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Identification of pesticides exposure biomarkers for residents living close to vineyards in France

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ABSTRACT

Biomonitoring can be relevant for assessing pesticides exposure of residents living close to vineyards (LCTV). However, because xenobiotics are generally present at low levels in human biological matrices and the sources of pesticide exposure are multiple, several challenges need to be overcome to reliably assess exposure in residents LCTV. This includes particularly identifying the most appropriate exposure biomarkers, the biological matrices in which they should be measured, and analytical methods that are sufficiently sensitive and specific to quantify them.

The aim of the present study was to develop a tiered approach to identify relevant biomarkers and matrices for assessing pesticide exposure in residents LCTV. We used samples from a biobank for 121 adults and children included in a national prevalence study conducted between 2014 and 2016 who lived near or far from vineyards. We analyzed five priority pesticides (folpet, mancozeb, tebuconazole, glyphosate, and copper) and their metabolites in urine and hair samples. We identified relevant biomarkers according to three criteria related to: i) the detection frequency of those pesticides and metabolites in urine and hair, ii) the difference in concentrations depending on residence proximity to vineyards and, iii) the influence of other environmental and occupational exposure sources on pesticide levels.

This tiered approach helped us to identify three relevant metabolites (two metabolites of folpet and one of tebuconazole) that were quantified in urine, tended to be higher in residents LCTV than in controls, and were not significantly influenced by occupational, dietary, or household sources of pesticide exposure. Our approach also helped us to identify the most appropriate measurement strategies (biological matrices, analytical methods) to assess pesticide exposure in residents LCTV. The approach developed here was a prerequisite step for guiding a large-scale epidemiological study aimed at comprehensively measuring pesticides exposures in French residents LCTV with a view to developing appropriate prevention strategies.

1. Introduction

There is growing evidence that residents living close to vineyards (LCTV) are exposed to pesticides through non-occupational pathways including spray drift and volatilization of pesticides beyond the treated area. Recent studies have shown that residential proximity to agricultural fields or crop acreage around households are associated with higher pesticide exposure in agricultural communities, based on measurements in household samples (dust, air) and in biological samples (urine, blood and hair) (Dereumeaux et al. 2020; Deziel et al. 2017;

Teysseire et al. 2021; Ward et al. 2006). Moreover, the scientific literature suggests an association between this proximity and a wide range of adverse health outcomes including cancer, neurological diseases, and fertility or pregnancy-related issues (Teysseire et al. 2020). In these epidemiological studies, pesticide exposure was generally not measured, and instead proximity to crops estimated by self-administered questionnaires, interviews or geographic information systems (GIS), was used as an indirect indicator of exposure. However, this type of indirect - or proxy - indicator is subject to potential misclassification when characterizing exposure because of a lack of knowledge about pesticide use

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in the surrounding area, recall bias, or errors in the geocoding and the assignment of proximity to crops (Rull et al. 2006a, b) leading to biased or weaker associations. Direct measurements of exposure to pesticides for residents LCTV are therefore needed when conducting large-scale epidemiological studies of such population.

Human biomonitoring (i.e. the measurement of biomarkers in human biological matrices such as urine, blood or hair) is a useful tool for measuring pesticide exposure (Yusa et al. 2015). However, there are several challenges to overcome in order to identify reliable exposure biomarkers. Relevant exposure biomarkers must be measurable in human biological media and must reliably estimate a specific environmental exposure (Angerer et al. 2007). As xenobiotics are generally found at low levels in human biological matrices, the challenge for measuring biomarkers is to maximize their detection frequency in the study population and to generate reliable data for their interpretation (Pirkle et al. 1995). This involves determining the most appropriate biomarkers (parent compound or metabolite), the biological matrices in which they should be determined, and the analytical methods that are sufficiently sensitive and specific to measure them (LaKind et al. 2017; Slimani et al. 2020; Vorkamp et al. 2021). Moreover, in the context of multi-source exposure to pesticides, it is also essential to identify biomarkers that evaluate the specific contribution of agricultural spray-drift exposure to total pesticide exposure, in order to develop and evaluate appropriate prevention strategies for reducing exposure. In this respect, the physicochemical properties of pesticides and metabolites must be considered as these properties can influence not only their mobility in the environment media but also their concentrations in biological samples according to their toxicokinetics (Mercadante et al. 2013; Mora et al. 2014; Silva et al. 2007).

A large-scale epidemiological study being planned in France to characterize pesticide exposure and associated health risks in residents LCTV, we conducted a preliminary study to identify appropriate and relevant exposure biomarkers. For this purpose, we developed a three-step tiered approach to identify relevant biomarkers from a prioritized list of pesticides, which aimed at answering the following questions:

- i) are the pesticides and metabolites of concern frequently detected in the study population?
- ii) does the distribution of their concentrations differ or not according to the proximity of residence to vineyards?
- iii) are the observed values influenced by determinants related to occupational or other environmental exposures?

This tiered framework was implemented using samples already available in a biobank in order to avoid collecting samples from participants for developmental purposes only and without guarantee of conclusive results.

2. Methods

To implement the biomarkers and matrices identification approach, we performed *ad hoc* pesticide measurements in urine and hair samples of individuals living near or far from vineyards (see parts 2.1 and 2.2). We analyzed a list of pesticides and metabolites prioritized with regard to their specificity in relation to wine crops (see part 2.3). We used socio-demographic data, and data on lifestyle, dietary habits, housing, and professional activities, to identify determinants other than proximity to vineyards that could influence the observed pesticide levels.

2.1. Study population

The study population comprised 60 adults and 61 children selected from the national human biomonitoring cross sectional survey Esteban, which was representative of the French population (2,503 adults 18–74 y.o and 1,104 children 6–17 y.o), conducted between April 2014 and March 2016 (Fillol et al. 2021b). We considered the distance between

Esteban participants' households and the nearest vineyard to select our study population. To do this, we used a composite indicator created in a previous study at the municipality scale, using CORINE Land Cover, the Land Parcel Information System (LPIS), and the reference areas of the Agricultural Census (RA) (De Crouy Chanel et al. 2014). We then combined this indicator with the French computerized vineyard register which contains registration of wine-producing enterprises in France, and the French graphical land parcel registration based on the declaration of farmers for the attribution of the common agricultural policy aids, to estimate the distance between an individual household and the nearest vineyard. Residents LCTV were defined as living within a radius of 500 m from a vineyard; persons who lived further away was considered as the control population. Each of the two groups comprised about 30 adults and 30 children whose biological samples in the Esteban biobank were of sufficient volume and quantity that they could be analyzed for our prioritized pesticides and their metabolites (see part 2.3). The size of these groups was designed to allow us to conduct the statistical analyses planned in the study. We controlled for gender in the overall study population (60 women and 61 men). However, the number of Esteban participants meeting our selection criteria (living near or far from vineyards, having sufficient biological samples in biobank) did not allow us to impose a perfect balance of the number of women / men within each subgroup of the population.

2.2. Data collection

All Esteban's participants answered a questionnaire survey, and underwent a biological sampling (blood, urine and hair) (Balicco et al. 2017). Details about the study are described in previous articles (Balicco et al. 2017; Fillol et al. 2021b).

Urine collection consisted in a single first morning void collected by the participant (150 mL for children and 200 mL for adults) in a polypropylene container. The urine sample was then either given to a nurse who made a home visit or brought to an examination center. Samples were conserved between + 4 °C and + 10 °C until they were aliquoted in polypropylene cryotubes. A hair sample was collected by trained nurses in homes or in an examination center, cutting a lock of hair three centimeters long from the occipital region as close as possible to the skin (needed for the analyses of pesticides and metabolites). Hair collection was optional for participants. All samples were then centrally stored in a biobank for long-term conservation. Urine samples were stored at –80 °C whereas hair samples were stored at room temperature.

Socio-demographic data, and data on housing (ventilation, cleaning of the house, etc.), lifestyle (tobacco consumption, leisure activities, etc.) and occupational exposure of the participant or other household members were collected in a face-to-face interview and self-administered questionnaires, given to adult participants and the children's responsible adults (i.e., parent, guardian, etc). Dietary information was collected using a Food Propensity Questionnaire (FPQ) which recorded average food intake over the previous twelve months (Subar et al. 2006), associated with a questionnaire about food buying and cooking methods and three 24-hour dietary recalls.

