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# Wastewater-based epidemiology to assess pan-European pesticide exposure

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36

## 37    **Abstract**

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

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

62

## 63 **1 Introduction**

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

73 Many “old and harmful” pesticides, such as p,p-dichlorodiphenyl-trichloroethane (p,p’-  
74 DDT), have been banned because of their toxicity and they were replaced by less-persistent  
75 pesticides, such as organophosphates and pyrethroids (Barr, 2008; López et al., 2005). Pesticides  
76 provide mankind with many benefits, but at the same time have the potential to pose risks for  
77 human health due to widespread use and high biological activity (Cooper and Dobson, 2007). For  
78 instance, pesticides exposure has positive association with the development of idiopathic  
79 Parkinson’s disease, neurobehavioral and neuropsychological disorders, respiratory symptoms or  
80 diseases, and sperm DNA damage (Allen and Levy, 2013; Mamane et al., 2015; Saillenfait et al.,  
81 2015; Stallones and Beseler, 2016). However, in the last two decades, the concept of “green  
82 chemistry” has been promoted and the agrochemical industry has focused on less toxic substances  
83 (Garrison, 2004).

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

86 the measurement of chemicals and/or their metabolites in body fluids or tissues (Barr, 2008; Yusa et  
87 al., 2015). The reliability of HBM depends on the selection of a proper biomarker that reflects the  
88 exposure to the parent compound, and is specific and detectable in the investigated matrices. Urine  
89 is the preferred human biological matrix, since it is easy to collect and non-invasive and it is also  
90 accessible in large volumes allowing the determination of very low concentrations of chemicals  
91 compared to other fluids (Wessels et al., 2003). Extensive HBM studies have analyzed the urine of  
92 thousands of individuals to investigate pesticide exposure in the general population (Barr et al.,  
93 2010, 2004; Heudorf and Angerer, 2001; McKelvey et al., 2013; Ye et al., 2015). Despite their  
94 power to evaluate exposure to chemicals, HBM studies suffer by limitations such as high costs for  
95 sample collection and analysis, ethical issues and data analysis to extrapolate individual results to  
96 the whole population. Moreover, urine sampling can reflect only a momentary snapshot of exposure  
97 due to sampling procedures (i.e. morning urine collection), and excretion profiles may vary  
98 throughout the day/days because of the short half-lives in the human body of most of pesticides.

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

110 The first exploratory study proposing WBE as a novel biomonitoring tool to evaluate the  
111 exposure of the general population to pesticides was recently performed (Rousis et al., 2016).  
112 Several metabolites of organophosphates, triazines and pyrethroids were detected in raw wastewater  
113 and their frequency of detection and abundance were in agreement with the profiles reported in  
114 urine of HBM studies (Rousis et al., 2016). Later three human urinary metabolites of pyrethroids  
115 were selected and used to back-calculate the population intake of pyrethroids in Italy (Rousis et al.,  
116 2017). This study indicated for the first time that WBE can be employed as a complementary  
117 biomonitoring tool to the HBM studies, but more data and a wider scale of investigation were  
118 necessary in order to confirm these preliminary results.

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

126

## 127 **2 Materials and methods**

### 128 **2.1 Chemicals and reagents**

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

134 chlorpyrifos (CPF, purity 99.9%), chlorpyrifos methyl (CPF-MET, purity 99.5%) and 3,5,6-  
135 trichloro-2-pyridinol (TCPY, purity 99.5%) were purchased from Chemical Research 2000 (Rome,  
136 Italy). Atrazine (ATZ, purity 97.5%), atrazine desethyl (DEA, purity 99.9%), terbutylazine desethyl  
137 (DES, purity 97.4%), atrazine desisopropyl (DIA, purity 95.4%), dimethyl chlorophosphate  
138 (DMCIP, purity 96%), dimethyl chlorothiophosphate (DMCITP, purity 97%), and O,O-diethyl  
139 thiophosphate (DETP, purity 98%) potassium salt were supplied by Sigma-Aldrich (Schnelldorf,  
140 Germany). Atrazine mercapturate (AM, purity 95.0%), 3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-  
141 cyclopropane)carboxylic acid (DCCA, purity 99.0%), 3-phenoxybenzoic acid (3-PBA, purity  
142 99.0%), 2-isopropyl-6-methyl-4-pyrimidinol (IMPY, purity 99.5%), cis-3-(2,2-dichlorovinyl)-2,2-  
143 dimethyl-(1-cyclopropane) carboxylic acid (cis-DCCA, purity 98%) and malathion monocarboxylic  
144 acid (MMA, purity 97.0%) were purchased from Lab Service Analytica (Bologna, Italy).  
145 Isotopically labeled compounds (deuterated or  $^{13}\text{C}$ -enriched) were used as internal standards (IS). 3-  
146 Phenoxybenzoic acid- $\text{C}_6$  (3-PBA- $^{13}\text{C}_6$ , phenoxy- $^{13}\text{C}_6$ , 99%; purity 98%) and 3,5,6-trichloro-2-  
147 pyridinol- $\text{C}_3$  (TCPY- $\text{C}_3$ , 4,5,6- $^{13}\text{C}_3$ , 99%; purity 97%) were obtained from Cambridge Isotope  
148 Laboratories, Inc. (Massachusetts, USA); atrazine- $\text{D}_5$  (ATZ- $\text{D}_5$ , 99.5%) from Sigma-Aldrich  
149 (Schnelldorf, Germany); and chlorpyrifos  $\text{D}_{10}$  (CPF- $\text{D}_{10}$ , 97.0%) from Lab Service Analytica  
150 (Bologna, Italy). Dimethyl phosphate (DMP) and dimethyl thiophosphate (DMTP) were  
151 synthesized by simple hydrolysis of DMCIP and DMCITP (Hernández et al., 2002; Rousis et al.,  
152 2016a).

