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Pesticides in the environment: analysis, occurrence, impact and recommendations for their attenuation

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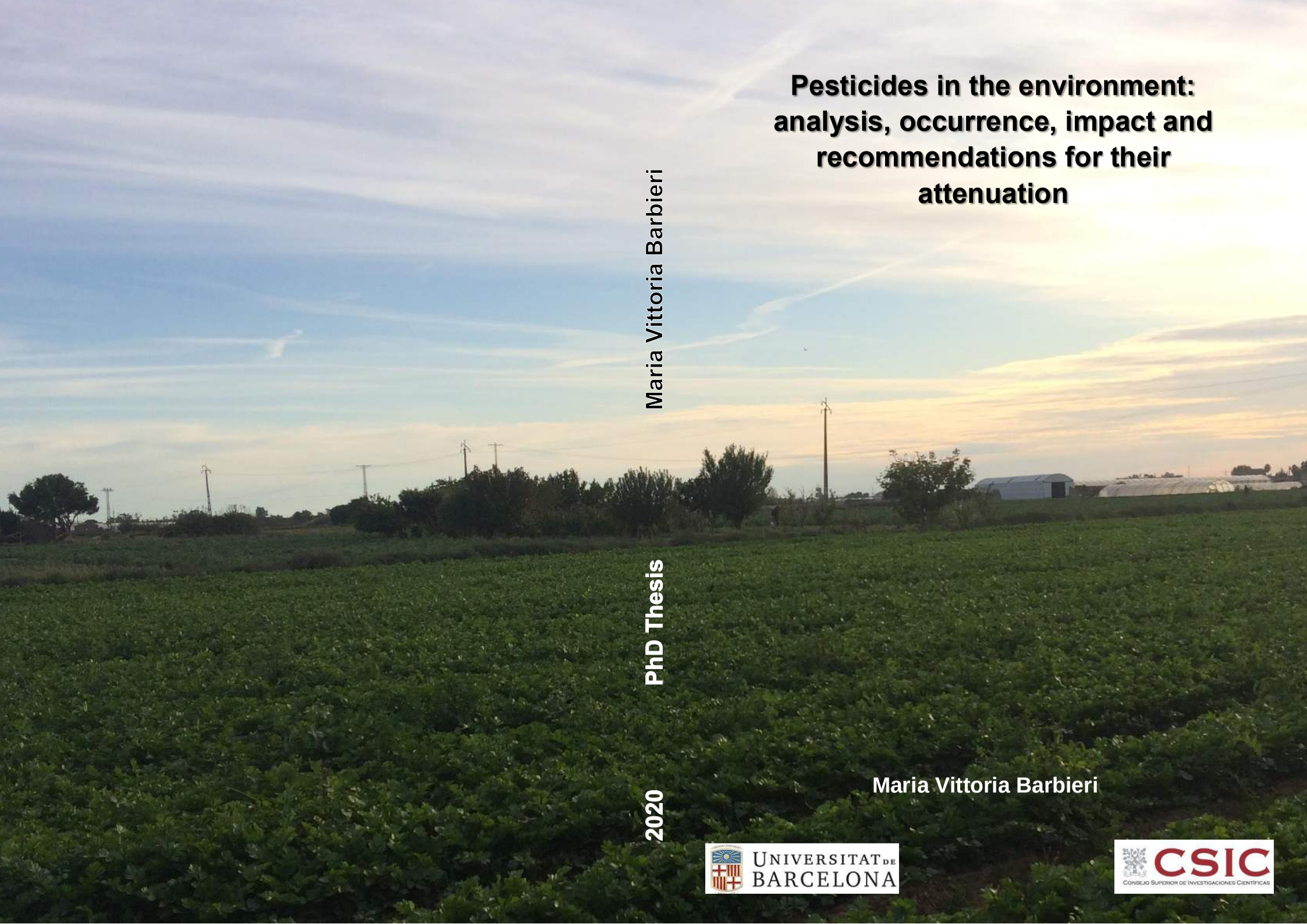

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PhD Thesis

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PESTICIDES IN THE ENVIRONMENT: ANALYSIS, OCCURRENCE, IMPACT AND RECOMMENDATIONS FOR THEIR ATTENUATION

Memoria presentada para optar al título de
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Barcelona, 25 de noviembre de 2020

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Certifican:

Que la presente memoria presentada para optar al título de Doctor, titulada "***Pesticides in the environment: analysis, occurrence, impact and recommendations for their attenuation***" ha sido realizada bajo nuestra dirección por la Sra. **Maria Vittoria Barbieri** en el Instituto de Diagnóstico Ambiental y Estudios del Agua (IDAEA), perteneciente al Consejo Superior de Investigaciones Científicas (CSIC), y que todos los resultados presentados son fruto del trabajo experimental realizado por el mencionado doctorando.

Barcelona, 25 de noviembre de 2020

Dra. Miren López de Alda Villaizán

Dra. Cristina Postigo Rebollo

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buena historia a cuestas y alguien a quien contársela.

Alessandro Baricco, Novecento

Table of Contents

Abbreviations and acronyms

AA	Annual Average
AChE	Acetylcholinesterase
AOP	Advanced Oxidation Process
APCI	Atmospheric Pressure Chemical Ionization
AR	Absolute Recovery
BCF	Bioconcentration Factor
BMP	Best Management Practice
DLLME	Dispersive Liquid–Liquid Microextraction
EC	European Commission
EI	Electron Ionization
EPA	Environmental Protection Agency
EQS	Environmental Quality Standards
ESI	Electrospray Ionization
EU	European Union
FAO	Food And Agricultural Organization Of The United Nations
GAP	Good Agricultural Practices
GUS	Groundwater Ubiquity Score
HILIC	Hydrophilic Interaction Liquid Chromatography
HLB	Hydrophilic-Lipophilic-Balance
HPLC	High-Performance Liquid Chromatography
HQ	Hazard Quotient
HRMS	High Resolution Mass Spectrometry
ILIS	Isotopically Labeled Internal Standards
K_{oc}	Soil Sorption Coefficient
K_{ow}	Octanol/Water Partition Coefficient
LC50	Lethal Concentration 50 %
LD50	Lethal Dose 50 %
LLE	Liquid-Liquid Extraction
LOD	Limit Of Detection
LODet	Limit Of Determination
LOQ	Limit Of Quantification
LPME	Liquid-Phase Microextraction

m/z	Mass To Charge Ratio
MAC	Maximum Allowable Concentration
MAE	Microwave-Assisted Extraction
MEC	Measured Environmental Concentration
MRLs	Maximum Residue Levels
MSPD	Matrix Solid-Phase Dispersion
MSPE	Magnetic Solid-Phase Extraction
NMJ	Neuromuscular Junction
NOAEC	No Adverse Effect Concentration
NOAEL	No Adverse Effect Level
PBT	Persistent, Bioaccumulative And Toxic
Pka	Acid Dissociation Constant
PLE	Pressurized Liquid Extraction
PNEC	Predicted No-Effect Concentration
PPPs	Plant Protection Products
QqLIT	Quadrupole-Linear Ion Trap
QqQ	Triple Quadrupole
QSAR	Quantitative Structure-Activity Relationship
QTOF	Quadrupole-Time-Of-Flight
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, And Safe Extraction
RfD	Reference Dose
RR	Relative Recovery
RSD	Relative Standard Deviation
SBSE	Stir Bar Sorptive Extraction
SLE	Solid-Liquid Extraction
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
SRM	Selective Reaction Monitoring
TPs	Transformation Products
UAE	Ultrasound Assisted Extraction
UPLC	Ultra-Performance Liquid Chromatograph
WFD	Water Framework Directive
WRF	White-Rot Fungi
WWTP	Wastewater Treatment Plant

Summary

Environmental pollution from agricultural, urban and industrial use of pesticides is known to be a threat to the healthy ecological functioning of aquatic ecosystems. The assessment of pesticide occurrence in different environmental compartments has become a matter of outstanding importance since its effects on exposed organisms may be considered a warning on the potential risk these substances may pose to human health. To accurately study pesticide exposure, the design of an effective monitoring program is required. This includes the selection of the appropriate compounds to be analyzed and the collection of representative samples. Pesticide monitoring is also essential to evaluate the effectiveness of remediation technologies and mitigation measures to reduce pesticide pollution.

In this context, and in the framework of this doctoral thesis, the presence and fate of 52 pesticides and transformation products (herbicides, insecticides, fungicides, biocides) belonging to 9 different chemical families were evaluated in surface water, groundwater, and sediments from three river basins in Catalonia (Ebro, Llobregat, and Ter) and biota (fish) from the Adige River (Italy), and the associated environmental risk was assessed. In addition, the performance of a fungi-based bioremediation technique for the removal of pesticides was evaluated.

The analysis of pesticides and their transformation products in the different environmental compartments requires the use of sophisticated analytical techniques, that are constantly evolving to meet the challenge of determining these chemicals at parts per trillion (ppt) levels or less in complex matrices with sufficient selectivity and reliability. To this objective, the methods developed in this doctoral thesis are based on the use of advanced extraction techniques such as on-line solid-phase extraction (SPE), pressurized liquid extraction (PLE) followed by SPE purification, and QuEChERS extraction for the recovery of the target pesticides from water, sediment, and biota samples, respectively, and analyte determination with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

The use of an isotope-dilution approach ensured the reliability of the results and the satisfactory performance of the methodologies developed in terms of accuracy

(relative recoveries between 80 and 120%), precision (relative standard deviations lower than 20%), and linearity (with calibration curves (r^2 > 0.99) expanding in most of the cases over several orders of magnitude). The extent of matrix effects changed depending on the complexity of the matrix and the target pesticides, and in the majority of the cases it appeared in the form of signal suppression. However, the use of isotopically-labeled internal standards also contributed to correct for matrix effects, as well as possible pesticide losses during the extraction process, and hence, allowed a reliable quantification of the target pesticides. The sensitivity of the methodologies developed, with limits of determination (LODets) below 40 ng/L in surface water, 63 ng/L in groundwater, 12 ng/g d.w. in sediment and 10 ng/g f.w. in biota, makes possible the determination of the selected pesticides at levels established as environmental quality standards by the European Commission in the field of water policy.

The most relevant pesticides detected in agricultural areas were the herbicides bentazone, MCPA and propanil, widely used in rice cultivation, and found at high concentrations in the Ebro River Delta (up to 180 µg/L). The Ter River, also impacted by intensive agricultural activities, resulted less contaminated by bentazone and MCPA. In urban and industrial areas like the lower Llobregat River basin, the most ubiquitous and abundant pesticides found were bromoxynil, diuron, linuron and terbutryn, used for both agricultural and non-agricultural purposes. Many of the pesticides found in this area presented levels above the limit of 100 ng/L set by the European Union for individual pesticides in waters intended for human consumption. Thus, they may pose a risk to human health, because the Llobregat surface water is used to produce the drinking water that is supplied to part of the city of Barcelona and its metropolitan area. In the sediments of the Llobregat River and the fish samples collected from the Adige River, the pesticides found (especially diazinon, dichlorvos, irgarol, quinoxyfen, and terbutryn) presented physical-chemical properties that make them more likely to accumulate in sediments or bioaccumulate in biota. Most of the pesticides detected in this matrices are currently banned, which suggests that their presence is not recent, and their persistence in the environment may pose a risk for aquatic ecosystem.

Regarding the environmental quality standards (EQS) for priority compounds in surface waters (Directive 2013/39/EC), only dichlorvos and irgarol exceeded the maximum

allowable concentrations established by the European Community, while the maximum acceptable limits of detection (LODs) established in the European Watch List (Commission Implementing Decision (EU) 2018/840) were surpassed by acetamiprid, imidacloprid, methiocarb and thiacloprid. In sediments, although EQS have not been established, the high levels of dichlorvos, irgarol and terbutryn, which were above the corresponding quality standards set for surface waters, are a warning of the necessity of monitoring and control the sediment contamination status. In biota, the pesticides found did not exceed the maximum residue level of 10 ng/g.

The studies conducted showed that the main factors contributing to the local pesticide pollution pattern in the aquatic environment include: the specific physical-chemical properties of the pesticides, the land use in the area, the level of irrigation, the issuance of restrictive regulations (prohibition, regulated use of some pesticides in specific food commodities), and pesticide application (e.g. type of pesticides, season and mode of application, etc.).

The assessment of the environmental risk that the pesticides detected may pose to aquatic organisms has highlighted the role of pesticides as relevant stressors in the aquatic environment, due to either their high presence or their high toxicity. In particular, the pesticides posing the highest risk in the investigated areas were: azinphos ethyl, bentazone, diazinon, dichlorvos, dicofol, diflufenican, imidacloprid, irgarol, methiocarb, MCPA, propanil, terbutylazine and terbutryn. Moreover, the co-occurrence of pesticides in the environmental samples investigated results in an increased risk in the environmental compartments, which makes necessary the adoption of measures to attenuate pesticide pollution.

For this purpose, the capability of the white-rot fungus *T. versicolor* to degrade malathion, acetamiprid and imidacloprid was explored. Results point at this organism as an efficient, green and economic alternative for pesticide removal during water treatment. Further pesticide pollution attenuation was attempted at local level by contributing to the design of participatory multi-actor events to increase the awareness of farmers and stakeholders on the issue of environmental pollution by pesticides, involve all water actors in the decision-making processes, and provide the farmers with practical knowledge to

motivate the implementation of mitigation measures and best management practices aimed at reducing pesticide release into the environment.

Resumen

La contaminación ambiental por el uso agrícola, urbano e industrial de plaguicidas supone una amenaza para el funcionamiento de los ecosistemas acuáticos. La evaluación de la presencia de plaguicidas en los diferentes compartimentos ambientales es de gran importancia ya que sus efectos en los organismos expuestos pueden considerarse una advertencia del riesgo potencial que estas sustancias pueden suponer para la salud humana. Para evaluar de forma precisa la exposición a plaguicidas es necesario diseñar un programa de vigilancia efectivo. Éste debe incluir los compuestos más indicados para su análisis en cada caso y la toma de muestras representativas. El análisis y control de plaguicidas también es esencial para evaluar la efectividad de las diversas tecnologías de remediación y medidas de mitigación propuestas para reducir este tipo de contaminación en el medio ambiente.

En este contexto, en el marco de esta tesis doctoral se ha estudiado la presencia y destino de 52 pesticidas y productos de transformación (herbicidas, insecticidas, fungicidas, biocidas) pertenecientes a 9 familias químicas diferentes en aguas superficiales, subterráneas y sedimentos de tres cuencas hidrográficas de Cataluña (Ebro, Llobregat, y Ter) y en biota (peces) del río Adige (Italia), y se ha evaluado el riesgo ambiental asociado. Además, se ha valorado la eficacia de una técnica de biorremediación basada en hongos para eliminar plaguicidas seleccionados.

El análisis de plaguicidas y sus productos de transformación en los diferentes compartimentos ambientales requiere el uso de técnicas analíticas sofisticadas, que están en constante evolución para afrontar el desafío de analizar estas sustancias a niveles de partes por trillón (ppt) o inferiores en matrices complejas con suficiente selectividad y fiabilidad. Es por ello, que los métodos desarrollados en el marco de esta tesis doctoral se basan en el uso de técnicas avanzadas de extracción como son la extracción en fase sólida (SPE) *on line* automatizada, la extracción mediante líquidos presurizados (PLE) seguida de purificación del extracto mediante SPE, y la extracción con QuEChERS para la preconcentración de los plaguicidas en agua, sedimento, y biota, respectivamente, y la

determinación de los compuestos objeto de estudio mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS).

El uso de dilución isotópica para la cuantificación garantiza la fiabilidad de los resultados y el rendimiento satisfactorio de los métodos desarrollados en términos de exactitud (recuperaciones relativas entre 80 y 120%), precisión (desviaciones estándar relativas inferiores al 20%) y linealidad (con curvas de calibrado (r^2> 0,99) que se expanden en la mayoría de los casos a lo largo de varios órdenes de magnitud). Los efectos de la matriz en el análisis variaron en función de la matriz y de los compuestos analizados, apareciendo en la mayoría de los casos en forma de supresión de la señal. Sin embargo, el uso de patrones internos marcados isotópicamente contribuyó a corregir estos efectos de la matriz, así como las posibles pérdidas de pesticidas durante los procesos de extracción, permitiendo así una cuantificación fiable de los plaguicidas objeto de estudio en las muestras investigadas. La sensibilidad de los métodos desarrollados, con límites de determinación (LODets) por debajo de 40 ng/L en agua superficial, 63 ng/L en agua subterránea, 12 ng/g de peso seco en sedimento y 10 ng/g de peso fresco en biota, posibilita la evaluación de los plaguicidas seleccionados a los niveles de los estándares de calidad ambiental marcados por la Comisión Europea en el ámbito de la política de aguas.

Los plaguicidas más relevantes detectados en las áreas agrícolas investigadas fueron los herbicidas bentazona, MCPA y propanil, ampliamente utilizados en el cultivo del arroz, y presentes a concentraciones muy elevadas en el delta del río Ebro (hasta 180 µg/L). El río Ter, también afectado por actividades agrícolas intensivas, resultó igualmente contaminado sobre todo por bentazona y MCPA, pero en mucha menor medida. En áreas urbanas e industriales como la cuenca baja del río Llobregat, los compuestos más ubicuos y abundantes fueron bromoxinilo, diurón, linurón y terbutrina, utilizados tanto con fines agrícolas como no agrícolas. Además, en esta zona, muchos de los plaguicidas encontrados superaban el límite de 100 ng/L establecido por la Unión Europea para plaguicidas individuales en aguas destinadas al consumo humano, lo que puede representar un riesgo para el hombre, dado que las aguas superficiales del río Llobregat se usan para la producción del agua potable que se suministra a parte de la ciudad de Barcelona y su área metropolitana. En los sedimentos del río Llobregat y las muestras de peces recogidas en el río Adige, los plaguicidas encontrados (sobre todo diazinón, diclorvos, irgarol, quinoxifeno

y terbutrina) presentan propiedades físico-químicas que los hacen más propensos a acumularse en sedimentos o bioacumularse en la biota. La mayoría de los pesticidas detectados en estas matrices están actualmente prohibidos, lo cual sugiere que su presencia no es reciente y su persistencia en el medio ambiente supone un riesgo para el ecosistema acuático.

En cuanto a las normas de calidad ambiental (NCA) para compuestos prioritarios en aguas superficiales (Directiva 2013/39/UE), solo diclorvos e irgarol superaron las concentraciones máximas admisibles establecidas por la Comunidad Europea, mientras que los límites máximos aceptables de detección (LODs) establecidos en la Lista de Observación europea (Decisión de ejecución (UE) 2018/840 de la Comisión) fueron superados por acetamiprid, imidacloprid, metiocarb y tiacloprid. En sedimentos, aunque no se han establecido NCA, los altos niveles de diclorvos, irgarol y terbutrina, por encima de los correspondientes estándares de calidad establecidos para aguas superficiales, son una advertencia de la necesidad de analizar y controlar el estado de contaminación de los sedimentos. En biota, ningún plaguicida superó el límite máximo de residuos de 10 ng/g.

Los estudios realizados mostraron que los principales factores que contribuyen al patrón local de contaminación por plaguicidas en el medio ambiente acuático incluyen: las propiedades físico-químicas específicas de los plaguicidas, el uso del suelo, la existencia de regulaciones restrictivas (prohibición, uso regulado de algunos plaguicidas en productos alimenticios específicos), el nivel de riego, y la aplicación de plaguicidas (por ejemplo, tipo de plaguicidas, temporada y modo de aplicación, etc.).

La evaluación del riesgo ambiental que los plaguicidas detectados pueden representar para los organismos acuáticos ha destacado el papel de los plaguicidas como estresores relevantes en el medio acuático, ya sea por su elevada presencia o por su elevada toxicidad. En particular, los plaguicidas de mayor riesgo en las áreas investigadas fueron: azinfos etilo, bentazona, diazinón, diclorvos, dicofol, diflufenican, imidacloprid, irgarol, metiocarb, MCPA, propanil, terbutilazina y terbutrina. Además, la co-ocurrencia de plaguicidas en las muestras ambientales investigadas resulta en un aumento del riesgo en los diversos compartimentos ambientales estudiados, lo que hace necesaria la adopción de medidas para atenuar la contaminación ambiental por plaguicidas.

Con este propósito, se exploró la capacidad del hongo de la podredumbre blanca *T. versicolor* para degradar malatión, acetamiprid e imidacloprid. Los resultados apuntan a este organismo como una alternativa eficaz, ecológica y económica para la eliminación de pesticidas del agua. Por otro lado, también se participó en la implementación de otras medidas para atenuar la contaminación por plaguicidas a nivel local. Para ello, se diseñaron eventos participativos con el fin de aumentar la conciencia de los agricultores y otras partes interesadas (gestores y usuarios del agua, representantes locales, etc.) sobre el problema de la contaminación ambiental por plaguicidas, involucrarlos en los procesos de toma de decisiones, y proporcionar a los agricultores los conocimientos prácticos para motivar la implementación de medidas de mitigación destinadas a reducir la liberación de plaguicidas al medio ambiente.

Thesis structure

The present thesis is structured in 5 chapters. In the first chapter, a general introduction about pesticides in the environment is presented, with a focus on medium to highly polar pesticides and their occurrence, distribution and fate in different environmental compartments. The most relevant pesticide-related legislation and its evolution in recent years is listed and explained, and the potential impact of pesticides in the environment to non-target organisms is overviewed. The state of the art in the analysis of pesticides in water, sediment and biota is also presented and, finally, the compounds that have been studied in this doctoral thesis are introduced, looking at their physical-chemical properties, use and current legislation status.

Chapter 2 presents the main objectives of the thesis.

Chapter 3 contains the results obtained. It consists of a compilation of the scientific publications completed in the framework of the doctoral thesis. This chapter is divided into 5 sections. Each section collects the experimental work done and the results obtained in the studies conducted during the doctoral period. The research done includes the development of analytical methodologies for the analysis of pesticides in surface water, groundwater, sediment, and biota, the evaluation of the occurrence and fate of pesticides in the aforementioned different matrices in various different scenarios, the assessment of the environmental risk that the concentrations found may pose to non-target organisms, and the evaluation of a bioremediation technique to reduce the presence of selected pesticides in the environment.

Finally, the discussion of all the results is presented in chapter 4, while chapter 5 collects the general conclusions obtained in this doctoral thesis, always seeking to integrate the main observations of the previous chapters. At the end of this document, the bibliography used is detailed together with the annexes corresponding to the indexes of tables and figures.

The scientific publications included in this thesis are distributed as follows:

Scientific publication #1: Barbieri M.V., Monllor-Alcaraz L.S., Postigo C., López de Alda M. (2020) Improved fully automated method for the determination of medium to highly polar

pesticides in surface and groundwater and application in two distinct agriculture-impacted areas. *Science of the Total Environment* 745, 140650.

Scientific publication #2: Barbieri M.V., Postigo C., Monllor-Alcaraz L.S., Barceló D., López de Alda M. (2019) A reliable LC-MS/MS-based method for trace level determination of 50 medium to highly polar pesticide residues in sediments and ecological risk assessment. *Analytical and Bioanalytical Chemistry* 411, (30):7981–7996.

Scientific publication #3: Barbieri M.V., Postigo C., Guillem-Argiles N., Monllor-Alcaraz L.S., Simionato J.I., Stella E., Barceló D. and López de Alda M. (2019) Analysis of 52 pesticides in fresh fish muscle by QuEChERS extraction followed by LC-MS/MS determination. *Science of the Total Environment* 653, 958-967.

Scientific publication #4: Barbieri M.V., Peris A., Postigo C., Moya-Garcés A., Monllor-Alcaraz L.S., Rambla-Alegre M., Eljarrat E., López de Alda M. (2020) Evaluation of the occurrence and fate of pesticides in a typical Mediterranean delta ecosystem (Ebro River Delta) and risk assessment for aquatic organisms. *Environmental Pollution* 115813, in press.

Scientific publication #5: Kaidi H., Barbieri M.V., López-García E., Postigo C., Caminal G., Montserrat S., López de Alda M. (2020) Degradation of selected medium to highly polar pesticides by the white-rot fungus *Trametes versicolor*. *Submitted to Environmental Science: Water Research & Technology (2020).*

CHAPTER 1 - INTRODUCTION

CHAPTER 1 - INTRODUCTION

1.1 Pesticides: definition and classification

Pesticides are individual substances or substance mixtures designed for preventing, destroying or mitigating any group of pests, unwanted species of plants or animals interfering with the production, processing, storage, transport or marketing of food, wood or animal feedstuffs. The term includes substances used as plant growth regulators, desiccants or defoliants, and applied to crops during harvest to protect their deterioration. A pesticide can be a chemical, a biological agent (such as virus or bacteria), an antimicrobial disinfectant, or a device used against pests on organisms such as insects, plant pathogens, weeds, or microbes (WHO, 2018).

Many different categorizations of pesticides have been established, taking into account certain characteristics such as their use, general mode of action, chemical family and molecular structure, or toxicity. Each specific classification takes into consideration different aspects that help us understand their chemical and physical properties and hence their behavior in exposed organisms. Table 1.1 includes the most relevant categorization.

Considering the different use of pesticides, they are classified according to the type of organism on which the compound performs its action, such as fungi (fungicides), weeds (herbicides), insects (insecticides) or all kind of living organisms (biocides). Moreover, according to their use, they present different modes of action on the targeted organisms. For instance, herbicides are usually residual; they are applied before the plant sprouts and they remain in the soil long enough to kill the weeds during its germination. These pre-emergence herbicides are considered a preventive treatment, while post-emergence pesticides are usually applied on weeds that have already grown.

The chemical structure of pesticides helps us to understand what they have in common at the molecular level, and dividing them by families we can predict the reactivity of these substances in the various environmental compartments. This categorization is also important from an analytical point of view, since analytes are addressed as groups with some similarities in their properties, which make them more or less amenable for their analysis by specific analytical methodologies.

Another important classification is based on their toxicity and focuses on the acute oral lethal dose 50 % (LD50) value, a statistical estimation of the number of mg of toxicant per kg of bodyweight required to kill 50 % of a large population of test animals.

Table 1.1. Examples of pesticide categories according to their different properties or characteristics.

Pesticide property	**Categories**
Use	Acaricide, biocide, fungicide, herbicide, insecticide, nematocide
Mode of action	Herbicide (contact, systemic, selective, non-selective, residual, non-residual) Fungicide (protective, eradicant) Insecticide (contact, inhalation, ingestion)
Chemical family	Acidics, azoles, carbamates, neonicotinoids, organochlorines, organophosphates, triazines, ureas
Toxicity	Ia - Extremely Hazardous Ib - Highly hazardous II - Moderately hazardous III - Slightly hazardous U - Unlikely to present acute hazard

The historical use of pesticides goes back over 2000 years, but the use of synthetic organic pesticides started in the decade of 1930, while the real establishment of the pesticide industry did not occur until 1942 when the insecticide DDT was introduced. Since then, many pesticide products have been introduced into the market, as a consequence of the continued population growth and the concomitant increased demand for food, which are considered the principal drivers of pesticide evolution. It has been estimated that in the past 50 years, the use of pesticides in agriculture has increased dramatically and currently amounts around 3 million tons per year (Silva et al., 2019). An additional source of pesticides comes from the non-agricultural uses, that in recent years received particular attention. The list includes industrial and urban applications (grass-management, industrial vegetation control, public-health), domestic uses, and non-agricultural crops (such as forestry or ornamental plants).

The Regulation 1185/2009/EC of the European Parliament and of the Council lists a total of more than 500 active ingredients available on the European market. Nowadays, the total sale of pesticides in Europe is estimated to be around 400,000 tons (Eurostat, 2020). Among the 20 European Union (EU) Member States for which complete data are available, Germany, Spain, France, and Italy reported over two-thirds of the total EU pesticide sales volume in 2018. Spain is ranked as the second country in Europe with the largest pesticide consumption: 72 Million kg per year on average in the period 2011-2018 (Eurostat, 2020). These countries are also the main agricultural producers in the EU. They collectively account for about one half (46 %) of the EU's total utilized agriculture area and one half (47 %) of the total arable land in the EU. The highest sales volumes in 2016 in terms of pesticide use were for fungicides and bactericides (46 %), followed by herbicides (29 %) and insecticides and acaricides (11 %) (Eurostat, 2018).

1.2 Pesticides in the environmental context

Although pesticides are meant to eradicate specific pests, a large amount of pesticides may reach other destinations than their targets, entering into the water, soil and food chain, including plants, animals and human beings, and thus contaminating the ecosystem (de Souza et al., 2020). The presence of pesticides in the aquatic environment is considered one of the main chemical stressors for organisms living in the aquatic ecosystems (Bunzel et al., 2013), and in particular, agriculture is considered one of the greatest causes of diffuse pollution of pesticides of surface water. Pesticide pollution of water may be caused by a variety of physical, biological, and chemical mechanisms, such as sorption–desorption into solid particles, chemical and biological degradation, surface run-off, soil leaching, plant uptake, volatilization, and atmospheric deposition (Figure 1.1). The extent to which these processes contribute to the overall fate of a pesticide in the environment is related to the physical and chemical properties of the pesticide (*e.g.* solubility, hydrophobicity), soil characteristics (*e.g.* organic matter content, microbial activity), environmental factors (*e.g.* salinity, temperature, precipitation events), and management practices (*e.g.* type of crops, time and rate of pesticide application) (Sarmah et al., 2004).

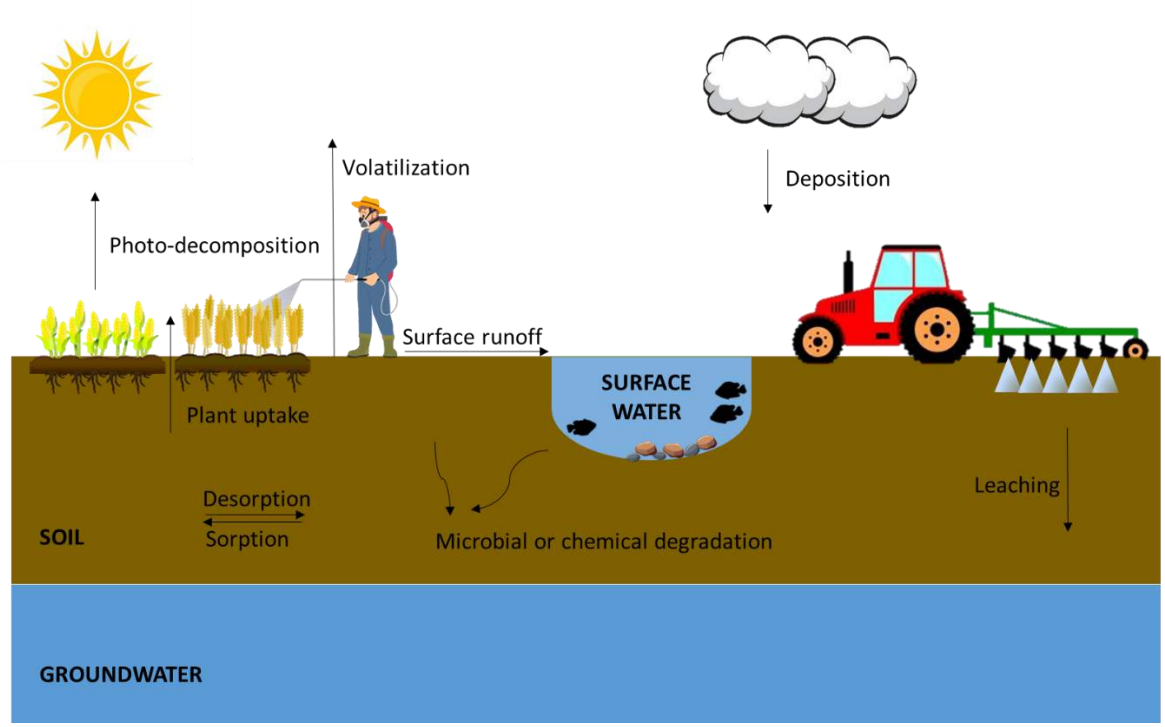


Figure 1.1. Scheme of the various processes responsible for the fate of applied pesticides in the environment.

Besides crops, industrial and domestic activities also contribute to the discharge of pesticides into the aquatic ecosystem and therefore, its contamination. Pesticide release from these activities is mainly done through wastewater treatment plant (WWTP) effluents. Unfortunately, WWTPs are not designed to efficiently eliminate pesticides and other organic pollutants from water (Petrović et al., 2003). Therefore, the discharge of WWTP effluents that contain pesticides and metabolites into the rivers, the runoff of rainwater and, to a lesser extent, the atmospheric deposition, contribute to the fact that continental surface waters present the highest levels of pesticides.

Schulz (2004) pointed out that between 1% and 10 % of pesticide losses from the agricultural fields and reach non-target areas. These values increase during rainfall events after pesticide application. This shows how environmental factors can also influence the presence and transport of pesticides in the various environmental compartments.

Different aspects can also influence the effects of pesticides on the aquatic communities. According to the Food and Agricultural Organization of the United Nations

(FAO) (FAO, 1997), the main factors that influence the ecological impacts of pesticides in these compartments are:

- the toxicity of pesticides itself, usually expressed by "dose descriptors" used to identify the relationship between a specific effect of a chemical and the dose at which it takes place. Dose descriptors are determined in toxicological studies about the risk of substances (i.e., lethal concentration 50 % (LC50), lethal dose 50 % (LD50), no observed adverse effect level/concentration (NOAEL/NOAEC), etc.). These dose descriptors are then used for deriving the no-effect threshold levels for human health (i.e., reference dose RfD) and the environment (i.e., predicted no-effect concentration PNEC);
- the persistence of the compounds, measured in terms of half-life (DT50), which is defined as the time (days) that takes to reduce the amount of a compound by half through degradation in an environmental compartment, which in turn is determined by biotic processes (biodegradation, metabolism) and abiotic processes (hydrolysis, photolysis, oxidation);
- the environmental fate of a pesticide in a certain compartment, which depends mainly on its physical-chemical properties (e.g. octanol/water partition coefficient (K_{ow}), soil sorption coefficient (K_{oc}), solubility, volatility, groundwater ubiquity score (GUS), etc.), as well as on the actual conditions of the medium (e.g., temperature, salinity, sunlight, etc.);
- the transformation products (TPs), generated from the pesticide breakdown once released into the environment, and which in many cases can be even more toxic than their parent compounds.

Additionally, many pesticides can persist for long periods in an ecosystem after their application. The so-called PBT (persistent, bioaccumulative, and toxic) pesticides, such as the organochlorine pesticides, for instance, were banned in the late 1970s, but they are still detectable in surface waters (Golfinopoulos et al., 2003), groundwater (Levy et al., 2017), soil (Cavanagh et al., 1999; Hong et al., 2006; Yang et al., 2013) and biota (García-Alvarez et al., 2014; Henríquez-Hernández et al., 2017; Panseri et al., 2019). In this regard, the soil is a major reservoir of organochlorine pesticides, and hence, responsible for their persistence in the environment. This pesticide class presents a high potential to adsorb onto particles, and long-term persistence and mobility, and thus they are able to enter

again into surface water or groundwater after leaching from soil particles or dissolution after sediment resuspension, or even entering again into food crops, causing a subsequent human exposure via plant uptake (Fantke et al., 2011; Liu et al., 2016). In the soil, they may be transformed by biotic or abiotic processes. Organochlorine pesticides have been also usually targeted in biota due to their high capacity to partition into lipids (high K_{ow}).

After the prohibition of using most organochlorine pesticides in developing countries, many other pesticides started to be synthesized and introduced into the market and are nowadays commonly used. In this regard, polar pesticides were presented as an attractive alternative to organochlorine pesticides, because of their high water solubility, low K_{oc} and K_{ow}, and low environmental persistence. This new generation of pesticides is transported primarily in the dissolved phase, and exhibit shorter half-lives than the organochlorine compounds. Thus, research on the environmental occurrence of medium to highly polar pesticides has been very much focused on the water compartment (Köck-Schulmeyer et al., 2019). However, their large application in agriculture and their continuous release into the aquatic systems has led to their ubiquitous presence in the environment. Despite the low K_{ow} values of the most polar compounds, they are also likely to accumulate in sediments via ion specific sorption mechanisms or bioaccumulate in aquatic organisms by exposure pathways and the ability of metabolization and elimination (Kah and Brown, 2006; Pérez-Parada et al., 2018)

Figure 1.2 summarizes the concentrations of individual medium to highly polar pesticides found in groundwater and surface water in different studies conducted in the past 10 years. In both matrices, the detected pesticides achieved very high concentrations, being in some cases in the µg/L level. For what concerns groundwater, at global level the highest concentrations are attributed to the presence of dimethoate (150 µg/L) in the Parbhani District (India) (Motekar Shrinivas, 2014). At Spanish level, the herbicide alachlor was found at concentrations of 10 µg/L in aquifers of Catalonia (Spain) in the study of Köck-Schulmeyer et al. (2014), and 60 % of the investigated pesticides were found to be present at concentrations above the limit of 100 ng/L set for individual pesticides in water intended for human consumption in the Drinking Water Directive (EC, 1998a) and the Groundwater Directive of 2006 (EC, 2006a). Likewise, in almost all the studies here reported, concentrations above 100 ng/L were measured for various compounds, like atrazine (180

ng/L) and bentazone (260 ng/L) in the study conducted in Switzerland by Kiefer et al. (2019), or atrazine (407 ng/L), desethylatrazine (385 ng/L) and simazine (104 ng/L) in French aquifers (Berho et al., 2013).

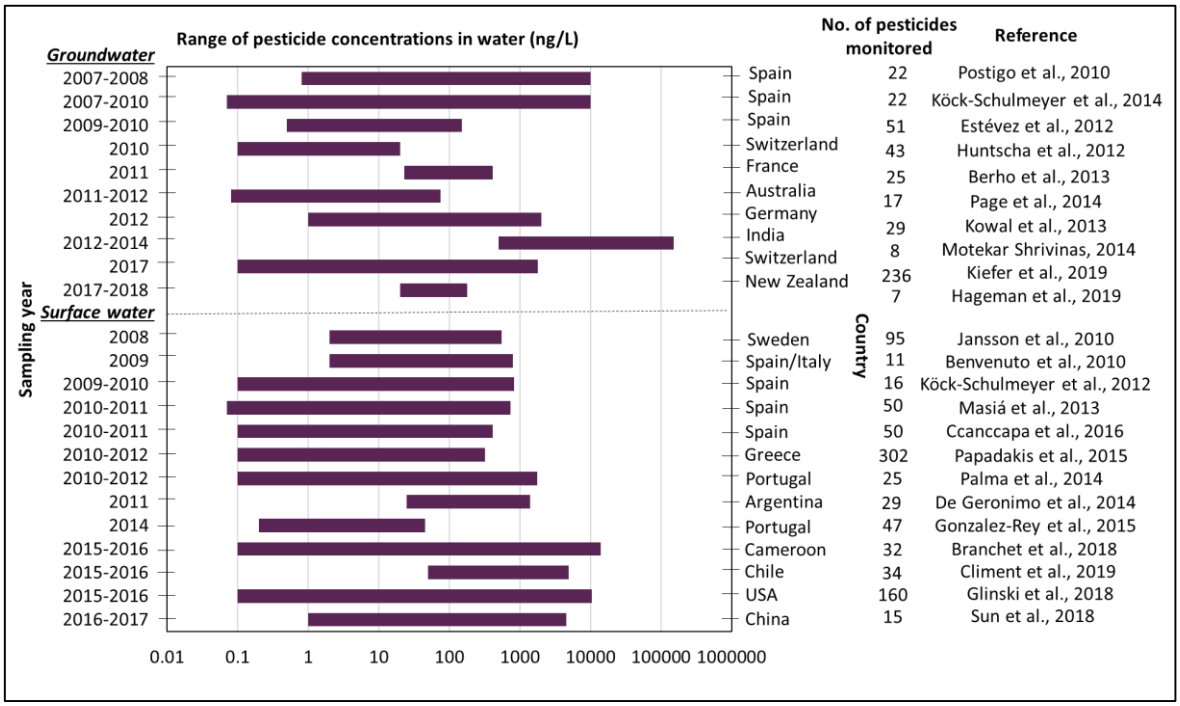


Figure 1.2. Concentration range (ng/L) of individual pesticides observed in various studies (chronological order of sampling) in groundwater and surface water.

In the case of surface water, some of the highest levels worldwide are attributed to the presence of diuron (14 µg/L) in the Mfoundi River Basin (Yaoundé, Cameroon), highly impacted by urban activities (Branchet et al., 2018), and to the presence of the herbicide metolachlor (10.5 µg/L) in an agriculturally impacted wetland area located in South Georgia (USA) (Glinski et al., 2018). High pesticides levels in the range of µg/L were also found in Shanghai's rivers (China) (Sun et al., 2018), with the insecticide acephate showing the highest concentration (4.5 µg/L). At Spanish level, the highest concentrations were measured for diuron (818 ng/L) and diazinon (132 ng/L) in the Llobregat River (Köck-Schulmeyer et al., 2012), in the metropolitan area of Barcelona (Catalonia, Spain). The use of both pesticides is mainly attributed to industrial and domestic activities. In the study of Ccanccapa et al. (2016), the analysis of surface waters from the Ebro River revealed that chlorpyrifos, diazinon, and carbendazim were the most ubiquitous pesticides (95, 95 and

70 % of the samples, respectively), while imazalil (410 ng/L) and diuron (150 ng/L) were the compounds present at the highest concentrations. In the Guadalquivir River, the highest levels reported corresponded to terbuthylazine (788 ng/L) and diazinon (457 ng/L) (Masiá et al., 2013). In this study, the authors suggested that the relatively constant concentrations of diazinon in almost all the samples could be indicative of an urban signal and could come, not only from agriculture but also from the developed areas of Seville and Cordoba. In a recent study, aimed at evaluating the influence of mixed urban and agricultural land use in the overall concentration dynamics of pesticides in surface waters, Wittmer et al. (2010) identified distinct concentration patterns for different compounds and sources, and classified diazinon among the compounds that showed elevated background concentrations throughout the year due to a constant household source and diuron due to a constant urban outdoor source.

Pesticide concentrations have been investigated also in sediments worldwide. Figure 1.3 summarizes the concentrations reported for this matrix in various studies. The highest concentrations were detected in Danube River sediments (Serbia), with peaks of 1222 ng/g for dimethoate and 392 ng/g in the case of atrazine. In Spain, chlorpyrifos was found at very high concentrations in almost all the reported studies. It was found at the maximum concentration of 560 ng/g in sediments from the Túria River Basin (Masiá et al., 2015b), at 113 ng/g and 36 ng/g in sediments collected from the Ebro River Basin by Farrè et al. (2014) and Ccanccapa et al. (2016), respectively, and in the study conducted in the Segre River by Köck-Schulmeyer et al. (2013a), in which it was the compound showing the highest concentration (66 ng/g). According to its physical-chemical properties, chlorpyrifos presents a high probability to be found in sediments, considering its high potential for accumulation (Log K_{ow} > 3) and extremely low mobility (K_{oc} > 4000). According to the Environmental Protection Agency (EPA), chlorpyrifos is one of the most widely used organophosphate insecticides in agriculture. In the other studies, maximum pesticide concentrations generally do not exceed 100 ng/g.

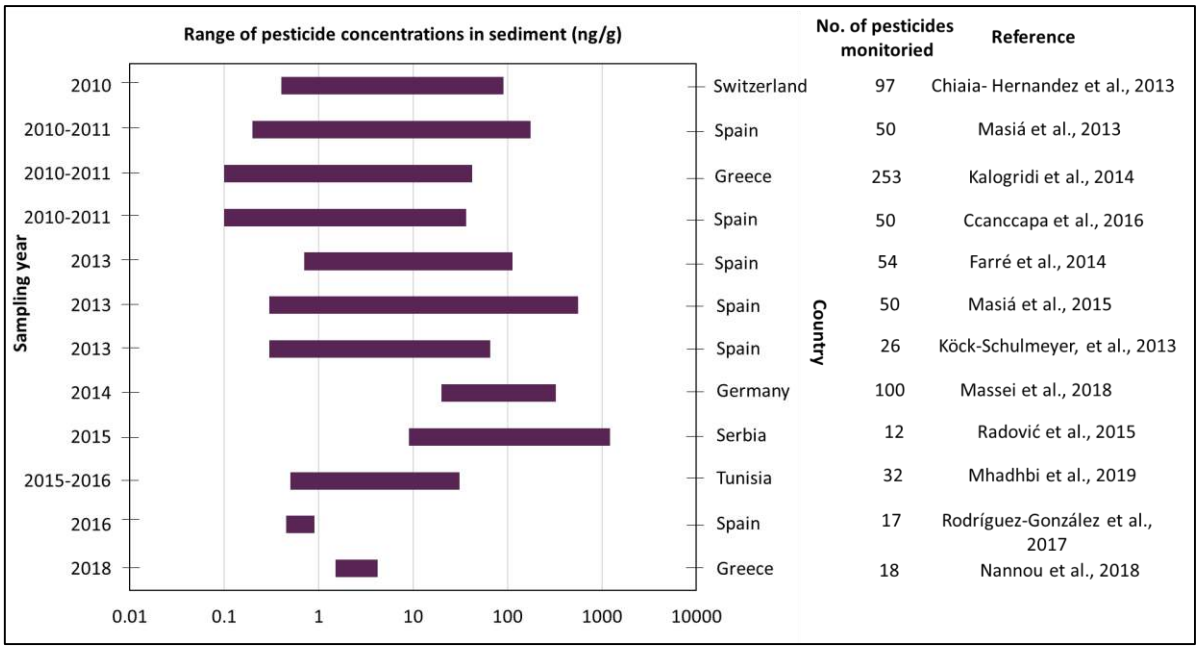


Figure 1.3. Concentration range (ng/g) of individual pesticides observed in various studies (chronological order of sampling) in sediments.

Finally, Figure 1.4 shows the pesticide concentrations in biota reported in the peer-reviewed literature. In the Spanish context, as in the case of sediments, the highest levels are related to those pesticides with more hydrophobic characteristics, more likely to accumulate in sediments or bioaccumulate in biota. This is the case of chlorpyrifos, found in fish samples from the Ebro River (Ccanccapa et al., 2016) at concentrations up to 840 ng/g in carp species, up to 169 ng/g in European catfish species, and up to 45 ng/g in fishes collected in the Llobregat River (Pico et al., 2019)). In the latter study, high concentrations of insecticides were also measured, e.g., the organothiophosphate insecticide azinphos ethyl (up to 106 ng/g) in the Llobregat River, the carbamate carbofuran (519 ng/g) in the Jucar River, and the pyrethroid cypermethrin (78 ng/g) in the Guadalquivir River. Comparatively lower pesticide concentrations were measured in shellfish species in the study of Álvarez-Muñoz et al. (2019). In this work, acetamiprid was detected at concentrations up to 9.5 ng/g in oysters collected from the Ebro Delta. Internationally, the highest pesticide concentrations were found by Ernst et al. (2018). In this work, they detected 30 out of the 72 investigated pesticides in 96 % of the fish samples collected from the Uruguay and Negro Rivers (Uruguay). Among them, the highest occurrence rates were found for the fungicide trifloxystrobin and pyraclostrobin and the herbicide metolachlor,

while chlorpyrifos presented the maximum value (194 ng/g). Another remarkable result was found by Cruzeiro et al. (2016), who studied the presence of pesticides in shellfish species from Ria Formosa Lagoon (Portugal) and found that 53 out of the 55 studied pesticides were present in a total of the 76 % investigated samples.

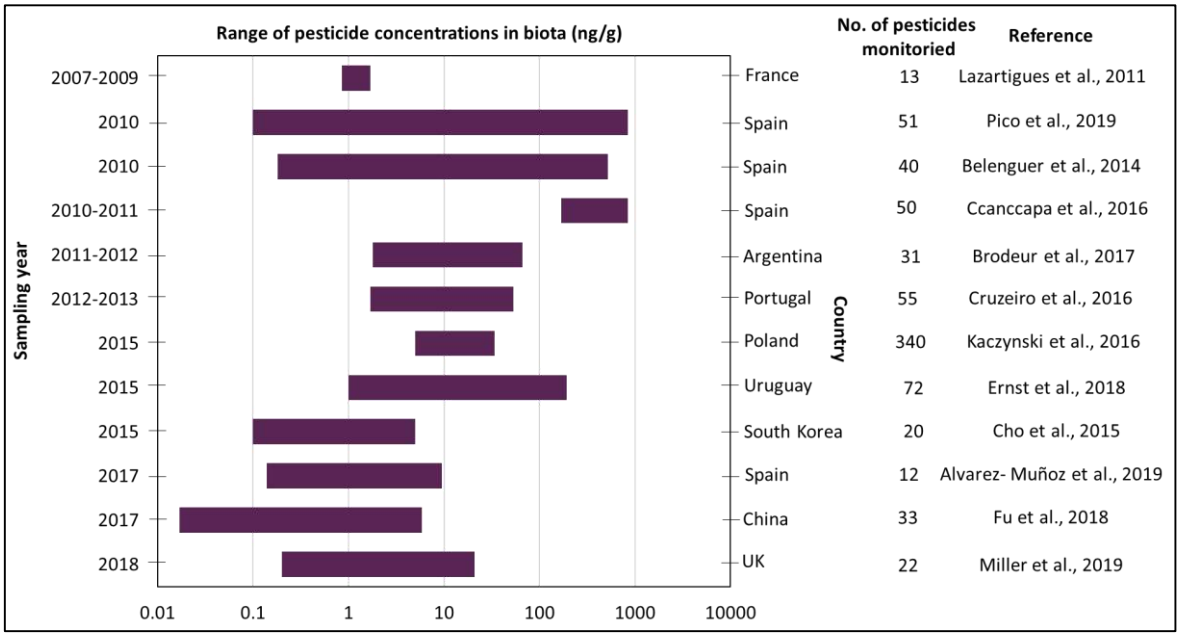


Figure 1.4. Concentration range (ng/g) of individual pesticides observed in various studies (chronological order of sampling) in biota samples.

These results outline the need for measuring polar pesticides even in matrices in which they are not normally likely to be found, since their presence may affect not only the environment, but also the reproductive capabilities of organisms, and may represent a risk for human health, via biomagnification processes and/or direct food consumption. The occurrence, fate and impact of polar pesticides in sediments and biota have not been extensively investigated and knowledge in this regard is nowadays still very limited. In addition, the presence of pesticide metabolites is also a matter of concern, since they are normally more polar and can be found at levels higher than the parent compounds (Ibáñez, 2017). Therefore, the study of polar pesticides in these compartments is essential to understand the role of these matrices as a potential source of these compounds, and to correctly assess the associated risk and establish updated pesticide regulations.

1.3 Pesticide-related legislation

Pesticide legislation varies widely worldwide since countries have different guidelines and legal limits for plant protection products (PPPs). Developed nations have stricter regulations than developing countries, which lack the resources and expertise to properly implement and enforce legislation. International parties have attempted to harmonize pesticide legislation, but there are still huge global differences (Handford et al., 2015). Nowadays, legislation in Europe is continuously being updated to regulate pesticide applications. Table 1.2 shows the most relevant directives of interest in this thesis.

Pesticide regulation was given little attention until the 1940s, when the use of synthetic pesticides such as DDT became more widespread in agriculture, with their application on major field crops. DDT was banned for all agriculture uses in developed nations by the 1980s, and the need for improved pesticide legislation was recognized. The European Commission introduced the first Directive in 1976 (Directive 76/464/EEC) regarding pollution caused by certain dangerous substances discharged into the aquatic environment (EEC, 1976). It includes a list of compounds considered dangerous, a selection based mainly on their toxicity, persistence and bioaccumulation (Annex to the Directive, List I), as well as compounds harming the aquatic environment (Annex to the Directive, List II). In the first list there are, among other compounds, organochlorine and organophosphate pesticides, and in the second, biocides and other pesticides not mentioned in list I. This Directive aimed to protect the aquatic environment from contamination, and in 2006 it was updated and repealed with the Directive 2006/11/EC (EC, 2006b).

As for the use of phytosanitary products in agriculture, in 1979 the European Commission initiated the control of their marketing, authorization and prohibition. The Council Directive 79/117/EEC of 21 December 1978 (EEC, 1978) prohibited the placing on the market and use of PPPs containing certain active substances, which includes persistent organochlorine compounds, such as aldrin, DDT, and heptachlor, among others. Subsequently, in 1991 and 1998, two European Directives regulated the placing of PPPs on the market (91/414/EEC) (EEC, 1991) and the placing of biocidal products for non-plant protection purposes on the market (98/8/EC) (EC, 1998b), respectively.

Table 1.2. European Directives, in chronological order, regarding the application and control of pesticides in the environmental matrices studied in this thesis.

Year	Regulation	Description
1976	Directive 76/464/EEC	regarding pollution caused by certain dangerous substances in the aquatic environment
1978	Directive 79/117/EEC	regarding the prohibition of placing on the market and use plant protection products containing certain active substances
1991	Directive 91/414/EEC	regarding the placement of plant protection products on the market
1998	Directive 98/8/EC	regarding the placement of biocidal products for non-plant protection purposes on the market
1998	Directive 98/83/EC	regarding the quality of water intended for human consumption
2000	Directive 2000/60/EC	establishing a European framework for Community action in the field of water policy
2005	Regulation (EC) No. 396/2005	establishing harmonised maximum residue levels (MRLs) for all foodstuff across Europe
2006	Directive 2006/118/EC	regarding the protection of groundwater against pollution and deterioration
2006	Directive 2006/11/EC	by which the Directive 76/464/EEC is updated and repealed
2008	Directive 2008/105/EC	establishing environmental quality standards in the field of water policy, amending Directive 2000/60/EC
2009	Regulation (EC) No. 1107/2009	regarding the marketing of phytosanitary products
2009	Directive 2009/128/EC	establishing a framework for Community action to achieve the sustainable use of pesticides
2012	Regulation (EU) No. 528/2012	regarding the marketing of biocides
2013	Directive 2013/39/EU	amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy
2015	Commission Implementing Decision (EU) 2015/495	establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC
2018	Commission Implementing Decision (EU) 2018/840	establishing a watch list of substances for Union-wide monitoring in the field of water policy repealing Commission Implementing Decision (EU) 2015/495
2019	Guidance document No. SANTE/12682/2019	establishing analytical quality control and method validation procedures for pesticide residues and analysis in food and feed

Currently, the marketing of phytosanitary products and biocides is controlled through the Regulation (EC) No. 1107/2009 (EC, 2009a) and the Regulation (EU) No. 528/2012 (EU, 2012), respectively.

More specifically in the field of water, in 1998 it was adopted the Council Directive 98/83/EC on the quality of water intended for human consumption (EC, 1998a), in which quality parameters were established for different pollutants. Parametric values set for individual and total pesticide concentrations were 100 ng/L and 500 ng/L, respectively. In this Directive, the category of pesticides included their relevant metabolites, degradation and reaction products. Then, in 2006 (EC, 2006a), the same limits were specified for pesticides in all groundwater bodies in the Directive 2006/118/EC on the protection of groundwater against pollution and deterioration. The measures provided in this Directive to fight against groundwater contamination included criteria to evaluate the chemical status of the waters, to determine trends in the increase of pollutant concentrations, as well as to regulate the prevention and limitation of indirect discharge of these pollutants in groundwater.

Later on, in 2000, the EC adopted the Directive 2000/60/EC (EC, 2000), a European framework for Community action in the field of water policy, or, for short, the EU Water Framework Directive (WFD). This framework directive has several objectives, based on the assumption that water is a heritage that must be protected and defended. These objectives included the prevention and reduction of pollution, the promotion of sustainable use of water, the protection of the environment, the improvement of the quality of the aquatic ecosystems, and the mitigation of the effects of floods and droughts. Approximately one year later, amending the Directive 2000/60/EC, the Decision 2455/2001/EC established the first list of priority substances in the field of water policy, which included 33 compounds (many of them pesticides) selected from amongst those presenting a significant risk to or via the aquatic environment. This first list was replaced by Annex II of the Directive 2008/105/EC (EC, 2008), also known as the Priority Substances Directive, which set environmental quality standards (EQS) for the priority substances in surface waters (river, lake, transitional and coastal), with the aim of achieving a good surface water chemical status. In Annex I, limits on the concentrations of 33 priority substances and 8 other pollutants were established, including also the possibility of applying EQS for sediment and biota. In 2013, the list of priority substances was updated in the Directive 2013/39/EU (EC,

2013) by identifying new substances for priority actions at European level, revising EQS for some substances based on scientific progress, and establishing EQS in biota for some others. At present, EQS are established in the form of annual average (AA) concentrations and maximum allowable concentration (MACs) for up to 45 priority substances. This list includes 24 pesticides or biocides. As for now, EQS have not been set yet for sediments, but the Directive establishes that this can be done at EU member state level and, in any case, long-term trend monitoring of priority substance concentrations in sediments has to be performed to prevent deterioration of surface water bodies.

Furthermore, 5 neonicotinoid pesticides, the carbamate methiocarb, and the semicarbazone metaflumizone are currently included in the Commission Implementing Decision (EU) 2018/840 (EC, 2018), establishing a Watch List of substances for Union-wide monitoring in the field of water policy, to gather information to make a decision regarding their consideration as priority substances.

In addition to monitoring pesticide residues in the environment, protecting public health also requires controlling pesticide residues in food or feed. For this purpose, maximum residue levels (MRLs), defined as “the upper legal levels of a concentration for pesticide residues (expressed in mg/kg) in or on food or feed, based on good agricultural practices (GAP) and to ensure the lowest possible consumer exposure”, have been established (EFSA, 2011). For the calculation of MRLs, toxicological studies determine the acute reference dose and acceptable daily intake of pesticides, and compare them with food consumption patterns, obtained from dietary intake surveys and residue data from rotational crop studies, supervised field trials, and, if available, monitoring data, to ensure that exposure does not exceed specified safety limits (FAO, 2016). The Regulation (EC) No. 396/2005 (EC, 2005) harmonized MRLs for all foodstuff across Europe. Pesticide MRLs have been set for pesticides currently in use or used in the past for food production, and 315 food products. However, MRLs of pesticides do not exist for all food or feed. For instance, MRLs do not exist in fish products, even though these organisms may be exposed continuously to pesticides released into the aquatic environment. The EU laws set a default lowest limit of analytical determination (LODet) value of 0.01 mg/kg when a pesticide or a matrix is not specifically mentioned, or when its use has not left detectable residues. Methods of sampling and sample analysis for the determination of pesticide residues for compliance with MRLs, as well as performance acceptability criteria of quantitative

methods, are outlined in the updated document No. SANTE/12682/2019, a Guidance Document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed (EC, 2019).

At the national level, every country establishes further regulations concerning the protection of water against pollution caused by pesticides from agricultural sources (*e.g.* packaging, inspection of the equipment of application of PPPs, etc.). In this regard, both the European and the national regulations (EC, 2009b; Ministerio de la Presidencia, 2012) force the professional farmers to apply the general principles of the integrated pest management (IPM) and keep records to demonstrate the application of these principles. The IPM record sheet must include, among others, a description of the characteristics of the farm and the production means, the phytosanitary treatments applied, farm product sales, and, in the cases established by the regulations, the PPPs applied. All these requirements are intended to establish the framework for action to achieve sustainable use of PPPs, adopting the necessary measures to promote the implementation of mitigation measures and best management practices (BMPs) in agriculture.

1.4 Environmental risk assessment of pesticide exposure

Several directives have been established to regulate the potential adverse consequences deriving from the use of pesticides, with the aim of safeguarding human health and the environment from the undesirable effects of these chemicals. For this purpose, there is a need to develop scientifically reliable tools that allow assessing the impact derived from the use of pesticides and can be then employed by environmental decision-makers and regulators.

In the last years, the risk assessment approach has been frequently used for ranking pesticides in terms of the hazard they may pose to the environment by the use of risk indexes. These indexes are calculated by giving scores to a set of physical–chemical, toxicological, and ecotoxicological properties of the substances under study, which are then combined through an algorithm to yield a number that indicates the possible environmental risk of the investigated compounds (Finizio and Villa, 2002).

The most important European and international research organizations started to use different tools for the calculation of risk assessment, with approaches greatly differing with respect to purposes (human or environmental impact) or the environmental compartment (groundwater, surface water, soil) and non-target organisms used for ecotoxicological studies considered representative of the different levels of the food chain (e.g. algae, daphnia and fish for surface water) (Finizio et al., 2001).

One of the main strategies applied by scientists is to use real contamination data in a given environmental compartment, and calculate the risk that pesticides concentrations may pose in specific organisms based on the predicted toxicity of these pollutants on them. The EPA recommends a hazard quotient (HQ) method (US EPA, 1997), which compares the measured environmental concentration (MEC) of each contaminant with its predicted no-effect concentration (PNEC), *i.e.*, the concentration at which no toxic effects are expected to occur, following the equation: HQ = MEC/PNEC. The PNEC values may be obtained experimentally on specific organisms via ecotoxicological assays, calculated by dividing toxicological dose descriptors by an assessment factor, or rather predicted by modeling software, which in recent years have become of strategic interest to regulatory authorities to support the risk assessment of transformation products or to complement missing experimental data for key species (Galimberti et al., 2020). A major advantage of *in silico* models is that they are useful to create prioritization lists for the general assessment of the potential pesticide toxicity, helping to choose pesticides that must be monitored in water bodies and minimizing the need for expensive and time-consuming *in vivo* and *in vitro* laboratory assays. *In silico* methods based on quantitative structure-activity relationship (QSAR) models are currently the most frequent approach to estimate the environmental impact of pesticides and their transformation products, and they are worldwide recognized alternatives to animal testing (Scholz et al., 2013; Villaverde et al., 2017).

1.5 Analysis of pesticides in the environment

Pesticides belong to many different chemical families with rather distinct properties, including polarity and volatility, and need to be determined in a variety of matrices of different composition. As expected, there is not a universal method that can be

applied to all pesticides and all environmental sample matrices. To date, several methods have been developed for pesticide detection based on conventional or advanced detection techniques. Generally, pesticide analysis involves different steps prior to analyte detection, including sample collection, sample pretreatment and storage, and sample extraction and/or clean-up. Pesticides separation and detection are conventionally performed with analytical methods based on gas chromatography (GC) and high-performance liquid chromatography (HPLC) coupled with various detectors. The data provided by this instrumentation needs to be treated for reliable identification and accurate quantification of results. The analytical procedures are constantly evolving to meet the challenge of developing multi-residue methods in different environmental samples, with increased automation, high-throughput, and sensitivity, using low sample volumes and at a low cost.

1.5.1 Collection and preservation of environmental samples

One of the most critical steps in environmental analysis is the sample collection, followed by sample preservation. As for the sample collection, the sampling needs to be designed to ensure the collection of representative samples. This contributes to developing reliable environmental data in relation to the sampling site. In this regard, an appropriate number of samples, as well as sufficient sample volume, should be collected to generate accurate estimations of the real pesticide concentration in the investigated area, (Ni et al., 2011). The sampling plan should cover field blanks, sample replicates, quality controls, and a cleaning protocol for the equipment used to collect the samples. The sampling material should be washed in warm water and detergent, and sequentially rinsed with clean water and an organic solvent (ethanol or acetone).

Samples of any matrix are either grab samples or composite samples. Grab samples are collected at one location and at one point in an exact time, whereas composite samples consist of multiple grab samples taken at different locations over an area (soil samples) or in one location at regular intervals over a period of time (water samples). While grab samples cannot individually be considered representative to evaluate the overall water quality, they can be useful to collect preliminary information to help determine if pesticide contamination is relevant in the area. Generally, grab sampling is applied for matrices

where the variation of the analyte concentration to be studied is assumed to be small (e.g., soil or sediments), or when the degradation rate of the analyte in the matrix is very high, and it is recommended by the WFD for the collection of waters (EC, 2000). In the case of surface water, a grab sample is typically taken manually, but automated samplers can be programmed to take grab samples at a specific time on a regular basis. However, automated samples are more commonly used to collect composite samples flow- or time-dependent. As for sediment collection, grab samplers like the Van Veen grab are commonly used as they are relatively easy to handle (Galanopoulou et al., 2005; Köck-Schulmeyer et al., 2013a; Opel et al., 2011).

In the case of water samples for the analysis of pesticides, it is recommended to use amber bottles to avoid photodegradation of the compounds. As for sediments, it is recommended to place collected sediment samples in a dark container, such as an aluminum tray, to avoid the exposition of the sample to direct light. Hydrolysis, biodegradation, photolysis, and evaporation are considered the main pathways for pesticide degradation (Ortiz-Hernández et al., 2013). Depending on the physical-chemical properties, each pesticide or class of pesticides presents different stability and therefore susceptibility to degradation. For instance, triazines and chloroanilides are overall more stable than carbamates and organophosphates. Thus, after collection, samples should be sealed, stored and kept in optimal conditions to avoid pesticide losses that can occur during collection and transport to the laboratory. Transport of the samples to the laboratory should be done as soon as possible and keeping the samples in the meantime cool and away from direct sunlight. Once in the laboratory, the best option is to preserve the samples frozen until their analysis.

Regarding biota sampling, the most reliable studies include the analysis of different species, since the different contamination among species can be a measure of the stress on the environment in relation to pesticide pollution. The target species are normally representative, recreationally important species for the waterbody being sampled, commonly taken by anglers for consumption. Samples are usually collected by electrofishing or other methods of collection such as netting, trotlines or angling, after obtaining the corresponding permit from the local authorities (US EPA, 2015). Samples are

then stored at cool conditions, transported to the laboratory, where, after pretreatment, they will be kept frozen until analysis.

1.5.2 Sample pretreatment

Pesticide residues in the water compartments are generally present at trace levels, so the sample pretreatment step is often necessary for the pre-concentration of the analytes to levels above the limit of detection (LOD) of the analytical instrumentation, the isolation of the analytes from the original matrix, and the removal of undesired interferences to improve the selectivity of the analytical procedure (L. Liu et al., 2018).

The analysis of pesticides in aqueous samples usually involves a filtration step to remove suspended particles. To date, different filtration materials have been used for minimizing losses during the determination of pesticides in water samples: cellulose acetate (Catalá-Icardo et al., 2014), cellulose nitrate (Herrero-Hernández et al., 2013), nylon (Gimeno et al., 2001; Hildebrandt et al., 2008), polytetrafluoroethylene (Rodríguez-González et al., 2016; Zhang et al., 2020), or simply glass microfiber (Gatidou et al., 2005; Orlikowska et al., 2015; Zambito Marsala et al., 2020). However, despite being the main technique for the removal of suspended particles, filtration presents some problems including filter clogging and/or analyte adsorption onto the filter and on the layer of particulate matter accumulating on the filter (Ademollo et al., 2012). Furthermore, the filtration step requires a large sample volume to obtain a sufficient amount of sample to be analyzed, increasing the risk of filter components release that may interfere later in the analysis.

Some studies report, in addition (Fenoll et al., 2011; Rodríguez-González et al., 2015) or alternatively (Habedank et al., 2017; Moro et al., 2018) to filtration, the centrifugation of the samples. This process avoids potential analyte losses that may occur during the filtration process. The main drawback can be the potential co-precipitation of the target analytes with suspended particles, but in this case the precipitate can be washed with appropriate solvents to improve the overall recovery (Locatelli et al., 2016). Centrifugation requires little sample volumes and reduces time and analysis costs. The addition of isotopically labeled standards before the filtration or the centrifugation step is

also useful to overcome the potential analyte losses and compensate for potential matrix effects.

Regarding the pretreatment of solid samples, sample preparation generally consists of a pre-drying step, in which the sample can be air-dried at room temperature (Zhao et al., 2013) or that can be performed by lyophilizing the previously frozen sample (Masiá et al., 2015b; Pico et al., 2019; Toan et al., 2013), followed by sample homogenization.

In the case of sediment, since it is a non-uniform mixture of particles of different sizes, it is essential to maximize the homogeneity of the sample, removing manually stones and other unwanted solids or using crushers and then pore sieves on the µm scale. In the study of Masiá et al. (2015b), sediment samples were freeze-dried with a lyophilizer at −65 °C and with a vacuum of 1–4 mT for 48 h, and then sieved to collect the fraction <125 µm. In other works (Gómez et al., 2011; Moreno-González and León, 2017), sediments were passed through finer mesh sieves, collecting the fraction <63 µm for chemical analysis. Mixing can be accomplished manually with standard laboratory tools (vortex, sonication) or with tools commonly used in household cooking.

As for biota samples, freeze-drying is widely used to eliminate water and determine the dry weight concentrations of the contaminants. After the lyophilization, samples are usually ground to homogenize the matrix for the analysis. The homogenization step is especially important due to the potential selective accumulation of pesticides in some tissues. In the case of analyzing the full body of the species, including different organs and tissue parts (muscle, fillet, gills, liver, intestine), the homogenization ensures the uniform distribution of pesticides in the sample (Álvarez-Ruiz and Picó, 2020). This can be done using stainless steel laboratory blenders (Darko et al., 2008; Ernst et al., 2018) or typical kitchen food processors (Baduel et al., 2015; Colazzo et al., 2019). In the work of Colazzo et al. (2019), frozen samples of fish muscle tissue (fillet) were chopped and homogenized with a stainless-steel kitchen cutter, while Darko et al. (2008) took the muscle tissue of the fish and grounded it in a waring blender to obtain a homogenous composite. Eel and shrimp samples were put in a blender and ground until they were mixed by Cho et al. (2015). The entire fish (including the different organs, muscles, skin and bones) was processed by Pico et al. (2019) for the analysis of 135 contaminants of emerging concern, including 25d pesticides.

Finally, there are studies in which the centrifugation of the sediment or ground fresh biota sample has been the technique adopted for sample preparation, as an alternative to lyophilization to eliminate water and other potential interferences. As an example, Xue et al. (2008) previously centrifuged the wet sediment samples at 4,000 rpm and 4 °C, and then extracted 18 pesticides by sonication (UAE; ultrasound-assisted extraction).

1.5.3 Sample extraction

Traditionally, extraction techniques were time consuming, laborious and used relatively high amounts of sample and organic solvents. Nowadays, the minimization of solvents and reagents use is a current topic for developing improved extraction methods and replacing the traditional procedures with more environmentally friendly ones.

Aqueous samples

Solid-phase extraction (SPE) is currently the most successful and widely used sample preparation technique, due to its ability to efficiently enrich and purify analytes from their liquid sample matrices. Some advantages of SPE are its versatility, resulting from the different types of sorbents available, and its automation, since it can be applied in both off-line and on-line modes. Furthermore, SPE allows reducing the extraction time and consumption of organic solvents, as compared to other extraction methods such as liquid-liquid extraction (LLE) (Chirila and Drăghici, 2013). Nowadays, new advanced techniques including minimal solvent usage are widely used, such as dispersive solid-phase extraction (dSPE), stir bar sorptive extraction (SBSE), magnetic solid-phase extraction (MSPE), solid-phase microextraction (SPME), liquid-phase microextraction (LPME) and dispersive liquid-liquid microextraction (DLLME). Table 1.3 presents a summary of the analytical techniques applied in the last 10 years (2010-2020) in various studies for pre-concentration and extraction of pesticides from water samples.

Table 1.3. Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in aqueous samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Separation/ detection method*	*Reference*
GW/SW	23	SPE	Oasis HLB	LC-MS/MS	(Loos et al., 2010)
DW/WW	13	SPE	Oasis HLB	UPLC-MS/MS	(Morasch et al., 2010)
WW	51	SPE	C18	GC-MS/MS	(Pitarch et al., 2010)
WW	52	SPE	Oasis HLB	UPLC-MS/MS	(Pitarch et al., 2010)
GW	22	on-line SPE	PLRPs/HySphere Resin GP	LC-MS/MS	(Postigo et al., 2010)
SW	22	on-line SPE	PLRPs/HySphere Resin GP	LC-MS/MS	(Ricart et al., 2010)
SW/WW	20	on-line SPE	Strata-X	LC-MS/MS	(Singer et al., 2010)
WW	11	on-line SPE	C18	LC-MS/MS	(Cahill et al., 2011)
SW	13	SPE	Strata-X	LC-MS/MS	(Lazartigues et al., 2011)
GW	4	direct injection/SPE	none/C18	LC-MS/MS	(Zhao et al., 2011)
GW	12	SPE	Oasis HLB	GC-MS	(Bono-Blay et al., 2012)
SW	4	SPE	C18	HPLC-DAD	(Cappelini et al., 2012)
GW	33	LLE	25 mL n-hexane	GC-MS/MS	(Estévez et al., 2012)
GW	18	SPE	Oasis HLB	LC-MS	(Estévez et al., 2012)
GW/SW/WW	43	on-line SPE	10 mg OASIS HLB, Strata X-AW, Strata X-CW, Isolute ENV+ (1:1:1.5)	HPLC-MS/MS	(Huntscha et al., 2012)
SW/DW	3	SDME	8 mL solution 10 % NaCl, w/v	HPLC-DAD	(Wang et al., 2012)
SW/DW/WW	40	on-line SPE	C18	UPLC-HRMS	(Wode et al., 2012)
GW	26	POCIS/SPE	Oasis HLB	UPLC-MS/MS	(Berho et al., 2013)
SW	7	SPE/SPME	C18	GC-MS	(Bonansea et al., 2013)
SW/DW	33	SPE	C18	LC-MS/MS	(Caldas et al., 2013)
SW	12	SPE	Strata-X	GC-MS	(Fernández-Gómez et al., 2013)
SW	18	LLE/SPE	Silica	GC-ECD	(Hellar-Kihampa et al., 2013)
GW/SW	58	SPE	Oasis HLB	LC-MS	(Herrero-Hernández et al., 2013)
SW/WW	43	SPE	Oasis HLB	LC-MS/MS	(Masiá et al., 2013)

Table 1.3. (Cont.) Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in aqueous samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Separation/ detection method*	*Reference*
SW	63	SBSE	100 mL NaCl	GC-MS	(Moreno-González et al., 2013)
SW	3	on-line SPE	HTLC C18	UPLC-MS/MS	(Quinete et al., 2013)
GW/SW	150	direct injection	none	LC-MS/MS	(Reemtsma et al., 2013)
SW/DW	13	SPE	C18	GC-MS	(Toan et al., 2013)
SW/DW	6	MSPE	C18 modified	GC-MS	(Xie et al., 2013)
SW	10	LLE/SPE	Alumina/Silica	GC-ECD	(Yang et al., 2013)
GW/SW	10	IPA-LLE	100 µL TBAHS, 1.5 mL ACN	HPLC-DAD	(Gure et al., 2014)
GW/SW	15	MSPE	HLB-MPNPs	HPLC/GC–µECD	(He et al., 2014)
SW	8	SPE	1D-PANIs	GC-ECD	(Jiang et al., 2014)
GW	32	on-line SPE	HySphere Resin GP/PLRPs	LC-MS/MS	(Köck-Schulmeyer et al., 2014)
GW/SW/WW	23	DLLME	50 µL carbon tetrachloride	GC-MS/MS	(Martins et al., 2014)
SW/DW	12	SPE	Oasis HLB	LC-MS/MS	(Montagner et al., 2014)
WW	400	SPE	Oasis HLB	LC-HRMS	(Robles-Molina et al., 2014)
SW/DW	10	MASE	n-hexane:ACE (9:1, v/v)	GC-MS/MS	(Shi et al., 2014)
DW	12	SPE	Oasis HLB	LC-MS/MS	(Tettenhorst and Shoemaker, 2014)
SW	5	DLLME	8 µL 1-dodecanol	GC-FID	(Wang et al., 2014)
SW	3	AALLME	30 µL 1-octanol	HPLC-DAD	(Wu et al., 2014)
SW	10	on-line SPE	Oasis HLB	LC-MS/MS	(Camilleri et al., 2015)
SW	200	SPE	Oasis HLB	LC-MS/MS, GC-MS/MS	(Charalampous et al., 2015)
SW	3	SPE	Polypropylene	UPLC–MS	(Deyerling and Schramm, 2015)
SW	8	SPME	SPME fiber tips	GC-MS	(Huang et al., 2015)
SW/DW	6	SPME	C18	GC-MS	(Li et al., 2015)
SW	5	SFODME	25 µL 1-dodecanol/p-xylene	GC-FPD	(Liu et al., 2015)
SW	6	DCF-EME	40 µL toluene	GC-ECD	(Molaei et al., 2015)

Table 1.3. (Cont.) Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in aqueous samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Separation/ detection method*	*Reference*
GW/SW/DW	13	SALLE	3.5 mL ACN	FI-MS/MS	(Nanita et al., 2015)
WW	8	SPE	Oasis HLB	UPLC–MS/MS	(Ribeiro et al., 2015)
SW	4	MSPME/IL-DLLME	M-β-CD/ATP	HPLC-UV	(Yang et al., 2015)
SW	15	HLLME	53 µL chloroform	GC-FPD	(Berijani et al., 2016)
DW	5	DLLME-SFO	250 µL 1-dodecanol	LC–MS/MS	(Bolzan et al., 2016)
SW	32	SD-DLLME	120 µL octanol and 750 µL ACE	LC–MS/MS	(Caldas et al., 2016)
SW	4	SPE-DLLME	C18	HPLC-UV	(Chen et al., 2016)
SW	15	SPE	PEP-SPE	APGC-QTOF-MS	(Cheng et al., 2016)
SW	6	ET-DLLME	110 µL 1,2-DBE	GC-FID	(Farajzadeh et al., 2016)
SW	4	MSPE	Fe_3O_4@SiO_2–MIL-101 microspheres	HPLC-DAD	(Ma et al., 2016)
SW	17	on-line SPE	Oasis HLB	UPLC-MS/MS	(Rodríguez-González et al., 2016)
GW/SW	26	SPME	PDMS/DVB 65 µm	GC-MS	(Rodriguez-Lafuente et al., 2016)
WW	44	SPE/direct injection	Oasis HLB	LC-MS/MS	(Rousis et al., 2016)
SW	14	SPE	Oasis HLB	HPLC-MS/MS	(Valls-Cantenys et al., 2016)
SW	4	DCF-EME	60 µL toluene	GC-MS	(Yao et al., 2016)
SW	3	MSPE	ZNCAHF	GC-MS	(Zare et al., 2016)
DW	30	d-SPE	200 µL EA	HPLC-MS/MS	(Zou et al., 2016)
WW	27	direct injection	none	UPLC–MS/MS	(Campos-Mañas et al., 2017)
SW	14	on-line SPME	PDMS-DVB	GC–HRMS	(Domínguez et al., 2017)
SW	62	RDSE	Oasis HLB	UPLC–MS/MS	(Donato et al., 2017)
GW/SW/DW	7	SPME	SPME stir bar	GC–ECD	(Gutiérrez-Serpa et al., 2017)
SW	20	UA-DLLME	200 µL ACE and 220 µL tetrachloroethene	GC-MS/MS	(Habedank et al., 2017)
WW	13	SPE	Strata-X	LC-MS/MS	(Jones et al., 2017)
GW/SW/DW	5	cop-CAE	$Al_2(SO_4)_3$ and SDS	HPLC-UV	(Mammana et al., 2017)

Table 1.3. (Cont.) Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in aqueous samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Separation/ detection method*	*Reference*
SW	23	TFME	PDMS/DVB	GC-MS	(Piri-Moghadam et al., 2017)
SW	17	SPE	Oasis HLB	LC-MS/MS	(Rodríguez-González et al., 2017)
SW/DW/WW	8	on-line SPE	HySphere C18 HD	LC–MS/MS	(Rubirola et al., 2017)
SW	5	MSPE	poly(pPDA-co-Th)@Fe_3O_4	GC-FID	(Targhoo et al., 2017)
SW	4	in-disk SPE	CNTs	GC-MS	(Vieira et al., 2017)
SW	6	in-disk SPE	CNTs	LC-DAD	(Zdolšek et al., 2017)
GW/SW/DW/ WW	5	DLLME	200 µL 1,2-dichloroethane and 3 mL 2-propanol	GC-MS	(Bulgurcuoğlu et al., 2018)
SW	215	SPE	Oasis HLB	LC-HRMS	(Casado et al., 2018)
WW	15	on-line HS-SPME	PA fiber	GC-HRMS	(Domínguez et al., 2018)
SW	5	CPE	100 µL NaOH 0.1 M	HPLC	(Kachangoon et al., 2018)
SW	13	DLLME	50 µL 1-undecanol	UPLC-MS/MS	(X. Liu et al., 2018)
SW	4	d-µ-SPE	50 mg HBPE	HPLC-UV	(C. Liu et al., 2018)
SW	4	MSPE	7 mg magnetic MOF-5	HPLC-DAD	(Ma et al., 2018)
SW	31	LLE	hexane, DCM and EA	GC-MS/MS	(Mondal et al., 2018)
SW/DW	9	on-line SPE	HyperSep Retain PEP	UPLC-MS/MS	(Montiel-León et al., 2018)
SW/DW	34	SPE	HLB and ENV	UPLC-HRMS	(Tröger et al., 2018)
SW	6	MSPE/SPE	magnetoliposomes/C18	GC–MS/MS	(Wang et al., 2018)
SW/WW	6	SPE/DLLME	C18	LC-MS/MS	(Zhao et al., 2018)
SW	20	UASE-HS-SPME	PDMS fiber	GC–MS/MS	(Cárdenas-Soracá et al., 2019)
SW	7	LTPE	ACN	LC-MS/MS	(de Barros et al., 2019)
SW	4	SPME	PDMS fiber	GC-MS	(Hu et al., 2019)
GW/SW/DW	9	SPE	100 mg NH2@COF	HPLC-DAD	(Ji et al., 2019)
WW	209	SPE	Oasis HLB Plus and Waters Sep-Pak Plus AC2	LC-QTOF-MS	(Kadokami and Ueno, 2019)
GW/SW	13	SPE	MSU-1	UPLC-MS/MS	(Kharbouche et al., 2019)

Table 1.3. (Cont.) Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in aqueous samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Separation/ detection method*	*Reference*
SW	26	SPE	Oasis HLB	LC-MS/MS	(Köck-Schulmeyer et al., 2019)
SW	5	UA-DLLME-DES	30 mg DES and 80 mg sodium chloride	HPLC-UV	(Liu et al., 2019)
SW/DW	6	VA-SI-LLME	400 µL of isopropanol/EA (1:2, v/v)	LC-MS/MS	(Pasupuleti et al., 2019)
GW/SW/DW	96	on-line SPE	C18	UPLC-MS/MS	(Quintana et al., 2019)
DW	124	SPE	Bond Elut Florisil	LC-MS/MS, GC-MS	(Schwanz et al., 2019)
SW	3	MSPE	MNPs coated with C18 functionalized silica	GC-MS/MS	(Srivastava et al., 2019)
GW	7	SPE-CSIA	Sepra ZT, LiChrolut EN and SDB-1	GC-IRMS	(Torrentó et al., 2019)
SW	28	SPE	Speedisk	UPLC-HRMS	(Vanryckeghem et al., 2019)
SW	13	SPE	Oasis HLB	UPLC-MS/MS	(Zaidon et al., 2019)
DW	3	SPE	Oasis HLB	HPLC-MS/MS	(Zhang et al., 2019)
GW/SW/DW	17	PRME	0.5 mL $CHCl_3$	GC-MS	(Biparva et al., 2020)
GW	9	SPE	PLRP-s	LC-MS/MS	(Blanchoud et al., 2020)
DW/WW	4	SS-LPME	0.5 mL *N,N*-dimethylbenzylamine/water (1:1, v/v)	GC-MS	(Bozyiğit et al., 2020)
SW	33	SBSE	PDMS adsorbent	GC-MS/MS	(Canlı et al., 2020)
SW/DW	8	BID	1 µL toluene/butyl acetate (3:2, v/v)	GC-MS	(Chullasat et al., 2020)
GW/SW/DW/ WW	5	SS-LPME	1 mL *N,N*-dimethylbenzylamine/water (1:1, v/v)	GC-MS/MS	(Durak et al., 2020)
WW	66	SPE	200 mg Oasis HLB, 150 mg Isolute ENV+, 100 mg Strata-X-AW and 100 mg Strata-X-CV	UPLC-QTOF-MS/MS	(Gago-Ferrero et al., 2020)

Table 1.3. (Cont.) Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in aqueous samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Separation/ detection method*	*Reference*
SW	18	SPE	Chromabond HR-X 200-mg	HPLC-MS/MS	(Paijens et al., 2020)
SW	29	SPE	HLB/WAX/WCX (2: 1: 1, w/w/w)	HPLC-MS/MS	(Tan et al., 2020)
GW/DW	11	SPE	PDMAT microbeads	GC-MS	(Tümay Özer et al., 2020)

AALLME: air-assisted liquid liquid–microextraction; ACE: acetone; ACN: acetonitrile; APGC: atmospheric pressure gas chromatography; BID: bubble-in-drop microextraction; CNTs: carbon nanotubes; Cop-CAE: coprecipitation-assisted coacervative extraction; CPE: amended-cloud point extraction; CSIA: compound-specific isotope analysis; DAD: diode array detection; DBE: dibromoethane; DCF-EME: dissolved carbon dioxide flotation-emulsification microextraction; DCM: dichloromethane; DES: deep eutectic solvent; DLLME: dispersive liquid-liquid microextraction; DVB: divinylbenzene; DW: drinking water; EA: ethyl acetate; ECD: electron capture detector; ET: elevated temperature; FI: flow injection; FPD: flame photometric detector; GP: general phase; GW: groundwater; HBPE: hyperbranched polyester; HLB: hydrophilic-lipophilic-balance; HLMME: homogeneous liquid–liquid microextraction; HRMS: high resolution mass spectrometry; IL: ionic liquid; IPA: ion-pair assisted; LPME: liquid-phase microextraction; LTPE: low-temperature partitioning extraction; MASE: membrane-assisted solvent extraction; MNP: magnetic nanoparticle; MSPE: magnetic solid-phase extraction; MSU: mesoporous silica material; M-β-CD/ATP: magnetic β-cyclodextrin/attapulgite; PDMAT: poly(divinylbenzene-N-methacryloyl-L-tryptophan methyl ester); PDMS: polydimethylsiloxane; PEP: polarity enhanced polymer; PLRP: polymeric reversed-phase sorbent; POCIS: polar organic chemical integrative sampler; PRME: promoted reaction microextraction; QTOF: quadrupole-time-of-flight ; RDSE: rotating disk sorptive extraction; SALLE: salting-out liquid-liquid extraction; SDME: single drop microextraction; SDS: sodium dodecyl sulfate; SFO: solidification of floating organic drop; SPME: solid-phase microextraction; SW: surface water; TBAHS: tetrabutylammonium hydrogensulfate; TFME: thin film microextraction; UASE: ultrasound-assisted solvent extraction; UPLC: ultra-performance liquid chromatography; VA-SI-LLME: vortex-assisted salt-induced liquid-liquid microextraction; WAX: weak anion exchange sorbent; WCX: weak cation exchange sorbent; WW: wastewater; ZNCAHF: zirconia nanoparticle/calcium alginate hydrogel fiber.

