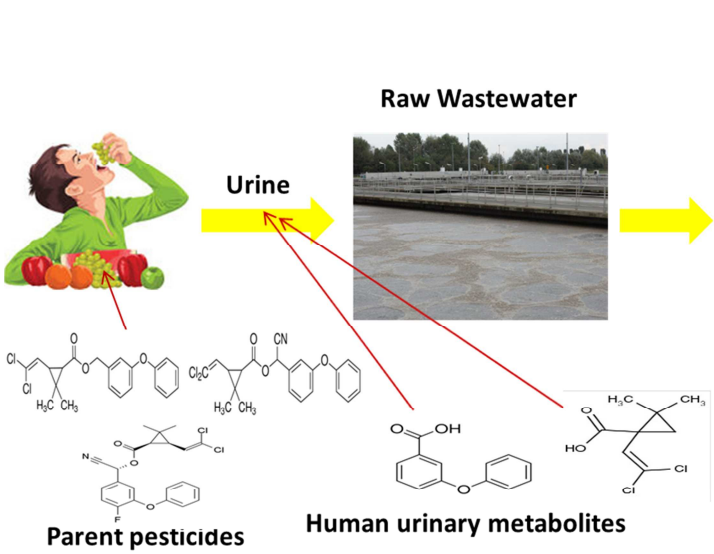

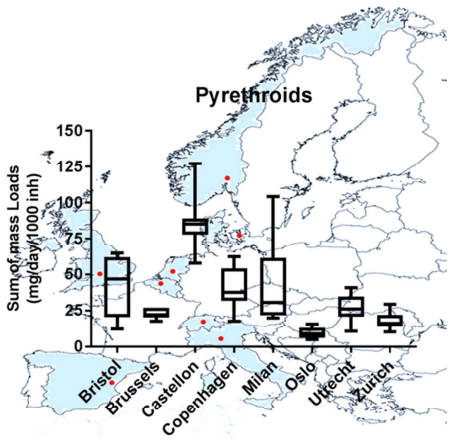

Wastewater-based epidemiology to assess pan-European pesticide exposure

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Abstract

Human biomonitoring, i.e. the determination of chemicals and/or their metabolites in human specimens, is the most common and potent tool for assessing human exposure to pesticides, but it suffers from limitations such as high costs and biases in sampling. Wastewater-based epidemiology (WBE) is an innovative approach based on the chemical analysis of specific human metabolic excretion products (biomarkers) in wastewater, and provides objective and real-time information on xenobiotics directly or indirectly ingested by a population. This study applied the WBE approach for the first time to evaluate human exposure to pesticides in eight cities across Europe. 24h-composite wastewater samples were collected from the main wastewater treatment plants and analyzed for urinary metabolites of three classes of pesticides, namely triazines, organophosphates and pyrethroids, by liquid chromatography-tandem mass spectrometry. The mass loads (mg/day/1000 inhabitants) were highest for organophosphates and lowest for triazines. Different patterns were observed among the cities and for the various classes of pesticides. Population weighted loads of specific biomarkers indicated higher exposure in Castellon, Milan, Copenhagen and Bristol for pyrethroids, and in Castellon, Bristol and Zurich for organophosphates. The lowest mass loads (mg/day/1000 inhabitants) were found in Utrecht and Oslo. These results were in agreement with several national statistics related to pesticides exposure such as pesticides sales. The daily intake of pyrethroids was estimated in each city and it was found to exceed the acceptable daily intake (ADI) only in one city (Castellon, Spain). This was the first large-scale application of WBE to monitor population exposure to pesticides. The results indicated that WBE can give new information about the "average exposure" of the population to pesticides, and is a useful complementary biomonitoring tool to study population-wide exposure to pesticides.

Keywords: Urban wastewater; Mass spectrometry; Pesticides; Human urinary metabolites; Biomonitoring; Human intake

1 Introduction

Pesticides play an important role in agriculture by protecting plants and plant products against harmful organisms and their action, and helping boost the growth of crops. Meeting the demand in food supply will be one of the great challenges in the near future, since the global population is expected to grow to nine billion by the middle of the century (Godfray et al., 2010). In order to raise food production, an increased pesticides use is expected. Taking into account that thousands of tons of pesticides are yearly applied in agriculture, homes, gardens, sports fields, and public areas (Grube et al., 2011), contamination of the environment most likely will further increase and human exposure to pesticides will continue being a matter of substantial concern in the near future.

Many "old and harmful" pesticides, such as p,p-dichlorodiphenyl-trichloroethane (p,p'-DDT), have been banned because of their toxicity and they were replaced by less-persistent pesticides, such as organophosphates and pyrethroids (Barr, 2008; López et al., 2005). Pesticides provide mankind with many benefits, but at the same time have the potential to pose risks for human health due to widespread use and high biological activity (Cooper and Dobson, 2007). For instance, pesticides exposure has positive association with the development of idiopathic Parkinson's disease, neurobehavioral and neuropsychological disorders, respiratory symptoms or diseases, and sperm DNA damage (Allen and Levy, 2013; Mamane et al., 2015; Saillenfait et al., 2015; Stallones and Beseler, 2016). However, in the last two decades, the concept of "green chemistry" has been promoted and the agrochemical industry has focused on less toxic substances (Garrison, 2004).

The general population is exposed to pesticides mainly through diet and household use (Aprea, 2012). Human biomonitoring (HBM) is the main tool for assessing exposure and consists in

the measurement of chemicals and/or their metabolites in body fluids or tissues (Barr, 2008; Yusa et al., 2015). The reliability of HBM depends on the selection of a proper biomarker that reflects the exposure to the parent compound, and is specific and detectable in the investigated matrices. Urine is the preferred human biological matrix, since it is easy to collect and non-invasive and it is also accessible in large volumes allowing the determination of very low concentrations of chemicals compared to other fluids (Wessels et al., 2003). Extensive HBM studies have analyzed the urine of thousands of individuals to investigate pesticide exposure in the general population (Barr et al., 2010, 2004; Heudorf and Angerer, 2001; McKelvey et al., 2013; Ye et al., 2015). Despite their power to evaluate exposure to chemicals, HBM studies suffer by limitations such as high costs for sample collection and analysis, ethical issues and data analysis to extrapolate individual results to the whole population. Moreover, urine sampling can reflect only a momentary snapshot of exposure due to sampling procedures (i.e. morning urine collection), and excretion profiles may vary throughout the day/days because of the short half-lives in the human body of most of pesticides.

