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Research paper

In vitro biotransformation and evaluation of potential transformation products of chlorinated paraffins by high resolution accurate mass spectrometry

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ABSTRACT

Chlorinated paraffins (CPs) are high production chemicals, which leads to their ubiquitous presence in the environment. To date, few studies have measured CPs in humans and typically at relatively low concentrations, despite indications that exposure may be high compared to various persistent organic pollutants. The aim of this study is to investigate the in vitro biotransformation of CPs by human liver fractions. We determined the changes of the CP concentrations after the enzymatic transformation with human liver microsomes using a two-tiered in vitro approach. CP concentrations decreased with human liver microsomes, with the decreases of 33–94% after incubating with different groups of enzymes for 2 h. The profiles of CP rapidly shifted after the incubation with human liver microsomes. In addition, the concentrations of CPs and the biotransformation products were tentatively measured using high-resolution mass spectrometric analysis, including very short CP (carbon chain length <10), alcohols, ketones, and carboxylic acids. C–C bond cleavage is a potential transformation pathway for CPs, and ketones are potential products of CP biotransformation, especially for long-chain CPs ($C_{>17}$). The ketone products may be investigated as CP exposure biomarker in biomonitoring studies.

1. Introduction

Short chain chlorinated paraffins (SCCPs, $C_{10–13}$) have been recently listed in the Stockholm Convention on Persistent Organic Pollutants (POPs), due to their bioaccumulation, long-range environmental transport and significant adverse effects on human health and the environment (U. United Nations Environment Programme, 2016). This leads to a cease of the manufacture of SCCPs in some countries, such as US, Japan, Canada and EU (van Mourik et al., 2016; Glüge et al., 2016). However, median (MCCPs, $C_{14–17}$) and long chain paraffins (LCCPs, $C_{18–21}$) are still produced and used worldwide (van Mourik et al., 2016; Tomy et al., 1998).

CPs are widely used as coolants and lubricants in metal working fluids, and as flame retardants and plasticizers in polymers, mainly in polyvinyl chloride (PVC) products (Lassen et al., 2014). Releases occur during production, storage, transportation, use, recycling and disposal of CPs, as well as CP containing products. Therefore, CPs have been detected in all types of environmental matrices (van Mourik et al., 2016; Wei et al., 2016). However, the studies on occurrences and concentrations of CPs in humans, as well as their fate in humans are very limited. To our knowledge, there are only few publications on the occurrence of CPs in human blood (Xu et al., 2019; Li et al., 2017; Chen et al., 2020; van Mourik et al., 2020) and in breast milk (Thomas et al., 2006; Xia et al., 2017; Zhou et al., 2020; Yang et al., 2018). A recent study has reported relatively low concentrations (i.e. several ng/mL) of CPs in human serum that were lower than what would be expected considering available monitoring data for CPs in the ambient including indoor environment (van Mourik et al., 2020; He et al., 2019). One explanation may be biotransformation in the liver. The liver plays an important role in metabolic processes in the human body, including for various persistent organic pollutants with relatively long half-lives in humans (Koonrungsesomboon et al., 2018). Enzymatic metabolism has been observed for many chemicals, and their related metabolites have been detected in urine, including chain shorten products, and oxidative

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products e.g. alcohols, ketones, and carboxylic acid (Van den Eede et al., 2015; Van den Eede et al., 2016). To date, only Dong et al. (2020) have reported the degradation of CPs using HLMs, but there is a lack of knowledge on the biotransformation products of CPs in liver, i.e. urinary biomarkers that can be used to assess the exposure to CPs, and whether exposure to CPs could be measured using urinary metabolites that are indicative of exposure.

The aims of this study are 1) to investigate the half-lives and biotransformation of CPs using an in vitro system, 2) to assess the change of profile of CPs due to the biotransformation, and 3) to evaluate the possible transformation products using high-resolution mass spectrometry.

2. Materials and methods

2.1. Chemicals

Commercial SCCP mixtures (Cl degree: 55.5%), MCCP mixtures (Cl degree: 52%), and LCCP mixtures (Cl degree: 49%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). ^{13}C-BDE 209 was purchased from Wellington Laboratories Inc (Guelph, ON, Canada). Pooled human liver microsomes (HLM), pooled human liver cytosol (HLCYT), 2,6-uridinediphosphate glucuronic acid (UDPGA), alamethicin (neat, purity > 99%), 3'-phosphoadenosine-5'-phosphosulfate (PAPS; purity > 60%) lithium salt hydrate, nicotinamide adenine dinucleotide phosphate (NADPH; purity > 99%), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Ultrapure water was obtained from a Milli-Q system (Merck Millipore, MA, USA). Gas chromatography grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany).

