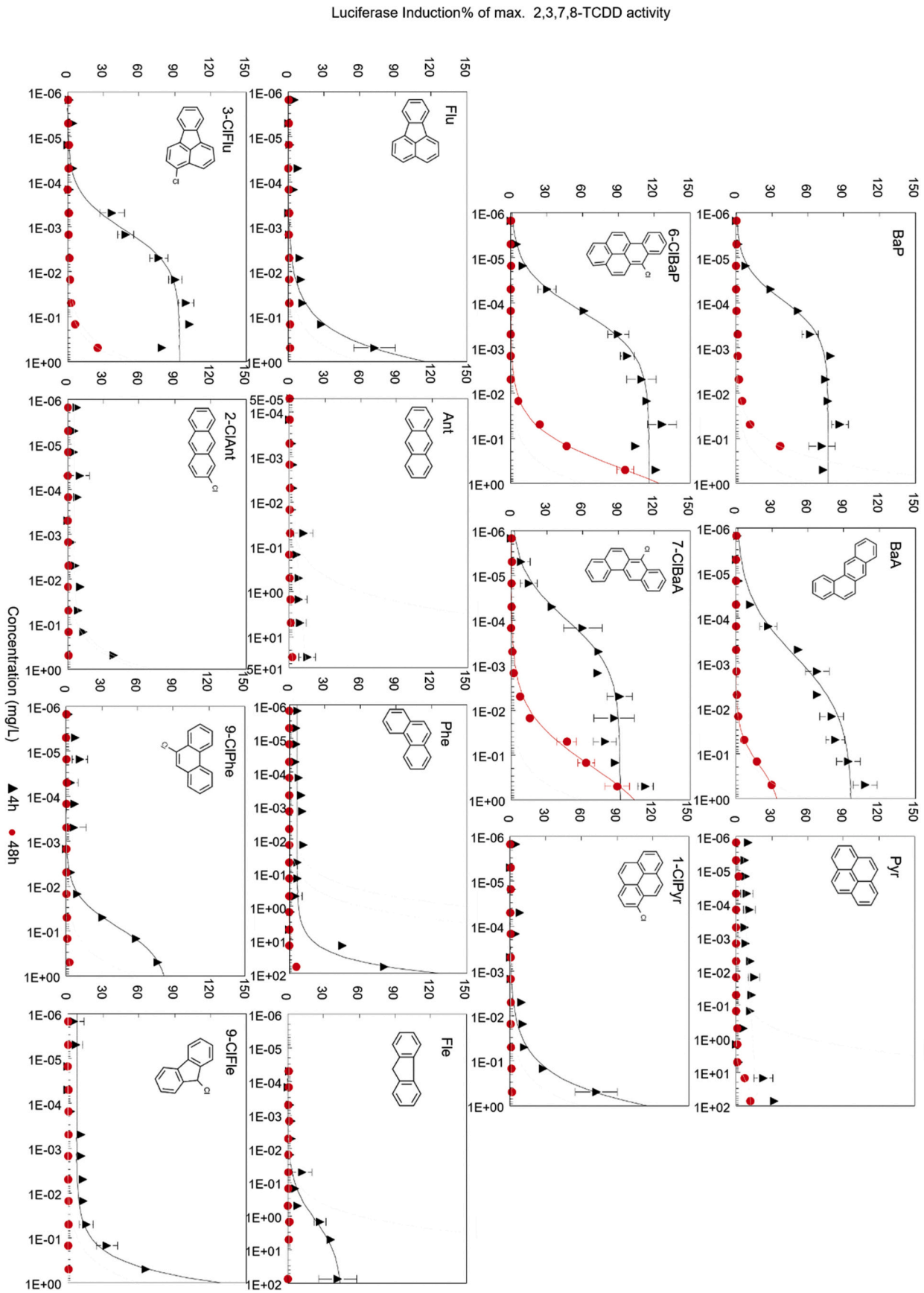

Fig. 7. Dose-response curves of 7 PAHs and their 7 corresponding Cl-PAHs at 4 h and 48 h of exposure as obtained in the DR-CALUX bioassay. The response is the mean of a triplicate ± standard deviations (SD).