Wastewater-based epidemiology (WBE) is a recent approach for the retrieval of epidemiological information from wastewater through the analysis of specific human metabolic excretion products (biomarkers) (Castiglioni et al., 2014). It can be described as a collective urine test, as the wastewater from a city pools the anonymous urine samples of thousands of individuals. WBE was originally developed in Italy to estimate illicit drug consumption in a population (Zuccato et al., 2008) and has later been applied worldwide with promising results (Banta-Green et al., 2009; Ort et al., 2014). New possibilities permit information on public health and lifestyles (Thomas and Reid, 2011; Venkatesan and Halden 2014). The main advantage of WBE is to provide objective, real-time information on substances directly or indirectly ingested daily by a population, with a clear potential to provide complementary data for epidemiological studies and to overcome some of the HBM limitations.

The first exploratory study proposing WBE as a novel biomonitoring tool to evaluate the exposure of the general population to pesticides was recently performed (Rousis et al., 2016). Several metabolites of organophosphates, triazines and pyrethroids were detected in raw wastewater and their frequency of detection and abundance were in agreement with the profiles reported in urine of HBM studies (Rousis et al., 2016). Later three human urinary metabolites of pyrethroids were selected and used to back-calculate the population intake of pyrethroids in Italy (Rousis et al., 2017). This study indicated for the first time that WBE can be employed as a complementary biomonitoring tool to the HBM studies, but more data and a wider scale of investigation were necessary in order to confirm these preliminary results.

The aim of the present study was to apply for the first time this new WBE approach in eight countries across Europe and to evaluate the pan-European human exposure to pesticides in order to validate the method by comparing results with international statistics. 24-h composite raw wastewater samples were collected and analyzed for organophosphate, triazine and pyrethroid metabolites. The results for the cities were compared and population-wide pyrethroid intake was estimated. To the best of our knowledge, this is the first WBE study designed to assess human exposure to pesticides at a European scale.

2 Materials and methods

2.1 Chemicals and reagents

Hydrochloric acid (HCl, 37%) and acetonitrile for liquid chromatography-mass spectrometry (LC-MS) were purchased from Riedel de Haen (Seelze, Germany); methanol (MeOH) for pesticide analysis from Carlo Erba Reagents (Italy); triethylamine and acetic acid from Fluka (Buchs, Switzerland). HPLC grade Milli-Q water was obtained with a Milli-RO Plus 90 apparatus (Millipore, Molsheim, France). Analytical standards for diethyl phosphate (DEP, purity 97.6%),

chlorpyrifos (CPF, purity 99.9%), chlorpyrifos methyl (CPF-MET, purity 99.5%) and 3,5,6-trichloro-2-pyridinol (TCPY, purity 99.5%) were purchased from Chemical Research 2000 (Rome, Italy). Atrazine (ATZ, purity 97.5%), atrazine desethyl (DEA, purity 99.9%), terbutylazine desethyl (DES, purity 97.4%), atrazine desisopropyl (DIA, purity 95.4%), dimethyl chlorophosphate (DMCIP, purity 96%), dimethyl chlorothiophosphate (DMCITP, purity 97%), and O,O-diethyl thiophosphate (DETP, purity 98%) potassium salt were supplied by Sigma-Aldrich (Schnelldorf, Germany). Atrazine mercapturate (AM, purity 95.0%), 3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane)carboxylic acid (DCCA, purity 99.0%), 3-phenoxybenzoic acid (3-PBA, purity 99.0%), 2-isopropyl-6-methyl-4-pyrimidinol (IMPY, purity 99.5%), cis-3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylic acid (cis-DCCA, purity 98%) and malathion monocarboxylic acid (MMA, purity 97.0%) were purchased from Lab Service Analytica (Bologna, Italy). Isotopically labeled compounds (deuterated or ^{13}C-enriched) were used as internal standards (IS). 3-Phenoxybenzoic acid-C_6 (3-PBA-$^{13}C_6$, phenoxy-$^{13}C_6$, 99%; purity 98%) and 3,5,6-trichloro-2-pyridinol-C_3 (TCPY-C_3, 4,5,6-$^{13}C_3$, 99%; purity 97%) were obtained from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA); atrazine-D_5 (ATZ-D_5, 99.5%) from Sigma-Aldrich (Schnelldorf, Germany); and chlorpyrifos D_{10} (CPF-D_{10}, 97.0%) from Lab Service Analytica (Bologna, Italy). Dimethyl phosphate (DMP) and dimethyl thiophosphate (DMTP) were synthesized by simple hydrolysis of DMCIP and DMCITP (Hernández et al., 2002; Rousis et al., 2016a).

2.2 Selection of exposure biomarkers

Specific urinary metabolites of pesticides were selected as biomarkers from HBM studies available in literature and official reports of the United States Environmental Protection Agency and the Centers for Disease Control and Prevention, as described elsewhere (Rousis et al., 2016). The biomarkers were chosen according to specific criteria: a) levels in urine; b) frequency of detection;

c) frequency of use of the respective classes of pesticides; d) risks for human health; e) specificity of the metabolites (human excretion *versus* environmental formation).

The selected biomarkers were three parent substances and 15 urinary metabolic products belonging to different pesticide classes. Among triazines, the parent atrazine and the metabolites DES, DIA, DEA and AM were selected. Among pyrethroids 3-PBA, the common metabolite of about 20 synthetic pyrethroids, and *cis*- and *trans*-DCCA, which are the specific metabolites of permethrin, cypermethrin and cyfluthrin were chosen. Among organophosphates, the four alkyl phosphates DEP, DETP, DMP and DMTP, which are common metabolites of a large group of organophosphates, chlorpyrifos, chlorpyrifos methyl and their specific metabolite TCPY, the metabolites of malathion (the α and β isomers of MMA) and the metabolite of diazinon (IMPY) were selected.

The reliability of back-calculation of the exposure to parent chemicals (pesticides) depends strictly on the selection of an appropriate WBE biomarker, which can be either the compound itself or one of its metabolites. Therefore, the selected metabolites were checked to fulfill the requirements of a WBE biomarker, which are: a) measurable in raw wastewater; b) released into sewers only as a result of human excretion; c) a well-defined excretion profile to avoid interference from other exogenous or endogenous sources; d) limited adsorption to suspended matter; e) stable in wastewater during in-sewer transit, sampling and storage (Gracia-Lor et al., 2016). The stability of each compound in wastewater was evaluated through specific laboratory tests (Rousis et al., 2016), and the specificity of each metabolite was assessed by checking the presence of sources other than human metabolism (i.e. any potential environmental transformation) (Rousis et al., 2017 and this study). The results for the selected substances are summarized in Table 1.

2.3 Samples and sampling method

Raw wastewater samples were taken from the inlet of the wastewater treatment plants (WWTPs) of eight European cities: Bristol, the United Kingdom; Brussels, Belgium; Castellon, Spain; Copenhagen, Denmark; Milan, Italy; Oslo, Norway; Utrecht, The Netherlands and Zurich, Switzerland (Figure 1).