2.2. In Vitro assays

In this study, a two-tiered approach for investigating the biotransformation of CPs with Phase I and Phase II enzymes was adopted from Lai et al. (2015). The purpose of Tier I was to assess the persistence and potential transformation of CPs, and different transformation rate using different groups of enzymes. Tier II was designed to assess the effects from different conditions, including incubation time, enzyme concentrations, and concentrations of CPs. A schematic flow of the in vitro approaches is shown in the Fig. 1.

To investigate the biotransformation of CPs with different groups of enzymes, CP standard solutions (experiment A: SCCP (Cl: 55.5), experiment B: MCCP (Cl: 52) and experiment C: LCCP (Cl: 49)) were incubated with HLM or HLCYT. In Tier I, Phase I transformation was investigated first, by activating different families of enzyme (i.e. cytochrome P450 (CYP), uridinediphosphate glucuronic acid transferase (UGT), and sulfotransferase (SULT)) separately. For the samples focusing on CYP enzymes, the reaction mixtures, containing 100 mM phosphate buffer (pH 7.4), HLM (0.5 mg/mL) and the substrate (100 nM) in a total volume of 1.0 mL were pre-incubated on a shaking bed for 5 min at 37 °C. The reaction was initiated by addition of 10 μL of NADPH solution (1 mM) in the mixture. To keep the NADPH concentration saturated, an extra aliquot (10 μL) was added every 30 min. The reaction was quenched after 2 h by adding 250 μL of 1% of formic acid in ice-cold ACN solution and 100 μL of ^{13}C-BDE 209 (used as internal standard). Samples were then vortexed for 30 s and centrifuged at 4000 rpm for 5 min, where after the supernatant was transferred to a glass tube, and concentrated to 200 μL under a gentle stream of nitrogen gas at 60 °C. For treatments where UGT enzymes were added the reaction mixture contained 100 mM phosphate buffer (pH 7.4), HLM (0.5 mg/mL), alamethicin (0.1 μg/mL) and the substrate (100 nM) in a total volume of 1.0 mL and UDPGA (1 mM) was used instead of NADPH as initiator. For treatment where SULT enzymes were added, the reaction mixture contained 100 mM phosphate buffer (pH 7.4), HLCYT (0.5 mg/mL) and the substrate (100 nM) in a total volume of 1.0 mL, while PAPS (1 mM) was used as an initiator. The other procedures were as described above for studies using the CYP.

For Phase II transformation, the products from CYP were further incubated with UGTs and SULTs. Phase I transformation was performed as described for Tier I. The reaction was quenched by keeping the samples on ice for 5 min, followed by centrifugation at 4000 rpm for 5 min. Then, the supernatant, containing the fraction of parent CPs and their transformation products generated by CYP enzymes, was transferred to a new vial containing a fresh aliquot of pooled HLM or HLCYT (0.5 mg/mL). Alamethicin and the appropriate cofactors were added at the concentrations and time intervals described above for Tier I samples.

In the Tier II experiment, three different sets of samples were prepared. In set 1, the reaction mixture (1 mL) consisted of 100 mM buffer, 0.5 mg/mL HLM/HLCYT, and 100 nM substrate. All the three enzyme families (i.e. CYPs, UGTs, and HLCYTs) were active in different samples separately. The reactions were stopped after 30, 60, and 120 min. In set

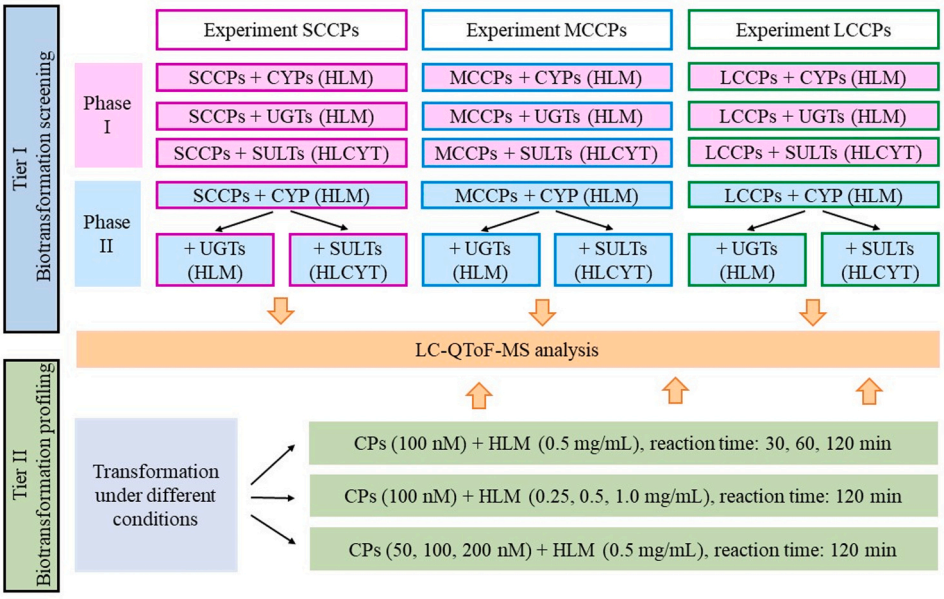

Fig. 1. Conceptual outline of the experimental design using two experimental stages (Screening stage in Tier 1 and profiling stage in Tier 2).