Looking at the reported extraction techniques, it can be observed that the most used technique is SPE, applied in 54 % of the studies, followed by DLLME (10 %) and SPME (9 %), mostly followed by LC or GC coupled with tandem mass spectrometry (MS/MS) detection (37 % and 26 % of the studies, respectively). As shown in Table 1.3, the most used cartridges for the analysis of pesticides in water samples are C18 and OASIS HLB. The C18 cartridges feature a highly retentive alkyl-bonded phase for nonpolar to moderately polar compounds. The OASIS HLB cartridges are prepared based on a universal polymeric reverse phase adsorbent that was developed for the extraction of a wide range of acidic, basic, and neutral compounds, allowing the extraction of both polar and less polar compounds. In two different studies (D'Archivio et al., 2007; Gervais et al., 2008), the comparison of five different sorbents for multiresidue SPE of pesticide in water showed that the best recoveries were obtained with the use of Oasis HLB cartridges.

Solid samples

The extraction of pesticides from solid environmental matrices such as sediments and biota is a critical step, due to the greater complexity of these matrices compared to water samples. The most traditional extraction system is solid-liquid extraction (SLE) (Chirila and Drăghici, 2013), which includes Soxhlet extraction. This technique requires the use of large volumes of solvents and in some cases is laborious and time-consuming. In recent years, new advanced techniques have been introduced: matrix solid-phase dispersion (MSPD) extraction, QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction, ultrasonic solvent extraction (USE), microwave-assisted extraction (MAE), and pressurized liquid extraction (PLE). Table 1.4 shows the analytical methods used in the last 10 years (2010-2020) in different studies for the analysis of pesticides in solid samples.

As can be seen, PLE and QuEChERS, used in more than half (60 %) of the reported studies, have received increasing attention and they are today the methods of choice for extraction of pesticides from sediments and biota. Compared with other techniques, PLE is a completely automated extraction technique that allows reducing the use of solvents and the analysis time, providing high-throughput and cost-effective sample preparation (Hoff and Pizzolato, 2018; Subedi et al., 2015; Vazquez-Roig and Picó, 2015).

Table 1.4. Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in solid samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Clean-up*	*Analytical method*	*Reference*
Biota (fish)	20	MSPD	Envi-Carb	SPE (Envi-Florisil)	GC-ECD	(Barriada-Pereira et al., 2010)
Sediment	28	PHWE	MeOH	SPME (PDMS/DVB)	GC-MS	(Concha-Graña et al., 2010)
Sediment	7	PLE	50 mL deionized water	SPE (Oasis HLB)	LC-MS/MS	(Degenhardt et al., 2010)
Sediment	3	LDMHLLE	10 mL MeOH	none	GC-MS	(Hassan et al., 2010)
Biota (fish)	17	LLE	n-Hexane/ACN(80:20, v/v)	SPE (silica gel–SCX)	GC-MS/MS	(Nardelli et al., 2010)
Sediment	7	PLE	DCM/ACE (1:1, v/v)	SPE (GCB/PSA)	GC-NPD, GC-ECD	(Wang et al., 2010)
Sediment	38	QuEChERS	C18	filtration (0.45 um filter)	GC-MS	(Yang et al., 2010)
Sediment	20	PLE	15 mL MeOH	SBSE	TD–GC–MS/MS	(Camino-Sánchez et al., 2011)
Sediment/ Biota (fish)	13	SLE	10 mL ACN/water (50:50, v/v); EA/cyclohexane (75:25, v/v)	none	LC-MS/MS	(Lazartigues et al., 2011)
Sediment	7	SFE-DLLME	17.0 µL Carbon tetrachloride and 1.0 mL ACN	none	GC-FID	(Naeeni et al., 2011)
Sediment / Biota (mussel)	6	UAE	DCM/hexane; DCM/ACE	Florisil (5 g) SPE cartridges	GC-MS/MS	(Sánchez-Avila et al., 2011)
Sediment	4	MAE	10 mL MeOH	MAE–SPE (EnvirElut pesticide cartridge (500 mg))	LC-MS/MS	(Sánchez-Rodríguez et al., 2011)
Sediment	10	PLE	Hexane/ACE (1:1, v/v)	SPE, GPC and AccuVap (Supelclean LC-Florisil)	GC-MS	(Han et al., 2012)
Biota (fish, squid)	15	SPE	Strata C18 ACN	Strata NH2	GC-MS	(Santhi et al., 2012)

Table 1.4. (Cont.) Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in solid samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Clean-up*	*Analytical method*	*Reference*
Sediment	97	PLE	EA and ACE (70:30, v/v)	QuEChERS modified	LC-HRMS	(Chiaia-Hernandez et al., 2013)
Sediment	7	UAE	MeOH, ACE	SPE (Supelclean™ ENVI-Carb™)	GC-MS	(Harrison et al., 2013)
Biota (fish/shellfish)	20	PLE	10 % DCM : hexane	Automated power-prep™	GC-MS	(Helaleh and Al-Rashdan, 2013)
Sediment	26	PLE	ACE:DCM (1:1, v/v) and FA (1 %, v/v)	SPE (Oasis HLB)	LC-MS/MS	(Köck-Schulmeyer et al., 2013a)
Biota (fish)	18	QuEChERS	ACN	d-SPE (zirconium-based sorbent)	LP-GC/MS–MS	(Sapozhnikova and Lehotay, 2013)
Sediment	2	UAE	15 mL MeOH and ACE (3:1, v/v)	SPE (STRATA C18)	LDTD-MS/MS	(Darwano et al., 2014)
Sediment Biota (fish)	54	QuEChERS	10 mL ACN	d-SPE (125 mg PSA, 750 mg anhydrous $MgSO_4$, and 15 mg GCB)	UPLC/LTQ-Orbitrap-MS	(Farré et al., 2014)
Sediment	253	MAE	25 mL ACE-hexane (1:1)	none	LC-MS/MS	(Kalogridi et al., 2014)
Sediment	12	UAE	McIlvaine buffer and ACN (1 : 1, v/v); $Mg(NO_3)_2$–$NH_3{\cdot}H_2O$ (96:4, v/v)	SPE (SAX and HLB)	HPLC-MS/MS	(Chen et al., 2015)
Sediment	18	MAE	15 mL tetrahydrofuran-hexane (9:1, v/v)	SPE (Florisil/Na_2SO_4)	GC-MS	(Merdassa et al., 2015)
Biota (fish)	24	QuEChERS	10 mL ACN (EN method)	dual d-SPE (PSA + SAX + NH2; PSA + SAX + NH2)	GC-MS	(Molina-Ruiz et al., 2015)
Sediment	9	QuEChERS	20 mL hexane/ACE, DCM/ACE	none	GC-MS	(Ben Salem et al., 2016)

Table 1.4. (Cont.) Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in solid samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Clean-up*	*Analytical method*	*Reference*
Sediment	18	PLE	DCM: hexane (4:3,v/v)	PLE in-cell clean-up (silica)	GC-MS/MS	(Duodu et al., 2016)
Biota (gammarids)	17	PuLE	ACN: water (1:1,v/v)	QuEChERS modified	LC-MS/MS	(Inostroza et al., 2016)
Sediment	31	PLE	DCM	PLE in-cell clean-up (alumina)	GC-MS/MS	(Pintado-Herrera et al., 2016)
Sediment/Biota (aquatic worms, bivalves)	6	SPLE	MeOH	On-line SPE (Oasis HLB)	UPLC-MS/MS	(Rodrigues et al., 2016)
Biota (fish)	340	QuEChERS and d-SPE one step	Chitin	None	LC-MS/MS	(Kaczyński et al., 2017)
Sediment	17	MSPD	ENVI-Carb	None	LC-MS/MS	(Rodríguez-González et al., 2017)
Biota (fish)	13	QuEChERS and DLLME-SFO	10 mL ACN	d-SPE (PSA)	GC-ECD	(Wang et al., 2017)
Biota (fish)	121	QuEChERS	water–ACN (50:50)	d-SPE (PSA)	LC-MS/MS	(Zhang et al., 2017)
Biota (fish)	11	US-DLLME-SFO	24 µL 1-undecanol	None	GC-ECD	(Asati et al., 2018)
Sediment	7	QuEChERS	EN method	d-SPE (AOAC method)	GC-MS	(Miossec et al., 2018)
Sediment	6	MAE/Soxhlet	10 mL hexane:water (3:2, v/v) / 180 mL ACE:hexane (1:1, v/v)	Silica gel column (hexane)	GC-MS	(Miyawaki et al., 2018)
Sediment	17	QuEChERS	AOAC method	d-SPE (150 mg $MgSO_4$, 50 mg PSA, 50 mg C18)	UPLC–Orbitrap MS/MS	(Nannou et al., 2018)
Biota (fish)	4	FUSLE	7 mL MeOH:Milli-Q water (95:5)	PES microextraction, Florisil SPE, LLE-HLB-SPE	LC-MS/MS	(Mijangos et al., 2019)

Table 1.4. (Cont.) Analytical methods reported in the last ten years (2010-2020) for the analysis of pesticides in solid samples.

Matrix	*Pesticides*	*Extraction method*	*Adsorbent/extraction solvent*	*Clean-up*	*Analytical method*	*Reference*
Biota (fish)	11	DLLME	10 mL ACN	SPE (alumina A)	GC-ECD	(Xu et al., 2019)
Sediment /Biota (fish)	9	PLE	DCM:hexane (1:1, v/v)	Multi-layer silica gel column (alumina, florisil)	GC-HRMS	(Zhao et al., 2019)
Biota (fish)	302	QuEChERS	ACN	Micro-SPE (45 mg of 20/12/12/1 (w/w/w/w) anh. $MgSO_4$, PSA, C18, CarbonX)	GC-MS/MS, UPLC-MS/MS	(Han and Sapozhnikova, 2020)
Sediment	6	SLE	MeOH:water (50:50, v/v)	SPE (stir disc)	UPLC-MS/MS	(Tomai et al., 2020)
Sediment	12	CSE	MeOH–MeOH–water	SPE (Oasis HLB)	HPLC-MS/MS	(Z. Wang et al., 2020)

CSE: continuous solvent extraction; FA: formic acid; FID: flame ionization detector; FUSLE: focused ultrasonic solid–liquid extraction; GCB: graphite carbon black; GPC: Gel-permeation chromatography; LDMHLLE: low density miniaturized homogenous liquid–liquid extraction; LDTD: Laser diode thermal desorption; LP: low pressure; LTQ: linear trap quadrupole; MeOH: methanol; NPD: nitrogen–phosphorus detector; PHWE: pressurized hot water extraction; PSA: primary secondary amine; PuLE: pulverised liquid extraction; SAX: strong anion exchange sorbent; SCX: strong cation exchange sorbent; SFE: supercritical fluid extraction; SPLE: selective pressurized liquid extraction.

PLE provides recoveries in the same range as, or greater than, other methods and is able to extract polar and non-polar analytes (Vazquez-Roig and Picó, 2015). On the other hand, over traditionxal extraction methods, QuEChERS offers high analyte recoveries and accurate results, combining the extraction of pesticides and clean-up, using a little amount of solvents and time, and demanding small lab-space and equipment requirements (Lehotay, 2011). Since the development and publication of the technique (Anastassiades et al., 2003), QuEChERS has gained significant popularity and has become the method of choice for food analysis (Blasco et al., 2011; Wilkowska and Biziuk, 2011). Nevertheless, PLE usually gives better reproducibility due to automation (Feng et al., 2013; Vazquez-Roig and Picó, 2015).

1.5.4 Sample analysis

Most analytical methodologies developed for the identification and detection of pesticides in environmental matrices are multi-residue approaches that provide high sensitivity and selectivity, and are based on GC or LC (either high performance (HPLC) or ultra-high performance LC (UPLC)) separation coupled in almost all cases with single (MS) or MS/MS detection (Tables 1.3 and 1.4).

Chromatographic separation

Due to the nonpolar nature of the early pesticides (*e.g.* organochlorine pesticides or pyrethroids), GC has been the most widely used technique for their analysis in environmental matrices (Concha-Graña et al., 2010; Duodu et al., 2016; Gutiérrez-Serpa et al., 2017; Helaleh and Al-Rashdan, 2013; Hu et al., 2019; Huang et al., 2015, 2020; Martins et al., 2014; Merdassa et al., 2015; Miyawaki et al., 2018; Molina-Ruiz et al., 2015; Nardelli et al., 2010; Santhi et al., 2012; Shi et al., 2014). However, for the determination of the new modern pesticides, which present medium-high polarity and low volatility, LC is a more suitable technique (Zwiener and Frimmel, 2004), since their analysis by GC requires the previous derivatization of the analytes to form more volatile and thermally-stable compounds. This process is very tedious and increases analysis time, and due to extensive sample handling, may overall negatively affect the reproducibility of the results.

Carbamates, phenylureas, organophosphates, triazines, neonicotinoids are some of the main classes of pesticides mostly analyzed with LC (Blanchoud et al., 2020; Chiaia-Hernandez et al., 2013; de Barros et al., 2019; Kalogridi et al., 2014; Köck-Schulmeyer et al., 2013a; Lazartigues et al., 2011; Mijangos et al., 2019; Montiel-León et al., 2018; Quintana et al., 2019; Rodríguez-González et al., 2017; Rousis et al., 2016; Rubirola et al., 2017; Tan et al., 2020). Most LC-based methods employ C18 reversed-phase columns and mobile phases consisting of water and polar solvents such as methanol or acetonitrile. In order to obtain better peak resolution and reduce peak tailing, a small amount of a volatile acid (*e.g.*, acetic or formic acid (v/v)), is often added to the water phase (Camilleri et al., 2015; Donato et al., 2017; Köck-Schulmeyer et al., 2013a; Morasch et al., 2010; Reemtsma et al., 2013; Ricart et al., 2010; Singer et al., 2010; Tomai et al., 2020; Tröger et al., 2018; Valls-Cantenys et al., 2016; Wode et al., 2012). However, the addition of modifiers to the mobile phase, despite being beneficial for some analytes, may limit the diversity of compounds that can be simultaneously analyzed in one analytical run. HPLC instruments normally use chromatographic columns packed with particles of an inner diameter of 3-5 μm for chromatographic separation, while UPLC systems can stand the high back-pressures that columns with smaller particles provide (< 2 μm) (Berho et al., 2013; Campos-Mañas et al., 2017; Kharbouche et al., 2019; Zaidon et al., 2019). Due to the high operating pressures (6,000 psi vs 15,000 psi), UPLC systems provide better peak resolution in shorter run times and allows reducing the solvent consumption. Nevertheless, the determination of highly polar pesticides in environmental samples is still a current issue, since it is not an easy task to selectively extract highly polar compounds from a polar matrix, and often interferences are co-extracted. Analytical methods based on hydrophilic interaction liquid chromatography (HILIC) have been considered as an alternative for highly polar compounds that poorly retain in reversed-phase columns (Buszewski and Noga, 2012; Danezis et al., 2016; Herrera López et al., 2019; Robles-Molina et al., 2017). For instance, HILIC was used for the analysis of 14 highly polar pesticides in five food matrices, making it possible to avoid its derivatization for proper detection (Herrera López et al., 2019).

Mass spectrometry detection

Mass spectrometry detection requires the ionization of the analytes present in the sample. For this, electron ionization (EI) is commonly used in GC-MS instruments (Han et al., 2012; Miossec et al., 2018; Miyawaki et al., 2018; Molina-Ruiz et al., 2015; Pintado-Herrera et al., 2016; Zare et al., 2016), while electrospray ionization (ESI) (Bolzan et al., 2016; Camilleri et al., 2015; Kalogridi et al., 2014; Montiel-León et al., 2018; Ribeiro et al., 2015; Rodríguez-González et al., 2016) and atmospheric pressure chemical ionization (APCI) (Blasco et al., 2004; Gimeno et al., 2001; Koal et al., 2003; Tomasini et al., 2012; Yang et al., 2018) are widely employed in LC-MS approaches.

The ionization of analytes with EI is achieved by bombarding the sample with a beam of electrons, which ejects an electron from the molecule, already in the gas phase, producing a radical ion. Thus, EI is ideal for ionizing highly volatile or semivolatile molecules, which are usually of small molecular weight and usually apolar character, and thus it is commonly used with GC approaches. EI is considered as an aggressive or hard ionization method that breaks up the molecule into different fragments, reducing to a minimum the presence or even completely destroying the molecular ion. However, it produces highly reproducible MS spectra, independent of the GC-MS instrument, when using the same ionization voltage (commonly 70 eV) and thus, allows the use of mass spectral libraries In ESI, a solvent spray is formed by applying a high voltage potential on a liquid stream. The solvent droplets from the spray evaporate in the ion source of the mass spectrometer, releasing ions to the gas phase for analysis in the MS. ESI, although highly versatile, is recommended for the analysis of medium or high polarity molecules, which makes it an ideal ionization technique for LC analysis. The APCI technique can be adapted to the two chromatographic techniques; however, its selective ionization limits it to the analysis of certain types of compounds, with specific volatility and polarity and molecular weight. In contrast to ESI, ions are not formed in solution or liquid phase, but are formed in the gas phase using a high voltage to ionize solvent molecules and analytes in the aerosol. Both, ESI and APCI are considered soft ionization techniques that preserve the molecular ion. However, the MS spectra that generate are very variable among LC-MS instruments (the configuration of the ionization source changes among vendors and this affects the ions formed and their intensity). Due to the semi-polar and polar nature (K_{ow} < 3) of the

pesticides selected for study in this doctoral thesis, the ESI technique is the most suitable ionization technique for the MS detection of these compounds in environmental samples. An evaluation of the analysis of over 75 pesticides by HPLC/MS, showed that some classes of pesticides, especially the more neutral and basic pesticides such as phenylureas and triazines, are more efficiently ionized and hence measured with higher sensitivity when using APCI, while others, e.g., cationic and anionic herbicides such as chlorophenoxyacid herbicides, alkyl sulfates, and acetanilide herbicide metabolites are better determined with ESI (Thurman et al., 2001). Nevertheless, they concluded that, whenever a compound works well by APCI, it also works well by ESI, but not necessarily *vice versa*, since the liquid-state basicity in ESI is related to proton affinity for APCI in the vapor state. However, positively charged solutes in solution are not volatile in the APCI source. A recent study, in which the efficiency of the two ion sources in LC-MS/MS systems was tested for the analysis of 22 pesticides in cabbage samples, revealed that both ionization methods were selective and exhibited good linearity, but a better response in terms of sensitivity (lower limits of quantification (LOQs)) was obtained with the ESI than with the APCI source (De O. Silva et al., 2019).

Ions formed are then introduced in the MS instrument, in which analyzers are responsible for separating the mixture of various ions according to their mass to detect them individually. The MS instruments most used for the analysis of pesticides in environmental samples are hybrid instruments that combine low-resolution analyzers such as quadrupole and linear ion traps, with high resolution (HR) analyzers like time of flight and Orbitraps: triple quadrupole (QqQ), quadrupole-linear ion trap (QqLIT), quadrupole-time of flight (QTOF), and quadrupole-Orbitrap (Q-Orbitrap) analyzers. Among them, the *target* analysis of pesticides mainly used QqQ. Figure 1.5 shows the typical QqQ configuration.

The QqQ analyzer is based on two in-line quadrupoles separated by a collision cell. Each of the quadrupoles is based on the use of an electric field generated on four parallel metal bars, through which the separation of the ions occurs according to their mass to charge (*m/z*) ratio. Depending on the electric field produced, only certain ions (precursor ions) are directed towards the collision cell and then, after dissociation, towards the next quadrupole, where there is a new selection of ions (product ions) that are led to the

detector, while the other ions are diverted towards the poles. QqQ offers the possibility of conducting different modes of acquisition. However, the selective reaction monitoring (SRM) mode, with the acquisition of the two most abundant and selective SRM transitions per compound, has become the modality of choice due to the high sensitivity and selectivity provided.

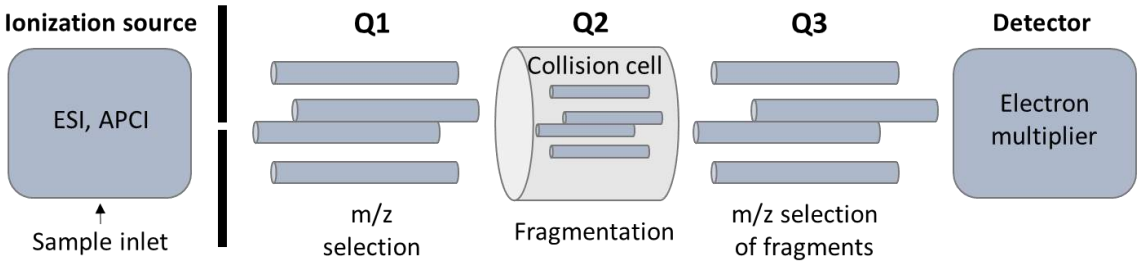


Figure 1.5. Schematic configuration of a triple quadrupole mass spectrometer.

While QqQ has been used mainly in the target analysis of pesticides, HR instruments have been mainly employed in *a non-target mode* for wide screening of pesticides and to identify and characterize unknown compounds (*e.g.* transformation products) in environmental samples as well as in degradation lab-scale experiments (Agüera et al., 2013; Deeb et al., 2017; Hernández et al., 2012; Matsushita et al., 2018; Picó and Barceló, 2015; Souissi et al., 2013; Temgoua et al., 2020; Vanryckeghem et al., 2019; X. Wang et al., 2020). Their identification is achieved through the acquisition of full spectra information with high mass-resolving power of parent molecule ions and their more characteristic fragment ions.

In a recent study by Wang et al. (2020), a HPLC-QTOF-MS-based methodology, applied in three Chinese wastewater treatment plants, allowed the identification of 60 pesticides by suspect screening (using compound lists) and 57 TPs, some of which presented higher concentration and toxicity than their parent compounds. An example of the application, in this case, of a hybrid Q-Orbitrap mass spectrometer Q-Exactive coupled with an LC system can be found in the work of Matsushita et al. (2018), who used this analytical instrumentation to estimate the removal of pesticides and their TPs during drinking water treatment simulated in laboratory-scale batch experiments.

Matrix effects

One of the most common problems in the analysis of environmental samples is that the extract typically contains a large number of components of the matrix, which can co-elute with the analytes, compete in the ionization process, and thus compromise the quantitative analysis (Petrovic et al., 2010). In the analysis of pesticides, the variations in the response induced by matrix components result in enhancement or suppression of the chromatographic signal and consequently in the loss of accuracy, precision, and sensitivity of the method that lead to incorrect quantifications and uncertain confirmation (Gosetti et al., 2010). To limit or correct matrix effects during the LC-MS determination of pesticides in environmental matrices, different approaches can be adopted. For complex matrices, the dilution of the sample helps to reduce the matrix effects, although in many cases, this approach leads to an overall reduction of sensitivity, affecting the LOD and LOQ of the analytical method (Moreno-González et al., 2017). An improvement of the clean-up of the extract also helps to reduce the matrix interferences. However, since matrix effects cannot be completely eliminated, especially in LC-MS-based methods with a high number of organic compounds simultaneously analyzed, the use of stable isotopically labeled internal standards (ILIS) allows to satisfactorily correct the matrix effects (Aszyk et al., 2018; Grimalt and Dehouck, 2016; Marín et al., 2009). In the ILIS method, an exact and known amount of ILIS is added to the calibration curve, blanks, controls, and samples, and the analyte concentration is obtained by plotting the signal *vs* the concentration ratios of the analyte and its corresponding ILIS. One of the methods of choice for the addition of ILIS is the isotope dilution approach. It consists of adding the ILIS at the beginning of the analytical protocol to correct for potential analyte losses during sample extraction and MS signal drift. The technique is, however, limited by the commercial availability and often high price of the required ILIS, which are isotopically labeled analogs of the target analytes, or failing that, isotopically labeled compounds presenting similar structure, retention time, and/or recoveries to the target analytes.

1.6 Investigated compounds

The compounds investigated within this thesis include 52 pesticides and TPs belonging to 9 different chemical groups. The list comprises: five acidic pesticides (2,4-D, bentazone, fluroxypyr, MCPA, and mecoprop), two anilides (diflufenican, and propanil), three carbamates (methiocarb, molinate, and triallate), two chloroacetanilides (alachlor, and metolachlor), five neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam), nine organophosphates (azinphos ethyl, azinphos-methyl and its metabolite azinphos-methyl oxon, chlorfenvinphos, diazinon, dichlorvos, dimethoate, fenitrothion and its metabolite fenitrothion oxon), nine organothiophosphates (chlorpyrifos, fenthion, and its metabolites fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, fenthion sulfone, and fenthion sulfoxide, malathion and its metabolite malaoxon), four phenylureas (chlortoluron, diuron, linuron, and isoproturon), eight triazines (atrazine, and its metabolites deisopropilatrazine and desethylatrazine, cyanazine, cybutryne, simazine, terbuthylazine, and terbutryn), and six pesticides of other chemical classes (bromoxynil, oxadiazon, pendimethalin, quinoxyfen, and thifensulfuron methyl).

Acidics: This family includes acid-derived pesticides, usually of the phenoxy type. Of these, the best known is 2,4-D, which, along with MCPA, has been used intensively for years around the world. These herbicides have complex mechanisms of action, affecting cell division, activating phosphate metabolism and modifying nucleic acid metabolism, also affecting protein synthesis.

Anilides and chloroacetanilides: These classes include pesticides that inhibit protein synthesis, affecting normal plant growth. They are used both in pre-emergence and post-emergence applications, being incorporated into the soil and absorbed by sprouts of germinated seeds or by the root system. They are usually quite toxic, as it is the case of alachlor, included in the list of priority substances in the field of water policy in the EU (EC, 2013).

Carbamates: These compounds are esters of carbamic acid that are commonly used as insecticides, although some derivatives of carbamic acid, thiocarbamic acid, and dithiocarbamic acid are also used as herbicides. Carbamates are well-known cholinesterase

inhibitors, which are able to inactivate acetylcholinesterase (AChE) by carbamylating the enzyme and cause overstimulation of the nervous system, producing a broad range of well-characterized symptoms of AChE poisoning.

Neonicotinoids: Neonicotinoids (literally "new nicotine-like insecticides") are insecticides derived from nicotine. They act by binding strongly to nicotinic acetylcholine receptors in the central nervous system of insects, causing overstimulation of their nerve cells, paralysis and death. Neonicotinoids are highly water-soluble, persistent in the environment, and they are systemic pesticides, which means that, unlike contact pesticides, which remain on the surface of the treated plant (*e.g.* leaves), they are taken up by the plant and transported through it (leaves, flowers, roots and stems, as well as pollen and nectar). Although their principal use is in agriculture for seed and soil treatment and on plant foliage, neonicotinoids may be used in home yards and gardens, golf courses, and for flea and tick treatments on dogs and cats. They are included in the Watch List of substances for Union-wide monitoring in the field of water policy (EC, 2018).

Organophosphates and organothiophosphates: The organophosphate class of pesticides, which includes the subclass of organothiophosphates, are esters of phosphoric acid. They are broad-spectrum compounds characterized by their high water solubility. This group of pesticides is highly applied worldwide due to their low persistence and high effectiveness, and they are generally regarded as safe for use on crops and animals due to their relatively fast degradation rates. However, they have high toxicity, as irreversibly inactivate AChE. Organophosphates and carbamates share a common mode of toxicological action associated with their ability to inhibit the AChE enzyme within the nervous tissue and at the neuromuscular junctions (NMJs), leading to high levels of acetylcholine in the NMJ and resulting in parasympathetic and sympathetic overstimulation, skeletal muscle paralysis, and sometimes respiratory failure. Overall, organophosphates effects are less reversible and more severe than carbamates. They are often used in combination, with the objective of achieving synergistic interaction and controlling a wide range of insects, including those that are considered highly resistant.

Phenylureas: these substances are generally herbicides, used for weed control in agricultural and non-agricultural practices (*e.g.* railroads and industrial areas). The herbicidal action of these compounds is based on their ability to inhibit photosynthesis, in

particular, most of them (*e.g.* diuron, isoproturon) are photosystem II inhibitors. They are relatively persistent in soils, where they can stay for a period of 3 to 6 months under favorable humidity and temperature conditions, with little or no leaching.

Triazines: This group of herbicides is a class of aromatic nitrogen-containing heterocycles, heavily used worldwide for control of broad-leaved weeds in agricultural production as well as in urban and recreational areas. They are well-known herbicides, extensively used during the last 40 years. Most triazines present low solubility in water (unlike their transformation products), which gives them high stability. Once incorporated into the soil, they can be absorbed by plants or degraded, although in general, they are highly persistent, causing possible contamination of nearby groundwater.

Selection of the target pesticides was made based on their feasibility for LC-MS analysis, their environmental relevance in terms of being considered as priority substances (EC, 2013) or included in the European Watch Lists (EC, 2018, 2015), their occurrence in the environment, and their extent of use at European level, with a special interest in those pesticides commonly applied in Spain, and particularly, in Catalonia, where most monitoring studies were conducted. The selected pesticides, representative of different chemical classes, present a very wide range of physical-chemical properties (K_{ow}, water solubility, volatility, etc.). Most of these compounds, due to their high toxicity on living organisms, are currently prohibited or subjected to European regulations which limit their use. The list of the target analytes and their main physical-chemical properties, together with their legal status, is provided in Table 1.5.

Table 1.5. List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, K_{oc}, K_{ow}, GUS, BCF, DT50, Pka) and regulated use at European level.

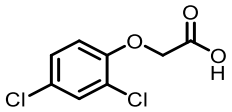
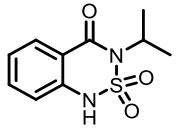
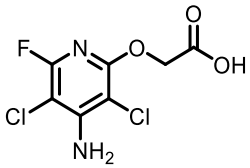
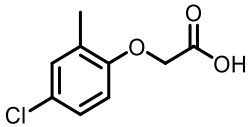
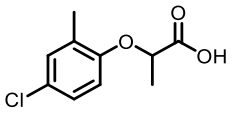
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Acidic	**2,4-D** 	94-75-7	221	24300	39	-0.82	3.82	10	7.7	3.40	✓
	Bentazone 	25057-89-0	240.3	7112	55	-0.46	1.95	21	80	3.51	✓
	Fluroxypyr 	69377-81-7	255	6500	10[β]	0.04	3.70	62.1	10.5	2.94	✓
	MCPA 	94-74-6	200.6	29390	29[β]	-0.81	2.98	1	13.5	3.73	✓
	Mecoprop 	7085-19-0	214.6	250000	47	-0.19	2.29	3	37	3.11	X

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

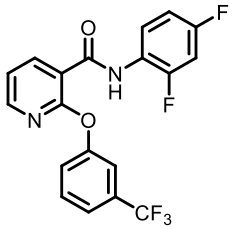
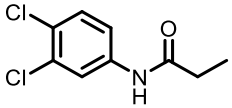
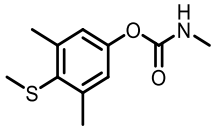
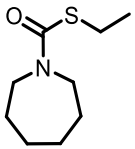
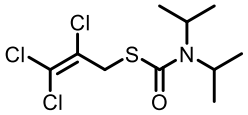
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Anilides	**Diflufenican** 	83164-33-4	394.3	0.05	5504	4.20	1.19	1276	175 [γ]	n/a	✓
	Propanil 	709-98-8	218.1	95	149	2.29	-0.51	111	1.2	19.1	X
Carbamates	**Methiocarb** 	2032-65-7	225.3	27	182 [β]	3.18	1.82	75	1.6	n/a	✓
	Molinate 	2212-67-1	187.3	1100	190	2.86	1.89	72	4	n/a	X
	Triallate 	2303-17-5	304.7	4.1	3034	4.06	0.61	1400	104	n/a	✓

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

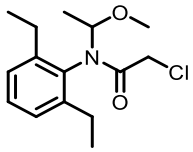
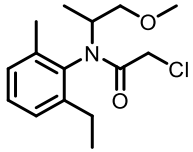
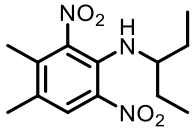
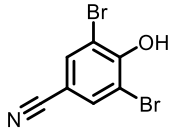
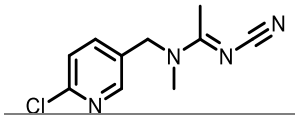
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Chloroacetanilides	**Alachlor** 	15972-60-8	269.8	240	335	3.09	0.80	39	2 [γ]	0.62	X
	Metolachlor 	51218-45-2	283.8	530	120	3.40	2.36	68.8	88	n/a	X
Dinitroanilines	**Pendimethalin** 	40487-42-1	281.3	0.33	17491	5.40	-0.28	5100	4	2.8	✓
Hydroxybenzonitrile	**Bromoxynil** 	1689-84-5	276.9	38000	302	0.27	1.71	28 [ζ]	13	3.86	✓
Neonicotinoids	**Acetamiprid** 	135410-20-7	222.7	2950	200	0.80	0.94	3 [ζ]	4.7	0.7	✓

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

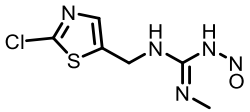
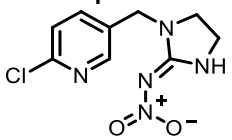
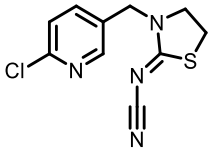
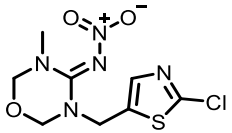
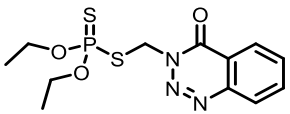
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Neonicotinoids	**Clothianidin** 	210880-92-5	249.8	340	123	0.90	3.74	3[ζ]	40.3	11.1	X
	Imidacloprid 	138261-41-3	25576	610	262[δ]	0.57	3.69	0.61	30	n/a	✓
	Thiacloprid 	111988-49-9	252.7	184	615[δ]	1.26	1.10	3[ζ]	1000	n/a	✓
	Thiamethoxam 	153719-23-4	291.7	4100	56	-0.13	3.58	3[ζ]	30.6	n/a	X
Organophosphates	**Azhinphos ethyl** 	2642-71-9	345.4	4.5	1500	3.18	1.40	101	50[Ω]	n/a	X

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

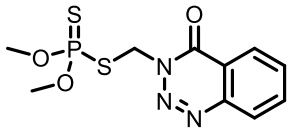
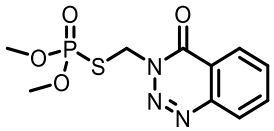
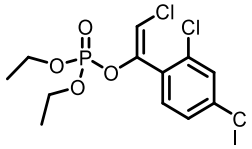
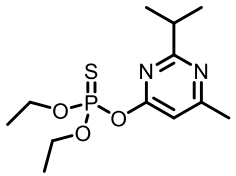
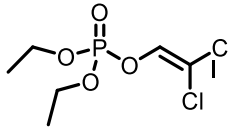
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Organophosphates	**Azinphos-methyl** 	86-50-0	317.3	28	1112	2.96	1.42	40	10[γ]	5	X
	Azinphos-methyl oxon[ε] 	961-22-8	301.3	2604[β]	10[β]	0.77[β]		-	n/a	n/a	-
	Chlorfenvinphos 	470-90-6	359.6	145	680	3.80	1.72	250	7	n/a	X
	Diazinon 	333-41-5	304.3	60	609	3.69	1.51	500	4.3	2.6	X
	Dichlorvos 	62-73-7	221	18000	50	1.90	0.69	0.8[ζ]	2[γ]	n/a	X

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

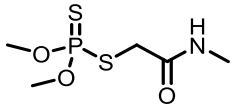
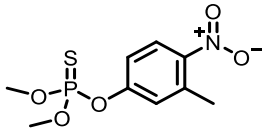
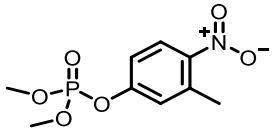
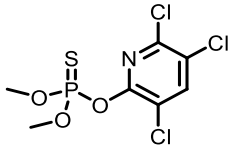
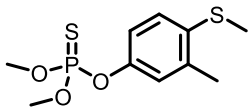
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Organophosphates	**Dimethoate** 	60-51-5	229.3	25900	25[β]	0.75	2.18	8	12.6	n/a	X
	Fenitrothion 	122-14-5	277.2	19	2000	3.32	0.48	29	1.1	n/a	X
	Fenitrothion oxon[ε] 	2255-17-6	261.2[β]	301[β]	21[β]	1.69[β]	-	-	n/a	n/a	-
Organothiophosphates	**Chlorpyrifos** 	2921-88-2	350.6	1.05	5509	4.70	0.58	1374	5	n/a	✓
	Fenthion 	55-38-9	278.3	4.2	1500	4.84	1.26	154	22[γ]	n/a	X

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

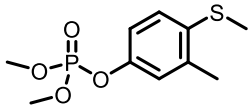
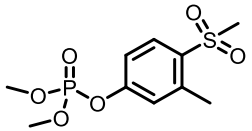
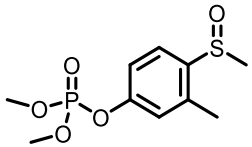
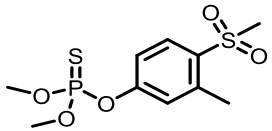
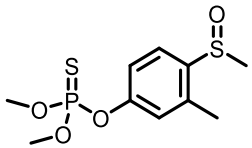
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Organothiophosphates	**Fenthion oxon**[ε] 	6552-12-1	262.3[β]	213.5[β]	57[β]	2.31[β]	-	-	n/a	n/a	-
	Fenthion oxon sulfone[ε] 	14086-35-2	294[β]	7602[β]	13[β]	0.28[β]	-	-	n/a	n/a	-
	Fenthion oxon sulfoxide[ε] 	6552-13-2	278.3[β]	1222[β]	11[β]	0.15[β]	-	-	n/a	n/a	-
	Fenthion sulfone[ε] 	3761-42-0	310.3[β]	190.4[β]	235	2.05[β]	-	-	n/a	n/a	-
	Fenthion sulfoxide[ε] 	3761-41-9	294.3[β]	3.72[β]	183	1.92[β]	-	-	n/a	n/a	-

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

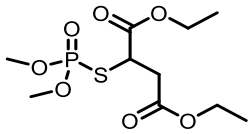
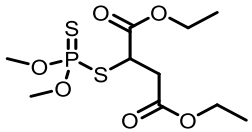
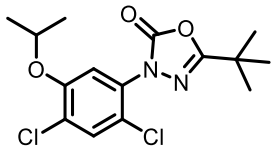
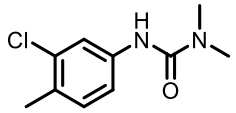
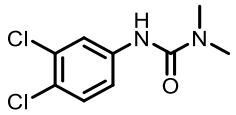
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Organothiophosphates	**Malaoxon**[ε] 	1634-78-2	314.3[β]	7500[β]	4650[β]	0.52[β]	-	3.2[ζ]	n/a	n/a	-
	Malathion 	121-75-5	330.4	148	1800	2.75	0.00	103	0.4	n/a	✓
Oxidiazole	**Oxadiazon** 	19666-30-9	345.2	0.57	3200	5.33	1.97	243	17.9	n/a	X
Phenylureas	**Chlortoluron** 	15545-48-9	212.7	74	196	2.50	2.62	40	42	n/a	✓
	Diuron 	330-54-1	233.1	35.6	680	2.87	2.65	9.45	8.8	n/a	✓

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

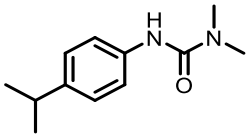
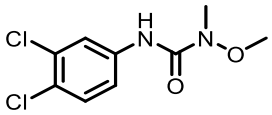
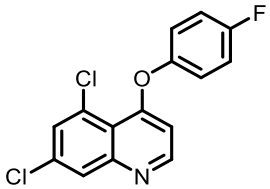
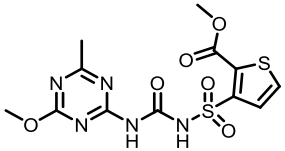
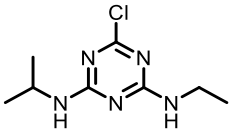
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Phenylureas	**Isoproturon** 	34123-59-6	206.3	70.2	251[β]	2.5	2.61	177	40	n/a	X
	Linuron 	330-55-2	249.1	63.8	843	3	2.11	49	13	n/a	X
Quinoline	**Quinoxyfen** 	124495-18-7	308.1	0.05	23[δ]	4.66	-0.80	5040	5	n/a	✓
Sulfonylurea	**Thifensulfuron methyl** 	79277-27-3	387.4	54.1	28	-1.65	3.05	0.8	22	4	✓
Triazines	**Atrazine** 	1912-24-9	215.7	35	100	2.70	2.57	4.3	75[γ]	1.7	X

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

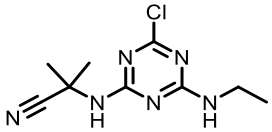
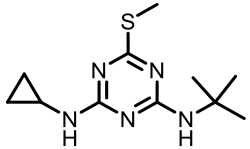
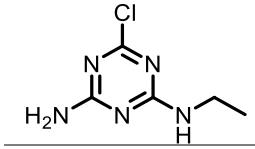
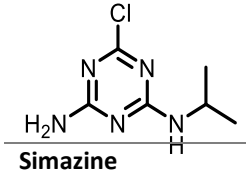
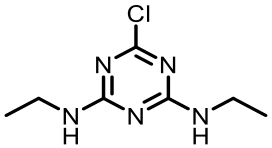
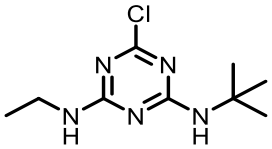
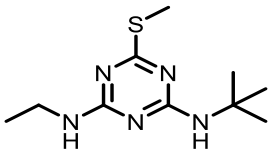
Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Triazines	**Cyanazine** 	21725-46-2	240.7	171	190	2.10	2.07	157	16[γ]	12.9	X
	Cybutryne (irgarol) 	28159-98-0	253.4	7	1569	3.95	-	160	n/a	n/a	X
	Deisopropilatrazine[ε] 	1007-28-9	173.6	980	130	1.15	-	-	n/a	n/a	-
	Desethylatrazine[ε] 	6190-65-4	187.6	2700	110	1.51	3.24	-	2.23[γ]	n/a	-
	Simazine 	122-34-9	201.7	5	130	2.30	2.20	221	46	1.62	X

Table 1.5. (Cont.) List of target pesticides and transformation products studied in this doctoral thesis, with details of their molecular structure, CAS number, molecular weight, physical-chemical properties (solubility, Koc, Kow, GUS, BCF, DT50, Pka) and regulated use at European level.

Class	Name	CAS number[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	BCF[α] (l kg^{-1})	DT50[α] (days)	Pka[α]	Regulated use[α]
Triazines	**Terbuthylazine** 	5915-41-3	229.7	6.6	329	3.40	2.19	34	6	1.9	✓
	Terbutryn 	886-50-0	241.4	25	2432	3.66	2.21	72.4	27	4.3	X

CAS number: CAS (chemical abstract service) unique numerical identifier for chemical substances; Solubility: solubility in water at 20 °C; K_{oc}: organic carbon partition coefficient; K_{ow}: octanol-water partition coefficient; GUS: leaching potential index; BCF: bioconcentration factor; DT50: degradation potential in water phase, expressed as half-life in days; Pka: dissociation constant at 25 °C; n/a: data not available; n/d: no dissociation.

[α] Information extracted from the Pesticide Properties Database (PPDB), https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm.
[β] Data estimated using the US Environmental Protection Agency EPISuite™.
[γ] Water-sediment DT50 or soil degradation DT50 values (in case that water phase DT50 data is not available).
[δ] Kegley, S.E., Hill, B.R., Orme S., Choi A.H., PAN Pesticide Database, Pesticide Action Network, North America (Oakland, CA, 2016), http://www.pesticideinfo.org.
[ζ] Information extracted from the PubChem Substances and Compound database (https://pubchem.ncbi.nlm.nih.gov/).
[ε] Metabolite.
✓ Approved for its use; X Not approved for its use

CHAPTER 2 - OBJECTIVES

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Pesticides are among the most used chemical substances worldwide. However, their investigation in the environment has very much focused on the so-called PBTs (persistent, bioaccumulative, and toxic compounds), whereas the more polar compounds have been little studied. Medium to highly polar pesticides, due to their physical-chemical properties (e.g., low hydrophobicity), are not expected to become adsorbed onto the soil and/or accumulated by non-target organisms, and hence, are unlikely to persist in the environment. However, their extensive use at present as a replacement of the more persistent and toxic pesticides used in the past, currently prohibited, has led to their widespread introduction into the aquatic environment and has conferred them a pseudo-persistent character.

After application, pesticides and a suite of transformation products may end up in the different environmental compartments through various transport mechanisms. In this regard, it becomes necessary to investigate their environmental fate, not only in water but also in other compartments, such as sediments and biota. The determination of pesticides in these complex matrices is challenging, and therefore, analytical methods for this purpose are at present very scarce.

In this context, the main objectives of the present thesis were:

- To develop and validate multi-residue analytical methodologies of high sensitivity and selectivity to determine medium and high polarity pesticides and their TPs in different environmental matrices, including water, sediment and biota.
- To apply the analytical methodologies developed to study the occurrence and fate of pesticides and TPs in different sites of interest.
- To assess the potential environmental impact of these contaminants on non-target aquatic organisms.
- To evaluate novel strategies to attenuate pesticide pollution and improve water quality, such as:

- The development of bioremediation techniques that could be implemented to biodegrade pesticides at large scale;
- The use of multi-actor participatory approaches to propose mitigation measures and best management practices in agriculture to reduce pesticide application.

CHAPTER 3 - RESULTS

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3.1 Analysis of medium to highly polar pesticides and metabolites in surface water and groundwater

The availability of high sensitivity and selectivity analytical technologies for the analysis of polar pesticide residues in environmental waters at the low concentrations at which they are often present and can exert toxic effects is essential. In this context, online-SPE-LC-MS/MS can be considered an attractive analytical technique, as it allows obtaining accurate and reproducible results using low sample volumes and minimizing sample processing and handling time.

In the past 10 yours, different methods based on online-SPE-LC-MS/MS have been developed in our research group for the analysis of medium to highly polar pesticides (22 compounds) in groundwater and wastewater (Köck-Schulmeyer et al., 2014, 2013a; Postigo et al., 2010). However, European legislation on pesticides is continuously evolving and being updated to include new relevant substances for priority action and hence prevent deterioration of water bodies. In the last years, several pesticides have been included in the list of priority substances and the European Watch Lists, and hence the existing analytical methodologies need to be adapted to cope with these new monitoring requirements.

For this reason, the online-SPE-LC-MS/MS methodology presented in the scientific publication #1 incorporates 29 additional medium to highly polar pesticides, including 8 TPs, in the list of target analytes for their investigation in surface water and groundwater. The method makes use of the isotopic dilution technique for quantification (ILIS for 88% of the analytes are added at the beginning of the analytical process). This approach is useful to correct matrix effects, and other possible sources of errors that may occur during the entire sample extraction and preparation process, providing, therefore, greater precision and accuracy, and hence, reliability of the results.

Improvements of the newly developed methodology in comparison with the aforementioned methods are the use of centrifugation instead of filtration, and the reduction of the analysis time, with all analytes being determined in a single analytical run,

thanks to the use of a generic sorbent for their simultaneous preconcentration and the switch of polarity ionization during MS acquisition.

Compared to previous fully automated methodologies published for the analysis of polar pesticides in water samples (Camilleri et al., 2015; Hurtado-Sánchez et al., 2013; Mann et al., 2016; Quintana et al., 2019; Rubirola et al., 2017; Singer et al., 2010), the presented method shows: good performance in terms of sensitivity, with equal or lower LODs and LODets, allowing the quantification of the pesticides in compliance with the EQS (EC, 2013, 2006a) and LODs established in the European Watch List (EC, 2018); validation results for 10 pesticides and TPs not previously investigated with this type of automated methodologies; and high reliability of results, due to the use of ILIS for almost all target pesticides.

As part of the validation, the method was applied to the determination of the selected pesticides in two different agriculture-impacted areas of Catalonia (Spain) and the assessment of their environmental risk for aquatic organisms.

The study here presented is part of the WaterProtect (European Union's Horizon 2020 - Research and Innovation Framework Programme, No. 727450), and BECAS (Spanish State Research Agency and European Regional Development Fund (ERDF), CTM2016-75587-C2-2-R) projects. WaterProtect is a multidisciplinary project aimed at developing new solutions and tools in areas where water pollution (nutrients and/or pesticides) from intensive agriculture and/or industrial and urban activities may affect the quality of the water used for drinking water production. BECAS is a project aimed at determining the presence of pesticides in water and soils and studying their biodegradation through new bioremediation processes.

Scientific publication #1:

"Improved fully-automated method for the determination of medium to highly polar pesticides in surface and groundwater and application in two distinct agriculture-impacted areas"

Maria Vittoria Barbieri, Luis Simón Monllor-Alcaraz,
Cristina Postigo, Miren López de Alda

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Improved fully automated method for the determination of medium to highly polar pesticides in surface and groundwater and application in two distinct agriculture-impacted areas.

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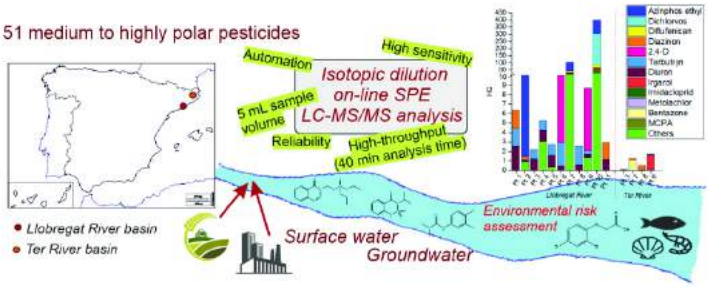
HIGHLIGHTS

- Automated, high throughput, simultaneous analysis of 51 pesticides in water.
- Reliable results regardless of matrix effects ensured by isotopic dilution method.
- Limits of detection for most compounds in compliance with European regulations.
- Twenty-eight pesticides detected in Llobregat River, seven pesticides in Ter River.
- Irgarol, dichlorvos, methiocarb, imidacloprid, thiacloprid above quality standards.

GRAPHICAL ABSTRACT



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ABSTRACT

Water is an essential resource for all living organisms. The continuous and increasing use of pesticides in agricultural and urban activities results in the pollution of water resources and represents an environmental risk. To control and reduce pesticide pollution, reliable multi-residue methods for the detection of these compounds in water are needed. In this context, the present work aimed at providing an analytical method for the simultaneous determination of trace levels of 51 target pesticides in water and applying it to the investigation of the target pesticides in two agriculture-impacted areas of interest. The method developed, based on an isotopic dilution approach and on-line solid-phase extraction-liquid chromatography-tandem mass spectrometry, is fast, simple, and to a large extent automated, and allows the analysis of most of the target compounds in compliance with European regulations. Application of the method to the analysis of selected water samples collected at the lowest stretches of the two largest river basins of Catalonia (NE Spain), Llobregat and Ter, revealed the presence of a wide suite of pesticides in the Llobregat basin, some of them at concentrations above the water quality standards (irgarol and dichlorvos) or the acceptable method detection limits (methiocarb, imidacloprid, and thiacloprid), and much cleaner waters in the Ter River basin. Risk assessment of the pesticide concentrations measured in the Llobregat River indicated high risk due to the presence of irgarol, dichlorvos, methiocarb, azinphos ethyl, imidacloprid, and diflufenican (hazard quotient (HQ) values>10), and moderate potential risk in the Ter River, associated to the occurrence of bentazone and irgarol (HQ > 1).

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1. Introduction

The extensive, and sometimes excessive, use of pesticides has led to significant undesired effects on the environmental and human health (Han et al., 2018; Islam et al., 2018; Mostafalou and Abdollahi, 2017). As a result, developed countries have withdrawn from the market the most toxic and persistent pesticides, such as the organochlorine ones, and have promoted the use of comparatively more polar substances, expected to degrade more rapidly and to be less toxic to non-target organisms (Sander et al., 2017). However, their use in large amounts has still resulted in their accumulation in the environment. Nowadays, over 500 active ingredients are available in the European market, with an estimated total turnover of around 400,000 tons (Eurostat, 2020). Spain is ranked as the first country in Europe with the largest pesticide consumption (according to pesticide sales (Eurostat, 2020), 72 millions kg per year on average in the period 2011–2018).

Water plays an important role in the environmental fate of pesticides because it transports these substances from agricultural fields to other areas, by flushing them away into the rivers *via* rainfall or irrigation runoff, or leaching them through the soil into groundwater bodies (Beitz et al., 2012). Pesticides applied in urban areas may also reach the aquatic environment after incomplete removal in wastewater treatment plants (WWTPs) (Köck-Schulmeyer et al., 2013; Rousis et al., 2017). Once applied or released in the environment, these compounds can be transformed by different natural processes (photodegradation, hydrolysis, oxidation, biotransformation). These processes hardly mineralize the parent compounds and, consequently, a wide spectrum of transformation products (TPs) are formed, some of them being even more toxic than the corresponding parent compound (Andreu and Picó, 2004; Richardson and Ternes, 2014).

To reduce contamination by pesticides and TPs in the environment and minimize their impact on aquatic organisms and human health, the European Commission has established guidelines that influence the selection and application of pesticides, as well as maximum allowable concentrations (MAC) in both surface water and groundwater. The groundwater Directive (EC, 2006a) sets maximum limits of 0.1 μg/L for individual pesticides and TPs and 0.5 μg/L for total pesticides in groundwater to preserve its quality, in line with the limits set in the drinking water Directive for waters intended for human consumption (EC, 1998). In surface water, the Directive 2013/39/EU (EC, 2013) establishes MACs for up to 45 priority substances, including 24 pesticides or biocides, in inland and other surface waters as well as in biota. Furthermore, five neonicotinoid pesticides, the carbamate methiocarb, and the semicarbamazone metaflumizone are currently included in the watch list of substances for Union-wide monitoring in the field of water policy (European Decision 2018/840 (EC, 2018)).

To meet European requirements, it is necessary to use analytical techniques that allow the monitoring of pesticide residues in surface and groundwater with high selectivity and sensitivity, such as liquid chromatography (LC) coupled to mass spectrometry (MS or MS^2) (Hernández et al., 2005; Picó and Barceló, 2015). However, to measure the low concentrations at which some pesticides are present and toxic in water, a sample enrichment step is still required. Solid phase extraction (SPE) is nowadays considered the method of choice for this purpose (Pérez-Fernández et al., 2017). SPE can be performed in off-line mode, but the fully automated on-line approach has become increasingly attractive as it requires minimum intervention of the operator, which results in reduced sample processing time and improved reproducibility and accuracy of the results. Moreover, it grants high sample throughput and high sensitivity using low sample amounts, which becomes very relevant in case of required sample shipment and/or small storage space (Rossi and Zhang, 2000; Singer et al., 2010). In spite of these advantages, only few methodologies published for the analysis of polar pesticides in water are fully automated (Camilleri et al., 2015; Hurtado-Sánchez et al., 2013; Mann et al., 2016; Quintana et al., 2019; Rubirola et al., 2017; Singer et al., 2010).

In this context, and with the final aim of advancing knowledge on the analysis and monitoring of regulated and non-regulated medium to highly polar pesticides in water bodies, the present work focused on developing and validating a fast and simple analytical methodology based on on-line SPE-LC-MS/MS to determine 51 pesticides in environmental waters at pg/L or ng/L levels in a single run. The selected pesticides belong to different chemical classes and the list includes pesticides of major concern included in the priority substances list or the watch list, some of their transformation products, and pesticides commonly applied in Spain, and particularly, in Catalonia. To the best of our knowledge, this work provides for the first time validation figures for the analysis of 10 pesticides and TPs (*i.e.*, azinphos ethyl, azinphos-methyl oxon, dichlorvos, diflufenican, fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, fenthion sulfone, fenthion sulfoxide, and oxadiazon) in water samples with the use of an on-line SPE-LC-MS/MS approach.

As a part of the validation process and to fulfil the overall aim of our work, the developed method was applied to the analysis of surface water and groundwater samples collected in two agriculture-impacted areas of Catalonia (NE Spain), to evaluate the occurrence and fate of the target pesticides and compliance of these water bodies with the current EQS. The results obtained were also used to assess the potential environmental risk that the pesticides found may pose for aquatic organisms in these areas.

2. Materials and methods

2.1. Standards and solvents

High purity (>96%) standards of the 51 pesticides selected as target analytes and stable isotope-labeled (SIL) analogs for 45 of them were purchased from Fluka (Honeywell Specialty Chemicals Seelze GmbH, Germany), Toronto Research Chemicals (North York, ON, Canada), Cambridge Isotope Laboratories (Tewksbury, MA, USA), Sigma Aldrich (Merck KGaA, Darmstadt, Germany) or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). The list of the target analytes and their main physical-chemical properties is provided in Table S1 as Supporting Information (SI).

Stock standard solutions of the individual analytes and SIL standards were prepared in methanol (MeOH), except in the case of simazine and its SIL analog that were prepared in dimethyl sulfoxide. All stock individual solutions (1000 μg/mL) were stored in amber glass bottles in the dark at −20 °C. Working standard solutions containing all analytes were prepared by appropriate dilution of the stock individual standard solutions at different concentrations (0.5 to 2000 ng/mL) in MeOH. A MeOH-based solution containing the mixture of SIL standards at a concentration of 1000 ng/mL was also prepared. These mixtures were used to prepare the aqueous standard solutions that defined calibration curves and in the validation studies. Pesticides-grade solvents MeOH, acetonitrile (ACN), and LC-grade water were supplied by Merck (Darmstadt, Germany).

2.2. On-line solid-phase extraction

On-line SPE of the water samples was performed with a commercial Prospekt-2 system (Spark Holland, Emmen, The Netherlands) connected in series with the LC-MS/MS instrument. Before automated on-line SPE and analysis, the water sample was fortified at a concentration of 200 ng/L with the mixture of SIL compounds that will be used as surrogate standards, and centrifuged at g-force of 2500 ×*g* (3500 rpm) and room temperature for 10 min to remove suspended particles (centrifuge 5810 R, Eppendorf Ibérica, Spain). Then, 5 mL of the sample, calibration solution and/or blank was delivered using a 2 mL high-pressure syringe onto a previously conditioned CHROspe cartridge Polymer DVB (divinylbenzene polymer, 10 mm × 2 mm i.d., 25–35 μm particle size) (Axel Semrau GmbH & Co. KG, Srockhövel, Germany) at

a flow rate of 1 mL/min. Conditioning of the cartridge was also performed *via* the high pressure dispenser (HPD) unit with 1 mL of ACN and 1 mL of LC-grade water (5 mL/min). Upon sample loading, the cartridge was washed with 1 mL of LC-grade water to complete sample transfer and remove highly polar components of the matrix, and the analytes were eluted with the LC mobile phase onto the LC analytical column. The system configuration allows the preconcentration of the next sample in a batch while the LC-MS analysis of the previously extracted sample takes place. The entire system was controlled through SparkLink Version 3.10 (Spark Holland).

2.3. LC-MS/MS analysis

LC-MS/MS analyses were performed using a 1525 binary HPLC pump connected in series with the Prospekt-2 system and a TQD triple-quadrupole mass spectrometer equipped with an electrospray (ESI) interface (Waters, Milford, MA, USA).

LC separation was carried out with a Purospher® STAR RP-18 endcapped column (100 mm × 2 mm i.d., 5 μm particle size) preceded by a guard column (4 mm × 4 mm i.d., 5 μm) of the same packing material (Merck, Darmstadt, Germany), and a linear gradient of ACN and water as mobile phase at a flow rate of 0.2 mL/min. The gradient started with an ACN composition of 10% that was increased to 50% in 5 min, to 80% in the next 20 min, and 100% in the following 6 min. Then, the chromatographic column was reequilibrated with the mobile phase initial conditions in the following 9 min. In total, the analysis time, including the sample extraction step, was 40 min.

MS/MS detection was performed in the selected reaction monitoring (SRM) mode, recording one SRM transition per SIL compound and two SRM transitions per target analyte, with the first one and more abundant being used for quantification and the second one for confirmation. A total of 146 SRM transitions were acquired in six separate retention windows to maximize the sensitivity of the MS instrument (Fig. S1 in SI). The ESI interface was operated in both positive (PI) and negative (NI) ionization modes according to the preferential ionization mode of the target analytes (43 were analyzed in PI and 8 in NI). Table 1 summarizes the optimum SRM transitions and ionization conditions for each selected analyte. Other specific optimized MS conditions were as follows: capillarity voltage, 3.5 kV; extractor voltage, 3 V; RF lens voltage, 1.8 V; source temperature, 150 °C; desolvation temperature, 450 °C. Nitrogen was used as cone gas (flow, 30 L/Hr) and desolvation gas (flow, 680 L/Hr); and argon was used as collision gas (flow, 0.19 mL/min). MassLynx 4.1 software from Waters was used to perform instrument control, data acquisition, and quantification.

2.4. Method performance

The analytical method was validated in terms of linearity, accuracy, precision, sensitivity, and matrix effects, in both surface water and groundwater. The validation in groundwater was carried out using a pooled sample of groundwater from various aquifers located in Catalonia (NE Spain). In the case of surface water, a pooled sample of water from three Catalonian rivers, namely, Segre, Llobregat, and Tordera, was used.

Eleven calibration solutions within the concentration range 0.5–2000 ng/L, constructed after appropriate dilution of the working standard solutions in LC-grade water, were used to evaluate the method linearity. Quantification was done using an isotope dilution approach, *i.e.*, considering the ratio between the peak area of each analyte and that of its corresponding SIL analog, except in the case of six compounds, for which SIL analogs were not available. These compounds were quantified using SIL standards presenting similar structure, retention time, and/or recoveries. Method linearity was expressed with the coefficient of determination (r^2) of the weighted linear regression model obtained for each analyte. $1/x^2$ was used as a weighting factor to reduce the influence of the high concentration data points in the model.

The accuracy and precision of the method in LC-grade water, surface, and groundwater were appraised with the analyte recovery and its repeatability after $n = 5$ replicated analyses of each matrix fortified at three different concentration levels (10 ng/L, 100 ng/L, and 1000 ng/L). Background concentration levels of each target pesticide in each matrix were taken into account in the calculations.

The method sensitivity was evaluated through the calculation of limits of detection (LODs), limits of quantification (LOQs), and limits of determination (LODets). LODs and LOQs were experimentally estimated from the analysis of the water matrices fortified at the lowest level (10 ng/L) as the analyte concentration giving a signal to noise ratio of 3 in the case of the LODs and 10 in the case of the LOQs. The LODet coincides with the minimum concentration of a compound that can be quantified (LOQ of SRM1) and confirmed (LOD of SRM2).

To evaluate the matrix effects produced by co-extracted matrix components, analyte peak areas obtained after on-line SPE-LC-MS/MS analysis of surface water and groundwater fortified at 100 ng/L were compared with those obtained after on-line SPE-LC-MS/MS analysis of LC-grade water fortified at equal concentration. Negative matrix effect values occur when the analyte signal in LC-grade water is higher than in fortified surface or groundwater, and indicate ionization suppression effects. On the contrary, positive matrix effect values occur when the analyte signal is higher in surface and groundwater than in LC-grade water, and indicate signal enhancement effects.

2.5. Sampling locations and water collection

The presence of the target pesticides was investigated in the last stretches of the two largest river basins of Catalonia (NE Spain), *i.e.*, the Llobregat and the Ter River basins. Surface water of these two basins is used to supply drinking water to about 4.5 million people in Barcelona and its metropolitan area (Postigo et al., 2018). The Llobregat River is located in an area with an important concentration of industries (*e.g.*, tannery, food products, textile, pulp, and paper industries), and high population density, and thus, with an important demand of water. This river is highly impacted by domestic and industrial wastewater discharges (> 30 wastewater treatment plants) and surface runoff from agricultural areas (González et al., 2012). On the contrary, the Ter River basin is characterized by a low population density and intense agricultural activities (*e.g.*, crops of rice, corn, alfalfa, and apple trees, among others). It also receives the impact of some metallurgic, pulp mill, textile, and tannery industries (Céspedes et al., 2006).

The sampling campaigns were conducted in February 2017 in the Llobregat River and in June 2018 in the Ter River. A total of 11 surface water samples were collected from the Llobregat River, and the same number (6 surface water and 5 groundwater samples) from the Ter River (Fig. 1). Grab sampling was done in all surface water locations. Groundwater samples were collected after pumping each well for few minutes (10–20 min) to remove stagnant water, at the minimum flow rate possible, and steady conditions of physical-chemical parameters (*i.e.* temperature, pH, and conductivity). All samples were collected in amber polyethylene terephthalate (PET) bottles and transported under cool conditions to the laboratory, where they were stored upon arrival at −20 °C in the dark until analysis.

2.6. Risk assessment

The potential environmental risk associated with the pesticides found in the investigated samples was assessed using the hazard quotient (HQ) approach (EPA, 1997), following the equation: HQ = MEC/PNEC. This approach compares the measured environmental concentration (MEC) for each compound with its predicted no-effect concentration (PNEC), *i.e.*, the concentration at which no toxic effects are expected to occur. To assess the worst-case scenario, the maximum pesticide concentration measured in the various investigated samples (MEC_{max}) was used as MEC, and the PNEC was the lowest PNEC value

Table 1
On-line SPE-LC-MS/MS conditions for the analysis of the 51 investigated pesticides and SIL analogs.

Analyte	Retention time (min)	SRMs (m/z) precursor ion>product ion	Cone (V)	Collision energy (eV)	SRM ratio (SRM1/SRM2)
		Negative ESI mode			
Bromoxynil	7.5	276 > 81 / 276 > 79	40	20 / 30	17
Bromoxynil $^{13}C_6$		*282 > 81*	*40*	*20*	
Bentazone	7.5	239 > 132 / 239 > 197	30	25 / 20	2.8
Bentazone d_6		*245 > 132*	*35*	*25*	
Fluroxypyr[a]	7.8	253 > 195 / 255 > 197	15	10 / 10	1.8
2,4-D	7.9	219 > 161 / 219 > 125	20	15 / 25	15
2,4-D d_3		*224 > 127*	*20*	*25*	
MCPA	7.9	199 > 141 / 201 > 143	25	10 / 10	2.7
MCPA d_3		*204 > 146*	*25*	*20*	
Mecoprop	7.9	213 > 141 / 213 > 71	25	10 / 10	10
Mecoprop d_3		*218 > 146*	*25*	*15*	
Propanil	14.9	216 > 160 / 218 > 162	25	20 / 20	1.4
Propanil d_5		*221 > 161*	*30*	*15*	
Fenitrothion	19.8	262 > 152 / 262 > 122	25	20 / 30	9.6
Fenitrothion d_6		*265 > 152*	*30*	*15*	
		Positive ESI mode			
Thifensulfuron methyl	7.3	388 > 167 / 388 > 141	25	15 / 20	6.4
Thifensulfuron methyl d_3		*391 > 167*	*20*	*15*	
Desethylatrazine (DEA)	7.4	188 > 146 / 188 > 79	30	15 / 25	12
Desethylatrazine d_6		*194 > 147*	*25*	*20*	
Fenthion oxon sulfoxide[b]	8.2	279 > 264 / 279 > 104	35	20 / 25	1.3
Deisopropilatrazine (DIA)	8.5	174 > 132 / 174 > 104	30	20 / 25	1.1
Deisopropilatrazine d_5		*179 > 101*	*40*	*20*	
Thiamethoxam	8.9	292 > 211 / 292 > 181	25	15 / 20	3.9
Thiamethoxam d_3		*295 > 214*	*30*	*15*	
Clothianidin	8.9	250 > 169 / 250 > 132	20	15 / 15	2.1
Clothianidin d_3		*254 > 172*	*20*	*10*	
Imidacloprid	9.0	256 > 175 / 256 > 209	25	20 / 15	1.3
Imidacloprid d_5		*261 > 214*	*25*	*25*	
Acetamiprid	9.2	223 > 126 / 223 > 56	15	15 / 20	1.9
Acetamiprid d_3		*227 > 126*	*35*	*20*	
Dimethoate	9.6	230 > 199 / 230 > 125	25	15 / 15	3.5
Dimethoate d_6		*236 > 131*	*30*	*15*	
Azinphos methyl oxon[c]	9.7	324 > 132 / 324 > 148	40	20 / 15	19
Thiacloprid	10.1	253 > 126 / 253 > 90	25	25 / 40	4.3
Thiacloprid d_4		*257 > 126*	*35*	*15*	
Dichlorvos	10.8	221 > 109 / 223 > 109	30	25 / 25	1.1
Dichlorvos d_6		*227 > 115*	*30*	*25*	
Simazine	10.8	202 > 124 / 202 > 71	30	25 / 20	3.0
Simazine d_{10}		*212 > 137*	*35*	*15*	
Cyanazine	10.9	241 > 214 / 241 > 174	30	15 / 20	13
Cyanazine d_5		*246 > 219*	*30*	*20*	
Malaoxon (MOX)[d]	11.0	315 > 99 / 315 > 127	20	25 / 15	1.4
Fenthion oxon sulfone	11.1	295 > 109 / 295 > 217	40	40 / 30	28
Fenthion oxon sulfone d_3		*298 > 104*	*35*	*25*	
Fenthion sulfoxide	11.2	295 > 109 / 295 > 125	40	30 / 35	4.3
Fenthion sulfoxide d_6		*301 > 108*	*35*	*30*	
Fenitrothion oxon	11.5	262 > 104 / 262 > 216	30	20 / 20	4.6
Fenitrothion oxon d_6		*268 > 106*	*30*	*25*	
Chlortoluron	11.9	213 > 72 / 213 > 140	25	15 / 30	47
Chlortoluron d_6		*219 > 78*	*35*	*15*	
Isoproturon	12.3	207 > 165 / 207 > 72	35	15 / 20	9.3
Isoproturon d_6		*213 > 171*	*30*	*20*	
Atrazine	12.4	216 > 174 / 216 > 132	35	15 / 20	6.5
Atrazine d_5		*221 > 179*	*35*	*15*	
Diuron	12.8	233 > 72 / 235 > 72	25	15 / 15	1.5
Diuron d_6		*239 > 78*	*25*	*25*	
Fenthion oxon	13.0	263 > 231 / 263 > 216	35	20 / 25	1.2
Fenthion oxon d_3		*266 > 234*	*30*	*15*	
Fenthion sulfone	14.2	311 > 125 / 311 > 109	35	30 / 40	2.7
Fenthion sulfone d_6		*317 > 115*	*45*	*30*	
Terbuthylazine	15.2	230 > 174 / 230 > 96	25	15 / 25	4.3
Terbuthylazine d_5		*235 > 179*	*30*	*20*	
Methiocarb	15.4	226 > 169 / 226 > 121	20	10 / 20	2.2
Methiocarb d_3		*229 > 169*	*25*	*10*	
Linuron	16.2	249 > 160 / 249 > 182	25	15 / 15	1.3
Linuron d_6		*255 > 185*	*30*	*15*	
Azinphos methyl	16.5	318 > 132 / 318 > 105	15	20 / 30	2.1
Azinphos methyl d_6		*324 > 132*	*35*	*15*	
Molinate[e]	17.4	188 > 126 / 188 > 83	30	15 / 20	1.5
Terbutryn	17.6	242 > 71 / 242 > 91	30	30 / 30	1.4

Table 1 (*continued*)

Analyte	Retention time (min)	SRMs (m/z) precursor ion>product ion	Cone (V)	Collision energy (eV)	SRM ratio (SRM1/SRM2)
Terbutryn d_5		*247 > 191*	*35*	*20*	
Irgarol	17.7	254 > 108 / 254 > 125	35	30 / 25	2.9
Irgarol d_9		*283 > 199*	*40*	*20*	
Metolachlor	18.3	284 > 176 / 284 > 73	25	25 / 25	6.5
Metolachlor d_{11}		*295 > 263*	*25*	*15*	
Alachlor	18.5	270 > 238 / 270 > 162	30	15 / 15	1.2
Alachlor d_{13}		*283 > 251*	*15*	*10*	
Malathion	18.9	353 > 195 / 353 > 227	30	15 / 15	3.5
Malathion d_{10}		*363 > 205*	*35*	*15*	
Chlorfenvinphos (CFP)	19.2	359 > 155 / 359 > 170	25	15 / 40	1.4
Chlorfenvinphos d_{10}		*369 > 101*	*25*	*30*	
Azinphos ethyl	19.8	346 > 132 / 346 > 104	15	20 / 35	2.9
Azinphos ethyl d_{10}		*356 > 132*	*15*	*20*	
Diazinon	21.9	305 > 153 / 305 > 97	35	20 / 30	1.7
Diazinon d_{10}		*315 > 170*	*35*	*20*	
Diflufenican	25.3	395 > 266 / 395 > 246	45	30 / 25	7.0
Diflufenican d_5		*398 > 268*	*35*	*25*	
Oxadiazon	28.3	345 > 220 / 345 > 177	35	30 / 20	1.1
Oxadiazon d_7		*352 > 221*	*35*	*20*	
Quinoxyfen[f]	28.6	309 > 245 / 309 > 150	50	30 / 30	1.1
Pendimethalin	29.2	282 > 212 / 282 > 194	15	10 / 15	27
Pendimethalin d_5		*287 > 213*	*20*	*10*	
Chlorpyrifos (CPF)	29.5	352 > 97 / 352 > 200	20	30 / 20	59
Chlorpyrifos d_{10}		*362 > 131*	*25*	*20*	
Triallate	29.6	304 > 86 / 304 > 143	25	15 / 30	3.8
Triallate $^{13}C_6$		*310 > 89*	*30*	*15*	

Italics highlight the conditions used for the analysis of stable isotope labeled compounds.
[a] Compound quantified using mecoprop d_3 as surrogate standard.
[b] Compound quantified using thiamethoxam d_3 as surrogate standard.
[c] Compound quantified using fenthion sulfoxide d_6 as surrogate standard.
[d] Compound quantified using chlortoluron d_6 as surrogate standard.
[e] Compound quantified using linuron d_6 as surrogate standard.
[f] Compound quantified using chlorpyrifos d_{10} as surrogate standard.

provided in the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/) (Dulio and von der Ohe, 2013). Furthermore, the overall effect of the pesticide mixtures present in the samples was evaluated using an additive model, *i.e.*, adding the individual HQs of the pesticides measured in each sample. It was considered that with an HQ < 0.1 no adverse effects are expected for the aquatic organisms, with 0.1 < HQ < 1 the risk is low but potential adverse effects cannot be fully dismissed, with HQ > 1 some adverse effects or moderate risk is probable, and with HQ > 10 a high risk is anticipated.

Additional risk assessment was conducted by comparing measured concentrations with quality standards in surface and groundwater.

3. Results and discussion

3.1. Method optimization

The analytical method developed is based on a methodology previously described for the analysis of 22 pesticides in environmental waters (Köck-Schulmeyer et al., 2014; Köck-Schulmeyer et al., 2013; Postigo et al., 2010). One of the analytical improvements incorporated in this methodology is the expansion of the list of the targeted pesticides with 29 additional medium to highly polar pesticides, including 8 TPs, *i.e.*, acetamiprid, azinphos ethyl, azinphos-methyl, azinphos-methyl oxon, bromoxynil, chlorfenvinphos, chlorpyrifos, clothianidin, dichlorvos, diflufenican, fenitrothion oxon, fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, fenthion sulfone, fenthion sulfoxide, fluroxypyr, imidacloprid, irgarol, malaoxon, methiocarb, oxadiazon, pendimethalin, quinoxyfen, terbutryn, thiacloprid, thiamethoxam, thifensulfuron methyl, and triallate, and SIL analogs for 24 of them. These pesticides and TPs were selected considering their feasibility for LC-MS analysis, their current use in Spain, and their inclusion in the EU legislation (as priority, watch list, or banned substances) (EC, 2013; EC, 2018) (Table S1 in SI). The current method, unlike the previous one, removes suspended particles by centrifugation instead of filtration. Moreover, this step is conducted after surrogate standard (SIL) addition to account for pesticides present in the whole matrix and reduce the loss of analytes due to adsorption during the centrifugation process. Compared to the previous method, the analysis time is reduced to half due to the determination of all targeted pesticides and TPs in a single analytical run. This was possible thanks to the use of a generic sorbent for the simultaneous preconcentration of all analytes, and the switch of polarity ionization during MS acquisition.