Composite 24-h samples of untreated wastewater were collected by automatic sampling devices (Table S1). Sampling was carried out over one week in March 2015. For each WWTP, seven consecutive 24-h samples were collected in high-density polyethylene bottles, transferred to Milan and stored at -20°C until sample treatment.

2.4 Sample pretreatment

The method for sample preparation was published in detail elsewhere (Rousis et al., 2016). Briefly, samples were filtered on a glass microfiber filter GF/A 1.6 μm (Whatman, Kent, U.K.) and on a mixed cellulose membrane filter 0.45 μm (Whatman, Kent, U.K.) before extraction. Solid phase extraction (SPE) was used to extract the target analytes using OASIS® HLB 3 cc/60 mg cartridges (Waters Corp., Milford, MA, USA) and an automatic GX-274 ASPEC (Gilson, Middleton, WI, USA) extractor. Samples (50 mL of untreated wastewater) were spiked with 2 ng of a mixture of internal standards and the pH was adjusted to 7.0-7.5, using diluted HCl (12%). Cartridges were conditioned with MeOH (5 mL) and Milli-Q water (3 mL) and samples were passed at a flow rate of 5 mL/min. The cartridges were dried under a nitrogen stream at a flow rate of 10 mL/min for 10 min and eluted with 3 mL of MeOH. Eluates were evaporated under a gentle nitrogen stream at room temperature and dried samples were reconstituted in 100 μL of Milli-Q water and transferred into glass vials for LC-MS/MS analysis.

The alkyl phosphate analytes DEP, DETP, DMP and DMTP were directly injected into the LC-MS/MS system; 500 μL of filtered samples were centrifuged at 2500 rpm for 2 min and 180 μL

of supernatant were collected, spiked with 2 ng of a mixture of internal standards and transferred into glass vials for LC-MS/MS analysis.

2.5 Instrumentation and analytical method

Chromatographic separation was done with an Agilent 1200 Series system (Agilent Technologies, Santa Clara, CA, USA) using an XSELECT^{TM} CSH^{TM} C18 (2.1 × 100 mm, 2.5 μm) column (Waters Corp., Milford, MA, USA). Mass spectrometric analysis done using an AB SCIEX Triple Quad^{TM} 5500 LC–MS/MS System (AB-Sciex, Thornhill, Ontario, Canada). Two or three most abundant product ions of the protonated pseudo-molecular ion of each substance were chosen for analysis which was done both in positive and negative ionization modes using the selected reaction monitoring mode (SRM). Quantification was performed by isotopic dilution. Method limits of detection and quantification are reported in Table S2. The method was fully validated in raw wastewater, as described elsewhere (Rousis et al., 2016).

2.6 Stability of biomarkers and parent pesticides in wastewater

Stability experiments aim to ensure that no degradation of the targeted compounds occurs in the sewage system and during sampling and storage, so no pre-analytical losses occur (McCall et al., 2016). The stability of parent pesticides is crucial, since degradation of these compounds could lead to formation of the targeted biomarker in wastewater, hence to overestimation of human exposure. The stability of metabolites in raw wastewater and the formation of pyrethroid metabolites from the degradation of parent pyrethroids were evaluated in previous studies (Rousis et al., 2016, 2017). The present study investigated the formation of triazine and some organophosphate metabolites after addition of the corresponding parent pesticides in raw wastewater, under different conditions. Parent triazine (atrazine, simazine, propazine, terbutylazine)

and organophosphate pesticides (chlorpyrifos, chlorpyrifos-methyl, malathion, diazinon) were spiked in wastewater to the maximum acceptable concentration (0.1 μg/L) for a single pesticide in groundwater, surface water and water intended for human consumption according to EU directives (Commision, 2008, 2006, 1998) to test their stability under controlled conditions (room temperature and 4°C). These temperatures were chosen in order to mimic conditions in the sewer system (room temperature, ~23°C; worst case scenario) and during the collection of the composite 24-h samples (occurring at 4°C). Each experiment was run in triplicate and samples were analyzed immediately after spiking (t_0), and after 6 (t_6) and 24 h (t_{24}). Unspiked samples were used as matrix blanks. Analysis of formed DEP, DETP, DMP and DMTP compounds following addition of parent pesticides in wastewater was not performed, since these metabolites are excretion or transformation products of a wide number of pesticides and other substances including flame retardants, plasticizers and industrial chemicals (Rousis et al., 2016).

2.7 Daily mass loads

Daily mass loads of biomarkers were calculated by multiplying the concentrations (ng/L) found in a 24h composite sample of raw wastewater by the daily wastewater flow rate (m^3/day) at the WWTPs (Table S1). Biomarker mass loads (mg/day) were then normalized to the number of people served by each WWTP (mg/day/1000 inhabitants), in order to compare results between different cities.

2.8 Pyrethroid intake and uncertainty evaluation

At present, pyrethroid metabolites (3-PBA and DCCA) were found to be the most suitable biomarkers of exposure according to the specific requirements of WBE (Table 1), so they were used to back-calculate population-wide intake of pyrethroids. Specific correction factors (CFs) were

developed by Rousis et al. (2017) and the following equation was used to estimate pyrethroids intake:

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

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

CFs were calculated taking into account the molar mass ratio between parent pesticide and target metabolite and the percentage of excretion of the target metabolite in human urine. Since each metabolite is common to more than one parent substance, the molar mass ratios were calculated using the arithmetic mean of the molecular weights of all parent substances divided by the molecular weight of each metabolite. All human urinary pharmacokinetic studies reporting the excretion rate of metabolites after a dose of the parent substances were considered. The weighted mean (WM) excreted fraction was calculated as the mean percentage of excretion weighted by the number of subjects in each study (Rousis et al., 2017). The following equation was used to calculate CFs:

$$CF = \frac{\frac{Mw\ (Parent\ pesticide)}{Mw\ (metabolite)}}{WM\ excreted\ fraction\ (metabolite)}$$

where: Mw is the molecular weight and WM is the weighted mean of the percentage of excretion of the targeted metabolites.

The procedure used to develop CFs has been described in detail elsewhere (Rousis et al., 2017). CFs were 6.95 for 3-PBA (used to estimate the intake of 20 pyrethroids) and respectively 3.67 and 5.45 for *trans*- and *cis*-DCCA (used to estimate the intake of permethrin, cypermethrin, and cyfluthrin) (Rousis et al., 2017). The intake levels of permethrin, cypermethrin and cyfluthrin (sum of *cis*- and *trans*- levels) estimated by WBE were compared with a toxicological indicator, the

acceptable daily intake (ADI), so as to evaluate the measured levels of exposure in relation to their potential effects on human health.