2, the mixture consisted of 100 mM buffer, 100 nM substrate, while the enzyme concentrations varied at 0.25, 0.50 and 1.0 mg/mL. The reaction time was 120 min for all the samples in set 2. In set 3, the mixture consisted of 100 mM buffer, 0.5 mg/mL HLM, and 50, 100 and 200 nM of substrate for different samples. The reaction time was 120 min

Negative control samples were included in this study to check the stability of CP without enzymatical transformation, by adding a particular substrate into a clean vial with buffer and HLM (no HLCYT). After 2 h incubation, 1% of formic acid in ice-cold ACN solution was also added. Blank samples were prepared as described above, but without cofactors and substrates, which were replaced by an equivalent volume of buffer.

2.3. Instrumental analysis

The analysis of CPs was conducted by a quadrupole time-of-flight high resolution mass spectrometer (QToF-MS, Triple TOF 5600$^+$ Sciex, Concord, Ontario, Canada) using Atmospheric Pressure Chemical Ionization (APCI) in negative mode. 10 μL of the sample extract was directly injected into the QToF-MS, without any chromatographic column. Dichloromethane (DCM) was used to improve the response of CPs in APCI mode. The mass spectrometer was operated in ToF-MS mode accruing HRMS full scan spectra across the range of *m/z* 250–1100. The minimal resolving power of the detector was 22,500 at *m/z* of 144.1030. The window used for extracting the *m/z* values was set as ± 0.0025. External mass calibration was performed every 10 samples with the Sciex APCI Negative Calibration solution 5600. Accurate mass spectra to 4 decimal places was used for tentative transformation product peak identification. Details on the parameters of QToF-MS have be published elsewhere (He et al., 2019; Brandsma et al., 2017).

2.4. QA/QC

Procedural blank samples were prepared by phosphate buffer (n = 3), then incubated and concentrated using the same protocol as real samples. The measured concentrations were 0.32, 0.98, 0.12 ng abs for SCCPs, MCCPs, and LCCPs, respectively. Blank correction was applied to all samples. Calibration curves, using six-point isotope dilution method were prepared in ACN, with the concentration ranging from 0.1 to 20 ng/μL for ∑S/M/LCCPs. The R^2 for the calibration curves were all greater than 0.99. In the negative control samples, the recoveries of internal standard (^{13}C-BDE-209) ranged from 87% to 109% and the accuracies, defined as the ratio between measured concentrations and spiked concentrations, ranged from 95% to 98% for different CP groups.

To check the potential interferences of the extraction windows for different analytes, the *m/z* values for all the analytes, including both quantification ions and quantitative ions, were compare. The minimal difference between any selected ions was 0.0178, for example, between $C_{12}H_{19}Cl_7O$ (460.8915) and $C_{13}H_{21}Cl_7$ (460.9093), which could be separated by mass spectrum at this resolution (Fig. S2). This suggested that there was no interference among the target analytes.

2.5. Data analysis

A quantitative method was established to estimate the concentrations of SCCPs, MCCPs, and LCCPs, using six-point calibration. Deconvolution method was used to calculate the concentrations of CP in this study Bogdal et al. (2015). The concentrations of SCCPs were also calculated using individual response factor of each congener group as an alternative quantification method (Yuan et al., 2017). The comparison of the data using these two quantification methods is shown in Table S1, where very similar results (average RSD of 7%) were found. Hence, we only used data from the deconvolution method for further discussion. Several groups of potential biotransformation products, including very short chain CPs (vSCCPs, $C_{<10}$), alcohols (OH-CPs, $C_nH_{2n-m+2}Cl_mO$), ketones (CO-CPs, $C_nH_{2n-2}{}_mCl_mO$), and carboxylic acids (COOH-CPs, $C_nH_{2n-2}{}_mCl_mO_2$) were assessed using accurate mass of the $[M+Cl]^-$ peaks. Internal standard corrected peak areas, defined as the ratio between peak area of a congener group ($Area_{CP}$) and the peak area of ^{13}C-BDE 209, were used for biotransformation product identification to calculate the MS responses before and after the biotransformation.