2.3. Selection of pesticides and metabolites for the study

2.3.1. Selection of priority pesticides

The pesticide selection process we used was derived from a previous study conducted as part of a French campaign on pesticides in ambient air (Hulin et al. 2021). First, with the contribution of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), we created an exhaustive list of active pesticide substances (APS) authorized in France for the treatment of vineyards from the French databank for authorized pesticides (ephy) (Anses 2021). Biocontrol products (bacteria, oils) and pesticides not sold in 2015 and 2016 (see part 2.1) according to the national databank for pesticides sales (BNVD) were excluded from this list (Ineris 2021). We then selected the priority APS

by categorizing each one according to the quantity used and its specificity to vineyards.

The quantity used in vineyards was evaluated from:

The tonnage of the given APS sold in 2015–2016 in winegrowing regions according to the BNVD;

The surface area of vineyards treated with the APS and the frequency of pesticide treatment in vineyard parcels according to the data from surveys on farming practices conducted by the French Ministry of Agriculture.

The specificity to wine crops was evaluated from:

The proportion of commercial products (i.e., products containing the active pesticide substance authorized for sale in France) based on the APS specifically used in vineyards according to ephy;

The proportion of the APS tonnage sold in winegrowing regions according to BNVD;

The use of the APS in other crops according to the EU pesticides database;

The domestic use of the APS according to the French survey on pesticide used at home (Anses 2019). (Subar et al. 2006)

Based on these criteria, we identified three priority APS widely used in vineyards (greater than 45 tons sold in winegrowing regions in

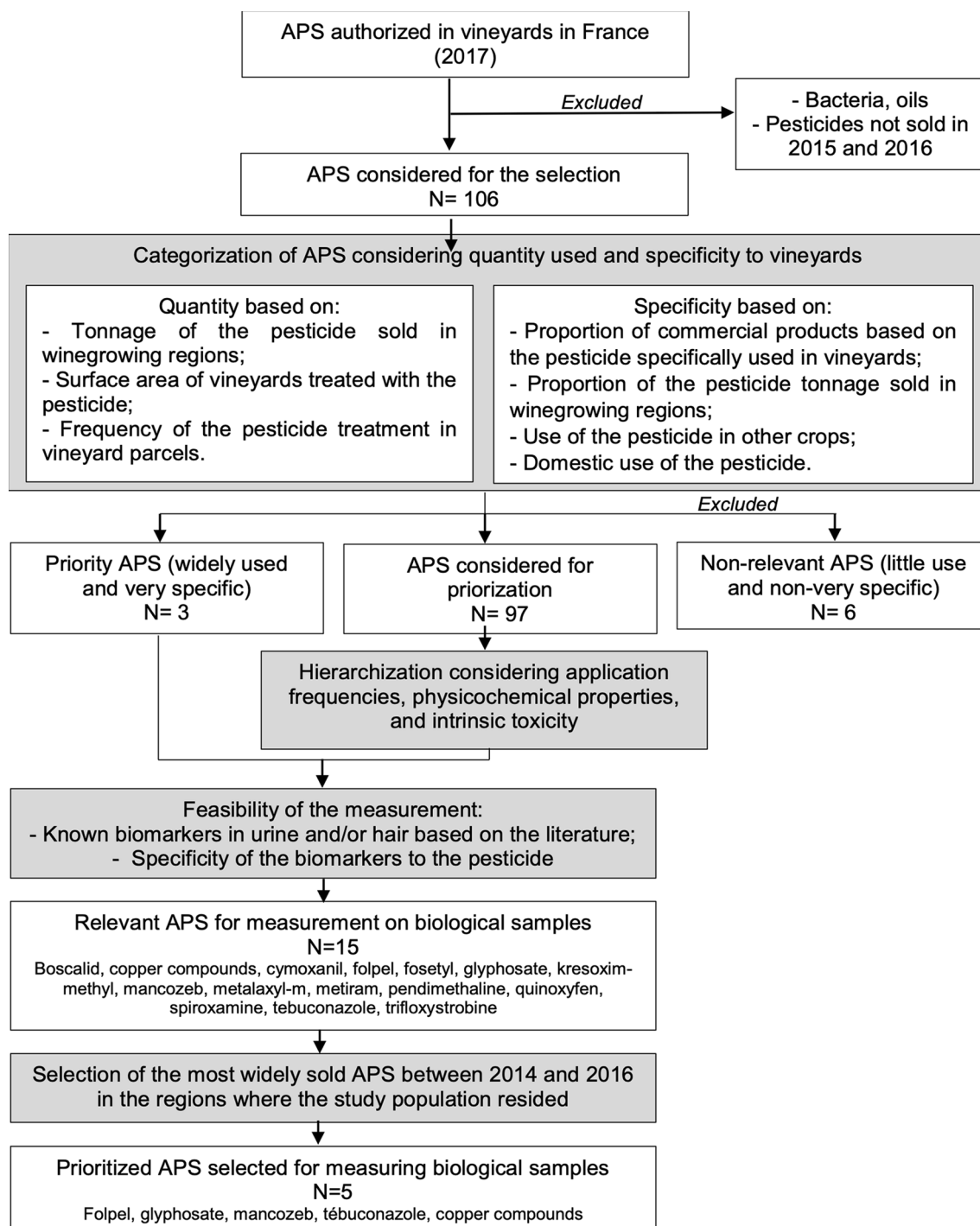


Fig. 1. Flow chart of prioritization of active pesticide substances used in vineyards and selected for measuring biological samples.

2015–2016, treated surface area greater than 150 000 ha) and very specific for this crop (>80% of the tonnage in winegrowing regions, >80% of the commercial products only used in vineyards, <20 commercial products used in other crops) (Fig. 1). Six APS were excluded because they were rarely used in vineyards (tonnage < 1 ton, treated surface area < 10 000 ha) and not specific to vineyards (<20% of the tonnage in winegrowing regions, <80% of the commercial products only used in vineyards, >20 commercial products used in other crops). The other pesticides (n = 97) were hierarchized considering their application frequencies, physicochemical properties, and intrinsic toxicity according to a method developed previously (Hulin et al. 2021). We then, analyzed scientific literature to identify urine and hair pesticide measurements related to those above prioritized. We considered the measured pesticides, their metabolite characteristics (i.e. half-life in urine and hair, specificity) and their analytical feasibility (i.e. conditions for collection and storage, availability of an analytical method) to refine the list of relevant APS for measurement on biological samples (n = 15). Finally, using the BNVD, we considered the quantity of APS sold between 2014 and 2016 in places where our study population resided (see part 2.2) in order to select pesticides with the highest priority for measuring biological samples in our study. Specifically, these were: glyphosate, folpet, mancozeb, tebuconazole, and copper compounds.

2.3.2. Identification of priority pesticides metabolites

On the basis of literature data, the toxicokinetics of priority APS were taken into account to identify molecules (parent substances or metabolites) that can be measured in human biological matrices. All metabolites, specific and nonspecific, resulting from the main APS metabolism route were considered for our study. Table 1 summarizes the toxicokinetic parameters of APS and their main metabolites which were selected for measuring biological samples in our study.

2.4. Pesticides and metabolites measurements

Folpet (phthalimide and phthalic acid), mancozeb (ethylene thiourea [ETU] and ethylene urea [EU]), tebuconazole (tebuconazole and hydroxytebuconazole [TEB-OH]), glyphosate (aminomethyl phosphonic acid [AMPA]), and copper were analyzed in urine samples for the selected adults and children.

Phthalimide, phthalic acid, tebuconazole and TEB-OH were analyzed by the LERES (Environmental and Health Research Laboratory - France), using gas chromatography coupled to tandem mass spectrometry (GC/MS/MS) after a solid phase extraction (SPE) step followed by a

derivatization procedure (trimethyl silylation). ETU and EU were also analyzed by the LERES, using GC/MS/MS after a supported liquid extraction (SLE) step followed by the same derivatization procedure. AMPA was analyzed by Labéo (France), using ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (LC/MS/MS) after a solid phase extraction (SPE) step. Copper was measured by Chemtox (France), using inductively coupled plasma mass spectrometry (ICP-MS). Creatinine was also analyzed by Chemtox Lab, using spectrophotometry at 546 nm according to the Jaffé method, which consists of measuring the intensity of the coloration of the red–orange complex formed by creatinine and picric acid in basic medium.

We were not able to measure TEB-COOH, another known metabolite of tebuconazole, due to the unavailability of a certified standard. We also encountered difficulties to determine phthalamic acid (unstable intermediate metabolite of folpet in urine), folpet (degradation of the molecule during the enzymatic deconjugation step using β -glucuronidase) and glyphosate in urine samples. Because of these difficulties, the determination of these compounds was not performed in urine.

Few children agreed to collect the lock of hair planned in the Esteban study, so due to the lack of availability of hair samples in the biobank, pesticides in hair were only measured in adults. Pesticides and their metabolites were analyzed by gas chromatography coupled to tandem mass spectrometry (GC/MS/MS) after a solvent extraction step followed by a derivatization procedure (trimethylsilylation).

