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Marie Mézière, Philippe Marchand, Sébastien Hutinet, Frédéric Larvor, Elisabeth Baéza, et al.. Transfer of short-, medium-, and long-chain chlorinated paraffins to eggs of laying hens after dietary exposure. Food Chemistry, 2021, 343, 10.1016/j.foodchem.2020.128491 . hal-03292410

HAL Id: hal-03292410
https://hal.inrae.fr/hal-03292410

Submitted on 2 Jan 2023

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Version of Record: https://www.sciencedirect.com/science/article/pii/S0308814620323530
Manuscript_8fefb0246ede4a2a4bb7750d16ff9cb3

Transfer of short-, medium-, and long-chain chlorinated paraffins to eggs of laying hens after dietary exposure

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Abstract

Chlorinated paraffins (CPs) are a complex family of contaminants. Lack of exposure data and an understanding of the fate of these chemicals in the environment affect our ability to reliably assess the human health risk associated with CP exposure. The present study focused on the evaluation of CP transfer from feed to eggs of laying hens exposed over 91 days. Laying hens were provided feed spiked with five technical mixtures of short-, medium- or long-chain CPs and featuring low or high chlorine contents, at concentrations of 200 ng/g each. Eggs were collected daily. All mixtures except the LCCPs with high chlorine content transferred into the eggs, with accumulation ratios increasing with the chain length and chlorine content. Concentrations at the steady-state varied between 41 and 1397 ng/g lw depending on the mixture. Additionally, the homologue-dependant transfer resulted in a change of pattern compared to that from the spiked feed.

Keywords (*6 max*)

chlorinated paraffin; homologue; transfer; kinetics; accumulation ratio; persistent organic pollutant

1. Introduction

Chlorinated paraffins (CPs) are described as complex synthetic mixtures of *n*-alkane chains ($C_xH_{2x+2-y}Cl_y$) with varying chlorination degrees in the range 30-70% *w/w* (European Food Safety Authority (EFSA) panel on contaminants in the food chain (CONTAM), 2020). They are used in numerous industrial applications depending on their chemical properties, such as lubricants in metal-working fluids, flame-retardants and plasticizers (van Mourik et al., 2016). Such diversity of applications explains the large volume of production worldwide, estimated at >1,000,000 tonnes per year (Glüge et al., 2016), which exceeds the total amount of PCBs produced before their ban in the 70s-80s.

For both regulation and analytical purposes, CPs are classically sub-categorized into short-chain CPs (SCCPs, C_{10}-C_{13}), medium-chain CPs (MCCPs, C_{14}-C_{17}), and long-chain CPs (LCCPs, $C_{\geq 18}$). The risk associated with SCCPs exposure had been the subject of a study by the International Agency for Research on Cancer in 1990, leading to their classification as substances possibly carcinogenic to humans (Group 2B) (International Agency for Research on Cancer (IARC) working group on the evaluation of carcinogenic risk to humans, 1990). Although toxicological studies remained scarce, it showed sufficient toxicity for their phasing out in North America and Europe in the 2010s and SCCPs were listed in the annex A of the Stockholm convention in 2017 (European Commission (EC), 2002; Government of Canada, 2009; United States Environmental Protection Agency (US EPA), 2015a; Persistent Organic Pollutants Review Committee (POPRC), 2017). Very recently in Europe, the European Food Safety Authority (EFSA) used available dietary studies and a benchmark modelling to estimate a reference dose of a 2.3 mg/kg body weight (bw) per day ($BDML_{10}$, 2019) (EFSA CONTAM panel, 2020). Recently, the application of novel analytical strategies showed their potential for endocrine and metabolism disruption with the main targets being the liver, the kidney and the thyroid gland (X. Wang et al., 2019), which could induce a higher toxicity in a long time range.

At the same time, risk assessment associated with MCCPs and LCCPs is less advanced, although MCCPs have been classified as persistent, bioaccumulative, and toxic substances (PBT) under the EU REACH regulation (European Union (EU), 2006) and by Environmental Canada (2008), and LCCPs are

58 suspected to exhibit similar properties (van Mourik et al., 2016). One factor hindering the MCCPs and
59 LCCPs risk assessment is certainly their challenging analysis which is extensively described elsewhere
60 (Krätschmer and Schächtele, 2019; Yuan et al., 2019). However, another factor might be the common
61 belief that SCCPs may be a greater risk for human health than MCCPs and LCCPs. Indeed, the toxicity
62 of CPs has been reported to be inversely proportional to the chain length (Tomy et al., 1998). Yet,
63 MCCPs were shown to induce increased liver weights, histopathological changes and liver necrosis at
64 high dose level (United States Environmental Protection Agency (US EPA), 2015b). Moreover,
65 recently, Ren et al. (2019) compared the impact of S, M, and LCCPs on human hepatic cell viability
66 and metabolism. They suggested that all three types of CPs induced oxidative stress and decreased
67 cell viability, although LCCPs cytotoxicity was acknowledged to differ slightly from SCCPs and MCCPs.
68 Overall, toxicity studies sometimes result in contradicting observations that show the complexity of
69 CPs toxicology, and enhance the need of thorough risk assessment for all CPs.
70 In order to move towards better and more comprehensive assessments, it is also crucial to study the
71 potential pathways of human exposure to CPs which occurs via food consumption (~85%) and air
72 inhalation (~15%) (van Mourik et al., 2016). Indeed, SCCPs have been detected in many types of food:
73 Iino et al. (2005) detected SCCPs in fats and oils, seafood, meat, dairy products, and vegetables at 14,
74 16-18, 7, 1, and 1.4-2.5 ng/g wet weight (ww), respectively, in supermarket products of Japanese
75 cities. Much higher concentrations of SCCPs were reported in a Chinese polluted area (898-5640,
76 881-2710, 397-3540, 1100 ng/g ww for seafood, meat, vegetables, and fats and oils, respectively)
77 (Chen et al., 2018). Fewer studies provided MCCPs concentrations, but when available, they were of
78 same level as SCCPs (Chen et al., 2018; Krätschmer et al., 2019; R. Wang et al., 2019). Unfortunately,
79 most of the analyses are performed with GC-based instrumentations, preventing the generation of
80 LCCPs occurrence data. It is however expected that due to the LCCPs high octanol-water partition
81 coefficient ($6.5<K_{ow}<12.7$), they may accumulate in highly lipidic matrices such as fats and oils, and
82 food of animal origin. To date, only one recent study reported trace levels of LCCPs in foodstuffs
83 (Darnerud, 2018). From the available occurrence data in food, it is clear that humans are exposed to

