Ministry of Environment and Food of Denmark
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Organophosphate metabolites in urine samples from Danish children and women

Measured in the Danish DEMOCOPHES population

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Foreword

The project ”DAP metabolites in Danish Children and Women” was conducted in 2014-2015 as part of the project “Pesticide Measurements in urine - establishment of analysis method and analysis of urine samples from Danish mothers and children collected as part of DEMOCHOPHES ” financed by the Danish Environmental Agency.

This report describes the concentrations of six dialkylphosphate metabolites (DAPs) used as biomarkers for organophosphate exposure. The DAPs have been measured in Danish school children and mothers participating in the European project DEMOCOPHES co-funded by the LIFE+ programme of EU (GD environment – LIFE09 ENV/BE/000410) and the Danish Environmental Agency, the Danish Veterinary and Food Administration and the Danish Health and Medicines Authority.

The project is a collaboration between Environmental Medicine, Institute of Public Health at University of Southern Denmark and section of Environmental Health at the Institute of Public Health, University of Copenhagen. The samples were analyses by the Flemish Institute for Technological Research NV (VITO) in Belgium in collaboration with professor Greet Schoeters.

We would like to thank the families participating in DEMOCOPHES and the collaborating schools taking part in the recruiting process.

Abbreviations

C, creatinine
COPHES, Consortium to perform human biomonitoring on a European scale
DAP, dialkyl phosphate
DEAP, diethyl alkylphosphates
DEDTP, Diethyl dithiophosphate
DEMOCOPHES, Demonstration of a study to coordinate and perform human biomonitoring on a European scale
DEP, Diethyl phosphate
DETP, Diethyl thiophosphate
DMAP, dimethyl alkylphosphates
DMDTP, Dimethyl dithiophosphate
DMP, Dimethyl phosphate
DMTP, Dimethyl thiophosphate
GM, geometric mean
LOD, limit of detection
NAAP, paracetamol
NHANES, National Health and Nutrition Examination Survey (US)
OP, organophosphate
PFBBr, pentafluorobenzylchloride
POPs, Persistent organic pollutants
SES, socioeconomic status
2.4-DCP, 2,4-dichlorophenol
2.5-DCP, 2,5-dichlorophenol
2-PP, 2-phenylphenol.

Summary and conclusion

Background
The use of organophosphate insecticides (OPs) in agriculture and as biocides in Denmark is very restricted due to their relatively high acute toxicity and neurotoxic effects. However, the population might still be exposed by ingestion of imported food items. Exposure to organophosphates can be estimated by measurements of dialkylphosphate (DAP) metabolites and in studies from the US urinary concentrations of DAPs have been associated with adverse neurobehavioural outcomes. Children with increased OP exposure may have higher risk of ADHD, and prenatal OP exposure was reported to be associated with structural brain anomalies and neurobehavioral deficits at school age. Urinary concentrations of DAP metabolites have only been measured in the Danish population in one study previously.

Objectives
The objectives of the present study was to investigate the urinary concentration of six unspecific DAP metabolites, which are commonly used as biomarkers for organophosphate exposure to pesticides such as dichlorvos, fenthion, dimethoat, malathion and chlorpyrifos and to compare the found concentrations with one previous Danish study and concentrations measured in other countries.

Methods
The urinary concentrations of Dimethyl phosphate (DMP), Dimethyl thiophosphate (DMTP), Dimethyl dithiophosphate (DMDTP), Diethyl phosphate (DEP), Diethyl thiophosphate (DETP), Diethyl dithiophosphate (DEDTP) were analyzed in 144 Danish school children and 145 mothers. The study persons were part of a large EU pilot project called DEMOCOPHES (Demonstration of a study to coordinate and perform human biomonitoring on a European scale), with focus on harmonization of human biomonitoring in Europe and which was ongoing in Europe from 2010 to 2012. In Denmark mother child pairs were recruited from an urban and a rural area and urine, hair and blood samples were collected from September to December 2011 The urine samples from the biobank of DEMOCOPHES in Denmark were used for the DAP analyses in the present study.

Results
At least one of the six DAP metabolites was detected in more than 90%, and four metabolites was detected in more than 30%, of both children and mothers. There was a tendency of higher DAP concentrations in children compared to their mothers. Furthermore, there was a tendency of higher concentrations in younger mothers and in children from families with higher socioeconomic status. The exposure source of the organophosphates in this study was difficult to determine as the study was not initially designed for this purpose. DAP concentrations were generally lower in participants from the rural compared to the urban area and the concentrations of the methylated DAPs were lower in children who often were eating homegrown fruit and vegetables, though only statistically significant for DMP. The levels of total DAP and DMAPs were lower in the investigated population than in Danish children investigated in 2007-08 indicating a decline in the total OP exposure level. However, the concentration of DEAPs was not similarly reduced. In general, the DAP levels were comparable with concentrations measured in other European countries in recent years, but the levels of total DAPs and DEAPs were higher than levels found in the US.

The concentration of the individual DAP metabolites were significantly correlated with each other in both mothers and children and in the mothers they were also significantly correlated with other chemicals associated with pesticide exposure (2.4-DCP, 2.5-DCP and 2-PP) measured in the same urine sample residues.

Conclusion

The findings of relatively high detection frequency of the DAP metabolites DEP, DETP, DMP and DMTP in a Danish group of children and their mothers, clearly indicate that there is still a widespread exposure to organophosphate pesticides in the Danish population, even though there have been major restrictions on their use in agriculture. The concentrations of the metabolites DEP and DETP found in the children and women of the present study are in line with what was found in Europe and previous measurements in Denmark. The methylated metabolites seem to have decreased in Denmark compared to previous measurements in 2007-08 and levels found in other European countries before 2007. However, the levels of total DAPs and DEAPs in the present study, as well as the levels found in other European countries, are higher than concentrations found in the biomonitoring program NHANES in the US, indicating higher exposure to some organophosphates in Europe. As the exposure to OPs has been associated with adverse health effects in some studies from the US population, there may also be a risk of adverse effects in Europe and Denmark, as we have found even higher levels. Although we do not know the specific organophosphates responsible for the relatively high concentrations of DEAPs in Denmark, chlorpyrifos is likely to be an important contributor since it is often found in samples of imported fruit and vegetables, which is considered the main exposure source for OPs in Denmark. Thus, further studies of potential adverse health effects related to organophosphate exposure in European populations are needed. Also identification of the main dietary exposure sources and related OPs is warranted in order to introduce adequate measures to reduce the exposure - especially for vulnerable population groups as children and pregnant women.

Resume og konklusion

Baggrund
Brugen af organofosfat-insekticider i landbruget og som biocider i Danmark er meget restriktiv på grund af deres relativt høje akutte toksicitet og neurotoksiske effekter. Den danske befolkning kan dog stadig være eksponeret for organofosfater via indtagelse af importerede fødevarer. Man kan estimere befolkningens udsættelse for organofosfater ved at måle dialkylfosfat (DAP) metabolitter i urinen. I undersøgelser fra USA er der fundet sammenhæng mellem urinkoncentrationen af DAP og negativ påvirkning af nervesystemets udvikling. Børn med højere organofosfateksponering synes at have øget risiko for at udvikle ADHD, og prænatal organofosfateksponering er fundet associeret med strukturelle ændringer i hjernen og adfærdsmæssige vanskeligheder i skolealderen. Koncentrationen af DAP-metabolitter er kun blevet målt i ét studie tidligere i Danmark.

Formål
Formålet med dette studie var at måle urinkoncentrationen af seks uspecifikke DAP-metabolitter, som bliver brugt som biomarkører for eksponering for organofosfat-insekticider som dichlorvos, fenthion, dimethoat, malathion and chlorpyrifos. Formålet var desuden at sammenligne de målte koncentrationer med koncentrationer fra et tidligere studie lavet i Danmark, samt koncentrationer målt i andre lande.

Metoder
Urin koncentrationen af Dimethyl phosphate (DMP), Dimethyl thiophosphate (DMTP), Dimethyl dithiophosphate (DMDTP), Diethyl phosphate (DEP), Diethyl thiophosphate (DETP og Diethyl dithiophosphate (DEDTP) blev analyseret i 144 danske skolebørn og 145 mødre. Forsøgspersonerne var en del af et større EU pilotprojekt omhandlende harmonisering af human biomonitering i Europa kaldet DEMOCOPHES (Demonstration of a study to coordinate and perform human biomonitoring on a European scale). Projektet kørte i Europa fra 2010 til 2012. Mor-barn par blev rekrutteret i Danmark i to områder, som henholdsvis repræsenterede et urbant og et ruralt område. Urin, hår og blodprøver blev indsamlet mellem september og december 2011. Urinprøver fra den indsamlede biobank fra den danske del af DEMOCOPHES blev brugt til analyse af DAP metabolitterne i dette studie.