153

## 154 **2.2 Selection of exposure biomarkers**

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



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

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

170 The reliability of back-calculation of the exposure to parent chemicals (pesticides) depends  
171 strictly on the selection of an appropriate WBE biomarker, which can be either the compound itself  
172 or one of its metabolites. Therefore, the selected metabolites were checked to fulfill the  
173 requirements of a WBE biomarker, which are: a) measurable in raw wastewater; b) released into  
174 sewers only as a result of human excretion; c) a well-defined excretion profile to avoid interference  
175 from other exogenous or endogenous sources; d) limited adsorption to suspended matter; e) stable  
176 in wastewater during in-sewer transit, sampling and storage (Gracia-Lor et al., 2016). The stability  
177 of each compound in wastewater was evaluated through specific laboratory tests (Rousis et al.,  
178 2016), and the specificity of each metabolite was assessed by checking the presence of sources  
179 other than human metabolism (i.e. any potential environmental transformation) (Rousis et al., 2017  
180 and this study). The results for the selected substances are summarized in Table 1.

181

## 182 2.3 Samples and sampling method

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

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

191

## 192 **2.4 Sample pretreatment**

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

205 The alkyl phosphate analytes DEP, DETP, DMP and DMTP were directly injected into the  
206 LC-MS/MS system; 500  $\mu\text{L}$  of filtered samples were centrifuged at 2500 rpm for 2 min and 180  $\mu\text{L}$

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

209

## 210 **2.5 Instrumentation and analytical method**

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

220

## 221 **2.6 Stability of biomarkers and parent pesticides in wastewater**

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

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

243

## 244 **2.7 Daily mass loads**

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

250

## 251 **2.8 Pyrethroid intake and uncertainty evaluation**

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

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

$$257 \quad \text{PYR}_{\text{intake}} = \frac{(\text{Conc.} \times F) \times CF}{P}$$

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

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

$$270 \quad CF = \frac{\frac{\text{Mw (Parent pesticide)}}{\text{Mw (metabolite)}}}{\text{WM excreted fraction (metabolite)}}$$

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

273 The procedure used to develop CFs has been described in detail elsewhere (Rousis et al.,  
274 2017). CFs were 6.95 for 3-PBA (used to estimate the intake of 20 pyrethroids) and respectively  
275 3.67 and 5.45 for *trans*- and *cis*-DCCA (used to estimate the intake of permethrin, cypermethrin,  
276 and cyfluthrin) (Rousis et al., 2017). The intake levels of permethrin, cypermethrin and cyfluthrin  
277 (sum of *cis*- and *trans*- levels) estimated by WBE were compared with a toxicological indicator, the

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

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

290

## 291 **2.9 Data elaboration**

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

298

## 299 **3 Results and Discussion**

### 300 **3.1 Stability of metabolites and parent pesticides**

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

308

### 309 **3.2 Occurrence of biomarkers in raw wastewater**

310 Concentrations of the biomarkers measured in wastewater are shown in Table 2 with their  
311 frequencies of detection. The substances most frequently observed were ATZ and DEA (detection  
312 rates 98.2% and 62.5%) among triazines; 3-PBA and *trans*-DCCA (detection rates 98.2% and  
313 96.4%) among pyrethroids; TCPY (detection rate 100%), IMPY (detection rate 87.5%), and DMP  
314 and DEP (detection rates 100% and 94.6%) among organophosphates. The other biomarkers had  
315 lower frequencies of detection (<40%), and chlorpyrifos, chlorpyrifos–methyl and DMTP were not  
316 detected. Mean concentrations ranged from a few ng/L (triazines) to 2.3 µg/L (DMP).