The optimization of the MS/MS conditions for the detection of the new analytes included in the methodology was performed by on-column injection of individual standard solutions of each compound. Full scan acquisition was used to select the molecular ion and the best ionization mode and optimum declustering potential for its detection, and product ion scan acquisition allowed obtaining the optimum collision energies to register the two most abundant and selective fragment ions (SRM transitions) in each case. MS/MS conditions for each pesticide and SIL analog are provided in Table 1. Up to six time-acquisition windows were established to maximize the acquisition time for each SRM transition and hence improve method sensitivity (Fig. S1 in SI). An example of the extracted ion chromatograms of the target compounds obtained after on-line SPE-LC-MS/MS analysis of a surface water sample fortified with the targeted pesticides at a concentration of 100 ng/L (500 ng/L for those compounds with LOD above 100 ng/L) is provided in Fig. S2 in SI.

3.2. Method validation

Table 2, Fig. 2, and Tables S2 and S3 in SI summarize the method performance in groundwater, surface water, and LC-grade water, in terms of linearity, recovery, repeatability, sensitivity, and matrix effects at the three concentration levels investigated (10 ng/L, 100 ng/L, and 1000 ng/L).

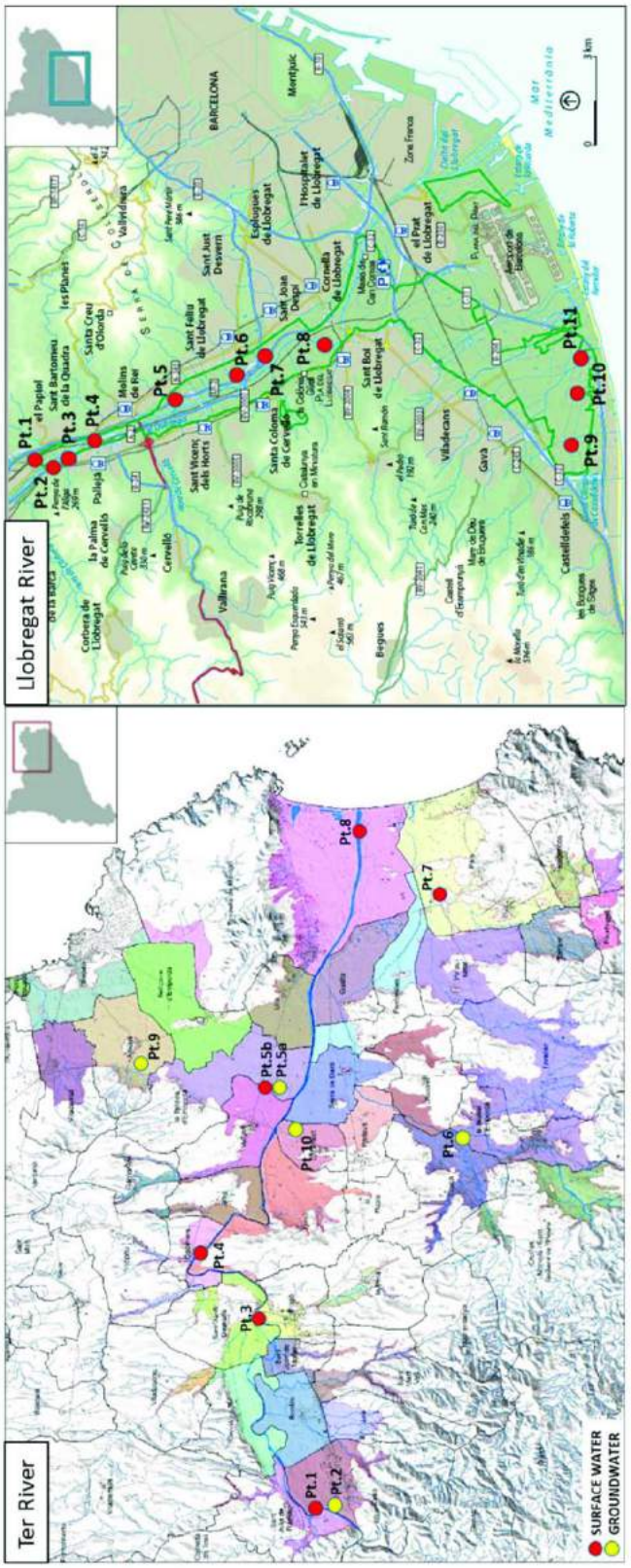

Fig. 1. Sampling locations in each of the river basins investigated. Maps courtesy of J. Montaner from IRTA (Ter) and V. Sola from CUADLL (Llobregat).

Table 2
Method performance in terms of linearity, recovery, repeatability (RSD, relative standard deviation), and sensitivity (limits of detection (LOD) and limits of determination (LODet)) for the target pesticides in surface water and groundwater.

Analyte	Linearity (r^2)	Groundwater			Surface water		
		Accuracy and precision (100 ng/L)	Sensitivity		Accuracy and precision (100 ng/L)	Sensitivity	
		Analyte recovery ± RSD(%)	LOD ng/L	LODet ng/L	Analyte recovery ± RSD(%)	LOD ng/L	LODet ng/L
2,4-D	0.9947	82 ± 10	4.0	13	104 ± 16	2.4	14
Acetamiprid	0.9900	115 ± 13	1.4	5.4	84 ± 7	1.0	4.0
Alachlor	0.9988	81 ± 12	2.1	7.1	116 ± 12	6.4	16
Atrazine	0.9964	93 ± 16	1.4	4.8	93 ± 3	1.9	6.7
Azinphos ethyl	0.9939	96 ± 10	2.6	8.7	118 ± 5	3.8	9.3
Azinphos methyl	0.9900	119 ± 15	1.7	5.6	81 ± 7	4.3	12
Azinphos methyl oxon	0.9978	81 ± 6	5.3	18	88 ± 15	15	27
Bentazone	0.9918	83 ± 9	2.5	9.3	101 ± 5	1.9	8.8
Bromoxynil	0.9901	84 ± 6	0.41	1.4	82 ± 11	8.5	22
Chlorfenvinphos	0.9988	86 ± 13	0.50	1.7	112 ± 4	0.59	2.9
Chlorpyrifos	0.9913	113 ± 13	1.7	5.6	97 ± 6	0.63	2.9
Chlortoluron	0.9970	85 ± 21	0.85	5.6	113 ± 4	2.5	10
Clothianidin	0.9912	83 ± 5	14	25	82 ± 13	18	30
Cyanazine	0.9990	119 ± 6	0.70	5.5	91 ± 10	1.6	8.8
DEA	0.9990	121 ± 8	4.0	13	100 ± 16	2.3	7.8
DIA	0.9983	114 ± 8	14	50	83 ± 10	7.3	22
Diazinon	0.9995	88 ± 10	0.17	0.58	108 ± 8	0.43	1.8
Dichlorvos	0.9916	100 ± 5	28	40	81 ± 3	6.8	20
Diflufenican	0.9951	80 ± 17	14	49	BLOD	120	260
Dimethoate	0.9926	99 ± 5	14	46	BLOD	180	330
Diuron	0.9949	117 ± 3	0.11	0.39	87 ± 17	0.57	2.5
Fenitrothion	0.9810	121 ± 6	13	44	BLOD	170	300
Fenitrothion oxon	0.9977	89 ± 11	1.6	5.3	85 ± 5	6.3	18
Fenthion oxon	0.9977	100 ± 4	0.38	1.7	99 ± 8	3.2	4.3
Fenthion oxon sulfone	0.9907	111 ± 10	6.1	20	93 ± 11	19	40
Fenthion oxon sulfoxide	0.9963	95 ± 9	2.2	7.4	121 ± 4	1.9	6.4
Fenthion sulfone	0.9830	88 ± 16	5.6	21	98 ± 15	17	39
Fenthion sulfoxide	0.9978	85 ± 13	1.4	4.7	127 ± 5	3.9	9.6
Fluroxypyr	0.9918	122 ± 6	13	42	89 ± 14	18	59
Imidacloprid	0.9949	117 ± 4	3.9	13	109 ± 5	4.0	10
Irgarol	0.9919	114 ± 6	0.86	2.9	97 ± 6	1.1	6.6
Isoproturon	0.9980	101 ± 5	1.1	1.9	80 ± 6	1.5	7.1
Linuron	0.9946	119 ± 19	3.7	15	89 ± 8	3.2	12
Malaoxon	0.9917	125 ± 17	0.88	2.5	109 ± 9	1.8	4.1
Malathion	0.9941	102 ± 10	13	44	101 ± 10	4.1	17
MCPA	0.9968	90 ± 12	2.3	7.6	91 ± 14	2.5	6.2
Mecoprop	0.9914	85 ± 17	3.3	11	91 ± 11	1.7	5.7
Methiocarb	0.9903	88 ± 11	0.28	0.95	106 ± 8	1.7	11
Metolachlor	0.9979	113 ± 5	0.84	2.8	119 ± 7	0.49	1.2
Molinate	0.9945	125 ± 3	16	63	BLOD	120	280
Oxadiazon	0.9914	122 ± 10	13	29	BLOD	130	440
Pendimethalin	0.9905	80 ± 3	11	37	BLOD	190	300
Propanil	0.9945	125 ± 17	2.1	7.2	100 ± 11	6.7	20
Quinoxyfen	0.9973	87 ± 18	0.66	2.6	109 ± 5	5.0	16
Simazine	0.9998	86 ± 4	2.4	6.7	102 ± 6	5.1	18
Terbuthylazine	0.9960	92 ± 6	0.20	0.76	83 ± 22	0.58	1.4
Terbutryn	0.9980	106 ± 8	0.16	0.54	122 ±	0.39	1.5
Thiacloprid	0.9994	112 ± 14	0.52	1.8	115 ± 4	0.30	0.79
Thiamethoxam	0.9991	87 ± 9	25	33	102 ± 8	1.8	5.9
Thifensulfuron methyl	0.9888	124 ± 19	1.3	4.4	88 ± 9	1.0	3.1
Triallate	0.9934	120 ± 20	2.6	8.6	121 ± 6	1.1	5.1

BLOD: Below limit of detection.

The linearity of the method expanded between 0.5 ng/L and 2000 ng/L for most of the compounds (1000 ng/L was the upper linearity range in the case of clothianidin, dichlorvos, diuron, fenitrothion oxon, fenthion sulfone, fenthion oxon sulfone, malathion, mecoprop, molinate, simazine, and thifensulfuron methyl). The weighted linear regression models presented a coefficient of determination (r^2) higher than 0.99 for all compounds except for fenitrothion (0.981) and fenthion sulfone (0.983) (Table 2).

Analyte recoveries observed in each of the investigated matrices were in general in good agreement at the three concentration levels (Table 2 and Tables S2 and S3). Analyte losses during extraction and variations in analyte ionization due to matrix effects were well compensated with the use of SIL standards, as indicated by the recoveries obtained, always between 80% and 120%, except in a few cases that slightly deviated from this range. Likewise, relative standard deviations (RSD) nearly always below 20%, or very close, indicated good repeatability, as corresponds to automated methodologies with minimal sample manipulation.

The average LODs and LODets obtained in surface water were between 0.3 and 19 ng/L and between 0.8 and 40 ng/L, respectively, for most of the compounds (86%), while in groundwater these limits ranged between 0.1 and 28 ng/L and from 0.4 to 63 ng/L for all compounds, respectively.

The extent of matrix effects in both surface and groundwater is shown in Fig. 2. Significant matrix effects (±20% variation of the signal) were observed for 90% of the compounds in both matrices. MS signal

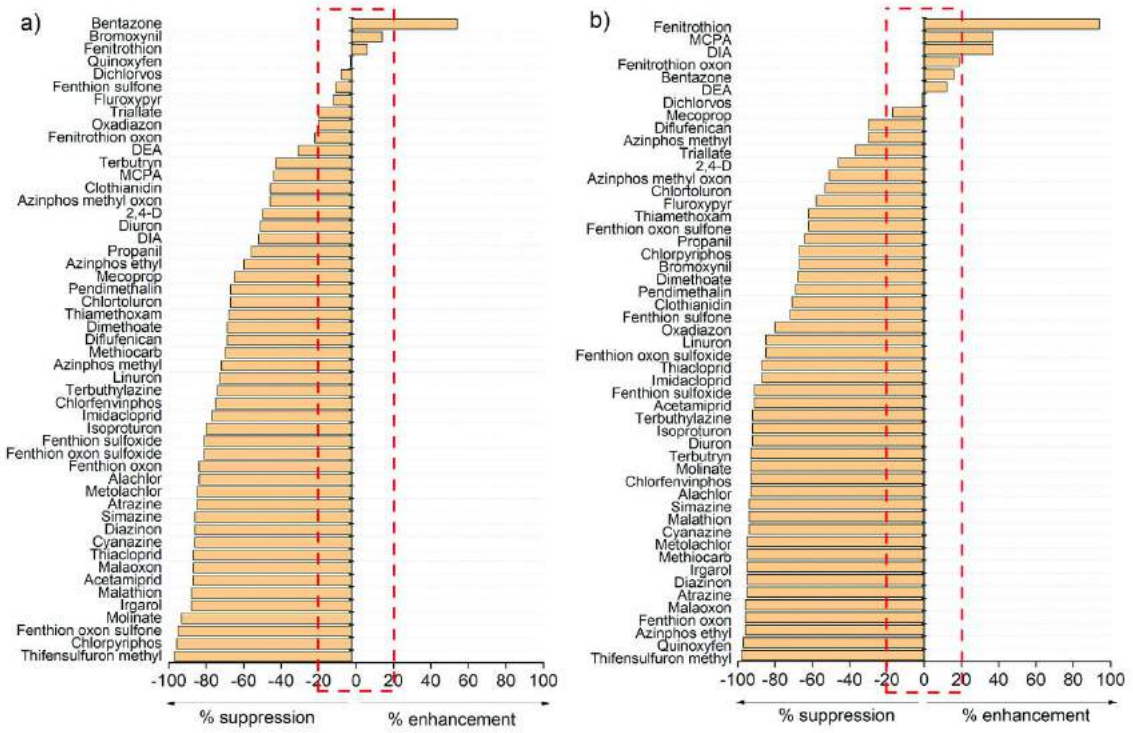

Fig. 2. Matrix effects observed at a concentration level of 100 ng/L in groundwater (a) and surface water (b).

enhancement was observed only for bentazone (+54%) in groundwater and for fenitrothion (+94%), DIA (+37%), and MCPA (+37%) in surface water. In all other cases, matrix effects occurred in the form of signal ionization suppression, with values up to −100%.

In the last ten years, of all the methodologies published in the peer-reviewed literature for the determination of non-polar and polar pesticides in water samples, only a few of them are fully automated (Camilleri et al., 2015; Hurtado-Sánchez et al., 2013; Mann et al., 2016; Quintana et al., 2019; Rubirola et al., 2017; Singer et al., 2010) (Table 3). Among them, the present method and the recent one described by Quintana et al. (2019) are the only capable of determining more than 50 pesticides in water samples, covering a wide spectrum of medium to highly polar compounds. A special feature of the methodology here presented as compared to the others is the large proportion of SIL analogs used for quantification (88%). The use of SIL compounds for almost all targeted analytes indeed requires an initial investment of economic resources for their acquisition; however, it is essential to correct for matrix effects and ensure the production of reliable results in any water matrix.

Overall, on-line methods allow lowering the LODs to a higher extent than off-line analytical approaches because the complete sample (5 mL in our case) is transferred into the LC-MS system. In comparison with other automated analytical methods available in the literature, the LODets obtained with the methodology developed, between 0.4 and 63 ng/L in groundwater and between 0.8 and 40 ng/L in surface water (for 86% of the compounds), are overall comparable or lower than those previously reported by other authors, for instance: LOQs from 0.3 to 33 ng/L reported by Hurtado-Sánchez et al. for 10 pesticides in surface water (Hurtado-Sánchez et al., 2013), from 3 to 100 ng/L reported by Singer et al. for 20 pesticides also in surface water (Singer et al., 2010), and LOQ values above 8 ng/L in groundwater and 10 ng/L in surface water as reported by Mann et al. (Mann et al., 2016). In this respect, it may be worth mentioning that the LODets in our method incorporate the confirmation by the SRM2, and thus could be higher than the LOQ of the SRM1 if the LOD of the SRM2 is above that value. Despite this, our automated approach provides the best sensitivity for the analysis of azinphos-methyl, bromoxynil, clothianidin, quinoxyfen, terbuthylazine, terbutryn, and thifensulfuron methyl, and to the best of our knowledge, this is the first time that an on-line SPE-LC-MS/MS-based method is validated for the analysis of azinphos ethyl, azinpho- methyl oxon, dichlorvos, diflufenican, fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, fenthion sulfone, fenthion sulfoxide, and oxadiazon in surface and groundwater samples. Moreover, the sensitivity of the presented methodology allows its application to monitor the target priority substances in surface waters below their respective lowest EQS (EC, 2013): alachlor, LOD = 6 ng/L *vs.* EQS = 300 ng/L; atrazine, 2 ng/L *vs.* 600 ng/L; chlorfenvinphos, 0.6 ng/L *vs.* 100 ng/L; chlorpyrifos, 0.6 ng/L *vs.* 30 ng/L; diuron, 0.6 ng/L *vs.* 200 ng/L; irgarol, 1 ng/L *vs.* 2.5 ng/L; isoproturon, 2 ng/L *vs.* 300 ng/L; quinoxyfen, 5 ng/L *vs.* 15 ng/L; simazine, 5 ng/L *vs.* 1000 ng/L; and terbutryn, 0.4 ng/L *vs.* 6.5 ng/L. Dichlorvos (LOD 6.8 ng/L) is the only target priority substance that cannot be detected with the proposed methodology below its lowest EQS (0.06 ng/L). The developed methodology also provides LODs in compliance with the maximum acceptable LODs established in the European watch list (EC, 2018) for the detection of methiocarb (1.7 ng/L *vs.* 2 ng/L) and four of the five neonicotinoids included in our study (imidacloprid 4 ng/L, thiacloprid 0.3 ng/L, acetamiprid 1 ng/L, thiamethoxam 1.8 ng/L, and as an exception clothianidin 18 ng/L, in all cases *vs* 8.3 ng/L). Regarding groundwater, all the studied pesticides can be detected at levels below 0.1 μg/L, which is the quality standard for individual pesticides established in the European Directive for the protection of groundwater against pollution and deterioration (EC, 2006a).

Table 3
On-line SPE LC-MS/MS methodologies published in the peer-reviewed literature in the last ten years for the simultaneous determination of medium to highly polar pesticides in groundwater and surface water.

Number of pesticides	Analyte overlap	Water matrix	Pre-extraction step	Quantification method	Accuracy (analyte recovery, %)	Precision (RSD, %)	Sensitivity (LOQ, ng/L)	Matrix effects	Reference
51		GW/SW	Centrifugation	Isotope dilution (88% of SIL)	80–127	<20	GW: 0.4–63 SW: 0.8–40 (86% of analytes)	± 100	This study
96	30	GW/SW/DW	Centrifugation	Isotope dilution (22% of SIL)	−40 to 42	<40	5–25	−161 to 100	(Quintana et al., 2019)
14	13	SW/DW/EWW	Filtration	Isotope dilution (86% of SIL)	<10 relative bias[a]	<10	SW: 0.3–2.1	± 100	(Rubirola et al., 2017)
23	8	GW/SW/DW	Filtration	Isotope dilution (22% of SIL)	72–198	<40	GW: 8–62 SW: 10–64	not provided	(Mann et al., 2016)
10	4	SW	Acidification (2.5‰ formic acid)	Not provided	86–114	<30	0.1–10	± 20	(Camilleri et al., 2015)
37	10	SW	Filtration	Standard addition	74–129	<14	0.3–33	not provided	(Hurtado-Sánchez et al., 2013)
20	9	SW/WW	Filtration	Isotope dilution (60% of SIL)	71–103	<20	SW: 3–100	± 100	(Singer et al., 2010)

GW, groundwater; SW, surface water; SIL, stable isotope-labeled analogs; DW, drinking water; EWW, effluent wastewater; WW, wastewater.
[a] Relative bias (%) = ((theoretical concentration − experimental concentration)/theoretical concentration) × 100.

It is also worthy to highlight that, contrary to our method that uses centrifugation for sample pre-treatment, almost all the automated methods evaluated filtrate the water to remove suspended particles before analysis. Centrifugation is as effective as filtration to remove suspended solids; however, it avoids the potential retention of the less polar compounds onto the filters and reduces time and analysis costs.

Besides increased sensitivity and repeatability, this methodology presents as additional advantages over other analytical methods available in the literature for the analysis of polar pesticides: i) full automation (which results in minimum sample preparation and manipulation requirements, *i.e.*, only 10 min centrifugation and SIL standards addition, ii) the use of a low sample volume (5 mL, which simplifies sample transport and storage), iii) high sample throughput (SPE of a sample is conducted during LC-MS/MS analysis of the previous sample in a batch, and the whole process takes only 40 min), iv) cost efficiency (due to low solvents consumption and avoidance of evaporation steps), and v) overall time saving (due to low maintenance and easy operation of the instrument, and automated data processing (MassLynx)).

3.3. Occurrence in water samples

The developed methodology was applied to the analysis of 22 water samples collected in two agriculture-impacted areas of Catalonia with different predominant crops and pressures. The results obtained (lowest and highest concentrations, average concentrations, and detection frequencies) are summarized in Table 4, while individual concentrations of the pesticides found in the investigated samples are provided as SI in Tables S4 and S5.

Of the 51 investigated compounds, 28 were detected in the Llobregat basin. The pesticide pattern observed in the studied area (Figs. 1 and 3), which includes small tributaries (Pt 1 and Pt 2) and their confluence into the main river (Pt 3), as well as irrigation and drainage channels that give service to surrounding farms (Pt 4–11) and in some points, receive the input of WWTP effluents (Pt 6 and Pt 9), was characterized by the generalized presence of diuron and terbutryn throughout the investigated stretch, with punctually high concentrations of other compounds in certain sites, such as bromoxynil in Pt 4 and linuron in Pt 7 and 10. A variety of compounds were present in some locations, *viz.*, Pt 7 and Pt 10, in line with the variety of small exploitations dedicated to the cultivation of different crops (Fig. 3). In this profile, it was also notable the presence of 2,4-D in the two sites most directly affected by the input of WWTP effluents (Pt 6 and Pt 9), which reflects a likely poor removal of this compound in the WWTPs.

As shown in Table 4, the herbicides diuron and terbutryn were the most ubiquitous pesticides, occurring in 100% and 91% of the samples analyzed, respectively. Diuron is an effective herbicide used to treat invasive vegetation on both agricultural and non-agricultural sites. It is also useful in removing mildew and killing algae. Thus, such a widespread occurrence may be associated with its use in agriculture but also in industrial and urban environments. The ubiquitous presence of diuron in this river has been already reported in previous studies (Köck-Schulmeyer et al., 2012; Masiá et al., 2015). On the other hand, terbutryn presence may be attributed to its release from the river sediments (Barbieri et al., 2019; Masiá et al., 2015) or nearby soils, where it may be accumulated, because the use of this herbicide/algaecide as a plant protection product has been banned for nearly a decade in the EU (EC, 2002). The sorption of terbutryn onto solid particles during its use in the past is supported by its low water solubility (25 mg/L) and its moderately high octanol-water partition coefficient (log Kow = 3.7) (Table S1 in SI).

Diuron was also one of the targeted pesticides that presented the highest concentrations (up to 500 ng/L), only surpassed by bromoxynil (1500 ng/L) and linuron (520 ng/L). Bromoxynil and linuron are both herbicides used to control annual broadleaf weeds on crop and non-crop sites.

In addition to diuron, bromoxynil, and linuron, various other pesticides, namely, 2,4-D, azinphos-ethyl, dichlorvos, diflufenican, imidacloprid, methiocarb, and terbutryn, were found at concentrations above the limit of 100 ng/L set for individual pesticides in water intended for human consumption (EC, 1998). This is a concern since the Llobregat River water is an important source of drinking water for the city of Barcelona and its metropolitan area. Total pesticide concentrations in the Llobregat River waters ranged between 205 and 1813 ng/L, being above the limit set for total pesticides of 500 ng/L in four out of the eleven investigated locations (Pt 1, Pt 4, Pt 7, and Pt 10) (Figs. 1 and 3). In Pt 1 and Pt 4, the exceedance is basically due to the large presence of one specific pesticide (diuron and bromoxynil, respectively), whereas in the other sites (Pt 7 and Pt 10) the exceedance is due to the concurrent presence of a variety of pesticides at low concentrations. Overall, the main groups contributing to total pesticide levels were triazines, organophosphophates, ureas, and neonicotinoids (Fig. 3).

Many of the pesticides detected are priority substances in the field of water policy (EC, 2013) or are included in the EU watch list (EC, 2018). EQS exceedances (EQS provided in Table S1 as SI) were only observed in the case of the antifouling agent irgarol in two locations (33 and 41 ng/L *vs* its EQS of 16 ng/L) and the insecticide dichlorvos in five locations (from 20 ng/L to 130 ng/L, far above its EQS of 0.7 ng/L). The use of irgarol and dichlorvos is currently prohibited in the EU (EC, 2006b; EC, 2009; EC, 2016). Both substances were also found in the Llobregat

Table 4
Minimum, maximum, and mean concentrations and detection frequency of the targeted pesticides in the investigated water samples.

Pesticides	Concentration (ng/L)			Detection frequency[b] (%)
	Min	Max	Mean[a]	
Llobregat River				
2,4-D	130	200	30	18
Alachlor	18	24	3.8	18
Atrazine	<6.7	21	5.6	55
Azinphos ethyl	10	110	17	27
Bromoxynil	<22	1500	150	27
Chlorfenvinphos	<2.9	67	12	55
Chlortoluron	18	67	13	36
Cyanazine	29	29	2.6	9
Diazinon	2.9	71	11	36
Dichlorvos	<20	130	16	45
Diflufenican	130	150	25	18
Diuron	24	500	170	100
Fenthion oxon	37	37	3.4	9
Fenthion sulfoxide	32	32	2.9	9
Imidacloprid	<10	190	19	27
Irgarol	<6.6	41	7.6	45
Isoproturon	<7.1	25	4.8	27
Linuron	130	520	100	27
Malaoxon	24	24	4.4	18
Malathion	<17	32	6	27
Methiocarb	50	130	17	18
Metolachlor	5.1	28	5.3	27
Molinate	27	33	5.5	18
Propanil	19	19	1.7	9
Simazine	16	20	3.3	18
Terbuthylazine	3.5	30	5.2	27
Terbutryn	8.9	160	67	91
Thiacloprid	<0.79	31	4.3	27
Ter River				
Bentazone	110	110	9.8	9
Diazinon	2.3	4.6	0.63	18
Diuron	14	14	1.3	9
Irgarol	5.4	5.4	0.49	9
MCPA	18	18	1.6	9
Metolachlor	15	24	3.6	18
Terbutryn	4.1	5.3	0.85	18

[a] Mean calculated considering values <LOQ as LOQ/2 and values <LOD as zero.
[b] % of positive samples (including values >LOD and <LOQ).

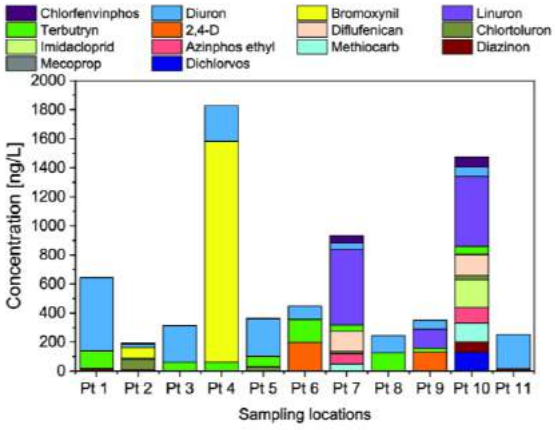

Fig. 3. Cumulative levels of the most abundant (>100 ng/L in at least one sample) and/or frequently detected (>36%) pesticides found in the lower basin of the Llobregat River. Alachlor, atrazine, cyanazine, fenthion sulfoxide, fenthion oxon, irgarol, isoproturon, malaoxon, malathion, metolachlor, molinate, propanyl, simazine, terbuthylazine, and thiacloprid, detected at lower concentrations in fewer samples, are not shown in the figure.

River sediments (Barbieri et al., 2019) which may be the source of these pollutants into the river water. While dichlorvos has not been previously investigated in the Llobregat River waters, irgarol was previously reported to occur in a tributary of the Llobregat River at a maximum concentration of 5 ng/L (Quintana et al., 2019).

Methiocarb and the neonicotinoids imidacloprid and thiacloprid were found at concentrations (up to 130, 190 and 31 ng/L, respectively) much higher than the maximum acceptable method LODs established in the watch list for the monitoring of these substances (2 ng/L for methiocarb and 8.3 ng/L for the neonicotinoids). Given that the acceptable method LODs provided by the implementing decision 2018/840 (EC, 2018) coincide with the substance-specific PNEC in water, the measured concentrations could affect aquatic organisms.

As for TPs, malaoxon was detected in two samples at similar concentration levels than its parent compound, malathion (24 ng/L in the case of malaoxon in both samples *vs* 25 and 32 ng/L of malathion). The presence of malaoxon is of concern, considering that it is 60 times more toxic than its parent compound (Jensen and Whatling, 2010). Moreover, the occurrence of malathion in drinking water sources is also worrisome as it may convert into malaoxon during chlorine-based disinfection of water (Ohno et al., 2008) if it survives to the water treatment train. Two TPs of the currently banned organophosphate insecticide fenthion, namely, fenthion oxon and fenthion sulfoxide, were also detected in one of the sampling locations (Pt.7, Fig. 1) at concentrations of 37 ng/L and 32 ng/L, respectively, while the parent compound was not detected in any sample.

In the Ter River, pesticide pollution was much less severe than in the Llobregat River (Fig. 4). In total, 7 pesticides (bentazone, diazinon, diuron, irgarol, MCPA, metolachlor, and terbutryn) were detected in this area (Table 4). Total concentrations of pesticides in groundwater were very low (up to 34 ng/L in Pt 9), being slightly higher in surface waters (up to 112 ng/L in Pt 7). Thus, pesticide levels did not exceed in any case the limit of 500 ng/L set in the European legislation for the sum of pesticides in groundwater (EC, 2006a) and waters intended for human consumption (EC, 1998). However, bentazone was found in one of the surface water samples (Pt 7) at a level (110 ng/L) higher than the standard of 100 ng/L set for individual pesticides. Bentazone is extensively used as an herbicide in agriculture and especially in rice fields, and likely to reach water bodies due to its high mobility in soils or *via* runoff (high water solubility = 7112 mg/L and low log K_{ow} = 0.46). The location where bentazone was found corresponded indeed with a drainage

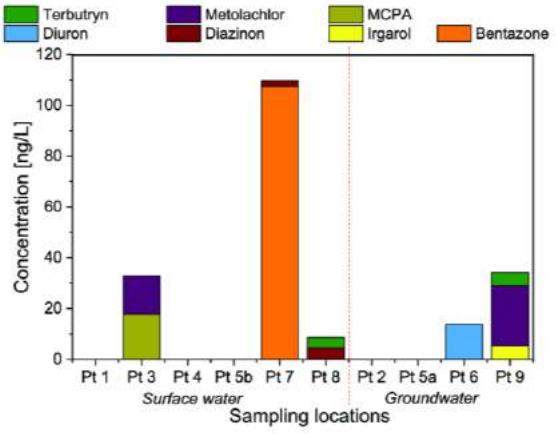

Fig. 4. Cumulative levels of the targeted pesticides measured in the Ter River water samples.

channel of a rice cultivation area, and was the most polluted among the investigated sites.

Regarding the occurrence of priority pesticides in the Ter surface waters, only three were found (terbutryn, irgarol, and diuron), but none of them at concentrations above its corresponding MAC (Tables S1 and S5 in SI).

Four compounds currently banned in Europe were also detected, including terbutryn, irgarol, metolachlor, and diazinon (EC, 2002; EC, 2006b; EC, 2016). Their presence may be due to improper use of the stock of these compounds or rather to their release by leaching or runoff from soils or sediments, where the contaminants could have accumulated over time.

Pesticide contamination in the Ter River has been scarcely investigated before. A study conducted in 2001 in the same area revealed the presence of atrazine, DEA, and metolachlor at levels below 100 ng/L in samples from the Ter River after water treatment for drinking purposes (Quintana et al., 2001). The use of these compounds is currently banned in Europe and this could explain the absence of atrazine and its metabolite desethyl atrazine (DEA) in our study, and the low levels of metolachlor (15 ng/L) found in surface water.

3.4. Environmental risk assessment

Hazard quotients (HQs) calculated for the various individual pesticides detected in the samples based on their maximum concentrations measured are provided in Table 5. In the case of the Llobregat River basin, six compounds, namely irgarol, dichlorvos, methiocarb, azinphos ethyl, imidacloprid, and diflufenican presented HQ values above 10, in Pt 10, and thus, they represent a potentially high risk for the aquatic organisms. This risk is associated with their very low PNEC values (≤ 0.01 μg/L) and relatively high concentrations measured in this water sample, which is also one of the most contaminated sites due to the co-occurrence of many pesticides. A moderate risk ($1 < HQ < 10$) was estimated for eight other pesticides: 2,4-D, bromoxynil, diazinon, diuron, linuron, malathion, terbutryn, thiacloprid. In the case of bromoxynil, diuron, and linuron, the risk is due to the elevated concentrations sporadically found (>500 ng/L).

In the Ter River basin, the only two compounds that exhibited a potential risk, although low, were bentazone ($HQ = 1.08$), the pesticide found at the maximum concentration, and irgarol ($HQ = 1.54$), which presents a very low PNEC (0.0035 μg/L).

Note that the above risk assessment considers the worst-case scenario (the highest pesticide concentration measured and the lowest concentration at which effects are not observed) and evaluates individual compounds. If the mixture of the pesticides present in each sample is considered by adding the corresponding HQs calculated based on the corresponding concentrations measured, through the so-called additive model, the sites Pt 2, Pt 6, Pt 7 and Pt 10 of the Llobregat River would be under high risk for the aquatic ecosystems ($HQ > 10$) (see Fig. S3 in SI). In the case of Pt 7 and Pt 10, the high risk is due to the presence of numerous compounds, with a greater contribution of azynphos ethyl, diflufenican and dichlorvos, while Pt 2 and Pt 6 presented high risk as a consequence of the presence mainly of azynphos ethyl and 2,4-D, respectively. These sites, particularly taking into account that contaminant mixtures may be more toxic than expected based on the sum of the toxicity of the single chemicals present (Hayes et al., 2019), deserve additional monitoring. On the other hand, no high risk was found in the Ter River samples (Fig. S3 in SI). Only moderate risk was calculated for Pt 7 and Pt 9, due to the presence of bentazone and irgarol, respectively.

4. Conclusions

A fast and simple analytical methodology based on on-line SPE-LC-ESI-MS/MS has been developed for the analysis of a wide range of medium to highly polar pesticides in surface water and groundwater.

Table 5
Hazard quotient (HQ) values calculated for the pesticides measured in water samples of the Llobregat and Ter River basins.

Pesticides	MEC[a] (μg/L)	PNEC[b] (μg/L)	HQ
Llobregat River			
2,4-D	0.20	0.02	9.86
Alachlor	0.024	0.3	0.08
Atrazine	0.021	0.6	0.03
Azinphos ethyl	0.11	0.0011	**96.34**
Bromoxynil	1.5	0.5	3.04
CFP	0.067	0.1	0.67
Chlortoluron	0.067	0.1	0.67
Cyanazine	0.029	0.19	0.15
Diazinon	0.071	0.01	7.08
Dichlorvos	0.13	0.0006	**216.30**
Diflufenican	0.15	0.009	**16.12**
Diuron	0.50	0.2	2.50
Fenthion oxon	0.037	0.2	0.18
Fenthion sulfoxide	0.032	–	–
Imidacloprid	0.19	0.0083	**23.40**
Irgarol	0.041	0.0035	**11.64**
Isoproturon	0.025	0.3	0.08
Linuron	0.52	0.1	5.24
Malaoxon	0.024	0.31	0.08
Malathion	0.032	0.006	5.30
Methiocarb	0.13	0.01	**13.06**
Metolachlor	0.028	0.2	0.14
Molinate	0.033	3.8	0.01
Propanil	0.019	0.2	0.09
Simazine	0.020	1	0.02
Terbuthylazine	0.030	0.06	0.51
Terbutryn	0.16	0.065	2.45
Thiacloprid	0.031	0.01	3.10
Ter River			
Bentazone	0.11	0.1	1.08
Diazinon	0.0046	0.01	0.46
Diuron	0.014	0.2	0.07
Irgarol	0.0054	0.0035	1.54
MCPA	0.018	0.5	0.04
Metolachlor	0.024	0.2	0.12
Terbutryn	0.0053	0.065	0.08

[a] MEC: maximum environmental concentration measured.
[b] PNEC: predicted no-effect concentration. Values extracted from [https://www.norman-network.com/nds/ecotox/].

Advanced aspects of the proposed method are its capability to determine in a single run a high number of multi-class pesticides (51) using a low sample volume (5 mL), in a considerably short analysis time (40 min) and with very high reliability of results (due to the use of isotopically labeled analogs of 45 out of the 51 target compounds for quantification by the isotope dilution method). For most of the initially targeted compounds, the method shows satisfactory performance in terms of accuracy and repeatability and provides enough sensitivity for their detection in groundwater and surface water in compliance with the current legislation.

The application of the method to real samples showed a very different contamination profile in the two investigated river basins. The average total pesticide concentration in the Ter River samples was 17.6 times lower than in the Llobregat River samples, and only 7 pesticides were found in the Ter River *versus* 28 detected in the Llobregat River. The list of pesticides found included priority and watch list substances, and even pesticides currently banned in Europe. The contamination pattern observed in the Llobregat River underlines the significant contribution of the urban and industrial activities conducted in the metropolitan area of Barcelona to pesticide pollution.

High risk for aquatic organisms was expected to be derived from the co-occurrence of many pesticides in specific locations, where pesticides at high concentrations or with very low PNEC values were present. These findings reveal that although less persistent than organochlorine

pesticides, medium to highly polar pesticides can be found in water at potentially harmful levels. Further research is needed to understand the sources of these compounds to control them, as well as to assess the real impact of pesticide co-occurrence on the health of the aquatic ecosystems.

CRediT authorship contribution statement

Maria Vittoria Barbieri: Investigation, Data curation, Formal analysis, Writing - original draft, Visualization. **Luis Simón Monllor-Alcaraz:** Investigation. **Cristina Postigo:** Supervision, Visualization, Writing - review & editing. **Miren López de Alda:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140650.

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Supporting Information

IMPROVED FULLY AUTOMATED METHOD FOR THE DETERMINATION OF MEDIUM TO HIGHLY POLAR PESTICIDES IN SURFACE AND GROUNDWATER AND APPLICATION IN TWO DISTINCT AGRICULTURE-IMPACTED AREAS.

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Number of pages: 15

Number of tables: 5

Number of figures: 3

List of Tables

List of Figures

Table S1. Target pesticides, main physical-chemical properties, and current legislative status.

Analyte	Chemical class	Formula‡	MM (g/mol)‡	Solubility (mg/L)‡	K_{oc} (mL/g)‡	K_{ow} logP‡	GUS‡	DT50‡ (days)	Legislative status‡	Currently used in Spain‡	EQSΩ (µg/L)	Method LODsε (ng/L)
2,4-D	Alkylchlorophenoxy	$C_8H_6Cl_2O_3$	221.04	24300	39	-0.82	3.82	7.7	✓	✓		
Acetamipridε	Neonicotinoid	$C_{10}H_{11}ClN_4$	222.67	2950	200	0.80	0.94	4.7	✓	✓		8.3
Alachlor×	Chloroacetamide	$C_{14}H_{20}ClNO_2$	269.77	240	335	3.09	0.8	-	X		0.7	
Atrazine×	Triazine	$C_8H_{14}ClN_5$	215.68	35	100	2.70	2.57	-	X		2	
Azinphos-ethyl	Organophosphate	$C_{12}H_{16}N_3O_3PS_2$	345.38	4.5	1500	3.18	1.4	-	X			
Azinphos-methyl	Organophosphate	$C_{10}H_{12}N_3O_3PS_2$	317.32	28	1112	2.96	1.42	-	X			
Azinphos-methyl-oxon	Metabolite	$C_{10}H_{12}N_3O_4PS$	301.26	2604*	10*	0.77*	-	-	-			
Bentazone	Benzothiazinone	$C_{10}H_{12}N_2O_3S$	240.30	7112	55	-0.46	1.95	80	✓	✓		
Bromoxynil	Hydroxybenzonitrile	$Br_2C_6H_2(OH)CN$	276.90	38000	302	0.27	1.71	13	✓	✓		
Chlorfenvinphos×	Organophosphate	$C_{12}H_{14}Cl_3O_4P$	359.60	145	680	3.80	1.72	7	X		0.3	
Chlorpyrifos×	Organophosphate	$C_9H_{11}Cl_3NO_3PS$	350.58	1.05	5509	4.70	0.58	5	✓	✓	0.1	
Chlortoluron	Phenylurea	$C_{10}H_{13}ClN_2O$	212.68	74	196	2.50	2.62	42	✓	✓		
Cyanazine	Triazine	$C_9H_{13}ClN_6$	240.69	171	190	2.10	2.07	-	X			
Clothianidinε	Neonicotinoid	$C_6H_8ClN_5O_2S$	249.68	340	123	0.90	3.74	40.3	X	✓		8.3
Deisopropylatrazine	Metabolite	$C_5H_8ClN_5$	173.60	980	130	1.15	-	-	-			
Desethylatrazine	Metabolite	$C_6H_{10}ClN_5$	187.63	2700	110	1.51	4.37	-	-			
Diazinon	Organophosphate	$C_{12}H_{21}N_2O_3PS$	304.35	60	609	3.69	1.51	4.3	X			
Dichlorvos×	Organophosphate	$C_4H_7Cl_2O_4P$	220.98	18000	50	1.90	0.69	-	X		7×10^{-4}	
Diflufenican	Carboxamide	$C_{19}H_{11}F_5N_2O_2$	394.29	0.05	5504	4.20	1.19	-	✓	✓		
Dimethoate	Organophosphate	$C_5H_{12}NO_3PS_2$	229.26	25900	25*	0.75	2.18	12.6	X	✓∞		
Diuron×	Phenylurea	$C_9H_{10}Cl_2N_2O$	233.09	35.6	680	2.87	2.65	8.8	✓	✓	1.8	
Fenitrothion	Organophosphate	$C_9H_{12}NO_5PS$	277.23	19	2000	3.32	0.48	1.1	X	✓		
Fenitrothion oxon	Metabolite	$C_9H_{12}NO_6P$	261.17*	301*	21*	1.69*	-	-	-			
Fenthion	Organophosphate	$C_{10}H_{15}O_3PS_2$	278.33	4.2	1500	4.84	1.26	-	X			
Fenthion oxon	Metabolite	$C_{10}H_{15}O_4PS$	262.26*	213.5*	57*	2.31*	-	-	-			
Fenthion oxon sulfone	Metabolite	$C_{10}H_{15}O_6PS$	294.03*	7602*	13*	0.28*	-	-	-			
Fenthion oxon sulfoxide	Metabolite	$C_{10}H_{15}O_5PS$	278.26*	1222*	11*	0.15*	-	-	-			

Fenthion sulfone	Metabolite	$C_{10}H_{15}O_5PS_2$	310.33*	190.4*	235	2.05*	-	-	-				
Fenthion sulfoxide	Metabolite	$C_{10}H_{15}O_4PS_2$	294.33*	3.72*	183	1.92*	-	-	-				
Fluroxypyr	Pyridine	$C_7H_5Cl_2FN_2O_3$	255.03	6500	10*	0.04	3.7	10.5	✓	✓			
Imidacloprid[ε]	Neonicotinoid	$C_9H_{10}ClN_5O_2$	255.66	610	6719	0.57	3.69	30	✓	✓			8.3
Irgarol[×]	Triazine	$C_{11}H_{19}N_5S$	253.37	7	1569	3.95	-	-	X			0.016	
Isoproturon[×]	Phenylurea	$C_{12}H_{18}N_2O$	206.28	70.2	251*	2.5	2.61	40	X	✓		1	
Linuron	Phenylurea	$C_9H_{10}Cl_2N_2O_2$	249.09	63.8	843	3	2.11	13	X	✓			
Malaoxon	Metabolite	$C_{10}H_{19}O_7PS$	314.29*	7500*	4650*	0.52*	-	-	-				
Malathion	Organophosphate	$C_{10}H_{19}O_6PS_2$	330.36	148	1800	2.75	0.00	0.4	✓	✓			
MCPA	Organophosphate	$C_9H_9ClO_3$	200.62	29390	29*	-0.81	2.98	13.5	✓	✓			
Mecoprop	Aryloxyalkanoic acid	$C_{10}H_{11}ClO_3$	214.65	250000	47	-0.19	2.29	37	X	✓			
Methiocarb[ε]	Carbamate	$C_{11}H_{15}NO_2S$	225.31	27	182*	3.18	1.82	1.6	✓	✓			2
Metolachlor	Chloroacetamide	$C_{15}H_{22}ClNO_2$	283.80	530	120	3.40	2.36	88	X				
Molinate	Thiocarbamate	$C_9H_{17}NOS$	187.30	1100	190	2.86	1.89	4	X	✓			
Pendimethalin	Dinitroaniline	$C_{13}H_{19}N_3O_4$	281.31	0.33	17491	5.40	-0.28	4	✓	✓			
Propanil	Anilide	$C_9H_9Cl_2NO$	218.08	95	149	2.29	-0.51	1.2	X	✓			
Quinoxyfen[×]	Quinoline	$C_{15}H_8Cl_2FNO$	308.13	0.05	23°	4.66	-0.8	5	✓	✓		2.7	
Simazine[×]	Triazine	$C_7H_{12}ClN_5$	201.66	5	130	2.30	2.2	46	X	✓		4	
Terbuthylazine	Triazine	$C_9H_{16}ClN_5$	229.71	6.6	329*	3.40	2.19	6	✓	✓			
Terbutryn[×]	Triazine	$C_{10}H_{19}N_5S$	241.36	25	2432	3.66	2.21	27	X			0.34	
Thiacloprid[ε]	Neonicotinoid	$C_{10}H_9ClN_4S$	252.72	184	615°	1.26	1.1	1000	✓				8.3
Thiamethoxam[ε]	Neonicotinoid	$C_8H_{10}ClN_5O_3S$	291.71	4100	56	-0.13	3.58	30.6	X	✓			8.3
Thifensulfuron methyl	Sulfonylurea	$C_{12}H_{13}N_5O_6S_2$	387.39	54.1	28	-1.65	3.05	22	✓	✓			
Triallate	Thiocarbamate	$C_{10}H_6Cl_3NOS$	304.70	4.1	3034	4.06	0.61	104	✓	✓			

[×] Compound included in the list of priority substances. EC Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regard priority substances in the field of water policy. Retrieved from: https://goo.gl/diHn8W.

[ε] Compound included in the European Watch List and corresponding maximum acceptable method detection limit (ng/L). EC Commission Implementing Decision (EU) 2018/840 of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495 (notified under document C(2018) 3362). Retrieved from: https://goo.gl/nR4ezg.

‡ The PPDB, Pesticide Properties Database. http://sitem.herts.ac.uk/aeru/footprint/index2.htm. - Lewis, K.A., Tzilivakis, J., Warner, D. and Green, A. (2016). An international database for pesticide risk assessments and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064.
* Data estimated using the US Environmental Protection Agency EPISuite™ http://www.Chemspider.com.
ˋ Kegley, S.E., Hill, B.R., Orme S., Choi A.H., PAN Pesticide Database, Pesticide Action Network, North America (Oakland, CA, 2016), http://www.pesticideinfo.org.
^ Calculated using the mathematical formula: GUS = log10 (half-life) x [4 - log10 (Koc)].
Ω Environmental Quality Standards (EQS) for priority substances in surface waters.
°° Commission implementing regulation (EU) 2019/1090 of 26 June 2019 concerning the non-renewal of approval of the active substance dimethoate. Member States shall withdraw authorizations for plant protection products containing dimethoate as active substance by 17 January 2020 at the latest.

MM: molecular mass; Solubility: solubility in water at 20 °C; K_{oc}: organic carbon partition coefficient; K_{ow}: octanol-water partition coefficient; GUS: leaching potential index; DT50: biodegradability, water phase only, expressed as *half-life* in days; Legislative status: ✓ approved, **X** not approved.

Table S2. Recovery and repeatability (RSD, relative standard deviation) obtained from the replicate (n=5) analysis of groundwater and surface water fortified with the target analytes at concentration levels of 10 and 1000 ng/L.

Analyte	Groundwater		Surface water	
	Analyte recovery ± RSD (%)		Analyte recovery ± RSD (%)	
	10 ng/L	1000 ng/L	10 ng/L	1000 ng/L
2,4-D	91 ± 15	95 ± 9	126 ± 7	127 ± 4
Acetamiprid	80 ± 19	101 ± 14	93 ± 5	80 ± 5
Alachlor	82 ± 11	80 ± 16	125 ± 5	98 ± 6
Atrazine	118 ± 10	119 ± 4	89 ± 5	94 ± 8
Azinphos ethyl	112 ± 17	92 ± 13	118 ± 17	85 ± 8
Azinphos methyl	114 ± 20	112 ± 18	113 ± 9	100 ± 10
Azinphos methyl oxon	121 ± 3	90 ± 5	BLOD	83 ± 23
Bentazone	107 ± 15	86 ± 16	104 ± 5	81 ± 8
Bromoxynil	82 ± 6	82 ± 18	121 ± 4	90 ± 5
Chlorfenvinphos	113 ± 17	90 ± 4	120 ± 14	93 ± 6
Chlorpyrifos	116 ± 11	89 ± 4	105 ± 7	122 ± 3
Chlortoluron	123 ± 16	88 ± 14	114 ± 11	87 ± 18
Clothianidin	BLOD	99 ± 7	BLOD	90 ± 12
Cyanazine	127 ± 24	120 ± 20	126 ± 4	120 ± 13
DEA	81 ± 6	93 ± 4	90 ± 6	102 ± 8
DIA	BLOD	104 ± 8	125 ± 3	112 ± 6
Diazinon	106 ± 10	104 ± 9	125 ± 18	106 ± 4
Dichlorvos	BLOD	87 ± 3	123 ± 7	95 ± 5
Diflufenican	BLOD	84 ± 20	BLOD	80 ± 17
Dimethoate	BLOD	84 ± 5	BLOD	110 ± 9
Diuron	111 ± 16	121 ± 4	85 ± 17	86 ± 11
Fenitrothion	BLOD	98 ± 3	BLOD	81 ± 5
Fenitrothion oxon	82 ± 17	88 ± 5	118 ± 8	91 ± 3
Fenthion oxon	107 ± 4	124 ± 20	102 ± 8	115 ± 6
Fenthion oxon sulfone	83 ± 5	106 ± 8	BLOD	94 ± 17
Fenthion oxon sulfoxide	99 ± 12	110 ± 16	98 ± 7	89 ± 8
Fenthion sulfone	116 ± 4	120 ± 4	BLOD	83 ± 11
Fenthion sulfoxide	84 ± 3	104 ± 8	95 ± 5	107 ± 11
Fluroxypyr	BLOD	97 ± 6	BLOD	79 ± 20
Imidacloprid	84 ± 4	83 ± 4	112 ± 15	118 ± 5
Irgarol	110 ± 15	122 ± 16	116 ± 10	102 ± 11
Isoproturon	104 ± 5	83 ± 5	107 ± 7	119 ± 13
Linuron	92 ± 4	106 ± 12	109 ± 6	86 ± 20
Malaoxon	120 ± 11	124 ± 10	125 ± 12	121 ± 13
Malathion	BLOD	80 ± 20	BLOD	88 ± 5
MCPA	115 ± 19	86 ± 13	93 ± 20	113 ± 13
Mecoprop	99 ± 16	86 ± 18	105 ± 14	106 ± 15
Methiocarb	111 ± 15	116 ± 19	88 ± 11	104 ± 12
Metolachlor	122 ± 10	114 ± 13	122 ± 19	118 ± 4
Molinate	BLOD	81 ± 8	BLOD	88 ± 7

Oxadiazon	BLOD	109 ± 20	BLOD	104 ± 7
Pendimethalin	BLOD	99 ± 4	BLOD	121 ± 4
Propanil	120 ± 4	85 ± 17	103 ± 19	110 ± 9
Quinoxyfen	95 ± 3	88 ± 21	73 ± 5	106 ± 19
Simazine	123 ± 12	86 ± 20	96 ± 4	79 ± 19
Terbuthylazine	115 ± 19	111 ± 13	111 ± 7	83 ± 4
Terbutryn	99 ± 3	100 ± 3	117 ± 13	95 ± 8
Thiacloprid	112 ± 15	99 ± 6	122 ± 3	81 ± 18
Thiamethoxam	BLOD	80 ± 6	88 ± 12	98 ± 12
Thifensulfuron methyl	108 ± 14	84 ± 19	115 ± 3	91 ± 11
Triallate	75 ± 19	113 ± 19	106 ± 20	87 ± 14

BLOD: Below limit of detection

Table S3. Recovery and repeatability (RSD, relative standard deviation) obtained from the replicate (n=5) analysis of LC-grade water fortified with the target analytes at concentration levels of 10, 100 and 1000 ng/L, and limits of detection (LOD) and determination (LODet) achieved.

Analyte	Analyte recovery ± RSD (%)			Sensitivity	
	10 ng/L	100 ng/L	1000 ng/L	LOD ng/L	LODet ng/L
2,4-D	79 ± 14	81 ± 12	88 ± 5	6.1	20
Acetamiprid	106 ± 9	81 ± 19	95 ± 9	0.16	0.53
Alachlor	93 ± 15	105 ± 1	97 ± 3	1.2	3.8
Atrazine	102 ± 5	123 ± 14	92 ± 2	0.14	0.88
Azinphos ethyl	113 ± 14	85 ± 6	98 ± 12	0.42	1.4
Azinphos methyl	81 ± 6	92 ± 13	121 ± 13	0.38	1.3
Azinphos methyl oxon	126 ± 10	100 ± 7	108 ± 11	3.1	10
Bentazone	76 ± 20	88 ± 20	113 ± 10	4.3	14
Bromoxynil	111 ± 5	99 ± 5	121 ± 8	2.6	8.6
Chlorfenvinphos	112 ± 11	106 ± 4	112 ± 15	0.24	0.80
Chlorpyrifos	123 ± 18	120 ± 10	104 ± 18	0.44	1.5
Chlortoluron	125 ± 20	98 ± 5	107 ± 14	0.13	0.42
Clothianidin	113 ± 11	100 ± 4	80 ± 5	2.3	7.5
Cyanazine	115 ± 7	112 ± 5	124 ± 3	0.081	0.28
DEA	90 ± 6	100 ± 26	102 ± 8	2.3	7.9
DIA	105 ± 21	120 ± 5	116 ± 13	4.4	15
Diazinon	82 ± 3	103 ± 5	125 ± 6	0.042	0.16
Dichlorvos	94 ± 20	120 ± 15	113 ± 14	5.4	18
Diflufenican	121 ± 11	96 ± 13	100 ± 19	1.2	4.0
Dimethoate	120 ± 9	117 ± 17	84 ± 19	0.76	2.6
Diuron	109 ± 4	127 ± 12	124 ± 3	0.13	0.43
Fenitrothion	106 ± 12	123 ± 5	120 ± 17	2.6	8.8
Fenitrothion oxon	120 ± 4	85 ± 8	112 ± 6	0.79	2.6
Fenthion oxon	110 ± 12	119 ± 3	122 ± 7	0.17	0.59
Fenthion oxon sulfone	99 ± 13	125 ± 4	112 ± 20	2.8	9.4
Fenthion oxon sulfoxide	110 ± 4	98 ± 5	109 ± 6	0.13	0.43
Fenthion sulfone	85 ± 20	109 ± 15	120 ± 6	4.2	14
Fenthion sulfoxide	89 ± 5	93 ± 20	106 ± 16	0.41	1.4
Fluroxypyr	BLOD	103 ± 18	102 ± 14	29	95
Imidacloprid	124 ± 20	103 ± 12	81 ± 18	0.87	2.9
Irgarol	89 ± 7	121 ± 16	87 ± 21	0.85	2.8
Isoproturon	91 ± 24	98 ± 16	90 ± 3	0.15	0.50
Linuron	122 ± 3	108 ± 8	114 ± 8	0.58	1.9
Malaoxon	90 ± 19	117 ± 16	123 ± 14	0.15	0.50
Malathion	83 ± 5	82 ± 12	82 ± 12	3.4	12
MCPA	118 ± 6	101 ± 20	82 ± 7	5.5	19
Mecoprop	92 ± 17	109 ± 4	81 ± 13	1.1	3.6
Methiocarb	110 ± 16	123 ± 11	108 ± 12	0.41	1.4
Metolachlor	112 ± 3	108 ± 7	114 ± 4	0.086	0.32
Molinate	93 ± 16	82 ± 17	122 ± 8	1.1	3.6

Oxadiazon	100 ± 4	82 ± 19	88 ± 19	1.3	4.5
Pendimethalin	BLOD	94 ± 4	93 ± 7	17	55
Propanil	122 ± 15	112 ± 10	112 ± 17	0.90	3.0
Quinoxyfen	109 ± 12	86 ± 3	92 ± 7	1.1	3.6
Simazine	113 ± 12	96 ± 20	104 ± 13	0.31	1.1
Terbuthylazine	122 ± 13	117 ± 6	115 ± 9	0.14	0.48
Terbutryn	92 ± 11	106 ± 4	120 ± 4	0.19	0.66
Thiacloprid	97 ± 3	110 ± 18	80 ± 14	0.059	0.21
Thiamethoxam	119 ± 22	120 ± 20	89 ± 1	1.8	6.0
Thifensulfuron methyl	83 ± 1	118 ± 12	80 ± 19	0.022	0.06
Triallate	114 ± 20	110 ± 14	118 ± 4	3.8	13

BLOD: Below limit of detection

Table S4. Concentrations (ng/L) of the individual pesticides and cumulative pesticide concentrations (TOTAL) measured in the water samples collected in the Llobregat River.

PESTICIDES	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10	Pt 11
2,4-D	n.d.	n.d.	n.d.	n.d.	n.d.	200	n.d.	n.d.	130	n.d.	n.d.
Alachlor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	18	n.d.	n.d.	24	n.d.
Atrazine	n.d.	13	<6.7	n.d.	n.d.	<6.7	17	n.d.	n.d.	21	<6.7
Azinphos ethyl	n.d.	10	n.d.	n.d.	n.d.	n.d.	69	n.d.	n.d.	110	n.d.
Bromoxynil	n.d.	74	<22	1500	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chlorfenvinphos	n.d.	4.8	n.d.	n.d.	4.8	<2.9	48	n.d.	n.d.	67	<2.9
Chlortoluron	n.d.	67	n.d.	n.d.	30	n.d.	18	n.d.	n.d.	27	n.d.
Cyanazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	29	n.d.
Diazinon	20	2.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	71	18
Dichlorvos	n.d.	<20	<20	n.d.	<20	n.d.	n.d.	<20	n.d.	130	n.d.
Diflufenican	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	130	n.d.	n.d.	150	n.d.
Diuron	500	24	250	250	260	91	42	120	61	63	240
Fenthion oxon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	37	n.d.	n.d.	n.d.	n.d.
Fenthion sulfoxide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	32	n.d.	n.d.	n.d.	n.d.
Imidacloprid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<10	n.d.	n.d.	190	<10
Irgarol	n.d.	n.d.	n.d.	<6.6	<6.6	n.d.	33	<6.6	n.d.	41	n.d.
Isoproturon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	24	<7.1	n.d.	25	n.d.
Linuron	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	520	n.d.	132	480	n.d.
Malaoxon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	24	n.d.	n.d.	24	n.d.
Malathion	n.d.	n.d.	n.d.	<17	n.d.	n.d.	25	n.d.	n.d.	32	n.d.
Methiocarb	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	50	n.d.	n.d.	130	n.d.
Metolachlor	5.1	n.d.	n.d.	n.d.	n.d.	n.d.	25	n.d.	n.d.	28	n.d.
Molinate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	33	n.d.	n.d.	27	n.d.
Propanil	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	19	n.d.	n.d.	n.d.	n.d.
Simazine	16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	20	n.d.
Terbuthylazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	24	n.d.	3.5	30	n.d.
Terbutryn	120	8.9	63	63	71	160	45	130	24	55	n.d.
Thiacloprid	<0.79	n.d.	n.d.	n.d.	n.d.	n.d.	16	n.d.	n.d.	31	n.d.
TOTAL	661	205	313	1813	366	451	1249	250	351	1805	258

n.d.: not detected

<LOQ: below limit of quantification

Total concentration calculated considering only values >LOQ.

Table S5. Concentrations (ng/L) of the individual pesticides and cumulative pesticide concentrations (TOTAL) measured in the water samples collected in the Ter River.

PESTICIDES	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5a	Pt 5b	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10
Bentazone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	110	n.d.	n.d.	n.d.
Diazinon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.3	4.6	n.d.	n.d.
Diuron	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14	n.d.	n.d.	n.d.	n.d.
Irgarol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.4	n.d.
MCPA	n.d.	n.d.	18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Metolachlor	n.d.	n.d.	15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	24	n.d.
Terbutryn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.1	5.3	n.d.
TOTAL	n.d.	n.d.	33	n.d.	n.d.	n.d.	14	112	8.7	34	n.d.

n.d.: not detected

Total concentration calculated considering only values >LOQ.

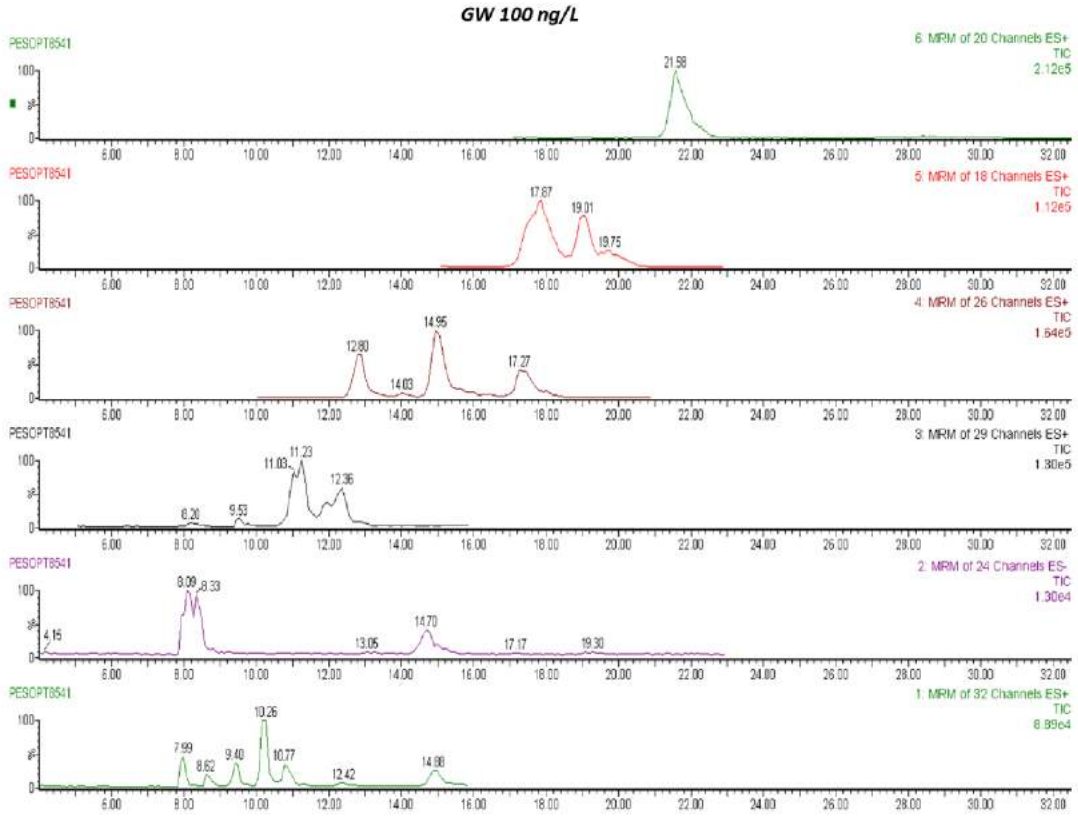

Figure S1. Total Ion Current (TIC) chromatograms obtained from the analysis of a fortified groundwater sample (100 ng/L) showing the acquisition of the 146 SRM transitions set for determination of the 51 target pesticides and their 45 SIL analogs in six different acquisition windows along the analytical run.

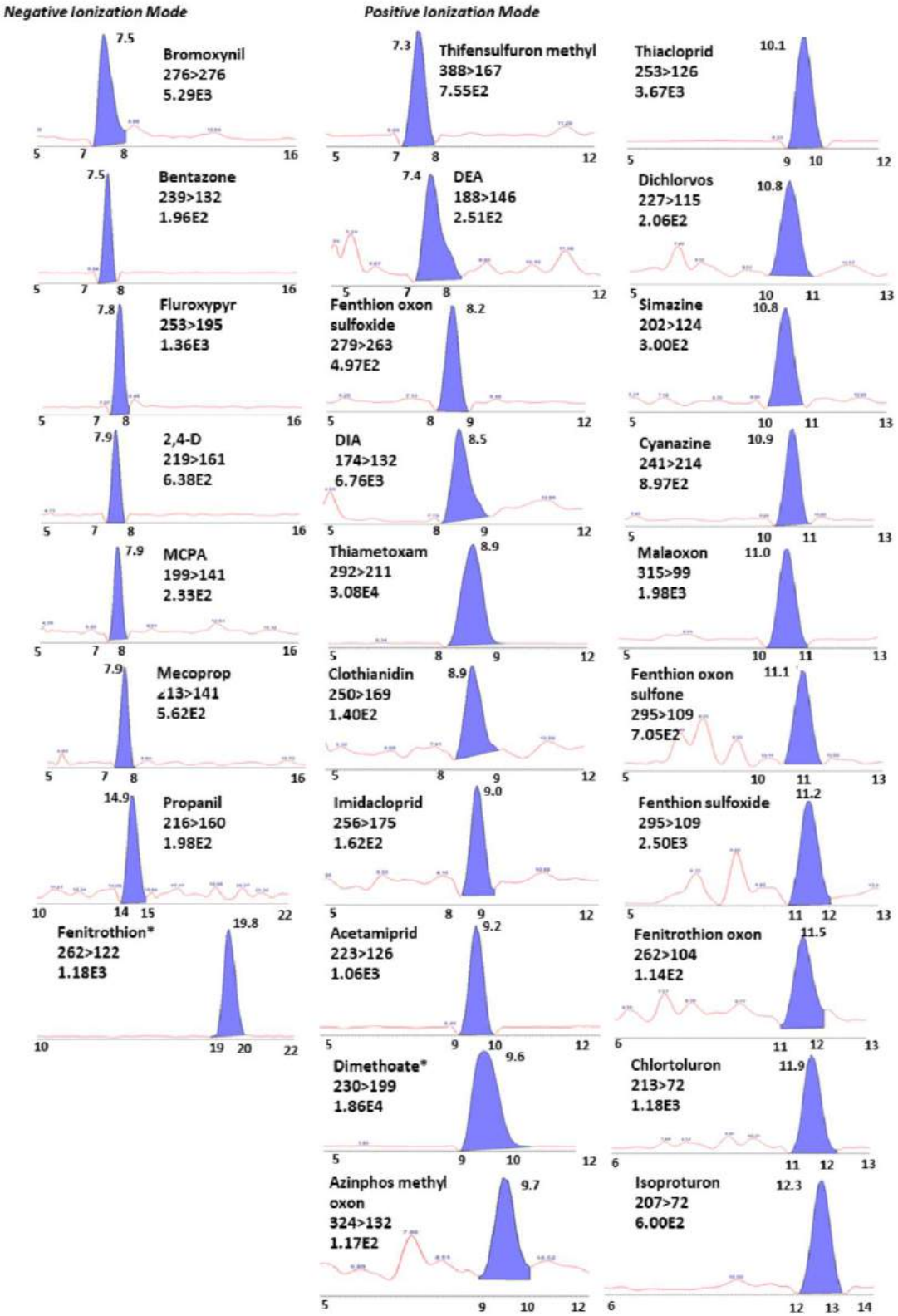

Figure S2. Extracted ion chromatograms (XIC) of the target pesticides after on-line SPE-LC-MS/MS analysis of a surface water sample fortified at a concentration of 100 ng/L (or 500 ng/L in the case of those compounds marked with *).

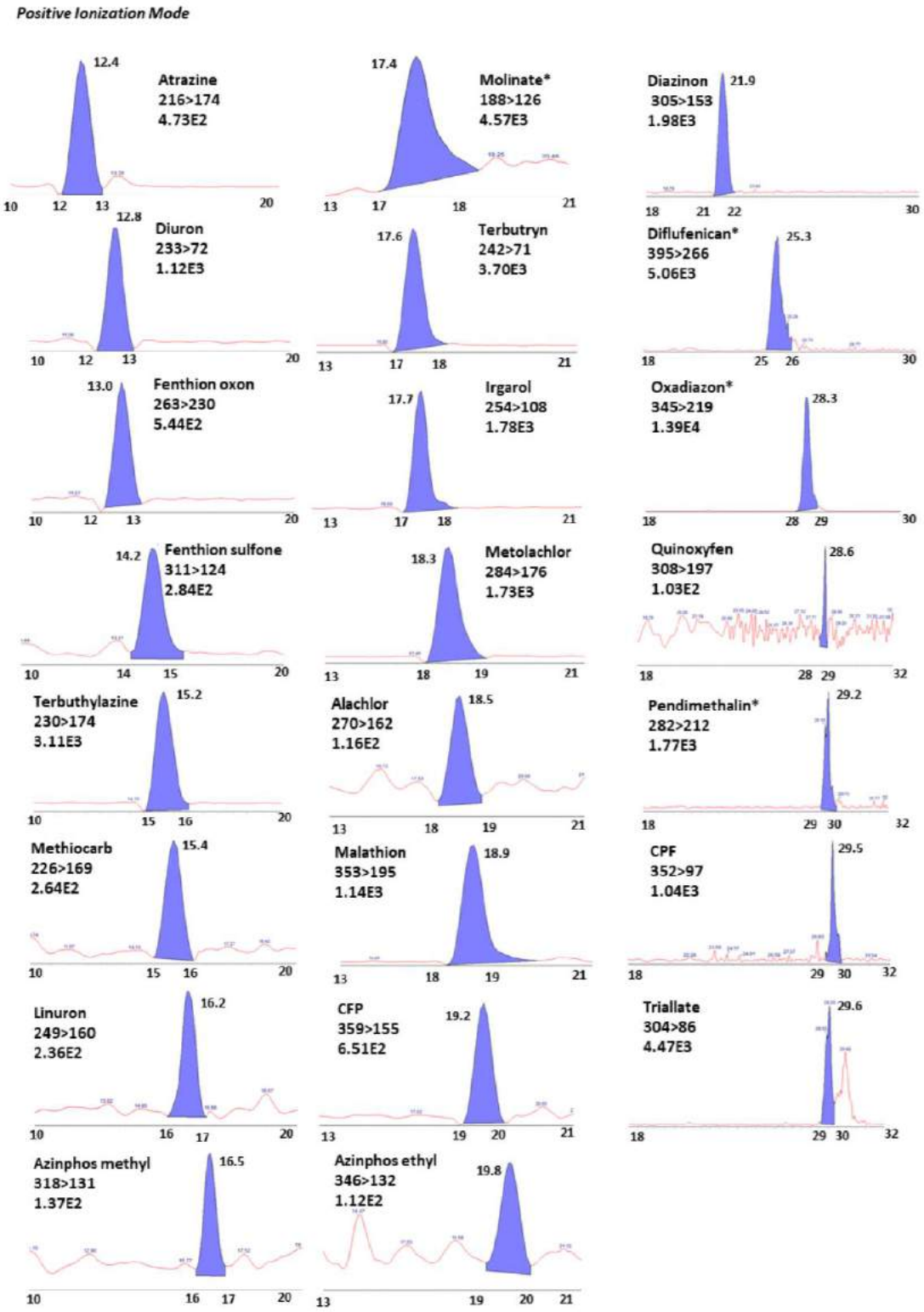

Figure S2. (continued).

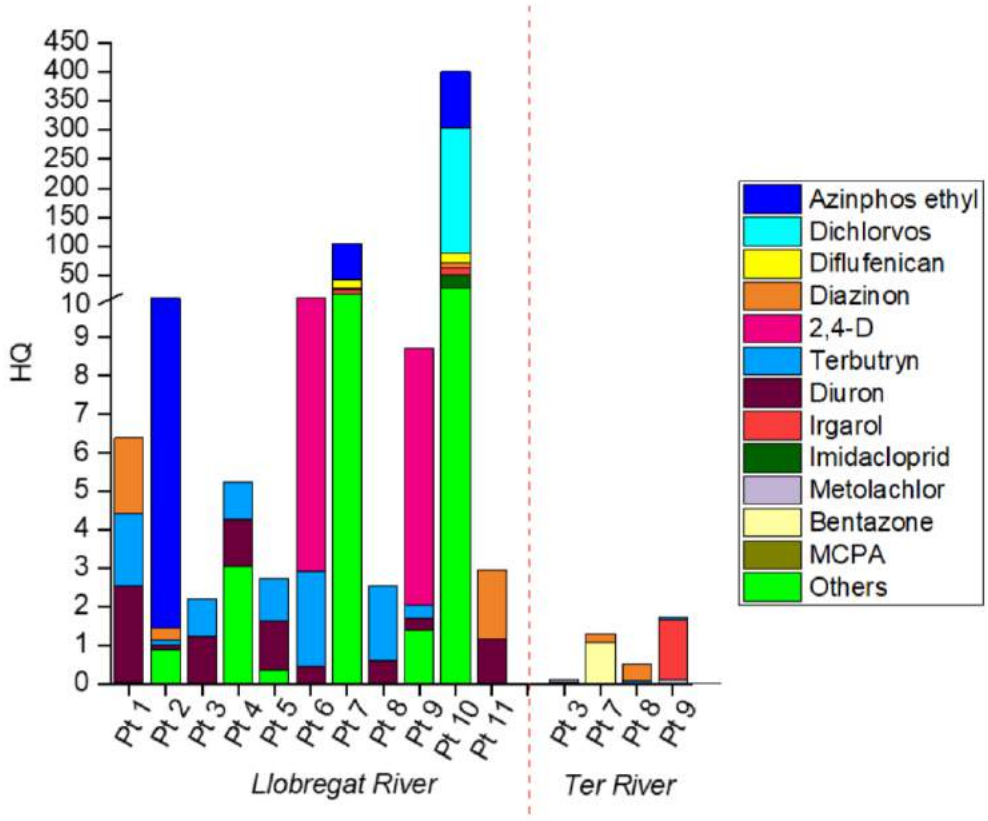

Figure S3. Hazard Quotients (HQ) in the samples analysed, based on the individual HQs of the pesticides measured in each sample. The HQs corresponding to those pesticides detected in the samples but not specified in the legend have been grouped as "*Others*".

3.2 Analysis of medium to highly polar pesticides and metabolites in sediments

The following article presents the development and validation of a multi-residue analytical method based on PLE, SPE clean-up and LC-MS/MS for the analysis of 50 medium to highly polar pesticides in sediments.

Sediment is a matrix of great importance since it can act as a sink of contaminants and provide information on historical contamination and pollution episodes.

Prior to this work, few studies had investigated the occurrence of medium to highly polar pesticides and TPs in sediments with the use of LC-MS/MS. Compared with these works, the method developed in the framework of this doctoral thesis (scientific publication #2) provides some improvements in terms of sensitivity, as it provides lower LOQs for some compounds, and reliability of results, as it uses ILIS for 85% of the target pesticides, whereas the other analytical approaches use highly labor-intensive quantification methods such as matrix-matched calibration curves. In the proposed methodology, the extraction is performed by PLE since it shows high efficiency and allows the automated extraction of multiple samples. A remarkable achievement of this method is the low signal suppression or enhancement observed during LC-MS/MS analysis (less than 10% in all cases), thanks to the efficiency of the clean-up process. Furthermore, this is one of the few works analyzing more than 50 pesticides in sediments, and the first that analyses the TPs azinphos-methyl oxon, fenitrothion oxon, and malaoxon in this matrix.

This work also aims at providing reference data on the occurrence of pesticides in sediments, as the EC recommends long-term trend analysis of concentrations of contaminants that can accumulate in sediments to ensure that their concentrations do not significantly increase in time.

The validated method was applied to investigate the occurrence of pesticides in sediment samples collected along the lower Llobregat River basin (Catalonia, Spain), in the framework of the WaterProtect and BECAS projects. Moreover, the evaluation of the potential risk that the pesticides concentrations found may pose for sediment-dwelling organisms was also assessed.

Scientific publication #2:

"A reliable LC-MS/MS-based method for trace level determination of 50 medium to highly polar pesticide residues in sediments and ecological risk assessment"

Maria Vittoria Barbieri, Cristina Postigo,
Luis Simón Monllor-Alcaraz, Damià Barceló, Miren López de Alda

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PAPER IN FOREFRONT

A reliable LC-MS/MS-based method for trace level determination of 50 medium to highly polar pesticide residues in sediments and ecological risk assessment

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Abstract
The occurrence of polar pesticides in sediments has not been extensively investigated because of their relatively poor hydrophobicity and apparently less persistence in the environment. However, their continuous release into the aquatic systems calls for the evaluation of their potential accumulation in sediments and the role of this matrix as a potential source of these compounds. Considering this, a method based on pressurized liquid extraction (PLE), extract clean-up by solid phase extraction (SPE), and analyte determination by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was developed and validated to analyze 50 relevant (frequently used and/or regulated or found in water) medium to highly polar pesticides in sediments. The method showed good performance regarding accuracy (relative recoveries between 76 and 124%), precision (relative standard deviation values < 20%), sensitivity (LODs in the low nanogram per gram for most compounds), linearity (coefficients of determination > 0.99), and matrix effects (negligible for all analytes). The use of an isotope dilution approach for quantification ensures result reliability. As a part of the validation process, the method was applied to the analysis of the target pesticides in sediments from the Llobregat River (NE Spain) showing the presence of five of them, namely, terbutryn, dichlorvos, terbuthylazine, diazinon, and irgarol. All 5 pesticides, due to both the concentrations found and their physical-chemical characteristics, demonstrate high potential for bioaccumulation and risk to aquatic organisms. Additional multi-disciplinary studies that investigate pesticide occurrence in different aquatic compartments and evaluate the potential risks for aquatic ecosystems are required to assess the environmental impact and significance of the presence of pesticides in sediments.

Keywords Phytosanitary products · Plant protection products · Analysis · Terbutryn · Terbuthylazine · Liquid chromatography-tandem mass spectrometry

Introduction

Pollutants may get adsorbed onto suspended particulate matter present in surface water. These particles may eventually deposit on the bottom and at the banks of water bodies, forming sediment layers. Thus, sediments play a fundamental role in the transport and fate of pollutants [1]. As a pollution sink, sediments also reflect long-term water pollution and can be used to evaluate the impact of human activities because changes in their pollution pattern are quite slow [2]. Pesticides are among those organic pollutants that may end up in freshwater sediments. However, their presence in sediments has been little studied and, when measured in aquatic systems, studies were focused in the freely dissolved phase.

Most of the few works that studied their fate in sediments focus on organochlorine pesticides [3–6], because of their low

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water solubility, high potential to adsorb on particles, persistence, and toxicity. However, in the late 1970s, most organochlorine pesticides started to be banned in developing countries and consequently many other pesticides started to be synthesized and introduced in the market and are nowadays commonly used. In this regard, polar pesticides were presented as an attractive alternative to organochlorine pesticides, because of their high water solubility, low organic carbon sorption coefficient (K_{oc}), and low environmental persistence. Nevertheless, their large use in agriculture, the main source of pesticide pollution in the environment [7], has led to their ubiquitous presence in water ecosystems. This makes the screening of these pesticides in sediments extremely important for understanding their environmental fate and assessing the potential risks that they may pose for aquatic organisms [2].

Several pesticides are included in the list of priority substances that determine the achievement, or not, of a good chemical status of surface water bodies in Europe. Consequently, their concentrations cannot exceed certain Environmental Quality Standards (EQS) established in surface waters, and in some cases also in biota [8]. As for now, EQS have not been set yet for sediments, but the Directive establishes that this can be done at EU member state level and, in any case, long-term trend monitoring of priority substance concentrations in sediments has to be performed in order to prevent deterioration of surface water bodies. Therefore, the development of high-sensitivity and reliable analytical methods to measure low levels of this type of organic pollutants in such a complex matrix is required for this purpose.

