Identification and Screening of Biomarkers of Human Exposure to Environmental and Food Toxicants in Sewage

Thesis

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by

Nikolaos Rousis

P.I. C7080473

DOCTOR OF PHILOSOPHY

Faculty: Environmental Chemistry

Affiliated Research Centre: IRCCS Istituto di Ricerche Farmacologiche

MARIO NEGRI

The Open University

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Nikolaos Rousis

Director of studies: Dr. Sara Castiglioni, IRCCS Istituto di Ricerche Farmacologiche MARIO NEGRI, Italy

Supervisor: Prof. Barbara Kasprzyk-Hordern, Department of Chemistry, University of Bath, United Kingdom

Third party monitor: Dr. Ettore Zuccato, IRCCS Istituto di Ricerche Farmacologiche MARIO NEGRI, Italy

Exam panel members

Exam panel chair: Dr. Roberto Fanelli, Head of Department "Environmental Health Science", IRCCS Istituto di Ricerche Farmacologiche MARIO NEGRI, Italy

Internal examiner: Dr. Mario Salmona, Head of Department "Molecular Biochemistry and Pharmacology", IRCCS Istituto di Ricerche Farmacologiche MARIO NEGRI, Italy

External examiner: Prof. Nick Voulvoulis, Faculty of Natural Sciences, Centre for Environmental Policy, Imperial College, London, United Kingdom

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Abstract

Pesticides are active substances with potentially adverse effects on human health, and therefore great effort is addressed to study the relation between their widespread use and human exposure. Human biomonitoring (HBM) is the most widely used and powerful tool to evaluate the exposure of the population to these substances. However, novel approaches are needed in order to give additional information on exposure at population level and overcome the limitations of HBM studies. A novel approach, called Wastewater-Based Epidemiology (WBE), was proposed as an alternative "biomonitoring tool" with the aim to assess the population exposure to organophosphates, triazines and pyrethroids. A specific analytical method based on liquid chromatography tandem mass spectrometry was developed and validated to measure human urinary metabolites of pesticides in influent wastewater. This method was applied to samples collected from wastewater treatment plants of fifteen European cities. Pyrethroids metabolites were suitable to back-calculate human exposure to this class. Generally, the results obtained from wastewater were in agreement with the urinary biomarker levels of HBM studies, taking into account the dilution of urine in wastewater. Spatial differences were observed on pesticide exposure in Italy and Europe and seasonal variations in human intake of pyrethroids were found, as expected, with higher intakes during spring/summer. Mass loads profiles of pesticides metabolites in the different European cities were in accordance with the use reported in the Eurostat official statistics. This novel WBE method can be used for obtaining objective and updated, direct information on the real levels of pesticide exposure in the general population, and can complement the findings of HBM studies. The method can also provide valuable information for public health organizations, such as the United States Environmental Protection Agency, the World Health Organization and the Centers for Disease Control and Prevention.

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List of abbreviations

3-PBA	3-Phenoxybenzoic acid
ADI	Acceptable daily intake
AM	Atrazine mercapturate
ANN	Artificial neural network
APCI	Atmospheric pressure chemical ionization
ATZ	Atrazine
ATZ-OH	Hydroxy atrazine
CAD	Collision gas
CE	Collision energy
CF	Correction factor
CID	Collision-induced dissociation
CPF	Chlorpyrifos
CPF-MET	Chlorpyrifos methyl
CUR	Curtain gas
CXP	Collision exit potential
DACT	Atrazine desethyl desisopropyl
DATM	Diaminotriazine mercapturate
DCCA	2,2-Dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid
DDT	Dichlorodiphenyltrichloroethane
DEA	Atrazine desethyl
DEAM	Atrazine desethyl mercapturate
DEA-OH	Hydroxy desethyl atrazine
DEP	Diethyl phosphate
DES	Terbuthylazine desethyl
DETP	O,O-Diethyl thiophosphate
DF	Frequency of detection
DIA	Atrazine desisopropyl
DIAM	Atrazine desisopropyl mercapturate
DMCIP	Dimethyl chlorophosphate
DMCITP	Dimethyl chlorothiophosphate
DMP	Dimethyl phosphate
DMTP	Dimethyl thiophosphate
DP	Declustering potential
EFSA	European Food Safety Authority
EP	Entrance potential
ESI	Electrospray ionization
FAO	Food and Agriculture Organization
GC	Gas chromatography
GS	Ion source gas
HBM	Human biomonitoring
HCl	Hydrochloric acid
HRMS	High-resolution mass spectrometry
IMPY	2-Isopropyl-6-methyl-4-pyrimidinol
IQL	Instrumental quantification limit

IS	Internal standard
JECFA	Joint FAO/WHO Committee on Food Additives
LC	Liquid chromatography
LOQ	Quantification limit
m/z	Mass-to-charge ratio
MDA	Malathion dicarboxylic acid
ME	Matrix effect
MeOH	Methanol
MMA	Malathion monocarboxylic acid
MRM	Multiple reaction monitoring
MS	Mass spectrometry
Mw	Molecular weight
NHANES	National Health and Nutrition Examination Survey
OP	Organophosphate
QqQ	Triple quadrupole
RSD	Relative Standard Deviation
SD	Standard Deviation
SPE	Solid phase extraction
SW	Surface water
TCPY	3,5,6-trichloro-2-pyridinol
TOF	Time of flight
TP	Transformation product
t_R	Retention time
US EPA	United States Environmental Protection Agency
WBE	Wastewater-based epidemiology
WHO	World Health Organization
WM	Weighted mean
WW	Wastewater
WWTP	Wastewater treatment plant

Preface

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. 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. In order to raise food production, an increased pesticides use is expected. Taking into account that thousands of tons of pesticides are already applied every year in agriculture, homes, gardens, sport fields, and public areas, contamination of the environment most likely will further increase and human exposure to pesticides will be a matter of substantial concern in the near future.

In fact in the last few decades, the production of pesticides has increased significantly and there is concern in the scientific community about their toxicity and the side effects they could cause to man and environment. Monitoring of exposure to pesticides is important for public health and this has been stressed by national and international organizations and committees. The United States Environmental Protection Agency (U.S. EPA), the European Food Safety Authority (EFSA), the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO) and the Joint FAO/WHO Committee on Food Additives (JECFA) are some of the leading organizations whose duty is to provide scientific advice on safety issues, to protect public health and the environment from risks posed by pesticides and to assess risks to human health and the environment.

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. A wide range of exposure effects on human health have been studied

extensively, 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, depending on the route and level of exposure.

Human biomonitoring (HBM) has been widely used in monitoring programs and epidemiological studies to explore biomarkers of pesticide exposure in the general population or relevant groups. HBM is the main tool for assessing exposure and consists in the measurement of chemicals and/or their metabolites in body fluids or tissues. To date, HBM is the most widely used and powerful tool to assess human exposure to pesticides and inform organizations evaluating human health. 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 urine analysis of few subjects is designed to represent the whole population of an area. Furthermore, urine sampling can reflect only a momentary snapshot of exposure due to sampling procedures (e.g. 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). 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 is a tool to monitor patterns of factors related to human habits, human exposure to environmental and food toxicants and health and illness status within a population. This approach was originally developed to estimate illicit drug consumption in a population and was first applied in Italy to measure cocaine, heroin, cannabis and amphetamine use.

Wastewater-based epidemiology is based on the principle that almost everything entering the human body is metabolized and subsequently excreted with the urine and/or feces into the sewage system as a mixture of metabolites and/or parent compounds. The amount of a specific metabolic residue (biomarker) measured in raw urban wastewater reflects the amount of the parent compound consumed by the population served by the plant. This approach can provide objective, real-time information on substances directly or indirectly ingested by a population and has therefore the potential to complement and integrate information from epidemiological studies.

The main aim of this doctoral thesis was to test WBE as an alternative biomonitoring tool to evaluate pesticide intake in a population. This PhD explored for the first time the possibility of monitoring urinary metabolites of pesticides in urban wastewater as markers of human exposure. A liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the determination of a heterogeneous set of pesticides urinary metabolites was developed and validated. A solid phase extraction (SPE) procedure and a direct injection procedure were used for sample preparation. Moreover, all the potential sources of pesticides in wastewater were investigated and finally this novel approach was applied in several Italian and European cities.

The present PhD was part of the European Marie Curie "SEWPROF" Initial Training Network (7^{th} Framework) project entitled "A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level". The main object of "SEWPROF" was to provide an integrated approach towards public health monitoring at a community level based on innovative sewage epidemiology techniques. SEWPROF aimed to advance knowledge of the epidemiology of (illicit) drug use with the ultimate goal of applying this innovative interdisciplinary approach in

epidemiological studies of community-wide health, disease and lifestyle (http://sewprof-itn.eu).

This doctoral thesis consists of four scientific articles published/submitted in peer-reviewed journals. The contribution of the PhD candidate to first and second published scientific articles (results are reported in Chapters 3 and 4) consisted of designing the studies. He performed literature investigation, developed and validated the analytical protocol, analyzed samples, compiled and evaluated data, drafted and revised the manuscripts. This research has been performed independently under the supervision of Dr. Sara Castiglioni and Dr. Ettore Zuccato.

The contribution of the PhD candidate to third scientific article (results are reported in Chapter 5) consisted of planning and designing the study. He coordinated sampling, performed the experiments and analysis, compiled and evaluated data, drafted and revised the manuscript. This research has been performed independently under the supervision of Dr. Sara Castiglioni and Dr. Ettore Zuccato. This study performed in collaboration with several universities and private institutes in Europe and the collection of the wastewater samples was organized by all authors (co-authors of third scientific article), namely Nikolaos I. Rousis, Emma Gracia-Lor, Richard Bade, Jose Antonio Baz-Lomba, Erika Castrignanò, Ana Causanilles, Adrian Covaci, Pim de Voogt, Félix Hernàndez, Barbara Kasprzyk-Hordern, Juliet Kinyua, Ann-Kathrin McCall, Benedek Gy. Plósz, Pedram Ramin, Yeonsuk Ryu, Kevin V. Thomas, Alexander van Nuijs and Zhugen Yang.

The contribution of the PhD candidate to fourth scientific article (results are reported in Chapter 6) consisted of planning the study. This study resulted from a collaboration with Research Institute for Pesticides and Water, University Jaume I (Castellón, Spain). The PhD candidate and Richard Bade performed the sample

pretreatment and analysis. The PhD candidate compiled and evaluated data, drafted and revised the manuscript. This research has been performed independently under the supervision of Prof. Dr Félix Hernàndez, Prof. Dr. Juan V. Sancho and Dr. Lubertus Bijlsma.

The PhD candidate (Nikolaos I. Rousis) acknowledges the European Union's Seventh Framework Programme under Grant Agreement No. [Marie Curie-FP7-PEOPLE Grant #317205 - SEWPROF] for supporting this study.

Chapter 1

General introduction

1.1 Pesticides use

According to the latest regulation of the European Parliament and Council (European Commission, 2009), pesticides or "Plant Protection Products" are products, in the form in which they are supplied to the user, consisting of or containing active substances, safeners or synergists, and intended for one of the following uses:

- protecting plants or plant products against all harmful organisms or preventing the action of such organisms;
- influencing the life processes of plants;
- preserving plant products;
- destroying undesired plants or parts of plants;
- checking or preventing undesired growth of plants.

Prior to the 1940s, crop protection was performed by mechanical means, natural pesticides, poisonous plants, inorganic materials, including salts of lead, copper and arsenic, sulfur, and oil. Research into synthetic pesticides started between 1930 and 1940, where the first important compound, dichlorodiphenyltrichloroethane (DDT; organochlorine insecticide), was introduced during World War II by Paul Muller (Nobel Prize in Medicine, 1948). Since then, thousands of pesticides were synthesized and used. However, many of them were banned as a result of their harmful effects on environment, wildlife and humans. The use of organochlorine pesticides was restricted and other compounds, such as organophosphates, carbamates and pyrethroids, with improved properties (i.e. lower toxicity, higher selectivity to target organisms and lower persistence in soil, plants and the environment) were produced (Garcia et al., 2012; López et al., 2005; Wheeler, 2002).

Pesticides have an enormous field of applications and this is the main reason of their ubiquity. The principal use of pesticides is in agriculture, but also their activity in

other fields is as important. They are used in public health (e.g. control of disease vectors such as malaria), treatment of large structures (e.g. public and private buildings), maintenance of green areas (e.g. parks and golf courses), maintenance of water reserves (e.g. ponds), livestock and domestic care animals (e.g. disinfection of sheep), industries (e.g. paints, resins and for the preservation of fresh foods) and homes (e.g. insect repellents) (Garcia et al., 2012). In fact billions of dollars are spent every year in all sectors relative to these compounds in USA, Australia and the whole world. Moreover, billions of kilos are used annually worldwide; world pesticide amount used was approximately 2.4 billion kilos in both 2006 and 2007 (Grube et al., 2011; Heffernan et al., 2016).

Pesticides provide many benefits to mankind and environment, but they pose a risk of poisoning when they are not used judiciously. There are many types of positive outcomes from pesticide use that could be distinguished in three main domains (social, economic and environmental) and could be operated at community, national or global scales. For instance, pesticides control pests and plant disease vectors and this help to an improved crop and livestock yields, which leads to food security and quality and finally to a better national agricultural economy (Cooper and Dobson, 2007).

However, unjustified use of pesticides could be hazardous to humans and other living organisms as they are designed to be poisonous to specific organisms (e.g. weeds and fungi). Many studies investigated the link between exposure to pesticides and adverse health effects. Exposure to pesticides has shown positive associations with various diseases such as the development of the idiopathic Parkinson’s disease, neurobehavioral and neuropsychological disorders, respiratory symptoms or diseases and sperm DNA damage (Allen and Levy, 2013; Kim et al., 2016; Mamane et al., 2015; Saillenfait et al., 2015; Stallones and Beseler, 2016).

In the last few decades, the scientific community is concerned about the increased production of pesticides, since their toxicity and the side effects on human health and environment are well established (Barr, 2008; Skevas et al., 2013). Considering that the food production will be enhanced as a result of the global population growth at 9 billion people by the middle of this century, the application of pesticides will be increased considerably (Godfray et al., 2010).

1.2 Evaluation of human exposure

Pesticide monitoring is important for public health and national and international organizations and committees have stressed this. Human biomonitoring (HBM) has been adopted as the main and more potent tool in monitoring programs and epidemiological studies for assessing pesticide exposure in the general population or relevant groups (Barr, 2008; Yusa et al., 2015). The exposure routes of a subject to pesticides can be oral (dietary or non-dietary), cutaneous and respiratory according to the place of living, type of work etc. However, the general population is exposed to pesticides through consumption of contaminated foodstuff and household use (Aprea, 2012; McKinlay et al., 2008).

To date, the main tool for assessing exposure to pesticides of the general population is HBM studies, where biomarkers (pesticides and/or metabolites) are measured in human specimens, such as urine, blood and hair (Barr, 2008; Yusa et al., 2015). The preferred human biological fluid is urine, since its collection is easier and non-invasive, greater volume can be collected and analyte concentrations are normally higher compared to other fluids (e.g. blood) (Wessels et al., 2003).

Pesticide exposure of a community (general population) is evaluated by large HBM studies, which analyze urine samples of thousands of individuals (Barr et al., 2010,

2004; Heudorf and Angerer, 2001; McKelvey et al., 2013; Ye et al., 2015). However, an extensive effort is required to collect a representative number of samples and to harmonize the strategies to conduct surveys (Casteleyn et al., 2015). Moreover, HBM studies are negatively influenced by the long realization time, high cost, complexity and ethical issues. Therefore, novel methodologies are needed in order to track human exposure to pesticides at population level, overcoming some of the limitations of HBM studies.

1.3 Wastewater-based epidemiology

Wastewater-based epidemiology (WBE) approach consists in the chemical analysis of the wastewater produced by a population and sampled at the inlet of an urban wastewater treatment plant (WWTP), to study the collective exposure of the community to chemicals. This approach is based on the known principle that traces of almost everything is eaten, smoked, drunk, ingested and/or absorbed by individuals, are excreted with urine or stool, either unchanged or as metabolites, into the sewage system. Therefore, monitoring wastewater has the potential to extract useful epidemiological information from qualitative and quantitative profiling of biomarkers entering the sewage system. Generally, wastewater can hold a wealth of data on chemical consumption and exposure and potentially even the health status of whole communities (Arnold, 2016; Thomas and Reid, 2011; Zuccato et al., 2008).

The theory of performing monitoring of drugs and assessing the human drug consumption, using influent wastewater samples taken from WWTPs was first proposed by Daughton (2001). This approach was implemented for the first time in several Italian cities in 2005 by Zuccato et al. (2005), using cocaine and its major metabolite, benzoylecgonine, as an example to estimate the levels of cocaine consumption on those

communities (Zuccato et al., 2005). Following the successful application of the approach on cocaine, WBE was further implemented to opiates, cannabis and amphetamines (Zuccato et al., 2008).

Estimation of consumption/exposure of a chemical by a community is composed by multiple steps and each one of them has its own uncertainties and difficulties. The first step includes the collection of a representative composite 24-hour sample. Then, the quantified concentrations of biomarkers are multiplied by the daily wastewater flow rate and the daily mass loads are estimated (g/day). Mass loads are normalized to the number of people served by each WWTP (g/day/1,000 inhabitants), in order to compare results among different cities. The population served by each WWTP is calculated from census data when available, or using the wastewater flow rate and the "biological oxygen demand" of the raw wastewater. The normalized mass loads of the biomarkers are used to quantify the amount of the parent chemical that is consumed by the population served by each WWTP. Specific correction factors are applied for this reason (e.g. as it was described for illicit drugs Zuccato et al. (2008)), which take into account the molar mass ratio between the parent pesticide and the metabolite and the percentage of the parent compound excreted in human urine as the selected metabolite. A schematic representation of WBE is shown in **Figure 1.1**.

Nowadays this approach is commonly applied in several cities and countries worldwide and it starts to be considered a potential tool to complement traditional epidemiological studies and to evaluate drug consumption in the population. Several studies are now available in the literature, reporting estimates of illicit drugs consumption in Belgium (van Nuijs et al., 2009), Italy (Zuccato et al., 2011), France (Karolak et al., 2010), Croatia (Terzic et al., 2010), Spain (Postigo et al., 2010), Norway (Reid et al., 2011), Czech Republic (Baker et al., 2012), Greece (Thomaidis et al., 2016), the USA (Banta-Green et al., 2009) and Australia (Lai et al., 2013a). Moreover, this approach was

simultaneously applied in many European cities, making it possible to directly compare illicit drug loads in Europe over a one week period (Ort et al., 2014; Thomas et al., 2012).

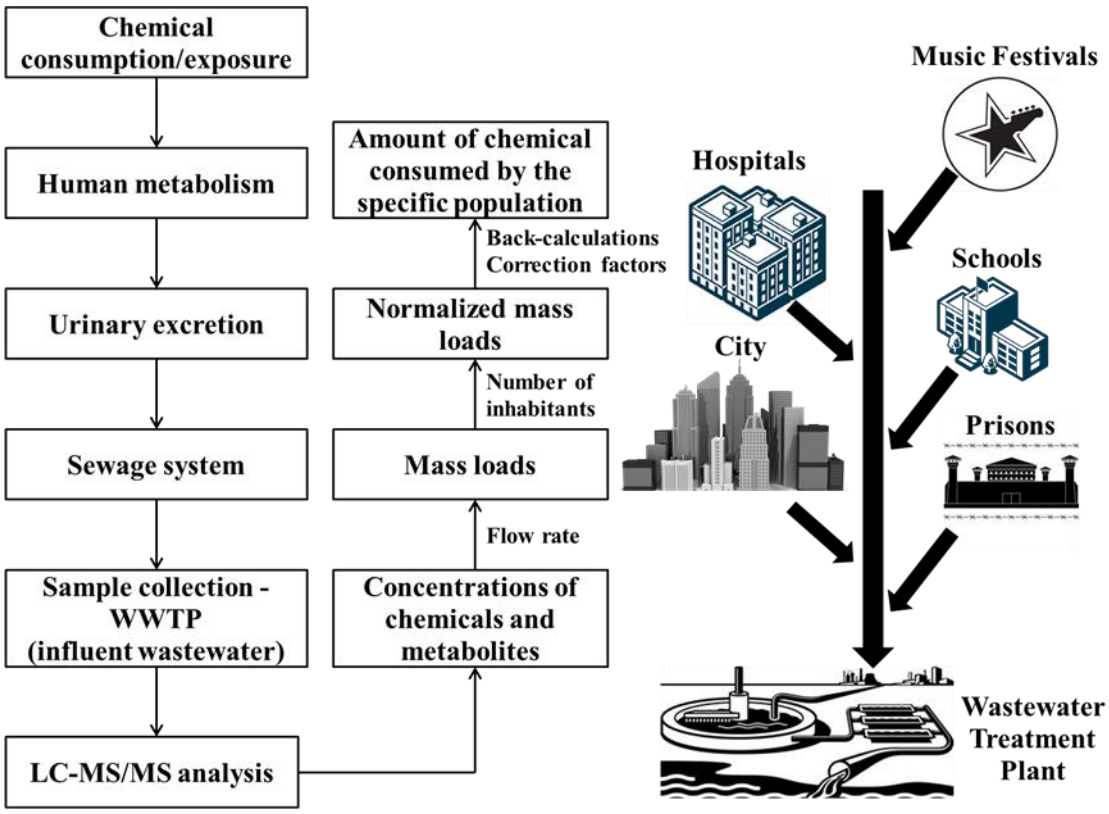

Figure 1.1: Schematic representation of wastewater-based epidemiology approach.

Wastewater-based epidemiology has also been applied to other chemical compounds like pharmaceuticals (Baker et al., 2014; van Nuijs et al., 2015), tobacco (Castiglioni et al., 2015) and alcohol (Mastroianni et al., 2014; Rodríguez-Álvarez et al., 2015) and many other potential wastewater biomarkers have been proposed (Chen et al., 2014; Daughton, 2012; Thomas and Reid, 2011). Furthermore, the WBE approach has been applied to smaller specific populations, such as schools (Panawennage et al., 2011), prisons (Postigo et al., 2011; van Dyken et al., 2016, 2014) and music festivals (Lai et al., 2013b).

There are many advantages of analyzing influent wastewater samples for human biomarkers. First of all, objective and real time information can be obtained, but at reduced complexity compared to other methodologies (Castiglioni et al., 2014). WBE approach is also able to identify rapidly any increase or/and decrease of particular substances within a surveyed area (Zuccato et al., 2011) and it could also be used as a tool to evaluate the effectiveness of preventive programs before, during and after the intervention (Burgard et al., 2014). Furthermore, wastewater analysis combined with chiral analysis can diagnose direct disposals of prescribed drugs in wastewater by measurements of active ingredients and their human urinary metabolites (e.g. case of the antidepressant fluoxetine) (Petrie et al., 2016).

On the contrary, there are several limitations or uncertainties of this approach that are associated with the stability of biomarkers in sewage (McCall et al., 2016), sampling mode of sewage (Ort et al., 2010), back-calculation methods (Gracia-Lor et al., 2016), reliability of analytical methods (Castiglioni et al., 2013; van Nuijs et al., 2011) and estimation of the population size (Rico et al., 2016). However, all these restrictions can be minimized using a "Best Practice Protocol". This common protocol of action was based on the current understanding of best-practice regarding sample collection, storage, and analytical procedure and developed within the SCORE network and it is now being used to conduct investigations at a European scale (Castiglioni et al., 2014, 2013). Finally, it has to be emphasized that WBE studies involve very low ethical risks (Prichard et al., 2014).

Generally, a wastewater treatment plant can be considered as the public "body" of a specific population and in principle the combination of information obtained through wastewater analysis with other epidemiological and demographic data can be used to monitor the health status of a population. The present approach has therefore the potential to be extended to other fields of investigation to track human consumption or exposure to

any chemical taken voluntarily or involuntarily by a subject, or to which the subjects are exposed. This can provide a valuable tool to obtain objective and direct information on the "real" levels of exposure of a specific population to different contaminants. WBE can be used as a biomonitoring tool that has the potential to monitor patterns of factors related to human habits, human exposure to environmental and food toxicants and health and illness status within a community (Thomas and Reid, 2011; Zuccato et al., 2008).

1.4 Wastewater-based epidemiology biomarkers

1.4.1 Requirements of specific WBE biomarkers

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. An ideal WBE biomarker should possess the following characteristics: a) be measurable in influent wastewater; b) be released into sewers only as a result of human excretion; c) have a well-defined excretion profile to avoid interference from other exogenous or endogenous sources; d) have a limited adsorption to suspended matter; e) be stable in wastewater during in-sewer transit, sampling and storage (Gracia-Lor et al., 2016).

Human urinary pharmacokinetic studies are essential for the development of correction factors, but there are several uncertainties related to these calculations (Castiglioni and Gracia-Lor, 2016). These studies require specific authorizations and adherence to strict ethical rules. Most of the available studies involve a small number of subjects, are quite old, do not include all the parent pesticides, and do not take into account the possible interactions between different pesticides. More research on this aspect should be done in order to refine and improve the estimates obtained through the WBE approach.

In WBE, the back-calculation currently used does not account for potential in-sewer transformation of the targeted WBE biomarkers. This can increase the uncertainty of estimates to an unknown degree. In-sewer stability experiments should account for all relevant processes occurring in sewer compartments: a) the bulk liquid (wastewater with suspended particulate matter); b) the biofilm growing on the sewer walls; c) the sediments; d) the sewer atmosphere in gravity sewers. It would be ideal to carry out in-sewer transformation studies in real sewers, but there are several factors making full-scale experiments often (too) laborious to obtain accurate results. Furthermore, due to varying environmental conditions that cannot be controlled during the experiments, several studies would be necessary (McCall et al., 2016).

