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Wastewater-based epidemiology as a novel tool to evaluate human exposure to pesticides: Triazines and organophosphates as case studies

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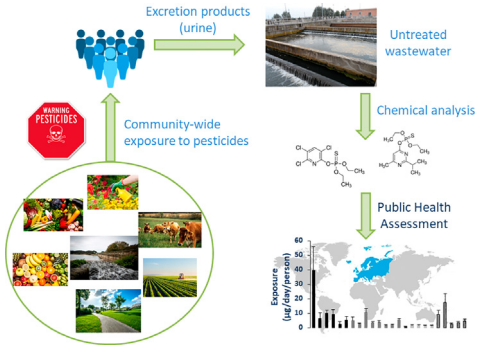
HIGHLIGHTS

- New biomarkers of triazines and organophosphates were investigated.
- TCPY and IMPY fulfilled the criteria of a WBE biomarker.
- The Urinary Factor was proposed.
- Spatial and temporal differences in human exposure were observed.
- Results were compared to the ADI to evaluate potential risks.

GRAPHICAL ABSTRACT



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ABSTRACT

Production and application of pesticides have risen remarkably in the last few decades. Even if they provide many benefits, they can be hazardous for humans and ecosystems when they are not used cautiously. Human exposure to pesticides is well documented, but new approaches are needed to boost the available information. This work proposes a new application of wastewater-based epidemiology (WBE) to assess the exposure of the general population to organophosphate and triazine pesticides (pyrethroid pesticides have already been validated). Several human urinary metabolites tested as WBE biomarkers, were suitable. Untreated wastewater samples from different European countries were analyzed by liquid chromatography-tandem mass spectrometry. Biomarker concentrations were converted to mass loads and used to back-calculate the local population's exposure to the parent pesticides, using specific correction factors developed in this study. Exposure to organophosphates and pyrethroids showed spatial and seasonal variations. Finally, pesticide exposure was estimated in twenty cities of ten European countries and compared with the acceptable daily intake, concluding that some populations might face health risks. The study confirms WBE as a suitable approach for assessing the average community exposure to pesticides and is a valuable complementary biomonitoring tool. WBE can provide valuable data for public health.

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1. Introduction

In recent decades the production and application of pesticides have risen remarkably, mainly as a result of enormous growth of food production (Evenson, 2003; Tilman et al., 2002). Food demand will rise further as the global population reaches nine billion people by 2050 (Godfray et al., 2010). Therefore, the production of pesticides is also expected to increase considerably, if no sustainable alternative practices are adopted (Tilman et al., 2002).

Pesticides offer many benefits for humans and the environment, but they pose a risk of poisoning when they are not used with care. Several positive social, economic and environmental outcomes from pesticide use can be obtained on community, national or global scales (Cooper and Dobson, 2007), but their unjustified or excessive use can be hazardous for humans and other living organisms (e.g. bees (Siviter et al., 2018)). Many studies report links among exposure to pesticides and adverse health effects, such as neurodegenerative disorders (Allen and Levy, 2013), respiratory diseases (Mamane et al., 2015), neuropsychological disorders (Stallones and Beseler, 2016), metabolic disorders and enhancement of adverse birth outcomes (Larsen et al., 2017).

Monitoring pesticide use is therefore important for public health, and different approaches are used to estimate human exposure. Firstly, the "external exposure" approach (ambient monitoring), which involves chemical analysis of environmental matrices such as house dust, indoor and outdoor air for the presence of agrochemicals and also the analysis of food and beverages intended for human consumption. Secondly, the "internal dose" approach (human biomonitoring, HBM), which involves measuring the levels of pesticides and/or their metabolites in human fluids (Angerer et al., 2007; Katsikantami et al., 2019).

HBM is currently the main tool for assessing exposure to pesticides in the general population. The preferred human biological sample is urine, since it can be easily collected under routine conditions, with no health risk for the individual (non-invasive technique). Urine is accessible in ample volumes, does not suffer exogenous contamination and pesticide concentrations are higher than in other fluids (e.g. blood) (Angerer et al., 2007; Wessels et al., 2003; Yusa et al., 2015). However, HBM studies present limitations related to high costs (sampling, chemical analysis and data processing), ethical issues, long times and the small number of collected samples compared to the whole population (Gracia-Lor et al., 2018). Other issues are related to sampling procedures and the different excretion profiles (throughout days) because of the short half-lives of many pesticides. Therefore, new methods are needed to track human pesticide exposure at the population level, overcoming some of these limitations.

Wastewater-based epidemiology (WBE) is a novel tool to assess human exposure to pesticides complementary to HBM and it can assess the exposure of whole population, combining the "external exposure" and "internal dose" approaches. WBE collects epidemiological information from wastewater through the analysis of human urinary metabolic products (biomarkers). It is described as a collective urine test, since the raw wastewater from a city pools the urine of all inhabitants. WBE is therefore a promising HBM tool to assess the patterns of exposure to a substance at population level (Gracia-Lor et al., 2018). There is already evidence that WBE can provide useful additional epidemiological information (Arnold, 2016; Gracia-Lor et al., 2018; Thomas and Reid, 2011; Zuccato et al., 2008).

The first WBE application to evaluate exposure to pesticides, reported in 2016 (Rousis et al., 2016), included triazines, organophosphates and pyrethroids. Later, three specific metabolites of pyrethroids were further investigated as WBE biomarkers for back-calculating the human intake of pyrethroids and were found suitable (Rousis et al., 2017a). This novel approach was then applied in a European study that included eight countries and gave results in line with national Eurostat statistics (Rousis et al., 2017b). Additional studies were done in the Caribbean island of Martinique, where human exposure to pyrethroids from indoor pesticide use was high (Devault et al., 2018) and in Norway, where 20% of the population was monitored in a nation-wide study (Rousis et al., 2020). The same approach was used to evaluate exposure to other substances such as phthalates (González-Mariño et al., 2021), phosphorus flame retardants (Been et al., 2018), mycotoxins (Gracia-Lor et al., 2020) and bisphenol A (Lopardo et al., 2019).

So far biomarkers for estimating human intake of pesticides have been fully validated only for pyrethroids (Rousis et al., 2017a). This work aimed to assay urinary metabolites from the triazine herbicides and organophosphate insecticides as suitable WBE biomarkers. Pesticide metabolites were tested to fulfill the requirements of a WBE biomarker (Gracia-Lor et al., 2016; Rousis et al., 2017a). The selected biomarkers were monitored in daily influent wastewater samples from four countries, Portugal, Spain, Italy and Switzerland. The new methodology was applied also to previous data to assess exposure to organophosphates and pyrethroids in twenty cities of ten European countries and results were compared with the acceptable daily intake (ADI).

