UNIVERSITAT DE BARCELONA

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PhD thesis

2023

CSIC
Consejo Superior de Investigaciones Científicas
Universitat de Barcelona

Programa de doctorado
"Química Analítica i Medi Ambient"

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Memoria presentada para optar al título de
Doctor por la Universidad de Barcelona

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Barcelona, mayo de 2023

This research work has been financially supported by the PRIMA-MED Programme within the EU Horizon2020 through the project MAGO (Project number 2022), by the European Union through the European Agricultural Fund for Rural Development through the project FitoFarmGest (Operational Group PDR2020-101-030926), by the Spanish State Research Agency of the Ministry of Economy and Competitiveness through the project BECAS (CTM2016–75587-C2–2-R), the Spanish Ministry of Science and Innovation through the project CEX2018-00794-S, and the Government of Catalonia (2017 SGR 01404 and 2021 SGR 00753).

Cover design by **Yaiza García Vara** and **Ane Agirre Bikuña**

"Tengo más imaginación que yo qué sé"

Anónimo

Agradecimientos

Bueno bueno, y yo qué se por dónde empezar. Y es que qué complicado a estas alturas ponerme a recordar a tanta buena gente que me ha acompañado en esta etapa que, dejando a un lado la tesis, me ha hecho madurar tantísimo y ser la personica que soy hoy.

Empezaré por mi directora de tesis, Miren, que me repescó cuando salía del IDAEA después de un año un poco turbulento y, cuando había decidido dejar mi etapa investigadora, me ofreció un doctorado con el que al principio tuve mis dudas, pero en el que pronto me di cuenta de la gran decisión que había tomado. Durante todos estos años he sentido gran autonomía, he podido trabajar a gusto, disfrutando de lo que hacía y creo que he acabado con un espíritu crítico tan necesario en la ciencia. Muchas gracias! A mi otra directora, Cris, que aun viviendo un embarazo, un parto, un cambio de puesto de trabajo y viviendo a mil kilómetros de distancia (así a ojo), siempre estaba ahí cuando la necesitaba, aportando todo lo que sabía y dándome siempre buenas ideas para mejorar los trabajos. Y lo confesaré, el inglés más fancy de los artículos se lo debo a ella.

A todxs lxs compañerxs que han pasado o siguen en el IDAEA con lxs que he compartido estos años de tesis. Desde la gente que me acogió y con lxs que disfruté innumerables casas rurales, rutas de bares, tardes de nocilla, congresos, viña rocks apocalípticos y rutas populares: Carlos, Simón, Esther, Dani, Gabri, Juan, Alba, Judit, Mavi, Mari Pau, Niko, Andrea y la tantísima gente que pasó temporadas por aquí), a la nueva generación Katerina, Rocío, Adri, Olga, Diana, Berta, Xiscu, Sandra, Julus, Maria, Xavi, Vega, Aleix y tantxs otrxs que, aunque yo ya estuviera más fuera que dentro, me han hecho sentir de nuevo como en casa. Y es que que tus compañerxs de curro sean también tus amigxs es casi más importante que el trabajo. A Dori, Alex y Roser, que han sufrido mi lamentable espíritu analítico en el masas. Y en especial, como no, quiero agradecer a Dana, que ha sido la mejor compañera que he podido tener para acabar nuestro último trabajo y a la que estoy muy agradecido de su empeño, sus ganas de aprender, su constancia y su alegría cada día. Estoy convencido de que será una gran investigadora pese a mi insistencia en abolir el trabajo. Y, por supuesto, A Cris, una de las mejores personas que he conocido y que tuve la suerte de conocer en el IDAEA y con la que pude compartir unos años preciosos llenos de alegrías y penas y a la que robé tantas bromas. Lástima que me pillaran, ya la gente me tiene calado…

A la Tresquera crew (o Corriols, o Tangent o a lxs garrotxerxs, yo qué ya!), Gemma, Inma, Celia, Miguel, Berta y Vity, que han dado una nueva oportunidad a la ciencia en mi vida para darle el sentido que nosotrxs

queremos. Aunque nos está costando un poquito, estoy seguro de que nuestra cabezonería sacará algo muy bonito de todo esto.

A mis antiguos compis de piso, Pep, Tania y Olga, con los que viví los primeros años de la tesis y con los que pasé el confinamiento más divertido que se ha podido tener. No quiero frivolizar, porque sé que hay gente que malvivió, y mucho, en esos meses, pero yo tuve la suerte de disfrutarlo. De hecho, esos meses me hicieron, por fin, encontrar en la lucha social y la vida en comunidad el camino por el que quería guiar el resto de mi vida. Por ello quiero agradecer a todxs lxs compañerxs del Sindicat de Barri del Poble Sec, el CRA y La Base por haber podido compartir tantas experiencias y aprendizajes en estos años. En especial, agradecer a Pep, mi mejor colega con el que tanto he compartido desde que nos conocemos (ya unos cuantos años), y que estoy seguro que seguiremos compartiendo mucho tiempo. Porque la subnormalidad une mejor que el celo, y eso que fue inventado para ir a la NASA, o era al espacio?

A mi familia gallega, en especial Alvaritiño y Rafa que, a pesar de los años y todo lo que hemos cambiado, nos hemos mantenido unidos. Han sido unos grandes confidentes y siempre he sabido que los tenía ahí para cuando hacía falta. Con ellos he aprendido a respetar (básicamente a huir) del licor café y creo que ellos, por fin, han aprendido a amar la mahou (como no puede ser de otra manera, por otra parte).

A mi familia poblesequina, que me ha atrapado en este barrio de manera irremediable. Ha tenido que ser casi a los 30 cuando por fin he encontrado lo que, de alguna manera, había buscado desde hacía muchísimo tiempo: una forma de vida en comunidad, otra idea de familia y de relacionarse, una gente maravillosa cada cual más personaje que la anterior que me hacen crecer y aprender día a día y que hacen muy difícil el sentirse solo. Y por qué no decirlo, es un privilegio poder vivir en una telenovela constante. En especial a Cris, la mejor compañera de vida. La verdad es que vivir con una amiga así, que además de amiga es consejera, psicóloga, emprendedora (de la que cualquier neoliberal estaría orgullosísima), y con la tengo tantos proyectos en marcha que no tendremos vida suficiente para llevarlos a cabo es un lujo que no quiero perder nunca. Capibros es el futuro!; a Nis (aka xula, xulasa, tronxi, croquetón, Goicoechea) que me ha enseñado que existe un mundo de la emocionalidad más allá de la racionalidad (tarea complicada para un cabeza-cuadrada como yo) y a la que sé que, aunque ahora sea neorural, siempre voy a tener ahí para lo que sea. Junto a mi terapeuta, a estas dos personas les debo mi crecimiento a nivel emocional y a las que estoy tan agradecido; a Silvie, una persona increíble con la que he disfrutado y aprendido un montón, desde aprender fragatí y conocer la franja

d’Aragó, a contruir una furgo y hacer mil viajes juntos (algo así como contratar una agencia de viajes), a descubrir una persona única, con un carácter y desparpajo magnéticos que espero no perder de mi vida ahora que nuestros caminos se han separado; a la Sossis crew, Julens, Pantxo, Míriam, Eia, Nis y Pep que, a través de nuestro amor por la montaña y por la bioconstrucción (bueno, quizás esto no tanto), compartimos tantísimas experiencias estos años y que, aunque Sossis se haya perdido, me quedo con las maravillosas personas que son, que al fin y al cabo es lo más importante, y con el equipo élite liderando, por supuesto; a Helena, Ane (gracias por tu aportación con el Indesgin, en qué momento me metí ahí!), Rosi, Anna, Miguel, Marie, Núria, y mucha más gente que seguro que me dejo en el tintero.

A mis colegas de Madrid Josete, Hugo, Alex, Sofía, Julio, Luisao, Zazo, Paula, Rubén y Ara, que, aunque no hemos compartido mucho en estos años, han hecho de alguna manera que llegue hasta aquí y sea como soy. La distancia no hace que me olvide de ellxs y una parte de mí siempre quedará allí.

Y a mis padres y mi hermana, que moldearon la persona que soy desde pequeñito (desde el pequeño sinvergüenza que era a la persona respetable y responsable que soy ahora…jejej vaaale no, sigo siendo un sinvergüenza) y a lxs que agradezco tanto que me hayan aguantado, tanto cuando estaba bien como cuando estaba mal, y todo a pesar de ser el descarriado de la familia. Espero con ansias el próximo viaje a Madrid para comernos las gallinejas prometidas! Invita Yaiza!

En fin, muchísima gente ha pasado por mi vida y, en mayor o menor medida, han influido en que sea quien soy. Y, aunque tengo la sensación de que lo que viene ahora va a ser genial, quiero agradecer a todxs el haber podido disfrutar de esta etapa predoctoral como lo he hecho porque en la vida no todo es trabajo y, cuanto menos lo sea, mejor.

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Acronyms and abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
APCI	Atmospheric-pressure Chemical Ionization
BAC	Biological Activated Carbon
C18	Octyldecylsilane
CAS	Conventional Activated Sludge
CEC	Contaminant of Emerging Concern
CI	Chemical Ionization
DEA	Desethylatrazine
DIA	Deisopropylatrazine
EC	European Commission
EC50	Half Maximal Effective Concentration
EFSA	European Food Safety Authority
EI	Electron Ionization
ESI	Electrospray Ionization
EU	European Union
FWHM	Full Width at Half Maximum
GAC	Granular Activated Carbon
GC	Gas Chromatography
GCB	Graphited Carbon Black
GDP	Gross Domestic Product
H2O2	Peroxidation
HLB	Hydrophilic-Lipophilic Balance
HRMS	High Resolution Mass Spectrometry
IPM	Integrated Pest Management
LC	Liquid Chromatography
LC50	Exposure concentration of a toxic substance lethal to 50% of the test animals
LDet	Limit of Determination
LOD	Limit of Detection
LOQ	Limit of Quantification
MBR	Membrane Biological Reactor
MCPA	2-Methyl-4-chlorophenoxyacetic acid
MF	Microfiltration
MRL	Maximum Residue Level
NF	Nanofiltration

PAC	Powdered Activated Carbon
PFAS	Per and PolyFluoroAlkyl Substances
PNEC	Predicted No-Effect Concentration
PSA	Primary Secondary Amines
QqQ	Triple Quadrupole
QSAR	Quantitative Structure Activity Relationship
QSTR	Quantitative Structure Toxicity Relationship
Q-TOF	Quadrupole Time-of-Flight
RO	Reverse Osmosis
RSD	Relative Standard Deviation
RQ	Risk Quotient
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
TP	Transformation Product
UF	Ultrafiltration
UHPLC	Ultra High Performance Liquid Chromatography
UV	Ultraviolet
WRF	White Rot Fungi
WWTP	Wastewater Treatment Plant

Abstract

Throughout the last century, the agricultural practices have suffered several changes to adapt to the socio-economic framework in which they are inserted. This has forced, in some regions, to intensify productions and unbalance the natural cycles by overexploiting resources such as water and soils. In addition, climate change is progressively reducing the quantity and quality of water resources, mostly in arid and semiarid regions, like the Mediterranean. To face this reality, water reuse reveals as an interesting alternative to freshwater for agricultural irrigation. Reclaimed water is conventionally-treated wastewater that undergoes an extra treatment to reach the minimum required standards to be reused. Despite the benefits of reclaimed water in terms of nutrients when used for irrigation purposes, it may also be a source of contamination. The contamination footprint associated to this water resource depends on its origin, i.e. urban or industrial, and it includes pharmaceuticals, personal care products, industrial chemicals, pesticides, etc. Pesticides also enter the environment due to their widespread use. These substances have become essential to maximize crop yield at the expense of other variables (as the environmental sustainability and safety). Thus, all these anthropogenic organic compounds that may be present in the environment at concentrations that may cause an ecological and/or human health impact, and are mostly not regulated, are the so-called contaminants of emerging concern (CECs).

This doctoral thesis aims at providing novel analytical tools for the environmental control of CECs in waters and crops and generating new knowledge about i) the occurrence and fate of CECs in the agricultural environment, and ii) the efficiency of bioremediation approaches to remove pesticides from water.

Firstly, analytical methodologies for the determination of up to 42 pesticides in five different plant origin food matrices (viz. corn, grapes, alfalfa, olives, and sunflower seeds) were developed and validated. The availability of highly sensitive and selective analytical methods for the determination of pesticides in plant origin food is crucial, considering the low concentrations at which they are normally found in these matrices. Moreover, Maximum Residue Levels (MRLs), the legal limits for pesticide residues in food and feed established by the Regulation (EC) 396/2005

are, in most cases, in the range of the low µg/kg, enforcing analytical methodologies to reach limits of detection below these values. The methods developed, based on QuEChERS extraction and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination, allowed detecting most of the target pesticides below their corresponding MRLs. The QuEChERS extraction procedures were cost-effectively optimized through a fractional factorial design of experiments taking into consideration each matrix idiosyncrasy. Moreover, to the authors' knowledge, these were the first analytical methodologies described for eleven (sunflower seeds), eighteen (olives), five (corn), thirteen (grapes), and twenty-four (alfalfa) pesticides in each indicated matrix. The analysis of real samples harvested at different locations along the Iberian Peninsula revealed the presence of a few insecticides and herbicides, some of them included in the Pesticide Action Network International List of Highly Hazardous Pesticides (acetamiprid, clothianidin, imidacloprid, and thiacloprid) and/or currently banned for use in the European Union (clothianidin, dimethoate, imidacloprid, terbutryn, thiacloprid, and thiamethoxam).

However, apart from the set of pre-selected agrochemicals investigated in plant products, typically chosen based on extent of use, many other CECs may be occurring in the agricultural environment, such as those coming from the reclaimed water used for irrigation that could eventually reach crops. In this context, the need of targeting the right substances at the various environmental and food matrices is clear. To address this need, a wide-scope suspect screening workflow and a prioritization procedure was developed and applied to identify the most relevant contaminants present in a reclaimed water-based irrigation system from a specific agricultural area in Catalonia, Spain. Aiming at a cost-effective and wide-scope approach to embrace as many contaminants as possible that could potentially be present in the investigated matrices (raw wastewater, reclaimed water, and irrigation water), a generic methodology was developed and implemented, based on the lyophilization of the water samples and the non-target analysis of the extracts with ultra-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS). More than one hundred CECs were found in the water used for irrigation (a mixture of surface water and reclaimed water). Based on their occurrence (semi-quantified levels) and ecotoxicity, CECs were prioritized in the investigated area. This approach allows a more rationale selection of the

organic contaminants to be included in monitoring programs than classical approaches, normally based on the CEC chemical class, in force legislation, toxic potency, physical-chemical properties, or volumes of usage, that may result in a CEC list that deviates from the real contamination footprint in a specific area. Moreover, the approach developed can be easily implemented in any location to detect site-specific pollutants that could be missed with the national or European regulations, rationally design monitoring and attenuation programs, and support legislators and water managers in their way to extend the safe application of water reuse.

Bearing in mind that CECs are not completely removed during conventional wastewater treatment, it is crucial to evaluate alternative treatment technologies, such as bioremediation technologies. In this doctoral thesis, the feasibility of white-rot fungi *Trametes versicolor* and microalgae-based systems to remove selected pesticides (bentazone, acetamiprid, and propanil) was investigated. The removal efficiencies were assessed, and the biodegradation pathways characterized, by identifying the TPs formed in each process using an UPLC-HRMS non-target screening approach. The degradation processes involved did not end up in the complete mineralization of the compounds (with the exception of propanil) and 19 TPs were formed during bentazone biodegradation by *Trametes versicolor* (eight of them tentatively identified), and two and four TPs were identified for propanil and acetamiprid, respectively, during the microalgae-based treatment. Ecotoxicological risk assessment based on the calculation of the Risk Quotients (RQs) of the TPs generated during the biodegradation processes revealed a few TPs, in the case of propanil and bentazone, to be more toxic for the aquatic environment than the parent compound.

Although further research is needed in this field, it is clear that there is not a unique wastewater treatment technology that efficiently removes all CECs and, thus, the combination of complementary treatments is suggested to remove a wider spectrum of CECs. In this context, bioremediation approaches, such as those studied in this doctoral thesis, have shown a good performance in terms of CEC removal, added to the benefits of using nature-based treatment technologies, and present a promising future to be implemented at real scale. CEC TPs formed during water treatment or natural biotic and abiotic processes (hydrolysis, biodegradation and photodegradation reactions) are still unknown to a

large extent and are relevant components of the CEC mixtures in the environment. Their identification and their inclusion in spectral libraries and compound databases are critical to advance in the characterization of the environmental contamination footprint using wide-scope screening approaches based on HRMS technologies.

Resumen

A lo largo del siglo pasado, la agricultura ha sufrido diferentes cambios para adaptarse al marco socioeconómico en el que se inserta. Esto ha obligado, en algunas regiones, a intensificar la producción y desequilibrar los ciclos naturales mediante la sobreexplotación de recursos como el agua y los suelos. Además, el cambio climático está reduciendo progresivamente tanto la cantidad como la calidad de los recursos hídricos, principalmente en las regiones áridas y semiáridas, como el Mediterráneo. Para enfrentar esta realidad, la reutilización del agua aparece como una alternativa interesante al agua superficial para usos agrícolas. El agua regenerada es agua residual tratada convencionalmente que se somete a un tratamiento adicional para alcanzar los estándares mínimos de calidad requeridos para ser reutilizada. A pesar de sus beneficios en cuanto a su contenido en nutrientes, el agua regenerada puede ser una fuente de contaminación para el medio agrícola. La huella de contaminación asociada a este recurso hídrico depende de su origen (por ejemplo, urbano o industrial), que incluye fármacos, productos de cuidado personal, productos químicos industriales, pesticidas, etc. Por otro lado, los pesticidas también entran en el medio ambiente debido a su uso generalizado en agricultura. Estas sustancias se han convertido en un elemento esencial para maximizar el rendimiento de los cultivos a expensas de otras variables (como la sostenibilidad ambiental y la seguridad alimentaria). Todos estos compuestos orgánicos de origen antropogénico, que pueden estar presentes en el medio ambiente en concentraciones que pueden causar un impacto ecológico y/o sobre la salud humana y que en su mayoría no están regulados, son los llamados contaminantes emergentes (CECs, por sus siglas en inglés).

Esta tesis doctoral tiene por objeto proporcionar nuevas herramientas analíticas para el control medioambiental de los CECs en aguas y cultivos y generar nuevos conocimientos sobre i) la presencia y el destino de los CECs en el entorno agrícola, y ii) la eficacia de los métodos de biorremediación para eliminar pesticidas del agua.

En primer lugar, se han desarrollado y validado metodologías analíticas para la determinación de hasta 42 pesticidas en cinco matrices alimentarias de origen vegetal (maíz, uvas, alfalfa, aceitunas y semillas

de girasol). La disponibilidad de métodos analíticos con alta sensibilidad y selectividad para la determinación de pesticidas en alimentos de origen vegetal es crucial, teniendo en cuenta las bajas concentraciones a las que éstos se encuentran normalmente en estas matrices. Además, los niveles máximos de residuos (MRLs, por sus siglas en inglés), es decir, los límites legales para los residuos de pesticidas en los alimentos y los piensos establecidos por el Reglamento (CE) 396/2005 se sitúan, en la mayoría de los casos, en el rango de los pocos µg/kg. Esto obliga a los métodos analíticos a alcanzar límites de detección por debajo de estos valores. Los métodos desarrollados en el marco de esta tesis, basados en la extracción por QuEChERS y análisis por cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS), permitieron detectar la mayoría de los pesticidas seleccionados por debajo de sus correspondientes MRL. Los procedimientos de extracción por QuEChERS fueron optimizados eficientemente a través de un diseño de experimentos factorial fraccional teniendo en cuenta la idiosincrasia de cada matriz. Además, hasta donde llega el conocimiento de los autores, éstas fueron las primeras metodologías analíticas descritas para once (semillas de girasol), dieciocho (aceitunas), cinco (maíz), trece (uvas), y veinticuatro (alfalfa) pesticidas en cada matriz indicada. El análisis de muestras reales recolectadas en diferentes lugares de la Península Ibérica reveló la presencia de algunos insecticidas y herbicidas, algunos de ellos incluidos en la Lista Internacional de Plaguicidas Altamente Peligrosos de la Red de Acción en Plaguicidas (acetamiprid, clothianidin, imidacloprid y thiacloprid) y/o actualmente prohibidos para su uso en la Unión Europea (clothianidin, dimethoate, imidacloprid, terbutryn, thiacloprid y thiametoxam).

Sin embargo, aparte del conjunto de agroquímicos preseleccionados que se investigaron en los alimentos de origen vegetal, que suelen elegirse en función del grado de uso, muchos otros CECs pueden estar presentes en el entorno agrícola, como los que provienen del agua regenerada utilizada para el riego, pudiendo estos llegar a los cultivos. Esta situación evidencia la necesidad de dirigir los análisis a las sustancias adecuadas según el contexto. Para hacer frente a esta necesidad, se desarrolló y aplicó un procedimiento de análisis *suspect screening* en un sistema de riego a base de agua regenerada en una zona agrícola en Cataluña, España, para llevar a cabo una priorización de los contaminantes más relevantes. Con el objetivo de poder analizar un espectro de contaminantes tan amplio como

fuera posible en las matrices investigadas (aguas residuales brutas, agua regenerada y agua de riego) de una forma eficiente, se elaboró y aplicó una metodología genérica, basada en la liofilización de las muestras de agua seguida de un análisis de tipo *suspect screening* de los extractos por cromatografía líquida de ultra-alto rendimiento acoplada a espectrometría de masas de alta resolución (UPLC-HRMS). Se encontraron más de cien CECs en el agua utilizada para el riego (mezcla de agua superficial y agua regenerada). Basándose en la concentración encontrada en las muestras (niveles semicuantificados) y su ecotoxicidad, se priorizaron los CECs más relevantes para la zona investigada. Este enfoque permite una selección más racional de los contaminantes orgánicos que deberían incluirse en los programas de monitoreo que los métodos clásicos, normalmente basados en el grupo químico de los CECs, la legislación vigente, su toxicidad, propiedades físico-químicas o su nivel de uso, que pueden dar lugar a una lista de CECs diferente de la huella de contaminación real en un área específica. Además, el enfoque desarrollado puede implementarse fácilmente en cualquier lugar para detectar contaminantes específicos de cada zona que podrían no estar contemplados en las regulaciones nacionales o europeas, así como diseñar racionalmente programas de vigilancia y atenuación y apoyar a las instituciones reguladoras en términos de seguridad en el uso de agua regenerada.

Teniendo en cuenta que los CECs no se eliminan completamente durante el tratamiento convencional de aguas residuales, es crucial evaluar tecnologías alternativas para el tratamiento de estas aguas, como las basadas en la biorremediación. En esta tesis doctoral, se investigó la viabilidad del uso del hongo *Trametes versicolor* y sistemas basados en microalgas para la eliminación de varios pesticidas (bentazona, acetamiprid y propanil). Se evaluó la eficacia de eliminación de dichos compuestos y se elucidaron las vías de biodegradación mediante la identificación de los productos de transformación (TPs, por sus siglas en inglés) formados en cada proceso utilizando un análisis *non-target* por UPLC-HRMS. Los procesos de degradación involucrados no finalizaron con la completa mineralización de los compuestos (con la excepción del propanil) ya que se formaron 19 TPs durante la biodegradación de la bentazona por *Trametes versicolor* (ocho de ellos identificados de forma tentativa), y dos y cuatro TPs en el caso de propanil y acetamiprid, respectivamente, durante el tratamiento basado en microalgas. La evaluación de riesgos

ecotoxicológicos basada en cálculo de los RQs de los TPs generados durante los procesos de biodegradación reveló que algunos TPs, en el caso del propanil y la bentazona, eran más tóxicos para el medio acuático que el compuesto original.

Aunque es necesario seguir investigando en este ámbito, es evidente que no existe una tecnología única de tratamiento de aguas residuales que elimine eficazmente todos los CECs y, por tanto, se sugiere la combinación de tratamientos complementarios para aumentar el espectro de eliminación de CECs. En este contexto, tecnologías de biorremediación, como las estudiadas en esta tesis doctoral, han mostrado un buen funcionamiento en términos de eliminación de CECs. Además, se añaden los beneficios de usar tecnologías de tratamiento basadas en la naturaleza, con menor impacto medioambiental, y presentan un futuro prometedor para implementarse a escala real. Los TPs formados durante el tratamiento de aguas o formados a través de procesos bióticos y abióticos naturales (hidrólisis, biodegradación y reacciones de fotodegradación) todavía son, en gran medida, desconocidos pese a que podrían ser componentes realmente relevantes en las mezclas de CECs presentes en el medio ambiente. Su identificación y su inclusión en bibliotecas espectrales y bases de datos de acceso libre son fundamentales para avanzar en la caracterización de la huella de contaminación ambiental utilizando análisis de cribado de amplio espectro basados en tecnologías de HRMS.

Chapter 1

Introduction

1.1. From traditional agriculture to the actual agroindustry

Since 1950, Spanish agriculture has been suffering a deep structural transformation. The rural production model that prevailed during the first half of the XX century (Figure 1.1) was progressively dismantled during the following decades. Thus, from a strong economical position in the Spanish production, hoarding almost 50% of the active population and representing the 30% of the Gross Domestic Product (GDP, PIB in its Spanish acronym) in the 50's, the agricultural sector evolved to employ only the 9% of the active population and represented the 3.5% of the total GDP in 1993 (Abad et al., 1994). In the last decade, it has maintained a GDP around 2-3% (O'Neill, 2022) and employed the 4% of the active population (Instituto Nacional de Estadística, 2022). The main reason for this decrease relays on the transition from a traditional agriculture, with almost closed productive cycles (from means of production to final commercialization) to an industrial-based agriculture, with the use of external means of production, including machinery, pesticides and fertilizers, post-processing of the goods, etc. Food industry has gained presence in the sector, becoming the main recipient of the agricultural production, for its transformation into either processed foodstuffs or cattle feed (Abad et al., 1994). Moreover, in the actual global market, the Spanish agroindustry has been placed with a strong exportation demand, mainly to the European Union (EU).

Figure 1.1. Farmhands mowing (extracted from http://jcdonceld.blogspot.com/2011/05/jornaleros-y-latifundios-en-la-espana.html)

With these premises, where the capitalist system moved the agricultural practices to maximize economic benefits at the expense of the rest of variables (working conditions and safety, environmental sustainability, open cycles, etc.), the widespread use of pesticides became essential. Pesticides are intended as any substance or a mixture of them used for the prevention, repelling, mitigation or the total elimination of any pest that may affect crops productivity. During the World War II, the pesticide industry boosted, introducing into the market compounds such as DDT, aldrin, dieldrin, parathion, among others, with a very optimistic view in their use, and no concerns on their potential risks to the environment or human health (Bernardes et al., 2015). It was not until the publication of "Silent Spring" (Carson, 1962), when the public awareness on the problems that pesticides were causing to the environment arose. Ten years later to this publication, DDT was banned in the United States. DDT ban was followed by other countries and extended to other pesticides with demonstrated toxicity.

Besides toxicity, the lack of selectivity in terms of activity, the low solubility in water and, consequently, the high potential to bioaccumulate, and the high persistence in the environment make classical pesticides very hazardous substances (Jayaraj et al., 2016). Therefore, in the following years, a new generation of pesticides with improved selectivity and low bioaccumulation potential was introduced into the market. However, these 2nd generation pesticides were not completely innocuous and may represent an important source of contamination to soils and water bodies that also endangers biodiversity. It is well known, for example, the relationship between the widespread use of neonicotinoids as insecticides and the regional mass mortality of wild and honey bees (Klingelhöfer et al., 2022). Due to their high water solubility, neonicotinoids are also ubiquitously present in ground and surface waters, affecting other non-target organisms or even humans.

Over the last decades, hundreds of compounds have been approved for their use as insecticides, herbicides, fungicides, nematicides or rodenticides, among other uses, and meanwhile others have been banned after scientific evidence of their toxicity. The European Commission, like other regulatory agencies worldwide, has issued policies aiming at the reduction of pesticide use. Nevertheless, sales statistics contradicted this

politic direction, showing that sales have not decreased in the last ten years (Eurostat, 2022). France, Spain, Germany and Italy represented two thirds of the total sales, being fungicides, herbicides and insecticides the most relevant pesticide groups in these statistics. These numbers reveal the ongoing farmers dependency on these compounds for their continuation on the global food market and the difficulties to apply promoted alternatives such as the agro-ecological farming.

1.2. Water resources

Water resources for irrigation are mainly constituted by surface and ground waters. Climate change and the associated unpredictable weather patterns and severe droughts are posing in risk the quality and quantity of these water resources. In addition, the need of water for irrigation also competes with the freshwater demand by the population and economic growth (Rosenzweig et al., 2004). In the last years, the situation has become critical, mostly in the arid and semi-arid regions. This includes the Mediterranean areas like the Iberian peninsula, as stated by Ungureanu et al. (Ungureanu et al., 2020), with Spain being affected by a medium-high water stress. The water stress index estimates the relationship between the water withdrawal from ground and surface waters and the renewed freshwater resources. This medium-high water stress suggests that water scarcity will be, year by year, an increasing problem.

The sustainable management of water is crucial to avoid open cycles and the overexploitation of the water resources. However, sometimes, economic profits prevail over the sustainable and ecological principles and, therefore, agricultural areas may present an unbalanced use of water due to an oversize of crops or even the increased presence of highly water-demanding crop varieties. The technological solutionism is addressing this situation by providing alternative water resources, such as desalinated water and reclaimed water, among others. A paradigmatic example of the situation above described is the case of the intensive horticultural systems in Almeria. With more than 31,000 ha of greenhouse farming, Almeria is Europe's main supplier of vegetables, mostly during winter. The high water demand in this region results in the overexploitation of 5 out of 6 natural water resources (Garcia-Caparros et al., 2017). Therefore, Almeria

is alternatively using the water from six desalination plants and water reclamation plants for crop irrigation, and developing other technological solutions to increase the efficiency in water use.

Reclaimed water is conventionally-treated wastewater that undergoes an extra treatment (this will be further explained in section 1.5) to be applied for diverse purposes. Although reclaimed water, used for irrigation purposes, is a nutrient source (especially nitrogen and phosphorus) and its use may reduce the need of adding commercial fertilizers, reclaimed water is also a source of contamination. Depending on its origin, i.e., urban or industrial, the contamination footprint associated to this water resource is different. It has been reported that irrigation with reclaimed water may lead to the accumulation of heavy metals in the soil or even the transmission of microbial pathogens (Rodriguez-Manzano et al., 2012). Furthermore, despite the purification efforts, irrigation with reclaimed water has been proven to be a source of organic pollutants such as the contaminants of emerging concern (CECs), that have reached soils, plants or aquifers in different agricultural areas (Ben Mordechay et al., 2022; Calderón-Preciado et al., 2011; Racar et al., 2020). Figure 1.2 shows the anthropogenic and natural water resources and their connections within the urban and natural water cycles to understand the potential contamination pathways in the agrarian environment.

In this thesis, a reclaimed water-based irrigation system was evaluated in terms of CECs occurrence to better understand the influence of this water resource in the contamination of the agricultural environment.

1.3. Contaminants of emerging concern (CECs)

CECs is the term used to describe the organic pollutants that have been detected in the various environmental compartments (water, soil, plants, animals, etc.), at concentrations that may cause an ecological or human health impact and, whose presence in the environment, in most cases, is not regulated (Dulio et al., 2018). CECs include diverse chemical groups, usually categorized in pharmaceuticals, personal care products, pesticides, drugs of abuse, industrial chemicals (involving flame retardants, per and polyfluoroalkyl substances (PFAS), plasticizers, among others), tire wear and road runoff chemicals, endocrine disruptors, etc., as well as their

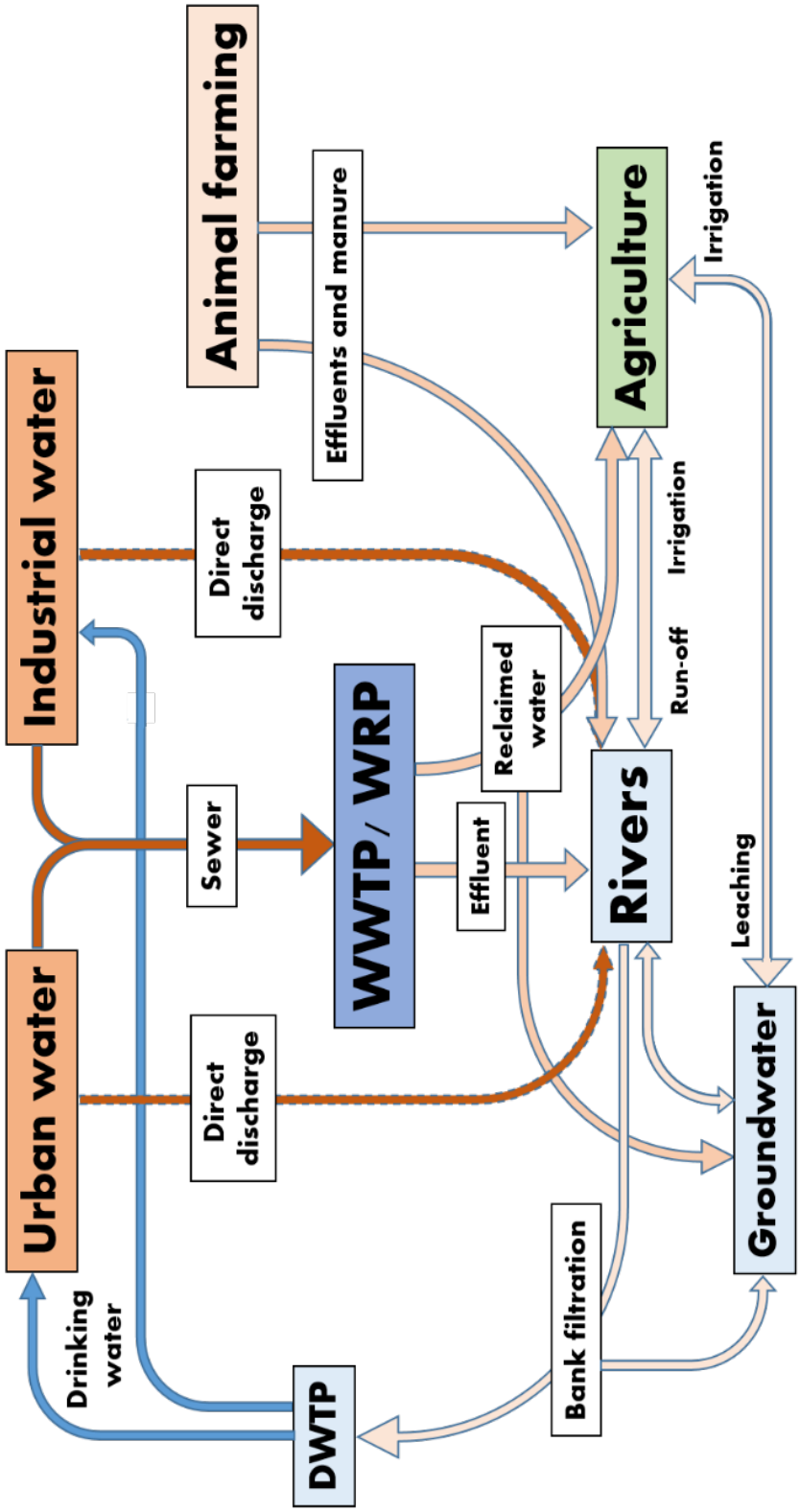


Figure 1.2. Anthropogenic and natural water resources and their connections. DWTP: drinking water treatment plant; WWTP: wastewater treatment plant; WRP: water reclamation plant (based on Petrović et al., 2003)

related transformation products (TPs). The list of compounds that could be considered CECs is continuously growing and, for example, according to the registered and pre-registered substances in REACH, between 30,000 and 50,000 industrial chemicals are present in daily-use products and, thus, they could end up in the environment (Dulio et al., 2018).

According to the European Commission (EC) public data, only 40% of the surface waters are in good environmental status and over one-third of the river basin districts in Europe are cross-border, which has implications in the management of surface water (European Commission, 2022c). European legislation regarding the regulation of CECs in water started in 2000 with the Water Framework Directive (European Union, 2000), followed by its daughter directives the 2008/105/EC Environmental Quality Standards Directive (European Union, 2008), amended by the Directive 2013/39/EU, including the final list of Priority Substances (European Union, 2013). This legislation encouraged all European Member States to extensively monitor relevant CECs and to establish River Basin Management Plans for all the EU river basins, pointing towards a good chemical and ecological status. The list of Priority Substances englobe 45 compounds for which Environmental Quality Standards (maximum concentration that can be found in surface water and biota) needs to be met and ensured by the EU member states. Directive 2008/105/EC included a watch list mechanism to intensify the monitoring programs of selected emerging pollutants that lack of sufficient information for their potential regulation. Four different watch lists have been published since 2015 including pharmaceuticals, pesticides or industrial chemicals (European Commission, 2015, 2018, 2020, 2022a). Now, in 2022, the EC adopted a proposal to revise the list of Priority Substances in surface waters and 24 CECs, including PFAS, pesticides, bisphenol A, and pharmaceuticals, are being considered as potential candidates (European Commission, 2022b). The protection of water resources from chemical pollution by the EU Water Framework Directive is complemented by the Groundwater Directive (2006/118/EC). This directive sets groundwater quality standards for nitrates (50 mg/L), and pesticides and their transformation products (100 ng/L for individual compounds and 500 ng/L for the sum of individual compounds).

Regarding pesticide use-related regulation, in 2009, the EC published the Sustainable Use of Pesticides Directive (Directive 2009/128/EC)

(European Commission, 2009a), in which the member states were required to adopt National Action Plans to reduce the risks and impacts associated to pesticide usage. This Directive indicates several measures to adopt to avoid pesticide pollution of the aquatic environment and drinking water, such as the banning of aerial spraying (only allowed in specific situations and under strict conditions), prioritization of non-chemical methods for pest control, promotion of organic farming or the implementation of the Integrated Pest Management (IPM) approach. The IPM is one of the tools for low-pesticide-input pest management that all professional users must implement. It involves actions pointing towards the prevention and/or suppression of crops harmful organisms by limiting, on a cost-effective way, the use of pesticides. An evaluation of the same EC in 2020 regarding the effectiveness and impact of the implementation of the Sustainable Use of Pesticides Directive stated that, despite the positive progress in terms of pesticide handling and farmers' awareness on the rational and sustainable use of pesticides, the lack of an economically competitive alternative to pesticides lead farmers to a dead-end. This was reflected earlier in section 1.1 when discussing the statistics of pesticide sales in the last decade.

Thus, it is not surprising that the legislation also extends to the control and management of pesticide residues in food and feed, mainly through the Regulation (EC) No 396/2005 (European Commision, 2005). Evidences of the pesticide contamination of food products are described in section 1.4. The EC and the member states, in association with the European Food Safety Authority (EFSA), set the legal limits for pesticide residues in food and feed, these are, the Maximum Residue Levels (MRLs). After a risk assessment evaluation performed by the EFSA, in which the active substances undergo ecotoxicological and human and animal toxicological tests, limits on the acceptable concentration of each compound (expressed in mg/kg) were set for each commodity evaluated. These MRLs have been set for pesticides currently in use or used in the past for food production and, at least, for 315 food products. However, not all existing commodities have MRLs for each active substance and not all pesticides currently in use were covered in these tests. Therefore, a default value of 0.01 mg/kg was established for these cases.

Now that the actual agricultural context and the contaminants of emerging concern have been described, the following sections will focus

on two major sources of organic contamination in the agricultural field: the use of pesticides and the use of reclaimed water for crop irrigation. The application of digested sludge as soil amendments, which is also a relevant source of organic contamination into the agricultural environment, is out of the scope of this doctoral thesis. Firstly, a brief literature review on the occurrence of CECs in the agricultural environment is performed. Then, an analysis of the state-of-the-art of advanced treatments for water regeneration and their efficacy in terms of CECs removal is done.

1.4. CECs in agricultural environments: occurrence and monitoring approaches

The research on this field has boosted in the last decade, with an increasing number of studies showing the ubiquitous presence of pharmaceuticals, pesticides, and other classes of CECs in the different compartments of the agricultural environment. These pollutants come from crop irrigation with either reclaimed water or other water sources and the direct application of plant protection products to the cultivated fields. Once released into the agricultural environment by any of the aforementioned sources, CECs are transferred to the soil, where they can stay stable and accumulate, leach to groundwater or move into water courses by runoff processes, photo- or bio-degrade leading to the formation of TPs, or even be taken up by crops. Depending on the physical-chemical properties of the compounds, in addition to the soil and plant properties and the pH of the aqueous solution, the availability of CECs to the different crops could vary (Malchi et al., 2014; Y. L. Zhang et al., 2014). CECs can be transferred from soils to the plant roots and then accumulate in the edible parts of the vegetables with the subsequent risk of human or animal exposure to them (Al-Farsi et al., 2017). However, depending on the chemicals and plant physiological properties, the bioaccumulation pattern may vary. Thus, Malchi et al. observed higher concentrations of non-ionic pharmaceuticals in crops than the ionic pharmaceuticals, as well as strong differences in pharmaceutical concentrations between roots and leaves (Malchi et al., 2014). Moreover, the long-term exposure of crops and soils to CECs (e.g. irrigation with reclaimed water for more than a single growing period) has been proven to facilitate the uptake of some of them by plants, showing higher concentrations year after year (Christou et al., 2017).

Table 1.1 summarizes the occurrence of CECs in the agricultural environment, based on representative studies that investigated the presence of these pollutants in the various agricultural matrices, from water to the edible parts of crops. Some of these studies show a relationship between the concentration detected in the water used for irrigation, the soil, and the crop growing in that soil (Ben Mordechay et al., 2021; Calderón-Preciado et al., 2011; Christou et al., 2017; Tao et al., 2021; Wu et al., 2014). Despite the low concentrations that are usually present in these compartments, the continuous release of CECs to the environment may potentially pose an ecotoxicological risk to aquatic and terrestrial organisms. In water, the concentrations found for these compounds range normally between the ng/L to the low µg/L level. Then, in soils and plants, these concentrations vary, depending on the physical-chemical properties of the compounds, from the ng/kg to the µg/kg level.

Table 1.1. CECs detected in the different agricultural environmental compartments, the contamination source and their concentrations.

Compound	Source	Water (ng L^{-1})	Soil (µg Kg^{-1})	Plant	Plant conc. (µg Kg^{-1})	Reference
Pharmaceuticals						
Diclofenac	RW	25-49	0.06-0.35	Tomato	1.3-11.6	(Christou et al., 2017)
	RW	0.7	-	-	-	(Wu et al., 2014)
	RW	1,524 (M)	n.d.	-	-	(Ben Mordechay et al., 2021)
Sulfamethoxazole	RW	25-55	0.38-0.98	Tomato	0.2 - 1.6	(Christou et al., 2017)
	RW	0.3	-	-	-	(Wu et al., 2014)
Trimethoprim	RW	22-73	0.15-0.62	Tomato	0.1 - 3.4	(Christou et al., 2017)
	RW	0.4	-	-	-	(Wu et al., 2014)
Acetaminophen	RW	1.9	-	-	-	(Wu et al., 2014)
Atenolol	RW	27	-	-	-	(Wu et al., 2014)
	RW	4,697 (M)	0.6 (M)	-	-	(Ben Mordechay et al., 2021)
Caffeine	RW	11		Celery	0.2	(Wu et al., 2014)
	RW - SF	238 - 791	-	Apple	0.02 – 15.5	(Calderón-Preciado et al., 2011)
	RW - SF	238 - 791	-	Alfalfa	<10.6 – 13.9	(Calderón-Preciado et al., 2011)

Table 1.1. continued

Compound	Source	Water (ng L^{-1})	Soil (µg Kg^{-1})	Plant	Plant conc. (µg Kg^{-1})	Reference
Carbamazepine	RW	4.2	-	Celery Lettuce Cucumber Cabbage	0.6 0.04 0.02 0.04	(Wu et al., 2014)
	RW	9,354 (M)	49.5 (M)	-	-	(Ben Mordechay et al., 2021)
Lamotrigine	RW	6,982 (M)	61.9 (M)	-	-	(Ben Mordechay et al., 2021)
Gemfibrozil	RW	0.4	-	-	-	(Wu et al., 2014)
Ibuprofen	RW	11	-	-	-	(Wu et al., 2014)
Naproxen	RW	0.4	-	Cabbage Cucumber	0.09 0.05	(Wu et al., 2014)
Diazepam	RW	20	-	-	-	(Wu et al., 2014)
4-amino antipyrine	RW	5,111 (M)	57.8 (M)	-	-	(Ben Mordechay et al., 2021)
Venlafaxine	RW	3,317 (M)	217.6 (M)	-	-	(Ben Mordechay et al., 2021)
Azithromycin	RW	1,368 (M)	n.d.	-	-	(Ben Mordechay et al., 2021)
Ciprofloxacin	RW	1,166 (M)	-	-	-	(Ben Mordechay et al., 2021)
Clarithromycin	RW	230 (M)	14.9 (M)	-	-	(Ben Mordechay et al., 2021)
Personal care products						
Triclosan	RW	3.2	-	Carrot	0.4	(Wu et al., 2014)
Galaxolide	RW	149 - 1023	-	Alfalfa	<0.01 - 16.9	(Calderón-Preciado et al., 2011)
Drugs of abuse						
Cotinine	RW	445 (M)	1.4 (M)	-	-	(Ben Mordechay et al., 2021)
Nicotine	RW	69,623 (M)	12 (M)	-	-	(Ben Mordechay et al., 2021)
Pesticides						
DEET	RW	181	-	Carrot	2.8	(Wu et al., 2014)
Dimetomorph	RW – SW - DA	-	-	Lettuce Tomato Broad beans	18.7 172 2.0	(Margenat et al., 2019)
Carbendazim	RW – SW - DA	-	-	Lettuce	1.0	(Margenat et al., 2019)
Indoxacarb	RW – SW - DA	-	-	Cauliflower Broad beans	3.63 2.15	(Margenat et al., 2019)

Table 1.1. continued

Compound	Source	Water (ng L^{-1})	Soil (µg Kg^{-1})	Plant	Plant conc. (µg Kg^{-1})	Reference
Acetamiprid	-	-	4.5 (m.)	Wheat	2.2 (m.)	(Tao et al., 2021)
	DA	-	-	Bell pepper Cabbage	n.d. – 50 n.d. - 100	(Jallow et al., 2017)
Imidacloprid	-	-	24.3 (m.)	Wheat	1.2 (m.)	(Tao et al., 2021)
	DA	-	-	Tomato Bell pepper Eggplant Cucumber Zucchini Apple Grapes	n.d. – 510 n.d. – 10 n.d. – 90 50 – 1,200 n.d. – 80 200 – 650 n.d. - 980	(Jallow et al., 2017)
Carbendazim	-	-	63.8 (m.)	Wheat	715 (m.)	(Tao et al., 2021)
Tebuconazole	-	-	123.7 (m.)	Wheat	4.4 (m.)	(Tao et al., 2021)
Hexaconazole	-	-	114.2 (m.)	Wheat	3.9 (m.)	(Tao et al., 2021)
Thiacloprid	-	-	-	Lettuce	140 – 3,300	(Algharibeh & AlFararjeh, 2019)
Dicofol	DA	-	-	Tomato	<LOQ - 130	(Salghi et al., 2012)
Endosulfan	DA	-	-	Tomato	9 – 1,200	(Salghi et al., 2012)
Cypermethrin	DA	-	-	Tomato	<LOQ - 300	(Salghi et al., 2012)
	DA	-	-	Tomato Eggplant Watermelon Grapes	20 – 240 n.d. – 130 n.d. – 90 n.d. - 280	(Jallow et al., 2017)
Deltamethrin	DA	-	-	Tomato	3 – 1,000	(Salghi et al., 2012)
	DA	-	-	Bell pepper Watermelon Apple Grapes	n.d. – 20 60 – 290 200 – 320 32 - 380	(Jallow et al., 2017)
Diazinon	DA	-	-	Apple	n.d. - 80	(Jallow et al., 2017)
Monocrotophos	DA	-	-	Tomato Cucumber Watermelon	n.d. – 20 n.d. – 40 n.d. - 20	(Jallow et al., 2017)
Propargite	-	-	-	Peach	1,800 – 6,000	(Algharibeh & AlFararjeh, 2019)

Table 1.1. continued

Compound	Source	Water (ng L^{-1})	Soil (µg Kg^{-1})	Plant	Plant conc. (µg Kg^{-1})	Reference
Thiamethoxam	-	-	-	Sweet pepper	100 – 1,500	(Algharibeh & AlFararjeh, 2019)
Industrial chemicals						
Benzotriazole	RW	26,238 (M)	053.5 (M)	-	-	(Ben Mordechay et al., 2021)
2-Mercapto-benzothiazole	RW – SW - DA	-	-	Lettuce	18.3	(Margenat et al., 2019)
Methylparaben	RW – SW - DA	-	-	Lettuce Tomato Broad beans	150 23.3 28.7	(Margenat et al., 2019)

RW: reclaimed water; SW: surface water; DA: direct application; PCP: personal care product; (M): maximum concentration; (m.): mean concentration; n.d.: not detected

However, this could only be the iceberg peak as there is a gap of knowledge regarding the existence and occurrence of TPs derived from the biotic and abiotic degradation of CECs that may occur from the outlet of the regeneration treatment plants to the point of irrigation, or once they are released into the agricultural fields. Diverse studies have been able to identify and detect some of this unknown compounds in reclaimed water, soils and plants (Beretsou et al., 2022; Martínez-Piernas et al., 2021). In addition to TPs, human metabolites of pharmaceuticals and drugs of abuse represent another type of "new" contaminants entering into the environment via wastewaters. In fact, some TPs or metabolites can be present in reclaimed water at concentrations comparable to those of the parent compounds (Bahlmann et al., 2014) and in some cases, they may even represent a higher ecotoxicological risk (W. L. Wang et al., 2018). For example, Bahlmann et al. detected eight different metabolites of the anticonvulsant pharmaceutical carbamazepine in wastewater and evaluated their removal efficiency during the wastewater treatment. Both carbamazepine and five metabolites showed negligible degradation during this process and one of the metabolites, 10,11-dihydro-10,11-dihydroxy-carbamazepine, was found at a higher concentration than its parent compound (Bahlmann et al., 2014). In another study, researchers demonstrated that metabolites of carbamazepine had lower sorption affinity and higher mobility in soils, resulting on a higher leach to groundwater and showing the importance of metabolites or TPs in the environment (Paz et al., 2016).