Resultater
Mindst en af de seks DAP metabolitter kunne måles i mere end 90% af forsøgspersonerne og fire af metabolitterne blev detekteret i mere end 30 % af både børn og mødre. Der var en tendens til højere DAP-koncentrationer i børnene sammenlignet med deres mødre. Desuden var der en tendens til højere koncentrationer i de yngste grupper af børn og mødre, samt i børn fra familier med højere socioøkonomisk status. Kilder til eksponeringen for organofosfater var svær at identificere ud fra dette studie, eftersom DEMOCOPHES projektet ikke initialt var designet til at undersøge udsættelsen for disse stoffer. DAP-koncentrationerne var generelt højere i deltagere fra byområdet sammenlignet med deltagere fra det mere landlige område og koncentrationen af de methylerede DAP'er (DMAPs) var højere i børn, der ikke spiste hjemmedyrkede fødevarer særlig ofte, dette var dog kun signifikant for DMP. Det samlede niveau af DAP-metabolitter og DMAP-metabolitter var lavere i dette studie sammenlignet med danske børn undersøgt i 2007-08, hvilket indikerer at der er sket et samlet fald i organofosfat-eksponeringen i Danmark. Koncentrationen af de ethylerede DAP'er (DEAPs) ser dog ikke ud til at være reduceret. Generelt var DAP koncentrationerne sammenlignelige med målinger fra andre Europæiske lande, men det samlede niveau af alle DAP-metabolitter og DEAP-metabolitter var højere end koncentrationer fundet i USA i

biomonitoreringsprogrammet NHANES. Koncentrationerne af de individuelle DAP metabolitter var signifikant korreleret med hinanden i både børn og mødre, og de var desuden korreleret med koncentrationerne af andre kemikalier, som også er associeret med pesticid eksponering (2,4-DCP, 2,5-DCP and 2-PP), som er målt tidligere i samme urinprøver.

Konklusion

Den relative høje detektionsfrekvens af DAP metabolitterne DEP, DETP, DMP and DMTP i danske børn og deres mødre, er en tydelig indikation på at der stadig i Danmark er en væsentlig udsættelse for organofosfat-pesticider på trods af nationale restriktioner på området.

De koncentrationer vi har målt i børn og mødre er generelt sammenlignelige med niveauer målt tidligere i Danmark samt målinger i andre Europæiske lande. Koncentrationen af de methylerede metabolitter ser dog ud til at være faldet siden målinger foretaget i Danmark i 2007-08 samt Europæiske målinger foretaget før 2007. Det totale DAP niveau samt niveauet af DEAP i dette studie, såvel som andre Europæiske studier, er højere end koncentrationer målt i det amerikanske biomoniteringsprogram NHANES, hvilket indikerer at eksponeringen for nogle organofosfater er højere i Europa end i USA. Eftersom organofosfat-eksponering i USA er fundet associeret med negative helbredseffekter, er der en risiko for, at vi i Europa og Danmark også kan have en forhøjet risiko for negative effekter ved det eksisterende eksponeringsniveau. Selvom vi ikke ved hvilke specifikke organofosfater, der er ansvarlige for den relativt høje koncentration af ethylerede metabolitter i Danmark, er det sandsynligt at chlorpyrifos bidrager væsentligt, da det er det organofosfat, som oftest måles i prøver af importeret frugt og grønt, som antages at være den vigtigste eksponeringskilde for organofosfater i Danmark. Der er derfor behov for flere studier af potentielle negative helbredseffekter forbundet med det nuværende eksponeringsniveau for organofosfater i Europa. Desuden er de vigtigste eksponeringskilder i kosten og hvilke organofosfater de bidrager med vigtigt at få undersøgt således at nødvendige foranstaltninger kan foretages for at reducere eksponeringen, - især i særligt sårbare grupper som børn og gravide.

1. Introduction

Organophosphates (OPs) are a group of insecticides widely used in agriculture and as biocides throughout the world. Because of their relatively high acute toxicity and neurotoxic properties, more bans or restrictions of use have been implemented in Europe and the US in the recent years accompanied of declining use. In Denmark it has only been legal to use few OPs after 2010. Dimethoate was banned in August 2013 and azamethiphos is still allowed in certain products (plates and cans) against ants and flies and is, to our knowledge, the only OP still in use in DK. Despite these reductions, residues of organophosphates are among the most frequently detected pesticides in imported food items on the Danish marked (Fødevareinstituttet 2014) and this is likely an important source of exposure for the Danish population. An important tool in the investigation of exposure to chemical substances is human biomonitoring, where the actual concentrations of the chemical of interest, or its metabolites, can be measured in suitable matrices. For OPs, the urinary concentration of specific or unspecific common metabolites, have often been used. In previous studies form different countries, metabolites of organophosphates have been detectable in urine samples from 70 - 90 % of the populations (Barr et al. 2004; Heudorf et al. 2004) with a tendency to decreasing levels in the most recent studies (Clune et al. 2012). The exposure level in the Danish population has only been measured in one previous study (Andersen et al. 2012) which demonstrated widespread exposure among school children in samples collected between 2007 and 2008. The urinary concentrations seemed to be higher than in samples collected in the same period among children of similar age in the US (Centers for Disease Control and Prevention 2009).

In 2011 the European pilot project DEMOCOPHES was conducted in 17 European project countries, with the purpose to exploit the possibility of a harmonized human biomonitoring setup across Europe. In all countries morning urine and hair samples were collected from school children and their mothers in two areas, representing an urban and a rural area, respectively. In Denmark, a blood sample was also collected from the majority of the participants. The urine samples were analyzed for phthalates, cadmium and cotinine, and the hair samples was analyzed for mercury. In Denmark a biobank was set up and additional biomarkers were analyzed. The blood was analyzed for persistent organic pollutants (POPs) and the urine was further analyzed for parabens, phenols and paracetamol. In the present study, the urine samples were analyzed for six unspecific DAP metabolites that are common metabolites for multiple OPs. Since OPs, like other pesticides used nowadays, are metabolized and excreted within few days, measurement of these unspecific common organophosphate metabolites are more useful to estimate the total OP exposure level in a population than measurements of specific metabolites of individual organophosphates. This approach has been used in several other studies which allow comparison with levels found in other countries.

Thus, the aim of this project was to investigate the urinary concentration of six unspecific DAP metabolites, which are commonly used as biomarkers for OPs such as dichlorvos, fenthion, dimethoat, malathion and chlorpyrifos and to compare the levels with one previous Danish study and concentrations measured in other countries.

2. Methods

2.1 Recruitment of study participants

The study persons of the present study was the Danish participation in the EU pilot project DEMOCOPHES/COPHES (Consortium to perform human biomonitoring on a European scale) and the recruitment was therefore performed in compliance with the COPHES protocol (Becker et al. 2014; Joas et al. 2012; Mørck et al. 2015a). In Denmark, the recruitment was done via local schools in two selected areas, representing urban and rural communities, respectively. An invitation to participate in the project was sent out to all parents of children in the age-group of 6 to 11 years of age and the project was also presented at parent meetings. Mothers could sign up for participation and book an appointment for sampling on our project webpage, where they also could find additional information about DEMOCOPHES. To reach the required number of participants the sampling was expanded to include additional schools in each area and articles in local newspapers were used to increase local attention to the project. The two areas of recruitment in Denmark were Gentofte (urban area), and Viby Sjælland (rural area). The areas were selected according to population density, where < 150 inhabitants/km2 was defined as rural. The following inclusion criteria were used: the child should be living with the mother a minimum of 16 days a month, the child and mother should have lived in the area for a minimum of 5 years, have sufficient Danish language knowledge and have normal kidney function and no metabolic disturbances. The goal of DEMOCOPHES was to reach 120 mother-child couples. In Denmark, 75 mother-child couples from the urban area and 70 couples from the rural area were recruited, resulting in 145 mother-child pairs in total. The children were equally distributed in age, gender and urban/rural location. All participants received written information about the study and gave informed consent before participating (for the child, all holders of custody should sign). Each mother-child pair received a voucher for two cinema tickets as a reward for their participation. The study was approved by the local regional ethics committee (H-3-2011-075 and H-1-2014-004) and the Danish data protection agency (2011-41-6607 and 2011-41-6766).
On the day of sampling, the completed questionnaire was handed in along with first morning urine samples. At the appointment hair samples were taken, and blood was drawn from the cubital vein. Mother-child pairs participating in the supplementary study on pain and self-medication were interviewed at the end of the visit. Sampling was conducted in parallel in the urban and the rural area from September to December 2011 to minimize seasonal variation.