2. Materials and methods

2.1. Wastewater sampling and study areas

Influent wastewater samples were collected from the inlets of wastewater treatment plants (WWTPs) in various European cities (Table S1): Castellon and Molina de Segura, Spain; Milan, Italy; Lugano, Switzerland and Porto, Portugal. Composite 24-h influent wastewater samples of a week were collected in March 2016 (Castellon, Milan and Lugano) and April 2016 (Molina de Segura and Porto). In Milan, sampling was also performed in five other periods (May 2014; February, March, June and September 2015), to assess any seasonal difference. Samples were frozen immediately after collection and were kept frozen until analysis.

2.2. Sample preparation and analysis

Wastewater samples were solid-phase extracted and analyzed by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). The analytical procedure and method validation are described in a previous publication (Rousis et al., 2016). Influent wastewater samples (50 mL) were kept at pH 7.0–7.5 and extracted by Oasis HLB 60 mg cartridges. The elution was done in methanol and dried samples were reconstituted in 100 μL of Milli-Q water (pre-concentration factor ×500).

The analysis of diethyl phosphate (DEP), diethyl thiophosphate (DETP), dimethyl phosphate (DMP) and dimethyl thiophosphate (DMTP) was done by adding deuterated standards to 180 μL of filtered wastewater and injecting 4 μL as described in a previous publication (Rousis et al., 2016).

Quantification was done using the isotopic dilution method with seven-point calibration curves prepared freshly before each analytical run. Instrumental and procedural blanks were included in each analytical batch to check for contamination. Stability experiments (Rousis et al., 2016, 2017a, 2017b) showed no noteworthy degradation for the selected metabolites under experimental conditions mimicking sampling and in-sewer conditions.

2.3. Pesticide exposure and risk assessment

Biomarker mass loads obtained from the concentrations and the daily flow rates of wastewater entering a WWTP were used to estimate the pesticide exposure of the population. New correction factors (CF) (Table 1) were developed as described for pyrethroids (Rousis et al., 2017a).

In some cases, a metabolite is an excretion product of more than one parent pesticides and therefore, the arithmetic mean of the molecular

Table 1
Selected metabolites to estimate population exposure to pesticides by wastewater analysis.

Metabolite (biomarker)	Parent pesticide	Molar mass ratio	Metabolite excretion (%)	Correction factor (CF)	Urinary factor (UF)
Triazines					
AM	Atrazine	0.63	17.7 (Buchholz et al., 1999)	3.56	1
Organophosphates					
TCPY	Chlorpyrifos, chlorpyrifos methyl	1.70	70.0 (Nolan et al., 1984)	2.43	0.20
IMPY	Diazinon	2.00	7.9 (Ross et al., 2008)	25.3	0.90
MDA	Malathion	1.20	8.7 (Bouchard et al., 2003)	13.8	0.90
MMA	Malathion	1.09	36.1 (Bouchard et al., 2003)	3.02	0.90
Pyrethroids (Rousis et al., 2017a)					
3-PBA	20 pyrethroids	1.98	28.5	6.95	1
trans-DCCA	*trans*-Permethrin, −cypermethrin, −cyfluthrin	1.98	53.9	3.67	1
cis-DCCA	*cis*-Permethrin, −cypermethrin, −cyfluthrin	1.98	36.3	5.45	1

Abbreviations: AM, atrazine mercapturate; TCPY, 3,5,6-trichloro-2-pyridinol; IMPY, 2-isopropyl-6-methyl-4-pyrimidinol; MDA, malathion dicarboxylic acid, MMA, malathion monocarboxylic acid; 3-PBA, 3-phenoxybenzoic acid; DCCA, 3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylic acid.

weights of all the parent pesticides was used for the CF calculation. In this work, the available human urinary pharmacokinetic studies were considered for the calculation of the excretion rate (Table 1). However, using the average values of molecular weights and excretion rates the potential variability of the CFs is not considered and the uncertainty due to these factors is lost. Therefore, for those pesticides whose above parameters are somewhat variable (RSD > 15%), a series of CFs were used to further evaluate the different exposure levels. This approach was applied for the pyrethroid metabolites.

Back-calculation of the population-wide intake of pesticides was done modifying the equation used by Rousis et al. (2017a), boosting the accuracy of the measured intakes by adding a "urinary factor", (UF). UF takes account of the percentage of a metabolite that comes from sources other than human metabolism and significantly affects the amounts measured in influent wastewater. Pesticide exposure was estimated according to the following equation:

$$PES_{intake} = \frac{(Conc. \times F) \times CF}{P} \times UF$$

where: Conc. is the concentration of each target analyte (ng/L) in untreated wastewater, F is the daily flow rate (m^3/day), CF is the specific correction factor, P is the population served by each WWTP and UF is the urinary factor.

UFs were calculated when sufficient data were available on sources other than human metabolism. For pesticides with no other exogenic sources, a UF equal to 1 was used. For pesticides with little available data and scarce evidence on sources other than human metabolism, or with low exogenic contamination, a UF equal to 0.9 was applied. It should be noticed that pesticides with little available data on other sources cannot be considered only as human-specific and thus a UF lower than 1 should be used. In addition, when the amounts in wastewater are highly affected by different sources other than human metabolism (e.g., metabolic mechanisms in foodstuff, presence of metabolites in tap water and household environment), a UF lower than 0.9 should be used. However, it would be necessary to evaluate whether these sources contribute to the measured amounts in untreated urban wastewater actually and to what extent. As a result, in the present study we applied three different values of UF according to the findings in the literature.

The estimated exposure to pesticides was compared with ADI, to evaluate the measured exposure levels in relation to their potential effects on human health. We used the lowest ADI for metabolites with more than one parent pesticide, as the "worst case" scenario and the highest ADI to obtain a range of exposure considering the corresponding uncertainty. The ADI was multiplied by the average man's weight (70 kg) and resulted in a threshold intake value (mg/person per day) that we called the WBE toxicological level (Table S2) (Rousis et al., 2017a).

