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Endocrine actions of pesticides measured in the Flemish environment and health studies (FLEHS I and II)

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Abstract Within the Flemish Environment and Health studies (FLEHS I, 2002–2006, and FLEHS II, 2007–2012), pesticide exposure, hormone levels and degree of sexual maturation were measured in 14–15-year-old adolescents residing in Flanders (Belgium). In FLEHS II, geometric mean concentrations (with 95 % confidence interval (CI)) of 307 (277–341) and 36.5 ng L^{-1} (34.0–39.2) were found for *p,p′*-dichlorophenyldichloroethylene (*p,p′*-DDE) and hexachlorobenzene (HCB). These values were respectively 26 and 60 % lower than levels in FLEHS I, 5 years earlier. Metabolites of organophosphorus pesticides (OPPs) and of *para*-dichlorobenzene were measured for the first time in FLEHS II, yielding concentrations of 11.4, 3.27 and 1.57 μg L^{-1} for the sum of dimethyl- and diethyl phosphate metabolites and 2,5-dichlorophenol (2,5-DCP), respectively. Data on internal exposure of HCB showed a positive correlation with sexual maturation, testosterone and the aromatase index for boys and with free thyroxine (fT4) and thyroid stimulating hormone (TSH) (both boys and girls). For both *p,p′*-DDE and HCB, a negative association with sexual development in girls was found. The OPP metabolites were negatively associated with sex hormone levels in the blood of boys and with sexual maturation (both boys and girls). The pesticide metabolite 2,5-DCP was negatively correlated with free T4, while a positive association with TSH was reported (boys and girls). These results show that even exposure to relatively low concentrations of pesticides can have significant influences on hormone levels and the degree of sexual maturation in 14–15-year-old adolescents.

Keywords Endocrine disruption · Pesticides · FLEHS · Hormones · Human biomonitoring · Sexual development

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Introduction

Flanders is one of the most densely populated areas in Europe, with a dense network of traffic roads, industrial activities and intensive farming close to habitation. The Flemish Environment and Health Study (FLEHS) of 1999, a preliminary small-scale biomonitoring study, provided evidence that levels of internal exposure to pollutants were different between a rural and an urban area and that differences in pollutant levels were associated with significant changes in effect

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markers (Den Hond et al. 2002; Van Den Heuvel et al. 2002; Koppen et al. 2002; Staessen et al. 2001; Van Larebeke et al. 2006). To investigate the complex relation between environmental pollution and human health, the Centre for Environment and Health in Flanders (Belgium) started in 2002 a human-biomonitoring programme (FLEHS I, 2002–2006). For 1,679 adolescents, residing in nine study areas with differing pollution pressure, exposure to the pesticide metabolite *p,p′*-dichlorophenyldichloroethylene (*p,p′*-DDE) and hexachlorobenzene (HCB), hormone levels and the degree of sexual maturation were measured. Possible confounding effects of lifestyle and personal characteristics were taken into account (Schroijen et al. 2008). In 2007, a second cycle of the Flemish human-biomonitoring programme (FLEHS II, 2007–2011) started. The main purpose was to generate reference values for several biomarkers, both of exposure and of effect, and establish dose–effect relationships. In this survey, in addition to the pesticides *p,p′*-DDE and HCB, also metabolites of organophosphate pesticides (OPPs) and *para*-dichlorophenol (2,5-DCP), a metabolite of *para*-dichlorobenzene, were measured.

The pesticides HCB and DDT are banned in Belgium since 1974, but due to their persistence in the environment, they are still pollutants of concern (Covaci et al. 2005). In the human body, these pesticides accumulate in lipid tissue, and, due to their long biological half-life times, they can still be measured in the blood/urine of a high proportion of the Flemish population. HCB is a known anti-estrogenic compound, while *p,p′*-DDE is anti-androgenic (Lemaire et al. 2004). Furthermore, DDT and several metabolites were reported to inhibit the aromatase activity in some cell types at high doses, while at lower concentrations, a stimulation of the aromatase activity was seen (Whitehead and Rice 2006). OPPs are active against a broad spectrum of insects. Several commercial formulations are also frequently used by the general population. They are metabolized in the human body to dialkyl (methyl or ethyl) phosphate metabolites (Barr et al. 2004). Measurement of these metabolites in urine reflects recent exposure. OPPs are especially known to be neurotoxic (Kamanyire and Karalliedde 2004; Wessels et al. 2003) but have also endocrine-disrupting effects. Several compounds, including the frequently used pesticide chlorpyriphos, show estrogenic activity (Kojima et al. 2004; Raun Andersen et al. 2002). A study from Kojima et al. (2004) found that 19 out of 56 tested OPPs were anti-androgenic. The chemical *para*-dichlorobenzene is used in moth balls, in toilet deodorizers and, previously, as an insecticidal fumigant. It is persistent in the environment, accumulates in lipid tissue and is classified as possibly carcinogenic (class 2B) by the International Agency for Research on Cancer (IARC) (IARC 1998). The metabolite *para*-dichlorophenol has a short half-life time in the human body and is excreted in urine over several days. To our knowledge, *p*-dichlorophenol has not been tested for potential estrogenic or androgenic activity. However, many organohalogens, including *p,p′*-DDE, HCB and *p*-dichlorobenzene, are known to affect the circulating thyroid hormone levels (Kodavanti and Curras-Collazo 2010).

