Supplementary Materials

Persistent organic pollutants in human milk of Belgian mothers: levels, time trend and exposure assessment for nursing infants

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1. Study – data collection

Following a guideline for STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) several aspects of an observational study need to be reported. Provided that the manuscript focuses on the presentation of the analytical results, next to the methodology described in the main text hereby we are providing the summary of the questionnaire used in the study. The participants were asked to complete the questionnaire with the following main sections:

a) SELECTION where the status of the participant was identified as "selected" or "reserve"; indication of the consent, birthday, height, weight and foreseen or real delivery date as well as the interest to be informed on the results of the study

b) Nutrition: food frequency questionnaire related to the main food groups, namely fish and fish products, meat, milk, eggs and food supplements

c) USE OF FOLIC ACID: in particular the participants were asked whether they consumed folic acid as a supplement and if they could provide more info on the food supplement

d) Historical data related to the pregnancy and breastfeeding of the mother

e) Socio -economic status and demography of the participants

f) Sampling where the dates were registered and how the milk was acquired (manually, with use of manual pump, with use of electric pump)

2. Fat extraction and fat content determination

Fat extraction was based on the prEN1528-2-1996 method (EN, 1996). To 10 g (+/- 0.05 g) of mother milk, 75 µL of internal PBDEs standard mixture (BDE-77, BDE-128, ^{13}C-BDE-209, and ^{13}C-PeCB), 0.5 mL of saturated NaCl water and 15 mL of a 1:2 mixture of n-hexane:acetone were added in a Falcon Tube of 50 mL. After vortexing for 30 min and centrifugation (10 min at 1700 rpm at 4°C), the upper organic layer was passed through an in-house prepared SPE cartridge containing 2 g Na2SO4. A second extraction was performed by adding 5 mL n-hexane followed by agitation for 10 min and centrifugation (10 min, 3900 rpm at 4°C). The upper organic layer was also transferred to the SPE cartridge and eluted. Further, the organic layer was evaporated first with a Kuderna-Danish and finally under a gentle air stream until 12 mL. From this, an aliquot (1/3 weight) was taken and sent for the analyses of PBDEs, PeCB, and BB-153, whereas the other aliquot (2/3 weight) was kept for OCP analyses. Fat percentage was assessed in each aliquot by solvent evaporation until dryness.

3. Analysis of PBDE, PeCB and BB-153 by GC-MS

The analyses of PBDEs, PeCB, and BB-153 in human milk samples were performed according to the methods described elsewhere (Dimitriadou et al., 2016) with slight modifications. The internal standards (BDE-77, BDE-128, 13C12-BDE-209, and 13C6-PeCB) have been already added to the milk samples before extraction (see section 2.4) and, after fat extraction, the 1/3 weight aliquot of fat extract was shipped to the Toxicological Centre (University of Antwerp). The concentrated extract was subjected to clean-up on 8 g acid silica (44%, w/w) and analytes were eluted with 20 mL hexane-dichloromethane (1:1, v/v). The cleaned extract was concentrated with a rotary evaporator, further evaporated under a nitrogen stream until dryness and reconstituted in 100 µL iso-octane. The extract was transferred to an

injection vial for GC-MS analysis. Quantification of POPs was done using GC-MS operated in electron-capture negative ionization mode (Dimitriadou et al., 2016; Malarvannan et al., 2013).

4. Analysis of HBCD by LC-MS/MS

Composite samples of human milk were prepared according to the WHO protocol and represented the pooled samples for each Belgian province. These samples were kept in glass containers since the first transport until the analyses and during any further storage.