3. Results and discussion

3.1. Targeted analysis of CPs

Different groups of CPs were investigated from C_{10} to C_{21} and Cl_3 to Cl_{21}. More details on the targeted chemicals are shown in Table S2 in the Supplementary Material. Overall, 67% of the groups were detected in this study.

The concentrations of SCCPs, MCCPs and LCCPs measured in their respective negative controls ranged from 94 to 100 nM, which were very close to the spiked concentrations (100 nM) (Table 1). These concentrations contributed >80% of the sum of the CPs in each experiment (i.e. 88% of SCCPs in Experiment SCCPs, 94% of MCCPs in Experiment MCCPs, and 88% of LCCPs in Experiment LCCPs). Notably we found that MCCPs contributed 12% to the sum CPs in the negative control of Experiment LCCPs (13 nM), which was in accordance with the distribution and concentrations that were detected in the standard (He et al., 2019). The distributions of different congener groups in negative controls are shown in Fig. S1, and are in good agreement with the distribution in the standard solutions, suggesting that the transformation of CPs in the negative controls was limited. Therefore, they were used as references to investigate the enzymatic transformation in this study.

3.2. Biotransformation of CPs

The concentrations of ∑CPs (sum of SCCPs, MCCPs, and LCCPs) detected in Tier I experiments with different groups of enzymes are also provided in Table 1. On average CYPs were the most efficient enzyme group on the transformation of CPs. After the incubation with CYP for two hours, we found a decrease of 85%, 98% and 73% for SCCPs, MCCPs and LCCP relative to their negative controls. CYPs are a unique family of enzymes that are capable of catalysing the oxidation of hydrocarbons, and are an efficient group of enzymes for many chemicals (O'Reilly et al., 2011). In addition, after Phase I and Phase II transformation, concentrations of different groups of CPs decreased more than 70%, suggesting a rapid transformation of CPs by HLMs.

In experiment SCCP, decreased (65–99%) concentrations of SCCPs were measured in all the samples after incubating with different groups of enzymes. Although the initial concentrations of MCCPs and LCCPs were lower, decreases were also measured in most of the samples, with a few exceptions for LCCPs. In the Experiment MCCP, the transformation of LCCPs were not clear, where the concentrations of LCCPs varied from 0.51 nM to 24 nM, resulting in increased concentrations in some samples, compared with the negative control. However, the concentrations of MCCPs consistently decreased, while SCCPs increased more than 80% in all the samples. In Experiment LCCP, the concentrations of LCCPs after the transformation decreased more than 70% in all the samples. MCCP concentrations were relatively stable, ranged from 1.0 nM to 18 nM with 13 nM in the negative control. SCCP concentrations increased more than 20 times, but note that the concentrations of SCCPs in samples after the biotransformation were still low. Overall, we found decreased concentrations of different CPs in their respective experiment, where their concentrations were higher than the other groups. Concentrations of SCCPs in Experiment MCCP and LCCP increased, and concentrations of MCCPs were relatively stable, suggesting that carbon chain shortening occurred in the biotransformation process of CPs. Our results consisted with those from Dong et al. (2020), where the concentrations of CPs decreased after the enzymatic treatment.

Table 1
Concentrations of ∑CPs detected in Tier I experiments.