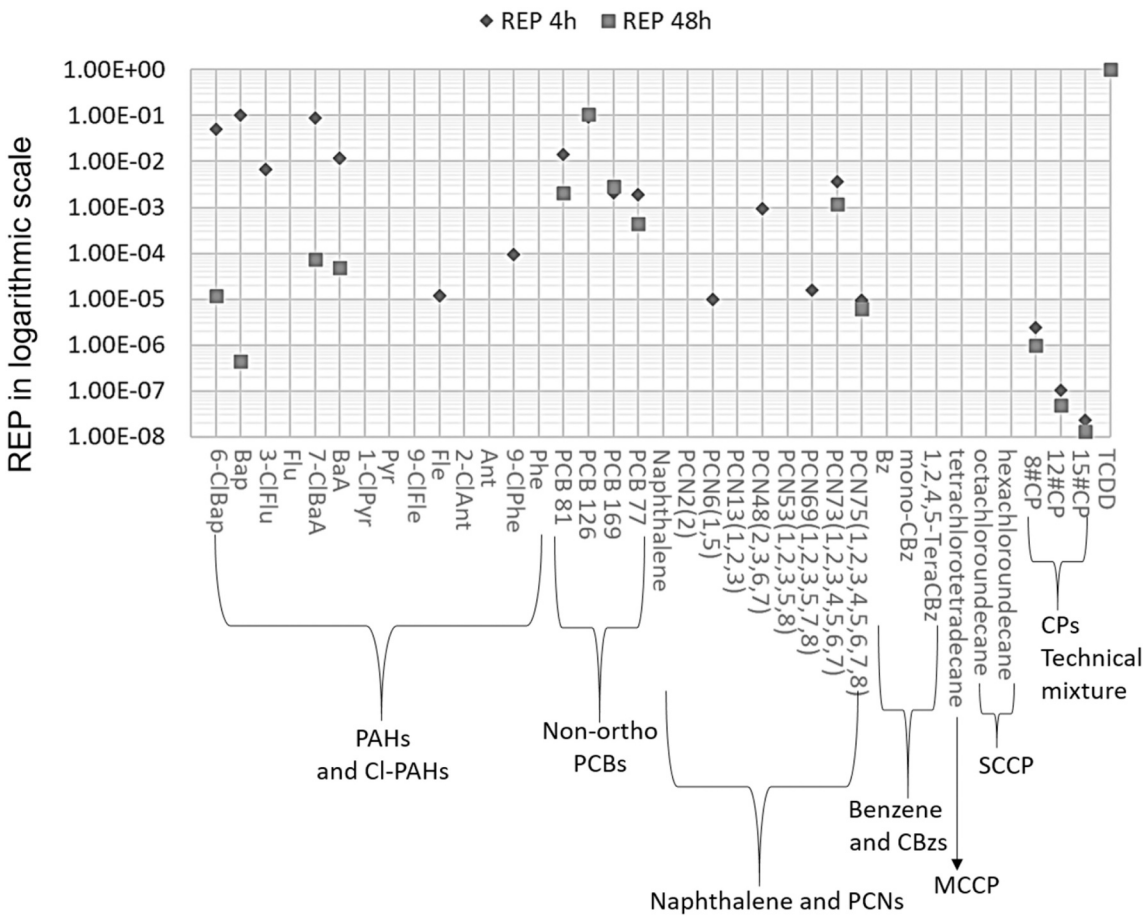

Fig. 8. Comparison of relative potencies (REPs) based on EC50 values of different groups of compounds after 4 h and 48 h exposure. MCCPs, SCCPs and ClBz and Bz, and Flu and (cl)Ant are not on the graph because they did not show any potency at relevant concentration.

exposure of 48 h. For most active PAHs and Cl-PAHs, the observed response at 4 h even fully disappeared after 48 h of exposure. Only high doses of BaP, 6-ClBap, BaA, and 7-ClBaA resulted in relative responses above 30% of the maximal response of 2,3,7,8-TCDD after 48 h of exposure.

4. Discussion

Three out of the 17 tested technical mixtures of chlorinated paraffins induced a response after 4 h exposure over 50% of the maximum response as obtained by the reference compound TCDD and to a far lesser degree after 8 h of exposure (Fig. 2). Tests with constitutionally defined CP materials (Fig. 3) show no AhR response. Therefore, it is assumed that CPs are not likely AhR-agonists themselves and that impurities in three of the tested mixtures are responsible for the obtained responses at 4 h. We compared the behaviors of different AhR-agonists in the DR-CALUX bioassay and compared those with the responses as obtained with these 3 CP technical mixtures.

Results of benzene and (poly)chlorobenzenes (Fig. 4) showed that these compounds did not elicit a clear dose-response in the DR-CALUX bioassay at either 4 or 48 h, in the concentration range as tested. These outcomes are as expected, as these compounds are not classic AhR-agonists when considering their structures. These substances show low structural similarity to the most potent AhR-agonist TCDD and the dioxin-like non-ortho-PCBs (Figs. 1 and 5).