1.4.2 Selection of potential WBE biomarkers

Recent results of large HBM studies found in the literature, such as the National Health and Nutrition Examination Survey in the USA and the Canadian Health Measures Survey in Canada, have reported that the main compounds commonly used as biomarkers of human exposure belong to different classes of pesticides (Barr, 2008). The main screened classes of pesticides were:

- Organophosphates: the six dialkyl phosphate metabolites which are products of the majority of organophosphate pesticides; the chlorpyrifos and chlorpyrifos-methyl specific metabolite, 3,5,6-trichloropyridinol; the malathion specific metabolites, malathion dicarboxylic acid and the a and b isomers of malathion monocarboxylic acid; diazinon specific metabolite 2-isopropyl-4-methyl-6-hydroxypyrimidine; and 4-nitrophenol, the non-specific metabolite of methyl and ethyl parathion and o-ethyl-o-(4-nitrophenyl) phenylphosphonothioate;

- Carbamates: the carbaryl non-specific metabolite 1-naphthol; 1- and 2-naphthol, the non-specific metabolites of naphthalene; the benomyl primary urine metabolite methyl-5-hydroxy-2-benzimidazole carbamate; the carbofuran metabolite 3-OH-carbofuran; the aldicarb major metabolites aldicarb sulphoxide and aldicarb sulphone; the hydroxypyrimidines 2-dimethylamino-5,6-dimethyl-4-hydroxy pyrimidine, 2-methylamino-5,6-dimethyl-4-hydroxy pyrimidine and 2-amino-5,6-dimethyl-4-hydroxypyrimidine, metabolites of pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yldimethyl carbamate);
- Pyrethroids: 3-phenoxybenzoic acid, a non-specific metabolite of about 20 synthetic pyrethroids; *cis*- and *trans*- isomers of 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid (*cis*- and *trans*-DCCA), the non-specific metabolites of permethrin, cypermethrin and cyfluthrin; cis-2,2-dibromovinyl-2,2-dimethylcyclopropane-1-carboxylic acid, a specific metabolite of deltamethrin; 4-fluoro-3-phenoxybenzoic acid, a specific metabolite of cyfluthrin;
- Triazines: the chlorotriazine herbicide metabolites hydroxyatrazine, atrazine mercapturate, diaminochlorotriazine, desethylatrazine, hydroxydesethylatrazine, desipropyl atrazine;
- The glyphosate metabolite aminomethylphosphonic acid.

In this doctoral thesis triazine, pyrethroid and organophosphate metabolites were selected as potential biomarkers of the WBE approach. The selection was performed according to specific criteria: a) concentration levels in urine; b) frequency of detection; c) most used classes of pesticides; d) potential human health effects; e) source of metabolites (human excretion and/or environmental).

The available information on human excretion profiles of these metabolites was collected, when available, in order to develop specific correction factors and evaluate human exposure through a WBE approach.

Atrazine is one of the most studied compounds of triazines and its urinary metabolism has been studied to both animals and humans (**Figure 1.2**). Atrazine is excreted with urine unmodified at 2% and its metabolites consist of 80% bi-dealkylated, 10% deisopropylated and 8% deethylated (Barr et al., 2007; Pozzebon et al., 2003). Some of the metabolites are also common products from other atrazine-related compounds, like simazine, propazine and terbuthylazine.

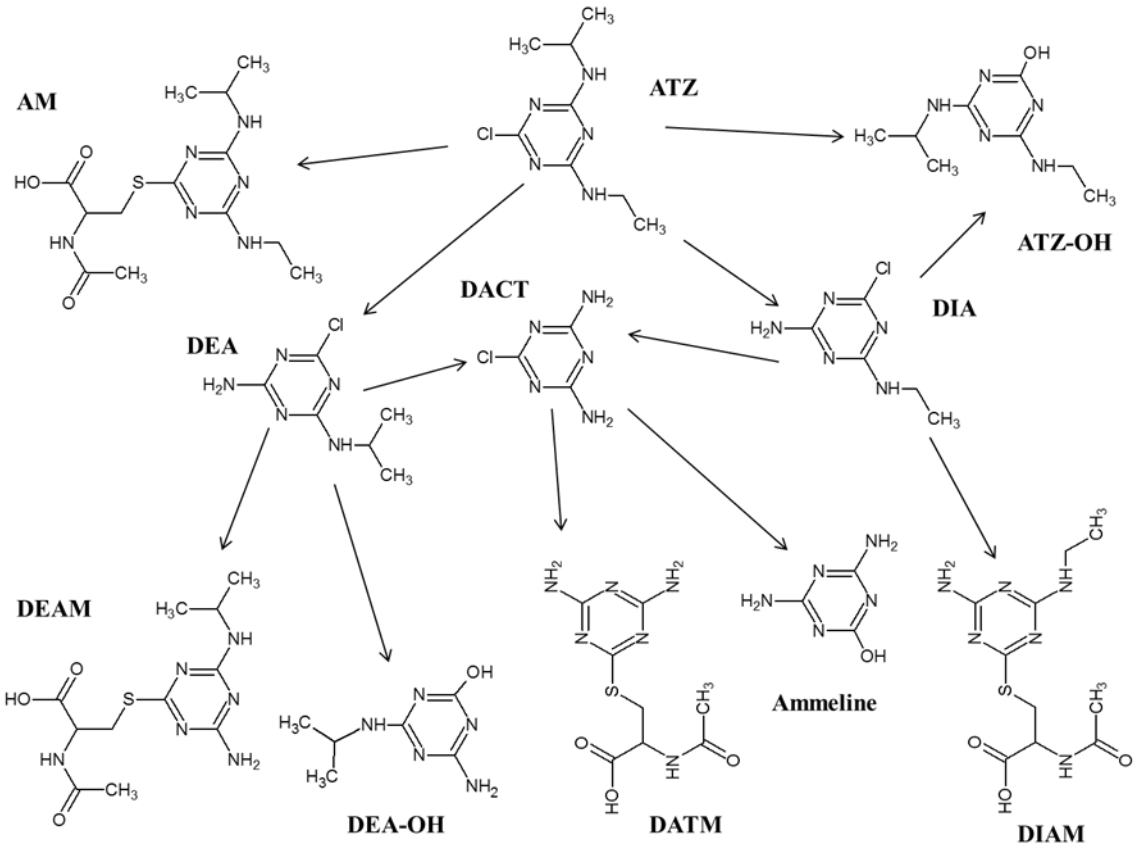

Figure 1.2: Proposed metabolism of atrazine: Atrazine, ATZ; Hydroxy-atrazine, ATZ-OH; Atrazine mercapturate, AM; Atrazine desethyl desisopropyl, DACT; Diaminotriazine mercapturate, DATM; Atrazine desethyl, DEA; Atrazine desethylmercapturate, DEAM; Hydroxy desethyl atrazine, DEA-OH; Atrazine desisopropyl, DIA; Atrazine desisopropyl mercapturate, DIAM (Barr et al., 2007).

Permethrin, cypermethrin and cyfluthrin have DCCA as a common urinary metabolite. The excretion rate, after an oral dose of the parent pesticide, varies from 19%

to 49% for *cis*-DCCA and 33-78% for *trans*-DCCA (Eadsforth et al., 1988; Eadsforth and Baldwin, 1983; Hays et al., 2009; Leng et al., 1997a; Ratelle et al., 2015a, 2015b; Woollen et al., 1992). Pharmacokinetic studies performed in human subjects have proved that following dermal administration of the parent pyrethroids, the *trans*- to *cis*-DCCA ratio varied from 0.85 to 1.2 (Woollen et al., 1992) and following oral and/or inhalation administration this ratio ranged from 1.5 to 3.3 (Eadsforth et al., 1988; Eadsforth and Baldwin, 1983; Kühn et al., 1999; Leng et al., 1997a, 1997b, Ratelle et al., 2015a, 2015b; Woollen et al., 1992).

Chlorpyrifos and chlorpyrifos-methyl are two broad-spectrum chlorinated organophosphate insecticides which are both metabolized to 3,5,6-trichloropyridinol. There is only one work available which has studied the human metabolism of chlorpyrifos and it was found that 70% is excreted as 3,5,6-trichloropyridinol in human urine (Nolan et al., 1984). Malathion is an organophosphate insecticide with many uses in different areas. Following oral exposure, it is further metabolized to the specific metabolites malathion dicarboxylic acid and malathion monocarboxylic acid in 9% and 36% respectively (Bouchard, 2003).

1.5 Chemical analysis

The occurrence and fate of emerging contaminants (e.g. pesticides) and their secondary products in the aquatic environment has attracted much attention over the years, since these compounds can cause known or suspected adverse ecological and/or human health effects. The principal goal of researchers is to develop sensitive methods for the quantification of these compounds at the low ng/L level.

Liquid chromatography (LC) and gas chromatography (GC) remain the primary analytical techniques combined mainly to mass spectrometry (MS) for water analysis.

One of the latest and hottest trends is the combination of high-resolution mass spectrometry (HRMS) with LC for identifying particular environmental transformation products (TPs). Solid phase extraction (SPE) continues to be the most popular technique of clean-up, concentration and extraction for emerging contaminants in waters. New SPE sorbents are developed, but Oasis HLB and MCX remain the most used types of cartridges.

Pesticides continue to be the focus of much environmental research. Lately the investigation has focused more on their TPs and/or metabolites, since are usually present in the environment at greater levels than the parent pesticides and can be as toxic or more toxic. LC-MS has become ideal for the detection of pesticide TPs and metabolites, since these compounds are generally more polar than the parent pesticides (Richardson and Kimura, 2016; Richardson and Ternes, 2014).

Furthermore, analytical chemistry is the strong base of the WBE approach. Quantification of WBE biomarkers in wastewater performed with advanced analytical instruments, such as LC-MS, which is one of the most powerful techniques to detect simultaneously different molecules in various complex matrices with high specificity and selectivity.

1.5.1 Sample pretreatment

Determination of pesticide metabolites in environmental water samples is not an easy task, since they are presented at very low concentrations. A further complication on their determination is the complexity of the matrix (e.g. influent wastewater samples). Therefore, a clean-up and pre-concentration step is usually applied before the instrumental analysis.

Influent wastewater samples contain a high amount both of organic material and solid particles that could cause many problems during the subsequent stages of analysis (e.g. clogging of the SPE cartridges). Consequently, a filtration or centrifugation step is mainly applied. There are different kind of filters, having different diameter, filter supports and physical form. The main limitation is that some analytes could be retained in the filter material leading to significant losses. Centrifugation is an additional and/or alternative technique to filtration, but potential analytes co-precipitation with suspended particles is a real possibility (Locatelli et al., 2016).

Next steps include pH adjustment, extraction, further clean-up and pre-concentration procedures in order to ensure that the analytes are found at a suitable concentration level. Rapid, inexpensive, efficient and environmentally friendly sample preparation techniques have been developed for these actions. Some of the techniques are SPE, solid phase microextraction, microwave assisted extraction and liquid-liquid extraction.

The most widely used sample preparation technique for environmental samples is SPE, since it was proved to be the most powerful tool for the isolation and purification of the analytes. The advantages include simplicity, flexibility, high selectivity, automation, rapidity, high enrichment factors, and the absence of emulsion and use of different sorbent materials. The main disadvantages of SPE include disposable cartridges and discs with a special manifold (Dimpe and Nomngongo, 2016).

The basic SPE procedure is composed of four steps (**Figure 1.3**). Conditioning and equilibration is the first one and consists of passing organic solvents and water through the column in order to "activate" the sorbent (increase effective surface area) and create an environment similar to the sample. Then the sample is loaded onto the solid phase, where the target analytes are retained and the undesired components are washed

away. The washing step (application of a solvent, different from the elution solvent) removes interferences, while the analytes are retained on the sorbent. Finally, the desired analytes are eluted with a suitable solvent into a collection tube (Andrade-Eiroa et al., 2016).

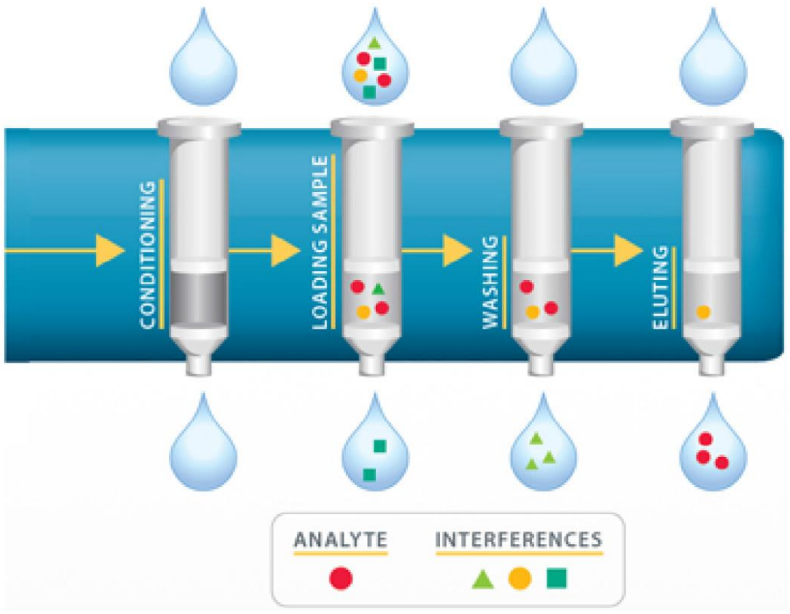

Figure 1.3: Typical steps of SPE (Andrade-Eiroa et al., 2016).

1.5.2 Liquid chromatography-tandem mass spectrometry

Liquid chromatography (LC) is a chromatographic separation technique in which the components to be separated are selectively distributed between two immiscible phases: a liquid mobile phase is flowing through a stationary phase bed. The chromatographic process occurs as a result of repeated adsorption/desorption steps during the movement of the analytes along the stationary phase. The separation is due to the differences in distribution coefficients of the individual analytes in the sample (Niessen, 2006).

The most widely used LC separation technique for pesticides is reverse-phase LC with non-polar stationary phases, since the new pesticides and their metabolites are more polar than the older ones. The correct choice of the column and mobile phase is important to achieve a good separation not only between analytes but also between analytes and some polar and apolar interfering compounds. The mobile phase is selected as a compromise between optimal chromatographic separation and good ionization efficiency and overall MS performance. The most used organic solvents in LC-MS applications are methanol and acetonitrile and the most popular additives are acetic acid, formic acid, ammonium hydroxide, ammonium acetate, and ammonium formate. These additives are added with the aim to enhance the ionization efficiency and consequently enhanced sensitivity and to improve the chromatography (e.g. better peak shape) (Botitsi et al., 2011; Masiá et al., 2014).

One of the most crucial parts of the LC-MS system is the interface, which is found among the LC and the MS analyzer. The most widely used atmospheric pressure ionization methods for determining pesticides in water samples are: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). ESI is considered more suitable for polar and ionic compounds with a wide range of molecular masses, while APCI is more adequate for non-polar and medium polarity compounds. However, both ESI and APCI allow the efficient ionization of a wide spectrum of compounds with varying polarities and wide linear dynamic ranges. Generally, ESI has found much wider use than APCI in pesticide multi-residue methods applied to water analysis; more than 88% of reviewed references are based on ESI sources interfaced to different mass analyzers (Botitsi et al., 2011).

ESI is a process that produces a fine spray of charged droplets under the influence of an intense electric field. Evaporation of the solvent converts those charged droplets into gas-phase ions (**Figure 1.4**). The sample solution in a suitable solvent mixture flows

continuously through a stainless steel capillary tube, whose tip is held at a high potential (3 to 4 kV). Due to the high voltage the solution is dispersed into charged droplets. Evaporation of the charged droplets is assisted by a flow of heated gas, generally nitrogen. Finally, the formed ions are transported from the atmospheric-pressure region to the high vacuum of the mass analyzer via a series of pressure-reduction stages (Dass, 2007).

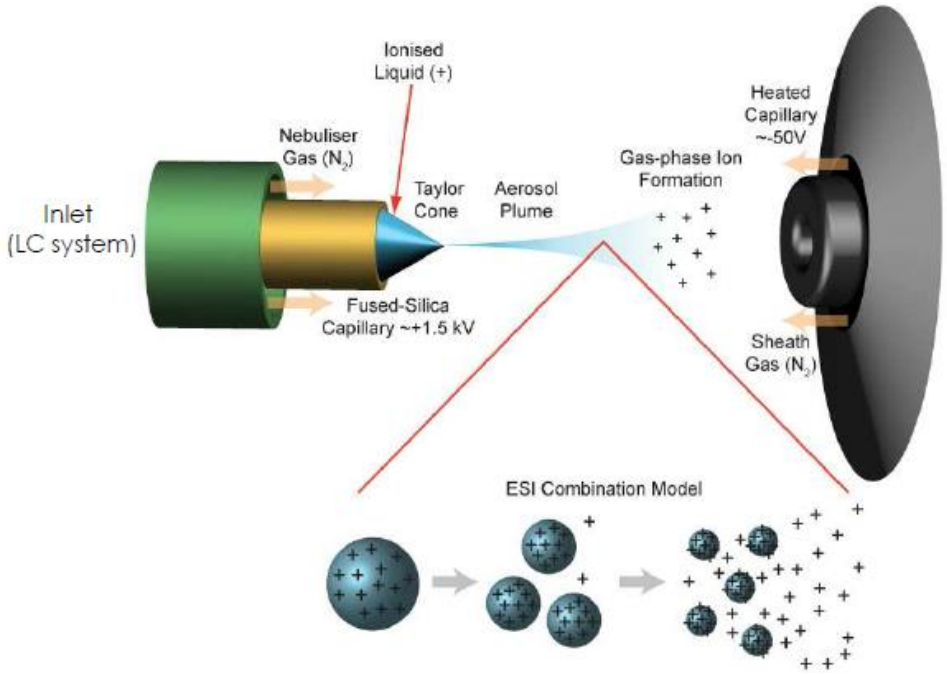

Figure 1.4: Principle of the ESI (www.lamondlab.com).

Mass spectrometry (MS) is an analytical technique that measures the molecular masses of individual compounds and atoms precisely by converting them into charged ions. MS measurements deal with ions because unlike neutral species, it is easy to manipulate the motion and direction of ions experimentally and detect them. MS analysis involves three basic steps (Dass, 2007):

1. Ionization converts analyte molecules or atoms into gas-phase ionic species. This step requires the removal or addition of an electron or proton(s). The excess

energy transferred during an ionization event may break the molecule into characteristic fragments.

2. The next step is the separation and mass analysis of the molecular ions and their charged fragments on the basis of their mass-to-charge (m/z) ratios.
3. The ion current due to these mass-separated ions is measured, amplified, and displayed in the form of a mass spectrum.

Many different mass analyzers have been successfully applied to pesticide determination, such as triple quadrupole (QqQ), ion trap, orbitrap and time-of-flight. All of them have different strengths and weaknesses depending on the requirements of the particular analysis (Masiá et al., 2014).

The most widely used mass analyzer for pesticide determination in water samples is QqQ. The main reasons of employing a QqQ are the high sensitivity, selectivity and specificity, wide linear dynamic range, low detection limits and precision (Botitsi et al., 2011; Kuster et al., 2006).

A QqQ consists of two mass analyzers (MS1 and MS2) with a collision cell in the middle. A selected ion in MS1 passes to collision cell, where an inert gas is applied and the ion produces fragment ions. This process is called collision-induced dissociation (CID). Fragment ions pass to MS2 where they are filtered or scanned. Different acquisition modes can be used, according to the objective of the study. The four main modes are presented in **Figure 1.5**.

Product ion scan: The first analyzer (MS1) is set to a value that selects one specific precursor ion at a time. The selected ion undergoes CID in the collision cell, and the resulting fragments are analyzed by the second analyzer (MS2). This process is repeated for different precursors (Domon, 2006).

Precursor ion scan: Precursor ion scanning sets the second analyzer (MS2) to transmit only one specific fragment ion to the detector. MS1 is scanned to detect all the precursor ions that generate this fragment. Typically, this method is used to detect a subset of compounds in a sample that contain a specific functional group (Domon, 2006).

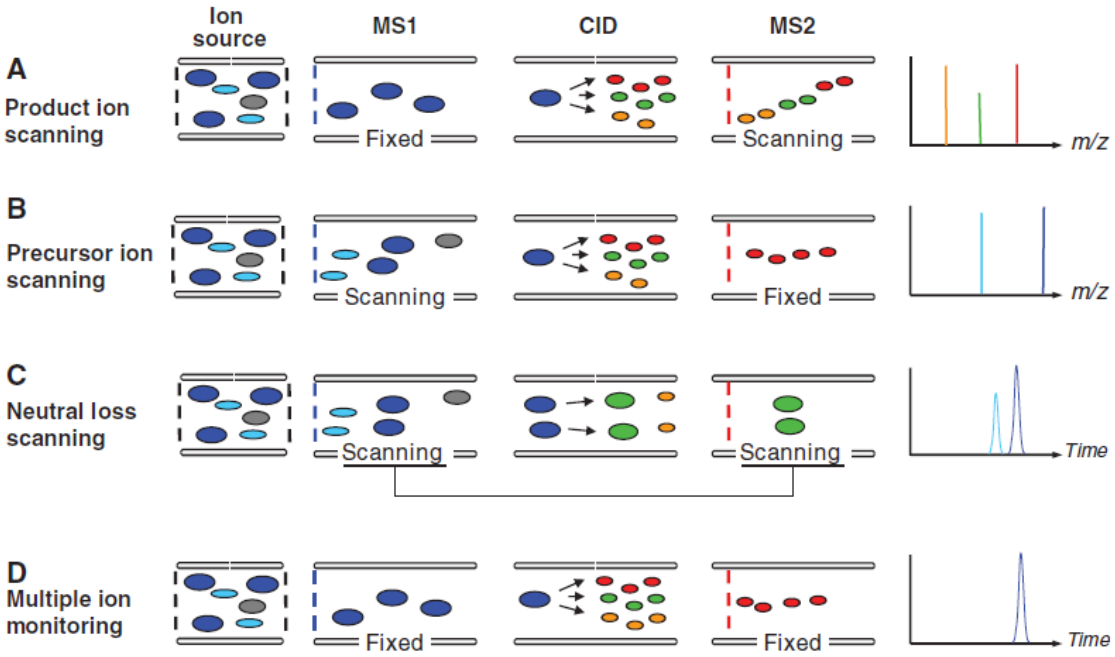

Figure 1.5: Main acquisition modes in QqQ (Domon, 2006).

Neutral loss scan: Neutral loss scanning scans both analyzers in a synchronized manner, so that the mass difference of ions passing through MS1 and MS2 remains constant. The mass difference corresponds to a neutral fragment that is lost from an ion in the collision cell. The neutral loss scan is therefore used to detect those compounds in a sample that contain a specific functional group (Domon, 2006).

Multiple ion monitoring: Multiple reaction monitoring (MRM) consists of a series of short experiments in which one precursor ion and one specific fragment characteristic for that precursor are selected by MS1 and MS2, respectively. Typically, the instrument cycles through a series of transitions (precursor-fragment pair) and records the signal as

a function of time (chromatographic elution). MRM is used for the detection of a specific analyte with known fragmentation properties in complex samples (Domon, 2006).

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Chapter 2

Aim of this Doctoral thesis

Aim

The main objective of this doctoral thesis was to develop novel applications of the wastewater-based epidemiology approach. The present study explored for the first time the possibility of monitoring urinary metabolites of pesticides in urban wastewater as markers of human exposure and measuring the collective exposure of the population to pesticides. Determination of human metabolic residues (biomarkers) in influent wastewater was performed using liquid chromatography-tandem mass spectrometry, in order to evaluate the collective exposure of a community to environmental chemicals and food toxicants.

The objectives of the thesis included:

- Selection of potential biomarkers of human exposure to chemicals. The selection was operated among different classes of environmental and food contaminants, particularly considering substances with endocrine disruptor activity. The main sources were human biomonitoring studies (articles in peer reviewed journals), priority pollutant lists of the European Union and the United States Environmental Protection Agency and reports from the Centers for Disease Control and Prevention.
- Development of an analytical methodology for the simultaneous determination of pesticides and their human urinary metabolites in influent wastewater.
- Optimization of the instrumental parameters (liquid chromatography and mass spectrometry) and the SPE technique.
- Validation of the developed method to ensure acceptable analytical quality criteria.
- Quantification of the selected biomarkers in influent wastewater, taken from WWTPs of many cities in Italy, covering virtually the whole country.

- Performance of stability tests to ensure that no losses of the biomarkers could be occurred before analysis and no spontaneous formation of the metabolites could be performed.
- Investigation of the source of the targeted biomarkers in influent wastewater.
- Development of novel wastewater-based epidemiology approaches. This phase was based on the development of new correction factors by considering the human urinary metabolism.
- Application of the novel WBE – Europe wide monitoring of biomarkers in influent wastewater.

Chapter 3

Monitoring population exposure to pesticides based on liquid chromatography-tandem mass spectrometry measurement of their urinary metabolites in urban wastewater: A novel biomonitoring approach

3.1 Introduction

One of the first objectives of this doctoral thesis was the development of an analytical method for the determination of pesticide metabolites at very low concentration levels in influent wastewater. In this chapter, is presented the development, validation and application of an advanced analytical methodology for the quantification of fifteen pesticide metabolites and three parent pesticides. These compounds were selected as potential biomarkers of exposure from the major classes of pesticides, namely triazines, organophosphates and pyrethroids. A liquid chromatography tandem mass spectrometry method, was used and a solid phase extraction procedure using Oasis HLB cartridges was applied for clean-up and pre-concentration of the complex samples. The developed method was employed for the analysis of influent wastewater samples collected in Italy.