The extraction was done according to the protocol described elsewhere (Dimitriadou et al., 2016; Malarvannan et al., 2013). Internal standards (13C-α-HBCD, 13C-β-HBCD, and 13C-γ-HBCD) were added to 5 g milk (pooled sample) and subsequently concentrated to approximately 0.5 mL under nitrogen. The concentrated extract was subjected to clean-up on 1 g acid silica (44%, w/w) on top of 0.5 g silica. A first fraction was eluted with 10 mL hexane and discarded, after which, a second fraction containing HBCDs was eluted with 10 mL dichloromethane. The second fraction was evaporated under a nitrogen stream until dryness and reconstituted in 100 µL methanol. The extract was transferred to an injection vial for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

The LC-MS/MS analysis was done on an Agilent 1200-6410 LC system coupled to mass spectrometer. The separation of HBCD isomers was done on Kinetex C18 (100 x 2.1 mm, 2.6 µm) column. The MS was operated in electrospray ionization mode. The acquisition was performed using the MRM mode. Two MRM transitions were monitored for the native HBCDs and for 13C-HBCDs used as internal standards. Following the validation of the method, the limit of quantification (LOQ) for each HBCD isomer was 5 pg/mL milk. Method precision and accuracy were tested by the analysis of standard reference material (SRM) 1945 (indicative values for HBCDs in whale blubber).

5. POPs listed to be analysed in national human milk sample

In UNEP-coordinated Survey of Human Milk for Persistent Organic Pollutants Guidelines for Organization, Sampling and Analysis a list of POPs identified in the Stockholm Convention (parent compounds and relevant transformation products) has been provided. These POPs have to be determined by the reference laboratory (CVUA) in the representative pooled national human milk samples

Compounds to be analysed in pooled national mothers milk samples by CVUA	
Initial POPs	
Aldrin	Aldrin
Chlordane	*cis*- and trans-chlordane; and *cis*- and trans-nonachlor, oxychlordane
DDT	4,4'-DDT, 2,4'-DDT and 4,4'-ODE, 2,4'-ODE, 4,4'-00 0, 2,4'-000
Dieldrin	Dieldrin
Endrin	Endrin
HCB	HCB
Heptachlor	Heptachlor and heptachlorepoxide
Mirex	Mirex
PCB	PCB_6(6 congeners):. 28, 52, 101, 138, 153, and 180; PCB with TEFs* (12 congeners): 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189
PCDD/PCDF	2,3,7,8-substituted PCDD/PCDF (17 congeners)
Toxaphene	Congeners P26, P50, P62
POPs listed at COP-4	
Chlordecone	Chlordecone
a-HCH	a-HCH
6-HCH	13-HCH
y- H CH	y-HCH
Hexabromobiphenyl	PBB 153
Pentachlorobenzene	PeCBz
c-penta BOEc-octa BOE	BOE 47, 99, 153, 154, 175/183 (co-eluting) Optional: BOE 100
POPS listed at COP-5	
Endosu lfan	a-, -endosulfan; and endosulfan sulfate
POPs listed at COP-6	
HBCD	a-HBCD, j3-HBCD, y-HBCD

* PCB with TEFs (Taxie Equivalency Factors) assigned by WHOin 1998

6 . Quality assurance/quality control

Efficiency of extraction, clean-up, and fractionation steps was evaluated by measurement of the absolute recoveries of the internal standards. Identification of the peaks was based on the following criteria: (1) the retention time matching that of the calibration standards within ±0.1 min and (2) ion ratio from sample extracts within 30% of average for the calibration standards. LOQ was calculated as three times the standard deviation of the mean of the blank measurements. Procedural blanks were analysed simultaneously with every batch of twenty samples to check for interferences or contamination from reagents and consumables. Procedural blanks were consistent (RSD < 30%) and therefore the mean value was calculated for each compound and subtracted from the values in the samples. LOQs ranged between 3 pg/mL milk for PBDEs and BB-153 and 20 pg/mL for PeCB. The analytical procedures (in particular for HBCD, PBDE's, and PeCB, BB-153) were validated through the analysis of standard reference material (SRM 1945 containing OCPs and PBDEs in whale blubber) for which deviations from certified values were < 15 % (Supplementary Table S3). To test the accuracy of fat determination, milk samples with a known lipid content purchased from a supermarket were used (Supplementary Table S4).

Supplementary Table 1. Human milk samples used for the analyses of POPs in Belgium in 2014.