317 The results were comparable with those of a previous study in seven Italian cities (Rousis et  
318 al., 2016). The profiles of the compounds most frequently detected were similar, besides a few  
319 exceptions; i.e. the frequency of detection of DES and *cis*-DCCA was higher in Italy (100% and  
320 73%) than in the other European cities (38% and 36%), and CPF was detected in one city in Italy  
321 (Rousis et al., 2016), but not in the EU cities (Table 2). The results for the other compounds were  
322 quite similar in both studies: AM, CPF-MET and DMTP were not detected; malathion and triazine  
323 metabolites were detected sporadically (frequency of detection <40%); and TCPY and DMP were  
324 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).

333

### 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



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

354 The mass loads of pyrethroids were higher than those of triazines, 3-PBA ranged between  
355 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*-  
356 DCCA mass loads were the lowest (3.6 - 10.5 mg/day/1000 inh). These specific metabolites were  
357 used to evaluate human exposure as described here below.

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

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

379

### 380 **3.4 Comparison of mass loads of insecticides with official sales statistics**

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

### 398 3.5 Back-calculation of pyrethroid intake

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

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

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

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

466

#### 467 **4 Conclusions**

468           WBE was applied here for the first time to assess human exposure to different classes of  
469 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 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.

482

## 483 **Contributions**

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

489

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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)
<b>Triazines</b>					
<b>DES</b>	Terbutylazine	Yes	Yes	Yes	No
<b>DIA</b>	Atrazine, terbutylazine, simazine, propazine	Yes	Yes	Yes	No
<b>DEA</b>	Atrazine, terbutylazine, simazine, propazine	Yes	Yes	Yes	No
<b>AM</b>	Atrazine	No	Yes	Yes	No
<b>Pyrethroids</b>					
<b>3-PBA</b>	20 pyrethroids <sup>a</sup>	Yes	Yes	Yes	No
<b>trans-DCCA</b>	Permethrin, cypermethrin, cyfluthrin	Yes	No	Yes	No
<b>cis-DCCA</b>	Permethrin, cypermethrin, cyfluthrin	Yes	No	Yes	No
<b>Organophosphates</b>					
<b>TCPY</b>	Chlorpyrifos, chlorpyrifos-methyl	Yes	Yes	Yes	No
<b>MMA</b>	Malathion	Yes	Yes	Yes	No
<b>IMPY</b>	Diazinon	Yes	Yes	Yes	No
<b>DEP</b>	Several organophosphate insecticides	Yes	Yes	Yes	- <sup>b</sup>
<b>DETP</b>	Several organophosphate insecticides	Yes	Yes	Yes	- <sup>b</sup>
<b>DMP</b>	Several organophosphate insecticides	Yes	Yes	No	- <sup>b</sup>
<b>DMTP</b>	Several organophosphate insecticides	No	Yes	Yes	- <sup>b</sup>

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

614 **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 (%)
<b>Triazines</b>									
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
<b>Pyrethroids</b>									
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
<i>trans</i> -DCCA	118 ± 65	65 ± 13	200 ± 60	44 ± 16	63 ± 34	15.1 ± 8.8	124 ± 54	31 ± 10	96.4
<i>cis</i> -DCCA	22 ± 11	<7.7	45 ± 11	<7.7	14 ± 11	<7.7	22.9 ± 8.3	<7.7	35.7
<b>Organophosphates</b>									
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
<b>Alkyl phosphates (Organophosphates)</b>									
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

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

626

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) <sup>§</sup>
<b>Bristol</b>	77 ± 37	126 ± 60	0.091
<b>Brussels</b>	41 ± 6	62 ± 11	<b>0.012</b>
<b>Castellon</b>	207 ± 47	227 ± 59	0.507
<b>Copenhagen</b>	57 ± 13	123 ± 50	<b>0.005</b>
<b>Milan</b>	75 ± 39	130 ± 101	0.209
<b>Oslo</b>	17 ± 5	26 ± 13	0.128
<b>Utrecht</b>	33 ± 8	90 ± 36	<b>0.001</b>
<b>Zurich</b>	29 ± 6	50 ± 22	<b>0.031</b>

629 \*Sum of *cis*- and *trans*-DCCA;<sup>§</sup> 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*
<b>Bristol</b>	0.126 ± 0.060	60
<b>Brussels</b>	0.062 ± 0.011	30
<b>Castellon</b>	0.227 ± 0.059	108
<b>Copenhagen</b>	0.123 ± 0.050	58
<b>Milan</b>	0.130 ± 0.101	62
<b>Oslo</b>	0.026 ± 0.013	12
<b>Utrecht</b>	0.090 ± 0.036	43
<b>Zurich</b>	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).