In the last 20 years, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been recognized as the technique of choice for the determination of polar compounds and their transformation products in environmental matrices [9, 10] and food [11, 12] because it provides good separation for ionic and small polar analytes (which includes most metabolites) and very good performance in terms of sensitivity and selectivity when operated in the selected reaction monitoring (SRM) mode. In the last decade, this technique has been selected to analyze these organic substances in sediments [13–18]. For this, a wide range of extraction procedures have been used: Soxhlet extraction [19], matrix solid phase dispersion (MSPD) extraction [15], QuEChERS (quick, easy, cheap, effective, rugged, and safe) approaches [18, 20, 21], ultrasonic solvent extraction [14, 22], microwave-assisted extraction (MAE) [17], and pressurized liquid extraction (PLE) [18, 23, 24]. The extraction of organic chemicals from sediments is always a critical step, due to the strong interactions, e.g., through ion exchange or covalent and H-bonding, that may occur between them and the organic matter and mineral surface of the sediment [25]. The precision of the extraction step can be improved with the use of automated techniques such as PLE. Besides, additional advantages that PLE presents over other extraction methods are the reduction of the extraction time and the requirement of low solvent volumes.

Thus, the main objective of this work was to develop and validate an analytical methodology based on PLE, solid phase extraction (SPE) purification, and LC-MS/MS analysis, for the simultaneous determination of 50 medium to highly polar pesticides in sediments. The list of compounds, which includes 40 pesticides and 10 transformation products, was designed taking into account three main criteria: (i) pesticide use at European level, (ii) environmental regulations, i.e., pesticides included in the list of priority substances in water [8] or in the European Watch List [26], and (iii) possibility of analysis by means of LC-MS/MS. The investigated pesticides are used for different purposes (herbicides, fungicides, biocides) and applied in different sectors (agricultural, urban, or industrial uses). They belong to different chemical classes (organophosphates, phenylureas, chloroacetamides, neonicotinoids, triazines, acidic pesticides, and other classes) and therefore present a wide range of physical-chemical properties (K_{oc}, K_{ow}, solubility, GUS index, etc.). Thus, a comprehensive study of their fate and impact in aquatic systems subject to different environmental stressors requires the development and application of multi-residue methods that allow their simultaneous analysis in the various environmental compartments, including sediments. Moreover, to the authors' knowledge, some of the target pesticides, namely, azinphos-methyl oxon, fenitrothion oxon, and malaoxon, were not previously investigated in sediment samples.

Finally, this method was applied to determine the occurrence of the selected pesticides in sediments of the Llobregat River basin (Catalonia, Spain). The concentrations observed were used to provide a first picture of the chemical status of this freshwater ecosystem and identify the most relevant threats so that appropriate mitigation measurements can be adopted.

Materials and methods

Chemicals

High-purity standards (96–99.9%) of the 50 pesticides and 44 isotopically labeled compounds used as surrogate standards were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany) or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). Table S1 in Electronic Supplementary Material (ESM) lists the target analytes including their chemical class, relevant physical-chemical properties (solubility in water, K_{oc}, K_{ow}, GUS index, and others), and actual legislative status regarding their use (approved or not approved). Stock individual standard solutions were prepared in methanol (MeOH), except in the case of simazine that was prepared in dimethyl sulfoxide, at a concentration of 1000 μg/mL, and stored in amber glass

bottles in the dark at − 20 °C. A mixture containing the surrogate standards at a concentration of 1000 ng/mL and working standard solutions containing the target pesticides at diverse concentrations (from 0.01 to 1000 ng/mL) were prepared by dilution of the stock individual solutions in MeOH. Pesticide-grade solvents MeOH, acetone (ACE), acetonitrile (ACN), dichloromethane (DCM), formic acid (FA), and LC-grade water were supplied by Merck (Darmstadt, Germany). Ottawa sand was purchased from Applied Separations (Allentown, PA) and alumina (Al_2O_3) from Merck (Darmstadt, Germany).

Extraction and clean-up of the sediment samples

For sample extraction, a 22-mL stainless steel extraction cell was prepared by placing sequentially at the bottom of the cell one cellulose filter (0.45-μm pore size), 1 g of sand, and 1 g of alumina, previously activated by heating it at 80 °C for at least 24 h (see Fig. 1). Then, an aliquot of 5 g of lyophilized and sieved (<125 μm) sediment sample was weighed directly into the cell, spiked with the surrogate standard mixture at a concentration of 50 ng/g and left overnight in a fume hood for 12 h to allow methanol evaporation and interaction of the surrogate standards with the matrix. The day after, 6 g of activated alumina was placed into the cell and mixed with the sediment to reduce the unintended extraction of matrix components other than the analytes of interest. Then, the cell was filled up to the top with sand, closed, and positioned in the PLE system, a Dionex accelerated solvent extraction (ASE) 350 apparatus (Vertex Technics S.L., Barcelona). PLE was performed using an acidified mixture of ACE and DCM (1:1 and 1% FA, v/v) as an extracting solvent. The extraction conditions were as follows: pressure, 1600 psi; temperature, 100 °C; heating time, 5 min; purge time, 90 s; static time, 5 min. Two extraction cycles were performed, resulting in a total extraction time of 20 min per cell. Upon extraction, the final extract (≈ 20 mL), to which 1 mL of MeOH was added, was evaporated under nitrogen to 1 mL using a Biotage TurboVap® LV workstation (Vertex Technics S.L., Barcelona). Then, the 1-mL extract was dissolved in 19 mL of LC-grade water for SPE clean-up. This was performed using a general-purpose Oasis HLB sorbent (500 mg, 6-cc cartridges, Waters, Milford, MA, USA). Before loading the aqueous extract, the SPE sorbent was conditioned with 5 mL of MeOH and DCM (1:1) and 5 mL of LC-grade water. After extract loading, the sorbent was washed with 5 mL of LC-grade water to complete sample transfer and remove matrix interferences. Then, the sorbent was dried for 30 min, and analytes were eluted using 4 mL of MeOH and DCM (1:1). The eluate was evaporated under nitrogen to approximately 1.5 mL and finally reconstituted with MeOH to 5 mL before its transfer to 2-mL vials for LC-MS/MS analysis.

LC-HESI-MS/MS analysis

Chromatographic separation was performed by means of an Aria™ LC system equipped with two Transcend quaternary pumps (Thermo Fisher Scientific Inc.), using a Purospher STAR RP-18e column (150 × 2.1 mm, 2-μm particle diameter) (Merck, Darmstadt, Germany) and a mobile phase consisting of ACN and water at a flow rate of 0.2 mL/min. The volume of extract injected was 10 μL. The organic gradient used was as follows: 10% at time (t) = 0, 50% at t = 2 min, 80% at t = 12 min, 100% at t = 13 min and maintained until t = 16 min. Initial conditions (10% ACN) were achieved in t=17 min and held for 8 min (until t = 25 min) for column re-equilibration.

MS/MS detection was carried out using a TSQ Quantiva triple-quadrupole mass spectrometer (Thermo Fisher Scientific Inc.) equipped with a heated electrospray ionization source (HESI). All analytes were determined in a single analytical run, being 43 compounds ionized in the positive (PI) mode and 7 compounds in the negative (NI) mode in one single acquisition window. The mass spectrometer was operated in the SRM mode, acquiring two SRM transitions per

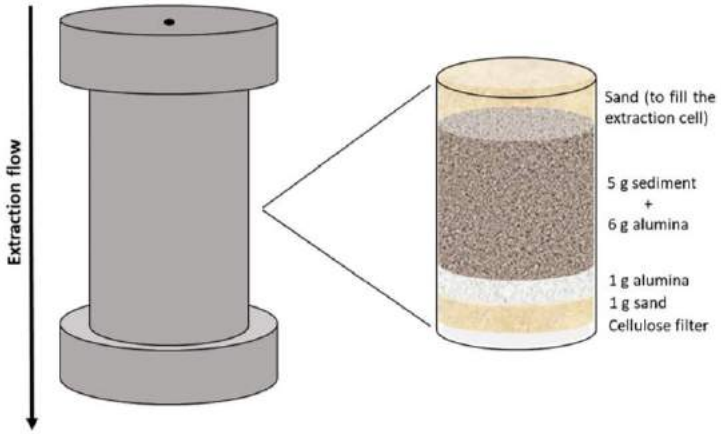

Fig. 1 Scheme of the PLE cell

target compound and one SRM transition per surrogate compound. MS acquisition conditions were as follows: ion spray voltage, 3500 V for PI and − 2500 V for NI; vaporizer temperature, 280 °C; ion transfer tube temperature, 350 °C. Nitrogen was used as the sheath gas, auxiliary gas, and sweep gas, and argon was used as the collision gas, at a pressure of 2.5 mTorr. Thermo Xcalibur 3.0.63 software (Thermo Fisher Scientific Inc.) was used for instrument control, data acquisition, and evaluation.

Method performance

The sediment sample used in the validation study was collected from the Guadalquivir River basin (south of Spain). Method validation was performed in terms of linearity, accuracy (recovery), precision (repeatability), sensitivity, and matrix effects.

Method linearity was evaluated in methanolic solutions containing the analytes within the concentration range 0.01–1000 ng/mL (equivalent to 0.01 ng/g d.w. and 1000 ng/g d.w., respectively, in sediment) and the surrogate standards at 50 ng/mL (50 ng/g in sediment). For this, eleven-point calibration curves using an isotope dilution approach were constructed. Linearity was expressed as the goodness of fit, i.e., the coefficient of determination (R^2), of the calibration data to a least squares linear regression model, obtained using $1/x^2$ as a weighting factor.

Method accuracy was appraised from analyte absolute and relative recoveries obtained after $n = 6$ replicate analysis of fortified sediment samples at three different concentration levels (low, 10 ng/g d.w.; medium, 50 ng/g d.w.; and high, 100 ng/g d.w.). The absolute recoveries were evaluated by comparing the analyte peak areas obtained in fortified sediment samples and methanolic solutions at equivalent concentrations. The relative recoveries were calculated by comparing the absolute recoveries of the analytes with those of their surrogates. Method precision was assessed from the repeatability of $n = 6$ replicate analysis of fortified sediment samples at the aforementioned concentration levels and expressed as the relative standard deviation (RSD) of the response obtained.

Appraisal of method sensitivity was done through the analytes limit of detection (LOD) and quantification (LOQ). These values were estimated from the analysis of sediment samples fortified at the lowest level (10 ng/g d.w.) as the concentration of analyte that provides a signal-to-noise (S/N) ratio of 3, in the case of LOD, and 10, in the case of LOQ. Method sensitivity was also assessed through the analyte limit of determination (LODet), i.e., the minimum concentration at which the analyte can be quantified using the first SRM transition (LOQ of SRM1) and confirmed with the second SRM transition (LOD of SRM2).

Evaluation of the effects that the matrix components had on analyte ionization, i.e., matrix effects (ME), was done by comparing the analyte peak areas obtained in $n = 3$ sediment samples fortified with the target analytes after their extraction (A_{matrix}) and in a methanolic solution (A_{std}) at equivalent concentrations (10 ng/mL). Ionization suppression effects were associated to negative ME values (smaller peak areas in A_{matrix} than in A_{std}), whereas ionization enhancement effects were associated to positive ME values (larger peak areas in A_{matrix} than in A_{std}).

Background concentrations of target analytes in the sediment sample used in the validation study were considered, if present, in all calculations done to evaluate method performance.

Study site and sample collection

The method was applied to evaluate the presence of the target pesticides in river sediments collected from the lower Llobregat River basin (Catalonia, Spain), one of the most important drinking water resources for the city of Barcelona and its metropolitan area (over 3 million people). The Llobregat River is a typical Mediterranean river. It presents flow fluctuations related to climate conditions and seasons, with drought periods, when dilution capacity of the river is decreased and consequently, the risk of contamination is increased [27], and peak rainfall events in spring (March–June) and autumn (September–December) that remove the river bottom. The intensive urban and industrial activities and surface runoff from agriculture also contribute to the flow variation and pollution of the river [28].

A sampling campaign was carried out in February 2017. Seven sites were sampled along the basin (to depict a downstream contamination profile) and land and concrete water channels adjacent to the main river (to assess the impact of agriculture), as shown in Fig. 2. Sediment samples were taken using a van Veen drag, placed in an aluminum tray and wrapped with aluminum foil, and transported to the laboratory under cool conditions. , and stored at − 20 °C. The samples were lyophilized for 36 h with a LyoAlfa 6-50 freeze-dryer (Telstar), sieved through a 125-μm mesh for homogenization, and stored at − 20 °C in the dark until their analysis.

Results and discussion

Method optimization

The method developed was based on a previous analytical method established by the same research group for the analysis of 26 pesticides in sediments [29] that was improved for additional performance, automation, and time-saving. Main modifications include the following:

Fig. 2 Map of the metropolitan area of Barcelona (Catalonia, Spain) with the Llobregat River basin and sediment sampling locations (source: Generalitat de Catalunya, Visor ACA, http://sig.gencat.cat/visors/VISOR_ACA.html)

1. Expansion of the list of target analytes with 24 pesticides and 20 isotopically labeled analogs to cover relevant substances in future monitoring programs. The newly added pesticides include the EU priority substances dichlorvos, irgarol, quinoxyfen, and terbutryn [8], the pesticides included in the EU Watch List methiocarb, and the neonicotinoids acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam [26]; and banned substances like azinphos ethyl [30], azinphos-methyl [31] and one of its metabolites (azinphos-methyl oxon), and fenthion [32] and five of its metabolites (fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, fenthion sulfone, fenthion sulfoxide). Moreover, pesticides currently used in Spain were also considered: bromoxynil [33], fluroxypyr [34], pendimethalin [35], thifensulfuron methyl [36], and diflufenican [37].
2. The use of the Dionex ASE 350 system instead of the PSE One system (Applied Separations, PA, USA), which allows increasing method automation. Contrary to the PSE One, in which only the extraction of one single cell could be programmed at a time, the Dionex ASE 350 allows the extraction of up to 24 different cells without intervention.
3. The use of a mass spectrometer with a fast acquisition rate (TSQ Quantiva) instead of the TQD triple-quadrupole mass spectrometer from Waters. This allows reducing the acquisition time allocated for each SRM transition so

that all transitions (144 in total) can be simultaneously acquired throughout the analytical run without losing sensitivity and reproducibility (obtaining sufficient points per peak). The optimum conditions for MS/MS determination of the compounds were individually optimized for each compound after injection of individual standard solutions in both PI and NI modes. The optimized conditions for the analysis of all analytes are provided in Table 1 and Table S2 in ESM.

4. The use of a chromatographic column with a smaller particle size (2 μm vs. 5 μm), which improves peak resolution, and hence, separation efficiency, and allows reducing the analysis time from 40 to 25 min. The composition of the LC mobile phase was maintained as it allows the analysis of all compounds in one single analytical run.

Method performance

Figures of merit of the developed methodology are summarized in Table 2 and Table S3 in ESM, and extracted ion chromatograms of the target pesticides in fortified sediment samples are shown in Fig. S1 in ESM.

As shown in Table 2, the linearity of the method expanded between 0.01 and 1000 ng/mL in most cases, up to 500 ng/mL in the case of bromoxynil. Shorter linearity ranges were observed for triallate and pendimethalin, because of their poor sensitivity. Coefficients of determination (r^2) were above 0.99 in all cases, except for fenitrothion (0.9847) and pendimethalin (0.9878).

Absolute recoveries, in general good agreement at the three concentration levels, ranged between 21% (fenitrothion oxon) and 99.7% (dimethoate) (average absolute recovery of the figures observed at the three investigated levels). Relative recoveries at any of the investigated concentration levels were always between 76 and 124%. This confirms that the use of surrogate standards (isotopically labeled analogs for 44 pesticides, and isotopically labeled pesticides similar in structure or analytical retention time and absolute recovery for the remaining 6 pesticides fluroxypyr, fenthion oxon sulfoxide, azynphos-methyl oxon, malaoxon, molinate, and quinoxyfen) allows compensating for the potential losses of the analytes during the extraction and clean-up steps and for ionization effects potentially caused by matrix components.

Relative standard deviation (RSD) values obtained after $n = 6$ replicate analysis of fortified samples at the three different concentration levels were always below 20%, except for fluroxypyr (24%) at the low concentration level tested and cyanazine (22%) and desethylatrazine (21%) at the medium concentration level investigated.

Results were good in terms of sensitivity for most of the compounds, with LODs between 0.01 and 4 ng/g d.w. and LODets between 0.03 and 12.4 ng/g d.w. for 80% of the investigated compounds. In the absence of legislation establishing maximum pesticide residues in sediments, the results obtained are satisfactory when compared with the value of 50 ng/g set by the European Commission as the desired LOQ for the analysis of pesticide residues in soil [38].

As for the priority substances considered, the method allows determining them in sediments at the low nanogram per gram level, that is at levels similar to the EQS established for these compounds in water (2013/39/EU) (i.e., alachlor (1.6 ng/g d.w. vs. 0.3 μg/L), atrazine (0.04 ng/g d.w. vs. 0.6 μg/L), chlorfenvinphos (1.9 ng/g d.w. vs. 0.1 μg/L), diuron (12 ng/g d.w. vs. 0.2 μg/L), irgarol (0.03 ng/g d.w. vs. 0.0025 μg/L), isoproturon (2.0 ng/g d.w. vs. 0.3 μg/L), simazine (0.6 ng/g d.w. vs. 1 μg/L), quinoxyfen (1.3 ng/g d.w. vs. 0.15 μg/L), and terbutryn (0.05 ng/g d.w. vs. 0.065 μg/L)), except in the case of dichlorvos (63.1 ng/g d.w. vs. 0.0006 μg/L). Besides dichlorvos, the worst performance of the method in terms of sensitivity was observed for bromoxinyl (25.6 ng/g d.w), linuron (31.9 ng/g d.w), fenitrothion oxon (62.2 ng/g d.w), fenthion sulfone (65.4 ng/g d.w), mecoprop (68.1 ng/g d.w), fenthion (71.6 ng/g d.w), pendimethalin (120 ng/g d.w), and triallate (121 ng/g d.w). As for the Watch List pesticides, the presented methodology works well to detect neonicotinoids (0.02–0.18 ng/g d.w. vs. 0.008 μg/L) and may not be sensitive enough in the case of methiocarb (1.2 ng/g d.w. vs. 0.002 μg/L), if maximum acceptable LODs suggested by the EU Decision 2018/840 for these compounds are considered. The effect of matrix components on the LC-MS/MS analysis of the target compounds is summarized in Fig. 3. As shown, the matrix components remaining after extraction did not have a strong effect on the ionization of the analytes, being the signal suppressed or enhanced by less than 10% in all cases.

The performance of this methodology was compared with that of the analytical LC-MS-based methods published in the peer-reviewed literature for the same purpose since 2015 (see Table 3) [14, 15, 17, 18, 20, 21]. The present methodology, together with the methods reported by Farré et al. [21], Masiá et al. [18], and Massei et al. [24], is one of the very few multiresidue analytical approaches currently available for the simultaneous determination of a large number (> 50) of pesticides belonging to different chemical classes. Furthermore, the target analytes included in the present method overlapped only by 50–60% with the analyte lists considered in the previously published multi-residue methods. This is the first time indeed that an analytical method is validated for the analysis of azinphos-methyl oxon, fenitrothion oxon, and malaoxon, in sediment samples. Malaoxon was included in the list of target pesticides analyzed by Kalogridi et al. [17] in sediments (253 pesticides in total); however, figures of merit of the method for

Table 1 MS/MS analysis of the target pesticides

Target pesticide	Precursor ion, m/z (RF Lens, V)	Product ion 1, m/z (CE, eV)	Product ion 2, m/z (CE, eV)	SRM1/ SRM2
Negative ionization mode (−)				
2,4-D	219 (35)	162 (16)	125 (28)	32.2
Bentazone	239 (68)	132 (28)	117 (33)	4.0
Bromoxynil	276 (82)	81 (30)	79 (30)	1.1
Fenitrothion	262 (52)	152 (21)	122 (34)	17.2
Fluroxypyr	255 (33)	197 (16)	235 (6)	47.2
MCPA	199 (38)	142 (17)	105 (30)	1.1
Mecoprop	213 (39)	142 (17)	140 (17)	3.2
Positive ionization mode (+)				
Acetamiprid[a]	223 (53)	126 (22)	90 (33)	5.5
Alachlor[b]	270 (40)	162 (21)	132 (42)	1.0
Atrazine	216 (58)	174 (18)	104 (28)	3.0
Azinphos ethyl	346 (37)	137 (25)	97 (32)	1.6
Azinphos methyl	318 (30)	132 (16)	261 (7)	1.6
Azinphos methyl oxon	324 (84)	132 (22)	148 (17)	15.5
Chlorfenvinphos[b]	359 (60)	170 (42)	99 (27)	1.6
Chlortoluron	213 (51)	140 (25)	104 (33)	3.2
Clothianidin[a]	250 (43)	169 (15)	132 (18)	1.2
Cyanazine	241 (59)	214 (18)	104 (30)	4.1
Desethylatrazine (DEA)	188 (66)	146 (18)	104 (25)	4.2
Deisopropylatrazine (DIA)	174 (58)	104 (23)	132 (18)	1.2
Diazinon	305 (64)	169 (22)	153 (22)	2.7
Dichlorvos[b]	221 (57)	109 (14)	145 (18)	6.9
Diflufenican	395 (60)	266 (24)	246 (34)	4.9
Dimethoate	230 (35)	125 (22)	157 (21)	11.0
Diuron[b]	233 (51)	160 (27)	133 (41)	1.2
Fenitrothion oxon	262 (66)	216 (19)	104 (22)	1.5
Fenthion	279 (63)	169 (20)	247 (13)	1.3
Fenthion oxon	263 (62)	231 (16)	216 (25)	2.3
Fenthion oxon sulfone	295 (74)	217 (20)	91 (34)	4.6
Fenthion oxon sulfoxide	279 (68)	264 (20)	262 (22)	6.2
Fenthion sulfone	311 (64)	125 (21)	233 (40)	3.0
Fenthion sulfoxide	295 (68)	280 (19)	109 (33)	1.5
Imidacloprid[a]	256 (51)	209 (20)	175 (20)	1.6
Irgarol[b] (cybutryne)	254 (57)	198 (19)	108 (30)	10.1
Isoproturon[b]	207 (51)	134 (23)	91 (37)	1.3
Linuron	249 (51)	160 (19)	133 (34)	1.2
Malaoxon	315 (48)	99 (23)	125 (33)	13.6
Malathion	353 (70)	227 (17)	306 (15)	4.3
Methiocarb[a]	266 (35)	169 (9)	121 (19)	1.3
Metolachlor	284 (48)	252 (16)	176 (26)	2.8
Molinate	188 (43)	126 (13)	98 (17)	4.1
Pendimethalin	282 (33)	212 (12)	194 (19)	7.4
Propanil	218 (53)	127 (27)	162 (16)	1.5
Quinoxyfen[b]	310 (102)	199 (34)	216 (36)	1.6
Simazine[b]	202 (61)	132 (20)	104 (25)	1.0
Terbuthylazine	230 (52)	174 (18)	104 (32)	6.1
Terbutryn[b]	242 (55)	186 (19)	158 (26)	14.2

Table 1 (continued)

Target pesticide	Precursor ion, m/z (RF Lens, V)	Product ion 1, m/z (CE, eV)	Product ion 2, m/z (CE, eV)	SRM1/ SRM2
Thiacloprid[a]	253 (59)	126 (23)	90 (35)	5.8
Thiamethoxam[a]	292 (47)	132 (24)	181 (23)	1.1
Thifensulfuron methyl	388 (57)	167 (18)	205 (27)	6.7
Triallate	304 (55)	142 (40)	83 (47)	1.7

[a] Compound included in the EU Watch List (2018/840/EC). [b] Compound included in the EU list of priority substances (2013/39/EC). *CE*, collision energy; *SRM*, selected reaction monitoring; *SRM1/SRM2*, peak area ratio

this and many other pesticides were not provided. Indeed, method performance was partially proven for only 10% of the target pesticides, which undermines results reliability [17].

All analytical approaches recently published to determine pesticides in sediments use matrix-matched calibration curves for quantification, except the one reported by Massei et al. [24]. This and the present method use isotopic labeled standards (ILS) for analyte quantification. While Massei et al. use 15 ILS to correct the response of 118 compounds, our method is the first one that uses isotopically labeled analogs for 85% of the 50 target pesticides. In the isotope dilution method, the ILS are added at a known concentration to the sample at the beginning of the extraction process and the analyte response is normalized to that of the corresponding ILS. Contrary to the matrix-matched calibration, which is also highly labor intensive, the isotope dilution method allows, as aforementioned, to correct potential analyte losses during sample preparation and matrix effects and signal drift during the MS analysis. This ensures extremely good precision and accuracy (see relative recoveries in Tables 2 and S3 in ESM) and hence reliability of the results.

As compared with the other analytical methods, the present one, despite being more labor demanding and time consuming than QuEChERS approaches, is highly effective in terms of removing matrix interferences.

Method sensitivity, with LOQs between 0.03 and 12.4 ng/g d.w. for most compounds, is similar to that reported in other LC-MS-based methods (see Table 3). The present method provides even lower LOQs for few compounds, namely acetamiprid, atrazine, fenthion oxon, fenthion oxon sulfoxide, irgarol, and terbutryn (< 0.1 ng/g d.w) as compared with previously published methods. Exceptionally low LOQs (0.1 and 0.01 ng/g) were reported by Kalogridi et al. [17]; however, as previously indicated, the method used in that study was not rigorously validated and few details were provided on LOQ calculation.

Application to real samples

The validated method was applied to the analysis of real sediment samples with a double objective: (i) to test its applicability and effectiveness and (ii) to assess the presence of the target pesticides in the lower Llobregat River basin (NE Spain). Only 5 out of the 50 compounds investigated, namely, terbutryn, dichlorvos, terbuthylazine, diazinon, and irgarol, were present in the seven sediment samples investigated (see Fig. 4). The most relevant compound in terms of abundance and ubiquity was the herbicide terbutryn that was found in all samples and showed a maximum concentration of 200 ng/g d.w. (Pt. 1). Terbutryn is an herbicide used as a pre-emergent and post-emergent control agent for grasses and broadleaf weeds in various cultivations (e.g., wheat, barley, sunflower, potatoes) and as an aquatic herbicide for the control of algae in water courses, reservoirs, and fish ponds. Previous studies already showed the presence of terbutryn at trace levels (5 ng/g d.w.) in sediment samples collected in the Llobregat River basin in 2011 [39], and in sediment samples from other river basins in Spain, like the Ebro River basin where it was found at a maximum concentration of 22 ng/g d.w. [40]. The results obtained in the present study reveal a significant increase of this pesticide in the Llobregat River sediment, in spite of the fact that the concentration of pollutants in this matrix may strongly depend on the season and the climatic conditions in which the samples are collected. Since terbutryn is one of the EU priority substances [8] with a maximum EQS of 34 ng/L in water, the evolution of its concentrations in the sediments of this basin should be regularly monitored.

The second most abundant pesticide detected in the lower Llobregat River basin sediments was the insecticide dichlorvos, present at a concentration of 44 ng/g d.w. in one sample (Pt. 1). This insecticide has various applications: insect control in the workplace and at home, in food-storage areas, greenhouses and vegetable crops; and parasite control in dogs, livestock, and humans. Dichlorvos is also considered as a priority substance in the EU, with a very low EQS (maximum allowable concentration of 0.07 ng/L in water). Although dichlorvos residues in sediments are not regulated, the concentration found in the present study exceeds by far the EQS in water. This insecticide was also reported to be present in sediments from the River Wuchuan in Southeast China (0.23 ng/g) [41] and in sediments from and around a highly eutrophic lake in Eastern China (up to 23.3 ng/g d.w.) [42].

Table 2 Method performance in terms of linearity (r^2), absolute and relative recoveries and repeatability at 50 ng/g, and sensitivity for the target pesticides in sediment

Analyte	Linearity (r^2)	Recovery[a]		Repeat.[b]	Sensitivity[c]	
		Abs. (%) 50 ng/g	Rel. (%) 50 ng/g	RSD (%) 50 ng/g	LOD ng/g d.w.	LODet ng/g d.w.
2,4-D	0.9901	34	99	20	0.59	1.97
Acetamiprid	0.9936	91	109	20	0.02	0.06
Alachlor	0.9917	49	95	4	0.59	1.57
Atrazine	0.9974	31	106	16	0.01	0.04
Azinphos ethyl	0.9901	99	108	7	2.19	12.4
Azinphos methyl	0.9916	22	88	10	10	15
Azinphos methyl oxon[x]	0.9928	81	96	5	0.09	0.32
Bentazone	0.9957	32	121	17	0.10	0.80
Bromoxynil	0.9959	32	86	3	11.6	25.6
Chlorfenvinphos	0.9945	49	83	4	0.57	1.9
Chlortoluron	0.9911	61	115	5	0.51	1.83
Clothianidin	0.9916	87	104	15	0.18	0.62
Cyanazine	0.9947	75	83	22	0.11	0.37
DEA	0.9990	48	89	21	0.04	0.14
DIA	0.9966	44	83	5	0.11	0.38
Diazinon	0.9949	69	76	3	0.03	0.10
Dichlorvos	0.9921	42	122	17	50	63.1
Diflufenican	0.9927	52	99	5	1.25	6.01
Dimethoate	0.9963	100	116	4	0.02	0.10
Diuron	0.9951	70	89	6	3.62	12.1
Fenitrothion	0.9847	24	117	13	2.18	7.26
Fenitrothion oxon	0.9948	22	88	14	48.6	62.2
Fenthion	0.9931	21	88	12	50	71.5
Fenthion oxon	0.9971	72	95	4	0.02	0.06
Fenthion oxon sulfone	0.9944	64	112	11	0.20	1.64
Fenthion oxon sulfoxide[x]	0.9963	24	85	10	0.02	0.07
Fenthion sulfone	0.9941	23	78	20	50	65.4
Fenthion sulfoxide	0.9911	31	86	20	0.20	0.68
Fluroxypyr ¤	0.9912	34	103	7	1.27	7.79
Imidacloprid	0.9915	74	89	17	0.06	0.17
Irgarol	0.9993	26	104	12	0.01	0.03
Isoproturon	0.9981	71	81	5	0.61	2.04
Linuron	0.9905	50	92	3	10.6	31.9
Malaoxon¤	0.9952	79	82	5	0.15	1.32
Malathion	0.9928	42	113	6	2.23	7.46
MCPA	0.9944	21	111	20	1.51	5.02
Mecoprop	0.9913	58	108	16	50	68.1
Methiocarb	0.9931	98	96	19	1.17	3.93
Metolachlor	0.9980	26	93	5	0.02	0.23
Molinate[δ]	0.9930	78	97	6	0.35	0.98
Pendimethalin	0.9878	23*	85*	17*	100	120
Propanil	0.9910	39	99	16	1.85	6.17
Quinoxyfen¤	0.9974	45	102	7	0.33	1.31
Simazine	0.9945	34	112	13	0.14	0.62
Terbuthylazine	0.9937	35	105	18	0.34	1.16

Table 2 (continued)

Analyte	Linearity (r^2)	Recovery[a]		Repeat.[b]	Sensitivity[c]	
		Abs. (%) 50 ng/g	Rel. (%) 50 ng/g	RSD (%) 50 ng/g	LOD ng/g d.w.	LODet ng/g d.w.
Terbutryn	0.9909	28	111	11	0.02	0.05
Thiacloprid	0.9927	106	117	19	0.03	0.13
Thiamethoxam	0.9990	76	120	9	0.11	0.33
Thifensulfuron methyl	0.9906	48	86	2	0.64	2.14
Triallate	0.9902	28*	85*	18*	100	121

[y] Compound quantified using fenthion sulfoxide-d_6 as surrogate standard

[x] Compound quantified using thiamethoxam-d_3 as surrogate standard

Compound quantified using mecoprop-d_3 as surrogate standard

Compound quantified using chlortoluron-d_6 as surrogate standard

[δ] Compound quantified using linuron-d_6 as surrogate standard

Compound quantified using methiocarb-d_3 as surrogate standard

[a] Average absolute recovery: comparison of peak areas obtained in $n = 6$ fortified samples and a methanol standard solution at equivalent concentrations. Average relative recovery: comparison of absolutes recoveries obtained for the analyte and its corresponding surrogate standard ($n = 6$)

[b] Repeatability: relative standard deviation of the relative recoveries observed in $n = 6$ fortified samples

[c] Sensitivity: limit of detection (LOD), estimated concentration that would result in a S/N of 3. LODet, limit of determination, minimum concentration at which the analyte can be quantified (LOQ of SRM1) and confirmed (LOD of SRM2). Calculated from sediment fortified samples at 10 ng/g, except for those compounds whose limits of detection were above 10 ng/g

*Values of recovery and repeatability calculated at 100 ng/g (analytes with LOD > 50 ng/g)

Irgarol, diazinon, and terbuthylazine were all found at trace levels (up to 0.2, 0.6, and 1.6 ng/g, respectively) in the investigated sediment samples. To the author's knowledge, this is the first time that irgarol, an algaecide used in antifouling paints for boats, is detected in Llobregat River sediments. However, since it was not previously investigated in this area, its previous presence cannot be discarded. The presence of diazinon and terbuthylazine in Llobregat River sediments has been studied in a few occasions, and trace levels of these pesticides were always found. In the case of diazinon, with past levels up to 4.6 ng/g d.w. [27, 29, 39], its presence (up to 0.6 ng/g in the present work) could be attributed to the proximity of the sampling locations to important urban zones (Barcelona and its metropolitan area), where diazinon could be extensively used for insect control. Terbuthylazine is an endemic pesticide used to control a broad spectrum of weeds, frequently found in the water of the Llobregat River basin [43, 1], while in sediment the only previous study conducted in this area [29] reported low levels (up to 1.4 ng/g d.w.) in the same range of those detected in the present study (up to 1.6 ng/g d.w.).

Environmental fate and ecotoxicity risk assessment

To better understand the fate of pesticides in the aquatic environment and the potential impact that they may pose to aquatic organisms, their physical-chemical properties need to be considered as they determine their mobility, adsorption and bioaccumulation capacity, and degradation, and hence their environmental concentrations. One of the most useful properties for assessing pesticide accumulation potential in organisms and adsorption potential onto particles is the octanol-water partition coefficient (K_{ow}). Compounds with high log K_{ow} values (> 3) have low affinity for water and are more readily sorbed onto particles. This process affects pesticide mobility in environmental systems [44]. Mobility of a pesticide is high if its solubility is high and its organic carbon-water partition coefficient (K_{oc}) is low. These properties, together with the degradation potential, expressed by the half-life (DT_{50}), determine the potential of a pesticide to move through soils and leach into groundwater (also assessed through the Groundwater Ubiquity Score (GUS) index [45]), or move by runoff to surface water bodies [46].

According to this, all pesticides detected in the sediment samples analyzed, except dichlorvos, present a log $K_{ow} > 3$ (see Table S1 in ESM) and can therefore potentially bioaccumulate in aquatic organisms. Many of the other pesticides investigated present also a high potential for accumulation (ESM Table S1), but they have not been detected, despite many of them (e.g., chlortoluron, diflufenican, isoproturon, linuron, and pendimethalin) have been largely applied in Catalonia. This could be explained by a distinct pattern of pesticide application in the investigated area compared with other Catalan regions. On the contrary, the non-detection of other pesticides extensively used also in the

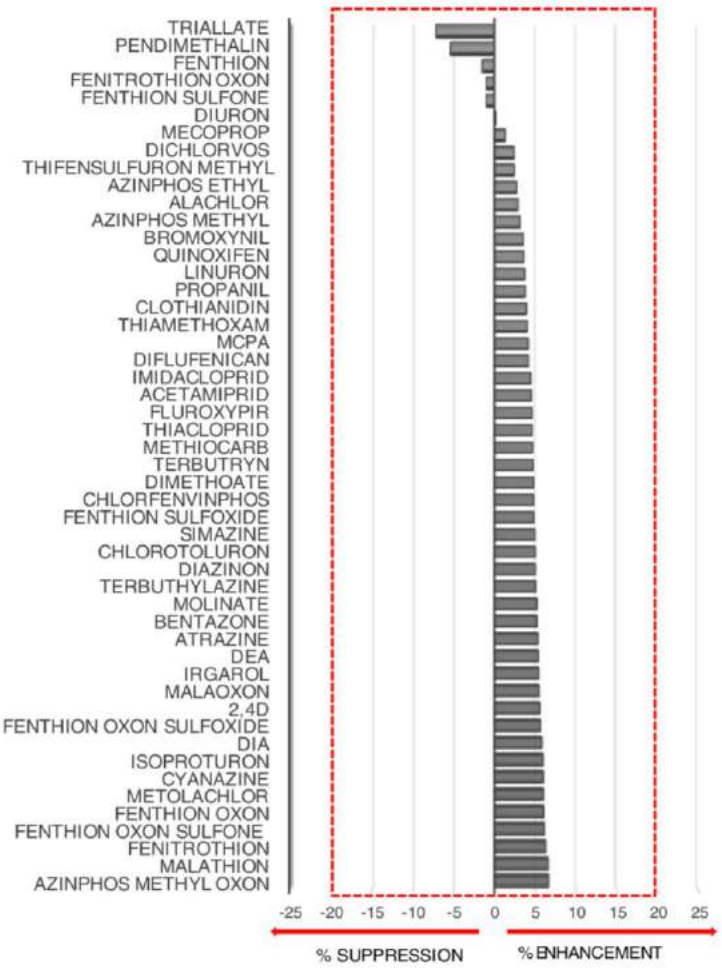

Fig. 3 Matrix effects calculated for the analysis of the target pesticides in sediment samples

investigated territory (i.e., 2,4-D, bentazone, bromoxynil, fluroxypyr, and MCPA) in the sediment samples analyzed could be attributed to their low log K_{ow} and high solubility.

Of all pesticides detected in the present study, terbuthylazine is the only one that exhibits a GUS index above 2.8 and is therefore likely to leach into groundwater [47]. Meanwhile, terbutryn and irgarol are the pesticides less likely to desorb from the sediments due to their high adsorption potential (with K_{oc} values of 2432 mL/g and 1569 mL/g, respectively) and low water solubility (25 mg/L in the case of terbutryn and 7 mg/L in the case of irgarol). Notwithstanding this, events such as changes in water flow or heavy rainfall and human activities could result in their eventual release in the water column.

Overall, the accumulation of pesticides in sediments can pose a direct toxicological risk for organisms living and feeding on river sediments due to chronic exposure. The potential ecotoxicity risk that the pesticides found may pose to aquatic organisms was assessed using the hazard quotient (HQ) approach. This approach compares the measured environmental concentrations (MEC) with predicted no-effect concentrations (PNEC) at which no toxic effects are expected. In this study, we used the maximum concentration measured for each pesticide in the investigated samples as MEC and the PNEC values in sediments ($PNEC_{sed}$) were extracted from the NORMAN Ecotoxicology Database. These values are derived from the corresponding PNECs of the pesticides in water (which are predicted by QSAR models or obtained experimentally) after applying an equilibrium partitioning approach [48]. According to this approach, pesticides showing HQ values < 1 are not considered hazardous for aquatic ecosystems, pesticides exhibiting HQ values between 1 and 10 are considered as potentially hazardous, and pesticides with HQ values > 10 are considered as the most hazardous to aquatic organisms.

Table 3 LC-MS-based methodologies published in the peer-reviewed literature since 2015 for the simultaneous determination of medium to highly polar pesticides in sediments

Number of pesticides	Analyte overlap	Extraction step		Extract purification[c]	Quantification method[f]	Accuracy (analyte recovery, %)	Precision, (RSD %)	Sensitivity (LOQ, ng/g)	Matrix effects	Reference
		Technique[a]	Solvent[b]							
50		PLE	Acet:DCM (1:1, 1% FA v/v)	SPE	Isotope dilution[d] (ILS for 85% of compounds)	21–99 absolute rec. 76–124 relative rec.	< 20	0.03–12.4 for 80% of analytes; 23–121 for the remaining 20%	± 10	This study
12	6	USE	MeOH:DCM (1:1)	None	Matrix-matched	12–125	< 26	3–10	± 30	[14]
17	7	MSPD	20 mL EtAc +5 mL ACN	None	Matrix-matched	61-100	< 0.73	0.23–4.26	Not provided	[15]
253[e]	31	MAE	ACN:hexane	8 g of Na_2SO_4	Not provided	67–123[e]	Not provided	0.009–0.072[e]	Not provided	[17]
50	27	QuEChERS	ACN	dSPE	Matrix-matched	39–120	< 25	0.1–15	0–250	[18]
18	7	QuEChERS	ACN (0.1% acetic acid)	dSPE	Matrix-matched	71–106	< 21	0.8–13	± 20	[20]
54	27	QuEChERS	7.5 mL H_2O + 10 mL ACN	None	Matrix-matched	59–113	< 20	0.1–2 ng/mL (LOD)	± 50	[21]
118	33	PLE	EtAc:Acet (1:1)+ Acet:FA 1% + MeOH:H_2O (9:1)	NPChrom or solvent exchange	Isotope dilution (15 ILS)	11–123 (overall)	< 20 (overall)	0.016–12.8	Not provided	[24]

[a] *MAE* microwave-assisted extraction, *MSPD* matrix solid phase dispersion extraction, *PLE* pressurized liquid extraction, *USE* ultrasonic solvent extraction

[b] *ACN* acetonitrile, *DCM* dichloromethane, *EtAC* ethyl acetate, *FA* formic acid, *MeOH* methanol

[c] *SPE* solid phase extraction, *dSPE* dispersive solid phase extraction, *NPChrom* normal phase chromatography

[d] Isotopically labeled analogs are available for 85% of the target compounds

[e] Data regarding method performance is provided for only 25 pesticides

[f] *ILS* isotopic labeled standards

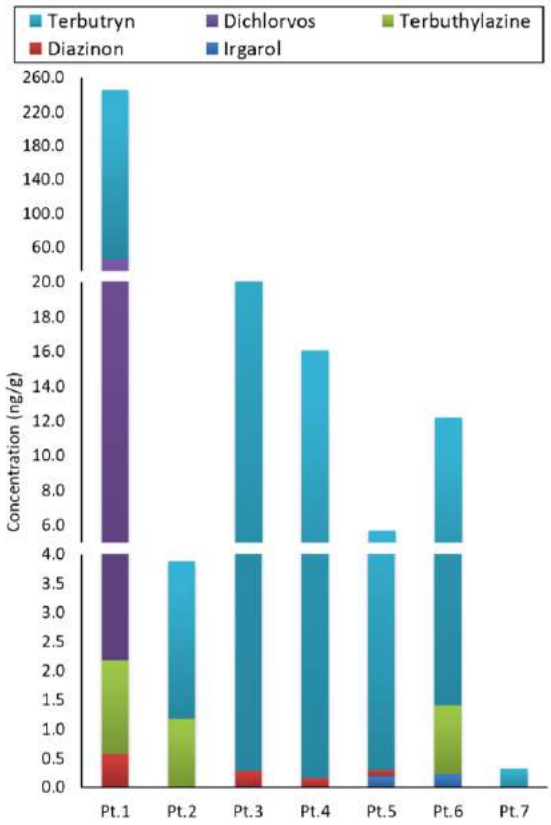

Fig. 4 Concentrations of pesticides found in sediment samples of the Llobregat River basin

HQ values calculated for the five pesticides detected in the sediments of the Llobregat River are shown in Table 4. In all cases, HQ values above 10 were obtained indicating high risk for aquatic organisms. This is due to the low PNEC values of the pesticides detected (< 0.1 ng/g) that result in HQ > 10 even at low environmental concentrations, as in the case of terbuthylazine. However, the extraordinarily high HQ values obtained for terbutryn (2000) and dichlorvos (45,250) are also explained by the relatively high levels of these pesticides measured in the sediments analyzed. These findings are of concern especially in the case of terbutryn because of its widespread use in the investigated area.

Table 4 Hazard quotient (HQ) values calculated for the pesticides detected in Llobregat River sediments

Analyte	MEC (ng/g)	$PNEC_{sed}$ (μg/kg)	HQ
Irgarol	0.2	0.005	42
Diazinon	0.6	0.016	35
Terbuthylazine	1.6	0.096	17
Dichlorvos	44	0.001	45,250
Terbutryn	200	0.1	2000

MEC maximum environmental concentration measured; $PNEC_{sed}$ values extracted from https://www.norman-network.com/nds/ecotox/ [48]

Conclusions

This study presents an analytical methodology based on PLE, SPE clean-up, and LC-HESI-MS/MS analysis for the determination of 50 moderately polar pesticides in sediment samples. The method was validated at three different concentration levels. Validation results indicate that the method is satisfactory in terms of sensitivity (with LODs below 4 ng/g d.w. and LODets below 12.4 for 40 of the 50 compounds), accuracy (with relative recoveries between 76 and 124% in all cases), and precision (with RSD nearly always below 20%). Besides, it is very effective in removing matrix interferences, which justifies the use of a long sample treatment approach that gets simplified and less labor intensive with the automation of the PLE process. Despite the fact that the described methodology presents similar sensitivity than previously published LC-MS-based methods, the use of an isotope dilution approach for quantification is a clear advantage over them, because results obtained in this way are highly reliable. This is the first time that azinphos-methyl oxon, fenitrothion oxon, and malaoxon are investigated in sediment samples.

The presented methodology allows evaluating a wide spectrum of pesticides belonging to many different classes and used for many different purposes, and due to its multi-residue character, the list of target analytes could be extended with additional pesticides belonging to these chemical classes covered in the current validated list. In this regard, evaluation of method performance of newly included pesticides is always recommended.

The application of the method to sediment samples collected at the lower Llobregat River basin revealed the presence of 5 pesticides, namely, terbutryn, dichlorvos, terbuthylazine, diazinon, and irgarol. All of them, but dichlorvos, present a high potential to sorb onto particles (log $K_{ow} > 3$). To the author's knowledge, this is the first evidence of the presence of irgarol in sediments of the Llobregat River. The presence of terbuthylazine in sediments is of concern, since it presents a high leaching potential into groundwater (GUS of 3.07) if released from the sediment. This pesticide has been continuously detected in the Llobregat River water since the 1990s, which may be attributed to its extended use for both urban and agricultural purposes. Likewise, the occurrence of terbutryn (low water solubility and high K_{oc}), which was the most ubiquitous and abundant compound, raises concern since sediments may act as a diffuse source of this pollutant into the water. According to the HQ approach, all 5 pesticides detected in the Llobregar River sediments pose an environmental risk for aquatic organisms

living and feeding on these sediments. The risk predicted is explained mainly by their low PNEC values, and in the case of terbutryn and dichlorvos also by the high levels detected. However, additional studies must be conducted to better understand the toxicity of pesticide-polluted sediments on aquatic communities.

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Compliance with ethical standards

This study did not involve any human participants or animals.

Conflict of interest The authors declare that they have no conflicts of interest.

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ELECTRONIC SUPPLEMENTARY MATERIAL

A reliable LC-MS/MS-based method for trace level determination of 50 medium to highly polar pesticide residues in sediments and ecological risk assessment

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List of Tables

List of Figures

Table S1. Target pesticides and main physical-chemical properties.

Analyte	Chemical class	Formula‡	Legislative status‡	MM (g mol^{-1})‡	Solubility (mg L^{-1}) ‡	K_{oc} (mL g^{-1}) ‡	K_{ow} logP‡	Henry´s (Pa m^3 mol^{-1}) ‡	GUS‡	DT50‡	Pka‡
2,4-D	Alkylchlorophenoxy	$C_8H_6Cl_2O_3$	✓	221.04	24300	39	-0.82	4.0×10^{-06}	1.69	4.4	3.40
Acetamiprid	Neonicotinoid	$C_{10}H_{11}ClN_4$	✓	222.67	2950	200	0.80	5.3×10^{-08}	0.40	1.6	0.7
Alachlor	Chloroacetamide	$C_{14}H_{20}ClNO_2$	X	269.77	240	335	3.09	3.2×10^{-03}	1.08	14	0.62
Atrazine	Triazine	$C_8H_{14}ClN_5$	X	215.68	35	100	2.70	1.5×10^{-04}	3.2	75	1.7
Azinphos-ethyl	Organophosphate	$C_{12}H_{16}N_3O_3PS_2$	X	345.38	4.5	1500	3.18	3.1×10^{-06}	1.4	50	n/a
Azinphos-methyl	Organophosphate	$C_{10}H_{12}N_3O_3PS_2$	X	317.32	28	1112	2.96	5.7×10^{-06}	1.42	10	5
Azinphos-methyl-oxon	Metabolite	$C_{10}H_{12}N_3O_4PS$	-	301.26	2604*	10 *	0.77*	6.2×10^{-13} *	-	-	n/a
Bentazone	Benzothiazinone	$C_{10}H_{12}N_2O_3S$	✓	240.30	7112	55	-0.46	7.2×10^{-05}	2.89	20	3.51
Bromoxinil	Hydroxybenzonitrile	$Br_2C_6H_2(OH)CN$	✓	276.90	38000	302	0.27	8.7×10^{-07}	0.03	1.04	3.86
Chlorfenvinphos	Organophosphate	$C_{12}H_{14}Cl_3O_4P$	X	359.60	145	680	3.80	1.4×10^{-01} *	1.83	40	n/a
Chlortoluron	Phenylurea	$C_{10}H_{13}ClN_2O$	✓	212.68	74	196	2.50	1.4×10^{-05}	3.02	45	n/a
Cyanazine	Triazine	$C_9H_{13}ClN_6$	X	240.69	171	190	2.10	6.6×10^{-06}	2.07	16	12.9
Clothianidin	Neonicotinoid	$C_6H_8ClN_5O_2S$	X	249.68	340	123	0.90	2.9×10^{-11}	4.91	545	11.1
Deisopropylatrazine	Metabolite	$C_5H_8ClN_5$	-	173.60	980	130	1.15	980	-	-	n/a
Desethylatrazine	Metabolite	$C_6H_{10}ClN_5$	-	187.63	2700	110	1.51	1.6×10^{-04}	4.37	2.23^	n/a
Diazinon	Organophosphate	$C_{12}H_{21}N_2O_3PS$	X	304.35	60	609	3.69	6.1×10^{-02}	1.14	9.1	2.6
Dichlorvos	Organophosphate	$C_4H_7Cl_2O_4P$	X	220.98	18000	50	1.90	2.6×10^{-02}	0.69	2	n/a
Diflufenican	Carboxamide	$C_{19}H_{11}F_5N_2O_2$	✓	394.29	0.05	5504	4.20	1.2×10^{-02}	1.51	94.5	n/a
Dimethoate	Organophosphate	$C_5H_{12}NO_3PS_2$	X	229.26	25900	25*	0.75	1.4×10^{-06}	1.01	2.5	n/a
Diuron	Phenylurea	$C_9H_{10}Cl_2N_2O$	✓	233.09	35.6	680	2.87	2.0×10^{-06}	1.83	146.6	n/a
Fenitrothion	Organophosphate	$C_9H_{12}NO_5PS$	X	277.23	19	2000	3.32	9.9×10^{-03}	0.48	2.7	n/a
Fenitrothion oxon	Metabolite	$C_9H_{12}NO_6P$	-	261.17*	301 *	21*	1.69*	4.0×10^{-1} *	-	-	n/a
Fenthion	Organophosphate	$C_{10}H_{15}O_3PS_2$	X	278.33	4.2	1500	4.84	2.4×10^{-02}	1.26	22	n/a
Fenthion oxon	Metabolite	$C_{10}H_{15}O_4PS$	-	262.26*	213.5*	57 *	2.31*	3.0×10^{-9} *	-	-	n/a
Fenthion oxon sulfone	Metabolite	$C_{10}H_{15}O_6PS$	-	294.03*	7602*	13*	0.28*	2.4×10^{-11} *	-	-	n/a
Fenthion oxon sulfoxide	Metabolite	$C_{10}H_{15}O_5PS$	-	278.26*	1222*	11*	0.15*	9.5×10^{-8} *	-	-	n/a
Fenthion sulfone	Metabolite	$C_{10}H_{15}O_5PS_2$	-	310.33*	190.4*	235	2.05*	1.1×10^{-8} *	-	-	n/a
Fenthion sulfoxide	Metabolite	$C_{10}H_{15}O_4PS_2$	-	294.33*	3.72*	183	1.92*	7.0×10^{-6} *	-	-	n/a
Fluroxypir	Pyridine compound	$C_7H_5Cl_2FN_2O_3$	✓	255.03	6500	10*	0.04	169×10^{-10}	2.42	13.1	2.94
Imidacloprid	Neonicotinoid	$C_9H_{10}ClN_5O_2$	✓	255.66	610	6719	0.57	1.7×10^{-10}	3.74	191	n/a

Irgarol	Triazine	$C_{11}H_{19}N_5S$	X	253.37	7	1569	3.95	1.3×10^{-07}*	-	-	n/a
Isoproturon	Phenyluera	$C_{12}H_{18}N_2O$	X	206.28	70.2	251*	2.5	1.5×10^{-05}	2.07	12	n/a
Linuron	Phenyluera	$C_9H_{10}Cl_2N_2O_2$	X	249.09	63.8	843	3	2.0×10^{-04}	2.21	57.6	n/a
Malaoxon	Metabolite	$C_{10}H_{19}O_7PS$	-	314.29*	7500*	4650*	0.52*	1.2×10^{-08} *	-	-	n/a
Malathion	Organophosphate	$C_{10}H_{19}O_6PS_2$	✓	330.36	148	1800	2.75	1.0×10^{-03}	-1.28	0.17	n/a
MCPA	Organophosphate	$C_9H_9ClO_3$	✓	200.62	29390	29*	-0.81	5.5×10^{-05}	2.94	24	3.73
Mecoprop	Aryloxyalkanoic acid	$C_{10}H_{11}ClO_3$	X	214.65	250000	47	-0.19	2.2×10^{-04}	2.29	8.2	3.11
Methiocarb	Carbamate	$C_{11}H_{15}NO_2S$	✓	225.31	27	182*	3.18	1.2×10^{-04}	0.55	2.94	n/a
Metolachlor	Chloroacetamide	$C_{15}H_{22}ClNO_2$	X	283.80	530	120	3.40	2.4×10^{-03}	2.10	90	n/a
Molinate	Thiocarbamate	$C_9H_{17}NOS$	X	187.30	1100	190	2.86	6.9×10^{-01}	2.49	28	n/a
Pendimethalin	Dinitroaniline	$C_{13}H_{19}N_3O_4$	✓	281.31	0.33	17491	5.40	2.7×10^{-03}	-0.32	182.3	2.8
Propanil	Anilide	$C_9H_9Cl_2NO$	X	218.08	95	149	2.29	4.4×10^{-04}	-0.51	0.4	19.1
Quinoxyfen	Quinoline	$C_{15}H_8Cl_2FNO$	X	308.13	0.05	23°	4.66	3.2×10^{-02}	-0.93	308	n/a
Simazine	Triazine	$C_7H_{12}ClN_5$	X	201.66	5	130	2.30	5.6×10^{-05}	2	60	1.62
Terbuthylazine	Triazine	$C_9H_{16}ClN_5$	✓	229.71	6.6	329*	3.40	3.2×10^{-03}	3.07	72	1.9
Terbutryn	Triazine	$C_{10}H_{19}N_5S$	X	241.36	25	2432	3.66	1.5×10^{-03}	2.4	74	4.3
Thiacloprid	Neonicotinoid	$C_{10}H_9ClN_4S$	✓	252.72	184	615°	1.26	5.0×10^{-10}	0.14	15.5	n/a
Thiamethoxam	Neonicotinoid	$C_8H_{10}ClN_5O_3S$	X	291.71	4100	56	-0.13	4.7×10^{-10}	4.69	50	n/a
Thifensulfuron methyl	Sulfonylurea	$C_{12}H_{13}N_5O_6S_2$	✓	387.39	54.1	28	-1.65	3.3×10^{-08}	0.44	1.39	4
Triallate	Thiocarbamate	$C_{10}H_6Cl_3NOS$	✓	304.7	4.1	3034	4.06	0.89	0.69	82	n/a

‡ The PPDB, Pesticide Properties Database. http://sitem.herts.ac.uk/aeru/footprint/index2.htm. - Lewis, K.A., Tzilivakis, J., Warner, D. and Green, A. (2016). An international database for pesticide risk assessments and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064.

*Data estimated using the US Environmental Protection Agency EPISuite™ http://www.Chemspider.com.

° Kegley, S.E., Hill, B.R., Orme S., Choi A.H., PAN Pesticide Database, Pesticide Action Network, North America (Oakland, CA, 2016), http://www.pesticideinfo.org.

∞ EU pesticides database. https://bit.ly/1oxd00K.

^ Calculated using the mathematical formula: GUS = log10 (half-life) x [4 - log10 (Koc)].

MM: molecular mass; Solubility: solubility in water at 20 °C ; K_{oc}: organic carbon partition coefficient; K_{ow}: octanol-water partition coefficient; Henry's: Henry's law constant at 25ºC; GUS: leaching potential index; DT50: soil degradation potential, expressed as *half-life* in days; Pka: dissociation constant at 25 °C; n/a: data not available.

Table S2. MS/MS analysis of the surrogate standards (isotopically labeled analogs were used for all analytes except 6).

Surrogate standards	Precursor ion, m/z (RF Lens, V)	Product ion, m/z (CE, eV)
Negative ionization mode (-)		
2,4-D-d_3	244 (32)	166 (17)
Bentazone-d_6	245 (79)	132 (29)
Bromoxynil-$^{13}C_6$	282 (90)	79 (30)
Fenitrothion-d_3	265 (51)	153 (22)
MCPA-d_3	204 (38)	146 (18)
Mecoprop-d_6	218 (38)	147 (18)
Positive ionization mode (+)		
Acetamiprid-d_3	226 (55)	126 (22)
Alachlor-d_{13}	283 (45)	251 (11)
Atrazine-d_5	221 (59)	179 (18)
Azinphos-ethyl-d_{10}	356 (39)	138 (25)
Azinphos methyl-d_6	324 (43)	132 (16)
Chlorfenvinphos-d_{10}	369 (58)	170 (41)
Chlortoluron-d_6	219 (58)	78 (19)
Clothianidin-d_3	253 (45)	172 (15)
Cyanazine-d_5	246 (59)	219 (18)
DEA-d_6	194 (59)	147 (20)
DIA-d_5	179 (57)	137 (18)
Diazinon-d_{10}	315 (67)	170 (23)
Dichlorvos-d_6	227 (69)	115 (19)
Diflufenican-d_3	398 (84)	268 (25)
Dimethoate-d_6	236 (44)	131 (23)
Diuron-d_6	239 (59)	160 (28)
Fenitrothion oxon-d_6	268 (66)	222 (19)
Fenthion-d_6	285 (62)	169 (20)
Fenthion oxon-d_3	266 (69)	234(17)
Fenthion oxon sulfone-d_3	298 (77)	218 (20)
Fenthion sulfone-d_6	317 (72)	131 (17)
Fenthion sulfoxide-d_6	301 (53)	286 (18)
Imidacloprid-d_5	261 (44)	214 (19)
Irgarol-d_9	263 (60)	199 (19)
Isoproturon-d_6	213 (53)	134 (23)
Linuron-d_6	255 (48)	185 (18)
Malathion-d_{10}	363 (70)	237 (18)
Methiocarb-d_3	229 (30)	169 (10)
Metolachlor-d_{11}	295 (48)	263 (17)
Pendimethalin-d_5	287 (43)	213 (12)
Propanil-d_5	223 (59)	128 (28)
Simazine-d_{10}	212 (63)	137 (21)
Terbuthylazine-d_5	235 (55)	179 (18)
Terbutryn-d_5	247 (59)	191 (19)
Thiacloprid-d_4	257 (60)	126 (23)
Thiamethoxam-d_3	295 (43)	214 (13)
Thifensulfuron methyl-d_3	391 (64)	167 (18)
Triallate-$^{13}C_6$	310 (55)	143 (29)

Table S3. Method performance in terms of absolute and relative recoveries and repeatability at 10 ng/g and 100 ng/g.

Analyte	Recovery[a]				Repeatability[b]	
	Absolute (%)		Relative (%)		RSD (%)	
	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g
2,4-D	31	53	114	115	10	13
Acetamiprid	76	102	116	113	13	19
Alachlor	46	64	113	94	10	14
Atrazine	62	31	116	97	2	20
Azinphos ethyl	57	73	87	99	12	3
Azinphos methyl	22	31	82	99	14	6
Azinphos methyl oxon[y]	102	102	111	104	12	19
Bentazone	24	31	80	103	8	18
Bromoxynil	BLOD	68	BLOD	87	-	11
Chlorfenvinphos	64	57	107	82	17	12
Chlorotoluron	83	67	92	88	15	7
Clothianidin	72	74	117	87	4	19
Cyanazine	99	109	100	111	15	15
DEA	39	61	90	92	19	11
DIA	64	45	113.3	108	2	8
Diazinon	52	81	114	85	18	14
Dichlorvos	BLOD	67	BLOD	89	-	4
Diflufenican	71	69	92	120	13	6
Dimethoate	105	94	120	102	2	19
Diuron	55	57	91	106	12	4
Fenitrothion	30	20	81	86	8	16
Fenitrothion oxon	BLOD	20	BLOD	76	-	18
Fenthion	BLOD	26	BLOD	81	-	17
Fenthion oxon	68	54	119	119	6	7
Fenthion oxon sulfone	99	89	121	103	1	18
Fenthion oxon sulfoxide[x]	55	51	89	105	1	4
Fenthion sulfone	BLOD	26	BLOD	90	-	16
Fenthion sulfoxide	33	22	87	93	10	13
Fluroxypyr[z]	27	46	121	88	24	19
Imidacloprid	84	88	98	98	14	15
Irgarol	39	24	93	88	7	4
Isoproturon	94	81	118	87	2	13
Linuron	BLOD	50	BLOD	102	-	12
Malaoxon[s]	102	75	111	78	1	14
Malathion	53	33	78	92	4	19
MCPA	26	34	81	97	6	5
Mecoprop	BLOD	42	BLOD	124	-	16
Methiocarb	110	88	87	117	17	16
Metolachlor	21	25	107	86	17	4
Molinate[δ]	88	77	88	84	8	4
Pendimethalin	BLOD	23	BLOD	85	-	17
Propanil	BLOD	41	BLOD	88	-	17
Quinoxyfen[ω]	46	38	82	110	3	7
Simazine	57	62	96	85	10	18
Terbuthylazine	54	40	117	92	15	11
Terbutryn	20	46	106	99	5	15
Thiacloprid	87	67	104	87	12	8
Thiamethoxam	96	85	120	96	12	12
Thifensulfuron methyl	68	56	120	86	5	13
Triallate	BLOD	28	BLOD	85	-	18

[y] *fenthion sulfoxide-d_6* used as surrogate standard; [x] *thiamethoxam-d_3* used as surrogate standard; [z] *mecoprop-d_3* used as surrogate standard; [s] *chlortoluron-d_6* used as surrogate standard; [δ] *linuron-d_6* used as surrogate standard [ω] *methiocarb-d_3* used as surrogate standard. BLOD: below limit of detection

[a] Average absolute recovery: comparison of peak areas obtained in n=6 fortified samples and a methanol standard solution at equivalent concentrations. Average relative recovery: comparison of absolutes recoveries obtained for the analyte and its corresponding surrogate standard (n=6). [b] Repeatability: relative standard deviation of the relative recoveries observed in n=6 fortified samples.

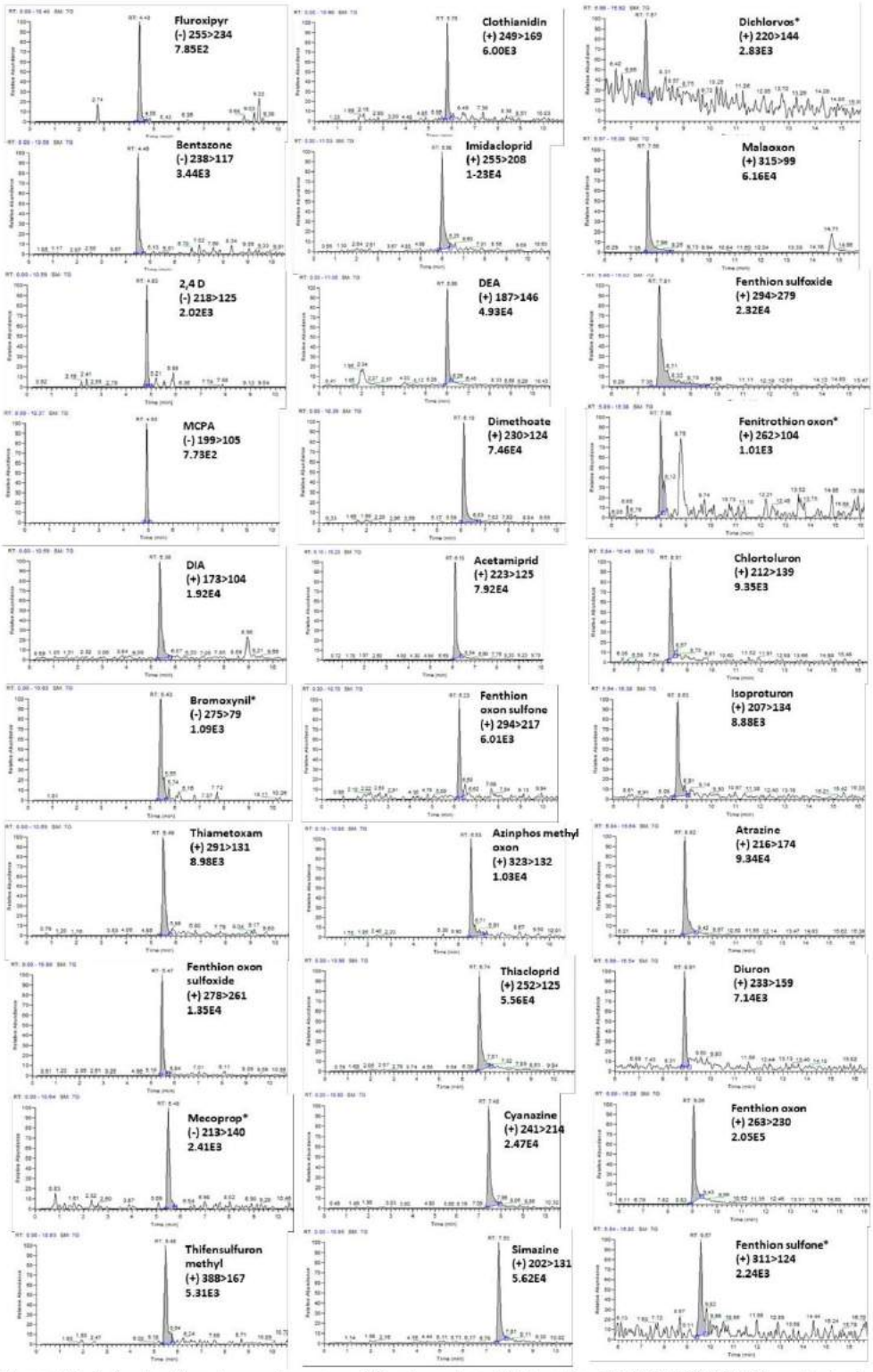

Figure S1. Extracted ion chromatograms of the target pesticides after PLE-SPE-LC-MS/MS analysis of a sediment sample fortified at a concentration of 10 ng/g (*50 ng/g for those compounds with LOD above 10 ng/g; **100 ng/g for those compounds with LOD above 50 ng/g).

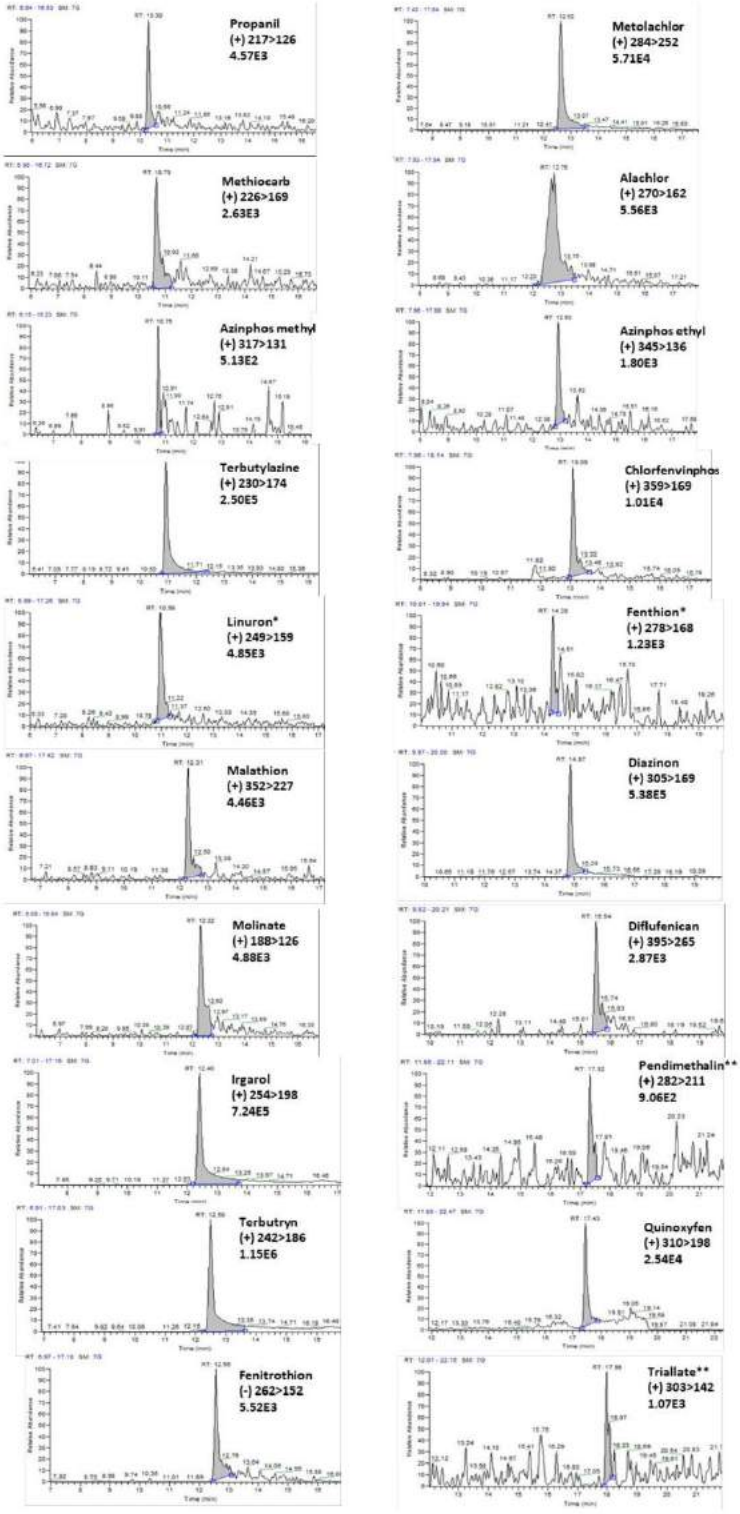

Figure S1. (continued).

3.3 Analysis of medium to highly polar pesticides and metabolites in biota

As for water and sediment, the environmental pollution also affects aquatic organisms, which may bioaccumulate pesticides and may be affected by their toxicity. Knowledge on the contamination of biota by pesticides is very important for their regulation and to guarantee the protection of the environment and public health. The EFSA has set MRLs for pesticides in 315 food products. However, fish products are not covered. This may be one of the reasons why the presence of pesticides in fish has not been extensively studied, but also the complexity of this matrix and the difficulty of developing efficient extraction and clean-up protocols for analysis may have contributed to this. The QuEChERS technique has recently gained attention for the extraction of organic compounds from complex matrices such as fish or vegetables, but there are only a few works available in the peer-reviewed literature for the analysis of medium to highly polar pesticides in fish.

The objective of the scientific publication #3 was the development and validation of an analytical method based on QuEChERS extraction and LC-MS/MS analysis for the determination of 52 pesticides in fish.

The methodology brings novelties compared to the previous ones because it targets 12 pesticides, including 3 TPs (i.e., 2,4-D, azinphos-methyl oxon, bentazone, bromoxynil, clothianidin, fenitrothion oxon, malaoxon, MCPA, mecoprop, oxadiazon, thifensulfuron-methyl, and triallate) never investigated before in fish samples, as well as other improvements in terms of analytical performance.

The validated methodology was applied to the analysis of pesticides in fish samples from the Adige River Basin (Italy), a river highly impacted by agricultural activity and hydropeaking for hydroelectricity production. This study provided for the first time a picture of the occurrence and fate of some of the analyzed pesticides in this matrix.

This work was part of the GLOBAQUA (EU Seventh Framework Programme (FP7), No. 265264) and SOLUTIONS (EU FP7, No. 603437) projects. GLOBAQUA aimed at identifying the prevalence of, and interaction between, stressors under water scarcity to improve knowledge of relationships between multiple stressors and to improve water

management practices and policies. SOLUTIONS aimed at producing consistent solutions for a large number of legacy, present and future emerging chemicals posing a risk to European water bodies with respect to ecosystems and human health.

Scientific publication #3:

"Analysis of 52 pesticides in fresh fish muscle by QuEChERS extraction followed by LC-MS/MS determination"

Maria Vittoria Barbieri, Cristina Postigo, Nuria Guillem-Argiles, Luis Simón Monllor-Alcaraz, Julianne Isabel Simionato, Elisa Stella, Damià Barceló, Miren López de Alda

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Analysis of 52 pesticides in fresh fish muscle by QuEChERS extraction followed by LC-MS/MS determination

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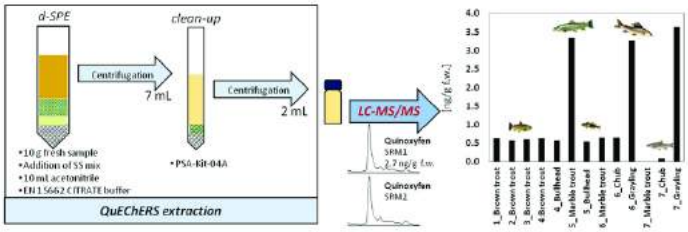
HIGHLIGHTS

- QuEChERS extraction and LC-MS/MS analysis for determining 52 pesticides in fish
- Satisfactory method performance according to SANTE guidelines for 44 pesticides
- Main advantages of the method: simplicity, high throughput, reliability of results
- Twelve pesticides investigated for the first time in fresh fish muscle

GRAPHICAL ABSTRACT



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ABSTRACT

Pesticide pollution in water has been well described; however, little is known on pesticide accumulation by aquatic organisms, and to date, most studies in this line have been focused on persistent organochlorine pesticides. For this reason, a method based on QuEChERS extraction and subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis has been developed and validated for the determination of 52 medium to highly polar pesticides in fresh fish muscle. Target pesticides were selected on the basis of use and occurrence in surface waters. Quantification is carried out following an isotope dilution approach. The method developed is satisfactory in terms of accuracy (relative recoveries between 71 and 120%), precision (relative standard deviations below 21%) and sensitivity (limits of determination in the pg/g or low ng/g f.w. range for most compounds). The application of the validated methodology to fish specimens collected from the Adige River (Italy) revealed the presence of trace levels of diazinon, dichlorvos and diuron, and measurable levels of metolachlor, quinoxyfen, irgarol, terbutryn, and acetamiprid, but in all cases at concentrations below the default maximum residue level of 10 ng/g established for pesticides not specifically regulated in fish intended for human consumption. Metolachlor and quinoxyfen were both the most ubiquitous and abundant pesticides, in agreement with their high potential for bioaccumulation. Both are toxic to aquatic organisms, and therefore, their potential effects on aquatic ecosystems should be further explored.

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1. Introduction

Pesticides are among the most used chemical substances worldwide, with an annual production of over 3 million tons (Roser and Ritchie, 2017). Their use in agriculture has allowed increasing the quality and the quantity of food production. However, regardless of their merits, they have been appointed as some of the most toxic substances in the environment and consequently represent a risk for ecosystems and human health (Furio et al., 2015). For this reason, and based on the available information, the protection of water resources and aquatic ecosystems from pesticide pollution has motivated the adoption of several regulatory measures. For instance, residues of selected pesticides that are considered priority substances in the environment must be strictly controlled in European water bodies and biota, so that their levels remain below established environmental quality standards (EQS) (EC, 2013).

Research on the environmental occurrence of medium to highly polar pesticides has been very much focused in the water compartment while PBT (persistent, bioaccumulative, and toxic) pesticides have been usually targeted in biota due to their high octanol-water partition coefficients (K_{ow}) and hence capacity to partition into lipids. However, ionizable and ionic pesticides, despite their low K_{ow} values, are also likely to bioaccumulate in aquatic organisms via ion specific sorption mechanisms. The knowledge on the bioaccumulation potential of this type of pesticides is nowadays very limited but essential for proper risk assessment and pesticide regulation. To gain insights in this respect pharmacokinetic models and novel screening tools for prediction of sorption of ionic chemicals in fish according to their physical-chemical properties are being developed (Bittermann et al., 2018). Moreover, analytical methods to study the concentrations of medium to highly polar pesticides in biota need also to be available so that the fate of these chemicals in different aquatic organisms can be evaluated and the aforementioned models validated.

Besides keeping pesticide residues in the environment low, protection of public health also requires controlling pesticide residues in food or feed. This has been achieved through the establishment of maximum residue levels (MRL), i.e., the highest pesticide levels legally tolerated after their correct application in food products (EC, 2005). MRLs set for the different pesticides by the European Food Safety Authority (EFSA) take into account the toxicity of the compound, the maximum levels expected on food, and the different diets in Europe. Such standards have been set for pesticides currently in use or used in the past for food production in or outside the European territory and for 315 food products in total. However, MRLs of pesticides do not exist for fish products despite the fact that these organisms may be exposed to trace pesticide concentrations continuously released into the aquatic environment. In this case, and in any other case where a pesticide is not specifically mentioned, a general default MRL value of 10 ng/g can be applied. Determination of pesticide residues in biota or in any other high fat content food sample, expected to contain low ng/g levels, requires the development of advanced multi-residue analytical methods with high sensitivity and selectivity instrumental technologies, such as gas chromatography (GC) or liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) (Picó, 2016; Villaverde et al., 2016). Because of the complexity of these matrices, efficient extraction and clean-up protocols are also needed. For analyte extraction, approaches based on Soxhlet extraction, solid-liquid extraction, pressurized solvent extraction (PSE) (Choi et al., 2016; Chung and Chen, 2011), or microwave assisted extraction (MAE) have been used (Chung and Chen, 2011; LeDoux, 2011). Solid phase extraction (SPE) has usually been the method of choice for clean-up of the extracts (Chung and Chen, 2011; LeDoux, 2011; Santhi et al., 2012). Many analytical approaches have also included as a first step a lipid removal approach so that fatty interferents were not co-extracted (Chung and Chen, 2011).

In recent years, extraction with QuEChERS (quick, easy, cheap, effective, rugged and safe) has received increasing use for preparation of complex samples like fish (Baduel et al., 2015; Belenguer et al., 2014; Farré et al., 2014; Kaczyński et al., 2017; Lazartigues et al., 2011; Morrison et al., 2016; Nácher-Mestre et al., 2014; Portolés et al., 2017). This could be attributed to the advantages that QuEChERS offer over traditional extraction methods, such as high analyte recoveries, accurate results, fast sample treatment, little use of solvent, and small lab-space and equipment requirements. However, as aforementioned, only a few methodologies are currently available for the analysis of medium to highly polar pesticides in fish samples.

In this context, this work presents a multi-residue target analytical method based on a QuEChERS extraction approach and LC-MS/MS analysis for the determination of 52 pesticides in fresh fish muscle. Selection of target pesticides was made based on their feasibility for LC-MS analysis, their environmental relevance in terms of being considered as priority substances (EC, 2013) or included in the European Watch List (EC, 2018), their occurrence in surface water, and their extent of use at European level (Eurostat, 2007). The list of investigated compounds contains ten pesticide transformation products. The compounds covered belong to many different chemical classes and present a very wide range of physical-chemical properties (K_{ow}, pK_a, water solubility, etc.). The list includes twelve organophosphates, six triazines, four phenylureas, two chloroacetamides, five neonicotinoids, four acidic pesticides, and nine compounds belonging to other chemical classes. This represents an analytical challenge in terms of developing a single multi-residue method for all of them.