2.2 Urine samples – dialkylphosphate (DAP) analysis

Urine was collected in 750 mL polyethylene containers, which were delivered to the participant's home the day prior to their appointment. The containers were prewashed in 10% nitric acid (>3 hours) and rinsed twice in purified water. The participants collected the total volume of the first morning urine void on the day of their appointment. To avoid decomposition of samples these were kept as cold as possible in the refrigerator or outdoors at the participants' home until the appointment. After collection of the samples, the urine was stored in a cooling box (4°C). At the laboratory the filled containers were weighed and 2 mL urine were transferred to 4 mL glass tubes with screw caps packed with aluminum foil and stored at -20 °C until further analyses.
At the Flemish Institute for Technological Research NV ("VITO") in Belgium, the urine samples were analyzed for six unspecific OP metabolites. The analyzed metabolites were:
Dimethyl phosphate (DMP), Dimethyl thiophosphate (DMTP), Dimethyl dithiophosphate (DMDTP), Diethyl phosphate (DEP), Diethyl thiophosphate (DETP), Diethyl dithiophosphate (DEDTP). The urine concentration of these metabolites was determined with a GC/MS method that

was developed and validated in-house at VITO. For a brief description of the method, urine samples were thawed and isotope-labeled internal standards were added to the samples. The samples were acidified with HCl and liquid/liquid extracted twice with a mixture of diethylether and acetonitrile (1:1). The organic layer was evaporated till dryness and the residue was dissolved in dehydrated acetonitrile. The OP metabolites were derivatized with pentafluorobenzylchloride (PFBBr). Hexane and water were added and the metabolites were transferred to the hexane fraction by shaking the tubes. The hexane fraction was concentrated and analyzed with GC/MS in SIM mode as follows: The alkyl phosphates are quantified with the internal standard method using the characteristic ions of the native and isotope labeled internal standard.
Samples were analyzed in sequences of 20 samples. Every sequence contains the necessary quality control samples: a procedural blank, a matrix spike (addition to one urine sample in the sequence), analysis in duplo (repetition of the analysis of one of the samples in the sequence) and calculation of the recovery of the isotopically labeled internal standards in each sample. The measured metabolites are listed in table 1 below, where examples of mother compounds and limit of detection (LOD) are also shown.
DAPs are stable in urine under storage at -20 °C (Tarbah et al. 2004). Six urine samples collected in 2007-08 and analyzed at Center of Disease Control in the US were re-analyzed at the VITO-laboratory in Belgium immediately before the analysis of the samples in this study and the results were very similar. This indicates that storage for up to six years at -20 °C does not affect the concentration. It also shows that results obtained in different laboratories are comparable.

Dialkyl phosphate metabolites (DAPs)	Organophosphate mother compounds	Limit of detection (µg/L)
Dimethyl alkylphosphates (DMAPs)		
DMP	Azinphos-methyl, chlorpyrifos-methyl, dichlorvos, dimethoate, fenthion, malathion, phosmet, pirimiphos-methyl	1.49
DMTP	Azinphos-methyl, chlorpyrifos-methyl, dimethoate, fenthion, malathion, phosmet, pirimiphos-methyl	0.30
DMDTP	Azinphos-methyl, dimethoat, malathion, phosmet	0.30
Diethyl alkylphosphates (DEAPs)		
DEP	Chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, terbufos	0.30
DETP	Chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, terbufos	0.30
DEDTP	Disulfoton, ethion, phorate, terbufos	0.30

TABEL 1
EXAMPLES OF ORGANOPHOSPHATE MOTHER COMPOUNDS OF THE MEASURED DAP METABOLITES AND LOD OF THE METABOLITES.

The results were expressed in µg/l for each of the six metabolites if the values were above LOD. For samples below LOD, a value of LOD/√2 was assigned. To account for urinary dilution, the DAP concentrations were adjusted for urinary creatinine content in the samples by the following formula:

$$UE_{crea}\left(\frac{\mu g}{g_{crea}}\right) = \frac{UC\left(\frac{\mu g}{L}\right) \times 1000\left(\frac{mg}{g}\right)}{UC_{crea}\left(\frac{mmol}{L}\right) \times MW_{crea}\left(\frac{mg}{mmol}\right)}$$

where UE_{crea} is the urinary concentration adjusted for creatinine, UC is the measured urinary concentration of each compound, UC_{crea} is the urinary concentration of creatinine, and MW_{crea} is the molecular mass (113 mg/mmol) for creatinine. Urinary concentrations of creatinine were determined previously by enzymatic detection on an Abbott Architect C8000 system.

Concentrations of the metabolites were converted to nmol/L (or nmol/g creatinin) by using their molecular weights (MW) as indicated below. Then DEAP (sum of diethyl alkylphosphates: diethylphosphate (DEP, MW: 154.1) anddiethylthiophosphate (DETP, MW: 170.2and DMAP (sum of dimethyl alkylphosphates: dimethylphosphate (DMP, MW:126.0) anddimethylthiophosphate (DMTP, MW: 142.1) were calculated. Dimethyldithiophosphate (DMDTP) and diethyldithiophosphate were not included in the summed variables due to none or very low detection frequency. Finally, the total DAP (dialkylphosphate) concentration was calculated as DEAP plus DMAP.

2.3 Hair and blood samples

In the Danish part of DEMOCOPHES, hair and blood samples were also collected. Hair samples were analyzed in a laboratory that participated actively in DEMOCOPHES and showed successful results in the hair analysis external quality assessment exercises (Esteban et al. 2015). The measurement of mercury in hair was determined on a Flow Injection Mercury System 400 (Perkin Elmer, Waltham, MA) in the 3 cm of the hair closest to the scalp according to the method previously described (Grandjean et al. 1992). The blood samples were analyzed for the persistent organic pollutants: polychlorinated biphenyls, perfluorinated alkyl substances and polybrominated diphenyl ethers (Mørck et al. 2014; Mørck et al. 2015a; Mørck et al. 2015b).

2.4 Questionnaires

The basic questionnaire and the urine-sampling related questionnaire developed within the COPHES framework were translated into Danish to ensure that there was no language barrier. Information on living conditions of the participants, dietary exposure and use of specific personal products and socio demographics were included in the questionnaire. The questionnaires were filled in at home and handed in at the day of the sampling with an on-site dialogue on potential non-response questions which were then resolved. Since measurement of organophosphates was not included in the original DEMOCHOPHES project, the questionnaire was not developed to investigate exposure sources for this group of chemicals. However, details on the consumption of rice, cereal and homegrown food as well as education, household income and living area were included in the present study.

2.5 Statistical analysis

Correlations between mothers and children and between the different chemicals were analyzed by Spearman's rho. The concentration variables were log-transformed for further statistical analyses to reach normal distributions of the values. Associations between DAP concentrations and different variables were analyzed by linear regression models using both unadjusted models (crude) and models adjusting for different covariates (e.g., household income and educational level) as indicated in the Tables. A p-value of ≤ 0.05 was used to determine statistical significance.

3. Results

3.1 Dialkylphosphate concentrations

DAP metabolites was measured in 145 mothers and 144 children. The children were aged 6-11 years old (8.5 ±1.7) and the mothers were between 31 and 52 years old (40.8 ± 4.3). The majority of the mothers have taken a higher education (127 out of 145) and 81% of the participants have a household incomes above the median household income in Denmark in 2011.

The geometric mean (GM), median and 95th percentile of the summed DAP metabolites (DMAP, DEAP and DAP) can be seen in table 2 below. Statistical significant differences and correlations between mothers and children are indicated with bold numbers. The concentrations and detection frequencies of the individual metabolites can be seen in tables 3 and 4, for children and mothers, respectively.

Concentration (nmol/L)		GM [95% CI]	Median (p95)	Spearman's ρ
DMAP	Children	57.7 [48.2-68.4]	59.5 (318)	0.121
	Mothers	45.5 [37.9-54.3]	50.7 (245)	
DEAP	Children	35.9 [30.8-41.7]	37.8 (150	0.107
	Mothers	29.6 [25.1-34.5]	29.8 (135)	
DAP	Children	**111 [96.7-126]***	106 (387)	0.086
	Mothers	**84.8 [72.7-98.2]**	92.3 (386)	
Creatinine corrected (nmol/g creatinine)				
DMAP	Children	**60.4 [49.5-72.5]***	63.5 (378)	***0.228****
	Mothers	**47.3 [39.8-55.5]**	48.2 (251)	
DEAP	Children	37.5 [32.4-43.6]	39.3 (151)	0.091
	Mothers	30.8 [26.9-35.2	31.6 (122)	
DAP	Children	**116 [99.8-133]****	106 (515)	***0.203****
	Mothers	**88.1 [77.6-100]**	81.9 (286)	

TABLE 2
GEOMETRIC MEANS (GM) WITH 95% CONFIDENCE INTERVALS (CI), MEDIANS AND 95 PERCENTILES OF THE SUMMED METABOLITES DMAP, DEAP AND DAP IN CHILDREN (N=144) AND MOTHERS (N=145). THE CORRELATION COEFFICIENT SPEAMAN'S RHO IS SHOWN FOR CORRELATIONS BETWEEN MOTHERS AND CHILDREN. VALUES BELOW LOD WERE SET TO LOD/√2, DAP WAS CALCULATED AS THE SUM OF DEAP (SUM OF DIETHYL ALKYLPHOSPHATES): DEP+ DETP AND DMAP (SUM OF DIMETHYL ALKYLPHOSPHATES): DMP+DMTP. SIGNIFICANT DIFFERENCES BETWEEN MOTHERS AND CHILDREN BY PAIRED T-TEST AND SIGNIFICANT CORRELATIONS MEASURED BY SPEARMAN'S RHO ARE MARKED IN BOLD. * SIGNIFICANCE LEVEL P<0.05, ** SIGNIFICANCE LEVEL P<0.01.