Details of the analytical methods, limits of detection (LOD) and limits of quantification (LOQ) are presented in supplementary materials (tables SM2 and SM3).

2.5. Data processing

Before analyzing the results of pesticides levels in urine and hair samples, we first examined the data distributions using Shapiro-Wilk normality tests (see table SM4 in supplementary materials). Each value below the LOD was replaced by a multiple imputation method by imposing a value between 0 and the LOD and a lognormal distribution (Royston and White 2011). The imputation was repeated ten times and the values were then combined to account for the uncertainty resulting from the multiple imputation method (Mislevy 1991). Concentrations measured between the LOD and LOQ were considered without imputation. The sum of the two ring metabolites of folpet (phthalimide and phthalic acid), expressed as PA equivalents (PAeq), was calculated and considered a folpet proxy of exposure.

We used the data collected in the Esteban questionnaires to create

Table 1
Toxicokinetic parameters of priority active pesticide substances (APS) in our study.

Active pesticide substance	Main metabolites retained for the study	Other metabolites non considered	Excretion rate	Elimination half-life
Folpet	Phthalimide (S*) Phthalamic acid (S) Phthalic acid (NS*) (Canal-Raffin et al. 2008; FAO/WHO 1970; Vermeulen et al. 2005)	Thiosphogene (NS) Thiazolidine-2-thione-4-carboxylic acid (TCCA) (NS) (Bernard and Gordon 2000; van Welie et al. 1991)	0,02% (phthalimide) 25% (phthalic acid) (Berthet et al. 2012a)	~30 h (phthalimide and phthalic acid) (Berthet 2011; Berthet et al. 2012a; Berthet et al. 2012b)
Mancozeb	Ethylene-bis-isothiocyanate (EBIS) (NS) ethylene thiourea (ETU) (NS) ethylene urea (EU) (NS) (FAO/WHO 1971; Houeto et al. 1995; Hurt et al. 2001; Somerville 1986)	Ethylmediamine (EDA) (NS) (ECHA 2017; Hurt et al. 2001)	7% (ETU) (ECHA 2017; Hurt et al. 2001)	~100 h (Hurt et al. 2001; Kurttio et al. 1990)
Tebuconazole	TEB-OH (S) TEB-COOH (S) (FAO/WHO 1994; Fustinoni et al. 2014)	–	17% – 21% (TEB-OH et TEB-COOH) (Fustinoni et al. 2014)	~8 to 16 h (TEB-OH) (Oerlemans et al. 2019)
Copper	–	–	12–34% (copper) (Turnlund et al. 1998)	13 to 33 days (Barceloux 1999)
Glyphosate	AMPA (NS) (FAO/WHO 1986; Grandcoin et al. 2017; Nowack 2003)	–	1% –20% (glyphosate) (EFSA 2015; Zoller et al. 2020) 23% (AMPA) (Zoller et al. 2020)	5 to 10 h (Connolly et al. 2019; Zoller et al. 2020)

S: specific metabolite of the active pesticide substance.

NS: non specific metabolite of the active pesticide substance.

indicators for other sources of pesticide exposures and factors influencing these exposures, including smoking status (smoker, non-smoker for adults and exposed to passive smoking / non-exposed to passive smoking for children), the frequencies of organic food intake, vegetable garden intake, home ventilation, and floor cleaning, the presence of pets at home, the recent use of pesticides at home (during the previous year or the previous seven days), gardening or floral composition and time spent outdoors (for children only). Occupational activities likely related to the use of pesticides (agriculture, florist, gardener, production of pesticides, nursing staff, wood processing) were also considered for adults and children's legal representatives. The data used to characterize potential pesticide exposures in our study population are presented in [supplementary materials \(table SM5\)](#).

2.6. Statistical analysis

Socio-demographic characteristics between residents LCTV and controls were compared using Chi-Square tests for categorical data and Student's t-tests for continuous data. These comparisons included additional data (data from the National Institute of Statistics and Economic Studies), the surface area of vineyards and other crops (data from the RA).

For each pesticide or metabolite, we described the detection frequency in urine and hair, and ran descriptive statistics, for both adults and children. When the proportion of results above the LOD was lower than 40%, geometric means (GM) were not calculated and were denoted as "NC". When a percentile estimate was under the LOD or LOQ, the percentile was not reported and was denoted, respectively, as "<LOD" or "<LOQ". Both crude values ($\mu\text{g/L}$) and creatinine-adjusted values ($\mu\text{g/g}$ creatinine) were used in data analyses for urinary pesticides. In this paper, we present results obtained with urinary creatinine adjustments. Crude values (in $\mu\text{g/L}$) are given in [supplementary materials](#).

We then tested whether log-transformed urinary metabolite levels differed between residents LCTV and controls using Student's t-tests. These were performed when the proportion of detected samples was above 40%. All the analyses were performed separately for adults and children. We then used generalized linear models to identify other factors affecting pesticide levels. These analyses were performed for exploratory purposes to implement our tiered approach only. Pesticide levels in urine ($\mu\text{g/L}$) were used as dependent variable and individual covariates, such as sex, educational level, recent use of pesticides, etc. were used as an independent variables. Creatinine was included in the models as a covariate (Barr et al. 2005). Concentrations were log-transformed to improve the normality of the model residuals. A two-step procedure was used to select the relevant covariates to be included in each model. First, age, sex, educational level and creatinine were included in the models. Akaike's information criterions (AIC and AICC) were then used to identify significant additional covariates (floor cleaning, home ventilation, recent use of pesticides, occupational activities likely related to the use of pesticides, etc.) relevant to each model. The statistical analyses were conducted using SAS Enterprise Guide 7.1.

3. Results

3.1. Results for adults

3.1.1. Adults characteristics

A summary of the characteristics of the adult population and comparison between residents LCTV and controls is reported in [supplementary materials \(table SM6\)](#).

There were 25 men and 35 women in our study population. Mean age was 55 years (min: 23; max: 74). Among adults included in the study, 83% lived with a partner. An average 2.5 persons lived in each household. Most adults were employees, executives or intermediate professionals, and 55% earned more than 15,000 € per year. A third (33%)

lived in agricultural areas. Participants generally lived in villages (45%) with a population of approximately 5,000 inhabitants. Most were non-smokers (75%). With regard to domestic exposure to pesticides, 75% declared using pesticides during the previous 12 months, and 18% during the previous 7 days. In terms of professional exposure to pesticides, six adults worked in sectors where pesticides can be used, of these, four declared they were exposed to or used pesticides in their work.

We compared socio-demographic, residential and lifestyle characteristics between residents LCTV and controls. Few differences were observed for most socio-demographic and lifestyle characteristics. Residents LCTV were more likely to be single, divorced or widowed (chi-square, $p = 0.0377$), and therefore, had fewer family members (t -test, $p = 0.014$). The surface area of vineyards (t -test, $p < 0.0001$) and of agricultural lands in the place of residence were higher in the group of participants LCTV than in the group of controls (t -test, $p < 0.0001$ and $p = 0.0024$ respectively). The mean distance between one's house and the closest vineyard was 148 m for participants LCTV and 6,030 m for the control group (t -test, $p < 0.0001$).

3.1.2. Pesticide concentrations in adults

Detection frequencies and distributions (GM and percentiles) of pesticides/metabolites levels, adjusted for creatinine (urine) and hair weight (hair), are given in [Table 2](#) (urine) and [Table 3](#) (hair). Crude distribution, in $\mu\text{g/L}$, are given in [supplementary materials \(table SM7\)](#). Results showed that copper and the metabolites of folpet (phthalic acid and phthalimide), mancozeb (EU/ETU), and glyphosate (AMPA) were detected in approximately 50% or more of the urine samples. In contrast, only phthalimide was detected in study participants' hair. However, the detection frequency of phthalimide in hair was higher than in urine, which may reflect the ability of hair to represent cumulative exposures over the previous several months (Pragst and Balikova 2006).

Considering the metabolites of folpet, the concentrations of phthalic acid were two orders of magnitude higher than those of phthalimide, which may be explained by higher urinary excretion rates of phthalic acid (25%) than phthalimide (0.02%) (Berthet et al. 2012a). With regard to mancozeb metabolites, EU and ETU presented very similar distribution levels.

The comparison between urinary pesticide concentrations among women and men generally showed no difference between sexes ([Figure SMI in supplementary materials](#)). Copper levels were significantly lower for men than women, which reflects findings in the Esteban study (Fillol et al. 2021a). On the contrary, phthalimide concentrations in hair were significantly lower in women.

