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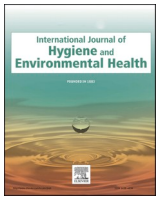

Assessment of exposure to pesticide mixtures in five European countries by a harmonized urinary suspect screening approach

Ilse Ottenbros [a,b], Erik Lebret [a], Carolin Huber [d,e], Arjen Lommen [f], Jean-Philippe Antignac [g], Pavel Čupr [h], Libor Šulc [h], Ondřej Mikeš [h], Tamás Szigeti [i], Szilvia Középesy [i], Inese Martinsone [j], Zanna Martinsone [j], Lasma Akulova [j], Olga Pardo [k,1,2], Sandra F. Fernández [k], Clara Coscollá [k], Susana Pedraza-Diaz [l], Martin Krauss [d], Laurent Debrauwer [m,n], Kévin Wagner [m,n], Rosalie Nijssen [f], Hans Mol [f], Chiara Maria Vitale [h], Jana Klanova [h], Borja Garlito Molina [o], Nuria León [k], Roel Vermeulen [a], Mirjam Luijten [c], Jelle Vlaanderen [a,*]

[a] *Institute of Risk Assessment Sciences, Utrecht University, Yalelaan 2, 3584CM, Utrecht, the Netherlands*
[b] *Center for Sustainability, Environment and Health, National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3721MA, Bilthoven, the Netherlands*
[c] *Center for Health Protection, National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3721MA, Bilthoven, the Netherlands*
[d] *Department of Effect-Directed Analysis, Helmholtz Centre for Environmental Research (UFZ), Permoserstr. 15, 04318, Leipzig, Germany*
[e] *Institute for Ecology, Diversity and Evolution, Goethe University Frankfurt Biologicum, Campus Riedberg, Max-von-Laue-Str. 13, 60438, Frankfurt am Mein, Germany*
[f] *Wageningen Food Safety Research, Part of Wageningen University and Research, Akkermaalsbos 2, 6708WB, Wageningen, the Netherlands*
[g] *Oniris, INRAE, LABERCA, 44300, Nantes, France*
[h] *RECETOX, Faculty of Science, Masaryk University, Kotlarska 2, Brno, 61137, Czech Republic*
[i] *National Public Health Center, Albert Flórián út 2-6, 1097, Budapest, Hungary*
[j] *Laboratory of Hygiene and Occupational Diseases, Rīga Stradiņš University, Latvia*
[k] *Foundation for the Promotion of Health and Biomedical Research of the Valencia Region, Av. Catalunya 21, Valencia, Spain*
[l] *National Centre for Environmental Health, Instituto de Salud Carlos III (ISCIII), Majadahonda, Spain*
[m] *Toxalim (Research Center in Food Toxicology), Toulouse University, INRAE UMR 1331, ENVT, INP-Purpan, Paul Sabatier University, 31027, Toulouse, France*
[n] *Metatoul-AXIOM Platform, National Infrastructure for Metabolomics and Fluxomics: MetaboHUB, Toulouse, France*
[o] *Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water (IUPA), Universitat Jaume I, Av. Sos Baynat S/N, 12071, Castelló de la Plana, Spain*

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ABSTRACT

Humans are exposed to a mixture of pesticides through diet as well as through the environment. We conducted a suspect-screening based study to describe the probability of (concomitant) exposure to a set of pesticide profiles in five European countries (Latvia, Hungary, Czech Republic, Spain and the Netherlands). We explored whether living in an agricultural area (compared to living in a peri-urban area), being a a child (compared to being an adult), and the season in which the urine sample was collected had an impact on the probability of detection of pesticides (-metabolites).

In total 2088 urine samples were collected from 1050 participants (525 parent-child pairs) and analyzed through harmonized suspect screening by five different laboratories. Fourty pesticide biomarkers (either pesticide metabolites or the parent pesticides as such) relating to 29 pesticides were identified at high levels of confidence in samples across all study sites. Most frequently detected were biomarkers related to the parent pesticides acetamiprid and chlorpropham. Other biomarkers with high detection rates in at least four countries related to the parent pesticides boscalid, fludioxonil, pirimiphos-methyl, pyrimethanil, clothianidin, fluazifop and propamocarb. In 84% of the samples at least two different pesticides were detected. The median number of

Abbreviations: HBM, Human Biomonitoring; HBM4EU, European Human Biomonitoring Initiative; LC-HRMS, Liquid chromatography coupled to High Resolution Mass Spectrometry; SPECIMEn, Survey on PEstiCIde Mixtures in Europe; SS, Suspect Screening.

* Corresponding author.
E-mail address: J.J.Vlaanderen@uu.nl (J. Vlaanderen).
[1] Present Address: Department of Analytical Chemistry, University of Valencia, Doctor Moliner 50, 46100, Burjassot, Spain.
[2] Present Address: Public Health Laboratory of Valencia, Av. Catalunya 21, Valencia, 46020, Spain.

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detected pesticides in the urine samples was 3, and the maximum was 13 pesticides detected in a single sample. The most frequently co-occurring substances were acetamiprid with chlorpropham (in 62 urine samples), and acetamiprid with tebuconazole (30 samples). Some variation in the probability of detection of pesticides (-metabolites) was observed with living in an agricultural area or season of urine sampling, though no consistent patterns were observed. We did observe differences in the probability of detection of a pesticide (metabolite) among children compared to adults, suggesting a different exposure and/or elimination patterns between adults and children.

This survey demonstrates the feasibility of conducting a harmonized pan-European sample collection, combined with suspect screening to provide insight in the presence of exposure to pesticide mixtures in the European population, including agricultural areas. Future improvements could come from improved (harmonized) quantification of pesticide levels.

1. Introduction

Humans are typically exposed to pesticides through multiple sources, including diet, occupational or environmental exposures (Damalas and Eleftherohorinos, 2011; Deziel et al., 2015). Growing evidence indicates that living in an agricultural area where pesticides are applied contributes to higher exposure than residents living away from agricultural areas (Dereumeaux et al., 2020; Figueiredo et al., 2021; Teysseire et al., 2020, 2021). Determinants contributing to this increased exposure include proximity to agricultural fields where pesticides are applied, crop acreage around the home, and season (Dereumeaux et al., 2020; Teysseire et al., 2021). Pesticide exposure has been linked to various short-term and chronic health effects such as respiratory or neurological development issues (Kim et al., 2017; Ntzani et al., 2013). Therefore a comprehensive characterization of the exposure to real-life mixtures of pesticides, which includes the contribution of living close to agricultural areas where pesticides are applied, is essential for human health risk assessment.

Most non-occupational pesticide exposure studies focus on selected sets of targeted pesticides for human biomonitoring (HBM), often based on *a priori* selected biomarkers related to e.g. the spraying activities in a certain area, the health outcome of interest, or practical considerations such as the commercial availability of standards (Dereumeaux et al., 2020; Teysseire et al., 2021). Currently, HBM for urinary pesticide biomarkers by targeted methods is limited to mostly pyrethroids and non-specific markers of organophosphorus pesticides. However, in real-life pesticide exposure often is already a mixture of multiple co-occurring compounds with repeated exposure timeframes (Crépet et al., 2019). With more than 450 active pesticides currently approved (plus 50 more currently pending) for use in the European Union (EU Database Pest, 2022), there is a growing need for information on the co-occurrence of these compounds in the human body. HBM of pesticides in urine is a useful method to assess the aggregate exposure of pesticides from various exposure sources and routes, by measuring the parent pesticide and/or the corresponding biotransformation products (Bonvallot et al., 2021). However, as the list of registered pesticides is long and they occur often highly metabolized in urine, a large number of targeted assays would be required to assess presence of all urinary pesticides and their metabolites in each sample. This is currently not feasible since many human urinary biomarkers of exposure (typically phase I/II metabolites) are often unknown, and the analytical reference standards are not readily available. Suspect screening (SS) approaches based on full scan High Resolution Mass Spectrometry (HRMS) emerge as an innovative way to assess the presence of a broad range of exposure markers and better capture the complexity of pesticide mixtures (Andra et al., 2017; Pourchet et al., 2020; Huber et al., 2022).

The study presented here, the Survey on PEstiCIde Mixtures in Europe (SPECIMEn), aimed to generate new pesticide exposure data in a harmonized pan-European setting (as part of the European Human Biomonitoring Initiative HBM4EU, www.hbm4eu.eu). This was done by analyzing 2088 urine samples collected in five countries through a multi-laboratory high-throughput SS approach. This study aimed at exploring co-occurrence (probability of exposure) of pesticide biomarkers across Europe and within each participating country. It also aimed at assessing differences of exposure patterns by location (living close to agricultural fields *versus* non-agricultural areas), seasons (differences in spraying activities), as well as age groups (adults *versus* children, of which the latter are more sensitive to health effects and usually have higher internal exposure levels, due to e.g. a higher food intake/kg body weight (Eskenazi et al., 1999; Sapbamrer and Hongsibsong, 2019). The study design therefore provides insight into local contributions, based on a broad combination of pesticides. Higher detection frequencies of pesticide markers might be expected for those pesticides applied on local crops during the spraying season in residents living close to the agricultural fields.

2. Material and methods

2.1. Sampling strategy

To create geographical coverage across Europe, study sites from five countries were included to provide insight into variations of pesticide exposure patterns across Europe, namely the Czech Republic, Hungary, Latvia, Spain and the Netherlands. Within each country, urine samples were collected simultaneously at two locations: agricultural and non-agricultural areas. Each address in the agricultural area was located within 250 m from an agricultural field where pesticides were typically applied, mainly focusing on tree-crops or so-called 'overhead cultures' (except Latvia where tree-crops were hardly grown). These 'overhead-cultures' will result in potentially higher exposure concentrations in the air due to machine-drawn air blast or a hand-held overhead spray, which are more prone to drift (Willenbockel et al., 2022). The crop types differed slightly between countries due to e.g. differences in climate. A detailed description of the area selection in all five country can be found in Supplementary Material F. In summary, Spain focused on residential areas close to citrus fruits, Czech Republic on apples, vineyards, peach, plums and apricots, Hungary on apples, the Netherlands on apples and pears, and Latvia mostly on winter and summer rapeseed, summer wheat and barley. Non-agricultural areas were defined as sub-urban areas at least 500 m away from any agricultural fields.