	%>LOD	GM [95% CI]	Min	P5	P50	P95	Max
Concentrations (µg/L)							
DMP	69	3.97 [3.27-4.74]	<LOD	<LOD	4.0	25.2	53.5
DMTP	76	2.42 [1.89-3.09]	<LOD	<LOD	3.2	23.2	57.5
DMDTP	1.4	-	<LOD	<LOD	<LOD	<LOD	6.2
DEP	96	4.54 [3.89-5.27]	<LOD	1.1	4.8	19.7	33.6
DETP	40	0.59 [0.47-0.73]	<LOD	<LOD	<LOD	5.2	57.9
DEDTP	0	-	<LOD	<LOD	<LOD	<LOD	<LOD
Creatinine corrected concentrations (µg/g creatinine)							
DMP		4.15 [3.41-5.04]	<LOD	<LOD	4.53	26.5	67.92
DMTP		2.52 [1.91-3.31]	<LOD	<LOD	3.42	29.29	88.96
DMDTP		-	<LOD	<LOD	<LOD	<LOD	9.59
DEP		4.75 [4.11-3.31]	<LOD	1.22	5.00	18.67	32.03
DETP		0.61 [0.50-0.77]	<LOD	<LOD	<LOD	5.66	57.12
DEDTP		-	<LOD	<LOD	<LOD	<LOD	<LOD

TABLE 3
URINARY CONCENTRATIONS OF DIALKYL PHOSPHATE METABOLITES CHILDREN (N=144). DETECTION FREQUENCY (%), GEOMETRIC MEAN WITH 95% CONFIDENCE INTERVALS (CI), MIN, 5, 50 AND 95 PERCENTILES ARE SHOWN

	%>LOD	GM [95% CI]	Min	P5	P50	P95	Max
Concentrations (µg/L)							
DMP	54	2.69 [2.30-3.16]	<LOD	<LOD	2.3	15.7	78.7
DMTP	73	2.17 [1.61-2.86]	<LOD	<LOD	3.1	24.3	58.8
DMDTP	2.1	-	<LOD	<LOD	<LOD	<LOD	1.7
DEP	97	3.78 [3.21-4.40]	<LOD	1.1	4.0	16.0	36.5
DETP	34	0.52 [0.41-0.65]	<LOD	<LOD	<LOD	6,2	56.1
DEDTP	0	-	<LOD	<LOD	<LOD	<LOD	<LOD
Creatinine corrected concentrations (µg/g creatinine)							
DMP		2.80 [2.37-3.31]	<LOD	<LOD	2.88	16.22	61.87
DMTP		2.26 [1.72-2.87]	<LOD	<LOD	3.22	22.6	61.87
DMDTP		-	<LOD	<LOD	<LOD	<LOD	0.96
DEP		3.93 [3.40-4.52]	<LOD	1.14	4.12	12.57	29.6
DETP		0.54 [0.43-0.66]	<LOD	<LOD	<LOD	5.3	29.46
DEDTP		-	<LOD	<LOD	<LOD	<LOD	<LOD

TABLE 4
URINARY CONCENTRATIONS OF DIALKYL PHOSPHATE METABOLITES IN MOTHERS (N=145). DETECTION FREQUENCY (%), GEOMETRIC MEAN WITH 95% CONFIDENCE INTERVALS (CI), MIN, 5, 50 AND 95 PERCENTILES ARE SHOWN

The mean concentration of total DAPs and DMAP was significantly higher in the children compared to their mothers for creatinine (C) corrected concentrations (DAP: *p=0.003,* DMAP*: p=0.038)* and for DAP also for the non-corrected values (*p=0.010)*. The same tendency was seen for all metabolites (see tables 3 and 4), although only significant at $p \leq 0.05$ level for DMP (C corrected and non-corrected). The urinary concentrations of total DAP were significantly correlated between mothers and children in C corrected values (see table 2). DMAP alone (see table 2) and the metabolite DMTP (Spearman's ρ: 0.218, p=0.009) were also significantly correlated between mothers and children.

3.2 DAP concentration in relation to age

The urinary concentrations of DAPs showed a trend of higher concentrations in younger age groups compared to the older age-groups for both the children (figure 1) and the mothers (Figure 2). For the children, the association was significant for the summed DAP (*p=0.002*) and DMAP (*p=0.009*) and for the metabolites DMP (*p=0.003)* and DMTP (*p=0.*037) for C corrected values. The same trend was also seen for the mothers, where the concentrations of DMAP (uncorrected) were negatively associated with age (*p=0.011*). However, this association disappeared after correction for creatinine. For the individual metabolites, the concentrations of DMP, DMTP and DEP seemed higher in the younger age group of the mothers compared to the older age groups, but the differences was only significant for DMP (*p=0.036)* before C-correction. No differences in urine DAP concentrations were seen between boys and girls in this study.

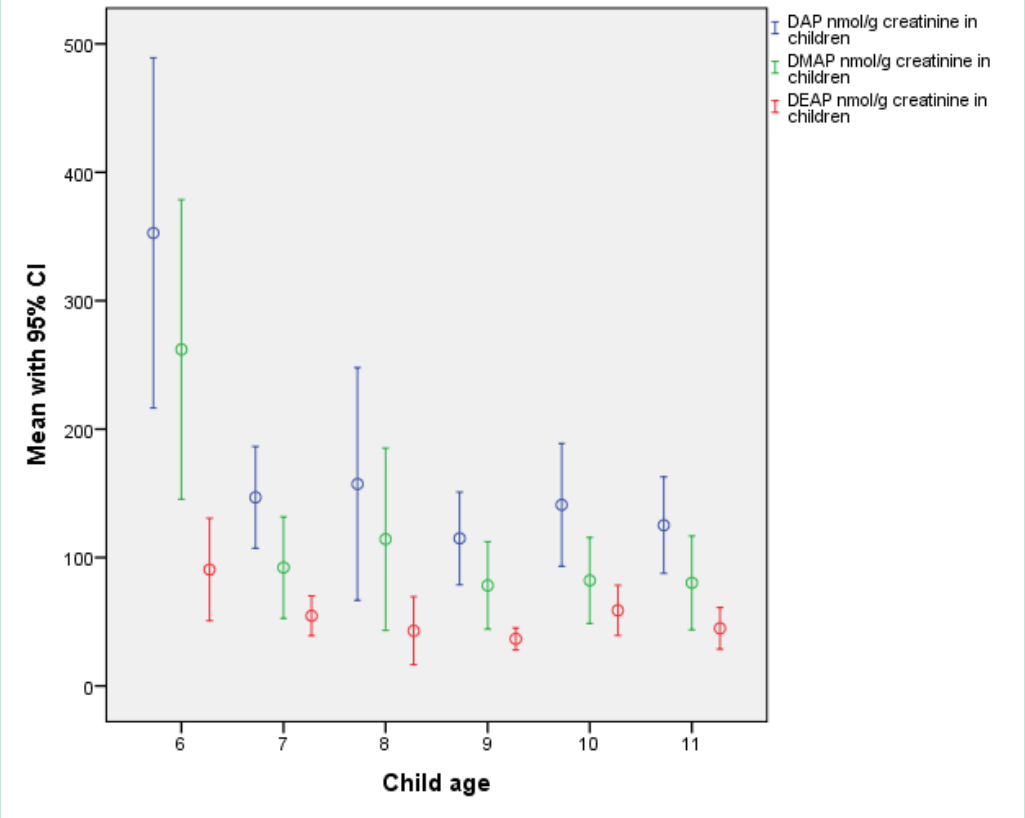
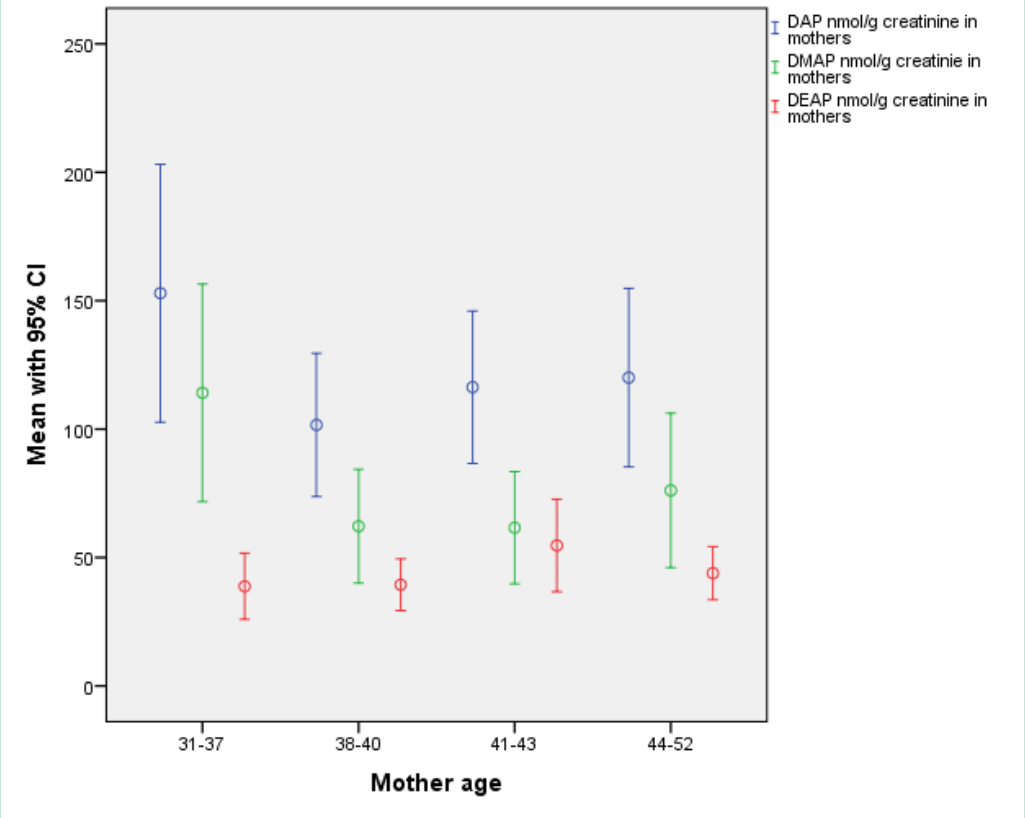