In this context, this thesis aimed at better understanding the fate of organic pollutants in the agrarian environment by, on one hand, developing and applying analytical methodologies for the determination of pesticides in plant origin foods and, on the other hand, characterizing the CEC footprint of a reclaimed water-based irrigation system in an agricultural area.

1.5. CEC removal with advanced and alternative wastewater treatments

Conventional treatment of urban wastewater in large communities consists of a pre-treatment (bar grating to remove large size objects), a primary treatment primary sedimentation) and a secondary treatment (conventional activated sludge (CAS) bioreactor and a secondary sedimentation with recycling sludge). Conventional wastewater treatment is mainly designed to remove the organic matter and, to some extent, nutrients (nitrogen and phosphorus). Sometimes, additionally, CAS continues with a disinfection stage to inactivate pathogens (Hendricks, 2010). The effluents of the wastewater treatment plants (WWTPs) are released in the natural water streams or the oceans under the current policy limitations, but no other reuse application is authorised for this water. In this context, the European Union has recently enabled an integrated regulation for all Member States to standardize the reuse of wastewater for agricultural irrigation, establishing strict quality requirements, mainly in terms of biological and chemical parameters, such as pathogens or the total suspended solids (The European Parliament and the Council, 2020). In Spain, the reuse of wastewater was previously regulated through the RD 1620/2007 (Ministerio de la Presidencia, 2007). In the following years, the use of reclaimed water increased for different purposes. Figure 1.3 shows the relative distribution of reclaimed water use in Spain during the period 2011-2020 (Instituto Nacional de Estadística, 2022). As can be observed, the use for agricultural irrigation has increased up to 70% in the last years, being the main destination for reclaimed water. However, regulation against CECs is scarce and limited to the WFD and their daughter directives. Taking into account that CAS treatments are not designed for the elimination of CECs, it is not surprising the fact that the effluents are permanently contaminated with dozens or even hundreds of these compounds (Petrie et al., 2014; Tang et al., 2020).

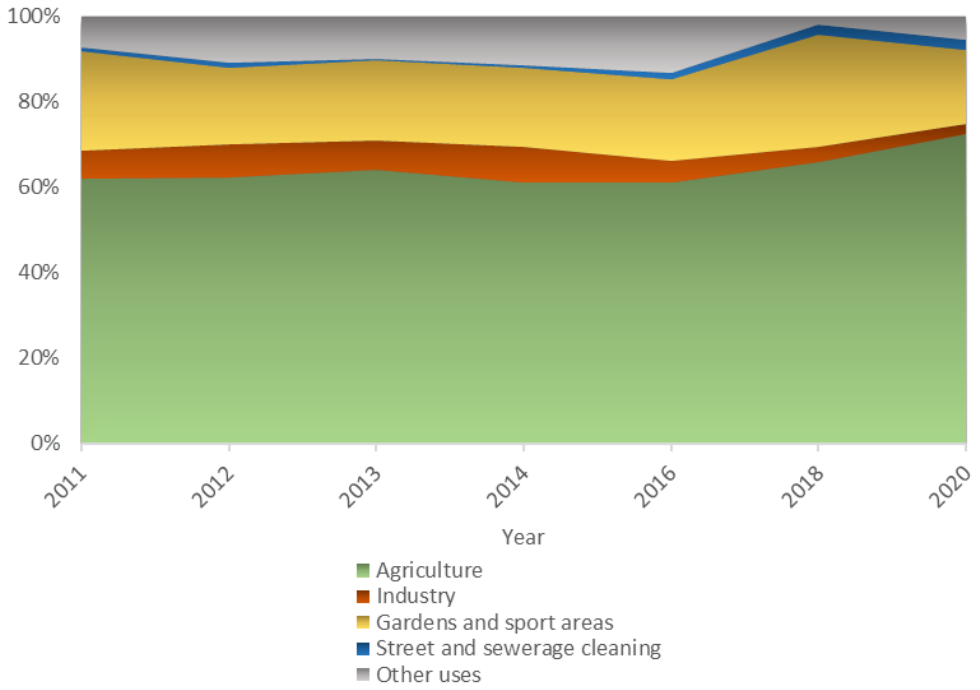


Figure 1.3. Relative distribution of reclaimed water use in Spain during the period 2011-2020 (Instituto Nacional de Estadística, 2022).

New water treatment technologies have been developed in the last years including improvements in the biological processes such as membrane biological reactors (MBR), biological aerated filters, moving bed biological reactors or granular sludge reactors. These advanced treatments are already being implemented at real scale allowing the reduction of sludge overproduction, while improving water quality (U.S. Environmental Protection Agency, 2013). In biological processes, polarity of CECs may be determining to estimate the removal efficiency. Thus, MBR systems show, in general, higher biodegradation efficiency for hydrophobic compounds than those with a high polarity, that are usually released in the effluent water (Roccaro et al., 2013). Some studies relate the removal efficiency of these systems to the rapid sludge adsorption of the most hydrophobic compounds relative to the hydraulic retention time (Xue et al., 2010).

Various advanced treatments are nowadays available to be implemented after the biological treatment in the so-called tertiary treatment, which contribute to achieve high water quality standards, in

terms of CEC removal. Table 1.2 summarizes the efficacy of CEC removal of the most commonly used tertiary and advanced treatments. Depending on the treatment methodology, chemicals may be bio or photo-transformed, chemically oxidized, adsorbed, filtrated or finally mineralized. Figure 1.4 outlines the processes involved in the different wastewater treatment methods.

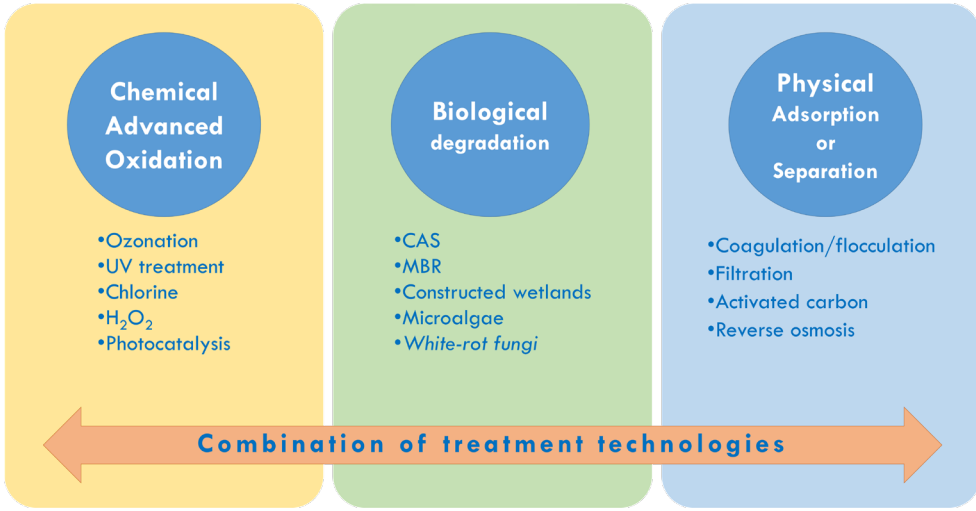


Figure 1.4. Scheme of the most typical processes involved in wastewater treatments.

Among other tertiary treatments, advanced oxidation processes have a great implementation in the WWTPs improving technologies. These treatments, based on the generation of strong radical oxidants (e.g. ·OH, SO4·-, etc.) or the use of highly reactive molecules (O3, Cl2), include catalytic ozonation, ultraviolet (UV) irradiation, and combined processes UV/ H2O2, UV/chlorine, among others (Salimi et al., 2017). Although these advanced treatments usually show great removal efficiencies, most of this success relies in the formation of oxidation by-products derived from the oxidation of the contaminants (Borowska et al., 2016; Choi et al., 2006). . Although UV irradiation has not been proved to be efficient in the elimination of pharmaceuticals itself, they have shown, in fact, promising results in the removal of some recalcitrant compounds such as N-Nitrosodimethylamine, for which, for example, reverse osmosis was not effective at all (I. Kim et al., 2009; Shad et al., 2019). A study compared the performance of UV and UV/ H2O2 on the removal of pharmaceuticals and personal care products, showing the lack of effectiveness of

the UV treatment for most of the compounds, while great removal efficiencies were obtained for the UV/ H2O2 combination (I. Kim et al., 2009). Thus, it seems that the combination of both technologies may be an interesting solution for wastewater purification in terms of CECs.

Membrane processes have been the most frequently used for water and wastewater treatment, including reverse osmosis, nanofiltration, ultrafiltration and microfiltration (S. Kim et al., 2022). In the case of reverse osmosis, size exclusion and electrostatic forces are supposed to be the most influent mechanisms regarding the efficiency of the treatment. Therefore, despite its great efficiency on CECs removal, in general, molecules with low molecular weight, including CECs as N-Nitrosodimethylamine, have shown poor elimination rates (Shad et al., 2019). Nevertheless, reverse osmosis alone or combined with biological processes usually show an excellent performance in CEC removal (Dolar et al., 2012). The main drawback relies on the high energy consumption of this technology that keeps it out of range for most of the WWTPs.

Adsorptive processes are also used in wastewater treatment, including the use of activated carbon, zeolite nanoparticles, among others (S. Kim et al., 2022). Although adsorption becomes the main removal process, some of these treatments combine the biological and adsorptive mechanisms when the sorbent becomes the basis structure for the growth of biofilms as it occurs in the granular activated carbon or the biological activated carbon. Different studies have evaluated the biodegradation capacity of these systems compared to the removal associated to the adsorption, concluding that both processes occurred simultaneously with different results depending on the chemical (Piai et al., 2020).

Taking into consideration the fact that no treatment in solitude has a fully efficient performance, some reviews in the field point towards the use of hybrid processes that combine CAS or MBR treatments with advanced oxidation processes or bioremediation alternatives as the use of White-Rot Fungi (WRF) (Grandclément et al., 2017) or microalgae-based systems, among others (H. T. Nguyen et al., 2021).

Table 1.2. Summary of the most typical tertiary and advanced treatments and CECs removal efficiencies.

CECs general removal*	CECs	Removal efficiency (%)	Reference
MBR			
Low	4-nonylphenol	75%	(Xue et al., 2010)
	17α-Ethinylestradiol	97%	
	4-Octylphenol	79%	
	Trimethoprim	>90%	
	Diclofenac	<20%	
	Carbamazepine	<20%	
	Sulpiride	<20%	
	Erythromycin	>90%	
	DEET	>90%	
	Acetaminophen	100%	(Kamaz et al., 2019)
	Amoxicillin	100%	
	Atrazine	<25%	
	Estrone	98%	
	Atenolol	77%	(M. Kim et al., 2014)
	Clarithromycin	-34%	
	Codeine	99%	
	Cotinine	98%	
	Diazepam	54%	
	Erythromycin	12%	
	Metformin	99%	
	Propranolol	16%	
	Sulfamethoxazole	66%	
	Atenolol	87%	(Dolar et al., 2012)
	Carbamazepine	51%	
	Metoprolol	71%	
	Lorazepam	48%	
	Propranolol	67%	
	Sulfamethoxazole	69%	
Coagulation			
No/negligible	Bisphenol A	0-3%	(Choi et al., 2006)
	Nonylphenol	4-7%	
GAC/PAC adsorption			
Low/High	4-nonylphenol	97%	MBR + GAC (L. N. Nguyen, Hai, Kang, et al., 2013)
	Acetaminophen	87%	
	Carbamazepine	32%	
	Diclofenac	15%	
	Metronidazole	40%	
	Naproxen	45%	
	Salicylic acid	98%	
	Bisphenol A	25-40%	PAC (Choi et al., 2006)
	Nonylphenol	25-40%	
	Bisphenol A	100%	GAC (Choi et al., 2006)
	Nonylphenol	100%	
NF/RO			
High	Atenolol	99-100%	RO (Dolar et al., 2012)
	Carbamazepine	99-100%	
	Clarithromycin	99-100%	RO (Dolar et al., 2012)
	Erythromycin	99-100%	
	Metoprolol	99-100%	
	Ofloxacin	99-100%	
	Clopidogrel	99-100%	
UV disinfection			
Low	Acetaminophen	~0%	(I. Kim et al., 2009)

Table 1.2. continued

CECs general removal*	CECs	Removal efficiency (%)	Reference
Low	Acetaminophen	~0%	(I. Kim et al., 2009)
	Atenolol	~30%	
	Azythromycin	~5%	
	DEET	~5%	
	Erythromycin	~10%	
	Indomethacin	~45%	
	Ketoprofen	~95%	
	Levofloxacin	~25%	
	Naproxen	~20%	
	Sulfamethoxazole	~85%	
	Theophylline	~50%	
UV/chlorine			
n.d.	Carbamazepine	49%	(Cerreta et al., 2020)
	Diclofenac	90%	
	Imidacloprid	90%	
	Sulfamethoxazole	90%	
UV/chlorine/ H_2O_2			
n.d.	Acridine	43%	(Kudlek, 2020)
	Benzocaine	22%	
	B-estradiol	80%	
	Ibuprofen	41%	
UV/chlorine/O_3			
n.d.	Acridine	55 %	(Kudlek, 2020)
	Benzocaine	55 %	
	B-estradiol	100 %	
	Ibuprofen	65 %	
UV/H_2O_2			
High	Atenolol	97%	MBR + UV/H_2O_2 (Augsburger et al., 2021)
	Carbamazepine	98%	
	Estrone	92%	
	Acetaminophen	90%	(I. Kim et al., 2009)
	Atenolol	100%	
	Azythromycin	95%	
	DEET	95%	
	Erythromycin	95%	
	Indomethacin	95%	
	Ketoprofen	100%	
	Levofloxacin	100%	
	Naproxen	100%	
	Sulfamethoxazole	100%	
	Theophylline	95%	
Ozonation			
High	Bisphenol A	60-100%	(Choi et al., 2006)
	Nonylphenol	89-100%	
	Cetirizine	92-95%	(Borowska et al., 2016)
	Fexofenadine	83-96%	
		86-99%	
	Hydrochlorothiazide	86-99%	(Borowska et al., 2016)
Photocatalysis (TiO_2 nanotubes)			
High	MCPA	88%	(Yin Ye et al., 2018)
	2,4-dichlorophenol	~100%	(Luo et al., 2018)
	Metoprolol	62-87%	(Y. Ye et al., 2018)
Constructed wetlands			
Low/Medium	Acetaminophen	90%	(Ávila et al., 2021)
	Ketoprofen	64-97%	

Table 1.2. continued

CECs general removal*	CECs	Removal efficiency (%)	Reference
Low/Medium	Diclofenac	64-97%	(Ávila et al., 2021)
	Gemfibrozil	92-100%	
	Carbamazepine	40-50%	
	Lorazepam	45-54%	
	Caffeine	~10-90%	(Reyes-Contreras et al., 2011)
	Naproxen	~15-65%	
	Triclosan	~30-65%	
	Galaxolide	~10-50%	
	Butylated hydroxytoluene	~5-30%	
	Carbamazepine	~5-30%	
Microalgae			
Medium	17β-estradiol	42-100%	(Hom-Diaz et al., 2017)
	17α-ethinylestradiol	60-100%	
	Acetaminophen	>99%	
	Ibuprofen	>98%	
	Naproxen	69%	
	Acetaminophen	>99%	(de Wilt et al., 2016)
	Diclofenac	40-60%	
	Ibuprofen	100%	
	Metoprolol	>99%	
	1H-benzotriazole	79%	(Gatidou et al., 2019)
	5-methyl-1H-benzotriazole	>97%	
	5-chlorobenzotriazole	52%	
	Xylytriazole	>42%	

GAC: Granular Activated Carbon; PAC: Powdered Activated Carbon; O_3: Ozonation; BAC: Biological Activated Carbon; MF: microfiltration; UF: Ultrafiltration; NF: Nanofiltration; RO: Reverse Osmosis; UV: Ultraviolet; H_2O_2: Peroxidation.
* Based on Roccaro (2018).

Among the above mentioned technologies for wastewater treatment, bioremediation strategies represent an interesting alternative for CEC removal. Bioremediation offers the possibility to destroy or render hazardous contaminants using natural biological activity and involves the application of microbes to attenuate the environmental pollution. It is usually a low-cost technology, with a limited maintenance requirements and a high public appreciation as a green technology (Vidali, 2001). Indigenous microorganisms found in contaminated locations hold the key to solving most of the challenges associated with biodegradation and bioremediation of hazardous chemicals (Verma & Jaiswal, 2016).. Microbial removal includes adsorption to cell surfaces, intracellular accumulation or biodegradation through the exo and endo-enzymatic system. For an optimal bioremediation effectiveness, some environmental parameters may be manipulated to allow microbial growth and degradation activity at a faster rate (Vidali, 2001). One of main advantages of bioremediation is that, normally, the natural process involved generates harmless residues (mainly containing carbon dioxide, water and cell biomass) and, once the

contaminant is degraded, the biodegradative population declines and, so, the process is auto-regulated (Vidali, 2001). However, some drawbacks may be attached to these strategies: it often takes longer than other treatments, it is limited to some specific contaminants and, further research is needed to scale-up from bench and pilot-scale studies to full-scale field operations. Nevertheless, diverse bioremediation systems have been tested for wastewater purification and some of them have already been applied at a full-scale WWTP. These systems include the phytoremediation-based constructed wetlands, the use of microalgae or white-rot fungi bioreactors.

Constructed wetlands are a consolidated green technology with a great settlement in small communities and rural areas (Álvarez et al., 2017). They are characterized by a low-cost maintenance and a great organic matter and nitrogen removal and the effective elimination of pathogenic microorganisms (Castillo-Valenzuela et al., 2017). Furthermore, despite the little research, a few studies have proven the removal of specific industrial contaminants such as nitrobenzene and some phenolic compounds (Kirui et al., 2016; Rossmann et al., 2012). These alternative engineered natural treatments have also the potential to remove a few pharmaceuticals (Ávila et al., 2021), although future research should focus on a more wide-scope screening of relevant wastewater-derived contaminants, and the plausible TPs formation during the treatment.

On the other hand, white-rot fungi (WRF) are a collection of fungal species part of the taxonomical division of basidiomycetes characterized by their ability to efficiently break down lignin to release its carbohydrates for a further metabolism. Lignin is a hardly degradable polymer that gives the structure to wood and WRF, combining extracellular ligninolytic enzymes (laccases, lignin peroxidases, manganese peroxidases, etc.) with organic acids, mediators and other supporting enzymes, are able to metabolize it (Mir-Tutusaus et al., 2018). This enzymatic cocktail makes these organisms a promising tool for contaminants removal.

As above mentioned, CECs are normally detected in wastewater streams at low concentrations. Microorganisms responsible of the biodegradation step in CAS or MBR systems typically use these compounds as growth substrates and, thus, low concentrations difficult this task as microbes will use another source of carbon for this growth (Harms et al.,

2011). Conversely, WRF degrade organic pollutants as part of its secondary metabolism and, therefore, it does not depend on the CECs concentration. Moreover, the non-specific enzymatic system broadens the substrate chemical range, allowing degrading several micropollutants, some of them highly recalcitrant. This has been tested for pharmaceuticals, and personal care products (Jelic et al., 2012; Marco-Urrea et al., 2010; Rodarte-Morales et al., 2011), hormones, UV-filters, industrial chemicals and pesticides (Blánquez & Guieysse, 2008; L. N. Nguyen et al., 2014; L. N. Nguyen, Hai, Yang, et al., 2013). Although bench studies have had promising performances for many of the evaluated contaminants, further research is needed at pilot and full-scale plants to adapt the processes to the real environment. Problems regarding the sterility of the process, the artificial supply of nutrients, the interaction of WRF with other microorganisms or the understanding of the biochemical pathways involved in CECs transformation should be addressed before the scale-up (Mir-Tutusaus et al., 2018).

In the last years, another biotechnological approach under development for wastewater treatment is based on the microalgae's ability to degrade and assimilate common organic and inorganic compounds, including organic micropollutants (H. T. Nguyen et al., 2021). The most typically used reactors are the high-rate algal ponds, open systems extensively employed in large-scale installations due to their low cost and low energy usage, easy scale-up and maintenance and, on the other hand, the closed systems such as tubular photobioreactors. These systems perform a better control of operational parameters, a higher light use efficiency, better mixing and lower risk of contamination (H. T. Nguyen et al., 2021). One of the advantages of these technologies are the capability of microalgae to sequestrate CO2 during photosynthesis, thus reducing greenhouse-gas emissions and the reuse of microalgae biomass as fertilizer in agriculture (de Wilt et al., 2016; Razzak et al., 2013). The CEC elimination efficiency of these systems is based on diverse removal processes including bioadsorption to biomass, bio and photodegradation and volatilization, depending on the physical-chemical properties of the target compounds (H. T. Nguyen et al., 2021). As other technologies, hydrophobic chemicals are prone to sorption and compounds with higher Henry's law constant will be to volatilization. Various studies have evaluated the removal efficiency of microalgae-based treatments, with dissimilar but promising results for pharmaceuticals, pesticides and

industrial chemicals (Table 1.2) (de Wilt et al., 2016; Gatidou et al., 2019; Hom-Diaz et al., 2017).

In this context, in the framework of this thesis, two of these bioremediation strategies, WRF and microalgae systems, were evaluated in the removal of a few selected pesticides of special concern.

1.6. Analytical methodologies

Under the scenario above described, and from the point of view of analytical chemistry, there is a clear need for obtaining high quality information on the occurrence of CECs and their TPs in environmental and food matrices. These data can be obtained by (1) quantitative target analysis of a limited number of chemicals and (2) qualitative or semi-quantitative non-target and suspect wide-scope screening of contaminants, which allows obtaining the contamination footprint of the sample. Both approaches demand technological improvements that allow developing methodologies with enough sensitivity and selectivity and obtaining accurate identification, confirmation and reliable quantification (especially in target methodologies) of CECs in complex matrices. Nowadays, these analytical requirements are fulfilled with the use of mass spectrometry (MS) coupled to gas chromatography (GC) or liquid chromatography (LC).

To guarantee the existence of tools for pesticide analysis, Regulation (EC) No. 1107/2009 (European Commission, 2009b) established the necessity of analytical methods for the measurement of residues of pesticides in/on food or feed, and their availability prior to the market authorisation of the corresponding pesticides. To reach sufficient sensitivity with respect to the MRLs established, analytical methods should be optimized in terms of sample preparation and the analytical procedure. This will be determined by the pesticides physical-chemical properties and the complexity of the commodity matrix. Sample preparation could be the most relevant step of the whole analytical process because it aims at removing the matrix interferences and selectively extracting the compounds of interest with minimal analyte losses (Villaverde et al., 2016). For this purpose, different techniques have been used, standing out solid phase extraction (SPE) with the use of a huge variety of selective sorbents (Picó et al., 2007), and the QuEChERS approach, developed by

Anastassiades et al. (Anastassiades et al., 2003), with the multiple variants that have improved this technique in the last years (Barbieri et al., 2019; Christia et al., 2015). These techniques have proven to reduce the use of extractive organic solvents, facilitate the technical procedure, and allow the multiresidue analysis. Lately, many studies have focused on the automation of the total process to ease the routine monitoring (Picó et al., 2007; J. Wang et al., 2017). QuEChERS methods are based on a sample extraction with solvent, a salting-out partitioning of water and a clean-up by dispersive SPE. Depending on the matrix nature and the polarity range of the selected pesticides, different buffered salts are used for the salting-out partitioning and different sorbents are employed for the clean-up, including primary-secondary amines (PSA), octyldecylsilane (C18) or graphited carbon black (GCB). Therefore, QuEChERS approach allows multiple combinations of extraction and clean-up sorbents, resulting on a practical and wide-scope option for many commodities and pesticides (Villaverde et al., 2016).

Diverse extraction methods have been described for the great variety of organic micropollutants typically detected in the water bodies (Calderón-Preciado et al., 2011; Daniels et al., 2020; Gago-Ferrero et al., 2020; Loos et al., 2010). These methods are normally SPE-based procedures that employ different sorbents or a combination of them, depending on the physical-chemical properties of the target analytes. SPE sorbents may be weak anion exchange (WAX), anion and cation exchange resins (Strata-X), non-polar sorbents (Isolute Env+), or the Oasis hydrophilic-lipophilic balanced (HLB), the most common sorbent for broad chemical enrichment (Daniels et al., 2020; Gago-Ferrero et al., 2020; Liu et al., 2019). Although these sorbents have been mainly used for target analysis of preselected compounds, in the last years, a stronger concern has risen around the need of wide-scope screening of CECs in the water bodies (Menger et al., 2020). SPE cartridges stacked in series with a combination of some of the sorbents above described has been optimised for this purpose (Daniels et al., 2020). Furthermore, non-selective methodologies with soft clean-ups are also an attractive alternative to wide-scope screening. With this premise, lyophilisation allows analyte enrichment and low loss of compounds during the extraction despite the gathering of higher matrix effects (Hu et al., 2014; Y. Zhang et al., 2021).

Regarding the chromatographic stage for pesticides analysis, GC has been used for decades, due to the chemical nature of the classical pesticides. However, the chromatographic analysis of the new agrochemicals, which are more polar compounds, requires shifting to LC or derivatizing these compounds for GC analysis (Villaverde et al., 2016). Improvements in GC include the optimization of the capillary columns, the gas flow, new designs of the ovens (Vaclavik et al., 2014), or the development of new GC alternatives as the low-pressure GC (González-Curbelo et al., 2014) and the two dimensional chromatography GC x GC (Botitsi et al., 2011). Advances in LC can be summarized by the development of the ultra high performance LC (UHPLC) (with higher back-pressures compared to classical HPLC and, thus, better peak capacity, sensitivity and resolution), and new stationary phases (Villaverde et al., 2016). Apart from the reverse phase chromatographic columns, traditionally based on a stationary phase mainly constituted by C18 chains, which only covers from low to medium polar compounds, newer developments have broaden the polarity range for LC analysis. Polar-modified reverse phase columns or Hydrophilic Interaction liquid chromatography (HILIC) are the principal alternatives for the separation of highly polar chemicals(Bieber & Letzel, 2020; Villaverde et al., 2016)(Figure 1.5).

Multiresidue analysis and wide-scope screening of CECs need the coupling of a chromatographic separation step with MS analysis for a selective, sensitive and reliable identification and/or quantification of the compounds of interest. Depending on the aims of the analysis, the choice of the MS instrumentation is crucial. Firstly, the ionization mode will determine the polarity range of chemicals that will be analysed. Electron impact (EI) and chemical ionizations (CI) are two ionization options mostly used in combination with GC for the analysis of small apolar compounds (Figure 1.5). Then, the development of the atmospheric-pressure chemical ionization (APCI), a soft ionization technique suitable for both GC and LC systems, broadened the polarity range and the molecular size (Agüera et al., 2013). Nevertheless, the most popular and generic ionization source for emerging pollutants determination is the electrospray ionization (ESI). This technology allows performing MS analysis for the widest range of chemicals, including the most polar compounds (Figure 1.5). With a soft ionization, very little molecular fragmentation happens during the ionization process, leaving this issue to the MS analyser. It is widely employed as the interface

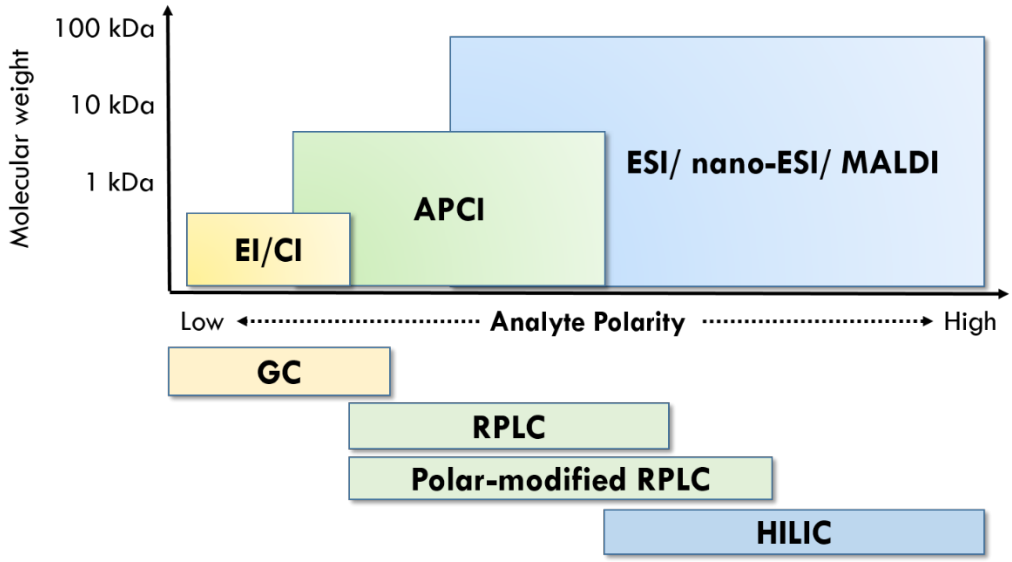


Figure 1.5. Instrumental capacity in terms of analyte polarity and molecular weight (based on Y. Wang et al., 2015)

between the LC and MS systems.

MS analysers can be divided into low resolution systems, mainly used for quantification of targeted compounds, and high resolution mass spectrometers for qualitative non-targeted wide-scope screening. For quantitative purposes, the low resolution triple quadrupole (QqQ) analyser has been the most reported analyzer in environmental water and wastewater (Agüera et al., 2013). With a great robustness and sensitivity, the selected reaction monitoring (SRM) mode allows the performance of a selective targeted quantitation, using two specific SRM transitions for each compound for its accurate confirmation in the sample. These SRM transitions should be as specific as possible to reliably confirm the compound of interest, and gather similar intensities in order not to hinder the identification of the compound when the second transition is not intense enough at low concentrations (Martínez Bueno et al., 2007). This is usually improved with a good sample preparation and the chromatographic separation. Another low resolution analyzer normally used for quantitative purposes is the hybrid triple quadrupole-linear ion trap, that gets to perform additional scan modes, increasing sensitivity (Agüera et al., 2013).

In contrast to low resolution, high resolution mass spectrometry (HRMS) has opened a new field in analytical chemistry enabling the possibility of obtaining the chemical profile of a sample, within the instrumental limitations, through untargeted data acquisition modes (Hollender et al., 2017). Hybrid systems as the quadrupole-time-of-flight (Q-TOF) or the orbitrap interfaced with a quadrupole (Q Exactive) or a linear ion trap (LTQ Orbitrap) are the main HRMS used for environmental analysis (Menger et al., 2020). These systems achieve mass accuracies below 5 ppm and mass resolution over 10,000 at full width at half maximum (FWHM) allowing the reliable calculation of elemental composition of detected features, the structure elucidation through the fragmentation pattern and reduce the necessity of reference standards for the confirmation of detected compounds (Moschet et al., 2013). Compound identification may be performed for known compounds, comparing HRMS data obtained with already created databases and reference libraries (suspect screening approach) or through the structure elucidation for unknown compounds and newly detected TPs (non-target screening and TPs identification) (Jelic et al., 2012; Liu et al., 2019; Newton et al., 2018).

Regarding the ecotoxicological risk assessment, many of these substances are being marketed with no or little information about their toxicity to non-target species. This ecotoxicological evaluation is indeed performed in some cases after entrance in the market. It is estimated that bioaccumulation potential data are only available for 1% of the chemicals registered in the European Union, while aquatic toxicity data are only accessible for 11% (Posthuma et al., 2019; Strempel et al., 2012). Therefore, an increasing effort is being made by researchers to provide enough information about the ecotoxicological risks associated to the presence of these compounds in the environment.

The studies conducted in this field with water matrices include effect-based monitoring for the detection of compounds with similar effects and the establishment of toxicity fingerprints, and effect-directed analysis to identify the drivers of toxicity (Dopp et al., 2019). Effect-based studies for the assessment of water quality measure effects caused by chemicals individually or in a mixture in cells or organisms through in vitro or in vivo bioassays. These activity assays provide data related with cytotoxicity,

genotoxicity, endocrine disruption, and stress responses (Dopp et al., 2019). However, the huge amount of compounds usually present in the environment, and considering the premise of the co-existence of unknown TPs, points towards the need of additional approaches that reduce the complexity and identify the toxicity-driving compounds: the effect-directed analysis. These methods connect the effect-based and chemical analysis of whole samples to identify those fractions or compounds responsible of any toxic activity. Moreover, in silico predictive tools based on quantitative structure-activity/toxicity relationship (QSAR/QSTR) models (Khan & Roy, 2022) for ecotoxicological risk assessment has gained popularity in the last years. These models contribute to cover the knowledge gaps regarding the persistence, bioaccumulation and toxicity properties of the chemicals present in the environmental mixtures, impossible to cover due to the diversity of the endangered species and the restrictions of toxicity testing, together with the complexity of the chemical footprint. Through all these in vivo, in vitro, and in silico methodologies, researchers evaluate the acute and chronic toxicity of chemicals and accumulate ecotoxicity data to end up in an estimation of concentration limits for each compound so to preserve a good ecological status.

1.7. Investigated pesticides

The pesticides investigated in this thesis include 42 compounds and metabolites belonging to ten different chemical groups (Table 1.3.). This list comprises: three acidic compounds (2,4-D, bentazone, and MCPA), five neonicotinoids (acetamiprid, chlothianidin, imidacloprid, thiacloprid, and thiamethoxam) , two chloroacetamide (alachlor, and metolachlor), eight triazines (atrazine, and its metabolites deisopropylatrazine and desethylatrazine, cyanazine, irgarol, simazine, terbuthylazine, and terbutryn), six organophosphates (azinphos ethyl, azinphos methyl, chlorfenvinphos, diazinon, dichlorvos, and dimethoate), eight organothiophosphates (fenthion, and its metabolites fenthion oxon, fenthion sulfone, fenthion sulfoxide, fenthion oxon sulfone, and fenthion oxon sulfoxide, malathion and its metabolite malaoxon), four phenylureas (chlortoluron, diuron, linuron, and isoproturon), two carbamates (methiocarb, and molinate), two anilides (diflufenican, and propanil), and one quinolone (quinoxyfen) and one hydroxybenzonitrile (bromoxynil).

These compounds present insecticide, herbicide and, to a lesser extent, fungicide, acaricide and molluscicide activities, among others.

This target list of pesticides was made based on their environmental relevance, their usage level in Europe, particularly those pesticides commonly used in Spain, their environmental occurrence, and their feasibility for LC-MS analysis. Compounds as alachlor, atrazine, chlorfenvinphos, dichlorvos, diuron, irgarol, isoproturon, quinoxyfen, and terbutryn were included in the Priority list in the field of water policy established in the Directive 2013/39/EU (European Union, 2013). On the other hand, neonicotinoid pesticides (acetamiprid, imidacloprid, thiamethoxam, thiacloprid, and chlothianidin), methiocarb, and diflufenican were included in, at least, one of the European Watch Lists (European Commission, 2015, 2018, 2022a).

Table 1.3. List of pesticides studied in this thesis, their molecular mass and Log K_{ow}, their main use, and European regulation

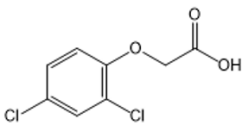
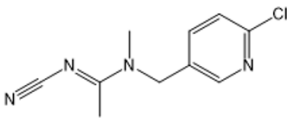
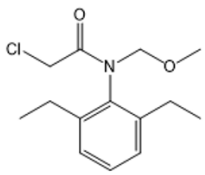
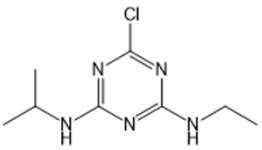
Name (structure)	CAS number [a]	Molecular mass [a] (g/mol)	Log K_{ow} [b]	Uses [b]	Regulated use [b]
2,4-D 	94-75-7	221	-0.82	Herbicide	Yes
Acetamiprid 	135410-20-7	222.7	0.80	Insecticide	Yes
Alachlor 	15972-60-8	269.77	3.09	Herbicide	No
Atrazine 	1912-24-9	215.68	2.70	Herbicide	No

Table 1.3. continued

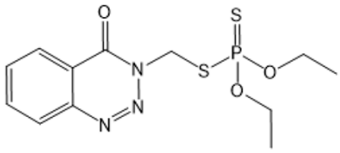
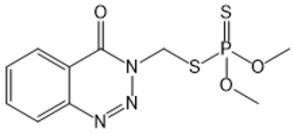
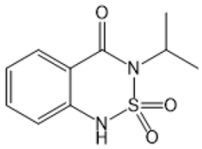
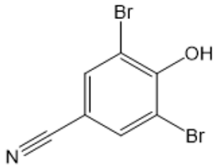
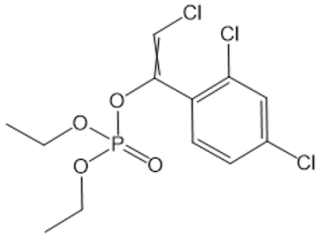
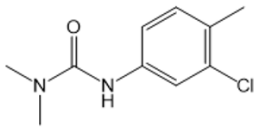
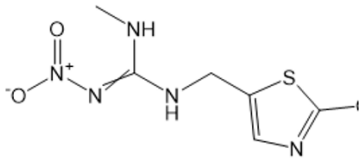
Name (structure)	CAS number [a]	Molecular mass [a] (g/mol)	Log K_{ow} [b]	Uses [b]	Regulated use [b]
Azinphos ethyl 	2642-71-9	345.38	3.18	Insecticide Acaricide	No
Azinphos methyl 	86-50-0	317.32	2.96	Insecticide	No
Bentazone 	25057-89-0	240.30	-0.46	Herbicide	Yes
Bromoxynil 	1689-84-5	276.90	0.27	Herbicide	Yes
Chlorfenvinphos 	470-90-6	359.60	3.80	Insecticide	No
Chlortoluron 	15545-48-9	212.68	2.50	Herbicide	Yes
Clothianidin 	210880-92-5	240.69	2.10	Insecticide	No

Table 1.3. continued

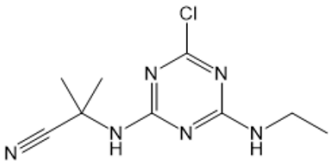
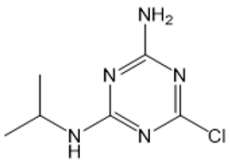
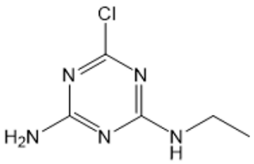
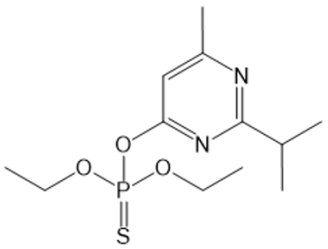
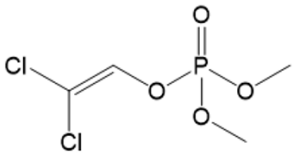
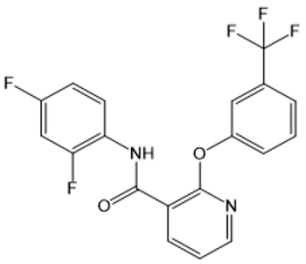
Name (structure)	CAS number [a]	Molecular mass [a] (g/mol)	Log K_{ow} [b]	Uses [b]	Regulated use [b]
Cyanazine 	21725-46-2	249.68	0.90	Herbicide	No
DEA 	6190-65-4	173.60	1.15	Metabolite	n.a.
DIA 	1007-28-9	187.63	1.51	Metabolite	n.a.
Diazinon 	333-41-5	304.35	3.69	Insecticide	No
Dichlorvos 	62-73-7	220.98	1.90	Insecticide	No
Diflufenican 	83164-33-4	394.29	4.20	Herbicide	Yes

Table 1.3. continued

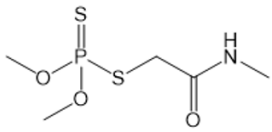
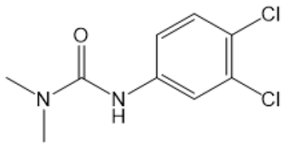
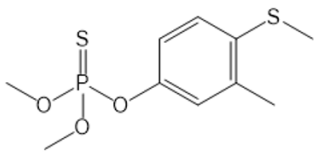
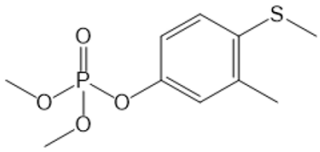
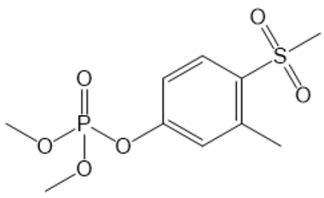
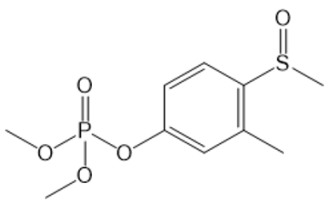
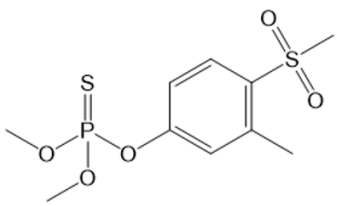
Name (structure)	CAS number [a]	Molecular mass [a] (g/mol)	Log K_{ow} [b]	Uses [b]	Regulated use [b]
Dimethoate 	60-51-5	229.26	0.75	Insecticide Acaricide	No
Diuron 	330-54-1	233.09	2.87	Herbicide	No
Fenthion 	55-38-9	278.33	4.84	Insecticide	No
Fenthion oxon 	6552-12-1	262.26 [c]	2.31 [c]	Metabolite	n.a.
Fenthion oxon sulfone 	14086-35-2	294.03 [c]	0.28 [c]	Metabolite	n.a.
Fenthion oxon sulfoxide 	6552-13-2	278.26 [c]	0.15 [c]	Metabolite	n.a.
Fenthion sulfone 	3761-42-0	310.33 [c]	2.05 [c]	Metabolite	n.a.