Per country at each agricultural and non-agricultural area, 50 parent-child pairs (50 households) were included (total of 100 parent-child pairs per country). Each parent-child pair was composed of one child aged 6–11 years at the time of inclusion, accompanied by one of their parents or legal guardians living in the same household. Adults who worked in the agricultural sector (i.e. farmers) were excluded from recruitment, since the sample size was too limited to distinguish occupational exposures. The same selection criteria were used in all five countries.

A minimum of 100 parent-child pairs per country (200 individuals) provided a first morning void urine sample, and completed a harmonized questionnaire. The admission of the questionnaire, sample collection procedures and timing of sampling was coordinated, and sampling materials such as cups and tubes were bought in bulk to avoid any batch differences. All collected urine samples were stored and transported refrigerated (at 4 °C), until samples were aliquoted and

stored at −80 °C (within 48 h of sample collection). Samples were transported to the laboratory of analysis after each season.

All households were visited twice: the first visit was made in winter 2019/2020 (season 1), the second in summer 2020 (season 2). The specific sampling dates (Supplementary Material A) differed slightly between study sites, partly due to differences in spraying season due to climate and the type of crop grown on the field. The sampling of the second season was slightly delayed (end of summer) due to the COVID-19 pandemic and accompanied uncertainties. The recruitment strategy differed between the study sites, a detailed description of the recruitment strategy per country can be found in Supplementary Material F. In summary, the Hungarian partner involved local public health officers to get in touch with the participants, while others sent out letters (the Czech Republic and the Netherlands), contacted colleagues as study participants (Spain and the Netherlands), conducted an online campaign (the Czech Republic and the Netherlands), and/or contacted participants through schools (Spain and Latvia). A detailed questionnaire was completed during the first season by the parent, and a subset of questions was asked again during the second season (Supplementary Material B). The joint questionnaire was developed in English, and subsequently translated to the local languages. The questionnaire covered personal and household characteristics, activities up to three days prior to sampling, potential pesticide exposure scenarios (occupational, usage of products containing pesticides), and the food consumption pattern of the day prior to sampling (origin of consumed foods as well as a food frequency table for food consumption 24 h prior to sampling).

All partner countries acquired approval from the appropriate local medical ethical committees, and written informed consent was obtained from all participants (parents and children separately). A description of the ethical approval procedure per country can be found in supplementary material F. A harmonized informed consent form was used for all participants, which was evaluated by an internal HBM4EU review board.

2.2. Suspect screening approach

A SS methodology was applied to analyze the urine samples, of which a detailed description can be found in Huber et al. (2022). Briefly, the applied analytical workflow from sample preparation, instrumental analysis, and data processing was conducted under harmonized conditions in five different laboratories across Europe, in the Netherlands, Germany, France, the Czech Republic and Spain (Vitale et al., 2022). Each laboratory analyzed approximately 400 urine samples originating from one of the five SPECIMEn study sites. Samples were analyzed after each season, and potential batch effects were addressed (Huber et al., 2022). The suspect database generation, MS data analysis and confirmation procedures were performed in a centralized way. Several consolidated quality assurance/quality control (QA/QC) dispositions, parameters and criteria were first implemented to ensure the consistency of the results obtained across the different participating laboratories as well as to document the applied method performances (Vitale et al., 2022). The applied analytical workflow was described in detail by Huber et al. (2022) and consists of i) SPE cleanup/concentration (5-fold) of the urine after pH adjustment, ii) measurement of the extracts by full scan liquid chromatography coupled to HRMS (LC-HRMS), iii) data pre-processing and analysis, iv) prioritization of putative detects, v) generation of a list of representative samples for follow up identification experiments using tandem mass spectrometry (MS/MS), and vi) final confirmation of putative detects by spectral comparison with reference standards either purchased/synthesized or generated *in vitro* by human liver S9 incubations. The curated suspect list of pesticides may include multiple metabolites originating from the same parent compound, resulting in a final datafile with potentially several metabolites that reflect exposure of the same parent compound. This redundancy is considered enhancing confidence. In the case of SPECIMEn, this list focused on pesticides and one aggregated list of known and predicted pesticide metabolites from all five laboratories was used as suspect database. 'Fully identified' were those with the highest level of confidence: Schymanski level 1 if a reference standard material is commercially available, or Schymanski 2 by diagnostic evidence acquired by human liver S9 incubation experiments (Schymanski et al., 2014). Biomarkers which were identified at a lower tier will end up in lower confidence levels, reflecting the level of uncertainty about the identity of that feature. In the context of the present paper, only biomarkers identified with confidence levels 1 and 2 were considered.

2.3. Statistical analysis

In line with the basic principle of the SS approach, the data generated in SPECIMEn are 'semi-quantitative', i.e. quantitative signal intensities for each representative spectrometric mass are reported per sample, yet these intensities cannot be considered as urinary pesticide concentrations and are not standardized across laboratories. The data was analysed by dichotomizing the intensities into 'detected' versus 'non-detected', which allows comparisons across study sites as well as inclusion of biomarkers with low detection rates in the statistical analysis.

The detection rate was calculated as the number of samples in which a particular biomarker was detected and identified with confidence levels 1 and 2 over the total number of samples collected, expressed in percentage. Based on the parent pesticides (if multiple metabolites and the parent pesticide were measured, these were considered as one), the patterns of co-occurrences were explored. First, the total number of pesticides per urine sample was evaluated. Secondly, with the usage of an UpSet plot it was evaluated which parent pesticide combinations co-occurred and how frequent. Thirdly, the correlation pattern in the total set of parent pesticides was evaluated for each study site with a weighted correlation network using the *IsingFit* R package v0.3.1 (van Borkulo et al., 2015). This package estimates the network based on the Ising model: combining L1-regularized logistic regression with EBIC model selection (gamma 0.25). On this network a clustering algorithm was applied (*walktrap)*, to detect communities of closely related features indicated by different colours in the network (Pons and Latapy, 2005).

To assess the influence of co-variates, logistic mixed effects regression models were applied, with participant ID and household ID as random effects. Our main model includes fixed effects for season (season 1/season 2), location (agricultural/non-agricultural) and age category (child/adult). We assessed the sensitivity for further adjustment for potential confounding by including body mass index (BMI) level, education of the parent, consumption of homegrown foods (yearly average percentage), and a summary indicator for pesticide usage in an extended model. The pesticide usage indicator indicates whether pesticide containing products were used up to three days prior to sampling either for human use, in the garden, indoors and/or for professional use. The estimates for season, location and age groups were transformed to Odds Ratios (OR) with 95% Confidence Intervals (CI) for both the main and extended models.

3. Results

3.1. Population characteristics

The description of the study population for the five study sites of the SPECIMEn study is provided in Table 1. In total 2088 urine samples were collected, which were equally spread across the five study sites and areas. The loss to follow-up of individuals between seasons was low, varying from 0.9 to 2.9%. Reasons for loss to follow-up were loss of contact, divorce and/or move to another location. The adult samples mainly originated from the mothers, while gender was equally divided across the children's samples. The mean age of the adults was comparable across all study sites, varying from 38 to 44 years. The mean BMI (self-reported) of the adults originating from Latvia and Hungary was

Table 1
Descriptive characteristics of the SPECIMEn study participants by study site and location.

Study site	ES[a]		LV[a]		HU[a]		CZ[a]		NL[a]	
Area	Agricultural	Non-Agricultural	Agricultural	Non-Agricultural	Agricultural	Non-Agricultural	Agricultural	Non-Agricultural	Agricultural	Non-Agricultural
Adult-child pairs[b], n	52	53	50	51	51	52	51	60	55	50
Urine samples, n	206	212	200	202	201	208	204	238	219	198
Season 1	104	106	100	102	102	104	102	120	110	100
Season 2	102	106	100	100	99	104	102	118	109	98
Gender, female, %										
Adults	50	87	90	82	94	85	71	60	71	66
Children	54	49	58	47	49	52	43	43	53	46
Mean age, years										
Adults	44	44	40	39	38	40	41	42	42	42
Children	8.2	8.7	8.9	8.4	9.7	9.2	8.8	9.1	8.6	8.6
Mean BMI										
Adults	25	24	26	26	26	26	24	24	24	23
Children	17	17	17	17	18	19	16	16	16	16.0
Educational level adult, %										
No or only primary education	0	0	2.0	0	40	5.8	0	1.7	1.8	0
Secondary education	7.8	17	30	12	28	20	2.0	3.3	5.5	2.0
Tertiary education (post-secondary)	25	17	8.0	7.8	23	26	26	10.0	18	18
University studies (BSc, MSc, PhD)	67	66	60	77	8.0	48	71	83	71	76
Don't Know/ NA	0	0	0	3.9	0	0	2.0	1.7	3.6	4.1
Usage of pesticide (-products) up to 3 days prior to sampling[c], n households										
Season 1	9	5	4	7	2	6	4	2	1	6
Season 2	27	8	12	8	22	7	14	12	10	4
Seasonal homegrown vegetables, fruit and/or herbs consumption, % of total consumption										
Winter	6.7	1.1	30	22	23	4.5	13	10	2.0	0.1
Spring	10	3.4	28	19	21	9.4	22	12	4.8	2.2
Summer	12	8.0	63	44	41	25	64	51	15	7.8
Autumn	8.9	6.0	63	45	39	17	45	40	8.3	4.5

[a] ES: Spain, LV: Latvia, HU: Hungary, CZ: Czech Republic, NL: the Netherlands.
[b] Number of individuals included in season 1.
[c] Summary indicator which includes: pesticides for human use, use indoors, use in garden, and professional use. For specification of the categories see Supplementary Material B.

slightly higher compared to the adults from other study sites. Most of the participants did not smoke, although in the agricultural areas of Spain and Hungary there was a substantial group of current smokers 35% and 45%, respectively (Supplementary Material B). Based on the total household income categories, participants of agricultural areas mostly earned less money than those living in non-agricultural areas. In all areas except the agricultural area in Hungary, the majority of the participants had a university education level. In Spain and Hungary, about half of the households in agricultural areas used pesticide products during summer season, which includes the use of consumer products, usage indoors, in the garden and/or professional use. These different categories are presented separately in Supplementary Material B. Overall, the homegrown food consumption percentage was higher in households in agricultural areas than those in non-agricultural areas, mostly during summer.

