CONCENTRATION LEVELS OF PCDD/F'S AND DIOXIN-LIKE PCB'S IN HUMAN MILK SAMPLES FROM THE RURAL AREAS OF FLANDERS (BELGIUM).

Croes K[1,5*], Vandermarken T[1], Colles A[2], Bruckers L[3], Koppen G[2], Govarts E[2], Nelen V[4], Van de Mieroop E[4], Schoeters G[2], Van Larebeke N[5], Denison MS[6], Kotz A[7], Van Langenhove K[1], Elskens M[1] , Baeyens W[1]

[1]Vrije Universiteit Brussel, Department of Analytical and Environmental Chemistry (ANCH), Pleinlaan 2, 1050 Brussels, Belgium; [2]VITO, Environmental Health and Risk, Boeretang 200, 2400 Mol, Belgium; [3]Interuniversity Institute for Biostatistics and Statistical Bioinformatics, Hasselt University, Belgium; [4]Provincial Institute for Hygiene, Antwerp, Belgium; [5]Ghent University Hospital, Study Centre for Carcinogenesis and Primary Prevention of Cancer, Ghent, Belgium; [6]Department of Environmental Toxicology, University of California, Davis, CA 95616, USA; [7]State Institute for Chemical and Veterinary Analysis of Food, Bissierstrasse 5, Freiburg, Germany

Introduction:
In the first Flemish Environment and Health survey run by the Flemish Centre of Expertise on Environment and Health (FLEHS I 2002-2006) increased concentrations of PCBs, dioxin-like substances and chlorinated pesticides (a metabolite of DDT and hexachlorobenzene) were observed in cord blood of newborns and in blood of 14-15 year-old adolescents and 50-65 year-old adults living in low populated rural communities of East and West Flanders and Flemish Brabant compared to other Flemish regions. Due to the health concern associated with chlorinated compounds follow-up of pollutant levels in this area is of importance. Therefore, human breast milk from mothers living in these regions was collected (2009-2010) for analysis of different POPs.
For the quantification of PCDD/Fs and/or dioxin-like PCBs in (human) milk samples both GC-HRMS and the CALUX bioassay are used in routine analysis. However, most CALUX methods use high amounts of milk (varying between 10 and 60 mL)[1, 2]. Since for the Flemish human breast milk survey in the rural areas not only PCDD/Fs and dioxin-like PCBs were analyzed, but also other POPs like p,p'-DDE, PBDEs, marker PCBs, HCB, HCH, perfluorinated compounds, etc., the amount of milk available for the each assay was limited. Therefore, the CALUX bioassay was used for the analysis of PCDD/Fs and dl-PCBs in the individual milk samples, while GC-HRMS was used to define the concentration level of a pooled human milk sample.

Materials and methods:
In this study, 5 mL human breast milk samples obtained from 84 18-35 year-old breastfeeding mothers residing in the rural areas of Flanders (24 low-populated rural communities in East and West Flanders), were analyzed for PCDD/Fs and dl-PCBs with a new sensitive CALUX mouse hepatoma cell line. Additionally, from each of the 84 individual samples, 10 mL was taken to compose a pooled sample, which was analyzed with both the CALUX assay (VUB, ANCH, Brussels, Belgium) and by GC-HRMS (WHO reference laboratory, State Institute for Chemical Analysis and Veterinary of Food, Freiburg, Germany). Details about inclusion criteria and characteristics of the study population are described by Colles et al., 2011[3].
CALUX analysis was performed at the VUB, using the methodology described by Croes et al. (2011 and submitted) [4,5]. Methods used for analysis of the pooled milk sample by the WHO reference laboratory (State Institute for Chemical Analysis of Food) in Freiburg, Germany, are described elsewhere [6,7,8].