84 substantial amounts of CPs via their diet, which calls for further assessment of the risk related to the
85 dietary exposure to those chemicals. In Europe, the EFSA estimated the risk related to SCCPs and
86 MCCPs dietary exposure to be rather low, but the risk assessment was at the time based on fish
87 consumption only as occurrence data in other European foodstuffs had not yet been submitted (EFSA
88 CONTAM panel, 2020). Moreover, the EFSA panel acknowledged the need for more data on the
89 transfer of CPs from feed to the food of animal origin for further and more robust assessment of the
90 risk related to dietary exposure to CPs.
91 Among the food categories, eggs and egg products are an important source of protein and are
92 consumed worldwide, with a mean consumption of 139 eggs per person in 2017 (Giannetto et al.,
93 2016). As a chicken egg yolk contains typically 30% ww of fat, it is likely that CPs, and even more
94 LCCPs, accumulate in this type of food. It is thus important to assess the potential contamination of
95 eggs, and especially the relationship between the laying hens CP intake and depuration via the eggs.
96 Indeed, it is well known that chickens are exposed to POPs via ingestion of contaminated feed or
97 consumption of soil in which POPs are accumulated. Particularly, soil was previously mentioned as an
98 important contamination source for free-ranged chicken, which are becoming more popular than
99 battery chicken for their more ethical rearing conditions (Jondreville et al., 2014; Polder et al., 2016).
100 The ubiquity of CPs suggests levels similar to those of other POPs, which, in turn, would lead to eggs
101 contamination. As a matter of fact, CP levels in chicken feed were already reported at least twice
102 (Dong et al., 2019; Sun et al., 2020) and chicken eggs of a Chinese polluted area featured extremely
103 high amounts of SCCPs and MCCPs (dozens of thousands ng/g ww) (Zeng et al., 2018).
104 Ueberschär et al. (2007) already showed that SCCPs accumulate in laying hen egg. In the present
105 study, we hypothesised that other types of CPs could be transferred to the egg. We exposed laying
106 hens to an exposure mixture including 2 SCCP, 1 MCCP and 2 LCCP technical mixtures to investigate
107 the influence of the carbon chain length and the chlorination degree of CPs on their transfer to eggs.
108 The targeted feed concentration was 5×200 ng/g ww, which was in the high end of total CPs amounts
109 previously reported in Chinese feeds (140-2000 ng/g) (Dong et al., 2019). Although the real

contamination differs slightly from this value, it stays in similar order of magnitude. The results provide valuable data on bio accessibility and transfer of CPs. In particular, to our knowledge it is the first time that LCCPs transfer to chicken eggs has been reported.

2. Material and methods

2.1. Chemicals

In order to cover the whole range of CPs, four technical mixtures were purchased from AccuStandard Inc. (New Haven, CT, USA), including Chlorowax™ 500C (SCCPs, low chlorine content, *SCCPs low %Cl*), Paroil™ 179-HV (SCCP, high chlorine content, *SCCPs high %Cl*), Unichlor™ 40-90 (LCCP, low chlorine content, *LCCPs low %Cl*) and CPW-100 (LCCP, high chlorine content, *LCCPs high %Cl*), as well as one MCCPs technical standard kindly provided by colleagues from EMPA, Switzerland (I-42, *MCCPs*). Details about their CP experimental homologue patterns are given in Figure S1. ^{13}C-γ-Hexabromododecane (HBCDD) and d_{18}-β-HBCDD were purchased from Wellington Laboratories Inc. (Ontario, Canada) and were used as internal (*IS*) and external (*ES*) standards, respectively.

Silica gel (70/230 mesh) and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Hexane, dichloromethane (DCM), and magnesium silicate (Florisil) were provided by LGC Promochem (Wesel, Germany). Sulphuric acid (H_2SO_4, 98%) was purchased from Panreac (Barcelona, Spain).

2.2. Feed, experimental design and sampling

The experimental feed basis was prepared from maize, wheat, soybean, and all the nutrients required for laying hens (Table S1). The target CP concentration of spiked feed was 200 ng/g for each of the five technical mixtures mentioned above (Figure S1), an environmentally relevant occurrence level (van Mourik et al., 2016). Thus, rapeseed oil was spiked to the level of 10,846 µg/kg for each technical mixture. Then, two feed batches were prepared by adding 1.8% *w/w* of control or spiked rapeseed oil. Both batches were subsequently pelleted (5 mm diameter).

The animal experiment was ethically approved by the French authorities under number APAFIS#17145-2018101712299769v2 and was conducted in an appropriate facility of the experimental unit PEAT (INRAE Nouzilly, France). Eighty laying hens (Isa Brown) were housed in individual cages and raised under conventional conditions of temperature and lighting, with control feed distributed *ad libitum* during one month. At the beginning of the experiment, the 25-week old hens were weighted, and 11 outlying hens were excluded from the experiment to limit individual variability. The remaining hens (n=69), weighing on average 1663 ± 105 g, were separated into 21 control and 48 exposed individuals, and were fed with control feed and spiked feed, respectively, during up to 91 days (13 weeks). Water was freely available, and feed intake was recorded weekly, by weighing feed allowance and refusals. Both control and exposed hens ingested an average of 107 ± 13 g feed daily along the experiment, corresponding to a targeted exposure of 5×21 µg of CPs per day.

It should be noted that the laying hens have been sacrificed over time, on a weekly basis, to further study their tissues distribution within a broader study framework than the one of the present study. Only eggs, collected and weighed daily, have been considered here (Figure S2). As the totality of CPs were expected in the yolk part (along with lipids), after egg cracking, the yolks were pooled per day for controls or exposed hens except for day 90 when they were kept as individuals to assess variability. Samples were then homogenised by mixing and about 100 g were stored at -20 °C until analysis.

