Carry over of PCDD/F and dl-PCBs from contaminated grass pellets into liver and fatty tissues of growing sheep

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Introduction

Dioxins (PCDD/F) and polychlorinated biphenyls (PCBs) are well known contaminants of the environment. The term Dioxins describes a group of dibenzo-p-dioxins and dibenzofuranes with 75 and 135 different chlorinated congeners. They are unintentional by-products, for example created during the production and use of leaded fuel, the industrial production of PCBs until the 1980s, chlorophenols as herbicides and fungicides, as well as sintering and refuse incineration. Contrary, PCBs are intentionally produced and widespread used in electronical, chemical and textile industry as hydraulic fluids, industrial oils, resolvents, flame retardants and plasticisers. Both groups of substances are lipophilic and persistent and therefore accumulative in the food chain. Food of animal origin is the main source for the consumer intake of these substances. Due to both, their accumulative and toxicological potentials, numerous toxicological reference values were defined and EU has set maximum levels for products of animal origin for these substances. For PCDD/F and dl-PCB congeners of toxicological relevance toxicity equivalence factors (TEF) are derived, reflecting their toxic potential in relation to 2,3,7,8 tetra-chlorodibenzo-p-dioxin. PCDD/F and dl-PCBs always occur together in technical mixtures. To assess their toxicological potential the toxic equivalent (TEQ) as the sum of the TEF multiplied by congener concentration, is calculated. Contrary to the decreasing background contamination levels, products from sheep and especially sheep liver often exceed the maximum levels set in Regulation (EC) No. 1881/2006[1]. Toxicokinetic findings and carry over data concerning dioxins, dl-PCBs and ndl-PCBs in sheep are published in a small number only[2,3]. Therefore the Federal Institute for Risk Assessment (BfR) and RIKILT Institute Wageningen performed a research study, where growing sheep were fed with grass pellets (GP) with background contamination of PCDD/F and dl-PCBs (blGP) and higher contaminated grass pellets (conGP), produced after a flooding on a riverside.

Materials and methods

48 male blackfaced sheep aged between 8 to 10 weeks were housed in on the experimental facilities of BfR. The animals were divided into 3 experimental groups with differing feeding concepts over 112 days. A settling period of 2 weeks was performed to accustom the sheep to pelleted feeding material and individual feeding. Slaughtering of 4 sheep after the settling period and directly before the first feeding during the experimental period shows the background contamination levels in liver and fatty tissues. The control group (n=8) was fed with blGP (0.27ng PCCD/F TEQ/kg, 0.06ng dl-PCB TEQ/kg, 454ng ndl-PCB/kg) and was slaughtered on days 56 and 113. The sheep of experimental group I (n=32) were fed 55 d with 5fold higher conGP (1.71ng PCDD/F TEQ/kg, 0.32ng dl-PCB TEQ/kg, 2328ng ndl-PCB/kg), followed by feeding with blGP for 57 d. The experimental group II (n=4) got conGP for 112d. ConGP were selected after a positive finding in the National Monitoring Program on feed and feed ingredients in the Netherlands. The animals were restrictively fed with 1 kg GP (as fed) for 1 hour (h)/d in single stables for sheep of the experimental groups, whereas control sheep were fed together as one group. The individual consumed amount of GP/d was documented after the reweighing of the remains of the feed. The intake of the bedding material was calculated mathematically after the experiment and verified by the scattered in amount of straw per d and sheep (0.5 kg). Weekly body mass development was checked and performed with empty stomach before the feeding. Slaughtering was performed on experimental days 7, 17, 29, 56 and after the changing of the feeding material on continuously numbered days 64, 71, 92 and 113 for 4 sheep on each date respectively with empty stomach. To anesthetize the sheep before their death by cutting the cervical vessels, a captive bolt was used. Samples were collected from liver, Musculus longissimus dorsi = intramuscular fat and kidney fat for all sheep. Omental fat and subcutaneous fat were additionally analyzed for control sheep and experimental group II sheep slaughtered on day 113.
Analyses were performed at RIKILT Institute and BfR, fulfilling the requirements of commission Regulations (EU) No 252/2012 and 278/2012. The sample preparation for the determination of PCDD/F and PCB in fatty