The results of this study have been published in:

- ✓ *Science of the Total Environment*, **2016**, 571, 1349-1357

3.2 Material and methods

3.2.1 Chemicals and reagents

Hydrochloric acid (HCl, 37%) and acetonitrile for 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 of diethyl phosphate (DEP, purity 97.6%), chlorpyrifos (CPF, purity 99.9%), chlorpyrifos methyl (CPF-MET, purity 99.5%), 3,5,6-trichloro-2-pyridinol (TCPY, purity 99.5%) and atrazine desethyl desisopropyl (DACT, purity 96.7%) were purchased from Chemical Research 2000 (Rome, Italy). Atrazine (ATZ, purity 97.5%), atrazine desethyl (DEA, purity 99.9%), terbuthylazine 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%), malathion monocarboxylic acid (MMA, purity 97.0%) and malathion dicarboxylic acid (MDA, purity 99.0%) were purchased from Lab Service Analytica (Bologna, Italy). Structures and molecular weights of the analytes are shown in **Figure 3.1**.

Isotopically labeled compounds (deuterated or ^{13}C-enriched) were used as internal standards (IS). 3-Phenoxybenzoic acid-C_6 (3-PBA-C_6, 99%; purity 98%) and 3,5,6-trichloro-2-pyridinol-C_3 (TCPY-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).

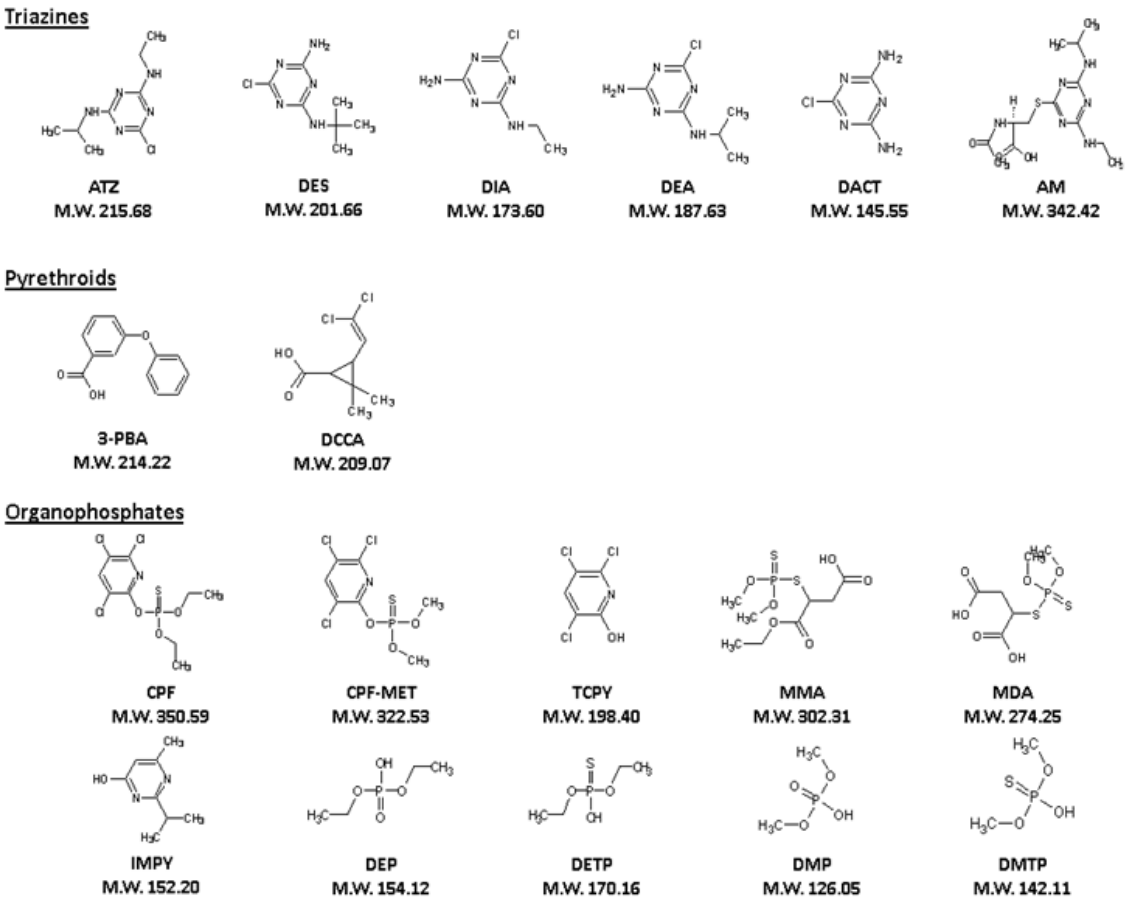

Figure 3.1: Molecular structures and molecular weights (M.W.) of the targeted compounds: Atrazine, ATZ; Terbuthylazine desethyl, DES; Atrazine desisopropyl, DIA; Atrazine desethyl, DEA; Atrazine desethyl desisopropyl, DACT; Atrazine mercapturate, AM; 3-Phenoxybenzoic acid, 3-PBA; 3-(2,2-Dichlorovinyl)-2,2-dimethyl-(1-cyclopropane)carboxylic acid, DCCA; Chlorpyrifos, CPF; Chlorpyrifos methyl, CPF-MET; 3,5,6-Trichloro-2-pyridinol, TCPY; Malathion monocarboxylic acid, MMA; Malathion dicarboxylic acid, MDA; 2-Isopropyl-6-methyl-4-pyrimidinol, IMPY; Diethyl phosphate, DEP; O,O-Diethyl thiophosphate, DETP; Dimethyl phosphate, DMP; Dimethyl thiophosphate, DMTP.

Dimethyl phosphate (DMP) and dimethyl thiophosphate (DMTP) were synthesized by simple hydrolysis of DMCIP and DMCITP (Hernández et al., 2002). Briefly, a solution of 740 μL Milli-Q water/acetonitrile (10:30), with 60 μL of triethylamine was prepared and stirred at 0°C (total volume 800 μL). Then 100 mg of DMCIP or DMCITP were dissolved in this solution and acetonitrile was added to a final

volume of 4 mL. The final concentrations of DMP and DMTP were 21,800 and 22,118 mg/L. The synthesis of DMP and DMTP was considered fully quantitative, because no chlorophosphate precursor ions (m/z 144 for DMP and m/z 160 for DMTP) were found in the mass spectra of the synthesized compounds.

3.2.2 Selection of analytes

The selected compounds were three widely used parent substances and 14 urinary metabolites: among triazines, ATZ and the metabolites DES, DIA, DEA, DACT and AM; among pyrethroids, the metabolite of about 20 synthetic pyrethroids (3-PBA) and the metabolite of permethrin, cypermethrin and cyfluthrin (cis- and trans-DCCA); among organophosphates, four alkyl phosphate metabolites (DEP, DETP, DMP and DMTP) which are metabolites to the majority of organophosphates; CPF, CPF-MET and their metabolite, TCPY; the metabolites of malathion (MDA and a and b isomers of MMA); and the metabolite of diazinon (IMPY).

Analytes were selected by screening the reports of HBM in the general population, to identify the main compounds commonly used as biomarkers of human exposure to the different classes of pesticides (Yusa et al., 2015). Although some of these substances can be formed in the environment as degradation products (**Table 3.1**), and can potentially affect the correlation with the exposure to the parent substances, they are still the best and most common used biomarkers to evaluate human exposure. This is also because no clear information is available about the amounts of biomarkers coming from environmental degradation. Thus, the same substances used in HBM were chosen to be detected in wastewater as biomarkers of exposure of an entire community. Following the main requirements to choose an appropriate biomarker, pesticide metabolites were selected according to: a) frequency of detection in the population; b)

concentration in urine; c) specificity from human excretion; d) most used types of pesticides (herbicides, insecticides) (**Table 3.1**).

Table 3.1: Main characteristics of the selected urinary metabolites including the urinary levels from HBM studies and their specificity to the parent substance

Pesticide metabolites selected as biomarkers of exposure	Urinary levels from HBM studies (range in µg/L)*	Specificity as biomarkers	
		Parent compounds	Other potential sources
Triazines			
DES	-	terbuthylazine atrazine,	-
DIA, DACT	‹LOD – 400	terbuthylazine, simazine, propazine	Environmental transformation products (Barr et al., 2007)
DEA		atrazine	
AM		atrazine	-
Pyrethroids			
3-PBA	‹LOD – 90	20 pyrethroids	Residential dust (Starr et al., 2008)
trans-DCCA	‹LOD – 121	Permethrin, cypermethrin, cyfluthrin	no information, to be assessed
cis-DCCA	‹LOD – 91	Permethrin, cypermethrin, cyfluthrin	no information, to be assessed
Organophosphates			
TCPY	‹LOD – 445	Chlorpyrifos, chlorpyrifos methyl	Dust, food (Wilson et al., 2003)
MMA	n.a.	Malathion	Degradation from parent substances after application (Chen et al., 2012)
MDA	‹LOD – 58	Malathion	Degradation from parent substances after application (Chen et al., 2012)
IMPY	‹LOD – 150	Diazinon	Environmental transformation product (Morgan et al., 2011)
DEP	‹LOD – 417		
DETP	‹LOD – 690	Several OP	OP flame retardants,
DMP	‹LOD – 660	insecticides	plasticizers and
DMTP	‹LOD – 505		industrial chemicals (Reemtsma et al., 2011)

*(Barr et al., 2007; Yusa et al., 2015)

The United States Environmental Protection Agency report on pesticides production and use in the U.S. and worldwide (Grube et al., 2011) reported in 2006 - 2007, herbicides and insecticides market was around 70% of the pesticide expenditure in the world and 80% in the U.S. The expenditures accounted 72% for the herbicides in the agriculture sector, and 55% for the insecticides in the non-agricultural sectors (industry/commercial/government and home and garden). In the U.S. agricultural market atrazine was one of the most used herbicides (ranked second to glyphosate) and chlorpyrifos was the most used insecticide, while in the non-agricultural sector pyrethroids, malathion and carbaryl were the most used insecticides. In the end, chlorpyrifos, malathion and diazinon were classified as the most commonly used organophosphate insecticides in both sectors in the U.S., ranking positions in the top ten of the list.

3.2.3 Sample collection

Raw wastewater samples were collected from the entrance of WWTPs in seven cities in Italy, covering virtually the whole country. Three cities (Cremona, Milan, Merano) are in the north of Italy, two (Florence, Terni) in the center and two (Bari, Palermo) in the south. The majority are “medium” size cities, with inhabitants from 50,000 to 500,000; Milan is the largest city with 1,300,000 inhabitants.

Composite 24-h samples of untreated wastewater were collected by automatic sampling devices from each plant. Sampling was carried out in May 2014 and seven consecutive samples were collected to check for inter-day variability, except for Cremona where only two samples were collected. Samples were collected in 500-mL polyethylene terephthalate bottles, frozen immediately and stored at -20°C until extraction.

3.2.4 Sample pretreatment

Samples were filtered on a glass microfiber filter GF/A 1.6 μm (Whatman, Kent, U.K.) then on a mixed cellulose membrane filter 0.45 μm (Whatman, Kent, U.K.) before extraction. SPE was used to extract the target analytes using disposable 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 fortified with 2 ng of a mixture of IS and the pH was adjusted to 7.0-7.5 using diluted HCl (12%) when necessary. Cartridges were conditioned with 5 mL of MeOH and equilibrated with 3 mL of Milli-Q water. Then the samples were passed through the cartridges at a flow rate of 5 mL/min, and finally the cartridges were dried under a nitrogen stream at a flow rate of 10 mL/min for 10 min. The analytes were eluted with 3 mL of MeOH and the eluates were then evaporated under a gentle nitrogen stream. Dried samples were reconstituted in 100 μL of Milli-Q water, centrifuged (2500 rpm; 2 min) and transferred into glass vials for LC-MS/MS analysis.

The highly polar alkyl phosphate analytes DEP, DETP, DMP and DMTP were poorly recovered on different SPE cartridges, so direct injection into the LC-MS/MS system was tested and adopted; 500 μL of filtered samples were centrifuged at 2500 rpm for 2 min and 180 μL of the supernatant were collected, spiked with 20 μL of 0.1 ng/μL IS and transferred into glass vials for LC-MS/MS analysis.

3.2.5 Liquid chromatography – tandem mass spectrometry

Chromatographic separation was done with an Agilent 1200 Series system (Agilent Technologies, Santa Clara, CA, USA) using an XSELECT™ CSH™ C18 (2.1 × 100 mm, 2.5 μm) column (Waters Corp., Milford, MA, USA). The chromatographic separation was performed using as mobile phase A Milli-Q water acidified with 0.1%

v/v acetic acid and mobile phase B 100% acetonitrile. The gradient began in the positive mode at 98% solution mobile phase A and decreased to 0% over 10 min. It was held for 4 min and then increased to 98% over 1 min. It remained for 10 min at 98% to condition the column. The total run time was 25 min. In the negative mode the gradient started at 99% mobile phase A, which was decreased linearly to 0% over 12 min. It was kept under these conditions for 4 min and then reverted to the initial conditions in 1 min. It remained for 9 min at 99% to condition the column. The entire time of a run was 26 min. The flow rate was set at 180 μL/min and the injection volume was 4 μL.

Mass spectrometric analysis was carried out using an AB SCIEX Triple Quad™ 5500 LC–MS/MS System (AB-Sciex, Thornhill, Ontario, Canada). The Turbo Ion Spray Source settings in positive ionization mode were: curtain gas (CUR) 25; collision gas (CAD) 7; source temperature 350°C; ion source gas 1 (GS1) 40; ion source gas 2 (GS2) 40; ion spray voltage 5500 V. For the negative ionization mode, parameters were the same except the ion spray voltage which was -4500 V. Nitrogen was used as CUR and CAD. The mass spectrometric analytical parameters were optimized by infusion experiments of the individual standards in MeOH/Milli-Q water acidified with acetic acid 0.1% (0.5 or 1 ng/μL, 50:50 v/v) in continuous-flow mode. For the analyses the two or three most abundant product ions of the protonated pseudo-molecular ion of each substance were chosen. Analysis was done in positive and negative ionization modes using the selected reaction monitoring mode under time-scheduled conditions and setting a time window of 180 s. Precursor and product ions for analytes and IS, with the optimized instrumental parameters and retention times are shown in **Table 3.2**.

Table 3.2: Analytical parameters optimized for MS/MS analysis of the selected analytes and retention time. Ions used as quantifiers are indicated in bold

Compound	t_R (min)	Ion mode	Internal standard	MRM transitions				
				Declustering potential (DP, V)	Entrance potential (EP, V)	Precursor ion, m/z	Product ions, m/z and collision energy (CE, eV)	Collision exit potential (CXP, eV)
Triazines								
ATZ	8.4	+	ATZ-D_5	60	10	216	**96 (32)** 174 (23)	12 12
DES	7.7	+	ATZ-D_5	45	10	202	**146 (21)** 79 (36)	12 12
DIA	5.5	+	ATZ-D_5	50	8	174	**68 (33)** 96 (24) 104 (31)	10 10 10
DEA	6.3	+	ATZ-D_5	60	12	188	**146 (23)** 79 (33) 104 (34)	10 10 10
DACT	4.1	+	ATZ-D_5	115	13	146	**68 (30)** 79 (24)	10 10
AM	6.5	+	ATZ-D_5	60	11	343	**214 (29)** 172 (40)	15 15
Pyrethroids								
3-PBA	12.0	-	3-PBA-C_6	-60	-10	213	**93 (-30)** 169 (-16)	-8 -10
DCCA	11.8/12.1	-	TCPY-C_3	-65	-10	207 209	**35 (-40)** 35 (-40) 37 (-40)	-15 -15 -15

Organophosphates								
CPF	12.1	+	$CPF\text{-}D_{10}$	55	12 11	350 352	**198 (27)** 200 (27)	12 11
CPF-MET	11.2	+	$CPF\text{-}D_{10}$	55	12 11	322 324	**125 (26)** 125 (27)	8 8
TCPY	13.0	-	$TCPY\text{-}C_3$	-55	-11	198 196	**37 (-44)** 35 (-44) 35 (-44)	-16 -16 -16
MMA (isomer 1)	10.8	-	$TCPY\text{-}C_3$	-35	-10	301	**142 (-42)** 157 (-18)	-10 -10
MMA (isomer 2)	10.8	-	$TCPY\text{-}C_3$	-35	-10	301	**126 (-44)** 141 (-15)	-10 -10
MDA	8.7	-	$TCPY\text{-}C_3$	-40	-10	273	**141 (-12)** 157 (-28)	-10 -10
IMPY	4.8	+	$ATZ\text{-}D_5$	60	12	153	**84 (24)** 70 (25)	10 10
DEP	6.2	-	$TCPY\text{-}D_3$	-50	-7	153	**79 (-28)** 125 (-15)	-7 -9
DETP	7.4	-	$TCPY\text{-}C_3$	-50	-10	169	**95 (-26)** 141 (-15)	-8 -8
DMP	5.0	-	$TCPY\text{-}C_3$	-50	-10	125	**79 (-35)** 63 (-23)	-8 -8
DMTP	6.0	-	$TCPY\text{-}C_3$	-50	-10	141	**79 (-19)** 63 (-41) 95 (-26)	-8 -8 -8
Internal standards								
$ATZ\text{-}D_5$	8.4	+		60	10	221	**179 (24)**	12
$CPF\text{-}D_{10}$	12.0	+		55	12	362	**99 (51)**	8
$TCPY\text{-}C_3$	13.0	-		-55	-11	199	**35 (-45)**	-16
$3\text{-}PBA\text{-}C_6$	12.0	-		-60	-10	219	**99 (-28)**	-8

3.2.6 Method validation and stability tests

The method was validated in terms of scope, specificity, accuracy, sensitivity, and repeatability. The recoveries and repeatability were tested in raw wastewater (triplicate analysis) by spiking 100 ng/L of the targeted analytes before extraction. Unspiked samples were analyzed in the same batch to correct the final concentrations for the amount of analytes already present in wastewater. Matrix effect (ME%) was checked by spiking 100 ng/L in a sample extract (after SPE) and calculating the spiked amount by comparing with an external calibration point.

Instrumental quantification limits (IQL) were determined by direct injection of picogram quantities of each substance as the concentrations giving peaks for which the signal-to-noise ratio was 10. The quantification limits (LOQ) for the whole method were calculated from wastewater samples as the concentrations giving peaks for which the signal-to-noise ratio was 10, or 5 in the case of direct injection.

The instrumental repeatability and precision were assessed by injecting five replicates of two standard mixtures (2 and 20 μg/L, 4 μL injected) and five replicates of a wastewater extract spiked at two levels (20 and 100 ng/L). Instrumental repeatability of DEP, DETP, DMP and DMTP was evaluated by injecting spiked raw samples (0.1 and 1 μg/L) five times.

Stability tests were conducted in raw wastewater samples spiked with a mixture of 1 μg/L of the analytes (except for DCCA which was spiked at 0.1 μg/L). One unspiked sample was used as a blank for the matrix. Samples were stored at room temperature and at 4°C and were analyzed immediately after spiking (t_0) and after 6 and 24 hours (t_6 and t_{24}). Samples stored at -20°C were analyzed after one and two months. Each analysis was done in triplicate.

3.2.7 Data analysis

Data were analysed using a MultiQuant™ 2.1 software package of Analyst® (AB-Sciex, Thornhill, Ontario, Canada). Statistical analysis was done with Excel (Microsoft, 2007). For the calculation of the means, concentrations below the LOQ were considered as half the LOQ.

3.3 Results and discussion

3.3.1 Sample extraction and analysis

Four different cartridges (OASIS HLB, WAX, MAX and MCX) were tested using specific conditions according to their different functional groups, i.e. cationic or anionic exchange groups and/or reverse-phase interactions. The majority of the compounds performed best using OASIS HLB cartridges (**Table 3.3**). Recoveries were mostly good, ranging from 75% (*cis*-DCCA) to 115% (CPF), but were slightly high for AM (133%), and very low for DACT (11.5%) and CPF-MET (22.0%). Relative standard deviations were lower than 13.1% for all the analytes indicating good precision of the method. MDA was the only compound not recovered with the HLB cartridges, but with a weak anion exchange cartridge (OASIS WAX) (**Table 3.3**). This substance was finally excluded from the method to avoid using two different extraction procedures and also considering that MDA was never detected using WAX cartridges.

The amounts recovered through direct injection (percentages) and the relative standard deviations of DEP, DETP, DMP and DMTP were obtained by spiking a raw wastewater sample in triplicate at 2.5 μg/L, before the instrumental analysis. The whole %R was close to 100%, with low %RSD, which indicated that the quantification of these compounds with TCPY-C_3 as IS was accurate (**Table 3.3**).

Table 3.3: Recoveries and their Relative Standard Deviations (RSD%), Instrumental Limits of Quantification (IQL) and method Limits of Quantification (LOQ)

Compound	Recoveries and RSD (%)	IQL (pg/injected)	LOQ (ng/L)
Triazines			
ATZ	104.3 (2.4)	0.29	0.99
DES	111.8 (7.1)	0.25	1.27
DIA	97.2 (4.4)	3.04	2.82
DEA	107.5 (7.9)	1.18	2.13
DACT	11.5 (12.2)	3.81	3.04
AM	133.4 (6.8)	0.08	1.00
Pyrethroids			
3-PBA	108.3 (2.0)	1.21	4.64
trans-DCCA	88.9 (4.3)	2.18	11.24
cis-DCCA	75.2 (5.8)	7.41	15.36
Organophosphates			
CPF	115.1 (6.6)	3.16	4.83
CPF-MET	22.0 (11.2)	5.06	7.09
TCPY	108.9 (2.5)	0.24	3.13
MMA (isomer 1)	84.1 (7.0)	2.09	7.75
MMA (isomer 2)	96.9 (3.5)	9.20	9.52
MDA*	120 (24)	0.63	2.58
IMPY	93.0 (5.2)	0.70	2.57
DEP§	102.5 (6.0)	0.62	62
DETP§	105.8 (13.1)	0.37	35
DMP§	85.5 (8.1)	0.31	68
DMTP§	90.2 (8.9)	8.60	790

*The recovery was obtained using OASIS WAX cartridges. § The recovery was obtained through direct injection.

Generally, ME % were close to 100% (**Table 3.4)**, e.g. no signal suppression was observed. ME was slightly high for AM (124%), and this could explain the high recovery, which is probably due to signal enhancement during the ionization that complex matrices such as raw wastewater can cause. ME was low for DACT (48%) and

CPF-MET (20%) and this might explain the low recovery for the latter (22%) which was different compared to CPF itself. The ion suppression observed for DACT can only partially explain its low recovery, so this substance was excluded from the method.

Table 3.4: Matrix effect for each compound in raw wastewater after SPE (OASIS HLB), expressed as the accuracy of the spiked level (100 ng/L)

Compound	Matrix Effect %	Compound	Matrix Effect %
ATZ	104.7	CPF	102.6
DES	103.8	CPF-MET	19.5
DIA	92.1	TCPY	110.2
DEA	98.4	IMPY	93.7
DACT	48.7	MMA (isomer 1)	89.5
AM	124.0	MMA (isomer 2)	103.9
3-PBA	100.6	IMPY	93.7
trans-DCCA	87.2		
cis-DCCA	81.3		

Separation was good for each analyte and typical chromatograms in raw wastewater are presented in **Figure 3.2**.

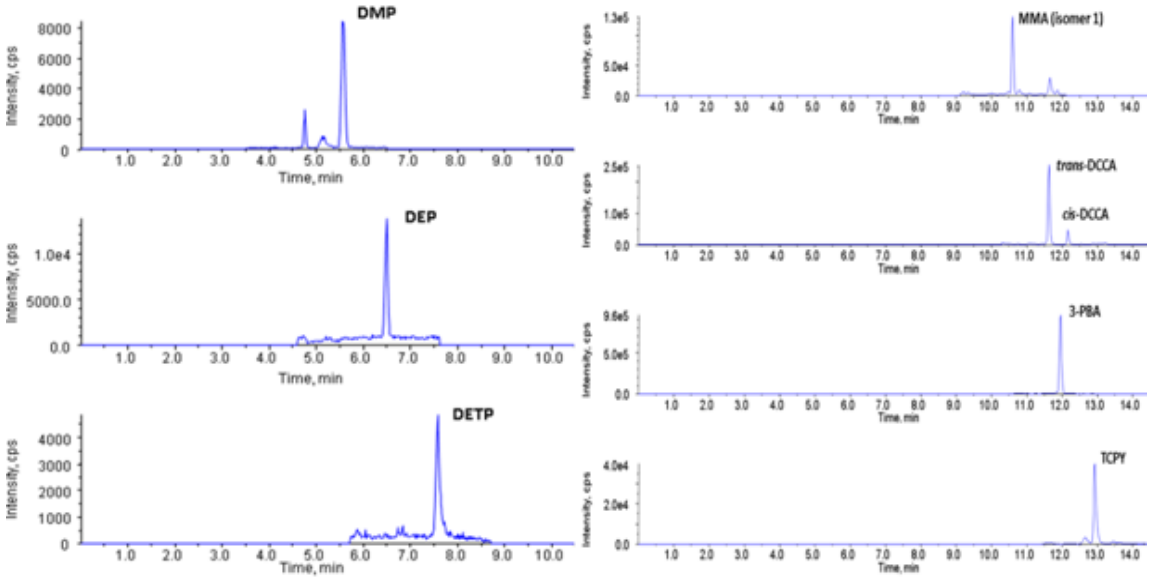

Figure 3.2: Characteristic chromatograms of some pesticide metabolites in influent wastewater (Concentrations (ng/L): DMP 483; DEP 206; DETP 70; MMA (isomer 1) 207; *trans*-DCCA 298; *cis*-DCCA 141; 3-PBA 181; TCPY 30).