2.4. Data analysis

The MultiQuant™ 2.1 software package of Analyst® (AB-Sciex, Thornhill, Ontario, Canada) was used for data analysis. GraphPad Prism was used for statistical analysis. The normality of the data was checked by the Shapiro-Wilk test. Then different tests at 95.0% confidence level were employed, such as the Kruskal-Wallis test followed by Dunn's test (not normal distribution) or the Mann–Whitney *U* test.

3. Results and discussion

3.1. Novel metabolites selected as WBE biomarkers

Pesticides are ubiquitous and are used in a vast field of applications such as agriculture, public health, maintenance of green areas and water reserves, livestock and domestic animals, industry and homes (Garcia et al., 2012). To select a specific WBE biomarker (in this case, a pesticide metabolite) it is therefore essential to investigate the existence of sources in influent wastewater besides human metabolism. Research should focus on metabolic mechanisms in plants, animals and foodstuff, transformation processes in the outdoor environment and metabolites in tap water and in the domestic environment (e.g., through analysis of dust). So far these requirements have been fulfilled for some pyrethroid metabolites, as reported in a previous publication (Rousis et al., 2017a). Several other metabolites, among triazines and organophosphates, were tested in this work.

3.1.1. Triazines

Triazine herbicides, such as atrazine, simazine, propazine and terbuthylazine have been widely used globally for decades, mainly in agriculture since they improve the yields of crops. However, symmetric triazines had adverse environmental effects due to their relatively high water-solubility, which meant they could leach from the soil to surface and ground waters. Humans are usually exposed to mixtures of chlorotriazines, so HBM studies include mainly non-compound-specific urinary metabolites such as urinary excretion products of bi-dealkylation, deisopropylation and deethylation reactions (Barr et al., 2007; Yusa et al., 2015).

This study investigated compound- and non-compound-specific triazine metabolites in order to test their suitability as WBE biomarkers. Atrazine was quantified in almost all the samples (Table S3) and had high frequencies of detection in other studies (Devault et al., 2018; Rousis et al., 2016, 2017b, 2020). Atrazine desisopropyl (DIA) and desethyl atrazine (DEA) were not detected in Spain (Castellon and Molina de Segura), but were found in many samples in the other cities

(Table S3). These compounds were not generally detected in previous WBE studies (Devault et al., 2018; Rousis et al., 2016, 2017b, 2020). The use of atrazine was banned in Europe in 2004 (European Commission, 2004) and its presence in water is related to the large volumes applied in the past and its persistence in soils and ability to leach with water (Barr et al., 2007; Tasca et al., 2018).

Atrazine was replaced in Europe by a structurally similar compound, terbuthylazine. Even though terbuthylazine has lower mobility due to its lower water solubility and higher hydrophobicity, it is found in many different water samples. Furthermore, terbuthylazine desethyl (DES), its metabolite and/or degradation product, is one of the most widely detected compounds in water systems (Tasca et al., 2018). DES was detected in all the samples of the present study (Table S3) and is reported at high frequency in other European cities, mainly in the south (Devault et al., 2018; Rousis et al., 2016, 2017b, 2020). It is well documented that chlorotriazine herbicides undergo several transformation processes in water systems to DES, DIA and DEA. Many studies have detected these compounds in different water systems (Bozzo et al., 2013; Fonseca et al., 2019; Rousis et al., 2017c; Tasca et al., 2018) and also in drinking water in Italy (Maggioni et al., 2013). Tap water was also analyzed in the present study and these compounds ranged 6–16 ng/L (sampling period: February and March 2015). These are omnipresent substances in the environment, so they are not reliable WBE biomarkers, since their presence in wastewater can be influenced by additional sources not related to human metabolism.

Atrazine mercapturate (AM) is the only specific human urinary metabolite of atrazine and can be used as a reliable indicator of occupational exposure. Determination of AM would be the only unequivocal indicator of exposure to atrazine (Barr et al., 2007; Mendaš et al., 2012). As regards using AM as a WBE biomarker, stability experiments show it is stable in influent wastewater (Rousis et al., 2017b; Rousis et al., 2016) and it was not found in tap water (Milan, Italy). However, AM was never detected in wastewater either in the present (Table S3) or previous studies (Devault et al., 2018; Rousis et al., 2020; Rousis et al., 2017b; Rousis et al., 2016). A CF was proposed (Table 1) considering an excretion factor of 17.7% (the excretion fraction was estimated using data published by Buchholz et al. (1999)).

3.1.2. Chlorpyrifos and chlorpyrifos-methyl

3,5,6-Trichloro-2-pyridinol (TCPY) is the specific human urinary metabolite of chlorpyrifos and chlorpyrifos-methyl and is used as a biomarker of exposure for the general population and other groups (e.g., exposed workers) in HBM studies (Fernández et al., 2020; Li et al., 2019; Roca et al., 2014). However, the levels of TCPY in urine and wastewater are greatly affected by its environmental contributions. The parent pesticides can be converted to TCPY in the environment and is detectable in different media such as soil, food, indoor air, outdoor air and dust (Morgan et al., 2011; Morgan et al., 2005; Peng et al., 2016; Wilson et al., 2003). Therefore, caution is needed when estimating human exposure to chlorpyrifos and chlorpyrifos-methyl from measurements of TCPY.

TCPY, investigated as a WBE biomarker, was detected in all the samples in the present study (Table S3) and in previous investigations (Devault et al., 2018; Rousis et al., 2020, Rousis et al., 2017b, Rousis et al., 2016), but not in tap water sampled in Milan. Furthermore, it was stable in influent wastewater (Rousis et al., 2017b; Rousis et al., 2016). A previous research study comparing results from HBM studies and wastewater found they were in agreement (Rousis et al., 2020). Considering that 80% of the urinary TCPY comes from environmental exposure to TCPY itself and the remaining 20% from exposure to the parent pesticides (Wilson et al., 2003), a UF of 0.2 was used. Human pharmacokinetics studies reported an 70.0% excretion for TCPY (Nolan et al., 1984) (Table 1).

3.1.3. Diazinon

Diazinon is an organophosphate pesticide widely used in the past in agriculture and indoors. Concern arose about both potential environmental damage and risks for human health, so some restrictions have been adopted (e.g., residential use in the USA). The implementation of specific programs and regulations in the USA led to an important reduction of its application (Wang et al., 2017).