	Description		Concentrations (nM)[a]			
			SCCPs	MCCPs	LCCPs	∑CPs
	Negative Control		97 ± 11	5.0 ± 1.6	6.6 ± 2.0	100
Experiment SCCP	Phase I	CYPs	15 ± 6.8 (−85%)	0.34 ± 0.17 (−93%)	0.29 ± 0.13 (−96%)	16 (−85%)
	Phase I	UGTs	24 ± 7.5 (−75%)	0.70 ± 0.35 (−86%)	3.7 ± 1.6 (−44%)	28 (−74%)
100 nM SCCP (Cl: 55.5%)	Phase I	SULTs	34 ± 10 (−65%)	0.91 ± 0.45 (−82%)	3.1 ± 2.0 (−53%)	38 (−65%)
	Phase II	CYP+UGTs	25 ± 8.8 (−74%)	0.52 ± 0.15 (−90%)	9.6 ± 5.7 (45%)	35 (−68%)
	Phase II	CYP+SULTs	0.98 ± 0.34 (−99%)	0.23 ± 0.097 (−95%)	14 ± 8.1 (110%)	15 (−86%)
	Negative Control		0.28 ± 0.11	94 ± 11	5.3 ± 3.0	100
Experiment MCCP	Phase I	CYPs	0.64 ± 0.31 (130%)	1.7 ± 0.82 (−98%)	3.0 ± 2.2 (−43%)	5.3 (−95%)
	Phase I	UGTs	0.62 ± 0.29 (120%)	32 ± 22 (−66%)	24 ± 19 (350%)	60 (−40%)
100 nM MCCP (Cl: 52%)	Phase I	SULTs	0.51 ± 0.20 (82%)	35 ± 13 (−63%)	7.7 ± 3.2 (45%)	43 (−57%)
	Phase II	CYP+UGTs	1.5 ± 0.71 (430%)	3.9 ± 0.96 (−96%)	0.51 ± 3.2 (−90%)	5.9 (−94%)
	Phase II	CYP+SULTs	0.91 ± 0.48 (230%)	0.87 ± 0.38 (−99%)	13 ± 6.0 (150%)	15 (−85%)
	Negative Control		0.0084 ± 0.0028	13 ± 4.8	100 ± 19	110
Experiment LCCP	Phase I	CYPs	0.25 ± 0.14 (2900%)	1.0 ± 0.40 (−92%)	27 ± 41 (−73%)	28 (−75%)
	Phase I	UGTs	1.2 ± 0.49 (14000%)	6.2 ± 2.9 (−52%)	8.4 ± 3.2 (−92%)	16 (−85%)
100 nM LCCP (Cl: 49%)	Phase I	SULTs	2.0 ± 0.68 (30000%)	6.6 ± 3.1 (−63%)	28 ± 13 (−71%)	37 (−68%)
	Phase II	CYP+UGTs	1.6 ± 0.85 (24000%)	20 ± 9.3 (11%)	14 ± 9.8 (−86%)	36 (−69%)
	Phase II	CYP+SULTs	1.6 ± 0.74 (25000%)	18 ± 8.6 (−2%)	9.0 ± 4.5 (−91%)	28 (−76%)

[a] : the percentage change compared with the concentrations in negative control were shown in brackets, where negative values indicated a decrease after the biotransformation.

3.3. Biotransformation profiling

In Tier II, we characterized the concentrations of different CP groups at different incubation time (30, 60, and 120 min) (Fig. 2). Similar to the results found in Tier I, concentrations of CPs in their respective experiment (i.e. SCCPs in Experiment SCCP, MCCPs in Experiment MCCP, and LCCPs in Experiment LCCP) decreased with incubation time. In the Experiment SCCP, concentrations of LCCPs, and MCCPs decreased after biotransformation. Since the mixture solution in Experiment SCCP were dominated by SCCPs, $C_{<10}$ chemicals (i.e. vSCCPs), rather than MCCP or LCCPs, were expected to be a major group of transformation products as the result of carbon chain shortening process (Huang et al., 2017; Qiao et al., 2018). In Experiment LCCP, concentrations of MCCPs and SCCPs increased significantly ($P < 0.05$) after the transformation, which suggests that carbon chain shortening is one of the transformation pathways for LCCPs causing formation of SCCPs and MCCPs when LCCP mixtures are transformed by such enzyme systems. Elevated concentration of SCCPs were also found in Experiment MCCPs, due to the transformation from MCCPs to SCCPs, indicating potential C-C bond cleavage in the biotransformation. Although there was no any similar studies in the transformation of CPs with HLMs, CPs were found to be partly degraded to CO_2 in quails and mice in previous in vivo studies (Biessmann et al., 1982; Darnerud and Brandt, 1982; Darnerud et al., 1982). Listed in the Stockholm Convention as a group of Persistent Organic Pollutants in 2017, there have been many restrictions on the applications and productions for SCCPs globally, but limited restriction on MCCPs or LCCPs and unlikely to have considered biotransformation to tightly regulated SCCPs. However, in this study, we found that MCCPs and LCCPs could be in vitro transformed to SCCPs. Thus, biotransformation of the LCCPs and MCCPs to SCCPs may be worth considering in regulatory assessment of MCCPs and LCCPs.

A comparison of carbon chain length and chlorine atoms before and after biotransformation are shown in Fig. 3 and Table S3. In experiment LCCP, C_{17} (45 μM) and C_{18} (34 μM) were the predominant congener groups in the mixture solution, but after transformation, their concentrations decreased to 6.7 and 3.6 μM, respectively, while the concentration of C_{12} congener groups increased from 0.097 to 21 μM. Similarly, in Experiment MCCP, the concentrations of C_{14} and C_{15} decreased significantly after the biotransformation (i.e. from 63 μM to 9.3 μM for C_{14} and 24–4.6 μM for C_{15}), while the congener groups with shorter chain length, i.e. C_{12} and C_{13} congeners increased. In the Experiment SCCP, in contrast, the concentrations of SCCPs decreased from 100 μM to 16 μM, but concentrations of MCCPs were stable or even slightly increased for C_{17} congeners. The increase of C_{17} congeners was partly from the transformation of LCCPs, and the change of the average carbon chain length indicated that the transformation rates might vary in different groups of CPs.