2.3. Extraction and Clean-up

Hen yolks from 5 (control group) and 17 (exposed group) selected days (Figure S2) were lyophilized and ground. About 0.8 g of dry yolk were extracted by pressurized liquid extraction (PLE, SpeedExtractor E-914/E-916, Büchi, France) with a mixture of toluene/acetone (7:3, *v/v*, 3 static cycles, 120 °C, 100 bar). Samples were concentrated to dryness and their lipid contents determined gravimetrically.

Aliquots of control and spiked feed were also collected at the beginning, middle and end of the experiment and analysed to verify the repeatability of the exposure throughout the experiment. About 2.5 g of feed were directly extracted by PLE, which recovered about 0.1 g of fat. Similarly, maize, wheat and soybean used for the feed basis were analysed separately. In each case, about 10 g of ingredient recovered about 0.3 g of fat.

The internal standard (^{13}C-γ-HBCDD, *IS*, 5 ng) was added in each extract prior to purification through a column packed with 10 g of neutral silica and 20 g of acidic silica gel at 44% H_2SO_4. Elution was performed with 60 mL of a mixture of DCM/Hexane (1:1, *v/v*). The extracts were reconstituted in 1 mL hexane and loaded onto a column packed with 6 g of Florisil deactivated with 3% H_2O. Elution was achieved with 20 mL of DCM. The extracts were reconstituted in 25 µL of acetonitrile containing d_{18}-β-HBCDD (*ES*, 0.2 ng/µL).

2.4. Data acquisition and data-treatment

Data acquisition and data-treatment were performed as described by Mézière, Cariou, et al. (2020) with a liquid chromatography – high resolution mass spectrometry (HRMS) coupling fitted with an electrospray ionisation source (Q-Exactive, Thermo Fischer Scientific, San José, CA, USA). Briefly, chromatographic separation was achieved using a Hypersil Gold analytical column (100 mm × 2.1 mm, 1.9 µm) (Thermo Fischer Scientific). Mobile phase consisted of 70% to 100% ACN in water. A mixture of DCM/ACN (1:1, *v/v*) was added post-column to enhance the formation of the targeted ions. HRMS data were acquired in the negative mode and in full scan mode over the *m/z* range [300-1500], at a resolving power set to 140,000 full width at half maximum at *m/z* 200. The open source programming *R* environment was used to extract and integrate specific $[M + Cl]^-$ adducts signals from CP homologue groups (± 5 ppm tolerance) within C_8-C_{36} chain length and Cl_4-Cl_{30} chlorine number (excluding homologue groups with $n_{Cl} > n_C+2$). Identification of homologues was controlled with two criteria: signals should feature an area > 1,000,000 AU and comply with the theoretical ion ratios between the two most intense ions of the isotopic profile (20% tolerance). Intensities were then

normalised to total homologue isotopic patterns to correct for the isotopic contribution of the quantifier ion.

2.5 Quantification and kinetics considerations at the CP subcategory level

External calibration was performed with standard solutions containing the five same CP technical mixtures in the dynamic range of 0.1-15 ng/µL, and the IS and ES at 0.2 ng/µL. The areas of the homologue groups relative to the IS were summed according to the delimitation of five CP subcategories (Figure S1) for quantification purpose (corresponding to eq. 2.1 of Yuan et al., 2019). As the calibration solutions did not follow a linear trend along the concentrations range, it was divided into two sub-ranges (0.5-2 and 2-15 ng/µL) which fitted adequately with linear curves (R^2>0.96, Figure S3).

The quantification accuracy was assessed by evaluating the profile similarities between the egg and feed samples and the quantification mixture. A least-square approximation with a non-negative constraint was calculated using the function *lsqnonneg* of the *pracma* package in the open-source programming *R* environment. The exposure mixture perfectly matched the samples when the parameter *a* of the equation $[S] = \mathrm{a} \times [M] + \mathrm{b}$ was equal to 1 (± 0.1), where $[S]$ and $[M]$ are the vectors of the homologues detected in the sample and the corresponding technical mixture, respectively.

Based on a preliminary data examination, a first-kinetic model was adjusted to the experimental egg concentrations using a non-linear regression with the function *nls* of the package *stats* in the *R* environment. The kinetics equation was expressed as in equation 1:

$$C_{CPs\ subcat.}(t) = C_{CPs\ subcat.}(egg, \infty) \times (1 - e^{-kt}) \quad \text{Eq.1}$$

where $C_{CPs\ subcat.}(t)$ and $C_{CPs\ subcat.}(egg, \infty)$ are the calculated concentration of the CPs subcategories in eggs (ng/g lw) for the exposed group eggs at day *t* and at steady-state, respectively, and k is the accumulation rate constant. From this equation, the accumulation ratios ($AR_{CPs\ mix}$) at

steady-state and corresponding carry-over rates ($COR_{CPs\ mix}$, in %) could be both calculated according to equation 2:

$$COR_{CPs\ subcat} = \frac{Q_{egg}}{Q_{feed}} \times \frac{C_{CPs\ subcat.}(egg,\infty)}{C_{CPs\ subcat.}(feed)} = \frac{Q_{egg}}{Q_{feed}} \times AR_{CPs\ subcat.} \quad \text{Eq.2}$$

where $C_{CPs\ subcat.}(feed)$ is the concentration of the CP subcategory in the spiked feed (ng/g ww), and Q_{egg} and Q_{feed} correspond to the amount of lipids excreted daily through eggs (g/day) and the amount of feed daily ingested (g/day) at the steady-state, respectively.