3.3.2 Performance of the method

Table 3.3 reports the IQLs calculated by direct injection of each substance in standard mixtures at the lowest concentrations of the calibration curve. IQLs were in the low pg/injected range for all the analytes, varying between 0.08 (AM) and 9.20 (MMA isomer) pg/injected. The LOQs of the method were calculated directly from wastewater chromatograms or by spiking wastewater samples at different levels. LOQs were generally lower than 16 ng/L. LOQs for DEP, DETP, DMP and DMTP, were 0.06, 0.04, 0.07 and 0.79 μg/L respectively.

Calibration curves were linear in the whole range tested for all the substances investigated (**Table 3.5**). The interday correlation factors, r^2, were >0.9991 for all the analytes. Instrumental repeatability was good with %RSD generally below 10% for all the analytes (**Table 3.5**).

Table 3.5: Linearity and instrumental variability in wastewater and standard mixtures

Compound	Linearity range, μg/L	Interday correlation factors, $r^2 \pm SD$	Standard mixtures % RSD		Spiked wastewater % RSD	
			2 μg/L	20 μg/L	20 ng/L	100 ng/L
Triazines						
ATZ	0-60	0.9997 ± 0.0002	1.50	1.53	3.42	5.25
DES	0-60	0.9999 ± 0.0001	2.89	1.68	16.63	6.28
DIA	0-60	0.9994 ± 0.0005	4.81	5.24	6.39	7.50
DEA	0-60	0.9996 ± 0.0003	3.21	3.65	2.09	2.84
AM	0-60	0.9998 ± 0.0003	3.70	2.36	6.42	4.61
Pyrethroids						
3-PBA	0-60	0.9996 ± 0.0002	3.07	3.54	6.57	5.97
trans-DCCA	0-60	0.9995 ± 0.0005	4.91	1.92	4.11	5.26
cis-DCCA	0-60	0.9991 ± 0.001	4.05	4.07	3.25	5.88
Organophosphates						
CPF	0-60	0.9999 ± 0.00002	6.09	5.51	3.35	3.64
CPF-MET	0-60	0.9996 ± 0.0003	8.43	7.77	6.61	7.86
TCPY	0-60	0.9997 ± 0.0004	3.59	2.18	3.08	1.69
MMA (isomer 1)	0-60	0.9991 ± 0.001	2.62	2.41	5.01	4.04
MMA (isomer 2)	0-60	0.9994 ± 0.0004	1.13	1.10	3.77	3.32
IMPY	0-60	0.9996 ± 0.0003	2.79	1.86	22.64	6.86
DEP	0-60	0.9999 ± 0.0002	2.40	3.06	2.66	6.86
DETP	0-60	0.9995 ± 0.0005	5.26	8.34	4.14	4.45
DMP	0-60	0.9996 ± 0.0002	2.39	3.14	3.24	8.15
DMTP	0-60	0.9996 ± 0.0005	3.20	3.15	14.95	15.40

Positives were confirmed by analyzing at least two transitions (parent compound/product) for each analyte (**Table 3.2**), and the conformity of the ion ratio between recorded transitions and retention time with those of standards was checked to be within the tolerance (± 30% for ion ratio and ± 0.2 min for retention times), according to the SANCO guideline (European Commission, 2014).

3.3.3 Stability in wastewater

One of the main requirements for a biomarker of exposure is its stability in the matrix to ensure that no losses occur before analysis (Castiglioni et al., 2013). Thus, the stability of the selected analytes in urban wastewater was assessed by analyzing wastewater samples stored in dark glass bottles for up to 24 hours. This test was run at room temperature, 4°C and -20°C to mimic "real conditions" respectively in the sewer system (residence time about 7 hours before entering WWTPs), during the collection of 24-h composite samples, and during storage before analysis.

Generally, no noteworthy variations were observed under the different conditions, except for DCCA, which was completely degraded during storage at -20°C. Triazine metabolites increased slightly at room temperature and at 4°C, while ATZ itself decreased slightly (6%) to the same extent in both conditions. The concentration of IMPY rose at room temperature (23.7%) and less at 4°C (14.6%). Generally, all the analytes, except the alkyl phosphate metabolites DMP and DMTP, were more stable at 4°C than at room temperature. It is therefore recommended to adopt refrigerated conditions for the sampling procedure, while no great losses are expected in the sewer system in view of the mean resident time (<10 hours). If analysis cannot be done immediately after sample collection, storage at -20°C is the most suitable. Most of the analytes were stable up to two months, but DCCA was completely degraded and AM

and DIA increased 34% and 24% respectively. Thus, the storage period should be shorter for these substances.

3.3.4 Analysis of wastewater samples

The method was applied to measure pesticides and their human urinary metabolites in 44 influent wastewater samples collected in May 2014 from seven cities of Italy (**Table 3.6**). CPF-MET, AM and DMTP were not detected in any of the samples, and CPF was detected only in one site. The most frequently detected compounds were DES, TCPY, 3-PBA, DMP and DEP which were detected in all the samples, followed by *trans*- and *cis*-DCCA (97.7% and 72.7%), IMPY (77.3%) and ATZ (56.8%). The target analyte concentrations ranged from a few ng/L to 1.6 μg/L.

The highest concentrations were for the alkyl phosphate metabolites DMP and DEP, followed by DETP, and this pattern reflects the concentrations found in human urine in biomonitoring studies (Aprea et al., 1996; Heudorf and Angerer, 2001b), except for DMTP that was detected at high concentrations in urine, but was not detected in wastewater probably due to its high LOQ. DMP and DEP are two of the six metabolites most frequently determined when assessing human exposure to a wide range of organophosphates (Barr, 2008). A more specific metabolite of CPF and CPF-MET, TCPY, was detected in all the samples and ranged between 12 and 280 ng/L. This metabolite was also found very frequently (>80%) in all the biomonitoring studies in the U.S. (Barr et al., 2005), Italy (Aprea et al., 1999) and Germany (Koch and Angerer, 2001). Another specific organophosphate metabolite, IMPY, which is the metabolite of diazinon, was found in most of the samples, though at lower concentrations (2-30 ng/L), except in one site (Cremona) where it reached 180 ng/L. Finally, the specific metabolite of malathion (MMA) was found sporadically at concentrations up to 50 ng/L. IMPY

was also found less frequently in HBM studies (30%) (Barr et al., 2005), while no information about the frequency of detection of MMA was available.

The metabolite of pyrethroids, 3-PBA, which is common to as many as 20 synthetic pyrethroids, was found in all the samples at concentrations up to 180 ng/L, and similar concentrations (7-220 ng/L) were found for the *cis* and *trans* isomers of DCCA, a specific metabolite common to permethrin, cypermethrin and cyfluthrin. These pyrethroids metabolites were also detected at high frequency (40-90%) in HBM studies in the U.S. (Barr et al., 2005) and Germany (Becker et al., 2006; Heudorf and Angerer, 2001a).

Triazines and the main related metabolites, were found only sporadically in wastewater samples and only in traces (< 10 ng/L), except DES which was found in all the samples up to 20 ng/L. Although the use of ATZ in Italy has been prohibited since the early 1990s (Meffe and de Bustamante, 2014), these residues are still in the environment, especially in groundwater where ATZ, terbuthylazine, DES and DIA are the most frequently detected compounds (ISPRA, 2013). DES is also one of the most ubiquitous substances in surface water in Italy according to a recent review by Meffe and de Bustamante, (2014). Moreover, ATZ can undergo several transformation processes in water systems and several metabolites such as DEA, DIA and DACT can be formed (Barr et al., 2007), thus indicating the presence of additional sources of these substances besides human metabolism (**Table 3.1**).

Table 3.6: Mean concentrations (ng/L), standard deviations and frequency of detection of pesticides and their metabolites in influent wastewater samples taken in seven Italian cities in May 2014

Compound	Cremona	Milan	Merano	Florence	Terni	Bari	Palermo	Frequency of detection (%)
Triazines								
ATZ	8.3 ± 1.4	8.6 ± 1.0	< LOQ	80 ± 98	0.7 ± 0.6	1.2 ± 0.5	0.6 ± 0.3	56.8
DES	19.2 ± 0.6	19 ± 16	3.5 ± 1.4	4.0 ± 2.6	3.5 ± 2.1	1.7 ± 0.4	2.8 ± 1.0	100
DIA	< LOQ	6.6 ± 0.5	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	15.9
DEA	7.9 ± 1.2	8.5 ± 0.8	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	20.5
AM	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0
Pyrethroids								
3-PBA	94 ± 12	53 ± 41	38 ± 15	181 ± 34	52 ± 42	50 ± 12	97 ± 71	100
trans-DCCA	156 ± 23	51 ± 25	27 ± 12	224 ± 49	52 ± 38	67 ± 15	96 ± 65	97.7
cis-DCCA	64 ± 11	15 ± 13	< LOQ	86 ± 33	15 ± 11	23 ± 5.9	31 ± 25	72.7
Organophosphates								
CPF	< LOQ	< LOQ	55 ± 48	< LOQ	< LOQ	< LOQ	< LOQ	15.9
CPF-MET	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0
TCPY	46 ± 4.9	32 ± 15	280 ± 120	28 ± 4.7	12.0 ± 7.1	47 ± 23	50 ± 16	100
MMA (isomer 1)	< LOQ	15 ± 13	< LOQ	43 ± 73	< LOQ	7.9 ± 10.7	5.9 ± 5.3	27.3
MMA (isomer 2)	< LOQ	8.3 ± 4.6	< LOQ	53 ± 99	< LOQ	11.1 ± 16.9	< LOQ	18.2
IMPY	179.2 ± 7.3	3.8 ± 3.9	3.1 ± 1.8	26 ± 14	1.8 ± 0.9	9.8 ± 5.0	2.5 ± 2.0	77.3
Alkyl phosphates								
DEP	175.0 ± 7.1	153 ± 92	717 ± 312	169 ± 34	88 ± 15	194 ± 45	117 ± 26	100
DETP	32 ± 18	< LOQ	132 ± 56	51 ± 24	< LOQ	< LOQ	< LOQ	31.8
DMP	105 ± 0	129 ± 15	1686 ± 307	337 ± 93	111 ± 27	277 ± 99	652 ± 440	100
DMTP	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0

3.4 Conclusions

To the best of our knowledge, this is the first method dealing with the determination of a wide range of human metabolites of pesticides in urban wastewater. Collective wastewater represents anonymous urine samples of thousands of individuals, thus this method is proposed as a novel biomonitoring approach to evaluate the exposure of the population to pesticides. The results obtained within the present study are preliminary but very promising, since the abundance of the metabolites were in line with the profiles reported in urine in HBM studies. This suggests that, measuring the concentrations of human metabolites of pesticides in raw urban wastewater might serve to evaluate the collective population exposure to pesticides. This novel method can be a valuable tool to obtain objective, direct information on the “real” levels of exposure of a specific population to pesticides and can provide additional information for HBM studies.

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Chapter 4

Wastewater-based epidemiology to assess human exposure to pyrethroid pesticides

4.1 Introduction

In this chapter, wastewater-based epidemiology was explored as a biomonitoring tool to evaluate pesticide intake in a population. This step is very critical in order to validate this novel approach and ensure that the selected biomarkers meet the main requirements of a biomarker of exposure. Pyrethroid metabolites were tested as suitable bomarkers of exposure in wastewater by studying their detectability and stability in raw wastewater, and by examining their source to ensure that they definitely come from human metabolism and therefore reflect human ingestion of the parent substances. The following compounds were used as biomarkers: 3-PBA, which is a common urinary metabolite of about 20 pyrethroids, and *cis*- and *trans*-DCCA which are specific urinary metabolites of permethrin, cypermethrin and cyfluthrin.

The results of this study have been published in:

- ✓ *Environment International*, **2017**, 99, 213-220

4.2 Material and methods

4.2.1 Wastewater sampling

Raw wastewater samples were collected from the inlet of the main WWTPs of six Italian cities, covering the whole country. Two cities (Milan and Merano) are in the north of Italy, two (Florence and Terni) in the center and two (Bari and Palermo) in the south. Composite 24-hour samples of untreated wastewater were collected by automatic sampling devices from each WWTP. The samples were taken daily for seven consecutive days to control for inter-day variability. Sampling was carried out in May 2014. In Milan, samples were also taken in four other periods (February, March, June and September 2015), in order to investigate potential seasonal differences in human pesticide intake.

4.2.2 Analytical method

The analysis of the samples was done with the developed and validated method, described in chapter 3.

4.2.3 Stability of biomarkers and parent substances in wastewater

One of the main requirements of a biomarker for WBE is its stability in the sewage system and during sampling and storage to ensure that no loss occurs before analysis. A biomarker should not be a degradation product formed in wastewater from the parent compound itself, to ensure that its source is only human metabolism. Therefore, the formation of the metabolites was investigated, after the addition of permethrin, cypermethrin and cyfluthrin in raw wastewater. The stability of 3-PBA and *cis*- and *trans*-DCCA is described in chapter 3.

To mimic conditions in the sewer system, the stability of the compounds was checked in the dark at room temperature for 24 h. A second experiment was conducted at 4 °C to mimic the collection of the composite 24 h samples. Permethrin, cypermethrin and cyfluthrin were spiked at a high concentration (2 μg/L) in wastewater and at 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). Unspiked samples were used as matrix blanks. Each experiment was run in triplicate and separate samples were analyzed immediately after spiking, and after 6 and 24 h.

4.2.4 From biomarker concentrations to pesticide intake

Daily mass loads of the selected biomarkers were calculated by multiplying the concentrations (ng/L) by the daily wastewater flow rate (m^3/day) at the WWTP. Mass loads (mg/day) were then normalized to the number of people served by each WWTP (mg/day/1,000 inhabitants), in order to compare results among different cities.

The mass loads of the metabolites were used to quantify pesticide intake of the population served by each WWTP. Correction factors (CF), which take into account the molar mass ratio between the parent pesticide and the metabolite and the percentage of the parent compound excreted in human urine as the selected metabolite (**Table 4.1**) were developed. The following equation was used to calculate a correction factor for each metabolite:

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

where: Mw is the molecular weight and WM is the weighted mean.

Table 4.1: Selected biomarkers to study population intake of pyrethroids by wastewater analysis

Parent pesticide	**Metabolite (biomarker)**	**Percentage of administered dose excreted as metabolite**	**Molar mass ratio**	**Correction factor**
		Mean		**Mean**
20 Pyrethroids	3-PBA	28.5	1.98	6.95
trans-Permethrin, -cypermethrin, -cyfluthrin	*trans*-DCCA	53.9	1.98	3.67
cis-Permethrin, -cypermethrin, -cyfluthrin	*cis*-DCCA	36.3	1.98	5.45

All the studies reporting the excretion rate of the three metabolites after a dose of the parent substances were considered (Eadsforth et al., 1988; Eadsforth and Baldwin, 1983; Hays et al., 2009; Leng et al., 1997a; Ratelle et al., 2015a, 2015b; Woollen et al., 1992). The weighted mean excreted fraction (i.e. percentage of excretion) in urine was calculated taking into account the number of subjects in each study (**Tables 4.2-4.3**). 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 the parent substances divided by the molecular weight of each metabolite. For instance, the weighted mean percentage of excretion of *trans*-permethrin, -cypermethrin and -cyfluthrin (mean molecular weight 414.00 g/mol) recovered in human urine as *trans*-DCCA (molecular weight 209.07 g/mol) is 53.9% and therefore a measured *trans*-DCCA excretion rate of 1 g/day/1,000 people will correspond to an intake of 1/0.54 × 414.00/209.07 = 3.67 g of *trans*-permethrin, -cypermethrin and -cyfluthrin per day per 1,000 people.

Table 4.2: Human dose excretion studies of pyrethroids – Urinary profile of 3-PBA

Pyrethroid	Subjects treated	Dose (mg)	3-PBA excretion (%)	SD	Reference
Cypermethrin	6	3.3	12.6	7.0	Woollen et al., 1992
Cypermethrin	6	0.1 mg/kg	27.2	17.2	Ratelle et al., 2015a
Permethrin	6	0.1 mg/kg	45.7	21.0	Ratelle et al., 2015b
ALL			**28.5**	**16.2**	**Present study**

4.2.5 Statistical analysis

GraphPad Prism (Version 6.03) was used for statistical analyses. The Shapiro-Wilk test was used to check the normality of the data. Then, since data distribution was not normal, the Kruskal-Wallis test at a 95.0% confidence level followed by Dunn's test were applied.

Table 4.3: Human dose excretion studies of pyrethroids – Urinary profile of DCCA

Pyrethroid	Subjects treated	Dose (mg)	Isomer *cis*-DCCA excretion (%)	SD	Isomer *trans*-DCCA excretion (%)	SD	Reference
Cypermethrim (50:50)	1 1 1 1 1 1	0.5 1 1.5 1 0.25 0.25	49	9.6	78	8.2	Eadsforth and Baldwin, 1983
cis-Cypermethrin	2 2 2	0.25 0.5 0.75	43	7.6			
	2 2 2	0.25 0.5 0.75	49	11			Eadsforth et al., 1988
Cypermethrim (50:50)	2 2 2	0.25 0.75 1.5	45	6.0	72	6.2	
Cypermethrin (50:50)	6	3.3	19	7.2	36	12.3	Woollen et al., 1992
Cypermethrin (trans/cis : 58:42)	6	0.1 mg/kg bw	26.2	12.3	43.9	15.2	Ratelle et al., 2015a
Permethrin (trans/cis : 60:40)	6	0.1 mg/kg bw	25.7	8.6	43.1	12.4	Ratelle et al., 2015b
Cyfluthrin (trans/cis : 58:42)	1	2.6	19.6		32.6		Hays et al., 2009; Leng et al., 1997a
ALL			**36.3**	**9.1**	**53.9**	**11.3**	**Present study**

4.3 Results

4.3.1 Stability of biomarkers and parent substances in wastewater

The degradation of 3-PBA and DCCA in wastewater after 24 h was less than 8% at room temperature and 4 °C (Rousis et al., 2016). Therefore no significant losses of these compounds were expected in the sewer system and during sampling. No spontaneous formation of the metabolites 3-PBA and DCCA was observed, in all the test conditions, after the addition of permethrin, cypermethrin and cyfluthrin in wastewater.

4.3.2 Comparison of WBE data and urinary biomarker levels from biomonitoring studies

Urinary biomarkers of pyrethroids were measured in wastewater in six cities covering the whole Italy, and were found in almost all the samples investigated (over 60 samples). Levels in wastewater were in the order of tens or hundreds of nanograms per liter for the three metabolites (3-PBA: median 38 ng/L, range 17 - 250; *trans*-DCCA: median 56 ng/L, range 6 - 300; *cis*-DCCA: median 19 ng/L, range 8 - 140) (**Table 4.4**). To check whether our results were in line with published findings, we compared the biomarker levels in raw wastewater measured in this study, with the levels in urine reported in HBM studies. Urinary levels in the general population collected from the literature were in the order of micrograms per liter, and ranged from not detected to 100 μg/L or higher (**Table 4.5**). A direct comparison of our results in wastewater with those from HBM studies is difficult, since no specific studies have been done in Italy. Furthermore, most of HBM studies were not performed in the general population, but on specific populations such as children and exposed workers, where concentrations are typically higher than in the general population. Nevertheless, the biomarker levels in urine and wastewater were in agreement considering the dilution factor of urine in wastewater.

Table 4.4: Wastewater concentrations of pyrethroid metabolites in the present study - Medians and ranges were calculated for 7 samples

Median (ng/L)			Range (ng/L)			WWTPs investigated
3-PBA	***trans*-DCCA**	***cis*-DCCA**	**3-PBA**	***trans*-DCCA**	***cis*-DCCA**	
36.7	27.6	n.d.[a]	20.8 – 62.7	5.6[b] – 44.0	n.d.	Merano
178.8	217.7	70.9	145.7 – 251.1	153.8 – 297.7	56.6 – 140.8	Florenze
30.4	33.7	7.7[b]	20.8 – 113.9	19.1 – 101.2	7.7 – 29.5	Terni
51.3	73.6	20.1	33.3 – 68.2	39.6 – 82.9	17.7 – 34.3	Bari
60.2	66.3	23.2	53.7 – 240.7	57.5 – 240.0	7.7 – 32.5	Palermo
36.1	40.4	7.7	30.2 – 145.6	36.6 – 104.8	7.7 – 46.8	Milan 2014
17.9	44.0	7.7	16.5 – 29.8	19.9 – 75.5	7.7 – 32.5	Milan February 2015
22.9	57.0	7.7	18.9 – 40.7	24.9 – 118.4	7.7 – 32.9	Milan March 2015
40.2	55.8	19.4	32.3 – 50.4	51.8 – 88.9	16.5 – 38.5	Milan June 2015
30.9	56.2	18.8	25.5 – 59.0	37.8 – 95.7	15.4 – 47.2	Milan September 2015

[a]n.d. not detected

[b]Concentrations below the limit of quantification (LOQ) were replaced with LOQ/2 (*trans*-$DCCA_{(LOQ/2)}$ = 5.6 ng/L; *cis*-$DCCA_{(LOQ/2)}$ = 7.7 ng/L)

Table 4.5: Urine concentrations of pyrethroid metabolites across selected studies

Mean / Median (μg/L)			Range (μg/L)			Reference
3-PBA	***trans*-DCCA**	***cis*-DCCA**	**3-PBA**	***trans*-DCCA**	***cis*-DCCA**	
5.0 / 1.9	3.6 / 1.1	2.2 / 0.6	0.1 – 89.3	0.5 – 85.7	0.5 – 94.9	Naeher et al., 2010
1.5	6.3	3.4	0.07 – 8.5	1.1 – 28.8	1.4 – 11.0	Davis et al., 2013
-	0.41 / 0.24	0.11	-	LOD – 17.8	LOD – 9.8	Heudorf and Angerer, 2001
2.1	2.9	1.8	0.22 - 170	0.5 - 380	0.5 - 88	Baker et al., 2004
-	-	-	LOD – 89.7	LOD - 121	LOD – 91.4	Yusa et al., 2015
1.2	1.9	-	-	-	-	Heffernan et al., 2016
2.9	-	-	-	-	-	Park et al., 2016
2.2 / 1.0	3.0 / 0.9 (total DCCA)		0.30 – 9.23	0.13 – 15.97 (total DCCA)		Tao et al., 2013
1.0 / 0.07	0.86 / 0.28	0.95 / 0.14	0.03 – 74.0	0.44 – 19.9	0.16 – 91.4	Panuwet et al., 2009
0.96 / 0.70	0.49 / 0.26	0.21 / 0.11	0.11 – 3.86	0.08 – 2.14	0.08 – 1.21	Wei et al., 2012
7.06 / 2.18	2.94 / 0.95	0.68 / 0.22	0.30 – 81.5	0.08 – 22.6	0.08 – 3.70	Wei et al., 2012

4.3.3 Mass loads of biomarkers in wastewater

Figure 4.1 reports mass loads of the biomarkers after normalization for the population, expressed as mg/day/1,000 inhabitants. The highest normalized mass loads of 3-PBA were in Florence (130.6 mg/day/1,000 inh.) followed by Palermo (37.5 mg/day/1,000 inh.), while loads were lower in Merano (11.0 mg/day/1,000 inh.), and Bari (12.0 mg/day/1,000 inh.). The patterns were similar for *cis*- and *trans*-DCCA. The highest values of *trans*- and *cis*-DCCA were in Florence (160.2 mg/day/1,000 inh. of *trans*- and 61.6 mg/day/1,000 inh. of *cis*-DCCA), while in Merano *cis*-DCCA was not detected and *trans*-DCCA was 7.9 mg/day/1,000 inh. The loads of *trans*-DCCA were always higher than those of *cis*-DCCA, as expected from HBM studies.

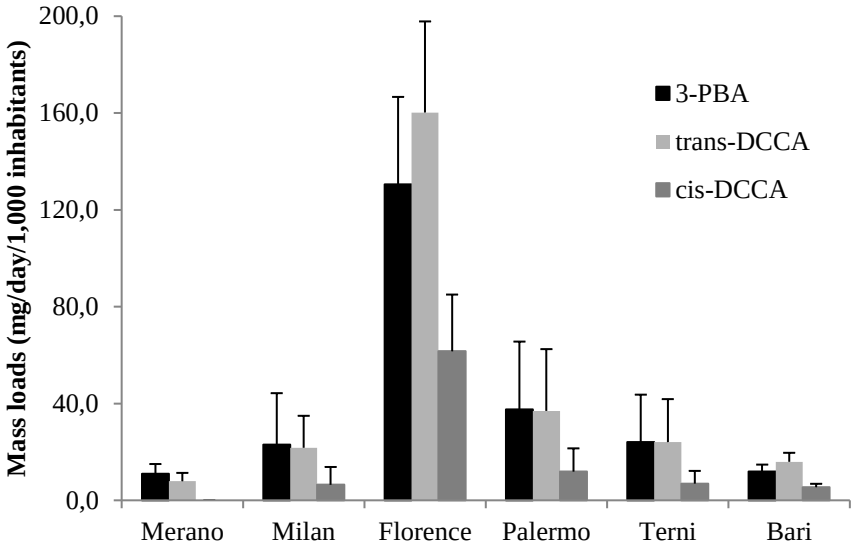

Figure 4.1: Normalized mass loads (mg/day/1,000 inhabitants) of 3-PBA, *trans*-DCCA and *cis*-DCCA in wastewater of the WWTPs of six Italian cities in May 2014 (mean ± SD of seven 24h samples).

Mass loads of biomarkers in Florence were much higher than in the other cities and no potential explanation could be found at present. Further research should be addressed to clarify this result.