Uncertainty was evaluated following the available best practice protocols for WBE (Castiglioni et al., 2014, 2013). Sampling procedures were selected to keep uncertainty below 10%, while the analytical procedure was optimized to have an analytical variability lower than 15% (Rousis et al., 2016). The variability of excretion profiles of pyrethroids metabolites was carefully evaluated to assess the uncertainty related to CFs and consequently to the back-calculation. It was calculated as the standard deviation of the percentages of excretion collected from the literature as shown previously (Rousis et al., 2017) and it was lower than 16%. Finally, data normalization to the population served by each WWTP was done considering the most reliable population estimation to keep uncertainty as lower as possible. Nevertheless, as described elsewhere, this is probably the most critical aspect of determining the variability (Castiglioni et al., 2013).

2.9 Data elaboration

Data were analysed using a MultiQuant™ 2.1 software package of Analyst® (AB-Sciex, Thornhill, Ontario, Canada). GraphPad Prism (Version 6.0) was used for figures elaboration and statistical analyses which was performed by using an unpaired t-test or a Mann-Whitney test according to the normality of data. All tests were done considering a statistical significance level of $p<0.05$. Concentrations below the Limit of Quantification (LOQ) were replaced with a value equal to half the LOQ.

3 Results and Discussion

3.1 Stability of metabolites and parent pesticides

The stability experiments showed no formation of triazine and organophosphate metabolites in any of the tested conditions (Table S3). Thus, the percentage variation of the concentration for each metabolite at t_6 and t_{24} respect to t_0 indicated that very small variations occurred for all metabolites. Even though these laboratory experiments were conducted under controlled conditions (pH = 7.0-7.5; room temperature and 4 °C) that are not reproducing the spatial and temporal variability in a sewer system, they can provide indicative information regarding the stability of a compound in wastewater.

3.2 Occurrence of biomarkers in raw wastewater

Concentrations of the biomarkers measured in wastewater are shown in Table 2 with their frequencies of detection. The substances most frequently observed were ATZ and DEA (detection rates 98.2% and 62.5%) among triazines; 3-PBA and *trans*-DCCA (detection rates 98.2% and 96.4%) among pyrethroids; TCPY (detection rate 100%), IMPY (detection rate 87.5%), and DMP and DEP (detection rates 100% and 94.6%) among organophosphates. The other biomarkers had lower frequencies of detection (<40%), and chlorpyrifos, chlorpyrifos–methyl and DMTP were not detected. Mean concentrations ranged from a few ng/L (triazines) to 2.3 µg/L (DMP).

The results were comparable with those of a previous study in seven Italian cities (Rousis et al., 2016). The profiles of the compounds most frequently detected were similar, besides a few exceptions; i.e. the frequency of detection of DES and *cis*-DCCA was higher in Italy (100% and 73%) than in the other European cities (38% and 36%), and CPF was detected in one city in Italy (Rousis et al., 2016), but not in the EU cities (Table 2). The results for the other compounds were quite similar in both studies: AM, CPF-MET and DMTP were not detected; malathion and triazine metabolites were detected sporadically (frequency of detection <40%); and TCPY and DMP were detected in all samples. The highest concentrations in both studies were measured for the alkyl

phosphate metabolites, DEP and DMP, which are metabolic products of most organophosphates, while the triazines group was found at the lowest concentrations (Rousis et al., 2016). The concentrations of *trans*-DCCA were always higher than those of *cis*-DCCA, in accordance with HBM studies, where the trans-isomer predominated (Rousis et al., 2017). The *trans*- to *cis*- DCCA ratio is used as an indicator of the route of human exposure and a ratio of 2:1 or higher indicates oral uptake and/or inhalation. This suggests that these metabolites in wastewater resulted mainly from human metabolism, since the ratio was higher than 2:1, as reported previously (Rousis et al., 2017).

3.3 Mass loads of biomarkers in the different cities

The mean mass loads of organophosphates, triazines and pyrethroids (parent and metabolites) expressed as mg/day/1000 inhabitants, are reported in Table S4.

The alkyl phosphates DMP and DEP gave the highest loads (up to 975 mg/day/1000 inh for DMP and 244 mg/day/1000 inh for DEP). These high mass loads were expected, since these substances are metabolic products of most of the organophosphate insecticides used in Europe. These substances also might originate from plasticizers or flame retardants following hydrolysis or from other industrial chemicals (Reemtsma et al., 2011) and are therefore not specific for human exposure. Among the other specific metabolites investigated, the loads of the diazinon metabolite IMPY ranged from 1.3 to 16 mg/day/1000 inh. and the metabolite of chlorpyrifos and chlorpyrifos-methyl, TCPY, ranged from 3.9 to 22 mg/day/1000 inh., suggesting different exposure to these organophosphates in the various countries.

Triazines had the lowest loads, ranging from 0.33 to 5.0 mg/day/1000 inh. Generally, the metabolite mass loads were of the order of magnitude of atrazine or slightly higher. Among the compounds investigated, only AM is a specific metabolite of atrazine that may indicate human

exposure, but it was never detected in wastewater. The other metabolites detected can also result from exposure to other triazines, particularly terbutylazine, which is the only chlorotriazine herbicide approved for use in EU, and DES, DIA and DEA can originate from degradation of the parent substances in the environment (Barr et al., 2007). It was therefore very difficult to correlate their occurrence in wastewater with human exposure.

The mass loads of pyrethroids were higher than those of triazines, 3-PBA ranged between 4.2 and 30 mg/day/1000 inh and *trans*-DCCA from 7.0 to 46 mg/day/1000 inh. In all the cities, *cis*-DCCA mass loads were the lowest (3.6 - 10.5 mg/day/1000 inh). These specific metabolites were used to evaluate human exposure as described here below.

The sum of the mass loads of the compounds measured for each class of pesticides was calculated as described in paragraph 2.7, in order to compare results from the different cities (Figure 2). Different patterns were observed among the cities and for the various classes of pesticides, but Utrecht and Oslo invariably had the lowest loads. The specific biomarkers of exposure to pyrethroids had the highest loads in Castellon (mean 86 mg/day/1000 inh) followed by Milan and Bristol (mean 43 mg/day/1000 inh), and Copenhagen (mean 41 mg/day/1000 inh). This may indicate a higher human exposure to pyrethroids in Spain due to either direct exposure or consumption of contaminated food, and fits with the fact that Spain is classified as one of the countries with the highest sales of pesticides in Europe (Eurostat, 2014). Regarding the specific metabolites of organophosphates, the highest loads were again in Castellon (mean 28 mg/day/1000 inh), Bristol (mean 26 mg/day/1000 inh) and also in Zurich (mean 21 mg/day/1000 inh). Among non-specific metabolites a direct correlation with exposure could not be performed. The highest levels were found for alkyl phosphates in Zurich (mean 1056 mg/day/1000 inh), followed by Bristol (mean 573 mg/day/1000 inh) and Brussels (mean 322 mg/day/1000 inh), and for triazines in Milan (mean 14 mg/day/1000 inh) Zurich and Brussels (mean 10 mg/day/1000 inh) (Figure 2).