Table 1.3. continued

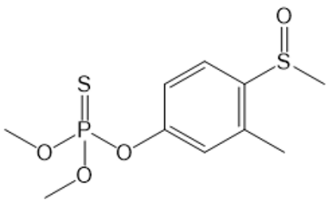
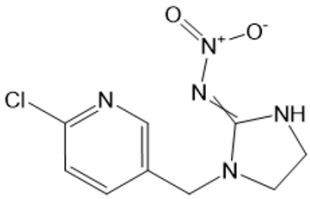
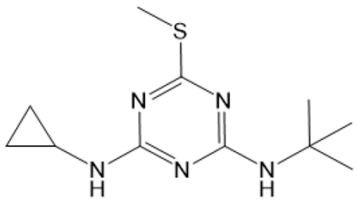
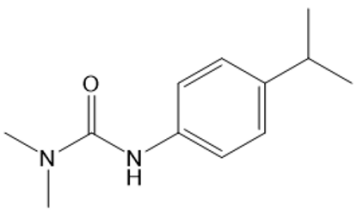
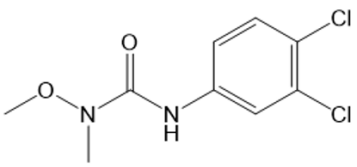
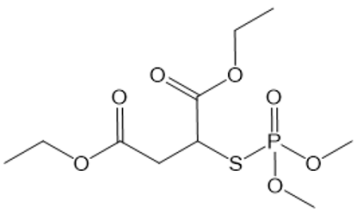
Name (structure)	CAS number [a]	Molecular mass [a] (g/mol)	Log K_{ow} [b]	Uses [b]	Regulated use [b]
Fenthion sulfoxide 	3761-41-9	294.33 [c]	1.92 [c]	Metabolite	n.a.
Imidacloprid 	138261-41-3	255.66	0.57	Insecticide	No
Irgarol 	28159-98-0	253.37	3.95	Herbicide Algicide	No
Isoproturon 	34123-59-6	206.28	2.5	Herbicide	No
Linuron 	330-55-2	249.09	3	Herbicide	No
Malaoxon 	1634-78-2	314.29[c]	0.52[c]	Metabolite	n.a.

Table 1.3. continued

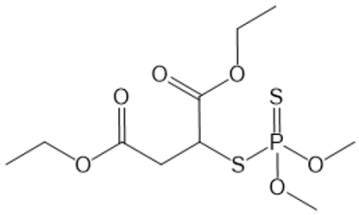
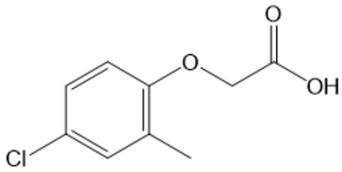
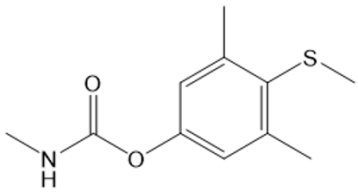
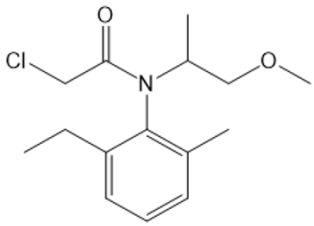
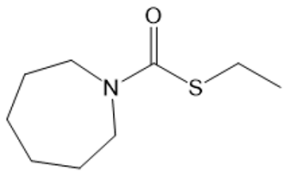
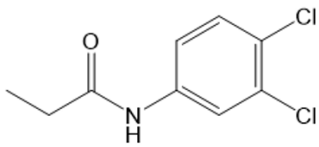
Name (structure)	CAS number [a]	Molecular mass [a] (g/mol)	Log K_{ow} [b]	Uses [b]	Regulated use [b]
Malathion 	121-75-5	330.36	2.75	Insecticide Acaricide	Yes
MCPA 	94-74-6	200.62	-0.81	Herbicide	Yes
Methiocarb 	2032-65-7	225.31	3.18	Moluscicide Insecticide	No
Metolachlor 	51218-45-2	283.80	3.40	Herbicide	No
Molinate 	2212-67-1	187.30	2.86	Herbicide	No
Propanil 	709-98-8	218.08	2.29	Herbicide	No

Table 1.3. continued

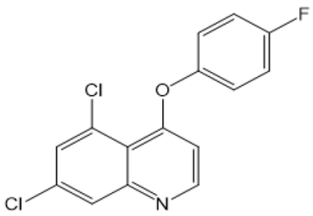
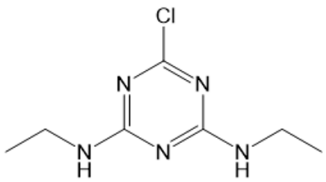
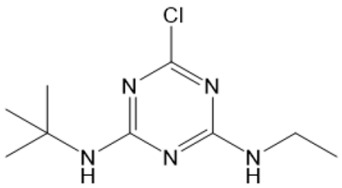
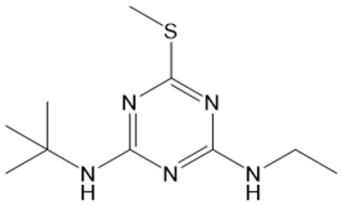
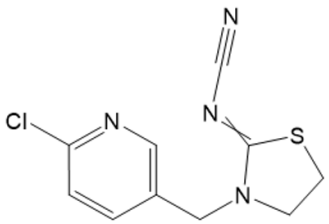
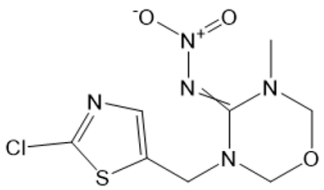
Name (structure)	CAS number [a]	Molecular mass [a] (g/mol)	Log K_{ow} [b]	Uses [b]	Regulated use [b]
Quinoxyfen 	124495-18-7	308.13	4.66	Fungicide	No
Simazine 	122-34-9	201.66	2.30	Herbicide	No
Terbuthylazine 	5915-41-3	229.71	3.40	Herbicide	Yes
Terbutryn 	886-50-0	241.36	3.66	Herbicide	No
Thiacloprid 	111988-49-9	252.72	1.26	Insecticide	No
Thiamethoxam 	153719-23-4	291.71	-0.13	Insecticide	No

Table 1.3. continued

CAS number: CAS (chemical abstract service) unique numerical identifier for chemical substances; Kow: octanol–water partition coefficient; n.a.: not applicable.
[a] Information extracted from the Pesticide Properties Database (PPDB), https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm
[b] Information extracted from the PubChem Substances and Compound database (https://pubchem.ncbi.nlm.nih.gov/).
[c] Data estimated using the US Environmental Protection Agency EPISuiteTM http://www.Chemspider.com

Chapter 2

Objectives and structure

2.1. Objectives

The main objective of this thesis is to better understand the fate of organic pollutants in the agrarian environment and the proposal of tools to monitor and reduce their levels. The attainment of this main objective implies the achievement of the following specific goals:

- The development and validation of target multiresidue methods based on QuEChERs extraction and LC-MS/MS analysis for determination of a selection of relevant pesticides in representative food matrices.
- The application of the developed methodologies to real samples to assess the presence and potential risk to human health of the selected pesticides.
- The development and application of a wide-scope screening approach based on LC-HRMS to characterize the CEC footprint in a reclaimed water-based irrigation system.
- The design of a cost-effective and simple prioritization approach to comprehensively select the most relevant site-specific pollutants to be considered in future monitoring programs.
- The assessment of bioremediation technologies to remove relevant pesticides from water, in terms of their efficiency and the potential formation of transformation products during the process.

2.2. Structure

This thesis is presented as a compendium of articles. It is divided into five chapters. Chapter 1 introduces the transition from the traditional agriculture to the actual agroindustry, the main sources of organic contamination, and the occurrence of CECs in the agrarian field. The main tertiary wastewater treatments and bioremediation approaches, as well as their efficiency in terms of CECs removal, are also summarized. Then, a brief explanation of the analytical chemistry role in the organic

contamination evaluation is also performed. Finally, the chapter ends with a list of the pesticides investigated and their main physical-chemical characteristics.

Chapter 2 explains the main and specific objectives of the thesis and its structure, broken down into chapters. Chapters 3, split into three sections, presents the articles published in the framework of this thesis that describe the experimental work conducted and the results obtained. This includes the development and application of target methods for the determination of pesticides in edible crops, the evaluation of the CEC footprint in a reclaimed water-based irrigation system with a wide-scope screening approach and the prioritization of site-specific pollutants, and finally, the assessment of bioremediation approaches to remove pesticides from water.

The articles included in each section of chapter 3 are:

Section 3.1:

- **Publication #1**: García-Vara, M., Postigo, C., Palma, P., Bleda, M. J., & López de Alda, M. QuEChERS-based analytical methods developed for LC-MS/MS multiresidue determination of pesticides in representative crop fatty matrices: Olives and sunflower seeds. Food Chemistry, 386 (2022), 132558. https://doi.org/10.1016/j.foodchem.2022.132558

- **Publication #2**: García-Vara, M., Postigo, C., Palma, P., & López de Alda, M. Development of QuEChERS-based multiresidue analytical methods to determine pesticides in corn, grapes and alfalfa. Food Chemistry, 405 (2023), 134870. https://doi.org/10.1016/j.foodchem.2022.134870

Section 3.2:

- **Publication #3**: García-Vara, M., Orlando-Véliz, D., Bonansea, R., Postigo, C., & López de Alda, M. Prioritization of the most relevant organic contaminants in a reclaimed water irrigation system through an LC-MS/HRMS-based suspect screening workflow. Submitted to Journal of Hazardous Materials.

Section 3.3:

- **Publication #4**: García-Vara, M., Hu, K., Postigo, C., Olmo, L., Caminal, G., Sarrà, M., & López de Alda, M. Remediation of bentazone contaminated water by Trametes versicolor: Characterization, identification of transformation products, and implementation in a trickle-bed reactor under non-sterile conditions. Journal of Hazardous Materials, 409 (2021), 124476. https://doi.org/10.1016/j.jhazmat.2020.124476

- **Publication #5**: Avila, R., García-Vara, M., López-García, E., Postigo, C., López de Alda, M., Vicent, T., & Blánquez, P. Evaluation of an outdoor pilot-scale tubular photobioreactor for removal of selected pesticides from water. Science of the Total Environment, 804 (2022), 150040. https://doi.org/10.1016/j.scitotenv.2021.150040

Chapter 4 includes a brief discussion of the results presented in chapter 3. Then, the general conclusions of this thesis are summarized in chapter 5. And, finally, a bibliographical section is included with the references cited throughout the different chapters.

Chapter 3

Results

3.1. Analytical multiresidue methodologies for pesticide determination in plant origin food

The availability of highly sensitive and selective analytical methods for the determination of pesticides in plant origin food is crucial because these substances are usually present at low concentrations in these matrices. Moreover, as stated in the introduction, MRLs established in Europe (Regulation No. 396/2005 (European Commision, 2005)) are, in most cases, in the low μg/kg range, enforcing analytical methodologies to reach limits of detection below these values. According to the EU Pesticides Database, there are 455 pesticides currently authorized for use in the EU territory, which are individually applied to crops or as a substance cocktail (European Commission, 2023). Therefore, there is a recognized need for developing multiresidue analytical methods. At present, although pesticides analysis remains challenging, advances and innovations in analytical instrumentation enable the simultaneous analysis of pesticide residues in food matrices.

In this context, the development of new multi-residue methodologies, improving the existing ones, in terms of analytes covered, sensitivity, cost and complexity, was one of the main objectives of the scientific publications #1 and #2. In this field, the QuEChERS extraction procedure has gained attention in the last decades, since its first implementation by Anastassiades et al. in 2003 (Anastassiades et al., 2003). QuEChERS methods allow the extraction of a broad range of pesticides in highly diverse matrices, in an easy and cost-effective way. Despite the extensive scientific knowledge that already exists on pesticides analysis in plant origin food matrices, optimization of the extraction and clean-up processes taking into consideration the matrix interferences of each commodity can improve the sensitivity of the analytical methods. Thus, optimization of the sample preparation procedure for food commodities of different characteristics (sunflower seeds, olives, grapes, corn, and alfalfa) was performed and described in the scientific publications #1 and #2.

Moreover, considering the huge number of legally approved pesticides that are employed in crops, analytical methodologies for many of them are yet not available. To the author's knowledge, the methods

developed in the framework of this doctoral thesis are the first ever described for the compounds and matrices indicated in table 3.1.

Table 3.1. Pesticides for which the methodologies described in the scientific publications #1 and #2 are the first analytical methods available in the peer-reviewed literature to determine their occurrence in the investigated food commodities.

Pesticide	Sunflower seeds	Olives	Corn	Grapes	Alfalfa
2,4-D	X	X		X	
Acetamiprid					X
Atrazine					X
Azynphos ethyl		X			
Azynphos methyl		X			X
Bentazone	X	X		X	X
Bromoxynil	X	X		X	X
Chlorfenvinphos		X		X	X
Chlothianidin	X				X
Cyanazine	X				
DEA	X	X			X
DIA	X	X			X
Diflufenican			X		X
Dimethoate					X
Fenthion oxon		X	X	X	X
Fenthion oxon sulfone	X		X	X	X
Fenthion oxon sulfoxide			X	X	X
Fenthion sulfone		X		X	X
Fenthion sulfoxide		X		X	X
Imidacloprid					X
Irgarol	X	X	X	X	X
Malaoxon		X			X
MCPA		X		X	X
Methiocarb		X			
Metholachlor					X
Molinate	X	X			X
Propanil		X		X	X
Quinoxyfen	X	X			
Terbuthylazine				X	
Terbutryn					X
Thiacloprid			X		

2,4-D: 2,4-Dichlorophenoxyacetic acid; DEA: desethylatrazine; DIA: deisopropylatrazine; MCPA: 2-methyl-4-chlorophenoxyacetic acid.

The methods, validated for up to 42 compounds, were applied to the analysis of sunflower seeds, olives, corn, grapes, and alfalfa samples harvested from crops located in Beja (Portugal), as part of the FitoFarmGest project, and Catalonia (Spain). The FitoFarmGest project (FitoFarmGest

Operational Group PDR2020-101-030926) aimed at improving the sustainable management of water and pesticides in different crops in the agrarian area of Beja.

3.1.1. Scientific publication #1

QuEChERS-based analytical methods developed for LC-MS/MS multiresidue determination of pesticides in representative crop fatty matrices: Olives and sunflower seeds

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Food Chemistry, 386 (2022), 132558

(DOI: https://doi.org/10.1016/j.foodchem.2022.132558)

Food Chemistry 386 (2022) 132558

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

QuEChERS-based analytical methods developed for LC-MS/MS multiresidue determination of pesticides in representative crop fatty matrices: Olives and sunflower seeds

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ARTICLE INFO

Keywords:
Pesticide residue analysis
Agrochemicals
Liquid chromatography-tandem mass spectrometry
Insecticides
Herbicides
Sample pre-treatment

ABSTRACT

Oilseed crops are greatly extended all over the world. Their high fat content can interfere during pesticide multiresidue analysis through liquid chromatography-tandem mass spectrometry (LC-MS/MS). This work aimed at overcoming this issue by developing and validating two QuEChERS-based methods for LC-MS/MS determination of 42 pesticides in two fatty food matrices: olives and sunflower seeds. Optimization of the extraction method was achieved following a 2^{6-2} fractional factorial design in a highly cost-effective way. Validation of the multi-residue methods demonstrated improved limits of detection, below the established maximum residue levels (MRLs) for almost all compounds, good precision, and trueness, in compliance with SANTE guidelines. Application of these methods to the analysis of real samples from the Iberian Peninsula showed the presence of some pesticides of relevant environmental concern, including four compounds contained in the Pesticide Action Network International list of highly hazardous pesticides, found at levels between 0.03 ng/g and 104 ng/g.

1. Introduction

In the last decades, European policies have aimed to reduce the use of pesticides for the sake of a more sustainable agriculture. Firstly, through the Common Agricultural Policy raised in 1962, and more recently through the European Directive 2009/128/EC (European Commission, 2009), the European Commission (EC) has established an Integrated Pest Management System and recommended reducing pesticide dependence in agriculture. However, in practice, these policies have not achieved their goals. The fact that pesticide sales have not decreased since 2011 indicates that farmers are still strongly relying on these chemicals for growing their crops (Eurostat, 2020). A recent report from the EC explicitly stated that "pesticides are a cause of pollution and have a direct effect especially on the state of biodiversity, water bodies, and soils" (European Commission, 2017). Moreover, many environmentally stable pesticides can also be harmful to humans due to their bioaccumulative and toxic properties (Bernardes, Pazin, Pereira, & Dorta, 2015). Among the EU member states, Germany, Spain, France, and Italy represent over 66% of the total EU pesticide sales volume. Huge amounts of these chemicals are released into the environment and, due to their well-known ubiquity, the monitoring of their levels on the different environmental compartments has become essential. In food, the EC has fixed Maximum Residue Levels (MRLs, the highest level of a pesticide residue that is legally tolerated in or on food), which vary depending on the commodity and the specific pesticide toxicity and environmental occurrence (European Commision, 2005). When a

Abbreviations: ACN, Acetonitrile; C_{18}, Octadecylsilane; D-SPE, Dispersive SPE; DLLME, Dispersive Liquid-Liquid Micro-Extraction; DOE, Design of Experiments; EMR, Enhanced Matrix Removal-Lipid; EC, European Commission; GCB, Graphitized Carbon Black; LOD, Limit of Detection; LOQ, Limit of Quantification; LDet, Limit of Determination; MeOH, Methanol; MRL, Maximum Residue Limit; MS, Mass Spectrometry; ND, below detection limit; PAN, Pesticide Action Network; PSA, Primary Secondary Amine; SPE, Solid Phase Extraction; SPME, Solid Phase Micro-Extraction; SRM, Selected Reaction Monitoring.

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https://doi.org/10.1016/j.foodchem.2022.132558
Received 19 August 2021; Received in revised form 23 February 2022; Accepted 23 February 2022
Available online 25 February 2022

Table 1
LC-MS/MS conditions for target pesticides and surrogate compounds and retention time (RT).

Target analyte	Abbrev.	HESI mode	SRMs (*m/z*)	RF lens (V)	CE (eV)	SRM1/SRM2	RT (min) Olives/Sunflower
DIA	DIA	+	174 > 104	58	22	1.1	5.27/5.12
			174 > 132	58	17		
	DIA-d_5	+	179 > 105	60	24		5.24/5.10
Fenthion oxon sulfoxide	FNOSX	+	279 > 264	68	19	4.1	5.31/5.23
			279 > 262	68	21		
	FNOSX-d_3	+	282 > 264	66	18		5.28/5.19
Thiamethoxam	THIAM	+	292 > 132	47	23	0.97	5.34/5.25
			292 > 181	47	22		
	THIAM-d_3	+	295 > 214	43	13		5.33/5.22
Bentazone	BEN	–	239 > 133	68	27	3.9	5.47/5.80
			239 > 117	68	33		
	BEN-d_6	–	245 > 132	63	25		5.42/5.75
Chlothianidin	CLOTD	+	250 > 169	43	14	1.89	5.70/5.62
			250 > 132	47	18		
	CLOTD-d_3	+	253 > 172	45	15		5.69/5.55
Imidacloprid	IMID	+	256 > 209	51	19	1.2	5.84/5.80
			256 > 175	51	20		
	IMID-d_4	+	260 > 213	63	19		5.81/5.78
DEA	DEA	+	188 > 146	66	18	4.9	5.86/5.82
			188 > 104	66	25		
	FNOSX-d_3	+	282 > 264	66	18		5.28/5.19
2,4D	2,4D	–	219 > 125	35	28	22.6	6.00/5.80
			219 > 162	35	16		
	2,4D-d_3	–	224 > 166	39	12		5.97/5.78
Acetamiprid	ACET	+	223 > 126	53	22	5.4	6.03/5.93
			223 > 90	53	33		
	ACET-d_3	+	226 > 126	55	22		5.98/5.89
Dimethoate	DIME	+	230 > 125	35	22	11.9	6.01/5.94
			230 > 157	35	20		
	DIME-d_6	+	236 > 131	44	22		5.98/5.91
Fenthion oxon sulfone	FENOXS	+	295 > 217	74	19	7.8	6.13/6.05
			295 > 91	74	33		
	FENOXS-d_3	+	298 > 218	77	20		6.10/6.01
MCPA	MCPA	–	199 > 142	38	29	1.1	6.39/6.17
			199 > 105	38	17		
	MCPA-d_3	–	204 > 146	41	11		6.36/6.11
Thiacloprid	THIAC	+	253 > 126	59	23	6.4	6.62/6.56
			253 > 90	59	34		
	THIAC-d_4	+	257 > 126	60	23		6.61/6.53
Cyanazine	CYANZ	+	241 > 214	59	18	5.6	7.30/7.25
			241 > 104	59	29		
	ATRZ-d_5	+	221 > 179	59	18		8.60
Simazine	SIMAZ	+	202 > 132	61	19	1.1	7.39/7.35
			202 > 104	61	25		
	ISOPR-d6	+	213 > 134	53	23		8.40
Fenthion sulfoxide	FENSOX	+	295 > 280	68	19	1.8	7.55/7.52
			295 > 109	68	32		
	FENSOX-d_6	+	301 > 286	53	18		7.53
Bromoxynil	BROMX	–	276 > 81	82	29	1.1	7.60/7.31
			276 > 79	82	29		
	THIAC-d_4	+	257 > 126	60	23		6.61/6.53
Dichlorvos	DICV	+	221 > 145	57	18	24.6	7.43/7.03
			221 > 109	57	13		
	DICV-d_6	+	227 > 115	69	19		7.38/6.98
Malaoxon	MALOX	+	315 > 99	48	22	15.2	7.50/7.46
			315 > 125	48	33		
	FENOX-d_3	+	266 > 234	69	17		8.85/8.82
Chlortoluron	CHLOR	+	213 > 140	51	24	3.9	8.17/8.12
			213 > 104	51	32		
	CHLOR-d_6	+	219 > 78	58	18		8.10/8.06
Isoproturon	ISOPR	+	207 > 134	51	23	1.7	8.46/8.43
			207 > 91	51	37		
	ISOPR-d_6	+	213 > 134	53	23		8.40
Atrazine	ATRZ	+	216 > 174	58	18	4.3	8.65/8.61
			216 > 104	58	28		
	ATRZ-d_5	+	221 > 179	59	18		8.60
Diuron	DIUR	+	233 > 160	51	27	1.3	8.75/8.71
			233 > 133	51	40		
	DIUR-d_6	+	239 > 160	59	28		8.68

(*continued on next page*)

Table 1 (*continued*)

Target analyte	Abbrev.	HESI mode	SRMs (*m/z*)	RF lens (V)	CE (eV)	SRM1/SRM2	RT (min) Olives/Sunflower
Fenthion oxon	FENOX	+	263 > 231	62	16	2.3	8.88/8.84
			263 > 216	62	24		
	FENOX-d_3	+	266 > 234	69	17		8.85/8.82
Fenthion sulfone	FENS	+	311 > 125	65	21	3.1	9.39/9.33
			311 > 233	84	17		
	FENS-d_6	+	317 > 131	72	21		9.32
Propanil	PROP	+	218 > 127	53	26	1.1	10.11/10.07
			218 > 162	53	16		
	PROP-d_5	+	223 > 128	59	28		10.05
Methiocarb	METCB	+	226 > 169	35	9	1	10.32/10.29
			226 > 121	35	18		
	METCB-d_3	+	229 > 169	30	9		10.30/10.25
Azinphos methyl	AZM	+	318 > 132	30	15	1.6	10.57/10.54
			318 > 261	30	5		
	AZM-d_6	+	324 > 132	43	15		10.51
Terbuthylazine	TERBZ	+	230 > 174	52	17	8.8	10.57/10.56
			230 > 104	52	31		
	TERBZ-d_5	+	235 > 179	55	17		10.51
Linuron	LINU	+	249 > 160	51	19	1.3	10.78/10.75
			249 > 133	51	34		
	LINU-d_6	+	255 > 185	48	18		10.70
Molinate	MOLI	+	188 > 126	43	12	4.8	12.02/11.99
			188 > 98	43	17		
	METCB-d_3	+	229 > 169	30	9		10.30/10.25
Malathion	MALA	+	353 > 227	70	16	4.9	12.10/12.10
			353 > 307	70	15		
	MALA-d_{10}	+	363 > 237	70	18		12.00
Alachlor	ALA	+	270 > 162	40	21	2.7	12.51/12.50
			270 > 132	40	41		
	ALA-d_{13}	+	283 > 251	45	10		12.35
Metolachlor	METO	+	284 > 252	48	15	2.4	12.39/12.39
			284 > 176	48	26		
	METO-d_{11}	+	295 > 263	48	17		12.25
Terbutryn	TBTN	+	242 > 186	55	19	15.2	12.85/12.19
			242 > 158	55	25		
	TBTN-d_5	+	247 > 191	59	19		12.81/12.16
Irgarol	IRGA	+	254 > 198	57	19	15.2	12.57/12.07
			254 > 108	57	30		
	IRGA-d_9	+	263 > 199	60	19		12.47/12.05
Azinphos ethyl	AZET	+	346 > 137	37	25	1.1	12.70/12.69
			346 > 97	37	31		
	CFVP-d_{10}	+	369 > 170	58	41		12.78
Chlorfenvinphos	CFVP	+	359 > 170	60	41	1.2	12.88/12.90
			359 > 99	60	27		
	CFVP-d_{10}	+	369 > 170	58	41		12.78
Fenthion	FEN	+	279 > 169	63	19	1	14.02/14.05
			279 > 247	63	13		
	FEN-d_6	+	285 > 169	62	19		13.94
Diazinon	DIAZ	+	305 > 169	64	22	1.9	14.62/14.64
			305 > 153	64	21		
	CFVP-d_{10}	+	369 > 170	67	21		12.78
Diflufenican	DIFLU	+	395 > 266	60	24	4.6	15.35/15.38
			395 > 246	60	34		
	DIFLU-d_3	+	398 > 268	84	25		15.31/15.36
Quinoxyfen	QUIN	+	310 > 199	102	33	1.9	17.38/17.31
			310 > 216	102	36		
	QUIN-d_4	+	312 > 162	112	44		17.32/17.27

HESI: Heated Electrospray Ionization; SRM: Selected Reaction Monitoring; RF Lens: Radio Frequency Lens; CE: Collision Energy; DIA: Desisopropylatrazine; DEA: Desethylatrazine; 2,4D: 2,4-Dichlorophenoxyacetic acid; MCPA: 2-methyl-4-chlorophenoxyacetic acid.

pesticide or a food commodity is not specifically classified, a general default MRL of 0.01 mg/kg is applied.

Pesticide multiresidue analysis in food represents a challenging task due to the frequently low concentrations of these chemicals and the complexity of the different matrices (Abbaspour et al., 2019; Parrilla Vázquez et al., 2016; Romero-González et al., 2014; Valverde et al., 2018). To overcome these difficulties, sample pre-treatment remains as one of the most important steps to be considered during method optimization. For the extraction of pesticides from food, diverse methods have been reported in the last years, including solid phase extraction (SPE) (Huo et al., 2016; Shamsipur et al., 2016), solid phase microextraction (SPME) (Choi et al., 2020; Kasperkiewicz & Pawliszyn, 2021; Liang et al., 2017; Pelit et al., 2015) and dispersive liquid–liquid micro-extraction (DLLME) (Chu et al., 2015; Farajzadeh et al., 2017; Ghoraba et al., 2018). However, over these extractive procedures, the QuEChERS method (acronym of *quick, easy, cheap, effective, rugged, and safe*), a two-step procedure consisting of a salting-out solid–liquid extraction and a dispersive-SPE (d-SPE) clean-up, has become the pre-treatment of choice for most laboratories worldwide (Barchanska et al., 2018). Two international standard organisations have in fact established two different versions of the original QuEChERS method as official methods for determination of pesticides in food: the European Committee for Standardisation CEN ((CEN) Standard Method EN 15662, n.d.) and the AOAC International (Lehotay & Collaborators, 2007). This

responds to the broad applicability of the methodology to a wide range of organic compounds and food matrices. In addition, QuEChERS extraction, when combined with chromatographic techniques coupled to mass spectrometry (MS), allows multiresidue analysis without compromising the method sensitivity and selectivity.

Oilseed crops are greatly extended all over the world as they play an important role in human nutrition. Their high fat content makes them a suitable energy source but, at the same time, represents a relevant source of interferences in the analysis of pesticide residues in these matrices. Therefore, great efforts have been made in the last years to improve clean-up procedures in such matrices (Madej et al., 2018). For instance, Cunha *et al.* evaluated different sorbents for the d-SPE of pesticides from olives, including $MgSO_4$, primary secondary amine (PSA), graphitized carbon black (GCB), and octadecylsilane (C_{18}), and concluded that the combination of all sorbents gave the cleanest extracts (Cunha et al., 2007). Another study showed better results with QuEChERS, as compared to matrix solid-phase dispersion, for the extraction of >100 pesticides from olives, using the aforementioned sorbents (Gilbert-López et al., 2010). Also in olives, negligible advantages were observed with the use of advanced sorbents such as Z-Sep or the novel Enhanced Matrix Removal-Lipid (EMR), obtaining a superior removal efficiency of co-extracts with the combination of PSA + C_{18} (López-Blanco et al., 2016). Freezing is also a common direct clean-up for fatty commodities, due to the different melting points of pesticides and fat. However, poor recoveries and the relatively long time of the procedure make this purification approach unsuitable for the analysis of low melting point pesticides (Kaczyński, 2017). Apart from this, most of the methods described in the literature for the analysis of pesticides in fatty food matrices, with few exceptions (Gilbert-López et al., 2010; Kaczyński, 2017), cover only a limited number of pesticides and they often belong to the same chemical class.

In this context, the objectives of this work were (1) to develop and validate a practical LC-MS/MS method for the multiresidue analysis of up to 42 moderately polar pesticides, including neonicotinoids, triazines, phenylureas, organophosphates or anilines, in two representative fatty commodities: olives and sunflower seeds, and (2) to apply the methodology developed to the analysis of various samples of different origin.

2. Materials and methods

2.1. Chemicals and reagents

High purity (96–99.9%) standards of 42 pesticides and 34 isotopically-labelled analogues used as surrogate standards for quantification were purchased from Fluka (Sigma–Aldrich, Steinheim, Germany) or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). Target analytes are shown in Table 1, whereas relevant physical–chemical properties (molecular mass, solubility, acid dissociation constant (pKa), and octanol–water partition coefficient (log 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 solutions at different concentrations (from 0.01 to 500 ng/mL) containing all analytes were prepared by appropriate dilution of the stock individual solutions in methanol. They were used to construct calibration curves and as spiking solutions during method development and in the validation study. A solution containing the surrogate standard mixture at a concentration of 2000 ng/mL was also prepared and used for method optimization, validation, and real sample quantification. Pesticide-grade solvents MeOH, acetonitrile (ACN), and LC-grade water were supplied by Merck (Darmstadt, Germany).

2.2. Sample preparation and extraction procedure

Olives were pitted and the edible part was milled in a coffee grinder. In the case of sunflower seeds, ultra-freezing with liquid nitrogen was performed prior to the grinding to obtain a better specific surface area to work with after milling. Then, samples were stored in the dark frozen at −20 °C. At the time of analysis, 7.5 g of pitted olives and sunflower seeds (containing kernel and husk) was weighed in 50 mL polypropylene centrifuge tubes. Then, 375 μL of the surrogate standard mixture was added to the samples (final concentration of 50 ng/g). In the case of sunflower seeds, 7.5 mL of deionized water was also added and manually shaken for matrix hydration (Kaczyński, 2017). The prepared samples were left under the hood for 60 min to allow the MeOH of the added surrogate standard solution evaporate. For extraction, 15 mL of ACN (with 1% formic acid) was added to the sample and vigorously mixed. Afterwards, the salting out step was performed with 6 g $MgSO_4$ and 1.5 g sodium acetate provided by Bekolut® GmbH & Co. KG (Hauptstuhl, Germany) as SALT-Kit-AC2. The sample was then manually and vortex shaken for 1 min and, after that, tubes were centrifuged for 5 min at 3220 Relative Centrifugal Force (RFC). Then, 7 mL of the supernatant was transferred into a 15-mL centrifuge tube for clean-up. Two different clean-up sorbents were used for each matrix: *a) Olives*: 1200 mg $MgSO_4$, 400 mg PSA, 400 mg GCB, 400 mg C18e (Bekolut® PSA-Kit-08A), *b) Sunflower seeds*: 900 mg $MgSO_4$, 150 mg PSA, 150 mg C18e (Bekolut® PSA-Kit-04). After vigorous manual and vortex shaking during 1 min, tubes were centrifuged for 5 min and 3220 RFC. Final extracts of olives and sunflower seeds, without any intermediate evaporation step with nitrogen, were acidified with 0.7% and 0.5% of formic acid, respectively, and transferred into 2 mL vials for LC-MS/MS analysis.

2.3. Analytical conditions

Chromatographic separation was carried out with an LC system Aria Mx equipped with two Transcend quaternary pumps (max pressure 600 bars) connected in series with a triple quadrupole TSQ Quantiva mass spectrometer, and interfaced with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific Inc.). A 10 μL volume of 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)) using a CTC Pal autosampler. Retained analytes were eluted using a mobile phase at a flow rate of 0.2 mL/min. Starting with 10% of ACN (organic constituent of the mobile phase) in water (aqueous constituent of the mobile phase), a linear organic gradient was established as follows: after 1 min in isocratic organic conditions, the organic phase proportion increased to 50% in 2.5 min. Then, during the following 10 min, the ACN proportion achieved 80% and, finally, 100% in 1 min. Afterwards, isocratic conditions were maintained for 2.5 min and then initial conditions were restored in 2 min and kept for 7.5 min to ensure complete re-equilibration of the column sorbent.

Selected reaction monitoring (SRM) was used for the MS/MS acquisition. Two SRM transitions were chosen for each target compound (one for quantification and another one for confirmation), and one SRM transition for each surrogate standard, based on the intensity and selectivity of the fragments. Positive and negative ionization modes were alternated, allowing the determination of all target pesticides in one single run. Optimum conditions of each transition, regarding collision energy, RF lens voltage, and m/z are summarized in Table 1. With respect to the MS general detection conditions, ion spray voltage was set to 3500 V in the positive ion mode, and −2500 V in the negative ion mode, ion transfer tube temperature was 350 °C, and vaporizer temperature was 280 °C. Nitrogen was used as sheath, sweep and auxiliary gas for the nebulization stage at the HESI, and argon was used as the collision gas at a pressure of 2.5 mTorr. Instrument setup and control, data acquisition and quantification were performed by Thermo Scientific Xcalibur v.4.1.31.9 software (Thermo Fisher Scientific Inc.).

2.4. Method optimization and validation

The extraction procedure was optimized through a 2^{6-2} fractional factorial design of experiments (DOE). This DOE was used to accommodate two 3-levels continuous factors with two 2-levels categorical factors (Table S2). A total of 16 randomized elemental experiments were performed. The regression model adjusted for each compound included lineal effects of all factors, quadratic effects of continuous factors and also their interaction (Table S2). Statistical evaluation of the optimization results was carried out with the software JMP 12.1.0 (SAS Institute Inc., Cary). Validation of the optimized analytical methods was performed in terms of compound recoveries, matrix effects, linearity, precision, and limits of detection and quantification (LOD and LOQ, respectively) following the guidelines described in Document No. SANTE/12682/2019 (European Commission, 2019). A representative (pool of various samples) matrix of olives and sunflower seeds was employed for the multiresidue method validation and, as no blank matrices were available, the background concentration of the target analytes, if present, was taken into consideration in the calculations. Quantification was performed by the internal standard method, in which surrogate standards are added at the beginning of the analytical method to account for all possible sources of errors throughout the method (analyte losses, poor recoveries and matrix effects). Surrogate compounds were the deuterated analogues of the target pesticides in most cases (Table 1). Method linearity was determined by the coefficient of determination (r^2) of the weighed (1/x) linear regression model obtained through the 11-point calibration curves built for each compound within the linearity range 0.01 ng/mL − 500 ng/mL (equivalent to 0.02 ng/g-1000 ng/g, respectively).

Analyte recoveries and precision of the method were calculated through the analysis of n = 6 replicate samples fortified at two different concentration levels (10 ng/g, as the default minimum MRL, and 100 ng/g). Absolute recoveries were obtained by comparing analyte peak areas in spiked samples and methanolic solutions at equivalent concentrations. Relative recoveries were calculated by comparing the absolute recovery of the analyte and that of the isotopically-labelled compound used as surrogate standard. The relative standard deviation (RSD %) of the n = 6 replicates analysed at both concentration levels (10 ng/g and 100 ng/g) was used to assess method precision.

Matrix effects were obtained by comparing analyte peak areas of the samples spiked after extraction, prior to LC-MS analysis, and methanolic standards at the same concentration (50 ng/mL, equivalent to 100 ng/g). Method LODs and LOQs were calculated through the signal to noise (S/N) method. A matrix-matched calibration curve in the low linearity range (0.1–10 ng/g) was generated for each compound and the S/N obtained in these solutions was used for LOD and LOQ calculation. Moreover, each integrated peak was visually checked for an extra confirmation. A S/N ratio of 3 was used for the LOD, and a S/N ratio of 10 was used for the LOQ. Then, the limit of determination (LDet) was established as the LOD of the SRM2 when higher than the LOQ of SRM1, and indicates the minimum concentration that can be quantified (SRM1) and confirmed (SRM2).

2.5. Real samples determination

After validation, the analytical method was applied and evaluated for the determination of the target pesticides in olives and sunflower seeds harvested from different sites of the Iberian Peninsula during 2018, 2019 and 2020. MCMG, SBG and JMG codes correspond to sunflower seed samples, whereas JMO (*Cordovil* variety), JMC (*Cordovil* variety), LBO (*Verdeal* variety), Farga, Sevillenc, Mor, and Sossis correspond to olive samples.

Table 2
Description of 16 elemental experiments of the 2^{6-2} fractional factorial DOE for extraction optimization.

Pattern	Factors			
	CAN (*formic acid*)[a]	Formic acid in final extract[b]	Extractive salts[c]	Clean-up sorbent[d]
− − − − − −	−1	−1	Citrate	4
− − − + + +	−1	0	Acetate	8A
− − + − + +	−1	0	Acetate	8A
− − + + − −	−1	+1	Citrate	4
− + − − + −	0	−1	Acetate	4
− + − + − +	0	0	Citrate	8A
− + + − − +	0	0	Citrate	8A
− + + + + −	0	+1	Acetate	4
+ − − − − +	0	−1	Citrate	8A
+ − − + + −	0	0	Acetate	4
+ − + − + −	0	0	Acetate	4
+ − + + − +	0	+1	Citrate	8A
+ + − − + +	+1	−1	Acetate	8A
+ + − + − −	+1	0	Citrate	4
+ + + − − −	+1	0	Citrate	4
+ + + + + +	+1	+1	Acetate	8A

Pattern: ACN (formic acid) and formic acid extract are continuous factors. "- -" corresponds to level −1, "-+" or "+-" corresponds to level 0, and "++" corresponds to level + 1. The first 2 symbols of the pattern determine the level of ACN (formic acid), the third and fourth determine the level of Formic acid extract and, then, the fifth and sixth determine the extractive salt and clean-up sorbent levels, respectively.
[a] level −1 represents 0% formic acid in ACN; level 0, 0.5% formic acid; level + 1, 1% formic acid.
[b] -1 represents 0% formic acid in the final extract; 0, 0.5% formic acid; +1, 1% formic acid.
[c] Citrate represents Bekolut® SALT-Kit-CIT; Acetate, Bekolut® SALT-Kit-AC2.
[d] 4 represents Bekolut® PSA-Kit-04; 8A, Bekolut® PSA-Kit-04.

3. Results and discussion

3.1. Method optimization

For the extraction optimization, a fractional factorial design of experiments was performed. Two 3-levels continuous factors and two 2-levels categorical factors were studied, conforming a 2^{6-2} fractional factorial design. For each continuous factor, a central point was established to evaluate possible quadratic effects. To accommodate factors with 3 levels, two artificial factors with 2 levels were used for each of them. Having a total of 6 factors with 2 levels each, a fraction of the complete 2^6 design was selected conforming the definitive 2^{6-2} fractional factorial design (Kuehl, 2000; Montgomery, 2001). During the extraction, different factors can affect the process efficiency and, considering the wide range of physical–chemical parameters of the target pesticides, optimum values for each factor should be adopted with an overall vision. Firstly, for the extractive salts, two buffered variants of

Table 3
Absolute and relative recoveries and repeatability of the method validation.

Pesticide	Absolute recoveries (%)				Relative recoveries (%)				Repeatability (% RSD)			
	Olives		Sunflower seeds		Olives		Sunflower seeds		Olives		Sunflower seeds	
	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g
2,4D	N.D.	N.D.	N.D.	57	N.D.	N.D.	N.D.	115	N.D.	N.D.	N.D.	5.6
Acetamiprid	9.2	8.1	63	56	87	87	89	108	4.8	6.3	3.6	4.2
Alachlor	74	39	85	73	94	80	87	105	8.2	6.6	5.7	4.6
Atrazine	42	53	128	87	75	81	85	104	2.0	3.8	6.0	2.4
Azinphos ethyl	N.D.	44	89	85	N.D.	77	67	100	N.D.	4.5	5.3	3.1
Azinphos methyl	N.D.	101	111	88	N.D.	91	86	104	N.D.	8.0	6.8	4.1
Bentazone	48	36	82	92	72	79	79	100	3.6	4	4.3	1.3
Bromoxynil	N.D.	N.D.	55	44	N.D.	N.D.	106	115	N.D.	N.D.	13	11
Chlorfenvinphos	77	66	104	87	88	116	79	102	2.3	5	7.6	4.3
Chlortoluron	26	22	61	66	111	120	82	117	7.7	5.8	5.3	2.5
Chlothianidin	N.D.	17	52	42	N.D.	100	100	108	N.D.	4.6	4.5	1
Cyanazine	64	76	125	94	102	82	83	112	5	2.8	3.7	2.6
DEA	43	53	169	121	129	113	89	111	3.3	6.4	4.1	3.1
DIA	27	32	105	79	116	132	77	101	0.9	5.4	6	2.9
Diazinon	70	80	123	83	118	133	82	104	9.1	13	6.1	2.9
Dichlorvos	N.D.	248	N.D.	N.D.	N.D.	92	N.D.	N.D.	N.D.	6.2	N.D.	N.D.
Diflufenican	57	44	130	84	93	89	78	116	17.8	7.3	5.0	2.9
Dimethoate	17	16	76	74	112	102	91	113	3.6	5.3	5.7	0.5
Diuron	29	26	66	70	96	91	72	103	5.6	6.3	9.2	3.7
Fenthion	N.D.	76	N.D.	89	N.D.	90	N.D.	118	N.D.	4.9	N.D.	10
Fenthion oxon	68	56	95	82	98	97	79	102	1.6	3	4.5	2
Fenthion oxon sulfone	51	32	132	119	101	93	95	109	4.4	3.7	4.7	2.3
Fenthion oxon sulfoxide	33	43	64	63	98	91	98	133	2.3	0.8	8.2	3
Fenthion sulfone	N.D.	46	N.D.	55	N.D.	71	N.D.	91	N.D.	10	N.D.	6.8
Fenthion sulfoxide	42	35	95	80	98	96	80	135	12	14	13	7.6
Imidacloprid	16	15	64	54	103	91	89	110	4.6	2.8	5.9	1.9
Irgarol	74	76	81	71	92	85	81	111	2.3	3.8	5.6	0.8
Isoproturon	49	53	80	75	106	108	87	114	2.1	7.5	4.5	1.9
Linuron	172	147	141	129	89	114	83	97	6.9	4.5	5.7	4.9
Malaoxon	73	52	95	81	104	101	128	145	4.5	3.2	8.3	3.1
Malathion	N.D.	19	39	36	N.D.	96	120	131	N.D.	13	8.1	2.8
MCPA	N.D.	N.D.	N.D.	9.2	N.D.	N.D.	N.D.	93	N.D.	N.D.	N.D.	18
Methiocarb	104	88	113	103	83	88	92	110	3.5	7	2.9	3.5
Metolachlor	73	54	79	72	98	96	78	99	1.5	14	4.3	1.6
Molinate	116	75	105	76	70	76	80	102	19	14	7.6	3.3
Propanil	209	169	175	166	113	113	89	103	10	7.7	7	5.4
Quinoxyfen	29	13	71	51	103	84	72	80	11.6	8.2	6.8	13
Simazine	37	49	138	95	80	97	82	99	1.4	3.8	4.5	2.8
Terbuthylazine	96	92	119	79	89	103	90	107	3	4.7	2.8	2
Terbutryn	80	59	80	77	97	100	79	109	1.3	1.4	2.6	0.9
Thiacloprid	13	12	58	51	75	88	87	106	7.1	8.5	5.2	1.3
Thiamethoxam	24	25	54	48	103	77	81	101	4.6	3.4	5.6	0.8

N.D.: not detected; RSD: relative standard deviation

QuEChERS methodology were studied: AOAC 2007 method, which uses sodium acetate as buffer (Lehotay & Collaborators, 2007), and CEN method, which uses citrate buffer instead ((CEN) Standard Method EN 15662, n.d.). Acid dissociation constant (pka) and log *P* are important parameters that can influence the salting out partitioning of pesticides between aqueous and organic phases, and, thus, pH adjustment could affect their extraction. Moreover, formic acid has been used for the stabilization of some pH-labile compounds during sample extraction (i.e. alachlor) (Kaczyński, 2017; Maštovská & Lehotay, 2004). Therefore, the proportion of formic acid in the extraction solvent (ACN) was investigated (levels: 0% – 1%, with a central point at 0.5%). Due to the complex composition of both matrices, i.e., medium–high proportion of fat (10–25% in the case of olives and 51% in the case of sunflower seeds (Garrido-Fernandez et al., 1997; Madej et al., 2018)), and a high presence of pigments (chlorophyll and carotenoids in the case of olives (Minguez-Mosquera & Garrido-Fernandez, 1989)), the selection of the clean-up sorbents could be determinant in the extraction efficiency. PSA is frequently used for the removal of fatty components, among others; non-polar compounds are effectively cleaned-up by C18e sorbent, while GCB remove planar compounds such as pigments (Madej et al., 2018). Thus, two different combinations of these sorbents were tested to cover both commodity matrices: Bekolut® PSA-Kit-08A, containing 1200 mg $MgSO_4$, 400 mg PSA, 400 mg GCB and 400 mg C18e in each tube and Bekolut® PSA-Kit-04, with 900 mg $MgSO_4$, 150 mg PSA, 150 mg C18e. In the end, the pH of the final extract could affect the ionization efficiency of the target pesticides at the HESI probe in the mass spectrometer. Consequently, the addition or not of 1% of formic acid to the final extract was also evaluated (levels: 0% – 1%, with a central point at 0.5%). A summary of the experimental design is shown in Table 2.

In sunflower seeds, the acetate buffer showed a clearly higher extraction efficiency than the citrate buffer and was thereby selected as optimum. Particularly, the acidic herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA), which usually represents an analytical challenge, showed a higher response with the acetate buffer than with the citrate buffer. In olives, given the overall similar performance obtained with both buffered salts, the acetate buffer was also selected. The use of formic acid in ACN during the extraction step favoured the process for almost all compounds, with a few negligible exceptions (bentazone, bromoxynil and malaoxon in olives; and MCPA, thiametoxam, and desethyl-atrazine (DEA) in sunflower seeds) and, so, 1% of formic acid in ACN was established as the optimum in both matrices. Regarding the clean-up, no significant differences were observed between both sorbent mixtures in the case of olives. Thus, Bekolut® PSA-Kit-08A was selected for the method according to the matrix components (high content of fats and pigments). Besides, clean-up optimization in sunflower seeds led to the use of Bekolut® PSA-Kit-04, as it showed comparatively better

Table 4
Linearity and sensitivity of the method and MRLs.