3.1.3. Comparison between residents LCTV and controls

[Fig. 2](#) presents the comparison of pesticide and metabolite levels (histograms and distributions) between residents LCTV (below) and controls (above). Box-plots and T-test results are also presented for each pesticide or metabolite. Concentration levels in residents LCTV and controls are given in [supplementary materials \(tables SM8, SM9, SM10\)](#).

These comparisons showed no difference between residents LCTV and controls, considering both detection frequencies and geometric means. However, folpet and tebuconazole metabolites levels above the 95th percentile in residents LCTV were more than twice as high as in controls. Adults who presented these highest folpet metabolites levels were residents LCTV who were included in the Esteban study during spring/summer season. Apart from one of these most exposed participants who declared using pesticides in the previous seven days, all others declared no known pesticide exposure through domestic or professional use (data not shown).

3.1.4. Other determinants of pesticide concentrations in adults

The associations between the levels of pesticides/metabolites and potential determinants related to occupational or environmental exposure to pesticides are presented in [Tables 4, 5 and 6](#). Determinants

Table 2

Distribution of urinary concentrations for folpet, mancozeb, tebuconazole, copper and AMPA in the adult population (in µg/g of creatinine).

Pesticide/metabolite	N	%>LOD	GM [CI 95%]	P25	P50	P75	P90	P95	Max
Phthalimide	60	48%	1.0 [0.8 – 1.2]	<LOD	<LOQ	1.6	2.7	4.2	17.3
Phthalic acid	60	100%	80.7 [63.0–103.2]	40.3	71.0	150.9	222.5	421.7	1,843.7
PA equivalents	60	–	82.0 [64.2–104.8]	41.4	71.7	152.5	224.4	426.0	1,846.0
EU	60	63%	0.4 [0.3 – 0.6]	<LOD	<LOQ	1.0	2.9	3.4	6.7
ETU	60	47%	0.5 [0.4 – 0.7]	<LOD	<LOQ	<LOQ	1.9	2.9	7.9
TEB-OH	60	37%	NC	<LOD	<LOD	<LOQ	6.6	10.6	22.5
Tebuconazole	60	0%	NC	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Copper	60	100%	12.4 [11.3 – 13.6]	9.5	11.6	15.6	20.8	24.5	31.5
AMPA	60	83%	0.07 [0.05–0.1]	<LOQ	0.08	0.14	0.27	0.62	2.22

Table 3

Distribution of hair concentrations for folpet, mancozeb and tebuconazole in the adult population (in ng/g of hair).

Pesticide/metabolite	N	%>LOD	GM [CI 95%]	P25	P50	P75	P90	P95	Max
Phthalimide	59	88%	18.6 [14.7 – 23.5]	<LOQ	20.0	31.0	45.0	48.0	160.0
TEB-OH	59	5%	NC	<LOD	<LOD	<LOQ	<LOQ	28.4	64.0
Tebuconazole	59	3%	NC	<LOD	<LOD	<LOD	<LOQ	<LOQ	113.0
Folpet	59	0%	NC	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
ETU	59	0%	NC	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
EU	59	0%	NC	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

influencing pesticide/metabolite levels were quite similar for all the pesticides studied.

Pesticide levels were minimally influenced by occupational activities where exposure was possible and by pesticide household use (during the previous 12 months or 7 days). In contrast, pesticide levels were influenced by diet (consumption of self-produced vegetables or organically grown foods), housing characteristics (ventilation, floor cleaning) and some outdoor leisure activities (gardening). These factors are likely to modulate exposure to pesticides from environmental origin. These results suggest that in our study, exposure to these pesticides was more likely to come from environmental sources, potentially related to agricultural activities, than from domestic or occupational sources. This reflects observations from previous studies (Teyssseire et al. 2021).

Pesticide levels were also associated with the season when biological samples were collected, which may suggest agricultural sources of exposure.

Smoking status was positively associated with all folpet metabolites in urine and hair, mancozeb metabolites in urine and copper in urine: smokers having systematically higher biological pesticides levels than non-smokers. This could be explained by direct exposure to pesticides used for treating tobacco leaves (Cai et al. 2002; Reay-Jones 2017) and by indirect exposure induced by hand-to-mouth actions from smoking (Kegel et al. 2014).

3.2. Results for children

3.2.1. Children's characteristics

A summary of the characteristics of the children in our study population and comparison between residents LCTV and controls is reported in supplementary materials (table SM11).

The study population included 36 boys and 25 girls in our study population. Mean age was 11 years (min: 6; max: 17). Among children included in the study, 92% lived with their parents as a couple. An average of 4.2 persons lived in each household. Households generally earned more than 15,000 € per year (57%). Two-fifths (39%) lived in agricultural areas, most lived in villages (62%) with an average population of 4,500 inhabitants. Most children were not exposed to passive smoking (77%). With regard to domestic exposure to pesticides, 67% of children's representatives declared using pesticides during the previous 12 months. In terms of professional exposure to pesticides, 11 children had at least one family member employed in sectors where pesticides can be used (agriculture, florist, nurse, wood processing). Of these, three

had family members who declared they were exposed to or used pesticides in their work.

As was observed for adults, most socio-demographic and lifestyle characteristics did not differ between the residents LCTV and control groups. There were fewer girls in the control group but not significantly so (chi-square, $p = 0.5$). Control children were more likely to live in a household that earned more than €15,000 per year per adult compared to children LCTV ($p = 0.0072$). Apart from the surface area of vineyards and of agricultural lands in the place of residence (t -test, $p = 0.0002$ and $p = 0.0933$ respectively), characteristics of the place of residence (i.e. housing estate, housing area, region and size of population in the place of residence) were similar for both groups. The mean distance between one's house and the closest vineyard was 202 m for children LCTV and 7,626 m for the control group (t -test, $p < 0.0001$).

3.2.2. Pesticide concentrations

Detection frequencies and distributions (GM and percentiles) of pesticides/metabolites levels are given in Table 7. Crude distributions, in µg/L, are given in supplementary materials (table SM12). As observed for adults, folpet metabolites, mancozeb metabolites, copper and AMPA were all detected in more than 50% of urine samples whereas TEB-OH was detected in only 7% of samples and tebuconazole was never detected.

Again, just as for adults, concentrations of phthalic acid were two orders of magnitude higher than those of phthalimide, and EU and ETU presented very similar distribution levels.

The comparison between urinary pesticide concentrations in girls and boys showed no difference between both sexes (see Figure SM2 in supplementary materials).

3.2.3. Comparison between residents LCTV and controls

Fig. 3 presents the comparison of pesticides and metabolite levels (histograms and distributions) between residents LCTV (below) and controls (above). Box-plots and Student's t -test results are also presented for each pesticide/metabolite. Concentrations levels in residents LCTV and controls are given in supplementary materials (tables SM13 and SM14).

Comparisons showed no difference in pesticide/metabolite detection frequencies between children LCTV and controls. However, they did show a slight difference between urinary phthalimide levels in residents LCTV and controls ($p = 0.0033$), with higher concentrations in the former group. Moreover, as observed for adults, folpet and tebuconazole