	Inhabitants*		Participants of the POPs study (n_{total} = 206)		Samples used to prepare Belgian pooled sample (n_{total}=50)	
Geographical parameter	n	%	n	%	n	%
Flemish region (provinces)						
Antwerp	1 764 773	16.1	30	14.6	7	14.0
East Flanders	1 445 831	13.2	25	12.1	6	12.0
West Flanders	1 164 967	10.6	21	10.2	5	10.0
Limburg	844 621	7.7	15	7.3	5	10.0
Flemish-Brabant	1 086 446	9.9	21	10.2	5	10.0
Walloon region (provinces)						
Brabant-Wallon	382 866	3.5	11	5.3	3	6.0
Namur	476 835	4.4	11	5.3	3	6.0
Hainaut	1 317 284	12.0	24	11.7	6	12.0
Liège	1 077 203	9.8	18	8.7	5	10.0
Luxembourg	271 352	2.5	8	3.9	1	2.0
Brussels Capital Region	1 119 088	10.2	22	10.7	4	8.0

*(n = 10 951 266 in Belgium, Jan 1st 2011, data NIS of NIHS)

Supplementary Table 2. MS parameters for analysis of OCPs.

Analyte	Scan Descriptor						Retention time (min)
	Parent Ion 1 (m/z)	Daughter Ion 1 (m/z)	Collision energy 1 eV)	Parent ion 2 (m/z)	Daughter Ion 2 (m/z)	Collision energy 2 (eV)	
DBOFB (surrogate)	296	227	5				6.64
HCB	249	214	10	284	249	15	7.37
HCH group							
α-HCH	181	145	15	217	181	5	7.26
γ-HCH	181	145	15	217	181	5	7.77
β-HCH	181	145	15	217	181	5	8.21
Chlordane group							
Oxychlordane	115	51	25	115	87	15	10.07
Chlordane-trans	373	266	15	272	237	15	10.68
Chlordane-cis	272	237	15	373	266	20	10.77
trans nonachlor	272	237	15	237	119	25	10.85
DDT group							
o,p' -DDE	246	176	30	248	176	30	10.41
p,p' -DDE	246	176	30	316	246	15	11.06
o,p' -DDD	235	165	20	235	199	5	11.24
o,p' -DDT	235	165	25	235	199	5	11.80
p,p' -DDD-	235	165	25	235	199	5	12.01
p-p'-DDT	235	165	25	235	199	5	11.97

Supplementary Table 3. Accuracy of fat extraction from cow milk samples containing various fat percentages. *measurements in duplicate.