3.2. Annotations and detection rates

The application and harmonization of the SS approach was performed on 2088 urine samples using the method described in detail in Huber et al. (2022). A total number of 498 tentative annotations of pesticide biomarkers was obtained and prioritized, of which 40 pesticide biomarkers were annotated with confidence level 1 or 2 (Table 2). These 40 related to a total of 29 parent pesticides. In addition to these 40, 54 other pesticide biomarkers (either pesticide metabolites or the parent pesticides as such) were detected with a lower confidence level (Schymanski levels 3–5) which are detailed in Supplementary Material C. These 54 are not further described in this paper and not used in the analyses.

For each annotated exposure marker (confidence levels 1 and 2), the overall detection rate per study site was calculated (Table 2). Overall, biomarkers were generally detected below 25% of the samples. The results evidenced a significant variability between study sites, with Latvia having generally the lowest number of detects and Spain the highest one. Overall, the metabolites related to the parent pesticides acetamiprid (N-demethylated metabolite) and chlorpropham (4-HSA metabolite) were most frequently detected in samples of all study sites. Other biomarkers that had detection rates of at least 10% (including both locations and both season) relate to the parent pesticides boscalid (not in Hungary), chlorpyrifos (only in Spain and Czech Republic), clothianidin (not in Latvia), cyprodinil (not in Latvia and Hungary), flonicamid (not in Latvia and Czech Republic), fluazifop (not in Latvia), fludioxonil (not in Hungary), imazalil (only in Spain and Latvia), imidacloprid (only in Spain), pirimiphos-methyl (not in Hungary), propamocarb (not in Latvia), pyrimethanil (not in Hungary), tebuconazole (not in Latvia), and thiamethoxam (only in Spain and Hungary). Biomarkers that were detected at low frequencies (<10%) across all study sites include 2,4-dichlorophenoxy acetic acid (2,4-D), ametoctradin,

Table 2
Annotated pesticide biomarkers with Schymanski confidence levels 1 and 2 (p = 40) and their overall detection frequency (%) per study site (Schymanski et al., 2014).

ID	Pesticide type[a]	Parent pesticide	Pesticide (metabolite) annotation[b]	Confidence level[c]	Overall Detection Frequency (%)				
					ES[d]	LV[d]	HU[d]	CZ[d]	NL[d]
P1	H	2,4-Dichlorophenoxyacetic acid	Parent	1	4.1	0	2.2	2.7	0
P2_a	I	Acetamiprid	-CH2	1	99	33	94	98	93
P3_a	F	Ametoctradin	-C2H6 +2O	1	5.0	2.7	1.2	4.8	2.9
P5_a	F	Boscalid	+O + SO3 [e]	2	36	18	3.9	23	33
P5_b			+O + SO3 [f]	2	7.2	0	0	0.5	0.2
P6	I	Chlorantraniliprole	+O	2	3.8	0.3	0.2	0	0.2
P8_a	H, GR	Chlorpropham	+O + SO3 (4-HSA)	1	56	32	31	34	75
P9_a	I	Chlorpyrifos (methyl)	TCPy	1	1.7	0	0.2	0.2	0.2
P9_b			-CH2	1	36	0	6.9	21.7	6.5
P10	H	Clopyralid	Parent	1	1.0	0	0	1.4	0.7
P11_a	I	Clothianidin (can come from thiamethoxam)	Parent	1	34	1.7	22	25	20
P11_b			-NO2 +H	1	0.5	0	0.2	0	0.2
P11_c			-CH2	2	21	0.8	9.8	6.6	3.1
P12_a	I	Cypermethrin, cyfluthrin, permethrin, transfluthrin	DCCA	1	0.5	0	0	0	0
P13_a	F	Cyprodinil	+O + SO3	2	14	7.7	2.7	10	26
P18_a	I	Flonicamid	Parent	1	1.7	0.8	2.0	2.7	5.7
P18_b			-C2HN	2	15	0.3	27	0.2	57
P19_a	H	Fluazifop	Parent [e]	1	20	2.5	11	18	21
P19_b			Parent [f]	1	8.1	1.5	4.9	5.2	8.2
P20	F	Fludioxonil	+O + C6H8O6	2	16	15	2.0	14	27
P21_a	F	Fluopyram	+O + SO3	2	3.6	0.5	0.2	1.1	1.0
P21_b			+O + C6H8O6	2	2.4	0.8	0.5	3.2	4.8
P21_c			-2H	2	11	6.7	0.5	3.4	3.1
P22_a	I	Flupyradifurone	Parent	1	2.6	0.3	0.5	0.7	2.2
P25_a	I, Ac	Fluvalinate	-C14H9NO	2	1.0	0	0.7	0.2	0
P27_a	F	Imazalil	+C6H8O6	2	19	11	8.3	4.5	4.6
P28_a	I	Imidacloprid	-NO2 +H	1	17	1.7	4.2	0.7	9.4
P32_a	F	Penconazole	+O + C6H8O6	2	6.5	1.7	2.2	2.0	2.4
P34_a	I, Ac	Pirimiphos-methyl	-CH2	1	85	10	6.6	24	48
P35_a	F	Propamocarb	Parent	1	9.6	1	11	5.0	23
P35_b			+O	2	21	5.5	18	12	43
P37	H	Propyzamide	+H2O3	2	8.6	0	0.5	0.9	1.0
P38_a	F	Pyrimethanil	+O + SO3	2	27	14	4.9	22	32
P38_b			+O	2	0.7	0	2.7	0	0.5
P40_a	F	Tebuconazole	-2H +2O	2	71	5.5	25	52	36
P41_a	F	Thiabendazole	+O + C6H8O6	2	0	0.8	0.2	0	0.5
P42_a	I	Thiacloprid	+O	2	8.4	0.8	2.9	7.9	4.6
P43_a	I	Thiamethoxam	Parent	1	0.7	0	2.4	0	0.5
P43_b			-NO2 +H	1	23	0	15	0	0.2
P46_a	F	Trifloxystrobin	-CH2 -CH2	2	0.7	0.5	0	3.6	3.8

[a] H: Herbicide, F: Fungicide, I: Insecticide, GR: Plant Growth Regulator, Ac: Acaricide.
[b] Metabolite annotation: "-CH2" means the molecular formula of the metabolite is that of the parent minus CH2 (corresponding to demethylation). Similarly, "+O" means the metabolite is the parent compound plus one oxygen atom (hydroxylation). "+SO3" and "+C6H8O6" indicate sulfation and glucuronidation, respectively.
[c] Schymanski confidence level, ranging from 1 to 5, (Schymanski et al., 2014).
[d] ES: Spain, LV: Latvia, HU: Hungary, CZ: Czech Republic, NL: the Netherlands.
[e] Positive precursor ion.
[f] Negative precursor ion.

chlorantraniliprole, clopyralid, fluopyram, flupyradifurone, fluvalinate, penconazole, propyzamide, thiabendazole, thiacloprid, trifloxystrobin, as well as the metabolite permethric acid (DCCA) (originated from parent pesticides cypermethrin, cyfluthrin, permethrin or transfluthrin).

3.3. Co-occurrence of pesticides

In order to assess how many pesticides were co-occurring within the same individual at a single time point, the number of detected parent pesticides per urine sample are presented in Fig. 1. In line with the detection ratios, the lowest number of detected pesticides were in samples originating from Latvia, with mostly less than 3 co-occurring pesticides per urine sample. Samples originating from Spain showed the highest numbers of co-occurring pesticides, with a median value of 7. In the majority of the samples the number of parent pesticides per samples typically ranged from 2 to 5. The maximum number of different pesticides detected in the same urine sample was 13, which was the case for two samples. The samples with no (n = 100) or only one (n = 225) detected pesticide add up to 16% of the total amount of samples, indicating that in a majority of the samples from the SPECIMEn study at least two different parent pesticides were detected.

The next step was to evaluate which pesticides were co-occurring in each urine sample, for which the most frequently (in 5 or more urine samples) co-occurrent pesticides or mixtures are presented in an UpSet plot in Fig. 2. These most frequent co-occurrences consisted of 44 different combinations based on 14 different pesticides. The majority of co-occurrences consisted of 2 or 3 pesticides, with minimal overlap across all study sites. The most common co-occurrence was acetamiprid with chlorpropham, detected in 62 samples although this combination was not detected in any sample originating from Spain. The second most frequently co-occurring pesticides were acetamiprid with tebuconazole, however this combination was not seen in the Netherlands. The only co-occurrence combination detected in all five study sites was acetamiprid with pirimiphos-methyl. The less frequent the co-occurrent pesticides the more variation in combinations were seen, which was even more pronounced when detected in just 2, 3 or 4 urine samples (see Supplementary Material D for the extended UpSet plot).