tissues of sheep combines freeze drying, fat extraction by column chromatography and clean up steps by PowerPrep using four different columns (silica gel coated with sulphuric acid, silica gel coated with sodium hydroxide, alumina and active carbon). The isotope labelled analogues of PCDD/F and PCB were added at the beginning of the fat extraction. The final extract was collected as two fractions containing mono-ortho and ndl-PCB for fraction A and PCDD/F and non-ortho PCB for fraction B by eluting with various solvents on the PowerPrep System. The measurements of the samples were performed by High Resolution Mass Spectrometry (HRMS) combined with two Gas Chromatographs (GC), one GC operated with DB-5 column for the analyses of PCDD/F and non-ortho PCB (fraction B) and the other GC with HT-8 column for the determinations of mono-ortho and ndl-PCB (fraction A).

Results and discussion

Figure 1 shows the results of animals of the control group (d 1, 113) and the experimental group II (d 113). During the whole experimental period the sheep nearly doubled their weights. The mean dry matter intake per d of grass pellets and straw was significantly higher in the control group in comparison to the experimental group.

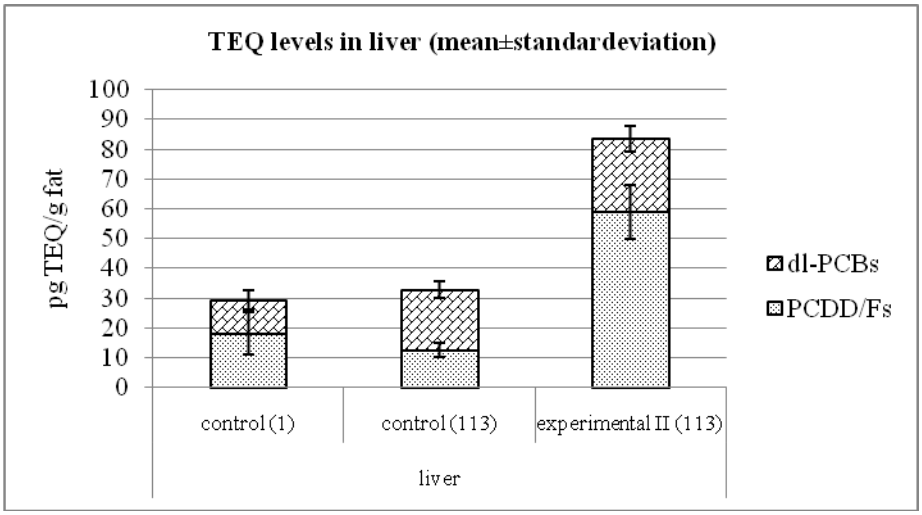

Figure 1: Mean TEQ levels (upper bound = ub), of dl-PCB and PCDD/F in livers of sheep of the control group (1, 113) and experimental group II (113).

The differences of the TEQ levels in liver between the control sheep slaughtered on day 1 and day 113 are significant in TEQ levels of dl-PCBs (p=0.029*) only. The TEQ levels in the liver of the experimental group II exposed over the whole period are significantly higher in the total TEQ (p=0.029*), the dl-PCB TEQ (p=0.029*) and the PCDD/F TEQ (p=0.029*) than the TEQ levels of the control group, respectively. Comparison of both groups slaughtered on d 113, shows significant differences between total TEQ and the PCDD/F TEQ (p=0.029*) only, but for dl-PCBs no significances were detectable (p=0.114). Maximum levels for sheep liver were re-evaluated in winter 2013. As a result the reference parameter was changed from "fat" (Regulation (EU) No. 1259/2011) to "wet weight (ww)" (Regulation (EU) No. 1067/2013). In this context, the maximum level for PCDD/F and sum of PCDD/F and dl-PCB in liver was increased in comparison to the older fat relation. Assessed after Regulation (EU) No. 1259/2011 the investigated livers of all sheep exceed the maximum levels for PCDD/F TEQ and the sum TEQ of PCDD/F and dl-PCBs based on fat, whereas at the assessment in conformity with Regulation (EU) No. 1067/2013 only livers of sheep of the experimental group II (113) exceed the maximum levels for PCDD/F TEQ, sum TEQ and ndl-PCB concentration based on ww (table1).