Since human exposure occurs mainly through the diet and can be related to direct exposure only in some cases (i.e. rural areas), the results obtained for the specific biomarkers of exposure can reveal new information about the "average exposure" of the population to these pesticides (pyrethroids and organophosphates). Regarding the other non-specific biomarkers, further investigation will be necessary to assess the main sources of these substances, and exclude the possibility of discharges from sources other than human metabolism.

3.4 Comparison of mass loads of insecticides with official sales statistics

Organophosphates and pyrethroids were the classes most frequently detected in wastewater, both of which are classified as insecticides. Wastewater results were therefore compared with the national sales statistics of insecticides reported by Eurostat (Eurostat, 2014). The sum of the specific biomarkers of insecticides was normalized to the population investigated in each city and the means are reported in Figure 3. Mass loads were the highest in Castellon, Bristol, Copenhagen and Milan and the lowest in Olso (Figure 3). These results mainly reflect the Eurostat official sales statistics (Figure 3), which reported that Spain, Italy and UK had the highest sales data of insecticides, and Norway had the lowest. Because human exposure to pesticides is mainly influenced by the diet, we can speculate that in the countries with a high sale of insecticides, and a consequent higher use in agriculture, there is also a major supply of products (vegetable and fruits) that leads to a higher exposure to these substances. This is supported by the fact that our study was focused on urban areas where direct exposure related to agricultural use can be excluded. In Spain and Italy the Mediterranean diet, which includes lots of fruits and vegetables, may also play an important role in the exposure to pesticides. Wastewater results seem to reflect also the available figures of vegetable and fruit supply and consumption in Europe which are reported to be higher in the South than in the North of Europe (EUFIC).

3.5 Back-calculation of pyrethroid intake

The daily intake by the general population was calculated for pyrethroids due to the suitability of wastewater biomarkers. The mass loads of biomarkers (3-PBA and *trans*- and *cis*-DCCA) were therefore used to back-calculate the intake of the corresponding parent substances. The mass loads of 3-PBA, which is the common urinary metabolic product of about 20 pyrethroids, were multiplied by its specific CF as previously described (Rousis et al., 2017). Pyrethroids highest intake was in Castellon (207 mg/day/1000 inh.) followed by Bristol (77 mg/day/1000 inh.) and Milan (75 mg/day/1000 inh.), and the lowest in Oslo (17 mg/day/1000 inh.) (Table 3).

The intake of *trans*- and *cis*- permethrin, cypermethrin and cyfluthrin was estimated using the mass loads of their specific metabolites *trans*- and *cis*-DCCA in wastewater and their specific CF (Rousis et al., 2017). Results are reported in Table 3 as the sum of the *cis*- and *trans*- DCCA isomers. The estimated intakes ranged between 227 mg/day/1000 inh in Castellon and 26 in Oslo. Similar intakes were found in UK (126 mg/day/1000 inh), Copenhagen (123 mg/day/1000 inh) and Milan (130 mg/day/1000 inh).

The intake profiles from both DCCA and 3-PBA were highest in Castellon and lowest in Oslo, indicating an extremely divergent exposure to this class of pesticides. These results are in accordance with the eEuropean statistics of fruit and vegetable consumption and also with national statistics of pesticides sales as previously discussed for the entire class of insecticides. The intake of pyrethroids estimated from DCCA was generally higher than those estimated from 3-PBA in all the cities (in several cases the difference was statistically significant, DCCA vs. 3-PBA) (Table 3). This may reflect different patterns of exposure to pyrethroids, which are excreted as the investigated biomarkers. Further research is therefore required to investigate the specific patterns of the household use of these substances and the food contamination.

421

422 **3.6 Comparison of estimated intake with the acceptable daily intake (ADI)**

423 The potential risk related to the intake of permethrin, cypermethrin and cyfluthrin was
424 assessed using the daily intake estimated from the loads of *trans*- and *cis*-DCCA measured in
425 wastewater. In order to compare these data with ADI values, the ADI of beta-cyfluthrin was used as
426 a worst case scenario, since it was the lowest for this class of compounds. An ADI of 0.003 mg/kg
427 body weight per day for a man of 70 kg resulted in an average consumption of 0.21 mg/person per
428 day (Rousis et al., 2017). The comparison between intakes estimated by WBE and the %ADI are
429 reported in Table 4. The estimated intake of permethrin, cypermethrin and cyfluthrin in the
430 population was generally lower than the ADI, and exceeded this reference value only in one case
431 (Castellon) (Table 4). As previously discussed, this area was found to have the highest exposure
432 level to insecticides (particulary pyrethroids) probably due to a combination of wide use of
433 pesticides and high consumption of contaminated food.

434 **3.7 Limitations and future research needs**

435 Up to date we checked the formation of metabolites from the parent substances through
436 laboratory tests performed in wastewater mimicking different temperature conditions during in-
437 sewer transport and sampling. Nevertheless, it would be ideal to perform transformation
438 experiments in real sewers, but many factors make troublesome to obtain accurate results in such
439 studies. Moreover, the stability of biomarkers in wastewater can be highly affected by “local”
440 conditions in a WWTP and may require specific investigations. Future research in this area should
441 take into account the main processes occurring in sewer compartments, and consequently the
442 potential presence of pesticides/metabolites in the different compartments: a) the bulk liquid
443 (wastewater with suspended particulate matter); b) biofilm growing on the sewer walls; c)
444 sediments; d) the sewer atmosphere (McCall et al., 2016).

The present study is the first one in which an attempt is made to correlate the mass loads of insecticides obtained from WBE with national sales statistics and vegetable and fruit consumption. A number of limitations must be considered to improve future comparisons of this kind of data. On one side, WBE results were obtained by measuring a few specific urinary metabolites that indicate the exposure to a limited number of parent substances within the entire class of insecticides. Furthermore, WBE was performed only in one city per country and for a limited period (seven consecutive days). Thus, results may not reflect longer periods of exposure. Under these conditions, the extrapolation of results to the whole country will be biased by the specific spatial and temporal profiles of that city. This was seen in previous studies, where significant differences in pesticide intake were found among cities within the same country (Rousis et al., 2016), and pesticides levels showed seasonal variations (Rousis et al., 2017). Thus, future WBE studies should include more cities per country and sampling should be repeated seasonally to improve the comparability of wastewater results with the available national statistics. On the other side, national sales statistics for pesticides may not reflect the actual use of these substances in a country and they are obviously not directly related to exposure, even if the first results suggest a correlation. Moreover, these data are referred to the sales of an entire class of substances, for instance insecticides in our case, registered in an EU database and collected over the whole year in each country, being therefore more comprehensive and aggregated than our information from WBE. Finally, food consumption can be measured in different ways and statistics can be obtained with different methods which are not directly comparable. Since National Authorities often adopt different methods to collect data, the comparability of international statistics should be carefully verified.