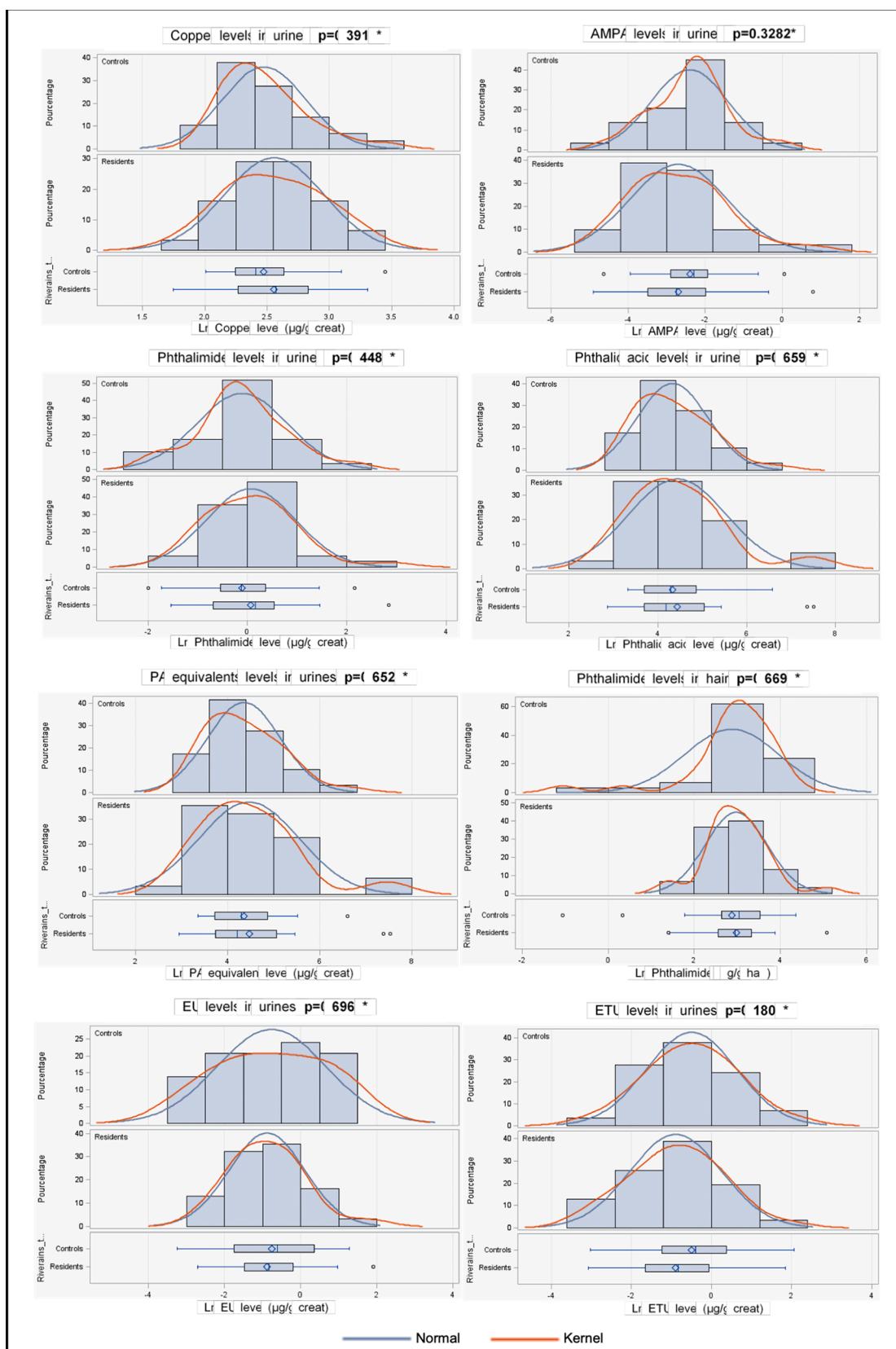


Fig. 2. Comparisons of folpet metabolite, mancozeb metabolite, copper and AMPA levels between adult residents LCTV and controls (log-transformed data). * *t*-test – equal variance – using log-transformed concentrations.

Table 4
Determinants associated with urine and hair folpet metabolite concentrations in the study's adult population.

Factors	n	Phthalic acid (urine)				Phthalimide (urine)				Phthalimide (hair)			
		Value	CI95%		p	Value	CI95%		p	Value	CI95%		p
Intercept	-	3.70	2.51	4.89	<0.0001	-0.48	-1.37	0.41	0.2866	3.21	2.07	4.35	<0.0001
Age*	-	0.01	0.00	0.03	0.0928	0.00	-0.01	0.01	0.9071	0.00	-0.02	0.01	0.564
Creatinine*	-	0.60	0.29	0.91	0.0002	0.16	-0.09	0.41	0.216	-	-	-	-
Sex*													
Men	25	0.05	-0.33	0.43	0.81	0.10	-0.23	0.43	0.5549	0.70	0.33	1.08	0.0002
Women	35	0.00	0.00	0.00	.	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Marital status*													
Married / unmarried couple	50	0.19	-0.31	0.69	0.4504	0.02	-0.39	0.42	0.9398	-0.56	-1.04	-0.08	0.0209
Single / divorced / widowed	10	0.00	0.00	0.00	.	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Education level*													
None/primary education	17	0.17	-0.29	0.63	0.4667	0.23	-0.15	0.61	0.244	0.01	-0.43	0.46	0.9562
Secondary education	20	-0.07	-0.51	0.37	0.7688	0.00	-0.36	0.37	0.9877	-0.45	-0.88	-0.02	0.0406
Higher education	23	0.00	0.00	0.00	.	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Smoking status													
Smoker	15	0.95	0.52	1.38	<0.0001	1.10	0.76	1.44	<0.0001	0.43	0.03	0.84	0.0367
Non-smoker	45	0.00	0.00	0.00	.	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Home ventilation													
Once a day or more	45	-0.48	-0.93	-0.02	0.0388	-0.53	-0.88	-0.18	0.0032				
Less than once a day	15	0.00	0.00	0.00	.	0.00	0.00	0.00	.				
Organic food product intake													
Once a week or more	21	-0.71	-1.14	-0.28	0.0012	-0.47	-0.81	-0.13	0.0074				
Between 1 and 4 portions per month	13	-0.24	-0.71	0.23	0.3121	-0.08	-0.46	0.30	0.6812				
Never or less than once a month	25	0.00	0.00	0.00	.	0.00	0.00	0.00	.				
Practiced gardening or floral composition													
No	27	0.43	0.04	0.83	0.0329	-	-	-	-	-0.33	-0.70	0.04	0.0844
Yes	33	0.00	0.00	0.00	.	-	-	-	-	0.00	0.00	0.00	.
Self-produced vegetables intake													
Once a month or more	14	-	-	-	-	0.41	0.08	0.75	0.0163	0.39	-0.04	0.81	0.076
Never or less than once a month	44	-	-	-	-	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Floor cleaning at home													
Once or twice a week	34	-	-	-	-	0.41	0.09	0.72	0.0108				
More than twice a week	26	-	-	-	-	0.00	0.00	0.00	.				
Season when biological samples taken													
Autumn / Winter	37	-0.33	-0.71	0.06	0.0955	-	-	-	-	0.44	0.07	0.81	0.0194
Spring / Summer	23	0.00	0.00	0.00	.	-	-	-	-	0.00	0.00	0.00	.
Scale		0.66	0.55	0.79		0.54	0.45	0.64		0.66	0.55	0.79	
AIC / AICC		146.95 / 156.49				120.55 / 130.32				136.27 / 142.14			
R ²		0.5695				0.5368				0.4138			

Table 5
Determinants associated with urine mancozeb metabolite concentrations in the study's adult population.

Factors	n	EU				ETU			
		Value	CI95%		p	Value	CI95%		p
Intercept	-	-1.72	-2.99	-0.45	0.008	-0.53	-1.68	0.62	0.3682
Age*	-	0.01	-0.02	0.03	0.6354	0.00	-0.02	0.02	0.7889
Creatinine*	-	0.48	0.04	0.93	0.0319	0.34	0.01	0.67	0.0441
Sex*									
Men	25	0.19	-0.38	0.76	0.511	0.51	0.09	0.93	0.0181
Women	35	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Marital status*									
Married / unmarried couple	50	-	-	-	-	-0.44	-0.98	0.10	0.1117
Single / divorced / widowed	10	-	-	-	-	0.00	0.00	0.00	.
Education level*									
None/primary education	17	-	-	-	-	-0.35	-0.83	0.13	0.1524
Secondary education	20	-	-	-	-	-0.11	-0.59	0.36	0.634
Higher education	23	-	-	-	-	0.00	0.00	0.00	.
Smoking status									
Smoker	15	1.32	0.70	1.93	<0.0001	0.76	0.30	1.22	0.0011
Non-smoker	45	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Season when biological samples taken									
Autumn / Winter	37	-	-	-	-	-0.42	-0.82	-0.01	0.0444
Spring / Summer	23	-	-	-	-	0.00	0.00	0.00	.
Scale		1.02	0.85	1.22	-	0.74	0.62	0.89	-
AIC / AICC		184.86 / 186.44				154.44 / 158.93			
R ²		0.3342				0.3778			

* Calibration covariate.

Table 6
Determinants associated with copper and AMPA concentrations in the study's adult population.