2.6 Kinetics considerations at the homologue level

Information on the homologue groups in the technical mixtures was not available, disallowing any homologue-level quantification. However, the homologue concentration could be expressed as a fraction of the concentration in the exposure mixture as described in equation 3:

$$C^S_{n,x} = F^{Cal}_{n,x} \times C^S_{\equiv Mix(n,x)} \quad \text{Eq.3}$$

where $C^S_{n,x}$ is the concentration of the homologue containing n carbons and x chlorines in the sample S (egg or feed), $F^{Cal}_{n,x}$ is the fraction of the homologue n,x in the exposure mixture, and $C^S_{\equiv Mix(n,x)}$ an equivalent total concentration of the CP exposure mixture in the sample that would give the corresponding homologue n,x response. Indeed, using the calibration solutions, a correlation between the relative response of the homologue to the IS in the CP exposure mixture and the equivalent total concentration of the CP exposure mixture, relative to the IS, could be plotted according to the equation 4:

$$\frac{A^S_{n,x}}{A^S_{IS}} = f_{n,x}\left(\frac{C^S_{\equiv Mix(n,x)}}{C^S_{IS}}\right) \quad \text{Eq. 4}$$

This equation could also, as the curves followed a bijective trend, be expressed as in equation 5:

$$C^S_{\equiv Mix(n,x)} = C^S_{IS} \times f^{-1}_{n,x}\left(\frac{A^S_{n,x}}{A^S_{IS}}\right) \quad \text{Eq. 5}$$

As the same calibration curve was used for feed and egg, the same relation between the relative responses and the equivalent concentration of the CP mixture could be applied for both matrices

(equations 3, 4 and 5). Hence, applying equations 3 and 5 in equation 2 enables the calculation of a homologue-dependant carry-over rate ($COR_{n,x}$, in %) as in equation 5 and 6:

$$COR_{n,x} = \frac{Q_{egg}}{Q_{feed}} \times \frac{C_{n,x}^{egg,\infty}}{C_{n,x}^{feed}} = \frac{Q_{egg}}{Q_{feed}} \times \frac{F_{n,x}^{Cal} \times C_{\equiv Mix(n,x)}^{egg}}{F_{n,x}^{Cal} \times C_{\equiv Mix(n,x)}^{feed}} \quad \text{Eq.6 (Eq.3 in Eq.2)}$$

$$COR_{n,x} = \frac{Q_{egg}}{Q_{feed}} \times \frac{Q_{IS}^{egg}}{Q_{IS}^{feed}} \times \frac{SS^{feed}}{SS^{egg}} \times \frac{f_{n,x}^{-1}\left(\frac{A_{n,x}^{egg}}{A_{IS}^{egg}}\right)}{f_{n,x}^{-1}\left(\frac{A_{n,x}^{feed}}{A_{IS}^{feed}}\right)} \quad \text{Eq.7 (Eq.5 in Eq.6)}$$

Where Q_{IS}^{egg} and Q_{IS}^{feed} are the quantities of internal standard in the analysed egg and feed sample, SS^{egg} is the egg sample size (in ng lipids) and SS^{feed} is the feed sample size (in ng wet weight). Additionally, the time needed to reach 95% of the steady-state concentration ($t_{SS(n,x)}$) was calculated from the first-order kinetic rate constant $k_{n,x}$ according to equation 8:

$$t_{SS(n,x)} = -\frac{1}{k_{n,x}} \times \ln(0.05) \quad \text{Eq.8}$$

The calculations were restricted to homologues containing 4 to 14 chlorines, and detected from day 2 of the experiment in order to fit the model. As a result, the $COR_{n,x}$ and $t_{SS(n,x)}$ were determined for 119 homologues.

2.7 QA/QC

All glassware was heated at 400 °C during 4 h before use. Egg and feed samples were analysed in distinct batches, along with a total of 9 procedural blanks and 7 quality control samples (QCs). The procedural blank extracts exhibited SCCPs low %Cl and MCCPs at trace levels within the range 3-28 ng/sample (Table S2). Thus, the relative areas from the blanks (average per batch) were subtracted from the samples for each homologue group before quantification. Recovery of CPs was determined independently from the experiment on QC samples spiked or not with the CP exposure mixture (n=2 each). Values were 115%, 122%, 66%, 151% and 79% for SCCPs low %Cl, SCCPs high %Cl, MCCPs, LCCPs low %Cl and LCCPs high %Cl, respectively. QCs originated from a mixture of exposed eggs from day 32 and day 88, further spiked with LCCPs high %Cl at the level of 250 ng/g. Variabilities inter-sequences and intra-sequence were acceptable considering the challenges of CPs

analysis and current interlaboratory coefficients of variation (van Mourik et al., 2018; Krätschmer and Schächtele, 2019; Mézière, Krätschmer, et al., 2020) (RSD within 5-32%, Table S3). In the present study, method detection limits were not calculated, as concentrations of homologue groups were not known and the limit of detection of a mixture was not clear (as homologues on the edge would not be detected but more abundant homologues could still be detected at the same technical mixture concentration). Instead, we defined the limit of quantification (LOQ) as the lowest point of the external calibration (0.5 ng/µL), corresponding to 25 ng/g lw for eggs and 5 ng/g ww for feed, for all CP subcategories.

3. Results and discussion

3.1. Feed

Spiked and control feeds collected at the beginning, middle and end of the experiment showed good repeatability over time (Table S4). Unfortunately, the control feed showed traces of LCCPs high %Cl mostly, followed by MCCPs, and SCCPs high %Cl (120, 57, and 25 ng/g ww, respectively). Such phenomenon had already been reported by the study of Dong et al. (2019) who measured SCCPs and MCCPs levels from 140 to 2,000 ng/g ww in feedstuffs of animal and plant origin, with the vegetal feeds being the less contaminated. Such contamination source is expected to originate not necessarily from the raw materials but also from the process (grounding, pelleting…). In the present study, the analysis of the feed's constituent raw materials revealed that SCCPs and MCCPs contamination profiles in wheat, maize and especially soybeans matched the one of the control feed, supporting a contamination from the raw seeds (Figure S4). However, although LCCPs high %Cl were also observed in the seeds, it was in relatively low amounts compared to the control feed. For this type of CPs, an additional contamination might have occurred during the processing of the feed.

The calculated concentrations in control and spiked feed matched the spiking levels since the difference (spiked feed-control feed) corresponded to the target spiking value of 200 ng/g ww within ± 10% error (Table S4) for all CP subcategories. The implemented experimental design therefore

corresponded to a real daily exposure of 24, 24, 25, 21, and 32 µg/day of SCCPs low %Cl, SCCPs high %Cl, MCCPs, LCCPs low %Cl and LCCPs high %Cl, respectively. Hence, although contamination of control feed was considered to have a limited impact on the spiked feed levels, the experimental concentrations values of the spiked feed were used for AR calculation purposes.