4 Conclusions

WBE was applied here for the first time to assess human exposure to different classes of pesticides across Europe. Several selected biomarkers of exposure to pesticides were measured in

raw wastewater and used as indicators of human exposure in the population. Mass loads suggested a different pattern of exposure to organophosphates, pyrethroids and triazines. Spatial differences in exposure to insecticides in the various cities were in line with national statistics related to pesticides exposure. Results suggested that in the countries with higher insecticides sales, there is also a major supply of products (vegetables and fruits) that leads to a higher exposure to these substances. WBE was able to provide new information about the "average exposure" of the population to pesticides. Moreover, the calculation of the daily intake of pyrethroids highlighted also a different pattern of exposure within this class. The comparison of daily intake calculated for permethrin, cypermethrin and cyfluthrin and a worst case ADI (the one from beta-cyfluthrin) indicated a potential risk for human health. This study suggest that WBE ~~as~~ can be a very promising complementary biomonitoring tool to evaluate population-wide exposure to pesticides. Some current limitations were also discussed in order to improve future applications.

Contributions

Nikolaos I. Rousis, Sara Castiglioni and Ettore Zuccato planned and designed the study. The collection of the wastewater samples was organized by all authors. Nikolaos I. Rousis analyzed the samples and interpreted the results with the input of Emma Gracia-Lor and Sara Castiglioni. Nikolaos I. Rousis and Sara Castiglioni drafted the manuscript, which was critically revised by all co-authors. All authors are aware of the content and accept responsibility, for the manuscript.

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498 doctoral contract.

499

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607

608

609 **Table 1.** Summary of the main characteristics of the metabolites selected as WBE biomarkers.

Metabolites selected as WBE biomarkers	Parent pesticides	Detection in wastewater (present study)	Other potential sources (Rousis et al., 2016)	Stability in wastewater (Rousis et al., 2016)	Formation from parent pesticides in wastewater (Rousis et al., 2017); present study)
Triazines					
DES	Terbuthylazine	Yes	Yes	Yes	No
DIA	Atrazine, terbuthylazine, simazine, propazine	Yes	Yes	Yes	No
DEA	Atrazine, terbuthylazine, simazine, propazine	Yes	Yes	Yes	No
AM	Atrazine	No	Yes	Yes	No
Pyrethroids					
3-PBA	20 pyrethroids[a]	Yes	Yes	Yes	No
***trans*-DCCA**	Permethrin, cypermethrin, cyfluthrin	Yes	No	Yes	No
***cis*-DCCA**	Permethrin, cypermethrin, cyfluthrin	Yes	No	Yes	No
Organophosphates					
TCPY	Chlorpyrifos, chlorpyrifos-methyl	Yes	Yes	Yes	No
MMA	Malathion	Yes	Yes	Yes	No
IMPY	Diazinon	Yes	Yes	Yes	No
DEP	Several organophosphate insecticides	Yes	Yes	Yes	-[b]
DETP	Several organophosphate insecticides	Yes	Yes	Yes	-[b]
DMP	Several organophosphate insecticides	Yes	Yes	No	-[b]
DMTP	Several organophosphate insecticides	No	Yes	Yes	-[b]

610 [a]Permethrin, cypermethrin, deltamethrin, fenvalerate, phenothrin, cyphenothrin, cyhalothrin, esfenvalerate,
611 fenpropathrin, allethrin, resmethrin, tralomethrin, flucythrinate, fluvalinate and their isomers; [b] not assessed
612 because these compounds come from multiple substances.
613

Table 2 Mean concentrations (ng/L) and standard deviations of the raw wastewater samples collected in eight European cities in March 2015.

Compound	Bristol	Brussels	Castellon	Copenhagen	Milan	Oslo	Utrecht	Zurich	Frequency of detection (%)
Triazines									
ATZ	4.4 ± 0.4	12.8 ± 1.3	2.0 ± 1.0	1.3 ± 0.1	7.9 ± 0.8	1.7 ± 0.2	2.1 ± 0.3	5.4 ± 0.6	98.2
DES	<0.6.	<0.6.	21.1 ± 3.7	<0.6.	12.2 ± 1.4	<0.6.	<0.6.	6.2 ± 0.8	37.5
DIA	<1.4	6.7 ± 2.0	<1.4	<1.4	8.9 ± 1.4	<1.4	<1.4	4.3 ± 0.2	36.2
DEA	7.5 ± 3.0	19.6 ± 5.5	4.5 ± 1.2	<1.1	7.7 ± 1.1	<1.1	<1.1	7.4 ± 0.9	62.5
AM	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0
Pyrethroids									
3-PBA	49 ± 25	22.4 ± 1.4	129 ± 32	12.4 ± 2.3	26.1 ± 9.3	5.3 ± 1.5	30.1 ± 7.4	9.6 ± 1.4	98.2
trans-DCCA	118 ± 65	65 ± 13	200 ± 60	44 ± 16	63 ± 34	15.1 ± 8.8	124 ± 54	31 ± 10	96.4
cis-DCCA	22 ± 11	<7.7	45 ± 11	<7.7	14 ± 11	<7.7	22.9 ± 8.3	<7.7	35.7
Organophosphates									
CPF	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4	0
CPF-MET	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	0
TCPY	43 ± 23	23.8 ± 2.7	93 ± 23	17.8 ± 2.3	20.1 ± 2.9	8.3 ± 1.3	28.3 ± 3.9	26.4 ± 3.1	100
MMA isomer 1	<3.9	<3.9	397 ± 966	<3.9	4.7 ± 2.3	<3.9	<3.9	<3.9	8.9
MMA isomer 2	<4.8	<4.8	285 ± 661	<4.8	<4.8	<4.8	<4.8	<4.8	7.1
IMPY	72 ± 48	4.9 ± 1.1	25 ± 11	3.6 ± 0.8	<1.29	6.5 ± 1.2	12.7 ± 2.8	19 ± 16	87.5
Alkyl phosphates (Organophosphates)									
DEP	1076 ± 670	180 ± 24	231 ± 56	110 ± 12	123 ± 20	46 ± 19	206 ± 13	187 ± 22	94.6
DETP	39 ± 19	<17.5	<17.5	<17.5	<17.5	<17.5	<17.5	<17.5	7.1
DMP	1388 ± 2228	1072 ± 1018	278 ± 77	280 ± 92	128 ± 22	233 ± 60	269 ± 43	2269 ± 630	100
DMTP	<395	<395	<395	<395	<395	<395	<395	<395	0

<LOQ/2 are reported as used for further calculation. LOQ values are reported in Table S2.