Results obtained with naphthalene and PCNs (Fig. 6) and with PAHs and Cl-PAHs (Fig. 7) show a similar activity profile as obtained with the three active technical mixtures of CPs (Fig. 2). When present as impurities in these CP mixtures, these compounds could contribute to the observed effect of the technical mixtures of CPs. The relative potency order of PCNs at 4 h of exposure was PCN 73(1,2,3,4,5,6,7-Cl) > PCN 48 (2,3,6,7-Cl) > PCN 69(1,2,3,5,7,8-Cl) ≈ PCN 75(1,2,3,4,5,6,7,8-Cl) (Figs. 6 and 8). PCNs with three or more chlorines at the 2,3,6,7 positions of the naphthalene skeleton presents structural similarities with 2,3,7,8 TCDD. These PCNs also elicit a higher response in the DR-CALUX bioassay. These outcomes are as expected and in line with Suzuki et al.'s in-vitro results and Puzyn et al.'s in-silico results (Puzyn et al., 2007; Suzuki et al., 2020). Even without chlorine in these four positions, such as PCN 6(1,5-Cl), the symmetric chlorinated structure also elicited an AhR-mediated activity in the DR-CALUX bioassay at 4 h exposure. PCNs were less stable than TCDD, resulting in decreasing responses and lower REPs after 48 h of exposure, probably due to metabolism in the liver cells.

Fig. 8 shows a summary of the determined REPs at 4 and 48 h of exposure of all compounds as tested in the present study. There are no marks in the chart for substances that lack REP values due to inactivity or a partial dose-response not allowing the calculation of an EC50 or EC_{20}. A higher position of the marker in the chart reflects a higher potency of the compound and a closer distance between the two markers of 4 h and 48 h reflects a more stable compound. The stable compounds TCDD, PCB 126 and PCB 169, and the relative stable PCB 77, PCB 81, PCN 73(1,2,3,4,5,6,7) and PCN 75(1,2,3,4,5,6,7,8) showed similar REP values for 4 and 48 h exposure. After 48 h of exposure, REP values of PCB 77, PCB 81, and PCB 126 were 0.0004, 0.0002, and 0.1 respectively, which are in line with the TEF values suggested by WHO (Van den Berg, 2005), while the REP of PCB 169 was 0.003, which was one order lower than WHO's TEF. Unstable compounds show higher EC50/20 values and lower REPs after a longer incubation time. The REP values at 4 h and 48

h exposure of PAHs and Cl-PAHs dropped by 2 to 6 orders, which is comparable to findings of others (Larsson et al., 2012; Masunaga et al., 2004). BaP, Fle, and 9-ClPhe presented the lowest stability. Overall, compared to TCDD, BaP, 7-ClBaA, and PCB 126 showed the highest REPs at 4 h exposure and PCB 126, PCB 169, and PCB 81 at 48 h exposure.

Although PAHs are relatively well studied, there has been limited information on the potencies of Cl-PAHs relative to that of 2,3,7,8-TCDD in DR-CALUX assays. Some Cl-PAH REP values were reported in the range of 10^{-5} or below (Horii et al., 2009), similar to the REP values we obtained at 48 h exposure (Fig. 8). The presence of chlorine in PAH's structure gave rise to a generally increased efficacy of the AhR induced transcription activation. This effect is observable especially for low mass weight PAHs such as Ant, Phe, and Fle, which elicit no AhR activities at 4 h of exposure, while their chlorinated analogs were able to activate the AhR (Fig. 7). These findings are in agreement with Horii et al.. Also, for BaA, Flu and Phe, the chlorinated analogs were more potent (Fig. 8), while for BaP and Fle, the chlorinated analogs displayed similar or even slightly lower REPs (Fig. 8). Similar to the PCNs, PAHs and Cl-PAHs are less stable than TCDD, resulting in decreasing responses and lower REPs after 48 h of exposure (Fig. 8), which is due to metabolism and the formation of less potent or even inactive metabolites (Larsson et al., 2012, 2014). In general, the stability of non-ortho PCBs and some dioxin-like PCNs was higher than those of PAHs and their corresponding Cl-PAHs.

When focusing on the CP technical mixtures, the three active CPs technical mixtures' exposure time profiles were more similar to some dioxin-like PCNs and PAHs/Cl-PAHs. However, it cannot be ruled out entirely that less stable non-ortho PCBs, like PCB 77, also contribute to the response as obtained by these three technical mixtures of CPs.

5. Conclusion

Three out of 17 technical CP mixtures show a response in the DR-CALUX assay. By utilizing the exposure time-dependence feature of the DR-CALUX bioassay for unstable compounds, it was predicted that the AhR-agonist impurities present in the three positive CP mixtures match best with those caused by dioxin-like PCNs or PAHs/Cl-PAHs. Nonetheless, a contribution of less stable non-ortho PCBs cannot be ruled out. This suggests that toxic impurities may occur in technical mixtures, and these should be considered when assessing the safety of CPs. In future work, we will develop multi-analyte gas chromatography and liquid chromatography high resolution mass spectrometric approaches for the identification and quantification of the impurities in the three positive technical mixtures that displayed the DR-CALUX response.

Declaration of Competing Interest

None.

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