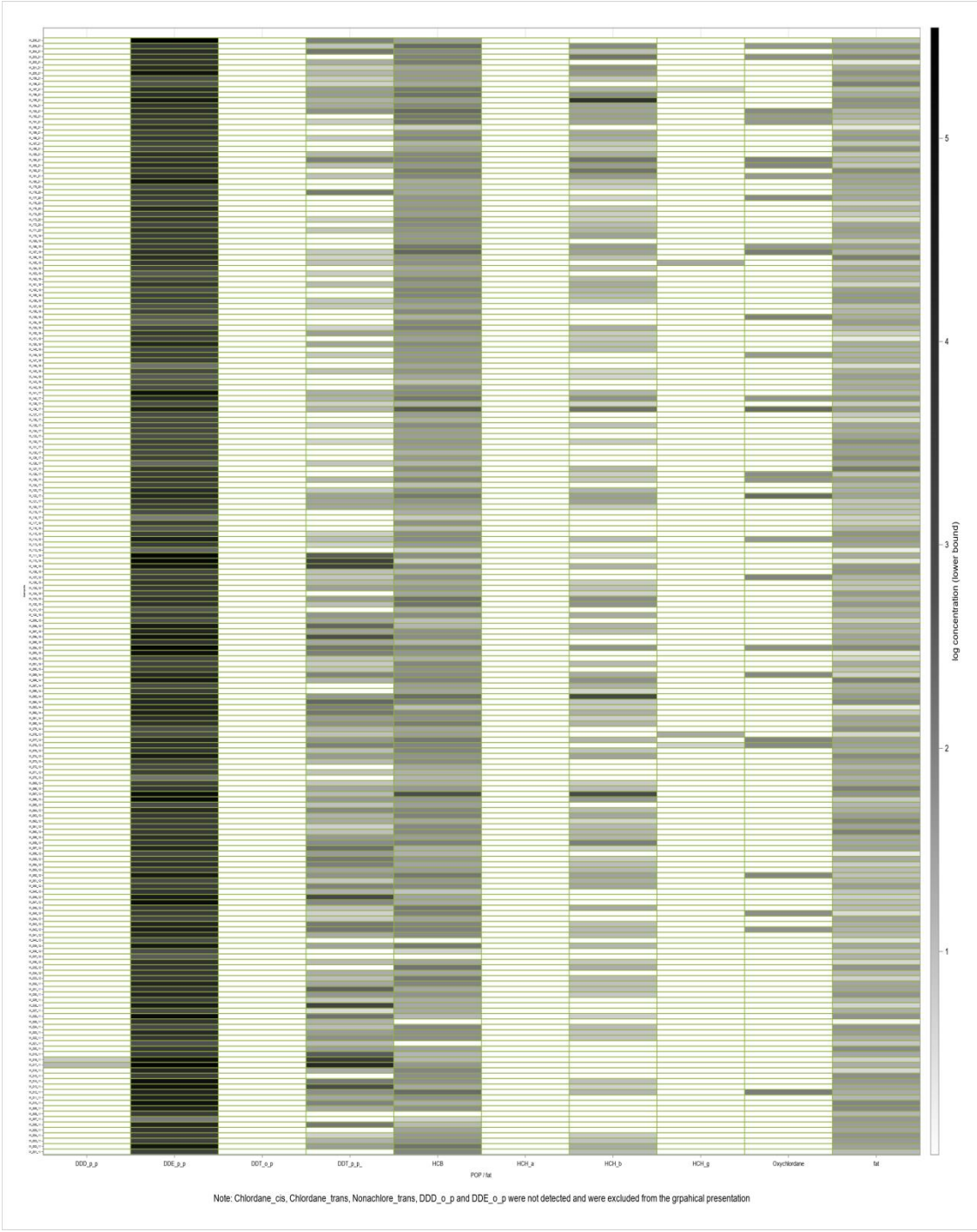
	Theoretical fat (%)	Measured fat (%)*	Accuracy (%)
Milk 1	1.5	1.5; 1.4	95; 90
Milk 2	3.6	3.5; 3.4	98; 94
Milk 3	5.0	5.0; 4.7	99; 93
Milk 4	10.0	9.7; 9.8	97; 98

Supplementary Table 4. Accuracy of standard reference material (SRM 1945). Ww: wet weight; SD: standard deviation.

	Average Concentration (ng/g ww)	SD	Certified Concentration (ng/g ww)	Uncertainty	Accuracy (%)
HCB	32	1	30.6	1.5	103
*p,*p'-DDE	511	5	497	19	103
p,p'-DDT	230	18	233	8	99
TN	187	6	198	16	95
CN	48	3	45.8	3.3	106
BDE-47	38	0	39.6	0.2	96
BDE-100	11	1	10.3	1.1	104
BDE-99	19	2	18.9	2.3	100
BDE-154	12	2	13.3	1.7	92
BDE-153	9	1	8.34	0.55	105

Supplementary Table 5. alpha-HBCD-concentrations (ng/g lw) in 11 pooled samples for the 10 Belgian provinces and for the Brussels Capital Region.

Region/Province	α-HBCD
Antwerpen	3.32
Limburg	3.64
East Flanders	4.97
West Flanders	0.90
Flemish-Brabant	1.61
Brabant-Wallon	2.06
Hainaut	2.46
Liège	2.36
Luxembourg	2.89
Namur	1.80
Brussels Capital Region	1.44
mean	*2.50*
min. – max.	*0.90 – 4.97*



Supplementary Figure 1. Heatmap of detected POPs concentrations in individual samples.

REFERENCES

L. Dimitriadou et al., “Levels and profiles of brominated and chlorinated contaminants in human breast milk from Thessaloniki, Greece,” Sci. Total Environ., vol. 539, pp. 350–358, Jan. 2016, doi: 10.1016/j.scitotenv.2015.08.137.

G. Malarvannan et al., “Distribution of persistent organic pollutants in two different fat compartments from obese individuals,” Environ. Int., vol. 55, pp. 33–42, May 2013, doi: 10.1016/j.envint.2013.02.012.