To the authors' knowledge, previous works that investigate highly to medium polar pesticides in fish samples within the last decade (Baduel et al., 2015; Belenguer et al., 2014; Ernst et al., 2018; Farré et al., 2014; Franco-Barrios et al., 2014; Kaczyński et al., 2017; Kaonga et al., 2015; Lazartigues et al., 2011; Morrison et al., 2016; Nácher-Mestre et al., 2014; Portolés et al., 2017; Shin, 2006; Vorkamp et al., 2014; Xiao et al., 2013) did not cover 23% of the pesticides selected in this study (i.e., 2,4 D, azinphos-methyl oxon, bentazone, bromoxynil, clothianidin, fenitrothion oxon, malaoxon, MCPA, mecoprop, oxadiazon, thifensulfuron-methyl, and triallate). In addition, the analytical methods used in these studies were in a few cases qualitative, developed for wide-scope pesticide screening (Nácher-Mestre et al., 2014; Portolés et al., 2017), and in other cases quantitative approaches that either used matrix-matched calibration curves (Belenguer et al., 2014; Farré et al., 2014; Lazartigues et al., 2011) or a few deuterated compounds as surrogate standards (Kaczyński et al., 2017; Lazartigues et al., 2011; Xiao et al., 2013) for quantification. In contrast, the analytical method proposed in the present study is based on the isotope dilution quantification method, which ensures the reliability of the results. Finally, the application of the method to real fish samples proved its suitability for the analysis of these compounds in this matrix and provided a first picture on the occurrence of some of the investigated pesticides in fish tissues.

2. Materials and methods

2.1. Chemicals and reagents

High purity (96–99.9%) standards of 52 pesticides and 45 isotopically-labeled compounds used as surrogate standards for quantification were purchased from Fluka (Sigma–Aldrich, Steinheim, Germany) or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). Table 1 lists the target analytes, whereas relevant physical-chemical properties (average molecular mass, solubility, and K_{ow} among others) are provided in Table S1 as Supporting information (SI). Stock standard solutions of the individual analytes were prepared in methanol (MeOH) (dimethyl sulfoxide in the case of simazine) and stored in amber glass bottles in the dark at −20 °C. Working methanolic solutions at different concentrations (between 0.01 and 1000 ng/mL) containing all analytes were prepared by appropriate dilution of the stock individual solutions. They were used to construct calibration curves and as spiking solutions

Table 1
LC-MS/MS conditions for target pesticides and surrogate compounds (in italics).

Target analyte	Abbrev.	RT (min)	HESI mode	SRMs (m/z)	RF lens (V)	CE (eV)	SRM1/SRM2
Fluroxypyr	FLUXP	4.3	−	255 > 197	33	16	2.7
				255 > 235		6	
Bentazone	BEN	4.4	−	239 > 132	68	28	20.3
				239 > 117		33	
	BEN-d_6	*4.4*	−	*245 > 132*	*79*	*29*	–
2,4 Dichloro phenoxy acetic acid	2,4D	4.9	−	219 > 162	35	16	20.1
				219 > 125		28	
	2,4D-d_3	*4.9*	−	*224 > 166*	*32*	*17*	–
4 Chloro 2 methylphenoxy acetic acid	MCPA	5.1	−	199 > 142	38	17	380
				199 > 105		30	
	MCPA-d_3	*5.0*	−	*204 > 146*	*38*	*18*	–
Deisopropylatrazine	DIA	5.2	+	174 > 104	58	23	2.3
				174 > 132		18	
	DIA-d_5	*5.1*	+	*179 > 137*	*57*	*18*	–
Thiamethoxam[a]	THIAM	5.3	+	292 > 132	47	24	1.1
				292 > 181		23	
	THIAM-d_3	*5.3*	+	*295 > 214*	*43*	*13*	–
Fenthion oxon sulfoxide	FNOSX	5.3	+	279 > 264	68	20	4.2
				279 > 262		22	
Mecoprop	MECO	5.6	−	213 > 142	39	17	2.9
				213 > 140		17	
	MECO-d_6	*5.6*	−	*218 > 147*	*38*	*18*	–
Clothianidin[a]	CLOTD	5.7	+	250 > 169	43	15	2.2
				250 > 132	47	18	
	CLOTD-d_3	*5.6*	+	*253 > 172*	*45*	*15*	–
Imidacloprid[a]	IMID	5.8	+	256 > 209	51	20	1.1
				256 > 175		20	
	IMID-d_5	*5.8*	+	*261 > 214*	*44*	*19*	–
Desethylatrazine	DEA	5.8	+	188 > 146	66	18	5.2
				188 > 104		25	
	DEA-d_6	*5.8*	+	*194 > 147*	*59*	*20*	–
Thifensulfuron methyl	THIFEN	6.0	+	388 > 167	57	18	6.2
				388 > 205	54	27	
	THIFEN-d_3	5.9	+	391 > 167	64	18	–
Dimethoate	DIME	6.0	+	230 > 125	35	22	13.8
				230 > 157		21	
	DIME-d_6	*5.9*	+	*236 > 131*	*44*	*23*	–
Acetamiprid[a]	ACET	6.0	+	223 > 126	53	22	5.5
				223 > 90		33	
	ACET-d_3	*6.0*	+	*226 > 126*	*55*	*22*	–
Bromoxynil	BROMX	6.0	−	276 > 81	82	30	1.1
				276 > 79		30	
	BROMX $^{13}C_6$	6.2	−	282 > 79	90	30	–
Fenthion oxon sulfone	FENOXS	6.1	+	295 > 217	74	20	9.9
				295 > 91		34	
	FENOXS d_3	6.1	+	298 > 218	77	20	
Azinphos methyl oxon	AZMO	6.4	+	324 > 132	84	22	17.1
				324 > 148		17	
Thiacloprid[a]	THIAC	6.6	+	253 > 126	59	23	6.8
				253 > 90		35	
	THIAC-d_4	6.6	+	257 > 126	60	23	–
Cyanazine	CIANZ	7.3	+	241 > 214	59	18	6.2
				241 > 104		30	
	CIANZ-d_5	*7.3*	+	*246 > 219*	*59*	*18*	–
Simazine[b]	SIMAZ	7.4	+	202 > 132	61	20	1.2
				202 > 104		25	
	SIMAZ-d_{10}	*7.3*	+	*212 > 137*	*63*	*21*	–
Dichlorvos[b]	DICV	7.4	+	221 > 109	57	14	11.4
				221 > 145		18	
	DICV-d_6	*7.3*	+	*227 > 115*	*69*	*19*	–
Fenthion sulfoxide	FENSOX	7.5	+	295 > 280	68	19	2.0
				295 > 109		33	
	FENSOX-d_6	*7.5*	+	*301 > 286*	*53*	*18*	–
Malaoxon	MALOX	7.5	+	315 > 99	48	23	16.4
				315 > 125		33	
Fenitrothion oxon	FENTOX	7.8	+	262 > 216	66	19	1.2
				262 > 104		22	
	FENTOX-d_6	*7.8*	+	*268 > 222*	*66*	*19*	–
Chlortoluron	CHLOR	8.1	+	213 > 140	51	25	5.0
				213 > 104		33	
	CHLOR-d_6	*8.0*	+	*219 > 78*	*58*	*19*	–
Isoproturon[b]	ISOPR	84	+	207 > 134	51	23	1.9
				207 > 91		37	
	ISOPR-d_6	*8.4*	+	*213 > 134*	*53*	*23*	–
Atrazine	ATRZ	8.7	+	216 > 174	58	18	4.6
				216 > 104		28	
	ATRZ-d_5	*8.6*	+	*221 > 179*	*59*	*18*	–

Table 1 (*continued*)

Target analyte	Abbrev.	RT (min)	HESI mode	SRMs (*m/z*)	RF lens (V)	CE (eV)	SRM1/SRM2
Diuron[b]	DIUR	8.7	+	233 > 160	51	27	1.4
				235 > 133		41	
	DIUR-d_6	*8.7*	+	*239 > 160*	*59*	*28*	–
Fenthion oxon	FENOX	8.9	+	263 > 231	62	16	2.3
				263 > 216		25	
	FENOX-d_3	8.8	+	266 > 234	69	17	–
Fenthion sulfone	FENS	9.4	+	311 > 125	64	21	3.0
				311 > 233		40	
	FENS-d_6	*9.3*	+	*317 > 131*	*72*	*17*	–
Propanil	PROP	10.1	+	218 > 127	53	27	1.0
				218 > 162		16	
	PROP-d_5	10.1	+	223 > 128	59	28	–
Methiocarb[a]	METCB	10.4	+	226 > 169	35	9	1.4
				226 > 121		19	
	METCB-d_3	*10.3*	+	*229 > 169*	*30*	*10*	–
Terbuthylazine	TERBZ	10.6	+	230 > 174	52	18	9.1
				230 > 104		32	
	TERBZ-d_5	*10.5*	+	*235 > 179*	*55*	*18*	–
Azinphos methyl	AZM	10.6	+	318 > 132	30	16	1.3
		10.6	+	318 > 261	30	7	
	AZM-d_6	*10.4*	+	*324 > 132*	*43*	*16*	–
Linuron	LINU	10.8	+	249 > 160	51	19	1.6
				249 > 133		34	
	LINU-d6	*10.7*	+	*255 > 185*	*48*	*18*	–
Molinate	MOLI	12.0	+	188 > 126	43	13	6.6
				188 > 98		17	
Irgarol[b] (Cybutryne)	IRGA	12.1	+	254 > 198	57	19	15.9
				254 > 108		30	
	IRGA-d_9	*12.0*	+	*263 > 199*	*60*	*19*	–
Terbutryn[b]	TBTN	12.2	+	242 > 186	55	19	16.1
				242 > 158		26	
	TBTN-d_5	*12.2*	+	*247 > 191*	*59*	*19*	–
Malathion	MALA	12.2	+	353 > 227	70	17	7.4
				353 > 306		15	
	MALA-d_{10}	*12.1*	+	*363 > 237*	*70*	*18*	–
Metolachlor	METO	12.5	+	284 > 252	48	16	2.8
				284 > 176		26	
	METO-d_{11}	*12.3*	+	*295 > 263*	*48*	*17*	–
Alachlor[b]	ALA	12.6	+	270 > 162	40	21	3.7
				270 > 132		42	
	ALA-d_{13}	*12.4*	+	*283 > 251*	*45*	*11*	–
Fenitrothion	FEN	12.5	–	262 > 152	52	21	1.0
				262 > 122		34	
	FEN-d_3	*12.4*	–	*265 > 153*	*51*	*22*	–
Azinphos-ethyl	AZET	12.8	+	346 > 137	37	25	1.6
				346 > 97		32	
	AZET-d_{10}	*12.7*	+	*356 > 138*	*39*	*25*	–
Chlorfenvinphos[b]	CFVP	13.0	+	359 > 170	60	42	1.2
				359 > 99		27	
	CFVP-d_{10}	*12.9*	+	*369 > 170*	*58*	*41*	–
Fenthion	FENT	14.1	+	279 > 169	63	20	1.3
				279 > 247		13	
	FENT-d_6	*14.0*	+	*285 > 169*	*62*	*20*	–
Diazinon	DIAZ	14.8	+	305 > 169	64	22	1.9
				305 > 153		22	
	DIAZ-d_{10}	*14.6*	+	*315 > 170*	*67*	*23*	–
Diflufenican	DIFLU	15.5	+	395 > 266	60	24	6.4
				395 > 246		34	
	DIFLU-d_3	15.4	+	398 > 268	84	25	–
Oxadiazon	OXDZ	16.8	+	345 > 303	60	13	1.5
			+	345 > 220		20	
	OXDZ-d_7	*16.8*	+	352 > 304	60	10	–
Quinoxyfen[b]	QUIN	17.4	+	310 > 199	102	34	1.9
				310 > 216		36	
Chlorpyrifos[b]	CPF	17.2	+	350 > 198	54	22	2.3
				350 > 97		40	
Pendimethalin	PENDI	17.3	+	282 > 212	33	12	7.5
				282 > 194		19	
	PENDI-d_5	17.3	+	287 > 213	43	12	–
Triallate	TLLT	18.0	+	304 > 142	55	40	1.7
			–	304 > 83		47	
	TLLT-$^{13}C_6$	*17.9*	+	310 > 143	55	29	–

CE: collision energy; HESI: heated electrospray ionization; RT: retention time; SRM: selected reaction monitoring, SRM1/SRM2: peak area ratio.

[a] Compound included in the EU watch list (2018/840/EC).

[b] Compound included in the EU list of priority substances (2013/39/EC).

during method development and in the validation study. A solution containing the surrogate standard mixture at a concentration of 1000 ng/mL was also prepared. Pesticide-grade solvents MeOH, acetonitrile (ACN), and LC-grade water were supplied by Merck (Darmstadt, Germany).

2.2. Extraction and clean-up

Ten grams of fresh fish muscle tissue were weighed into a 50 mL polypropylene centrifuge tube for QuEChERS extraction containing the extraction salts used in the EN 15662 CITRATE buffered method (4 g $MgSO_4$, 1 g NaCl, 1 g NaCitrate and 0.5 g disodium citrate sesquihydrate (DCS)) provided by Bekolut® GmbH & Co. KG (Hauptstuhl, Germany) as Bekolut® Citrate-Kit-01. Then the sample was spiked with the surrogate standard mixture at a concentration of 50 ng/g and manually shaken. The dispersive solid phase extraction of the sample was carried out using 10 mL of acetonitrile as extraction solvent (1:1 w/v). After vortex extraction at 2500 revolutions per min (rpm) for 1 min, tubes were centrifuged for 5 min at 4000 rpm. Seven milliliters of the supernatant was transferred into a 15 mL centrifuge tube for clean-up with Bekolut® PSA-Kit-04A (400 mg primary secondary amine (PSA), 400 mg C18 and 1200 mg $MgSO_4$). The use of PSA sorbent aims at removing sugars and fatty acids, whereas C18 is added to remove remaining lipids. The role of $MgSO_4$ is to remove water (necessary for GC–MS applications) and to increase the process efficiency. After vortex extraction (1 min at 2500 rpm) and centrifugation (5 min, 4000 rpm) the supernatant was transferred into a 2 mL vial for LC-MS/MS analysis.

2.3. LC-MS/MS analysis

The instrumentation used for analysis consisted of an LC system Aria Mx with two Transcend quaternary pumps (max pressure 600 bars) coupled to a TSQ Quantiva triple-quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific Inc.). Sample injection was carried out by means of a CTC pal autosampler. For LC-MS/MS analysis, 20 μL of the final QuEChERS extract was injected into the chromatographic column, a Purospher STAR RP-18e column (150 × 2.1 mm, 2 μm particle diameter) from Merck (Darmstadt, Germany). Chromatographic separation was achieved with an organic linear gradient using ACN and water as the mobile phase at a flow rate of 0.2 mL/min. The starting composition of the mobile phase was 10% ACN in water. The organic phase proportion was increased to 50% in 2 min, to 80% in the following 10 min, and finally to 100% in 1 min. Pure organic conditions were maintained for 3 min to ensure column cleaning, and then initial conditions were achieved in 1 min and maintained for 8 min to ensure column equilibration.

MS/MS detection was carried out in the selected reaction monitoring (SRM) mode acquiring two SRM transitions per target pesticide and one SRM transition per surrogate compound. The analytical system allowed switching between positive and negative ionization modes without affecting method sensitivity, and consequently, all analytes were determined in a single analytical run (45 compounds in the positive ionization mode and 7 compounds in the negative ionization mode). Table 1 shows the optimum conditions for MS/MS determination of the target pesticides. Other MS detection conditions were as follows: ion spray voltage, 3500 V (positive ionization mode) and 2500 V (negative ionization mode); ion transfer tube temperature, 350 °C; vaporizer temperature, 280 °C. Nitrogen was used as sheath gas, auxiliary gas, and sweep gas; whereas argon was used as collision gas, 2.5 mTorr. Instrument control and data acquisition and evaluation were performed by Thermo Xcalibur 3.0.63 software (Thermo Fisher Scientific Inc.).

2.4. Method validation performance

The performance of the analytical methodology was evaluated in terms of linearity, sensitivity, accuracy, matrix effects, and precision according to the guidelines set for pesticide residues analysis in food and feed (EC, 2017). The validation study was carried out using fresh muscle from a trout individual purchased at a nearby supermarket. For method validation, twenty pesticides were added to the initial pesticide list (32 pesticides, see Table S2 in SI) investigated during the optimization of the extraction approach.

Quantification was performed by the internal standard method, based on the peak areas obtained for each analyte and its corresponding surrogate compound, which was a deuterated analog in most cases. Up to eleven-point calibration curves were constructed using least squares linear regression models within the concentration range 0.01 ng/mL–1000 ng/mL (equivalent to 0.01 and 1000 ng/g f.w., respectively). Method linearity was expressed by the coefficient of determination (r^2) of the linear model obtained for each analyte.

Analyte recovery and repeatability were calculated from the analysis of n = 6 fortified fish muscle samples at two different concentration levels (10 ng/g f.w. and 500 ng/g f.w.). Absolute recovery values were calculated by comparing analyte peak areas in fortified fish samples and methanolic solutions with the standards mixture at equivalent concentrations (10 ng/mL and 500 ng/mL). Background concentrations in the fish tissue used for the recovery study were taken into account for the calculations. Relative recovery values were calculated by comparing the absolute recovery of the analyte and that of the corresponding isotopically-labeled compound used as surrogate standard.

Method repeatability was calculated as the relative standard deviation (RSD) of the analyte peak areas obtained after the replicate (n = 6) analysis of fish muscle samples fortified with the standard mixture at concentrations of 10 ng/g f.w. and 500 ng/g f.w.

The method limits of detection (LODs) and quantification (LOQs) were estimated from the analyte signals observed after LC-MS/MS analysis of fortified (10 ng/g f.w.) fish muscle samples as the concentration that provided a signal to noise ratio of 3 in the case of the LOD and 10 in the case of the LOQ. The LOQ of the analyte SRM1 and the LOD of the analyte SRM2 were considered in the provision of the analyte limit of determination (LODet), which is the minimum concentration of analyte that can be quantified with the SRM1 and confirmed with the SRM2.

Matrix effects were evaluated comparing analyte peak areas obtained after analysis of samples fortified after the extraction process and standard solutions at equivalent concentrations (500 ng/g f.w.), according to the following equation: $((A_{matrix} - A_{std})/A_{std}) * 100$, where A_{matrix} is the analyte area obtained in a fish sample extract fortified with the standard mixture after QuEChERS extraction and A_{std} is the analyte area obtained in a methanolic standard solution. Negative values indicate ionization suppression matrix effects whereas positive values indicate ionization enhancement matrix effects.

2.5. Sample collection

The validated method was applied to the analysis of the target pesticides in fish muscle tissue of individuals collected from the Adige River basin (Italy). This river basin is highly impacted by agricultural activities, such as apple tree cultivation, and also by hydroelectric energy production. Thirteen fishes were caught during a sampling campaign carried out in July 2015. Sampling locations were distributed along the basin as shown in Fig. 1.

Riverine brown trout (*Salmo trutta fario*) or marble trout (*Salmo trutta marmoratus*), bullhead (*Cottus gobio*) or grayling (*Thymallus thymallus*), and chub (*Leuciscus cephalus*) were collected as representative species of predator, benthivorous and omnivorous ecological groups, respectively (Kračun-Kolarević et al., 2016). Table S3 in SI shows the details of the species sampled in the different sampling locations.

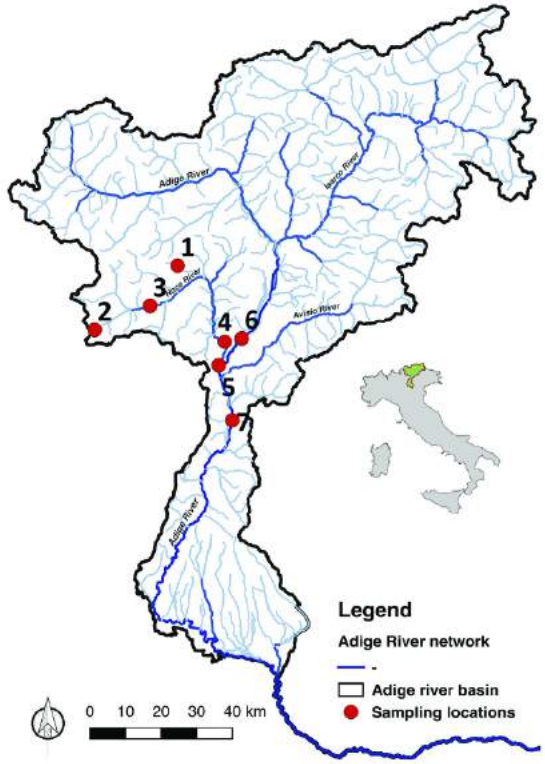

Fig. 1. The Adige River basin and a detailed map of fish sampling sites.

Fish were caught by net and/or electrofishing after obtaining the corresponding permit from local authorities. Samples were kept at 4 °C and transported to the laboratory. Fish muscle (350 g tissue of one individual or 350 g of tissue blend of small individuals) was pulverized in a mixer, separated in different portions in polyethylene vessels and stored in the dark at −20 °C. Samples were shipped on dry ice to the analytical laboratory and upon reception, they were also stored at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of the extraction procedure

In the present work, a fractional factorial planning (4-1) was used to determine the best approach to extract pesticides from fish muscle tissue samples. To this end, different extraction salts, extraction solutions, and agitation times were evaluated (see Table S4 in SI). These experiments were carried out only with 32 of the target compounds.

As for the extraction salts, the original QuEChERS unbuffered methodology that uses $MgSO_4$ (6 mg) and NaCl (1.5 g) (Anastassiades et al., 2003), and two variations of this methodology, i.e., the method AOAC 2007.01 that uses NaAc (1.5 g) instead of NaCl (AOAC, 2007), and the European method EN15662 that requires the use of $MgSO_4$ (4 mg), NaCl (1 g), NaCitrate (1 g) and DCS (0.5 g) (EURL-SRM, 2015), were tested. A clean-up process based on PSA (PSA-kit-04A, BEKOlut®) was applied in all cases because it was the clean-up recommended for cleaning high lipid content extracts. As extraction solvent, acetonitrile with and without acetic acid (1%) were tested.

Pareto charts were constructed for each pesticide with the absolute recoveries obtained in the extraction optimization study so that the significance of each of the tested parameters and the effects of the interactions among them on analyte recovery could be evaluated. Pareto charts for average recovery for 20 selected pesticides are provided as supplementary information (Figs. S1–S21).

Overall, the parameters tested did not significantly affect the recovery of most of the target analytes. Only linuron extraction was affected by the salt used in the process, azinphos-methyl extraction was affected by the extraction time, and chlortoluron extraction was affected by the pair salt-agitation time during the clean-up process. The use of longer times (e.g., 5 min) might have resulted in improved extraction of some analytes, but since samples were manually processed, this option was discarded. Although the original unbuffered extraction approach using $MgSO_4$ and NaCl followed by PSA-based cleanup is the one usually applied for pesticide analysis in fish in the peer-reviewed literature (Belenguer et al., 2014; Ernst et al., 2018; Nácher-Mestre et al., 2014), the method recommended by European Union (EU) regulations, i.e., the citrated buffered extraction, was selected because linuron, a pesticide with relatively poor MS sensitivity, was best recovered under those conditions.

3.2. Optimization of LC-MS/MS conditions

Optimum conditions for MS/MS determination of the target pesticides were obtained after flow injection of individual standard mixtures in positive and negative HESI modes. The identification of the molecular (parent) ions and the optimum RF lens voltage for their detection was carried out in full scan mode over an m/z range of 50–500, while the identification of the most abundant product ions and the optimum collision energies was achieved by means of product-ion scan. The MS/MS method was created in compliance with European Commission Decision 2002/657/EC (EC, 2002) that establishes the need of acquiring 2 SRM transitions per analyte (one for surrogate standard) to obtain enough identification points. All transitions, regardless of their ionization mode, were recorded within one single acquisition window.

Analyte peak shape and chromatographic separation with different organic linear gradients were evaluated by injecting 20 μL of a pesticide standard mixture (200 ng/mL). The composition of the mobile phase (ACN and water with no modifiers) was selected on the basis of previous optimization tests performed in the lab for LC-MS/MS analysis of 26 pesticides in sediment samples (Köck-Schulmeyer et al., 2013). This composition allows the simultaneous analysis of all target pesticides during one unique chromatographic run, regardless of their different physical-chemical properties. Extracted ion chromatograms of the target analytes obtained after LC-MS/MS analysis of a fortified fish muscle tissue sample at 10 ng/g f.w. are shown in Fig. S22 in SI.

3.3. Method performance

Method performance for the different analytes in terms of linearity, trueness (recovery), precision, and sensitivity is summarized in Table 2. Calibration curves constructed within the concentration range 0.01 ng/mL–1000 ng/mL presented coefficients of determination (r^2) above 0.99 for all analytes, except fenthion (0.9886), fluroxypyr (0.9877) and pendimethalin (0.9746). The linearity range, including only calibration solutions not deviating ±20% from theoretical concentrations (as suggested by SANTE/11813/2017 guidelines), expanded from the analyte LOQ to 1000 ng/mL in all cases, with the exception of 2,4 D, azinphos-methyl, diuron, fenthion, fenthion sulfoxide, fluroxypyr, MCPA, mecoprop, oxadiazon, pendimethalin, and thiamethoxam (only up to 500 ng/mL).

Analyte absolute and relative recoveries were in general in good agreement at both investigated concentration levels (10 ng/g f.w. and 500 ng/g f.w). Average absolute recovery values ranged from 21% (deisopropylatrazine (DIA)) to 106% (bentazone), being below 50% for

Table 2
Parameters of method performance: linearity (r^2), absolute and relative recoveries and repeatability at 10 ng/g and 500 ng/g, and method sensitivity for the different compounds in fish samples.

Analyte	Linearity (r^2)	Recovery[a]				Repeatability[b]		Sensitivity[c]		
		Absolute (%)		Relative (%)		RSD (%)		ng/g f.w.		
		10 ng/g	500 ng/g	10 ng/g	500 ng/g	10 ng/g	500 ng/g	LOD	LOQ	LODet
2,4 D	0.9930	24	22	107	103	10.4	11.6	1.28	4.29	9.41
Acetamiprid	0.9993	45	61	86	111	6.1	3.9	0.02	0.07	0.13
Alachlor	0.9985	51	41	94	107	16.3	5.9	0.48	1.59	1.59
Atrazine	0.9959	50	61	88	118	5.5	4.4	0.02	0.05	0.06
Azinphos ethyl	0.9915	77	80	87	114	15.2	11.2	1.07	2.49	2.49
Azinphos methyl	0.9921	39	41	107	108	9.3	19.8	1.70	5.68	5.68
Azinphos methyl oxon[y]	0.9979	33	35	93	88	9.3	8.4	0.05	0.17	0.44
Bentazone	0.9982	79	106	115	116	5.0	12.4	0.36	1.20	1.49
Bromoxynil	0.9912	38	39	95	83	8.0	3.3	0.81	2.73	2.73
Chlorfenvinphos	0.9981	81	71	87	98	9.2	4.2	0.08	0.27	0.27
Chlorotoluron	0.9976	59	60	106	110	7.6	7.2	0.16	0.53	0.53
Chlorpyrifos[b]	0.9989	BLOD	48	BLOD	81	–	14.9	14.2	28.5	28.5
Clothianidin	0.9963	38	49	105	119	9.4	5.3	0.32	1.05	1.05
Cyanazine	0.9987	49	60	86	115	8.9	3.6	0.03	0.11	0.20
DEA	0.9963	30	38	89	106	4.0	8.0	0.08	0.28	0.42
DIA	0.9960	21	40	71	103	16.6	16.3	0.22	0.73	0.73
Diazinon	0.9960	59	58	91	105	2.4	6.3	0.01	0.04	0.04
Dichlorvos	0.9979	28	81	80	107	18.9	12.6	0.32	3.73	3.73
Diflufenican	0.9939	38	37	81	83	16.3	7.8	0.34	1.13	1.13
Dimethoate	0.9977	46	58	94	117	3.6	3.78	0.03	0.11	0.29
Diuron	0.9943	60	59	102	103	16.7	9.7	0.20	1.23	1.23
Fenitrothion	0.9952	BLOD	76	BLOD	92	–	10.6	22.4	87.1	87.1
Fenitrothion oxon	0.9933	BLOD	86	BLOD	113	–	10.9	12.5	18.2	18.2
Fenthion	0.9886	BLOD	57	BLOD	110	–	13.4	21.5	49.8	49.8
Fenthion oxon	0.9933	65	66	100	116	6.3	5.3	0.01	0.04	0.04
Fenthion oxon sulfone	0.9973	55	62	75	108	15.4	18.6	0.05	0.18	0.52
Fenthion oxon sulfoxide[x]	0.9952	62	69	93	114	3.6	6.3	0.01	0.04	0.06
Fenthion sulfone	0.9924	BLOD	69	BLOD	113	–	9.8	18.0	35.1	60.0
Fenthion sulfoxide	0.9977	62	66	96	117	4.0	6.3	0.02	0.08	0.08
Fluroxypyr[z]	0.9877	BLOD	27	BLOD	100	–	6.8	23.3	77.5	77.5
Imidacloprid	0.9941	46	50	100	118	10.9	9.3	0.15	0.49	0.49
Irgarol	0.9938	74	71	109	110	7.9	3.6	0.01	0.05	0.06
Isoproturon	0.9988	73	69	110	112	11.9	5.5	0.12	0.39	0.39
Linuron	0.9974	51	57	95	101	18.2	7.4	1.05	1.48	1.48
Malaoxon[x]	0.9935	47	62	83	115	7.0	9.2	0.32	1.07	1.07
Malathion	0.9953	59	54	84	88	15.8	7.4	0.50	0.92	0.92
MCPA	0.9916	29	25	110	118	16.8	16.3	0.72	2.43	9.89
Mecoprop	0.9924	33	31	109	115	19.5	12.0	0.35	1.18	1.22
Methiocarb	0.9937	43	48	85	93	11.9	4.0	0.34	1.15	1.15
Metolachlor	0.9989	65	53	89	90	5.0	6.6	0.01	0.04	0.04
Molinate[a]	0.9980	58	52	107	92	15.4	7.0	0.30	1.02	1.02
Oxadiazon	0.9903	BLOD	61	BLOD	120	–	6.6	50.0	100	100
Pendimethalin	0.9746	BLOD	54	BLOD	103	–	20.6	50.0	150	150
Propanil	0.9930	44	48	78	83	13.0	10.1	0.60	2.00	2.00
Quinoxyfen[w]	0.9921	31	36	80	82	10.6	17.2	0.79	2.65	2.65
Simazine	0.9957	36	51	91	119	6.6	5.4	0.20	0.50	0.50
Terbuthylazine	0.9964	41	48	89	115	3.4	4.3	0.02	0.06	0.13
Terbutryn	0.9984	71	73	89	108	5.5	4.7	0.01	0.05	0.06
Thiacloprid	0.9956	47	55	89	117	3.4	4.5	0.02	0.06	0.15
Thiamethoxam	0.9939	55	51	117	103	10.5	3.5	0.33	1.10	1.10
Thifensulfuron methyl	0.9960	27	31	108	120	11.5	1.1	0.23	0.78	0.78
Triallate	0.9906	64	48	116	96	4.3	12.0	2.93	9.79	9.79

[a]Compound quantified using *fenthion sulfoxide-d_6* as surrogate standard.
[b]Compound quantified using *triallate-$^{13}C_6$* as surrogate standard.
[x]Compound quantified using *thiamethoxam-d_3* as surrogate standard.
[a]Compound quantified using *mecoprop-d_3* as surrogate standard.
[y]Compound quantified using *chlortoluron-d_6* as surrogate standard.
[z]Compound quantified using *linuron-d_6* as surrogate standard.
[w]Compound quantified using *methiocarb-d_3* as surrogate standard.

[a] Average absolute recovery: comparison of peak areas obtained in n = 6 fortified samples and methanol standard solutions at equivalent concentrations. Average relative recovery: comparison of absolutes recoveries obtained for the analyte and its corresponding surrogate standard (n = 6).

[b] Repeatability: relative standard deviation of the relative recoveries observed in n = 6 fortified samples.

[c] Sensibility: limit of detection (LOD) and limit of quantification (LOQ) – estimated concentration that would result in a S/N of 3 and 10, respectively. Calculated from fish fortified samples at 10 ng/g. LODet: limit of determination, minimum concentration at which the analyte can be quantified (LOQ of SRM1) and confirmed (LOD of SRM2). BLOD: below limit of detection.

38% of the analytes. Although poor absolute recoveries affect negatively method sensitivity, the use of isotopically labeled compounds serves to compensate the two possible causes behind them, i.e., poor extraction efficiency and/or matrix ionization suppression effects, which would eventually lead to inaccurate results. The six compounds for which isotopically labeled analogs were not available were quantified using isotopically labeled compounds presenting as far as possible similar absolute recoveries, structure, and retention times. Average relative recoveries

were found to range between 71 and 120% for all compounds. Recovery rates in replicate (n = 6) samples were found to be consistent, with RSD values <15% in most cases, and below 20% in all cases with the exception of pendimethalin at 500 ng/g f.w. Accuracy and precision values thus overall complied with SANTE/11813/2017 guidelines for performance acceptability criteria of quantitative methods (average recovery between 70 and 120% and repeatability at each spiking level with RSD below 20%) (EC, 2017).

As for sensitivity, the method allows detecting and quantifying most of the target pesticides at concentrations in the pg/g f.w. or the low ng/g f.w. range, thus covering the values expected for this type of compounds in fish. LODs below 3 ng/g f.w. and LODets below the general MRL of 10 ng/g applied to pesticides in food, were obtained for 44 out of the 52 pesticides investigated. Compounds showing higher LODets and therefore not sufficing the necessary sensitivity required for their determination in this matrix according to legislation were: chlorpyrifos, fenitrothion, fenitrothion oxon, fenthion, fenthion sulfone, fluroxypyr, oxadiazon, and pendimethalin. In contrast, many other relevant pesticides, such as those included in the list of priority substances in water alachlor (LODet 1.59 ng/g f.w.), atrazine (0.06), chlorfenvinphos (0.27), diuron (1.23), isoproturon (0.39), simazine (0.50), quinoxyfen (2.65), irgarol (0.06), dichlorvos (3.73) and terbutryn (0.06) (EC, 2013), and those of the Watch List methiocarb (1.15), imidacloprid (0.49), thiacloprid (0.15), thiamethoxam (1.10), clothianidin (1.05) and acetamiprid (0.13) (EC, 2018), could be determined at levels down to 0.06–3.73 ng/g f.w.

Fig. 2 summarizes the extent of matrix effects affecting pesticide analysis in trout tissue with the described methodology. Matrix effects were not significant for 11 of the 52 target analytes (±20% variation of the signal). Except in the case of six pesticides, that showed some signal enhancement, matrix effects occurred in the form of signal ionization suppression caused by co-eluting matrix components. The compounds affected the most by signal suppression effects were quinoxyfen (−98%), DIA (−72%), and dichlorvos and oxadiazon (−64%). On the contrary, signal enhancement was significant for azinphos-ethyl (+23%) and fenitrothion oxon (+32%). As previously mentioned, the use of isotopically labeled standards compensates for these effects that may also differ among fish species due to their different fat and protein content. Low matrix effects (<20%) for LC-MS analysis of pesticides in hake tissues have been reported by Belenguer et al. (2014), whereas strong ionization suppression effects were observed in carp and perch muscle (up to −94%) by Lazartigues et al. (2011).

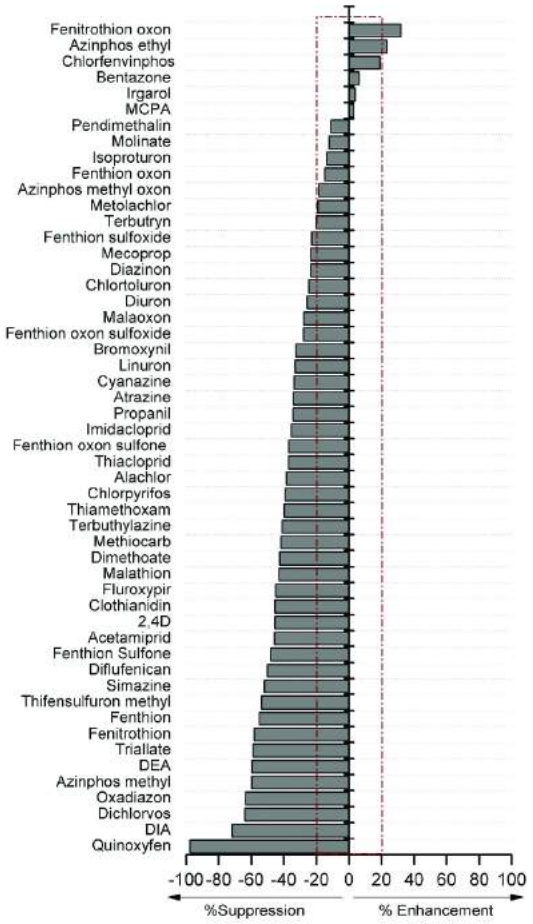

Fig. 2. Matrix effects found in the analysis of the target pesticides in fish (trout) tissue samples.

3.4. Occurrence of target pesticides in real samples

As part of the validation process, the applicability of the method was evaluated through the analysis of the target pesticides in 13 fish muscle tissue samples from the Adige River basin (Italy). Positive identification followed the criteria established by the SANTE guidelines (EC, 2017) regarding pesticide analysis in food: SRM transitions for one analyte fully overlapped, the analyte SRM ratio in the sample extract was within ± 30% of the average SRM ratio observed in the calibration standards for the same analytical batch, and the analyte retention time in the extract corresponded to that of the calibration standard within ±0.1 min. Larger retention time deviations were accepted when the corresponding deuterated analog retention time was also shifted due to matrix components.

Results obtained (concentrations above LOQ in ng/g f.w.) are shown in Fig. 3. Twelve out of the 13 analyzed samples were found to contain measurable quantities of the target pesticides. However, only 5 compounds were quantified: quinoxyfen, terbutryn, metolachlor, acetamiprid and irgarol. Trace levels below the method limit of determination of diazinon (<0.04 ng/g f.w.), dichlorvos (<3.73 ng/g f.w.), and diuron (<1.23 ng/g f.w.) were detected in a few samples.

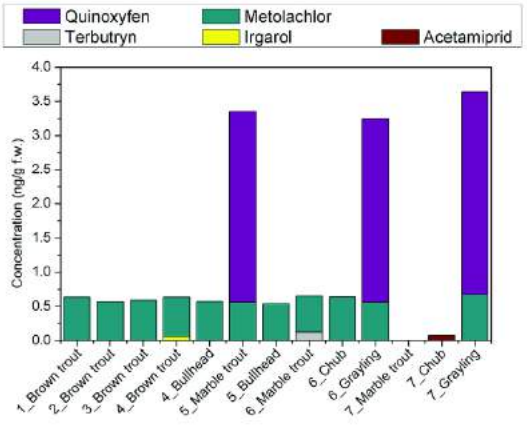

Fig. 3. Concentrations of pesticides (ng/g f.w.) quantified in fish muscle tissue samples.

Metolachlor followed by quinoxyfen were the most ubiquitous compounds, being present in 11 and 10 samples, respectively. Quinoxyfen was below its LOQ in 7 out of the 10 samples that contained it. Irgarol, terbutryn and acetamiprid were found in only one sample.

The compound showing the maximum concentration was quinoxyfen (2.97 ng/g f.w. in a grayling (*Thymallus thymallus* spp.) sample collected in sampling location 7), a quinoline fungicide very used in agriculture for disease control in grapes and hops. This compound presented similar concentrations in the other two fish tissue samples where it was quantified. The second most abundant compound was metolachlor, a chloroacetanilide herbicide used for the control of many kinds of grass and broadleaf weeds in crops. Its average value was 0.59 ng/g f.w. Quinoxyfen levels in river fish muscle and liver samples have been also investigated by Kaczyński et al. (2017), and were found to be below the method LOD. As for metolachlor, the levels found in this study were slightly lower than those measured in fish muscle tissue by Kaczyński et al. (2017) (up to 11 ng/g f.w) and Belenguer et al. (2014) (4 ng/g d.w).

Irgarol and terbutryn, measured at trace levels in trout individuals, are booster biocides used in antifouling paints of boats, and thus fish exposure to these compounds may be of concern. Irgarol has been previously detected in liver samples from sea mullets at concentrations up to 6.9 ng/g (Franco-Barrios et al., 2014). Terbutryn is included in the EU list of potential endocrine disruptors within category 1: substances for which endocrine activity has been documented in at least one study of a living organism (Petersen et al., 2007). These substances are given the highest priority for further studies.

All pesticides detected except acetamiprid present a high bioaccumulation potential (Log K_{ow} from 3.5 to 4.7) (see Table S1 in SI). However, the high solubility of acetamiprid (2950 mg/L) may result in a constant exposure of fish to this compound and hence its bioaccumulation, which could occur through sorption mechanisms different from those expected for less polar neutral pesticides. Acetamiprid and other neonicotinoids were investigated in eels by Xiao et al. (2013), but were not found at levels above the method LOD in any of the analyzed samples.

Some of the investigated pesticides were found in the benthic species grayling (*Thymallus thymallus* spp.) and bullhead (*Cottus gobio* spp.). This indicates that species living and feeding at the river bottom may also find there a source of pesticides (Belenguer et al., 2014).

Finally, it may be worth noting that five out of the eight detected pesticides are included in either the list of priority substances in water (diuron, dichlorvos, irgarol, quinoxyfen and terbutryn) (EC, 2013) or in the Watch List of substances for Union-wide monitoring in the field of water policy (acetamiprid) (EC, 2018).

4. Conclusions

An analytical methodology, based on QuEChERS extraction and LC-HESI-MS/MS detection has been developed for analysis of 52 pesticides belonging to different chemical classes in fish muscle tissue. The method described shows overall satisfactory performance in terms of repeatability (with RSD values below 20.6%) and trueness (average relative recoveries between 71 and 120%). The method, with LODets in the low ng/g f.w. range for most compounds, allows determining 44 of the 52 target pesticides at levels below the general MRL of 10 ng/g that applies to pesticides in food when no specific MRLs are regulated. Additional advantages of the methodology developed that make it attractive for regular monitoring and quality control are simplicity and high throughput, if compared to methodologies based on pressurized liquid extraction or ultrasound assisted extraction and solid-phase extraction clean-up, and relatively low cost. Surrogate standards are costly and increase the initial economical investment needed to apply the proposed method. However, their use makes unnecessary the performance of other time-consuming methods of calibration and quantification like matrix-matched calibration or standard addition, and is essential to generate accurate and reliable results in the analysis of complex samples like fish where matrix variability and effects can be very large. Another advantage of using surrogate standards is that their presence in the sample extracts at the expected MS signal response provides evidence that the analytical process has taken place correctly. This obviates the need also for performing replicate analyses of the samples. Application of the method to fish individuals collected in the Adige basin revealed the presence of five of the target pesticides (i.e., irgarol, metolachlor, quinoxyfen, terbutryn, acetamiprid) at quantifiable levels. Trace levels of diazinon, diuron, and dichlorvos were also detected. These findings indicate that these compounds do not need to be as persistent as organochlorine compounds to accumulate in aquatic organisms. However, additional and more extensive monitoring studies are needed to understand the potential of these compounds to bioaccumulate and the potential effects of this bioaccumulation, so that aquatic ecosystems can be preserved.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.10.289.

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Supporting Information

Analysis of 52 pesticides in fresh fish muscle by QuEChERS extraction followed by LC-MS/MS determination.

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List of Tables

List of Figures

Table S1. Main physical-chemical properties of the target pesticides.

Pesticide	Type	MM ($g\ mol^{-1}$)‡	Solub ($mg\ L^{-1}$) ‡	Koc ($mL\ g^{-1}$) ‡	Kow logP‡	Henry´s ($Pa\ m^3\ mol^{-1}$) ‡	GUS‡	Pka‡
2,4-D	Alkylchlorophenoxy	221.04	24300	39	-0.82	4.0×10^{-06}	1.69	3.40
Acetamiprid	Neonicotinoid	222.67	2950	200	0.80	5.3×10^{-08}	0.40	0.7
Alachlor	Chloroacetamide	269.77	240	335	3.09	3.2×10^{-03}	1.08	0.62
Atrazine	Triazine	215.68	35	100	2.70	1.5×10^{-04}	3.2	1.7
Azinphos-ethyl	Organophosphate	345.38	4.5	1500	3.18	3.1×10^{-06}	1.4	n/a
Azinphos-methyl	Organophosphate	317.32	28	1112	2.96	5.7×10^{-06}	1.42	5
Azinphos-methyl-oxon	Metabolite	301.26	2604*	10*	0.77*	6.2×10^{-13} *	-	n/a
Bentazone	Benzothiazinone	240.30	7112	55	-0.46	7.2×10^{-05}	2.89	3.51
Bromoxinil	Hydroxybenzonitrile	276.90	38000	302	0.27	8.7×10^{-07}	0.03	3.86
Chlorfenvinphos	Organophosphate	359.60	145	680	3.80	-	1.83	n/a
Chlorpyrifos	Organophosphate	350.58	1.05	5509	4.70	0.478	3.63	n/a
Chlortoluron	Phenylurea	212.68	74	196	2.50	1.4×10^{-05}	3.02	n/a
Cyanazine	Triazine	240.69	171	190	2.10	6.6×10^{-06}	2.07	12.9
Clothianidin	Neonicotinoid	249.68	340	123	0.90	2.9×10^{-11}	4.91	11.1
Desisopropylatrazine	Metabolite	173.60	980	130	1.15	980	-	n/a
Desethylatrazine	Metabolite	187.63	2700	110	1.51	1.6×10^{-04}	4.37	n/a
Diazinon	Organophosphate	304.35	60	609	3.69	6.1×10^{-02}	1.14	2.6
Dichlorvos	Organophosphate	220.98	18000	50	1.90	2.6×10^{-02}	0.69	n/a
Diflufenican	Carboxamide	394.29	0.05	3.19°	4.20	1.2×10^{-02}	1.51	n/a
Dimethoate	Organophosphate	229.26	25900	25*	0.75	1.4×10^{-06}	1.01	n/a
Diuron	Phenylurea	233.09	35.6	813	2.87	2.0×10^{-06}	1.83	n/a
Fenitrothion	Organophosphate	277.23	19	2000	3.32	9.9×10^{-03}	0.48	n/a
Fenitrothion oxon	Organophosphate	261.17*	301*	21*	1.69*	4.0×10^{-1} *	-	n/a
Fenthion	Organophosphate	278.33	4.2	1500	4.84	2.4×10^{-02}	1.26	n/a
Fenthion oxon	Metabolite	262.26*	213.5*	57*	2.31*	3.0×10^{-9} *	-	n/a
Fenthion oxon sulfone	Metabolite	294.03*	7602*	13*	0.28*	2.4×10^{-11} *	-	n/a
Fenthion oxon sulfoxide	Metabolite	278.26*	1222*	11*	0.15*	9.5×10^{-8} *	-	n/a
Fenthion sulfone	Metabolite	310.33*	190.4*	542*	2.05*	1.1×10^{-8} *	-	n/a
Fenthion sulfoxide	Metabolite	294.33*	3.72*	466*	1.92*	7.0×10^{-6} *	-	n/a
Fluroxypir	Pyridine compound	255.03	6500	-	0.04	169×10^{-10}	2.42	2.94
Imidacloprid	Neonicotinoid	255.66	610	6719	0.57	1.7×10^{-10}	3.74	n/a
Irgarol	Triazine	253.37	7	1569	3.95	1.3×10^{-07} *	-	n/a
Isoproturon	Phenylurea	206.28	70.2	251*	2.5	1.5×10^{-05}	2.07	n/a
Linuron	Phenylurea	249.09	63.8	843	3	2.0×10^{-04}	2.21	n/a
Malaoxon	Organophosphate	314.29*	7500*	4650*	0.52*	1.2×10^{-08} *	-	n/a
Malathion	Organophosphate	330.36	148	1800	2.75	1.0×10^{-03}	-1.28	n/a
MCPA	Metabolite	200.62	29390	29*	-0.81	5.5×10^{-05}	2.94	3.73
Mecoprop	Aryloxyalkanoic acid	214.65	250000	47	-0.19	2.2×10^{-04}	2.29	3.11
Methiocarb	Carbamate	225.31	27	182*	3.18	1.2×10^{-04}	0.55	n/a
Metolachlor	Chloroacetamide	283.80	530	120	3.40	2.4×10^{-03}	2.10	n/a
Molinate	Thiocarbamate	187.30	1100	190	2.86	6.9×10^{-01}	2.49	n/a
Oxadiazon	Oxidiazole	345.2	0.57	3200	5.33	3.8×10^{-02}	2.40	n/a
Pendimethalin	Dinitroaniline	281.31	0.33	17491	5.40	2.7×10^{-03}	-0.32	2.8
Propanil	Anilide	218.08	95	149	2.29	4.4×10^{-04}	-0.51	19.1
Quinoxyfen	Quinoline	308.13	0.05	23°	4.66	3.2×10^{-02}	-0.93	n/a
Simazine	Triazine	201.66	5	130	2.30	5.6×10^{-05}	2	1.62
Terbuthylazine	Triazine	229.71	6.6	329*	3.40	3.2×10^{-03}	3.07	1.9
Terbutryn	Triazine	241.36	25	2432	3.66	1.5×10^{-03}	2.4	4.3
Thiacloprid	Neonicotinoid	252.72	184	615°	1.26	5.0×10^{-10}	0.14	n/a
Thiamethoxam	*Neonicotinoid*	*291.71*	*4100*	*56*	*-0.13*	4.7×10^{-10}	*4.69*	*n/a*
Thifensulfuron methyl	Metabolite	387.39	54.1	28	-1.65	3.3×10^{-08}	0.44	4
Triallate	Thiocarbamate	304.7	4.1	3034	4.06	0.89	0.69	n/a

MM: molecular mass; Solub: solubility in water at 20 °C ; Koc: organic carbon partition coefficient; Kow: octanol–water partition coefficient; Henry's law constant at 25ºC; GUS: leaching potential index; Pka: dissociation constant at 25 °C; n/a: data not available

‡ The PPDB, Pesticide Properties Database. http://sitem.herts.ac.uk/aeru/footprint/index2.htm - Lewis KA, Tzilivakis J, Warner D, Green A. (2016). An international database for pesticide risk assessments and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064. *Data estimated using the US EPA EPISuite™ http://www.Chemspider.com. ° Kegley SE, Hill BR, Orme S, Choi AH, PAN Pesticide Database, Pesticide Action Network, North America (Oakland, CA, 2016), http://www.pesticideinfo.org

Table S2. Recovery rates of pesticides in fish muscle tissue.

Absolute recovery (%)	1	2	3	4	5	6	7	8	9
Alachlor	38	28	31	31	23	30	28	32	46
Atrazine	71	90	120	115	100	94	100	73	133
Azynphos methyl	26	22	16	23	10	20	25	28	35
Azynphos ethyl	52	50	56	77	34	56	45	47	59
Chlorfenvinphos	26	27	24	41	15	35	31	46	61
Chlorpyrifos	10	8	11	10	6	10	10	9	20
Chlortorluron	55	51	73	72	64	57	61	51	76
Cyanazine	89	62	76	66	69	60	72	67	81
Deisopropylatrazine	52	52	54	38	68	62	43	28	51
Desethyl atrazine	40	45	64	51	47	45	57	47	51
Diazinon	31	30	38	41	33	39	38	41	52
Dichlorvos	84	70	70	83	57	84	79	92	64
Dimethoate	34	33	52	43	38	31	43	29	36
Diuron	31	28	40	33	33	33	42	28	42
Fenitrothion	5	1	5	2	1	0	2	3	2
Fenthion oxon	37	32	54	42	44	40	52	40	52
Fenthion oxon sulfone	10	30	166	13	15	109	22	32	8
Fenthion oxon sulfoxide	179	128	140	95	155	130	150	85	144
Fenthion sulfone	25	22	26	27	18	20	18	11	31
Fenthion sulfoxide	94	77	97	100	85	86	86	86	98
Irgarol	45	48	56	73	47	59	48	56	72
Isoproturon	77	84	91	96	89	92	102	89	120
Linuron	37	50	46	52	44	55	48	47	59
Malaoxon	100	66	76	80	68	70	82	71	82
Malathion	4	-7	0	11	-6	1	3	7	14
Metholachlor	47	40	46	51	33	44	40	46	62
Molinate	23	25	31	42	32	44	25	29	40
Propanil	2	2	2	2	2	2	3	2	3
Quinoxyfen	9	8	13	14	7	10	9	12	17
Simazine	56	35	49	45	42	41	39	41	43
Terbuthylazine	35	46	47	61	39	50	45	46	61
Terbutryn	40	47	56	70	45	53	45	52	64

Table S3. Fish species sampled in the different sampling points, with details of diet habits (predator, benthivorous or omnivorous) and the code assigned to classify them.

CODE	SITE	SPECIE	CLASS
1_Brown trout	1	*Salmo trutta fario*	Predator
2_Brown trout	2	*Salmo trutta fario*	Predator
3_Brown trout	3	*Salmo trutta fario*	Predator
4_Brown trout	4	*Salmo trutta fario*	Predator
4_Bullhead	4	*Cottus gobio*	Benthivorous
5_Marble trout	5	*Salmo trutta marmoratus*	Predator
5_Bullhead	5	*Cottus gobio*	Benthivorous
6_Marble trout	6	*Salmo trutta marmoratus*	Predator
6_Chub	6	*Leuciscus cephalus*	Omnivorous
6_Grayling	6	*Thymallus thymallus*	Benthivorous
7_Marble trout	7	*Salmo trutta marmoratus*	Predator
7_Chub	7	*Leuciscus cephalus*	Omnivorous
7_Grayling	7	*Thymallus thymallus*	Benthivorous

Table S4. Experimental planning to optimize the QuEChERS extraction procedure.

EXP.	EXTRACTING SALTS	EXTRACTING SOLVENT	AGITATION TIME (sec)	
			EXTRACTION	CLEAN-UP
1	AOAC – ACETATE	ACN/1%HOAc	30	30
2	EN – CITRATE	ACN/1%HOAc	30	90
3	AOAC – ACETATE	ACN	30	90
4	EN – CITRATE	ACN	30	30
5	AOAC – ACETATE	ACN/1%HOAc	90	90
6	EN – CITRATE	ACN/1%HOAc	90	30
7	AOAC – ACETATE	ACN	90	30
8	EN – CITRATE	ACN	90	90
9	ORIGINAL - CHLORIDE	ACN/0.5%HOAc	60	60

AOAC – ACETATE: 6 mg $MgSO_4$, 1.5 g NaAc
EN – CITRATE: 4 mg $MgSO_4$, 1 g NaCl, 1 g NaCitrate, 0.5 g DCS
ORIGINAL-CHLORIDE: 6 mg $MgSO_4$, 1.5 g NaCl

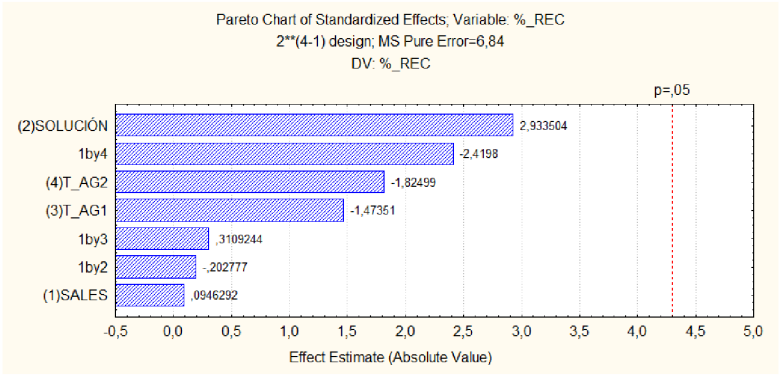

Figure S1. Pareto Chart for the standardize effect of each parameter tested on the average recovery of all pesticides investigated (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

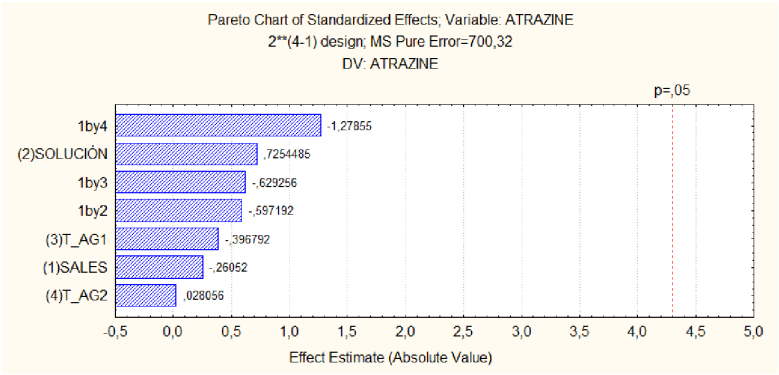

Figure S2. Pareto Chart for the standardize effect of each parameter tested on the average recovery of atrazine (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

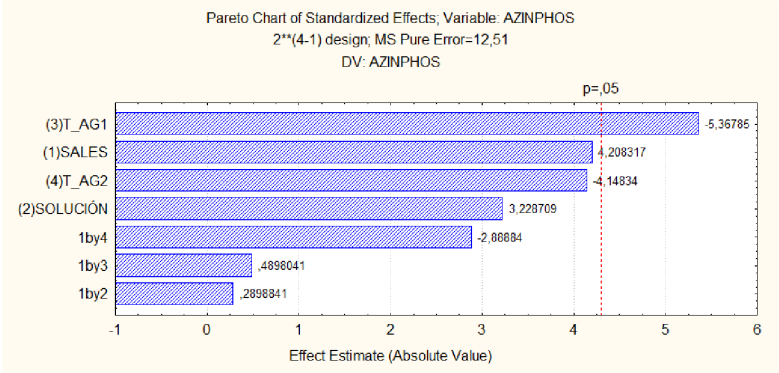

Figure S3. Pareto Chart for the standardize effect of each parameter tested on the average recovery of azinphos (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

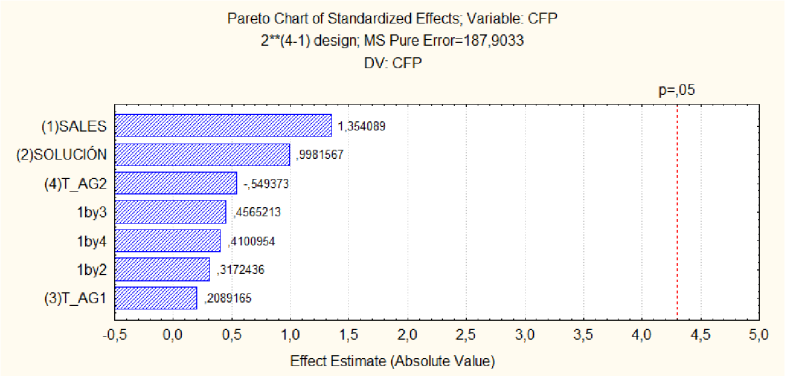

Figure S4. Pareto Chart for the standardize effect of each parameter tested on the average recovery of chlorfenvinphos (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

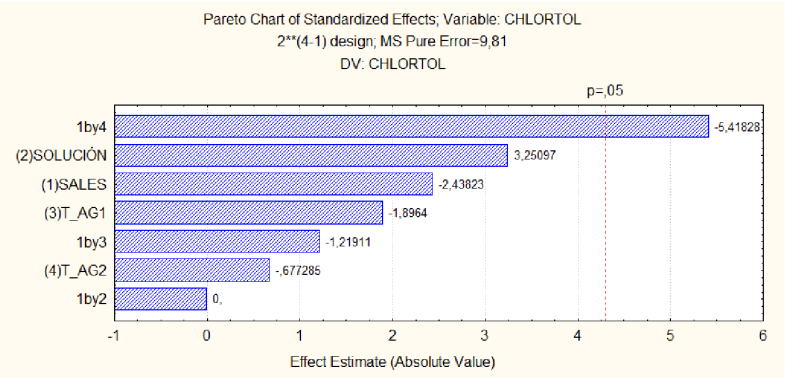

Figure S5. Pareto Chart for the standardize effect of each parameter tested on the average recovery of chlortoluron (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

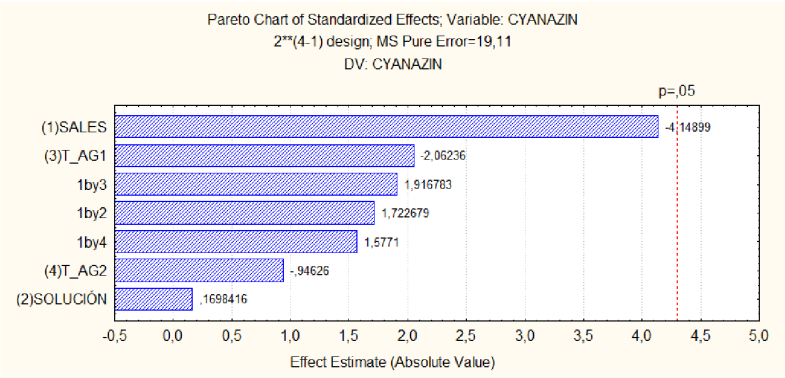

Figure S6. Pareto Chart for the standardize effect of each parameter tested on the average recovery of cyanazine (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

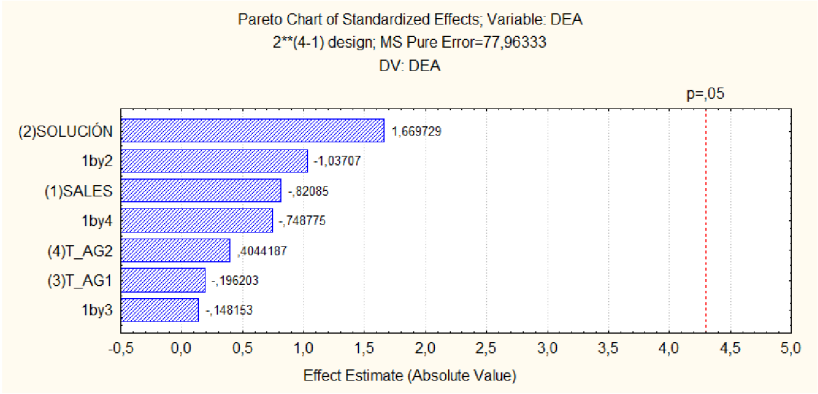

Figure S7. Pareto Chart for the standardize effect of each parameter tested on the average recovery of DEA (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

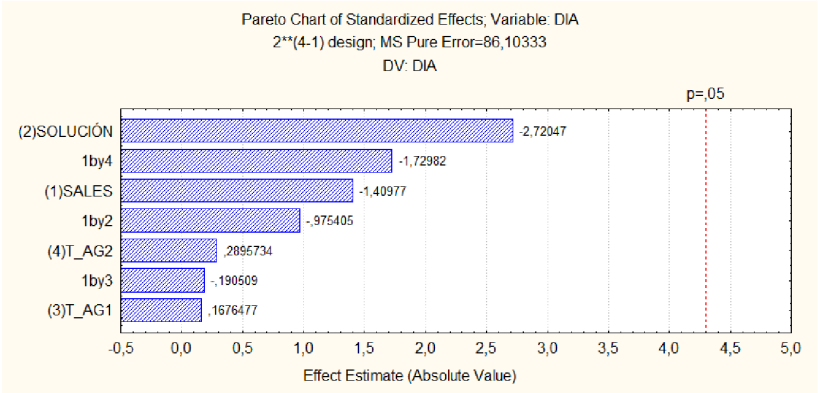

Figure S8. Pareto Chart for the standardize effect of each parameter tested on the average recovery of DIA (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

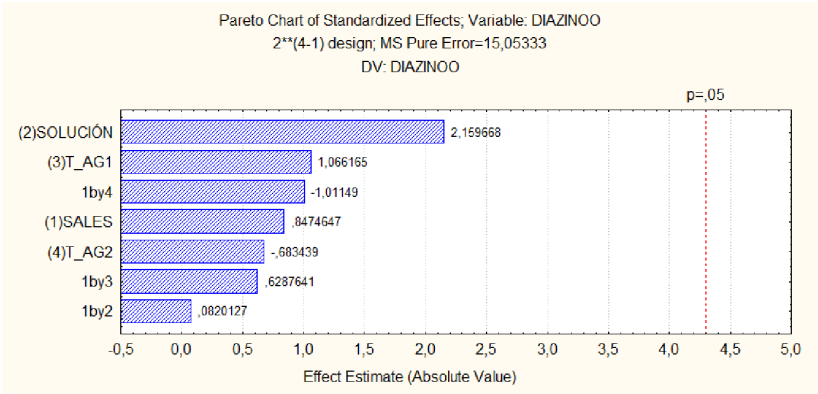

Figure S9. Pareto Chart for the standardize effect of each parameter tested on the average recovery of diazinon (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

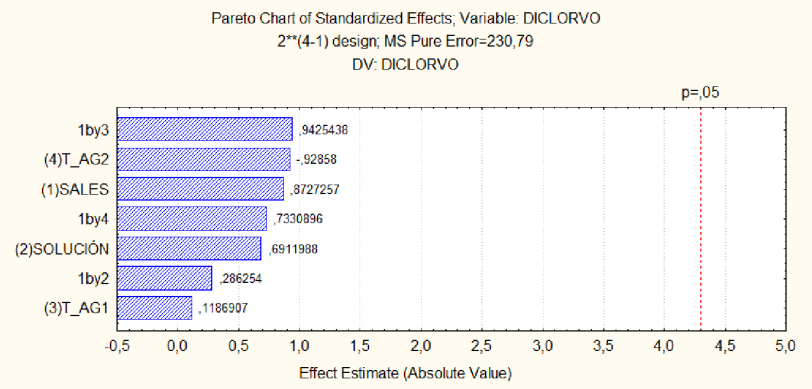

Figure S10. Pareto Chart for the standardize effect of each parameter tested on the average recovery of dichlorvos (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

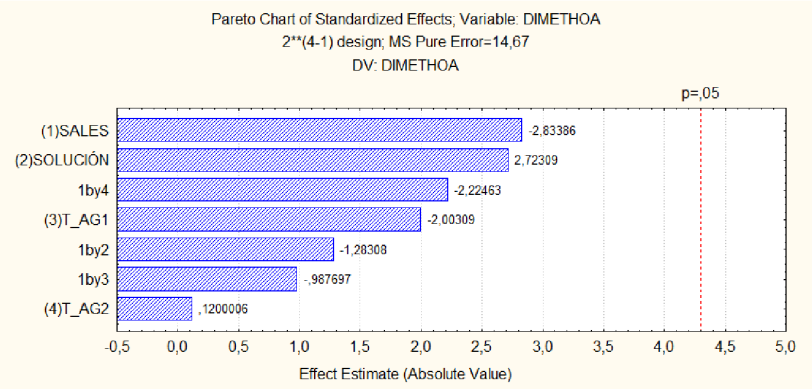

Figure S11. Pareto Chart for the standardize effect of each parameter tested on the average recovery of dimethoate (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

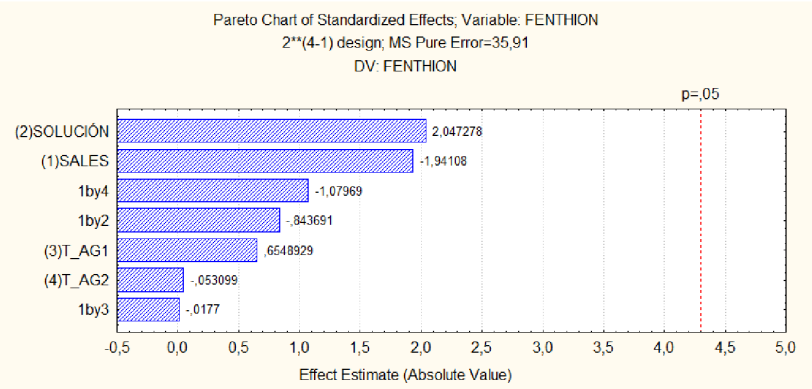

Figure S12. Pareto Chart for the standardize effect of each parameter tested on the average recovery of fenthion (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

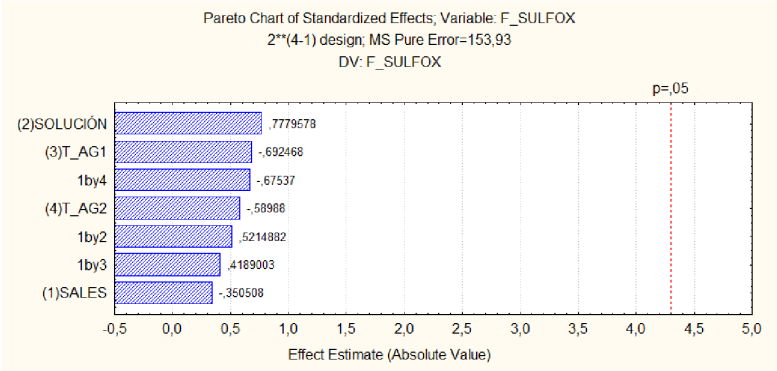

Figure S13. Pareto Chart for the standardize effect of each parameter tested on the average recovery of fenthion sulfoxide (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

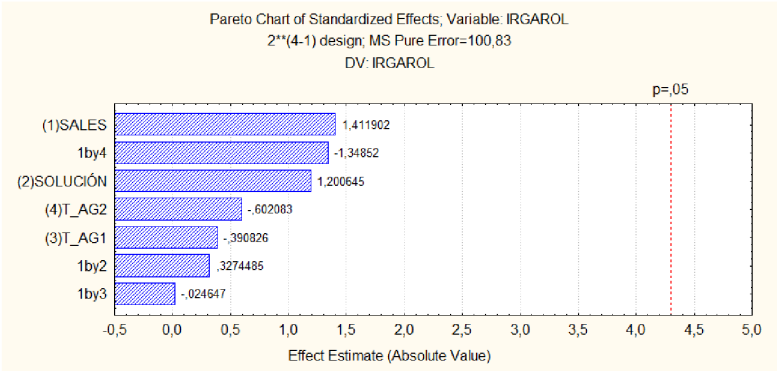

Figure S14. Pareto Chart for the standardize effect of each parameter tested on the average recovery of irgarol (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

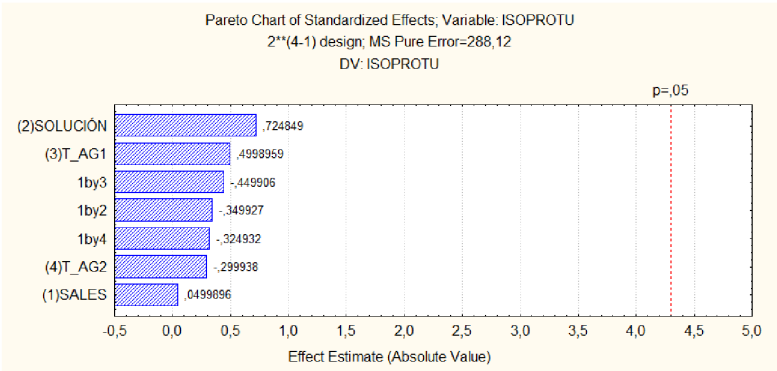

Figure S15. Pareto Chart for the standardize effect of each parameter tested on the average recovery of isoproturon (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

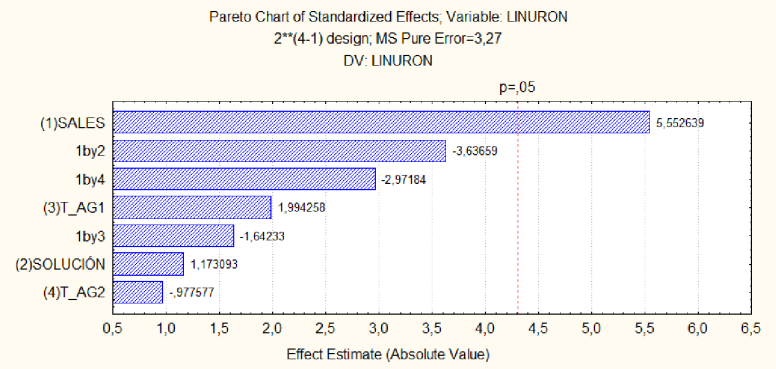

Figure S16. Pareto Chart for the standardize effect of each parameter tested on the average recovery of linuron (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

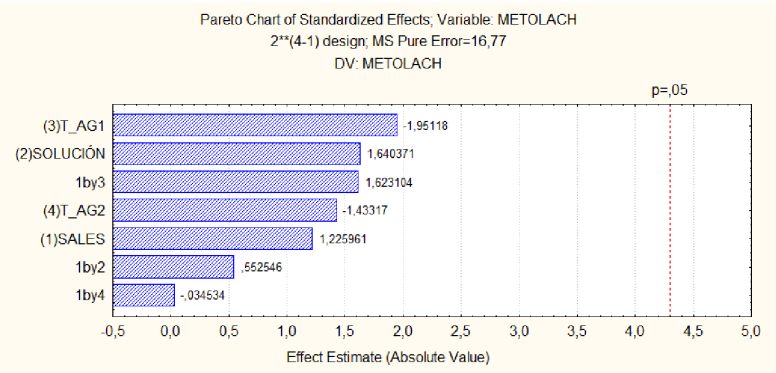

Figure S17. Pareto Chart for the standardize effect of each parameter tested on the average recovery of metolachlor (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

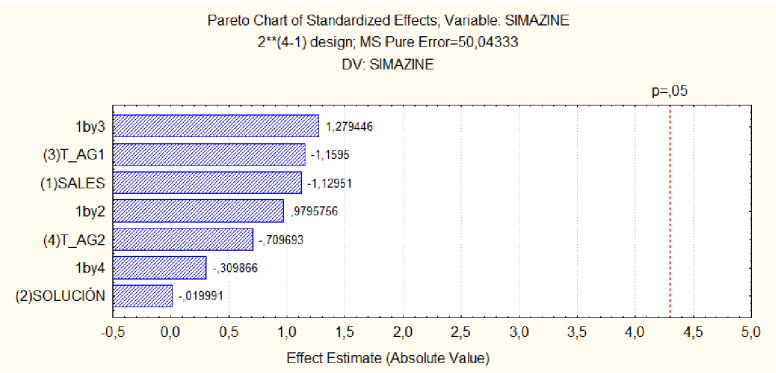

Figure S18. Pareto Chart for the standardize effect of each parameter tested on the average recovery of simazine (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

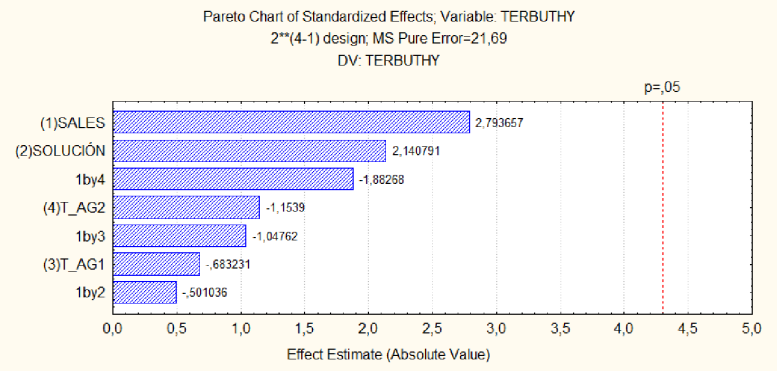

Figure S19. Pareto Chart for the standardize effect of each parameter tested on the average recovery of terbuthylazine (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

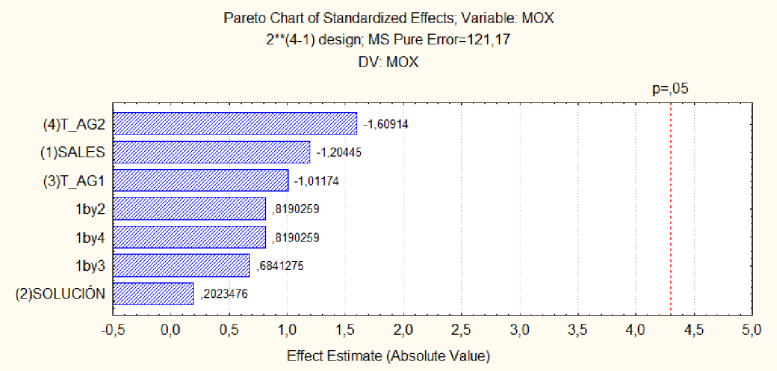

Figure S20. Pareto Chart for the standardize effect of each parameter tested on the average recovery of malaoxon (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

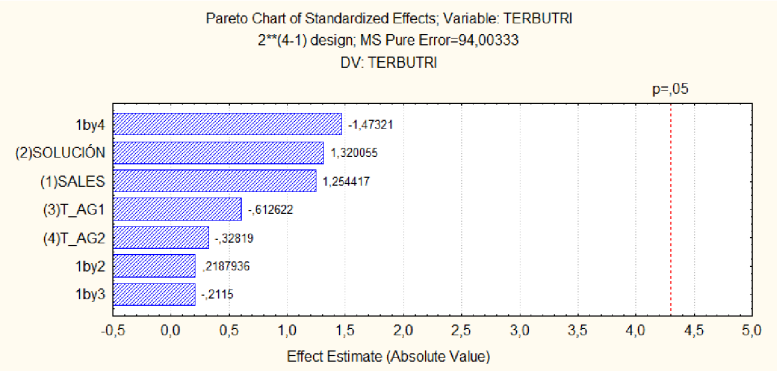

Figure S21. Pareto Chart for the standardize effect of each parameter tested on the average recovery of terbutryn (SALES: extraction salt, SOLUCIÓN: extracting solvent, T_AG1: agitation time for extraction, T_AG2: agitation time for clean-up).

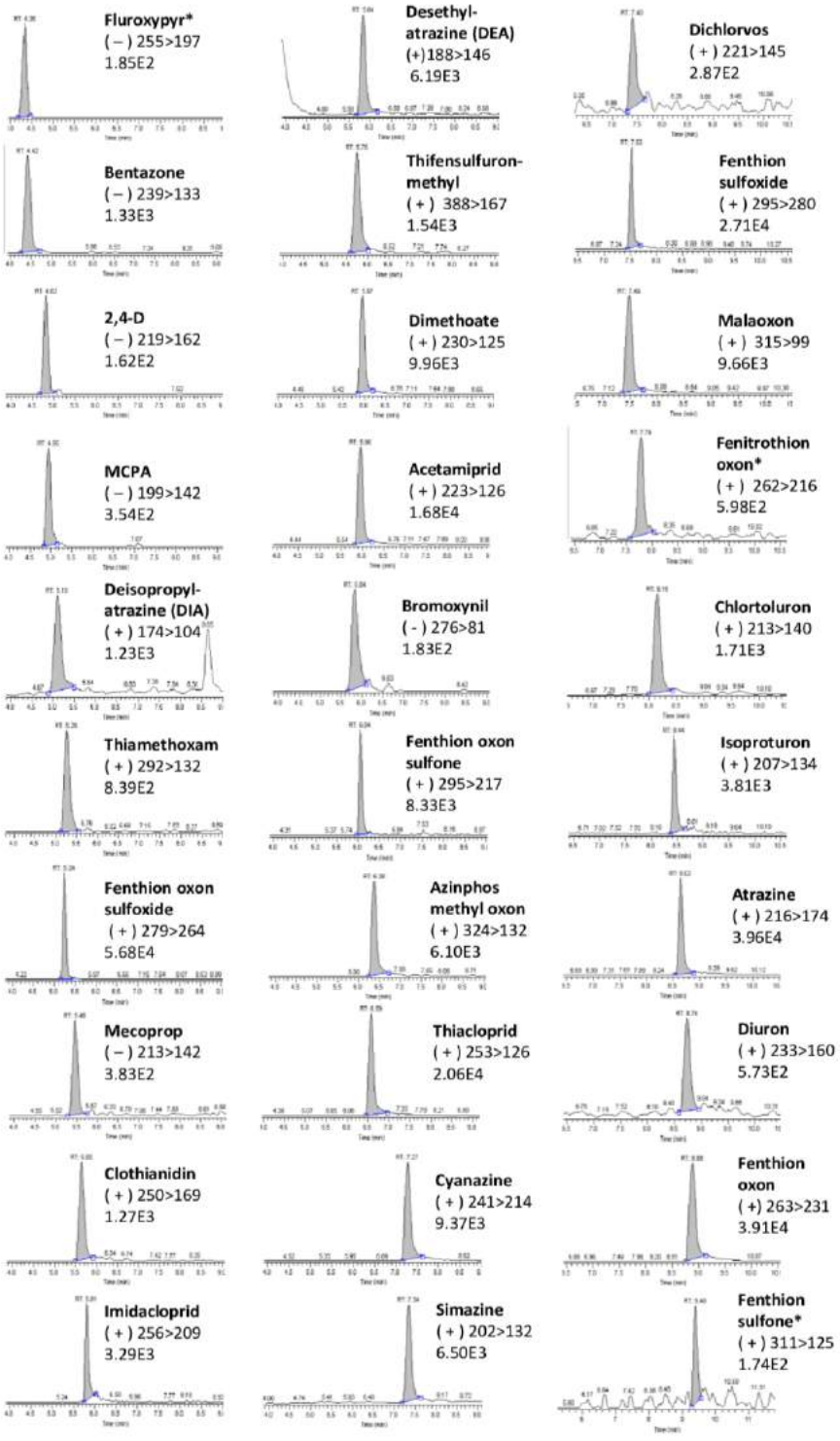

Figure S22. Extracted ion chromatograms of the target pesticides after QuEChERs and LC-MS/MS analysis of a fish muscle tissue sample fortified at a concentration of 10 ng/g f.w.(*50 ng/g for those compounds with LOD above 10 ng/g f.w.)