The goals of this paper are to give an overview of the pesticide concentrations, measured in the serum and urine of the Flemish adolescents between 2003 and 2011, and to investigate the association with possible health effects on endocrine disruption.

Methods and materials

Selection and recruitment of the participants

In both the FLEHS I and II studies, 14–15-year-old adolescents were recruited in Flanders (Belgium). In FLEHS I, 1,679 adolescents were recruited in nine areas in Flanders with a different pollution pressure (two industrial sites, two harbours, two cities, a rural area, a zone around waste incinerators and a fruit cultivating area). In FLEHS II, a representative sample of the general population (n=210) and inhabitants of two industrial hotspots (n=396) were recruited. Sampling was performed between October 2003 and July 2004 (FLEHS I) and between May 2008 and February 2011 (FLEHS II). For both surveys, inclusion criteria were (1) residing at least 10 years in Flanders or at least 5 years in the selected hotspot, 2) giving written informed consent and (3) being able to fill in an extensive Dutch questionnaire.

The study design was approved by the medical–ethical committee of the University of Antwerp.

More detailed information on the selection and recruitment of the participants in the FLEHS I and II studies was described earlier (Schoeters et al. 2012a, b; Croes et al. 2009; Schroijen et al. 2008).

Analysis of exposure and effect markers

During both surveys, the pollutants *p,p′*-DDE and HCB were analyzed in human serum by GC-MS at the University of Antwerp (Belgium), according to the protocols described by Covaci and Schepens (2001) and by Covaci and Voorspoels (2005). The OPP metabolites and 2,5-DCP were analyzed at VITO (Mol, Belgium). OPP metabolite analysis was based on the method of Hardt and Angerer (2000), using derivatization and GC-MS detection. 2,5-DCP was measured with GC-MS after enzymatic treatment with β-glucuronidase, solid phase extraction and derivatization.

The pesticides *p,p′*-DDE and HCB were analyzed in the serum of the adolescents participating the FLEHS I (n=1,679) and FLEHS II (n=606) studies. The OPP metabolites (dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP) and diethyl

dithiophosphate (DEDTP)) were measured only in the urine of the adolescents of the FLEHS II reference campaign (n=210). The OPP metabolites diethyl phosphate (DEP) and diethyl thiophosphate (DETP) and the pesticide metabolite 2,5-DCP were measured during the FLEHS II (n=210) and FLEHS I (eight pooled samples) surveys. The involved laboratories had to fulfil standard quality assurance and quality control (QA/QC). Validation reports were required, and participation to international ring tests was requested. The limit of quantification (LOQ) in urine samples was 3 μg L^{-1} for DMP; 1 μg L^{-1} for DMTP, DMDTP and DETP; 2 μg L^{-1} for DEP and DEDTP and 0.4 μg L^{-1} for 2,5-DCP. The LOQs for HCB and *p,p′*-DDE were 20 ng L^{-1} serum.

All effect markers were analyzed in FLEHS I and FLEHS II, using the same techniques. Commercial immunoassays were used to determine serum levels of total testosterone (Medgenix, Fleurus, Belgium), luteinizing hormone (LH), sex-hormone-binding globulin (SHBG) (Orion Diagnostica, Espoo, Finland) and total 17β-estradiol (Clinical Assay, DiaSorin s.r.l., Saluggia, Italy; adapted protocol with use of double amount of serum). The free fractions of testosterone and estradiol were calculated from the levels of the total testosterone, respectively, estradiol and the SHBG concentration, assuming a fixed albumin concentration (Vermeulen et al. 1999). The sex hormones were only measured in the serum of boys. The thyroid hormones free 3,5,3′-triiodothyronine (fT3), free thyroxine (fT4) and thyroid stimulating hormone (TSH) were determined in the serum of boys and girls by direct chemoluminescence immunoassay on a Modular E170 (T0470) autoanalyzer. fT3 and fT4 assays were labelled antibody methods involving competitive immunoassay; the TSH assay is a two-site sandwich method (Cobas Elecsys Line; Roche Diagnostics, Vilvoorde Belgium). Data on sexual development were provided by the Centre for Guidance of Pupils. The time period between the blood sampling and the health investigation was less than 10 months. In boys genital and pubic hair development was assessed, while in girls breast and pubic hair development was scored using the international scoring criteria of Marshall and Tanner, where 1 is used for the start of puberty, while at stage 5, the adult stage is reached (Marshall and Tanner 1969, 1970). The assessment of pubertal development was performed by experienced school doctors who all had received a specific training designed to standardize the protocols and reduce inter-individual variability.