Pesticide	Olives					Sunflower seeds				
	Linearity	Sensitivity (ng/g)			MRLs* (ng/g)	Linearity	Sensitivity (ng/g)			MRLs* (ng/g)
	r^2	LOD	LOQ	LDet		r^2	LOD	LOQ	LDet	
2,4D	N.D.	N.D.	N.D.	N.D.	50	0.997	2.0	6.6	91	50
Acetamiprid	0.995	0.22	0.75	5.8	3000	0.999	0.06	0.21	0.32	10
Alachlor	0.995	0.24	0.79	3.8	20	0.999	0.91	3.1	3.05	20
Atrazine	0.995	0.05	0.18	0.18	50	0.998	0.03	0.11	0.11	50
Azinphos ethyl	0.997	6.6	22	22	20	0.995	1.8	6.0	6.0	20
Azinphos methyl	0.997	3.9	13	13	50	0.990	2.2	7.3	7.3	50
Bentazone	0.999	0.07	0.24	0.24	30	0.998	0.21	0.70	0.70	30
Bromoxynil	N.D.	N.D.	N.D.	N.D.	10	0.997	2.9	9.6	9.6	10
Chlorfenvinphos	0.999	0.23	0.78	0.78	20	0.998	0.08	0.26	0.26	20
Chlortoluron	0.998	0.47	1.6	2.9	20	0.997	0.45	1.49	1.49	20
Chlothianidin	0.997	14	45	45	90	0.997	1.1	3.8	7.5	20
Cyanazine	0.998	0.09	0.31	0.64	10	0.995	0.03	0.11	0.11	10
DEA	0.983	0.83	2.8	3.4	10	0.976	0.42	1.4	1.4	10
DIA	0.996	1.1	3.8	13	10	0.993	0.60	2.0	2.0	10
Diazinon	0.998	0.23	0.75	0.75	20	0.998	0.03	0.10	0.10	20
Dichlorvos	0.998	13	42	42	10	N.D.	N.D.	N.D.	N.D.	10
Diflufenican	0.998	0.65	2.2	2.1	600	1.000	0.51	1.7	1.7	10
Dimethoate	0.999	2.2	7.3	7.3	3000	0.998	0.12	0.49	1.3	10
Diuron	0.998	2.5	8.4	8.4	20	0.997	1.9	6.2	6.2	20
Fenthion	0.992	12	39	39	10	0.998	12	40	40	20
Fenthion oxon	0.998	0.06	0.20	0.20	10	0.996	0.06	0.21	0.21	20
Fenthion oxon sulfone	1.000	1.9	6.4	29	10	0.998	0.50	1.6	6.6	20
Fenthion oxon sulfoxide	0.996	0.21	0.71	0.94	10	0.997	0.19	0.64	0.64	20
Fenthion sulfone	0.992	18	59	59	10	0.996	12	41	41	20
Fenthion sulfoxide	0.997	0.40	1.3	1.3	10	0.996	0.23	0.77	0.77	20
Imidacloprid	0.999	1.5	5.1	5.1	1000	0.990	0.3	0.98	0.98	100
Irgarol	0.998	0.19	0.62	0.62	10	0.993	0.11	0.38	0.38	10
Isoproturon	0.998	0.49	1.6	2.5	10	0.995	5.9	19	19	10
Linuron	0.998	1.0	3.4	3.4	50	1.000	0.6	2.0	2.0	100
Malaoxon	0.999	0.18	0.61	0.61	20	0.999	0.08	0.27	0.27	20
Malathion	0.997	1.6	5.3	5.2	20	0.997	1.6	5.5	18	20
MCPA	N.D.	N.D.	N.D.	N.D.	50	0.998	5.1	17	17	100
Methiocarb	0.991	0.80	2.7	4.4	200	0.992	0.22	0.73	0.73	100
Metolachlor	0.998	0.08	0.27	0.27	50	0.998	0.05	0.18	0.18	50
Molinate	0.997	0.26	0.85	0.85	20	0.999	0.25	0.82	1.3	20
Propanil	0.996	0.60	2.0	2.0	50	0.994	0.19	0.62	0.62	50
Quinoxyfen	0.999	1.5	4.9	4.9	20	0.996	0.08	0.28	0.28	50
Simazine	0.994	0.16	0.5	0.53	10	0.996	0.11	0.37	0.37	20
Terbuthylazine	0.996	0.01	0.03	0.03	50	0.995	0.03	0.08	0.08	100
Terbutryn	0.999	0.51	1.7	1.7	10	0.997	0.09	0.30	0.30	10
Thiacloprid	0.999	0.66	2.2	3.9	4000	0.998	0.06	0.19	0.19	20
Thiamethoxam	0.995	1.9	6.2	6.2	400	0.995	0.91	3.0	3.0	20

r^2: coefficient of determination, LOD: Limit of Detection, LOQ: Limit of Quantification, LDet: Limit of Determination, N.D.: *not detected.* MRL: *Maximum Residue Level.*
*Extracted from http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=product.selection&language=EN

performance for pesticides such as 2,4-dichlorophenoxyacetic acid (2,4D), bromoxynil, diflufenican and quinoxyfen, among others. Finally, the acidification of extracts presented quadratic effects and, considering the overall desirability results, maximum effects were set at 0.7% and 0.5% of formic acid for olives and sunflower seeds, respectively. These conditions only affected negatively to bromoxynil, for which the presence of protons in the medium could probably hinder the ionization of its hydroxyl group. Prediction profiles for each compound and desirability plots are shown in Figs. S1-S2.

3.2. Method validation

Method performance for the different target compounds under the optimum conditions is shown in Table 3 and Table 4 in terms of trueness (recoveries), sensitivity, repeatability and linearity. As described in the guideline SANTE/12682/2019 (European Commission, 2019), a linearity range was established from the LOQ to 500 ng/mL (1000 ng/g) for each analyte, through the methanolic solutions used to construct calibration curves that did not deviate ± 20% from the theoretical values. The coefficient of determination (r^2) was used for linearity evaluation, showing values above 0.99 for every pesticide analysed, except for DEA (0.983 in olives and 0.976 in sunflower seeds).

As shown in Table 3, absolute recoveries were calculated for both matrices, showing good agreement at the two concentrations tested in the validation study. Extraction recoveries were considerably better in sunflower seeds than in olives: 88% and 57% of the target pesticides presented absolute recoveries above 50% after sunflower seed and olives extraction, respectively. Many matrix components from olives co-elute with the target pesticides (Cunha et al., 2007; López-Blanco et al., 2016) diminishing the extraction efficiency rates and subsequent MS response. In sunflower seeds, MCPA is the only pesticide with a highly affected recovery (9%), while in olives low recoveries were obtained for all neonicotinoids (from 9 to 25%), diuron (24%), and dimethoate (17%). Nevertheless, as shown in Table 4, LOQs for these compounds were below the MRLs established by the EC. Moreover, the use of isotopically labelled compounds as surrogates compensates for the losses occurred during extraction and analysis, and thus average relative recoveries (n = 6) obtained for both commodities are in compliance with the SANTE guidance (European Commission, 2019). As shown in Table 3, relative recoveries were between 70 and 120% for all compounds with the exception of azinphos ethyl, fenthion oxon sulfoxide, fenthion sulfoxide, malathion and malaoxon in sunflower seeds (67, 133, 134, 131 and 145%, respectively), and desisopropylatrazine (DIA) and diazinon in olives (132 and 133%, respectively). However, precision

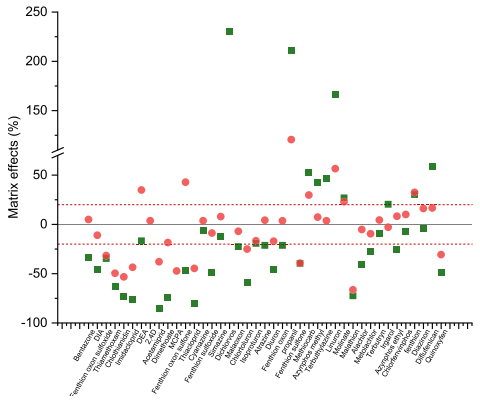

Fig. 1. Matrix effects (%) calculated for olives (green squares) and sunflower (red dots) seeds during method validation. Red dash reference lines represent ± 20%. Compounds ordered according to retention time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was consistent for these compounds with RSD values below 8% (except diazinon, RSD 13.5%). In general, RSD values were below 20% for all analytes, with a 90% of the target pesticides below 10% RSD.

In terms of sensitivity, LDets in olives ranged from 0.03 to 59 ng/g, whereas in sunflower seeds LDets oscillated from 0.08 to 42 ng/g. LDets below the European MRLs established for each commodity were obtained for 37 (olives) and 38 (sunflower seeds) out of the 42 target pesticides (Table 4). Higher LDets were obtained for fenthion and fenthion sulfone (in both matrices), azinphos ethyl, DIA, and dichlorvos in the case of olives, and 2,4D and isoproturon in sunflower seeds. On the other hand, the lowest LODs were achieved for bentazone, cyanazine, metolachlor, and terbuthylazine (in olives), and acetamiprid, atrazine, chlorfenvinphos, cyanazine, diazinon, fenthion oxon, malaoxon, metolachlor, quinoxyfen, terbuthylazine, terbutryn, and thiacloprid (in sunflower seeds), reaching values below 0.1 ng/g. In general, this responds to an appropriate extraction procedure, which led to good recoveries, and lower matrix interferences enhancing the ionization efficiency in the ESI probe. Moreover, improved LODs were obtained for most of the compounds studied in previously developed methods (Table S3) (Anagnostopoulos & Miliadis, 2013; Gilbert-López et al., 2010; Gómez-Almenar & García-Mesa, 2015; Kaczyński, 2017; López-Blanco et al., 2016; Sánchez-Hernández et al., 2016).

As expected, comparatively lower matrix effects were obtained for sunflower seeds due to the matrix complexity of olives. Fig. 1 shows the distribution of matrix effects in both commodities, where, in olives, suppression effects affected to 74% of the compounds and only 18% had a soft matrix effect (±20%). In the case of sunflower seeds, 53% of the pesticides had no significant matrix effects. In this matrix, half of the target compounds presented a signal ionization enhancement, being propanil the most affected (120%). In contrast, malathion was the pesticide that suffered from highest suppression (-66%). In olives, the maximum ionization suppression was determined for acetamiprid (-85%), imidacloprid (-76%), thiacloprid (-80%) or dimethoate (-74%), which is in accordance with the results of absolute recoveries obtained previously, while dichlorvos, propanil and linuron presented ionization enhancement effects above 100%. As shown in Fig. 1, the use of clean-up sorbents such as C18e, focused on the removal of fatty components, resulted on a higher suppression effect for the most polar compounds (lower RT).

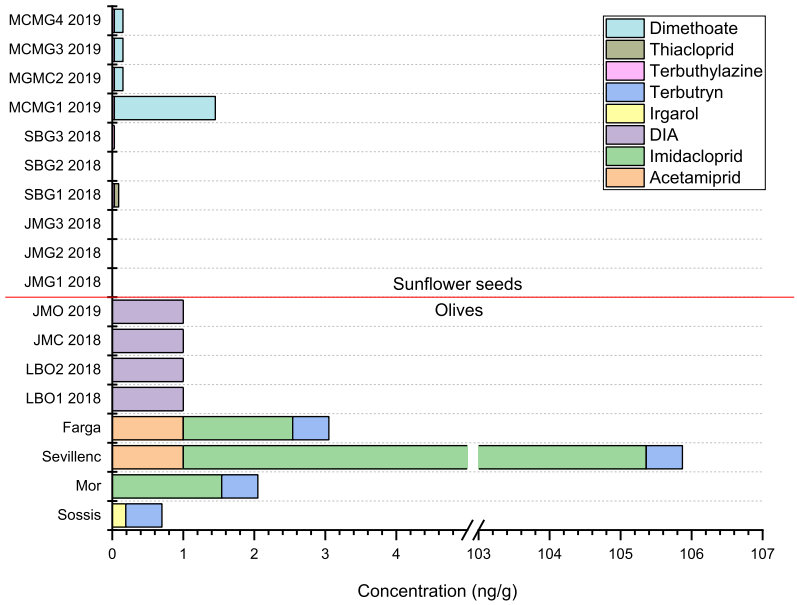

Fig. 2. Pesticide concentrations detected in real samples. LODs are shown for those analytes detected at a level below LDet. MCMG, SBG and JMG samples correspond to sunflower seeds. JMO, JMC, LBO, Farga, Sevillenc, Mor and Sossis samples correspond to olives.

3.3. Method application to real samples

Olive and sunflower crops from Serpa (South of Portugal) harvested during 2018 and 2019, together with samples taken from different fields of Tarragona (NE Spain) during 2020 were analysed as a part of the method evaluation. For the positive identification of the target pesticides in a sample, both SRM transitions should completely overlap regarding retention time (RT) and peak shape, and RT of the extract should correspond to that of the calibration standard in the same batch within ± 0.1 min. If longer RT shift occurred among samples in the same sequence, the corresponding isotopically-labelled compound should have been affected with the same shift to be accepted. Furthermore, the ratio between both SRM transitions in the sample should be within ± 30% of that observed in calibration standards from the same sequence (SANTE/12682/2019).

With these premises, a few pesticides were detected (Fig. 2). For these kinds of oilseed crops, the use of pesticides is predominantly covered by herbicides, insecticides and, to a lesser extent, fungicides (Amvrazi & Albanis, 2009; Debaeke et al., 2017). The neonicotinoid imidacloprid was the pesticide found at the highest concentration in the investigated samples, surpassing 100 ng/g in one olive sample, the "*sevillenc*" variety. This compound, together with DIA and terbutryn, were the most frequently detected pesticides in olives. Besides, acetamiprid and irgarol were also found at concentrations over the LOQ of their SRM1 but below the LOD of their SRM2, thus preventing quantification. In the case of sunflower seeds, the organophosphorus insecticide dimethoate was present in 4 out of 10 samples, showing the highest concentration at 1.42 ng/g in one of the Portuguese samples from the 2019 campaign. Moreover, terbuthylazine was also detected at very low concentrations in 6 out of 10 sunflower seed samples and the insecticide thiacloprid in one sample.

Thiacloprid, imidacloprid, dimethoate, and terbutryn are included in the Pesticide Action Network (PAN) International list of Highly Hazardous Pesticides (Pesticide Action Network International, 2019). High honeybee toxicity is attributed to dimethoate and imidacloprid, together with a moderate acute human toxicity. Moreover, ecotoxicity of dimethoate includes moderate poisoning of mammals and moderate to very high toxicity to birds. Regarding the neonicotinoid thiacloprid, it represents a well-known moderate hazard to humans and it has been classified as a carcinogen (Pesticide Action Network International, 2019).

4. Conclusions

Two QuEChERS-based analytical methods have been developed and validated for the determination of 42 pesticides, including triazines, organophosphates, phenylureas, anilines, neonicotinoids and others, in two representative food fatty matrices: olives and sunflower seeds. Method extraction was optimized through a fractional factorial design of experiments, which allowed optimizing the method conditions in a cost-effective way. Four different factors were evaluated, including the acidification of the extraction solvent, the type of extractive salts, the type of clean-up salts, and the acidification of the final extract. The optimized methods were successfully validated in terms of linearity, repeatability (with RSD values below 20%) and trueness (with average relative recoveries between 70 and 120% for almost all analytes), in compliance with SANTE guidelines. The sensitivity achieved with the methods, with LOQs in the range of pg/g or low ng/g, allowed determining concentrations below the established MRLs for>90% of the target compounds. Although the use of isotopically-labelled surrogates for the quantification of the target pesticides represents a high initial economical cost, numerous advantages, such as control of the well performance of the analytical method throughout the sequence, compensation of matrix effect regardless of its variability between samples, accuracy of results, and easier quantification (without the need to use recovery factors) are obtained. Thus, the use of this kind of surrogate compounds in the analysis of complex matrices, such as olives and sunflower seeds, are extremely useful to obtain highly reliable results. Furthermore, the advantages of using QuEChERS over other conventional extraction methods are well-known: fast, cheap and safe method performance, beside the wide applicability for multiresidue analysis.

These methods were applied to the analysis of a few real samples from different locations across the Iberian Peninsula. Their application showed the presence of some herbicides and insecticides of relevant environmental concern, including four pesticides from the Highly Hazardous Pesticides list from the PAN International.

CRediT authorship contribution statement

Manuel García-Vara: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Cristina Postigo:** Methodology, Validation, Writing – review & editing. **Patricia Palma:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **María José Bleda:** Methodology, Formal analysis, Resources, Writing – review & editing. **Miren López de Alda:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration.

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.

Acknowledgements

This work has been funded by the European Union through the European Agricultural Fund for Rural Development (FitoFarmGest Operational Group PDR2020-101-030926), the Spanish Ministry of Science and Innovation (Project CEX2018-00794-S) and the Government of Catalonia (2017 SGR 01404). BEKOlut is acknowledged for the gift of QuEChERS kits.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132558.

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Supporting Information

QuEChERS-based Analytical Methods Developed for LC-MS/MS Multiresidue Determination of Pesticides in Representative Crop Fatty Matrices: Olives and Sunflower Seeds

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

Pesticide	Type	MM (g mol^{-1}) ‡	Solub (mg L^{-1}) ‡	pKa ‡	Kow logP ‡	Koc (mL g-1) ‡	GUS ‡	Henry´s constant (Pa m^3 mol^{-1}) ‡
2,4D	Alkylchlorophenoxy acid	221.04	24300	3.40	-0.82	39	1.69	4.0 X 10^{-06}
Acetamiprid	Neonicotinoid	222.67	2950	0.7	0.80	200	0.40	5.3 X 10^{-08}
Alachlor	Chloroacetamide	269.77	240	0.62	3.09	335	1.08	3.2 X 10^{-03}
Atrazine	Triazine	215.68	35	1.7	2.70	100	3.2	1.5 X 10^{-04}
Azinphos ethyl	Organophosphate	345.38	4.5	n/a	3.18	1500	1.4	3.1 X 10^{-06}
Azinphos methyl	Organophosphate	317.32	28	5	2.96	1112	1.42	5.7 X 10^{-06}
Bentazone	Benzothiazinone	240.30	7112	3.51	-0.46	55	2.89	7.2 X 10^{-05}
Bromoxynil	Hydroxybenzonitrile	276.90	38000	3.86	0.27	302	0.03	8.7 X 10^{-07}
Chlorfenvinphos	Organophosphate	359.60	145	n/a	3.80	680	1.83	-
Chlortoluron	Phenylurea	212.68	74	n/a	2.50	196	3.02	1.4 X 10^{-05}
Chlothianidin	Triazine	240.69	171	12.9	2.10	190	2.07	6.6 X 10^{-06}
Cyanazine	Neonicotinoid	249.68	340	11.1	0.90	123	4.91	2.9 X 10^{-11}
DEA	Metabolite	173.60	980	n/a	1.15	130	-	980
DIA	Metabolite	187.63	2700	n/a	1.51	110	4.37	1.6 X 10^{-04}
Diazinon	Organophosphate	304.35	60	2.6	3.69	609	1.14	6.1 X 10^{-02}
Dichlorvos	Organophosphate	220.98	18000	n/a	1.90	50	0.69	2.6 X 10^{-02}
Diflufenican	Carboxamide	394.29	0.05	n/a	4.20	3.19°	1.51	1.2 X 10^{-02}
Dimethoate	Organophosphate	229.26	25900	n/a	0.75	25*	1.01	1.4 X 10^{-06}
Diuron	Phenylurea	233.09	35.6	n/a	2.87	813	1.83	2.0 X 10^{-06}
Fenthion	Organophosphate	278.33	4.2	n/a	4.84	1500	1.26	2.4 X 10^{-02}
Fenthion oxon	Metabolite	262.26*	213.5*	n/a	2.31*	57 *	-	3.0x10^{-9} *
Fenthion oxon sulfone	Metabolite	294.03*	7602*	n/a	0.28*	13*	-	2.4 x 10^{-11} *
Fenthion oxon sulfoxide	Metabolite	278.26*	1222*	n/a	0.15*	11*	-	9.5 x 10^{-8} *
Fenthion sulfone	Metabolite	310.33*	190.4*	n/a	2.05*	542*	-	1.1x10^{-8} *
Fenthion sulfoxide	Metabolite	294.33*	3.72*	n/a	1.92*	466*	-	7.0x10^{-6} *
Imidacloprid	Neonicotinoid	255.66	610	n/a	0.57	6719	3.74	1.7 X 10^{-10}
Irgarol	Triazine	253.37	7	n/a	3.95	1569	-	1.3 x 10^{-07}*
Isoproturon	Phenylurea	206.28	70.2	n/a	2.5	251*	2.07	1.5 X 10^{-05}
Linuron	Phenylurea	249.09	63.8	n/a	3	843	2.21	2.0 X 10^{-04}
Malaoxon	Organophosphate	314.29*	7500*	n/a	0.52*	4650*	-	1.2 X 10^{-08} *
Malathion	Organophosphate	330.36	148	n/a	2.75	1800	-1.28	1. 0 X 10^{-03}
MCPA	Metabolite	200.62	29390	3.73	-0.81	29*	2.94	5.5 X 10^{-05}
Methiocarb	Carbamate	225.31	27	n/a	3.18	182*	0.55	1.2 X 10^{-04}
Metolachlor	Chloroacetamide	283.80	530	n/a	3.40	120	2.10	2.4 X 10^{-03}
Molinate	Thiocarbamate	187.30	1100	n/a	2.86	190	2.49	6.9 X 10^{-01}
Propanil	Anilide	218.08	95	19.1	2.29	149	-0.51	4.4 X 10^{-04}
Quinoxyfen	Quinoline	308.13	0.05	n/a	4.66	23**	-0.93	3.2 X 10^{-02}
Simazine	Triazine	201.66	5	1.62	2.30	130	2	5.6 X 10^{-05}
Terbuthylazine	Triazine	229.71	6.6	1.9	3.40	329*	3.07	3.2 X 10^{-03}
Terbutryn	Triazine	241.36	25	4.3	3.66	2432	2.4	1.5 X 10^{-03}
Thiacloprid	Neonicotinoid	252.72	184	n/a	1.26	615**	0.14	5.0 X 10^{-10}
Thiamethoxam	Neonicotinoid	291.71	4100	n/a	-0.13	56	4.69	4.7 X 10^{-10}

MM: molecular mass; Solub: solubility in water at 20 oC ; 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/ppdb/en/index.htm - Lewis, K.A., Tzilivakis, J., Warner, D. and Green, A. (2016). An international database for pesticide risk assessment and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064. *Data estimated using the US Environmental Protection Agency EPISuiteTM 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

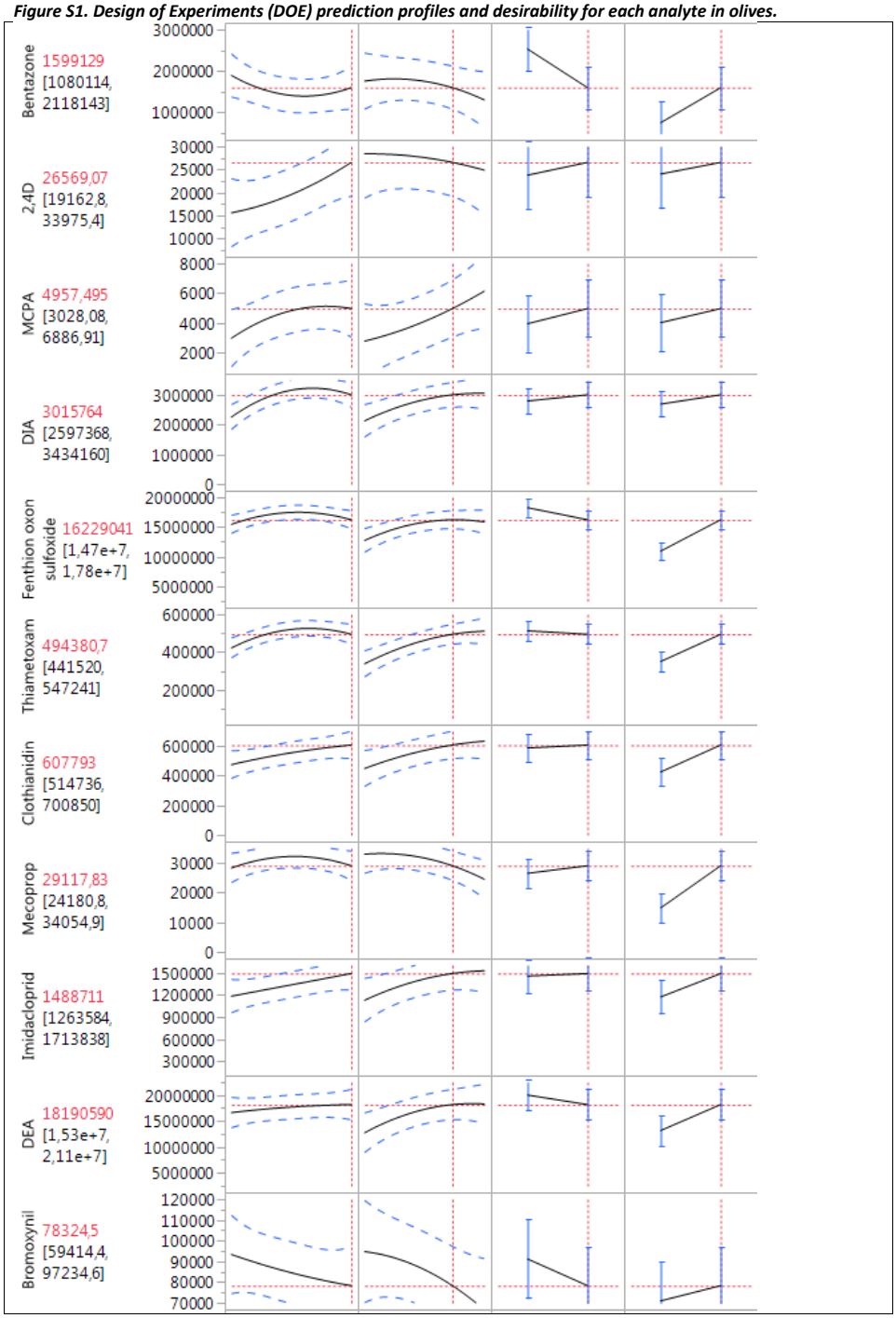
Table S2. Details of the regression model adjusted for each compound in the design of experiments.

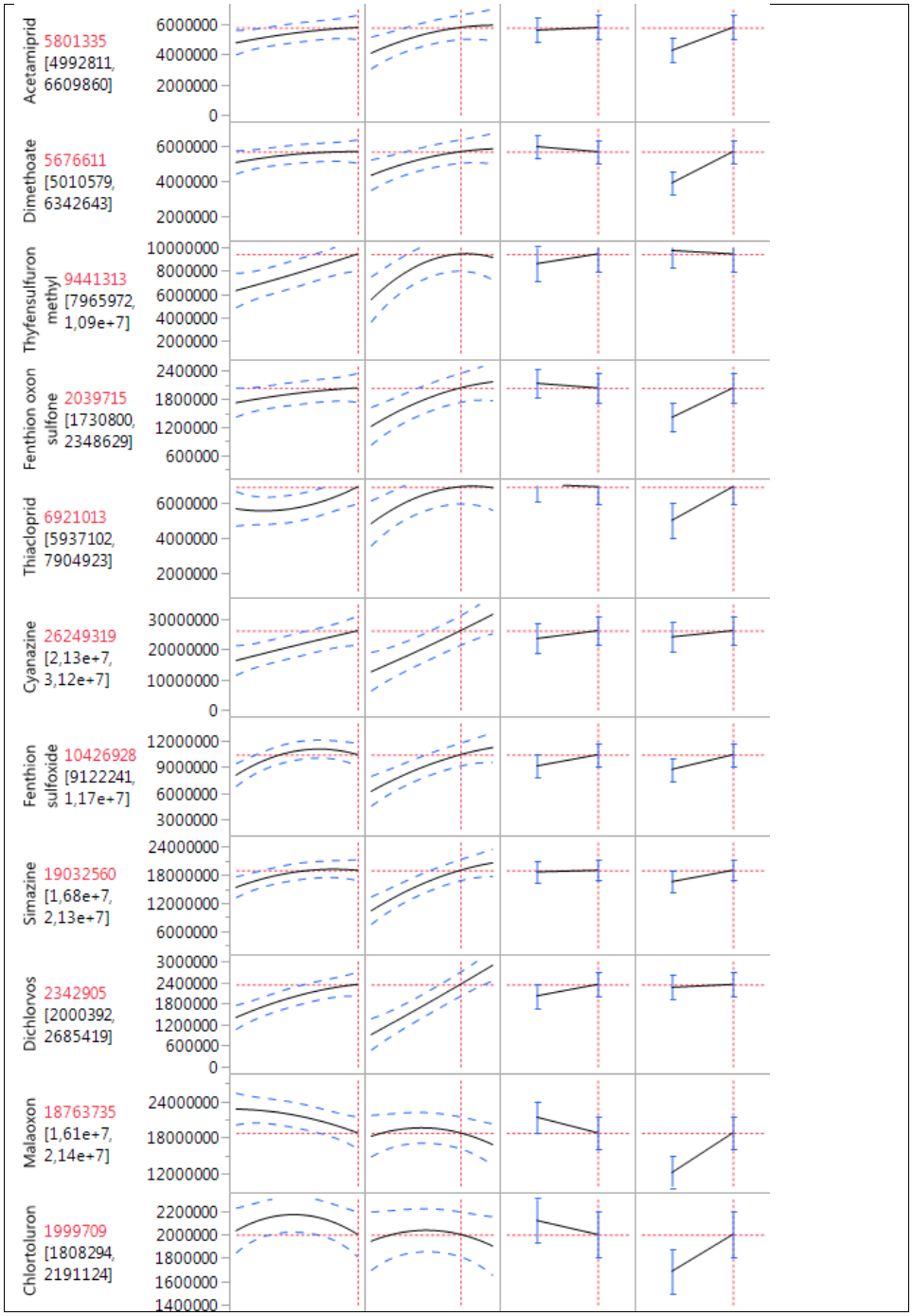
Factors	Type	Name	Number of levels	Low level	Medium level	High level
ACN (formic acid)	Continuous	X_1	3	0%	0.5%	1%
Formic acid final extract	Continuous	X_2	3	0%	0.5%	1%
Extractive salts	Categorical	F_1	2	Acetate		Citrate
Clean-up sorbent	Categorical	F_2	2	4		8A
Adjusted regression model for compound *Y*						
$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 F_1 + \beta_4 F_2 + \beta_5 X_1^2 + \beta_6 X_2^2 + \beta_7 X_1 X_2$						

Table S3. Comparison of LODs (ng/g) with commensurate methods found in the literature.

	Olives					Sunflower seeds		
Compound	**LOD**	[1]	[2]	[3]	[4]	**LOD**	[5]	[6]
2,4D	N.D.					2.0		
Acetamiprid	0.22		0.8	3.0		0.06		0.3
Alachlor	0.24	20	3.3	3.0		0.91	1.2	
Atrazine	0.05		0.7			0.03	0.06	
Azinphos ethyl	6.6	6.0		3.0		1.8		
Azinphos methyl	3.9		7.0			2.2		
Bentazone	0.07					0.21		
Bromoxynil	N.D.					2.9		
Chlorfenvinphos	0.23	0.50	1.4	3.0		0.08		
Chlortoluron	0.47		1.3	3.0		0.45	0.25	
Chlothianidin	14					1.1		1.2
Cyanazine	0.09			3.0		0.03	0.87	
DEA	0.83					0.42		
DIA	1.1					0.60		
Diazinon	0.23	0.06	5.0	3.0		0.03		
Dichlorvos	13					N.D.		
Diflufenican	0.65		21		0.40	0.51	0.06	
Dimethoate	2.2		2.7	3.0	0.40	0.12		
Diuron	2.5		5.7		0.30	1.9	0.12	
Fenthion	12		22			12		
Fenthion oxon	0.06			3.0		0.06		
Fenthion oxon sulfone	1.9					0.50		
Fenthion oxon sulfoxide	0.21			3.0		0.19		
Fenthion sulfone	18			3.0		12		
Fenthion sulfoxide	0.40		2.3	3.0		0.23		
Imidacloprid	1.5		2.8	3.0		0.30		0.60
Irgarol	0.19					0.11		
Isoproturon	0.49		6.0	3.0		5.9	0.12	
Linuron	1.0					0.60	0.05	
Malaoxon	0.18			3.0		0.08		
Malathion	1.6	1.6	1.3			1.6		
MCPA	N.D.					5.1		
Methiocarb	0.80					0.22		
Metolachlor	0.08	0.20				0.05	0.07	
Molinate	0.26					0.25		
Propanil	0.60					0.19		
Quinoxyfen	1.5					0.08		
Simazine	0.16		1.0			0.11	0.05	
Terbuthylazine	0.01		2.8		0.03	0.03	0.10	
Terbutryn	0.51					0.09	0.15	
Thiacloprid	0.66		2.1	3.0		0.06		0.40
Thiamethoxam	1.9		3.7	3.0		0.91		0.60

Figure S1. Design of Experiments (DOE) prediction profiles and desirability for each analyte in olives.

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4000000
2000000
0
Dimethoate
5676611
[5010579, 6342643]
6000000
4000000
2000000
Thyfensulfuron methyl
9441313
[7965972, 1,09e+7]
10000000
8000000
6000000
4000000
2000000
Fenthion oxon sulfone
2039715
[1730800, 2348629]
2400000
1800000
1200000
600000
Thiacloprid
6921013
[5937102, 7904923]
6000000
4000000
2000000
Cyanazine
26249319
[2,13e+7, 3,12e+7]
30000000
20000000
10000000
0
Fenthion sulfoxide
10426928
[9122241, 1,17e+7]
12000000
9000000
6000000
3000000
Simazine
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[1,68e+7, 2,13e+7]
24000000
18000000
12000000
6000000
Dichlorvos
2342905
[2000392, 2685419]
3000000
2400000
1800000
1200000
600000
0
Malaoxon
18763735
[1,61e+7, 2,14e+7]
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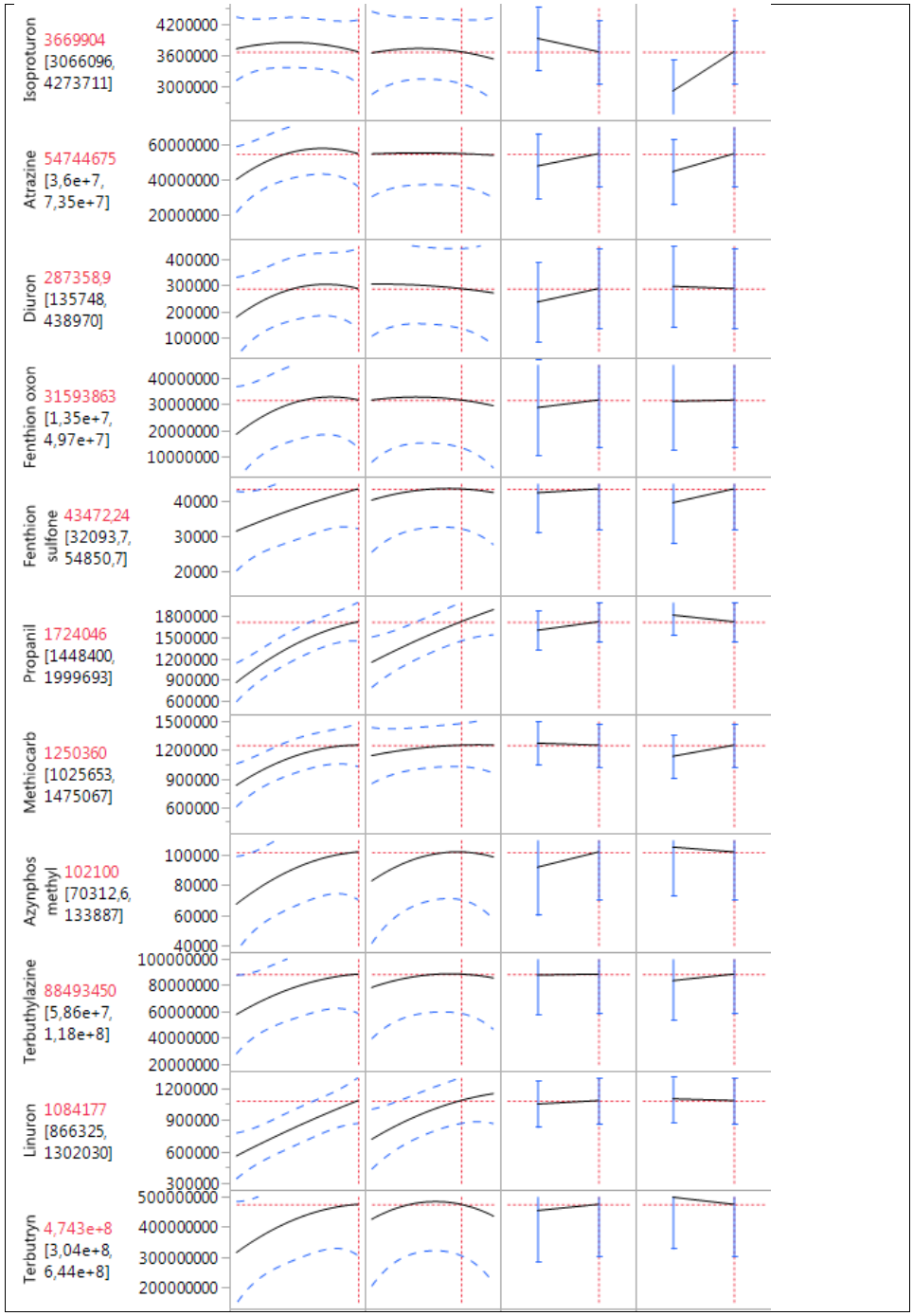
18000000
12000000
Chlortoluron
1999709
[1808294, 2191124]
2200000
2000000
1800000
1600000
1400000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3000000
Atrazine
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60000000
40000000
20000000
Diuron
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[135748, 438970]
400000
300000
200000
100000
Fenthion oxon
31593863
[1,35e+7, 4,97e+7]
40000000
30000000
20000000
10000000
Fenthion sulfone
43472,24
[32093,7, 54850,7]
40000
30000
20000
Propanil
1724046
[1448400, 1999693]
1800000
1500000
1200000
900000
600000
Methiocarb
1250360
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1500000
1200000
900000
600000
Azynphos methyl
102100
[70312,6, 133887]
100000
80000
60000
40000
Terbuthylazine
88493450
[5,86e+7, 1,18e+8]
100000000
80000000
60000000
40000000
20000000
Linuron
1084177
[866325, 1302030]
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600000
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Terbutryn
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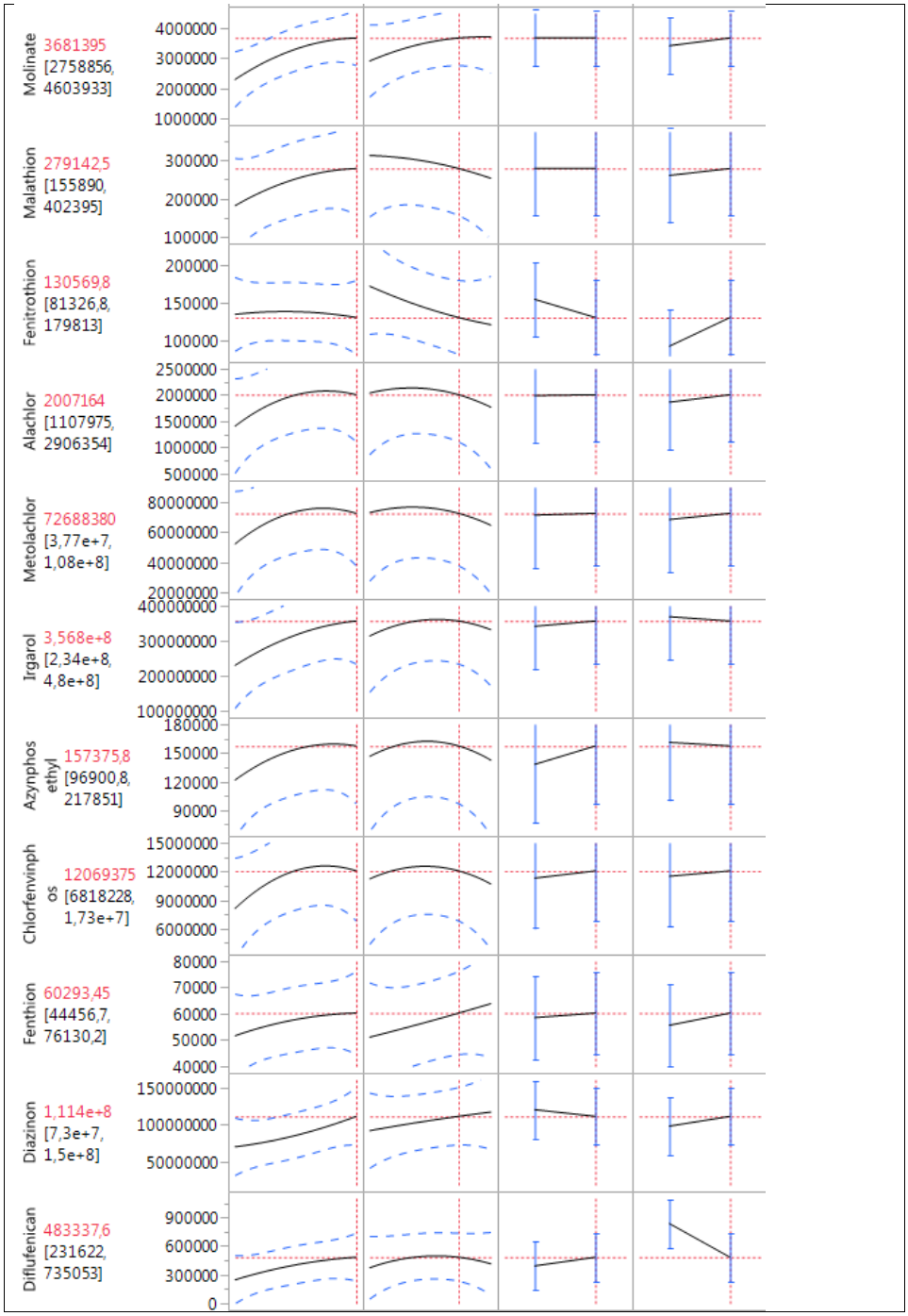
300000000
200000000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[2758856, 4603933]
4000000
3000000
2000000
1000000
Malathion
279142,5
[155890, 402395]
300000
200000
100000
Fenitrothion
130569,8
[81326,8, 179813]
200000
150000
100000
Alachlor
2007164
[1107975, 2906354]
2500000
2000000
1500000
1000000
500000
Metolachlor
72688380
[3,77e+7, 1,08e+8]
80000000
60000000
40000000
20000000
Irgarol
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300000000
200000000
100000000
Azynphos ethyl
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150000
120000
90000
Chlorfenvinphos
12069375
[6818228, 1,73e+7]
15000000
12000000
9000000
6000000
Fenthion
60293,45
[44456,7, 76130,2]
80000
70000
60000
50000
40000
Diazinon
1,114e+8
[7,3e+7, 1,5e+8]
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100000000
50000000
Diflufenican
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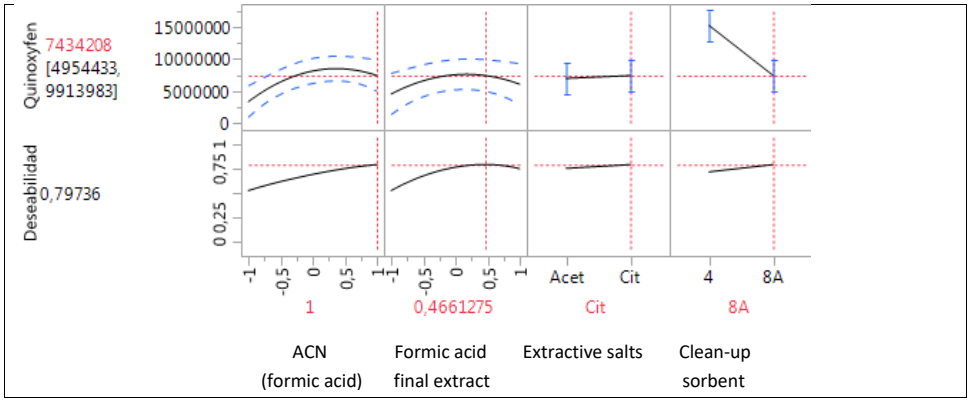
[231622, 735053]
900000
600000
300000
0


Quinoxyfen
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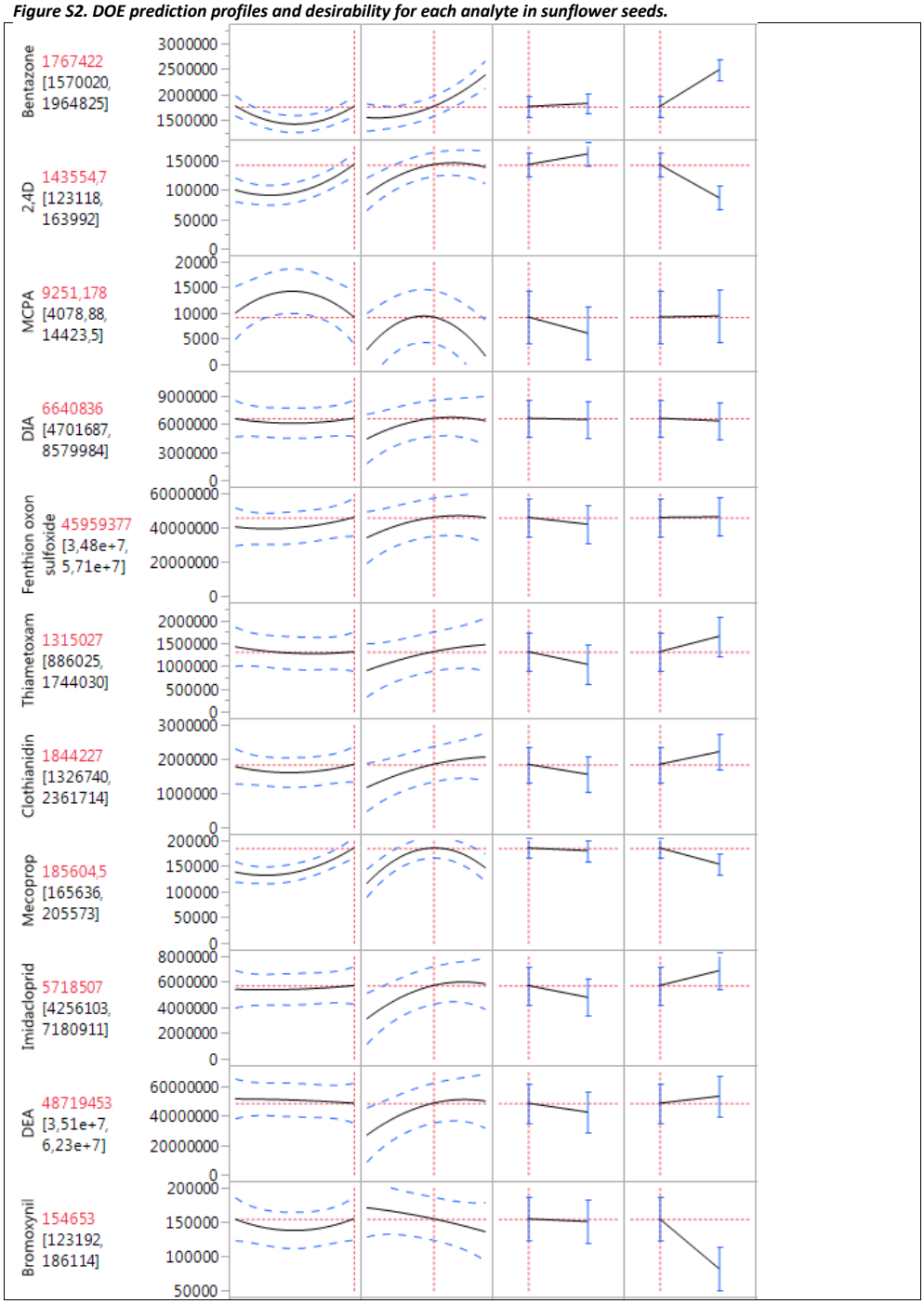
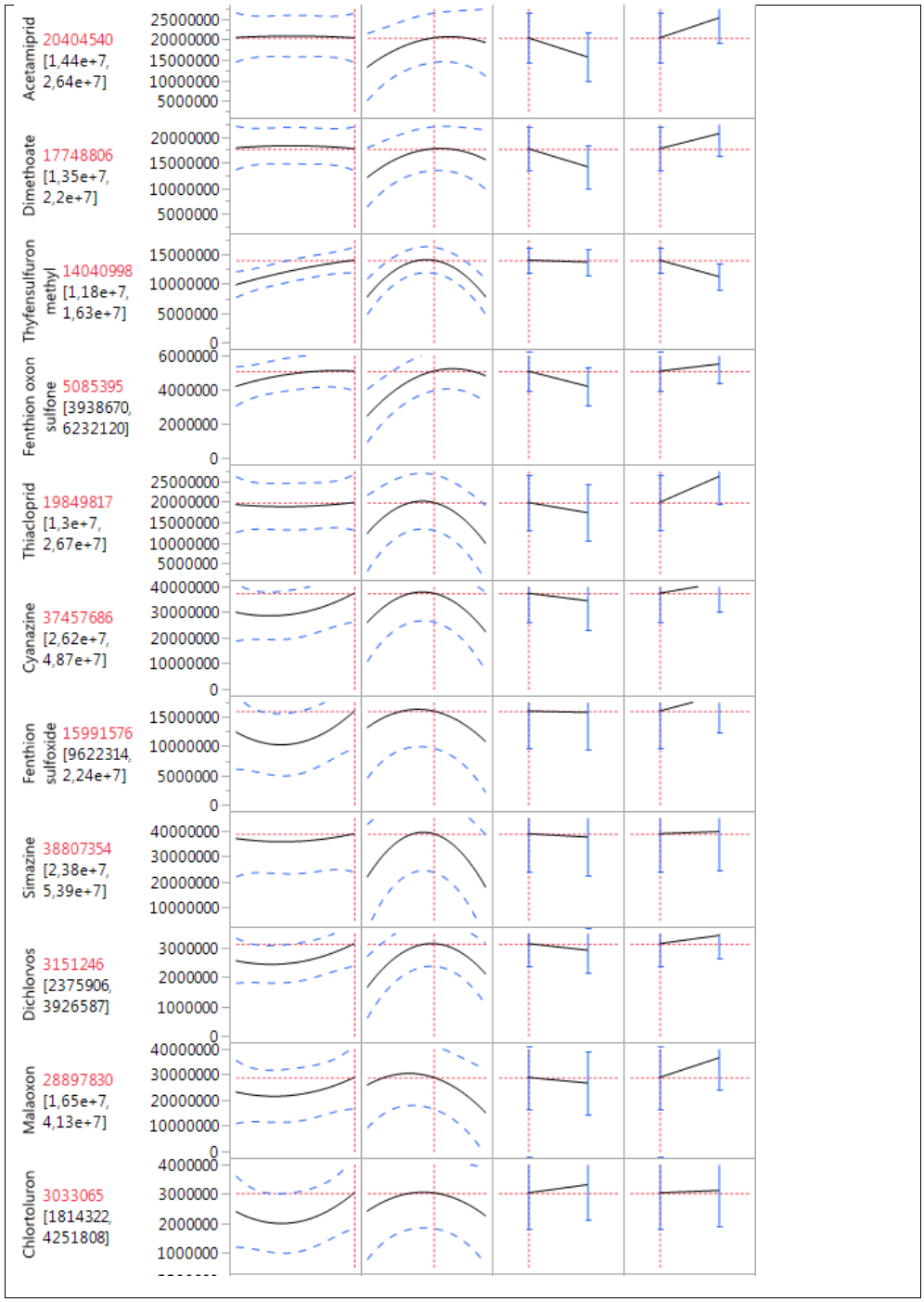
[4954433,
9913983]
Deseabilidad
0,79736
15000000
10000000
5000000
0
0
0,25
0,75
1
-1
-0,5
0
0,5
1
Acet
Cit
4
8A
1
0,4661275
Cit
8A
ACN
(formic acid)
Formic acid
final extract
Extractive salts
Clean-up
sorbent

Figure S2. DOE prediction profiles and desirability for each analyte in sunflower seeds.