FIGURE 1
MEAN CONCENTRATIONS WITH 95%CONFIDENCE INTERVALS OF DAP, DMAP AND DEAP IN THE DIFFERENT AGEGROUPS
OF THE CHILDREN (N=144)



FIGUR 2
MEAN CONCENTRATIONS WITH 95%CONFIDENCE INTERVALS OF DAP, DMAP AND DEAP IN THE DIFFERENT AGEGROUPS
OF THE MOTHERS (N=144)

3.3 Impact of Socioeconomic status

For the children, a significant positive association was found between the uncorrected total concentrations of DAP (p=0.040) and DMAP (*p=0.024*) and educational level of the mother. This was also significant for the DM metabolites alone (DMP: *p=0.019* and DMTP: *p=0.048*, DMAP: *p=0.024*) and for the DETP metabolite in mothers (*p=0.029*). The significance disappeared when the concentrations were corrected for creatinine, except for DETP in mothers (*p=0.038)*. For children the concentration of DETP (creatinine corrected) also increased with increasing monthly household income (*p=0.017*). The concentrations of DAP, DMAP and DEAP (nmol/ g creatinine) I mothers and children in relation to educational level of the mother and household income can be seen in table 5 below.

3.4 Food frequency questionnaire

Among potential relevant questions in relation to OP exposure, the questionnaire contained information on the frequency of eating rice, cereals and homegrown food. Homegrown food was explained in the questionnaire as: fruit and vegetables, inclusive potatoes, grown in your own, friends', or family's garden or from a local producer nearby. For the children 37% ate rice several times a week or more, 64% ate cereal daily and 33% reported to eat homegrown food 2-3 times a month or more. In mothers these numbers were 34%, 52% and 35%, respectively. For the intake of rice and cereal no significant association with DAP metabolites – neither for individual metabolites nor for total-DAPs, DMAP, or DEAPs was found. For the intake of homegrown food, the concentration of DMP in children eating homegrown food 2-3 times a month or more was significantly lower than children eating homegrown food once a month or less. The difference was found in both creatinine corrected (*p=0.027)* and non-corrected (*p=0.020)* values and persisted after adjusting for age, gender, household income and education of the mother. No difference was

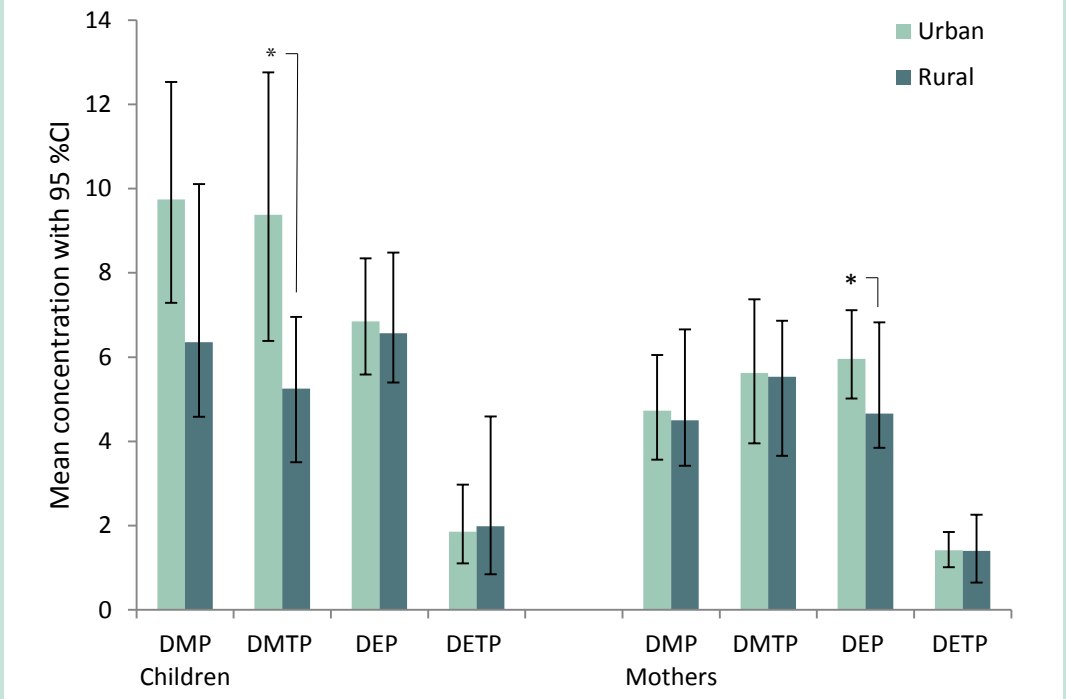
seen for other metabolites or total-DAPs, DMAP, or DEAPs. For the mothers, intake of homegrown food was not significantly associated with DAP concentrations. The concentrations of the summed variables DMAP, DEAP and DAP in relation to intake of rice, cereal and homegrown food can be seen in table 5 below.

		Children (GM [95%CI in nmol/g creatinine])				Mothers (GM [95%CI in nmol/g creatinine])			
Variables	**Categories**	**N**	**DMAP**	**DEAP**	**DAP**	**N**	**DMAP**	**DEAP**	**DAP**
Household income	< 450.000 a year	49	67.5	34.1	118	50	48.2	30.4	88.8
			[46.9-93.7]	[26.4-43.3]	[89.0-152]		[35.9-63.0]	[23.7-38.5]	[70.5-110.4]
	> 450.000 a year	91	58.3	40.4	118	91	47.0	31.2	88.1
			[45.1-73.2]	[33.9-48.2]	[99.7-139]		[38.4-57.9]	[26.0-37.3]	[75.4-103]
Educational level*	ISCED 2-4	17	53.7	34.6	105	17	45.0	30.1	91.6
			[29.05-99.5]	[24.6-49.0]	[68.8-160]		[31.6-79.5]	[19.8-43.9]	[66.4-128]
	ISCED 5 or 6	126	61.8	38.3	118	127	46.7	30.9	87.4
			[50.3-75.5]	[32.6-44.7]	[102-137]		[39.6-55.9]	[27.1-35.8]	[76.8-101]
Rice	Several times a week or more	53	71.0	39.7	128	49	42.6	32.2	83.3
			[52.3-96.5]	[32.5-49.2]	[104-158]		[32.3-56.3]	[26.6-38.9]	[68.2-101]
	Once a week or less	91	55.0	36.3	109	95	49.2	30.3	89.8
			[43.1-70.6]	[29.4-44.1]	[91.1-160]		[40.3-58.9]	[25.3-36.4]	[76.5-104]
Cereal	Daily	93	62.0	38.0	120	75	44.2	30.9	84.2
			[48.3-79.2]	[31.3-45.7]	[101-145]		[35.5-54.8]	[25.4-38.1]	[70.0-100]
	Several times a week or more	50	55.6	35.6	105	69	49.9	31.0	91.3
			[40.5-77.4]	[28.0-45.1]	[83.0-134]		[40.0-62.9]	[25.9-37.0]	[76.9-109]
Homegrown food	2-3 times a month or more	47	51.6	41.4	109.2	50	46.6	33.1	89.4
			[36.0-74.8]	[31.3-56.4]	[83.8-145]		[35.2-61.8]	[26.9-40.7]	[73.0-108.9]
	Once a month or less	97	65.2	35.8	119	95	47.7	29.6	87.4
			[52.0-81.5]	[30.2-42.2]	[102-140]		[39.1-57.9]	[24.8-35.2]	[74.2-101]