Statistical data treatment

Geometric means with 95 % confidence intervals were calculated for the reference populations of, respectively, 1,679 (FLEHS I) and 210 (FLEHS II) adolescents using SAS 9.2. To determine the factors that influence the pesticide concentrations, univariate regression relationships were first calculated. To establish reference values for Flanders, linear multiple regression analysis with correction for confounders was performed. Confounders for the pesticides HCB and *p,p′*-DDE were sex, age, BMI, smoking behaviour and amount of blood fat when expressed per volume of serum. Confounders for the OPP metabolites (sum of all ethyl metabolites (sumDE) and sum of all methyl metabolites (sumDM)) and 2,5-DCP were sex, age and concentration of creatinine when expressed per volume of urine.

Dose–effect relationships were established using stepwise multiple regression analysis with correction for pre-defined confounders (that were fixed in the model) and selected covariates. For the binary effect markers (Table 5), logistic multiple regressions were used, while for all other effect markers (Tables 4 and 6), linear multiple regression models were applied. Covariates with a p value below 0.20 in univariate analysis were used in the multiple regression model but only stayed in the model when significant ($p<0.05$). Confounders of testosterone, reaching the adult stage of total and free testosterone (i.e. concentrations >320 and >6 ng dL^{-1}, respectively), estradiol and the aromatase index (ratio testosterone/estradiol) were age, smoking, hour of blood sampling and BMI. The parameters 'illness during the last 14 days' and season were added as covariates to the multiple regression models. Confounders of LH and FSH were age, BMI and smoking. Confounders of SHBG were age, BMI, smoking and fasting before sampling of the blood, while alcohol consumption was added as a covariate. Confounders of sexual development were age, BMI and smoking. Confounders of thyroid hormones were age, BMI, sex and illness during the last 14 days. The continuous effect markers were ln-transformed for multiple regression analysis, while the biomarkers of exposure were put in the model both as non-transformed and ln-transformed marker. In Tables 4, 5 and 6, results are given for the non-transformed exposure markers, unless indicated differently. For samples below the LOQ, half of the LOQ was used for statistics.

Results and discussion

Pesticide concentrations in Flanders

In both surveys, *p,p′*-DDE and HCB could be measured in more than 90 % of the serum samples. The concentration levels of these pesticides were lower in the FLEHS II study compared to the levels found 5 years earlier (FLEHS I, 2002–2006).

Geometric, corrected mean concentrations (with 95 % confidence interval (CI)) of 307 (277–341) and 36.5 ng L^{-1} (34.0–39.2) were found for, respectively, *p,p′*-DDE and HCB in FLEHS II. These values were respectively 26 and

60 % lower ($p<0.001$, ANOVA testing) compared to the mean values of the Flemish population obtained 5 years earlier in FLEHS I. When comparing these data to other international studies (Table 1), much lower concentrations of *p,p′*-DDE were observed in the German Environmental Survey (GerES) study (12–14-year-old adolescents, 2003–2006) (Becker et al. 2008), while in the US National Health and Nutrition Examination Survey (NHANES) study (12–19-year-old adolescents, 2003–2004) (CDC 2009), concentrations of *p,p′*-DDE were comparable to the values measured in the FLEHS I study. For HCB, the German average values were comparable to the concentrations measured in FLEHS I, while the US concentrations were in between the results reported in FLEHS I and FLEHS II. A further decrease in time of these historical pollutants can thus be expected. For both *p,p′*-DDE and HCB, a positive association with socio-economic status (measured as the highest parental educational attainment) was found: Significantly higher concentrations were measured for adolescents with a higher socio-economic status (Morrens et al. 2012). These social gradients are consistent with literature (Becker et al. 2008) and could partly be explained by the higher prevalence of breastfeeding by mothers with a higher educational attainment.