The stability of the co-occurrences at each study site can be evaluated with correlation networks, which are presented in Fig. 3A–E. Similar to the findings of Fig. 2, mostly small groups (two to four biomarkers) of

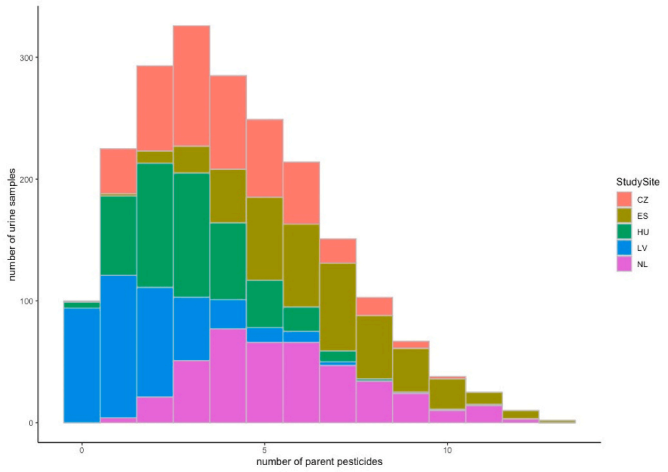

Fig. 1. Number of parent pesticides (p = 29) detected per urine sample (n = 2088), with the five different study sites indicated in different colors (CZ=Czech Republic, ES = Spain, HU=Hungary, LV = Latvia, NL=Netherlands). Multiple metabolites and/or parent compounds related to the same parent pesticide were considered as one.

co-occurrent pesticides were found. Consistent across all study sites was the positive relation between cyprodinil (P13) and fludioxonil (P20), both fungicides, although sometimes together with other pesticides and/or part of a different community. Also, in both Spain and the Czech Republic, imazalil (P27) was related to pyrimethanil (P38), which are both fungicides. Finally, in Spain and Hungary chlorpyrifos-methyl (P9) was related to pirimiphos-methyl (P34), which are both insecticides. Interestingly, the relations of acetamiprid (P2) with chlorpropham (P8) or tebuconazole (P40) were not detected in the networks.

3.4. Changes in occurrence of pesticides by location, season, age category

To explore the differences in occurrence of the pesticide biomarkers by location, season, and age category, logistic mixed effects models were constructed. The main model includes the covariates for location, season and age category, the extended model was also corrected for pesticide usage (self-reported), BMI, level of education and homegrown food consumption. Results of the models of the biomarkers detected in at least four study sites are shown in Table 3, the full table with estimates for all exposure markers associated with confidence levels 1 and 2 can be found in Supplementary Material E.

In Spain, no effect of location was detected in the models, except for clothianidin which was less frequently detected in agricultural areas compared to non-agricultural areas. Chlorpropham, chlorpyrifos, clothianidin, fluazifop, fludioxonil, imazalil, imidacloprid, pyrimethanil, and tebuconazole were most frequently detected during the first sampling season. These effects were not influenced by inclusion of the additional predictors in the extended model. Between the group of parents and children in Spain, the biomarkers related to boscalid, and cyprodinil were most frequently detected among parents, while chlorpropham, chlorpyrifos, chlothianidin, pirimiphos-methyl, tebuconazole, and thiacloprid were more frequently detected among children. The extended models confirmed most of these effects (not for clothianidin and cyprodinil).

In Latvia, propamocarb was the only biomarker more frequently detected at the agricultural area. Acetamiprid, fluopyram, imazalil, and propamocarb were more frequently detected in the first season (winter), while pyrimethanil and tebuconazole were more frequently detected during the second season (summer). Only the effects related to propamocarb and pyrimethanil were confirmed with the extended models. Chlorpropham, pirimiphos-methyl, and propamocarb were more frequently detected among the Latvian children compared to adults, while imazalil was more frequently detected within Latvian parents (not in extended model).

In Hungary, both biomarkers related to clothianidin were more frequently detected at the agricultural areas. On the other hand, chlopyrifos, pirimiphos-methyl, propamocarb, tebuconazole, and thiacloprid were most frequently detected at the non-agricultural areas. Chlorpyrifos, chlothianidin, pirimiphos-methyl, propamocarb, and tebuconazole were most frequently detected during the second season. While, in contrary, chlorpropham and imazalil were most frequently detected during the first season. Acetamiprid, chlorpropham, chlorpyrifos, chlothianidin, fluazifop, pirimiphos-methyl, propamocarb, and tebuconazole, were most frequently detected among the Hungarian children. Of which chlorpropham, fluazifop, pirimiphos-methyl, propamocarb and tebuconazole were confirmed in both models.

In the Czech Republic, the metabolite of ametoctradin was more frequently detected at the agricultural areas, although this effect disappeared in the extended model. The biomarkers related to cyprodinil and fludioxonil were more frequently detected at the non-agricultural locations (only cyprodinil confirmed with the extended model). The chlorpropham metabolite (4-HSA) was more frequently detected during the second season. While the biomarkers related to ametoctradin, imazalil, and pyrimethanil showed an opposite effect, and were more frequently detected during the first season. Of these three, only the effect of pyrimethanil was confirmed with the extended model. Seven different biomarkers were found to be more detected among children compared to adults: boscalid, chlorpropham, chlorpyrifos, flonicamid, pirimiphos-methyl, tebuconazole, and thiacloprid. The extended model confirmed the effects seen for chlorpropham and tebuconazole.

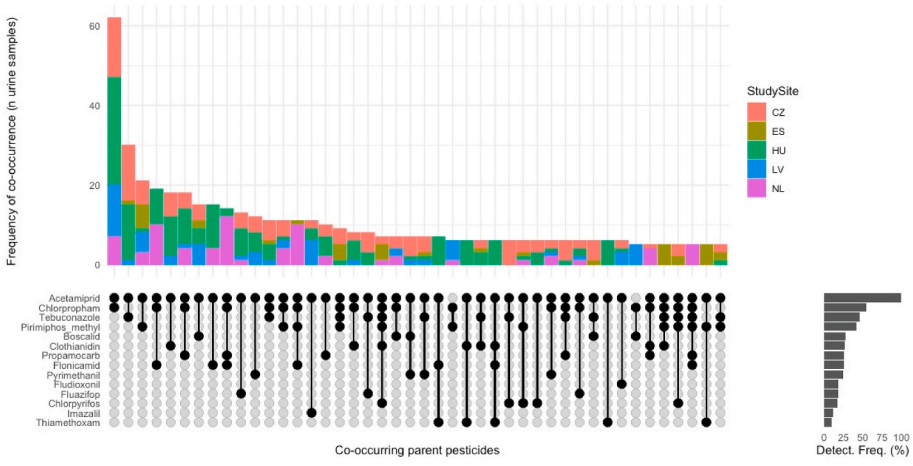

Fig. 2. Frequency (number of urine samples, n = 2088) of co-occurrent parent pesticides; the most frequent (in 5 or more urine samples) co-occurrences are shown. Different study sites are indicated by colors (CZ=Czech Republic, ES = Spain, HU=Hungary, LV = Latvia, NL=Netherlands), the detection frequency (%) of the listed parent pesticides is given on the right. Pesticides are co-occurring in the same sample when both have a black connected dot. Multiple metabolites and/or parent compounds related to the same parent pesticide were considered as one.

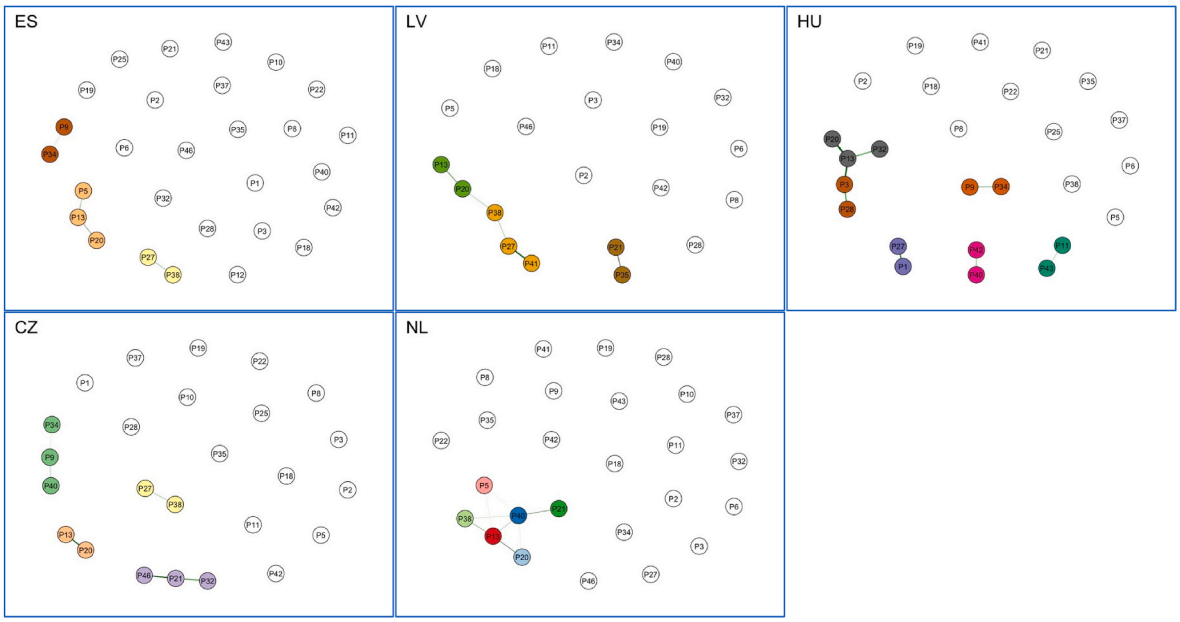

Fig. 3. Weighted correlation networks per study site based on the parent pesticides. Relationships between markers are indicated by a line (green = positive, red = negative). The colours indicate the different communities or groups of more closely related markers. **ES)** Spain (p = 28), **LV)** Latvia (p = 21), **HU)** Hungary (p = 26), **CZ)** Czech Republic (p = 25), **NL)** the Netherlands (p = 26). See Table 2 for a description of the used ID numbers for each pesticide. Multiple metabolites and/or parent compounds related to the same parent pesticide were considered as one.