Analytes contenting 5–7 chlorine atoms were the predominant both before and after the transformation. Note that the concentrations of Cl_3 congeners increased in all the experiments (i.e. from <0.01–4.0 μM, from <0.01–1.5 μM, and from <0.01–2.1 μM in Experiment S/M/LCCP). It was possible that the increase of Cl_3 was caused by carbon chain shortening, as the cleavage resulted in fragmentation with chlorine atoms. In an oral administration study, increased levels of lower chlorinated CPs were also observed in rats' urine after a single oral dose of SCCPs (Geng et al., 2016).

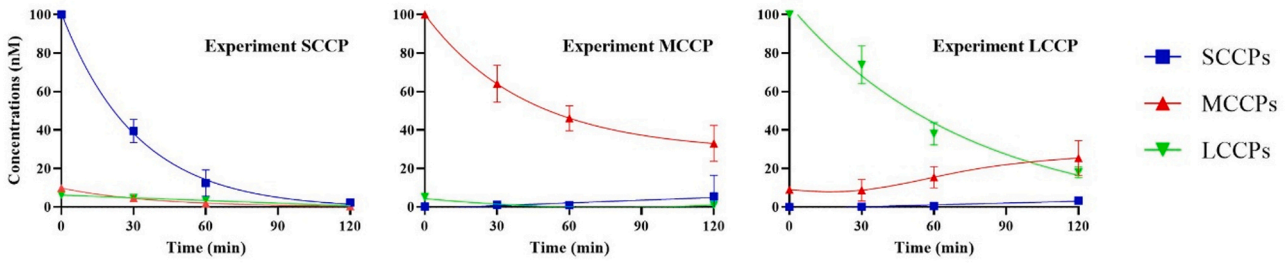

Fig. 2. Biotransformation of CPs in Tier II experiment, where 0.1 μM of SCCPs (experiment SCCPs), MCCPs (experiment MCCPs), and LCCPs (experiment LCCPs) were spiked separately.

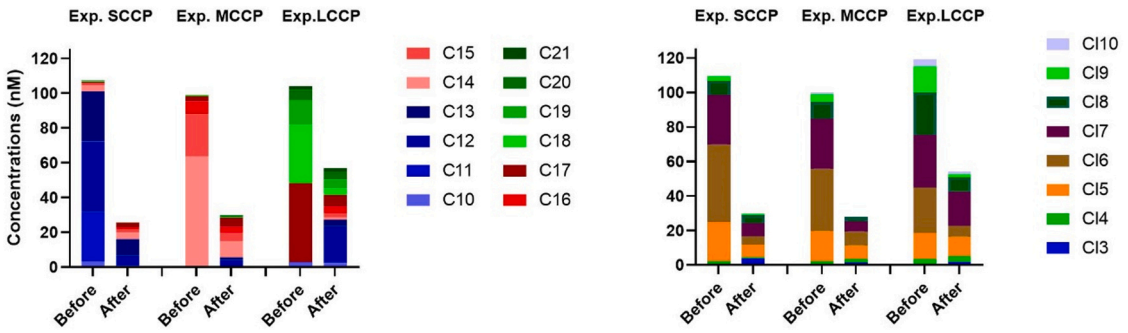

Fig. 3. Change of the profile of CPs following biotransformation. Left panel showed the concentrations of congener groups with different carbon chain length, while the right panel showed the concentrations of congener groups with different numbers of chlorine atoms. Contribution from $Cl_{>10}$ were not shown due to the very low concentrations detected both before and after incubation (<1% of the contribution).

3.4. Assessment of potential transformation products using HRMS

To assess the potential biotransformation products of CPs, we calculated the peak area of their potential products, including vSCCPs, OH-CPs, CO-CPs, and COOH-CPs, with carbon chain length between 6 and 21 and chlorine atoms between 3 and 10. These potential transformation products were based on the postulated pathway by Allpress and Gowland (1999), which involves initial attack at two adjacent non-halogenated sites by oxygenase, followed by chain shortening via β-oxidation. In total, 351 ions of a certain mass estimate from the CPs of interest replacing –Cl or –H with $^{-}$O or –OH were investigated, where 328 of them were detected in the samples after enzymatic transformation. It is interesting to note that some of the masses were also detected in the standards. To investigate if these products were formed during the biotransformation, we firstly compared the peak area of each group of product in the sample after the transformation ($Area_{C,tran}$) and its peak area in negative control ($Area_{C,nc}$). In total, 33% of vSCCPs, 39% of OH-CPs, 50% of CO-CPs, and 49% of COOH-CP congener groups showed increased peak area in samples after transformation (Table 2). The formation of the sum of specific groups of chemicals in the assay over multiple time points are shown in Table S4, where all the groups of products increased with the concentrations of substrate.