Results and discussion:
Five millilitre milk samples were analyzed with the CALUX bioassay for all 84 mothers participating in the study. All samples were above the LOQ, both for the PCDD/Fs and the dl-PCBs. The geometric mean PCDD/F and dioxin-like PCB concentrations (raw data) in the total study population were respectively 10.4 (95% CI: 9.4-11.4) pg CALUX-BEQ per g fat or 0.41 (95% CI: 0.37-0.45) pg CALUX-BEQ per g milk for the PCDD/Fs and 1.73 (1.57-1.91) pg CALUX-BEQ per g fat or 0.07 (95% CI: 0.06-0.08) pg CALUX-BEQ per g milk for the dioxin-like PCBs.

Univariate analysis of the raw data showed that participants who lost weight after the pregnancy had significant higher values of dioxin-like PCBs ($p=0.02$) and PCDD/Fs ($p=0.002$) compared to participants who gained

weight. The CALUX-BEQ values increased with the age of the mother (p=0.001 for PCDD/Fs and p=0.048 for dl-PCBs). PCDD/F levels were also higher for mothers consuming local eggs (p=0.023). A non-significant trend was observed between smoking and the measured activity of the dioxin-like PCBs and PCDD/Fs. Levels of dioxin-like activities were higher in milk samples from current smokers (active and passive) compared to non-smokers. This can be expected since dioxins are present in cigarette smoke. For the PCDD/Fs BEQ values of 10.7, 11.7 and 9.85 pg BEQ per g fat were found for respectively active, passive and non-smokers. For the dl-PCBs BEQ values of 1.93, 1.98 and 1.57 pg BEQ per g fat were found for respectively active, passive and non-smokers. The small (non-significant) differences between dioxin levels in active and passive smokers can be due to amount of cigarettes smoked a day. When only taking into account active smoking, higher BEQs (for both PCDD/Fs and dl-PCBs) were found for ex-smokers compared to non-smokers or current smokers. After exclusion of outliers, PCDD/F levels were borderline significantly higher in mothers with lower BMI (p=0.047). No association was found between dl-PCBs and PCDD/Fs on the one hand and the education level of the mother, highest education level in the family and household income on the other hand. Sex of the baby, the number of years living in the study area and parity were also not significantly associated with the concentration of dl-PCBs or PCDD/Fs. It is however expected that the total concentration of POPs is lower in multiparous breastfeeding mothers compared to primiparous breastfeeding women, since dioxin and PCBs are off-loaded to the infant during gestation and lactation. Lorber and Philips (2002)[9] estimated that maternal PCB/dioxin body burdens decrease 20% to 70% during six months of exclusive breastfeeding. This means that a mother will transfer more POPs to her first child compared to the later born children. In our study, also lower CALUX-BEQ values were found in the human milk sample of mother giving birth to a second child compared to the milk samples from primiparous women (2.1 versus 1.8 pg BEQ per g lipid with p=0.20 for dl-PCBs and 12.2 versus 10.5 pg BEQ per g lipid with p= 0.17 for PCDD/Fs).

In *multiple regression analysis* (after correction for predefined confounders and covariates that were significant in a stepwise model) no significant relationships were found for the dl-PCBs. A borderline non-significant relation was found between smoking before pregnancy and PCDD/Fs (p=0.05). The BEQ-levels were higher in ex-smoker compared to current smokers or women who never smoked. For the PCDD/Fs also significant relationships were found with weight change after pregnancy (p=0.003 for ln PCDD/Fs and p=0.0001 for the non-transformed PCDD/Fs in the model, both expressed in pg BEQ per g lipid) and consumption of local eggs. Participants who lost weight after pregnancy relative to their weight before pregnancy had significant higher values of PCDD/Fs compared to participants who gained weight. Weight losses result in a decrease of adipose tissue and thus in a release of POPs into blood and human milk. Participant who never consumed local eggs showed significantly lower PCDD/F BEQ values (expressed per g lipid), with p-values of respectively 0.05 and 0.04 for the Ln-transformed and the non-transformed exposure marker.