Finally, in the Netherlands, the metabolites of chlorpropham were most frequently detected at agricultural areas. While biomarkers related to cyprodinil and pyrimethanil had higest detection frequencies at the non-agricultural areas. Chlorpropham, fluazifop, and thiacloprid were more frequently detected during the second season, while acetamiprid, chlorpyrifos, clothianidin, imazalil, and pyrimethanil had highest detection rates during the first season. The biomarkers related to ametoctradin, cyprodinil, flonicamid, fludioxonil, pirimiphos-methyl, and tebuconazole were more frequently detected among children. Of these, the effects seen for pirimiphos and tebuconazole were confirmed in the extended models. While on the other hand, propamocarb was more frequently detected among adults (only in extended model).

Overall, almost no biomarkers were more frequently detected in both agriculture areas and (summer) season 2. Only exceptions were chlorpropham (4-HSA metabolite) in the Netherlands, and clothianidin (parent compound and the N-demethylated metabolite) in Hungary.

4. Discussion

This study reports on the co-occurrence patterns of 40 different pesticide biomarkers at study sites from five European countries, and identifies whether proximity to agrigultural fields, season, and age category impacted the probability of detection of these biomarkers. The developed application of a harmonized SS methodology allowed screening for 1000s of suspects (pesticides and their known/predicted phase I/II metabolites), and enabled detection of many pesticides/metabolites at different levels of confidence in urine. As such, this study should be seen as the first step towards a more complete assessment of the pesticide mixture exposure in the general European population.

4.1. Detected pesticides and the impact of location, season and age category

The most frequently detected biomarkers across all study sites were related to the parent pesticides acetamiprid and chlorpropham. Acetamiprid is a neonicotinoid (insecticide), is approved in the EU and commonly used on fruit trees such as apples, pears and citrus, but also on e.g. potatoes and rapeseed (Allema et al., 2017; EU Database Pest, 2022). All study sites included agricultural areas where these crops are grown. However since we did not find a difference between areas for acetamiprid, this high detection frequency is likely due to other factors such as diet. For Latvia and the Netherlands, acetamiprid was less frequently detected during the second season (summer), arguing that additional exploration is needed on for example the change of diet between seasons. Chlorpropham is a plant growth regulator and herbicide, commonly used on e.g. onions and potatoes to prevent sprouting. In the Netherlands only, chlorpropham had a higher probability of detection in the summer season, which is consistent with an earlier study on flower bulb fields in the Netherlands (Gooijer et al., 2019; Oerlemans et al., 2021). Although chlorpropham has no longer been approved as pesticide since 2019 in the EU, still high probabilities of detection were seen in both seasons (EU Database Pest, 2022). This is not unexpected due to periods of grace until October 2020, which overlaps with both sampling periods of the current study. Interestingly, in Spain and Hungary chlorpropham was more frequently detected during the first season, while in Czech Republic and the Netherlands highest frequencies were seen in the second season. Chlorpropham also had higher probabilities of detection in children compared to adults, which could be related to food consumption: children have a larger food intake per kg of bodyweight; also, biological elimination mechanisms may differ between children and adults (Arena et al., 2017).

Also, high detection rates in SPECIMEn were found for the biomarkers related to pirimiphos-methyl and tebuconazole, which are in good agreement with other targeted studies (Norén et al., 2020; Yusà et al., 2022). For these and other highly detected pesticides, no consistent effect across all countries of season or location was found, in contrast with expectations based on previous findings (Dereumeaux et al., 2020; Teysseire et al., 2020). Differences in study sites might occur due to different crop types. Detected differences are most likely influenced by a set of other covariates not included in the current regression models, such as diet. Dietary habits of participants may differ between the countries, locations within countries, seasons and age groups. Also, there might be differences in percentage of consumption of imported foods, and percentage of homegrown food consumption. These aspects make the variety of exposure due to diet complex and subject to many changes; therefore future work needs to focus on the actual consumed

Table 3
Results of logistic mixed effects models, main and extended. Results are presented as Odds Ratios (OR) with 95% confidence intervals (CI). Significance levels based on p-value: '***' <0.001, '**' <0.01, '*' <0.05. Random effects are household and participant ID. Main model includes the predictors: location, season, and age category. Extended model includes additional predictors for pesticide usage, BMI, level of education and homegrown food consumption. Results are shown of features detected in at least 4 study sites.

ID	Parent pesticide	Category	SP		LV		HU		CZ		NL	
			Main OR (95% CI)[a]	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)
P2_a	Acetamiprid	Agricultural vs Non-Agricultural[b]	NA[c]	NA[d]	1.0 (0.6; 1.6)	1.1 (0.7; 1.9)	1.2 (0.1; 2.7)	1.4 (0.5; 3.5)	0.5 (0.1; 2.2)	0.6 (0.1; 3.0)	2.0 (0.3; 14)	2.4 (0.9; 6.2)
		Season 2 vs 1[b]	0.5 (0.1; 2.7)	0.5 (0.1; 2.7)	0.6 (0.4; 1.0)	0.6 (0.4; 1.0) *	1.2 (0.5; 2.7)	1.3 (0.5; 3.1)	0.6 (0.1; 2.5)	0.8 (0.2; 3.9)	0.2 (0.0; 0.8) *	0.5 (0.2; 1.2)
		Parent vs Child[b]	0.2 (0.0; 1.7)	0.2 (0.0; 2.8)	0.8 (0.5; 1.3)	0.7 (0.4; 1.5)	0.4 (0.2; 1.0) *	0.5 (0.2; 1.5)	1.0 (0.2; 4.1)	12 (1.0; 149)	1.3 (0.2; 9.0)	1.7 (0.3; 8.0)
P3_a	Ametoctradin	Agri. vs Non-Agri.	0.3 (0.0; 2.8)	0.4 (0.1; 1.0)	1.8 (0.5; 6.3)	1.3 (0.3; 5.3)	1.6 (0.3; 9.5)	NA[d]	3.2 (1.1; 9.5) *	3.0 (1.0; 9.4)	0.8 (0.03; 20)	NA[d]
		Season 2 vs 1	0.4 (0.1; 1.5)	0.6 (0.2; 1.4)	0.6 (0.2; 2.0)	0.5 (0.2; 1.9)	0.7 (0.1; 4.1)	NA[d]	0.4 (0.1; 1.0) *	0.4 (0.1; 1.1)	0.3 (0.1; 1.6)	NA[d]
		Parent vs Child	2.1 (0.3; 17)	3.0 (0.9; 10)	0.8 (0.2; 2.8)	2.3 (0.3; 15)	4.0 (0.4; 37)	NA[d]	0.6 (0.2; 1.5)	0.2 (0.0; 1.3)	0.1 (0.02; 0.9) *	NA[d]
P5_a	Boscalid	Agri. vs Non-Agri.	1.0 (0.6; 1.9)	1.0 (0.5; 1.8)	1.2 (0.5; 2.6)	1.4 (0.6; 3.1)	0.8 (0.2; 2.2)	0.5 (0.1; 1.5)	1.4 (0.8; 2.5)	1.4 (0.8; 2.7)	0.6 (0.3; 1.0)	0.5 (0.3; 1.0)
		Season 2 vs 1	0.7 (0.4; 1.0)	0.6 (0.4; 1.0)	0.9 (0.5; 1.6)	0.9 (0.5; 1.6)	0.6 (0.2; 1.7)	0.6 (0.2; 1.9)	0.7 (0.4; 1.2)	0.8 (0.5; 1.3)	1.4 (0.9; 2.2)	1.4 (0.9; 2.2)
		Parent vs Child	2.9 (1.8; 4.6) ***	2.5 (1.3; 4.9) **	1.3 (0.7; 2.4)	1.0 (0.4; 2.6)	2.1 (1.0; 9.9)	2.4 (0.6; 9.3)	2.1 (1.3; 3.5) **	2.5 (1.0; 6.1)	1.4 (0.9; 2.1)	1.1 (0.4; 2.6)
P8_a	Chlorpropham	Agri. vs Non-Agri.	0.7 (0.4; 1.3)	0.7 (0.4; 1.3)	1.3 (0.7; 2.7)	1.2 (0.6; 2.5)	1.3 (0.7; 2.5)	1.5 (0.7; 3.2)	1.0 (0.6; 2.0)	1.0 (0.5; 2.0)	2.1 (1.1; 3.9) *	2.1 (1.1; 4.1) *
		Season 2 vs 1	0.4 (0.3; 0.7) ***	0.4 (0.3; 0.7) ***	1.6 (1.0; 2.6)	1.5 (0.9; 2.4)	0.5 (0.3; 0.8) **	0.5 (0.3; 0.9) *	2.1 (1.3; 3.3) **	1.9 (1.2; 3.1) *[e]	2.8 (1.7; 4.7) ***	2.7 (1.6; 4.6) ***
		Parent vs Child	0.4 (0.2; 0.6) ***	0.3 (0.2; 0.6) ***	0.3 (0.2; 0.6) ***	0.4 (0.2; 1.0) *	0.5 (0.3; 0.7) **	0.4 (0.2; 0.7) **	0.4 (0.2; 0.6) ***	0.3 (0.1; 0.8) *	0.6 (0.4; 1.1)	0.5 (0.2; 1.2)
P9_a	Chlorpyrifos (/methyl)	Agri. vs Non-Agri.	0.8 (0.5; 1.3)	0.8 (0.5; 1.3)	ND[f]	ND[f]	0.2 (0.1; 0.7) *	0.3 (0.1; 1.0)	1.3 (0.7; 2.4)	1.3 (0.7; 2.4)	1.2 (0.4; 3.2)	1.2 (0.4; 3.3)
		Season 2 vs 1	0.2 (0.1; 0.4) ***	0.2 (0.1; 0.4) ***	ND[f]	ND[f]	2.5 (1.0; 6.1) *	2.7 (1.1; 6.5) *	0.6 (0.4; 1.0)	0.6 (0.4; 1.0)	0.5 (0.2; 1.1)	0.4 (0.1; 1.0) *
		Parent vs Child	0.5 (0.3; 0.7) ***	0.4 (0.2; 0.7) **	ND[f]	ND[f]	0.5 (0.2; 1.1)	0.2 (0.1; 0.8) *	0.5 (0.3; 0.7) **	0.7 (0.3; 1.7)	0.8 (0.3; 1.8)	0.9 (0.2; 5.2)
P11_a	Clothianidin (can come from thiamethoxam)	Agri. vs Non-Agri.	0.5 (0.3; 0.8) **	0.4 (0.3; 0.7) ***	1.4 (0.3; 6.3)	0.9 (0.2; 4.6)	2.8 (1.6; 4.7) ***	2.8 (1.5; 5.1) **	1.3 (0.8; 2.2)	1.4 (0.8; 2.5)	1.3 (0.7; 2.3)	1.4 (0.8; 2.7)
			0.6 (0.4; 0.9) **	0.5 (0.3; 0.8) **	6.3 (0.7; 53)	5.7 (0.7; 50)	3.1 (1.8; 5.2) ***	3.5 (2.0; 6.1) ***	0.6 (0.4; 1.0)	0.7 (0.4; 1.1)	0.6 (0.4; 1.0)	0.6 (0.4; 1.0)
		Season 2 vs 1	0.6 (0.5; 0.9) *	0.6 (0.3; 1.0)	0.2 (0.0; 1.4)	0.3 (0.0; 5.7)	0.6 (0.4; 1.0)	0.4 (0.2; 0.7) **	1.0 (0.6; 1.5)	1.1 (0.5; 2.7)	0.9 (0.5; 1.5)	1.7 (0.6; 4.6)
P11_c			1.4 (0.8; 2.8)	1.4 (0.7; 2.8)	ND[f]	ND[f]	4.4 (1.8; 11) ***	5.5 (2.1; 14) ***	1.3 (0.6; 2.7)	1.3 (0.6; 2.8)	1.5 (0.0; 38)	1.4 (0.7; 2.6)
		Parent vs Child	0.9 (0.5; 1.5)	0.8 (0.5; 1.4)	ND[f]	ND[f]	1.9 (0.9; 3.9)	2.5 (1.2; 5.5) *	0.5 (0.2; 1.1)	0.5 (0.2; 1.1)	0.02 (0.0; 0.3) **	0.6 (0.4; 1.0)[e and g]