3.2 Laying performance

The body weight of hens at slaughter (1645 ± 139 g) and the number of eggs produced daily (0.96 ± 0.05) remained stable over time. The average weight of the eggs slightly increased from 55.3 ± 2.8 and 54.8 ± 3.6 g (day 1) to 61.2 ± 1.5 and 57.9 ± 4.6 g (day 91) for the control and the exposed groups, respectively (Figure S5). The increase in egg weight over time was statistically less pronounced for the exposed compared to the control hens after day 42 for all hens or day 49 when considering only the 6 control and 8 exposed hens slaughtered at the end of the experiment ($P < 0.05$). Such observation may indicate that CPs exposure induces a physiological effect on egg weight. As previous studies suggested that CPs might affect the lipid metabolism (Gong et al., 2019), the egg weight could originate from smaller yolks. However, no significant changes were observed between the control and exposed groups regarding the yolk proportion or lipids content in yolk (Figure S6). Thus, in the present study, the decrease of egg weight does not seem to be related to the yolk content, suggesting that CPs affect the laying process in a more global way. Should such observation be confirmed, the exposure of laying hens to CPs would strongly impact agronomic performances. The influence on the egg nutritional qualities should also be further investigated.

3.3. Homologue profiles in control and exposed hen eggs

The CPs homologue response profiles at the steady-state were compared between the feed and egg samples for the control and exposed groups (Figure 1). The overall profiles looked similar, confirming the transfer capability of most CP homologues to the eggs. However, two main differences could be pointed out between the feed and egg samples.

312 Firstly, the LCCPs high %Cl relative area contributed to about 51% and 6% of the total area in the
313 control feed and spiked feed, respectively, and included homologues with up to 34 carbons and 30
314 chlorines. In the eggs of the control group and exposed group, this contribution dropped to 1% and
315 2%, respectively, and only homologues up to 25 carbons and 24 chlorines were detected. These types
316 of CP homologues are usually not targeted in CP transfer studies. Hence, no data is available on their
317 occurrence in animal tissues. One possible reason of their absence in the eggs is their high molecular
318 weight (700-1500 Da). As compounds of molecular weight above 1000 Da are very unlikely to be
319 absorbed by the gastro-intestinal tract (EFSA, 2008), CPs with higher molecular weight may be
320 directly excreted through faeces rather than absorbed and transferred to eggs. However,
321 interestingly, some homologues with $m/z > 1000$ were detected in the eggs, although in relatively
322 low amounts. As the absorption is driven by the size and shape of the molecules as well as the
323 molecule physico-chemical properties such as the octanol-water partition coefficient (K_{ow}), the limit
324 of 1000 Da might not be so strict. Because the amounts of LCCPs high %Cl in eggs were relatively low,
325 this CP subcategory was not further considered for kinetics evaluation.
326 Secondly, a shift towards higher chlorine contents in the egg compared to feed CP pattern could be
327 observed for SCCPs low and high %Cl, MCCPs, and LCCPs low %Cl (Figure 1, Figure S7). The influence
328 of this shift on the similarities between the quantification mixtures and the feed and eggs patterns
329 was evaluated by calculating a least square approximation between the samples and the exposure
330 mixture as reference (Table 1). For all CP subcategories, the control feed contamination patterns
331 were quite distinct from the exposure mixture, and the spiked feed pattern was very similar to that
332 of the exposure mixture, with factors a reaching 1 ± 0.1. Comparatively, the exposed eggs
333 contamination patterns showed a noticeable difference ($0.56 < a < 0.88$), except for SCCPs high %Cl
334 which perfectly matched ($a = 1.02$). This phenomenon could be expected, as the chain length and
335 degree of chlorination have already been demonstrated as influencing factors on the behaviour of CP
336 homologue groups in animals (Zhou et al., 2018; Castro et al., 2019). Particularly, Hilger et al. (2011)
337 observed a positive correlation between the chlorination degree and the log K_{ow} for the range 55-

70% Cl, that would imply a higher affinity of highly chlorinated homologues for lipophilic matrices such as egg yolk.

3.4. CP subcategory level kinetics

A subset of 5 control and 17 exposed group egg pools was selected for analysis. The quantification of 4 CP subcategories (SCCPs low and high %Cl, MCCPs, LCCP low %Cl) was based on an external calibration from the exposure mixture (Table S5). Concentrations in the exposed hens increased from <LOQ to 32 ng/g lw, from 49 to 173 ng/g lw, from 49 to 88 ng/g lw and from <LOQ to 1275 ng/g lw for SCCPs low %Cl, SCCPs high %Cl, MCCPs and LCCPs low %Cl, respectively. The individual variability assessed at day 90 were acceptable (RSD = 19-34%) except for SCCPs low %Cl (67%), which indicated that the concentration increase was due to accumulation of those 4 CP subcategories in the eggs. However, these concentration values should be considered with caution since the homologue patterns in the eggs diverges significantly from those of the analytical standards. Indeed, in our analytical method, the homologue ionisation efficiency increases with the chlorination degree. In order to circumvent this phenomenon, the concentrations in the exposed group were expressed with respect to those of the control group (Figure 2). At the beginning of the experiment, the concentrations were similar between the control and exposed group for all CPs, with a ratio $Conc._{exposed} / Conc._{controls}$ from 0.5 to 1.5, except for SCCPs low %Cl which were more concentrated in the exposed eggs. However, it increased to reach a steady-state (t_{ss}) in roughly 14 days, visually fitting a first-order kinetic model. At the steady-state, the concentrations of CPs were 3 to 111 times higher in the exposed group than in the control group, depending on the subcategories considered. The highest concentrations were measured for LCCPs low %Cl. As all CP mixtures were administered at similar concentrations to the hens, a higher concentration of LCCPs low %Cl in the eggs indicates towards a higher potential of this subcategory for transfer in eggs.