The internal standard corrected peak area of ∑vSCCPs increased from an approximate peak area of 7.9×10^2 to 7.0×10^3 by almost 800%, suggesting they might be formed during the enzymatic transformation. It is noteworthy that estimating relative increases of vSCCPs remains a challenge because of the poor ionisation of vSCCPs as well as a relatively high background for such low molecular weight compounds. Nevertheless, the change of carbon chain length profile that was found above suggested that the cleavage of C–C bonds as a relevant metabolism pathway of CPs. While no other studies exist on the biotransformation of CPs with HLMs, carbon chain decomposition of 1,1,1,3,8,10,10,10-octachlorodecane was reported in the atmosphere (Li et al., 2019).

An increase in peak area of approximate 130% were observed for CO-CPs following biotransformation, especially for CO-LCCPs. In experiment LCCP, the peak area of all the subgroups of CO-CPs increased more than 10 times, suggesting CO-CPs might be an important group of metabolites of LCCPs. Heeb et al. (2019) found that a dehydrohalogenase expressed in bacteria could transform SCCPs to chlorinated olefins.

Overall, the total peak area of OH-CPs only showed increases in 39% of the congener groups, and on average the peak areas decreased by 61% across all the different congener groups. This indicates that OH-CPs were not a major group of transformation products and might be intermediates in the metabolism of CPs. Similarly, in our experiment, COOH-CPs were not found to be a major group of transformation products of CPs either, since the total peak area decreased by 47% after biotransformation.

In the Tier II experiment, a series of HLMs with different CP concentrations were performed. At t = 60 min, the concentrations of ∑SCCPs, ∑MCCPs, and ∑LCCPs decreased by 63%, 75%, and 60%, respectively. Due to the co-existence of the CPs and their potential biotransformation products in the standards, the increased peak areas compared with the negative controls with the same concentrations of substrates were used to evaluate the net formation of different groups of CP biotransformation products (Fig. 4). Overall, the net formation of transformation products, i.e. vSCCPs, OH-CPs, CO-CPs, COOH-CPs, increased with the substrate concentrations. The transformation rates

Table 2
Evaluation of CP potential transformation products, i.e. vSCCPs and oxidative products.

	No. of chemicals investigated	$\sum Area_{tran} > \sum Area_{nc}$ (%)[a]	$\sum Area_{C,nc}$	$\sum Area_{C,tran}$	Transformation rate (%)[b]
∑vSCCPs	20	33	7.9×10^2	7.0×10^3	783
OH-vSCCPs	21	36	1.0×10^4	4.0×10^4	304
OH-SCCPs	31	31	5.4×10^5	8.2×10^4	-85
OH-MCCPs	32	35	2.0×10^5	8.0×10^4	-60
OH-LCCPs	32	53	5.7×10^4	1.2×10^5	104
∑OH-CPs	116	39	8.1×10^5	3.2×10^5	-61
CO-vSCCPs	21	42	2.9×10^3	1.8×10^4	304
CO-SCCPs	32	40	2.0×10^5	3.5×10^4	-83
CO-MCCPs	32	44	7.9×10^4	1.4×10^5	81
CO-LCCPs	32	71	2.4×10^4	2.7×10^5	1022
∑CO-CPs	117	50	2.0×10^5	4.6×10^5	131
COOH-vSCCPs	22	40	2.1×10^3	8.6×10^3	315
COOH-SCCPs	32	45	3.1×10^5	1.8×10^5	-44
COOH-MCCPs	32	42	3.4×10^5	1.1×10^5	-69
COOH-LCCPs	32	66	8.8×10^4	1.0×10^5	15
∑COOH-CPs	118	49	7.4×10^5	3.9×10^5	-47

[a] : $\sum Area_{tran} > \sum Area_{nc}$ (%): the percentage of chemicals with increased peak area after biotransformation.
[b] : Transformation rate =($\sum Area_{tran}$ - $\sum Area_{nc}$)/ $\sum Area_{nc}$, positive values suggested an increase after the biotransformation.