(*continued on next page*)

Table 3 (*continued*)

ID	Parent pesticide	Category	SP		LV		HU		CZ		NL	
			Main OR (95% CI)[a]	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)
			0.7 (0.4; 1.3)	0.8 (0.4; 1.8)	ND[f]	ND[f]	0.8 (0.4; 1.5)	0.3 (0.1; 0.8) *	0.9 (0.4; 2.0)	1.2 (0.3; 4.6)	1.6 (0.0; 42)	1.8 (0.7; 4.9)
P13_a	Cyprodinil	Agri. vs Non-Agri.	1.4 (0.7; 2.7)	1.4 (0.7; 2.7)	1.1 (0.4; 2.8)	1.1 (0.4; 2.9)	1.9 (0.1; 44)	1.8 (0.1; 61)	0.3 (0.1; 0.6) **	0.3 (0.1; 0.6) **	0.6 (0.3; 0.9) *	0.5 (0.3; 0.9) *
		Season 2 vs 1	1.2 (0.7; 2.1)	1.2 (0.6; 2.2)	0.7 (0.3; 1.5)	0.7 (0.3; 1.5)	0.3 (0.1; 2.0)	0.4 (0.1; 2.9)	0.9 (0.5; 1.6)	0.9 (0.5; 1.7)	0.8 (0.5; 1.2)	0.8 (0.5; 1.2)
		Parent vs Child	2.0 (1.6; 5.6) ***	2.3 (1.0; 5.4)	0.9 (0.4; 2.1)	1.5 (0.5; 5.1)	6.2 (0.9; 40)	2.9 (0.3; 26)	0.8 (0.4; 1.5)	1.0 (0.3; 3.0)	0.5 (0.3; 0.8) **	1.2 (0.5; 2.8)
P18_a	Flonicamid	Agri. vs Non-Agri.	2.6 (0.5; 14)	2.6 (0.4; 15)	ND[f]	ND[f]	1.0 (0.3; 4.3)	2.3 (0.5; 11)	2.7 (0.1; 84)	NA[d]	0.4 (0.1; 1.5)	0.4 (0.1; 1.3)
		Season 2 vs 1	1.4 (0.3; 6.3)	1.4 (0.3; 7.1) [e]	ND[f]	ND[f]	3.1 (0.6; 16)	3.8 (0.7; 20)	1.0 (0.2; 5.3)	NA[d]	1.6 (0.6; 4.0)	1.5 (0.6; 3.8)
		Parent vs Child	6.2 (0.7; 52)	7.8 (0.6; 96)	ND[f]	ND[f]	0.3 (0.1; 1.6)	0.2 (0.0; 1.5)	0.1 (0.0; 0.4) **	NA[d]	0.3 (0.1; 0.8) *	0.3 (0.0; 2.3)
P19_a	Fluazifop	Agri. vs Non-Agri.	1.0 (0.6; 1.9)	1.1 (0.6; 2.1)	4.2 (0.9; 20)	3.1 (0.6; 16)	1.1 (0.5; 2.2)	0.9 (0.4; 2.1)	1.1 (0.6, 2.1)	1.3 (0.7; 2.4)	1.4 (0.7; 3.0)	1.5 (0.7; 3.1)
			0.5 (0.3; 0.8) **	0.5 (0.3; 0.9) *	0.7 (0.2; 2.4)	0.6 (0.1; 2.3)	1.1 (0.6; 2.0)	0.9 (0.4; 1.7)	0.9 (0.5; 1.4)	0.8 (0.5; 1.4)	1.0 (0.6; 1.6)	1.0 (0.6; 1.7)
		Season 2 vs 1	1.0 (0.6; 1.7)	0.7 (0.3; 1.5)	1.0 (0.3; 3.5)	1.2 (0.2; 7.8)	0.6 (0.3; 1.2)	0.4 (0.2; 1.0) *	0.7 (0.4; 1.2)	0.5 (0.2; 1.3)[e]	0.7 (0.4; 1.2)	0.5 (0.2; 1.5)
P19_b			1.6 (0.7; 3.6)	1.6 (0.6; 4.1)	5.2 (0.6; 45)	4.6 (0.5; 44)	2.1 (0.3; 17)	2.4 (0.8; 7.2)	0.9 (0.3; 2.3)	0.9 (0.3; 2.5)[e]	1.4 (0.2; 10)	3.1 (0.3; 29)
		Parent vs Child	0.8 (0.4; 1.6)	0.7 (0.3; 1.6)	0.5 (0.1; 2.7)	0.4 (0.1; 2.6)	1.4 (0.1; 4.5)	1.0 (0.3; 2.6)	2.5 (1.0; 6.3)	2.4 (0.9; 6.3)	7.2 (1.6; 32) **	4.5 (1.1; 17) *
			1.0 (0.5; 2.1)	0.4 (0.1; 1.2)	0.5 (0.1; 2.7)	1.9 (0.1; 29)	0.2 (0.0; 0.9) *	0.3 (0.1; 0.9) *	1.3 (0.6; 3.2)	1.0 (0.2; 4.6)	0.6 (0.1; 4.1)	0.0 (0.0; 2.9)
P20	Fludioxonil	Agri. vs Non-Agri.	1.1 (0.6; 2.1)	1.0 (0.5; 2.0)	0.8 (0.4; 1.7)	0.9 (0.4; 1.9)	0.6 (0.1; 2.6)	0.9 (0.2; 4.8)	0.5 (0.3; 0.9) *	0.6 (0.3; 1.1)	0.9 (0.5; 1.6)	0.8 (0.5; 1.4)
		Season 2 vs 1	0.5 (0.3; 0.9) *	0.5 (0.3; 0.9) *	0.8 (0.4; 1.4)	0.8 (0.4; 1.4)	1.7 (0.4; 7.2)	1.7 (0.4; 7.5)	0.7 (0.4; 1.2)	0.7 (0.4; 1.2)	0.6 (0.3; 0.9) *	0.6 (0.4; 0.9) *
		Parent vs Child	1.5 (0.8; 2.8)	0.9 (0.4; 2.2)	1.1 (0.6; 2.1)	0.8 (0.3; 2.1)	1.0 (0.2; 4.0)	0.9 (0.2; 5.5)	0.7 (0.4; 1.2)	1.1 (0.4; 3.1)	0.5 (0.3; 0.9) *	0.8 (0.3; 1.8)
P21_c	Fluopyram	Agri. vs Non-Agri.	1.4 (0.6; 3.4)	1.5 (0.6; 4.0)	0.6 (0.1; 4.3)	0.7 (0.2; 2.2)	ND[f]	ND[f]	1.3 (0.2; 10)	1.9 (0.2; 20)	1.0 (0.1; 22)	NA[d]
		Season 2 vs 1	1.0 (0.5; 1.9)	1.1 (0.5; 2.2)	0.2 (0.0; 0.8) *	0.4 (0.2; 1.1)	ND[f]	ND[f]	1.8 (0.5; 6.4)	1.8 (0.5; 7.2)	4.3 (0.5; 36)	NA[d]
		Parent vs Child	1.5 (0.8; 3.0)	0.9 (0.3; 2.6)	0.9 (0.1; 6.1)	1.1 (0.3; 4.3)	ND[f]	ND[f]	0.8 (0.2; 2.8)	1.0 (0.1; 12)	0.5 (0.2; 12)	NA[d]
P27_a	Imazalil	Agri. vs Non-Agri.	1.0 (0.5; 2.0)	1.1 (0.6; 2.2)	1.7 (0.6; 4.9)	0.7 (0.2; 2.2)	0.6 (0.2; 2.0)	0.7 (0.2; 2.3)	0.9 (0.1; 11)	1.0 (0.4; 2.6)	1.6 (0.6; 4.2)	1.6 (0.6; 4.1)
		Season 2 vs 1	0.2 (0.1; 0.3) ***	0.2 (0.1; 0.4) ***	0.4 (0.2; 0.8) *	0.4 (0.2; 1.1)	0.2 (0.1; 0.5) ***	0.2 (0.1; 0.5) **	0.1 (0.0; 0.6) *	0.4 (0.2; 1.2) [e]	0.3 (0.1; 1.0) *	0.4 (0.1; 1.0) [e]
		Parent vs Child	1.1 (0.7; 2.0)	0.8 (0.4; 1.9)	2.4 (1.1; 5.2) *	1.1 (0.3; 4.3)	0.5 (0.2; 1.2)	0.3 (0.1; 1.1)	2.9 (0.2; 44)	3.1 (0.6; 15)	1.4 (0.6; 3.6)	2.2 (0.4; 11)
P28_a	Imidacloprid	Agri. vs Non-Agri.	1.5 (0.7; 3.1)	1.2 (0.6; 2.6)	1.4 (0.3; 6.2)	1.1 (0.2; 5.7)	0.9 (0.2; 3.3)	1.0 (0.3; 3.7)	ND[f]	ND[f]	1.2 (0.6; 2.5)	1.4 (0.6; 2.9)