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2,64e+7]
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10000000
5000000
Dimethoate
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2,2e+7]
20000000
15000000
10000000
5000000
Thyfensulfuron methyl
14040998
[1,18e+7,
1,63e+7]
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10000000
5000000
0
Fenthion oxon sulfone
5085395
[3938670,
6232120]
6000000
4000000
2000000
0
Thiacloprid
19849817
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2,67e+7]
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20000000
15000000
10000000
5000000
Cyanazine
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[2,62e+7,
4,87e+7]
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30000000
20000000
10000000
0
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5,39e+7]
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30000000
20000000
10000000
Dichlorvos
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3926587]
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2000000
1000000
0
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4,13e+7]
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0
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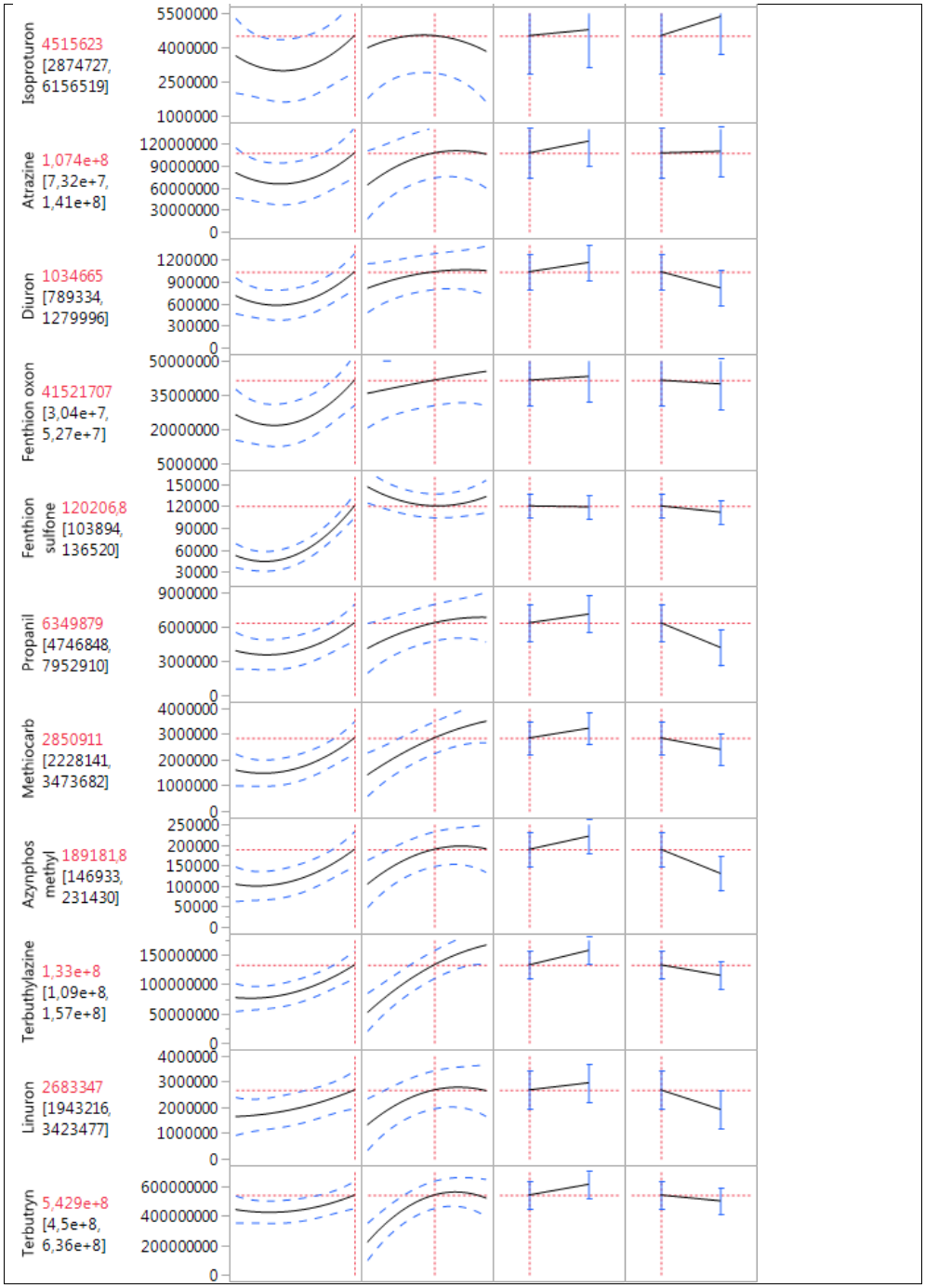
3000000
2000000
1000000

11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6156519]
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4000000
2500000
1000000
Atrazine
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1,41e+8]
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90000000
60000000
30000000
0
Diuron
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1279996]
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600000
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5000000
Fenthion sulfone
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136520]
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120000
90000
60000
30000
Propanil
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6000000
3000000
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2000000
1000000
0
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231430]
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150000
100000
50000
0
Terbuthylazine
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1,57e+8]
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100000000
50000000
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Linuron
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6,36e+8]
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200000000
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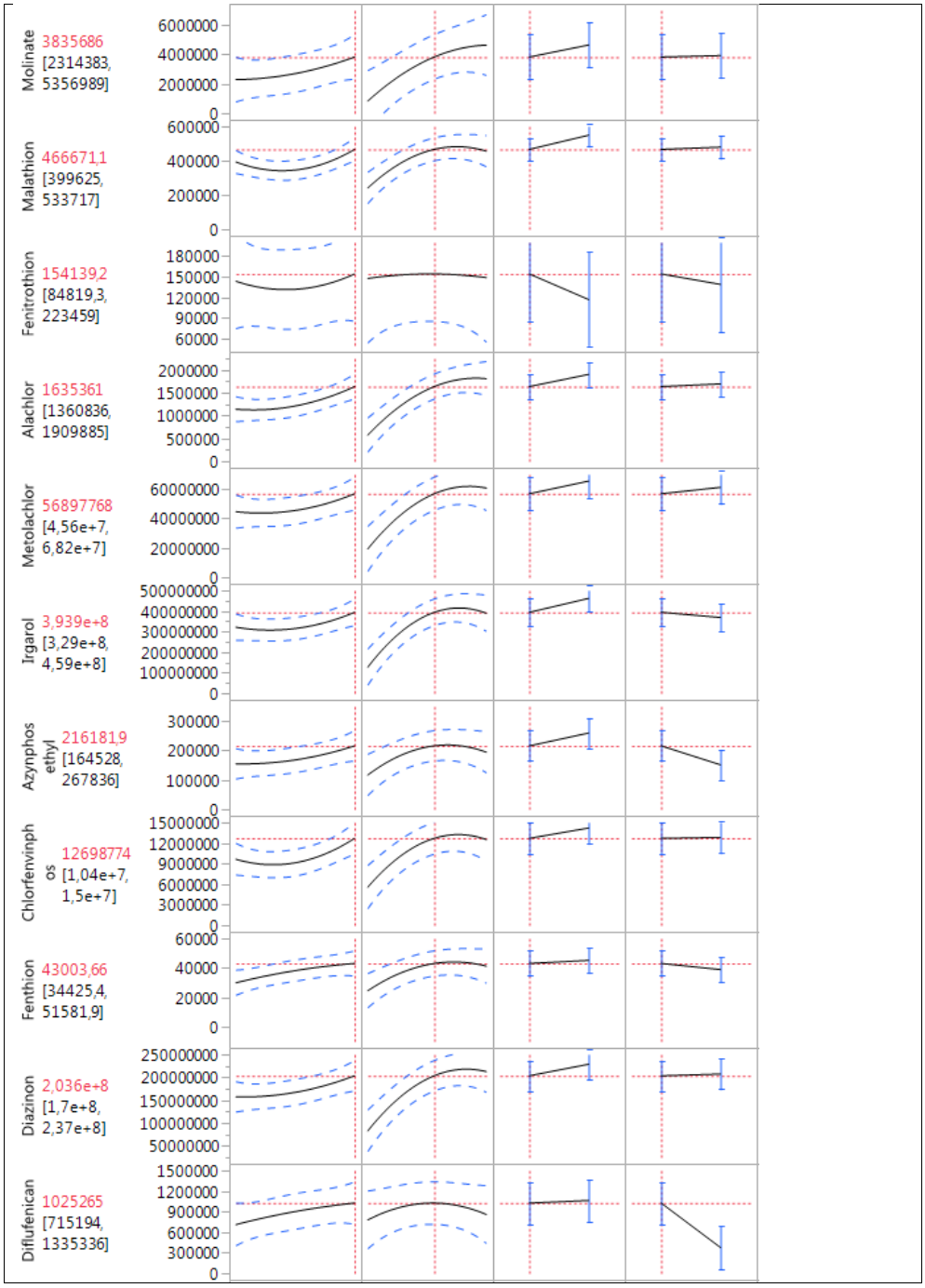
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200000
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223459]
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150000
120000
90000
60000
Alachlor
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1500000
1000000
500000
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6,82e+7]
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Irgarol
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300000000
200000000
100000000
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267836]
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200000
100000
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Chlorfenvinphos
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1,5e+7]
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12000000
9000000
6000000
3000000
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51581,9]
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40000
20000
0
Diazinon
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2,37e+8]
250000000
200000000
150000000
100000000
50000000
Diflufenican
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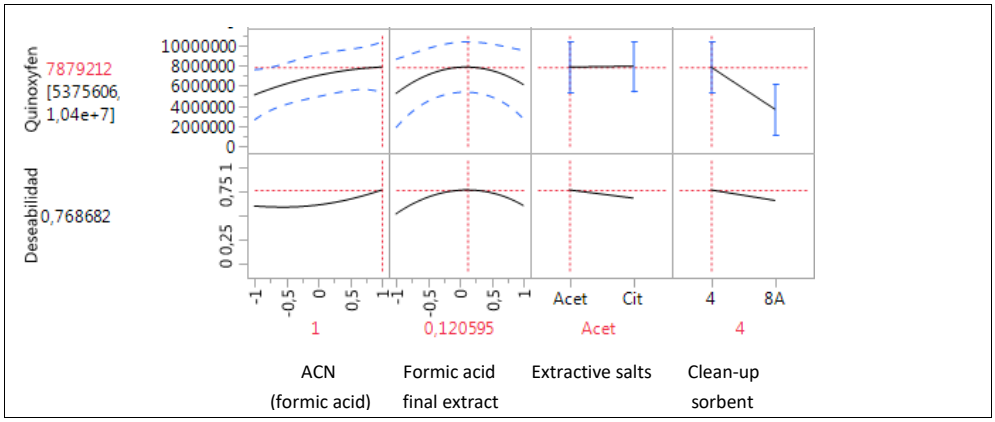
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Deseabilidad
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2000000
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0,25
-1
-0,5
0
0,5
1
Acet
Cit
4
8A
1
0,120595
Acet
4
ACN
(formic acid)
Formic acid
final extract
Extractive salts
Clean-up
sorbent

3.1.2. Scientific publication #2

Development of QuEChERS-based multiresidue analytical methods to determine pesticides in corn, grapes and alfalfa

Manuel García-Vara

Cristina Postigo

Patrícia Palma

Miren López de Alda

Food Chemistry, 405 (2023), 134870

(DOI: https://doi.org/10.1016/j.foodchem.2022.134870)

Food Chemistry 405 (2023) 134870

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Development of QuEChERS-based multiresidue analytical methods to determine pesticides in corn, grapes and alfalfa

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ARTICLE INFO

Keywords:
Pesticide residue analysis
Agrochemicals
Liquid chromatography-tandem mass spectrometry
Insecticides
Herbicides
QuEChERS

ABSTRACT

Analytical methods based on QuEChERS and liquid chromatography-tandem mass spectrometry were developed for the determination of 42 polar and semi-polar pesticides in three representative vegetable matrices, *viz.* corn, grapes and alfalfa. A 2^{6-2} fractional factorial experimental design was used to cost-effectively optimize the extraction procedure. The optimal analytical approaches were validated in terms of linearity, sensitivity, trueness, and precision. Most of the target pesticides showed a relative recovery of 70–120 %. Moreover, limits of detection (between 0.01 ng/g and 20 ng/g) were below the maximum residue levels set for the target analytes in nearly all cases. Analysis of real samples showed the presence of five pesticides in grapes and alfalfa at concentrations between the method LOQs and 40 ng/g. To the authors' knowledge, these methods are the first ever described for the determination of 6, 13 and 24 of the 42 target pesticides measured in corn, grapes and alfalfa, respectively.

1. Introduction

Pest control has existed for thousands of years (Unsworth, 2010). However, the negative consequences of the indiscriminate use of pesticides were publicly reported for the first time by Rachel Carson in 1962 (Carson, 1962). This work was the spur of a global concern on the environmental impact of pesticides that led to the development of a newer generation of, in principle, less toxic and less persistent pesticides. Although currently used pesticides have greater selectivity and lower environmental and toxicological impact, they are not completely innocuous and may represent an important source of contamination to soils and water bodies that also endangers biodiversity. To prevent potential damage, regulatory agencies worldwide, including the European Commission, have issued policies that aim at reducing pesticide use. However, farmers are still strongly dependent on these substances, as highlighted by the pesticide sales statistics in the last decade in Europe (Bernardes, Pazin Pereira, & Dorta, 2015; Pesticide Use in Europe | PAN Europe., n.d.). Among the EU member states, Germany, Spain, France and Italy buy more than two thirds of the total share of pesticides commercialized in Europe (Eurostat, 2020). To protect public health, the European Commission (EC) established Maximum Residue Levels (MRLs), mostly in the order of μg/kg or mg/kg, for pesticides in or on food (European Commision, 2005). Although MRLs differ depending on the commodity or the specific environmental occurrence and toxicological profile of the pesticide, a general MRL of 10 μg/kg is established when an MRL value is not specified (EU Pesticides Database, 2022). Therefore, the monitoring of pesticide residues in food becomes essential to accomplish with these MRLs.

Weeds, insects, or fungi, among others, are prone to control by spreading different pesticides to crops. The huge variety of chemicals that are legally approved for use requires the use of multiresidue analytical methods for their determination. However, the need of reaching low limits of detection in line with the stablished MRLs and the complexity of some food matrices complicate this task (Parrilla Vázquez et al., 2016; Valverde et al., 2018; Viera et al., 2017). Moreover, pesticides belong to different chemical classes, and consequently, present diverse physical–chemical properties. Therefore, the design of an extraction method efficient for many of them becomes a challenge. A myriad of sample pre-treatments for pesticide analysis in food commodities have been reported in literature, including solid-phase micro-

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https://doi.org/10.1016/j.foodchem.2022.134870
Received 19 May 2022; Received in revised form 2 November 2022; Accepted 3 November 2022
Available online 8 November 2022

extraction (SPME) (Madej et al., 2018; Naccarato & Tagarelli, 2019), solid–liquid extraction (SLE) (Madej et al., 2018; Valverde et al., 2018), pressurized liquid extraction (PLE) (Hoff & Pizzolato, 2018; Kinross et al., 2020), matrix solid-phase dispersion (MSPD) (Gilbert-López et al., 2010; Hoff & Pizzolato, 2018), solid-phase extraction (SPE) (Madej et al., 2018), microwave-assisted extraction (MAE) (Farajzadeh et al., 2019), or QuEChERS-based procedures (Barbieri et al., 2019; Gilbert-López et al., 2010; Madej et al., 2018), among others. Over the last few years, QuEChERS (acronym of *quick, easy, cheap, effective, rugged,* and *safe*) has gained ground in laboratories worldwide due to its broad applicability on food matrices and its good extraction efficiency for a wide number of organic compounds (Barchanska et al., 2018). QuEChERS-based methods consist on the analyte extraction via liquid partitioning using a small volume of acetonitrile (ACN), the subsequent collection of the organic fraction after a salting-out step, and the clean-up of the organic extract using dispersive solid-phase extraction (d-SPE). The versatility of this approach allows the use of different buffered salts for the salting-out step depending on the matrix and the pesticides properties (Lehotay et al., 2007; Standarization, 2018). Moreover, relying upon the matrix components, modified QuEChERS-based methods have been also developed through the optimization of the clean-up step. Primary secondary amines (PSA) (Barbieri et al., 2019; Cunha et al., 2007; López-Blanco et al., 2016), graphited carbon black (GCB) (Cunha et al., 2007; Lee et al., 2008), or octadecylsilane (C_{18}) (Barbieri et al., 2019; Cunha et al., 2007; López-Blanco et al., 2016) are the main sorbents used for clean-up. As regards analysis, the determination of residues of lowly polar, volatile and semi-volatile pesticides in food is commonly achieved by means of gas chromatography coupled to mass spectrometry (GC–MS) (Farajzadeh et al., 2019; Lee et al., 2008; Pico et al., 2020). In contrast, liquid chromatography combined with mass spectrometry (LC-MS) is the preferred technique for the determination of semi-polar and polar pesticides. For this purpose, the use of triple quadrupole mass spectrometers working in the selected reaction monitoring mode (SRM) is widely extended (López et al., 2020; Narenderan et al., 2020; Pico et al., 2020).

In this context, the objectives of this study were: *a)* to develop and validate highly sensitive, simple, reliable and practical analytical methods based on QuEChERS and LC coupled to tandem mass spectrometry (MS/MS) for the multiresidue determination of up to 42 polar and semi-polar pesticides, including neonicotinoids, triazines, phenylureas, organophosphates and anilines, among others, in three representative vegetable matrices, viz., corn (cereals), grapes (fruits) and alfalfa (green leaves); and *b)* to apply these methodologies to the analysis of real samples harvested from different regions to explore the occurrence of the target pesticides. Corn, grapes and alfalfa were chosen for the study as they represent rotary (corn) and permanent crops (alfalfa, vineyards), and they were irrigated cultures throughout the study period. Moreover, these crops, used for human food (grapes), animal food (alfalfa) or both (corn), represent a large agricultural surface in the area under study (Serpa, Portugal). To the authors' knowledge, these are the first analytical methods ever published for some of the target compounds in the considered matrices: diflufenican, fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, irgarol, and thiacloprid in corn (14 % of the target pesticides); 2,4-D, bentazone, bromoxynil, chlorfenvinphos, fenthion oxon, fenthion oxon sulfone and sulfoxide, fenthion sulfone and sulfoxide, irgarol, MCPA, propanil, and terbuthylazine in grapes (31 % of the target pesticides); and acetamiprid, atrazine, deisopropylatrazine (DIA), desethylatrazine (DEA), azinphos methyl, bentazone, bromoxynil, clothianidin, chlorfenvinphos, dimethoate, diflufenican, fenthion oxon, fenthion oxon sulfone and sulfoxide, fenthion sulfone and sulfoxide, imidacloprid, irgarol, malaoxon, molinate, metolachlor, MCPA, propanil, and terbutryn in alfalfa (57 % of the target pesticides).

2. Materials and methods

2.1. Chemicals and reagents

High purity (96–99.9 %) standards for 42 pesticides and 34 isotopically-labelled analogues were purchased from Fluka (Sigma–Aldrich, Steinheim, Germany) or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). Table 1 shows the target analytes with the optimal LC-MS/MS conditions used for their determination, while their relevant physical–chemical properties (molecular mass, solubility, acid dissociation constant (pKa), and octanol–water partition coefficient (log K_{ow}), among others) are provided in Table S1 as supporting information (SI). Stock individual standard solutions of the analytes were prepared in methanol (MeOH), except simazine (prepared in dimethyl sulfoxide), and stored in amber glass bottles in the dark at −20 °C. Working solutions with all target pesticides were prepared at different concentrations (from 0.01 to 500 ng/mL) by appropriate dilution of the stock individual solutions. A solution containing only the isotopically-labelled analogues at a concentration of 2000 ng/mL was also prepared and used as surrogate standard mixture during method optimization, validation, and quantification of real samples. Pesticide-grade solvents MeOH, ACN, and LC-grade water were supplied by Merck (Darmstadt, Germany).

2.2. Sample pre-treatment and extraction

Corn, grape and alfalfa samples (approximately 100 g each) were ultra-frozen with liquid nitrogen, milled in a coffee grinder to increase the specific surface area, and stored at −20 °C in the dark until extraction. For this purpose, 5 g of corn and alfalfa, and 7.5 g of grapes were weighed and introduced into 50 mL polypropylene centrifuge tubes. The surrogate standard solution was then added to the tube to a final concentration of 50 ng of surrogate pesticides per g of food sample. In the case of corn, 5 mL of LC-grade water was also added at this stage and vortexed until complete homogenization. Afterwards, this mixture was left under the hood for one hour to allow MeOH evaporate and water properly hydrate the corn sample. Then, different QuEChERS-based extraction methods were applied to the samples depending on the matrix composition:

a) *Corn*: 10 mL of ACN (0.5 % of formic acid) was added to the centrifuge tube, which was then both manually and vortex shaken for 1 min. A citrate buffer, which includes 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, and 0.5 g disodium salt sesquihydrate (Bekolut® Citrate Kit 01, Bekolut® GmbH & Co. KG 129, Hauptstuhl, Germany), was used for salting out. The tube was centrifuged for 5 min (3220 Relative Centrifugal Force (RFC)) and the organic-phase supernatant was then transferred into a 15-mL polypropylene centrifuge tube, where clean-up was performed by d-SPE. For this purpose, the sorbent mixture 900 mg $MgSO_4$, 150 mg PSA, and 150 mg C_{18} (Bekolut® PSA-Kit-04) was added to the extract, and subsequently manually shaken for 1 min. Afterwards, the tube was centrifuged for 5 min at 3220 RFC. The final extract was acidified with 1 % of formic acid and transferred into a 2 mL vial for LC-MS/MS analysis.
b) *Grapes:* the extraction process was equal to the above described for corn, but with the following modifications: 15 mL of ACN (1 % of formic acid) was added for the initial extraction. Then, the salting out was performed with an acetate buffer (6 g $MgSO_4$, and 1.5 g sodium acetate (Bekolut® Salt-Kit-AC2)), and the d-SPE was done with 900 mg $MgSO_4$, 150 mg PSA, and 45 mg GCB (Bekolut® PSA-Kit-08). The final extract was acidified with 0.7 % of formic acid prior to LC-MS/MS analysis.
c) *Alfalfa:* the extraction reproduces the same process as for corn with the following modifications: 10 mL of ACN (0.37 % of formic acid) was added for the initial extraction, and d-SPE was done with 900 mg $MgSO_4$, 150 mg PSA, and 45 mg GCB (Bekolut® PSA-Kit-08)

Table 1
LC-MS/MS conditions for the target pesticides and the surrogate compounds.

Target analyte	Abbrev.	RT (min)	HESI mode	SRMs (*m/z*)	SRM1/ SRM2	RF lens (V)	CE (eV)
DIA	DIA	5.21	+	174→104	1.1	58	22
				174→132		58	17
	DIA-d_5	5.19	+	179→105		60	24
Fenthion oxon sulfoxide	FNOSX	5.30	+	279→264	4.1	68	19
				279→262		68	21
	THIAM-d_3	5.33	+	295→214		43	13
Thiamethoxam	THIAM	5.34	+	292→132	1	47	23
				292→181		47	22
	THIAM-d_3	5.33	+	295→214		43	13
Bentazone	BEN	5.34	–	239→133	4	68	27
				239→117		68	33
	BEN-d_6	5.44	–	245→132		63	25
Clothianidin	CLOTD	5.69	+	250→169	1.9	43	14
				250→132		47	18
	CLOTD-d_3	5.69	+	253→172		45	15
Imidacloprid	IMID	5.88	+	256→209	1.2	51	19
				256→175		51	20
	IMID-d_4	5.87	+	260→213		63	19
DEA	DEA	5.88	+	188→146	4.9	66	18
				188→104		66	25
	DIA-d_5	5.19	+	179→105		60	24
2,4-D	2,4D	5.96	–	219→125	22.6	35	28
				219→162		35	16
	2,4D-d_3	5.96	–	224→166		39	12
Acetamiprid	ACET	6.01	+	223→126	5.4	53	22
				223→90		53	33
	ACET-d_3	5.99	+	226→126		55	22
Dimethoate	DIME	6.01	+	230→125	11.9	35	22
				230→157		35	20
	DIME-d_6	5.98	+	236→131		44	22
Fenthion oxon sulfone	FENOXS	6.11	+	295→217	7.8	74	19
				295→91		74	33
	FENOXS-d_3	6.10	+	298→218		77	20
MCPA	MCPA	6.35	–	199→142	1.1	38	29
				199→105		38	17
	MCPA-d_3	6.33	–	204→146		41	11
Thiacloprid	THIAC	6.62	+	253→126	6.4	59	23
				253→90		59	34
	THIAC-d_4	6.60	+	257→126		60	23
Cyanazine	CYANZ	7.31	+	241→214	5.6	59	18
				241→104		59	29
	SIMAZ-d_{10}	7.30	+	212→137		63	21
Simazine	SIMAZ	7.38	+	202→132	1.1	61	19
				202→104		61	25
	SIMAZ-d_{10}	7.30	+	212→137		63	21
Fenthion sulfoxide	FENSOX	7.55	+	295→280	1.8	68	19
				295→109		68	32
	FENSOX-d_6	7.52	+	301→286		53	18
Bromoxynil	BROMX	7.53	–	276→81	1.1	82	29
				276→79		82	29
	2,4D-d_3	5.96	+	224→166		39	12
Dichlorvos	DICV	7.43	+	221→145	24.6	57	18
				221→109		57	13
	DICV-d_6	7.39	+	227→115		69	19
Malaoxon	MALOX	7.52	+	315→99	15.2	48	22
				315→125		48	33
	CHLOR-d_6	8.12	+	219→78		58	18
Chlortoluron	CHLOR	8.19	+	213→140	3.9	51	24
				213→104		51	32
	CHLOR-d_6	8.12	+	219→78		58	18
Isoproturon	ISOPR	8.48	+	207→134	1.7	51	23
				207→91		51	37
	ISOPR-d_6	8.42	+	213→134		53	23
Atrazine	ATRZ	8.68	+	216→174	4.3	58	18
				216→104		58	28
	ATRZ-d_5	8.63	+	221→179		59	18
Diuron	DIUR	8.77	+	233→160	1.3	51	27
				233→133		51	40
	DIUR-d_6	8.70	+	239→160		59	28
Fenthion oxon	FENOX	8.91	+	263→231	2.3	62	16
				263→216		62	24
	FENOX-d_3	8.87	+	266→234		69	17

(*continued on next page*)

Table 1 (*continued*)

Target analyte	Abbrev.	RT (min)	HESI mode	SRMs (*m/z*)	SRM1/ SRM2	RF lens (V)	CE (eV)
Fenthion sulfone	FENS	9.42	+	311→125	3.1	65	21
				311→233		84	17
	FENS-d_6	9.35	+	317→131		72	21
Propanil	PROP	10.14	+	218→127	1.1	53	26
				218→162		53	16
	PROP-d_5	10.07	+	223→128		59	28
Methiocarb	METCB	10.37	+	226→169	1	35	9
				226→121		35	18
	METCB-d_3	10.32	+	229→169		30	9
Azinphos methyl	AZM	10.62	+	318→132	1.6	30	15
				318→261		30	5
	AZM-d_6	10.54	+	324→132		43	15
Terbuthylazine	TERBZ	10.61	+	230→174	8.8	52	17
				230→104		52	31
	TERBZ-d_5	10.54	+	235→179		55	17
Linuron	LINU	10.81	+	249→160	1.3	51	19
				249→133		51	34
	LINU-d_6	10.73	+	255→185		48	18
Molinate	MOLI	12.04	+	188→126	4.8	43	12
				188→98		43	17
	METCB-d_3	10.32	+	229→169		30	9
Malathion	MALA	12.13	+	353→227	4.9	70	16
				353→307		70	15
	MALA-d_{10}	12.02	+	363→237		70	18
Alachlor	ALA	12.54	+	270→162	2.7	40	21
				270→132		40	41
	ALA-d_{13}	12.35	+	283→251		45	10
Metolachlor	METO	12.44	+	284→252	2.4	48	15
				284→176		48	26
	METO-d_{11}	12.29	+	295→263		48	17
Terbutryn	TBTN	12.52	+	242→186	15.2	55	19
				242→158		55	25
	TBTN-d_5	12.46	+	247→191		59	19
Irgarol	IRGA	12.32	+	254→198	15.2	57	19
				254→108		57	30
	IRGA-d_9	12.20	+	263→199		60	19
Azinphos ethyl	AZET	12.74	+	346→137	1.1	37	25
				346→97		37	31
	TBTN-d_5	12.46	+	247→191		59	19
Chlorfenvinphos	CFVP	12.93	+	359→170	1.2	60	41
				359→99		60	27
	CFVP-d_{10}	12.82	+	369→170		58	41
Fenthion	FEN	14.07	+	279→169	1	63	19
				279→247		63	13
	FEN-d_6	14.01	+	285→169		62	19
Diazinon	DIAZ	14.67	+	305→169	1.9	64	22
				305→153		64	21
	DIAZ-d_{10}	14.53	+	315→171		67	21
Diflufenican	DIFLU	15.33	+	395→266	4.6	60	24
				395→246		60	34
	DIFLU-d_3	15.39	+	398→268		84	25
Quinoxyfen	QUIN	17.47	+	310→199	1.9	102	33
				310→216		102	36
	DIFLU-d_3	15.39	+	398→268		84	25

RT: *LC retention time*; HESI: *Heated Electrospray Ionization;* SRM: *Selected Reaction Monitoring;* RF Lens: *Radio Frequency Lens;* CE: *Collision Energy;* DIA: *Desisopropylatrazine;* DEA: *Desethylatrazine;* 2,4D: *2,4-Dichlorophenoxyacetic acid;* MCPA: *2-methyl-4-chlorophenoxyacetic acid.*

sorbents. The final extract was acidified with 0.7 % of formic acid prior to LC-MS/MS analysis.

2.3. LC-MS/MS conditions

Chromatographic separation was carried out with an LC system Aria Mx equipped with two Trascend quaternary pumps (max pressure 600 bars) (Thermo Fisher Scientific Inc., Waltham, MA) coupled with a triple quadrupole TSQ Quantiva mass spectrometer, equipped with a heated electrospray ionization (HESI) source (also from Thermo Fisher Scientific Inc.). A Purospher STAR RP-18e column, 150 × 2.1 mm, 2 μm particle diameter (Merck, Darmstadt, Germany), was used for the chromatographic separation, with a flow rate of 0.2 mL/min. Injection volume was 10 μL. The sample was introduced in the LC-MS/MS system using a CTC PAL autosampler. Starting with 10 % of ACN (organic mobile phase) in water (aqueous mobile phase), a linear organic gradient was established as follows: after 1 min in isocratic organic conditions, the organic phase proportion increased to 50 % in 2.5 min. Then, during the following 10 min, the ACN proportion achieved 80 % and, finally, 100 % in 1 additional min. Afterwards, isocratic conditions were maintained for 2.5 min and then initial conditions were restored in 2 min and kept for 7.5 min to ensure complete re-equilibration of the column sorbent.

Regarding the MS conditions, SRM was set as the acquisition mode. For this, two SRM transitions were recorded for each target analyte (quantifier and qualifier), and one SRM transition for each surrogate standard. These transitions were chosen based on the intensity and selectivity for each compound. Positive and negative ionization modes

were alternated, allowing the determination of all target pesticides in one single run. Table 1 shows the surrogate standards used for quantification, retention times (RT), HESI mode, selected SRM transitions and their ratio, and the corresponding optimal radio frequency (RF) lens voltages and collision energy voltages. *Figure S4* shows the extracted ion chromatograms of both transitions for each compound. The positive and negative ion spray voltages were set at 3500 V and −2500 V, respectively. The ion transfer tube temperature was set at 350 °C, and the vaporizer temperature was set at 280 °C. Nitrogen gas was used as sheath, sweep and auxiliary gas for the nebulization stage at the HESI, and argon was chosen as collision gas at a pressure of 2.5 mTorr. Instrument setup and control, data acquisition and quantification were performed with Thermo Scientific Xcalibur v.4.1.31.9 software (from Thermo Fisher Scientific Inc.).

2.4. Method development and validation

To maximize extraction efficiencies, various key aspects of the extraction procedure were optimized. A 2^{6-2} fractional factorial design of experiments (DOE) was performed for this purpose, including two 3-level continuous factors (concentration of formic acid in ACN and in the final extract) with two 2-level categorical factors (extraction buffered salts and clean-up sorbents, *Table S2 and S3*). Through a total of 16 randomized elemental experiments, a regression model was adjusted for each analyte including the effects of all factors, the quadratic effects of continuous factors and their interactions. Each commodity was studied individually to build matrix-fitted methods. Statistical analysis of the optimization results was performed with the software JMP 12.1.0 (SAS Institute Inc., Cary, NC).

Upon method optimization, validation experiments were conducted to establish compound recoveries, matrix effects, linearity, precision, and sensitivity following the guidelines described in Document No. SANTE 11312/2021 (European Commission, 2021). We could not obtain blank matrices without the presence of any targeted pesticide, hence a representative matrix of each commodity (a pool of various samples) was prepared and used for method validation. Background concentrations of the target pesticides present in the blank matrices were then taken into consideration in the calculation of the recoveries and matrix effects by subtracting the peak area found in the blank matrix from the peak area obtained in the pesticide-fortified matrix (*Fig. S5, S6 and S7*). Surrogate standards added at the beginning of the analytical method were used for the quantification, as established in the isotope dilution method. This aids in correcting all potential errors or analyte losses during sample manipulation and/or analysis. For this purpose, most of the surrogate standards were deuterated analogues of the target compounds (Table 1). The dynamic range of the method was evaluated between 0.01 ng/mL and 500 ng/mL (equivalent to 0.02 ng/g and 1000 ng/g, respectively), and established by the coefficient of determination (R^2) of the weighed (1/x) linear regression model obtained from the calibration curve built for each compound.

Matrix effects were calculated by comparing analyte peak areas of the samples spiked after the extraction procedure, just before the LC-MS/MS analysis, and a standard mixture solution in methanol at the same concentration (50 ng/mL, equivalent to 100 ng/g). Pesticide recoveries and precision of the method were determined by the analysis of n = 6 replicates spiked with the target analytes at two different concentration levels (10 ng/g, corresponding to the generic minimum MRL, and 100 ng/g). Absolute recoveries were calculated by comparing analyte peak areas of the fortified samples with methanolic standard solutions at equivalent concentrations. Then, relative recoveries were obtained by comparing absolute recoveries of the target pesticides and their corresponding surrogate standards. Relative standard deviations (RSD %) of the 6 replicates of both studied concentration levels were used for assessing method precision. Limits of detection and quantification (LOD and LOQ, respectively) were calculated through the signal to noise (S/N) method. S/N ratios obtained from a matrix-matched calibration curve constructed in the low concentration range of the method linearity were used for the determination of LOD (S/N ratio of 3) and LOQ (S/N ratio of 10). The LOD and LOQ estimates were visually confirmed in the chromatograms obtained. Moreover, the limit of determination (LDet) was derived from the LOD of the second MS transition of each analyte (used for the confirmation of the target compound), when this is higher than the LOQ of the first MS transition (used for the quantification of the target compound). Thus, the LDet establishes the minimum concentration that can be quantified and confirmed with both transitions (European Commission, 2021).

2.5. Real samples analysis

Once validated, the developed analytical methods were applied to samples from 6 crops located in the Brinches-Enxoé hydro-agricultural area (Serpa, Southern Portugal), harvested during 2018 and 2019. A total of 21 samples were analysed (7 samples of corn, 8 samples of grapes, and 6 samples of alfalfa) to evaluate the performance of the method in the routine analysis of food samples and assess the presence of the target pesticides in the selected matrices. The grape samples included: (i) two samples of Aragonês variety from the same field (MCV1 and MCV2); and (ii) two samples of Antão Vaz variety from two fields (SBV, SPV), collected in September (Antão Vaz variety) and October (Aragonês variety) of 2018 and 2019. As for alfalfa, the samples analysed were all from the same field, three samples collected in July 2018 and three samples collected in July 2019 (NBL1, 2, 3). Four of the corn samples analysed were collected from the same field in November 2018 (MCM1, 2, 3, 4); and three from another field in December 2019 (SBM 1, 2, 3). The agricultural practices in the fields investigated in the two consecutive years were not changed throughout the sampling period.

3. Results and discussion

3.1. Method development

To develop each analytical method in a cost-effective way, a fractional factorial DOE was set up, including two continuous factors, with three levels each, and two categorical factors with two levels each (2^{6-2} fractional factorial DOE). Due to the diverse physical–chemical properties of the target compounds, added to the complexity of the studied matrices, each step of the pre-treatment process can be critical for achieving an efficient extraction. Therefore, the four most relevant stages of this process were studied and optimal conditions were selected for subsequent validation. Moreover, aiming for the highest method capabilities and considering the different matrices contexts, each commodity was optimised individually. In a first stage, the acidification of the acetonitrile used for the extraction was investigated. Some compounds are pH-labile (e.g., acidic pesticides) and, consequently, the presence of formic acid may stabilize them and enhance their extraction (Kaczyński, 2017; Maštovská & Lehotay, 2004). For this purpose, three levels were evaluated (0 % – 1 %, with a central point at 0.5 % of formic acid). The addition of 1 % of formic acid significantly enhanced the extraction efficiency in grapes (with the exception of a few polar compounds, *Fig. S2*), while dissimilar results were obtained for corn and alfalfa samples. However, quadratic effects of both DOEs showed maximum responses around 0.5 % and, thus, to simplify the method, this value was set as the optimum for both corn and alfalfa extraction (*Fig. S1 and S3*). Phenoxy acid herbicides like 2,4-D and MCPA were best extracted using acidified acetonitrile as previously described by Guo et al. (2019). The second aspect to optimize was the selection of the extractive salt for *salting-out* during the extraction with ACN. Two buffered salts were evaluated: the citrate buffer (Standarization, 2018), and the sodium acetate buffer (Lehotay et al., 2007), to study the influence of physical–chemical parameters as the acid dissociation constant (pKa) and log *P* on the biphasic partitioning of each compound. Minor differences were found for corn and alfalfa, although citrate

Table 2
DOEs method optimization results.

	ACN (*formic acid*)	Extractive salts	Clean-up sorbent	Formic acid in final extract
Corn	0.5 %	Citrate buffer	900 mg $MgSO_4$, 150 mg PSA, 150 mg C_{18}	1 %
Grapes	1 %	Acetate buffer	900 mg $MgSO_4$, 150 mg PSA, 45 mg GCB	0.7 %
Alfalfa	0.5 %	Citrate buffer	900 mg $MgSO_4$, 150 mg PSA, 45 mg GCB	0.7 %

buffer was selected due to the improved response achieved in the case of low sensitivity compounds such as MCPA, dichlorvos, fenthion, azinphos ethyl or propanil in corn (*Fig. S1*), and 2,4-D, MCPA, dichlorvos and azinphos methyl in alfalfa (*Fig. S3*). In the case of grapes, the acetate buffer was found to perform better than the citrate buffer, particularly for polar compounds such as bentazone, fenthion oxon sulfoxide, and neonicotinoid pesticides (*Fig. S2*). Similar conclusions were achieved in a previous study when comparing both buffers for the extraction of a selection of pesticides in grapes (Christia et al., 2015).

Grapes, corn, and alfalfa are highly complex matrices containing pigments (chlorophyll and carotenoids), fibers, minerals, proteins, sugars, fatty acids, vitamins, and many other substances that represent an analytical problem in terms of matrix effects during electrospray ionization. Thus, the clean-up step has to be also optimised to achieve acceptable matrix effects values. Different sorbents were tested including PSA, which effectively removes the most polar fatty acids,

Table 3
Linearity and sensitivity of the methods and EC established MRLs.