Overall, the $ARs_{CPs\ subcat.}$ were calculated to be 0.2, 0.8, 0.5 and 7.1 for SCCPs low %Cl, SCCPs high %Cl, MCCPs and LCCPs low %Cl, respectively, although this may be an overestimate. However,

the $AR_{SCCPs\ low\ \%Cl}$ was close to that previously reported by Ueberschär et al. (2007) (0.08 according to ww which is equivalent to 0.26 according to lw), confirming their findings and extending it to CPs of higher chlorination degree and of longer C-chain lengths. Interestingly, in their study, steady-state was reached after 42 days. This difference might be due to different experimental conditions as well as higher dose used in their study (100 vs 5×0.2 mg/kg of feed). It also should be noted that in the present study, exposed group eggs of day 15 and day 19 were quantified to higher values, which we attributed to analytical bias. This high concentration may influence the kinetics modelling, hence shortening the time to reach the steady-state. The corresponding $CORs_{CPs\ subcat.}$ were calculated to be 1%, 4%, 2% and 33% for SCCPs low %Cl, SCCPs high %Cl, MCCPs and LCCPs low %Cl, respectively. Carry-over rates in eggs of several chlorinated pesticides and environmental contaminants were reported by Kan and Meijer (2007), and varied between <0.1% to 80%. In the present study, CPs covered the nearly the whole range of these $CORs$, emphasizing the diversity of physico-chemical properties from SCCPs to LCCPs. According to Kan and Meijer classification, SCCPs and MCCPs could be classified as molecules of low to moderate accumulation, and LCCPs could be considered as highly cumulative.

Compared to the classically targeted SCCPs low %Cl, the SCCPs high %Cl, MCCPs and especially LCCPs low %Cl were transferred to eggs at a higher rate, proving the capability of longer chains and higher chlorinated CPs to accumulate in lipophilic matrices. The comparison of the CP subcategories distribution in the other laying hens' compartments would allow completing the accumulation assessment of CPs.

3.5. Homologue-level kinetics

The transfer potential of CPs was evaluated more finely by calculating the homologue carry-over rates ($COR_{n,x}$) and the time necessary to reach 95% ($t_{ss(n,x)}$) of the steady-state at the homologue level. These estimations suffered neither from the profile bias nor from the fact that the relative contributions to the complex mixtures remains unknown. The $t_{ss(n,x)}$values varied between 10 to 24

390 days for most of CP homologues, which encompasses the rough estimate of 14 days at the CP
391 subcategory level (Table S6). However, these $t_{ss(n,x)}$values were highly dependent on the
392 concordance of the model with the experimental values.. Indeed, $t_{ss(n,x)}$ values inferior to 10 days
393 were obtained only for homologues that were also detected in the control eggs (Figure S8), and the
394 highest $t_{ss(n,x)}$ were determined for homologues not detected in the exposed group at day 2, or with
395 a rather low intensity. From this observation, it can be assumed that the shortest and longest $t_{ss(n,x)}$
396 may be under or overestimated due to analytical bias, and that mean $t_{ss(n,x)}$ could be fixed around
397 14 days.
398 The $COR_{n,x}$ increased with both the chain length and chlorine content, and reached a maximum for
399 the $C_{30}Cl_9$ homologue (Figure 3). At the maximum, the $COR_{n,x}$ was superior to 100%, which is
400 unlikely. It indicates an overestimation of the $COR_{n,x}$, possibly due to matrix effects, at least for the
401 LCCPs. It should also be reminded that the CP homologues present in the control feed were not taken
402 into account in the $COR_{n,x}$ calculation, thus could alter slightly the $COR_{n,x}$. In the chain length
403 perspective (Figure 3, left), the $COR_{n,x}$ continuously increased with a slope that becomes steeper
404 with higher chain lengths. For a fixed chlorine number, $COR_{n,x}$ optimums were reached from C_{27}
405 ($C_{27}Cl_5$) to C_{30} ($C_{30}Cl_9$) forming a ridge of $COR_{n,x}$ optimums (Table S7). Similarly, in the chlorine
406 number perspective (Figure 3, right, Table S7), $COR_{n,x}$ increased up to an optimum around Cl_{10}-Cl_{11}
407 for SCCPs and MCCPs, versus Cl_7-Cl_9 for long chain lengths. This shift of optimum chlorine number
408 and chain length indicated that both influence interdependently the homologue accumulation
409 capacity.
410 As both the chlorine content and the chain length are related to the lipophilicity of the compounds
411 (Ma et al., 2014), an attempt to correlate the coefficient log K_{ow} with the $COR_{n,x}$ was performed on
412 SCCPs (Figure S9). Although the homologue log K_{ow} was only a rough estimation as it was based on
413 measurements of single-chain CP mixtures performed by (Hilger et al., 2011), a positive linear
414 correlation with the homologue accumulation ratio was observed for C_{11}, C_{12}, and C_{13} chain lengths
415 (R^2 = 0.77, 0.93 and 0.99, respectively). Thus, as the log K_{ow} of CPs have been estimated to increase

from 5.1-8.1 for SCCPs, to 8.7-12.7 for LCCPs (Muir et al., 2000), it confirms the high potential of LCCPs to transfer into eggs. However, as discussed previously, the $COR_{n,x}$ is also driven by the molecular size and shape of the molecule. Beyond a certain limit, that we can relate in this study to the $C_{27\text{-}30}/Cl_{9\text{-}11}$ homologues, although the lipophilicity still increases, it is unlikely that the homologues cross the intestinal barrier.

Overall, the transfer capability of CP homologues was closely related to their chemical structure and related lipophilic properties, and caused a variation in the homologue response pattern (§3.3) both in chlorine and chain length distributions. This is supported by the previously observed shift towards longer chains and higher chlorination degree for animal-origin products compared to vegetables (Li et al., 2020). This profile shift is interesting because the differences between CP technical mixtures and CP patterns in animals could be indicators of the CP bioaccumulation/biomagnification in animals, although the contribution of the animals' environments (e.g. air, soil) need to be taken in account.

4. Concluding remarks and perspectives

In the present study, the transfer of CPs to hen eggs after dietary exposure during 91 days was studied, at both the sum CPs and homologue levels. Two main particular points stand out from this study: first, the transfer capability of CP homologues increased with the chain length and the degree of chlorination, hence variating the response pattern between feed and egg matrices - with consequences on quantification accuracy to be expected. Second, LCCPs with low chlorine content were found highly concentrated in eggs compared to the other CP subcategories, confirming their bioavailability and capacity for transfer to eggs, while so far this type of CPs remains relatively poorly studied. Unfortunately, the experimental design did not allow the study of the depuration rate through egg excretion route.