The CALUX-BEQ values on the pooled breast milk sample were in good agreement with the GC-HRMS data for both the PCDD/F and the dl-PCB fractions (Table 1). The PCDD/F BEQ values were 32 % and 60 % higher with CALUX compared to GC-HRMS, when using respectively the 1998 and 2005 TEF scheme. For the dl-PCBs a better agreement was found between the two techniques with the 2005 TEF scheme (a CALUX/GC-HRMS ratio of 0.58 compared to 0.37 for the 1998 TEFs). As already reported by various researchers, the lower dl-PCB values are probably due to the difference between the WHO-TEFs and the CALUX-REPs, while the differences obtained for the PCDD/Fs are probably due to other AhR agonists. The concentrations levels (in pg per g lipid) for some PBDD/F congeners, analyzed in the pooled human milk sample with GC-HRMS, are shown in Table 2. Although only a few congeners were measured, it is clear that these compounds have a relatively low contribution to the total TEQ/BEQ. When using the same TEF/REP values as for the chlorinated analogs, the TEQ would raise 0.42, 0.34, 0.41 pg TEQ per g lipid (medium bound, using the WHO-TEFs of 1998 or 2005, or the CALUX-REPs from Brown et al. (2001)[10], respectively). This would mean an increase of only 5 % to the total PCDD/F WHO-TEQ value and an adapted CALUX/GC-HRMS ratio of 1.26/1.53/1.24.
In a human milk study in Sweden (samples collected in 2002-2003), also low concentration levels of brominated compounds were found. The levels of 2,3,7,8-TBDF and 2,3,4,7,8-PeBDF were detected at concentrations of respectively 0.55 pg per g fat and 0.33 pg per g fat, which is comparable to the levels found in the Flemish pooled milk sample. 1,2,3,7,8-PeBDF was not detected in the Swedish milk samples (Flemish rural sample: 0.2 pg per g lipid), while 1,2,3,4,7,8-/1,2,3,6,7,8-HxBDF was found at a concentration of 3.8 pg per g fat (Flemish

rural sample: below LOQ)[11]. Kotz et al. (2005)[12] reported mean upper and lower bound PBDD/F TEQ levels of 1.08 and 0.32 in human milk samples from the third round of the WHO human milk survey (2001-2002). Dominant PBDD/Fs congeners were 2,3,7,8-TBDF (average concentration 0.7 pg per g fat, range < 0.1 - 2.7 pg per g fat) and 2,3,4,7,8-PeBDF (average 0.23 pg per g fat, range < 0.1 - 1.1 pg per g fat). Some other congeners, e.g. 2,3,7,8-TBDD (concentrations 0.06 - 0.28 pg per g fat), 1,2,3,7,8-PeBDD (0.14 - 1.0 pg per g fat), 1,2,3,7,8-PeBDF and 1,2,3,4,7,8-HxBDF could only be quantified in some of the samples. The PBDD/Fs contributed about 12 % to the total WHO-TEQ. In human adipose tissue, PBDD/Fs were found in a Swedish study from 2007. Depending on the TEFs/REPs used for calculating the upper and lower bound TEQ, the PBDD/Fs contributed 1 - 15 % to the total PCDD/F WHO-TEQ[11].

The concentrations of the PCDD/Fs and dl-PCBs measured in the pooled sample of the rural population in 2009-2010 by the WHO reference lab (CVUA, Freiburg, Germany) followed the ongoing declining trend that was observed in the WHO-coordinated human milk surveys (1989-2006).
In all Belgian campaigns, it was seen that the concentrations of dl-PCBs in human milk were much lower than the concentration levels of the PCDD/Fs. When comparing the Flemish PCDD/F levels, measured in the rural area, to the results from other European countries participating the 4th WHO human milk survey from 2006 (Figure 1), the Belgian levels are still quite high despite the declining trend[13]. The dl-PCBs levels measured in this study were lower compared to other European and non-European studies[14].