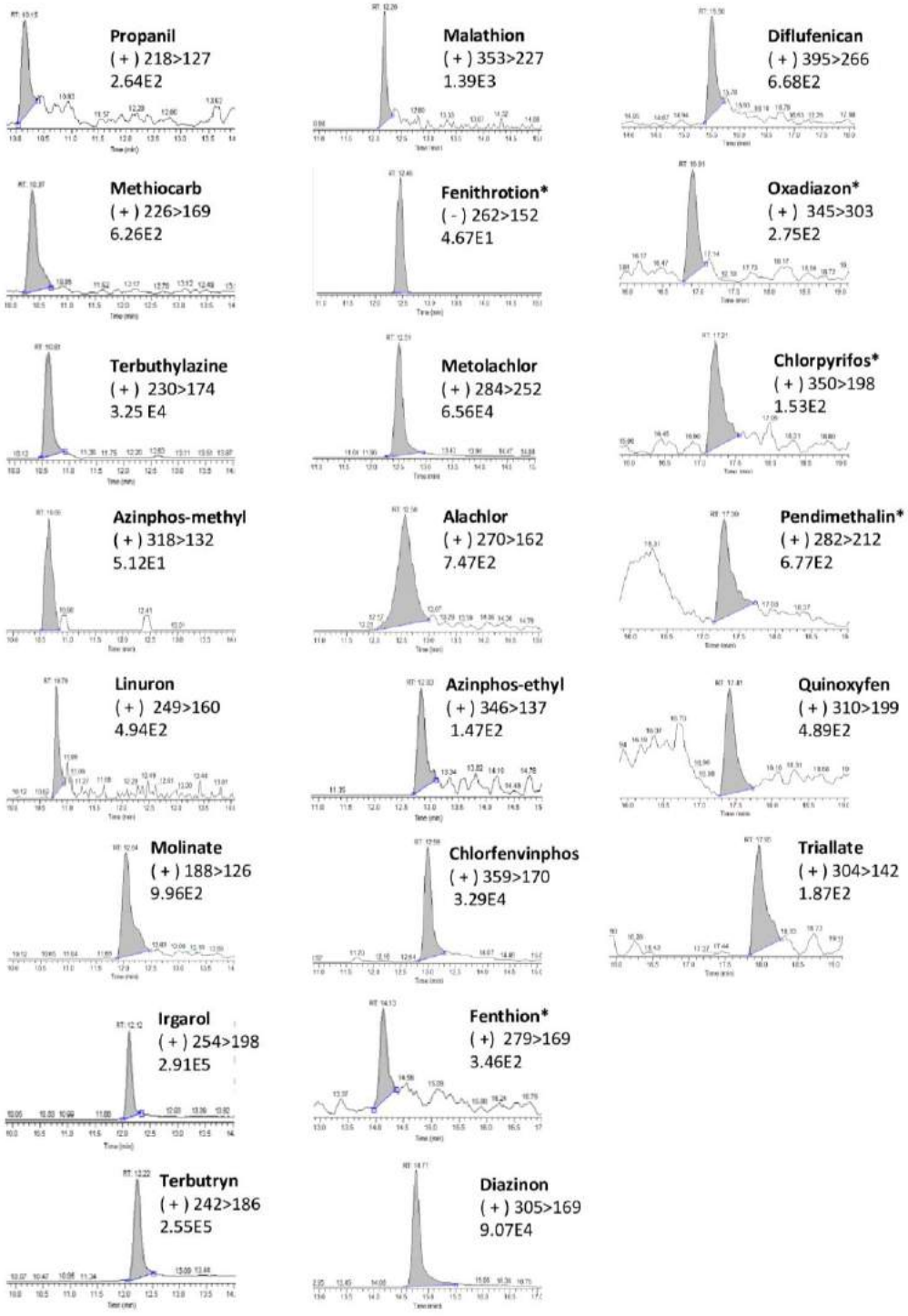

Figure S22. (continued).

3.4 Evaluation of the presence and impact of medium to highly polar pesticides in the Ebro River Delta waters

Agriculture is considered the main source of pesticide pollution in the environment (Rasheed et al., 2019). Wetlands, besides being relevant habitats to preserve biodiversity, are often under intensive agricultural pressure, and thus subject to a high pesticide pollution risk.

The Ebro River Delta, located at the eastern coast of Spain, is one of the largest wetland areas in the western Mediterranean region and hosts numerous beaches, marshes, and salt pans that provide habitat for over 300 species of birds. The modern Delta is nowadays in intensive agricultural use for rice, fruit, and vegetables and it has become a relevant economic area also for seafood production. Therefore, the monitoring of pollution by pesticides in this area is of great interest to evaluate their fate in this ecosystem and the main hazards for aquatic organism and seafood production.

For these reasons, in the scientific article #4 the occurrence of 66 pesticides of different polarities was investigated in the Ebro River Delta, and their fate and potential risk for aquatic organisms assessed. To this end, not only the individual risk of the single compounds was investigated, but also the cumulative risk derived from the pesticide mixtures, which very often is not considered in toxicity studies in spite that it has been shown that synergistic effects of different compounds can lead to higher toxicity on non-target organisms. Results on pesticide occurrence were statistically analysed to identify different spatial pollution patterns and sources.

The results showed the presence of 35 pesticides, and high concentrations of bentazone, MCPA and propanil, whose use is strongly related to rice cultivation. They, together with dicofol, imidacloprid, and irgarol, may represent a high risk for the aquatic ecosystem.

This study was conducted in the framework of the BECAS project (Spanish State Research Agency (AEI) and the European Regional Development Fund (ERDF), CTM2016-75587-C2-2-R).

Scientific publication #4:

"Evaluation of the occurrence and fate of pesticides in a typical Mediterranean delta ecosystem (Ebro River Delta) and risk assessment for aquatic organisms"

Maria Vittoria Barbieri, Andrea Peris, Cristina Postigo,
Alba Moya-Garcés, Luis Simón Monllor-Alcaraz,
Maria Rambla-Alegre, Ethel Eljarrat, Miren López de Alda

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Evaluation of the occurrence and fate of pesticides in a typical Mediterranean delta ecosystem (Ebro River Delta) and risk assessment for aquatic organisms☆

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ABSTRACT

Delta ecosystems are areas of high ecologic and economic values, where wildlife commonly shares the territory with intensive agricultural activities, particularly, rice cultivation and seafood production. This work aimed at evaluating the occurrence of a wide spectrum of pesticides and transformation products in the water of irrigation and drainage channels of the Ebro River Delta (NE Spain) during the main rice-growing season, when pesticide application is at its peak. Furthermore, the impact that these contaminants may have on local ecosystems and seafood production activities was assessed. A total of 35 pesticides, mainly associated with rice cultivation, out of the 66 analyzed were detected. Bentazone, propanil, MCPA, acetamiprid, and triallate were found at the μg/L level. Cybutryne, despite being banned in the European Union, was measured for the first time in the area and at concentrations above its environmental quality standard (11–49 ng/L). Sixteen additional banned pesticides were also detected at trace levels, likely due to their desorption from soil and sediment particles. Despite its dilution when discharged into the bay, this study demonstrates that the agricultural use of pesticides may have important effects on water quality and may cause a serious hazard for aquatic non-target organisms, although other factors such as temperature and salinity may play also a relevant role. Bentazone, cybutryne, dicofol, imidacloprid, MCPA, and propanil may pose a moderate to high risk for aquatic organisms at the concentration levels measured during the rice-growing season. The co-occurrence of pesticides may result in a high risk for aquatic organisms in all sampling locations. The finding of the EU Watch List insecticides imidacloprid and acetamiprid at concentrations above their maximum acceptable method detection limit calls for control of their use and revision of their legal status.

1. Introduction

Pesticides have been widely used since the mid-twentieth century to control pests and improve agricultural production. However, the global use of pesticides has resulted in their widespread presence in the environment (Fenner et al., 2013). In regions with intensive agricultural activities, thousands of pesticide residues are continuously released into the aquatic environment. Pesticide pollution of water is ruled by different mechanisms, *viz.*, physical-chemical and biological degradation, sorption-desorption into solid particles, surface run-off, soil leaching, plant uptake, volatilization, and atmospheric deposition. The extent of these mechanisms is linked to the pesticide properties (*e.g.*, solubility, hydrophobicity), environmental factors (*e.g.*, salinity, temperature, precipitation events), type of soil/sediment (*e.g.*, organic carbon content, microbial activity), and agricultural management practices

☆ This paper has been recommended for acceptance by Maria Cristina Fossi.

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(*e.g.*, type of crops, pesticide application rate) (de Souza et al., 2020; Geissen et al., 2015; Hintze et al., 2020).

Wetlands, often used for intensive agriculture, play also an important role in removing excess pesticides (Lizotte et al., 2012), although with some limitations, because the fate and effects of pesticides in these ecosystems are largely unpredictable and far from being fully known (Rooney et al., 2020). Therefore, the monitoring of these contaminants in these ecosystems and adjacent bays remain a subject of scientific interest, provided that pesticides can persist in water, accumulate into sediments and biota, and hence, affect non-target organisms (Bustos et al., 2019; de Souza et al., 2020; Iturburu et al., 2019).

The Ebro River Delta (Catalonia, NE Spain) is one of the largest wetlands in the western Mediterranean (320 Km^2). Similarly to other Mediterranean areas, this delta has been used for agricultural purposes. Most of the pesticides used in this area are commonly employed also at European level for the control of pests in rice crops, *e.g.*, the herbicides bentazone, molinate, and propanil, mixtures of MCPA with propanil or bentazone (EC, 2003), or the pyrethroid insecticides cypermethrin and deltamethrin (Feo et al., 2010), and occasionally fenitrothion and malathion (Kuster et al., 2008). These pesticides are less persistent and bioaccumulative than the classical organochlorine pesticides, whose use has been banned for years in many countries. However, because they are produced and applied in high quantities, they still represent a potential threat to the aquatic ecosystems (Aguilar et al., 2017; Montiel-León et al., 2019; Parsons et al., 2010). Their behaviour in the environment is strongly linked to their physical-chemical properties. As a general rule, polar compounds are likely to remain in the aqueous phase and potentially leach into aquifers, while less polar compounds are persistent and tend to be sorbed onto sediments and bioaccumulate in living organisms. Moreover, once in the environment, all pesticides can degrade through chemical, physical or biological processes and transform into other compounds (Fenner et al., 2013; Ji et al., 2020), which are usually more polar, and hence more mobile. In some cases, these transformation products (TPs) are more persistent and even more toxic for the aquatic environment than their corresponding parent compound (Bustos et al., 2019; Buttiglieri et al., 2009; Gutowski et al., 2015; Hensen et al., 2020; Sinclair and Boxall, 2009).

In the context of the Water Framework Directive (Directive 2000/60/EC) for the protection of freshwater resources (EC, 2000), 24 pesticides or biocides have been identified as hazardous substances for the environment and are considered as priority substances (EC, 2013). This means that their concentrations in inland surface waters and biota should be below established environmental quality standards (EQS). Furthermore, six additional pesticides are included in the European Watch List of substances for Union-wide monitoring (EC, 2018) that aims at gathering sufficient information to support the decision on their consideration as priority substances. All the existing regulations issued to protect water quality are focused on the presence of single compounds and do not consider the co-occurrence of multiple contaminants. Indeed, most of the studies conducted to assess pesticide toxicity deal with individual pesticides, and hence, neglect possible cumulative or synergic exposure effects caused by compounds of different nature (Cedergreen, 2014; Verro et al., 2009). In this regard, some works have already demonstrated that the toxicity effects caused by the co-exposure to a pesticide mixture can be much higher than those derived from the corresponding additive exposure to the individual compounds (Backhaus et al., 2004; Gatidou et al., 2015; Junghans et al., 2006). Thus, the real ecotoxicological impact of pesticide mixtures is still largely unknown to date (Kuzmanović et al., 2016).

Within this context, the objectives of this study were to (i) investigate the simultaneous presence of 66 pesticides in the Ebro River Delta freshwaters, in terms of their spatial distribution and fate in the study area, and (ii) assess the potential ecotoxicological risk that individual pesticides and pesticide mixtures found during the rice-growing season pose to exposed organisms and hence the seafood production that takes place in the Ebro River Delta.

2. Materials and methods

2.1. Chemicals and standards

High purity (96–99.9%) standards of the 66 selected pesticides and 48 isotopically labeled compounds used as internal standards (IS) were purchased from Fluka (Honeywell Specialty Chemicals Seelze GmbH, Germany), Sigma Aldrich (Merck KGaA, Darmstadt, Germany), Toronto Research Chemicals (North York, ON, Canada), Cambridge Isotope Laboratories (Tewksbury, MA, USA), or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). The target compounds are listed in Table S1 in Supplementary Material (SM). The list includes: five acidic pesticides (2,4-D, bentazone, fluroxypyr, MCPA, mecoprop), two anilides (diflufenican, propanil), two azoles (cyproconazole, triadimefon), three carbamates (methiocarb, molinate, triallate), two chloroacetanilides (alachlor, metolachlor), two dinitroanilines (pendimethalin, trifluralin), five neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid, thiamethoxam), nine organochlorides (2,4′-DDD, 4,4′-DDD, 2,4-DDE, 4,4′-DDE, 2,4′-DDT, 4,4′-DDT, dicofol, heptachlor epoxide, oxadiazon), twelve organophosphates (azinphos ethyl, azinphos-methyl, azinphos-methyl oxon, chlorfenvinphos, chlorpyrifos, diazinon, dichlorvos, dimethoate, fenitrothion, fenitrothion oxon, malaoxon, malathion), six organothiophosphates (fenthion and its metabolites fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, fenthion sulfone, fenthion sulfoxide), four phenylureas (chlortoluron, diuron, linuron, isoproturon), two pyrethroids (cyhalothrin, cypermethrin), eight triazines (atrazine, cyanazine, cybutryne, deisopropilatrazine, desethylatrazine, simazine, terbuthylazine, terbutryn), and four pesticides of other chemical classes (bromoxynil, oxyfluorfen, quinoxyfen, thifensulfuron methyl). The 66 target pesticides were selected considering their current legislation and use in Europe, Spain, Catalonia, and rice crops according to previous studies and information from local authorities.

Individual stock solutions were prepared at a concentration of 1000 μg/mL in methanol (MeOH) in the case of polar compounds or 100 μg/mL in ethyl acetate (EtAc) in the case of nonpolar compounds. As an exception, simazine and its IS analog were prepared in dimethyl sulfoxide to overcome solubility problems. Intermediate working mixture solutions containing all the standards and/or the IS were prepared by appropriate dilution of the individual stock solutions in MeOH or EtAc. These mixtures were used for the preparation of the calibration solutions. All these solutions were stored in amber glass bottles at −20 °C protected from light. Pesticide-grade solvents used, *i.e.*, MeOH, EtAc, acetonitrile (ACN), dichloromethane (DCM), hexane, and LC-grade water were supplied by Merck (Darmstadt, Germany).

2.2. Extraction procedures and instrumental analysis

Two different analytical approaches had to be employed to cover all analytes. Medium to highly polar compounds were analyzed according to Barbieri et al. (2020), using a method based on on-line solid-phase extraction-liquid chromatography-tandem mass spectrometry (on-line SPE-LC-MS/MS). Briefly, water samples (5 mL) were preconcentrated onto previously conditioned CHROspe cartridges (divinylbenzene polymer, 10 mm × 2 mm i.d., 25–35 μm particle size) (Axel Semrau GmbH & Co. KG, Srockhövel,

Germany) using an automated on-line SPE sample processor Prospekt-2 (Spark Holland, Emmen, The Netherlands) at a flow rate of 1 mL/min. After sample loading, the cartridges were washed with 1 mL of LC-grade water and the analytes were eluted with the LC mobile phase onto the chromatographic column. LC-MS/MS analysis was performed using a reversed-phase Purospher® STAR RP-18 end-capped column (100 mm × 2 mm i.d., 5 μm particle size) from Merck, a 1525 binary HPLC pump (Waters, Milford, MA, USA), and a TQD triple-quadrupole mass spectrometer (Waters) equipped with an electrospray ionization (ESI) source operated in both positive and negative modes. Mass acquisition was done in the selected reaction monitoring (SRM) mode.

Nonpolar pesticides were analyzed following a previously optimized method based on liquid-liquid extraction (LLE) and gas chromatography-tandem mass spectrometry (GC-MS/MS) detection (Peris and Eljarrat, 2020). Water samples (50 mL) were manually extracted twice with 25 mL of EtAc/chloroform (1:1) mixture by classical LLE in a 100 mL separatory funnel. The extract obtained was evaporated under a gentle stream of nitrogen: firstly, to an approximate volume of 1 mL using a Turbovap (Biotage, Sweden), and then, to dryness using a needle evaporator (ReactiVap III, Pierce, USA). The dried extract was reconstituted in 50 μL of EtAc (1000x concentration factor). GC-MS/MS determination was performed in SRM mode using a 7890B GC system coupled to a 7000C triple quadrupole (Agilent Technologies, Santa Clara, CA, USA) detector. For chromatographic separation, a DB-5MS column (30 m × 250 μm x 0.25 μm) was used.

Table S2 in SM summarizes the analytical limits of detection (LODs) and quantification (LOQs) achieved for the target pesticides with the methodologies employed. Table S3 in SM reports the main on-line SPE-LC-MS/MS and GC-MS/MS acquisition parameters.

2.3. Study area

The Ebro River Delta is an area of high ecological and agricultural value. This delta is characterized by two lateral spit bars NW and SW of the river mouth that form two bays, namely Fangar (NW) and Alfacs (SW). Although fishing and aquaculture are economic activities of relevance, agriculture is the main occupation, with 80% of the land devoted to rice cultivation (Köck et al., 2010). Rice cultivation in the Ebro River Delta extends for 22,000 ha and as for this, an extensive network of irrigation and drainage channels has been constructed. Two main channels, located on each side of the river, move water into the fields for irrigation, and also collect the water excess (Ochoa et al., 2012). Thus, this channel network plays an important role in the transport of pesticides from rice crops to the bays, where aquaculture is also an important activity, especially for the cultivation of oysters and mussels. Moreover, there are several chemical industries in the area that may also affect the quality of the water in the Delta (Gusmaroli et al., 2019). In addition to the impact that these activities may have on the Delta ecosystem, hydrological changes derived from climate change have also an effect on the chemical status of its water (Batalla et al., 2004; Ccanccapa et al., 2016). In this regard, the Ebro Delta, due to its Mediterranean character, is a very vulnerable area that has faced major changes since the last century as a consequence of global warming (Taller d'Enginyeria Ambiental, 2008; Somoza and Rodríguez-Santalla, 2014).

2.4. Sampling

Sampling was conducted in June 2017, during the main rice-growing season, and hence the highest use of pesticides in the area, reflecting the worst-case scenario. The Ebro Delta area and the sampling sites are shown in Fig. 1 (coordinates of sampling locations are provided in Table S4 as SM). The freshwater sites sampled included nine locations in the northern part (Fangar bay) and nine in the southern part (Alfacs bay). Most of these sampling sites corresponded with drainage channels (fourteen samples), whereas five of them were irrigation channels. The irrigation channels were sampled at different sites with an increasing proportion of water recovered from the agricultural fields (Terrado et al., 2007).

Samples were collected in amber polyethylene terephthalate (PET) bottles, transported in refrigerated containers to the laboratories, and frozen at −20 °C until their analysis. Before sample extraction, the samples were spiked with the mixture of IS, and, for the analysis of medium to highly polar compounds, they were also centrifuged at 3500 rpm at room temperature for 10 min to remove suspended particles (centrifuge 5810 R, Eppendorf Ibérica, Spain). Therefore, concentrations in the aqueous phase were only considered for the pesticides measured using the on-line SPE-LC-MS/MS approach.

2.5. Statistical analysis

For statistical purposes, the concentration of non-detected values was set to half the LOD, while the non-quantifiable values (<LOQ) were assigned a concentration of LOQ/2. Furthermore, those pesticides with low detection frequencies (<30%) were excluded. A detailed analysis of the investigated variables and further details on the statistical analyses performed are provided as supporting information (Text S1 and Table S5).

The potential relationships among pesticide occurrence in the study area were investigated through pairwise correlations using the Spearman's rank test. A significance level of 0.05 was established. Principal-component analysis (PCA) was used to extract useful information from the data, *e.g.*, to investigate multivariate correlations between the concentrations of the different pesticides, and their geographical distribution.

All statistical analyses were performed using the R statistical software interface R-Studio (R version 3.6.3). The R code used is stored in the GitHub repository https://github.com/albamgarces/PCA_EbrePesticides_2020.

2.6. Risk assessment

The environmental risk that the presence of pesticides in the freshwater samples may pose to aquatic organisms was assessed using the hazard quotient (HQ) approach. The risk quotient of a single pesticide (HQ_i) was calculated using the equation $HQ_i = \frac{MEC}{PNEC}$, where MEC is the measured environmental concentration and PNEC is the lowest concentration at which toxic effects are not expected (predicted no-effect concentration). In this study, the average concentration and the maximum concentration measured for each pesticide were used as MEC to assess the general risk and to evaluate the worst-case scenario, respectively. PNEC values were obtained from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/) (Dulio and Von der Ohe, 2013), and corresponded to the lowest value predicted by QSAR models or obtained experimentally. Moreover, to have a complete view of water pollution by pesticides in the area and the associated ecotoxicological risk, an additive model was applied. For this, the HQ of each sampling site (HQ_s) was calculated as the sum of the HQ of the various pesticides present in the corresponding sample (HQ_i), following the equation: $HQ_s = \sum_{i=1}^{n} HQ_i$. Such an additive model allows to evaluate the potential ecotoxicological risk derived from the co-occurrence of various pesticides in a specific location, although with some limitations, as it does not consider

Fig. 1. Map of the Ebro River Delta, with a detail of the sampling locations. F: Fangar zone; A: Alfacs zone; CD: drainage channel (red spots); CE: irrigation channel (blue spots). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unpredictable synergistic or antagonistic effects. HQ values below 1 indicate zero or low risk, while HQ values between 1 and 10 anticipate moderate risk, and HQ values above 10 suggest high environmental risk.

3. Results and discussion

3.1. Occurrence of individual pesticides

The occurrence of the investigated pesticides in surface waters of the Ebro River Delta is summarized in Table 1 and detailed in Table S8 in the SM. Moreover, the pairwise correlations among pesticide concentrations in the investigated samples (Fig. 2; Figures S1 and S2 and Table S6) have been also evaluated to further explain the results observed.

More than half of the target pesticides (35 out of 66) were detected in the Ebro River Delta surface waters. It is important to highlight that in the case of medium and highly polar pesticides, their occurrence was investigated only in the aqueous phase, as suspended particles were removed before sample extraction. However, in the case of apolar pesticides, amounts sorbed onto suspended particles may have eventually been recovered during the sample extraction process, although the determination in the solid phase of the freshwater system was not the objective of the study.

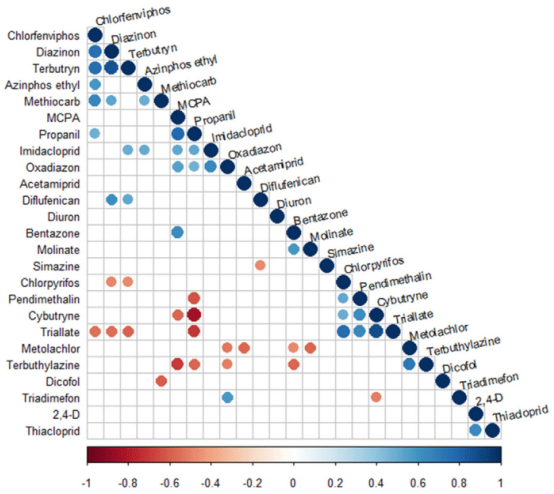

Fig. 2. Pairwise correlations between pesticide concentrations in the study area (after Spearman's Rank test, $\alpha = 0.05$).

Table 1
Minimum, maximum, and mean concentration in ng/L of the detected individual pesticides and frequency of detection in the investigated water samples.

Class	Name	Concentration (ng/L)			
		Min	Max	Mean[α]	Frequency[β] (%)
Acidics	2,4-D	10	440	41	50
	Bentazone	150	180×10^3	53×10^3	100
	MCPA	130	8210	1700	61
Anilides	Diflufenican	2.0	19	4.2	50
	Propanil[a]	21	61×10^3	9000	83
Carbamates	Methiocarb	0.74	3.3	1.0	56
	Molinate[a]	5.7	48	16	61
	Triallate	41	1000	310	100
Chloroacetanilides	Alachlor[a]	1.4	1.6	0.17	11
	Metolachlor[a]	10	73	38	100
Dinitroaniline	Pendimethalin	1.0	1.0	0.61	61
Neonicotinoids	Acetamiprid	0.25	4000	420	67
	Imidacloprid	23	700	130	61
	Thiacloprid	0.11	2.7	0.43	44
Organochlorines	4,4′-DDD[a]	1.2	1.2	0.07	6
	Dicofol[a]	3.7	3.7	1.8	50
	Oxadiazon[a]	0.35	47	18	89
	Triadimefon[a]	2.0	4.9	1.2	50
Organophosphates	Azinphos ethyl[a]	0.70	5.6	0.94	33
	Chlorfenvinphos[a]	0.40	6.3	1.9	72
	Chlorpyrifos	0.75	27	15	100
	Diazinon[a]	1.0	4.8	2.1	89
	Malaoxon	0.25	0.57	0.060	17
Organothiophosphates	Fenthion oxon	0.81	2.5	0.41	28
	Fenthion oxon sulfoxide	0.22	3.2	0.11	28
	Fenthion sulfoxide	0.70	4.5	0.43	22
Phenylureas	Chlortoluron	7.5	14	1.2	11
	Diuron	5.2	12	5.7	67
	Isoproturon[a]	13	13	0.70	6
	Linuron[a]	1.0	13	1.1	22
Triazines	Atrazine[a]	0.45	2.5	0.19	17
	Cybutryne[a]	11	49	29	100
	Simazine[a]	0.55	6.7	1.5	44
	Terbuthylazine	11	41	21	72
	Terbutryn[a]	1.7	6.6	2.7	89

[α] Mean calculated considering values < LOQ as LOQ/2 and values < LOD as zero.
[β] % of positive samples, including compounds with values < LOQ.
[a] Compounds currently prohibited for their use in Europe. The exceptional use of propanil is annually allowed in Spain.

The compound that was found at the highest concentration was the herbicide bentazone, present in all investigated samples and measured at the maximum concentration of 18 × 10^4 ng/L. Individual concentrations of propanil, MCPA, acetamiprid, and triallate also reached the μg/L level, with maximum values of 61 × 10^3, 8200, 4000, and 1000 ng/L, respectively. Imidacloprid and 2,4-D could be also highlighted among the most abundant pesticides in the investigated area, with concentrations above 100 ng/L, and occurrence peaks of 700 and 440 ng/L, respectively. The herbicide 2,4-D, largely used in cereal crops, presented an average concentration of 41 ng/L in this study. This herbicide was also found at similar concentrations in previous studies conducted in this area by Terrado et al. (2007) (mean of 22 ng/L) and Barta et al. (2007) (mean of 24 ng/L).

Among the most abundant pesticides aforementioned, bentazone and triallate were the two most widely distributed herbicides, found in all sampling sites. In addition to bentazone and triallate, other pesticides like chlorpyrifos, cybutryne, and metolachlor can be classified among the most ubiquitous in the area of study (detection frequency of 100%), but they presented much lower concentrations (up to 27, 49 and 73 ng/L, respectively) than the aforementioned pesticides bentazone and triallate. The remaining most abundant compounds were detected in more than half of the samples (61–83%), except 2,4-D that was present in 50% of the samples (Table 1). The presence of these pesticides is related to their agricultural use in rice crops. Bentazone, propanil, and MCPA are indeed among the herbicides mostly applied at the European level for the control of pests in rice crops and, consequently, their presence in the Ebro Delta surface waters has been previously documented. Similar bentazone concentrations (up to 13 × 10^4 ng/L) were reported in a study conducted in this area during the rice-growing season ten years ago (Kuster et al., 2008). This finding suggests a repeated pattern of high bentazone levels in the Ebro Delta during the same period throughout the years. Its physical-chemical properties (Table S1 in SM) - high solubility (7112 mg/L) and polarity (log K_{ow} −0.46), relatively high half-life time in water (DT_{50}) (80 days), and rather low organic carbon-water partition coefficient (K_{oc} 55) - denote its preference to remain in the water, and thus, justify the high concentrations found after its application. Similar conclusions could be drawn for MCPA and propanil, pesticides continuously found in the Ebro Delta surface waters (Köck et al., 2010; Kuster et al., 2008). Despite propanil was withdrawn from the EU market in 2008 (EC, 2008), its detection is still possible because the Spanish Government annually issues an exceptional authorization for the use of this active substance in rice crops during the growing season (May to July) (Spanish Ministry of Agriculture, 2017).

Although bentazone concentrations were not strongly correlated with those found for MCPA or propanil, these two showed a significant and strong positive correlation (Fig. 2 and Figures S1 and S2). This could suggest a similar application pattern of MCPA and propanil in this area, which differs from that of bentazone. Note that contrary to MCPA (auxin synthesis inhibitor), bentazone and propanil share a common mode of action (photosynthesis inhibition).

MCPA and propanil were negatively correlated with triazine herbicides like terbuthylazine and cybutryne, and the carbamate herbicide triallate. Terbuthylazine and triallate may be more commonly applied in crops other than rice in the area, while cybutryne is no longer approved for use, and therefore residual concentrations may be released from the sediments/soils where it may have been accumulated during its past use in antifouling paints for ships and boats. Triallate, scarcely investigated in the Ebro Delta freshwaters in previous studies, was recently reported to occur in this area at very low concentrations (<2 ng/L, in 24% of the analyzed samples) (Gusmaroli et al., 2019). This herbicide showed also a high and significant correlation with cybutryne and chlorpyrifos (Table S6, Fig. 2 and Figures S1 and S2). While the correlation with cybutryne is difficult to explain, since its use is banned, that with chlorpyrifos, an organophosphate insecticide used to control foliage and soil-borne insect pests in a variety of crops, especially in corn and other cereal fields, could be explained by the simultaneous application of these two pesticides in the study area. Chlorpyrifos has already been reported as one of the most commonly detected pesticides in the Ebro River in several studies (Claver et al., 2006; Navarro et al., 2010), where it has been found at concentrations higher than those measured in the present work, in spite that it presents a low sloubility in water (1.05 mg/L) and is considered not persistent in the water phase (DT50 of 5 days). The concentration found in this study were always below the EQS of 100 ng/L set for this pesticide in surface water (EC, 2013), in contrast to the concentrations up to 312 ng/L reported by Claver et al. (2006). On the contrary, the herbicidal biocide cybutryne, from the triazines group, exceeded its EQS of 16 ng/L in 72% of the sampled locations. As previously mentioned, the presence of this compound is not linked to agriculture practices, but to its application as an antifouling agent in paints for boats and other water vessels to control slimes, molds, mosses, and algae (Lewis et al., 2006). Since 2016 its use in antifouling products is no longer allowed in the European Union (EC, 2016) and, therefore, its detection could be attributed to illegal use of cybutryne stocks or, what it is more likely, to resuspension from the Delta sediments. According to its properties (low water solubility 7 mg/L, high log K_{ow} 3.95, and high K_{oc} 1569) this compound is likely to sorb onto particles, which is supported by the report of its presence in freshwater sediments of Mediterranean areas (Barbieri et al., 2019). To the best of our knowledge, cybutryne has never been investigated before in the Ebro River Delta and hence the results obtained cannot be compared with historical data. Metolachlor, an herbicide usually found in the Ebro Delta freshwaters, was also detected in this study, at concentrations similar to those reported in previous works (Claver et al., 2006; Köck et al., 2010), even though it is no longer commercially available in the EU market (EC, 2002).

A large proportion of the pesticides identified in the study area are currently banned for use (17 out of 35 detected pesticides). Strong positive correlations were also found between banned pesticides (chlorfenvinphos + diazinon, chlorfenvinphos + terbutryn, or diazinon + terbutryn) (Table S6, Figure 2, and Figures S1 and S2). Like cybutryne, these positively correlated pesticides present a Log K_{ow} above 3.6, and thus, a high tendency to sorb onto soil and sediment particles, and a GUS index above 1.5 (Table S1). Thus, their presence in the area could also result from desorption of solid particles where they may be accumulated.

As for the neonicotinoid insecticides detected at the highest concentrations in this study, *viz*., imidacloprid (up to 700 ng/L) and acetamiprid (up to 4 × 10^3 ng/L), the former was also found in previous studies conducted in the Ebro Delta, at maximum concentrations of 182 ng/L (Gusmaroli et al., 2019), 16 ng/L (Borrull et al., 2019) and 15 ng/L (Ccanccapa et al., 2016), while the latter was measured for the first time at maximum concentrations of 58 ng/L in a few samples in the recent study conducted by Gusmaroli et al. (2019). Since acetamiprid is the neonicotinoid with the shortest half-life DT50 (4.7 days) and imidacloprid is the neonicotinoid with the highest K_{oc} (6719) and moderate water solubility (610 mg/L), the high concentrations detected indicate that these two insecticides are extensively applied in the Ebro Delta to control sucking insects on crops like rice, cereals, potatoes, and sugar beet.

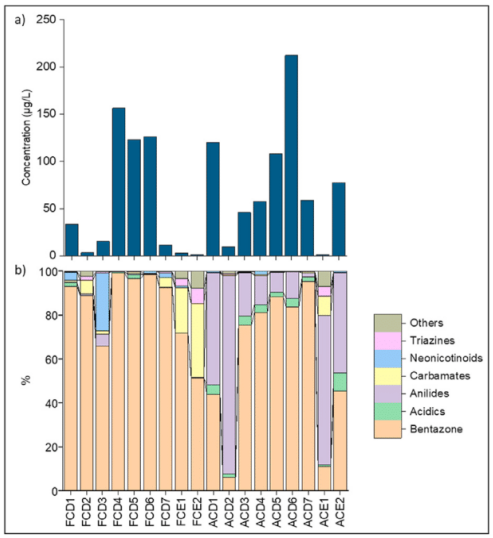

Fig. 3. a) Total concentration of pesticides in the analyzed water samples of the Ebro River Delta; b) contribution of each class to the total pesticide levels. Pesticides included under "Others": azoles, chloroacetanilides, organochlorines, organophosphates, organothiophosphates, pendimethalin, phenylureas. Bentazone is shown outside the class of acidic pesticides to graph its amount separately. (Sample codes: F: Fangar zone, A: Alfacs zone, CD: drainage channel, CE: irrigation channel).

3.2. Spatial distribution of pesticide contamination patterns in the Ebro River Delta

The contribution of each pesticide class to the total pesticide levels in the investigated samples is illustrated in Fig. 3. As can be seen, the profile of pesticide contamination in the Ebro Delta is overall characterized by the dominant presence of acidic pesticides (85% contribution to total pesticide levels on average, with 82% corresponding to bentazone) and anilides (14%). The contribution of all the remaining pesticide classes was lower than 2% in each case. Triazines and organophosphate pesticides, although at lower levels than acidic pesticides and anilides, were also among the most detected and ubiquitous pesticide groups in the investigated waters, with 2–5 different compounds present in each sample. PCA was used to statistically investigate pesticide contamination patterns (Fig. 4) and the geographical distribution of the identified contamination patterns in the Ebro Delta during the main rice-growing season (Fig. 5) (further details in SM, Figures S3 and S4). Up to 65% of the data variance could be explained with four principal components (PCs) (Table S7). Overall, diffuse contamination patterns were identified by each PC, with several different pesticides (including banned pesticides) contributing in each case. However, all four PCs describe a contamination pattern mainly coming from rice-growing fields (due to the presence of MCPA, bentazone and/or propanil) that is inversely correlated to pesticides coming from other sources (different agricultural activities in the area or main river transport from upstream activities) (Fig. 4). In the case of PC4, which explains only 9% of the total variance, pesticides used for rice cultivation were inversely correlated, and thus, this PC may describe a contamination pattern generated by small local changes in the use of pesticides (Fig. 4).

Although the spatial distribution of pesticide pollution was variable (Figure 5), overall waters in the Alfacs bay (south of the Delta) were more contaminated than in the Fangar bay (north), in terms of co-occurrence of pesticides and total pesticides loads. PCA scores plots also indicate indeed a different pesticide pattern in Alfacs bay compared to Fangar bay (Fig. 5). Overall, all PCs point a contamination pattern coming from rice-growing fields in most sampling locations of the Alfacs bay, although some locations of the Fangar bay were also exceptionally included in each case. This type of contamination was found in both drainage and irrigation channels. PC4 indicated that MCPA and propanil use was relevant in ACD1, ACD6 and ACE2 locations of the Alfacs bay, while bentazone application was predominant in other locations of this bay (ACD2, ACD3, and ACD5) and in FCD3 of the Fangar bay (Fig. 4).

The most contaminated sites of the Alfacs bay were ACD1 and ACD6, which correspond with drainage channels located close to the main course of the river and south of Deltebre town that collect water from the fields located nearby. Both sites were highly contaminated with bentazone, MCPA, and propanil, due to the use of these pesticides for rice and cereal cultivation in the surrounded areas. The pesticide contamination pattern described by PC3 and PC4 was similar in these two locations, but those described in PC1 and PC2 were exclusive for ACD1 and ACD6, respectively (Fig. 5). This could be explained because there is less rice cultivated area upstream (ACD1) than downstream (ACD6). The farmland upstream is also devoted to the cultivation of fruit trees, especially citrus fruits such as orange and tangerine, which would also explain the presence of pesticides that are not commonly applied in rice field. This hypothesis could be confirmed by the increasing bentazone and MCPA concentrations in the downstream direction.

In the irrigation and drainage system network further south, water pollution by pesticides increased in the direction to the bay (from ACD2 to ACD5 sampling sites). Each water sample collected in the Alfacs drainage channels presented a total co-occurrence of more than 20 pesticides, which reflects the high use of pesticides in the area during the sampling period. While ACD4 receives water from ACD2, which may explain the increased levels found in ACD4 (both showing positive scores in PC1), ACD3 and ACD5 are independent channels that do not receive water from the aforementioned channels, and thus, pesticide pollution found in their waters has its origin on the drained crop fields (although ACD3 has a similar contamination pattern than ACD2 and ACD4 according to PC1, and ACD5 has a similar contamination pattern than ACD4 according to PC2) (Fig. 5).

The least contaminated site on the SW side of the delta was the irrigation channel ACE1. In this site, the water is not affected by pesticide application because it comes directly from the right-hand channel of the Ebro River without receiving any input (Terrado et al., 2007) from drainage channels. On the other hand, and contrary to expected, the irrigation channel ACE2, coming directly from Sant Carles de la Rapita town, presented considerable contamination, which may result from runoff events from nearby agricultural fields.

Overall, pesticide contamination patterns in the Fangar bay (NW) was driven by herbicides other than bentazone, MCPA and propanil (*e.g.*, triallate in PC1 and PC3, terbuthylazine in PC2) and a variety of other pesticides. Overall, higher pesticide loads were present in the drainage channels, particularly, in those located nearby the bay, than in the irrigation ditches. The water from FCD5 and to a smaller extent the water from FCD6 feed a green filter designed to improve the quality of the water drained from the rice fields into the bay. The effluent of the filter is discharged into FCD4; however, the sampling location was located before the discharge point, and therefore the results observed cannot be used to evaluate the performance of the filter in terms of pesticide removal.

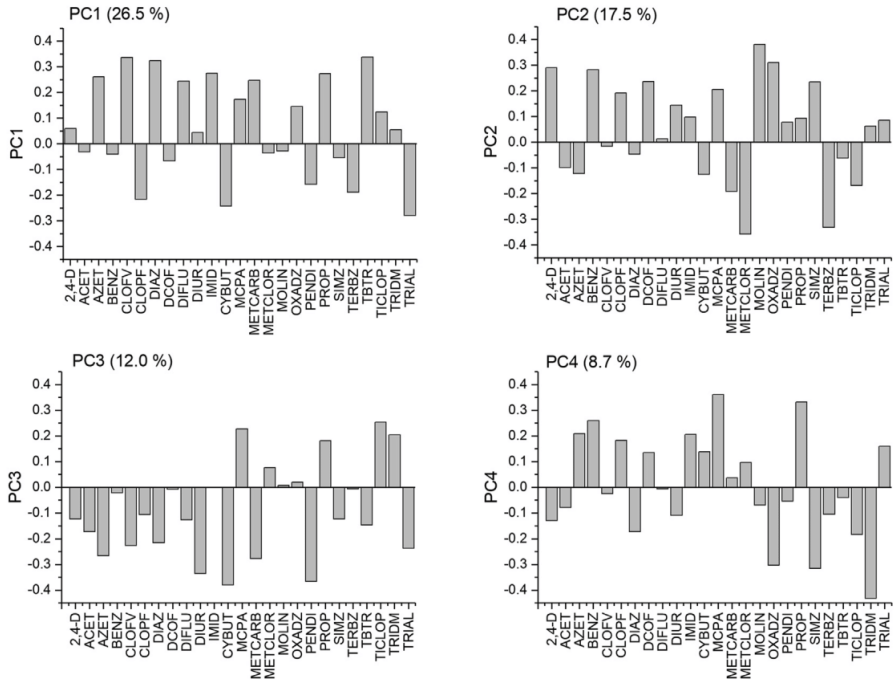

Fig. 4. Amount of variance explained by each PC and loading plots showing the main pesticide contamination patterns identified by PCA in the Ebro Delta.

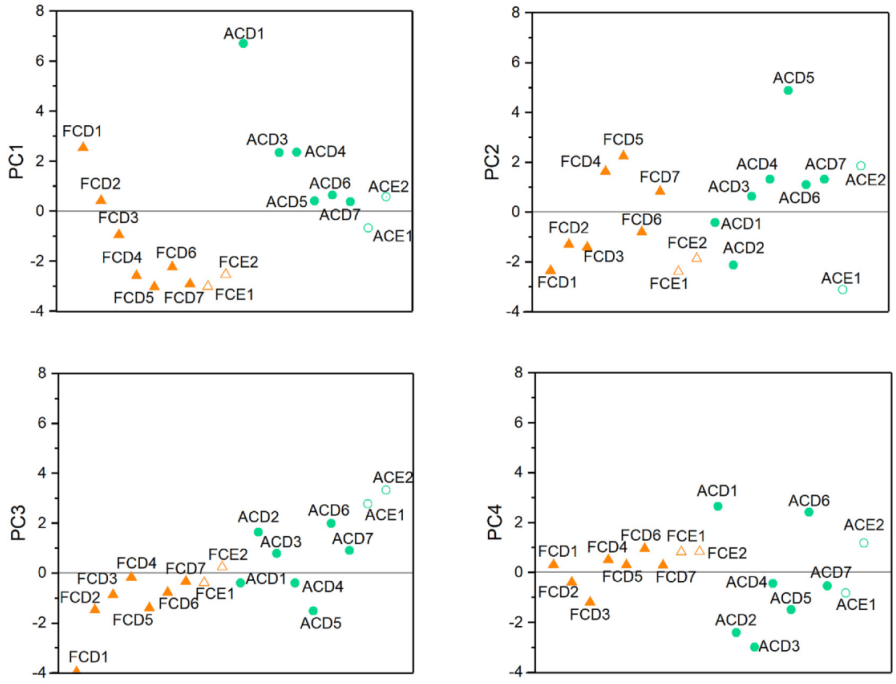

Fig. 5. Scores plot of the four contamination patterns identified by PCA showing the spatial distribution of pesticide pollution.

3.3. Pesticides in the Ebro Delta under the current legal framework

Nowadays, 17 of the 35 detected compounds (see Table 1 and S1 in SM) are currently banned by the European Commission for their use. Of these, propanil is the only one whose exceptional use in rice crops is annually authorized in Spain during the growing season. Thus, the presence of the remaining banned pesticides in the Ebro Delta waters is unexpected and could be attributed to illegal use of existing stock solutions or what is more likely, their release from soils/sediments. Looking at the physical-chemical properties of these compounds (Table S1) (*i.e.*, low water solubility, high octanol-water partition coefficient (log K_{ow}), high organic carbon-water partition coefficient (K_{oc}) and a long half-life time (DT50)), they are not expected to be found in the aqueous phase and they are likely to sorb onto suspended particles and accumulate into soil and sediments. This is particularly true, in the case of the banned pesticides with high log K_{ow} values (>3), *i.e.*, 4,4-DDD, alachlor, azinphos ethyl, chlorfenvinphos, diazinon, dicofol, linuron, oxadiazon, terbutryn, and triadimefon (Table S1). Due to the apolar character of these compounds, they are also likely to bioaccumulate in aquatic organisms, which points out the importance of assessing not only the environmental risk associated with their occurrence in water but also their occurrence in aquatic organisms, paying special attention to those intended for human consumption.

The only priority pesticide found to occur at concentrations above the established EQS in surface water was the herbicide cybutryne (EQS of 16 ng/L) (100% of detection frequency and concentration > 16 ng/L in 72% of the samples) (Table 1). Cybutryne contributed moderately to the pesticide pollution pattern described by PC1 and PC3, observed mainly in most of the Fangar bay samples (Figs. 4 and 5). The remaining priority pesticides targeted were either not detected or measured at a concentration below their corresponding EQS (EC, 2013).

As for the pesticides included in the Watch List, the neonicotinoids imidacloprid and acetamiprid, with maximum measured concentrations of 700 ng/L and 4×10^3 ng/L, respectively, largely exceeded the maximum acceptable LOD of 8.3 ng/L set for them in the regulation (EC, 2018). Also, methiocarb was found to exceed its LOD (2 ng/L) in two sampling locations (up to 3.3 ng/L, frequency of detection of 50%). LOD values set in the legislation for the detection of these substances match their PNECs in water, and therefore, undesired effects on aquatic organisms at the measured concentrations could be expected.

3.4. Environmental risk assessment

To evaluate the impact of the pesticides in the Ebro River Delta ecosystem, the hazard quotient approach was employed, by comparing the maximum and mean measured concentrations of each pesticide with its corresponding lowest PNEC (extracted from the NORMAN ecotoxicology database) (Dulio and Von der Ohe, 2013). The results obtained have been summarized in Table 2. Only 10 out of the 35 pesticides detected in the Ebro Delta presented a certain risk in both investigated contamination scenarios. Bentazone, dicofol, imidacloprid, and propanil exhibited the highest HQ values (HQ > 10) under normal (average) and worst-case contamination scenarios, whereas MCPA and cybutryne only may pose a high risk under the worst-case contamination scenario. The potential high risk obtained for these pesticides is mostly attributed to the high concentrations measured in the samples, except for dicofol and cybutryne, detected at relatively low concentrations (<7.3 ng/L in the case of dicofol and 11–49 ng/L in the case of cybutryne). Thus, the high-risk values obtained for these pesticides are mainly driven by their very low PNEC values (0.032 ng/L for dicofol and 3.5 ng/L for cybutryne).

A moderate risk (HQ > 1) was obtained for MCPA and cybutryne under a normal (average) contamination scenario and for 4,4′-DDD, acetamiprid, azynphos ethyl, and diflufenican under the worst-case contamination scenario. Except for acetamiprid, which was detected at high concentrations in the investigated area, the risk associated with 4,4′DDD, azynphos ethyl, and diflufenican can be attributed to their low PNEC values (<10 ng/L).

Further risk assessment analyses were conducted in this study to evaluate the environmental risk associated with the pesticide mixtures present in each sampling site. As shown in Fig. 6 (detailed in Table S9 in the SM), the HQ values obtained suggest a high risk in all sampling locations (HQ > 10), even in those with the lowest pesticide loads. The approach used is a simple additive model, and consequently, it may underestimate the real risk because synergistic effects that may occur among co-occurring contaminants are not considered. Despite this, the findings obtained highlight the need for conducting risk assessment studies with pollutant mixtures. For mixtures of substances that do not share a common mechanism of action, effects on joint toxicity can be expected. This hypothesis has been already confirmed in several studies that investigated the co-exposure to pesticide mixtures and their synergistic effects on non-target organisms. For instance, the exposure of carps to organophosphates and carbamate pesticides produced neurotoxicity (Wang et al., 2015), by inhibiting the activity of acetylcholinesterase (AChE) and interfering with the normal behavior of this species. The combined presence of organochlorine and organophosphate pesticides resulted in a synergistic effect that decreased the immune response capacity of white shrimps (Abad-Rosales et al., 2019; Bautista-Covarrubias et al., 2020). A recent study has also demonstrated that mixtures of pyrethroids and neonicotinoids exhibit a synergistic effect in the enzyme activity and gene expression of embryonic zebrafish (Wang et al., 2020). Additional evidence of additive toxicity has been observed after exposure of duckweeds to phenylureas and algal plants to triazines, which resulted in the blockage of the transport of photosynthetic electrons at the level of the photosystem II (Faust et al., 2001; Gatidou et al., 2015).

In the Ebro River Delta, two studies have investigated the potential toxic effects of some pesticides on non-targeted organisms (Álvarez-Muñoz et al., 2019; Ochoa et al., 2012). In 2017, analysis of shellfish specimens dead in the course of mortality episodes that took place in the Ebro Delta between April and November showed the presence of small concentrations of metolachlor, atrazine, bentazone, and acetamiprid. In that study, mortality events were not associated with any particular chemical present in the water but with other causes such as the presence of potential pathogens (Álvarez-Muñoz et al., 2019). In a previous study, a similar approach, conducted in this case to find out the possibles causes of mortality events affecting oysters cultivated in the Ebro Delta bays, showed the presence of bentazone and propanil in the dead organisms and its relation with markers of tissue damage during DNA strand breakage (Ochoa et al., 2012). However, many other factors need to be considered when assessing possible causes that lead to organism mortality, since aspects such as temperature, salinity, runoff, as well as temporal trends of exposure may influence the synergistic effects of pesticides and therefore, their risk to aquatic organisms.

4. Conclusions

The monitoring of 66 pesticides in the Ebro River Delta during the rice-growing season in 2017 revealed the presence of 35 compounds in the Ebro Delta surface waters. Bentazone was found to be the herbicide with the highest concentrations (up to 18×10^4 ng/L) in all the samples analyzed, followed by propanil, MCPA,

Table 2
Hazard Quotient (HQ) for the worst-case (HQ-Max) and the normal (HQ-mean) contamination scenarios.

Class	Name	PNEC[a] (μg/L)	MEC[b]-Max (μg/L)	MEC[b]-Mean (μg/L)	HQ-Max	HQ-Mean
Acidics	2,4-D	12.4	0.441	0.041	0.036	0.003
	Bentazone	0.1	177.4	53.04	**1774**	**530**
	MCPA	0.5	8.212	1.704	**16.4**	**3.41**
Anilides	Diflufenican	0.009	0.019	0.004	**2.07**	0.463
	Propanil[a]	0.2	61.21	8.968	**306**	**44.8**
Carbamates	Methiocarb	0.01	0.003	0.001	0.332	0.097
	Molinate[a]	3.8	0.048	0.016	0.013	0.004
	Triallate	10	1.011	0.306	0.101	0.031
Chloroacetanilides	Alachlor[a]	0.3	0.002	0.0002	0.005	0.001
	Metolachlor[a]	0.2	0.073	0.038	0.364	0.189
Dinitroaniline	Pendimethalin	0.018	0.001	0.001	0.056	0.034
Neonicotinoids	Acetamiprid	3.74	3.993	0.421	**1.07**	0.112
	Imidacloprid	0.0083	0.703	0.127	**84.7**	**15.3**
	Thiacloprid	0.01	0.003	0.0004	0.266	0.043
Organochlorines	4,4′-DDD[a]	0.0005	0.001	0.0001	**2.4**	0.135
	Dicofol[a]	0.000032	0.004	0.002	**114.1**	**57.03**
	Oxadiazon[a]	0.088	0.047	0.018	0.532	0.207
	Triadimefon[a]	1.86	0.005	0.001	0.003	0.001
Organophosphates	Azinphos ethyl[a]	0.0011	0.006	0.001	**5.13**	0.851
	Chlorfenvinphos[a]	0.1	0.006	0.002	0.063	0.019
	Chlorpyrifos	0.03	0.027	0.015	0.900	0.513
	Diazinon[a]	0.01	0.005	0.002	0.481	0.211
	Malaoxon	0.31	0.001	0.0001	0.002	0.0002
Organothiophosphates	Fenthion oxon	0.2	0.003	0.0004	0.013	0.002
	Fenthion oxon sulfoxide	n/a	0.001	0.00011	–	–
	Fenthion sulfoxide	10	0.004	0.0004	0.0004	0.00004
Phenylureas	Chlortoluron	7.25	0.014	0.001	0.002	0.0002
	Diuron	0.2	0.012	0.006	0.059	0.029
	Isoproturon[a]	0.3	0.013	0.001	0.042	0.002
	Linuron[a]	0.1	0.013	0.001	0.131	0.011
Triazines	Atrazine[a]	0.6	0.002	0.0002	0.004	0.0003
	Cybutryne[a]	0.0035	0.049	0.029	**14.03**	**8.294**
	Simazine[a]	1	0.007	0.001	0.007	0.001
	Terbuthylazine	0.06	0.041	0.021	0.686	0.352
	Terbutryn[a]	0.065	0.007	0.003	0.101	0.042

n/a: data not available.
[a] Lowest PNEC extracted from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php).
[b] MEC: Measured environmental concentration

acetamiprid, triallate, imidacloprid, and 2,4-D. The occurrence of all these pesticides in the waters is related to their agricultural use and has continuously been documented in this area. Different diffuse pesticide contamination patterns were identified using PCA. All PCs obtained describe a contamination pattern mainly coming from rice-growing fields (due to the presence of MCPA, bentazone and/or propanil) that was inversely correlated to pesticides coming from other sources (different agricultural activities in the area or main river transport from upstream activities). According to the last decade data, total loads of pesticides show an increasing trend, particularly associated with the presence of acidic pesticides and anilides. The neonicotinoids acetamiprid and imidacloprid were measured at concentrations that largely exceeded the LOD established for the analysis of Watch List substances in water. Thus, this study provides relevant information for the revision of the Commission Implementing Decision (EU) 2018/840 (EC, 2018), and based on the results obtained, reduced and controlled use of the neonicotinoids imidacloprid and acetamiprid is recommended. Cybutryne was the only pesticide found above its EQS in surface water, despite being banned. A total of 17 banned pesticides (including propanil, whose use is exceptionally authorized in Spain) were found in Ebro Delta waters, at trace concentrations. Their presence is explained by desorption from soil and sediment particles, where they are likely accumulated due to their physical-chemical characteristics.

The environmental risk assessment carried out indicates that bentazone, propanil, MCPA, imidacloprid, dicofol, and cybutryne pose a moderate to high risk for aquatic organisms at the average

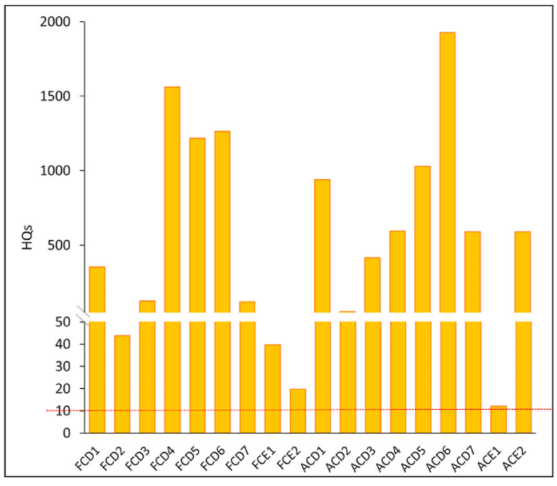

Fig. 6. Cumulative hazard quotients (HQs) calculated for the various investigated sampling locations as the sum of the HQ of each pesticide found in the sample, following the equation: $HQ_s = \sum_{i=1}^{n} HQ_i$.

contamination levels found. The co-occurrence of different pesticides results in a high potential risk (HQ > 10) for organisms in all investigated sites, even in those with the lowest pesticide loads. Although the approach applied to investigate mixture toxicity is a simple additive model and does not consider synergistic effects, it highlights the need of evaluating the effect of all contaminants present in a sample. This work, to the best of our knowledge, is the most complete assessment of pesticide contamination in waters from a delta ecosystem because it assesses the co-occurrence of low to highly polar pesticides and some of their TPs. The results of this study demonstrate that the agricultural use of pesticides has important effects on water quality and may pose a serious hazard for aquatic non-target organisms. However, many other factors need to be considered to link pesticide occurrence with mortality episodes of aquatic organisms in the area, because aspects like temperature and salinity may be also relevant, and even affect the toxic effects of pesticides. Long-term toxicological studies are required to assess the real risk of the pesticide mixtures for the health of wetlands ecosystems.

Credit author statement

Maria Vittoria Barbieri: LC-MS/MS analysis of samples, data treatment, elaboration of tables and figures, writing of the original manuscript draft, and revision of the manuscript. Andrea Peris: sampling, analysis of samples using GC-MS/MS, and data treatment. Cristina Postigo: supervision, support on data treatment, provision of critical feedback of the manuscript, and manuscript submission. Alba Moya Garcés: statistical analysis. Luis Simón Monllor-Alcaraz: sampling and LC-MS/MS analysis of samples. Maria Rambla Alegre: sampling and valuable information on sampling locations, and manuscript feedback. Ethel Eljarrat: supervision of the GC-M/MS-based work and critical feedback of the manuscript. Miren López de Alda: conceptualization and coordination of the study, sampling, supervision, and revision of the manuscript. All authors have read and approved the final article.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115813.

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Supplementary material

EVALUATION OF THE OCCURRENCE AND FATE OF PESTICIDES IN A TYPICAL MEDITERRANEAN DELTA ECOSYSTEM (EBRO RIVER DELTA) AND RISK ASSESSMENT FOR AQUATIC ORGANISMS

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Number of figures: 4

Number of pages: 25

List of Tables

List of Figures

Table S1. List of target compounds with details of their physical-chemical properties, regulated use and environmental quality standards (EQS).

Class	Name	Molecular formula[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	DT50[α] (days)	Pka[α]	Regulated use[α]	EQS (µg/L)[*]	Method LOD[^] (ng/L)
Acidic	2,4-D	$C_8H_6Cl_2O_3$	221	24300	39	-0.82	3.82	7.7	3.40	✓		
	Bentazone	$C_{10}H_{12}N_2O_3S$	240.3	7112	55	-0.46	1.95	80	3.51	✓		
	Fluroxypyr	$C_7H_5Cl_2FN_2O_3$	255	6500	10[β]	0.04	3.70	10.5	2.94	✓		
	MCPA	$C_9H_9ClO_3$	200.6	29390	29[β]	-0.81	2.98	13.5	3.73	✓		
	Mecoprop	$C_{10}H_{11}ClO_3$	214.6	250000	47	-0.19	2.29	37	3.11	X		
Anilides	Diflufenican	$C_{19}H_{11}F_5N_2O_2$	394.3	0.05	5504	4.20	1.19	175[Ω]	n/a	✓		
	Propanil	$C_9H_9Cl_2NO$	218.1	95	149	2.29	-0.51	1.2	19.1	X		
Azoles	Cyproconazole	$C_{15}H_{18}ClN_3O$	291.8	93	1198[β]	3.09	3.04	1000[Ω]	n/d	✓		
	Triadimefon	$C_{14}H_{16}ClN_3O_2$	293.8	70	300	3.18	1.59	12	n/a	X		
Carbamates	Methiocarb	$C_{11}H_{15}NO_2S$	225.3	27	182[β]	3.18	1.82	1.6	n/a	✓		2
	Molinate	$C_9H_{17}NOS$	187.3	1100	190	2.86	1.89	4	n/a	X		
	Triallate	$C_{10}H_6Cl_3NOS$	304.7	4.1	3034	4.06	0.61	104	n/a	✓		
Chloro-acetanilides	Alachlor	$C_{14}H_{20}ClNO_2$	269.8	240	335	3.09	0.80	2[Ω]	0.62	X	0.7	
	Metolachlor	$C_{15}H_{22}ClNO_2$	283.8	530	120	3.40	2.36	88	n/a	X		
Dinitroanilines	Pendimethalin	$C_{13}H_{19}N_3O_4$	281.3	0.33	17491	5.40	-0.28	4	2.8	✓		
	Trifluralin	$C_{13}H_{16}F_3N_3O_4$	335.3	0.221	15800	5.27	0.13	13	n/d	X	Not/Ap	
Diphenyl ether	Oxyfluorfen	$C_{15}H_{11}ClF_3NO_4$	361.7	0.116	3.99 x 10^{4}[β]	4.86	0.23	35[Ω]	n/d	✓		
Hydroxy-benzonitrile	Bromoxynil	$Br_2C_6H_2(OH)CN$	276.9	38000	302	0.27	1.71	13	3.86	✓		
Neonicotinoids	Acetamiprid	$C_{10}H_{11}ClN_4$	222.7	2950	200	0.80	0.94	4.7	0.7	✓		8.3
	Clothianidin	$C_6H_8ClN_5O_2S$	249.8	340	123	0.90	3.74	40.3	11.1	X		8.3
	Imidacloprid	$C_9H_{10}ClN_5O_2$	25576	610	262°	0.57	3.69	30	n/a	✓		8.3
	Thiacloprid	$C_{10}H_9ClN_4S$	252.7	184	615°	1.26	1.10	1000	n/a	✓		8.3
	Thiamethoxam	$C_8H_{10}ClN_5O_3S$	291.7	4100	56	-0.13	3.58	30.6	n/a	X		8.3

Table S1. (continued)

Class	Name	Molecular formula[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	DT50[α] (days)	Pka[α]	Regulated use[α]	EQS (µg/L)[*]	Method LOD[^] (ng/L)
Organo-chlorines	2,4'-DDD	$C_{14}H_{10}Cl_4$	320	0.09	131000	6.02	-2.46	1000[Ω]	n/d	X		
	4,4'-DDD	$C_{14}H_{10}Cl_4$	320	0.09	131000	6.02	-2.46	1000[Ω]	n/d	X		
	2,4'-DDE	$C_{14}H_8Cl_4$	318	0.12	1.17 x 10^{5}[β]	6.51	-	5000[Ω]	n/d	X		
	4,4'-DDE	$C_{14}H_8Cl_4$	318	0.12	1.17 x 10^{5}[β]	6.51	-	5000[Ω]	n/d	X		
	2,4'-DDT	$C_{14}H_9Cl_5$	354.6	0.006	151000	6.91	-3.89	6200[Ω]	n/a	X		
	4,4'-DDT	$C_{14}H_9Cl_5$	354.6	0.006	151000	6.91	-3.89	6200[Ω]	n/a	X		
	Dicofol	$C_{14}H_9Cl_5O$	370.5	0.8	6064	4.3	0.36	29[Ω]	n/a	X	Not/Ap	
	Heptachlor epoxide	$C_{10}H_5Cl_7O$	389.3	0.2	22485	4.56[β]	-	3204[β]	n/a	-	$3x10^{-4}$	
	Oxadiazon	$C_{15}H_{18}Cl_2N_2O_3$	345.2	0.57	3200	5.33	1.97	17.9	n/a	X		
Organo-phosphates	Azhinphos ethyl	$C_{12}H_{16}N_3O_3PS_2$	345.4	4.5	1500	3.18	1.40	50[Ω]	n/a	X		
	Azinphos-methyl	$C_{10}H_{12}N_3O_3PS_2$	317.3	28	1112	2.96	1.42	10[Ω]	5	X		
	Azinphos-methyl oxon[ε]	$C_{10}H_{12}N_3O_4PS$	301.3	2604[β]	10[β]	0.77[β]	-	n/a	n/a	-		
	Chlorfenvinphos	$C_{12}H_{14}Cl_3O_4P$	359.6	145	680	3.80	1.72	7	n/a	X	0.3	
	Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	350.6	1.05	5509	4.70	0.58	5	n/a	✓	0.1	
	Diazinon	$C_{12}H_{21}N_2O_3PS$	304.3	60	609	3.69	1.51	4.3	2.6	X		
	Dichlorvos	$C_4H_7Cl_2O_4P$	221	18000	50	1.90	0.69	2[Ω]	n/a	X	$7 x 10^{-4}$	
	Dimethoate	$C_5H_{12}NO_3PS_2$	229.3	25900	25[β]	0.75	2.18	12.6	n/a	X		
	Fenitrothion	$C_9H_{12}NO_5PS$	277.2	19	2000	3.32	0.48	1.1	n/a	X		
	Fenitrothion oxon[ε]	$C_9H_{12}NO_6P$	261.2[β]	301[β]	21[β]	1.69[β]	-	n/a	n/a	-		
	Fenthion	$C_{10}H_{15}O_3PS_2$	278.3	4.2	1500	4.84	1.26	22[Ω]	n/a	X		
	Malaoxon[ε]	$C_{10}H_{19}O_7PS$	314.3[β]	7500[β]	4650[β]	0.52[β]	-	n/a	n/a	-		
	Malathion	$C_{10}H_{19}O_6PS_2$	330.4	148	1800	2.75	0.00	0.4	n/a	✓		

Table S1. (continued)

Class	Name	Molecular formula[α]	Molecular weight (g mol^{-1})	Solubility[α] (mg l^{-1})	K_{oc}[α]	Log K_{ow}[α]	GUS[α]	DT50[α] (days)	Pka[α]	Regulated use[α]	EQS (µg/L)[*]	Method LOD[^] (ng/L)
Organo-thiophosphates	Fenthion oxon[ε]	$C_{10}H_{15}O_4PS$	262.3[β]	213.5[β]	57[β]	2.31[β]	-	n/a	n/a	-		
	Fenthion oxon sulfone[ε]	$C_{10}H_{15}O_6PS$	294[β]	7602[β]	13[β]	0.28[β]	-	n/a	n/a	-		
	Fenthion oxon sulfoxide[ε]	$C_{10}H_{15}O_5PS$	278.3[β]	1222[β]	11[β]	0.15[β]	-	n/a	n/a	-		
	Fenthion sulfone[ε]	$C_{10}H_{15}O_5PS_2$	310.3[β]	190.4[β]	235	2.05[β]	-	n/a	n/a	-		
	Fenthion sulfoxide[ε]	$C_{10}H_{15}O_4PS_2$	294.3[β]	3.72[β]	183	1.92[β]	-	n/a	n/a	-		
Phenylureas	Chlortoluron	$C_{10}H_{13}ClN_2O$	212.7	74	196	2.50	2.62	42	n/a	✓		
	Diuron	$C_9H_{10}Cl_2N_2O$	233.1	35.6	680	2.87	2.65	8.8	n/a	✓	1.8	
	Isoproturon	$C_{12}H_{18}N_2O$	206.3	70.2	251[β]	2.5	2.61	40	n/a	X	1	
	Linuron	$C_9H_{10}Cl_2N_2O_2$	249.1	63.8	843	3	2.11	13	n/a	X		
Pyrethroids	Cyhalotrhin	$C_{23}H_{19}ClF_3NO_3$	449.8	0.004	180000	6.8	-2.20	57[Ω]	9	X		
	Cypermethrin	$C_{22}H_{19}Cl_2NO_3$	416.3	0.009	307558	5.55	-1.99	3	n/d	✓	6×10^{-4}	
Quinoline	Quinoxyfen	$C_{15}H_8Cl_2FNO$	308.1	0.05	23°	4.66	-0.80	5	n/a	✓	2.7	
Sulfonylurea	Thifensulfuron methyl	$C_{12}H_{13}N_5O_6S_2$	387.4	54.1	28	-1.65	3.05	22	4	✓		
Triazines	Atrazine	$C_8H_{14}ClN_5$	215.7	35	100	2.70	2.57	75[Ω]	1.7	X	2	
	Cyanazine	$C_9H_{13}ClN_6$	240.7	171	190	2.10	2.07	16[Ω]	12.9	X		
	Cybutrine	$C_{11}H_{19}N_5S$	253.4	7	1569	3.95	-	n/a	n/a	X	0.016	
	Deisopropilatrazine[ε]	$C_5H_8ClN_5$	173.6	980	130	1.15	-	n/a	n/a	-		
	Desethylatrazine[ε]	$C_6H_{10}ClN_5$	187.6	2700	110	1.51	4.37	2.23[µ]	n/a	-		
	Simazine	$C_7H_{12}ClN_5$	201.7	5	130	2.30	2.20	46	1.62	X	4	
	Terbuthylazine	$C_9H_{16}ClN_5$	229.7	6.6	329	3.40	2.19	6	1.9	✓		
	Terbutryn	$C_{10}H_{19}N_5S$	241.4	25	2432	3.66	2.21	27	4.3	X	0.34	

[*] Environmental Quality Standards (EQS) for priority substances included in the Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Retrieved from: https://goo.gl/diHn8W.

[^] Maximum acceptable method detection limit (ng/L) for compounds included in the European Watch list. Commission Implementing Decision (EU) 2018/840 of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive

2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495 (notified under document C (2018) 3362). Retrieved from: https://goo.gl/nR4ezg.

[ε] Metabolite.

[α] The PPDB, Pesticide Properties Database, http://sitem.herts.ac.uk/aeru/footprint/index2.htm. - Lewis, K.A., Tzilivakis, J., Warner, D. and Green, A. (2016). An international database for pesticide risk assessments and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064.

[β] Data estimated using the US Environmental Protection Agency EPISuite™, http://www.Chemspider.com.

[μ] Calculated using the mathematical formula: GUS = log10 (half-life) x [4 - log10 (Koc)].

[°] Kegley, S.E., Hill, B.R., Orme S., Choi A.H., PAN Pesticide Database, Pesticide Action Network, North America (Oakland, CA, 2016), http://www.pesticideinfo.org.

[Ω] Water-sediment DT50 or soil degradation DT50 values (in case that water phase DT50 data is not available).

Solubility: solubility in water at 20 °C;
K_{oc}: organic carbon partition coefficient;
Log K_{ow}: octanol-water partition coefficient;
GUS: leaching potential index;
DT50: degradation potential in water phase, expressed as *half-life* in days;
Pka: dissociation constant at 25 °C;
n/a: data not available;
n/d: no dissociation;
Not/Ap: not applicable.

Table S2. Analytical technique and method sensitivity in LC-grade water (limits of detection (LODs) and limits of quantification (LOQs)) for the determination of the various target pesticides.

Analyte	Sensitivity LOD (ng/L)	Sensitivity LOQ (ng/L)
LC-MS/MS analysis		
2,4-D	6.1	20
Acetamiprid	0.16	0.53
Alachlor	1.2	3.8
Atrazine	0.14	0.88
Azinphos ethyl	0.42	1.4
Azinphos methyl	0.38	1.3
Azinphos methyl oxon	3.1	10
Bentazone	4.3	14
Bromoxynil	2.6	8.6
Chlorfenvinphos	0.24	0.80
Chlorpyrifos	0.44	1.5
Chlortoluron	0.13	0.42
Clothianidin	2.3	7.5
Cyanazine	0.081	0.28
Cybutryne	0.85	2.8
DEA	2.3	7.9
DIA	4.4	15
Diazinon	0.042	0.16
Dichlorvos	5.4	18
Diflufenican	1.2	4.0
Dimethoate	0.76	2.6
Diuron	0.13	0.43
Fenitrothion	2.6	8.8
Fenitrothion oxon	0.79	2.6
Fenthion oxon	0.17	0.59
Fenthion oxon sulfone	2.8	9.4
Fenthion oxon sulfoxide	0.13	0.43
Fenthion sulfone	4.2	14
Fenthion sulfoxide	0.41	1.4
Fluroxypyr	28	95
Imidacloprid	0.87	2.9
Isoproturon	0.15	0.50
Linuron	0.58	1.9
Malaoxon	0.15	0.50
MCPA	5.5	18
Mecoprop	1.1	3.6
Methiocarb	0.41	1.4
Metolachlor	0.090	0.32
Molinate	1.1	3.6
Propanil	0.90	3.0
Simazine	0.31	1.1
Terbuthylazine	0.14	0.48
Terbutryn	0.19	0.66
Thiacloprid	0.063	0.21
Thiamethoxam	1.8	6.0
Thifensulfuron methyl	0.022	0.064
GC-MS/MS analysis		
2,4'-DDD	1.1	3.8
2,4'-DDE	1.2	4.0
2,4'-DDT	1.7	5.8
4,4'-DDD	0.73	2.4
4,4'-DDE	1.6	5.3
4,4'-DDT	1.0	3.3
Cyhalothrin	7.8	26
Cypermethrin	1.5	4.9
Cyproconazol	1.6	5.3
Dicofol	2.2	7.3
Fenthion	1.5	4.9
Heptaclor epoxide	0.53	1.6
Malathion	0.42	1.2
Oxadiazon	0.23	0.72
Oxyfluorfen	7.0	23
Pendimethalin	0.62	2.0
Quinoxyfen	0.72	2.2
Triadimefon	1.2	4.0
Triallate	2.1	7.0
Trifluralin	15	51

Table S3. LC-MS/MS and GC-MS/MS acquisition parameters: target compounds, internal standard (IS) used for the quantification of each target analyte, retention time, SRM transitions (precursor and fragment ions) and corresponding collision energies (CE) (*).

Target compound	IS	t_R (min)	Precursor ion (m/z)	Fragment ion 1 (m/z)	CE 1 (EV)	Fragment ion 2 (m/z)	CE 2 (EV)
LC-MS/MS							
2,4-D	*2,4-D-d_3*	7.9	219	161	15	125	25
Acetamiprid	*Acetamiprid- d_3*	9.2	223	126	15	56	20
Alachlor	*Alachlor- $d13_3$*	18.5	270	238	15	162	15
Atrazine	*Atrazine-d_5*	12.4	216	174	15	132	20
Azinphos ethyl	*Azinphos ethyl-d_{10}*	19.8	346	132	20	104	35
Azinphos methyl	*Azinphos methyl- d_6*	16.5	318	132	20	105	30
Azinphos methyl oxon	*Azinphos methyl- d_6*	9.7	324	132	20	148	15
Bentazone	*Bentazone- d_6*	7.5	239	197	25	132	20
Bromoxynil	*Bromoxynil-$^{13}C_6$*	7.5	276	81	20	79	30
Chlorfenvinphos	*Chlorfenvinphos-d_{10}*	19.2	359	155	15	170	40
Chlorpyrifos	*Chlorpyrifos-d_{10}*	29.5	352	97	30	200	20
Chlortoluron	*Chlortoluron- d_6*	11.9	213	72	15	140	30
Clothianidin	*Clothianidin- d_3*	8.9	250	169	15	139	15
Cyanazine	*Cyanazine-d_5*	10.9	241	214	15	174	20
Cybutrine	*Cybutrine-d_9*	17.7	254	108	30	125	25
DEA	*DEA- d_6*	7.4	188	146	15	79	25
DIA	*DIA-d_5*	8.5	174	132	20	104	25
Diazinon	*Diazinon- d_{10}*	21.9	305	153	20	97	30
Dichlorvos	*Dichlorvos- d_6*	10.8	221	109	25	115	25
Diflufenican	*Diflufenican-d_5*	25.3	395	266	30	246	25
Dimethoate	*Dimethoate- d_6*	9.6	230	199	15	125	15
Diuron	*Diuron-d_6*	12.8	233	72	15	78	15
Fenitrothion	*Fenitrothion-d_6*	19.8	262	152	20	122	30
Fenitrothion oxon	*Fenitrothion oxon -d_6*	11.5	262	104	20	216	20
Fenthion oxon	*Fenthion oxon-d_3*	13.0	263	231	20	216	25
Fenthion oxon sulfone	*Fenthion oxon sulfone-d_3*	11.1	295	217	30	109	40
Fenthion oxon sulfoxide	*Thiamethoxam-d_3*	8.2	279	264	20	104	25
Fenthion sulfone	*Fenthion sulfone-d_6*	14.2	311	125	30	109	40
Fenthion sulfoxide	*Fenthion sulfoxide-d_6*	11.2	295	109	30	125	35
Fluroxypyr	*Mecoprop-d_3*	7.8	253	197	10	195	10
Imidacloprid	*Imidacloprid-d_5*	9.0	256	209	20	175	15
Isoproturon	*Isoproturon-d_6*	12.3	207	165	15	72	20
Linuron	*Linuron-d_6*	16.2	249	160	15	182	15
Malaoxon	*Chlortoluron- d_6*	11.0	315	127	15	99	25
MCPA	*MCPA-d_3*	7.9	199	143	10	141	10

Mecoprop	*Mecoprop-d_3*	7.9	213	141	10	71	10
Methiocarb	*Methiocarb-d_3*	15.4	226	169	10	121	20
Metolachlor	*Metolachlor-d_{11}*	18.3	284	176	25	73	25
Molinate	*Linuron-d_6*	17.4	188	126	15	83	20
Propanil	*Propanil-d_5*	14.9	216	162	20	160	20
Simazine	*Simazine-d_{10}*	10.8	202	124	25	71	20
Terbuthylazine	*Terbuthylazine-d_5*	15.2	230	174	15	96	25
Terbutryn	*Terbutryn-d_5*	17.6	242	71	30	91	30
Thiacloprid	*Thiacloprid-d_4*	10.1	253	126	25	90	40
Thiamethoxam	*Thiamethoxam-d_3*	8.9	292	211	15	181	20
Thifensulfuron methyl	*Thifensulfuron methyl-d_3*	7.3	388	167	15	141	20
Triallate	*Triallate-$^{13}C_6$*	29.6	304	143	15	86	30
GC-MS/MS							
2,4'-DDD	*Oxadiazon-d_7*	20.8	235	199	20	165	20
4,4'-DDD	*Oxadiazon-d_7*	22.3	235	199	20	165	20
2,4'-DDE	*Oxadiazon-d_7*	19.2	246	211	20	176	40
4,4'-DDE	*Oxadiazon-d_7*	20.6	246	211	20	176	40
2,4'-DDT	*Oxadiazon-d_7*	22.3	235	199	20	165	20
4,4'-DDT	*Oxadiazon-d_7*	23.7	235	199	20	165	20
Cyhalothrin	*Fenoxi-cyhalothrin-d_5*	29.1	181	152	20	77	40
Cypermethrin	*Fenoxi-fenvalerate-d_5*	33.0	163	127	5	91	5
Cyproconazol	*Oxadiazon-d_7*	21.4	222	125	20	82	10
Dicofol	*Chlorfenvinphos-d_{10}*	16.9	139	111	20	75	20
Fenthion	*Fenthion-d_6*	16.5	278	169	10	109	20
Heptachlor epoxide	*Chlorfenvinphos-d_{10}*	18.0	353	263	10	217	40
Malathion	*Malathion-diethyl-d_{10}*	16.0	173	117	5	99	10
Oxadiazon	*Oxadiazon-d_7*	20.7	175	112	10	76	40
Oxyfluorfen	*Oxadiazon-d_7*	20.9	252	224	10	177	40
Pendimethalin	*Chlorfenvinphos-d_{10}*	17.7	252	191	5	162	5
Quinoxyfen	*Oxadiazon-d_7*	23.5	237	208	40	181	40
Triadimefon	*Fenthion-d_6*	16.8	208	181	5	75	40
Trifluralin	*Trifluralin-d_{14}*	9.5	306	264	5	43	20

(*) Information extracted from:

Barbieri, M.V., Monllor-Alcaraz, L.S., Postigo, C., de Alda, M.L., 2020. Improved fully automated method for the determination of medium to highly polar pesticides in surface and groundwater and application in two distinct agriculture-impacted areas. Sci. Total Environ. 140650. https://doi.org/10.1016/j.scitotenv.2020.140650

Peris, A., Eljarrat, E., 2020. Multi-residue methodologies for the analysis of non-polar pesticides in water and sediment matrices by GC-MS/MS. Submitted to Anal. Bioanal. Chem.

Table S4. Geolocation of sampling sites in the Ebro River Delta.

SAMPLING POINT	Coordinate X	Coordinate Y
Fangar zone		
FCD1	N 40º 42.905'	E 000º 38.281'
FCD2	N 40º 43.149'	E 000º 39.312'
FCD3	N 40º 44.134'	E 000º 38.446'
FCD4	N 40º 46.440'	E 000º 42.709'
FCD5	N 40º 45.628'	E 000º 43.504'
FCD6	N 40º 45.819'	E 000º 44.243'
FCD7	N 40º 45.566'	E 000º 47.519'
FCE1	N 40º 45.480'	E 000º 42.657'
FCE2*	N 40º 45.064'	E 000º 46.749'
Alfacs zone		
ACD1	N 40º 43.048'	E 000º 39.971'
ACD2	N 40º 39.611'	E 000º 40.884'
ACD3	N 40º 37.902'	E 000º 38.841'
ACD4	N 40º 38.826'	E 000º 42.434'
ACD5	N 40º 38.762'	E 000º 43.033'
ACD6	N 40º 42.044'	E 000º 45.737'
ACD7	N 40º 39.672'	E 000º 47.342'
ACE1	N 40º 39.611'	E 000º 40.884'
ACE2	N 40º 37.946'	E 000º 38.716'

CD: drainage channel; CE: irrigation channel;
* reference site

Text S1. Statistical analyses.

Preliminary data pretreatment was performed before conducting basic descriptive statistics: values <LOD = LOD/2, values <LOQ = LOQ/2, removal of variables (pesticides) with low detection frequencies (<30%). In total, 25 compounds were considered for statistical analysis.