4.3.4 Pyrethroid intake estimated by measuring 3-PBA in wastewater

A good biomarker should be specific. This means that in wastewater it should only derive from the metabolic transformation of a single compound in man and its excretion in wastewater. With this in mind 3-PBA cannot be considered an exclusive biomarker of a single pesticide, since it is a common urinary metabolic product of about 20 pyrethroids that cannot be distinguished. Therefore, it was not possible to estimate an exposure rate for each of them, but only a total exposure rate for the whole group, considering a mean molar mass ratio between parent pesticides and 3-PBA and a mean metabolic excretion rate in humans. The total intake for this group of pyrethroids was estimated using the normalized excretion rates of 3-PBA, as previously described, with the correction factor reported in **Table 4.1**. The highest intake was in Florence (907.5 mg/day/1,000 inh.) and the lowest in Merano (76.2 mg/day/1,000 inh.). Generally the intake was lower than 260 mg/day/1,000 inh (**Table 4.6**).

Table 4.6: Pyrethroid intake (mg/day/1,000 inhabitants, mean and standard deviation) estimated by measuring 3-PBA and *cis*- and *trans*-DCCA in the wastewater of six Italian cities

WWTP	Group of pyrethroids (3-PBA)	*cis*-Permethrin, *cis*-cypermethrin and *cis*-cyfluthrin (*cis*-DCCA)	*trans*-Permethrin, *trans*-cypermethrin and *trans*-cyfluthrin (*trans*-DCCA)
Merano	76.2 ± 28.0	-	29.0 ± 12.6
Milan	160.1 ± 147.6	35.0 ± 40.1	79.7 ± 48.5
Florence	907.5 ± 250.7	335.5 ± 127.7	588.0 ± 138.1
Palermo	260.8 ± 195.0	64.7 ± 52.3	135.6 ± 93.6
Terni	167.3 ± 136.1	37.7 ± 28.6	88.3 ± 65.1
Bari	83.1 ± 19.5	29.6 ± 7.6	58.4 ± 13.6

4.3.5 Permethrin, cypermethrin and cyfluthrin intake estimated by measuring DCCA in wastewater

The *trans*- and *cis*-DCCA daily mass loads were used to measure the intake of *trans*- and *cis*- permethrin, cypermethrin and cyfluthrin by multiplying the normalized mass loads by the respective correction factors. The estimated intakes for *cis*-DCCA ranged between below the LOQ and 335 mg/day/1,000 inh, while *trans*-DCCA intake was higher and ranged between 30 and 590 mg/day/1,000 inh (**Table 4.6**). The pattern of DCCA mass loads was the same as for 3-PBA with the highest values in Florence and the lowest in Merano, where *cis*-DCCA was below the LOQ.

The intake profiles for these three compounds (from *cis* and *trans*-DCCA) and for the general group of pyrethroids (from 3-PBA) were similar (Florence ›› Palermo › Terni › Milan › Bari › Merano) (**Table 4.6**). The intakes for both DCCA isomers were similar to those estimated from 3-PBA alone in Florence and Bari, but lower in the other cities, indicating additional intake from other pesticides which are also excreted as 3-PBA.

4.3.6 Seasonal changes of pyrethroid intake

Five sampling campaigns in 2014-15 were done in Milan, with the aim of investigating seasonal differences in human intake of pyrethroids. The frequency of detection (DF) of 3-PBA and *trans*-DCCA was 100% (7/7) in all the samples, while for *cis*-DCCA it was 7/7 in June and September, but only 2/7 in May, February and March.

The levels of 3-PBA in wastewater were highest during the spring/summer (May 2014 and June 2015) (**Figure 4.2**). This might be ascribed to a high intake of pyrethroids by the population, possibly because of increased consumption of contaminated fruits and vegetables in this season. In September, mass loads of 3-PBA decreased substantially

(about 32%), and in winter (February and March) the mass loads were even lower (50-54%).

Significant differences were found for 3-PBA between June and February (p = 0.0019), June and March (p = 0.0142) and May and February (p = 0.0190). Mass loads of *trans*-/*cis*-DCCA were also slightly higher in June than in the other months but differences were not significant (**Figure 4.2**).

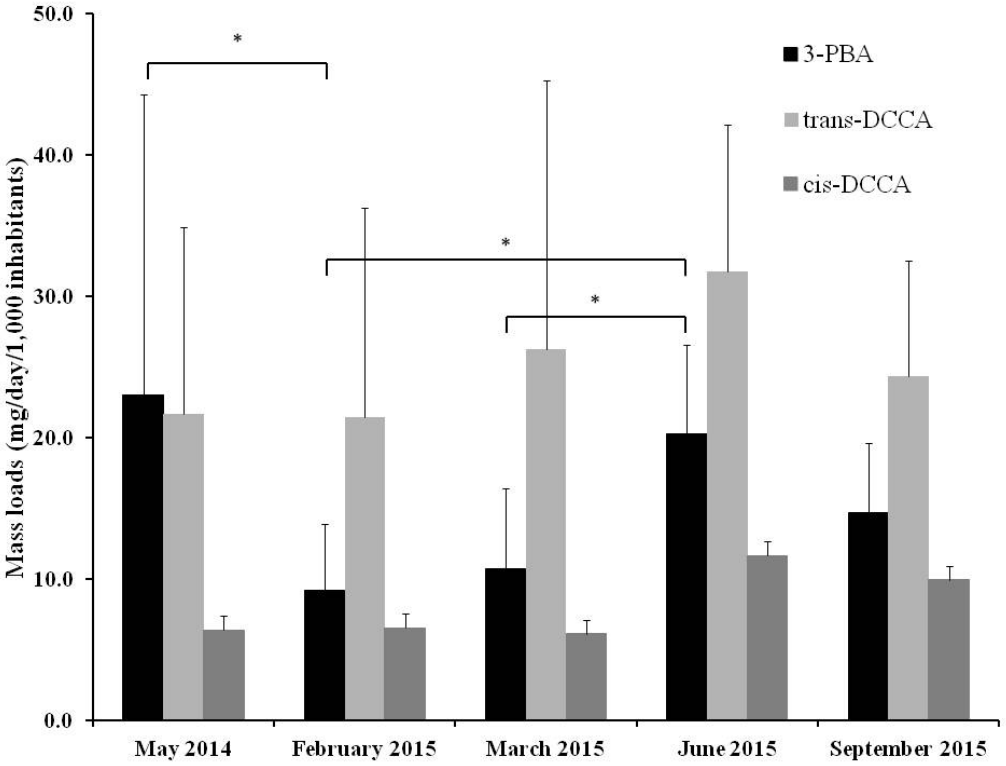

Figure 4.2: Seasonal patterns of the mass loads of pyrethroid metabolites in wastewater in Milan.

4.3.7 Evaluation of risks related to the human intake of permethrin, cypermethrin and cyfluthrin

In order to evaluate if the found levels of exposure through wastewater analysis could have potential effects for human health, the intake levels estimated from *trans* and *cis*-DCCA were summed and compared to the acceptable daily intake (ADI) (**Table 4.7**).

This was a preliminary attempt to evaluate our results in the framework of an established methodology to perform risk assessment. The ADI of beta-cyfluthrin was selected for comparison, because it is the lowest ("worst case" scenario) for this class of compounds. An ADI of 0.003 mg/kg bw per day for a man of 70 kg resulted in an average consumption of 0.21 mg/person per day.

The estimated intake of permethrin, cypermethrin and cyfluthrin in Florence found in this study exceeded the ADI and in Palermo was close to the ADI (**Table 4.7**). The mean estimated intake, obtained by considering the weighted mean excretion percentage of each metabolite, is therefore a suitable value to evaluate the potential risks for human health.

Table 4.7: Estimated intake of permethrin, cypermethrin and cyfluthrin of the population living in different cities in Italy and comparison with the ADI of beta-cyfluthrin

WWTP	Permethrin, cypermethrin and cyfluthrin (mg/day/person)*	% ADI (0.21 mg/day/person)
	Mean	Mean
Merano	0.029 ± 0.013	14
Milan	0.115 ± 0.089	55
Florence	0.924 ± 0.259	440
Palermo	0.200 ± 0.145	95
Terni	0.126 ± 0.093	60
Bari	0.088 ± 0.019	42

* *trans*-DCCA and *cis*-DCCA are presented as the sum

4.4 Discussion

In 2016, Rousis et al. (2016) explored for the first time the possibility to measure pesticide metabolites in urban wastewater as indicators of human exposure to different

classes of pesticides. This is the first study to apply WBE as a novel tool for assessing human exposure to pyrethroid pesticides in the general population. The selected biomarkers of exposure were detected in urban wastewater at concentrations reflecting those found in human urine in HBM studies. This indicates that analysis of wastewater collecting these substances after human excretion is a suitable means of assessing pesticide intake by the general population.

Much research has been devoted to studying the main pathways of population exposure to pyrethroid pesticides. Schettgen et al. (2002) suggested that the general population is exposed to pyrethroids mainly through the diet. HBM studies excluded the exposure to dust in residential environments as a significant source and concluded that pyrethroid metabolites in urine of the residents were due to their use in gardening and/or to their uptake with the diet (Berger-Preiß et al., 2002; Heudorf and Angerer, 2001; Yusa et al., 2015). The New York City Health and Nutrition Examination Survey reported that the difference in 3-PBA urinary levels among participants who reported pesticide use at home was relatively small compared to those who did not (McKelvey et al., 2013). Generally children have higher metabolite concentrations in urine than adolescents and adults (Barr et al., 2010; Couture et al., 2009; Heudorf and Angerer, 2001) and this can be explained by differences in the diet or by greater exposure in the domestic environment. For instance, a thorough investigation of children in Ohio concluded that the routes of exposure to permethrin could be ranked as: Dietary › Indirect (daily environments) ›› Dermal ››› Inhalation (Morgan et al., 2007). People eating vegetables five times a week or more had higher 3-PBA urine levels than those eating less (Fortes et al., 2013), and adults eating more than four portions of fruits and vegetables a day had higher 3-PBA and DCCA urine levels than those eating fewer (Couture et al., 2009). The bulk of evidence therefore points to a priority role of the diet in the exposure of the general population to pyrethroid pesticides.

Human excretion studies have shown that cypermethrin, permethrin and cyfluthrin are extensively metabolized in the body and the metabolites are excreted mainly and rapidly with the urine. The urinary metabolite *trans*-DCCA is produced in the body primarily from the *trans*-isomers of cypermethrin, -permethrin and -cyfluthrin, and the *cis*-DCCA metabolite from the *cis*-isomers. Pharmacokinetic studies suggested that the *trans*- to *cis*-DCCA ratio could be used as an indicator of the route of exposure. The ratio is 1:1 after dermal exposure and 2:1 or higher after oral uptake and/or inhalation (Woollen et al., 1992). **Table 4.8** reports the *trans*- to *cis*-DCCA ratios in urine after a test dose administered by different routes and during HBM studies, and in wastewater collecting human sewage. The *trans*- to *cis*-DCCA ratios in wastewater ranged between 2.6 and 3.5, in line with the ratios in HBM studies in the general population. This suggests that pesticide metabolites in urban wastewater reflect the urinary content originating from human metabolism and that the oral or inhalatory routes might be the main sources of exposure.

Another important issue for WBE is how to exclude the presence of additional sources of both parent substances and metabolites. For instance, pesticides in foods might undergo metabolic processes with spontaneous formation of metabolites. There are a few studies dealing with the determination of 3-PBA and DCCA in foods. Baby food (Radford et al., 2014), fruits and vegetables (Li et al., 2016) and tea samples (Tsumura et al., 1994) were analyzed, and 3-PBA was never detected, while DCCA was detected only in one baby food sample (organic banana) (Radford et al., 2014) and one lettuce sample (Li et al., 2016) at concentrations close to the LOQ. The domestic environment is another substantial route of exposure, and some studies have estimated human exposure to organic pollutants in residential environments by analyzing vacuum cleaner dust (Starr et al., 2008) and dust collected on kitchen floors and windows in the main living area (Clifton et al., 2013). 3-PBA and DCCA were detected in 67% and 81% of the samples,

but at concentrations close to or below the LOQ. Thus, it can be concluded that the formation of these metabolites in food or in the environment is not a common event and the potential for human ingestion of these substances is therefore very low.

Table 4.8: The *trans-/cis*-DCCA ratio in urine or wastewater in different studies, in relation to the route of exposure

***trans*- to *cis*-DCCA ratio**	**Comments**	**Reference**
Human dose excretion studies (urine)		
0.85 – 1.2	dermal administration	Woollen et al. 1992
1.8 – 2.3	oral intake	Woollen et al. 1992
1.7	oral intake	Ratelle et al. 2015a
1.7	oral intake	Ratelle et al. 2015b
1.5 – 2.2	inhalation	Leng et al. 1997b
2.3	oral intake	Leng et al. 1997a
1.5 – 3.3	inhalation	Kühn et al. 1999
1.4 – 2.0	oral intake	Eadsforth and Baldwin 1983
1.6	oral intake	Eadsforth et al. 1988
Biomonitoring studies (urine)		
‹ 1	dermally exposed textile workers	Lu et al. 2013
1.5 – 3.2	pest control operators	Leng et al. 1997a
2.3	people with no known exposure	Le Grand et al. 2012
1.6	people with no known exposure	Baker et al. 2004
1.9	people with no known exposure	Davis et al. 2013
3.0	Canadian Health Measures Survey	Oulhote and Bouchard 2013
1.9	GerES IV Pilot Study	Becker et al. 2006
1.6	children	Naeher et al. 2010
3 – 4	NHANES	Barr et al. 2010
2	urban population without exposure	Heudorf and Angerer 2001
Wastewater treatment plants (influent)		
2.6	Florence	Present study
3.5	Terni	Present study
2.9	Bari	Present study
3.1	Palermo	Present study
2.9	Milan	Present study

Furthermore, pyrethroids occurring in wastewater might degrade spontaneously to metabolites such as 3-PBA and DCCA. The stability experiments within the present study were done in the laboratory using unfiltered wastewater samples, under constant pH and temperature conditions. These experiments can mimic some in sewer conditions,

but are not able to reproduce the real conditions occurring in the sewer, including anaerobic or partially aerobic conditions. Nevertheless, DCCA and 3-PBA were not formed from permethrin, cypermethrin and cyfluthrin in any of the tested condition in wastewater and additional monitoring studies on influent and effluent wastewater, surface water, groundwater, and tap water failed to detect these parent substances (Bernarda et al., 2015; Carro et al., 2012; Casas et al., 2006; Chang et al., 2010; Gómez et al., 2007; Li et al., 2009; Moschet et al., 2014; Sengupta et al., 2014; Weston et al., 2013). This can suggest that the spontaneous formation of DCCA and 3-PBA in raw wastewater from pyrethroid degradation is unlikely, hence their levels in sewage might reflect mostly their excretion with urine after intake of pyrethroids.

4.5 Conclusions

This novel application of WBE was developed to study the collective exposure of a population to pyrethroid pesticides. Three human pyrethroid metabolites were measured in wastewater and the loads of these biomarkers were used to estimate the exposure of the population to a group of 20 pyrethroids, by measuring their common metabolite 3-PBA, and to permethrin, cypermethrin and cyfluthrin, by measuring their specific metabolites *cis*-and *trans*-DCCA. This new approach detected spatial and temporal differences in human intake of pyrethroids, and comparison with the ADI was useful to detect potential health risks related to this exposure. Results in this study were in line with findings from HBM studies reported in the literature.

This novel method could complement the findings of HBM studies and could become a valuable tool for obtaining objective, direct information on the real levels of exposure to pyrethroids in different populations almost in real time.

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Chapter 5

Wastewater-based epidemiology to assess pan-European pesticide exposure

5.1 Introduction

In this chapter, wastewater-based epidemiology was applied, for the first time, as an alternative biomonitoring tool to evaluate human exposure to pesticides in eight cities across Europe. Influent wastewater samples were collected and analyzed for organophosphate, triazine and pyrethroid metabolites by the analytical method developed and validated in this doctoral thesis. Furthermore, pyrethroid intake of population was estimated for all cities. The results of this work were compared with official data of Eurostat in order to validate this novel WBE approach. This study confirmed WBE as a very promising complementary biomonitoring tool to evaluate population-wide exposure to pesticides.

The results of this study have been submitted in a peer-reviewed journal.

5.2 Material and methods

5.2.1 Samples and sampling method

Raw wastewater samples were taken from the inlet of the 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 5.1**).

Composite 24-h samples of untreated wastewater were collected by automatic sampling devices. 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.

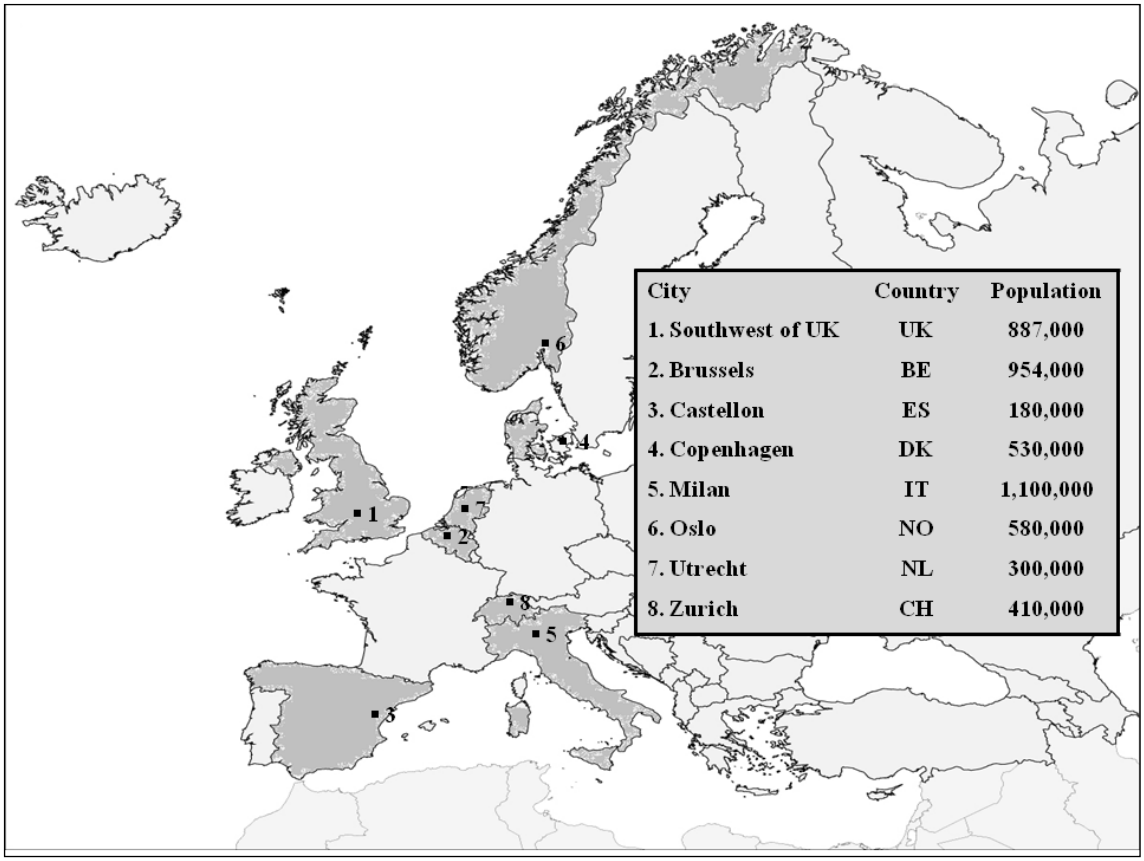

Figure 5.1: Cities investigated in the present study.

5.2.2 Analytical method

The analysis of the samples was done with the developed and validated method, described in chapter 3.

5.2.3 Stability of biomarkers and parent pesticides in wastewater

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 Chapters 3 and 4. 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 triazines (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 to test their stability under controlled conditions (room temperature and 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. Formation of DEP, DETP, DMP and DMTP metabolites 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).

5.2.4 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, so they were used to back-calculate population-wide intake of pyrethroids. The procedure used to develop correction factors is described in detail in Chapter 4. Correction factors were 6.95 for 3-PBA (used to estimate the intake of 20 pyrethroids) and 3.67 and 5.45, respectively for the *trans*- and the *cis*-DCCA (used to estimate the intake of permethrin, cypermethrin, and cyfluthrin).

The intake levels of permethrin, cypermethrin and cyfluthrin (sum of *cis*- and *trans*- levels) estimated by wastewater analysis were compared with a toxicological indicator, the ADI. This aimed to evaluate the measured levels of exposure in respect of the potential effects on human health.

Uncertainty was evaluated following the available best practice protocols for WBE (Castiglioni et al., 2014). 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 correction factors and consequently to back-calculation, and it was lower than 16% (Rousis et al., 2017). 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 point and high variability can be expected (Castiglioni et al., 2014).

5.3 Results and discussion

5.3.1 Stability of metabolites and parent pesticides

The stability experiments performed in the present study showed that no spontaneous formation of the triazine and organophosphate metabolites was noticed after the addition of the parent compounds in the different tested conditions. These experiments were done in the laboratory using unfiltered raw wastewater samples, under constant pH and temperature conditions. Despite they can give information on the stability of a compound in wastewater, they cannot reproduce the real conditions occurring in the sewer system.

5.3.2 Biomarkers in raw wastewater

Concentrations of the biomarkers measured in wastewater are shown in **Table 5.1** with their frequencies of detection. The most frequent substances 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 obtained in seven Italian cities (Chapter 3). The profiles of the most frequently detected compounds were similar, besides a few exceptions; e.g. 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, but not in the EU cities. 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 the majority of organophosphates, while the triazines group was found at the lowest concentrations. The concentrations of *trans*-DCCA were always higher than those of *cis*-DCCA, in accordance with HBM studies, where the trans-isomer predominated. 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 (Chapter 4).

Table 5.1: Mean concentrations (ng/L) and standard deviations of the seven raw wastewater samples collected in the eight European cities investigated (March 2015)

Compound	Bristol	Utrecht	Copenhagen	Zurich	Milan	Castellon	Brussels	Oslo	Frequency of detection (%)
Triazines									
ATZ	4.4 ± 0.4	2.1 ± 0.3	1.3 ± 0.1	5.4 ± 0.6	7.9 ± 0.8	2.0 ± 1.0	12.8 ± 1.3	1.7 ± 0.2	98.2
DES	< LOQ	< LOQ	< LOQ	6.2 ± 0.8	12.2 ± 1.4	21.1 ± 3.7	< LOQ	< LOQ	37.5
DIA	< LOQ	< LOQ	< LOQ	4.3 ± 0.2	8.9 ± 1.4	< LOQ	6.7 ± 2.0	< LOQ	36.2
DEA	7.5 ± 3.0	< LOQ	< LOQ	7.4 ± 0.9	7.7 ± 1.1	4.5 ± 1.2	19.6 ± 5.5	< LOQ	62.5
AM	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0
Pyrethroids									
3-PBA	49 ± 25	30.1 ± 7.4	12.4 ± 2.3	9.6 ± 1.4	26.1 ± 9.3	129 ± 32	22.4 ± 1.4	5.3 ± 1.5	98.2
trans-DCCA	118 ± 65	124 ± 54	44 ± 16	31 ± 10	63 ± 34	200 ± 60	65 ± 13	15.1 ± 8.8	96.4
cis-DCCA	22 ± 11	22.9 ± 8.3	< LOQ	< LOQ	14 ± 11	45 ± 11	< LOQ	< LOQ	35.7

LOQ (ng/L) (Chapter 3): ATZ, 0.99; DES, 1.27; DIA, 2.82; DEA, 2.13; AM, 1.00; 3-PBA, 4.64; *trans*-DCCA, 11.24; *cis*-DCCA, 15.36

Compound	Bristol	Utrecht	Copenhagen	Zurich	Milan	Castellon	Brussels	Oslo	Frequency of detection (%)
Organophosphates									
CPF	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0
CPF-MET	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0
TCPY	43 ± 23	28.3 ± 3.9	17.8 ± 2.3	26.4 ± 3.1	20.1 ± 2.9	93 ± 23	23.8 ± 2.7	8.3 ± 1.3	100
MMA isomer 1	< LOQ	< LOQ	< LOQ	< LOQ	4.7 ± 2.3	397 ± 966	< LOQ	< LOQ	8.9
MMA isomer 2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	285 ± 661	< LOQ	< LOQ	7.1
IMPY	72 ± 48	12.7 ± 2.8	3.6 ± 0.8	19 ± 16	< LOQ	25 ± 11	4.9 ± 1.1	6.5 ± 1.2	87.5
Alkyl phosphates (Organophosphates)									
DEP	1076 ± 670	206 ± 13	110 ± 12	187 ± 22	123 ± 20	231 ± 56	180 ± 24	46 ± 19	94.6
DETP	39 ± 19	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	7.1
DMP	1388 ± 2228	269 ± 43	280 ± 92	2269 ± 630	128 ± 22	278 ± 77	1072 ± 1018	233 ± 60	100

LOQ (ng/L) Chapter 3): CPF, 4.83; CPF-MET, 7.09; TCPY, 3.13; MMA isomer 1, 7.75; MMA isomer 2, 9.52; IMPY, 2.57; DEP, 62; DETP, 35; DMP, 68; DMTP, 790

5.3.3 Mass loads of biomarkers in the different cities

The alkyl phosphates DMP and DEP gave the highest loads (up to 975 mg/day/1,000 inh for DMP and 244 mg/day/1,000 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/1,000 inh. and the metabolite of chlorpyrifos and chlorpyrifos-methyl, TCPY, ranged from 3.9 to 22 mg/day/1,000 inh., suggesting different exposure to these organophosphates in the various countries.