Human exposure to diazinon is usually assessed in HBM studies by measuring its specific metabolite, 2-isopropyl-6-methyl-4-pyrimidinol (IMPY) (Li et al., 2019; Yusa et al., 2015). This metabolite is also reported as an environmental degradation product (Bavcon et al., 2003; Morgan et al., 2011), but not enough data are available on this so far. However, one study investigated the preschool children exposure to different pesticides and indicated the diet as the most important route of exposure (Wilson et al., 2010).

IMPY was detected in wastewater in the present study (Table S3) and in previous investigations (Devault et al., 2018; Rousis et al., 2020, Rousis et al., 2017b, Rousis et al., 2016). This metabolite was not detected in tap water in Milan. Moreover, no formation was observed after adding diazinon to influent wastewater in laboratory stability experiments (Rousis et al., 2017b). Consequently, IMPY is a good candidate WBE biomarker and a correction factor was proposed based on an excretion fraction of 7.9% (Ross et al., 2008) (Table 1).

3.1.4. Malathion

Malathion is an organophosphate insecticide widely used in agriculture, homes, and in public health pest control programs. The specific malathion metabolites, malathion monocarboxylic acid (MMA) and malathion dicarboxylic acid (MDA), are usually used in HBM studies to evaluate human exposure (Katsikantami et al., 2019; Li and Kannan, 2018; Li et al., 2019; Muñoz-Quezada et al., 2019). So far, WBE has investigated only the α and β isomers of MMA, and not MDA because of the poor performance of the analytical method for this compound (Rousis et al., 2016).

A few studies have investigated the formation of these two metabolites in environmental media and concluded that they can be produced in plants and in the environment (Chen et al., 2012; Radford et al., 2016). However, there is not enough data in the literature to support this hypothesis and/or to quantify the levels of the exogenous sources in urine. MMA was rarely found in wastewater (<9% of samples) (Devault et al., 2018; Rousis et al., 2020; Rousis et al., 2017b), except in Italy (Rousis et al., 2016) where it was more frequent (up to 27%). In-lab stability experiments concluded that MMA was stable in wastewater (Rousis et al., 2017b, Rousis et al., 2016). Even though malathion metabolites were not frequently detected in influent wastewater, two correction factors were proposed here (Table 1) considering the excretion factors published by Bouchard et al. (2003).

3.1.5. Dialkyl phosphates (DAPs)

Organophosphate pesticides have been investigated in numerous HBM studies as markers of exposure. The most common markers of cumulative human exposure to the organophosphates are the non-specific DAP metabolites: the dimethyl phosphates (DMP, DMTP, dimethyl dithiophosphate) and the diethyl phosphates (DEP, DETP, diethyl dithiophosphate) (Kavvalakis and Tsatsakis, 2012).

DAP metabolites can be produced by plants and as degradation products in the environment, leading to their presence in food and environmental media. Various research studies found DAPs at high concentrations (even higher than those of parent pesticides) in different media such as fruits, vegetables, fresh fruit juices and house dust (Chen et al., 2012; Krieger et al., 2012; Lu et al., 2005; Quirós-Alcalá et al., 2012; Sudakin and Stone, 2011; Weerasekera et al., 2009; Zhang et al., 2008). Thus, exogenous sources other than human metabolism can affect DAPs metabolite levels in wastewater, so they may be attributed to the parent pesticides and/or to the derivatives themselves, not related to human exposure. The reliability of these metabolites as WBE biomarkers is therefore limited and can result into overestimation of human exposure and inaccurate risk assessment.

Wastewater studies reported DEP and DMP very frequently (present study and (Devault et al., 2018; Rousis et al., 2020, Rousis et al., 2017b, Rousis et al., 2016)) at relatively high concentrations (Table S4). The levels of the same metabolites in human urine ranged from 0.7 to 14 μg/L (Haines et al., 2017; Katsikantami et al., 2019; Ock et al., 2020; Yusa et al., 2015), so taking into account the dilution factor in wastewater (100–400), the concentrations in wastewater cannot be assigned only to human metabolism. The comparison of HBM and WBE results in a recent study noted that DAP levels in wastewater were significantly affected by other sources (Rousis et al., 2020). Consequently, these metabolites should not be used as WBE biomarkers.

3.2. Mass loads of biomarkers in wastewater

Mean mass loads of triazines, pyrethroids and organophosphates are shown in Table 2. The lowest loads were for triazines and ranged from 0.26 to 4.8 mg/day/1000 inhabitants, similar to those reported in other studies (Devault et al., 2018; Rousis et al., 2020; Rousis et al., 2017b). The sum of the mean mass loads of triazines were 9.7 and 1.8 mg/day/1000 inhabitants respectively in Milan and Castellon (sampling period March 2016) and were lower than those reported 1 year earlier in the same cities (14 and 6.4 mg/day/1000 inhabitants in Milan and Castellon) (Rousis et al., 2017b). The sum of triazine mean mass loads was highest in Switzerland, Lugano (this study) and Zurich (Rousis et al., 2017b), where contamination of water bodies with atrazine and terbuthylazine was also documented (Moschet et al., 2014). AM was not found in wastewater in Europe and this may be due to the ban of atrazine use. However, atrazine ranked second after glyphosate as the most used pesticide active ingredient in the USA (Environmental Protection Agency, 2017) and is one of the most widely used herbicides in Australia (Australian Pesticides and Veterinary Medicines Authority, 2017). Therefore, analysis of influent wastewater in the USA and Australia could give interesting information on human exposure and may prove the suitability of AM as WBE biomarker.

The mean mass loads of pyrethroids (Table 2) were 4.1–42 mg/day/1000 inhabitants for 3-PBA, 13–88 mg/day/1000 inhabitants for *trans*-DCCA and 3.9–40 mg/day/1000 inhabitants for *cis*-DCCA. These results were lower in Milan than those found 1 year earlier (Rousis et al., 2017b), while DCCA was slightly higher in Castellon. Mass loads were higher in Milan in May 2014 (Rousis et al., 2017a), indicating a potential seasonal change. Mass loads in Spain were always higher than in the other countries. Both metabolites are excretion products of various parent compounds and although some of these pesticides are not approved for use in Europe, it is still difficult to identify the exact compound responsible for the measured levels in wastewater. Several banned pesticides are still used and others are reported in several studies (e.g., water and food analysis) and some of them present isomeric forms; therefore, it is difficult to assign the exact compound.

The mean mass loads of the specific organophosphate metabolite TCPY ranged from 5.5 to 36 mg/day/1000 inhabitants (Table 2). The levels in Castellon remained stable in the two campaigns and no large changes were seen in Italy and Switzerland (present study and (Rousis et al., 2017b)).