627 **Table 3** Pyrethroid intake (mg/day/1000 inhabitants; mean and standard deviation) back-calculated
628 from 3-PBA and *cis*- and *trans*-DCCA.

WWTP	Group of pyrethroids (3-PBA)	Permethrin, cypermethrin and cyfluthrin (DCCA*)	Statistical analysis (p-values)[§]
Bristol	77 ± 37	126 ± 60	0.091
Brussels	41 ± 6	62 ± 11	**0.012**
Castellon	207 ± 47	227 ± 59	0.507
Copenhagen	57 ± 13	123 ± 50	**0.005**
Milan	75 ± 39	130 ± 101	0.209
Oslo	17 ± 5	26 ± 13	0.128
Utrecht	33 ± 8	90 ± 36	**0.001**
Zurich	29 ± 6	50 ± 22	**0.031**

629 *Sum of *cis*- and *trans*-DCCA;[§] unpaired t-test or Mann-Whitney test were performed considering a
630 statistical significance for $p<0.05$.

631

632

633 **Table 4** Estimated intake of permethrin, cypermethrin and cyfluthrin of the population living in
634 different European cities and comparison with the acceptable daily intake (ADI) for beta-cyfluthrin
635 (0.21 mg/day/person).

WWTP	Intake of permethrin, cypermethrin and cyfluthrin (mg/day/person)	% ADI*
Bristol	0.126 ± 0.060	60
Brussels	0.062 ± 0.011	30
Castellon	0.227 ± 0.059	108
Copenhagen	0.123 ± 0.050	58
Milan	0.130 ± 0.101	62
Oslo	0.026 ± 0.013	12
Utrecht	0.090 ± 0.036	43
Zurich	0.050 ± 0.022	24

636 *Permethrin, cypermethrin and cyfluthrin intake percentage compared to the ADI of beta-cyfluthrin and
637 expressed in %.