Factors	n	Copper			AMPA				
		Value	CI95%	p	Value	CI95%	p		
Intercept	-	2.06	1.70	2.43	<0.0001	-2.33	-3.60	-1.06	0.0003
Age*	-	0.00	0.00	0.01	0.1576	-0.01	-0.03	0.01	0.4249
Creatinine*	-	0.69	0.57	0.80	<0.0001	0.47	0.04	0.89	0.0318
Sex*									
Men	25	-0.19	-0.34	-0.04	0.0112	-0.19	-0.75	0.37	0.5043
Women	35	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Marital status*									
Married / unmarried couple	50	0.11	-0.07	0.30	0.2384	-	-	-	-
Single / divorced / widowed	10	0.00	0.00	0.00	.	-	-	-	-
Education level*									
None/primary education	17	-	-	-	-	-	-	-	-
Secondary education	20	-	-	-	-	-	-	-	-
Higher education	23	-	-	-	-	-	-	-	-
Smoking status									
Smoker	15	0.16	0.00	0.32	0.0502	-	-	-	-
Non-smoker	45	0.00	0.00	0.00	.	-	-	-	-
Presence of pets in home									
Yes	39	0.06	-0.09	0.21	0.4074	-	-	-	-
No	21	0.00	0.00	0.00	.	-	-	-	-
Self-produced vegetables intake									
Once a month or more	14	0.10	-0.06	0.26	0.2359	0.82	0.21	1.43	0.0082
Never or less than once a month	44	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Scale		0.26	0.22	0.31	-	1.00	0.83	1.20	-
AIC / AICC		26.27 / 30.02				176.66 / 178.31			
R ²		0.2612				0.2282			

* Calibration covariate.

Table 7
Distribution of urinary concentrations for folpet metabolites, mancozeb metabolites, tebuconazole, copper and AMPA in the study's child population (in µg/g of creatinine).

Pesticide/metabolite	N	%>LOD	GM [CI 95%]	P25	P50	P75	P90	P95	Max
Phthalimide	61	48%	0.7 [0.5 – 0.8]	<LOD	<LOQ	<LOQ	2.0	2.6	4.6
Phthalic acid	61	100%	49.6 [40.5 – 60.7]	29.1	46.8	70.6	148.1	164.6	1,237.3
PA equivalent	61	-	50.5 [41.3 – 61.8]	29.7	47.4	72.9	149.0	169.2	1,239.9
EU	61	53%	0.2 [0.2 – 0.3]	<LOD	<LOQ	<LOQ	1.0	1.5	3.2
ETU	61	47%	0.4 [0.3 – 0.6]	<LOD	<LOD	<LOQ	2.0	3.1	5.9
TEB-OH	61	7%	NC	<LOD	<LOD	<LOD	<LOQ	<LOQ	5.1
Tebuconazole	61	0%	NC	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Copper	61	100%	13.6 [12.1 – 15.1]	9.9	12.4	19.2	23.3	26.1	36.5
AMPA	61	88%	0.15 [0.12 – 0.18]	0.09	0.16	0.27	0.41	0.47	0.70

metabolites levels above the 95th percentile in children LCTV were slightly higher than in controls. Children who had the highest folpet metabolite levels were included in the Esteban study during the spring/summer season. No responsible adult declared pesticide exposure through domestic or professional use (data not shown).

3.2.4. Other determinants of pesticide concentrations

The associations between pesticide levels and potential determinants related to occupational or environmental exposures to pesticides are presented in Tables 8-10.

Determinants influencing pesticide levels were quite similar for all pesticides studied and similar to those observed for the study's adult population.

Levels were moderately influenced by determinants related to their responsible adult's occupational or environmental exposure. AMPA levels in urine tended to be lower in children whose responsible adult did not use pesticides in an occupational activity. However, we found no association with the use of pesticides at home (during the previous 12 months or 7 days) or with organic food product intake.

As observed for adults, pesticide levels in children were more often associated with factors that could modulate environmental exposure: home ventilation, floor cleaning, time spent outdoors, and the presence of pets. Urinary phthalic acid levels tended to be lower in biological samples collected during autumn or winter than during spring or

summer. Unlike what we observed for adults, pesticide levels in children were not influenced by their smoking environment.

4. Discussion

4.1. Identification of relevant biomarkers of exposure to pesticides used in vineyards and matrices for their biomonitoring

With regard to the frequency of detection of pesticides/metabolites in urine and hair samples, the results of the study showed that copper and the metabolites of folpet (phthalic acid and phthalimide), mancozeb (EU/ETU) and glyphosate (AMPA) were frequently detected in the urine of study participants. The metabolite of tebuconazole (TEB-OH) was less frequently detected in urine, especially in children. This low detection rate could be explained by the short half-life of this metabolite in the body (between 8 h and 16 h (Oerlemans et al. 2019) which could limit its presence in the first morning urine, and by the LOD reached in our study, which was four times higher than that reached in urine by Fustinoni et al (Fustinoni et al. 2014), and ten times higher than that reached in hair (Mercadante et al. 2018; Polledri et al. 2019). In hair, only phthalimide could be detected in the analyzed samples but with a higher detection rate than that observed in urine, which could reflect a greater ability of hair to reflect cumulative exposures over time (Pragst and Balikova 2006). The higher detection of folpet metabolites in urine and hair could

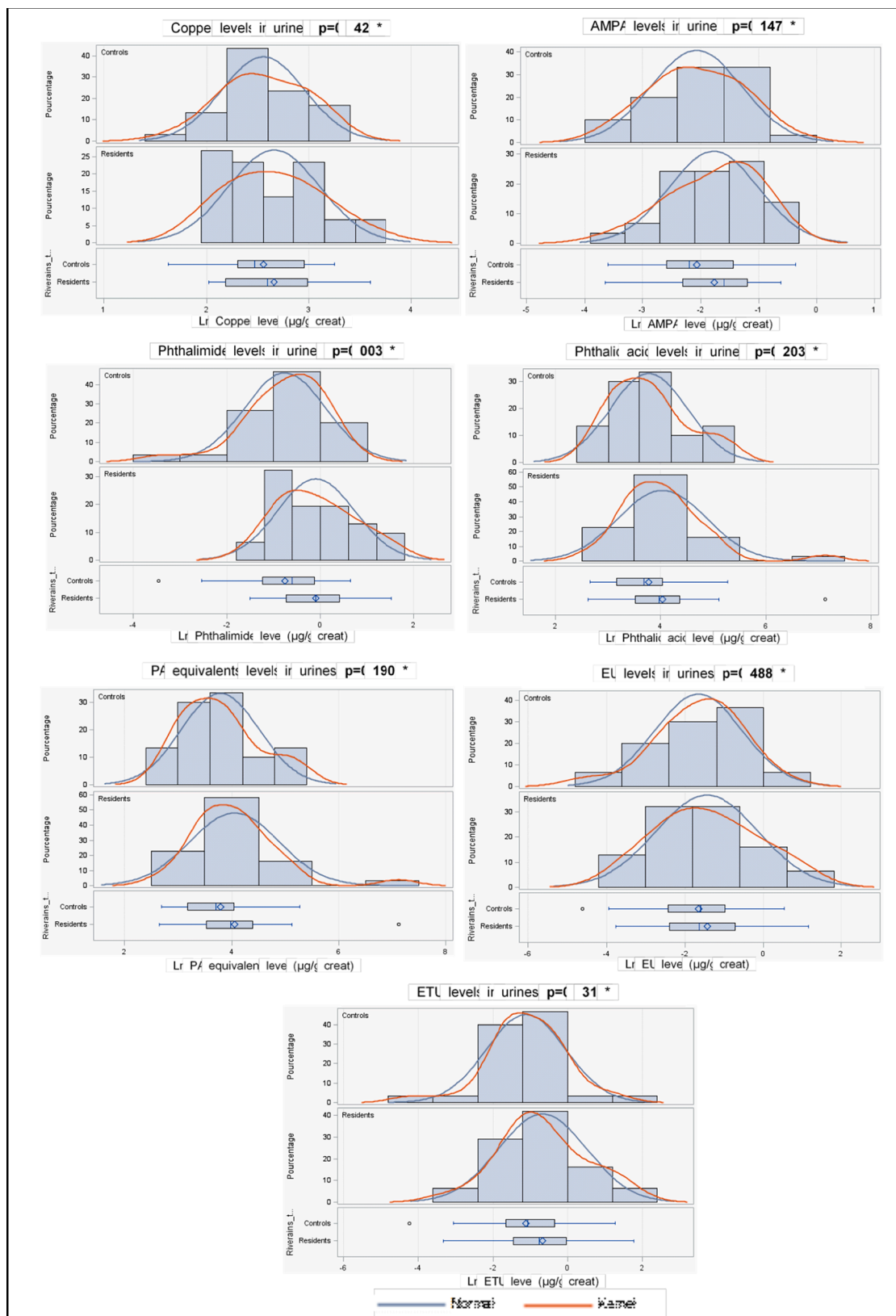


Fig. 3. Comparisons of folpet metabolites, mancozeb metabolites, copper and AMPA levels between residents LCTV and controls in the study's child population (log-transformed data). * t-test – equal variance – using log-transformed concentrations.

Table 8

Determinants associated with urinary folpet metabolite concentrations in the study's child population.