Pesticide	Corn					Grapes					Alfalfa				
	Linearity	Sensitivity (ng/g)			MRLs*	Linearity	Sensitivity (ng/g)			MRLs*	Linearity	Sensitivity (ng/g)			MRLs*
	r^2	LOD	LOQ	LDet	(ng/g)	r^2	LOD	LOQ	LDet	(ng/g)	r^2	LOD	LOQ	LDet	(ng/g)
2,4-D	0.9886	2.6	8.9	34	50	0.9884	4.5	15	40	100	0.9974	1.5	5.1	20	50
Acetamiprid	0.9991	0.06	0.18	0.18	10	0.9966	0.25	0.82	0.82	500	0.9959	0.13	0.42	0.56	3000
Alachlor	0.9964	0.55	1.8	1.8	10	0.9969	0.69	2.3	2.3	10	0.9936	0.73	2.4	2.4	10
Atrazine	0.9958	0.06	0.22	0.22	50	0.9991	0.05	0.16	0.16	50	0.9912	0.05	0.17	0.17	50
Azinphos ethyl	0.9951	1.4	4.8	4.8	50	0.9986	2.2	7.4	7.4	20	0.9912	1.3	4.2	4.2	20
Azinphos methyl	0.9945	3.7	12	12	50	0.9981	5.7	19	19	50	0.9858	3.1	10	10	50
Bentazone	0.9991	0.02	0.08	0.20	200	0.9960	0.12	0.39	0.39	30	0.9997	0.35	1.17	1.1	30
Bromoxynil	0.9915	2.2	7.5	7.5	100	0.9930	10	31	31	10	0.9967	20	66	66	10
Chlorfenvinphos	0.9964	0.12	0.39	0.39	10	0.9988	0.20	0.68	0.68	10	0.9969	0.07	0.23	0.23	10
Chlortoluron	0.9952	0.4	1.3	1.2	10	0.9916	0.10	0.32	0.61	10	0.9973	0.28	0.95	1.1	10
Clothianidin	0.9990	0.3	1.1	2.0	20	0.9994	0.25	0.83	0.83	700	0.9980	0.95	3.2	13	10
Cyanazine	0.9974	0.05	0.16	0.16	N.A.	0.9990	0.04	0.15	0.15	N.A.	0.9962	0.08	0.28	0.28	N.A.
DEA	0.9945	4.3	14	14	N.D.	0.9956	0.14	0.48	0.52	N.D.	0.9841	0.89	3.0	3.0	N.D.
DIA	0.9982	0.20	0.07	1.0	N.D.	0.9993	0.81	2.7	2.7	N.D.	0.9926	1.5	5.2	22	N.D.
Diazinon	0.9921	0.05	0.17	0.17	10	0.9983	0.13	0.42	0.42	10	0.9956	0.05	0.16	0.16	10
Dichlorvos	0.9990	9.9	33	33	10	0.9979	1.1	3.7	21	10	0.9970	2.4	8.1	10	10
Diflufenican	0.9992	0.86	2.8	2.8	10	0.9993	0.78	2.6	2.6	10	0.9968	0.19	0.64	0.64	10
Dimethoate	0.9992	0.07	0.24	0.59	10	0.9991	0.06	0.20	0.41	10	0.9968	0.28	0.92	0.92	10
Diuron	0.9952	0.92	3.0	3.05	10	0.9970	0.42	1.4	1.4	10	0.9962	1.0	3.4	3.4	10
Fenthion	0.9916	13	45	45	10	0.9885	5.6	19	19	10	0.9953	7.3	24	24	10
Fenthion oxon	0.9982	0.04	0.14	0.14	10	0.9988	0.04	0.12	0.12	10	0.9998	0.15	0.50	0.50	10
Fenthion oxon sulfone	0.9991	0.57	1.9	33	10	0.9986	0.13	0.43	2.18	10	0.9965	0.89	3.0	3.0	10
Fenthion oxon sulfoxide	0.9975	0.02	0.07	0.07	10	0.9989	0.05	0.18	0.18	10	0.9914	0.08	0.27	2.3	10
Fenthion sulfone	0.9990	17	57	57	10	0.9942	7.2	24	24	10					10
Fenthion sulfoxide	0.9997	0.09	0.29	0.31	10	0.9958	0.04	0.14	0.14	10	0.9936	0.14	0.46	0.50	10
Imidacloprid	0.9969	0.36	1.2	1.2	100	0.9981	0.09	0.29	0.29	1000	0.9978	1.6	5.5	5.5	2000
Irgarol	0.9957	0.18	0.61	0.61	N.D.	0.9980	0.01	0.02	0.23	N.D.	0.9920	0.08	0.28	0.28	N.D.
Isoproturon	0.9924	0.23	0.78	2.0	10	0.9945	0.22	0.74	0.74	10	0.9951	0.69	2.3	20	10
Linuron	0.9974	0.52	1.7	1.7	50	0.9952	0.59	1.9	1.9	50	0.9961	0.17	0.56	0.72	50
Malaoxon	0.9933	0.06	0.20	0.20	8000	0.9968	0.05	0.16	0.23	20	0.9967	0.14	0.48	0.56	20
Malathion	0.9959	1.5	4.9	13.6	8000	0.9943	0.67	2.2	4.2	20	0.9905	0.10	0.33	0.33	20
MCPA	0.9973	0.90	3.0	3.0	50	0.9922	4.6	15	15	50	0.9882	9.2	31	31	50
Methiocarb	0.9903	0.89	2.9	2.9	10	0.9928	0.77	2.6	2.6	300	0.9989	6.6	22	22	1000
Metolachlor	0.9996	0.04	0.13	0.13	50	0.9967	0.07	0.25	0.25	50	0.9944	0.05	0.16	0.16	50
Molinate	0.9956	0.61	2.0	2.0	10	0.9971	0.08	0.27	0.27	100	0.9958	0.11	0.38	0.38	10
Propanil	0.9927	1.5	4.9	4.9	10	0.9985	0.69	2.3	2.3	10	0.9913	0.13	0.44	0.44	10
Quinoxyfen	0.9901	1.6	5.5	5.4	20	0.9906	1.1	3.8	3.8	1000	0.9928	0.08	0.26	0.26	20
Simazine	0.9951	0.11	0.36	0.36	10	0.9924	0.09	0.30	0.30	0	0.9949	0.15	0.51	0.51	10
Terbuthylazine	0.9987	0.05	0.18	0.18	100	0.9952	0.17	0.56	0.56	100	0.9949	0.10	0.35	0.35	50
Terbutryn	0.9988	0.13	0.42	0.42	N.A.	0.9984	0.35	1.2	1.2	N.A.	0.9981	0.07	0.23	0.23	N.A.
Thiacloprid	0.9990	0.06	0.20	0.20	10	0.9994	0.04	0.14	0.14	10	0.9970	0.05	0.16	0.19	10
Thiamethoxam	0.9967	0.66	2.2	2.2	50	0.9978	0.42	1.4	1.4	400	0.9959	1.8	6.2	22	10

2,4-D: *2,4-dichloro phenoxy acetic acid*. DIA: *desisopropylatrazine*. DEA: *desethylatrazine*. MCPA: *4-chloro 2-methylphenoxy acetic acid.* r^2: *coefficient of determination*, LOD: *limit of detection*, LOQ: *limit of quantification*, LDet: *limit of determination*, N.D.: *no data.* N.A.: *not approved in UE.* MRL: *maximum residue level.* *Extracted from https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en.

sugars, and other components forming hydrogen bonds, GCB, which adsorbs non-polar substances and planar compounds as pigments (Li et al., 2009; Madej et al., 2018), and C_{18}, responsible of the removal of non-polar lipids (López-Blanco et al., 2016; Madej et al., 2018). The same clean-up sorbent (Bekolut® PSA-Kit-08), which included 900 mg $MgSO_4$, 150 mg PSA, and 45 mg GCB, was chosen for grapes and alfalfa with preference to the other tested kits (Bekolut® PSA-Kit-04 and Bekolut® PSA-Kit-06, respectively) as a result of the DOE outcome. PSA is used for the elimination of polar compounds as sugars, which is one of the main components of grapes (Grimalt & Dehouck, 2016). Both grapes and alfalfa contain a high proportion of pigments for which GCB would be responsible of their removal. Previous studies have observed the same results when comparing different clean-up protocols in grapes (Grimalt & Dehouck, 2016; Schenck et al., 2002). Moreover, a study comparing different extraction methods for alfalfa leaves, also determined the necessity of GCB for a colourless extract (Kinross et al., 2020), although other sorbents are also needed for fatty acids removal. In the case of alfalfa, PSA is also known for reducing the amount of the most polar fatty acids (Anastassiades et al., 2003). Compared to grapes and alfalfa, with less than 1 % of fat content, corn is a cereal that contains between 3 and 18 % of fatty acids (Bathla et al., 2020). This fact led to the selection of the Bekolut® PSA-Kit-04 (with C_{18} as a clean-up sorbent in addition to PSA) over the other tested Bekolut® PSA-Kit-08 for improved extraction of pesticides from the corn matrix. The QuEChERS methods described by Wang et al. (2017) also ended up with the employment of similar clean-up adsorbents for pesticide analysis in grape and corn matrices (Wang et al., 2017).

Finally, acidification of the final extract was optimized to enhance the chromatographic separation and ionization in the subsequent LC-MS/MS analysis. Three levels were set in the DOE, ranging from 0 % to 1 % of formic acid, with a central point at 0.5 %. The acidification of the three matrices at different proportions optimized the ionization of most of the target compounds. Only some acidic compounds as bromoxynil or 2,4-D were not benefited due to their detection under negative electrospray ionization. The summarized results of the DOEs for each commodity are presented in Table 2. More detailed information

Table 4
Absolute and relative recoveries and precision of the method validation.

Pesticide	Absolute recoveries (%)						Relative recoveries (%)						Precision (% RSD)					
	Corn		Grapes		Alfalfa		Corn		Grapes		Alfalfa		Corn		Grapes		Alfalfa	
	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g	10 ng/g	100 ng/g
2,4-D	N.D.	99	N.D.	79	N.D.	42	N.D.	88	N.D.	108	N.D.	125	N.D.	4.0	N.D.	11.9	N.D.	9.6
Acetamiprid	31	34	70	55	23	40	79	89	105	97	90	112	1.9	1.5	10.0	2.2	3.8	1.8
Alachlor	136	116	108	97	61	69	94	94	94	94	107	122	3.7	2.8	7.6	2.6	9.3	8.5
Atrazine	122	133	93	112	59	68	77	90	83	93	88	106	3.6	2.3	4.5	1.6	2.7	3.1
Azinphos ethyl	111	92	167	124	47	49	71	82	106	92	69	82	18.2	4.1	11.4	3.0	7.1	2.6
Azinphos methyl	N.D.	85	N.D.	139	97	59	N.D.	80	N.D.	63	104	116	N.D.	9.5	N.D.	19.5	12.5	5.4
Bentazone	217	205	183	182	25	33	82	88	82	91	89	102	2.3	0.8	6.4	4.8	4.7	4.2
Bromoxynil	68	46	N.D.	61	N.D.	55	64	47	N.D.	88	N.D.	109	19.4	9.3	N.D.	10.3	N.D.	12.7
Chlorfenvinphos	69	98	140	107	54	55	83	80	91	104	80	92	3.7	2.2	3.2	3.5	2.6	3.6
Chlortoluron	58	57	69	77	36	48	80	85	83	105	84	102	3.4	5.6	4.9	4.7	5.9	5.8
Clothianidin	34	34	74	66	17	20	76	83	113	115	99	108	1.3	2.2	4.6	2.2	10.1	2.3
Cyanazine	124	144	117	113	53	61	80	96	121	116	130	118	1.6	12.3	4.2	2.9	9.4	13.7
DEA	80	89	118	78	38	53	77	87	76	103	92	105	2.4	4.9	7.6	3.9	16.4	17.5
DIA	89	81	96	121	27	29	85	79	89	105	99	89	2.5	0.5	5.5	2.8	6.7	6.2
Diazinon	58	113	132	115	104	90	72	91	88	97	94	101	1.5	2.4	6.7	4.0	7.3	6.3
Dichlorvos	N.D.	908	N.D.	440	86	86	N.D.	95	N.D.	107	111	111	N.D.	2.5	N.D.	4.0	5.7	3.0
Diflufenican	154	109	202	166	106	111	84	96	96	108	94	125	4.8	4.9	3.7	3.9	3.4	4.0
Dimethoate	51	52	65	72	40	62	84	95	95	110	71	118	2.3	1.3	3.8	1.5	3.5	2.8
Diuron	107	78	83	88	49	65	81	77	78	90	83	93	6.8	6.3	6.5	4.0	3.3	1.7
Fenthion	N.D.	198	N.D.	202	N.D.	74	N.D.	110	N.D.	98	N.D.	126	N.D.	5.0	N.D.	14.1	N.D.	12.8
Fenthion oxon	99	66	113	109	61	68	79	94	99	105	99	111	4.3	3.1	4.0	1.9	1.4	2.5
Fenthion oxon sulfone	129	120	121	112	37	54	77	87	91	103	90	106	3.6	2.3	2.0	2.3	1.6	1.9
Fenthion oxon sulfoxide	36	46	62	55	54	48	89	125	78	88	102	126	3.2	3.3	3.3	3.7	11.8	7.4
Fenthion sulfone	N.D.	357	N.D.	188	N.D.	N.D.	N.D.	86	N.D.	97	N.D.	N.D.	N.D.	4.9	N.D.	7.3	N.D.	N.D.
Fenthion sulfoxide	70	95	101	100	48	57	95	128	124	125	117	111	4.0	11.3	5.1	5.0	3.3	5.2
Imidacloprid	26	29	47	51	20	26	85	101	88	102	100	116	4.5	1.2	5.7	2.4	6.7	3.7
Irgarol	122	131	147	133	71	73	76	84	88	99	86	112	1.9	2.8	3.4	1.1	3.1	3.9
Isoproturon	55	64	76	83	41	54	81	92	87	106	87	111	3.5	2.5	5.5	1.5	4.7	2.4
Linuron	574	501	306	275	120	81	71	93	86	107	77	110	3.7	2.5	5.9	3.0	2.7	2.7
Malaoxon	67	81	104	86	119	76	90	109	119	110	91	116	3.2	2.4	6.0	4.8	3.3	2.1
Malathion	45	69	81	66	78	72	103	121	101	105	98	118	7.3	19.2	4.9	3.3	3.4	3.5
MCPA	58	62	N.D.	76	N.D.	25	74	89	N.D.	106	N.D.	125	8.7	2.5	N.D.	10.7	N.D.	11.3
Methiocarb	230	171	166	197	N.D.	18	79	90	83	96	N.D.	127	4.0	1.0	3.0	4.2	N.D.	16.1
Metolachlor	89	71	111	96	44	50	83	80	99	98	79	100	3.8	3.8	3.1	2.1	3.9	3.4
Molinate	184	126	142	133	75	68	126	126	112	116	48	92	6.6	10.5	8.8	6.1	13.5	10.9
Propanil	N.D.	843	546	465	133	86	N.D.	84	74	111	79	108	N.D.	2.8	8.0	5.0	4.4	4.6
Quinoxyfen	79	96	161	136	91	93	73	84	89	93	82	105	10.9	8.2	24.1	26.0	8.8	10.4
Simazine	98	132	92	108	42	59	72	88	86	108	107	117	4.4	1.4	2.6	1.6	8.3	8.8
Terbuthylazine	139	122	100	123	87	65	90	87	77	96	76	108	1.5	3.2	2.4	2.0	16.4	2.8
Terbutryn	126	120	120	137	74	78	81	84	77	102	96	110	1.2	1.1	5.3	4.0	2.9	2.0
Thiacloprid	32	38	72	73	25	36	76	93	94	105	93	113	1.3	3.0	3.8	1.6	2.3	4.0
Thiamethoxam	34	35	72	68	N.D.	22	84	94	91	109	N.D.	107	4.4	4.0	2.4	2.8	N.D.	4.0

2,4-D: *2,4-dichloro phenoxy acetic acid*. DIA: *desisopropylatrazine*. DEA: *desethylatrazine*. MCPA: *4-chloro 2-methylphenoxy acetic acid*. N.D.: not detected; RSD: relative standard deviation.

about each compound optimization is broken down through the prediction profiles and desirability plots in *Figures S1-S3* in SI.

3.2. Method validation

The validation of the three optimised methods was performed following the SANTE 11312/2021 guidelines (European Commission, 2021) with regard to linearity, sensitivity, precision, and trueness (recoveries). This validation was carried out for the 42 target analytes in each commodity, except for fenthion sulfone in alfalfa for which sensitivity was not enough. The validation figures have been summarized in Tables 3 and 4. Table 3 also shows the MRL set for each pesticide in each food commodity.

The linearity of the methods ranged from the analyte LOQ to 500 ng/mL (equivalent to 1000 ng/g of fresh sample) with a coefficient of determination (R^2) over 0.990 in almost all cases. Exceptions were 2,4-D in corn and grapes, azinphos methyl, DEA, and MCPA in alfalfa, and fenthion in grapes, which presented R^2 values between 0.9841 and 0.9886 (Table 3).

In terms of trueness, absolute and relative recoveries were determined for each compound and food matrix as described in the SANTE guidance (European Commission, 2021). Results referring recoveries are outlined in Table 4 showing, in general, higher values for grapes and corn than for alfalfa (100 % and 83 % of the analytes presented an absolute recovery over 50 % in the case of grapes and corn, respectively, while for alfalfa this percentage was 68 %). A particularly low recovery was obtained for neonicotinoids (acetamiprid, imidacloprid, thiacloprid, clothianidin and thiamethoxam) in the three matrices; however, their good instrumental detection allowed to achieve LODs much lower than their respective MRLs (Table 3). The use of surrogate standards corrected the analyte losses that occurred during the extraction procedure as well as the deviations in the MS signals due to matrix effects (which are shown as absolute recoveries), resulting in the actual relative recoveries. The given values correspond to the average relative recoveries (n = 6) of the samples analysed at both high and low concentration levels investigated in each matrix. In this case, almost all target pesticides complied with the SANTE guidance, showing an average relative recovery in the range of 70–120 %, with a few exceptions presenting values between 60 and 130 %. Only bromoxynil in corn and molinate in alfalfa showed poor relative recoveries (48 and 47 %, respectively). However, the performance of all methods provided a steady precision for all evaluated analytes with RSD values under 20 %, excluding quinoxyfen in grapes, for which the RSD was 26 % (Table 4).

Sensitivity was measured by the calculation of LODs, LOQs and LDets for each compound. LODs oscillated from 0.01 ng/g to 10 ng/g in grapes, 0.02 ng/g to 17.12 ng/g in corn, and 0.05 ng/g to 20 ng/g in alfalfa, where >75 % of LODs reached values below 1 ng/g (Table 3). In terms of EU MRLs, 97 % of the studied pesticides could be determined accomplishing the required limits. The exceptions were bromoxynil in alfalfa, and fenthion and fenthion sulfone in corn (Table 3). The suitability of the extraction procedures together with a good ionization efficiency in the HESI probe allowed reaching this optimal sensitivity. With these methods, improved sensitivity was obtained for most compounds compared to similar published methods (*Table S4*).

Finally, matrix effects were also evaluated for the three matrices, as shown in Fig. 1, finding a similar matrix interference behaviour in all of them. As occurred with absolute recoveries, matrix suppression was stronger in alfalfa extracts affecting to 95 % of the target analytes. In the case of corn and grapes, 48 % and 32 %, respectively, of the target pesticides were negatively influenced by matrix effects during MS ionization. Around 30 % of the target analytes were not strongly affected by matrix interferences, showing values within ± 20 %. Regression lines in Fig. 1 show slightly higher matrix suppression effects at lower retention times, mostly for grapes and alfalfa. The high complexity of both matrices containing pigments and polar compounds that co-elute at the beginning of the chromatographic separation could explain these results.

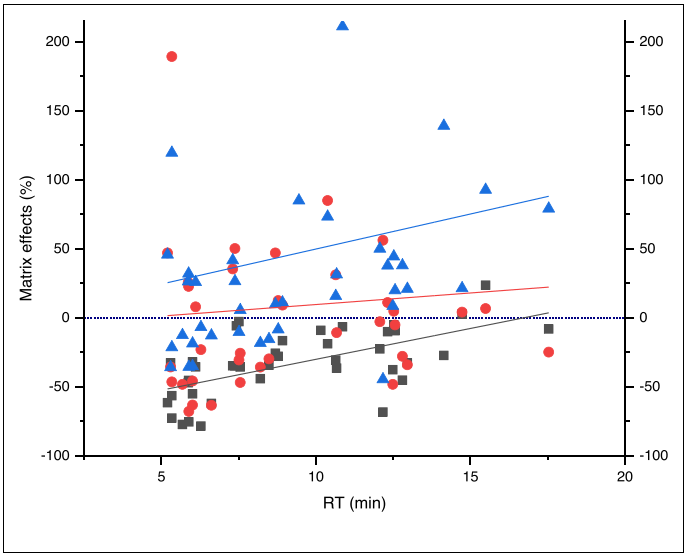

Fig. 1. Matrix effects (%) of all matrices throughout the chromatogram (RT: retention time (min)). *Red dots*, *blue triangles*, and *grey squares* represent each target compound for corn, grapes and alfalfa extracts, respectively. Multiple linear regression lines were performed for each matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

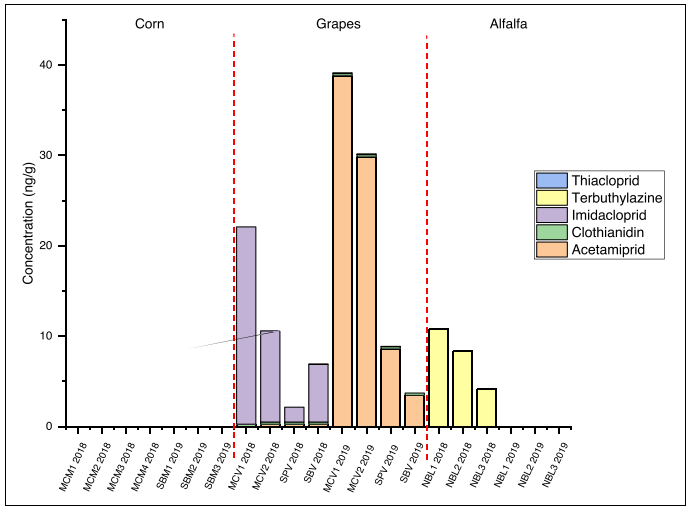

Fig. 2. Target pesticides detected and/or quantified in real samples. For compounds detected at concentrations below LDets, LODs are shown. MCM1, 2, 3, 4 and SBM1, 2, 3 are corn samples. MCV1, 2, SPV, and SBV are grape samples. NBL 1, 2, 3 are alfalfa samples.

3.3. Analysis of real samples

Samples of corn, grape and alfalfa harvested in Serpa (southern Portugal) at different campaigns during 2018 and 2019 were examined to evaluate methods performance. To avoid false positives, both SRM transitions of any target compound should match in retention time and peak shape. Moreover, retention time shifts should not be longer than 0.1 min between the sample extract and calibration standard unless the corresponding isotopically-labelled surrogate had also been affected with the same time shift. In addition, as indicated in the SANTE guidance (European Commission, 2021), SRM ratios between transitions should not exceed ± 30 % of that observed in calibration standards from the same batch sequence. Fig. 2 shows the detected and quantified pesticides in the different samples. While no target analyte was detected in corn, at least one pesticide was detected above its LOQ in all grape samples and half of the alfalfa extracts. Acetamiprid and imidacloprid were found at concentrations up to 38.8 and 21.8 ng/g, respectively, in grapes. These neonicotinoids are typical insecticides used to prevent plants from leafhoppers, grape moth, and other small insects (Grimalt & Dehouck, 2016). Clothianidin and thiacloprid, other neonicotinoids, were also detected below LOQ in all grape samples. In the case of alfalfa, terbuthylazine was observed at concentrations ranging from 4 to 11 ng/g. This triazine herbicide is recommended by suppliers for weed control for the second and subsequent years after planting alfalfa crops (Seeds, 2018). None of the detected pesticides overpassed authorised MRLs; however, neonicotinoids are well-known insecticides highly toxic to honeybee, most of them included in the Pesticide Action Network (PAN) International list of Highly Hazardous Pesticides (Pesticide Action Network International, 2019), and with a moderate acute toxicity for exposed humans. In particular, thiacloprid has been characterized as a moderate hazardous pesticide and carcinogen. Concentration values for the detected pesticides are presented in *Table S5*.

4. Conclusions

Three multiresidue methods based on QuEChERS extraction and LC-MS/MS have been developed to detect and quantify up to 42 polar and semi-polar pesticides, including organophosphates, triazines, phenylureas, anilines, and neonicotinoids largely employed in the EU, in three representative plant origin foods, such as corn (cereals), grapes (fruits) and alfalfa (green leaves). To the authors' knowledge, this is the first analytical method available to determine the targeted pesticides in alfalfa, with just a few exceptions (Kinross et al., 2020; Lehotay et al., 2010). The optimization of the extraction procedure through a fractional factorial DOE that considered four critical factors (acidification of the extractive solvent, the buffered salts, the type of clean-up sorbents, and the acidification of the final extract) provided matrix-fitted methods in a cost-effective way. The optimised methods were validated in terms of linearity, sensitivity, trueness, and precision, following the SANTE guidelines. Although a large number of pesticides were simultaneously extracted and analysed, extremely low LODs were reached, overcoming the MRLs established by the European Commission, and improving the LODs achieved in other works in most cases. These matrix-based QuEChERS methods represent an economical and secure way to perform multiresidue determination covering a wide range of compounds. Moreover, the use of isotopically-labelled surrogate standards permits a higher control of the correct operation of the whole analytical method during the sequence, a more accurate matrix effect compensation despite its fluctuations between samples, and a faster quantification, avoiding the use of recovery factors.

The good performance of the methods in real samples harvested in different places from the south of Portugal revealed the presence of some insecticides and herbicides in grapes and alfalfa although calculated concentrations were below the regulatory limits. Nevertheless, two of the detected compounds (imidacloprid and thiacloprid) are part of the PAN International List of Highly Hazardous Pesticides.

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.

Data availability

Data will be made available on request.

Acknowledgements

This work has been funded by the European Union through the European Agricultural Fund for Rural Development (FitoFarmGest Operational Group PDR2020-101-030926), the Spanish Ministry of Science and Innovation (grant CEX2018-00794-S, funded by MCIN//AEI/ 10.13039/501100011033) and the Government of Catalonia (2017 SGR 01404). BEKOlut is acknowledged for the gift of QuEChERS kits.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134870.

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Supplementary Information

Development of QuEChERS-based multiresidue analytical methods to determine pesticides in corn, grapes and alfalfa

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This supplementary material includes tables with the physical-chemical properties of the target analytes, details of the regression models resulting from the design of experiments, concentrations found in real samples and sensitivity provided by similar methods, as well as figures with the prediction profiles and desirability obtained in the design of experiments for each analyte in each matrix, and extracted ion chromatograms of the target analytes in analysed food samples.

Number of pages: 40

Number of tables: 5

Number of figures: 7

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

Pesticide	Type	MM (g mol^{-1}) ‡	Solub (mg L^{-1}) ‡	pKa ‡	Kow logP ‡	Koc (mL g-1) ‡	Henry´s constant (Pa m^3 mol^{-1}) ‡
2,4-D	Alkylchlorophenoxy acid	221.04	24300	3.40	-0.82	39	4.0 X 10^{-06}
Acetamiprid	Neonicotinoid	222.67	2950	0.7	0.80	200	5.3 X 10^{-08}
Alachlor	Chloroacetamide	269.77	240	0.62	3.09	335	3.2 X 10^{-03}
Atrazine	Triazine	215.68	35	1.7	2.70	100	1.5 X 10^{-04}
Azinphos ethyl	Organophosphate	345.38	4.5	n/a	3.18	1500	3.1 X 10^{-06}
Azinphos methyl	Organophosphate	317.32	28	5	2.96	1112	5.7 X 10^{-06}
Bentazone	Benzothiazinone	240.30	7112	3.51	-0.46	55	7.2 X 10^{-05}
Bromoxynil	Hydroxybenzonitrile	276.90	38000	3.86	0.27	302	8.7 X 10^{-07}
Chlorfenvinphos	Organophosphate	359.60	145	n/a	3.80	680	-
Chlortoluron	Phenylurea	212.68	74	n/a	2.50	196	1.4 X 10^{-05}
Clothianidin	Triazine	240.69	171	12.9	2.10	190	6.6 X 10^{-06}
Cyanazine	Neonicotinoid	249.68	340	11.1	0.90	123	2.9 X 10^{-11}
DEA	Metabolite	173.60	980	n/a	1.15	130	980
DIA	Metabolite	187.63	2700	n/a	1.51	110	1.6 X 10^{-04}
Diazinon	Organophosphate	304.35	60	2.6	3.69	609	6.1 X 10^{-02}
Dichlorvos	Organophosphate	220.98	18000	n/a	1.90	50	2.6 X 10^{-02}
Diflufenican	Carboxamide	394.29	0.05	n/a	4.20	3.19°	1.2 X 10^{-02}
Dimethoate	Organophosphate	229.26	25900	n/a	0.75	25*	1.4 X 10^{-06}
Diuron	Phenylurea	233.09	35.6	n/a	2.87	813	2.0 X 10^{-06}
Fenthion	Organophosphate	278.33	4.2	n/a	4.84	1500	2.4 X 10^{-02}
Fenthion oxon	Metabolite	262.26*	213.5*	n/a	2.31*	57 *	3.0x10^{-9} *
Fenthion oxon sulfone	Metabolite	294.03*	7602*	n/a	0.28*	13*	2.4 x 10^{-11} *
Fenthion oxon sulfoxide	Metabolite	278.26*	1222*	n/a	0.15*	11*	9.5 x 10^{-8} *
Fenthion sulfone	Metabolite	310.33*	190.4*	n/a	2.05*	542*	1.1x10^{-8} *
Fenthion sulfoxide	Metabolite	294.33*	3.72*	n/a	1.92*	466*	7.0x10^{-6} *
Imidacloprid	Neonicotinoid	255.66	610	n/a	0.57	6719	1.7 X 10^{-10}
Irgarol	Triazine	253.37	7	n/a	3.95	1569	1.3 x 10^{-07}*
Isoproturon	Phenylurea	206.28	70.2	n/a	2.5	251*	1.5 X 10^{-05}
Linuron	Phenylurea	249.09	63.8	n/a	3	843	2.0 X 10^{-04}
Malaoxon	Organophosphate	314.29*	7500*	n/a	0.52*	4650*	1.2 X 10^{-08} *
Malathion	Organophosphate	330.36	148	n/a	2.75	1800	1. 0 X 10^{-03}
MCPA	Metabolite	200.62	29390	3.73	-0.81	29*	5.5 X 10^{-05}
Methiocarb	Carbamate	225.31	27	n/a	3.18	182*	1.2 X 10^{-04}
Metolachlor	Chloroacetamide	283.80	530	n/a	3.40	120	2.4 X 10^{-03}
Molinate	Thiocarbamate	187.30	1100	n/a	2.86	190	6.9 X 10^{-01}
Propanil	Anilide	218.08	95	19.1	2.29	149	4.4 X 10^{-04}
Quinoxyfen	Quinoline	308.13	0.05	n/a	4.66	23**	3.2 X 10^{-02}
Simazine	Triazine	201.66	5	1.62	2.30	130	5.6 X 10^{-05}
Terbuthylazine	Triazine	229.71	6.6	1.9	3.40	329*	3.2 X 10^{-03}
Terbutryn	Triazine	241.36	25	4.3	3.66	2432	1.5 X 10^{-03}
Thiacloprid	Neonicotinoid	252.72	184	n/a	1.26	615**	5.0 X 10^{-10}
Thiamethoxam	Neonicotinoid	291.71	4100	n/a	-0.13	56	4.7 X 10^{-10}

MM: molecular mass; Solub: solubility in water at 20 oC ; 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/ppdb/en/index.htm - Lewis, K.A., Tzilivakis, J., Warner, D. and Green, A. (2016). An international database for pesticide risk assessment and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064. *Data estimated using the US Environmental Protection Agency EPISuiteTM 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

Table S2. Details of the regression model adjusted for each compound in the design of experiments for corn and grapes.

Factors	Type	Name	Number of levels	Low level	Medium level	High level
Formic acid ACN	Continuous	X_1	3	0%	0.5%	1%
Formic acid final extract	Continuous	X_2	3	0%	0.5%	1%
Extractive salts	Categorical	F_1	2	Acetate		Citrate
Clean-up sorbent	Categorical	F_2	2	4		8
Adjusted regression model for compound *Y*						
$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 F_1 + \beta_4 F_2 + \beta_5 X_1^2 + \beta_6 X_2^2 + \beta_7 X_1 X_2$$						

Table S3. Details of the regression model adjusted for each compound in the design of experiments for alfalfa.

Factors	Type	Name	Number of levels	Low level	Medium level	High level
Formic acid ACN	Continuous	X_1	3	0%	0.5%	1%
Formic acid final extract	Continuous	X_2	3	0%	0.5%	1%
Extractive salts	Categorical	F_1	2	Acetate		Citrate
Clean-up sorbent	Categorical	F_2	2	6		8
Adjusted regression model for compound *Y*						
$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 F_1 + \beta_4 F_2 + \beta_5 X_1^2 + \beta_6 X_2^2 + \beta_7 X_1 X_2$$						

Table S4. Comparison of the LOQs (ng/g) provided by similar LC-MS-based methods.

	Corn							Grapes						Alfalfa	
Compound	**LOQ (Method Developed)**	(Viera et al., 2017)	(Abbaspour et al., 2019)	(Guo et al., 2019)	(Wang et al., 2017)	(Rong et al., 2018)	(He et al., 2015)	**LOQ (Method Developed)**	(Banerjee et al., 2007)	(Montiel-León et al., 2019)	(Ortelli et al., 2004)	(Afify et al. 2010)	(Kasperkiewicz & Pawliszyn, 2021)	**LOQ (Method Developed)**	(Tsiplakou et al., 2010)
2,4-D	8.9	160		0.2				15						5.1	10
Acetamiprid	0.18		1.0		2			0.82	2.5	3.3	10	10	1	0.42	
Alachlor	1.8				2		5	2.3	2.5					2.4	10
Atrazine	0.22	16			2		5	0.16		5.1		10		0.17	
Azinphos ethyl	4.8							7.4	2.5			10		4.2	10
Azinphos methyl	12				2			19				10		10	
Bentazone	0.08	160						0.39						1.17	
Bromoxynil	7.5							31						66	
Chlorfenvinphos	0.39				2		5	0.68						0.23	
Chlortoluron	1.3							0.32					2.5	0.95	10
Clothianidin	1.1				2	10		0.83	2.5	3.6		10	2.5	3.2	
Cyanazine	0.16	16						0.15		3.9				0.28	10
DEA	14				2		5	0.48		3.9				3.0	
DIA	0.07				2		5	2.7		3.9				5.2	
Diazinon	0.17				2		5	0.42	1			10		0.16	10
Dichlorvos	33				2			3.7	2.5			10		8.1	10
Diflufenican	2.8							2.6				10		0.64	
Dimethoate	0.24	16			2		5	0.20	1	3	10	10	1	0.92	10
Diuron	3.0	16						1.4				10	1	3.4	
Fenthion	45	16			2		10	19	2.5			10		24	10
Fenthion oxon	0.14							0.12						0.50	
Fenthion oxon sulfone	1.9							0.43						3.0	
Fenthion oxon sulfoxide	0.07							0.18						0.27	
Fenthion sulfone	57						5	24							
Fenthion sulfoxide	0.29						5	0.14						0.46	
Imidacloprid	1.2	16			2			0.29	2.5	4.8	10	10	2.5	5.5	
Irgarol	0.61							0.02						0.28	
Isoproturon	0.78							0.74	2.5			10	2.5	2.3	
Linuron	1.7	16						1.9		5.6		10	1	0.56	10
Malaoxon	0.20						50	0.16	1			10		0.48	
Malathion	4.9				2		5	2.2	1			10		0.33	10
MCPA	3.0			0.2				15						31	
Methiocarb	2.9							2.6			10	10	2.5	22	10

Metolachlor	0.13		2		5	0.25						0.16		
Molinate	2.0	16	2		5	0.27						0.38		
Propanil	4.9	16	2		5	2.3						0.44		
Quinoxyfen	5.5		2		5	3.8					10	0.26	10	
Simazine	0.36	16			10	0.30	5	3.6				0.51	10	
Terbuthylazine	0.18	16			5	0.56						0.35	10	
Terbutryn	0.42		2		10	1.2					1	0.23		
Thiacloprid	0.20					0.14	1	4.2	10		0.5	0.16	10	
Thiamethoxam	2.2	16	2	10		1.4	2.5	3.9			5	6.2	10	

Table S5. Concentration in ng/g of detected target pesticides in real samples.

		Acetamiprid	**Clothianidin**	**Imidacloprid**	**Terbuthylazine**	**Thiacloprid**
Corn	MCM1 2018	n.d.	n.d.	n.d.	n.d.	n.d.
	MCM2 2018	n.d.	n.d.	n.d.	n.d.	n.d.
	MCM3 2018	n.d.	n.d.	n.d.	n.d.	n.d.
	MCM4 2018	n.d.	n.d.	n.d.	n.d.	n.d.
	SBM1 2019	n.d.	n.d.	n.d.	n.d.	n.d.
	SBM2 2019	n.d.	n.d.	n.d.	n.d.	n.d.
	SBM3 2019	n.d.	n.d.	n.d.	n.d.	n.d.
Grapes	MCV1 2018	n.d.	<LDet	22	n.d.	n.d.
	MCV2 2018	<LDet	<LDet	10	n.d.	n.d.
	SPV 2018	<LDet	<LDet	1.6	n.d.	n.d.
	SBV 2018	<LDet	<LDet	6.4	n.d.	<LDet
	MCV1 2019	39	<LDet	<LDet	n.d.	n.d.
	MCV2 2019	30	<LDet	<LDet	n.d.	n.d.
	SPV 2019	8.6	<LDet	n.d.	n.d.	<LDet
	SBV 2019	3.5	<LDet	n.d.	n.d.	n.d.
Alfalfa	NBL1 2018	n.d.	n.d.	n.d.	11	n.d.
	NBL2 2018	n.d.	n.d.	n.d.	8.4	n.d.
	NBL3 2018	n.d.	n.d.	n.d.	4.2	n.d.
	NBL1 2019	n.d.	n.d.	n.d.	n.d.	n.d.
	NBL2 2019	n.d.	n.d.	n.d.	n.d.	n.d.
	NBL3 2019	n.d.	n.d.	n.d.	n.d.	n.d.
	<LDet: Compounds detected with a concentration below LDet. *n.d.:* not detected					

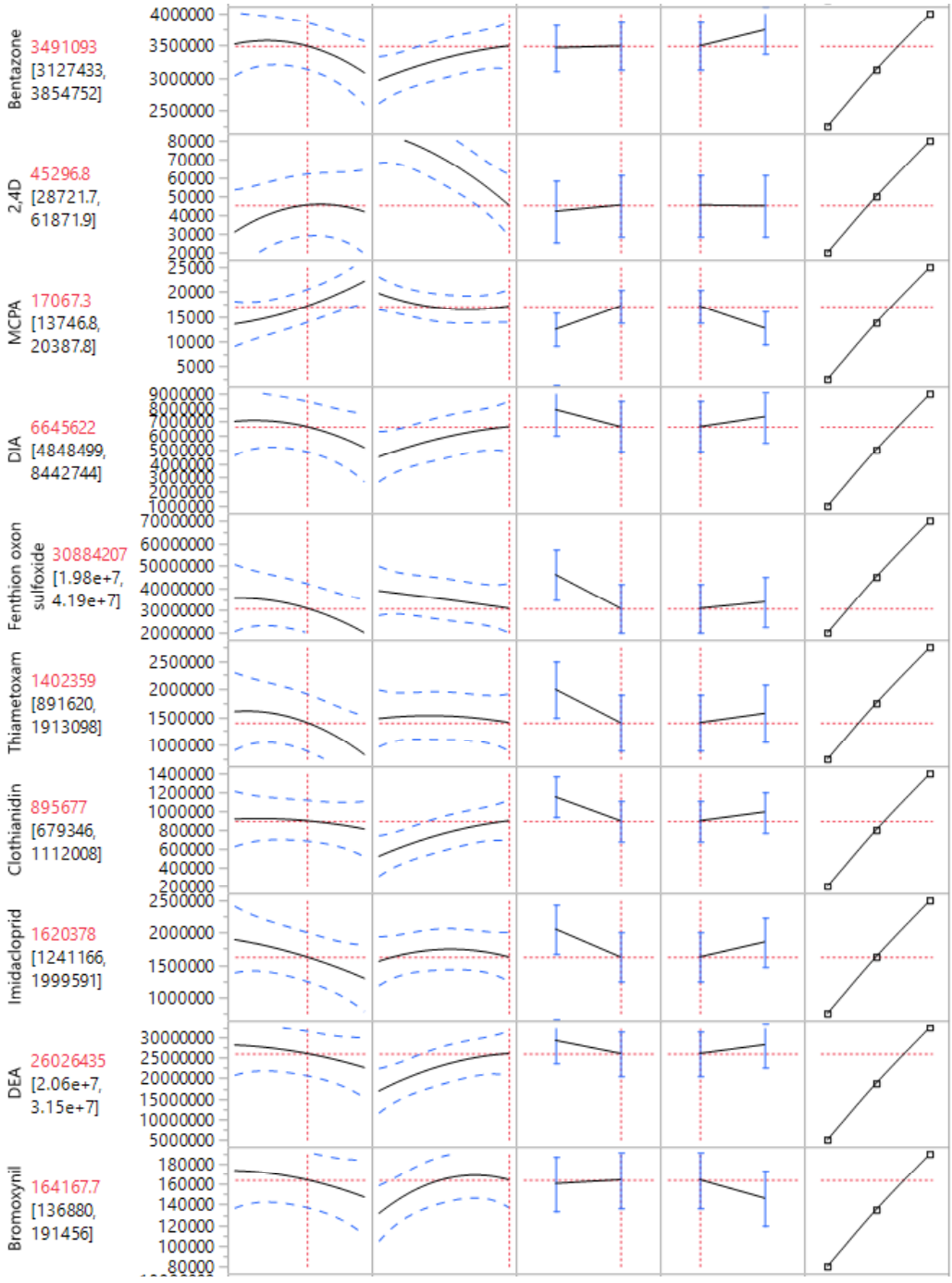
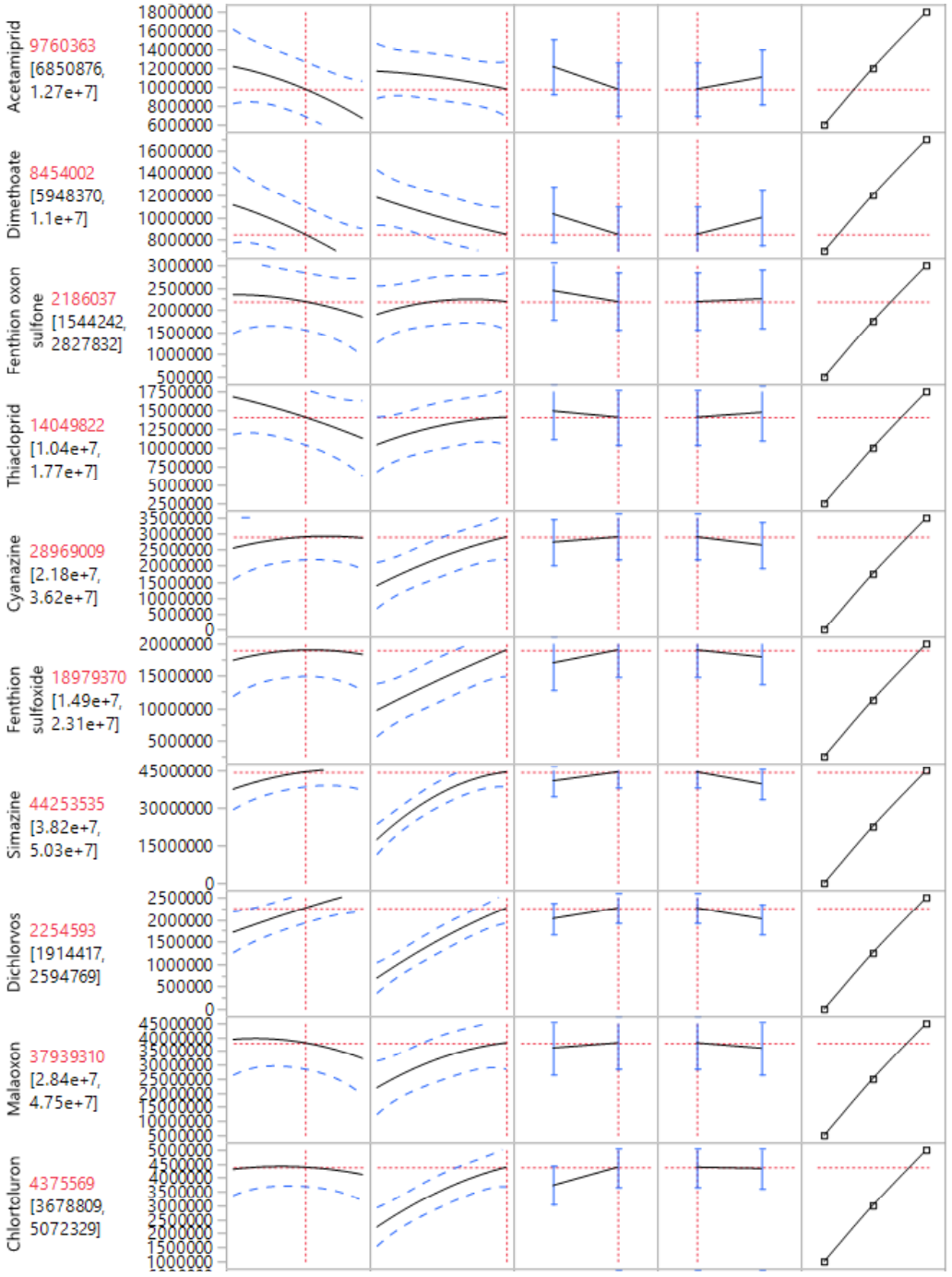


Figure S1. DOE prediction profiles and desirability for each analyte in corn.