The authorization for using chlorpyrifos and chlorpyrifos-methyl in Europe was not renewed in February 2020 (European Commission, 2019a, 2019b). Therefore, WBE could be useful to evaluate the effectiveness of this regulatory action in Europe. Furthermore, chlorpyrifos was the most used insecticide in the USA (Environmental Protection Agency, 2017), so TCPY measurements in wastewater in the USA could give valuable information on human exposure to chlorpyrifos and chlorpyrifos-methyl.

The mean mass loads of the specific diazinon metabolite, IMPY, were 0.6–31 mg/day/1000 inhabitants (Table 2). Mass loads were highest in Molina de Segura, and 15 times those in Castellon. Spatial differences within a country were also observed in Norway (Rousis et al., 2020).

The highest mass loads were for the dialkyl phosphates, DEP and DMP (Table 2), as in previous studies (Devault et al., 2018; Rousis et al., 2020, Rousis et al., 2017b). This is likely due to the fact they are metabolic/transformation products of many insecticides and other chemicals and can also be produced from industrial chemicals (Reemtsma et al., 2011).

3.3. Seasonal changes of pesticide exposure

A sampling campaign in Milan (2014–2015) investigated seasonal variations in human exposure to pesticides over 5 months in the four seasons. TCPY was found in all the samples (Fig. 1). The TCPY mass loads were lowest in March and were significantly different from May ($p = 0.0198$), June ($p = 0.0363$) and September ($p = 0.005$). The second lowest mass loads were in February and were significantly different from September ($p = 0.0048$). HBM and ambient monitoring studies found no significant differences between seasons during a year of human exposure to chlorpyrifos, with slightly higher exposures from April to September (Bakke et al., 2009; Smith et al., 2017; Wilson et al., 2010).

IMPY was found in all the samples (7/7) in June, in 4/7 in May, but never in September, February and March. The mass loads of IMPY were significantly different ($p = 0.0378$) in June (2.6 ± 1.2 mg/day/1000 inhabitants) and May (1.6 ± 1.4 mg/day/1000 inhabitants).

Table 2
Normalized mass loads (mg/day/1000 inhabitants) of triazines, pyrethroids and organophosphates in influent wastewater (mean ± standard deviation of seven 24-h composite samples).

Compound	Castellon	Molina de Segura	Milan	Lugano	Porto
Triazines					
ATZ	0.26 ± 0.07	0.26 ± 0.13	2.61 ± 0.18	3.57 ± 0.50	4.8 ± 1.4
DES	1.55 ± 0.43	0.56 ± 0.30	4.16 ± 0.58	3.76 ± 0.16	1.10 ± 0.17
DIA	Not detected	Not detected	1.10 ± 0.26	1.86 ± 0.30	0.56 ± 0.16
DEA	Not detected	Not detected	1.87 ± 0.28	2.28 ± 0.30	1.54 ± 0.43
Pyrethroids					
3-PBA	19.5 ± 3.6	42.1 ± 15.7	5.38 ± 0.36	4.07 ± 0.36	11.8 ± 5.5
trans-DCCA	58.9 ± 17.2	88.4 ± 25.8	12.7 ± 1.7	13.4 ± 2.2	51.2 ± 29.3
cis-DCCA	15.2 ± 3.9	39.7 ± 11.3	3.9 ± 1.4	not detected	12.1 ± 5.3
Organophosphates					
TCPY	19.1 ± 5.6	36.2 ± 12.3	5.52 ± 0.91	7.2 ± 1.4	9.7 ± 2.4
IMPY	2.02 ± 0.99	30.9 ± 25.2	0.57 ± 0.11	0.83 ± 0.21	1.34 ± 0.66
Dialkyl phosphates					
DEP	not analyzed	not analyzed	83 ± 18	133 ± 38	63 ± 13
DMP	not analyzed	not analyzed	79 ± 48	186 ± 149	69 ± 24

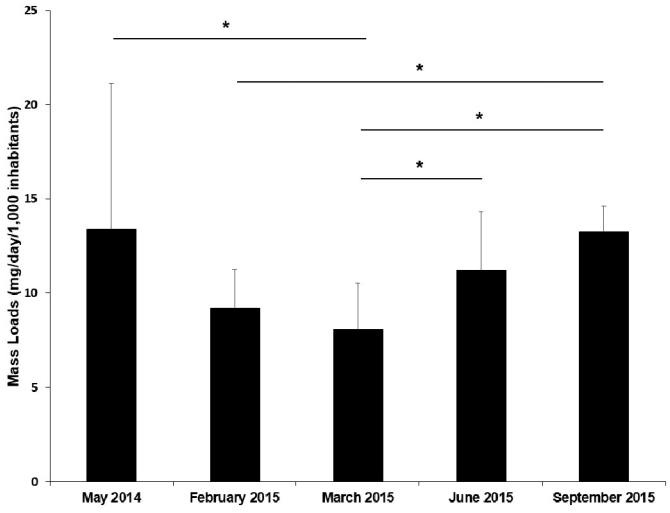

Fig. 1. Seasonal patterns of the TCPY mass loads in wastewater in Milan, Italy (*$p < 0.05$).

Analysis of IMPY in HBM studies and other environmental media highlighted seasonal differences, with higher values in autumn (Smith et al., 2017; Wilson et al., 2010). This WBE study found IMPY in wastewater only in spring-summer but included only one city. Furthermore, the observed differences could be explained by agricultural and non-agricultural uses in dormant and non-dormant periods of the year (Wang et al., 2017). The period of application is diverse throughout the year for diazinon (Smith et al., 2017) and thus more samples from each season should be analyzed. Larger studies in different areas will also help improve information for comparison with HBM data.

3.4. Back-calculation of pesticide exposure

The mass loads of the selected metabolites 3-PBA, DCCA, TCPY and IMPY were used to back-calculate human exposure to the parent pesticides. The exposure to pyrethroids was estimated from 3-PBA using the proposed CF (Table 1). The highest values were in Spain in Molina de Segura (0.29 ± 0.11 mg/day/person) where they were double those in Castellon (0.14 ± 0.03 mg/day/person) (Fig. 2). Differences among cities in the same country were also observed in Italy and Norway (Rousis et al., 2020; Rousis et al., 2017a). Pyrethroid exposure in Lugano was the same as in Zurich sampled 1 year earlier. However, in Milan the estimated exposure (0.037 ± 0.002 mg/day/person) was lower than the exposure calculated in May 2014 (0.16 mg/day/person (Rousis et al., 2017a)) and March 2015 (0.075 mg/day/person (Rousis et al., 2017b)). Exposure, in the present study, was also evaluated using different CFs considering the variability in molecular weights (molar mass ratio 1.98 ± 0.43) and excretion rates. Three CFs were finally applied (19.1, 6.95 and 3.39) and the results are found in Table S5. These CFs are slightly different from those applied in a previous study, since the authors had considered only the variation of excretion rates (Rousis et al., 2017a).