	Maximum value ww (pg TEQ/g)	Control (113)	Experimental II (113)	**Maximum value fat (pg TEQ/g fat)**	Control 113	Experimental II (113)
PCDD/F	**1.25**	0.57 ±0.1	2.65 ±0.40	**4.5**	12.75 ±2.28	58.92 ±8.86
Sum PCDD/F +	**2.00**	1.47 ±0.18	3.76 ±0.58	**10.0**	32.73 ±3.98	83.50 ±12.87

dl-PCB						
	Maximum level (ng/g wet weight)	Control 113	Experimental 113	**Maximum level (ng/g fat)**	Control 113	Experimental 113
ndl-PCB	**3.00**	1.31 ±0.43	4.43 ±1.38	**75.0**	29.14 ±9.55	98.47 ±30.68

Table 1: Mean TEQ levels (ub) of PCDD/F and sum PCDD/F + dl-PCB as well as mean concentrations of ndl-PCB in livers (mean ± standard deviation) of control group (113) and experimental group II (113) and maximum values with altered reference parameters.

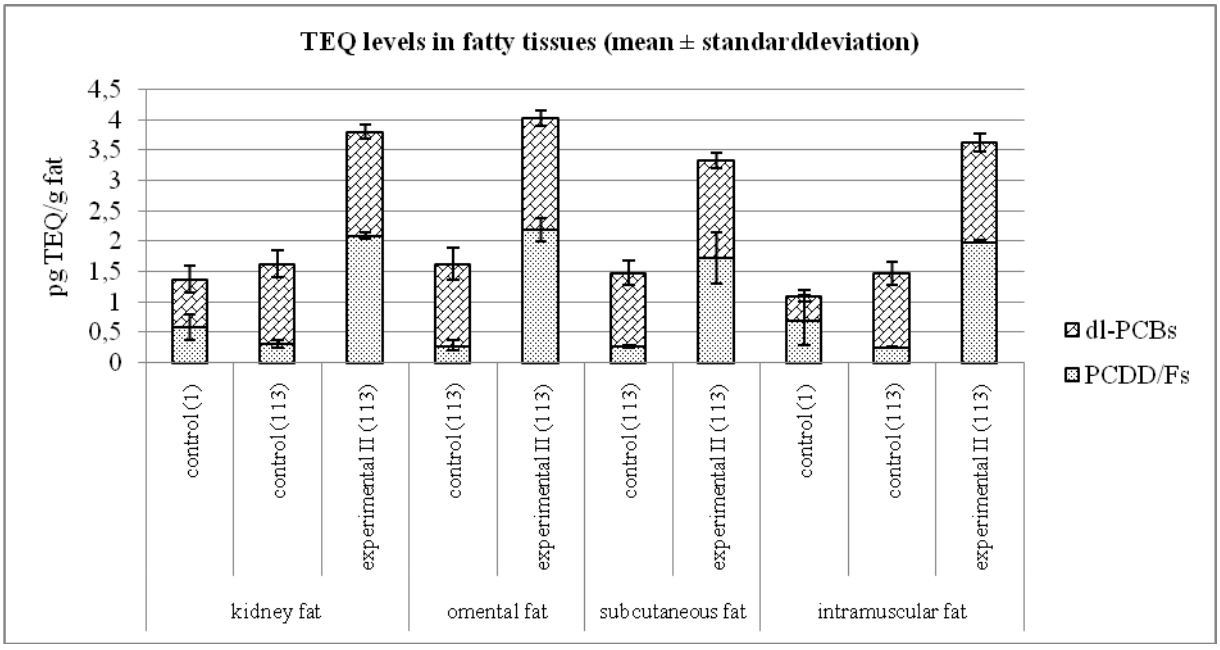

Figure 2: Mean TEQ levels (ub) and the contribution of PCDD/F and dl-PCBs to total TEQ for kidney, omental, subcutaneous and intramuscular fat for the control group (1, 113) and the experimental group II (113).