Triazines had the lowest loads, ranging from 0.33 to 5 mg/day/1,000 inh. Generally, the metabolite mass loads were of the order of magnitude of atrazine or slightly higher. Only the specific metabolite of atrazine, AM, was not detected in wastewater, while the other metabolites could also result from exposure to other triazines, particularly terbutylazine, which is the only chlorotriazine herbicide approved for use in EU. Furthermore, these substances 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/1,000 inh and *trans*-DCCA from 7 to 46 mg/day/1,000 inh. In all the cities, *cis*-DCCA mass loads were the lowest (3.6 - 10.5 mg/day/1,000 inh).

The sum of the mass loads of each class of pesticides was calculated and normalized by the population served by each WWTP in order to compare results from the different cities (**Figure 5.2**). Different patterns were observed among the cities and for

the various classes of pesticides, but the cities of Utrecht and Oslo had always the lowest loads. The specific biomarkers of exposure to pyrethroids had the highest loads in Castellon (mean 86 mg/day/1,000 inh) followed by Milan and Bristol (mean 43 mg/day/1,000 inh), and Copenhagen (mean 41 mg/day/1,000 inh). This may indicate a higher human exposure to these pesticides in Spain due to either direct exposure and 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/1,000 inh), Bristol (mean 26 mg/day/1,000 inh) and also in Zurich (mean 21 mg/day/1,000 inh). Among non-specific metabolites a direct correlation with exposure could not be performed. The highest levels were found for alkylphosphates in Zurich (mean 1056 mg/day/1,000 inh), followed by Bristol (mean 573 mg/day/1,000 inh) and Brussels (mean 322 mg/day/1,000 inh), and for triazines in Milan (mean 14 mg/day/1,000 inh) Zurich and Brussels (mean 10 mg/day/1,000 inh).

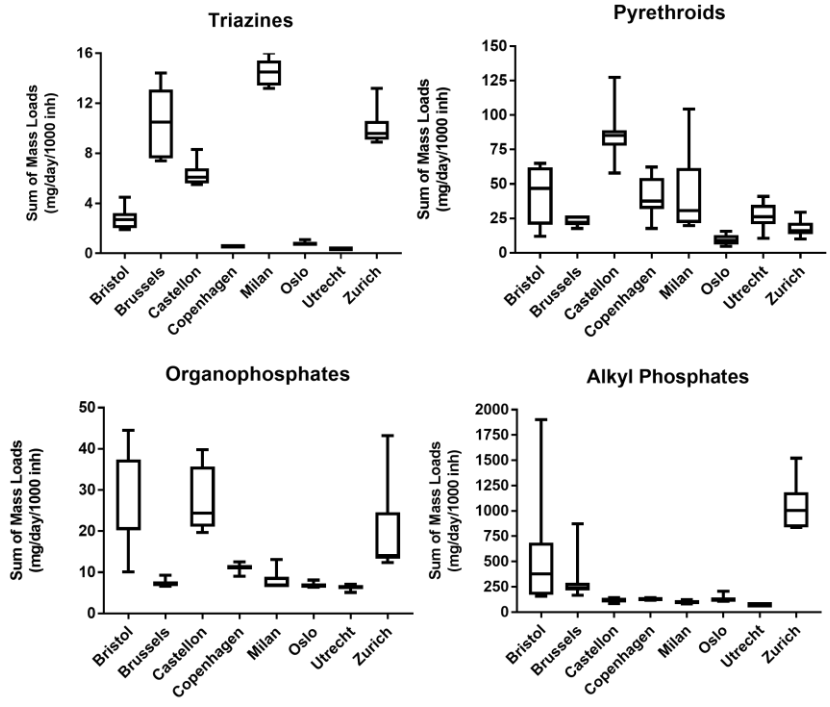

Figure 5.2: Sum of the normalized mass loads (mg/day/1,000 inhabitants) of organophosphates, triazines, pyrethroids and alkyl phosphates in eight European cities.

Since human exposure occurs mainly through the diet and can be related to direct exposure only in some cases (e.g. rural areas), the results obtained for the specific biomarkers of exposure can reveal new information about the "real local 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.

5.3.4 Comparison of mass loads of insecticides with official sales statistics

Organophosphates and pyrethroids were the classes most frequently detected in wastewater, which are both 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 were normalized to the population investigated in each city and the means are reported in **Figure 5.3**. Mass loads were the highest in Castellon, Bristol, Copenhagen and Milan and the lowest in Oslo. These results mainly reflect the Eurostat official sales statistics, which reported that Spain, Italy and UK had the highest sales data of insecticides, and Norway the lowest. Since human exposure is mainly influenced by the diet, it was speculated that in 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 reflect also the 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).

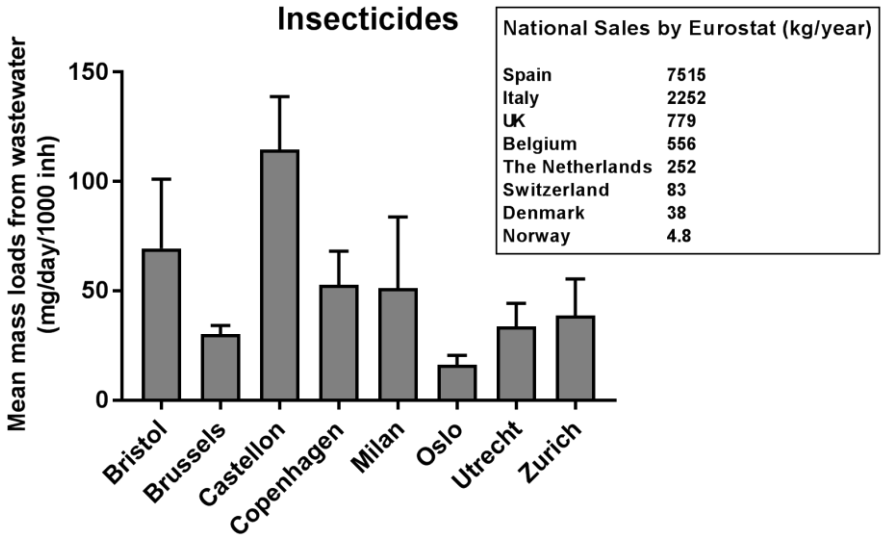

Figure 5.3: Sum of the mass loads of insecticides (mg/day/1,000 inhabitants) estimated from wastewater in eight European cities and national sales from Eurostat.

This was a first attempt to correlate results of pesticides exposure obtained from WBE and several national statistics related to pesticides exposure such as pesticides sales and vegetable and fruit consumption. A number of limitations must be taken into account to improve future comparisons of this kind of data. On one side, WBE results were obtained by measuring few specific urinary metabolites that indicate the exposure to a limited number of parent substances. Furthermore, WBE was performed only in one city per country for a limited period (seven consecutive days). Thus 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 in the same country (Rousis et al., 2016), and pesticides levels showed seasonal variation (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, the national sales statistics of pesticides may not reflect the actual use of these substances 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 than our information from WBE.

5.3.5 Back-calculation of pyrethroid intake and comparison with the ADI

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. Pyrethroids highest intake was in Castellon (207 mg/day/1,000 inh.) followed by Bristol (77 mg/day/1,000 inh.) and Milan (75 mg/day/1,000 inh.), and the lowest in Oslo (17 mg/day/1,000 inh.) (**Table 5.2**).

Table 5.2: Pyrethroid intake (mg/day/1,000 inhabitants, mean and standard deviation) back-calulated from 3-PBA and *cis*- and *trans*-DCCA

WWTP	Group of pyrethroids (3-PBA)	Permethrin, cypermethrin and cyfluthrin (DCCA)
Oslo	17 ± 5	26 ± 13
Bristol	77 ± 37	126 ± 60
Utrecht	33 ± 8	90 ± 36
Copenhagen	57 ± 13	123 ± 50
Zurich	29 ± 6	50 ± 22
Milan	75 ± 39	130 ± 101
Castellon	207 ± 47	227 ± 59
Brussels	41 ± 6	62 ± 11

The estimated intakes of *trans*- and *cis*- permethrin, cypermethrin and cyfluthrin ranged between 227 mg/day/1,000 inh in Castellon and 26 in Oslo. Similar intakes were found in UK (126 mg/day/1,000 inh), Copenhagen (123 mg/day/1,000 inh) and Milan (130 mg/day/1,000 inh).

The intake profiles from both DCCA and 3-PBA were highest in Castellon and lowest in Oslo, indicating an extremely different exposure to this class of pesticides. These results are in accordance with European statistics of fruit and vegetable consumption and also with national statics of pesticide sales as previously discussed for the entire class of insecticides. The intake of pyrethroids estimated from DCCA was higher than those estimated from 3-PBA in all the cities. This may reflect different patterns of exposure to pyrethroids, which are excreted as the investigated biomarkers.

The comparison between intakes estimated by WBE and the %ADI of beta-cyfluthrin are reported in **Table 5.3**. The estimated intake of permethrin, cypermethrin and cyfluthrin in the population was generally lower than the ADI, and exceeded this reference value only in one case (Castellon). As previously discussed, this area was found to have the highest exposure level to insecticides (particulary pyrethroids) probably due to a combination of a high use of pesticides and consumption of contaminated food.

Table 5.3: Estimated intake of permethrin, cypermethrin and cyfluthrin of the population living in different European cities and comparison with the ADI of beta-cyfluthrin

WWTP	Permethrin, cypermethrin and cyfluthrin (mg/day/person)*	% ADI (0.21 mg/day/person)
Oslo	0.026 ± 0.013	12
Bristol	0.126 ± 0.060	60
Utrecht	0.090 ± 0.036	43
Copenhagen	0.123 ± 0.050	58
Zurich	0.050 ± 0.022	24
Milan	0.130 ± 0.101	62
Castellon	0.227 ± 0.059	108
Brussels	0.062 ± 0.011	30

* *trans*-DCCA and *cis*-DCCA are presented as the sum

5.4 Conclusions

Wastewater-based epidemiology was applied for the first time to assess human exposure to different classes of pesticides across Europe. Several biomarkers of 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, such as pesticides sales and vegetable and fruit consumption. Results suggested that in the countries with a 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 "real local 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.

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Chapter 6

Monitoring a large number of pesticides and transformation products in water samples from Spain and Italy

6.1 Introduction

In this chapter, a large number (450) of pesticides and transformation products was monitored in wastewater and surface water of Spain and Italy, which are two countries with high pesticide use. In the present study a high resolution mass spectrometry technique was applied in order to overcome the main disadvantage of triple quadrupole (Chapter 1), which is the limited number of compounds that can be determined in a single run. Furthermore, many compounds are ignored in the analysis as they are not part of the target list. The main focus was to identify pesticides which are highly used and had not been included in our first list (Chapter 1), and propose them and/or their metabolites as potential human exposure biomarkers. This study was performed entirely at the Research Institute for Pesticides and Water, University Jaume I in Spain.

The results of this study have been submitted in a peer-reviewed journal.

6.2 Material and methods

6.2.1 Selection of analytes and study areas

Pesticides were selected on the basis of the priority pollutant list of the EU and the United States Environmental Protection Agency (US-EPA) and the United Nations list of persistent organic pollutants (Stockholm Convention). The database was built based on our experience with environmental and food samples LC-MS/MS analysis (Díaz et al., 2012). Lists of pesticides are found at the end of this chapter (**Tables S6.1-S6.2**).

Spain and Italy were chosen for the study since pesticides were one of the most frequently detected classes of micropollutants in waters (Hernández et al., 2015; Meffe and de Bustamante, 2014). Eurostat data showed that pesticide use in Spain in 2014, when the sampling was done, reached 78.8×10^6 kg, making Spain the country with the highest

use of pesticides in Europe. Italy ranked third, after France, applying 64.1 × 10^6 kg of pesticides in the same year (Eurostat, 2014).

6.2.2 Sample collection

Fourteen wastewater samples (seven IWW and seven EWW) were taken from the WWTP of Castellón, Eastern Spain, and four wastewater samples (two IWW and two EWW) from Cremona, Northern Italy. Composite 24-h samples of wastewater were collected by automatic sampling devices from each plant, in March 2014 (Castellón) and in May 2014 (Cremona). Samples were collected in high-density polystyrene bottles, frozen immediately and stored at -20°C until extraction.

Five surface water (SW) samples (grab samples) were taken from the Valencia region, Eastern Spain: Almenara, Burriana Clot, Nules and two sites in Albufera Natural Park. All samples were stored in high-density polystyrene bottles at 4°C for less than 48 h, until extraction.

6.2.3 Analytical method

Samples were filtered and solid phase extracted using OASIS HLB 3 cc/60 mg cartridges. The analysis was performed with a Waters Acquity UPLC system (Waters, Milford, MA, USA) interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), using a Z-Spray ESI interface operating in positive ion mode. The chromatographic separation was done using a Waters Acquity UPLC BEH C_{18} (100 × 2.1 mm, 1.7 μm) column (Bade et al., 2015a).

6.2.4 Criteria for detection/identification

The detection and identification of the compounds was based on the confidence levels for small molecules in HRMS analysis proposed by Schymanski et al. (2014) and the European Commission Decision 2002/657/EC (Commision, 2002).

Standard available

A compound was classified as "*detected"* when the accurate-mass protonated molecule $[M+H]^+$ (mass error <5 ppm) was found and the retention time was in agreement with the reference standard (± 0.2 min).

A compound was classified as "*identified"* when the accurate-mass protonated molecule $[M+H]^+$ (mass error <5 ppm) was found together with at least one fragment ion at accurate mass (mass error <5 ppm) and the retention time was in agreement with the reference standard (± 0.2 min).

Standard not available

Compounds were classified as "*tentatively detected/identified"* when a protonated molecule $[M+H]^+$ at accurate mass (<5 ppm) was found, and at least one fragment ion could be justified (<5 ppm).

Retention time predictor

A retention time (t_R) prediction approach, based on a previously developed artificial neural network (ANN) method using 544 compounds (Bade et al., 2015b), was employed to aid in the tentative detection and/or identification of compounds, when no reference standards were available.

6.3 Results and discussion

The pesticides were initially investigated using the information from standards about fragmentation and retention times according to the criteria described above. Examples of a substance *"detected"* and one *"identified"* are reported in **Figure 6.1**. Occasionally, the isotopic pattern of chlorine was used for additional confirmation and proved advantageous, especially when no other data were available. Many compounds containing chlorine(s) were considered false-positive and removed (i.e. phosfon, tepraloxydim), since no Cl isotopic pattern was displayed.

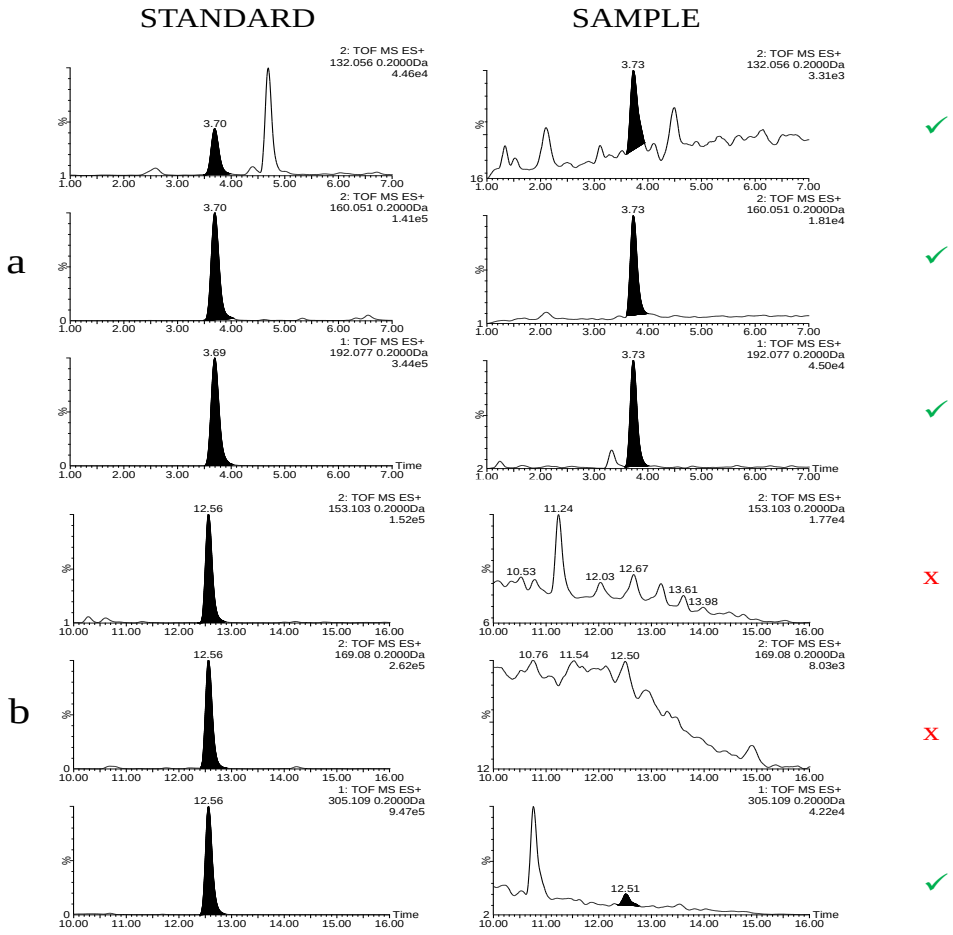

Figure 6.1: a) Identification of carbendazim (m/z 192.077, t_R 3.73) from a SW sample (RIGHT), with fragment ions 165.051 and 132.056 and retention time comparable to the standard (LEFT); b) Detection of diazinon (m/z 305.109, t_R 12.51) from a EWW sample (RIGHT). Neither of the fragment ions (169.08 and 153.103) of the standard (LEFT) could be seen in the sample.

From the initial list of 164 compounds (**Table S6.1**), seventeen pesticides and transformation products were detected and identified in the samples (**Tables 6.1-6.2**). These were fungicides (imazalil, metalaxyl, propiconazole, thiabendazole, carbendazim), herbicides (metolachlor, molinate, simazine, terbutylazine, terbutryn), insecticides (carbaryl, diazinon, imidacloprid) and transformation products (2-hydroxy-simazine, 2-hydroxy-terbutylazine, deethyl terbutylazine, deethyl terbumeton). In most cases, once a compound was detected, it could also be identified using the reference standards (fragment ions). However, in some cases only the exact mass of the protonated molecule and the t_R could be used to assess the presence of the substance. Although these findings cannot be classified as level 1 (Schymanski et al., 2014) and more research is needed for reliable confirmation, the information obtained (e.g. low mass errors and low t_R deviation) gave sufficient confidence to report these compounds as detected.

Table 6.1: Compounds detected/identified in influent (IWW) and effluent (EWW) wastewater samples from Spain and Italy

Compound	**Spain IWW (n = 7)**	**Spain EWW (n = 7)**	**Italy IWW (n = 2)**	**Italy EWW (n = 2)**
2-OH-Simazine		3/1		
2-OH-Terbuthylazine		4/0	1/0	2/1
Carbaryl		1/0		
Carbendazim		7/7		
Deethylterbumeton		1/0		
Diazinon		5/0		
Imidacloprid		1/1		
Metolachlor				1/1
Terbuthylazine			2/2	2/2
Terbutryn		7/7	1/1	1/1
Thiabendazole		6/6		
Total	0/0	9/5	3/2	4/4

Table 6.2: Compounds detected/identified in surface water samples from Spain

Compound	Burriana Clot	Nules	Almenara	Albufera Natural Park 1	Albufera Natural Park 2
2-OH-Terbuthylazine	1/1	1/1		1/1	
Carbendazim	1/1			1/1	1/1
Deethyl terbuthylazine	1/1	1/1		1/1	1/1
Deethylterbumeton	1/1	1/1		1/1	1/1
Imazalil				1/0	1/1
Metalaxyl		1/1		1/1	1/1
Molinate				1/1	
Propiconazole				1/1	1/1
Simazine	1/0			1/0	
Terbuthylazine	1/1	1/1		1/1	
Terbutryn		1/1			1/1
Thiabendazole	1/1	1/1		1/1	1/1
Total	7/6	7/7	0/0	11/9	8/8

The second list of pesticides (**Table S6.2)** was also screened and seven masses were frequently detected (**Table 6.3**). These could be assigned to a *"tentatively detected"* compound according to the exact mass, since no fragments were available in our database. Furthermore, the retention times were the same in all the samples for each of these seven compounds and the mass errors were always lower than 5 ppm. Unfortunately, these substances could not be fully identified only on the basis of the exact mass and analytical standards would be needed to confirm their identity. However, t_R prediction was used as a way to increase the confidence for these tentatively detected compounds, using a 2-min window (Bade et al., 2015b, 2015c) with 5-OH-clethodim sulfon found within this threshold. The "classical" pesticides in the priority pollutant lists of EU, US-EPA and United Nations, and many organophosphorus compounds widely used in recent years, were not detected in the samples analyzed.

Table 6.3: Compounds (exact masses) detected in different samples with high frequency and intensity and the potential corresponding pesticides

	Spain			Italy		Potential corresponding pesticides (according to Table S6.2)	Retention time (min)	
Exact mass $[M+H]^+$	IWW (n = 7)	EWW (n = 7)	SW (n = 2)	IWW (n = 2)	EWW (n = 2)		Sample	Predicted
408.1248	4	5			2	5-OH-clethodim sulfon	5.30	5.77
282.2797	7	7	5	2	2	Dodemorph	15.90	12.56
190.1266	4		2			EPTC	8.76	10.87
304.2640			5	2	2	Fenpropimorph	15.90	12.10
165.1028	5	3			1	Fenuron	1.70	5.39
204.1025	7	7		2		Hormodin	1.45	8.83
203.0933	3		1	2		Metamitron	8.18	4.03

6.3.1 Results in wastewater

In total, eleven pesticides and transformation products were detected and identified in WW samples from Spain and Italy (**Table 6.1**). A few compounds were found in IWW: terbutryn, terbutylazine and its transformation product 2-hydroxy-

terbutylazine were detected in Italy, while none of the selected pesticides were found in Spain. Respectively nine and four pesticides were detected in EWW samples from Spain and Italy (**Table 6.1**). In Italy the compounds found in IWW were also found in EWW, indicating that removal was not complete during wastewater treatment. Metolachlor was only identified in EWW. Generally, in both countries more compounds were detected in EWW than IWW.

Nine substances (bendiocarb, desmethylprimicarb, dibenzylamine, ethofenprox, ethoxyquin, kresoxim-methyl, spiroxamine, thiofanox, thiofanox sulfone) were tentatively detected from the first database (**Table S6.1**), but reference standards were no longer available to confirm them. Therefore, a t_R prediction model was applied, utilizing a ± 2-min window, as in previous works (Bade et al., 2015b, 2015c) and concluded that seven of the nine were within the window. The strength of t_R prediction as a complementary tool is thus underscored, as two compounds (bendiocarb and thiofanox) could be removed from further investigation, without the need for reference standards.

A number of factors explain why more substances were detected in EWW than IWW. First of all, raw wastewater (IWW) is a complex matrix and therefore harder to analyze than EWW. Commonly, matrix effects are much higher than in EWW, and sensitivity in analysis is poorer. Considering that pesticide concentrations in wastewater are usually very low, the full MS scan option used in HRMS can fail to identify multiple substances, thus giving a false-negative result due to the lack of sensitivity compared to, for example, LC-MS/MS with QqQ. The frequency of detection was better in EWW which is a cleaner matrix. Another hypothesis relates to the behavior of pesticides during the treatment processes; if a compound is retained in the activated sludge or reverts to the parent form from conjugated forms during treatments, its concentration may be higher in EWW than in IWW. Since our method is based on qualitative detection and identification of compounds, and no concentrations were available, the behavior of pesticides during

the WWTP processes could not be fully evaluated (Campo et al., 2013; Kock-Schulmeyer et al., 2013). Other possible reasons are related to the sampling procedure, the wastewater treatment technology, environmental conditions (e.g. rainfall, high temperature), and hydrolysis and transformation during treatments (Kasprzyk-Hordern et al., 2009; Kock-Schulmeyer et al., 2013; Luo et al., 2014; Moschet et al., 2014; Ort et al., 2010).

6.3.2 Results in surface water

Surface water samples were taken from five areas in Spain, and twelve pesticides and transformation products were detected and identified (**Table 6.2**). In almost all the cases the compounds detected were also identified, except for simazine (Burriana Clot and Albufera Natural Park 1) and imazalil (Albufera Natural Park 1), probably because of their very low concentrations. Up to eleven pesticides were found in Albufera Natural Park compared to the other areas. Some differences were observed among the two sites of this park; 2-hydroxy-terbutylazine, molinate, simazine and terbutylazine were found only in the first site, while terbutryn was detected only in the second. Seven substances were found in Burriana Clot and Nules, while in Almenara none of the substances investigated were found.

6.3.3 Comparison of results in Spain and Italy

In general, more compounds were detected (9) and identified (5) in Spanish EWW than Italian EWW (4 detected and 4 identified) (**Table 6.1**). Terbutryn and 2-hydroxy-terbutylazine were found in both countries, but terbutylazine, the parent compound of 2-hydroxy-terbutylazine, was identified only in Italy. The chloroacetanilide herbicide metolachlor was only identified in one EWW sample from Italy, and 2-hydroxy-simazine,

carbendazim, imidacloprid and thiabendazole were only identified in Spanish EWW. In addition, carbaryl, deethylterbumeton and diazinon were also detected only in the Spanish samples.

More pesticides were found in SW than WW. This is in line with the fact that additional sources of contamination can affect SW, such as direct runoff from cultivations, while WW is mainly affected by the urban use of these substances. Eleven compounds were found in Spanish SW. Five of these were also found in Spanish EWW samples. The compounds deethyl terbutylazine, imazalil, metalaxyl, molinate, propiconazole and simazine were identified only in Spain and in SW, indicating that their main source was agriculture.