All variables except the pesticides pendimethalin, chlorpyrifos, diuron, and terbuthylazine, presented positively skewed distributions (with positive asymmetry coefficients), especially in the case of 2,4-D, propanil, acetamiprid, imidacloprid, and azinphos ethyl (asymmetry coefficient >2) (Table S5). Positively skewed distributions have a long right tail and the median of the data falls well below the mean value. In our study, this is attributed to a large proportion of small concentration values and only a few high concentrations within each pesticide variable.

Table S5. Basic descriptive statistics of the variables investigated.

Class	Name	Median (ng/L)	Standard deviation (ng/L)	Kurtosis	Asymmetry coefficient
Acidics	2,4-D	6.6	103	15	3.8
	Bentazone	34958	56966	-0.14	1.0
	MCPA	733	2451	2.1	1.7
Anilides	Diflufenican	1.3	6.0	0.39	1.3
	Propanil	879	16337	5.9	2.4
Carbamates	Methiocarb	0.72	1.0	0.07	0.99
	Molinate	15	15	-0.95	0.47
	Triallate	240	244	2.9	1.5
Chloroacetanilides	Metolachlor	0.60	0.30	6.3	2.7
Dinitroaniline	Pendimethalin	36	17	-0.16	0.67
Neonicotinoids	Acetamiprid	1.0	0.35	-1.99	-0.50
	Imidacloprid	61	956	13	3.4
	Thiacloprid	50	211	3.5	2.1
Organochlorines	Dicofol	0.03	0.82	2.6	1.9
	Oxadiazon	2.4	1.3	-2.3	0.00
	Triadimefon	15	17	-1.3	0.40
Organophosphates	Azinphos ethyl	1.3	1.1	4.5	1.8
	Chlorfenviphos	0.21	1.8	3.3	2.1
	Chlorpyrifos	1.4	1.9	0.15	0.96
	Diazinon	15	7.9	-0.92	-0.08
Phenylureas	Diuron	1.9	1.4	-0.81	0.32
Triazines	Cybutryne	6.7	4.6	-1.52	-0.20
	Simazine	25	15	-1.9	0.21
	Terbuthylazine	0.16	2.0	0.99	1.3
	Terbutryn	23	15	-1.4	-0.35

Pairwise correlation of pesticide concentrations

Pairwise correlations coefficients were calculated using the Spearman's Rank correlation test, as the variables under consideration were not normally distributed. Table S6 summarizes the results obtained, where strong (>|0.4|) and significant (α=0.05) correlations have been highlighted. All correlations above |0.5| were found to be statistically significant. The highest significant correlations were ranked (Figure S1) and a network plot of the correlations observed (Figure S2) was elaborated to better evaluate those pesticides or pesticide classes that were strongly correlated.

As shown in Figures S1 and S2, the herbicides MCPA (phenoxy-carboxylic acid) and propanil (anilide), two of the pesticides found at the highest concentrations in this study, are strongly positively correlated. This could be explained by their extensive use in rice-crops in the investigated area, as they present different mode of action, propanil, like bentazone, acts as a photosynthesis inhibitor, while MCPA inhibits auxin synthesis. These herbicides were negatively correlated with triazine herbicides like terbuthylazine and cybutryne, and the carbamate herbicide triallate. Thus, terbuthylazine and triallate may be more commonly applied in crops other than rice in the area, while cybutryne is no longer approved for use and residual concentrations may be released from the sediments/soils from past use as an antifouling paints for ships and boats. Strong positive correlations were also found between banned pesticides (chlorfenvinphos + diazinon, chlorfenvinphos + terbutryn, or diazinon + terbutryn). All of them present a Log K_{ow} above 3.6, and thus, a high tendency to sorb onto soil and sediment particles, and a GUS index above 1.5. Thus, their presence in the area could result from leaching of these particles. The herbicide triallate showed a high correlation with cybutryne and chlorpyrifos. While the correlation with cybutryne is difficult to explain, since its use is banned, that with chlorpyrifos, an organophosphate insecticide used to control foliage and soil-borne insect pests in a variety of crops, especially in corn and other cereal fields, could be explained by the simultaneous application of these two pesticides.

Table S6. Cross-correlation table after pairwise correlation evaluation (* indicate significant correlations, with a signficance level α=0.05).

	2,4-D	ACET	AZET	BENZ	CLOFV	CLOPF	DIAZ	DCOF	DIFLU	DIUR	IMID	CYBUT	MCPA	METCARB	METCLOR	MOLIN	OXADZ	PENDI	PROP	SIMZ	TERBZ	TBTR	TICLOP	TRIDM	TRIAL
2,4-D	1.00																								
Acetamiprid	-0.16	1.00																							
Azinphos ethyl	0.01	-0.02	1.00																						
Bentazone	0.11	0.45	-0.02	1.00																					
Chlorfenviphos	0.10	0.05	0.59*	0.08	1.00																				
Chlorpyrifos	0.05	0.00	-0.45	0.35	-0.41	1.00																			
Diazinon	0.32	0.18	0.42	0.02	**0.75***	-0.48*	1.00																		
Dicofol	0.44	0.01	-0.03	0.25	-0.36	0.31	-0.29	1.00																	
Diflufenican	0.14	0.28	0.44	0.23	0.29	-0.13	**0.62***	0.07	1.00																
Diuron	0.12	0.28	-0.08	0.45	0.27	0.08	0.33	0.01	-0.04	1.00															
Imidacloprid	0.07	0.41	0.50*	0.30	0.40	-0.09	0.45	0.12	0.44	0.11	1.00														
Cybutryne	-0.32	0.29	-0.25	0.01	-0.40	0.50*	-0.31	-0.01	-0.15	0.21	-0.21	1.00													
MCPA	0.06	0.19	0.28	**0.63***	0.41	0.02	0.20	0.17	0.34	0.08	0.52*	-0.58*	1.00												
Methiocarb	-0.22	0.01	0.50*	-0.14	**0.66***	-0.30	0.53*	**-0.61***	0.15	0.24	0.34	0.07	-0.04	1.00											
Metolachlor	-0.25	-0.57*	-0.08	-0.48*	-0.07	-0.12	-0.04	-0.29	-0.12	-0.08	-0.36	0.12	-0.42	0.31	1.00										
Molinate	0.07	0.03	0.10	0.58*	0.05	0.24	-0.20	0.25	-0.20	0.34	0.17	-0.12	0.36	-0.24	-0.57*	1.00									
Oxadiazon	0.30	0.10	0.26	0.14	0.35	0.06	0.36	0.24	0.31	0.17	**0.64***	-0.44	0.54*	0.03	-0.52*	0.35	1.00								
Pendimethalin	-0.05	0.10	-0.13	-0.03	-0.26	0.52*	-0.11	0.34	0.12	0.19	-0.08	**0.63***	-0.31	-0.22	-0.10	0.15	0.11	1.00							
Propanil	0.29	0.12	0.22	0.36	0.48*	-0.40	0.39	0.05	0.20	0.02	0.52*	**-0.84***	**0.78***	-0.02	-0.32	0.21	0.48*	**-0.63***	1.00						
Simazine	0.18	-0.07	-0.19	0.01	0.20	0.16	-0.07	-0.06	-0.47*	0.22	-0.22	0.05	0.08	-0.01	-0.35	0.24	0.19	0.08	0.01	1.00					
Terbuthylazine	-0.42	-0.28	-0.25	-0.57*	-0.36	-0.03	-0.35	-0.34	-0.44	-0.02	-0.41	0.44	**-0.70***	0.20	**0.71***	-0.47	-0.50*	-0.03	-0.58*	-0.14	1.00				
Terbutryn	0.14	0.15	0.43	-0.18	**0.75***	-0.48*	**0.87***	-0.21	0.52*	0.09	0.50*	-0.36	0.28	0.43	-0.03	-0.28	0.42	-0.04	0.43	0.03	-0.35	1.00			
Thiacloprid	**0.64***	-0.32	-0.12	-0.12	0.22	0.07	0.25	0.12	0.08	-0.14	-0.04	-0.46	0.02	-0.09	0.06	-0.09	0.25	-0.13	0.29	-0.07	-0.25	0.14	1.00		
Triadimefon	0.17	-0.09	-0.06	-0.04	-0.09	-0.15	0.04	0.05	0.15	0.05	0.11	-0.50*	0.25	-0.15	-0.18	0.14	0.58*	-0.17	0.33	-0.05	-0.06	-0.05	0.23	1.00	
Triallate	-0.21	0.19	-0.38	0.26	-0.55*	**0.77***	-0.55*	0.27	-0.26	0.24	-0.19	**0.89***	-0.35	-0.21	-0.04	0.19	-0.27	**0.65***	**-0.70***	0.13	0.27	-0.59*	-0.38	-0.35	1.00

ACET: acetamiprid, AZET: azinophos ethyl, BENZ: bentazone, CLOFV: chlorfenvinphos, CLOPF: chlorpyrifos, CLORT: chlortoluron, DIAZ: diazinon, DCOF: dicofol, DIFLU: diflufenican, DIUR: diuron, IMID: imidacloprid, CYBUT: cybutryne, METCARB: methiocarb, METCLOR: metolachlor, MOLIN: molinate, OXADZ: oxadiazon, PENDI: pendimethalin, PROP: propanil, SIMZ: simazine, TERBZ: terbuthylazine, TBTR: terbutryn, TICLOPR: thiacloprid, TRIDM: triadimefon, TRIAL: triallate.

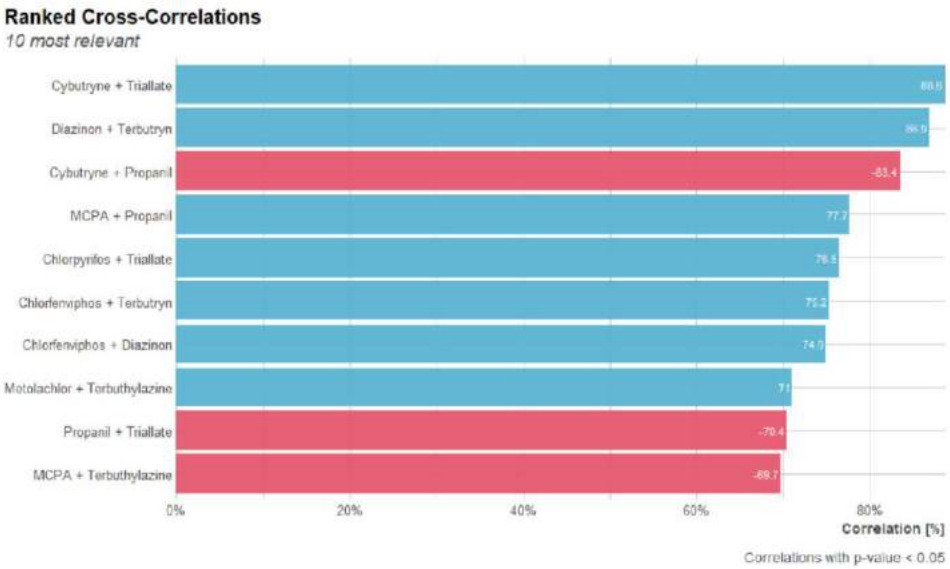
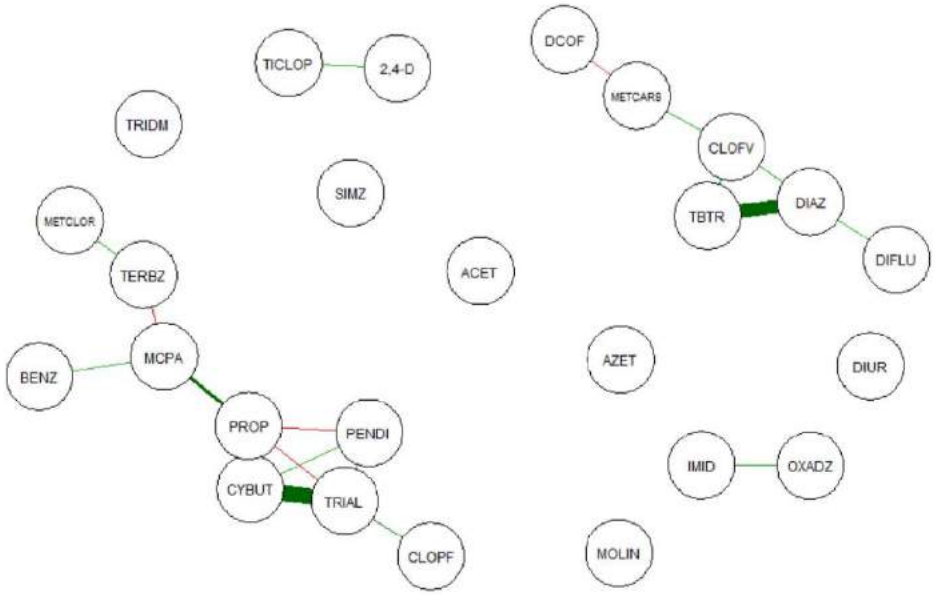


Figure S1. Ranking of the highest correlated variables obtained.



ACET: acetamiprid, AZET: azinophos ethyl, BENZ: bentazone, CLOFV: chlorfenvinphos, CLOPF: chlorpyrifos, CLORT: chlortoluron, DIAZ: diazinon, DCOF: dicofol, DIFLU: diflufenican, DIUR: diuron, IMID: imidacloprid, CYBUT: cybutryne, METCARB: methiocarb, METCLOR: metolachlor, MOLIN: molinate, OXADZ: oxadiazon, PENDI: pendimethalin, PROP: propanil, SIMZ: simazine, TERBZ: terbuthylazine, TBTR: terbutryn, TICLOPR: thiacloprid, TRIDM: triadimefon, TRIAL: triallate.

Figure S2. Network plot of the correlation dataframe.

Principal component analysis (PCA)

Principal-component analysis (PCA) was used to extract useful information from the data, e.g., to investigate multivariate correlations between the concentrations of the different pesticides, and identify their geographical distributions [1]. After PCA, the original variables (loadings) are reduced to several uncorrelated orthogonal variables (principal components or PC), which are used to calculate new orthogonal axes where the samples (scores) are represented and in which the explained data variance is shown in a decreasing order. Thus, the loadings identify the chemical composition of the main sources of the data variance, and the scores describe the contribution of these sources in the investigated samples, enabling sample mapping of these sources and description of the distribution of potential spatial contamination patterns. Before applying PCA, data were autoscaled by mean-centering and division the standard deviation of the corresponding variable to reduce potential collinearity problems [2].

Four principal components (PCs) explained 65 % of the total data variance (Table S7). Therefore, these four were selected to describe the main spatial variation trends of pesticide concentrations in the Ebro Delta during the main rice-growing season. The variable loadings and sampling site scores list for the four first PCs are shown in the main manuscript in Figures X and X, respectively. To try to relate these PCs with pesticide pollution patterns, biplots were also built, including only pesticide variables (Figure S3). The magnitude of the vectors (lines) shows the strength of their contribution to each PC. Vectors pointing in similar directions indicate positively correlated variables, vectors pointing in opposite directions indicate negatively correlated variables,

[1] Joliffe, I.T., 1986. Principal-component analysis. Springer, New York. https://doi.org/10.1007/978-1-4757-1904-8

[2] Massart, D.L., Vanderginste, B.G.M., Buydens, L.M.C., de Jong, S., Lewi, P.J., Smeyers Verbeke, J., 1997. Handbook of chemometrics and qualimetrics: parts A and B. Elsevier, Amsterdam. https://doi.org/10.1021/ci980427d

and vectors at approximately right angles indicate low or no correlation. Finally, PCA biplots are provided in Figure S4.

Table S7. Proportion of the total data variance explained by each principal component.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15	PC16	PC17
Standard deviation	2.57	2.09	1.73	1.47	1.32	1.31	1.19	0.94	0.92	0.79	0.73	0.62	0.55	0.45	0.35	0.25	0.17
Proportion of Variance	***0.27***	***0.18***	***0.12***	***0.09***	0.07	0.07	0.06	0.04	0.03	0.03	0.02	0.02	0.01	0.01	0.01	0.00	0.00
Cumulative Proportion	***0.27***	***0.44***	***0.56***	***0.65***	0.72	0.79	0.84	0.88	0.91	0.93	0.96	0.97	0.98	0.99	1.00	1.00	1.00

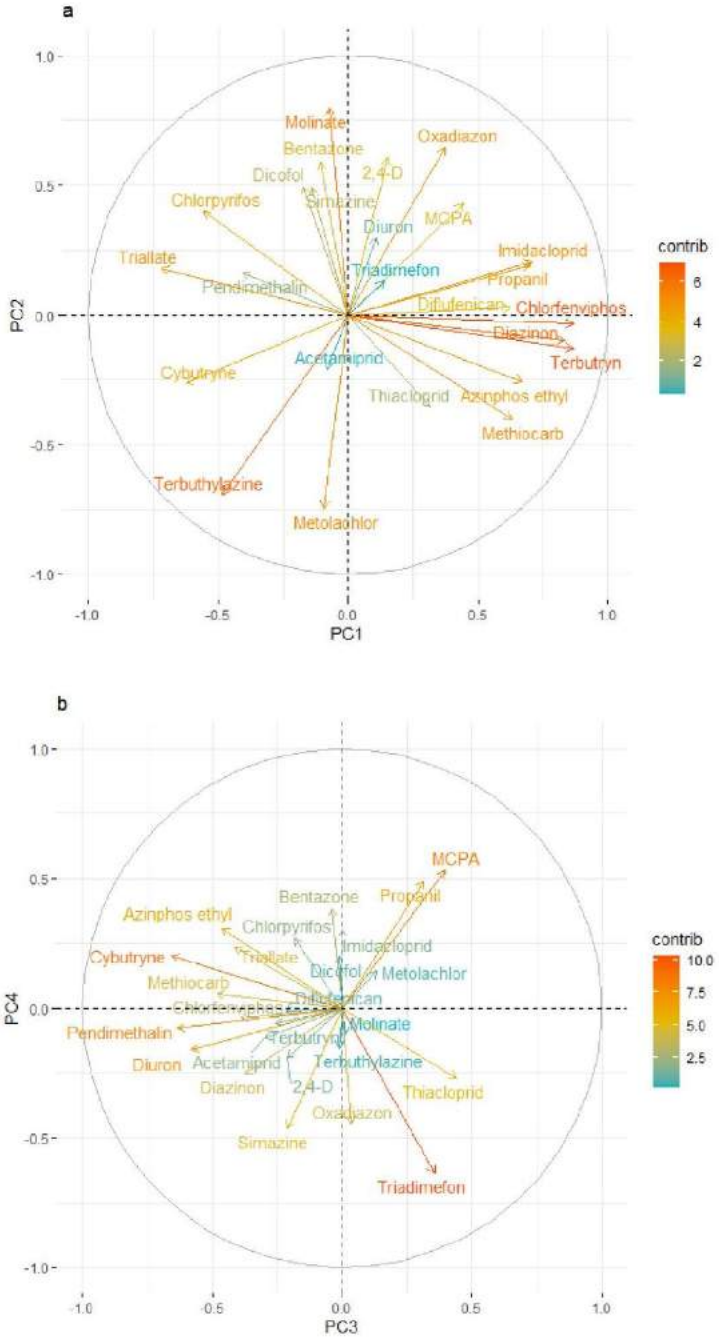


Figure S3. Contribution of each pesticide to the different PC.

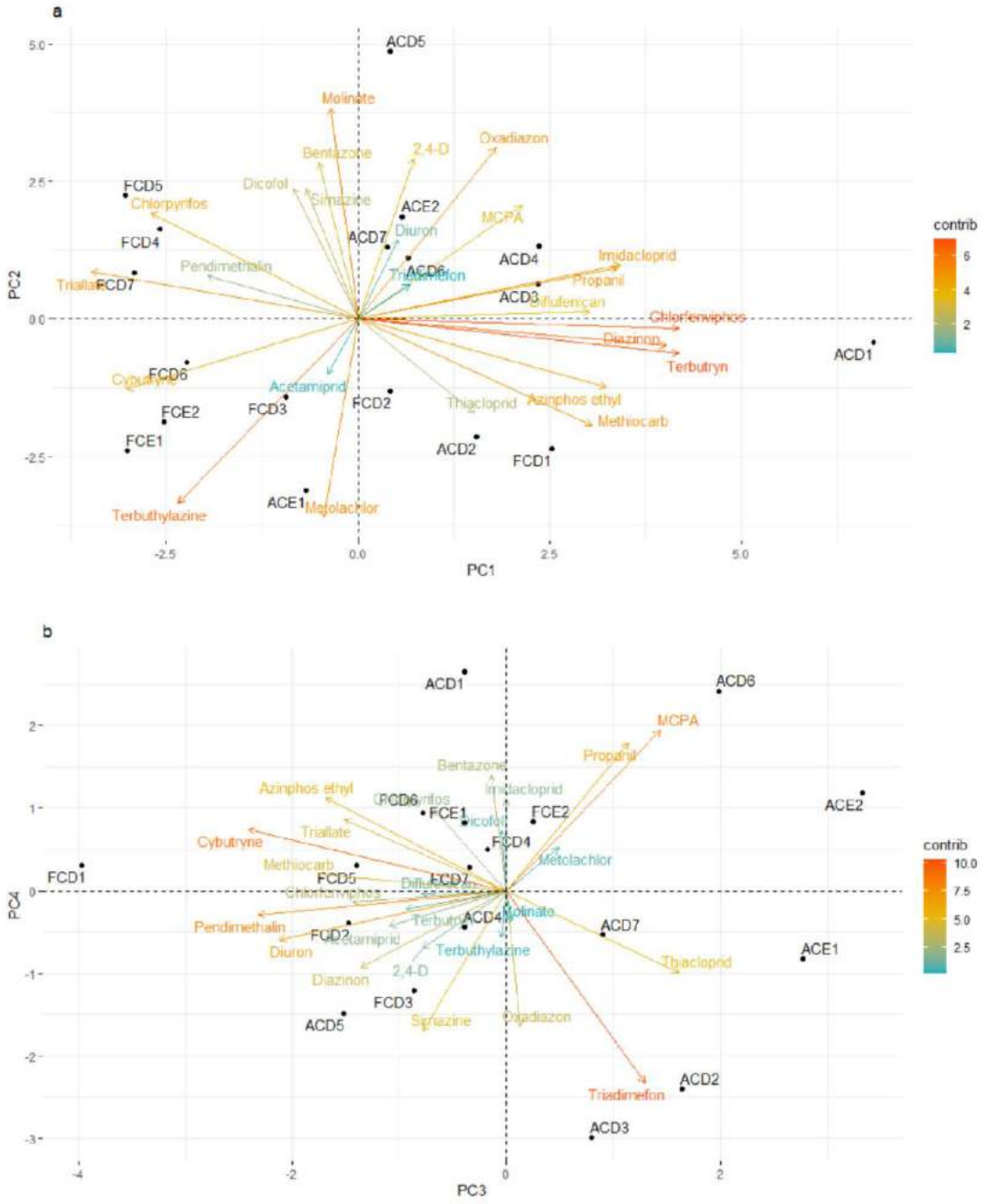


Figure S4. PCA biplots of the investigated samples along a) PC1 and PC2 and b) PC3 and PC4.

PCA loadings enable characterization of the main patterns of contamination by pesticides in the Ebro Delta. Overall, a diffuse contamination pattern is explained by the different PCs, as many pesticides contribute to each. PC1 explains 27% of the total variance, and describes a contamination pattern ruled by the presence of azinphos ethyl, chlorfenvinphos, diazinon, diflufenican, imidacloprid, methiocarb, MCPA, propanil, terbutryn (all of them with positive loadings), and chlorpyrifos, cybutryne, and triallate (negative loadings). According to PC1 scores, a contamination pattern mainly coming from rice-growing fields, due to the presence of MCPA and propanil was observed in the Alfacs bay (especially in ACD1, one of the sites with the highest pesticide loadings in this bay, and to a minor extent, in other locations of the bay (ACD2, ACD3 and ACD4)) and exceptionally in FCD1 in the Fangars bay (positive scores), while triallate, cybutryne, and chlorpyrifos governed the pesticide contamination pattern in most locations in the Fangars bay (negative scores), which may be attributed to other agricultural activities.

PC2, explaining 18% of the total variance, describes a contamination pattern governed by bentazone, dicofol, MCPA, molinate, oxadiazon, simazine (positive loadings), metolachlor, and terbuthylazine (negative loadings). Again, the presence of bentazone and MCPA in the contamination pattern may be characteristic of rice cultivation activities. This type of contamination was again mainly found in the Alfacs bay (drainage channels ACD4, ACD5, ACD6 and ACD7, and even irrigation channel ACE2), but was also characteristic of FCD4 and FCD5 in the Fangars bay (positive scores). In contrast, metholachlor and terbuthylazine governed the contamination pattern in most Fangars bay drainage (FCD1, FCD2, FCD3) and irrigation channels (FCE1, FCE2), but also in drainage channels ACE1 and ACD2 of the Alfacs bay.

The contamination pattern explained by PC3, which explained 12% of the total variance, has composition in which is attributed to the presence of azinphos ethyl, chlofenvinphos, diazinon, diuron, cybutryne, methiocarb, pendimetalin, and triallate (negative loadings) were inversely

correlated with MCPA, thiacloprid and triadimefon (positive loadings). As in the aforementioned PCs, the presence of MCPA may be indicative of the rice fields as relevant pesticide source in Alfacs bay (ACD2, ACD6, ACE1 and ACE2), while the other pesticides, that could be transported into the Delta via the main river, and thus, have different sources to the one aforementioned, were relevant to position some locations in the Fangars bay FCD1, FCD2, FCD5 and ACD5.

Finally, PC4 that explained only 9 % of the total variance, described a contamination pattern in which imidacloprid, MCPA and propanil (positive loadings) were inversely correlated with azinphos ethyl, bentazone, oxadiazon, simazine, and triadimefon (negative loadings). In this PC, pesticides used for rice cultivation are inversely correlated, which may indicate small local changes in the use of these pesticides. MCPA and propanil use was relevant in ACD1, ACD6 and ACE2, while bentazone application was predominant in other locations of the Alfacs bay (ACD2, ACD3, and ACD5 and in FCD3).

Table S8. Concentrations (ng/L) of individual pesticides measured in water samples belonging to sampling sites located in Fangar bay and Alfacs bay of the Ebro River Delta. Pesticides are grouped by chemical class.

Pesticides	*Fangar bay*									*Alfacs bay*									*SUM**
	FCD1	FCD2	FCD3	FCD4	FCD5	FCD6	FCD7	FCE1	FCE2	ACD1	ACD2	ACD3	ACD4	ACD5	ACD6	ACD7	ACE1	ACE2	
Acidics																			
2,4-D	nd	nd	<20	48	nd	nd	48	nd	nd	102	<20	33	nd	441	nd	33	11	nd	**720**
Bentazone	31×10^3	3000	10×10^3	15×10^4	12×10^4	12×10^4	10×10^3	2000	620	52×10^3	600	35×10^3	47×10^3	96×10^3	18×10^4	56×10^3	150	35×10^3	**95×10^4**
MCPA	660	nd	nd	810	2400	nd	nd	nd	nd	5300	130	1900	2000	1700	8200	1100	nd	6300	**31×10^3**
Anilides																			
Diflufenican	14	nd	<4.0	<4.0	nd	nd	nd	nd	<4.0	13	nd	10	12	nd	<4.0	19	nd	nd	**67**
Propanil	29	21	850	43	490	330	nd	nd	nd	61×10^3	8700	9000	7700	9700	26×10^3	1100	910	35×10^3	**16×10^4**
Azoles																			
Triadimefon	nd	nd	<4.0	<4.0	<4.0	nd	nd	nd	nd	nd	<4.0	4.9	<4.0	nd	nd	<4.0	<4.0	<4.0	**4.9**
Carbamates																			
Methiocarb	3.3	2.1	nd	nd	0.74	1.9	nd	1.0	nd	2.9	1.5	1.8	1.6	nd	nd	nd	<1.4	nd	**17**
Molinate	nd	31	nd	28	26	25	5.7	nd	nd	nd	nd	19	23	48	12	27	nd	40	**280**
Triallate	290	180	230	460	1000	540	480	600	410	70	41	74	180	300	170	250	110	120	**5500**
Chlorooacetanilides																			
Alachlor	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.4	1.6	nd	nd	nd	nd	nd	nd	nd	**3.2**
Metolachlor	37	38	36	39	33	37	10	73	62	51	52	22	27	18	28	25	70	25	**680**
Dinitroanilines																			
Pendimethalin	<2.0	<2.0	<2.0	<2.0	<2.0	nd	<2.0	<2.0	<2.0	nd	nd	nd	<2.0	<2.0	nd	<2.0	nd	nd	**nd**
Neonicotinoids																			
Acetamiprid	1100	nd	4000	nd	200	1070	240	nd	nd	<0.53	nd	49	270	100	390	72	nd	42	**7600**
Imidacloprid	120	nd	53	nd	47	75	nd	23	nd	700	nd	92	610	110	nd	91	nd	350	**2300**
Thiacloprid	nd	nd	nd	<0.21	nd	nd	<0.21	nd	nd	1.3	2.7	nd	<0.21	<0.21	nd	1.3	2.1	nd	**7.4**
Organochlorines																			
4,4'-DDD	nd	nd	nd	nd	<2.4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**nd**
Dicofol	nd	nd	<7.3	<7.3	<7.3	nd	<7.3	nd	<7.3	<7.3	nd	nd	nd	<7.3	nd	<7.3	nd	<7.3	**nd**
Oxadiazon	17	<0.72	14	15	30	nd	11	<0.72	<0.72	16	24	43	47	42	nd	31	<0.72	39	**330**

Table S8. (continued)

Pesticides	*Fangar bay*									*Alfacs bay*									*SUM*
	FCD1	FCD2	FCD3	FCD4	FCD5	FCD6	FCD7	FCE1	FCE2	ACD1	ACD2	ACD3	ACD4	ACD5	ACD6	ACD7	ACE1	ACE2	
Organophosphates																			
Azinphos ethyl	5.6	3.2	nd	nd	nd	nd	nd	nd	nd	5.5	nd	<1.4	nd	nd	nd	<1.4	nd	1.1	**15**
Chlorfenviphos	5.6	3.5	nd	nd	1.4	0.90	nd	nd	nd	6.3	3.3	3.1	3.1	3.2	1.7	1.5	0.95	<0.80	**35**
Chlorpyrifos	13	5	<1.5	27	27	17	26	25	18	9.4	5.9	7.7	19	21	12	18	13	11	**280**
Diazinon	4.3	2.5	2.7	1.5	nd	1.2	0.83	0.88	1.0	4.8	3.1	3.8	3.4	3.0	1.8	2.0	1.2	nd	**38**
Malaoxon	<0.50	0.57	nd	nd	nd	nd	nd	nd	nd	nd	nd	<0.50	nd	nd	nd	nd	nd	nd	**0.57**
Organothiophosphates																			
Fenthion oxon	nd	0.77	0.81	nd	nd	nd	nd	nd	nd	nd	1.9	nd	1.5	2.5	nd	nd	nd	nd	**7.4**
Fenthion oxon sulfoxide	nd	nd	<0.43	nd	nd	nd	nd	nd	nd	<0.43	1.2	nd	<0.43	nd	nd	nd	nd	<0.43	**1.2**
Fenthion sulfoxide	nd	1.8	nd	nd	nd	<1.4	nd	nd	nd	nd	nd	4.5	nd	nd	nd	nd	<1.4	nd	**6.3**
Phenylureas																			
Chlortoluron	nd	nd	nd	nd	nd	nd	nd	nd	14	nd	nd	nd	nd	nd	nd	nd	7.5	nd	**21**
Diuron	10	7.4	8.9	12	11	11	nd	nd	nd	6.3	6.9	6.8	6.6	12	5.2	nd	nd	nd	**103**
Isoproturon	nd	13	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**13**
Linuron	nd	<1.9	nd	nd	nd	nd	nd	nd	nd	4.6	13	nd	nd	nd	<1.9	nd	nd	nd	**18**
Triazines																			
Atrazine	nd	nd	nd	nd	<0.88	nd	nd	nd	2.5	nd	nd	nd	nd	<0.88	nd	nd	nd	nd	**2.5**
Cybutrine	48	33	41	30	47	49	47	49	41	14	11	15	17	20	17	18	15	11	**520**
Simazine	nd	2.1	nd	nd	4.8	nd	3.6	1.7	nd	nd	3.1	3.6	nd	6.7	<1.1	nd	nd	nd	**26**
Terbuthylazine	31	23	29	23	26	39	11	41	38	nd	33	22	nd	nd	nd	nd	40	23	**380**
Terbutryn	5.0	3.2	3.3	nd	1.7	nd	0.85	2.0	2.5	6.6	3.6	3.6	4.0	3.7	2.8	2.2	2.1	1.8	**49**
SUM*	**34x10^3**	**3400**	**15x10^3**	**16x10^4**	**12x10^4**	**13x10^4**	**11x10^3**	**2900**	**1200**	**12x10^4**	**9600**	**46x10^3**	**58x10^3**	**11x10^4**	**21x10^4**	**59x10^3**	**1300**	**77x10^3**	

nd: not detected

*Sum calculated considering values >LOQ.

Table S9. Hazard quotients (HQs) for each compound and hazard index for each sample.

Pesticides	*Fangar bay*									*Alfacs bay*								
	FCD1	FCD2	FCD3	FCD4	FCD5	FCD6	FCD7	FCE1	FCE2	ACD1	ACD2	ACD3	ACD4	ACD5	ACD6	ACD7	ACE1	ACE2
Acidics																		
2,4-D	n.a.	n.a.	0.001	0.004	n.a.	n.a.	0.004	n.a.	n.a.	0.008	0.001	0.003	n.a.	0.036	n.a.	0.003	0.001	n.a.
Bentazone	314.1	29.94	101.2	1549	1186	1238	106.5	20.72	6.245	525.7	5.948	346.7	469.5	956.1	1774	562.6	1.467	352.5
MCPA	1.312	n.a.	n.a.	1.616	4.860	n.a.	n.a.	n.a.	n.a.	10.59	0.254	3.893	4.077	3.448	16.42	2.262	n.a.	12.61
Anilides																		
Diflufenican	1.594	n.a.	0.222	0.222	n.a.	n.a.	n.a.	n.a.	0.222	1.421	n.a.	1.081	1.274	n.a.	0.222	2.074	n.a.	n.a.
Propanil	0.146	0.104	4.234	0.216	2.439	1.655	n.a.	n.a.	n.a.	306.1	43.47	45.03	38.46	48.77	130.0	5.388	4.559	176.6
Azoles																		
Triadimefon	n.a.	n.a.	0.001	0.001	0.001	n.a.	n.a.	n.a.	n.a.	n.a.	0.001	0.003	0.001	n.a.	n.a.	0.001	0.001	0.001
Carbamates																		
Methiocarb	0.332	0.197	n.a.	n.a.	0.074	0.187	n.a.	0.104	n.a.	0.297	0.148	0.175	0.157	n.a.	n.a.	n.a.	0.070	n.a.
Molinate	n.a.	0.008	n.a.	0.007	0.007	0.007	0.001	n.a.	n.a.	n.a.	n.a.	0.005	0.006	0.013	0.003	0.007	n.a.	0.010
Triallate	0.029	0.018	0.023	0.046	0.101	0.054	0.048	0.060	0.041	0.007	0.004	0.007	0.018	0.030	0.017	0.025	0.011	0.012
Chloroacetanilides																		
Alachlor	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.005	0.005	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Metolachlor	0.185	0.190	0.179	0.194	0.166	0.183	0.050	0.364	0.308	0.254	0.261	0.112	0.137	0.089	0.139	0.123	0.351	0.124
Dinitroanilines																		
Pendimethalin	0.056	0.056	0.056	0.056	0.056	n.a.	0.056	0.056	0.056	n.a.	n.a.	n.a.	0.056	0.056	n.a.	0.056	n.a.	n.a.
Neonicotinoids																		
Acetamiprid	0.306	n.a.	1.068	n.a.	0.053	0.286	0.064	n.a.	n.a.	0.000	n.a.	0.013	0.074	0.027	0.104	0.019	n.a.	0.011
Imidacloprid	14.34	n.a.	6.395	n.a.	5.706	9.087	n.a.	2.742	n.a.	84.72	n.a.	11.03	73.72	13.89	n.a.	10.91	n.a.	42.66
Thiacloprid	n.a.	n.a.	n.a.	0.010	n.a.	n.a.	0.010	n.a.	n.a.	0.127	0.266	n.a.	0.010	0.010	n.a.	0.134	0.211	n.a.
Organochlorines																		
4,4'-DDD	n.a.	n.a.	n.a.	n.a.	2.430	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Dicofol	n.a.	n.a.	114.1	114.1	114.1	n.a.	114.1	n.a.	114.1	114.1	n.a.	n.a.	n.a.	114.1	n.a.	114.1	n.a.	114.1
Oxadiazon	0.192	0.004	0.159	0.167	0.342	n.a.	0.122	0.004	0.004	0.180	0.272	0.489	0.532	0.472	n.a.	0.348	0.004	0.444

Table S9. (continued)

Pesticides	*Fangar bay*									*Alfacs bay*								
	FCD1	FCD2	FCD3	FCD4	FCD5	FCD6	FCD7	FCE1	FCE2	ACD1	ACD2	ACD3	ACD4	ACD5	ACD6	ACD7	ACE1	ACE2
Organophosphates																		
Azinphos ethyl	5.128	2.946	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	5.006	n.a.	0.636	n.a.	n.a.	n.a.	0.636	n.a.	0.963
Chlorfenviphos	0.056	0.035	n.a.	n.a.	0.014	0.009	n.a.	n.a.	n.a.	0.063	0.033	0.031	0.031	0.032	0.017	0.015	0.009	0.004
Chlorpyrifos	0.445	0.166	0.025	0.890	0.900	0.578	0.849	0.842	0.598	0.313	0.197	0.257	0.645	0.702	0.412	0.596	0.438	0.380
Diazinon	0.427	0.248	0.269	0.146	n.a.	0.121	0.083	0.088	0.105	0.481	0.311	0.381	0.337	0.303	0.176	0.200	0.117	n.a.
Malaoxon	0.001	0.002	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.001	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Organothiophosphates																		
Fenthion oxon	n.a.	0.004	0.004	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.009	n.a.	0.007	0.013	n.a.	n.a.	n.a.	n.a.
Fenthion oxon sulfoxide	n.a.	0.0002	n.a.	n.a.	n.a.	0.0001	n.a.	n.a.	n.a.	n.a.	n.a.	0.0004	n.a.	n.a.	n.a.	n.a.	0.0001	n.a.
Phenylureas																		
Chlortoluron	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.002	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.001	n.a.
Diuron	0.048	0.037	0.044	0.059	0.056	0.055	n.a.	n.a.	n.a.	0.032	0.034	0.034	0.033	0.057	0.026	n.a.	n.a.	n.a.
Isoproturon	n.a.	0.042	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Linuron	n.a.	0.010	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.046	0.131	n.a.	n.a.	n.a.	0.010	n.a.	n.a.	n.a.
Triazines																		
Atrazine	n.a.	n.a.	n.a.	n.a.	0.001	n.a.	n.a.	n.a.	0.004	n.a.	n.a.	n.a.	n.a.	0.001	n.a.	n.a.	n.a.	n.a.
Cybutrine	13.84	9.474	11.80	8.648	13.34	13.91	13.52	14.03	11.80	3.937	3.217	4.141	4.810	5.600	4.769	5.201	4.223	3.037
Simazine	n.a.	0.002	n.a.	n.a.	0.005	n.a.	0.004	0.002	n.a.	n.a.	0.003	0.004	n.a.	0.007	0.001	n.a.	n.a.	n.a.
Terbuthylazine	0.523	0.390	0.480	0.385	0.425	0.651	0.189	0.686	0.635	n.a.	0.550	0.369	n.a.	n.a.	n.a.	n.a.	0.661	0.384
Terbutryn	0.077	0.050	0.050	n.a.	0.027	n.a.	0.013	0.031	0.038	0.101	0.055	0.056	0.062	0.057	0.042	0.034	0.032	0.027
Hazard index*	**353**	**44**	**126**	**1561**	**1217**	**1264**	**121**	**40**	**20**	**939**	**55**	**414**	**594**	**1030**	**1926**	**590**	**12**	**590**

n.a.: not available, since environmental concentration was <LOD.

*Sum calculated considering only HQs corresponding to concentration values >LOQ.

3.5 Degradation of pesticides using bioremediation techniques

Pesticides, according to the few studies carried out, are very poorly eliminated in treatment plants (Köck-Schulmeyer et al., 2013b). Hence, the interest in developing new elimination processes for these compounds that can also be applied on-site in agricultural fields, or even within the aquifers. Some non-biological processes have been proposed for the treatment of these pollutants, such as their adsorption onto granular activated carbon (Portillo et al., 2004). This approach provided excellent performances but at a very high cost. Also, advanced oxidation processes (AOPs) have been explored for pesticide elimination. However, the results obtained depend on the AOP used and the chemical characteristics of the matrix (Carra et al., 2016).

In comparison to chemical and physical remediation approaches, bioremediation techniques present major advantages in terms of sustainability and low cost. Among the known bioremediation techniques, the capacity of white-rot fungi (WRF) in the degradation of xenobiotic pollutants of environmental interest has been pointed out in the scientific literature over the last few years. Their enzymatic system gives these fungi the versatility to degrade different xenobiotics and makes them especially interesting for their use in environmental applications. In the particular case of pesticides, it has been shown that some of them can be degraded by different WRF species.

In this context, the objectives of this study, performed within the BECAS project, were: i) to evaluate the ability of the fungus *Trametes versicolor* in the degradation of three widely used insecticides, malathion, acetamiprid and imidacloprid, recognized as toxic to non-target aquatic organisms, ii) to explore the enzymatic system involved in the degradation process, iii) to elucidate the degradation pathways from the main TPs formed during the process, and iv) to assess the toxicity of treated waters, and hence that of the main TPs identified.

The results demonstrated the capacity of the *T. versicolor* to degrade the selected pesticides. Although a slight increase in the toxicity of the treated waters was observed, the main TPs formed were overall less toxic than the parent compounds. These results highlight the potential of the investigated WRF species to treat pesticide-contaminated waters.

Scientific publication #5:

"Degradation of selected medium to highly polar pesticides by the white-rot fungus *Trametes versicolor*"

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Degradation of selected medium to highly polar pesticides by the white-rot fungus *Trametes versicolor*

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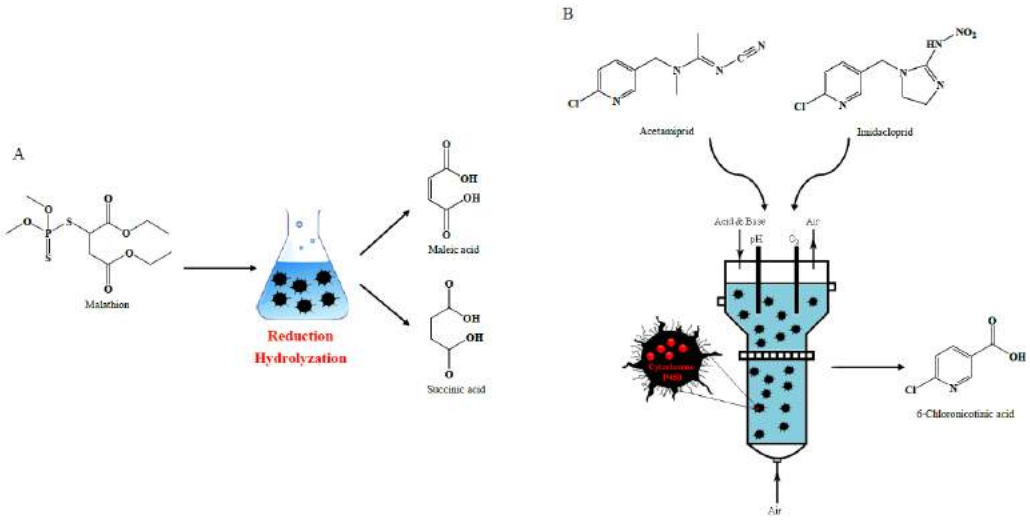
Highlights

- *T. versicolor* could effectively degrade malathion, imidacloprid and acetamiprid
- Cytochrome P450 is involved in acetamiprid and imidacloprid degradation
- Degradation pathways proposed based on metabolites identification
- Predicted toxicity of metabolites is lower than that of the parent compounds.

Graphical Abstract



Abstract

The massive use of pesticides represents one of the main causes of environmental deterioration, as they produce adverse effects on non-target organisms. Thus, the development of technologies capable of reducing their release into the environment is urgent. This study reports for the first time the white-rot fungus *Trametes versicolor* as an alternative towards the degradation of the medium to highly polar pesticides the organophosphate malathion, and the neonicotinoids acetamiprid and imidacloprid. Specifically, *T*. *versicolor* could completely remove 1 mg L^{-1} of malathion in Erlenmeyer within 48 h, whilst experiments of acetamiprid and imidacloprid (4 mg L^{-1}), conducted in air-pulse fluidized bioreactors, resulted in degradation percentages of 20% and 64.7%, respectively, after 7 days of operation. Enzymatic exploration studies revealed that the cytochrome P450 system, instead of the extracellular enzyme laccase, is involved in the degradation of acetamiprid and imidacloprid. The degradation pathways were proposed based on the main transformation products (TPs) formed in the solutions: seven in the case of malathion, and two and one in the case of imidacloprid and acetamiprid, respectively. Although the TPs identified were predicted to be less toxic than the investigated pesticides, the toxicity of the individual solutions slightly increased throughout the degradation process, according to the Microtox assay. However, the solution toxicity was always below the threshold established in the local regulation. Although additional research is needed to implement this treatment at a pilot plant scale, this work highlights the potential of *T. versicolor* to bio-remediate pesticide-contaminated waters.

Keywords: Micropollutants; agrochemicals; fungal bioremediation; degradation metabolites; non-target high-resolution mass spectrometry.

1. Introduction

Among all known environmental micropollutants, those that have become persistent due to, not only their high volumes of production and consumption, but also their poor elimination rates in conventional wastewater treatment plants (WWTPs), are of great concern. In addition, many of them have bioactive properties and pose a real risk to organisms living in the receiving water bodies[1]. In this regard, pharmaceuticals, personal care products and pesticides are among the most relevant bioactive micropollutants.

The release of pesticides into the environment is considered the main trigger of environmental deterioration[2]. Although harmful for non-target organisms, their use has continuously increased over time in order to improve crop yield and cover the increasing food demand[3]. To reduce the risk of pesticide use, the persistent, bioaccumulative and toxic organochlorine pesticides have already been banned for years in many countries and substituted by more polar and less persistent substances of relatively low toxicity[3]. However, due to their extended use, the new pesticides placed in the market are not completely innocuous for the environment, and may also produce undesired effects in non-target organisms, especially considering that a substantial amount of the applied chemical is not uptaken by the crop and hence remains in the different environmental compartments[4-6].

Among these new pesticides, the organophosphate malathion [diethyl 2-dimethoxyphosphinothioyl sulfanylbutanedioate], and the neonicotinoids acetamiprid [(E)-N-(6-chloro-3-pyridylmethyl-N'-cyano-N-methylacetamidine] and imidacloprid [(E)-1-(6-chloro-3-pyridylmethyl-N-nitroimidazolidin-2-ylideneamine] are largely used for insect control in public health, agricultural as well as domestic applications. Malathion, despite being less toxic than other organophosphate pesticides[7], has been classified as probably carcinogenic to humans by the International Agency for Research on Cancer[8], and has been proven to be highly toxic to aquatic species[7]. As an alternative to organophosphate and carbamate insecticides, neonicotinoid insecticides were introduced. Their development was once considered as a milestone in agrochemical research because these substances presented high selectivity for insects[9]. However, there are growing evidence of their high persistence in the environment and negative effects on non-target organisms and human health[4, 10-13]. These three pesticides are indeed rated as moderately (acetamiprid) or highly

(imidacloprid and malathion) toxic for bees[14]. Pesticide exposure has indeed been appointed as one of the causes of global pollinator decline, which represents a severe threat to biodiversity conservation and the maintenance of ecosystem services[11, 15-17]. Thus, the development of elimination technologies targeting such pollutants is strongly motivated and imperative.

Bioremediation techniques are considered as a low-cost, eco-friendly, and efficient alternative to physical and chemical technologies currently available for the abatement of a broad range of pollutants from the environment[18]. Among the existing bioremediation techniques, those based on white-rot fungi (WRF) have been proven effective to remove a variety of micropollutants, some of them recalcitrant to other microorganisms. The versatility and effectivity of this type of fungi can be attributed to its well-developed enzymatic systems, which include extracellular ligninolytic enzymes that confer the fungus a high tolerance to toxic compounds[19]. So far, different WRF species, such as *P. ostreatus*[20], *P. chrysoporium*, *B. adusta,* and *C. gallica*[21, 22] have been reported to harbor the capability of degrading malathion and other organophosphate pesticides, but limited knowledge has been generated regarding their performance with neonicotinoids. In this regard, the degradation of neonicotinoid pesticides has been explored only with *Phanerochaete* spp.[23-26].

The main objective of the present work was to assess the ability of the WRF species *Trametes versicolor* in degrading malathion, acetamiprid, and imidacloprid, and to explore the contribution of its two most relevant enzymatic systems (i.e., laccase and cytochrome P450) in the degradation of neonicotinoid, aspects scarcely addressed to date, in particular for the WRF species selected in this study. The main transformation products (TPs) formed during the process were also identified, which allowed elucidating the biodegradation pathways. Furthermore, the toxicity of the treated waters and that of the TPs formed was explored to assess the environmental safety of the treatment. Thus, this work aimed at providing valuable knowledge on the WRF-degradation of the selected pesticides that can be used for future implementation of this technology on a large scale.

2. Materials and Methods

2.1. Microorganisms and media

T. versicolor ATCC 42530 was acquired from American Type Culture Collection and maintained by subculturing every 30 days on 2% (w/v) malt extract plates (pH 4.5) at 25 °C. Blended mycelial suspensions and pellets were prepared using malt extract following the methodology described elsewhere[27].

The composition of the defined medium (pH 4.5) used for degradation experiments was: glucose (8 g L^{-1}), ammonium tartrate (3.3 g L^{-1}), dimethyl succinate (1.68 g L^{-1}), micronutrients (10 mL L^{-1}), macronutrients (100 mL L^{-1})[28]. The medium pH was adjusted using HCl (1 M) and NaOH (1 M).

2.2. Chemicals and reagents

High purity (≥ 98%) analytical standards of malathion, acetamiprid, imidacloprid and their isotopically labeled analogs (malathion-d_{10}, acetamiprid-d_3, and imidacloprid-d_5) used as internal standards (IS) for quantitative analysis, commercial laccase purified from *T. versicolor* (20 AU mg^{-1}), laccase mediator 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 98%), 2,6-dymetoxyphenol (DMP, 99%) and cytochrome P450 inhibitor 1-aminobenzotriazole (purity, 98%) were purchased from Sigma-Aldrich (Barcelona, Spain). Microtox bioassay kits were supplied by Strategic Diagnostics Inc. (Newark, USA). Chromatographic grade acetonitrile was purchased from Carlo Erba Reagents S.A.S (Barcelona, Spain). High-performance liquid chromatography (HPLC)-grade water, acetonitrile and methanol, and formic acid (> 98%) used as a mobile phase modifier, were obtained from Merck (Darmstadt, Germany). Stock solutions (5 mg mL^{-1}) of each pesticide for degradation experiments were prepared by appropriate dilution of each substance in ethanol, while those for analytical purposes, standard solutions were prepared in methanol. All stock and working standard solutions were stored in the dark at – 20 °C until use.

2.3. In vivo degradation batch experiments

All *in vivo* experiments were conducted using *T. versicolor* pellets as inoculum and in sterile conditions.

Degradation experiments for malathion were performed in 500 mL Erlenmeyer flasks containing 100 mL of fresh defined medium and a pesticide concentration of 1 mg L^{-1}. Fungal pellets were carefully transferred into the flasks, thereby achieving a concentration of approximately 2.4 g dry weight (DW) L^{-1}. Afterward, the cultures were incubated at 25 °C under continuous shaking (135 rpm) for 7 days, and experiments were run in triplicate.

Degradation experiments for acetamiprid and imidacloprid were conducted in Erlenmeyer flasks, but also in an air pulsed fluidized bed reactor (1.5 L). This reactor carried 1.3 L of defined medium and an equivalent concentration of inoculum (2.4 g DW L^{-1}). The pesticide concentration was set to 4 mg L^{-1}. The reactor was operated at 25ºC for 7 days at a constant pH value of 4.5 (maintained with HCl and/or NaOH). Fluidized conditions in the reactor were sustained by 1 s air pulses generated by an electrovalve every 4 s. Glucose was regularly monitored and added to a concentration of 4 g L^{-1} before it was completely consumed.

Abiotic (uninoculated) as well as heat-killed culture (121 °C, 30 min) reactors containing the working concentration of each pesticide were used as controls and conducted in 500 mL Erlenmeyer flasks, to evaluate the stability of contaminants and their potential adsorption onto the biomass. Triplicates were set for control group and all tested sets were run in the dark to avoid photodegradation. Two mL samples were withdrawn periodically for the analysis of the residual concentrations of the selected pesticide and glucose, and laccase activity.

2.4. Experiments to assess the enzymatic system involved in neonicotinoids degradation

The involvement of cytochrome P450 system in the fungal degradation of organophosphorus pesticides, including malathion, has been already well described[21], and thus experiments to characterize the enzymatic system involved in biodegradation were only conducted for acetamiprid and imidacloprid.

Laccase-mediated *in vitro* degradation experiments were performed in 250 mL Erlenmeyer flasks containing 20 mL laccase-sodium malonate dibasic monohydrate solution (250 mM, pH 4.5) at a final enzyme activity of 1000 AU L^{-1}, and a pesticide concentration of 10 mg L^{-1}. The effect of having the lacasse mediator ABTS in the system was evaluated by comparing the results of culture that contained 0.8 mM ABTS with those Erlenmeyer flasks where ABTS was not added[29]. Abiotic conditions (only the pesticide) were also explored. All experiments

were run in triplicate. The flasks were incubated for 24 h on an orbital shaker (135 rpm) at 25 °C. At designated times, 1 mL aliquots were collected and mixed with 100 µL of 1 M HCl to stop the reaction. They were filtered with a Millipore Millex-GV unit equipped with a polyvinylidene difluoride (PVDF) membrane (0.22 µm) before the analysis of the residual pesticide concentration.

For the evaluation of the role of the cytochrome P450 system in the degradation, *in vivo* experiments were carried out in an air-pulsed bioreactor containing 4 mg/L of the pesticide, in the presence (4 mM) and the absence of 1-aminobenzotriazole, an inhibitor of the P450 system[29]. The reactor was inoculated with 2.4 g DW L^{-1} of *T. versicolor* pellets and run for 7 days at the identical operational conditions described in section 2.3. One mL aliquots were collected daily and filtered (PVDF, 0.22 µm) before the analysis of the residual pesticide concentration.

2.5. Experiments for the identification of degradation products

The experiments conducted to evaluate the transformation products generated during the degradation process were essentially analogous to those described in section 2.3, except that the initial pesticide concentration was 1 mg L^{-1} in all cases. At selected times (0, 2, and 7 days), 4 mL of the culture was withdrawn from the bioreactor and centrifuged (17,700 × g, 4 min) at room temperature. Then, 1.5 mL of the supernatant was transferred into a 2-mL amber vial that contained 0.75 µg of the corresponding deuterium-labeled pesticide that was used as IS in ultra-high performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) analysis. The samples were kept at – 20 °C until analysis.

2.6. Analytical procedures

2.6.1. Laccase

Laccase activity was measured through the oxidation of DMP (µM in one min) by the enzyme in the absence of a cofactor as described elsewhere[30]. For this, a molar extinction coefficient of DMP 24.8 mM^{-1} cm^{-1} was used. Laccase activity is expressed as the activity units per liter (AU L^{-1}).

2.6.2. Pesticide Analysis

Malathion residual concentration was determined using UPLC-HRMS (further details provided inText 1 in supporting information (SI)).

Residual concentrations of acetamiprid and imidacloprid were determined using HPLC and UV detection (HPLC, Ultimate 3000, Dionex, USA). HPLC analysis was performed with a mobile phase consisting of 0.01% (v/v) formic acid in water and acetonitrile (60:40, v/v) at a flow rate of 0.7 mL min^{-1}, and a C18 reversed-phase column (Phenomenex®, Kinetex® EVO C18 100 Å, 4.6 mm × 150 mm, 5 µm) set at 30 °C. The injection volume was 40 µL. The detection wavelengths for acetamiprid and imidacloprid were 242 and 270 nm, respectively.

2.6.3. Evaluation and identification of transformation products

The TPs formed during the degradation process were evaluated using UPLC-HRMS using an Acquity system (Waters, Milford, MA, USA) connected in series with a hybrid quadrupole-Orbitrap mass spectrometer (QExactive) (Thermo Fisher Scientific, USA) using both electrospray polarity modes, positive and negative. The HRMS analysis was conducted in the data-dependent acquisition (DDA) mode: full scan over the *m/z* range from 70 to 1,000 and data-dependent MS/MS scan events with a 40% normalized collision energy for the five most intense ions detected in each scan. Further details on the methodology used are provided in Text 1 of the SI. Data acquisition was controlled by Xcalibur 2.2 software (Thermo Fisher Scientific).

UPLC-HRMS data were processed using Compound Discover 3.1 software (Thermo Fisher Scientific). Experimental samples collected at time = 2 and 7 days were compared with samples at t = 0 to identify newly formed peaks or features. After peak alignment and deconvolution (using a retention time window of 2 min and 5 ppm of mass tolerance), the features (*m/z* ions) detected were grouped and plausible elemental compositions were assigned to each peak. In parallel, a search by molecular formula or exact mass was performed in various MS libraries and compound databases (ChemSpider, mzCloud, mzVault) to assign a potential compound identity to each peak. Then, the list of potential candidates generated was manually filtered to identify TPs, i.e., those peaks that were only present in samples collected after 2 and/or 7 days of degradation, and evaluate the

molecular structures proposed by the software. The latter was done using the elemental composition of the molecular and fragment ions, logical fragment rationalization, and considering the presence of isotopic patterns.

2.6.4. Other analyses

Biomass was determined by the dry weight of the pellets, obtained after filtrating the culture and drying the residue at 100 °C to a constant weight.

Glucose concentration was measured using a biochemistry analyzer (2700 select, Yellow Springs Instrument, USA) after filtrating the sample with a nylon filter (0.45 µm pore size).

2.7. Toxicity assessment

2.7.1. Microtox Test

The acute toxicity of experimental samples after 7 days of incubation (see section 2.5) and abiotic controls was measured using the Microtox test. This assay allows monitoring the natural emission (in the range of the visible light, with a maximum intensity at 490 nm) of the marine bioluminescent bacterium *Vibrio fischeri* after exposure to selected samples. Toxicity data, corresponding to the 50% effective concentration (EC_{50}), were based on 5 and 15 min incubation of bacteria with filtered diluted samples (pH 7) at 25 °C. Toxicity was expressed as toxicity units (TU), calculated by TU =100/EC_{50}.

2.7.2. QSAR-prediction of toxicity

The aquatic toxicity of the investigated pesticides and their degradation products was predicted by employing the Ecological Structure-Activity Relationships (ECOSAR) predictive model (v2.0), validated and developed by the US Environmental Protection Agency (EPA)[31]. This software estimates a chemical's acute (short-term) toxicity and chronic (long-term or delayed) toxicity to aquatic organisms, such as fish, aquatic invertebrates, and aquatic plants, by using computerized Structure-Activity Relationships (SARs). This approach, routinely used by the US EPA in a regulatory context for evaluating aquatic toxicity, is also cited as a potential non-testing method by EFSA[32], and included in the OECD QSAR toolbox (http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm).

In this work, ECOSAR software was used to predict the acute toxicity values LC50 (50% lethal

concentration for fish and daphnia after exposure for 96 h and 48 h, respectively) and EC50 (50% effective concentration for green algae growth inhibition after 96 h) of the investigated pesticides and related TPs. For this, the information on their molecular structure was introduced into ECOSAR using either their CAS number or their SMILES code when the CAS number was not available or recognized by the software. Since an overestimation of the toxicity is preferable from a regulatory perspective, the lowest (i.e. most conservative) QSAR-predicted LC50 or EC50 value for each compound was considered, following EFSA recommendations[32].

2.8. Data analysis

The removal rate constant (K_d) [mg (L d)$^{-1}$] was determined through a first-order kinetics model (Eq. (1))

$$\ln S = -tK_d + \ln S_0 \quad (1)$$

where t is the removal period (d), S is the residual concentration of the substrate (mg L^{-1}) at time t, and S_0 is the initial concentration of the substrate at time 0 (mg L^{-1}).

The half-life ($T_{1/2}$) of the substrate was calculated using Eq. (2):

$$T_{1/2} = \frac{\ln 2}{K_d} \quad (2)$$

3. Results and discussion

3.1. Degradation of selected pesticides by T. versicolor

As shown in Figure 1a, *T. versicolor* was able to completely remove malathion in defined medium within 48 h. Up to 20% of malathion was removed in culture-killed control experiments, which evidenced that adsorption played also a relevant role in this elimination process. Removal of acetamiprid and imidacloprid in Erlenmeyer flask showed much lower removals ($p < 0.05$) (data not shown), probably due to the effect of the low pH (pH < 4 after 3 d) generated from the acid secretion by the fungi that further hindered their metabolism. Simultaneously, high initial spiked concentration (10 mg L^{-1}) may also cause toxicity to fungus. Therefore, an air-pulsed fluidized reactor connected to a pH controller was adopted to explore the degradation of neonicotinoid pesticides at lower concentration (4 mg L^{-1}).

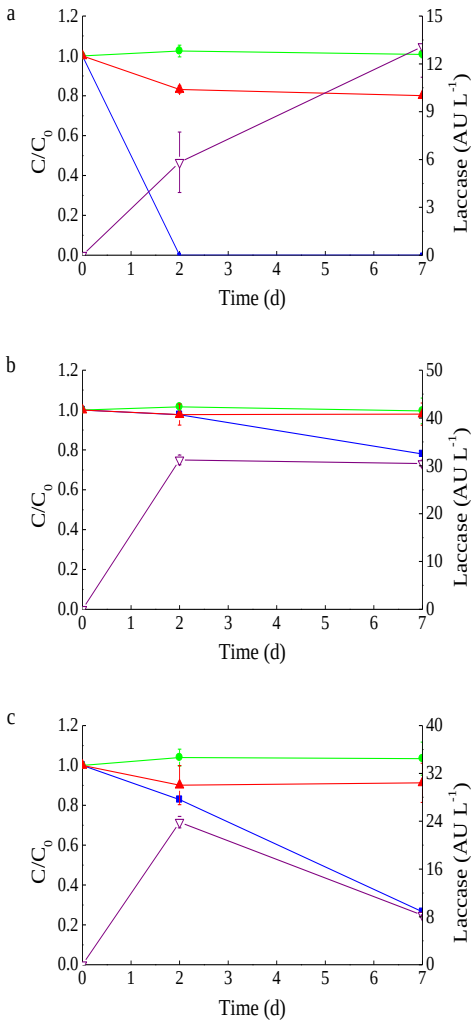


Figure 1. Time-course of pesticide degradation by *T. versicolor*. a. malathion; b. acetamiprid; c. imidacloprid. C represents the residual concentration of the pesticide in the sample (mg L^{-1}), and C_0 corresponds to the initial concentration of the pesticide in the sample (mg L^{-1}); *Blue lines with filled squares*, experimental reactor; *red lines with filled triangles*, killed control; *green lines with filled circles*, abiotic control; *purple lines with inverted empty triangles*, laccase activity in the experimental reactor.

In this reactor, *T. versicolor* was capable of degrading 20% and 65% of acetamiprid and imidacloprid, respectively (Figure 1b and 1c). As expected, due to their physical-chemical properties (Table S1 in SI), neonicotinoids (low octanol-water (Log Kow), and organic carbon-water partition coefficients (Koc)) were less adsorbed onto biomass than malathion. On the other hand, a sustained increase of laccase activity was observed in the malathion culture system, while it remained constant in the case of acetamiprid or even apparently dropped in imidacloprid experiments after 48 h of incubation.

These insecticides were satisfactorily removed by different microbes, including different bacteria strains and fungi species[20, 24, 33-36]. However, this is the first study that confirms *T. versicolor* as a degrader of imidacloprid, acetamiprid and malathion, thus enriching the candidates of organisms that can be used in bioremediation treatments.

3.2. Role of laccase and cytochrome P450 inhibitor in the degradation of acetamiprid and imidacloprid

As mentioned above, laccase activity was detected to some extent during degradation experiments of acetamiprid and imidacloprid using *T. versicolor*. Therefore, we investigated whether this extracellular enzyme was involved in the degradation of these neonicotinoid pesticides. However, it turned out that these pesticides did not degrade using commercial laccase in the absence and presence of the laccase mediator. Thus, no firm connection between biodegradation and laccase can be proposed. Similar findings have been reported with this particular fungus when dealing with other contaminants[27, 37, 38].

Based on these findings, the possibility that a different enzymatic system, namely the cytochrome P450 system, participates in the degradation of imidacloprid and acetamiprid was also evaluated. The results are provided in Table 1.

Table 1. Degradation kinetics parameters of acetamiprid and imidacloprid by *T. versicolor* under the effect of cytochrome P450 inhibitor 1-aminobenzotriazole.

Pesticide	Treatment	Regression equation	$T_{1/2}$ (d)	K_d [mg (L d)$^{-1}$]	R^2
Acetamiprid	With inhibitor	ln S = − 0.012 t + 1.372	57.8	0.012	0.921
	Inhibitor-free	ln S = − 0.047 t + 1.637	14.8	0.047	0.955
Imidacloprid	With inhibitor	ln S = − 0.008 t + 1.011	86.6	0.008	0.873
	Inhibitor-free	ln S = − 0.049 t + 1.273	14.2	0.049	0.938

The degradation process could be fitted with a pseudo-first-order kinetic equation due to the low pollutant concentration (4 mg L^{-1}) with respect to the Michaelis-Menten kinetics (K_M>>S). The $T_{1/2}$ of acetamiprid and imidacloprid increased markedly in the presence of the cytochrome P450 inhibitor, 1-aminobenzotriazole. This observation is indicative of the indispensable contribution of the cytochrome P450 to the substrate depletion, and it is in line with previous studies in which cytochrome P450 was also reported to play a key role in

the biotransformation of different neonicotinoid insecticides by different strains of the fungus *Phanerochaete* species, including acetamiprid[24], clothianidin[23], nitenpyram, and dinotefuran[26].

3.3 TPs generated during the degradation of selected pesticides by T. versicolor

The TPs identified during the degradation of malathion, acetamiprid, and imidacloprid by *T. versicolor* are summarized in Table 2. A total of ten compounds were identified as TPs; however, logical tentative structures could be only proposed for eight of them with a confidence level of 3 according to the Schymanski scale[39] since they could not be confirmed with analytical standards.

During malathion degradation, seven TPs were identified including TP172 (diethyl maleate), TP174 (diethyl succinate), TP128 (ethyl (2E)-4-oxo-2-butenoate), TP144 (monoethyl maleate), TP118 (succinic acid), TP132 (monoethyl succinate), and TP160 (ethyl methyl succinate). All of them were found to be present after 7 days of treatment showing an increasing trend by the end of the experiment, except TP132, which could be considered as an intermediate byproduct that was further degraded (Figure S1 in SI). Given the obtained formulae, we speculate that C-S bond cleavage was the first step in the presence of a proton donor (a base or a reductase), generating diethyl maleate and diethyl succinate, respectively (Figure 2). Subsequently, diethyl maleate underwent reduction or hydrolyzation, yielding ethyl (2E)-4-oxo-2-butenoate or monoethyl maleate, which could be further converted into maleic acid, although the last one has not been identified as TP. Meanwhile, diethyl succinate either underwent serial hydrolysis or got through a demethylation process followed by hydrolyzation, until it transformed into succinic acid (Figure 2).

The hydrolysis of the phospho-ester bond has been reported as the first and also the most significant step in detoxification of organophosphorus pesticides (i.e., chlorpyrifos, glyphosate, and malathion, etc.) by either bacteria or fungi[22, 40]. Our findings point at the cleavage of the S-C bond as the main step. This pathway has been also documented by Paris et al. using heterogeneous bacterial populations consisting of *F. meningosepticum*, *Xanthomonas* sp., *C. terrigeri,* and *P. cepacia*[41].

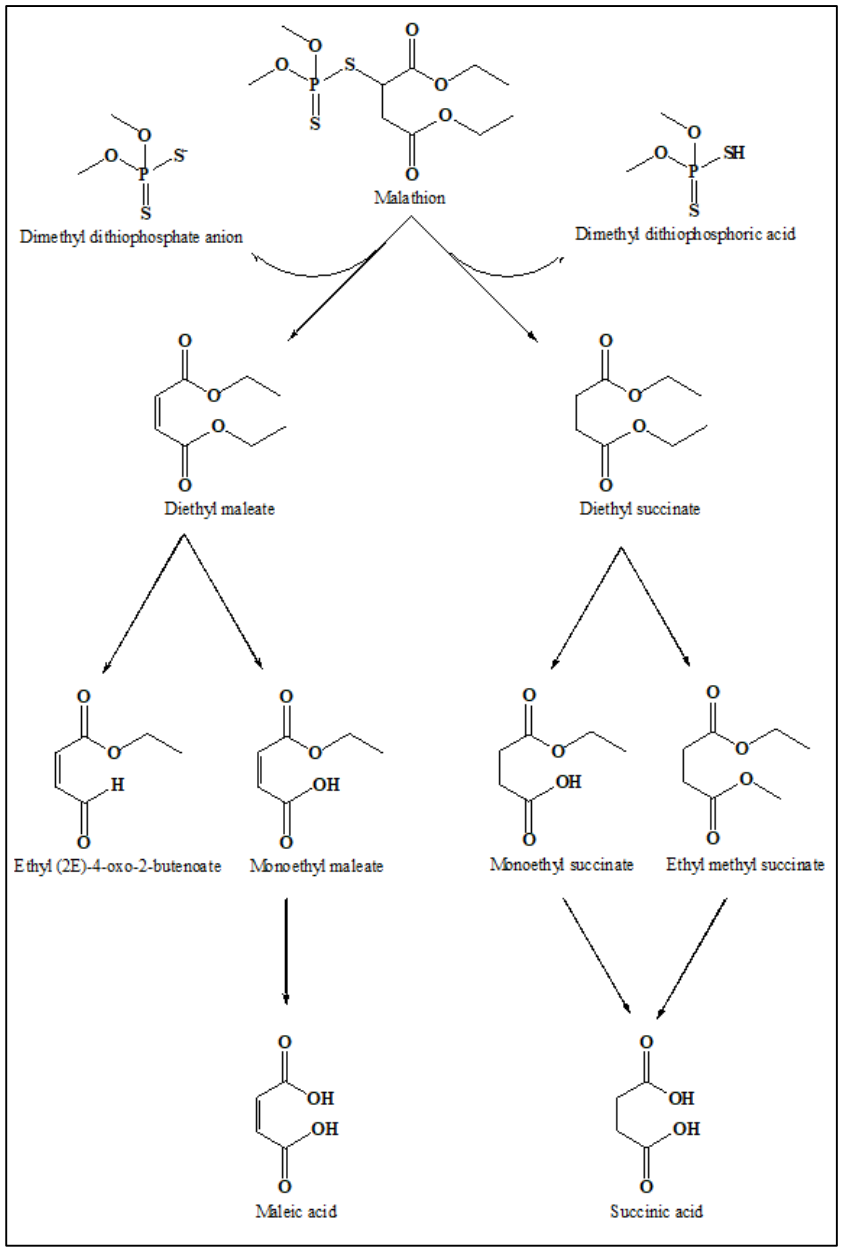


Figure 2. Proposed pathway of malathion degradation by *T. versicolor.*

On the other hand, the hydrolysis of the carboxylic ester bonds, probably by carboxylesterases, resulted in the mono- and diacid metabolites, and considerably contributed to malathion degradation. This is also in agreement with previous reports[35, 36, 42]. Although demethylation has been reported in malathion detoxification using two fungi species including *A. niger* and *P. rotatum*[43], in our study demethylation takes place on diethyl succinate, rather than on the dimethyl dithiophosphoric acid moiety, leading to the formation of ethyl methyl succinate which is reported for the first time. Identification of the circumstances behind this different pathway would require further research.

Table 2. Transformation products formed during the degradation of selected pesticides by *T. versicolor.*

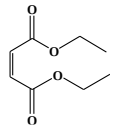
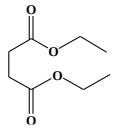
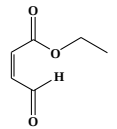
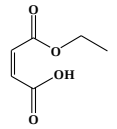
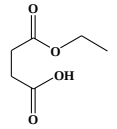
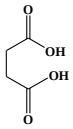
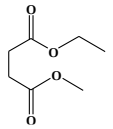
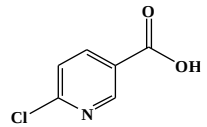
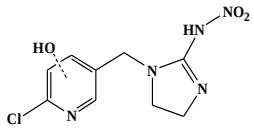
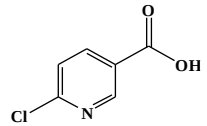
Pesticide	Number	t_R min)	HESI mode	Full Scan				MS/MS				Suspect identity (Confidence level)	Chemical structure
				m/z	Formula	RDB	Δm (ppm)	*m/z*	Formula	RDB	Δm (ppm)		
Malathion	TP172	6.9	+	173.0816	$C_8H_{13}O_4$	2.5	4.2	129.0552	$C_6H_9O_3$	2.5	4.5	Diethyl maleate (CL3)	
	TP174	7.5	+	175.0971	$C_8H_{15}O_4$	1.5	3.68	143.0708 115.0761	$C_7H_{11}O_3$ $C_6H_{11}O_2$	2.5 1.5	4.0 6.2	Diethyl succinate (CL3)	
	TP128	7.5	+	129.0552	$C_6H_9O_3$	2.5	4.9	101.0605	$C_5H_9O_2$	1.5	8.4	Ethyl (2E)-4-oxo-2-bute--noate (CL3)	
	TP144	6.2	+	145.0501	$C_6H_9O_4$	2.5	3.9	145.0501 99.0449 71.0500	$C_6H_9O_4$ $C_5H_7O_2$ C_4H_7O	2.5 2.5 1.5	3.9 8.0 12.4	Monoethyl maleate (CL3)	
	TP132	3.9	–	131.0331	$C_5H_7O_4$	2.5	– 6.4	n.i.				Monoethyl succinate (CL5)	
	TP118	3.6	+	119.0346	$C_4H_7O_4$	1.5	6.0	n.i.				Succinic acid (CL5)	

Table 2. (cont) Transformation products formed during the degradation of selected pesticides by *T. versicolor.*

Pesticide	Number	t_R (min)	HESI mode	Full Scan				MS/MS				Suspect identity (Confidence level)	Chemical structure
				m/z	Formula	RDB	Δm (ppm)	*m/z*	Formula	RDB	Δm (ppm)		
Malathion	TP160	7.5	+	161.0814	$C_7H_{13}O_4$	1.5	3.5	115.0761	$C_6H_{11}O_2$	1.5	6.3	Ethyl methyl succinate (CL3)	
								101.0605	$C_5H_9O_2$	1.5	8.2		
Acetamiprid	TP157	7.6	+	158.0009	$C_6H_5O_2NCl$	4.5	3.5	122.0234	$C_6H_4O_2N$	5.5	– 2.1	6-Chloronicotinic acid (CL3)	
								78.0346	C_5H_4N	4.5	9.3		
Imidacloprid	TP271	7.0	+	272.0551	$C_9H_{11}O_3N_5Cl$	6.5	2.3	228.0540	$C_9H_{11}O_2N_3Cl$	5.5	2.5	Hydroxyl-imidacloprid (CL3)	
								225.0544	$C_9H_{10}ON_4Cl$	6.5	2.8		
								126.0111	C_6H_5NCl	4.5	3.9		
								144.0216	C_6H_7ONCl	3.5	3.6		
	TP157	7.7	+	158.0010	$C_6H_5O_2NCl$	4.5	4.10	n.a.				6-Chloronicotinic acid (CL5)	

t_R: chromatographic retention time, HESI, heated-electrospray ionization; Δm, mass measurement error; RDB, ring and double bond equivalents;
n.i.: no fragments with plausible formula identified;
n.a.: MS2 data were not acquired for this ion, and m/z ions for fragments were thus not available.

Concerning acetamiprid and imidacloprid, two compounds TP157 (6-chloronicotinic acid) and TP271 (hydroxyl-imidacloprid) were identified as TPs with a confidence level of 3. Both metabolites were found to be present after 7 days of treatment, showing an increasing trend by the end of the experiment (Figure S2). The TP157 was generated during the degradation of both pesticides, which is consistent with the degradation experiments conducted with *Mycobacterium* sp. isolated from soil[34]. The formation of 6-chloronicotinic acid is generated by oxidative cleavage of the C-N bond that leaves out the 2-chloro-5-methylpyridine moiety, and it seems to be a common TP of neonicotinoids that contain a pyridinyl ring[34, 44]. In the case of imidacloprid, another hydroxylated byproduct, possibly mediated by cytochrome P450[44] was identified. However, it is interesting to point that MS^2 data revealed that the hydroxylation occurred in the pyridinyl ring (Figure 3) instead of in the heterocyclic spacer as previously documented[44-47].

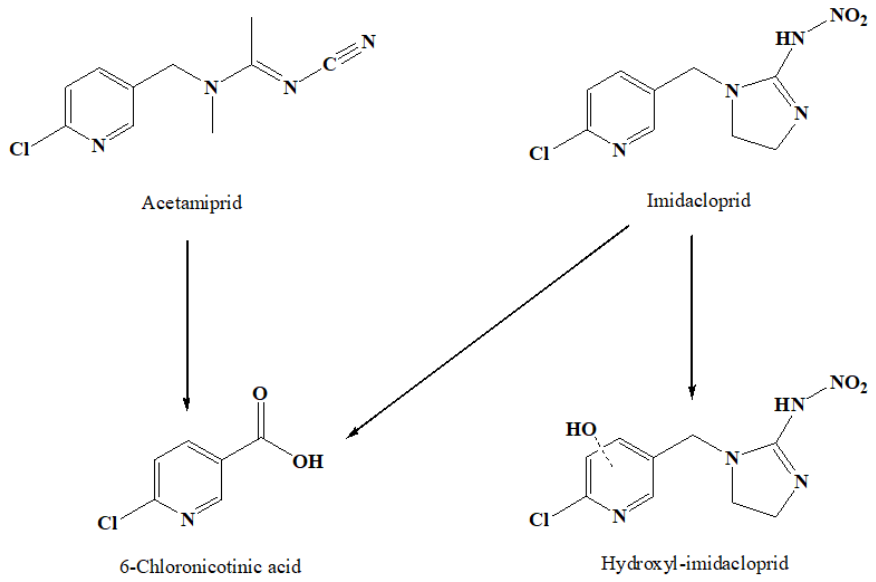


Figure 3. Degradation pathways of neonicotinoid pesticides by *T. versicolor.*

3.4 Toxicity assessment

3.4.1 Microtox test

Toxicity data obtained from the Microtox test performed on the 7-days-culture samples using the *Vibrio fischeri* bacterium showed a general increase of TU in the experimental

culture in comparison to the abiotic control. A nearly double effect on TU value was observed in the case of malathion and its degradation products, increasing from 0.65 to 1.27. Although both values were lower than the wastewater discharge limit (25) set in Catalonia industrial areas[48], the results indicate that some of the metabolites generated in the biotransformation process are more toxic than malathion.

Similar results were observed in the case of imidacloprid and acetamiprid, where TU values increased from 0.54 to 3, and 0 to 0.86, respectively, which supports previously published results indicating that the TP157 (6-chloronicotinic acid) possesses higher toxicity than its parent compounds imidacloprid and acetamiprid[4]. In any case, the maximum discharge limit was not achieved, despite the fact that the initial pesticide concentration in these experiments was dramatically higher than the environmental levels (normally in the ng or µg/L range).

3.4.2 QSAR-prediction of toxicity

The QSAR-predicted LC50 and EC50 values for the three representative aquatic species, *viz.*, fish, daphnia, and green algae are shown in Table 3. The interpretation of the results was based on a simple comparison with the threshold values given by the Globally Harmonized System of Classification and Labelling of Chemicals[49]. According to these values, the toxicity data obtained were divided into four levels (i.e., very toxic, toxic, harmful, and not harmful) (Table S2). Malathion is predicted to be very toxic for daphnia after 48 h exposure (LC50 < 1 mg L^{-1}), toxic for fish (LC50 between 1 and 10 mg), and just harmful for green algae (EC50 between 10 and 100 mg L^{-1}) after 96 h of exposure. In contrast, the toxicity of its degradation products was comparatively lower. Most metabolites generated in the first stage of the degradation pathway are classified as toxic or harmful (LC50/EC50 values between 10 and 100 mg L^{-1}), while a general no harmful potential is expected for all three tested organisms for metabolites formed at a later stage, which present LC50 or EC50 values above 100 mg L^{-1}.