TABEL 5
THE CONCENTRATIONS OF THE SUMMED VARIABLES DMAP, DEAP AND DAP IN RELATION TO HOUSEHOLD INCOME, EDUCATIONAL LEVEL OF THE MOTHER AND INTAKE OF RICE, CEREAL AND HOMEGROWN FOOD, RESPECTIVELY. * EDUCATIONAL LEVEL ISCED 2-4: FINISHED 10TH GRADE, HIGH SCHOOL LEVEL, OR SUPPLEMENTS TO HIGH SCHOOL. ISCED 5 AND 6: SHORT, MIDDLE OR LONG UNIVERSITY DEGREE AND PH.D. DEGREE

3.5 Urban and rural differences

When comparing the two areas, we generally found highest concentrations of DAPs in participants from the urban area. The concentration of total DAPs before C correction (*p=0.049*), the summed DMAP (*p=0.029*), and the metabolite DMTP alone (*p=0.019*) were significantly higher in the children from the urban area compared to children from the rural area. It should be noted that the rural area in this study (Viby-Sjælland) is not an agricultural region but rather a village area. The same tendency was found for DMP, however this was only borderline significant (*p< 0.10*) (see figure 1). In mothers, the differences in total DAP were not significantly different between the areas, but significantly higher levels of DEAP (*p=0.017*) and the individual metabolite DEP (*p=0.012*) were found in urban living mothers compared to rural mothers in creatinine corrected values. The differences in DEP (before C correction) and DETP were also borderline significant. The differences between areas remained after adjusting for age, educational level of the mother, and household income. The differences in DAP metabolites between the two areas may be partly explained by the frequency of intake of homegrown food as 40% of children and mother in the rural area reported to eat homegrown food 2-3 times a month or more compared to 27% and 29% of children and mothers in the urban area.



FIGUR 3
MEAN (95% CI) CONCENTRATIONS OF CREATININE CORRECTED VALUES IN MOTHERS AND CHILDREN FROM URBAN AND RURAL AREAS.

The correlation between the individual DAP metabolites was significant for DEP, DETP, DMP, DMTP in mothers. In children significant correlations were only found between the related metabolites – DEP/DETP and DMP/DMTP, respectively. Furthermore, the concentrations of the individual DAPs in the mothers significantly correlated with the levels of some other industrial chemicals 2,4-dichlorophenol (2,4-DCP), 2,5- dichlorophenol (2,5-DCP) and 2-phenylphenol (2-PP) measured in the same participants. No significant correlations were found between DAP concentrations and paracetamol (NAAP) or mercury in hair for neither children nor mothers.

	DAPs				Other chemicals measured in DEMOCOPHES				
	DMP	**DMTP**	**DEP**	**DETP**	**2,4-DCP**	**2,5-DCP**	**2-PP**	**NAAP**	**Mercury**
Children									
DMP	1.000	**0.567****	**0.192***	0.042	0.032	-0.018	-0.065	0.066	0.060
DMTP		1.000	0.114	0.131	0.113	0.022	-0.089	-0.008	0.015
DEP			1.000	**0.477****	0.109	0.032	0.078	0.144	-0.055
DETP				1.000	0.056	-0.006	-0.006	0.063	-0.067
Mothers									
DMP	1.000	**0.450****	**0.290****	**0.226****	0.076	0.179*	**0.178***	0.002	-0.02
DMTP		1.000	**0.409****	**0.453****	**0.305****	**0.294****	**0.313****	0.015	0.015
DEP			1.000	**0.624****	**0.320****	**0.265****	**0.223****	0.018	-0.031
DETP				1.000	**0.246****	**0.254****	**0.213***	0.041	-0.016

TABEL 6
CORRELATIONS BETWEEN THE MEASURED DAPS, 2,4-DCP, 2,5-DCP AND 2-PP IN CHILDREN (N=144) AND MOTHERS (N=145). * P<0.05, **P<0.01

4. Discussion

Although the urine samples analyzed in this study were collected after organophosphates were almost phased out for agricultural and biocidal use in Denmark, most urine samples had detectable concentrations of at least one DAP metabolite. The metabolite DEP was detected in 96% of the samples from the children and 97% of the samples from the mothers and the concentration of DEP and the total concentration of DEAPs was at the same level as in urine samples collected among Danish children of similar age in 2007-08 (Table 7). The levels were similar to DEP and DEAP concentrations reported in other European studies performed in recent years but higher than levels reported in studies from the US (Table 7 and 8). In contrast, the concentration of methylated metabolites, DMAPs, and the total DAP concentration was markedly lower than in the Danish samples from 2007-08 and lower than levels measured in Germany, France, and the Netherlands between 2002 and 2007 but higher than among Spanish children from the Valencia region measured in 2010 (Table 7 and 8).

Overall, the DAP levels found in this study and in other European studies seem to be higher than levels measured in the US National Health and Nutrition Examination Survey (NHANES) and for DEP and the total DEAPs, the concentration seem to be higher in Denmark than in most other countries with available data (Table 7 and 8).

To enable comparison with other studies, the results are presented both as μg or nmol per L of urine and as μg or nmol per gram of creatinine (to account for inter-individual differences in urine dilution). In adults, the daily mass of excreted creatinine is considered to be fairly constant (Mage et al. 2008). The creatinine excretion in children is more variable because of their growth but despite that, presentation of creatinine corrected concentrations has been s recommended for both adults and children. However, for children creatinin variability has to be taken into consideration if the urine concentrations are used for calculation of the daily dose of contaminants (Mage et al. 2008).

In studies from both the US, Australia, and Canada, the main source of organophosphate exposure in the general population has been demonstrated to be the diet (Curl et al. 2015; Oates et al. 2014; Ye et al. 2015). This is also likely the case in Europe inclusive Denmark, although no specific studies on this issue have been published. In our study, DAP levels were in general lower among those living in the more rural area and among those reporting to eat homegrown fruit or vegetables 2-3 times a month or more although the later association was only statistically significant for DMP. In a study from Australia, higher DAPs were found in children from rural areas compared to urban areas (Babina et al. 2012) probably due to agricultural use of OPs in the rural area. The associations between the levels in mothers and children, which were found statistically significant for DAP and DMAP, also indicates that the exposure is related to a common source within the family, very likely from the consumption of the same diet containing more or less organophosphate contaminated food.

According to results from the Danish monitoring program of pesticide residues in food samples on the Danish marked for the 2004-2011 period, foreign produced fruit and vegetables most often contained detectable pesticide residues and most often contained concentrations above the Maximum Residue Limits (MRLs) (Petersen et al. 2013) especially those produced outside EU. Chlorpyrifos was the second most frequently detected pesticide and was found in approximately 10% of 12.500 samples of fruit and vegetables analyzed. For some commodities the detection

frequency of chlorpyrifos was especially high e.g., 63% for mandarins/clementines, 43% for oranges, 37% for grapefruit, 36% for lemons, 25% for apples, 19% for peaches and 12% for table grapes. Compared to the period 1998-2003, the detection frequency of several OPs, inclusive chlorpyrifos-methyl and chlorpyrifos, was reported to have increased. Malathion was also often detected during the 2004-2011 period but in less than 5% of the samples and the detection frequency was unchanged compared to 1998-2003. For cereals, pesticide residues were found in 39% of the samples. Primiphos-methyl was detected in 5.2% of the samples and was the second most detected pesticide in cereals (Petersen et al. 2013). Thus, this data support that intake of imported fruit and vegetables is likely to be the main source for OP exposure in this cohort. The data also indicate that chlorpyrifos may contribute considerably to the relatively high level of DEP and DEAPs seen in this study.