Factors	n	Phthalic acid			Phthalimide				
		Value	CI95%	p	Value	CI95%	p		
Intercept	-	4.91	3.99	5.83	<0.0001	0.11	-0.81	1.03	0.8168
Creatinine*	-	0.75	0.43	1.07	<0.0001	0.03	-0.27	0.34	0.8305
Age*	-	-0.08	-0.15	-0.01	0.0187	-0.05	-0.12	0.01	0.1076
Sex*									
Boy	36	0.06	-0.28	0.40	0.7297	0.06	-0.27	0.38	0.7339
Girl	25	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Education level*									
None/primary/secondary education	23	0.04	-0.32	0.40	0.8406	0.17	-0.16	0.50	0.3148
Higher education	38	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Presence of pets at home									
Yes	40	0.33	-0.04	0.70	0.0826	0.18	-0.16	0.51	0.3073
No	17	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Home ventilation									
Once a day or more	47	-	-	-	-	-0.35	-0.76	0.05	0.0863
Less than once a day	14	-	-	-	-	0.00	0.00	0.00	.
Season when biological samples taken									
Autumn / Winter	44	-0.55	-0.94	-0.16	0.0061	-	-	-	-
Spring / Summer	17	0.00	0.00	0.00	.	-	-	-	-
Scale		0.65	0.54	0.78		0.60	0.50	0.72	
AIC / AICC		130.76 / 133.70				121.66 / 124.60			
R²		0.3047				0.1111			

* Calibration covariate.

Table 9

Determinants associated with urinary mancozeb metabolite concentrations in the study's child population.

Factors	n	EU			ETU				
		Value	CI95%	p	Value	CI95%	p		
Intercept	-	0.75	-0.79	2.30	0.3402	-1.08	-2.35	0.19	0.0958
Creatinine*	-	0.77	0.27	1.27	0.0026	0.40	-0.06	0.86	0.092
Age*	-	-0.07	-0.17	0.04	0.2053	0.04	-0.06	0.14	0.4584
Sex*									
Boy	36	-0.58	-1.12	-0.05	0.0331	-0.44	-0.96	0.07	0.0913
Girl	25	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Education level*									
None/primary/secondary education	23	-0.60	-1.17	-0.04	0.0365	0.11	-0.46	0.68	0.7124
Higher education	38	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Presence of pets at home									
Yes	40	0.47	-0.08	1.03	0.0955	0.77	0.24	1.30	0.0045
No	17	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Home ventilation									
Once a day or more	47	-1.16	-1.84	-0.48	0.0009	-	-	-	-
Less than once a day	14	0.00	0.00	0.00	.	-	-	-	-
Floor cleaning at home									
Twice a week or less	33	-0.70	-1.26	-0.14	0.0137	-	-	-	-
More than twice a week	25	0.00	0.00	0.00	.	-	-	-	-
Self-produced vegetables intake									
Once a month or more	32	-	-	-	-	-0.43	-0.95	0.09	0.1054
Never or less than once a month	26	-	-	-	-	0.00	0.00	0.00	.
Time spent outdoors (garden, park, etc.)									
Less than 2 h per day	22	-	-	-	-	-0.72	-1.36	-0.07	0.0287
Between 2 and 3 h per day	17	-	-	-	-	-0.38	-1.04	0.27	0.2544
More than 3 h per day	22	-	-	-	-	0.00	0.00	0.00	.
Scale		0.99	0.82	1.19					
AIC / AICC		181.38 / 185.13				178.27 / 182.95			
R²		0.3097				0.2804			

* Calibration covariate.

also be explained by folpet being one of the most widely and specifically used substances in vineyards. The comparison of levels measured in residents LCTV and controls showed that, with the exception of phthalimide, the mean concentrations of pesticides/metabolites measured in participants were not influenced by the presence of vineyards near their place of residence. However, larger differences were observed between residents and controls with higher folpet and tebuconazole exposure (concentrations above the 95th percentile). This suggests that while average exposure levels between residents LCTV and controls were similar overall, residents with the highest exposure to folpet and

tebuconazole were more exposed than controls. The results of the study showed that the concentrations of pesticides/metabolites measured in the participants were little influenced by occupational, dietary or household sources of exposure. They were more influenced by factors that could modulate environmental exposure to pesticides (consumption of self-produced foods, ventilation or cleaning of the home, gardening, time spent outdoors, presence of animals in the home, season of biological collection). These factors were generally consistent with those observed in previous studies of people living near crops (López-Gálvez et al. 2019; Teyssseire et al. 2021) and thus might suggest that the

Table 10
Determinants associated with urinary copper and AMPA concentrations in the study's child population.

Factors	n	Copper			AMPA				
		Value	CI95%	p	Value	CI95%	p		
Intercept	-	3.37	3.05	3.69	<0.0001	-0.92	-1.85	0.02	0.0553
Creatinine*	-	0.75	0.62	0.88	<0.0001	0.86	0.53	0.19	<0.0001
Age*	-	-0.08	-0.11	-0.06	<0.0001	-0.06	-0.13	0.01	0.0914
Sex*									
Boy	36	0.00	-0.14	0.15	0.97	-0.28	-0.64	0.07	0.1195
Girl	25	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Education level*									
None/primary/secondary education	23	0.15	-0.01	0.30	0.0609	0.37	-0.02	0.75	0.0608
Higher education	38	0.00	0.00	0.00	.	0.00	0.00	0.00	.
Time spent outdoors (garden, park, etc.)									
Less than 2 h per day	22	0.18	0.00	0.35	0.0473	-	-	-	-
Between 2 and 3 h per day	17	0.10	-0.07	0.28	0.2516	-	-	-	-
More than 3 h per day	22	0.00	0.00	0.00	.	-	-	-	-
Presence of pets at home									
Yes	40	-	-	-	-	-0.05	-0.42	0.33	0.80
No	17	-	-	-	-	0.00	0.00	0.00	.
Use of pesticides at home during the last 12 months									
Never or less than twice a year	20	-	-	-	-	0.28	-0.12	0.69	0.1639
More than three times per year	41	-	-	-	-	0.00	0.00	0.00	.
Occupational activity related with the use of pesticides (responsible adult)									
No	11	-	-	-	-	-0.42	-0.92	0.08	0.1008
Yes	50	-	-	-	-	0.00	0.00	0.00	.
Scale		0.27	0.22	0.32	-	0.65	0.54	0.78	-
AIC / AICC		28.01 / 30.84				128.86 / 132.77			
R²		0.0634				0.3953			

* Calibration covariate.

pesticides/metabolites measured in the study would reflect environmental exposure to pesticides potentially related to agricultural use. However, as these analyses were performed for exploratory purposes, the main goal of which was to implement the exposure biomarker identification approach, the interpretation of the analytical results requires great precautions.

Thus, considering the three criteria of our tiered approach, we identified two relevant biomarkers (phthalimide and phthalic acid) that met all the defined criteria. We also identified five biomarkers (TEB-OH, ETU, EU, and AMPA, copper) that met one or two criteria and which could therefore be of potential interest to complete the assessment of pesticide exposures in residents LCTV.

Besides this identification approach, additional information, including metabolic and toxicokinetic data, is needed to demonstrate the utility of these biomarkers for pesticide exposure assessment related to spray-drift events (Silva et al. 2007). Among the seven relevant or of potential interest biomarkers identified, only phthalimide and TEB-OH are specific to their parent pesticide. With regards to the others, phthalic acid is also a metabolite of phthalates (Vermeulen et al. 2005), ETU and EU are also metabolites of other ethylene bis-dithiocarbamate pesticides (Houeto et al. 1995; Somerville 1986), AMPA is also a metabolite of other phosphonates (Grandcoin et al. 2017; Nowack 2003), and copper may have other origins of exposure besides pesticides (Aspin and Sass-Kortsak 1981; Barceloux 1999). Nevertheless, previous studies have shown that these non-specific metabolites could be relevant biomarkers for measuring the exposures to pesticides used in vineyards. Indeed, two studies showed that although phthalic acid is not specific to folpet, as its excretion rate is higher than phthalimide, joint measurement of these two biomarkers is more accurate in reflecting exposure to folpet (Berthet et al. 2012a; Berthet et al. 2012b). Elsewhere, a study confirmed that TEB-OH is a specific biotransformation product of tebuconazole in humans and a promising candidate for biomonitoring tebuconazole exposure in winegrowers (Fustinoni et al. 2014). Two other studies have shown that ETU can be considered a relevant biomarker of mancozeb exposure in vineyard workers (Colosio et al. 2002; Mandic-Rajcevic et al. 2019). Finally, copper and AMPA have been widely measured in general population biomonitoring studies (Conrad et al. 2017; Gillezau et al. 2019; Haines et al. 2017) but only

two studies have used these biomarkers to characterize exposure in a vineyard context. A study conducted in Slovenia measured AMPA in urine samples of children living in rural areas, including near vineyards, but did not distinguish between dietary exposure and exposure from glyphosate uses in the residential environment (Stajniko et al. 2020). A study conducted in Brazil measured copper in the serum of wine growers and showed that copper concentrations were twice as high in this population than a non-rural population not directly exposed to heavy metals from pesticide handling (Rocha et al. 2015), copper-based products being the most frequently used pesticides among wine growers.