638

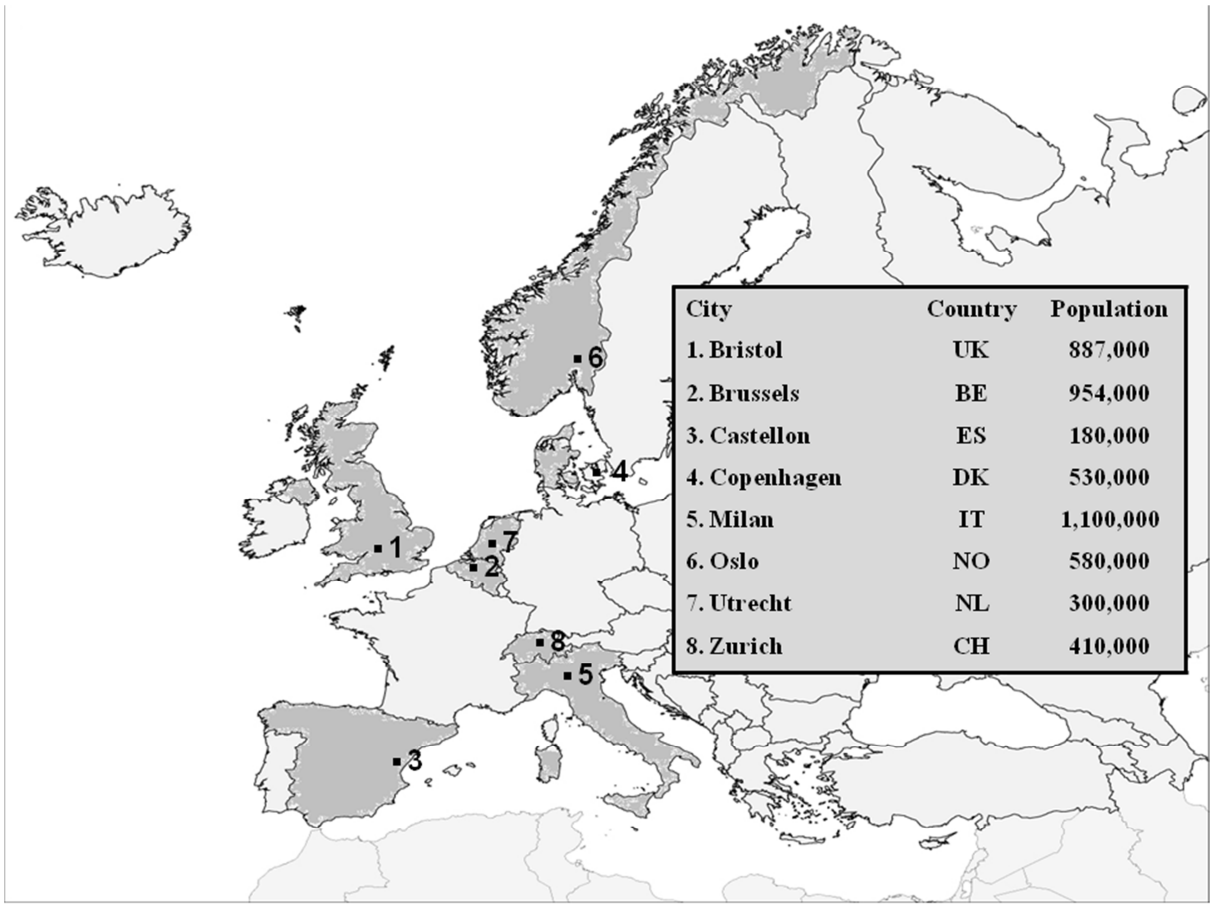
639

640 **Figure Legends**

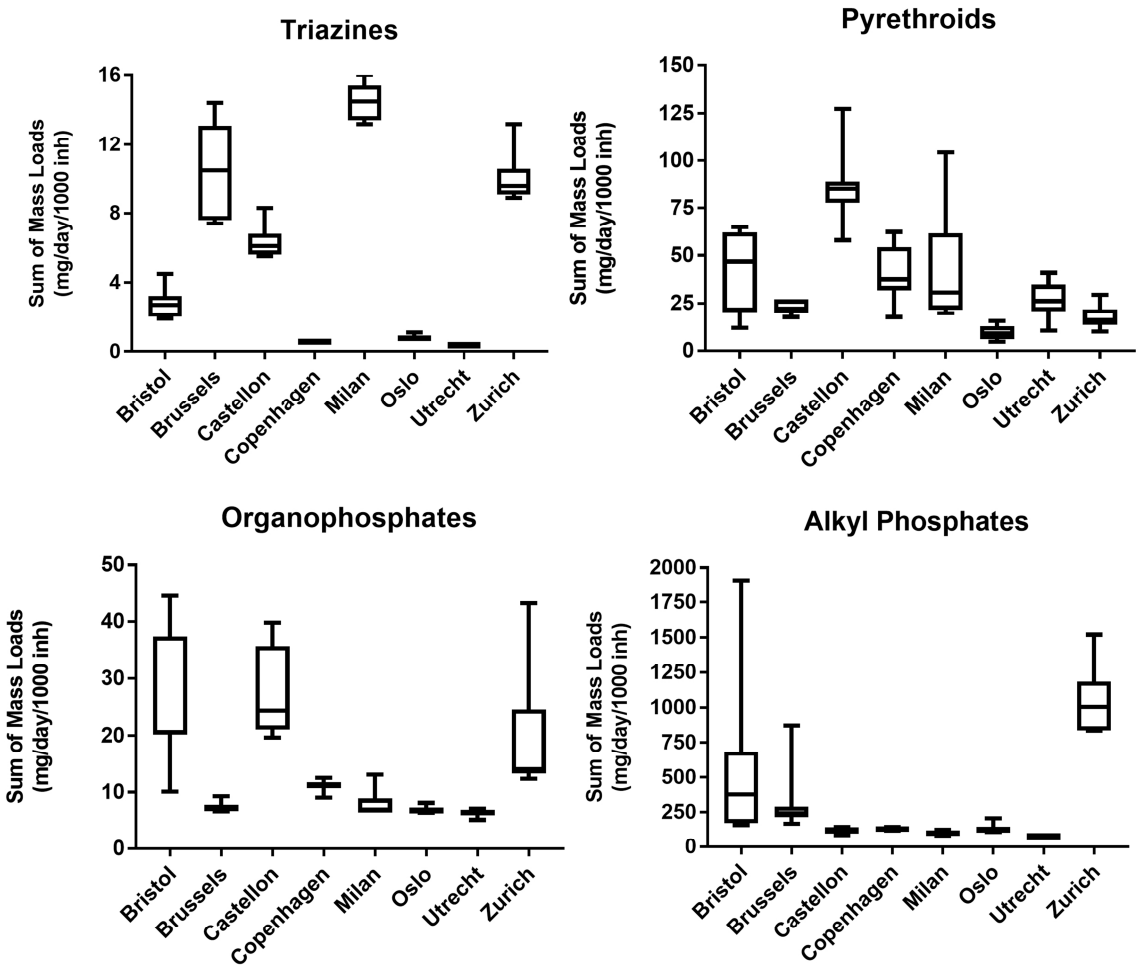
641 **Fig. 1.** Cities investigated in the present study in Europe.

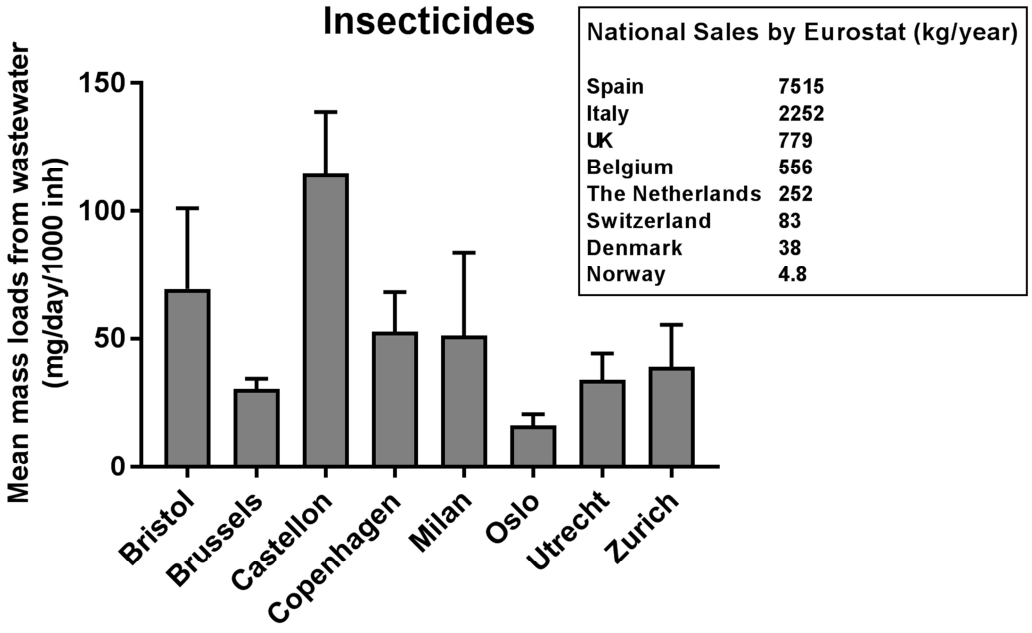
642 **Fig. 2.** Sum of the mass loads (mg/day/1000 inhabitants) of organophosphates, triazines,
643 pyrethroids and alkyl phosphates in eight European cities.

644 **Fig.3.** Sum of the mass loads of insecticides (mg/day/1000 inhabitants) estimated from wastewater
645 in eight European cities and national sales from Eurostat (2014).



City	Country	Population
1. Bristol	UK	887,000
2. Brussels	BE	954,000
3. Castellon	ES	180,000
4. Copenhagen	DK	530,000
5. Milan	IT	1,100,000
6. Oslo	NO	580,000
7. Utrecht	NL	300,000
8. Zurich	CH	410,000


Triazines
Sum of Mass Loads (mg/day/1000 inh)
16
12
8
4
0
Bristol
Brussels
Castellon
Copenhagen
Milan
Oslo
Utrecht
Zurich
Pyrethroids
Sum of Mass Loads (mg/day/1000 inh)
150
125
100
75
50
25
0
Bristol
Brussels
Castellon
Copenhagen
Milan
Oslo
Utrecht
Zurich
Organophosphates
Sum of Mass Loads (mg/day/1000 inh)
50
40
30
20
10
0
Bristol
Brussels
Castellon
Copenhagen
Milan
Oslo
Utrecht
Zurich
Alkyl Phosphates
Sum of Mass Loads (mg/day/1000 inh)
2000
1750
1500
1250
1000
750
500
250
0
Bristol
Brussels
Castellon
Copenhagen
Milan
Oslo
Utrecht
Zurich



Highlights

- WBE was applied for the first time to assess human exposure to pesticides in Europe
- Different patterns were observed among the cities and the classes of pesticides
- Results were in line with the national statistics related to pesticides exposure
- This study gives new information about the “average exposure” of the population