The exposure to permethrin, cypermethrin and cyfluthrin was calculated using the sum of the mass loads of *trans*- and *cis*-DCCA and their CFs (Table 1). Exposure was highest in Spain (Molina de Segura, 0.54 ± 15 mg/day/person; Castellon, 0.30 ± 0.08 mg/day/person) and Portugal (Porto, 0.25 ± 0.13 mg/day/person) (Fig. 3). The exposure in Lugano (0.07 ± 0.01 mg/day/person) was higher than in Zurich (0.05 ± 0.02 mg/day/person (Rousis et al., 2017b)), contrary to the results for 3-PBA. In Milan the estimated exposure (0.07 ± 0.01 mg/day/person) was lower than in May 2014 (0.12 mg/day/person (Rousis et al., 2017a)) and March 2015 (0.13 mg/day/person (Rousis et al., 2017b)). Finally, low and high CFs were used to estimate intake

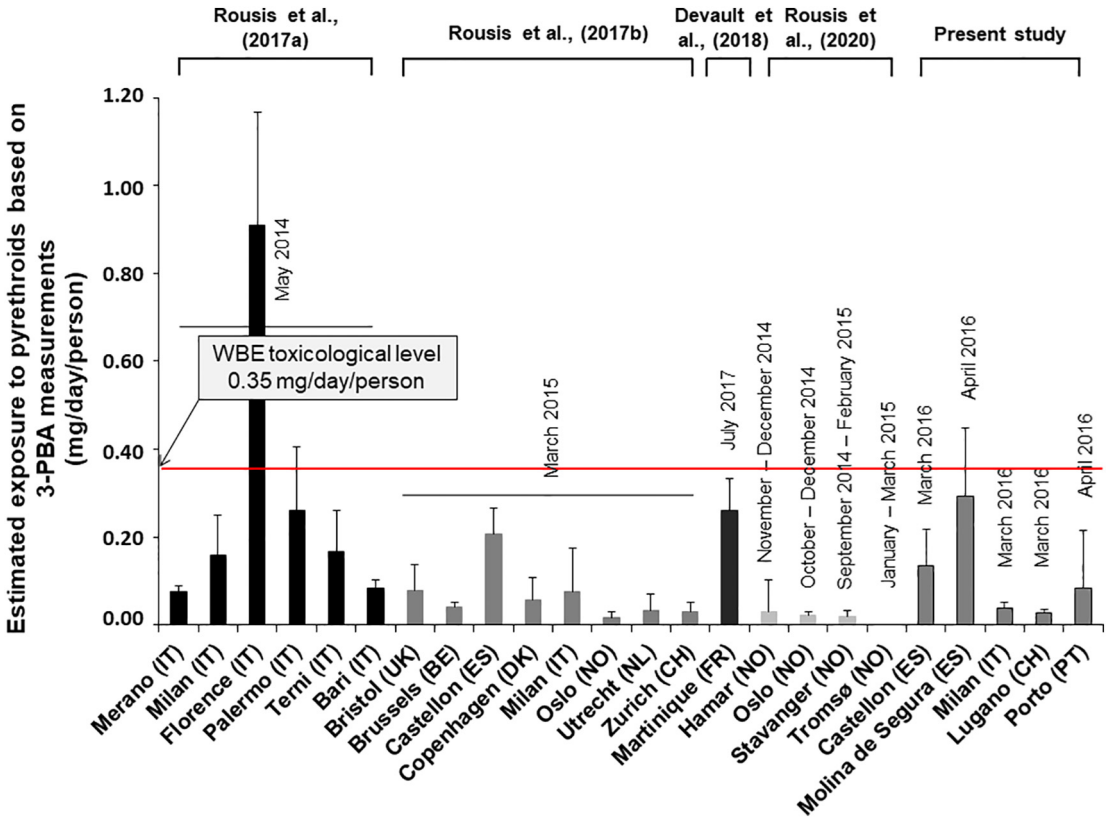

Fig. 2. Pyrethroid exposure (mg/day/person) back-calculated from 3-PBA in influent wastewater and comparison with the WBE toxicological level. The correction factor equal to 6.95 and the ADI of fluvalinate were used.