The pesticide metabolite 2,5-DCP and the OPP metabolites DEP and DETP were measured in eight pooled samples during the FLEHS I survey and in 210 adolescent samples during FLEHS II. The other dimethyl- and diethyl phosphate metabolites were measured for the first time in the Flemish population ($n=210$) during FLEHS II. The metabolites DEP, DMP, DMTP and 2,5-DCP could be measured in most urine samples (respectively, 55, 68, 95 and 89 % of the samples above the LOQ), while for the metabolites DETP, DEDTP and DMDTP, only a small percentage of the samples could be quantified (respectively, 23, 5 and 34 %). Geometric, corrected mean concentrations (with 95 % CI) of 2.54 (2.20–2.90), 4.96 (4.31–5.71), 5.71 (5.03–6.50) and 1.54 μg L^{-1} (1.30–1.82) were found for DEP, DMP, DMTP and 2,5-DCP, respectively, (Table 2). For the other metabolites, more than 50 % of the samples were below LOQ, and no GM was thus calculated. For the sum of all dimethyl- and diethyl OPP metabolites, geometric means of 11.4 and 3.27 μg L^{-1} were reported. The Flemish mean concentrations of OPP metabolites were lower compared to the German GerES IV study, but higher than in the US NHANES survey. The concentrations of DEP and DETP in FLEHS II were also comparable to the results found in the pooled samples in the FLEHS I survey. In other international studies, the major metabolites were also DMP and DMTP, while DEDTP and DMDTP could hardly be quantified (Aprea et al. 2000; Becker et al. 2006; CDC 2009; Saieva et al. 2004). The mean concentration of 2,5-DCP (1.54 μg L^{-1} or 1.16 μg g^{-1} creatinine) in FLEHS II was comparable to the results found in the pooled samples, 5 years earlier (median 1.02 μg g^{-1} creatinine; mean 1.28 μg g^{-1} creatinine; minimum–maximum range 0.77 and 4.15 μg g^{-1} creatinine; unpublished results). The concentration of this metabolite is still twice as high compared to the German results (Becker et al. 2006), but significantly lower (five to ten times) than in the different surveys (between 2003 and 2010) of US NHANES (CDC 2009).

For all OPP metabolites, significantly higher concentrations were measured in the urine of girls compared to boys ($p<0.0001$ for DEP, $p=0.002$ for DETP, $p=0.048$ for DMP). This was also found in the GerES survey (significant only for DMTP) (Becker et al. 2006). Significantly lower concentrations of DEP were found in the winter and autumn compared to spring ($p=0.03$): a finding that was also observed in several other studies (Bradman et al. 2003; Becker et al. 2006) and could be linked to the use of OPPs in agriculture and home gardens. Adolescents residing in a rural area also yielded significantly higher concentrations of DMP ($p=0.007$) compared to participants residing in an urban environment.

Table 1 Reference values of the pesticides *p,p′*-DDE and HCB in the Flemish population (FLEHS I and II) and in the US NHANES and the German GerES IV surveys

Compounds	GM concentration (95 % CI) (ng g^{-1} lipid)	GM concentration (95 % CI) (ng L^{-1})	Age of participants (years)	Sampling period	Study
p,p′-DDE	94 (89–99)	418 (396–440)	14–15	2003–2004	FLEHS I, Flanders
	70 (63–78)	307 (277–341)	14–15	2008–2011	FLEHS II, Flanders
	105 (85–129)	516 (419–635)	12–19	2003–2004	NHANES, USA
	–	190 (177–204)	12–14	2003–2006	GerES IV, Germany
HCB	21 (20–21)	91 (87–92)	14–15	2003–2004	FLEHS I, Flanders
	8.33 (7.77–8.93)	36.5 (34.0–39.2)	14–15	2008–2011	FLEHS II, Flanders
	13.3 (12.5–14.1)	65 (62–69)	12–19	2003–2004	NHANES, USA
	–	91 (86–95)	12–14	2003–2006	GerES IV, Germany

Source: CDC 2009 and Becker et al. 2006. FLEHS II $n=210$ and FLEHS I $n=1679$

GM geometric mean, *CI* confidence interval

Table 2 Reference values of the organophosphate pesticide metabolites and 2,5-DCP in the Flemish population (FLEHS I and II) and in the US NHANES and the German GerES IV surveys