Recently, the EFSA proposed a first decision on CPs risk assessment, emphasising that it was based on relatively few data and thus acknowledging the relatively low robustness of the evaluation (EFSA

442 CONTAM panel, 2020). According to this assessment, benchmark doses were estimated at 2.3 and 36
443 mg/kg bw/day for SCCPs and MCCPs, respectively. The margin of exposure calculated based on
444 available European occurrence data at the time indicated no health effect for both types of CPs. In
445 our study, we exposed hens to 5×26 µg/day of CPs in total, which is equivalent to 0.13 mg/kg bw/day,
446 and observed a physiological change between the exposed and the control hen eggs. Although we do
447 not know whether this change is related to one CP type in particular or to the global exposure as a
448 whole, nor if it reflects a toxic effect, it does indicate an incidence of CPs exposure in the hen
449 metabolism. Hence exposure and accumulation studies should be further performed to improve the
450 CPs risk assessment.
451 The target analysis strategy used in this study did not allow the investigation of potential
452 biotransformation products, though CPs were demonstrated to degrade into shorter CPs or even
453 metabolize into their hydroxylation, aldehyde or carboxylated products (HO-CPs, CO-CPs or COOH-
454 CPs) (He, 2019). This kind of information could reveal new potential molecules to target in the goal of
455 CPs dietary exposure assessment.
456 Finally, in order to complete the present study, excreta and various hen compartments (body fat,
457 liver, muscle, blood) were sampled and will provide information on related accumulation ratios and
458 mass balance.
459

460 **Acknowledgements**

461 The authors acknowledge the French Ministry of Agriculture and Food, General Directorate for Food
462 (DGAL) for its financial support. The authors are grateful to (i) Juliane Glüge and Lena Schinkel for
463 kindly providing the I-42 technical mixture, (ii) to the technical staff of the experimental unit PEAT
464 (INRAE, Nouzilly, France) and particularly Nicolas Besné and Philippe Didier for the preparation of
465 feed, rearing hens and collecting eggs, (iii) Thierry Bordeau and Pascal Chartrin for their implication in
466 sample collection, as well as (iv) to Lucie Ravot and Daniela Magalhães for help in sample
467 preparation.

468

469 **Conflict of interest**

470 The authors declare no financial or personal competing interest.

471

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Figure captions

Figure 1.

2D-map representing 95% of the total areas of CP homologue signals in feed (n=3) and the eggs for control (n=1) and exposed (n=1) groups at day 89. Blue and dashed green lines: egg and feed, respectively; Blue dot and green diamond: signal barycentre for egg and feed, respectively; MW: molecular weight.

Figure 2.

Concentrations of CP subcategories in eggs in the exposed group relative to the control group according to time. Individual variability was assessed at day 90.

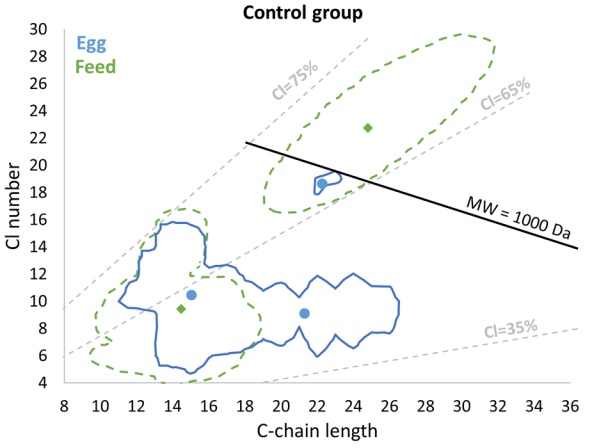
Figure 3.

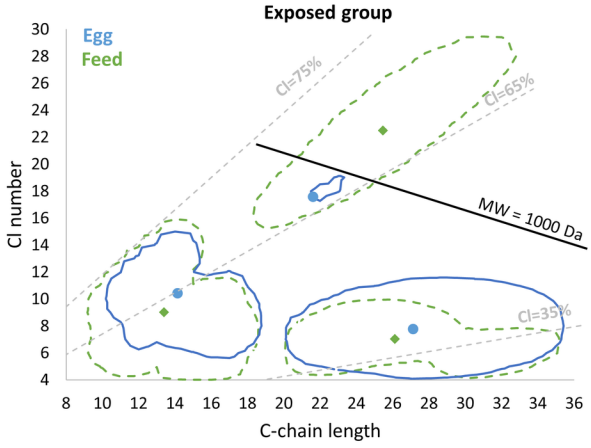
3D-representation of the homologue-level carry-over rates ($COR_{n,x}$).