In fatty tissues of sheep TEQ concentrations are generally lower compared to those of liver. Due to small amounts of fatty tissues in sheep slaughtered on day 1, analyses were made from 3 of 4 animals.
There are only statistically significant differences among the omental fat and the subcutaneous fat for sum TEQ (p= 0.026*) and TEQ of PCDD/F (p=0.040*) of the experimental group II (113), whereas in the control group no significances can be seen for the sum TEQ, as well as the dl-PCB TEQ values and the PCDD/F TEQ values among the fatty tissues. Mann-Whitney Test shows significant differences between the dl-PCB TEQ and the PCCD/F TEQ of control (113) and experimental group II (113) (p=0.050*), respectively.
The high values of dl-PCBs in the control and the experimental group II (113) are a consequence of the high levels of dl-PCBs in the bedding material (0.15ng PCDD/F TEQ/kg, 0.31ng dl-PCB TEQ/kg, 1092ng ndl-PCB/kg) and its intake. Calculated dry matter intake of straw per day was about 0.33±0.035 kg for control group (113) and 0.24±0.038 kg for the experimental group II (113).
One of the most dominant congeners for the composition of TEQ in the straw is dl-PCB 126 due to its high Toxic Equivalence Factor (TEF) (0.1). In straw dl-PCB 126 contribute to about 65% to TEQ sum, in comparison to only 16 % in blank grass pellets and 71% in kidney fat of the control sheep on d 113. The ratio of this congener in absolute concentrations between grass pellets and straw, is higher for straw (control 113: 0.18:1; experimental 113: 0.80:1) and therefore the straw intake has a higher influence for the exposition in both groups. The absolute concentration of this congener shows no significant differences in the intramuscular, subcutaneous and kidney fat between the control and the experimental group II, nevertheless the experimental group shows significantly higher levels in the omental fat as the control group on d 113.
The TEQ values in control sheep slaughtered on day 1 are possibly a reflection of the mother's burden, passed on to the lambs by the milk and during pregnancy via the placenta[4,5]. The milk of sheep is one of the fattest

(7.4%) in comparison to milk of other livestock species, and so it is predestined for higher transfer of lipophilic substances to their suckling offspring[6]. Due to the process of growing and increasing mass of the fatty tissues, PCDD/F body burden of control sheep slaughtered after 112 d of feeding lowered in comparison to the lambs of day 1(p=0,034*), whereas the total TEQ levels didn't show significant differences (p=0,289) [7,8]. Dl-PCBs burden of the fatty tissues increased (p=0,034*) in comparison to sheep of day 1 because of the intake of bedding material. The liver was verified to be the organ of the main accumulation, especially for PCDD/F, whereas dl-PCB shows higher accumulation potentials for the fatty tissues. The significant differences among omental and subcutaneous fatty tissue in the experimental group can possibly be explained by dissimilarities in perfusion, but also by the specific growing rates of these tissues, as mentioned in Kempster et al. 1981[9,10]. They assumed, that subcutaneous fat grows faster than intermuscular fat and growing rates of kidney knob and channel fat are more variable. As shown in cattle, subcutaneous fat is a suitable tissue to predict dioxin and PCBs levels in intramuscular fat, this could be a possibility for growing sheep too, because of no significant differences among subcutaneous and intramuscular fat neither in the control nor in the experimental group II[11].

In conclusion, liver of sheep was verified as the organ of main accumulation especially for PCDD/F whereas the fatty tissues of growing sheep show significant lower levels. The livers of sheep fed with conGP over the whole period exceed the maximum levels set in Regulation (EU) No. 1067/2013, amending Regulation (EU) No. 1881/2006. There are no differences in TEQ levels among the intramuscular and subcutaneous fatty tissues in growing sheep, so the usage of the easily taken subcutaneous fat as a predictor for the TEQ levels in the eatable intramuscular fat might be possible. The effect of growing is shown as a dilution of TEQ levels of PCDD/F in the control sheep slaughtered after 113 d in comparison to those slaughtered at the beginning of the experimental period. The highly dl-PCB contaminated straw used as bedding material has a prominent effect on the exposure of sheep.

Acknowledgements
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