The higher concentrations of DAPs in the children than their mothers are in accordance with other studies as for example NHANES (Barr et al. 2004)(see also Table 6) and related to a relatively higher food intake (food per kilogram of bodyweight) in children than adults which is more pronounced for the youngest children. The positive association between socioeconomic status (SES; maternal education level and household income) and urinary DAP concentrations among the children might be explained by dietary habits related to SES as both education level and income are determinants of fruit and vegetable intake (Groth et al. 2001).

Health benefits of eating fruits and vegetables are well-documented but whether pesticide residues in non-organic produce can compromise the health beneficial effects are discussed (Baranski et al. 2014; Forman and Silverstein 2012). However, it is noteworthy that the levels in the Danish and European studies in general are higher than in the US studies in which neurobehavioural outcomes have been associated with DAP concentrations in urine samples. A cross-sectional study based on data from the NHANES has demonstrated that, within the range of exposure in the general US population in 2002-04, the odds of ADHD for 8- to 15-year old children increased 55% with a 10-fold increase in urinary concentrations of DMAPs (Bouchard et al. 2010). Among 5 years old children of farm workers in Salinas Valley in California (the CHAMACOS cohort) a 10-fold increase in child urinary DEAP concentration doubled the odds in an ADHD indicator variable although this association was not seen for the total DAP concentration (Marks et al. 2010). The cross-sectional study design of these studies does not allow conclusion about causality but effects on attention and other neurobehavioural outcomes have also been reported from longitudinal studies, especially related to prenatal exposures. In longitudinal birth cohort studies based on the CHAMACOS-cohort and residents of New York City, maternal exposure to chlorpyrifos and other organophosphate insecticides in pregnancy was associated with neurobehavioural deficits in the children at least through 7 years of age (Bouchard et al. 2011; Eskenazi et al. 2007; Marks et al. 2010; Rauh et al. 2011; Rauh et al. 2006). Additionally, prenatal exposure to chlorpyrifos was associated with structural brain anomalies at school-age including a thinner cortex and disruption of normal sexual dimorphisms in brain structure demonstrated by magnetic resonance imaging (Rauh et al. 2012). Based on these studies, chlorpyrifos was recently categorized as a human developmental neurotoxicant (Grandjean and Landrigan 2014). These findings are also supported by results from experimental studies of low dose gestational exposure to chlorpyrifos (De Felice et al. 2014; Mullins et al. 2015), parathion (Levin et al. 2010) or diazinon (Slotkin et al. 2008) showing long-lasting cognitive effects in the offspring. Most reviews of neurodevelopmental effects of OPs in humans suggest that exposure during pregnancy, at levels found among groups of the general population, may have negative effects on children's neurodevelopment (Gonzalez-Alzaga et al. 2014; Munoz-Quezada et al. 2013; Ross et al. 2013) while others find the evidence less convincing (Burns et al. 2013; Reiss et al. 2015). The discrepancy is likely related to the large variability in study designs and in methodologies used for assessing exposure and neurodevelopmental outcomes across studies as well as differences in the procedure used for including studies in the reviews. However, the OP exposure has been declining in the US, and a recent study found no negative impact of gestational OP-exposure on neurobehavioral outcomes in early infancy (four weeks of age) (Yolton et al. 2013).

This might indicate that the exposure level in the general US population has reached a no-adverse-effect-level. Hopefully this cohort will be followed further during childhood where more sensitive neurobehavioural test can be performed. To our knowledge no European studies have investigated associations between urinary DAP concentrations and health outcomes, however, as the European levels exceeds the US levels where adverse health outcomes have been found in some studies, such studies are recommended.

We have previously measured the urinary concentrations of the 2,4-DCP and 2,5-DCP and 2-PP in the same batch of urine samples (Frederiksen et al. 2013). 2,4-DCP, 2,5-DCP and 2-PP are intermediates in the production of dichlorophenols pesticides (e.g., phenoxy acid herbicides such as 2.4-D and 2,4,5-T, some OPs such as dichlofention and prothiofos, and p-dichlorobenzene used as insecticide) but also degradation products of these pesticides as well as of chlorinated phenols used as biocides and for disinfection (e.g., triclosan) (Bomhard et al. 2002; Casas et al. 2011; Wei et al. 2014). The concentrations found in the mothers in this study significantly correlate with the DAP metabolites measured in the present study. This indicates that some exposure sources of 2,4-DCP, 2,5-DCP and 2-PP are similar to OP exposure and might be related to intake of fruits and vegetables imported from countries with less restrictions on pesticide use in agriculture.
The present study has some limitations. First, the study was not planned and designed to investigate determinants of OP exposure and therefore the questionnaire did not include detailed questions on relevant food items or other potential exposure sources such as use of pesticides in the homes or gardens. However, since OPs were almost phased out in Denmark in 2011, information about residential pesticide use seem less important. The finding, that intake of home-grown food was associated with reduced urinary DAP concentrations may be an indication of lower intake of imported fruit and vegetables although we cannot make final conclusion on this.
The use of unspecific DAP metabolites as exposure biomarkers does not allow us to identify which OPs that constitute the majority of the exposure and although the total level seem higher than in studies from the US, the composition of the exposure may differ. Besides, part of the DAPs measured may be from intake of OP metabolites preformed in food items and in the environment. This will cause an overestimation of the exposure to the parent toxic compound and hamper the conclusion on the health risk associated with the exposure level. However, the same approach has been used in studies reporting associations between urinary DAP concentrations and adverse health outcomes (Eskenazi et al. 2014). Although some OPs are more potent than others, they all target the same organ (nervous system) and share common mechanisms (e.g., cholinesterase inhibition) indicating additive effects and therefor the total exposure level seem to be of most relevance for public health. From a precautionary point of view, a risk assessment assuming that the exposure consists of the most potent OPs identified from the food monitoring program would be obvious.
Formation of DAPs from other chemicals or endogenous compounds would also affect the urinary concentrations and cause an overestimation of the OP exposure. However, in intervention studies where groups of individuals have been offered organic food, a marked reduction in urinary DAP concentrations were reported (Bradman et al. 2015; Oates et al. 2014) indicating that OPs are the main source for these metabolites. The herbicide glyphosate, which is also an organophosphate, does not seem to contribute to the DAP concentrations, as this compound is rapidly converted to aminomethylphosphonic acid (AMPA) and glufosinate to 3-(methylphosphinyl) propionic acid (3-MPPA). These very polar metabolites are excreted in the urine (Motojyuku et al. 2008; Raina-Fulton 2014).
An additional limitation is the use of spot urine samples. Since OP and their metabolites have short half-lives of few days, DAP concentrations in spot urine samples will only reflect exposures occurring few days prior to sampling. Accordingly, the within-individual variation is high, and sometimes higher, than between individuals (Attfield et al. 2014; Bradman et al. 2013; Spaan et al. 2015). Therefore, these measurements should only be used to compare OP exposure at population level whereas for individual exposure information repeated sampling or analysis of pooled urine samples collected during a period would be more valid.