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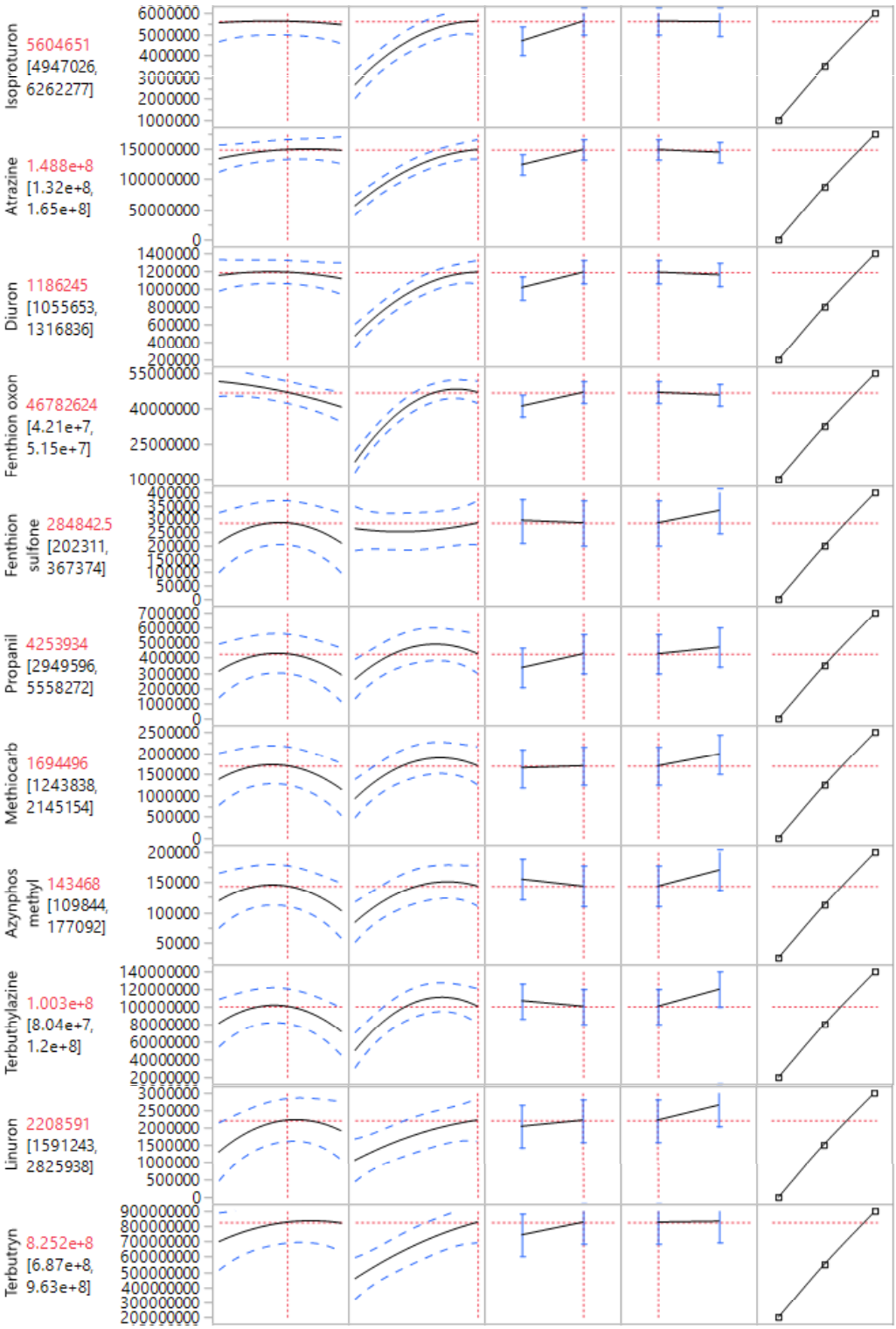

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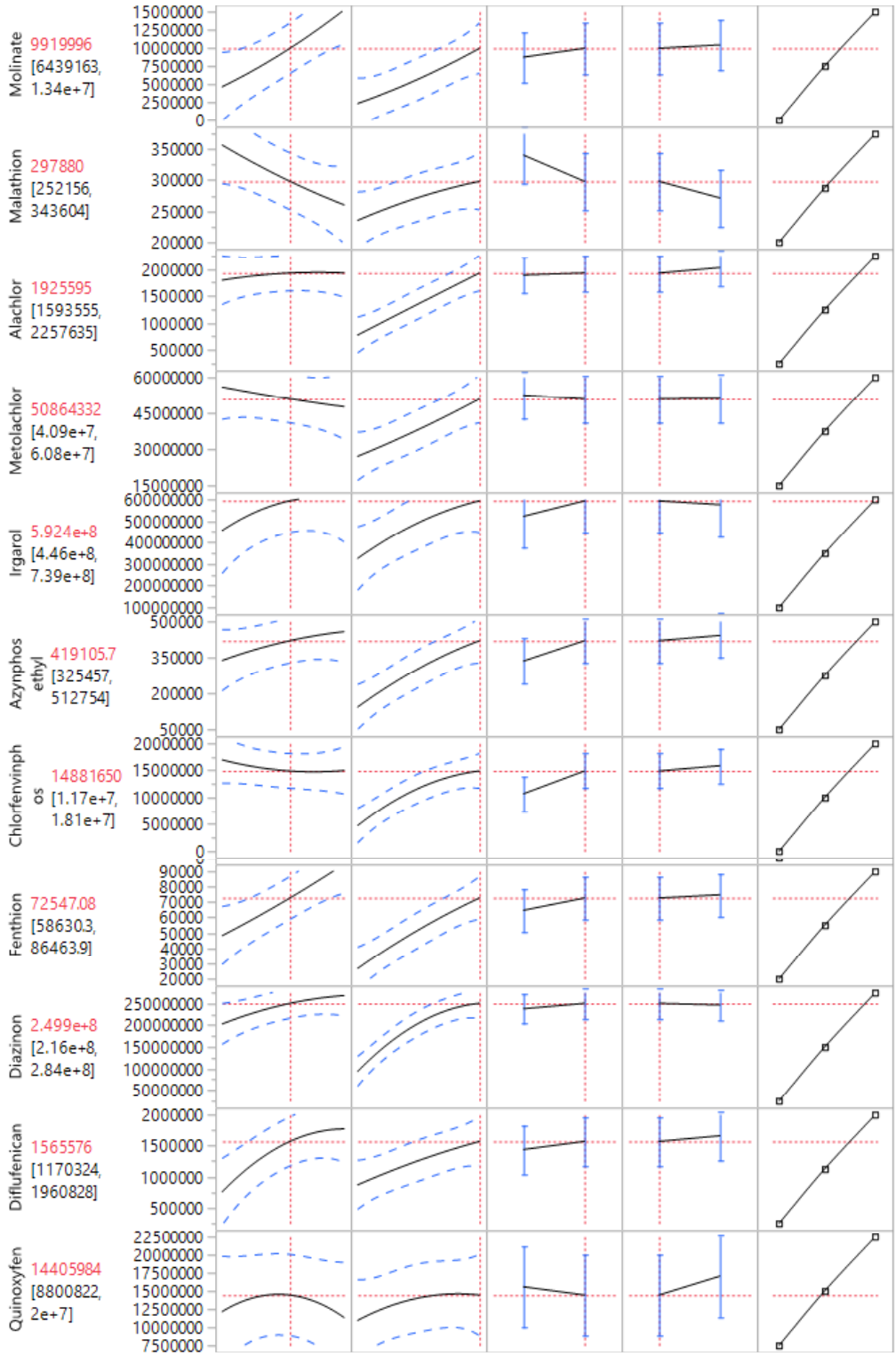

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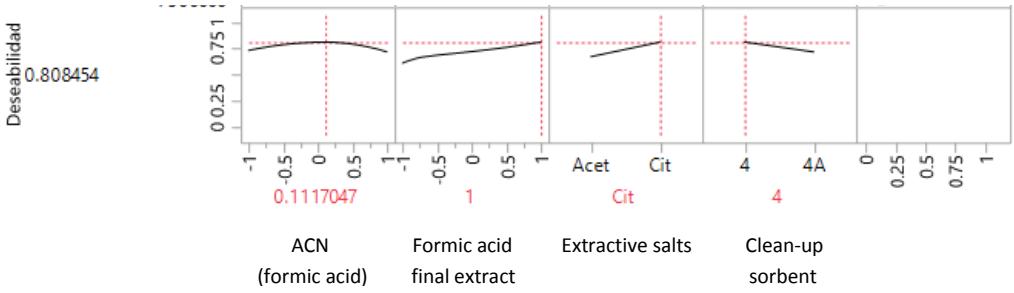


Figure S1. (Continued).

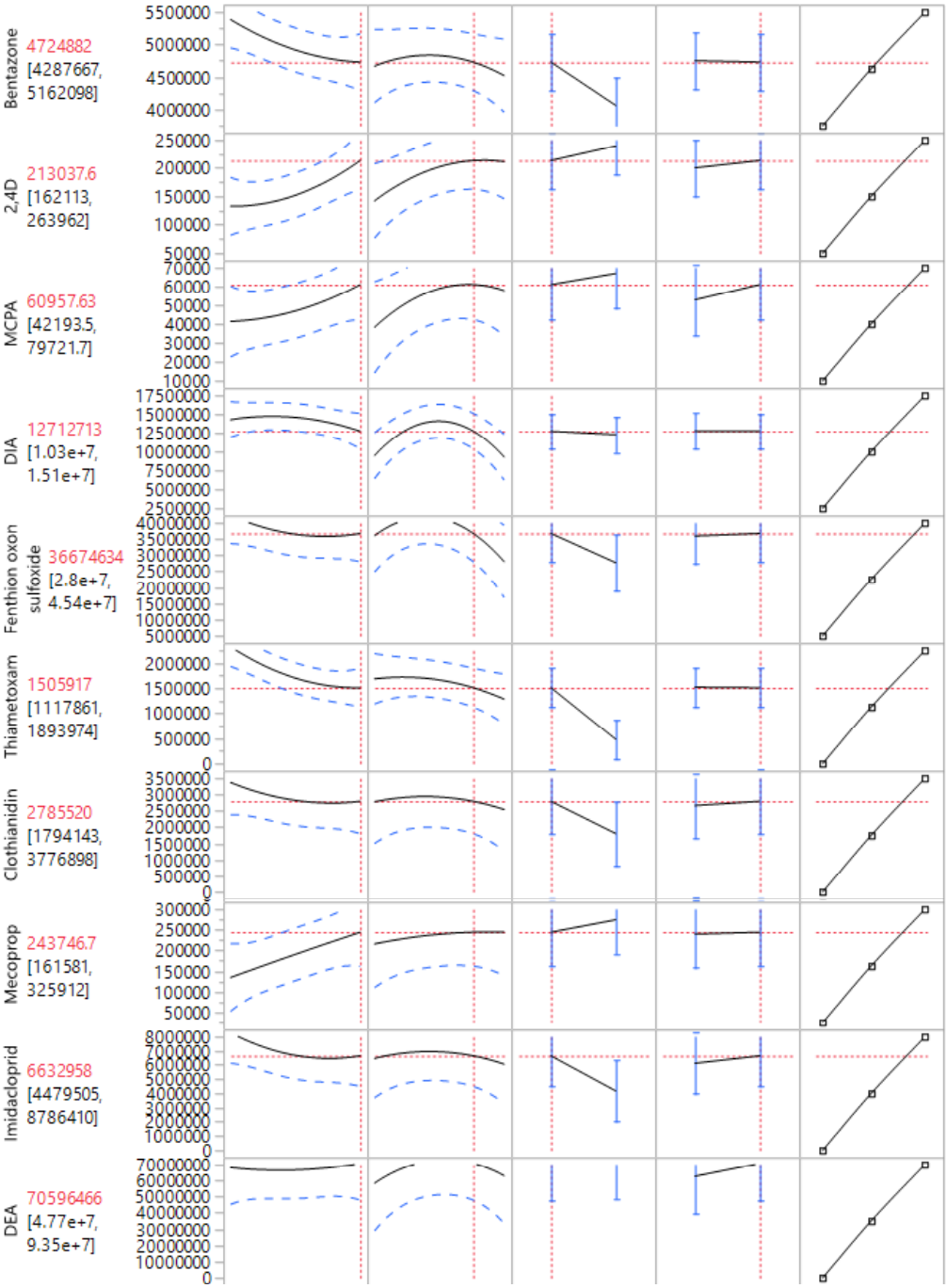


Figure S2. DOE prediction profiles and desirability for each analyte in grapes.

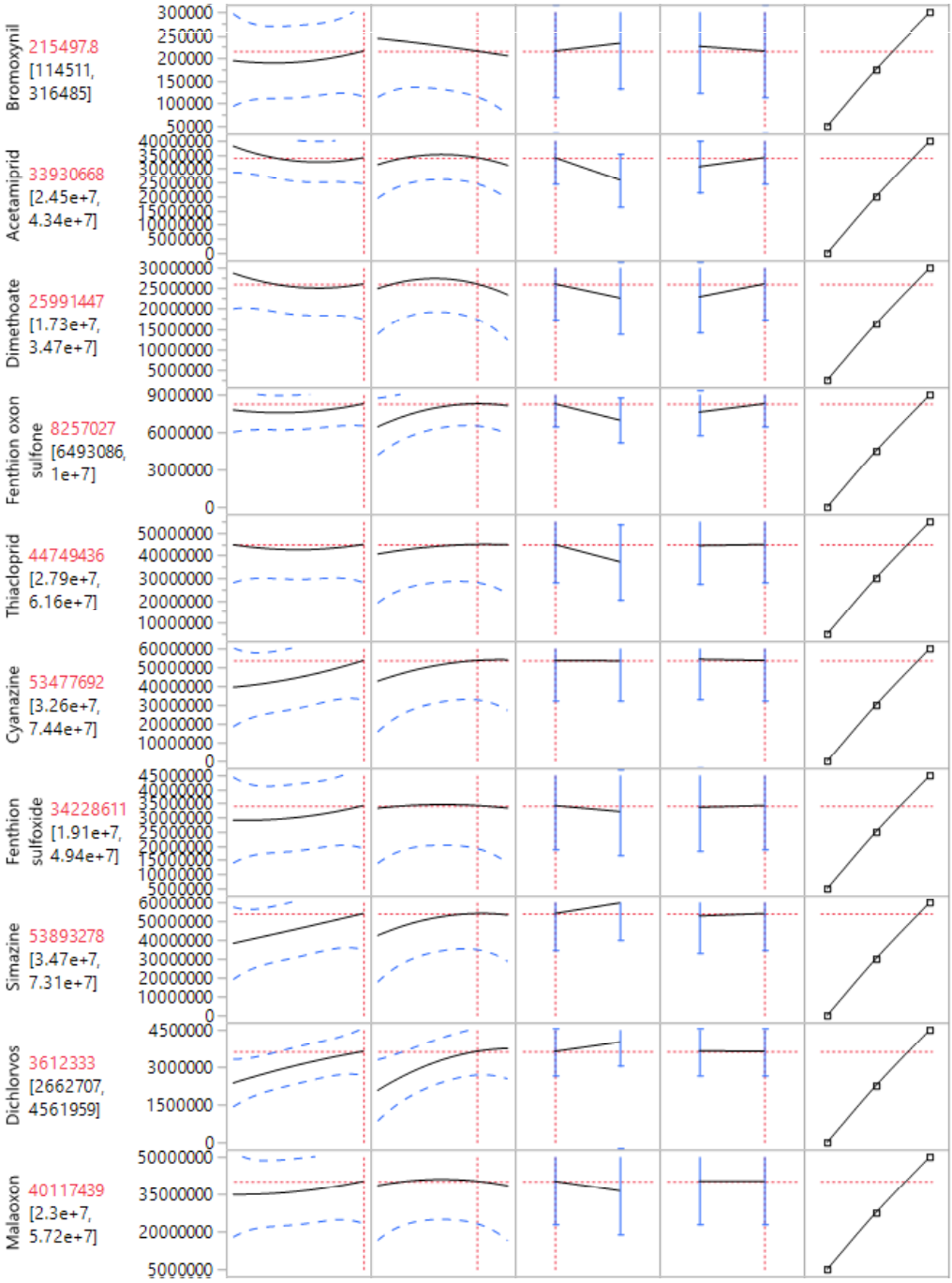


Figure S2. (Continued).

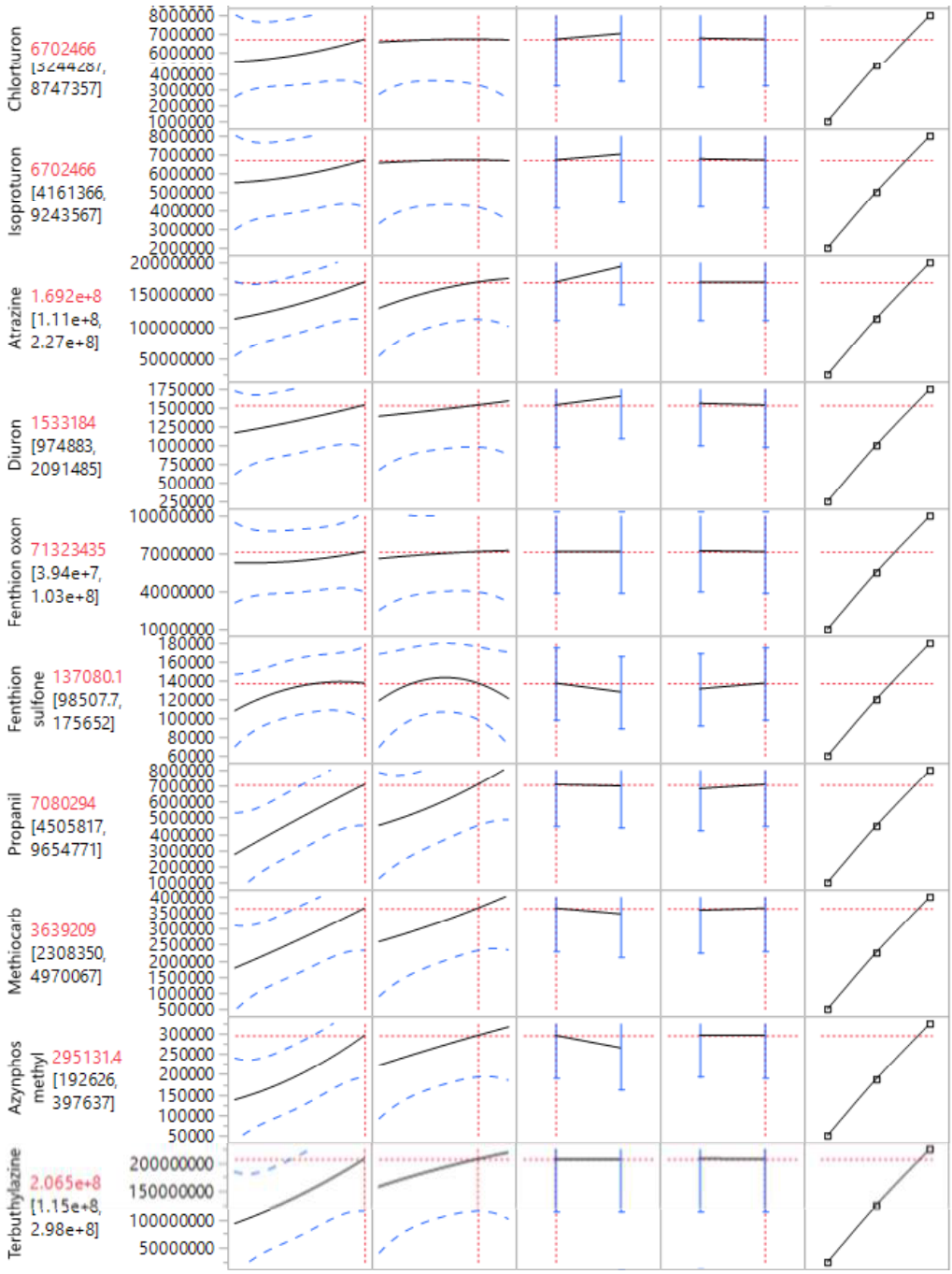
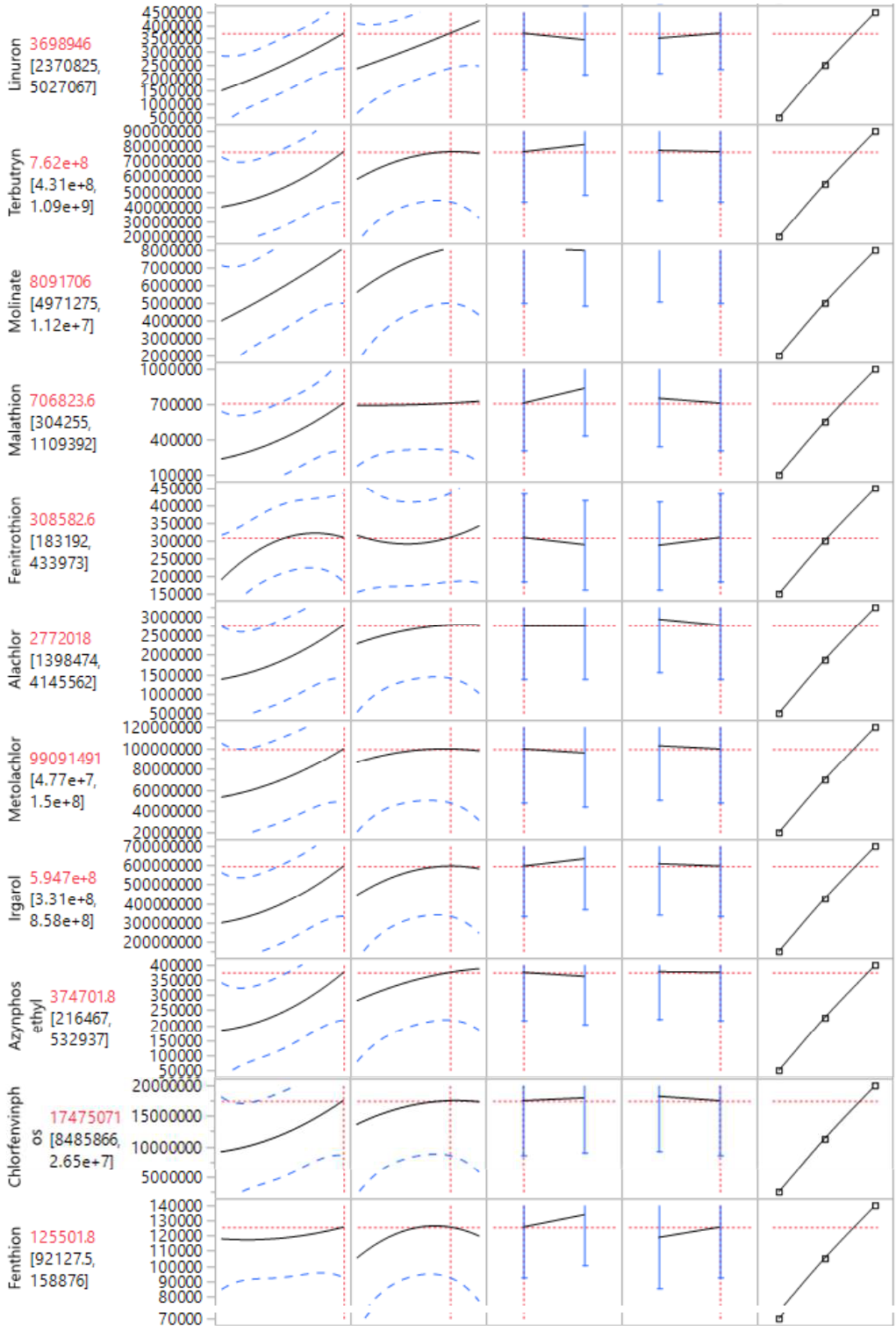


Figure S2. (Continued).


Linuron
3698946
[2370825, 5027067]
Terbutryn
7.62e+8
[4.31e+8, 1.09e+9]
Molinate
8091706
[4971275, 1.12e+7]
Malathion
706823.6
[304255, 1109392]
Fenitrothion
308582.6
[183192, 433973]
Alachlor
2772018
[1398474, 4145562]
Metolachlor
99091491
[4.77e+7, 1.5e+8]
Irgarol
5.947e+8
[3.31e+8, 8.58e+8]
Azynphos ethyl
374701.8
[216467, 532937]
Chlorfenvinphos
17475071
[8485866, 2.65e+7]
Fenthion
125501.8
[92127.5, 158876]

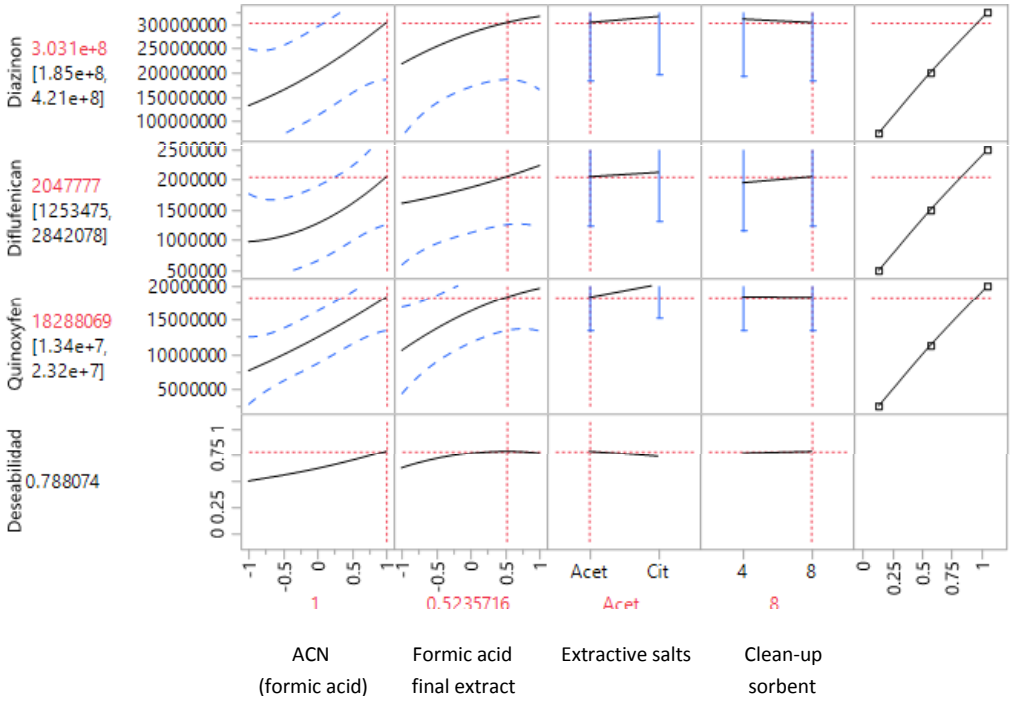


Figure S2. (Continued).

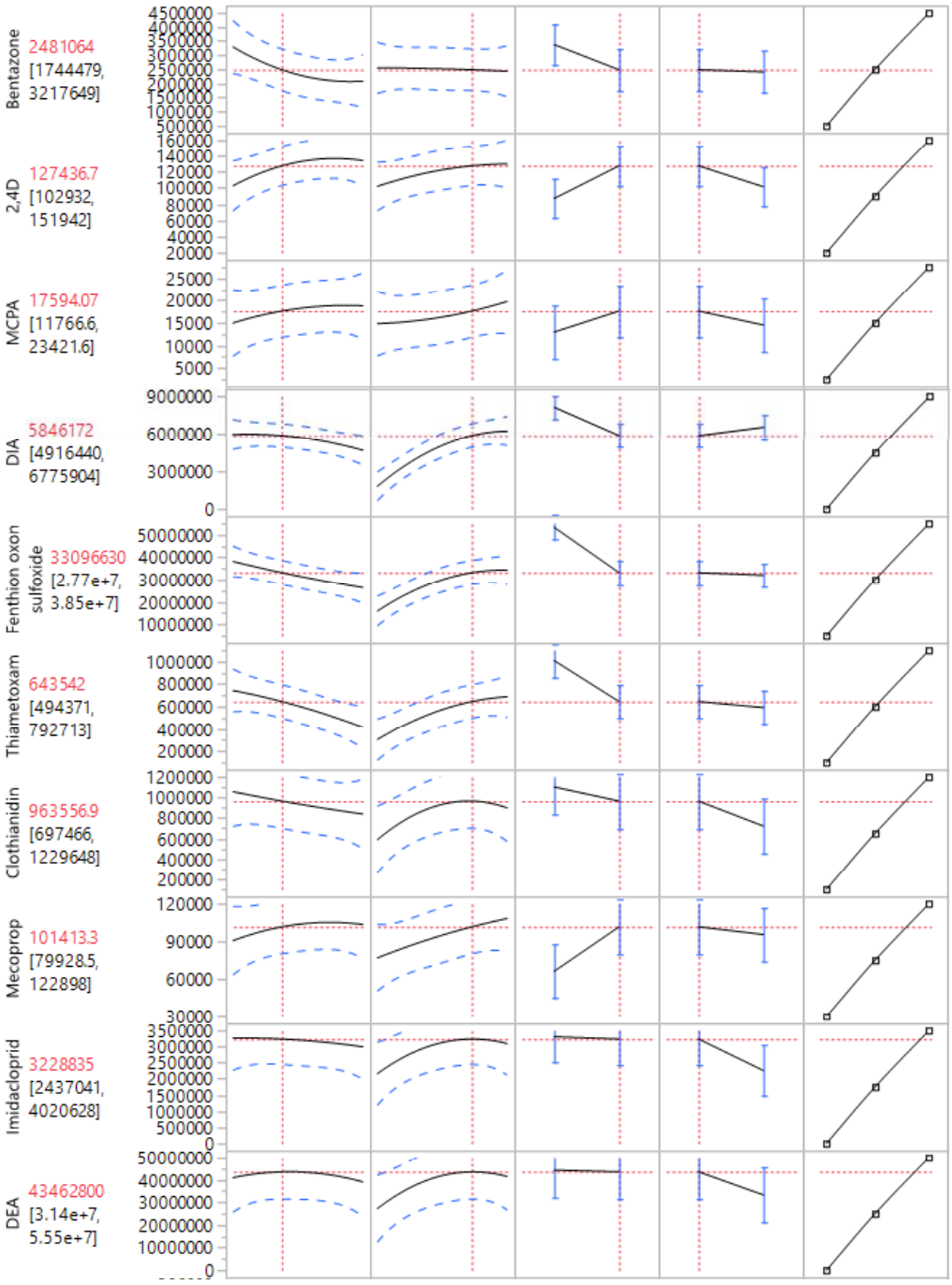


Figure S3. DOE prediction profiles and desirability for each analyte in alfalfa

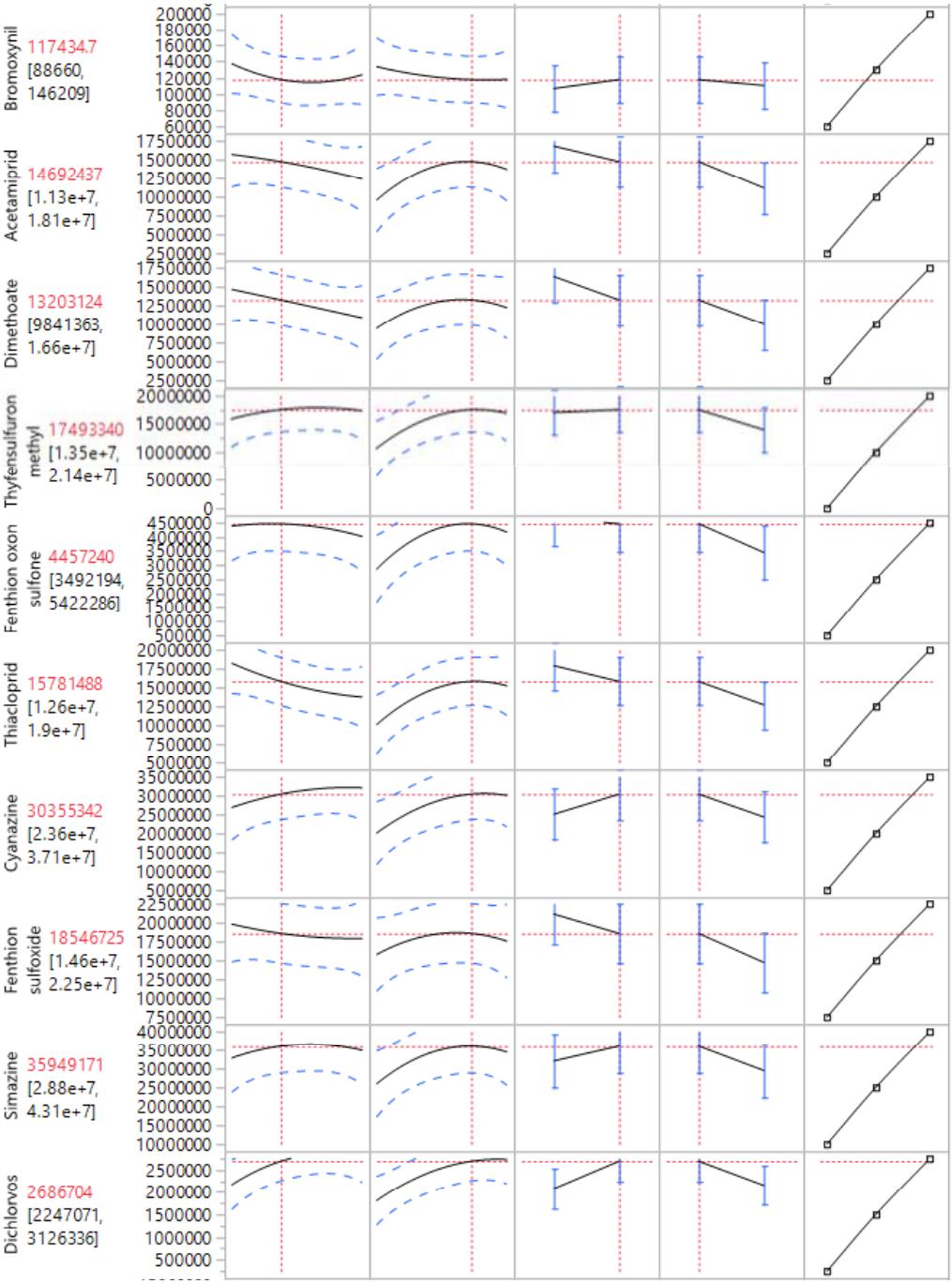


Figure S3. (Continued).

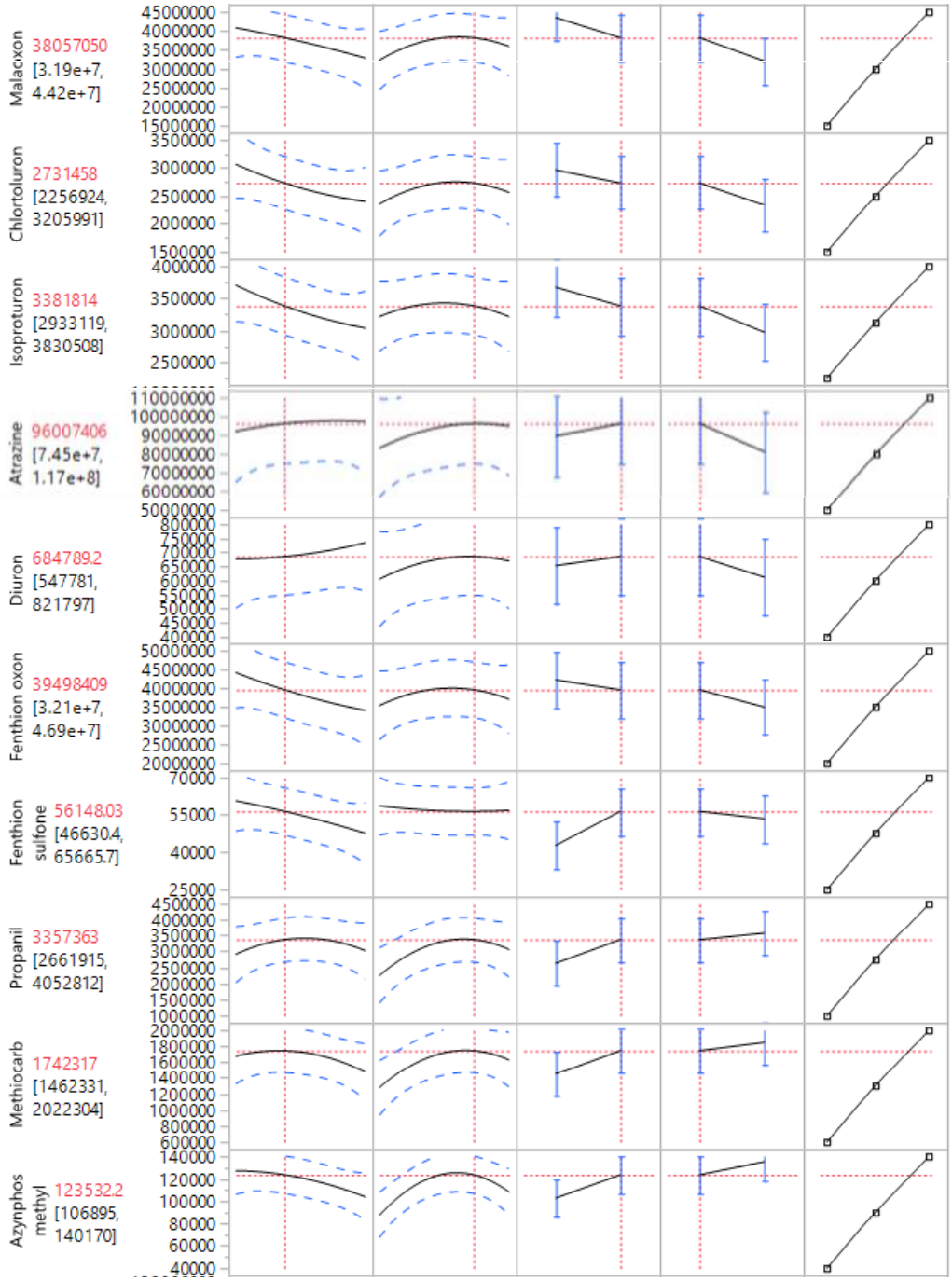
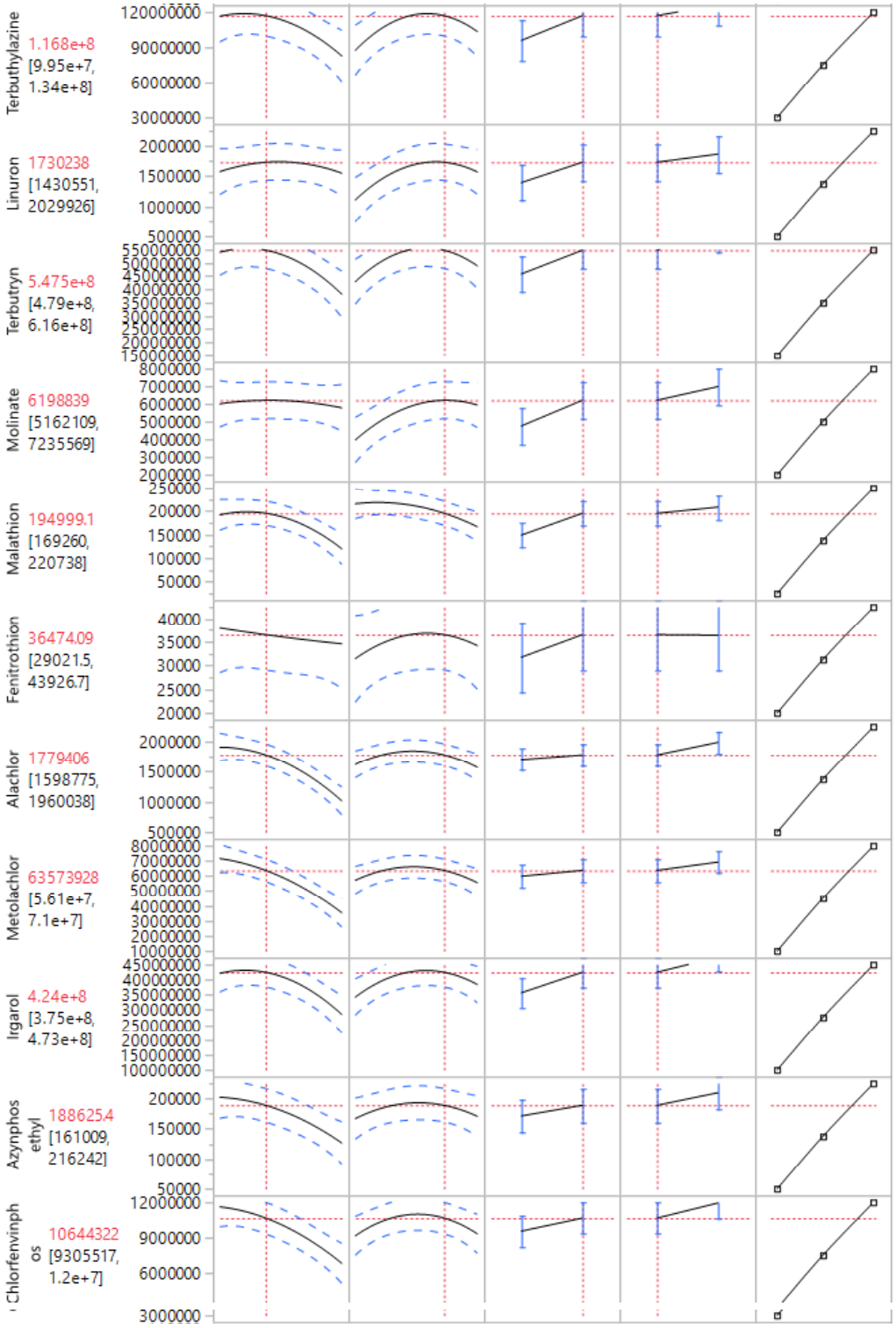


Figure S3. (Continued).


Terbuthylazine
1.168e+8
[9.95e+7, 1.34e+8]
Linuron
1730238
[1430551, 2029926]
Terbutryn
5.475e+8
[4.79e+8, 6.16e+8]
Molinate
6198839
[5162109, 7235569]
Malathion
194999.1
[169260, 220738]
Fenitrothion
36474.09
[29021.5, 43926.7]
Alachlor
1779406
[1598775, 1960038]
Metolachlor
63573928
[5.61e+7, 7.1e+7]
Irgarol
4.24e+8
[3.75e+8, 4.73e+8]
Azynphos ethyl
188625.4
[161009, 216242]
Chlorfenvinphos
10644322
[9305517, 1.2e+7]

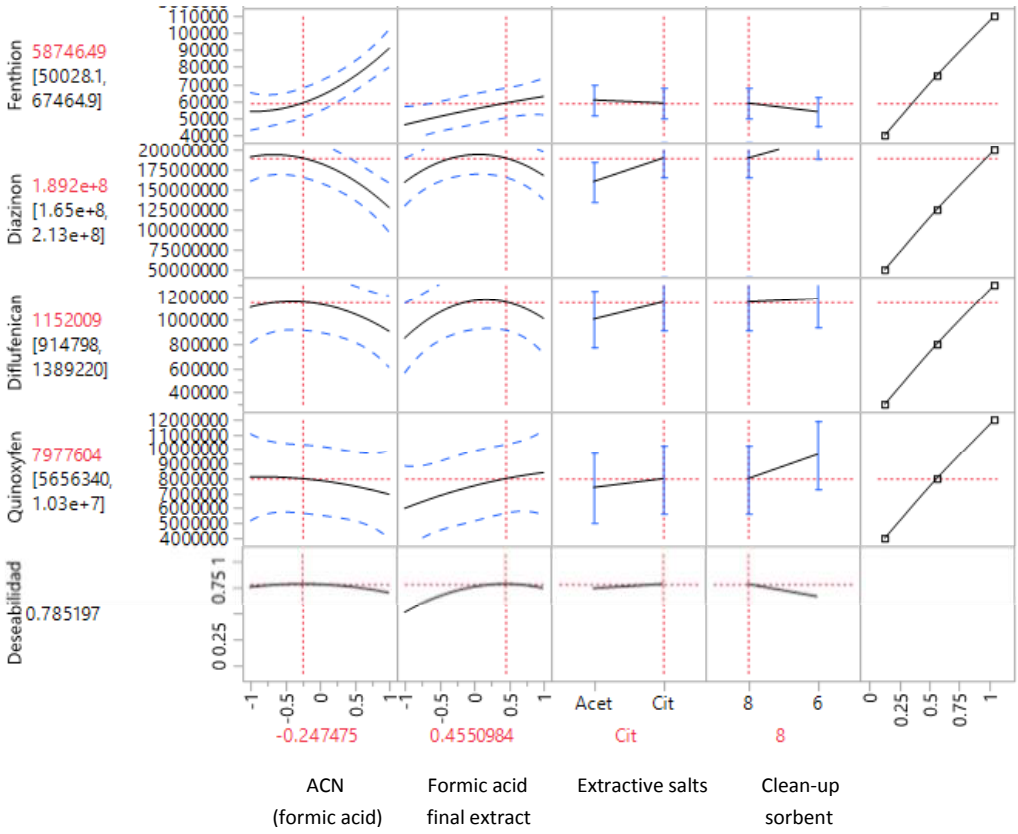


Figure S3. (Continued).

DIA

Fenthion oxon sulfoxide

Bentazone

Clothianidin

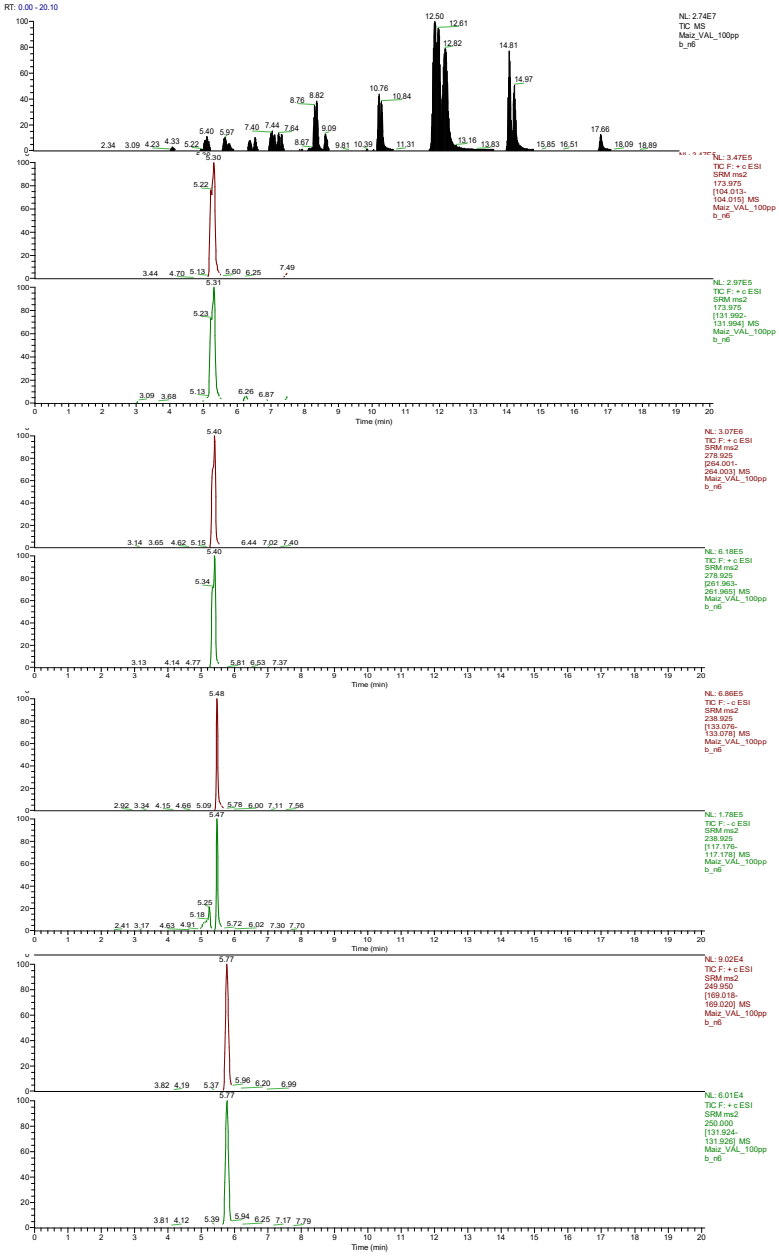


Figure S4. Extracted ion chromatograms of all the targeted compounds (TIC (black), SRM transition 1 (red chromatogram) and 2 (green chromatogram) of each compound) from the corn samples spiked at 100 ng/g used for method validation.