Compounds	GM concentration (95 % CI) (μg g^{-1} creatinine)	GM concentration (95 % CI) (μg L^{-1})	Age participants (years)	Sampling period	Study
2,5-DCP	Range 0.77–4.15 Median 1.02	Range 1.12–5.60 Median 1.53	14–15	2003–2004	FLEHS I (eight pools), Flanders
	1.16 (0.98–1.37)	1.54 (1.30–1.82)	14–15	2008–2011	FLEHS II, Flanders
	12.7 (8.50–18.9) 8.88 (6.34–12.4)	16.9 (11.1–26.0) 11.9 (8.47–16.8)	12–19	2003–2004 2005–2006	NHANES, USA
	8.79 (6.81–11.4)	11.3 (8.78–14.5)		2007–2008	
	6.44 (4.40–9.42)	8.01 (5.53–11.6)		2009–2010	
	–	0.72 (0.61–0.86)	12–14	2003–2006	GerES IV, Germany
DMPs	–	–	14–15	2003–2004	FLEHS I, Flanders
	DMP 3.79 (3.29–4.37) DMTP 4.34 (3.81–4.94)	DMP 4.96 (4.31–5.71) DMTP 5.71 (5.03–6.50)	14–15	2008–2011	FLEHS II, Flanders
	DMDTP <LOQ[b]	*DMDTP <LOQ*[b]			
	DMTP 1.66 (1.37–2.03) DMTP 1.62 (1.27–2.06)	DMTP 2.21 (1.81–2.70) DMTP 2.10 (1.68–2.61)	12–19	2003–2004 2007–2008	NHANES, USA[a]
	–	DMP 14.3 (12.4–16.6) DMTP 12.8 (10.9–15.1) DMDTP 0.41 (0.34–0.49)	12–14	2003–2006	GerES IV, Germany
DEPs	DEP: range <LOQ–2.75; median 2.26 DETP range <LOQ–1.03; median 0.8	DEP: range <LOQ–4.13; median 3.39 DETP: range < LOQ–1.54; median 1.2	14–15	2003–2004	FLEHS I (8 pools), Flanders
	DEP 1.93 (1.69–2.20) *DETP <LOQ*[b]	DEP 2.54 (2.20–2.90) *DETP <LOQ*[b]	14–15	2008–2011	FLEHS II, Flanders
	DEDTP <LOQ[b]	*DEDTP <LOQ*[b]			
	<LOD	<LOD	12–19	2003–2004	NHANES, USA
	–	DEP 5.32 (4.51–6.29) DETP 0.84 (0.69–1.02) DEDTP 0.025 (0.020–0.031)	12–14	2003–2006	GerES IV, Germany

Source: CDC 2009 and Becker et al. 2006. FLEHS II n=210 and FLEHS I n=1679 (or eight pools for 2,5-DCP, DEP and DETP)

GM geometric mean, *CI* confidence interval

[a] Only DMTP could be quantified; all other metabolites (DMPs and DEPs) were below the LOQ (varying between 0.10 and 0.56 μg L^{-1})

[b] For DETP, DEDTP and DMDTP, more than 60 % of the samples were below the LOQ, and no GM was calculated

Dose–effect relationships

Sex hormones and degree of sexual maturation

The sex hormones were only measured in the blood of the boys participating in the studies. Data on sexual maturation was obtained for both boys (pubic hair and genital development) and girls (pubic hair and breast development and age at reaching menarche) (Table 3).

In the FLEHS II survey, HCB concentrations in boys were positively correlated with total testosterone (p=0.004), reaching the adult stage of testosterone (p=0.04, odds ratio (OR)=1.29), the aromatase index (p=0.007) and pubic hair development (p=0.052, OR=1.77). For girls, a negative association with reaching menarche at the age of 14–15 years old (p=0.02, OR=0.35) was reported. The pesticide metabolite *p,p′*-DDE was negatively correlated with breast development in girls (p=0.03, OR=0.74) (Tables 4 and 5). A positive association between HCB and free (p=0.002) and total (p=0.0001) testosterone, the aromatase index (p=0.0007) and pubic hair development (p<0.0001) was also found for the boys in the FLEHS I study (Dhooge et al. 2011). In FLEHS I, also positive relationships between *p,p′*-DDE and pubic hair development (p=0.002) and genital development (p=0.001) in boys and between HCB and total estradiol (p=0.0001) in boys were observed, but this could not be confirmed in the FLEHS II survey. In a study in Canada (girls aged 10–17 years), a negative association was found between age at menarche and the sum of four estrogenic PCBs (PCB 52, 70, 101 and 187), but no relationship was found with HCB and *p,p′*-DDE (Schell and

Table 3 Hormone levels and data on sexual development for the whole study population from FLEHS II (n=600)