With regard to metabolic, toxicokinetic and other data published in the literature, it appears that besides the two urinary folpet biomarkers that met all three criteria in our study, TEB-OH is also a relevant biomarker to measure pesticide exposures in residents LCTV. Measurements of ETU, AMPA and copper may also be of interest but would require more careful interpretation of results.

These biomarkers are readily excreted in the urine, making this an appropriate matrix for their determination (see Table 1). However, their elimination half-life is short, which means that a more frequent urine sample collection is necessary to accurately reflect vineyard treatment-related exposures which are brief and irregular (approximately 20 treatments per year in France, performed mainly between March and August (Simonovici 2020)). For this purpose, the collection of repeated urine samples over several days seems particularly suitable to better take into account the short-term evolution in biomarker levels (individual variability and vineyard treatment event) (Berthet et al. 2012c; Faÿs et al. 2020; Philippat and Calafat 2021; Verner et al. 2020).

In addition, our study results suggested that hair could be an interesting matrix to characterize semi-chronic exposure to folpet. Hair presents several benefits in terms of collection (non-invasive, easy to transport and store) and shows promising results in terms of detecting substances (including when conducting multi-residue analyses), and reflecting sub-chronic exposures (i.e. exposures in the previous weeks or months) (Hardy et al. 2015; Hardy et al. 2021; Liu et al. 2015; Polledri et al. 2019; Vorkamp et al. 2021). However, interpreting results for hair concentrations could be unclear and requires further research on the relationship to internal exposure, individual variability, external contamination, etc. (Vorkamp et al. 2021). In addition, the collecting of

hair samples cannot be performed in people who are bald or have very short hair. Therefore, measurements of pesticides/metabolites in hair can only be considered as a parallel and complementary method to measuring relevant biomarkers in other biological samples (urine or blood).

4.2. Insights and perspectives for future large-scale studies

Biomonitoring studies are confronted with the high cost of analyzing substances in biological matrices. A thorough prioritization of the substances to be analyzed is therefore crucial in order to determine and satisfy the most important needs with respect to the study objectives. While some methods have been proposed to prioritize substances of interest for biomonitoring (Fillol et al. 2014; Ougier et al. 2021), few have also included the identification of relevant biomarkers and biomarker/matrix pairings. As part of the European biomonitoring program HBM4EU, Vorkamp et al. recently proposed a method to identify the most appropriate biomarkers for a prioritized list of substances along with the biological matrices and analytical methods best suited for their biomonitoring (Vorkamp et al. 2021). The method was based exclusively on the analysis of literature data and was confronted with the lack of information for some emerging compounds (certain metabolites of phthalates, bisphenols, flame retardants, etc.). In addition, the approach was designed to identify biomarker/matrix pairings to be included in the studies conducted as part of the HBM4EU program and was therefore intended to meet not only the most important needs of European policy makers and risk assessors, but also the common national needs of the program's participating countries (Ougier et al. 2021). The tiered approach we propose highlights the advantage of developing methods to characterize very specific environmental exposure (in our case, exposure to pesticides in people living near vineyards in France), which combines both data from the literature (data on toxicokinetics, metabolism, and biological measurements in previous studies in a vineyard context) and *ad hoc* measurements made from samples available in a biobank. In the present study, these *ad hoc* measurements provided us with the possibility to consider the usefulness of selecting lesser-known biomarkers (metabolites of folpet, tebuconazole and mancozeb) - some of which were found to be relevant - and to develop their analysis in hair, a non-invasive matrix that is still under used in biomonitoring studies. In addition, the approach we developed here can be adapted to identify biomarkers and relevant biomarker/matrix pairings for other biomonitoring studies in different exposure contexts, both in France and elsewhere.

Nevertheless, our results show that the tiered approach may have been too restrictive for TEB-OH, a metabolite that has been found to be relevant for assessing pesticide exposures in residents LCTV. This findings could be explained by several limitations of our study. First, given the specificity of the exposure studied, it was difficult to identify individuals with appropriate biobank biological samples who met the selection study criteria from the national prevalence study Esteban. For example, although the selected residents living in the immediate vicinity of vineyards (175 m on average) and the controls living far from vineyards (6.8 km on average), it was not possible to ensure that they did not live near other types of crops. In addition, the available urine samples were a single collection from 1st morning void, which imperfectly characterizes snapshot and variable exposure to pesticides with short elimination half-lives (Brunekreef et al. 1987; Perrier et al. 2016). As mentioned in other studies, because urinary levels are susceptible to significant variations, the use of repeated or 24-hour urine samples would be more adequate to assess exposure to pesticides used on crops (Berthet 2011; Berthet et al. 2012c; Philippat and Calafat 2021). Elsewhere, the Esteban study questionnaires were designed to provide information on potential exposures during the previous year, which may not be suitable for the time frame relevant to assessing vineyard treatment-related exposures. Questionnaires to identify potential pesticide exposures in the last few days, to characterize urine measurements,

and in the last few weeks, to characterize hair measurements, would be more suitable for a large-scale study to assess pesticide exposures in residents LCTV. Finally, our study was conducted on a relatively small number of participants (120) and used multi-residue methods (except for copper and AMPA) which generally implies higher LODs than with more specific methods. Thus, we observed that the LOD of TEB-OH in our study were too high to allow us to perform further analysis. These methodological limitations may explain why the pesticide / metabolite concentrations measured in urine and hair in our study were low and failed to show a significant difference between residents LCTV and controls. This findings suggest that a more sensitive strategy for identifying relevant biomarkers appears necessary to overcome limitations in future studies. For example, the use of biological samples collected specifically for a pilot study would be more appropriate than the posteriori use of biobank samples collected for a prior study designed for other purposes. Furthermore, the combination of specific, multi-residue and even suspect screening analyses should be considered to test the feasibility and relevance of less known biomarkers that can be measured in a large-scale epidemiological study (Chaker et al. 2020; Wang et al. 2021).

We remind the reader that the analyses performed in this study were conducted for exploratory purposes only in order to implement the tiered approach for identifying relevant biomarkers. Our results cannot be used to characterize pesticide exposure of residents LCTV or the conditions of this exposure related to vine growing in France. However, our findings do give us insights and perspectives for the design of a future large-scale study to assess pesticides exposures in residents LCTV in France. Measuring relevant biomarkers can only provide a conclusive assessment of pesticide exposure in residents LCTV - and the origin of this exposure- if they are part of a comprehensive biomonitoring design when measurements are performed at different periods of the year, in various regions covering different environmental conditions (meteorology, topography, soil nature), in a group of residents and controls, and where other sources of exposure to pesticides during the last few days or weeks (environmental and occupational) and factors influencing these exposures are taken into account (Dereumeaux et al. 2020; Teysseire et al. 2021). Analyses should be based primarily on specific targeted methods for the determination of biomarkers in urine with good sensitivity to maximize detection frequencies in the study population. Complementary, multi-residue analyses of these biomarkers, in hair for instance, should also be considered, although this approach still requires improvement (quantity of available hair, sample preparation, etc.) (Polledri et al. 2019).

Finally, the whole process required more than two years of work, a long time when one considers that the use of pesticides in agriculture evolves rapidly. That is why, the final list of relevant biomarkers should consider the potential changes in the use of pesticides in vineyards.

5. Conclusion

We developed a tiered approach to identify relevant biomarkers for assessing pesticide exposure in residents LCTV. This approach led us to identify three relevant biomarkers (two of folpet and one of tebuconazole) that were quantified in urine, tended to be higher in residents LCTV than in controls, and were not significantly influenced by occupational, dietary, or household sources of pesticide exposure. However, our results also showed that the identification of relevant biomarkers alone cannot effectively assess exposure and must be supported by appropriate measurement strategies and a suitable study design. The approach developed here was a prerequisite step for guiding a large-scale epidemiological study aiming at comprehensively measure pesticide exposure in residents LCTV with a view to evaluating health risks, and developing appropriate prevention strategies. Our approach may also inspire future research in other exposure contexts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2021.107013>.

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