(*continued on next page*)

Table 3 (*continued*)

ID	Parent pesticide	Category	SP		LV		HU		CZ		NL	
			Main OR (95% CI)[a]	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)	Main OR (95% CI)	Extended OR (95% CI)
		Season 2 vs 1	0.6 (0.3; 1.0) *	0.5 (0.3; 1.0) *	0.2 (0.0; 1.4)	0.2 (0.0; 1.4)	0.9 (0.3; 2.5)	1.1 (0.4; 3.0)	ND[f]	ND[f]	1.1 (0.5; 2.1)	1.1 (0.5; 2.1)
		Parent vs Child	1.4 (0.8; 2.5)	1.8 (0.8; 4.2)	1.4 (0.3; 6.1)	0.7 (0.1; 6.2)	0.9 (0.3; 2.4)	0.5 (0.1; 2.0)	ND[f]	ND[f]	0.8 (0.4; 1.7)	0.9 (0.2; 3.3)
P32_a	Penconazole	Agri. vs Non-Agri.	0.7 (0.3; 1.7)	0.8 (0.3; 1.9)	1.4 (0.3; 6.1)	1.6 (0.3; 7.7)	0.5 (0.1; 2.1)	0.7 (0.0; 38)	2.4 (0.6; 9.7)	2.2 (0.5; 9.3)	0.9 (0.3; 3.2)	0.8 (0.2; 3.0)
		Season 2 vs 1	1.1 (0.5; 2.5)	1.2 (0.5; 2.6)	0.8 (0.2; 3.4)	0.8 (0.2; 3.4) [g]	3.6 (0.7; 18)	9.5 (1.0; 93)	0.5 (0.1; 2.0)	0.6 (0.1; 2.5)[e,g]	1.0 (0.3; 3.6)	1.0 (0.3; 3.6) [g]
		Parent vs Child	2.2 (0.9; 5.0)	2.9 (0.9; 9.0)	0.8 (0.2; 3.4)	0.6 (0.1; 6.0)	1.3 (0.3; 4.8)	0.7 (0.0; 12)	2.0 (0.5; 8.3)	2.9 (0.3; 30)	1.5 (0.4; 5.5)	0.5 (0.1; 4.5)
P34_a	Pirimiphos-methyl	Agri. vs Non-Agri.	0.7 (0.3; 1.4)	1.1 (0.5; 2.5)	1.0 (0.5; 1.9)	1.5 (0.3; 7.4)	0.1 (0.02; 0.4) ***	0.2 (0.1; 0.8) *	1.5 (0.8; 2.7)	1.3 (0.7; 2.5)	1.4 (0.8; 2.7)	1.4 (0.8; 2.7)
		Season 2 vs 1	0.8 (0.4; 1.4)	1.0 (0.5; 2.0)	1.5 (0.8; 2.9)	0.8 (0.2; 3.8)	3.6 (1.4; 9.4) **	4.1 (1.5; 11) **	0.6 (0.4; 1.0)	0.6 (0.4; 1.1)	0.7 (0.4; 1.0)	0.7 (0.4; 1.0)
		Parent vs Child	0.4 (0.2; 0.9) *	0.2 (0.1; 0.7) *	0.4 (0.2; 0.9) *	0.6 (0.1; 5.4)	0.2 (0.1; 0.5) **	0.1 (0.0; 0.3) ***	0.5 (0.3; 0.8) **	0.6 (0.2; 1.5)	0.3 (0.2; 0.5) ***	0.3 (0.1; 0.8) *
P35_a	Propamocarb	Agri. vs Non-Agri.	1.3 (0.5; 3.1)	1.0 (0.4; 2.4)	ND[f]	ND[f]	0.5 (0.2; 1.0)	0.5 (0.2; 1.2)	0.5 (0.1; 4.5)	0.5 (0.2; 1.9)	1.6 (0.8; 3.0)	1.9 (1.0; 3.6)
			1.9 (0.9; 4.1)	1.7 (0.8; 3.5)	ND[f]	ND[f]	1.1 (0.6; 2.1)	1.1 (0.6; 2.2)	1.5 (0.4; 6.0)	1.4 (0.5; 3.5) [e]	1.4 (0.9; 2.4)	1.5 (0.9; 2.5)
		Season 2 vs 1	1.1 (0.5; 2.5)	1.2 (0.4; 3.4)	ND[f]	ND[f]	0.4 (0.2; 1.0) *	0.3 (0.1; 0.9) *	0.5 (0.1; 3.9)	1.5 (0.2; 10)	1.0 (0.6; 1.6)	3.1 (1.1; 8.5) *
P35_b			1.3 (0.7; 2.4)	1.2 (0.7; 2.2)	4.2 (1.1; 16) *	4.0 (1.0; 17)	0.5 (0.3; 0.9) *	0.5 (0.2; 1.0) *	0.7 (0.3; 1.9)	0.8 (0.3; 2.1)	1.2 (0.7; 1.9)	1.3 (0.8; 2.2)
		Parent vs Child	1.2 (0.7; 1.9)	1.1 (0.7; 1.9)	0.3 (0.1; 0.9) *	0.3 (0.1; 0.8) *	2.1 (1.2; 3.6) *	2.3 (1.3; 4.1) **	1.4 (0.7; 2.6)	1.4 (0.7; 2.8)	1.0 (0.6; 1.5)	1.0 (0.7; 1.5)
			1.1 (0.7; 1.9)	1.1 (0.5; 2.3)	0.4 (0.1; 1.1)	0.2 (0.0; 1.0) *	0.5 (0.3; 0.9) *	0.4 (0.2; 0.8) *	0.5 (0.3; 1.0)	0.9 (0.2; 3.3)	1.2 (0.8; 1.8)	3.2 (1.4; 7.3) **
P38_a	Pyrimethanil	Agri. vs Non-Agri.	0.8 (0.5; 1.3)	0.8 (0.5; 1.3)	1.2 (0.6; 2.4)	1.1 (0.5; 2.3)	0.4 (0.1; 2.0)	0.3 (0.0; 3.1)	0.9 (0.5; 1.7)	1.0 (0.5; 1.8)	0.6 (0.4; 1.0) *	0.6 (0.4; 0.9) *
		Season 2 vs 1	0.6 (0.4; 1.0) *	0.7 (0.4; 1.0)	2.1 (1.1; 3.8) *	2.1 (1.1; 3.8) *	1.3 (0.5; 3.8)	1.6 (0.5; 5.2)	0.4 (0.3; 0.7) **	0.5 (0.3; 0.8) **	0.6 (0.4; 1.0) *	0.6 (0.4; 1.0) *
		Parent vs Child	0.8 (0.5; 1.2)	0.7 (0.3; 1.3)	1.0 (0.6; 1.8)	1.3 (0.5; 3.2)	2.3 (0.8; 7.2)	1.7 (0.8; 7.4)	1.4 (0.9; 2.3)	2.1 (0.9; 5.3)	0.8 (0.5; 1.2)	1.1 (0.5; 2.4)
P40_a	Tebuconazole	Agri. vs Non-Agri.	0.9 (0.5; 1.7)	0.7 (0.4; 1.4)	1.5 (0.4; 5.3)	1.5 (0.6; 4.0)	0.5 (0.3; 1.0)	0.5 (0.2; 1.0) *	1.0 (0.6; 1.5)	1.1 (0.7; 1.7)	0.8 (0.4; 1.5)	0.8 (0.4; 1.6)
		Season 2 vs 1	0.5 (0.3; 0.9) *	0.5 (0.3; 0.8) **	3.3 (1.1; 9.3) *	2.8 (1.1; 7.4)	2.1 (1.2; 3.5) **	2.2 (1.3; 3.8) **	0.7 (0.5; 1.0)	0.7 (0.4; 1.0)	0.6 (0.4; 1.0)	0.6 (0.4; 1.1)
		Parent vs Child	0.3 (0.2; 0.4) ***	0.2 (0.1; 0.4) ***	0.8 (0.3; 2.1)	2.9 (0.7; 12)	0.3 (0.2; 0.5) ***	0.5 (0.2; 0.9) *	0.2 (0.2; 0.4) ***	0.4 (0.2; 0.7) **	0.1 (0.1; 0.2) ***	0.2 (0.1; 0.5) ***
P42_a	Thiacloprid	Agri. vs Non-Agri.	1.6 (0.6; 4.4)	1.6 (0.6; 4.5)	ND[f]	ND[f]	0.1 (0.0; 0.7) *	0.1 (0.0; 0.5) *	1.6 (0.6; 4.7)	2.1 (0.8; 5.8)	0.4 (0.1; 3.5)	0.5 (0.1; 4.0)
		Season 2 vs 1	0.9 (0.4; 2.0)	1.1 (0.5; 2.4)	ND[f]	ND[f]	3.2 (0.8; 12)	3.5 (0.9; 14)[g]	0.9 (0.4; 2.0)	1.2 (0.6; 2.7)	6.5 (1.5; 29) *	6.3 (1.3; 30) *[e]
		Parent vs Child	0.4 (0.2; 1.0) *	0.3 (0.1; 1.2)	ND[f]	ND[f]	0.3 (0.1; 1.2)	0.6 (0.1; 3.3)	0.3 (0.1; 0.7) **	0.2 (0.1; 1.1)	0.6 (0.2; 2.1)	2.4 (0.2; 27)

[a] OR: Odds Ratio, CI: Confidence Interval.
[b] First mentioned is the reference category.
[c] 100% detected in one of the categories, no estimate could be provided.
[d] Due to low detection rate no extended model possible.
[e] Model not corrected for level of Education, separation issue.
[f] ND: Not detected or low detection rate (<1%), no model possible.
[g] Model not corrected for Pesticide usage, separation issue.

diet and their pesticide residue levels versus the suspect screening patterns. For example, the consumption of organic foods has been linked to lower exposure concentrations of several pesticides such as organophosphates and pyrethroids (Baudry et al., 2019; Hyland et al., 2019).