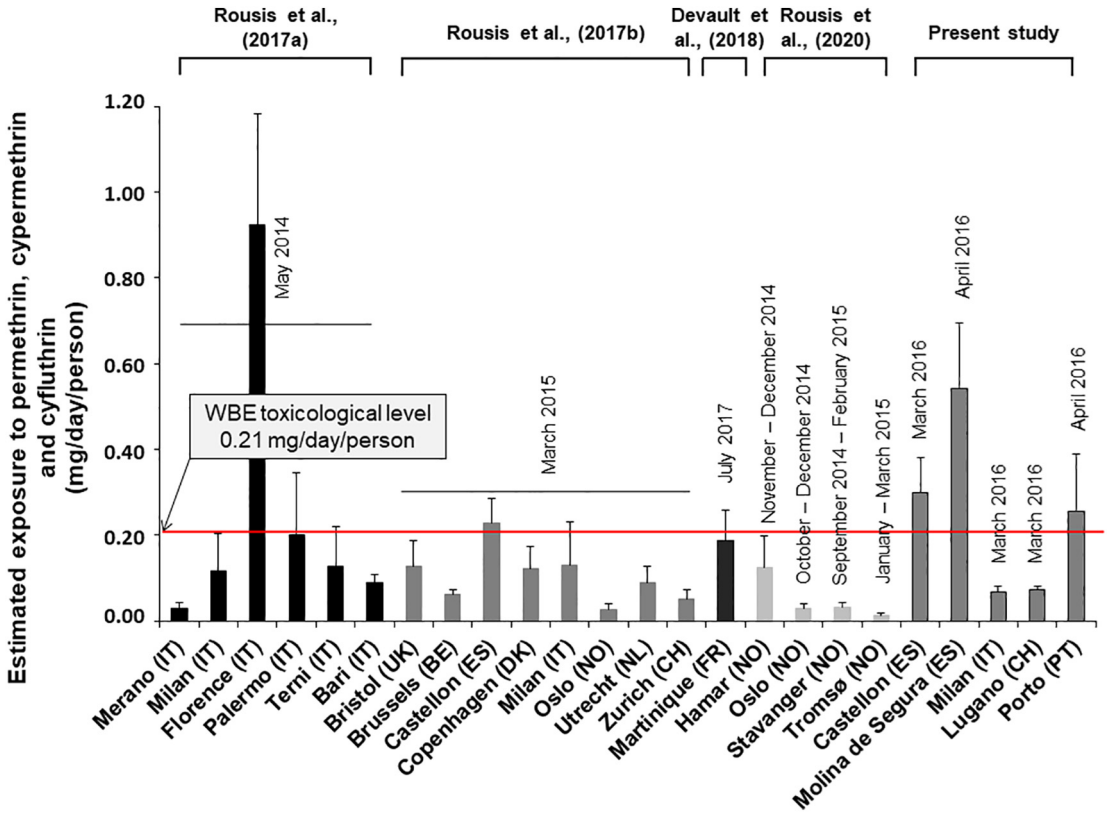

Fig. 3. Permethrin, cypermethrin and cyfluthrin exposure (mg/day/person) back-calculated from DCCA in influent wastewater and comparison with the WBE toxicological level. The correction factors 3.67 and 5.45 and the ADI of cyfluthrin were used.

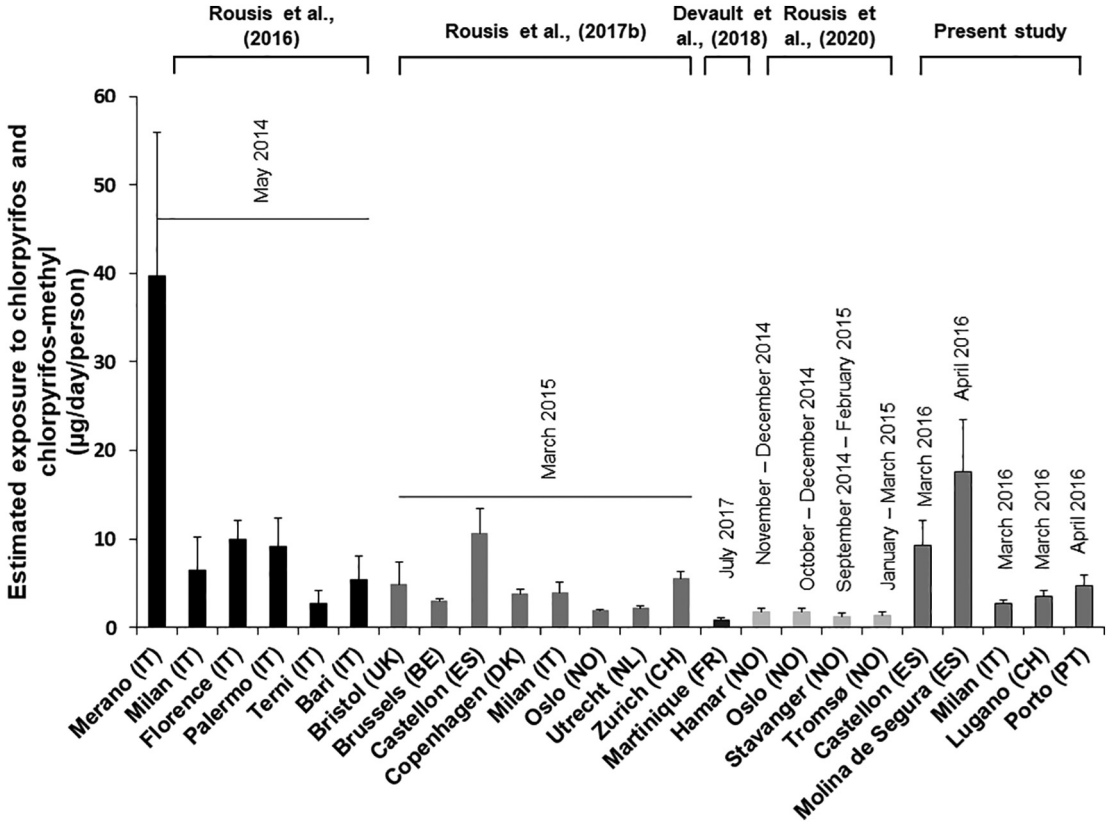

Fig. 4. Chlorpyrifos and chlorpyrifos-methyl exposure (µg/day/person) back-calculated from TCPY in influent wastewater. The data were obtained from the literature for the estimation of exposure. A WBE toxicological level of 0.7 mg/day/person was considered.

variability, taking into account different excretion rates (Rousis et al., 2017a). The different molecular weights were not considered for the calculation of CFs, as the variability was very low (RSD = 5%). The results are presented in Table S6.

The exposure to chlorpyrifos and chlorpyrifos-methyl was estimated for the first time using their specific metabolite, TCPY, and the proposed CF (Table 1). Except in a few cases, the estimated exposure was lower than 10 μg/day/person (Fig. 4). The highest exposures were found in Spain, Molina de Segura, 17.6 ± 6.0 μg/day/person and Castellon, 9.3 ± 2.7 μg/day/person (Fig. 4). The results in Castellon were similar in both the campaigns (present study and Rousis et al., 2017b). In Italy, the highest exposure was in Merano, an area famous for growing apples (Rousis et al., 2016), while the lowest exposure was in Norway (Rousis et al., 2020) (Fig. 4).

The exposure to diazinon was estimated from the mass loads of its main metabolite IMPY (Table 1). The highest exposure was found in Molina de Segura (Spain) followed by Hamar (Norway), Florence (Italy) and Bristol (United Kingdom) and exposures ranged 0.3–0.7 mg/day/person. However, the majority of cities had values lower than 0.1 mg/day/person (Fig. 5). In Milan, IMPY was detected only in one sample in March 2016, while it was not detected in March 2015 (Rousis et al., 2017b), and was detected in four out of seven samples in May 2014 (Rousis et al., 2016). These results may reflect seasonal differences in diazinon use, and wider monitoring campaigns would be useful to investigate the human exposure profile to this substance better.