Acknowledgements:
The authors acknowledge all mothers who kindly accepted to participate to this study, as well as the fieldworkers, midwifes, gynecologists, doctors and directors of the participating maternities, whose cooperation was essential for the successful results of this study.
This study was commissioned, financed and steered by the Environment, Nature and Energy Department of the Flemish government.
The authors are grateful to M. Denison and the other members of the University of California-Davis (USA) for generously providing our laboratory with the H1L7.5c1 cell lines which was developed with funding from a Superfund Research Program grant (ES04699) from the National Institute of Environmental Health Sciences.

References:
1. Leng J-H, Kayama F, Wang P-Y, Nakamura M, Nakata T, Wang Y. (2009); *Chemosphere* 75: 634–639
2.Van Overmeire I, Van Loco J, Roos P, Carbonnelle S, Goeyens L. (2004); *Talanta* 63: 1241–1247
3. Colles A, Koppen G, Van De Mieroop E, Covaci A, Croes K, Kotz A, Mampaey M, Schoeters G. (2011); *Organohalogen Compounds* 73: 1555-1558.
4. Croes K, Vandermarken T, Colles A, Bruckers L, Koppen G, Govaerts E, Nelen V, Van de Mieroop E, Schoeters G, Van Larebeke N, Denison M, Kotz A, Van Langenhove K, Elskens M, Baeyens W. (2011); *Organohalogen Compounds* 73:1582-1585.
5. Croes K, Colles A, Koppen G, Govarts E, Bruckers L, Van de Mieroop E, Nelen V, Covaci A, Dirtu A.C, Thomsen C, Haug L.S, Becher G, Mampaey M, Schoeters G, Van Larebeke N, Baeyens W. (2012); *Chemosphere* submitted.
6. Malisch R, van Leeuwen FXR. (2002); *Organohalogen Compounds* 56: 317-320
7. Kotz A, Malisch R, Kypke K, Oehme M. (2005); *Organohalogen Compounds* 67: 1540-1544
8. Hui LL, Hedley AJ, Kypke K, Cowling BJ, Nelson EAS, Wong TW, van Leeuwen FXR, Malisch R. (2008); *Chemosphere* 73: 50-55
9. Lorber M., Phillips L. (2002); *Environmental health perspectives* 110: A325.
10. Brown D.J, Chu M.D, Van Overmeire I, Chu A, Clark G. (2001); *Organohalogen Compounds* 53: 211-214.
11. Ericson Jogsten I, Hagberg J, Lindström G, Bavel B. (2010); *Chemosphere* 78: 113-120.
12. Kotz A, Malisch R, Kypke K, Oehme M. (2005); *Organohalogen Compounds* 67: 1540-1544.
13. World Health Organization (2009); *Fact Sheet 4.3*; European Environment and Health Information System (ENHIS)
14. Malisch R, Kypke K, Van Leeuwen R, Moy G, Park S. (2008); *Organohalogen Compounds* 70, 228-232.

pg WHO-TEQ/g lipid	GC-HRMS	GC-HRMS	Mean	Ratio: mean

and pg BEQ/ g lipid	(TEF 1998)	(TEF 2005)	CALUX-BEQ (n=2)	CALUX/GC-HRMS (TEF 1998/2005)
PCDD/Fs	8.41	6.95	11.09	1.32/1.60
dl-PCBs	5.80	3.77	2.17	0.37/0.58
Total dl-compounds	14.21	10.72	13.26	0.93/1.24

Table 1: The CALUX-BEQ and GC-HRMS WHO-TEQ values, expressed per g lipid, for the PCDD/F and dl-PCB fractions of a pooled human milk sample. TEF values from 1998 and 2005 were used.