Compounds	Number of participants	Mean concentration	95 % CI
Estradiol (pg mL^{-1})	322	23.4	22.4–24.4
Free estradiol (pg mL^{-1})	303	0.39	0.36–0.42
Testosterone (ng dL^{-1})	321	415	395–435
Free testosterone (ng dL^{-1})	321	7.05	6.57–7.54
Aromatase	321	18.8	17.7–19.8
LH (mU mL^{-1})	322	3.56	3.33–3.79
FSH (mU mL^{-1})	322	4.50	4.04–4.96
SHBG (nmol L^{-1})	321	42.9	40.7–45.0
TSH (μU mL^{-1})	599	2.36	2.27–2.45
fT4 (ng dL^{-1})	599	1.24	1.23–1.26
fT3 (pg mL^{-1})	599	4.15	4.11–4.19
	Number of participants	**Result**	
Reaching menarche (girls)	279	92.1 %	
Reaching adult phase of testosterone (boys)	321	71.3 %	
Reaching adult phase of free testosterone (boys)	321	58.3 %	
Stadium of breast development (number of girls)	152	B1 (0) B2 (0) B3 (6) B4 (50) B5 (96)	
Stadium of pubic hair development (number of girls)	145	P1 (0) P2 (0) P3 (5) P4 (50) P5 (90)	
Stadium of genital development (number of boys)	154	G1 (0) G2 (7) G3 (18) G4 (67) G5 (62)	
Stadium of pubic hair development (number of boys)	155	P1 (5) P2 (8) P3 (14) P4 (67) P5 (61)	

CI confidence interval

P1–P5 and G1–G5 and B1–B5: stadium of pubic hair and genital and breast development according to Marshall and Tanner

Gallo 2010). A study on 9-year-old girls in the USA also found no associations between *p,p′*-DDE concentrations and pubic hair or breast development (Wolff et al. 2008). Rylander et al. (2006) reported a significant negative association between estradiol and *p,p′*-DDE in adult men. *p,p′*-DDE is a known antiandrogenic compound (Gray et al. 2001), but few studies report significant effect on pubertal development. HCB showed antiestrogenic characteristics in animal studies (Alvarez et al. 2000; Foster et al. 1995), but few data are available from adolescent studies. This indicates the need for more research, concerning the relationship between these endocrine-disrupting pesticides and sexual maturation of adolescents, to confirm these findings and establish possible mechanistic pathways.

The sum of OPP metabolites (both methyl and ethyl metabolites) were significantly negatively correlated with free-estradiol concentrations in the blood of the boys (p=0.03 for the methyl group and p=0.01 for the ethyl group, both after ln transformation). The sum of the ethyl OPP metabolites was also negatively associated with free testosterone (p=0.04, after ln transformation) and reaching the adult stage of free testosterone (p=0.04, OR=0.53, after ln transformation). For the girls, negative associations were found between the sum of ethyl OPP metabolites and breast development (p=0.048, OR=0.78), while for the boys, a negative relation between the sum of methyl OPP metabolites and genital development was observed (p=0.04, OR=0.46) (Tables 4 and 5). In

Table 4 Dose–effect relationships (FLEHS II) between pesticide concentrations (exposure) and sex hormones, measured only for boys (effect, all ln-transformed)

Exposure	Effect	Confounders	Covariates	Estimate[a] (95 % CI)	IQR	*p* value
SumDE (μg g^{-1} creatinine)	Free estradiol (pg mL^{-1})	Age, blood collection before 11 h, BMI, smoking	Season, illness last 14 days	0.77 (0.63; 0.95)	3.04	0.01 [b]
SumDM (μg g^{-1} creatinine)			–	0.74 (0.57; 0.96)	9.57	0.03[b]
HCB (ng g^{-1} lipid)	Testosterone (ng dL^{-1})	Age, blood collection before 11 h, BMI, smoking	–	1.04 (1.01; 1.07)	4.84	0.004
SumDE (μg g^{-1} creatinine)	Free testosterone (ng dL^{-1})	Age, blood collection before 11 h, BMI, smoking	Illness last 14 days	0.80, (0.66; 0.98)	3.04	0.04[b]
HCB (ng g^{-1} lipid)	Aromatase	Age, blood collection before 11 h, BMI, smoking	–	1.05 (1.01; 1.08)	4.84	0.007

IQR interquartile range, *sumDM* sum DMP and DMTP, *sumDE* sum DEP, DETP and DEDTP

[a] Interpretation estimate (regression coefficient): If the exposure increases with the IQR, the mean effect is multiplied with the estimate

[b] *p* value for the exposure marker in a non-transformed scale is borderline not significant, but significant relationships are found for the marker on an ln-transformed scale

literature, most studies report negative associations between OPP metabolite concentrations in urine and sex hormones, although the relationships are not always straightforward. Furthermore, in most studies, an adult population was selected, while in our studies, only adolescents participated. Blanco-Muñoz et al. (2010) found a significant negative relationship between DMP, DEP, DETP and their sum and inhibin B; between DETP and LH and between DEP and FSH and a borderline significant positive association between DEP and testosterone ($p=0.06$) in a group of adult men. Meeker et al. (2006) reported a significant negative correlation between urinary 3,5,6-trichloro-2-pyridinol (TCPY, a metabolite of the OPP chlorpyriphos) and serum testosterone concentrations, while Larsen et al. (1999) found that farmers using OPPs had lower concentrations of testosterone in their blood compared to organic farmers (not using OPPs). A study from Straube et al. (1999) showed that testosterone concentrations in the blood were lower when resulting from acute exposure, while higher concentrations could be found when resulting from chronic exposure. Furthermore, acute exposure was related to lower estradiol levels. Another study also reported a significant negative correlation between the chlorpyriphos metabolite TCPY in urine of adult men and estradiol concentrations (Meeker et al. 2008). To our knowledge, our survey is the first study reporting associations between OPPs and sexual development in adolescents.