The use of simazine, carbaryl, carbendazim, terbumeton, diazinon, metolachlor, molinate and terbutryn is currently prohibited in EU (European Commission, 2016). Nevertheless, the presence of carbendazim and molinate can probably be explained by the fact that during the sampling year (2014) their use was still permitted. The other compounds in water might imply spills and disposals of unused pesticides, transportation through the wind, illegal use of banned pesticides, high environmental persistence, transportation with foodstuffs and/or application during storage and transport from countries in which their use is allowed (Barco-Bonilla et al., 2013b; Botero-Coy et al., 2015; Coscollà et al., 2013; Wittmer et al., 2010).

6.3.4 Potential wastewater-based epidemiology biomarkers

Analysis of water samples from Spain and Italy resulted to detection of pesticides and transformation products belonging to the classes of funficides, herbicides and insecticides. Some of them have already been tested as WBE biomarkers and the rest could be investigated thoroughly for this purpose. In **Table 6.4** are presented the found

parent pesticides and their human urinary metabolites that could be used as WBE biomarkers.

Table 6.4: Compounds detected and/or identified in water samples from Spain and Italy and their potential WBE biomarkers

Compound	Human urinary metabolite Potential WBE biomarker	Comment*
Funficides		
Imazalil	1-(2,4-Dichlorophenyl)-2-(1H-imidazole-1-yl)-1-ethanol (DCPI)	
Metalaxyl	Ethylenethiourea	Mancozeb
Thiabendazole	5-OH-thiabendazole	
Herbicides		
Metolachlor	Metolachlor mercapturate	
Molinate	4-OH-molinate	
Insecticides		
Carbaryl	1-Naphthol	Naphthalene
Imidacloprid	6-Chloronicotinic acid	Acetamiprid

*The presented parent pesticides share the same human urinary metabolite with the pesticide found in water samples.

6.4 Conclusions

A large number of pesticides was investigated in WW and SW in Spain and Italy by HPLC-QTOF MS. Seventeen pesticides and transformation products belonging to different classes (fungicides, herbicides and insecticides) were found. The detected pesticides and their human urinary metabolites could be tested as biomarkers of human exposure based on the developed WBE approach. The wide-scope screening method based on HRMS was an efficient tool for screening a large number of pesticides and selecting priority substances to be investigated in a subsequent quantitative target, where more sensitive methods are required (i.e. based on LC-QqQ-MS/MS).

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Table S6.1: Compounds, related fragment ions and associated molecular formulas for pesticides screening

Compound	Formula	Molecular Formula of Fragments and Adducts					
2-hydroxy-atrazine	C8H15N5O	C5H9N5O	C4H7N3O	C4H4N2O	C2NH3N3O	C2N2O	
2-hydroxy-simazine	C7H13N5O	C4H7N3O	C2H2N5	C2N2O	C2H3N3O	C3H6N2	
2-hydroxy-terbuthylazine	C9H17N5O	C5H9N5O	C4H7N3O	C3H2N3O	C2H3N3O		
3,4-Dichloroaniline	C6H5Cl2N	C6H5NCl	C6HCl				
Acephate	C4H10NO3PS	C4H9NO3PSNa					
Acetamiprid	C10H11N4Cl	C10H10N4ClNa	C6H4NCl				
Acetochlor	C14H20ClNO2	C14H19NaClNO2	C12H14NOCl	C9H10N	C8H6N		
Acrinathrin	C26H21F6NO5	C26H20F6NO5Na					
Alachlor	C14H20ClNO2	C14H19NaClNO2	C13H16NOCl	C12H15N	C11H15N	C10H12N	C10H11N
		C9H9N	C8NO	C8H7N	C8H6N	C3H4NCl	C8NO
Aldicarb	C7H14N2O2S	C7H13NaN2O2S	C5H9NS	C4H8S			
Aldicarb sulfoxide	C7H14N2O3S	C7H13NaN2O3S	C2H4N2O2	C5H9NOS	C3H2N2OS	C5H13N2O2S	C5H4N2O
		C4H8S					
Aldicarb sulfone	C7H14N2O4S	C7H13NaN2O4S	C5H13N2O2S	C6H7O2S	C3HN2OS	C6H6NO	C4H7NO
	C2H5NO2						
Amidosulfuron	C9H15N5O7S2	C9H14N5O7S2Na					
Atrazine	C8H14N5Cl	C5H8N5Cl	C3H4N5Cl	C4H6N3Cl	C2H2N3Cl	C4H5N3	CH3N2Cl
Azinphos-ethyl	C12H16N3O3PS2	C9H11N3O2PS2	C7H7N3O2PS2	C5H11O2PS2	C8H5N3O	C8H5NO	C7H5N
		HO2PS	C7H4OS				
Azinphos-methyl	C10H12N3O3PS2	C10H11N3O3PS2Na	C8H5N3O	C8H5NO	C7H4O	C7H5N	C6H4
Azoxystrobin	C22H17N3O5	C22H16NaN3O5	C21H13N3O4	C20H13N3O3	C19H10N3O3		
Bendiocarb	C11H13NO4	C11H12NaNO4					
Benfuracarb	C20H30N2O5S	C20H29N2O5SNa	C7H9N2O4	C3H3NOS	C5H10N2O4S		
Bensulide	C14H24NO4PS3	C14H23NO4PS3Na	C6H7NO2S	C6H4O2S	C6H4		
Bifenazate	C17H20N2O3	C17H19N2O3Na	C13H11NO	C12H11N	C12H10NO	C12H7	C4H6N2O2
		C12H8N					
Boscalid	C18H12Cl2N2O	C6H2NOCl	C18H11N2OCl	C18H11N2O	C18H10N2O		
Bromacil	C9H13BrN2O2	C9H12NaBrN2O2	C5H5N2O2Br	C5H3N2O2Br	C4H2NOBr		
Bupirimate	C13H24N4O3S	C11H17N3O3S	C8H11N3	C11H19N3O	C2H5NO2S	C8H11N3O	
Buprofezin	C16H23N3OS	C7H7N	C9H16N2OS	C5H9NS	C5H8N2OS	C4H7NO	
Butocarboxim	C7H14N2O2S	C7H13N2O2SNa	C3H6S				
Butoxycarboxim	C7H14N2O4S	C7H13NaN2O4S					
Carbaryl	C12H11NO2	C12H10NaNO2	C10H8O	C10H6			

Carbendazim	C9H9N3O2	C8H5N3O	C7H5N3	C6H4N2			
Carbofuran	C12H15NO3	C12H14NaNO3	C10H12O2	C7H6O2			
Carbofuran-3-OH	C12H15NO4	C12H14NaNO4	C12H13NO3	C10H12O3	C10H10O2	C10H8O	C9H10O
Chlorantraniliprole	C18H14BrCl2N5O2	C7H6O C18H13NaBrCl2N5O2	C17H9BrCl2N4O2	C9H3N3OCl Br	C9H4NO2Cl		
Chlorfenvinphos	C12H14Cl3O4P	C12H13NaCl3O4P	C12H14O4PCl3	C7H3OPCl2	C8H3Cl2	C2H7O4P	C4H11O4P
Chlorpropham	C10H12ClNO2	C10H11ClNO2Na					
Chlorsulfuron	C12H12N5O4SCl	C12H11N5O4SClNa	C6H6N4O2	C5H8N4O	C6H6N5OS	C3H6N4O3S	C3H4N5O
Chlorpyrifos	C9H11Cl3NO3PS	C6H5Cl C9H10NaCl3NO3PS	C5H7N4ONa				
Clothianidin	C6H8ClN5O2S	C6H7ClN5O2SNa	C6H8N4S	C4H2NSCl	C4H4N2S	C5H7N3	C4H5N3
Coumafos	C14H16ClO5PS	C3H2S C12H12O5PSCl	C10H8O5PSCl	C10H7O2SCl	C10H6O4PSCl	C10H7O3Cl	C10H6O2Cl
Cyprodinil	C14H15N3	C13H11N3	C8H8N2	C7H5N2	C7H9N	C6H6N	
DDVP (dichlorvos)	C4H7Cl2O4P	C4H6Cl2O4PNa					
Deethyl atrazine	C6H10N5Cl	C3H4N5Cl	C3H3N5	C2H2N3Cl	CH3N2Cl	C2HN3	
Deethyl-2-hydroxy-terbuthylazine	C7H13N5O	C2H3N3O	C3H5N5O				
Deethyl terbumeton	C8H15N5O	C4H7N5O	C2H3N3O	C3H5N3O			
Deethyl terbuthylazine	C7H12N5Cl	C3H4N5Cl	C3H3N5	C2H2N3Cl	CH3N2Cl		
Deethyl terbutryn	C4H7N5S	C8H15N5S	C2H3NS				
Deisopropylatrazine	C5H8N5Cl	C3H4N5Cl	C4H6N3Cl	C2H2N3Cl	C4H5N3	C2HN3	CH3N2Cl
Deltamethrin	C22H19NO3Br2	C22H18NO3Br2Na					
Demethon-S-methyl	C6H15O3PS2	C6H14O3PS2Na					
Desmedipham	C16H16N2O4	C16H19N3O4					
Desmethyl pirimicarb	C10H16N4O2	C10H15NaN4O2					
Diazinon	C12H21N2O3PS	C8H12N2S	C8H12N2O	C2H5O2PS	H3O3PS	HO2PS	C4H5NO
Dibenzylamine	C14H15N	C14H12	C7H7N	C7H6			
Dichlofenthion	C10H13Cl2O3PS	C10H12NaCl2O3PS C6H4O23PSCl	C8H9O3PSCl2 O2PS	C6H5O3PSCl 2	C6H3O2PSCl2	C6H5OPSCl2	C6H4OCl2
Diethofencarb	C14H21NO4	C14H20NO4Na	C11H15NO4	C10H13NO2	C8H9NO2	C6H5NO2	C6H5NO
Diflubenzuron	C14H9ClF2N2O2	C14H8ClF2N2O2Na	C8HN2O2F	C7H5NOF2	C7H2OF2	C6H2F2	C7H3NF2
Diflufenican	C19H11F5N2O2	C13H6NO2F3	C13H5NO2F2	C12H6NOF3			
Dimethoate	C5H12NO3PS2	C5H11NaNO3PS2	C2H5O2PS	C2H6O2P	CH4O2P		
Dimethomorph	C21H22NO4Cl	C21H21NO4ClNa	C17H13O3Cl	C16H13O2Cl	C15H10O2Cl	C15H10OCl	C9H8O3

Diphenylamine	C12H11N	C9NO C6H6N					
Disulfoton	C8H19O2PS3	C8H18O2PS3Na					
Diuron	C9H10N2OCl2	C3H5NO	C7H3NOCl2	C6H3NCl2			
Epoxiconazole	C17H13ClFN3O	C8H5F	C8H4				
Ethion	C9H22O4P2S4	C9H21NaO4P2S4	C5H11O2PS2	C5H11O2PS3	C3H7O2PS2	HOPS	CH3O2PS2
Ethiofencarb	C11H15NO2S	C11H14NaNO2S	C9H9NO2	C7H6	C7H6O		
Ethiofencarb sulfone	C11H15NO4S	C11H14NaNO4S	C7H6	C7H6O	C6H5		
Ethiofencarb sulfoxide	C11H15NO3S	C11H14NaNO3S	C6H5	C7H6	C7H6O		
Etofenprox	C25H28O3	C25H27NaO3	C13H10O	C12H7O	C7H8O		
Ethofumesate	C13H18O5S	C13H17NaO5S C8H8O2	C11H14O5S C9H8O	C11H12O4S C9H8	C8H8O C7H7	C10H10O3	C10H8O2
Etoxazole	C21H23F2NO2	C7H2OF2					
Ethoxyquin	C14H19NO	C13H15NO	C12H15NO	C11H11NO	C10H9NO	C9H7NO	C8H7NO
Fenarimol	C17H12Cl2N2O	C17H10N2Cl2 C7H3OCl	C16H10NOCl C4H4N2	C15H8Cl2	C16H8OCl	C15H8NCl	C15H7Cl
Fenhexamid	C14H17Cl2NO2	C14H16Cl2NO2Na	C7H12				
Fenitrothion	C9H12NO5PS	C8H8NO4PS	C7H4NO3PS	C8H8O2PS	C7H5OPS	C2H5O2PS	C2H5O3P
Fenoxaprop	C16H12ClNO5	C16H11NaClNO5 C15H10NO3Cl	C16H12NO5Cl C6H3O	C8H6O	C13H8NO3Cl	C7H3NO2Cl	C9H9O4
Fenoxon sulfone	C10H15PSO6	C10H14NaPSO6	C9H11O5PS	C9H13O3P	C8H9O3PSCl2	C6H6O5	C7H6
Fenoxon sulfoxide	C10H15O5PS	C9H12O5PS	C9H11O4PS	C9H12O4P	C2H5O3P		
Fenoxycarb	C17H19NO4	C17H18NaNO4	C3H5NO2				
Fenthion	C10H15O3PS2	C9H11O2S2P	C7H5O3P	C8H8OS	C8H9S	C2H5O3P	
Fenthion oxon	C10H15O4PS	C10H14NaO4PS	C9H11O3PS	C8H8O3PS	C7H6O2PS	C8H8S	C7H6
Fenthion sulphone	C10H15O5PS2	C6H4S					
Fenthion sulfoxide	C10H15O4PS2	C10H14NaO4PS2	C9H12O4PS2	C6H4S	C6H6OS	C9H12O3PS	
Fluazifop-P-butyl	C19H20F3NO4	C19H19NaF3NO4 C12H6NOF3	C15H12NO4F3 C13H8NO3	C14H9NO4 C8H6O	C14H10NO2F3 C7H6	C15H11NO3	C14H8NO4
Fludioxonil	C12H6F2N2O2	C12H5NaF2N2O2	C12H5N2O2F				
Flufenoxuron	C21H11ClF6N2O3	C21H11ClF6N2O3Na	C14H5NO2F3Cl	C7H5NOF2	C7H2OF2		
Fluroxypyr	C7H5Cl2FN2O3	C7H4Cl2FN2O3Na	C7H3N2O2FCl2	C5H3N2FCl2	C6H3N2OFCl2		
Furathiocarb	C18H26N2O5S	C18H25NaN2O5S					
Haloxyfop-2-ethoxyethyl	C19H19ClF3NO5	C19H18ClF3NO5Na	C14H9NO2F3Cl	C14H6NO3Cl	C15H10NO3Cl		
Haloxyfop-methyl	C16H13ClF3NO4	C16H12NaClF3NO4	C14H9NO2F3Cl	C15H10NO3 Cl	C14H6NO3Cl	C8H6O	
Hexythiazox	C17H21ClN2O2S	C17H20NaClN2O2S	C10H10NOSCl	C10H8NOCl	C9H10NCl	C9H7Cl	C9H5Cl

		C9H7	C9H6				
Imazalil	C14H14Cl2N2O	C11H8N2OCl2	C9H6OCl2	C10H6NCl	C7H4Cl2	C3H4N2	C8H6Cl2
		C4H4N2					
Imidacloprid	C9H10ClN5O2	C9H9NaClN5O2	C9H9N4Cl	C9H10N4	C9H9N4	C9H8N3	C8H7N3
		C7H6N3	C6H6NCl	C6H4NCl	C3H5N3		
Indoxacarb	C22H17ClF3N3O7	C22H16NaClF3N3O8					
Iprodione	C13H13Cl2N3O3	C13H12Cl2N3O3Na	C9H6N2O2Cl2	C7H5NCl2	C6H5NCl2	C10H2N3O3Cl2	
Isoproturon	C12H18N2O	C12H17NaN2O	C3H5NO				
Kresoxim-methyl	C18H19NO4	C18H18NaNO4	C17H15NO3	C17H4O3	C11H11NO3	C10H11NO	C8H5N
Linuron	C9H10Cl2N2O2	C8H6N2OCl	C6H4NCl2	C6H3NCl2	C7H5N2Cl	C6H3NCl	C6H3N
		C5H2Cl2					
Malathion	C10H19O6PS2	C10H18NaO6PS2	C2H5O2PS	C4H2O3	CH3O2P	C3H2O2	
Mesotrione	C14H13NO7S	C14H12NO7SNa	C14H12O5S	C8H5NO5S	C11H5O5S	C13H9O3	C6HNO
Metalaxyl	C15H21NO4	C15H20NaNO4	C14H17NO3	C13H17NO2	C10H10N	C14H18NO3	C11H13N
		C12H17NO	C8H6N				
Methamidophos	C2H8NO2PS	CH4NO2P	C2H5O2PS				
Methidathion	C6H11N2O4PS3	C6H10N2O4PS3Na	C4H4N2O2S	C3H4N2O			
Methiocarb	C11H15NO2S	C11H14NaNO2S	C9H12OS	C8H9O	C7H6O	C7H8	C7H6
		C8H9O	C6H4				
Methiocarb sulfone	C11H15NO4S	C11H14NO4SNa	C9H12O3S	C8H9O	C7H6O		
Methiocarb sulfoxide	C11H15NO3S	C11H14NO3SNa	C9H12O2S	C8H9O2S	C9H11OS	C8H8OS	C8H9O
		C7H6O					
Methomyl	C5H10N2O2S	C5H9N2O2SNa	C3H7NONaS	C3H7NOS	C3H5NS	C4H3N2OS	C3HN2OS
		C2H2NS					
Metolachlor	C15H22ClNO2	C15H21NaClNO2	C14H18NOCl	C13H17N	C12H17N	C11H13N	C9H11N
		C8H8N					
Metribuzin	C8H14N4OS	C7H14N4S	C3H6N4S	C2H4N2S	C2H3NS		
Molinate	C9H17NOS	C9H16NaNOS	C7H11NO	C6H12N	C6H10		
Monocrotophos	C7H14NO5P	C7H17N2O5P	C7H13NaNO5P	C6H9O5P	C5H11O4P	C5H6NOP	C5H7NO
Omethoate	C5H12NO4PS	C5H11NaNO4PS	C3H7O3PS	C2H5O2PS	C2H5O3P	CH3O2P	
Oxadixyl	C14H18N2O4	C14H17N2O4Na	C12H14N2O2	C11H13NO2	C9H9N	C8H6N	
Oxamyl	C7H13N3O3S	C7H12N3O3SNa	C3H1N2OS	C3H5NO			
Paclobutrazol	C15H20N3OCl	C8H7Cl	C7H5Cl	C5H6N3	C5H10O	C2H3N3	
Parathion-ethyl	C10H14NO5PS	C8H10NO5PS	C6H6NO5PS	C3H8O3PS	C6H5NO3	C2H6O3P	C6H5O
Parathion-methyl	C8H10NO5PS	C7H6NO4PS	C7H6O2PS	C2H5O2PS	C2H5O3P	CH3O2P	
Pendimethalin	C13H19N3O4	C8H9N3O4	C8H7N3O3				

Phosmet	C11H12NO4PS2	C11H11NaNO4PS2	C9H5NO2	C8H4O2	C7H4O	C11H18N4O2	
Pirimicarb	C11H18N4O2	C11H17NaN4O2	C9H15N3O	C7H8N2O	C6H8N2	C4H8N2	C3H5NO
Pirimiphos-methyl	C11H20N3O3PS	C9H16N3O3PS	C9H13N3	C7H9N3	C2H5O2PS	C5H5N3	C5H6N2
Prochloraz	C15H16Cl3N3O2	C12H12NO2Cl3	C9H6NO2Cl3	C8H5OCl3	C6HOCl3	C6H3OCl2	C4H7N
		C3H3NO					
Promecarb	C12H17NO2	C12H16NaNO2	C7H8O	C6H5O	C10H14O		
Propanil	C9H9NOCl2	C9H7NCl2	C6H5NCl2	C9H7NO	C6H5NCl		
Propazine	C9H16ClN5	C6H10N5Cl	C3H5N5Cl	C3H3N5	C2HN3		
Propiconazole	C15H17Cl2N3O2	C7H4Cl2					
Propoxur	C11H15NO3	C11H14NO3Na	C8H9NO3	C9H12O2	C6H6O2	C6H4O	C7H2NO3
		C7H2NO2					
Pymetrozine	C10H11N5O	C6H4N2					
Pyraclostrobin	C19H18ClN3O4	C19H17NaClN3O4	C9H8NO2	C8H6NO2	C8H7NO		
Pyridaphenthion	C14H17N2O4PS	C14H16N2O4PSNa	C10H8N2O2	C9H6N2	C6H5N	C10H8N2OS	
Pyrifenox	C14H12Cl2N2O	C6H6N					
Pyriproxyfen	C20H19NO3	C15H14O2	C12H8O2	C5H5NO	C9H10O	C8H6O	
Quizalofop-ethyl	C19H17ClN2O4	C16H11N2O2Cl	C14H7N2OCl	C10H9NO3			
Quizalofop-methyl	C18H15ClN2O4	C15H11N2OCl					
Reserpine	C33H40N2O9	C23H28N2O4	C10H10O4	C8H13O4			
Simazine	C7H12ClN5	C5H8N5Cl	C6H9N3	C4H6N3Cl	C2H2N3Cl	C4H5N3	C2HN3
Spinosyn A	C41H65NO10	C8H15NO					
Spinosyn D	C42H67NO10	C8H15NO					
Spiroxamine	C18H35NO2	C8H17NO	C6H13N				
Tebuconazole	C16H22ClN3O	C9H7Cl	C7H5Cl	C2H3N3			
Tebufenozide	C22H28N2O2	C44H55N4O4Na	C22H27N2O2Na	C18H20N2O2	C9H8O	C8H8	
Tebufenpyrad	C18H24ClN3O	C18H23ClN3ONa	C7H7N2OCl	C6H9N2Cl	C4H5N2Cl		
Teflubenzuron	C14H6Cl2F4N2O2	C14H5Cl2F4N2O2Na					
Terbumeton	C10H19N5O	C6H11N5O	C4H7N5O	C5H9N3O	C4H7N3O	C3H5N3O	C3H2N3O
		C2H3N3O	C4H5N3	C2H6N2O	C3H3N5	C2HN3	C5H7N5
Terbuthylazine	C9H16ClN5	C5H8N5Cl	C3H4N5Cl	C4H6N3Cl	C2H2N3Cl	C4H5N3	CH3N2Cl
Terbutryn	C10H19N5S	C6H11N5S	C4H7N5S	C5H9N3S	C6H11N5S	C3H3N5	C3H5N3S
		C2H6N2S	C4H5N3	C3H6N2	C2HN3		
Tetraconazole	C13H11Cl2F4N3O	C7H4Cl2	C2H3N3				
Thiabendazole	C10H7N3S	C9H6N2S	C8H6N2				
Thiacloprid	C10H9ClN4S	C10H8ClN4SNa	C6H4NCl				
Thiamethoxam	C8H10ClN5O3S	C8H9ClN5O3SNa	C8H10N4OS	C4H2NSCl	C7H8N4S	C6H7N3	C6H5N3S

		C8H9N4OS	C3N4O2	C3HN3O2	C5H5N3	C3H4N2
Thiobencarb	C12H16ClNOS	C12H15NaClNOS	C7H5Cl			
Thiodicarb	C10H18N4O4S3	C10H17NaN4O4S3	C3H5NS	C4H4N2S	CH3N2O2S	
Thiofanox	C9H18N2O2S	C9H17N2O2SNa				
Thiofanox-sulfone	C9H18N2O4S	C9H21N3O4S				
Thiofanox-sulfoxide	C9H18N2O3S	C9H21N3O3S				
Thiophanate-methyl	C12H14N4O4S2	C12H13N4O4S2Na	C11H10N4O3S2	C7H6N2S	C8H5N3O	
Tolclofos-methyl	C9H11Cl2O3PS	C8H7OSPSCl2	C7H4OCl2	C2H5O2PS	C6H3Cl	CH3O2P
Tolylfluanid	C10H13Cl2FN2O2S2	C10H12Cl2FN2O2S2Na	C8H6NSCl2F	C7H6NS		
Triadimenol	C14H18N3O2Cl	C14H17N3O2ClNa	C12H15O2Cl	C6H10O	C2H3N3	
Trichlorfon	C4H8Cl3O4P	C4H7NaCl3O4P				
Triflumizole	C15H15ClF3N3O	C12H11NOF3Cl	C8H3NF3Cl	C11H4N2Cl	C7H2F3Cl	
Triflumuron	C15H10ClF3N2O3	C15H9ClF3N2O3Na				
Triflusulfuron-methyl	C17H19F3N6O6S	C17H18F3N6O6SNa				

Table S6.2: Compounds and associated molecular formulas for pesticides screening

Compound	Molecular Formula
1-Naphthylacetamide (1-NAD)	C12H11NO
1-Naphthylacetic acid (1-NAA)	C12H10O2
2,4,5-T	C8H5Cl3O3
2,4,5-T Isopropyl ester	C11H11Cl3O3
2,4-D	C8H6Cl2O3
2,4-D Butyl ester	C12H14O3Cl2
2,4-D isopropyl ester	C11H12Cl2O3
2,4-D methyl ester	C9H8Cl2O3
2-Aminobenzimidazole	C7H5N2O
2-Naphthoxyacetic acid	C12H10O3
2-Phenoxypropionic acid	C9H10O3
3,4,5-Trimethacarb	C11N15NO2
3-Hydroxy-carbofuran	C12H15NO4
5-Hydroxy-imidacloprid	C9H10ClN5O3
5-OH-clethodim-sulfon	C17H26NO6SCl
6-Chloro-4-hydroxy-3-phenyl-pyridazin	C10H7N2OCl
8-Hydroxyquinoline	C9H7NO
Abscidic acid	C15H20O4
Acibenzolar-S-methyl	C8H6N2OS2
Aclonifen	C12H9ClN2O3
Alanycarb	C17H25N3O4S2
Ametryn	C9H17N5S
Aminocarb	C11H16N2O2
Amitraz	C19H23N3
Anilazine	C9H5N4Cl3
Anilofos	C13H19ClNO3PS2
ANTU	C11H10N2S
Avermectin B1a (abamectin)	C48H72O14
Avermectin B1b (abamectin)	C47H70O14
Azamethiphos	C9H10N2O5PSCl
Azadirachtin	C35H44O16
Benalaxyl	C20H23NO3
Benomyl	C14H18N4O3
Bensulfuron-methyl	C16H18N4O7S
Bensultap	C17H21NO4S4
Benzoximate	C18H18ClNO5
Bifenox	C14H9NO5Cl2
Bifenthrin	C23H22ClF3O2
Bitertanol	C20H23N3O2
Bromoxynil	C7H3Br2NO