In the case of acetamiprid and imidacloprid, the predicted LC50 or EC50 values (between 2.8 and 51) indicate overall a moderate toxicity for all three aquatic organisms. Compared to them, the corresponding metabolites show in general similar or slightly higher LC50 or EC50 values, meaning less toxicity. The only exception is hydroxyl-imidacloprid that,

according to its predicted LC50/EC50 values, would be very toxic to daphnia after 48 h exposure (LC50 of 0.336 mg L^{-1}), and slightly more toxic to algae after 96 h exposure (EC50 of 10.2 mg L^{-1}) than imidacloprid (EC50 of 50.7 mg L^{-1}).

These results suggest that *Trametes versicolor*-based bioremediation can be a useful tool for the treatment of waters contaminated with malathion and acetamiprid, while in the case of imidacloprid the formation of its, more toxic, hydroxylated TP (TP271), would require additional investigation.

Table 3. Predicted toxicity of malathion, acetamiprid, imidacloprid, and their degradation products by the ECOSAR program*.

Compounds	No. CAS	LC50 fish 96h (mg L^{-1})	LC50 daphnia 48h (mg L^{-1})	EC50 green algae 96h (mg L^{-1})
Malathion	121-75-5	1.39	0.0028	15.0
Diethyl maleate	141-05-9	17.7	36.0	14.9
Diethyl succinate	123-25-1	53.1	118	55.3
Ethyl (2E)-4-oxo-2-butenoate	2960-66-9	52.6	120	58.1
Monoethyl maleate	3052-50-4	736	1.72E+03	866
Monoethyl succinate	1070-34-4	1.27E+03	3.06E+03	1.62E+03
Succinic acid	110-15-6	2.89E+05	1.27E+05	3.31E+04
Ethyl methyl succinate	627-73-6	94.5	221	112
Acetamiprid	135410-20-7	16.6	8.11	4.34
6-Chloronicotinic acid	5326-23-8	21.1	12.8	n/a
Imidacloprid	138261-41-3	5.13	2.81	50.7
Hydroxyl-imidacloprid	380912-09-4	11.6	0.336	10.2
6-Chloronicotinic acid	5326-23-8	21.1	12.8	n/a

*Toxicity classes were highlighted with colors: red: LC50/EC50 ≤ 1, very toxic; orange: 1 < LC50/EC50 ≤ 10, toxic; blue: 10 < LC50/EC50 ≤ 100, harmful; green: LC50/EC50 > 100, not harmful.
n/a: not available.

4. Conclusions

This study demonstrates that *T. versicolor* is capable of degrading, at least to some extent, medium to highly polar pesticides like malathion, acetamiprid, and imidacloprid. The removal percentages obtained in air-pulsed bioreactors in the case of neonicotinoids and Erlenmeyer flasks in the case of malathion varied from 20% (acetamiprid) to 100% (malathion). Enzymatic exploration studies revealed that the cytochrome P450 is substantially involved in the degradation of acetamiprid and imidacloprid by *T. versicolor*. According to the main TPs identified in the solution, both reduction and hydrolyzation

played relevant roles in malathion detoxification, whereas oxidative cleavage represented a trigger reaction in the depletion of neonicotinoid insecticides. The presence of TPs in the solution indicates that pesticides were not mineralized during the process. Therefore, future research involving *T. versicolor* should seek for strategies leading to the mineralization of these compounds to avoid the potential toxicity of treated waters.

In the specific case of the pesticides investigated in this study, the Microtox test indicated a slight increase in the toxicity of the treated solutions. However, the ECOSAR-predicted toxicity of the TPs identified to aquatic organisms (daphnia, fish, and green algae) was overall lower than that of the corresponding parent compound. Such difference could be attributed to the formation of highly toxic unidentified minor TPs or mixture toxicity effects. However, since the overall toxicity of the solution is below the established toxicity threshold for industrial effluents, treatment with *T. versicolor* can be a valuable tool for bioremediation of waters contaminated with medium to highly polar pesticides such as those investigated in the present study.

Acknowledgments

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Author contributions

Kaidi Hu: Writing, original draft. Investigation. Formal analysis. Visualization

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Conflict of interest

We declare that no conflict of interest exists in the submission of this manuscript.

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Supporting information

Degradation of selected medium to highly polar pesticides by the white-rot fungus *Trametes versicolor*

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Number of figures: 2

Table of Contents

Text 1. UPLC-ESI-HRMS analyses

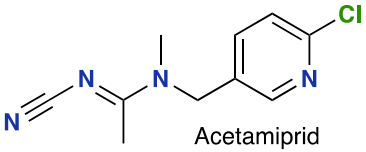
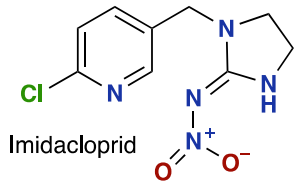
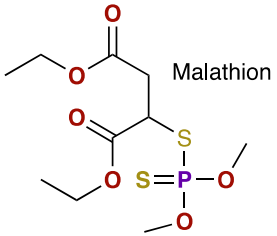
An ultra-high performance liquid chromatography (UPLC) system (Acquity, Waters, Milford, MA, USA) coupled to a high-resolution mass spectrometer (HRMS) (a hybrid quadrupole-Orbitrap analyser Q Exactive, Thermo Fisher Scientific, San Jose, CA, USA) was used to identify the biodegradation products formed during the fungal degradation process and malathion abatement.

Chromatographic separation of 10 µL of the sample extract was performed with a Purospher® STAR RP-18 endcapped Hibar® HR (150 × 2.1 mm, 2 µm) column (Merck, Darmstadt, Germany) and a linear organic gradient. Two chromatographic runs were performed to analyse independently the samples under the electrospray positive ionization (ESI+) and negative ionization (ESI-) modes. The mobile phase used for ESI+ analyses consisted of water with and methanol, both with 0.1% of formic acid at a flow rate of 0.2 mL/min. In the ESI- analyses, a mobile phase consisting of water and acetonitrile at a flow rate of 0.3 mL/min was used. The linear organic gradient used was as follows: 5% for 1 min, increased to 20% in 2 min, to 80% in 4 min, and to 100% in 1 more min. Pure organic conditions were maintained for 2 min and then decreased to the initial conditions (5%) in 0.5 min, held for 4.5 min for column re-equilibration.

The ESI interface was operated using the following specific conditions: spray voltage, 3.0 kV; sheath gas and auxiliary gas flow rates, 40 and 10 arbitrary units, respectively; capillary and vaporizer temperatures, 350 ºC and 400ºC, respectively. HRMS acquisition was conducted in data-dependent acquisition (DDA) mode. For this, a full scan over the m/z range 70 to 1,000 with a full width at half maximum (FWHM) resolution of 70,000 (at m/z 200) was done, and data-dependent MS/MS scan events (FWHM resolution of 17,500 at m/z 200) using a 40% normalized collision energy were recorded for the most intense ions detected in each scan (with an Automatic Gain Control (AGC) target of 10e5 or a maximum IT of 50 ms). Data acquisition was controlled by Xcalibur 2.2 software (Thermo Fisher Scientific).

Malathion was analysed in the positive ionization mode and quantified using the full scan response of its molecular ion [M+H]+. The quantification of malathion residual concentrations in the samples was performed by the internal standard method using its deuterated analogue (malthion-d10), and a 6-point solvent-based calibration curve.

Table S1. Structure and physical-chemical properties of the investigated pesticides.

Analyte	MW (g/mol)	Molecular formula	CAS number	IUPAC name	Solubility[α]	K_{oc}[α]	K_{ow}[α]	GUS[α]	Henry's law constant[α]	DT50[α]
 Acetamiprid	222.67	$C_{10}H_{11}ClN_4$	135410-20-7	*N*-[(6-chloro-pyridin-3-yl) methyl]-*N*'-cyano-*N*-methyl-ethanimidamide	2950	200	0.80	0.40	5.30 X 10^{-08}	1.6
 Imidacloprid	255.66	$C_9H_{10}ClN_5O_2$	138261-41-3	(*NE*)-*N*-[1-[(6-chloropyridin-3-yl)methyl]imidazolidin-2-ylidene]nitramide	610	262[β]	0.57	3.69	1.7 X 10^{-10}	191
 Malathion	330.36	$C_{10}H_{19}O_6PS_2$	121-75-5	diethyl 2-dimethoxyphosphinothioylsulfanylbutanedioate	148	1800	2.75	0.00	1.00 X 10^{-03}	0.17

[α] The PPDB, Pesticide Properties Database, http://sitem.herts.ac.uk/aeru/footprint/index2.htm. - Lewis, K.A., Tzilivakis, J., Warner, D. and Green, A. (2016). An international database for pesticide risk assessments and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064.

[β] Kegley, S.E., Hill, B.R., Orme S., Choi A.H., PAN Pesticide Database, Pesticide Action Network, North America (Oakland, CA, 2016), http://www.pesticideinfo.org.

MW: molecular weight; Solubility: solubility in water at 20 °C; K_{oc}: organic carbon partition coefficient; K_{ow}: octanol-water partition coefficient; GUS: leaching potential index; Henry's law constant: Henry's law constant at 25 °C (Pa m^3 mol^{-1}); DT50: soil degradation potential, expressed as *half-life* in days.

Table S2. Toxicity classification according to the Globally Harmonized System of Classification and Labelling of Chemicals.

Toxicity range (mg/L)	Interpretation
LC50/EC50 [a] ≤ 1	**Very toxic**
1 < LC50/EC50 ≤ 10	Toxic
10 < LC50/EC50 ≤ 100	Harmful
LC50/EC50 > 100	Not harmful

[a] LC50, 50% lethal concentration; EC50, 50% effective concentration.

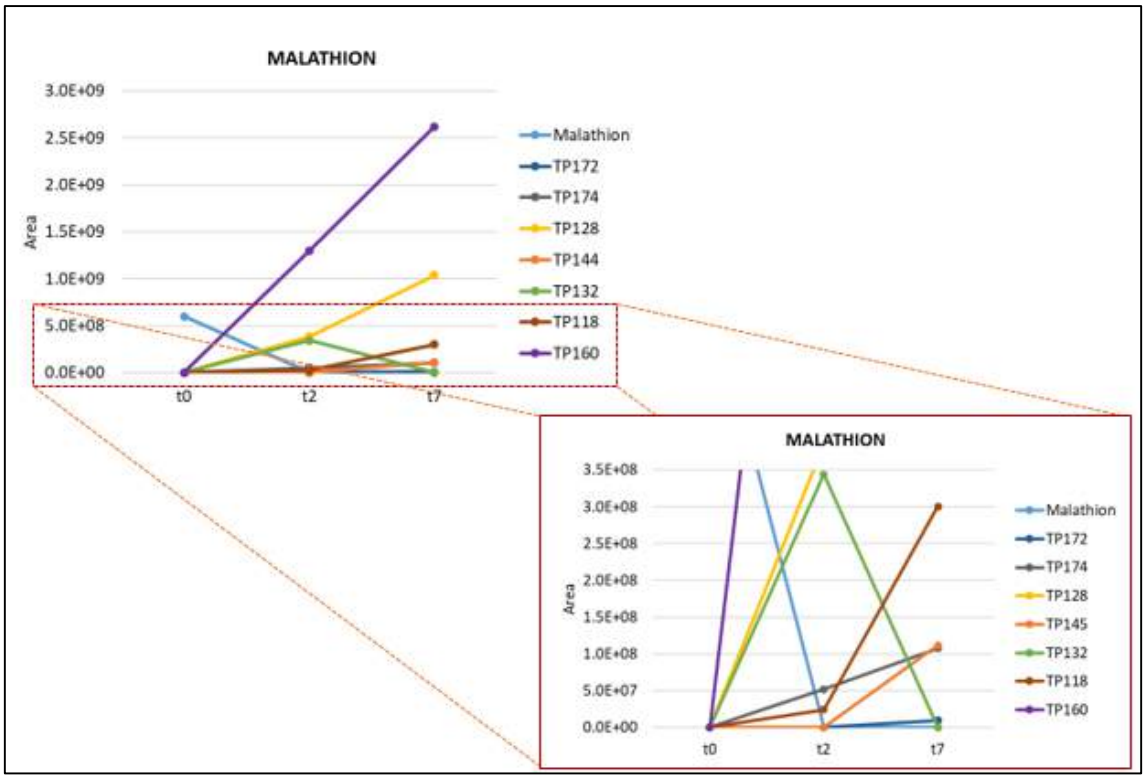


Figure S1. Evolution of the TPs identified during *T.versicolor*-mediated degradation of malathion.

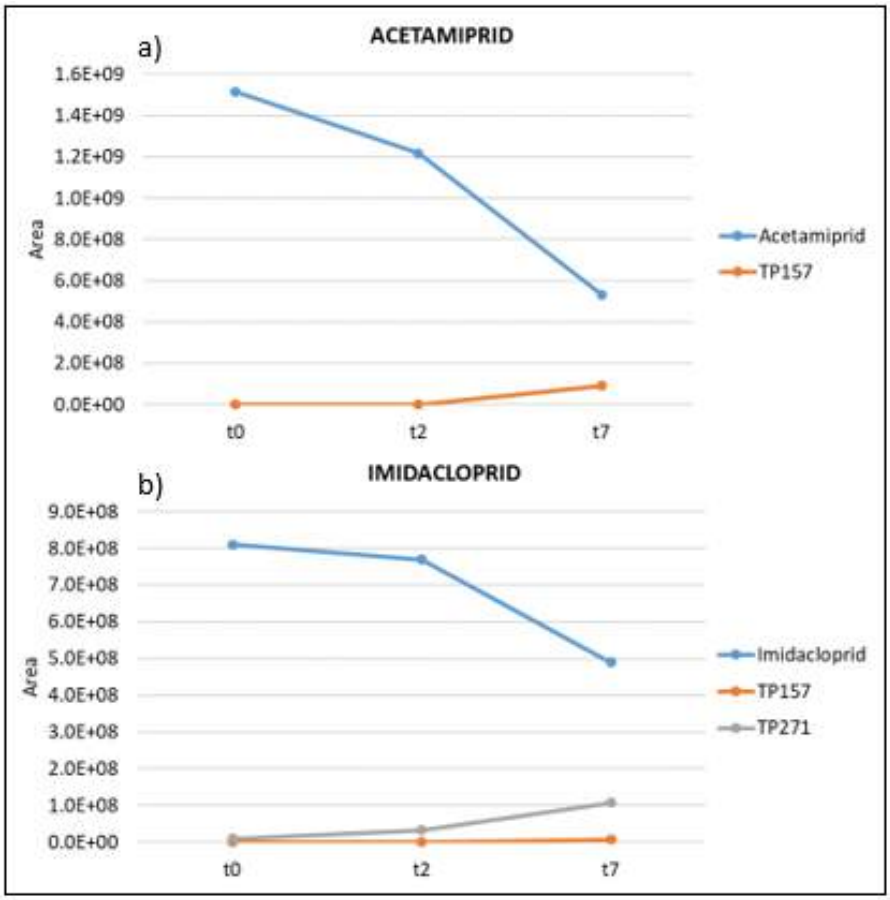


Figure S2. Evolution of the TPs identified during *T.versicolor*-mediated degradation of a) acetamiprid and b) imidacloprid.

CHAPTER 4 - DISCUSSION

CHAPTER 4 - DISCUSSION

4.1 Analytical methodologies

In this thesis, different analytical methodologies were developed and validated, for the determination of pesticides in environmental waters, sediment and biota. As part of the validation process, they were applied to study the occurrence and fate of the target pesticides in different agricultural areas, and to evaluate the associated environmental risk and bioremediation technologies for their attenuation. All analytical methods target the same pesticides, except in the case of chlorpyrifos and oxadiazon that were analyzed in water and biota but not in sediment, and fenthion that was analyzed in sediment and biota, but not in water.

Since the matrices investigated are very different, specific extraction techniques were applied in each case. For the analysis of pesticides in surface water and groundwater, the on-line approach was selected since it allows the automatization of the sample extraction process, and hence high sample throughput. Additional advantages of on-line approaches in comparison with off-line SPE methods include the elimination of analyte losses by evaporation or by degradation during the extract preconcentration step, and the improvement of the accuracy (recovery) and precision (repeatability) of the results. In the case of sediments, analyte extraction was conducted using PLE, and subsequent purification of the extract to remove interferences by means of off-line SPE. Although an intensive-labour process was needed to recover pesticides from this matrix, the automatization of the first part of the extraction process reduced significantly the total analysis time. As for biota (fish muscle), a fast and simple QuEChERS protocol was applied.

As expected, the analytical performance of the developed methodologies in terms of linearity, accuracy, precision, sensitivity, and matrix effects for the different target pesticides varied among matrices. Regarding accuracy, average relative recoveries obtained with the three methodologies were always between 80% and 120%, except on a few occasions that slightly deviated from this range. The results obtained are in agreement with the analytical acceptability parameters indicated in the SANTE/12682/2019 document, which describes analytical quality control and method validation procedures for pesticide residues analysis in food and feed (EC, 2019). According to these specifications,

when the isotopic dilution method is not used, the absolute recovery (AR) of each analyte should preferably remain above 50%; however, when using ILIS, AR below 50% are acceptable, provided that:

- the relative recovery (RR) of the analytes as a function of the recovery of the corresponding ILIS is between 80 and 120%;

- the repeatability, calculated as the relative standard deviation (RSD) of replicate measurements of RR, is good (<20%);

- LODets are low enough to allow detection of analytes at acceptable levels.

Concerning the calibration curves, quantification by the internal standard method allows working intervals greater than four orders of magnitudes for most compounds without losing quality (r^2> 0.99), since the use of ILIS allows the correction of the signal variation of the target analytes, common in MS analysis. In all cases, least-squares linear regression models were constructed. $1/x^2$ was used as a weighting factor to reduce the influence of the high concentration data points in the model. On the other hand, while for the analysis of sediments and biota, solvent-based calibration curves were used, the calibration solutions used for the analysis of water consisted of HPLC-grade water fortified at different levels with the target analytes and processed in the same way as samples.

As for the matrix effect, ion suppression was observed in most cases. Figure 4.1 shows a comparison of the matrix effects caused by the different studied matrices (surface water, groundwater, sediment, and biota).

As shown, matrix effects (60% of cases) mainly occurred in the form of signal ionization suppression (matrix effect < 20%) caused by co-eluting matrix components, while only in 3% of cases, they occurred in the form of signal enhancement (matrix effect > 20%). It should be noticed that the effects caused by the matrix are more evident in the case of water or biota than in sediment. In this matrix, no significant interferences in the ionization of all the target pesticides were indeed observed (±20% variation of the signal). This is due to the high selectivity of the extraction and purification processes performed, which in fact include the performance of two purification processes: one inside the PLE cell through the addition of activated alumina, and another one afterwards via SPE of the PLE extract.

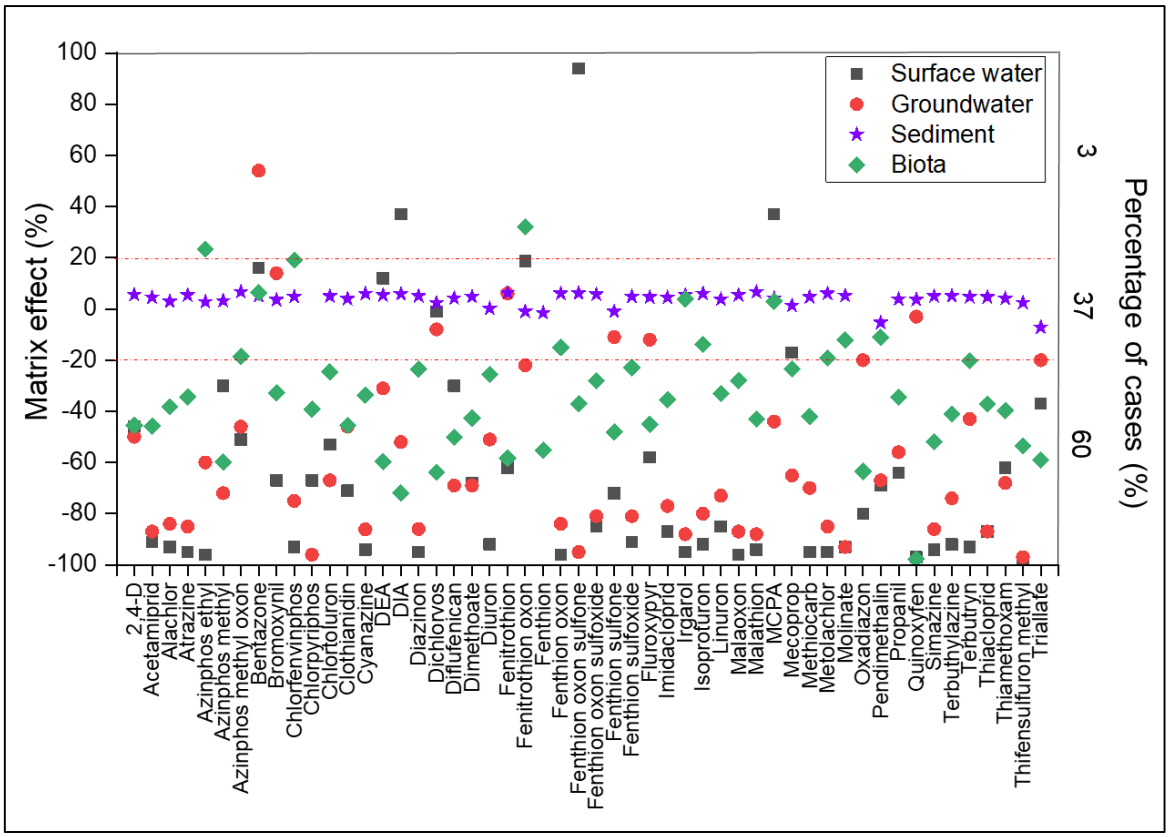


Figure 4.1. Matrix effects found in the analysis of the target pesticides in surface water, groundwater, sediment and biota.

In the case of water, it has been reported that on-line SPE may lead to higher matrix effects than off-line SPE methods due to the lower flexibility existing in the selection of the solvents to be used in the SPE process, that in online systems must be compatible with the LC mobile phase (Rodriguez-Mozaz et al., 2007). As for biota, the presence of matrix effects could be attributed to the high amount of organic matter in this matrix and a low selectivity of the fast QuEChERS method to remove it.

As shown in Figure 4.1, matrix effects depend also on the analyte. The heterogeneity of the matrix effects, which are a function of the matrix and/or the analyte, makes very difficult to find analytical methods capable of correcting the effects for all target compounds in multi-residue methodologies like the ones developed in this doctoral thesis. Solutions proposed to overcome the problem of matrix effects in the analysis of pesticides by LC-based methods include the reduction of the sample volume (Sancho et al., 2004), or the use of HILIC, which allows highly polar analytes to elute away from the LC solvent front (Richardson and Ternes, 2005). For many authors, reliable quantification has been obtained

using internal standards (Blanchoud et al., 2020; Herrera López et al., 2019; Huertas Pérez et al., 2015; Yu et al., 2019), and, preferably, the use of the ILIS analogue for each analyte (as they are expected to behave equally during the analysis), although this is not always feasible due to the high cost of ILIS and the lack of availability of many of them, especially for TPs. It is important to note that the use of ILIS, in contrast to matrix-matched calibration, permits to correct matrix effects regardless of their variability between samples, which greatly improves the accuracy and precision of the results obtained. The use of ILIS also helps to correct the slight variations in the retention time of the analytes in the samples that may also occur due to the matrix.

Regarding sensitivity, Figures 4.2 and 4.3 show the method LOD and LODet for the target pesticides in each of the five matrices under study: surface water, groundwater, HPLC-grade water, sediment and biota.

The limit of detection of each analyte depends on the signal intensity of the first transition (SRM1) and the effects caused on its ionization by the matrix components present in the extract. LODs of the target pesticides in the three water matrices investigated were comprised between 0.042 (diazinon) and 28 (dichlorvos) ng/L, except in a few cases (4%) where they raised up to 190 ng/L (pendimethalin). For most analytes, LODs were below 1 ng/L (34%) and between 1 and 10 ng/L (50.7%) (Figure 4.2a). As expected, HPLC-grade water presented the lowest LODs (up to 29 ng/L). The higher dispersion of the LODs in the case of surface water and groundwater indicates the degree of vulnerability of the analytes to matrix effects. For example, terbutryn with a LOD range that expands from 0.16 to 0.39 ng/L (0.23 units), shows less vulnerability to matrix effects than dimethoate, with a LOD range from 0.76 to 180 ng/L (179 units). The LODets (Figure 4.2b) of the target analytes followed a dispersion similar to that observed for their LODs, although at higher levels. All LODets for pesticides in water were below 100 ng/L, except in a few cases (4%), and most of them were between 1 and 10 ng/L (49.3%).

The availability of reliable analytical methodologies with sufficient selectivity and sensitivity is necessary to generate data that can contribute to current and future regulations.

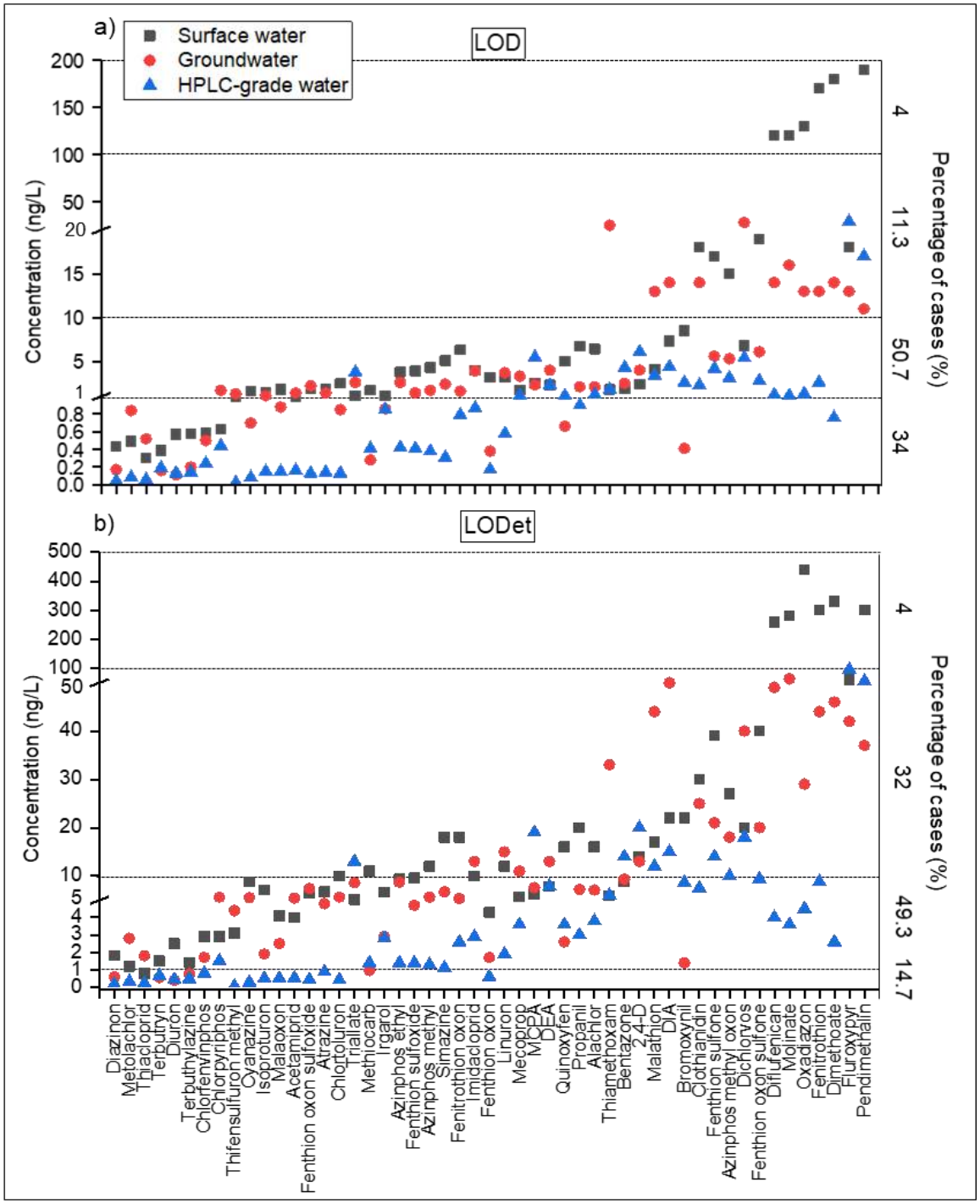


Figure 4.2. LODs (a) and LODets (b) of the target pesticides in surface water, groundwater, and HPLC-grade water.

Overall, the methodology developed for the determination of pesticides in environmental waters was able to comply with the monitoring and analytical demands established for these compounds in the European legislation, reaching the EQS for priority pesticides (EC, 2013), the LODs established in the European Watch List (EC, 2018), and the

quality standard of 100 ng/L for individual pesticides set for the protection of groundwater (EC, 2006a) and in water intended for human consumption (EC, 1998a). As already shown in Table 3 of the scientific publication #1, elaborated to compare the method developed with other fully automated methodologies available at the moment for the analysis of pesticides in water samples (Camilleri et al., 2015; Hurtado-Sánchez et al., 2013; Mann et al., 2016; Quintana et al., 2019; Rubirola et al., 2017; Singer et al., 2010), the method developed during this doctoral thesis shows similar or better sensitivity than the reviewed methods, with lower LODs and LODets for the analysis of azinphos-methyl, bromoxynil, clothianidin, quinoxyfen, terbuthylazine, terbutryn, and thifensulfuron methyl, and introduced for the first time validation results for 10 pesticides and TPs (azinphos ethyl, azinphos-methyl oxon, dichlorvos, diflufenican, fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, fenthion sulfone, fenthion sulfoxide, and oxadiazon) in surface and groundwater. Compared to off-line SPE methodologies where only an aliquot of the extract is transferred into the analytical system, on-line SPE procedures allows achieving lower LOD due to the injection and analysis of the whole sample.

In the case of sediment and biota, the LODs and LODets obtained showed similarities in both matrices for the analyzed compounds (Figure 4.3), as in the case of irgarol, which presented the lowest LOD in both matrices (0.01 ng/g), or pendimethalin, resulting to be the analyte with the highest LOD in both sediment (100 ng/g d.w.) and biota (50 ng/g f.w.).

Overall, very high sensitivity in sediment and biota was obtained, with LODs lower than 1 ng/g for the majority of compounds (69.8%) (Figure 4.3a), and LODets below 10 ng/g for 80.4% of cases (Figure 4.3b), which is the MRL set by the EU (EC, 2005) in food and feed of plant and animal origin for compounds not specifically mentioned in the regulation. In the case of sediment, with no legislation establishing maximum pesticide residues, a sensitivity threshold of 50 ng/g may be considered satisfactory, as this is the value set for the analysis of pesticide residues in soil (EC, 2010).

The highest LODs and LODets were associated with low-polarity (Log K_{ow} > 3) compounds, hardly amenable to LC-MS/MS analysis, in particular, chlorpyrifos, fenthion, oxadiazon, pendimethalin, and triallate (Log K_{ow} > 4). In these cases, better sensitivity could be achieved employing GC-MS/MS (Andreu and Picó, 2004; Pintado-Herrera et al., 2016).

Pendimethalin, together with fluroxypyr, were the pesticides showing the worst sensitivity also in the method developed for the analysis of environmental waters.

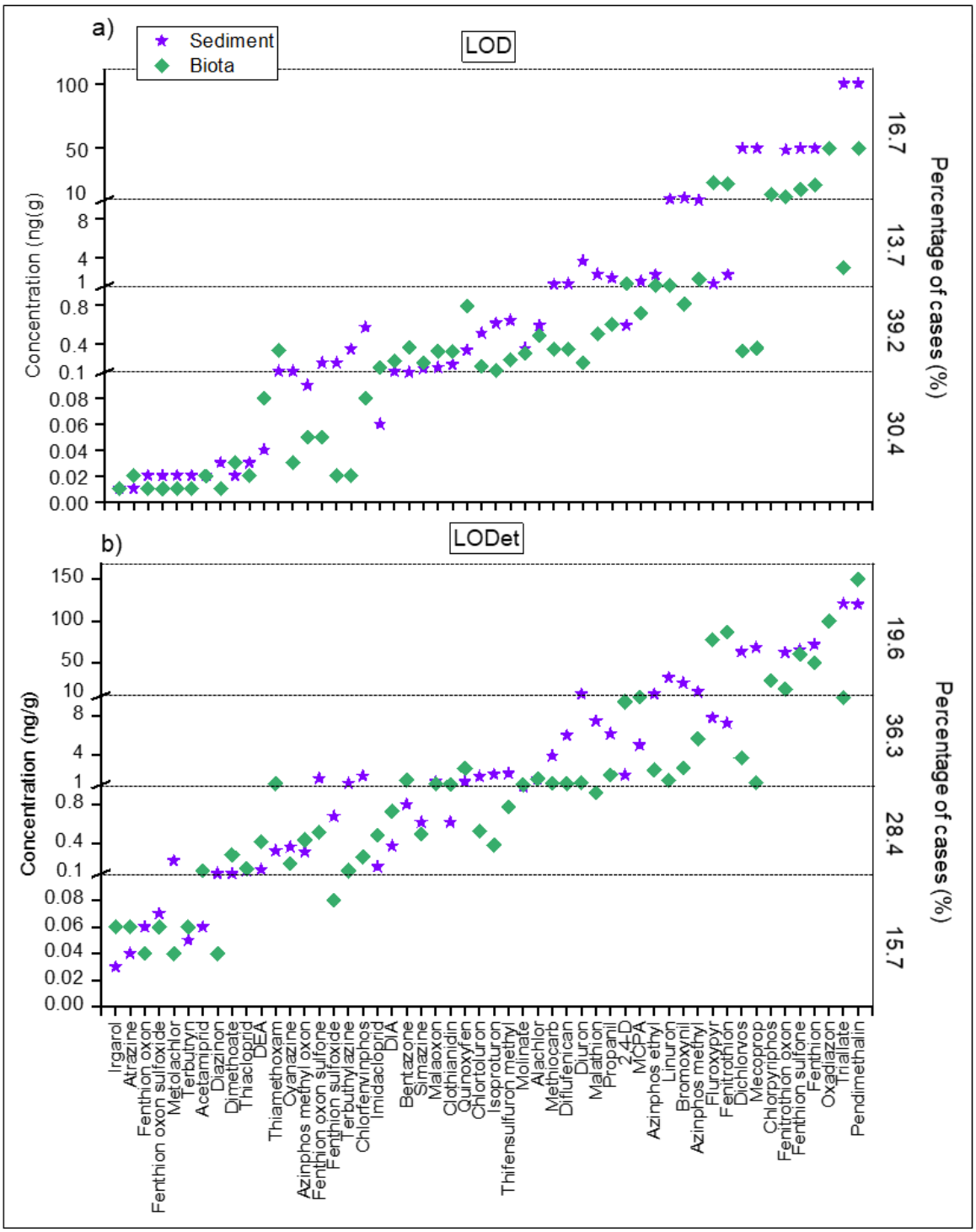


Figure 4.3. LODs (a) and LODets (b) of the target pesticides in sediment (ng/g d.w.) and biota (ng/g f.w.).

4.2 Environmental occurrence and fate of medium to highly polar pesticides in the investigated matrices and compliance with regulations

In chapter 3, the presence of pesticides in different environmental matrices is evaluated in separate scientific publications on the basis of their physical-chemical characteristics, and considering their transport and fate within the different compartments analyzed and the activities carried out in each study area. Moreover, the study of the presence of pesticides in areas impacted by different stressors such as agricultural, urban or industrial activities, has made possible to trace a general contamination profile in the aquatic ecosystem. Table 4.1 shows a qualitative summary of the detection frequencies and concentrations of the target pesticides in the various investigated matrices and study areas.

As it can be observed, and could be expected from the polar nature of the majority of the target pesticides, the highest average concentrations and diversity of pesticides were found in water samples, especially in those from the Ebro Delta and Llobregat River as compared to the Ter River. These three rivers are located in Catalonia (NE Spain) and are affected by different environmental stressors, which result in a diverse presence and distribution of pesticides based on the anthropogenic pressures prevailing in the area.

Bentazone, MCPA and propanil were the only three pesticides showing very high average concentration (> 1000 ng/L) in the Ebro River Delta. Their presence is strongly related to their widespread use in the area, being herbicides extensively applied in rice cultivation, the main crop in the Ebro Delta (80% of agricultural land), and to their moderately-high water solubility (> 50 mg/L) and relatively low octanol-water partition coefficient (Log K_{ow} < 3) (see Table 1.5). Because of this, these pesticides were also among the pesticides that presented the highest frequencies of detection in the area, indicating a diffuse pollution pattern. The occurrence of these pesticides in the Ebro Delta was already reported in previous studies (Köck et al., 2010; Kuster et al., 2008), which confirm their continuous release into this ecosystem.

Table 4.1. Qualitative comparison of the presence (average concentration and detection frequency) of the pesticides found in the investigated matrices and study areas.

Pesticides	Average concentration [α]					Frequency of detection [β]				
	Water Ebro	Water Llobregat	Water Ter	Sediment Llobregat	Biota Adige	Water Ebro	Water Llobregat	Water Ter	Sediment Llobregat	Biota Adige
2,4-D										
4,4'-DDD*										
Acetamiprid										
Alachlor										
Atrazine										
Azinphos ethyl										
Bentazone										
Bromoxynil										
Chlorfenviphos										
Chlorpyrifos										
Chlortoluron										
Cyanazine										
Diazinon										
Dichlorvos										
Dicofol*										
Diflufenican										
Diuron										
Fenthion oxon										
Fenthion oxon sulfoxide										
Fenthion sulfoxide										
Imidacloprid										
Irgarol										
Isoproturon										
Linuron										
Malaoxon										
Malathion										
MCPA										
Methiocarb										
Metolachlor										
Molinate										
Oxadiazon										
Pendimethalin										
Propanil										
Quinoxyfen										
Simazine										
Terbuthylazine										
Terbutryn										
Thiacloprid										
Triadimefon*										
Triallate										

Not analyzed
Not detected
Low frequency, ≤ 25 %
Medium frequency, >25% and ≤ 50 %
High frequency, >50% and ≤ 75 %
Very high frequency, >75% and ≤ 100 %
Low concentration, ≤ 10 ng/L or ≤ 1 ng/g
Medium concentration, >10 and ≤ 100 ng/L or >1 and ≤ 20 ng/g
High concentration, >100 and ≤ 1000 ng/L or >20 and ≤ 50 ng/g
Very high concentration, > 1000 ng/L or > 50 ng/g

[α] Average concentration calculated considering values <LOQ as LOQ/2 and values <LOD as zero.
[β] % of positive samples, including compounds with values <LOQ.
* Compounds analyzed with a GC-MS/MS methodology (Peris and Eljarrat, 2020).

Bentazone and MCPA were also found in the Ter River, together with other five compounds, although at relatively low average concentrations (< 10 ng/L). The Ter River area is characterized by low population density, the presence of some industries (metallurgic, pulp mill, textile, and tannery) and intensive agricultural activities, which include rice fields and other crops (e.g. corn, alfalfa, and apple trees).

Contrariwise, they have not been detected in the Llobregat River, where agriculture is mainly characterized by the cultivation of horticulture, vegetables and fruit trees (*e.g.*, artichoke, cucumber, tomato, and Brassica species) typically spread over multiple small extension properties, and other anthropogenic pressures, such as industrial and urban activities have been also identified to affect the quality of the surface water resources (Postigo et al., 2021). The lower basin of the Llobregat River, which corresponds to the investigated area, is located southern to the Barcelona Metropolitan area, and is affected by large infrastructures (*i.e*., highways, roads, railways, Barcelona harbor and airport) and is highly impacted by the presence of 30 WWTP effluent discharges along the upstream river course. Bromoxynil and diuron were the most ubiquitous herbicides in the Llobregat River, found at the highest average concentration (150 and 172 ng/L, respectively). Both are herbicides used to control a wide range of annual broadleaved weeds, either on crop or non-crop land. While bromoxynil is still used as a plant protection product in the area, diuron is not currently authorized for use in agriculture; therefore, its finding must be linked to different urban and/or industrial purposes. Moreover, the presence of diuron has been previously reported in the Llobregat River waters at similarly high concentrations (Köck-Schulmeyer et al., 2012; Masiá et al., 2015a).

It is interesting to observe the comparison of these findings with the results obtained from the analysis of pesticides in the Llobregat River sediments, which were collected during the same sampling campaign and belong to the same sampling sites of surface waters. The sediment contamination profile is marked by the presence of fewer contaminants as compared to water (5 pesticides in sediments *vs.* 28 in waters). However, pesticides detected in sediments were found to be widely distributed, with terbutryn being detected in all the samples analyzed, and diazinon and irgarol being present in more than half of the samples. Looking at their physical-chemical properties (see Table 1.5), these compounds present a high probability to be found in sediments, due to their relatively high

Log K_{ow} (> 3), a good indicator of the tendency of an organic compound to adsorb onto soil and/or bioaccumulate in living organisms. Moreover, their high soil sorption coefficient (K_{oc} > 500), and low-moderate solubility (< 60 mg/L) confer these pesticides low mobility and consequently, they are most likely to be found in the river sediment than in the water phase. Even if at low-medium average concentrations, terbutryn, diazinon and irgarol were also detected in the Llobregat water, despite being currently banned in Europe, suggesting their possible release after desorption from sediment where they may have accumulated during past applications. In fact, the insecticide diazinon and the herbicide terbutryn, both used on crop sites and for urban purposes, were already reported in a previous study in both Llobregat waters and sediments (Masiá et al., 2015a), confirming this hypothesis and pointing out the different anthropogenic pressures from which their presence can originate. On the contrary, the presence of irgarol, an industrial herbicidal biocide used as an antifouling agent in paints for boats and other water vessels, was also previously detected in the water of a tributary of the Llobregat River (Quintana et al., 2019); however, it had never been investigated in the Llobregat sediments before.

Similarly to sediments, the occurrence of medium to highly polar pesticides in biota (fish muscle) was very rare. Only eight compounds were identified at low average concentrations and detection frequencies, except in the case of the fungicide quinoxyfen and the herbicide metolachlor, found in almost all the analyzed samples (> 75%) (Table 4.1). As discussed for sediments, the compounds detected in biota are used for both agricultural and urban/industrial uses, and they all, except acetamiprid, present high bioaccumulation potential (Log K_{ow} > 3), which can explain their finding in the analyzed fish samples. The Adige River basin (Italy) is an important area continuously subjected to different stressors originated by agriculture, in particular intensive apple tree cultivation, and industrial and urban activities, with the main associated stressors being hydropeaking for hydroelectricity production and wastewater effluents, respectively. It is interesting to notice that most pesticides in fish samples were found in the benthic species grayling (*Thymallus thymallus spp.*) and bullhead (*cottus gobio spp.*), both living and feeding at the river bottom (sediments) where they may find a source of pesticides. This finding points out the persistence of some of the target pesticides in aquatic ecosystems and the constant exposure of fish to them, which leads to their bioaccumulation.

As far as transformation products is concerned, malaoxon (TP of malathion), was detected in the Ebro Delta and the Llobregat River waters at low concentrations, while malathion was found only in the waters of the Llobregat River at low levels. Malaoxon is a metabolite 60 times more toxic than malathion (Jensen and Whatling, 2010), which makes its formation in the water compartment worrying. Fenthion oxon and fenthion sulfoxide were also detected in the Ebro Delta and the Llobregat River, and fenthion oxon sulfoxide was only detected in the Ebro Delta water, while their parent compound fenthion was not detected in any of the investigated samples. Considering that fenthion is currently banned, these findings suggest a possible degradation of the parent compound and the formation of its TPs after leaching from sediments where it may have accumulated during its use in the past.

Considering all samples and matrices investigated, diazinon, irgarol and terbutryn were found in all the studies and in some of them, they were among the most widespread compounds. As aforementioned, they present hydrophobic characteristics and hence, they are more likely to accumulate in sediment or bioaccumulate in aquatic organisms that to dissolve in the aqueous phase. Nevertheless, water pollution by these contaminants can also occur as a result of soil leaching, desorption from soils and sediments or other biological or chemical degradation mechanisms, thus making these pesticides, prohibited due to their harmful consequences in the environment, always available in the aqueous compartment.

Taking into consideration the presence of pesticides in relation to the current EU regulation, Figure 4.4 shows the percentage of cases of pesticides surpassing the limits established in the European legislation. The only compounds that surpassed their EQS in surface waters (EC, 2013) were dichlorvos and irgarol. In the Llobregat surface waters, dichlorvos and irgarol were detected at concentrations above the EQS in 45% and 18% of the cases, respectively, while in the Ebro Delta waters, irgarol was found to exceed its EQS in 72% of the samples.

The presence of dichlorvos and irgarol was also found in the sediments collected in the Llobregat River; however, since pesticides in sediment are not yet regulated, the EQS values set for surface waters were used as reference to get a general idea of their contamination. In this regard, dichlorvos concentrations (ng/g) exceeded in 14% of cases

the EQS value of 0.7 (ng/L) set for its presence in surface water, while irgarol concentrations surpassed the EQS of 16 (ng/L) in 43% of cases, representing a possible environmental risk due to further release from sediment after desorption or sediment resuspension. The insecticide dichlorvos, used in a range of crops and for non-agricultural purposes, is currently not approved for use in the EU, considering that it has a high tendency to bioaccumulate and it is highly toxic to mammals and honeybees, posing in consequence negative effects in the overall ecosystem biodiversity. Similarly, the presence of terbutryn in sediments is of concern, since it was found in 100% of the sediment samples at concentrations above its EQS of 340 ng/L in water. Its use, like that of dichlorvos and irgarol, is currently not allowed. Thus, the presence of these three pesticides in sediments confirms this environmental compartment as a source of these substances in water.

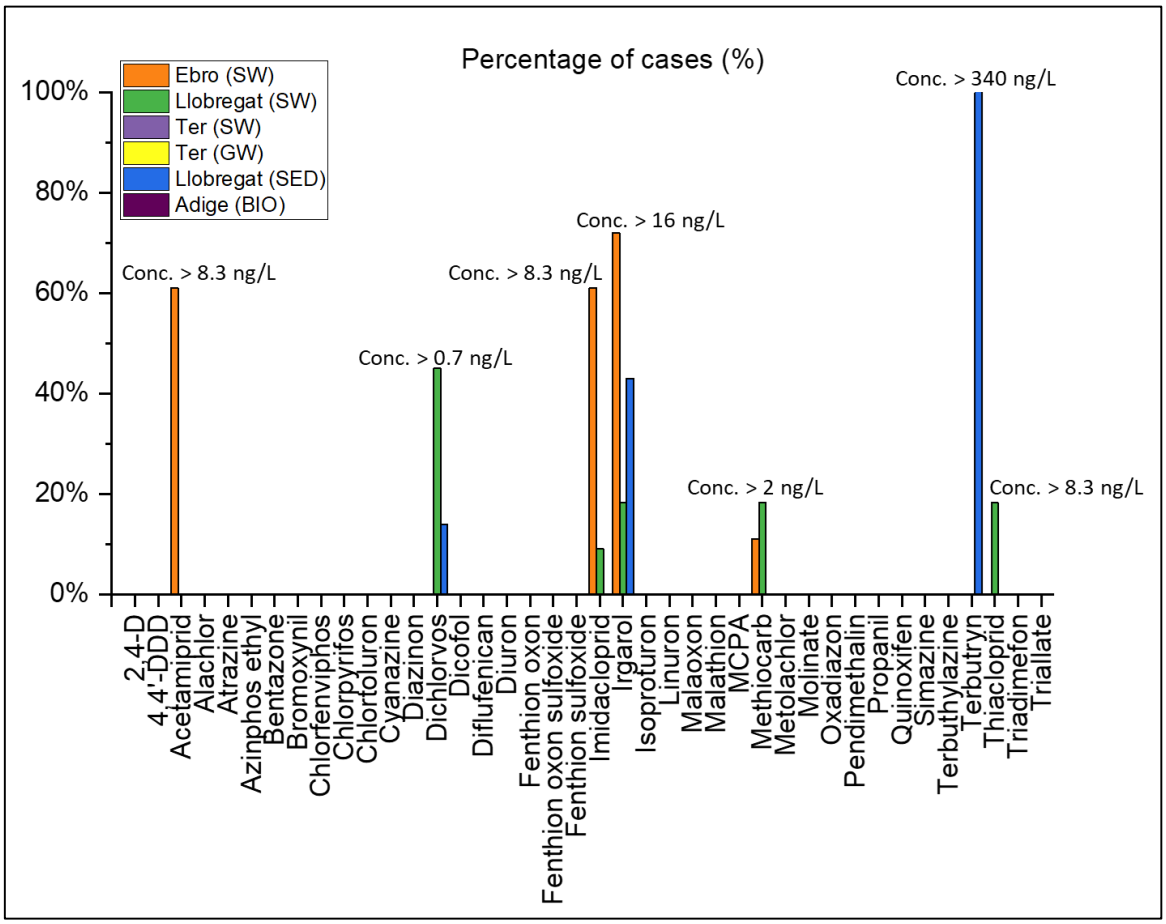


Figure 4.4. Summary of the percentage of cases (%) of pesticides exceeding their quality standards (EC, 2013, 2006a), maximum acceptable LODs (EC, 2018) or MRLs (EC, 2005) in each of the matrix under study.

As for the presence of pesticides included in the Watch List of substances for Union-wide monitoring (EC, 2018) and currently used in the European context, methiocarb and the neonicotinoids acetamiprid, imidacloprid, and thiacloprid were detected at levels above their maximum acceptable LOD in the Llobregat River and/or the Ebro Delta surface waters (Figure 4.4). This is a relevant finding, since the substances in the Watch List are selected from amongst those considered of high risk to or via the aquatic environment, but for which monitoring data are insufficient. In particular, neonicotinoids are highly toxic to many invertebrates, including beneficial insects such as bees, affecting their nervous system and causing sublethal and, occasionally, lethal effects on these organisms (Blacquière et al., 2012; Mitchell et al., 2017). Therefore, these results contribute to increase knowledge about their occurrence in the environment and point out their high use at European level and the need for their continuous monitoring and control.

The quality standards of 100 ng/L for individual pesticides and 500 ng/L for the sum of pesticides (EC, 2006a) were not exceeded in groundwater samples, collected from the Ter River. However, as the same limits are set for the presence of pesticides in waters intended for human consumption (EC, 1998a), high concentrations above these limits may also be of concern in surface water used to produce drinking water. As we saw in section 3.1, the high concentration of bentazone in the Ter River (119 ng/L), as well as the concentration above 100 ng/L in the case of 2,4-D, azinphos ethyl, bromoxynil, dichlorvos, diflufenican, diuron, imidacloprid, linuron, methiocarb and terbutryn and above 500 ng/L in four out of the 11 samples analyzed in the Llobregat River is worrying, since the two rivers serve as water resources (24% in the case of Ter River and 54% in the case of Llobregat River) to produce the drinking water supplied to over 4.5 million people in Barcelona and its metropolitan area (Quintana et al., 2019).

As observed in Figure 4.4, in the case of biota none of the analyzed pesticides surpassed the general MRL of 10 ng/g established as the highest pesticide level legally tolerated after their correct application in food products (EC, 2005). However, pesticide presence in fish should be regulated separately in future legislation, as it happens with other foods for which specific MRLs already exist, since fish can be continuously exposed to pesticides present in the aquatic environment and thus represent a risk to human health.

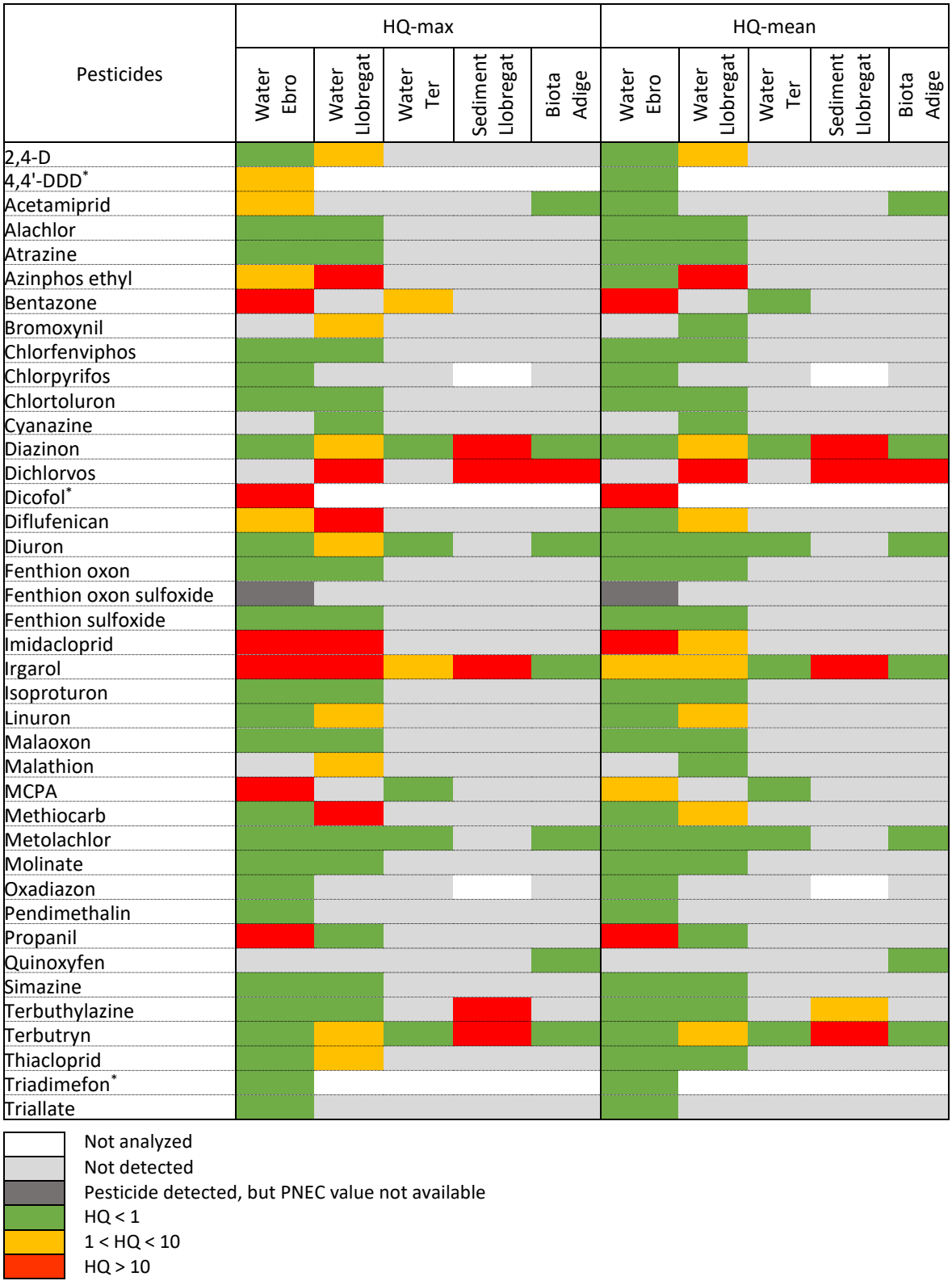
4.3 Environmental risk assessment: pesticides of highest concern

It should be noted that the interpretation of the monitoring results in section 4.2 was made considering to a large extent the highest concentrations found in the various studies and those pesticides for which there are sufficient toxicity data to establish EQS, as their exposure levels in past works indicated a potential environmental hazard. However, in many cases, the lack of information about the toxic properties of many other pesticides makes difficult to interpret their potential impact on the aquatic ecosystems. One way of assessing the environmental risk of the pesticide mixture detected in environmental samples is by applying the hazard quotient approach. This method allows to predict the potential risk of a specific compound on the basis of its measured environmental concentration (MEC) and lowest predicted concentration at which toxic effects are not expected (PNEC), using the equation: HQ = MEC/PNEC. In this thesis, the maximum concentration and the average concentration were selected as MEC to calculate the worst-case scenario (HQ-max) and the general risk (HQ-mean), respectively. The PNEC values for water and sediment samples were extracted from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/) (Dulio and Von der Ohe, 2013), while the PNEC value for biota was derived, as suggested by NORMAN, using the bioconcentration factor (BCF) approach according to the equation: $PNEC_{biota/fish} = PNEC_{water} * BCF$. HQ values below 1 indicate zero or low risk, while HQ values between 1 and 10 anticipate moderate risk, and HQ values above 10 suggest high environmental risk.

Table 4.2 illustrates a comparison between the HQ-max and HQ-mean of the measured pesticides in the analyzed matrices, while the PNEC values are shown in Table 4.3.

As shown, the majority of compounds presented a low risk (HQ <1) in all matrices in both the worst and the normal contamination scenarios, mostly due to their relatively low concentration. Nevertheless, 18% of the compounds exhibited high risk (HQ >10) as a result of their high concentration in the specific matrix/study, as it is the case for bentazone, MCPA and propanil in the Ebro Delta water, or terbutryn in the Llobregat River sediments, or their very low PNEC values.

Table 4.2. Quality comparison of the Hazard Quotient (HQ) for the worst-case (HQ-Max) and the normal (HQ-mean) contamination scenarios calculated for the detected pesticides in the investigated matrices.



*Compounds analyzed with a GC-MS/MS methodology (Peris and Eljarrat, 2020).

This was the case of azynphos ethyl, found in the Llobregat water at an average concentration of 0.017 µg/L and with a PNEC of 0.0011 µg/L, which resulted in high both HQ-max and HQ-mean values. Indeed, the low PNEC is correlated to the high toxicity of azynphos ethyl, an organophosphate insecticide highly toxic to mammals, birds, fish and aquatic invertebrates. Additional pesticides with very low PNECs, and thus, highly toxic to aquatic organisms are irgarol and dichlorvos, with PNEC values in freshwater of 0.035 µg/L and 0.0006 µg/L, respectively.

In the case of biota, high HQ values may originate from low PNEC values in water, low BCFs or both. In this way, dichlorvos was found in a fish sample at a concentration below its LODet (< 3.73 ng/g), but its very low PNEC in water (0.0006 µg/L) together with its low BCF (0.8 L/kg), resulted in a very high risk in both the normal and the worst-case scenarios. As already mentioned in section 4.2, dichlorvos is highly toxic to a variety of non-target organisms, including fish (Nan et al., 2013). These findings suggest that some pesticides, even if found at low levels in the environment, may present high toxicity and pose a risk for the ecosystem. In all the other cases, pesticides in biota exhibited low risk.

Overall, the waters of the Ebro Delta and the Llobregat River are those that present the highest number of high-risk pesticides (6 out of 35 and 28, respectively). In the case of the Llobregat sediments, all 5 compounds show moderate or high HQ, suggesting a high impact of these contaminants in sediment-dwelling organisms. However, we must consider that the $PNEC_{sed}$ collected in the NORMAN Database are calculated following the equation: $PNEC_{sed} = PNEC_{water} * 2.6 * (0.615+0.019*K_{oc})$, where the K_{oc} was given a value of zero to consider the most conservative scenario.

In this case, the HQ results that would be obtained considering the specific K_{oc} of each compound would certainly be lower. As an example, the PNEC of irgarol would be higher (0.28 ng/g instead of 0.006 ng/g) if calculated considering its high soil sorption coefficient (K_{oc} = 1569), which would result in an HQ value lower than 1 in both the normal and the worst-case scenarios, indicating a low risk for this compound in sediment. Similar conclusions would be drawn in the case of the other 4 pesticides found in sediment (see Table 4.4).

Table 4.3. PNEC values for the detected pesticides in the analyzed matrices.

Pesticides	$PNEC_{water}$ [a] (µg/L)	$PNEC_{sed}$ [b] (ng/g)	$PNEC_{sed}$ [c] (ng/g)	$PNEC_{biota/fish}$ [d] (ng/g)
2,4-D	0.02	0.032	0.07	0.2
4,4'-DDD	0.0005	0.001	3.24	25
Acetamiprid	3.74	5.98	43	11
Alachlor	0.3	0.48	5.44	12
Atrazine	0.6	0.96	3.92	2.58
Azinphos ethyl	0.0011	0.002	0.083	0.11
Bentazone	0.1	0.16	0.43	2.1
Bromoxynil	0.5	0.8	8.26	14
Chlorfenviphos	0.1	0.16	3.52	25
Chlorpyrifos	0.03	0.048	8.21	41
Chlortoluron	0.1	0.16	1.13	4
Cyanazine	0.19	0.3	2.09	30
Diazinon	0.01	0.016	0.32	5
Dichlorvos	0.0006	0.001	0.0024	0.0005
Dicofol	0.000032	0.0001	0.01	0.32
Diflufenican	0.009	0.014	2.46	11
Diuron	0.2	0.32	7.04	1.89
Fenthion oxon	0.2	0.32	0.88	n/a
Fenthion oxon sulfoxide	n/a	n/a	n/a	n/a
Fenthion sulfoxide	10	16	106	n/a
Imidacloprid	0.0083	0.013	0.12	0.01
Irgarol	0.0035	0.006	0.28	0.56
Isoproturon	0.3	0.48	4.20	53
Linuron	0.1	0.16	4.32	4.9
Malaoxon	0.31	0.5	72	0.99
Malathion	0.006	0.01	0.54	0.62
MCPA	0.5	0.8	1.52	0.5
Methiocarb	0.01	0.016	0.11	0.75
Metolachlor	0.2	0.32	1.51	14
Molinate	3.8	6.08	42	274
Oxadiazon	0.09	0.14	14	21
Pendimethalin	0.018	0.029	15	92
Propanil	0.2	0.32	1.79	22
Quinoxyfen	0.15	0.24	0.41	756
Simazine	1	1.6	8.02	221
Terbuthylazine	0.06	0.096	1.07	2.04
Terbutryn	0.065	0.1	7.91	4.71
Thiacloprid	0.01	0.016	0.32	0.03
Triadimefon	1.86	2.97	30	119
Triallate	10	16	1514	14000

[a] $PNEC_{water}$ extracted from the NORMAN Database (https://www.norman-network.com/nds/ecotox/).

[b] $PNEC_{sed}$ extracted from the NORMAN Database, calculated following the formula: $PNEC_{sed} = PNEC_{water} * 2.6 * (0.615+0.019*K_{oc})$ considering $K_{oc} = 0$.

[c] $PNEC_{sed}$ calculated following the formula: $PNEC_{sed} = PNEC_{water} * 2.6 * (0.615+0.019*K_{oc})$ considering the specific K_{oc} of each pesticide.

[d] $PNEC_{biota/fish}$ calculated following the formula: $PNEC_{biota/fish} = PNEC_{water} *$ BCF considering the specific BCF of each compound.

Table 4.4. HQ-max and HQ-mean calculated in sediment samples using $PNEC_{sed}$ considering the specific K_{oc} of each pesticide.

Pesticides	MEC-max (ng/g)*	MEC-mean (ng/g)*	HQ-max	HQ-mean
Diazinon	0.56	0.16	1.78	0.51
Dichlorvos	43	6.2	17793	2542
Irgarol	0.23	0.06	0.82	0.23
Terbuthylazine	1.6	0.57	1.5	0.53
Terbutryn	200	38	25	4.8

*MEC: measured environmental concentration

Dichlorvos, imidacloprid and irgarol showed medium or high risk in all basins where they were found, denoting great concern due to both their concentration and ecotoxicity characteristics. In the Ter River, only bentazone and irgarol were at concentrations that could pose a medium risk to aquatic organisms in the worst-case contamination scenario.

Apart from the study of the risk of individual pesticides, further analyses were conducted to evaluate the overall risk that pose the pesticide mixture found in the investigated samples to aquatic organisms. For this, an additive model was used, which consisted on adding the HQ value of the various pesticides present in each sample. With these data, the risk of each matrix and study area investigated was assessed as the relative percentage of samples that presented a low, medium or high risk.

As seen in Figure 4.5, even if the majority of individual pesticides showed low HQs (Figure 4.4), many samples presented a moderate or high risk due to the co-occurrence of these substances. This is particularly noticeable in the Ebro Delta and the Llobregat River water samples, where a high number of pesticides was detected. In the case of the Ter River, only few compounds were identified and their cumulative HQ did not exceed the value of 10 in any sampled location. Similarly, in biota only the sample in which dichlorvos was detected showed an HQ >10, while in sediment samples the risk was considerably high according to the moderate-high HQs of all individual pesticides detected in these samples.

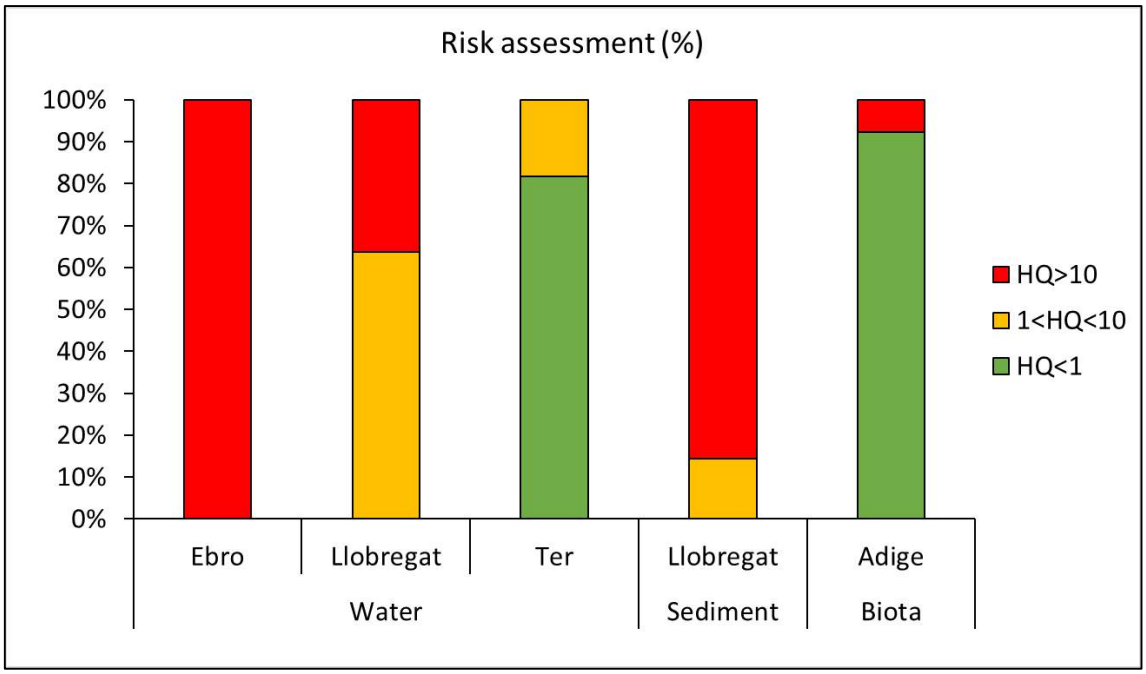


Figure 4.5. Overall risk of the pesticide mixture in each matrix and study area investigated, given as the relative percentage (%) of samples that presented low, medium or high risk.

These results are particularly important as it can be seen that the risk of the single compound taken separately does not affect river basins as much as that given by the sum of several pesticides in the entire matrix. However, no definitive conclusions can be drawn since the simple additive method does not consider the synergistic and/or antagonistic effects that may appear among co-occurring contaminants. Moreover, assessment of the risk using an additive model would be more accurate when considering only compounds with the same toxicity mode of action. In spite of this, the HQ approach is likely to underestimate the real risk, since calculations are based on few pre-selected compounds, and other biological active chemicals present in these matrices are overlooked (e.g., pharmaceuticals, personal care products, endocrine disrupting compounds, and other pesticides). In any case, this type of analysis is necessary to increase the understanding on the harmful properties of pesticides and to support the revision and modification of current legislation.

4.4 Attenuation of pesticide pollution

4.4.1 Assessment of innovative technologies for pesticide removal

The results obtained from the study of the occurrence and risk assessment of the 52 pesticides investigated within this thesis project showed a different contamination pattern in the analyzed matrices and high risk associated to the presence of specific pesticides. An outcome of particular relevance is the detection of highly toxic pesticides, including European priority substances (EC, 2013) and Watch List compounds (EC, 2018), and some of them exceeding the quality standards established for their presence in the environment. According to the risk assessment analysis, some of the pesticides detected may pose a high risk for aquatic organisms in the different environmental compartments. This was particularly evident in the Ebro Delta, where there was a high proportion of pesticides with high occurrence and risk. Among the compounds detected in the different studies conducted during this doctoral thesis, we selected three insecticides, namely malathion, acetamiprid, and imidacloprid for bioremediation studies (scientific publication #5), since they are among the most toxic pesticides, but have not been extensively studied and hence information on their occurrence and elimination is still scarce. Hence, the bioremediation capacity of the white-rot fungus *Trametes versicolor* in the degradation of these three insecticides was studied in section 3.5 to investigate its efficiency and the possible formation of their TPs and their toxicity as compared to that of the parent compounds.

The results obtained from this study revealed an efficient degradation by *T. versicolor*, with a complete malathion removal after 48h experiments in Erlenmeyer flask, and degradation of 20% and 64.7% of acetamiprid and imidacloprid after 7 days in air-pulse fluidized bioreactors. The study also allowed to investigate the main enzymatic systems (intracellular cytochrome P450 system or extracellular laccase system) involved in the biodegradation of the selected pesticides and the elucidation of the biodegradation pathways, according to the main TPs generated in solution, which could be identified using UHPLC-HRMS in a non-target manner. The biodegradation process produced five TPs in the case of malathion, one in the case on acetamiprid, and two in the case of imidacloprid.

Although the aim of the bioremediation treatment is detoxifying the water, the formation of these TPs may result in a higher toxicity of the water, because sometimes the species formed are more toxic than the original compounds. This aspect was evaluated in the scientific publication #5. While a slight increase in toxicity was observed in treated water as compared to non-treated water using the bioluminescent bacterium *Vibrio fischeri* (Microtox assay), the toxicity of the malathion TPs, as predicted with the ECOSAR QSAR model, was in general lower than that of malathion and decreased for those metabolites generated at a later stage of the degradation pathway. In the case of acetamiprid and imidacloprid, TPs were predicted to pose a similar or slightly higher toxicity to aquatic organisms than the parent compound (see Table 3 of scientific publication #5 for further details), and therefore, a significant increase of the water toxicity is not expected. The increased toxicity observed in the Microtox assay could be explained by the formation of highly toxic unidentified minor TPs or to mixture toxicity effects. However, the toxicity of treated waters was always below the wastewater discharge limit of 25 established in industrial areas of Catalonia under the DECREE 130/2003, which approves the Regulation of public sanitation services (DOGC, 2003).

The use of QSAR models to predict toxicity of chemicals is a valuable tool to initially explore the toxicity of newly discovered chemicals such as biodegradation TPs. *In silico* toxicity tests are in fact a green, fast and low-cost alternative to *in vivo* and *in vitro* laboratory assays for the determination of pesticides toxicity.

Although there exist physical and chemical approaches to remove pesticides from water, fungal-based bioremediation systems present major advantages in terms of sustainability, as they are low energy demanding systems, and require low operational costs (no nutrients need to be supplemented, no light is required). Satisfactory results have been obtained also in the case of other pesticides, such as diuron (Hu et al., 2020) and bentazone (García-Vara et al., 2020). In this works, diuron was almost completely removed (83%) after seven days of treatment, and bentazone was completely eliminated after three-day incubation. However, further research is still needed to optimize relevant operational conditions and make these bioremediation techniques implementable in the field at real scale.

4.4.2 Implementation of mitigation measures and best management practices in agriculture

One of the aims of the doctoral thesis, in line with the objectives of the WaterProtect project, was to effectively implement best management practices and mitigation measures to improve water quality and protect drinking water supplies from agriculture pollutants. Within the WaterProtect project, multi-actor activities were organized to (i) evaluate and improve the water governance in the lower Llobregat River basin, and (ii) establish participatory monitoring strategies to investigate pesticide and nitrate pollution sources in this area. Besides exploring novel bioremediation techniques that could be applied in agricultural fields to reduce pesticide release into the environment (as discussed in 4.4.1), mitigation measures and BMPs that can be successfully implemented by farmers were proposed and promoted in this area. For the identification of these BMPs, a multi-actor approach was used, in which farmers, plant protection associations, research entities and management authorities of the Agrarian Park participated. In these activities, information regarding existing mitigation measures and the actual implementation of BMPs in the area was gathered, as well as the willingness of farmers to implement additional, innovative measures, depending on costs and benefits.

Despite that water resources in the lower Llobregat are more impacted by urban and industrial activities than by agricultural activities (according to the research conducted within the WaterProtect project), the selected BMPs were focused on the reduction of agricultural pollution sources. These BMPs were chosen considering those that have the best chances for implementation on a local scale.

Considering the characteristics of the catchment, and the agriculture scenario in the Baix Llobregat, the five BMPs chosen for their, expected successful, implementation in the study area were:

1. The calibration of the sprayers for appropriate and optimum application of the PPPs
2. The disposal of obsolete PPPs by an authorized waste collection company
3. The use of safe filling and cleaning places for the spraying equipment
4. The safe disposal of spraying liquid residues

5. The use of alternative systems to chemical use for pest control

For each BMP, different actions were undertaken by the project partners, together with other local entities in the area, to advise farmers and demonstrate the applicability and feasibility of the selected BMPs. Hence, informative brochures for dissemination were edited for distribution among farmers and stakeholders, together with the organization of different workshops, conferences and training sessions with experts for the explanation of the appropriate BMPs application. Examples of the actions taken can be viewed on the WaterProtect project website (https://water-protect.eu/en) and the Baix Llobregat action lab webpage (https://www.protect-baixllobregat.com/en1/) created for local dissemination.

The lesson learned from the inclusion of farmers and stakeholders in the decision-making process, as expressed in the final WaterProtect deliverable on BMPs (Kuczyńska et al., 2020), is that raising awareness about environmental problems, together with the dissemination of monitoring results, is very beneficial for the effectiveness of actions. In such wise, a participatory monitoring strategy was implemented with the engagement of all interested actors (i.e., industry, water management authorities, drinking water producers, etc.). Historical water quality data of the Baix Llobregat and newly acquired data to cover information gaps identified within the participatory monitoring process were gathered in the collaborative tool GISEL (https://gisel.cuadll.org/geoportal/public). This online tool provides farmers and all water actors with a quick and easy overview of water quality and quantity data in the area, including pesticide pollution of surface water and groundwater in the catchment area, as well as hydrological information, interfaced with geographical information systems. As concluded in the final WaterProtect deliverable about the implementation of a participatory monitoring strategy (Postigo et al., 2020), the engagement of the different water actors in the design of monitoring activities is effective in generating trust, awareness and credibility of the monitoring results, and the harmonization of data in the collaborative tool is effective in helping farmers to take appropriate measures for the protection of the local water resources, and provides valuable information to be used during the discussion and decision making with local stakeholders.

CHAPTER 5 - CONCLUSIONS

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The main results and conclusions of the work carried out during the doctoral thesis can be summarized as follows:

- The analytical methodologies developed for the analysis of medium to highly polar pesticides and their metabolites in surface water and groundwater (on-line SPE-LC-MS/SMS), sediment (PLE followed by SPE and LC-MS/MS), and biota (QuEChERS followed by LC-MS/MS), show high sensitivity, with limits of determination for most compounds below 40 ng/L in surface water, 63 ng/L in groundwater, 12 ng/g d.w. in sediment, and 10 ng/g f.w. in biota, being overall comparable or lower than LODets previously reported in peer-reviewed methods. The sensitivity obtained allows the determination of compounds in compliance with the legislation set by the EU for their presence in the different environmental matrices. The significant matrix effect observed in surface water, groundwater, and biota matrices was corrected with the use of ILIS analogues for most of the target analytes (88%), ensuring the reliability of results. Other advantages of the developed methodologies are their versatility (analysis of different families of compounds with different polarities), relative simplicity and speed (due to the automation of several steps of the analysis), with increased sample throughput.

- The analysis of surface and groundwater samples collected from three main river basins of Catalonia has revealed diverse contamination patterns driven by the main pressures and the different use of pesticides in the investigated sites. Based on this consideration:

  - Pesticide pollution in the Llobregat River is mostly motivated by urban and industrial pressures, being diuron and terbutryn the most ubiquitous compounds, and bromoxynil, diuron and linuron the compounds found at the highest levels (up to 1500 ng/L). Besides, 2,4-D, azinphos-ethyl, dichlorvos, diflufenican, imidacloprid, methiocarb, and terbutryn, were found at concentrations above the limit of 100 ng/L set for individual pesticides in waters intended for human consumption in the EU, which raises concern considering that the Llobregat River serves as a drinking water resource for a high proportion of Barcelona and its metropolitan area.

- In agricultural areas dedicated to rice cultivation, as it is the case of the Ebro River Delta, the most abundant and ubiquitous pesticides were bentazone, MCPA and propanil (up to 180 µg/L), all used in rice cultivation.

- In the Ter River, an area with low population density and the presence of a modest number of industries and intensive agricultural activities, bentazone and MCPA were found at low concentrations nearby rice cultivation fields, while other five compounds (diazinon, diuron, irgarol, metolachlor, terbutryn) were occasionally detected at low levels in both surface water and groundwater samples.

- In sediments collected from the Llobregat River, only 5 pesticides (diazinon, dichlorvos, irgarol, terbuthylazine, and terbutryn) were detected. Terbutryn showed the highest concentration (200 ng/g) and ubiquity (100% of the samples). Pesticide occurrence in sediments was related to the organic matter content of the sediment and the physical-chemical properties of the pesticides, such as water solubility, mobility, and sorption potential. Indeed, the pesticides detected present high probability to be found in sediments. The detection of some of these pesticides in water and sediment samples of the same sampling locations, despite being banned for years in Europe, suggests that they may have accumulated in sediments during their application in the past and are subsequently released after sediment desorption processes.

- The results obtained for biota samples collected in the Adige River suggest fish contamination due to both agricultural and industrial/urban pesticide use, in agreement with the land use of the area. The high Log K_{ow} of almost all the detected compounds (acetamiprid, diazinon, dichlorvos, diuron, irgarol, metolachlor, quinoxyfen, terbutryn) justifies their bioaccumulation in biota, and thus their persistence in the environment.

- The most important factors governing the occurrence and distribution of pesticides in the different environmental compartments are: intensive land use (agricultural, urban or industrial), and the physical-chemical properties of the pesticides (for example, high solubility in surface water, high K_{oc} in sediments, and high K_{ow} in biota). Other factors such as peak rainfall events, changes in water flow or irrigation runoff should also be considered.

- Concerning the general status of waters, results showed that dichlorvos and irgarol were the only compounds exceeding their EQS set by the Directive 2013/39/EC. They were also found, together with terbutryn, in sediment samples at levels that would be above EQS, if values were set in this matrix from extrapolation of those existing in surface waters. These findings call for the need of establishing EQS for pesticides in sediment, with the aim of increasing the knowledge on their contamination and preventing deterioration of water bodies. Pesticides included in the Watch List (acetamiprid, imidacloprid, thiacloprid, and methiocarb) were also detected in waters at levels above their maximum acceptable LOD, established on the basis of their toxicity for their monitoring in the legislation. These results should be useful for the drafting of future regulations. As for fish samples, the general MRL of 10 ng/g was not surpassed in any case. Nonetheless, the continuous release of pesticides into the aquatic system makes necessary the formulation of specific MRLs for fish, considering that their contamination may affect human health.

- The assessment of the risk posed to aquatic organisms by individual pesticides in the studied areas have pointed out pesticides with a high risk (HQ > 10) in the different environmental compartments. The most relevant compounds in this respect are: bentazone, dicofol, imidacloprid, irgarol, MCPA, and propanil in the Ebro River Delta; azinphos ethyl, dichlorvos, diflufenican, imidacloprid, irgarol, and methiocarb in the waters of the Llobregat River; diazinon, dichlorvos, irgarol, terbutryn, and terbuthylazine in the Llobregat sediment samples; dichlorvos in fish samples. This risk may originate from the high concentrations measured, the very low PNEC values, or both. Most of the pesticides posing a high risk in the investigated areas are currently prohibited due to their toxicity, or included in the list of priority substances or the European Watch List for their monitoring, which confirms the usefulness of the risk assessment analysis for a better understanding of the implications for the ecosystems.

- The risk assessment results given by the sum of the HQs of the individual compounds revealed an increased risk in most samples, demonstrating that co-occurrence of compounds imply a greater risk for the aquatic ecosystem.

- Following the results obtained for the presence of toxic and high-risk pesticides in the analyzed water compartments, the bioremediation assays performed with the white-rot fungus *Trametes versicolor* have demonstrated its capability in degrading the selected insecticides malathion, acetamiprid and imidacloprid, without any relevant increase in the toxicity of the treated waters. These outcomes are very promising since they can be considered for the implementation of more efficient and sustainable water treatment techniques for the removal of organic contaminants from waters.

- Finally, the implementation of mitigation measures and BMPs for pesticide attenuation should be identified at local level together with farmers and stakeholders, considering the different pollution pressures and pesticide sources of the study area. The knowledge about water quality derived from the dissemination of monitoring results, together with the growing awareness about environmental problems coming from the contribution of different actors to the decision-making process, constitute a step forward in the implementation of effective measures aimed at reducing environmental contamination, from which the whole community will benefit.

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ANNEXES

Annexes

Annex I: Index of Tables

Annex II: Index of Figures