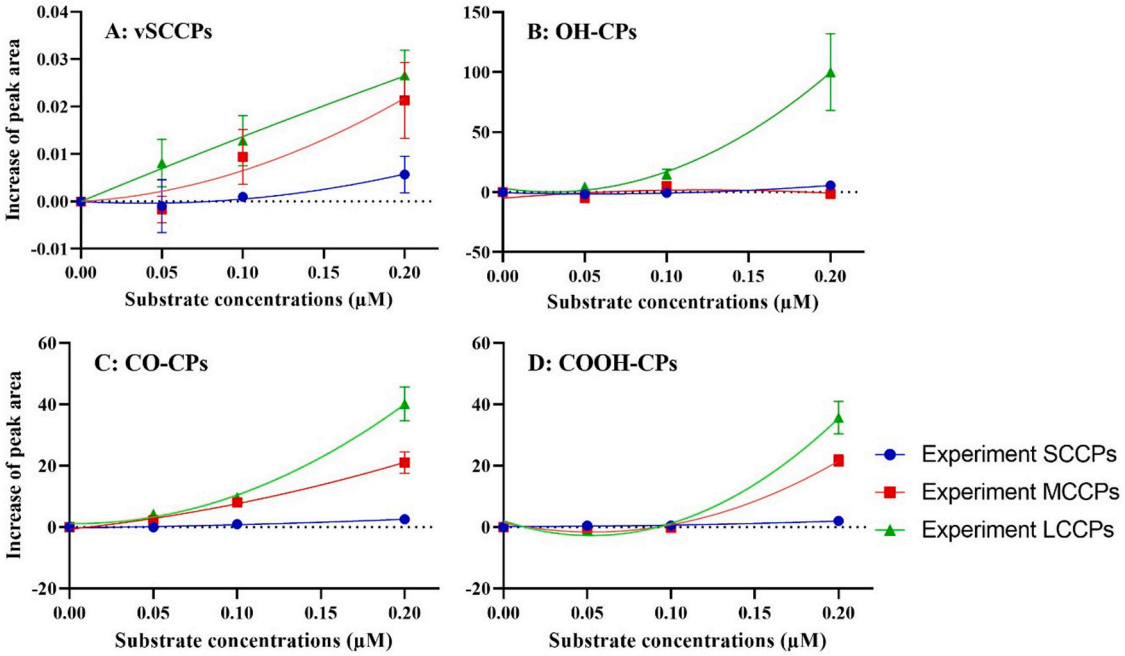

Fig. 4. CP biotransformation products formation with HLMs at various substrate concentrations.

of all the transformation products in Experiment LCCPs were higher than that in the other two, possibly because of lower areas for these chemicals in the LCCP experiments prior to the transformation experiment (i.e. in the LCCP standards). Net gains were found for vSCCPs and CO-CPs in most samples, suggesting the in vitro biotransformation rates were higher than the degradation rates for vSCCPs and CO-CPs, which thus could be useful as biomarkers in urine to assess human exposure to CPs.

Assuming that the potential transformation product groups have similar average molecular weight and response on the mass spectrometer under the operational condition when compared to the spiked CPs, in Experiment LCCPs, the increases of vSCCP, OH-CP, CO-CPs and COOH-CP after the biotransformation accounted for 2%, 5%, 60% and −10%, respectively, while the increase in MCCPs and SCCPs accounted for 20% and 5% of the concentrations of the spiked LCCPs, respectively. However, the concentrations of all the potential transformation products targeted only make up of less than 20% of the spiked chemicals in Experiment SCCPs and MCCPs which may indicate that there were other products resulting from the biotransformation of SCCPs and MCCPs, including CO_2, and olefins.

3.5. Limitations

To the best of our knowledge, this is the first study to assess the biotransformation products of CPs using HLMs.

One of the limitations of this study was that we were not able to identify all of the potential transformation products of CPs. Non-target analysis is one of the best ways for the transformation products identification, and it requires high purity standards. In the case of CPs, the major transformation products were also detected in the standards, which resulted in interference for the products identification. Also, the use of a LC column (Li et al., 2017) could also add to separation of biotransformation products from the original compounds, however it should be noted that this would increase the analysis time from a few minutes to 15–20 min.

No authentic standards are available for the transformation products. Therefore, we are unable to quantify the concentrations in the samples. In this study, we used the peak area of the related ions with mass windows of ±0.0025 for tentative calculation of the increase or decrease of these products. Once these standards are produced, it is necessary to (further) develop an analytical method and to measure the occurrence and concentrations of CP transformation products in urine samples. Follow up with the in vitro experiment, in vivo studies are expected to confirm the biotransformation of CPs in human bodies.

CRediT authorship contribution statement

Chang He: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft. **Louise van Mourik:** Methodology, Validation, Writing - original draft. **Shaoyu Tang:** Resources, Data curation, Writing - original draft. **Phong Thai:** Data Curation, Writing - review & editing. **Xianyu Wang:** Data curation, Writing - review & editing. **Sicco H. Brandsma:** Methodology, Writing - review & editing. **Pim E.G. Leonards:** Conceptualization, Writing - review & editing, Supervision. **Kevin V. Thomas:** Conceptualization, Writing - review & editing, Supervision. **Jochen F. Mueller:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.124245.

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