Polybrominated dioxins (pg/g lipid)		Polybrominated furans (pg/g lipid)	
2,3,7,8-TBDD	0.05	2,3,7,8-TBDF	0.7
1,2,3,7,8-PeBDD	<0.07	1,2,3,7,8-PeBDF	0.2
		2,3,4,7,8-PeBDF	0.4
1,2,3,4,7,8/1,2,3,6,7,8-HxBDD	<0.07	1,2,3,4,7,8/1,2,3,6,7,8-HxBDF	<0.3
1,2,3,7,8,9-HxBDD	<0.04	1,2,3,7,8,9-HxBDF	Na
		2,3,4,6,7,8-HxBDF	Na
1,2,3,4,6,7,8-HpBDD	Na	1,2,3,4,6,7,8-HpBDF	Na
		1,2,3,4,7,8,9-HpBDF	Na
OBDD	Na	OBDF	Na

Table 2: Overview of the concentration levels (in pg/g lipid) of PBDD/F congeners, measured in the pooled human milk sample. < are concentration levels below the LOQ. These concentrations were set at half the LOQ for TEQ determination. Na= result not available.

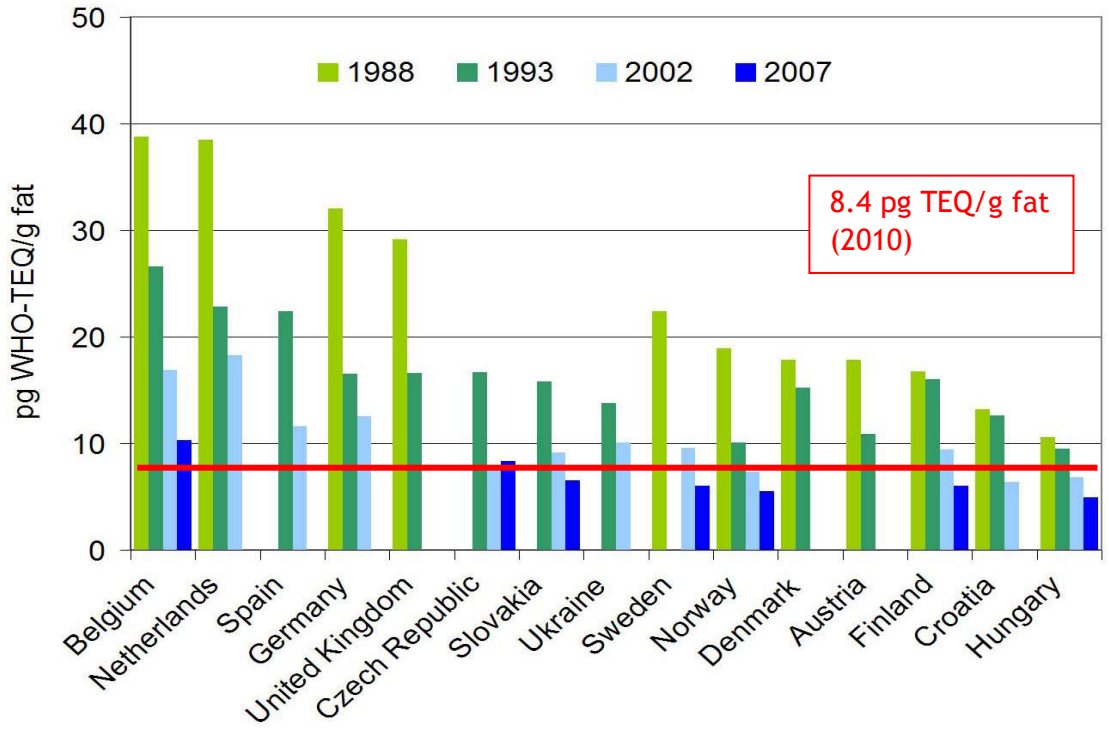

Figure 1: overview of the PCDD/F results of the four WHO-coordinated human milk surveys in different European countries, and comparison with the TEQ found in this study. Measurements were done with GC-HRMS and WHO-TEFs from 1998 were used the calculate the TEQ (World Health Organization, 2009).