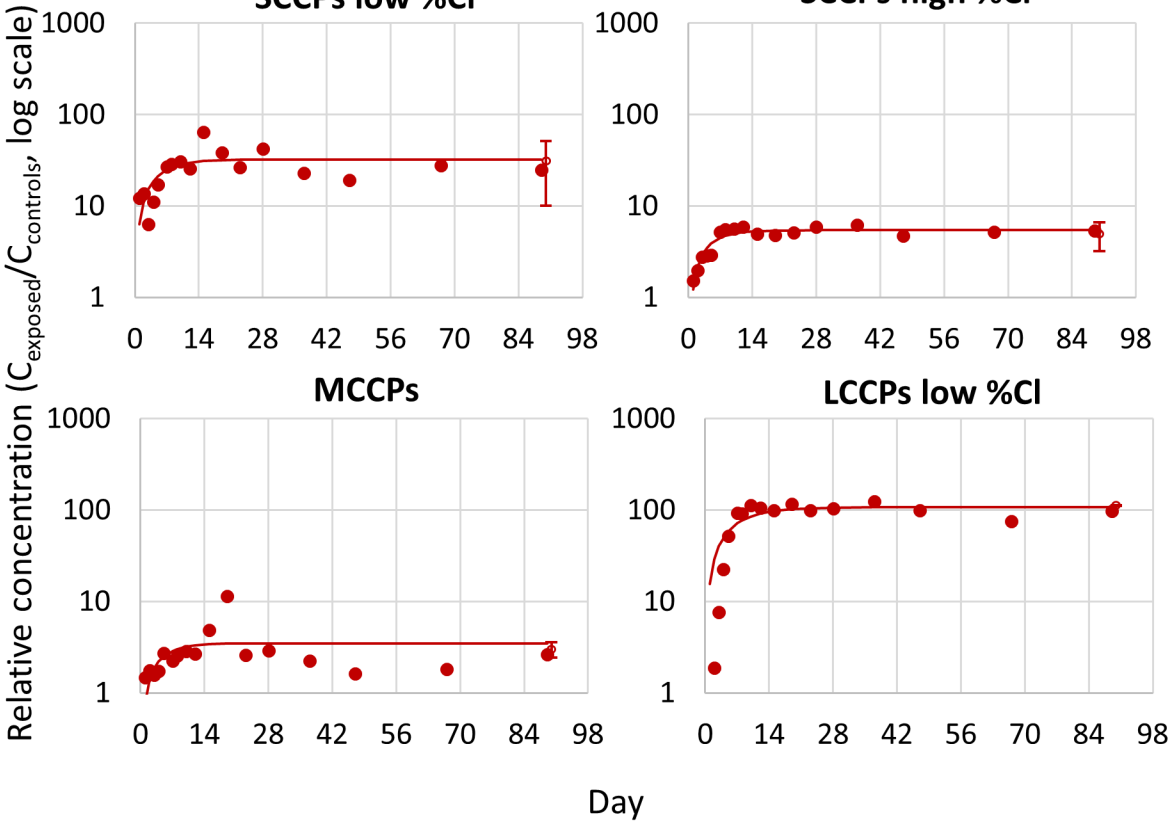
Table 1.

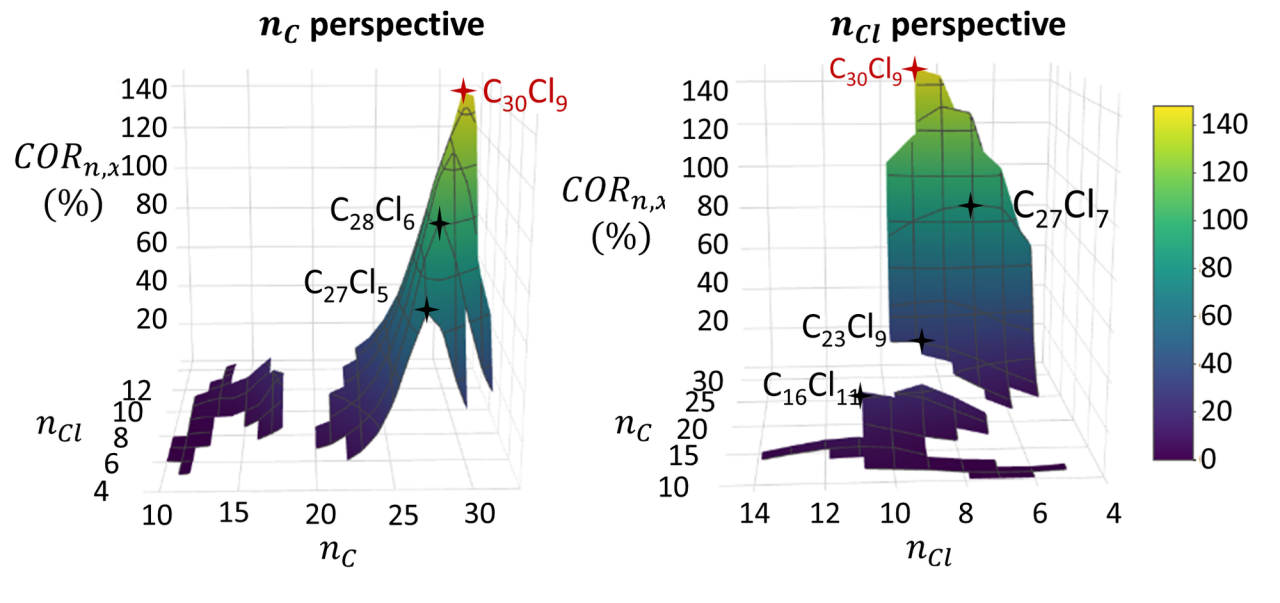
Parameter *a* of the least-squares approximation with a non-negative constraint, of equation $[\mathrm{S}] = a \times [\mathrm{M}] + b$, for the feed (mean) and eggs (day 89) of the control and exposed groups compared to the exposure mixture.

	Control group		**Exposed group**	
	Feed	**Eggs**	**Feed**	**Eggs**
Entire CP profile	0.59	0.70	0.94	0.56
SCCPs low %Cl	0.59	0.77	0.98	0.88
SCCPs high %Cl	0.67	0.80	0.96	1.02
MCCPs	0.81	0.51	0.91	0.57
LCCPs low %Cl	0.50	0.40	0.94	0.87
LCCPs high %Cl	0.84	0.90	0.92	0.52


Control group
Egg
Feed
Cl=75%
Cl=65%
Cl=35%
MW = 1000 Da
Cl number
C-chain length


Exposed group
Egg
Feed
Cl=75%
Cl=65%
Cl=35%
MW = 1000 Da
Cl number
C-chain length


SCCPs low %Cl
SCCPs high %Cl
MCCPs
LCCPs low %Cl
Relative concentration (C_exposed/C_controls, log scale)
Day
1000
100
10
1
0
14
28
42
56
70
84
98


n_C perspective
$COR_{n,x}$ (%)
140
120
100
80
60
40
20
$C_{30}Cl_9$
$C_{28}Cl_6$
$C_{27}Cl_5$
n_{Cl}
12
10
8
6
4
10
15
20
25
30
n_C
n_{Cl} perspective
$C_{30}Cl_9$
$COR_{n,x}$ (%)
$C_{27}Cl_7$
$C_{23}Cl_9$
$C_{16}Cl_{11}$
n_C
30
25
20
15
10
14
12
10
8
6
4
n_{Cl}
140
120
100
80
60
40
20
0