Thyroid hormones

Dose–effect relationships on thyroid hormones (TSH, free triiodothyronine (fT3) and fT4) were established for boys and girls together.

In the FLEHS II survey, the pesticides *p,p′*-DDE and HCB were positively correlated with fT4 ($p=0.02$ and 0.08,

Table 5 Dose–effect relationships (FLEHS II) between pesticide concentrations (continuous exposure marker) and sexual development (binary effect marker)

Exposure	Effect	Confounders	Covariates	Odds ratio[a] (95 % CI)	IQR	*p* value
HCB (ng g^{-1} lipid)	Reaching menarche (%) girls	Age, BMI, smoking	–	0.35 (0.15; 0.84)	4.84	0.02
p,p′-DDE (ng g^{-1} lipid)	Breast development girls	Age, BMI, smoking	–	0.74 (0.57; 0.98)	53.3	0.03
SumDE (μg g^{-1} creatinine)				0.78 (0.61; 1.00)	3.03	0.048
SumDE (μg g^{-1} creatinine)	Reaching the adult stage of free testosterone (%) boys	Age, blood collection before 11 h, BMI, smoking	Illness last 14 days	0.53 (0.29; 0.96)	3.04	0.04[b]
HCB (ng g^{-1} lipid)	Reaching adult stage of testosterone (%) boys	Age, blood collection before 11 h, BMI, smoking	–	1.29 (1.01; 1.65)	4.84	0.04
HCB (ng g^{-1} lipid)	Pubic hair development boys	Age, BMI, smoking	–	1.77 (1.00; 3.14)	4.84	0.052
SumDM (μg g^{-1} creatinine)	Genital development boys	Age, BMI, smoking	–	0.46 (0.22; 0.96)	9.57	0.04

sumDM sum DMP and DMTP, *sumDE* sum DEP, DETP and DEDTP

[a] Interpretation of odds ratio (OR): If the exposure increases with the IQR, the odds for the effect marker are multiplied with the factor OR

[b] *p* value for the exposure marker in a non-transformed scale is borderline not significant, but significant relationships are found for the marker on an ln-transformed scale

respectively), while for 2,5-DCP a negative association was found (p=0.001). Serum HCB (p=0.02) and urinary 2,5-DCP (p=0.02) were positively associated with TSH (Table 6). No significant relationships with fT3 were found in the FLEHS II, but a positive association between HCB and fT3 (p=0.006 for girls and p=0.046 for boys; unpublished results) was reported in FLEHS I. In most animal studies and in a large part of the published human surveys, negative associations between chlorinated pesticides (especially HCB) and total and free T4 are described (Boas et al. 2006 and references herein; Alvarez et al. 2005; Sala et al. 2001). On the other hand, Turyk et al. (2007) reported a positive association between *p,p′*-DDE and total T4 in the blood of adult women. A positive association between *p,p′*-DDE and total T3 and fT4 was also found by Meeker et al. (2007) in adult men, while Schell et al. (2008) reported a negative correlation between HCB and total T4 in a study on Canadian adolescents, aged between 10–17 years old. Langer et al. (2007) also found a negative association between HCB and free T4, while *p,p′*-DDE was positively correlated with total T3 (adult men, Slovakia). Freire et al. (2012) reported a positive correlation between 17 different chlorinated pesticides (including *p,p′*-DDE and HCB) and total T3 levels in the blood of children (0–14 years old), while no significant trends were found between *p,p′*-DDE or HCB and TSH and free T4 concentrations. It seems thus that the complex relation with endocrine-disrupting substances, like *p,p′*-DDE and HCB, and thyroid function is not completely understood. It is also likely that the effect of endocrine-disrupting compounds, such as HCB and *p,p′*-DDE, varies in function of several physicological parameters and of additional exposures. This might be consistent, and partly explainable, by the occurrence of non-monotonic dose–responses to endocrine-disrupting compounds (Vandenberg et al. 2012). Furthermore, relationships could be different in adolescents compared to adults, who have reached steady-state concentration levels of thyroid hormones. Additional large-scale studies in adolescents in which not only (free) T4, (free) T3 and TSH but also thyroxine-binding globulin (TBG), transthyretin (TTR, or pre-albumin) and albumin are measured, could possibly aid at a better understanding.