3.5. Potential risks for human health

The ADIs of the parent pesticides were used to evaluate the potential risk for human health. WBE intakes were compared with the WBE toxicological levels (Table S2). Intake of 3-PBA was estimated from the lowest and highest ADI of all potential parent compounds. A high exposure level was found only in Florence (Italy) when it was compared to the lowest ADI (Fig. 2). When the highest ADI was used, all calculated exposures were below this value, even when using the high CFs. Furthermore, when the high CFs were applied, in addition to Florence, the cities of Milan and Terni (Italy; sampling campaign 2014), Castellon (Spain; sampling campaign 2015), Martinique (France) and Castellon and Molina de Segura (Spain; sampling campaign 2016) exceeded the lowest WBE toxicological level. In the case of DCCA the ADI of cyfluthrin was used as a worst case scenario. Intakes in Castellon and Molina de Segura (Spain), Florence (Italy) and Porto (Portugal) exceeded the WBE toxicological level, suggesting a potential risk for the local population (Fig. 3). Exposure in Castellon also exceeded this level in a study one year earlier (Rousis et al., 2017b). However, when compared to the highest ADI, all cities were below this value, even when the high CFs were applied for back-calculations. In addition, using the high CFs, two more cities exceeded the lowest WBE toxicological level (0.21 mg/person per day), namely Palermo (Italy) and Martinique (France). The exposure to chlorpyrifos and chlorpyrifos-methyl was lower than the WBE toxicological level (0.7 mg/day/person) in all the cases, indicating no need for the general population (Fig. 4). The human intake of diazinon estimated by WBE was higher than the WBE toxicological level in Molina de Segura, Florence, Bristol, Zurich and Hamar (Fig. 5), suggesting potential risks for the population in these cities.

4. Conclusions

This study shows that measurements of human urinary metabolites of herbicides and insecticides in untreated wastewater of urban areas may be useful to assess the population exposure to specific pesticides. This study tested several new pesticide metabolites as WBE biomarkers and the organophosphates TCPY and IMPY fulfilled the criteria. These new biomarkers were used to assess exposure in different communities. This WBE application indicates for the first time spatial and temporal

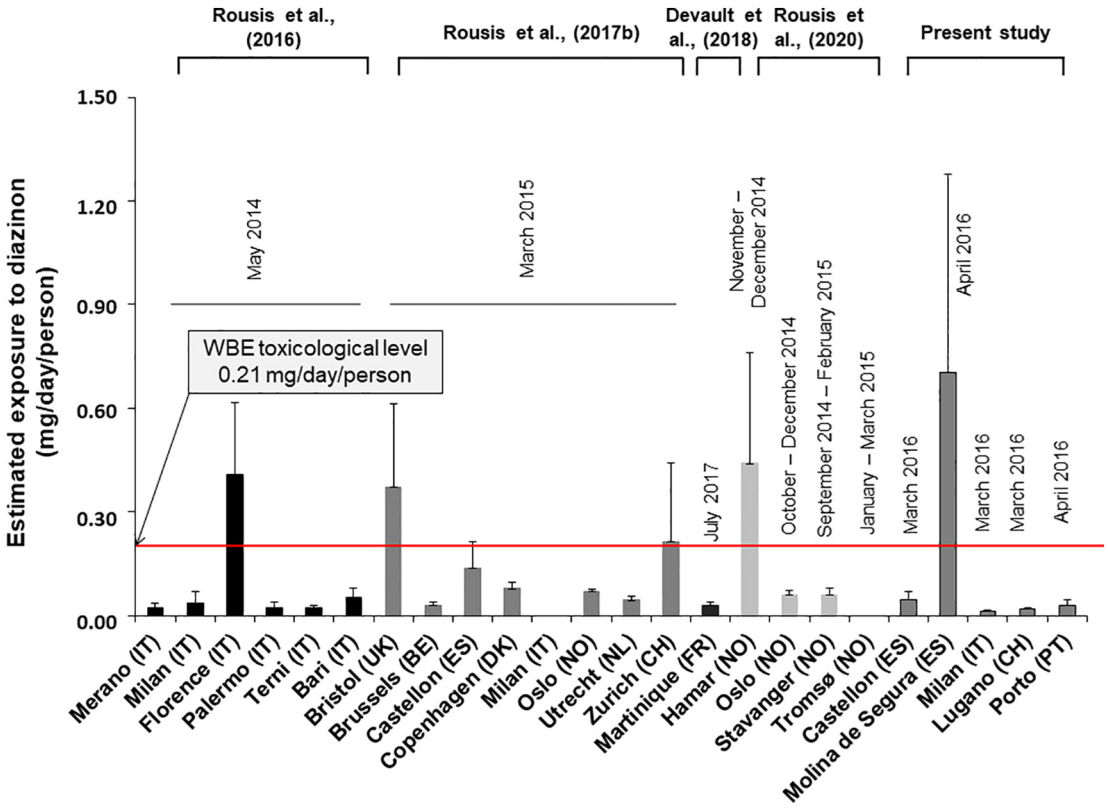

Fig. 5. Diazinon exposure (mg/day/person) back-calculated from IMPY in influent wastewater and comparison with the WBE toxicological level. The data were obtained from the literature for the estimation of exposure.

differences in human exposure to organophosphates and extends information on exposure to pyrethroids. The estimated intakes were compared with the ADI and potential risks for human health came to light in some communities. We propose for the first time the use of a complementary factor, namely "urinary factor, UF", for a more accurate assessment of human exposure to pesticides. The application of UF was considered to improve the exposure back-calculations using the WBE approach, given that some pesticide metabolites are derived from sources other than human metabolism. However, the use of UF is subjected to some limitations, as a function of previous data available and thus further research is needed to establish a robust methodology for its calculation.

This study confirms that WBE can serve as a complementary biomonitoring tool to make available valuable data for public health, that may be useful for policy-making and national and international agencies.

CRediT authorship contribution statement

Nikolaos I. Rousis: Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Emma Gracia-Lor:** Formal analysis, Investigation, Resources, Writing – review & editing. **Félix Hernández:** Resources, Writing – review & editing. **Francesco Poretti:** Resources, Writing – review & editing. **Miguel M. Santos:** Resources, Writing – review & editing. **Ettore Zuccato:** Conceptualization, Writing – review & editing, Funding acquisition. **Sara Castiglioni:** Conceptualization, Writing – review & editing, Funding acquisition.

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.scitotenv.2021.148618.

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