Bromuconazole	C13H12N3OCl2Br
Butoxycarboxim-sulfoxid	C7H14N2O3S
Buturon	C12H13ClN2O
Captan	C9H8NO2SCl3
Carbetamide	C12H16N2O3
Carbosulfan	C20H32N2O3S
Carboxin	C12H13NO2S
Chlorbromuron	C9H10N2O2BrCl
Chlorfenapyr	C15H11BrClF3N2O
Chlorfenvinphos-Met	C10H10Cl3O4P
Chlorfluazuron	C20H9Cl3F5N3O3
Chloridazon	C10H8N3OCl
Chlorophenoxyacetic acid	C8H7ClO3
Chloropicrin	CCl3NO2
Chlorotoluron	C10H13ClN2O
Chloroxuron	C15H15ClN2O2
Chlorpyrifos-methyl	C7H7Cl3NO3PS
Chromafenozide	C24H30N2O3
Cinosulfuron	C15H19N5O7S
Clethodim	C17H26NO3SCl
Clethodim-imin-sulfon	C14H23NO4S
Clethodim-imin-sulfoxide	C14H23NO3S
Clethodim-sulfon	C17H26NO5SCl
Clethodim-sulfoxid	C17H26NO4SCl
Clodinafop-propargyl	C17H13ClFNO4
Clofentezine	C14H8Cl2N4
Clomazone	C12H14ClNO2
Clopyralid	C6H3Cl2NO2
Coroxon	C9H10N2O2BrCl
Cyanazine	C9H13ClN6
Cyanazine acid	C9H14ClN5O2
Cyanazine amide	C9H15N6OCl
Cyanofenphos	C15H14NO2PS
Cyanofenphos oxygen	C15H14NO3P
Cyazofamid	C13H13ClN4O2S
Cycloate	C11H21NOS
Cycloheximide	C15H23NO4
Cycloxydim	C17H27NO3S
Cyfluthrin	C22H18Cl2FNO3
Cymoxanil	C7H10N4O3
Cyproconazole	C15H18ClN3O
Cyromazine	C6H10N6
Dacthal	C10H6Cl4O4
Daminozide	C6H12N2O3

Dazomet	C5H10N2S2
Deethyl ametryn	C7H13N5S
Deethyl cyanazine	C7H9ClN6
Deethyl cyanazine acid	C7H10N5O2Cl
Deethyl cyanazine amide	C7H11N6OCl
Deethyl-deisopropyl-2-hydroxy-Atrazine	C3H5N5O
Deethylhydroxyatrazine	C6H11N5O
Deethylsymetrine	C6H11N5S
Deisopropyl-2-hydroxy-atrazine	C5H9N5O
Deisopropylprometryne	C7H13N5S
Demeton-S-methyl sulphone	C6H15O5PS2
Demeton-S-methyl sulfoxide	C6H15O4PS2
Demethyl fluometuron	C9H9F3N2O
Demethyl isoproturon	C11H16N2O
Demethyl monuron	C8H9ClN2O
Diafenthiuron	C23H32N2OS
Dialifos	C14H17ClNO4PS2
Diallate	C10H17Cl2NOS
Dichlofluanid	C9H11Cl2FN2O2S2
Dichlone	C10H4Cl2O2
Dichlorprop	C9H8Cl2O3
Diclobutrazol	C15H19Cl2N3O
Diclofop-methyl	C16H14Cl2O4
Dicloran	C6H4Cl2N2O2
Dicrotophos	C8H16NO5P
Dicryl	C10H9Cl2NO
Dieldrin	C12H8Cl6O
Difenoconazole	C19H17Cl2N3O3
Difenoxuron	C16H18N2O3
Dimefuron	C15H19ClN4O3
Dimethachlor	C13H18NO2Cl
Dimethylvinphos	C10H10Cl3O4P
Diniconazole	C15H17Cl2N3O
Dinocap	C18H24N2O6
Diphacinone	C23H16O3
Diquat dibromide monohydrate	C12H14N2Br2O
Dixanthogen	C6H10O2S4
Dodemorph	C18H35NO
Dodine	C15H33N3O2
Edifenphos	C14H15O2PS2
Endrin	C12H8Cl6O
EPN	C14H14NO4PS
EPTC (S-dipropylthiocarbamate)	C9H19NOS

Ethephon	C2H6ClO3P
Ethiprole	C13H9Cl2F3N4OS
Ethoprophos	C8H19O2PS2
Etrimfos	C10H17N2O4PS
Famoxadone	C22H18N2O4
Fenamiphos	C13H22NO3PS
Fenazaquin	C20H22N2O
Fenbuconazole	C19H17N4Cl
Fenfuram	C12H11NO2
Fenoxaprop-ethyl	C18H16NO5Cl
Fenpiclonil	C11H6Cl2N2
Fenpropathrin	C22H23NO3
Fenpropidin	C19H31N
Fenpropimorph	C20H33NO
Fenpyroximate	C24H27N3O4
Fensulfothion	C11H17O4PS2
Fensulfothion-sulfone	C11H17O5PS2
Fenuron	C9H12N2O
Flamprop	C16H13NO3FCl
Flamprop isopropyl	C19H19ClFNO3
Flamprop methyl	C17H15ClFNO3
Flazasulfuron	C13H12N5O5SF3
Flonicamid	C9H6F3N3O
Florasulam	C12H8F3N5O3S
Fluacrypyrim	C20H21F3N2O5
Fluazifop	C15H12F3NO4
Flucythrinate	C26H23F2NO4
Flufenace	C14H13F4N3O2S
Fluometuron	C10H11F3N2O
Fluquinconazole	C16H8Cl2FN5O
Fluridone	C19H14F3NO
Flurtamone	C18H14F3NO2
Flusilazole	C16H15N3F2Si
Folpet	C9H4Cl3NO2S
Fonofos	C10H15OPS2
Forchlorfenuron	C12H10ClN3O
Formetanate	C11H15N3O2
Fosthiazate	C9H18NO3PS2
Fuberidazole	C11H8N2O
Gibberellic acid	C19H24O6
Haloxyfop	C15H11ClF3NO4
Haloxyfop-ethoxyethylester	C19H19ClF3NO4
Heptenophos	C9H12ClO4P
Hexaconazole	C14H17Cl2N3O

Hexazinone	C12H20N4O2
Hormodin	C12H13NO2
Hydroxy atrazine	C8H15N5O
Imazameth	C14H17N3O3
Imazamethabenz-methyl	C16H20N2O3
Iprodione desisopropyl	C10H7Cl2N3O3
Iprovalicarb	C18H28N2O3
Isazofos	C19H17ClN3O3PS
Isofenphos	C15H24NO4PS
Isoxaflutole	C12H15NO4SF3
Isoxathion	C13H16NO4PS
Lufenuron	C17H8Cl2F8N2O3
Malaoxon	C10H19O7PS
MCPA methylester	C10H11ClO3
Mecarbam	C10H20NO5PS2
Mepanipyrim	C14H13N3
Merphos	C12H27PS3
Metamitron	C10H10N4O
Metazachlor	C14H16N3OCl
Metconazole	C17H22N3OCl
Methabenzthiazuron	C10H11N3OS
Methfuroxam	C14H15NO2
Methoxyfenozide	C22H28N2O3
Metobromuron	C9H11N2O2Br
Metosulam	C14H13N5O4SCl2
Metoxuron	C10H13ClN2O2
Metsulfuron	C14H15N5O6S
Mevinphos	C7H13O6P
Monolinuron	C9H11ClN2O2
Monuron	C9H11ClN2O
Myclobutanil	C15H17N4Cl
N-2,4-Dimethylphenyl-N'-methylformamidine	C10H14N2
Naled	C4H7Br2Cl2O4P
Napropamide	C17H21NO2
Neburon	C12H16Cl2N2O
Nicosulfuron	C15H18N6O6S
Nicotine	C10H14N2
Nitenpyram	C11H15ClN4O2
N-m-Tolylphthalamic acid	C15H13NO3
Nuarimol	C17H12FN2OCl
Ofurace	C14H16ClNO3
Oxamide	C15H15N2O3Cl
Oxycarboxin	C12H13NO4S

Oxydemeton-methyl	C6H15O4PS2
Oxyfluorfen	C15H11ClF3NO4
Paraoxon	C10H14NO6P
Paraoxon-methyl	C8H10NO6P
Paraquat dichloride	C12H14N2Cl2
Parathion	C10H14NO5PS
Pebulate	C10H21NOS
Penconazole	C13H15Cl2N3
Pencycuron	C19H21ClN2O
Phenmedipham	C16H16N2O4
Phenyl mercuric acetate	C8H8HgO2
Phorate	C7H17O2PS3
Phorate oxygen analogue	C7H17O3PS2
Phorate sulfoxide	C7H17O4PS2
Phorate-sulfone	C7H17O5PS2
Phosalone	C12H15NO4PS2Cl
Phosfon	C20H34Cl2P
Phosphamidon	C10H19NO5PCl
Phoxim	C12H15N2O3PS
Picolinafen	C19H12F4N2O2
Pirimiphos ethyl	C13H24N3O3PS
Primisulfuron-methyl	C15H12F4N4O7S
Procymidone	C13H11Cl2NO2
Profenofos	C11H15BrClO3PS
Prometon	C10H19N5O
Prometryn	C10H19N5S
Propachlor	C11H14ClNO
Propamocarb	C9H20N2O2
Propargite	C19H26O4S
Propetamphos	C10H20NO4PS
Propham	C10H13NO2
Propyzamide	C12H11NOCl2
Prosulfuron	C15H16F3N5O4S
Prothiofos	C11H15Cl2O2PS2
Pyrazophos	C14H20N3O5PS
Pyridaben	C19H25ClN2OS
Pyridate	C19H23ClN2O2S
Pyrimethanil	C12H13N3
Quinalphos	C12H15N2O3PS
Quinmerac	C11H8ClNO2
Quinoclamine	C10H6ClNO2
Quinoxyfen	C15H8FNOCl2
Rimsulfuron	C14H17N5O7S2
Rotenone	C23H22O6

Sethoxydim	C17H29NO3S
Silvex	C9H7Cl3O3
Simetryn	C8H15N5S
Strychnine	C21H22N2O2
Sulfallate	C8H14ClNS2
Sulfosulfuron	C19H21N3O7S2
Sulfotep	C8H20O5P2S2
Sulprofos	C12H19O2PS3
Tebuthiuron	C9H16N4OS
Tepraloxydim	C17H24ClNO4
Terbufos	C9H21O2PS3
Terbufos sulfone	C9H21O4PS3
Terbufos sulfoxide	C9H21O3PS3
Tetrachlorvinphos	C10H9Cl4O4P
Thifensulfuron-methyl	C12H13N5O6S2
Thiophanate-ethyl	C14H18N4O4S2
Thiram	C6H12N2S4
Triadimefon	C14H16N3O2Cl
Triasulfuron	C14H16ClN5O5S
Triazophos	C12H16N3O3PS
Tribenuron-methyl	C15H17N5O6S
Tricyclazole	C9H7N3S
Trietazine	C9H16ClN5
Trifluralin	C13H16F3N3O4
Triticonazole	C17H20N3OCl
Vamidothion	C8H18NO4PS2

Chapter 7

Research limitations and future perspectives

Research limitations and future perspectives

The present doctoral thesis demonstrated that the measurement of human urinary metabolites of pesticides in influent wastewater serve to evaluate the collective population exposure to pesticides. This novel method can be a valuable tool for obtaining objective, direct information on the real levels of pesticide exposure of the general population, almost in real time and can complement the findings of HBM studies.

Analytical chemistry is the strong base of the WBE approach, as it was also emphasized in this thesis. For the first time, a new very sensitive analytical method was developed for the determination of pesticide metabolites, which have attracted much attention, in wastewater taken from urban areas. This method applied LC-MS/MS with QqQ in a limited number of compounds. Therefore, new methodologies could be introduced for the improvement of this approach. The main disadvantage of the applied technique is the restricted number of compounds that can be determined in a single run and the fact that many compounds are ignored in the analysis as they are not part of the target list. Thus, a HRMS instrument could be used as an additional tool for the identification of "unknown" substances (without reference standards), such as metabolites. Additionally, a retention time prediction model combined with HRMS can help for the tentative identification of these compounds. Furthermore, development of chiral analytical methods would be potentially a powerful tool to allow one to differentiate the sources of pesticides to wastewater. Chiral methods can be applied for instance to malathion and its metabolites.

Stability during in-sewer transit, sampling and storage is a very crucial aspect of the WBE, since it has to be verified that the measured amounts of the biomarkers are not formed or degraded during these procedures and therefore are resulted from the human metabolism. The stability experiments within the present study were done in the

laboratory using unfiltered wastewater samples, under constant pH and temperature conditions. These experiments can mimic some in sewer conditions, but are not able to reproduce the real conditions occurring in the sewer. Future investigations should be followed taking into account abiotic and biotic transformation processes, sorption to suspend particulate matter and sediments, and the presence of biofilms on the sewer walls. Ideally in-sewer transformation studies in real sewers should be carried out, in order to investigate the stability and the spontaneous formation of the WBE biomarkers.

Pharmacokinetic studies are substantial for the development of correction factors, which are used to estimate the population exposure to parent pesticides. In the present study, all the available human urinary pharmacokinetic studies published in peer-reviewed journals were considered in order to obtain suitable correction factors. Most of these studies involve a small number of subjects, are quite old, do not include all the parent pesticides (e.g. pyrethroids), and do not take into account the possible interactions between different pesticides. Moreover, there are not pharmacokinetic studies for all the pesticides investigated in this doctoral thesis. Therefore, new human urinary pharmacokinetic studies are required, including adequate number of subjects, all the parent pesticides (individually and together) and realistic doses of exposure in order to refine and improve the estimates obtained through the WBE approach.

Another important issue for WBE is how to exclude the presence of additional sources of both parent substances and metabolites in wastewater. A WBE biomarker should be unique to human metabolism, ensuring that its presence in wastewater only derives from human excretion and not from other exogenous sources. Hence, the total measured amount results from the human exposure. Otherwise, the estimated population exposure is overestimated. In the present study, this was done by reviewing papers in the literature. In the future, experiments dealing with the determination of the WBE biomarkers in foodstuff (e.g. pesticides in foods might undergo metabolic processes with

spontaneous formation of metabolites) and the domestic environment (e.g. analyzing dust samples) are recommended. Furthermore, the main use of pesticides is in agriculture and thus, studies investigating their metabolism in plants can give important information for other potential exogenous sources. Some pesticides are also applied to livestock and domestic care animals (e.g. disinfection of sheep) and therefore the metabolism of the selected pesticides to the targeted metabolites should be investigated. Another way to exclude additional sources of the metabolites to wastewater is analysis of tap water, since its major part ends into sewage. For instance, terbuthylazine desethyl has been found in drinking water of many Italian cities and consequently cannot be used as WBE biomarker. Finally, analysis of influent wastewater and urine samples taken from the same area at the same period of time, would give useful information about the reliability of the applied approach to selected biomarkers and the existence of additional sources.

This novel application of the WBE approach could be further applied to other compounds having as a scope the study of the population exposure. It can be expanded to other classes of pesticides and also to other exposure WBE biomarkers from environment and food, such as mycotoxins, parabens, UV-filters, plasticizers and brominated flame retardants. Furthermore, this approach can be expanded to other categories of biomarkers, such as lifestyle and substance use biomarkers (e.g. new psychoactive substances), health biomarkers (e.g. antibiotics) and population biomarkers (e.g. artificial sweeteners).

Chapter 8

Conclusions

Conclusions

The main outcome of this doctoral thesis is the development of a novel application of the wastewater-based epidemiology approach based on pesticide metabolites as target category. The application was developed by measuring human urinary metabolites in influent wastewater, at very low concentrations, by liquid chromatography tandem mass spectrometry with a triple quadrupole analyzer. This novel approach was suitable to evaluate the exposure of the general population to pesticides. Several metabolites were tested as target biomarkers in wastewater in order to evaluate the exposure to the parent substances and some of them resulted suitable to be used as biomarkers of exposure. A WBE application could be developed and a good correlation between human urinary biomonitoring studies and wastewater results was found. This approach was proposed as a new biomonitoring tool and can provide important information for public health that can be of interest for national and international organizations and committees, such as the United States Environmental Protection Agency, the World Health Organization and the Centers for Disease Control and Prevention.

Several other specific outcomes were provided:

- ✓ A new liquid chromatography coupled to mass spectrometry method was developed and validated for the determination of a wide range of pesticide human urinary metabolites in influent wastewater.
- ✓ Sample pretreatment is an important step of wastewater analysis, because of the complexity of the matrix and the fact that compounds are usually found at very low concentrations. Solid phase extraction was proved to be essential for sample clean-up and pre-concentration for the majority of analytes. The investigated alkyl phosphate metabolites were determined without SPE, fulfilling the analytical criteria.

- ✓ One of the main requirements of WBE is the stability of the targeted compounds, in order to ensure that the measured amounts are not formed or degraded from the toilet to the lab. Stability experiments showed that refrigerated conditions should be adopted for the sampling procedure, while no great losses are expected in the sewer system.
- ✓ Storage at the lab is a crucial aspect that has to be investigated, since it is not always possible to perform the chemical analysis immediately after sampling. Therefore, storage at -20 °C was found to be the most suitable procedure for the larger part of compounds up to two months, except for DCCA, AM and DIA which are not stable for such a long period.
- ✓ It is important to test the stability of the parent compounds in relation to the formation of the targeted metabolites, with the aim to verify the uniqueness of the biomarkers in wastewater. No spontaneous formation of 3-PBA and DCCA in the sewer system and during sampling from degradation of permethrin, cypermethrin and cyfluthrin is expected.
- ✓ A compound can be characterized as WBE biomarker (one of the requirements) when it is detected frequently and at quantified concentrations. The most frequently detected compounds in Italy (seven cities) and Europe (eight cities) were: the specific metabolite of chlorpyrifos and chlorpyrifos-methyl, TCPY; the metabolite of diazinon, IMPY; the alkyl phosphate metabolites, DMP and DEP; the generic metabolite of the class of pyrethroids, 3-PBA; and the metabolite of permethrin, cypermethrin and cyfluthrin, DCCA.
- ✓ The compounds chlorpyrifos-methyl, AM and DMTP were not detected in any of the Italian (seven cities) and European (eight cities) samples.
- ✓ The highest concentrations were found for the alkyl phosphate metabolites DMP and DEP, followed by DETP. High concentrations were also estimated for the

pyrethroid metabolites, while very low concentrations were found for triazine metabolites (Italian and European samples).

- ✓ Mass loads of insecticides were found to be lower in Norway (Oslo) and Denmark (Copenhagen), compared to the United Kingdom (Bristol), Italy (Milan) and Spain (Castellon). These results were in accordance with the Eurostat official statistics.
- ✓ Temporal and spatial changes are components of the most significant advantages of the WBE approach, which can be used mainly for comparison reasons. Seasonal variations on pesticide intake (i.e. pyrethroids) were found in Milan, Italy.
- ✓ Spatial differences on pesticide exposure were observed across Italy.
- ✓ Spatial differences on exposure to pesticides in the various European cities were in accordance with official sales data (Eurostat) of these substances.
- ✓ It is very important to ensure that the measured amounts of the biomarkers are coming exclusively from the human metabolism and therefore all the potential sources in wastewater should be explored. Investigation of the source of the targeted compounds showed that the selected triazine metabolites might not be good WBE biomarkers, since they are formed also in the environment and the measured amounts in wastewater are not derived exclusively from human metabolism. On the contrary, the investigated pyrethroid metabolites found in influent wastewater are mainly originated from human metabolism.
- ✓ A comparison of the wastewater results with an existing risk indicator (ADI) was successfully applied for first time in WBE.

CURRICULUM VITAE

Name: Nikolaos I. Rousis

Date of Birth: 5th of June 1987

Nationality: Greek

Place of Birth: Athens, Greece

E-mail: nrousis@chem.uoa.gr

Education

11/2013 – 01/2017: PhD in Environmental Chemistry (Marie Curie ESR), IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy.

2010 – 2012: MSc "Chemical Analysis-Quality Control", Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece.

2009 – 2010: Certificate of Enology, Department of Chemistry, University of Athens, Greece.

2005 – 2010: BSc in Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, Greece.

Articles in Peer Reviewed Journals

1. V.P. Kalantzis, **N.I. Rousis**, I.N. Pasias, N.S. Thomaidis and E.A. Piperaki. "Evaluation of different modifiers for the determination of arsenic in leachate samples from sanitary landfills by electrothermal atomic absorption spectrometry" *Analytical Letters*, **2012**, 45, 592-602.
2. A.K. Psoma, I.N. Pasias, **N.I. Rousis**, K.A. Barkonikos and N.S. Thomaidis.

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Poster Presentations

1. V. Kalantzis, **N.I. Rousis**, I.N. Pasias, N.S. Thomaidis and E.A. Piperaki. "Evaluation of different modifiers for the determination of arsenic in drainage samples from solid waste dump areas by electrothermal atomic absorption spectrometry" Proceedings of the 7th Aegean Analytical Chemistry Days, Mytilene **2010**.

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2. **N.I. Rousis**, S. Castiglioni and E. Zuccato. "Determination of biomarkers of exposure in raw wastewater by liquid chromatography-tandem mass spectrometry – Pesticides as the case study" 2nd International Conference on "Wastewater-based drug epidemiology" – Testing the Waters, Ascona, Switzerland 11-15 October **2015**.

Professional Experience

03/2016 – Present: Research Associate IRCCS Mario Negri Institute for Pharmacological Research, Department of Environmental Health Sciences, Milan, Italy (Dr. Ettore Zuccato and Dr. Sara Castiglioni).

04/2014 – Present: WG member, "Sewage biomarker analysis for community health assessment" Sewage Analysis CORe group Europe (SCORE), COST Action ES1307.

03/2013 – 02/2016: Marie Curie Early Stage Researcher (https://sewprof-itn.eu/), IRCCS Mario Negri Institute for Pharmacological Research, Department of Environmental Health Sciences, Milan, Italy (Dr. Ettore Zuccato and Dr. Sara Castiglioni).

04/2015: Research Associate "Occurrence of pesticides and their metabolites in raw wastewater in Norway" Norwegian Institute for Water Research, NIVA, Oslo, Norway (Dr. Kevin Thomas).

10-11/2014: Research Associate "Enantiomeric profiling of chiral pesticides in wastewater" Department of Chemistry, University of Bath, England (Prof. Dr. Barbara Kasprzyk-Hordern)

06/2014: Research Associate "Use of high resolution instruments for the screening of pesticides, pharmaceuticals and illicit drugs in natural water and wastewater" Research Institute for Pesticides and Water, University Jaume I, Spain (Prof. Dr. Félix Hernàndez).

09/2010 – 02/2013: Research Associate, Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece (Prof. Dr. Nikolaos S. Thomaidis).

06/2011 – 06/2012: Research Associate, Chemistry Laboratory, Center of Biological Research of Armed Forces, Ministry of National Defense, Greece (Dr. Paul Nisianakis).

Educational Activities

2010 – 2011: Teaching Assistant of laboratory in "Instrumental Analysis II" course (4th semester, Chemistry Department; Prof. Dr. Nikolaos S. Thomaidis)

2011 – 2012: Teaching Assistant of laboratory in "Analytical Chemistry II" course (4th semester, Pharmacy Department; Prof. Dr. Michael A. Koupparis)

Training Courses

22-24/04/2013: "Human Health and the Environment" University of Bath, Bath, England.

09-10/09/2013: "Sampling, Sample Handling Storage and Sample Preparation" Norwegian Institute for Water Research, NIVA, Oslo, Norway.

10-11/04/2014: "Analytical Techniques for Biomarkers Analysis" KWR Water cycle Research Institute, Utrecht, the Netherlands.

16-18/09/2014: "Assessing Human Health and Lifestyle by Sewage Epidemiology" Mario Negri - Institute for Pharmacological Research, Milan, Italy.

29-30/04/2015: "Method Validation and Quality Control" University Jaume I, Castelló de la Plana, Spain.

Brief description of research experience - Analytical Skills

Experience in techniques: CRC-ICP-MS, ETAAS, FAAS, AES, LC-ESI-MS/MS (QqQ), UHPLC-QOTF-MS, Orbitrap, GC-MS, Chiral analysis

Main sample preparation techniques: microwave digestion, solid phase extraction, solid liquid extraction

Other analytical skills: development and validation of methods, uncertainty measurements, ISO 17025, Microsoft Office, software "STATGRAPHICS Centurion XV.I"

Scientific Committees

- Member of the Association of Greek Chemists
- Member of the Trace Analysis and Mass Spectrometry Group

Reviewer

Analytical Letters

International Journal of Environmental Analytical Chemistry

Journal of Analytical Atomic Spectrometry

Science of the Total Environment

Languages

Greek (Native speaker)

English

Italian