Only one study was found concerning the effect of dichlorobenzene on thyroid hormones. A rat study showed that the urinary concentrations of dichlorobenzene (2,5-DCP is the most important metabolite of this compound) were associated with lower T4 levels in the blood of the animals (den Besten et al. 1991). Furthermore, Van den Berg (1990) demonstrated that all chlorinated phenols (including 2,5-DCP) were competitors for the T4 binding site of TTR, a carrier of thyroxine.

Long-term health effects

It is generally known that, especially during the last decades, sperm quality of men has decreased dramatically in Flanders and Europe (Comhaire et al. 2007). Also, the sex ratio has decreased in many countries (Davis et al. 2007), while the incidence of cancer (including breast, prostate and testis cancer) has increased. For example, De Coster and Van Larebeke (2012) showed that in Great Britain the incidence of breast has increased by 57 %, while prostate cancers have tripled during the last 30 years. Lifetime exposure to numerous endocrine-disrupting compounds, including the pesticides studied in this paper, can definitely contribute to this type of health deficits. Recently, several studies showed that HCB and the OPPs malathion and parathion have significant effects on the endogenous estradiol levels in human breast cells (Calaf and Roy 2008; García et al. 2010), while a study in California (USA) showed that women who were heavily exposed to DDT as a child had a higher risk for developing breast cancer before the age of 50 (Cohn et al. 2007). Also, in men endocrine health effects can be associated to pesticide exposure. Giannandrea et al. (2011) found a significant positive relationship between the occurrence of testis cancer and the use of household insecticides and between testis cancer and serum levels of *p,p′*-DDE and HCB. Dimethyl methylphosphonate, an OPP, seemed to impair fertility in male rats (Chapin et al. 1984). These examples show that monitoring concentration levels of these pesticides in the human body during follow-up studies (with focus on fertility problems and cancer) can provide important information.

Table 6 Dose–effect relationships (FLEHS II) between pesticide concentrations (exposure) and thyroid hormones (effect, ln-transformed)

Exposure	Effect	Confounders	Covariates	Estimate[a] (95 % CI)	IQR	p value
HCB (ng g^{-1} lipid)	TSH (μU ml^{-1})	Age, BMI, sex, illness last 14 days	–	1.03 (1.01; 1.05)	4.84	0.02
2,5-DCP (μg g^{-1} creatinine)				1.003 (1.001; 1.006)	2.16	0.02
HCB (ng g^{-1} lipid)	Free T4 (ng dL^{-1})	Age, BMI, sex, illness last 14 days	–	1.02 (1.01; 1.03)	4.84	0.002[b]
p,p′-DDE (ng g^{-1} lipid)				1.003 (1.001; 1.006)	53.3	0.02
2,5-DCP (μg g^{-1} creatinine)				0.9993 (0.9996; 0.9998)	2.16	0.001

[a] Interpretation of estimate (regression coefficient): If the exposure increases with the IQR, the mean effect is multiplied with the estimate

[b] p value for the exposure marker in a non-transformed scale is borderline not significant, but significant relationships are found for the marker on an ln-transformed scale

Conclusions

During 5 years between the FLEHS I and FLEHS II surveys, concentration levels of HCB and *p,p′*-DDE in the Flemish adolescent population have decreased drastically. Furthermore, population mean values were established for 2,5-DCP and OPP metabolites in FLEHS II. Most of these compounds could be measured in the majority of the samples with concentrations within the normal range found in literature. Data on internal exposure of the pesticides *p,p′*-DDE and HCB indicated that exposure to these compounds was associated with a faster sexual maturation in boys, while for girls signs of a delayed development were found. Thyroid hormones, especially fT4, showed positive associations with these persistent, chlorinated pesticides. These observations were also found for the marker PCBs (Croes et al. 2014) and several hydroxylated PCBs (results not published), indicating similar mechanisms of action. Dose–effect relationships for OPP metabolites showed associations with delayed sexual development for both boys and girls, while the pesticide metabolite 2,5-DCP seemed to have an influence on the concentrations of thyroid hormones in the blood of boys and girls, such as a negative effect on fT4 and a positive association with TSH. These results show that even exposure to relatively low concentrations of pesticides can have significant influences on hormone levels and the degree of sexual maturation in 14–15-year-old adolescents. Furthermore, follow-up studies on this cohort would provide interesting information concerning infertility and development of cancer in relation with internal concentration levels of pollutants.

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