	Years of sampling	Value	DMP	DMTP	DMDTP	DEP	DETP	DEDTP	Reference
Studies presenting creatinin corrected concentrations (µg/g creatinine)									
Denmark, DGCC (6-11 years), n=174	2007-08	M	6.37	4.14	0.36	3.83	0.84	0.31	(Andersen et al. 2012)
Spain (6-11 years), n=125	2010	M	<LOD	<LOD	<LOD	2.28	<LOD	<LOD	(Roca et al. 2014)
France, ENNS (18-74 years), n=392	2006-07	M	8.04	5.95	0.54	3.66	1.12	0.02	(Fréry et al. 2011)
Australia (2.5-6 years) n urban area = 115 **n periurban area= 111** **n rural area = 114**	2003-06	M	- - -	6.8 24.8 37.3	7.2 6.9 7.2	4.1 15.4 24.7	2.7 7.9 13.8	- - -	(Babina et al. 2012)
Israel (20-74 years), n=247	2011	M	10.0	5.2	0.2	1.5	0.5	0.02	(Berman et al. 2013)
USA NHANES (> 6 years), n=3016 **(6-11 years), n=576**	2001-02	M	< LOD 1.93	0.93 2.16	< LOD < LOD	< LOD 0.89	0.57 0.64	< LOD < LOD	(CDC 2013)
USA NHANES (> 6 years), n=2491 **(6-11 years), n=310**	2003-04	M	< LOD < LOD	1.98 3.41	< LOD < LOD	< LOD < LOD	< LOD < LOD	< LOD < LOD	(CDC 2013)
USA NHANES (> 6 years), n=2634 **(6-11 years), n=350**	2005-06	M	< LOD < LOD	1.75 2.78	< LOD < LOD	< LOD < LOD	< LOD < LOD	< LOD < LOD	(CDC 2013)
USA NHANES (> 6 years), n=2591 **(6-11 years), n=385**	2007-08	M	< LOD < LOD	2.00 4.28	< LOD < LOD	< LOD < LOD	< LOD < LOD	< LOD < LOD	(CDC 2013)
USA, NYC HANES (aged > 20 years), n= 876	2004	M	< LOD	< LOD	< LOD	0.52	< LOD	< LOD	(McKelvey et al. 2013)
Puerto Rica (pregnant women), n=54 (154 samples)	2010-12	M	< LOD	1.0	< LOD	< LOD	< LOD	< LOD	(Lewis et al. 2014)
Ecuador (6-9 years old), n=92	2008	M	1.65	0.70	< LOD	0.53	0.24	< LOD	(Harari et al. 2010)
Studies presenting volume based concentrations (µg/L urine)									
Denmark, DGCC (6-11 years), n=174	2007-08	M	8.40	5.60	<LOD	5.16	1.02	<LOD	(Andersen et al. 2012)
Germany, GerES IV (3-14 years), n=599	2003-06	M	15.2	15.9	0.5	6.0	1.0	0.02	(Schulz et al. 2012)
Canada, CHMS (6-79 years), Males (n=2653) **Females (n=2814)**	2007-09	GM	3.02 2.91	2.07 1.99	<LOD <LOD	2.38 2.22	<LOD <LOD	<LOD <LOD	(Haines and Murray 2012)
Canada, MIREC, (pregnant women), n=1850	2008-11	M	3.25	3.43	0.39	2.38	0.65	<LOD	(Colapinto et al. 2015)
Caribbean Islands (pregnant women), n=150	2011-12	M	1.40	0.80	-	1.50	< LOD	-	(Forde et al. 2015)
Ecuador (pregnant women), n=26 (incl. 16 rose workers)	2011	M	3.41	40.6	0.45	0.92	0.41	0.38	(Handal et al. 2015)

TABEL 7 URINARY CONCENTRATIONS OF DIALKYL PHOSPHATE METABOLITES (DAPS) IN DIFFERENT STUDIES CONDUCTED AFTER 2000. VALUES ARE PRESENTED AS MEDIAN (M) OR GEOMETRIC MEAN (GM). LOD BETWEEN 0.01 AND 0.5 DEPENDENT ON CHEMICAL, LABORATORY, AND YEAR OF ANALYSIS.

	Years of sampling	Values	DEAP	DMAP	DAP	Reference
Studies presenting creatinine corrected concentrations (nmol/g creatinine)						
Danish children (6-11 years old), n=174	2007-08	M (5-95 pcts)	36.1 (5.0-255.9)	96.7 (10.0-891.6)	141.4 (22.4-1070.9)	(Andersen et al. 2012)
The Netherlands, Generation R Study (pregnant women), n=106	2002-06	M (5-95 pcts)	33.4 (8.9-158.4)	204.6 (37.1-611.5)	261.7 (50.3-698.2)	(Spaan et al. 2015)
Canada, CHMS (6-79 years)	2007-09	GM (95% CI)	25.0 (23.0-27.2)	57.2 (50.1-65.3)	93.2 (84.3-103.1)	(Ye et al. 2015)
USA, CHAMACOS, Salinas valley, (5-years old), n=274	2004-05	GM (95% CI)	11.1 (9.1-13.5)	114 (95.8-136.0)	147 (124.2-173.9)	(Quiros-Alcala et al. 2011)
USA, NYC HANES (aged > 20 years), n= 876	2004	M (95 pcts)	6.4 (90.0)	57.8 (992.6)	83.1 (1003.9)	(McKelvey et al. 2013)
USA, HOME-study (pregnant women), n=350	2003 - 06	GM (95% CI)	9.4 (8.0-11.1)	46.4 (40.2-53.7)	73.5 (64.8-83.4)	(Yolton et al. 2013)
Ecuador (6-9 years old), n=92	2008	M (5-95 pcts)	6.6 (1.8-58.5)	23.7 (5.2-267.1)	32.2 (7.2-343.0)	(Harari et al. 2010)
Studies presenting volume based concentrations (nmol/L urine)						
USA, NHANES (6-11 years old), n=471	1999-2000	Least-squares GM[A](95% CI)	17.4 (11.1-27.3)	72.8 (54.3-97.5)	109.6 (83.3-144.3)	(Barr et al. 2004)
USA, NHANES (8-15 years old), n=1139	2000-04	GM (IQR	11.0 (2.1-35.0)	41.3 (10.1-130.7)	68.3 (24.4-186.0)	(Bouchard et al. 2010)
USA, CHAMACOS, Salinas valley, (pregnant women), n=348	1999-2000	GM (95% CI)	17.7 (16.1-19.4)	76.8 (69.3-85.0)	109.0 (99.4-119.6)	(Marks et al. 2010)
USA, CHAMACOS, Salinas valley, (5-years old), n=320	2004-05	GM(95% CI)	7.2 (6.0-8.7)	72.4 (61.0-86.0)	92.6 (78.6-109.0)	(Marks et al. 2010)
Canada, CHMS (6-11 years), n=1035	2007-09	M (IQR)	25.0 (10.5-51.3)	62.0 (18.7-192.8)	99.2 (34.3-273.3)	(Oulhote and Bouchard 2013)
Ecuador (pregnant women), n=26 (incl. 16 rose workers)	2011	GM	8.26	51.6	83.6	(Handal et al. 2015)

TABEL 8
URINARY CONCENTRATIONS OF DIALKYL PHOSPHATE METABOLITES (DAPS) IN DIFFERENT STUDIES. RESULTS ARE PRESENTED AS MEDIANS (M) WITH PERCENTILES (PCTS) OR GEOMETRIC MEANS (GM) WITH CONFIDENCE INTERVALS (CI) OR INTERQUARTILE RANGE (IQR). DEAP (SUM OF DIETHYL ALKYLPHOSPHATES): DEP+ DEP+DETP; DMAP (SUM OF DIMETHYL ALKYLPHOSPHATES): DMP+DMTP+DMDTP; DAP: DEAP + DMAP. [A]ADJUSTED FOR AGE, SEX, RACE/ETHNICITY, AND CONCENTRATIONS OF SERUM COTININE AND URINARY CREATININE.

5. Conclusion

The findings of relatively high detection frequency of the DAP metabolites DEP, DETP, DMP and DMTP in a Danish group of children and their mothers, clearly indicate that there is still a widespread exposure to organophosphate pesticides in the Danish population, even though there have been major restrictions on their use in agriculture and as biocides. The concentrations of the metabolites DEP and DETP found in the children and women of the present study are similar to levels found among Danish children in 2007-08 and as levels found in other recent European studies. The methylated metabolites seem to have decreased in Denmark compared to previous measurements in 2007-08 and levels found in other European countries before 2007. However, the levels of total DAP and DEAP in the present study, as well as the levels found in other European countries, are higher than concentrations found in the US in a national survey (NHANES), indicating higher exposure to organophosphates in Europe compared to the US. As the exposure to organophosphates has been associated with adverse effects in some studies from the US, there may also be a risk of adverse effects in Europe and Denmark, as we have found even higher exposure levels. Although we do not know the specific organophosphates responsible for the relatively high concentrations of DEAPs in Denmark, chlorpyrifos is likely to be one of the most important since it is often found in samples of imported fruit and vegetables – also in the latest published survey of pesticide residues in food samples on the Danish marked (Fødevareinstituttet 2014). Thus, studies of potential adverse health effects related to organophosphate exposure in European populations are needed. Also identification of the main dietary exposure sources and the related OPs is warranted in order to introduce adequate measures to reduce the exposure - especially for vulnerable population groups.

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Organophosphate metabolites in urine samples from Danish children and women

This report describes the concentrations of six dialkylphosphate metabolites (DAPs) used as biomarkers for organophosphate exposure in Danish school children and mothers participating in the European project DEMOCOPHES.

The findings of relatively high detection frequency of DAP indicate that there is still a widespread exposure to organophosphate pesticides in the Danish population, even though there have been major restrictions on their use in agriculture. Chlorpyrifos is likely to be an important contributor to the DAP found since it is often found in samples of imported fruit and vegetables, which is considered the main exposure source for OPs in Denmark.

The concentrations of the DAPs found in the children and women of the present study are in line with what was found in Europe and previous measurements in Denmark. However, methylated metabolites seem to have decreased in Denmark compared to previous measurements in 2007-08 and levels found in other European countries before 2007.

Exposure to OPs has been associated with adverse health effects in some studies in USA, hence, there may also be a risk of adverse effects in Europe and Denmark, as we have found even higher levels of exposure.

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