As a final remark on the detected pesticides, the SS methodology is only recently being applied in large scale studies to assess exposure to pesticides, and only a few HBM studies have previously applied SS approaches to complement for example targeted monitoring programs (Gerona et al., 2018; Pellizzari et al., 2019; Plassmann et al., 2015; Wang et al., 2018). Within a cohort of approximately 300 pregnant women in France, Bonvallot et al. (2021) performed a large targeted pesticide exposure study which was extended with the application of suspect screening. This SS approach resulted in the most frequent detection of the parent pesticides azoxystrobin, fenpropimorph, phenmedipham, fluazifop(/butyl) and chlorpyrifos. From these, only the metabolites of fluazifop(/butyl) and chlorpyrifos overlapped and were also detected in the samples of the SPECIMEn study. This is due to among others differences in the suspect database, for example fenpropimorph was not included in our current study because it didn't contain Cl, Br or PO3 (Huber et al., 2022). Another interesting point is the difference in detection frequency between the TCPy and -CH2 biomarkers of chlorpyrifos (methyl) in Spain and Czech Republic. TCPy can originate from both parent compounds chlorpyrifos and methyl-chlorpyrifos. -CH2 is not a human metabolite of chlorpyrifos, and its detection is likely due to exposure through diet. Also, a higher sensitivity for -CH2 compared to TCPy at an individual instrument level might have contributed to this difference.

4.2. Co-occurrence

To explore the exposure to pesticide mixtures in the general population, it was assessed which parent pesticides co-occurred in the same urine sample. With the current work we were able to assess the probability of detection of 29 different parent pesticides simultaneously. In a large majority of the samples (84%) two or more different pesticides were detected. Our findings confirm the presence of mixtures and the necessity of assessing co-occurrent exposures, which is a topic of high concern in risk assessment (European Commission, 2020; Socianu et al., 2022; Luijten et al., 2022). The number of co-occurring pesticides typically ranged from 2 to 5, with a maximum of 13 different pesticides (2 urine samples). These two urine samples originate both from the Spanish non-agricultural area, one from a child of the first season, the other of an adult of the second season. Both individuals had a lower number of co-occurring pesticides during the other season, resepectively 8 and 10 pesticides.

Based on the 14 most frequent co-occurrent pesticides, 44 different combinations could be made, resulting in highly individualized exposure profiles. The most common combination of acetamiprid with chlorpropham, occurred in just 3% (n = 62) of the urine samples. Also, assessment of the co-occurrence patterns at country level (network analysis), did not result in strong relations and hardly any overlap across countries was seen. The underlying correlations between these probabilities of detection were also low, generally below 0.3. These results indicate that, qualitatively, pesticide mixtures might be highly variable between individuals. Nevertheless, the combined exposures may still pose a concern in terms of public health, especially when the different components of a chemical mixture share modes of action underlying toxicity (Rotter et al., 2018). Acetamiprid and chlorpropham seem to induce different toxicological effects (Arena et al., 2017; EFSA, 2016). Acetamiprid has been reported to mainly target the liver (EFSA, 2016), where it may cause, at least in rodents, oxidative stress leading to mitochondrial dysfunction (EL-Hak et al., 2022; S. Li et al., 2021). Exposure to chlorpropham rather leads to adverse effects on the hematopoietic system (Arena et al., 2017; Fujitani et al., 2000, 2004). Hemotoxicity such as hemolytic anemia, however, is considered to be due to oxidative stress (Rokushima et al., 2007; Sivilotti, 2004). Chlorpropham belongs to the family of carbamates, which have been reported to induce oxidative stress in occupationally exposed workers (Saad-Hussein et al., 2022). The other frequently observed combination of co-occurring substances involved acetamiprid and tebuconazole. Tebuconazole is a fungicide that mainly affects the liver and the adrenals (EFSA, 2014). Additionally, it has been reported to induce oxidative stress in the liver and endocrine disruption including anti-androgenic effects (Taxvig et al., 2007; Yang et al., 2018). Follow-up studies involving a larger number of participants and targeted biomarkers for these substances are needed to better assess the composition of the relevant mixtures and associated health risks.

4.3. Strengths and limitations

With the uniform design of our study a comparison could be made across Europe between agricultural and non-agricultural areas, seasons, and adults and children. Close collaborations with partners from all five countries resulted in the harmonized data collection, with little loss to follow up. The collection of the urine samples required a minimal invasive protocol, reducing the burden of citizens to participate in this survey, and opening up possibilities for scaling-up studies in future endeavours. A novel SS approach was harmonized and standardized across laboratories, with extensive QA/QC procedures (Vitale et al., 2022). Such harmonization is crucial to compare SS data and results coming from different laboratories and countries, a situation that is often unavoidable in large-scale studies. The applied SS approach allows for a relatively cost-effective way of providing semi-quantitative measurements of a large number of pesticides. A clear strength of the SPECIMEn study is that information is obtained on (putative) internal exposure to pesticides not or hardly monitored before, and on simultaneous exposure to multiple pesticides. Across countries, different pesticides targeted to different controls on different crops are likely to have been applied at the time of sampling, of which the variation is covered with the SS approach. As such, this study should be seen as the first step towards a more complete assessment of the pesticide mixtures that the general European population is exposed to. Further in-depth screening of the collected data and further methodological developments will increase the number of biomarkers that can be detected in the collected urine samples. This allows an increasingly more complete coverage of all pesticides that are present in these samples as well as the detection of other biomarkers that might potentially interact with the pesticide mixture. Also, future more quantitative analysis of signal intensities will allow for a semi-quantitative interpretation, both in co-occurrence patterns and in the role of determinants of pesticide levels.

Although the current study yields many new insights and perspectives on pesticide occurrence and mixtures, several limitations need to be addressed. From an analytical methodology point of view, the suspect screening approach is less sensitive than targeted methods (Pourchet et al., 2020), and the data mining was biased towards halogenated pesticides (Huber et al., 2022). Despite harmonized methods between

the involved laboratories, differences in sensitivity between the instruments used by the labs did occur, potentially introducing variability between countries which should be interpreted with care (Vitale et al., 2022, Huber et al., 2022). Importantly, data generated by the SS approach applied in the SPECIMEn can currently not be related to urinary pesticide concentration levels in the traditional quantitative way as in targeted analysis, but rather as semi-quantitative intensities as indicators of exposure.

With regards to the sample collection, it should be kept in mind that samples of the second season were collected during the COVID-19 pandemic, while the first sample collection was not affected by the pandemic. Activity patterns or diet of participants might have been altered, and differences between seasons should be interpreted with caution. Also, the different seasons cannot be interpreted as 'non-spraying' and 'spraying', since the timing of the actual spraying activities (and spraying techniques) most likely differed between countries and crop types. Since the applied study design was not timed with an actual spraying activity, the detected exposures might be an underestimation as compared to what has been reported in the literature (Dereumeaux et al., 2020; Teysseire et al., 2020). Agricultural areas were selected based on national databases on land-use (see Supplementary Material F for a description of the area selection per country), due to which the application of pesticides during the time of sampling could not be confirmed. Within SPECIMEn, only first morning void urines were collected. Due to the rapid excretion of many pesticides, the detected pesticides in the morning voids likely do not reflect the total daily exposure (A. J. Li et al., 2019; Scher et al., 2007). Finally, with respect to the performed logistic regression models, no correction for multiple testing was performed, since we wanted to detect any possible effects, accepting the risk of false-positive results. The inclusion of both location and season could have lead to an over-correction, especially since no difference between seasons at the non-agricultural locations would be expected due to any spraying activity (although diet might still differ between the seasons).

5. Conclusions

The current survey demonstrates the feasibility of conducting a harmonized pan-European sample collection combined with suspect screening (SS) to provide insight in the co-occurrence of pesticide mixtures in European agricultural areas. The application of a novel LC-HRMS based SS approach harmonized between different laboratories, resulted in detection of 40 biomarkers related to 29 parent pesticides with high levels of confidence. Some effects of living close to agricultural fields or season were detected, but these effects were not common at a European level. This study is a first step in addressing pesticide mixture exposure under real-life conditions. Combined with a suspect screening approach, this approach is a promising strategy for pesticide mixture risk assessment in the European population, that can guide the prioritization of pesticide (metabolites) to be measured using quantitative targeted methods.

Credit author statement

Conceptualization and design (IO, JV, EL, RV, JA); Investigation (IO, JV, EL, PČ, LŠ, OM, TS, SK, IM, ZM, LA, OP, SF, CC, SP)); Analytical methodology (JA, CH, AL, OP, SF, MK, LD, KW, RN, HM, CM, JK, BG, NL); Formal analysis (IO, JV); Writing - Original Draft (IO, JV, EL); Writing – Review and Editing (all authors); Visualization (IO); Supervision (ML, RV); Project administration (IO, JV, EL); All authors read and approved the final manuscript.

Declaration of competing interest

Declarations of interest: none.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114105.

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