Imidacloprid

DEA

2,4-D

Acetamiprid

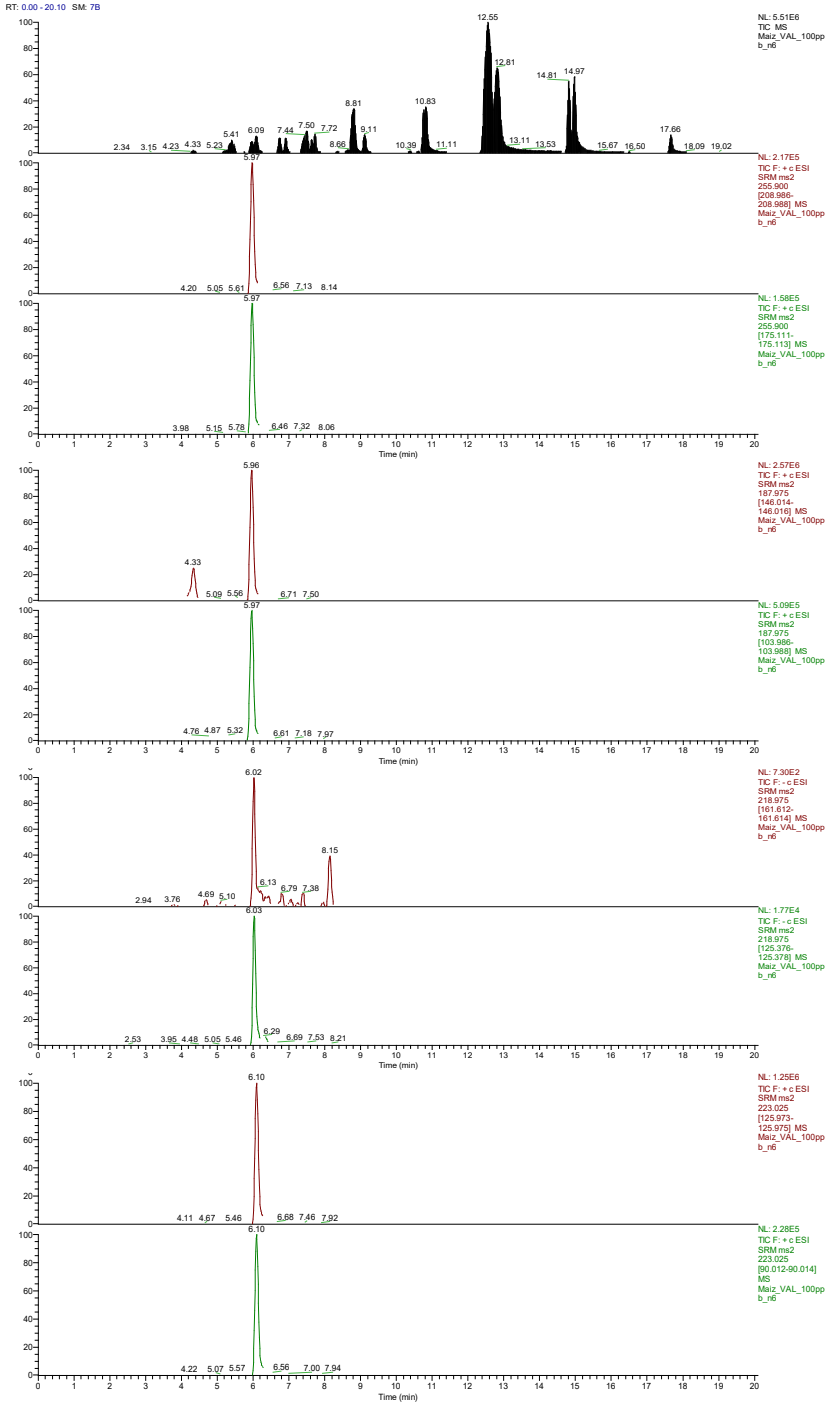


Figure S4. (Continued)

Dimethoate

Fenthion oxon sulfone

MCPA

Thiacloprid

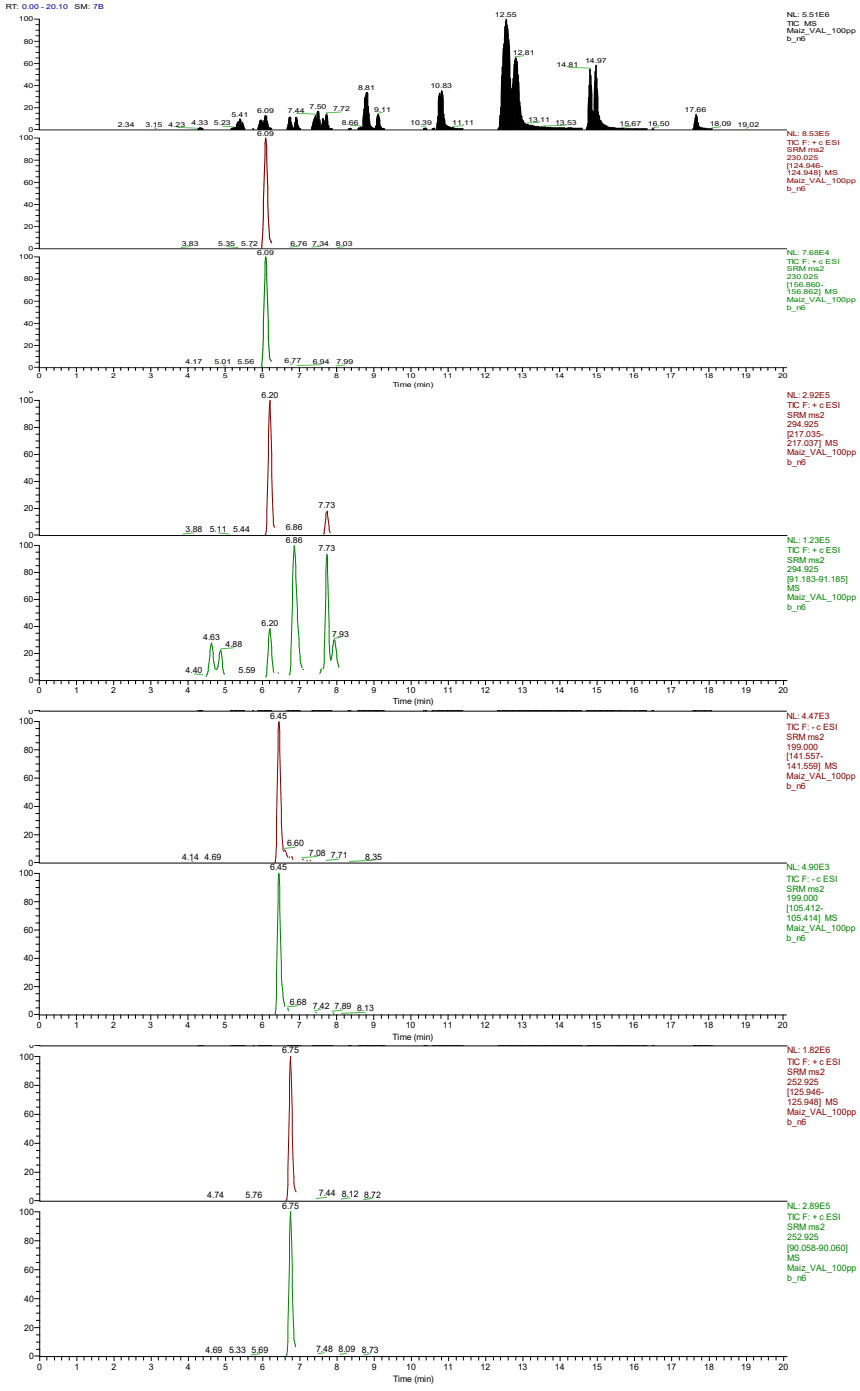


Figure S4. (Continued)

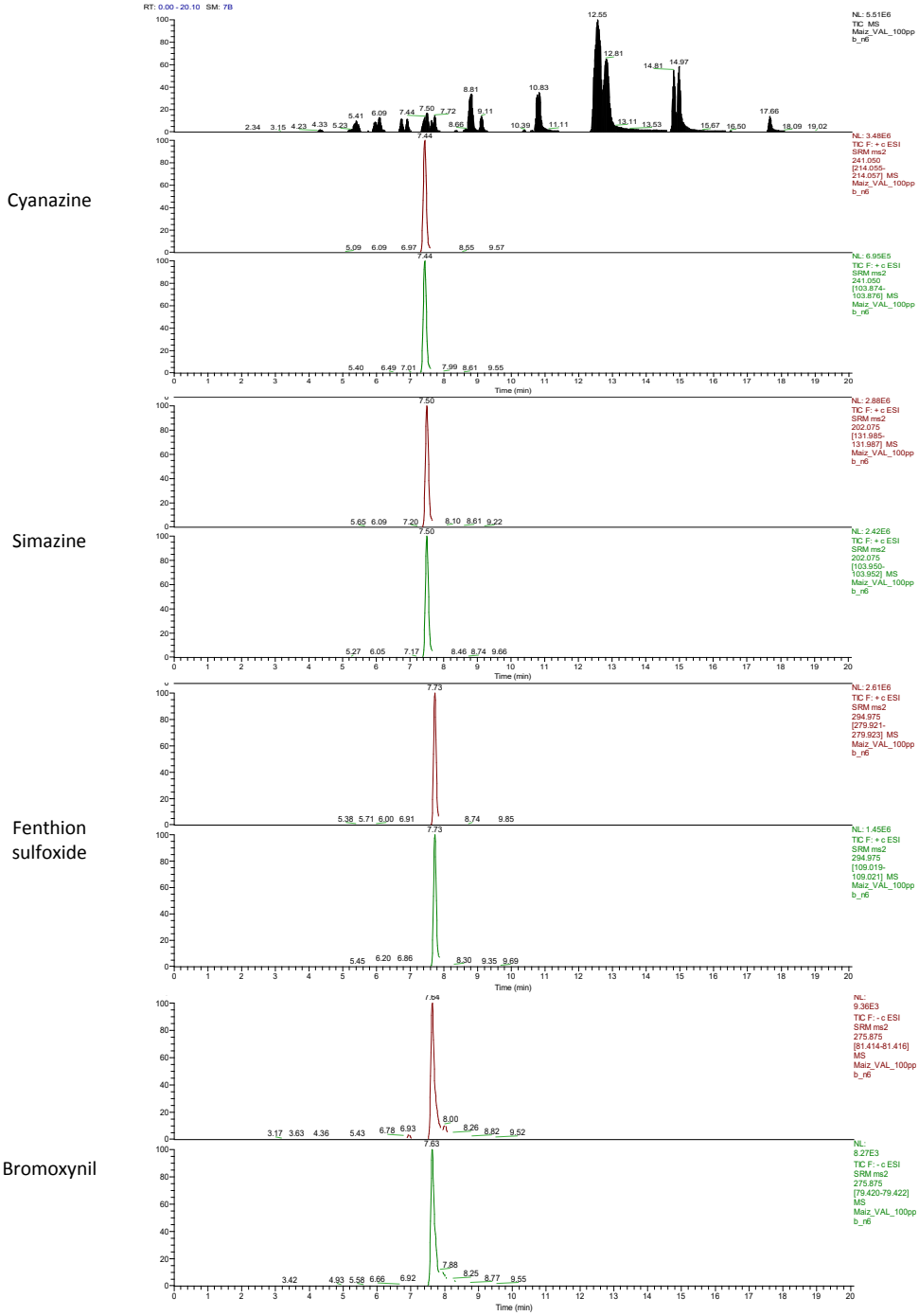


Figure S4. (Continued)

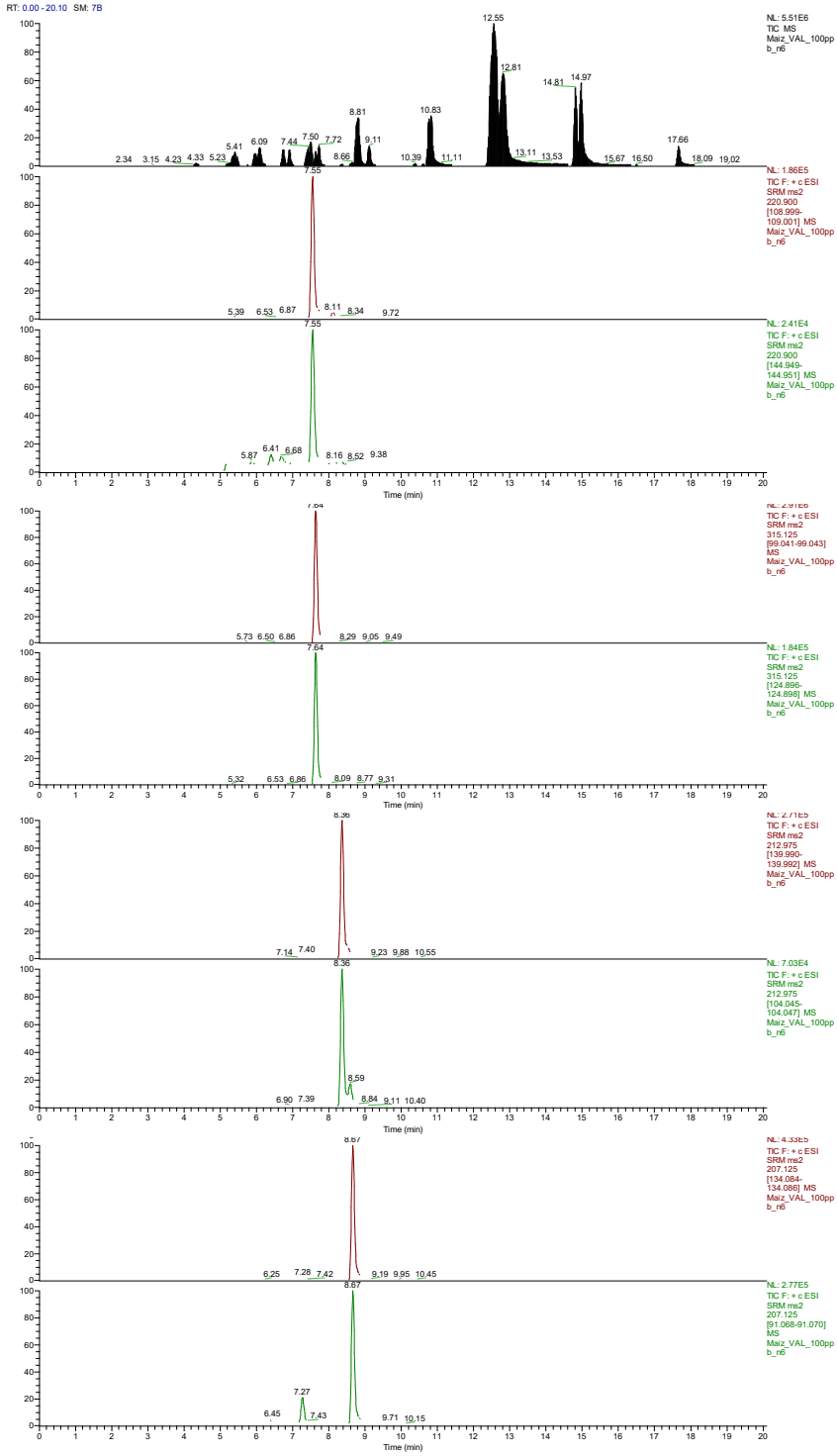


Figure S4. (Continued))

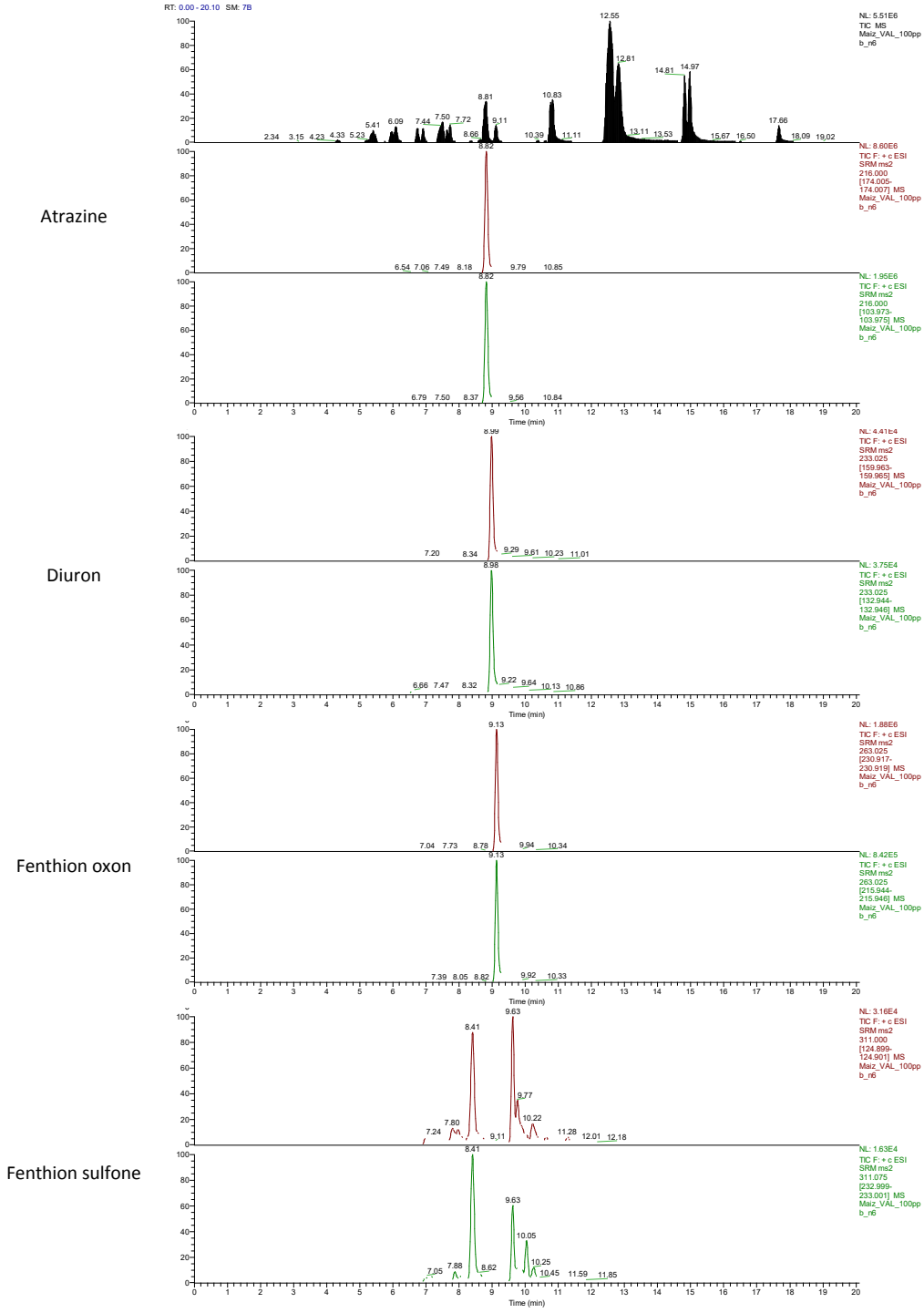


Figure S4. (Continued)

Propanil

Methiocarb

Azinphos methyl

Terbuthylazine

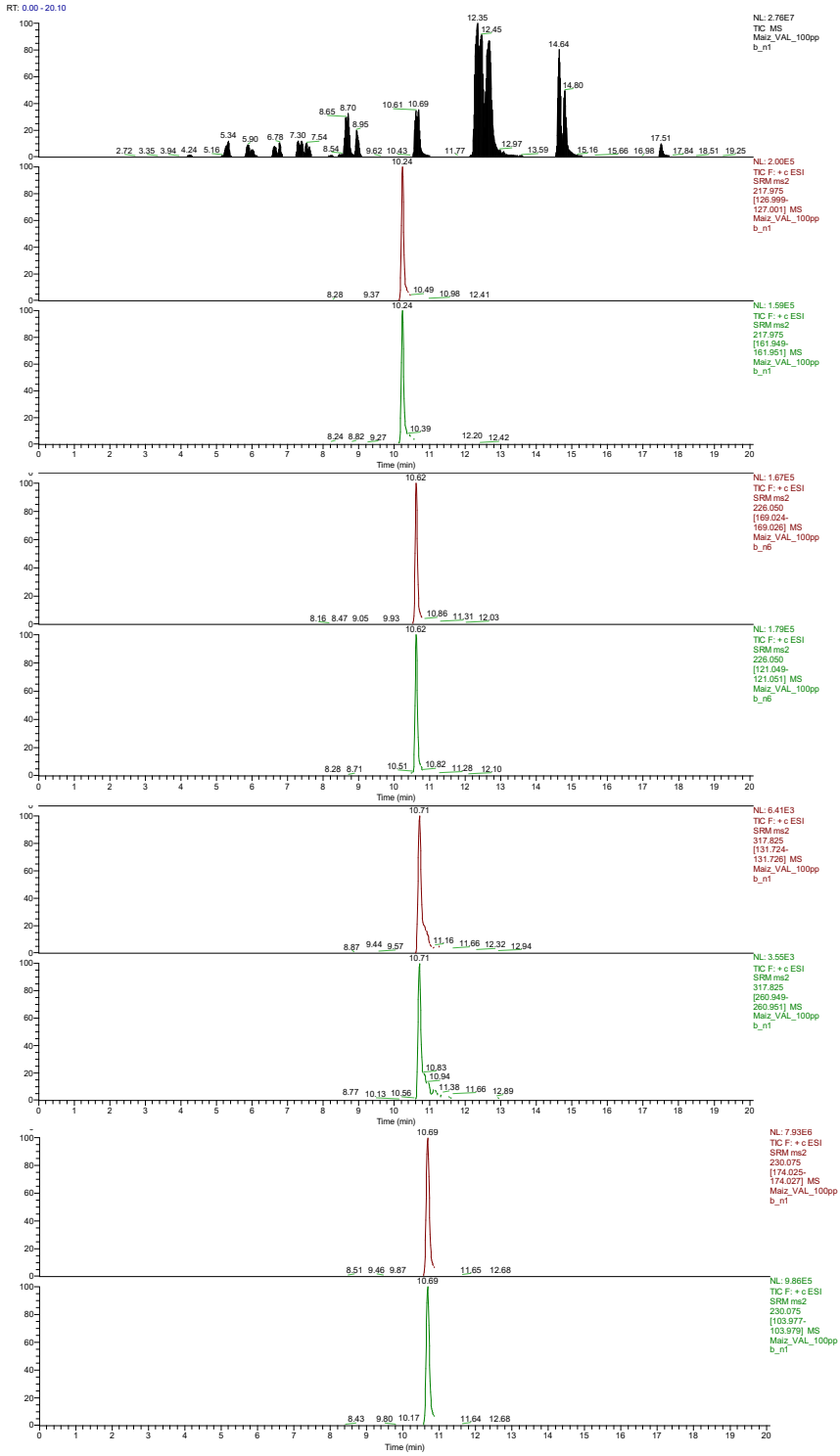


Figure S4. (Continued)

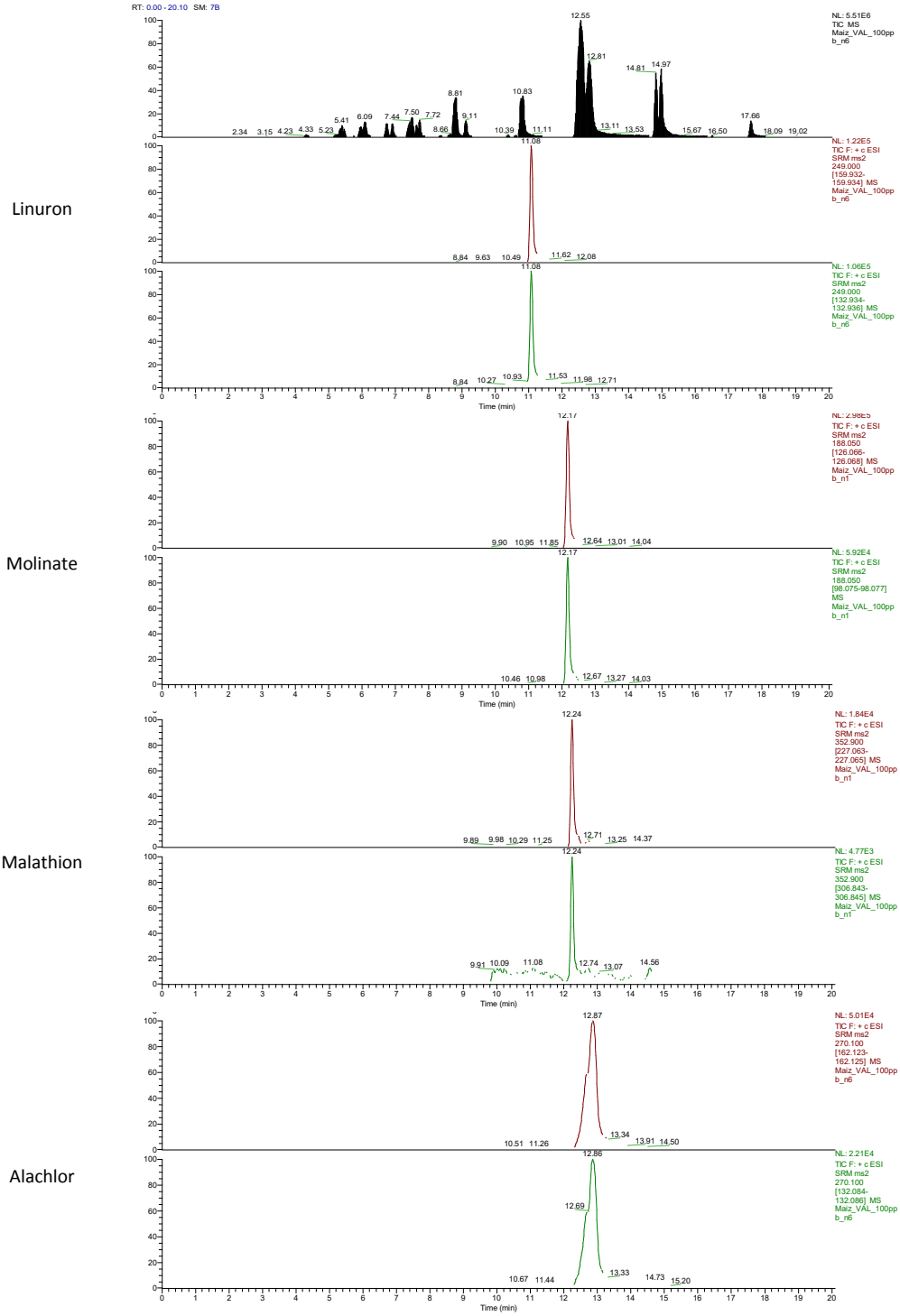


Figure S4. (Continued)

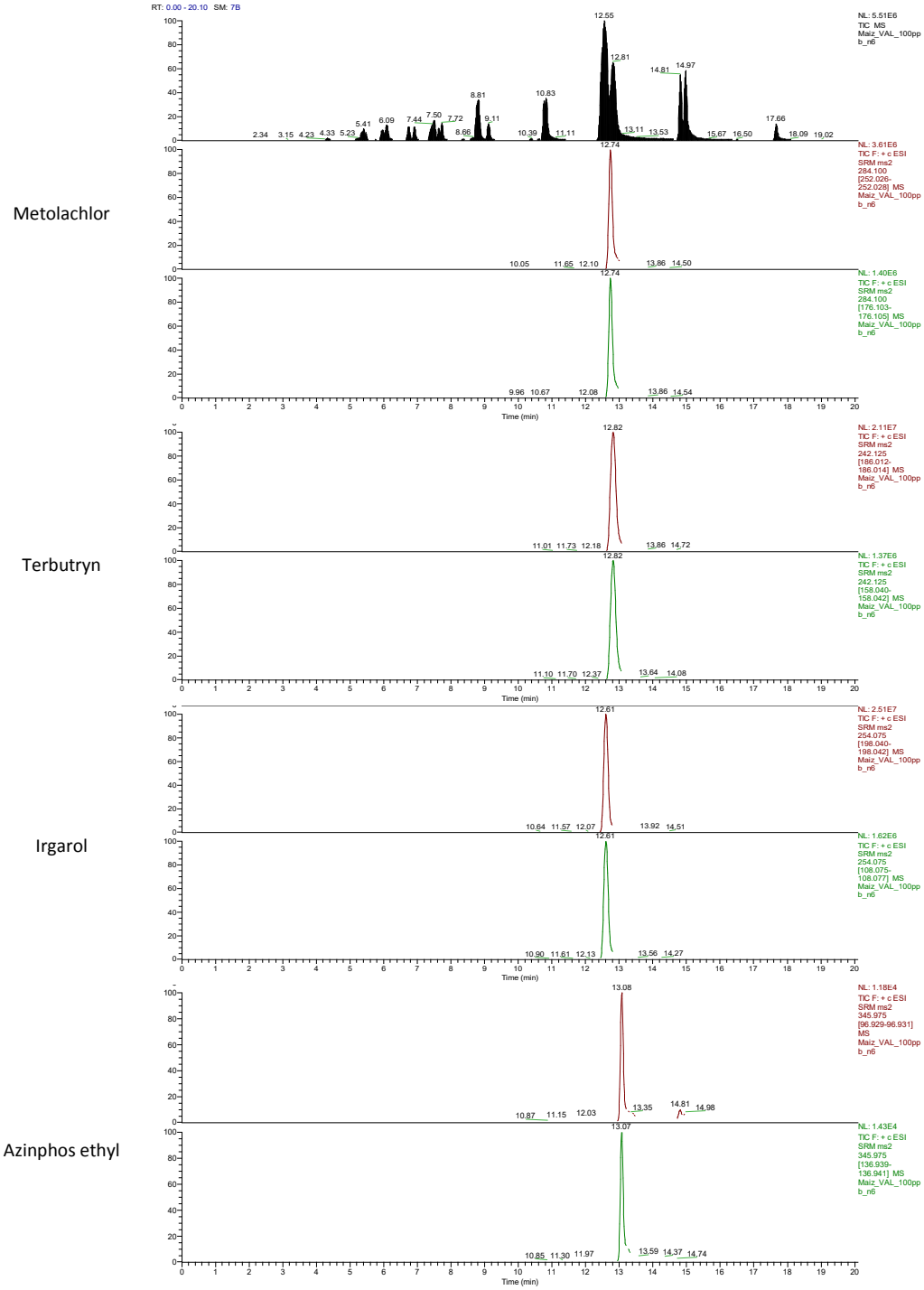


Figure S4. (Continued)

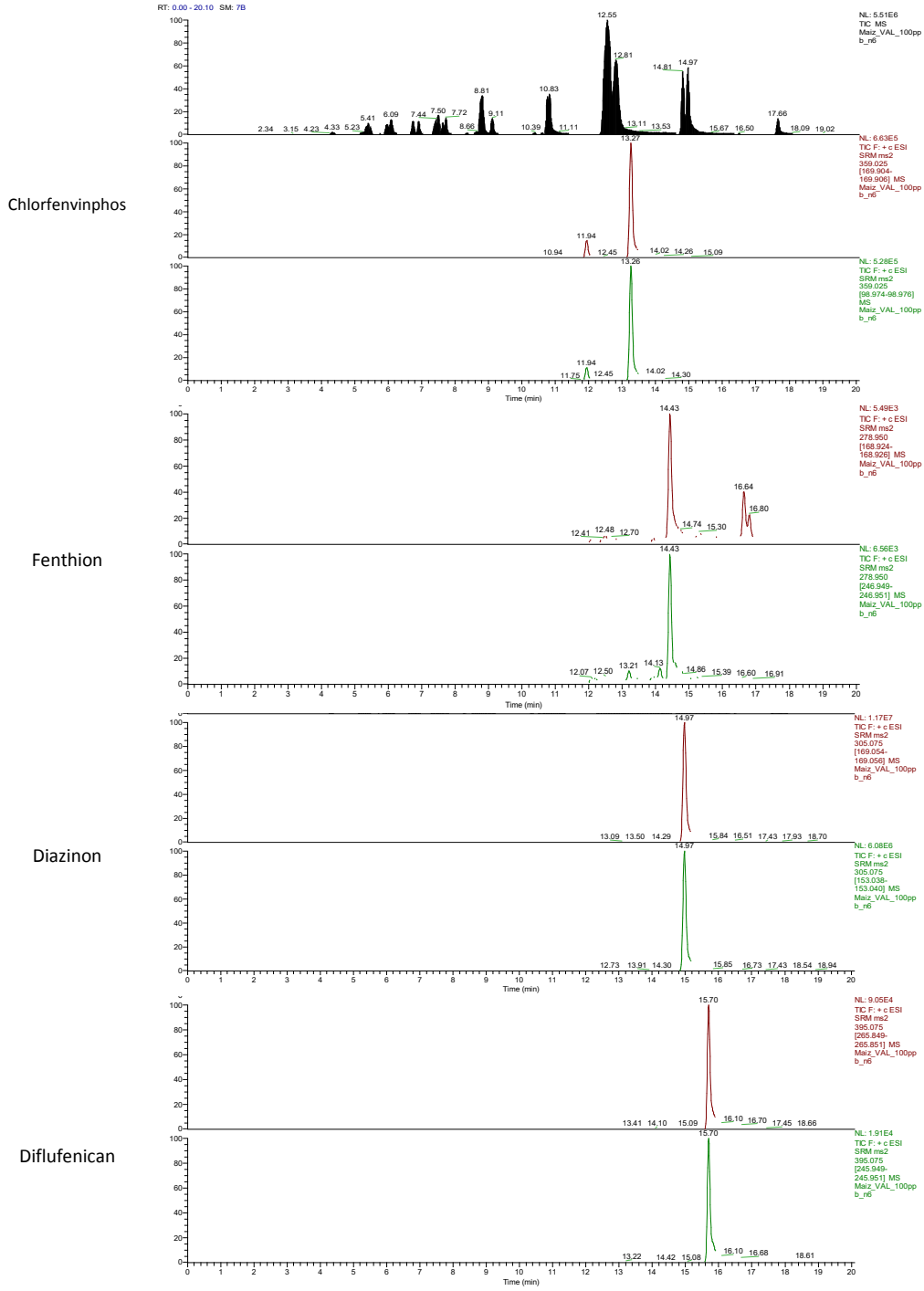


Figure S4. (Continued)

Quinoxyfen

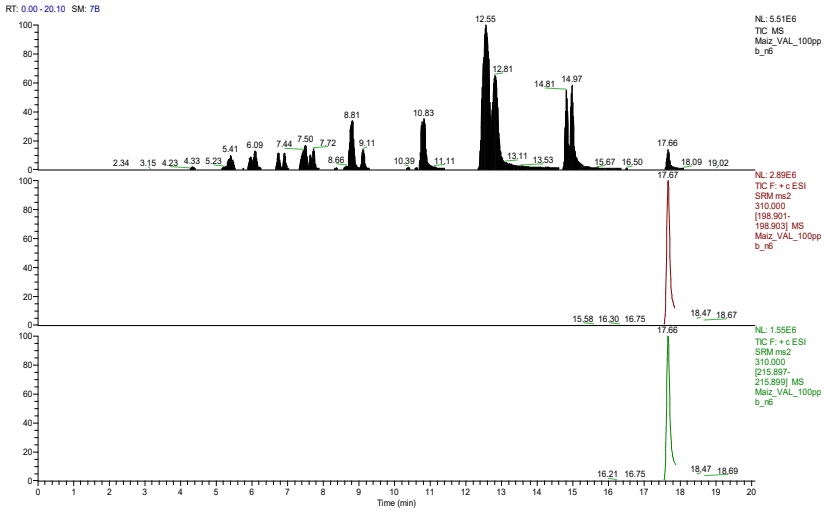


Figure S4. (Continued)

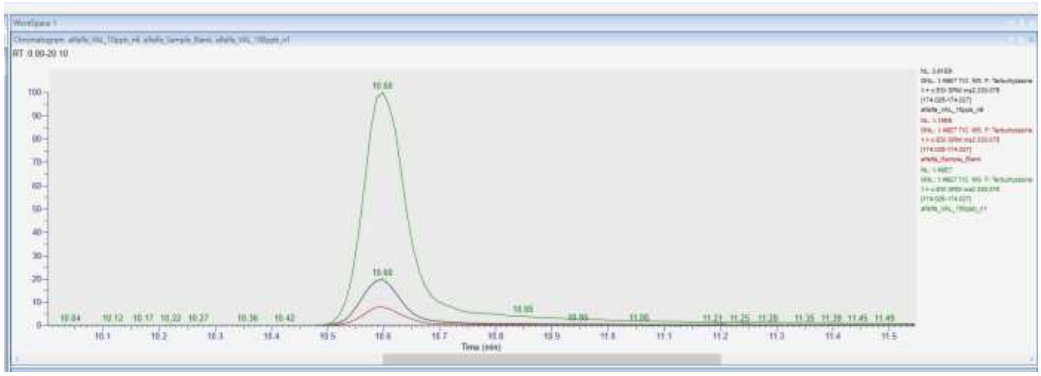


Figure S5. Extracted ion chromatograms corresponding to the analysis of terbuthylazine (quantification SRM transition m/z 230 -> 174) in the alfalfa sample used for method validation (red) and the same sample spiked with the compound at 10 (black) and 100 ng/g (green).

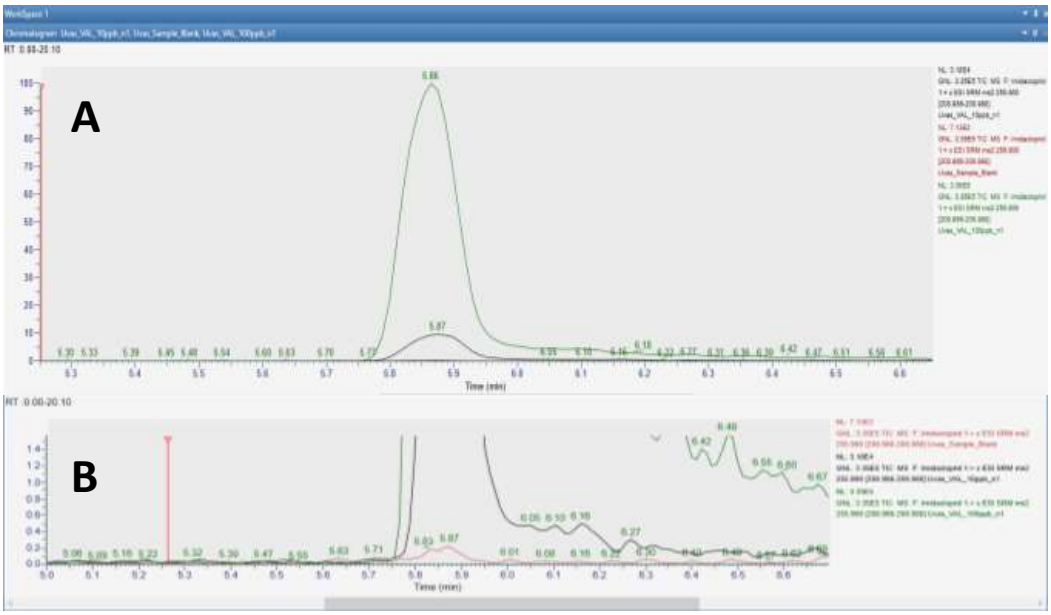


Figure S6. A. Extracted ion chromatograms corresponding to the analysis of imidacloprid (quantification SRM transition m/z 256 -> 209) in the grape sample used for method validation (red, not visible at this scale) and the same sample spiked with the compound at 10 (black) and 100 ng/g (green). B. Zoom of the same extracted ion chromatograms.

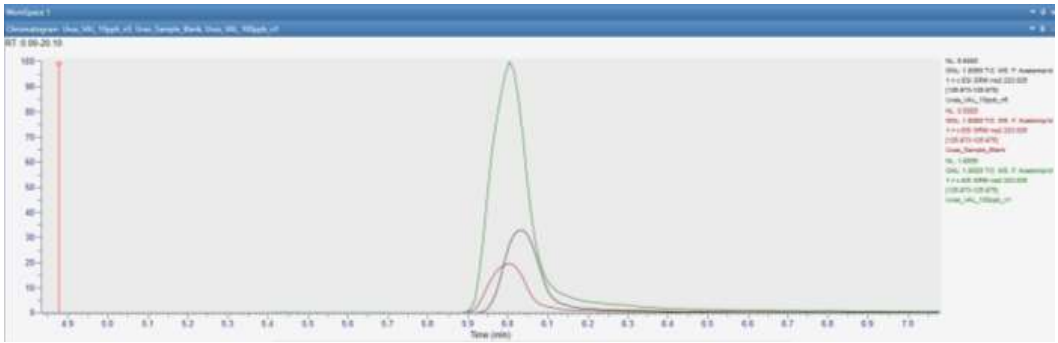


Figure S7. Extracted ion chromatograms corresponding to the analysis of acetamiprid (quantification SRM transition m/z 223 -> 125) in the grape sample used for method validation (red) and the same sample spiked with the compound at 10 (black) and 100 ng/g (green).

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3.2. HRMS-based suspect screening approach for CECs prioritization in a reclaimed water-based irrigation system

For many years, environmental chemistry has mainly focused its efforts on providing analytical tools for the determination of specific pollutants with a pre-established environmental or toxicological concern. These analytical tools were mostly based on target methodologies limited to a small number of contaminants due to the technological capabilities. GC and LC coupled to low resolution (tandem) mass spectrometry have been the preferred techniques for CEC analysis due to their high sensitivity, robustness, selectivity, and excellent performance for quantitative analysis (Agüera et al., 2013). However, as stated in the introduction, the list of chemicals considered as CECs grows every day and many of them, including metabolites and TPs, continue uncovered to date. Moreover, there are increasing concerns about the combined effects of this multitude of compounds permanently found in the environment.

At this point, targeted strategies present some limitations when we intend to characterize the contamination footprint of a specific area. Thus, new trends in analytical chemistry based on HRMS suspect and non-target screening offers the opportunity to perform this characterization in a more holistic way, as they allow the determination in a single analytical run of thousands of chemicals (Gago-Ferrero et al., 2018). This was one of the main objectives of the work described in the scientific publication #3: to develop a generic HRMS-based analytical protocol to determine the chemical footprint in various water matrices from an agrarian area irrigated with reclaimed water. Since CECs are only partially removed during water regeneration processes, reclaimed water may contain a remarkable number of CECs that could arrive to crops. To control the presence of the most relevant CECs in regenerated water and support local managers in the eventual implementation of mitigation actions if needed, tools that allow the prioritization of these compounds in water are needed. In this work, a prioritization procedure based on the compound ecotoxicity and occurrence was developed and implemented to identify the priority CECs in the water used for irrigation. For this purpose, a semi-quantification approach based on an ionization efficiency model was applied to the compounds detected in this water matrix.

This work was done in the framework of the MAGO project, which aims at creating a link between research results with real market needs and end-users demand to address food security and water management in the Mediterranean Region, and finding novel solutions to enhance integrated water resources management for sustainable agriculture.

3.2.1. Scientific publication #3

Prioritization of the most relevant organic contaminants in a reclaimed water irrigation system through an LC-MS/HRMS-based suspect screening workflow

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Dana Orlando Véliz

Rocío Bonansea

Cristina Postigo

Miren López de Alda

Submitted to *Journal of Hazardous Materials*

(Ref. No.: HAZMAT-D-23-03499)

Prioritization of the most relevant organic contaminants in a reclaimed water irrigation system through an LC-MS/HRMS-based suspect screening workflow

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Abstract

A prioritization procedure was developed and implemented at local level to identify the most relevant organic contaminants of emerging concern (CECs) in an agricultural area irrigated with reclaimed water. A wide-scope screening methodology based on UPLC-HRMS analysis was applied to holistically characterize the CEC footprint in water and its spatial and temporal variations. One hundred sixty-two CECs, including pharmaceuticals, industrial chemicals, and pesticides, among others, were identified with a confidence level of 2 in the water samples investigated. After water treatment in the reclamation plant and transport within the irrigation channel network, more than a hundred compounds were still detected at the location where water is abstracted for crop irrigation. Compound ecotoxicity and occurrence (semi-quantified concentrations or peak intensity) were the parameters used to prioritize CECs in the water used for irrigation. Results pointed at venlafaxine, O-desmethyl-venlafaxine, galaxolidone, theophylline/paraxanthine, oxybenzone, and N-phenyl-1-naphtylamine, among others, as CECs of concern in the investigated area. This study provides a simple and cost-effective approach to detect site-specific priority pollutants that could be otherwise overlooked by national or European regulations. The prioritization tool provided contributes to rationally design monitoring and attenuation programs and efficiently manage water resources, by ensuring the safety of reclaimed water applications.

Keywords: Contaminants of emerging concern, non-target screening, water reuse, circular economy, micropollutant, water analysis, agriculture, ecotoxicological risk assessment.

1. Introduction

In some regions, climate change and the unbalanced use of water between territories due to an unsustainable agricultural system force to irrigate crops or refill aquifers with reclaimed water. In terms of scarcity and quality, the water crisis is already affecting arid and semi-arid regions worldwide. In these areas, water reuse becomes a compulsory alternative to surface and groundwater abstraction to fulfill water demand despite its potential risks to human and environmental health [1]. Some of the risks associated with this practice derive from the possible

presence of contaminants of emerging concern (CECs). The removal of these substances from wastewater represents one of the most important challenges for wastewater treatment technologies [2]. A simple search of the terms "Contaminants of emerging concern" + "Wastewater" + "Occurrence" in Web of Science returned 344 entries, including 92 review articles, at the date when these lines were written. This proves the relevance and wide knowledge about the footprint of CECs in wastewater and, thus unavoidably, surface and ground waters [3–6]. Depending on the pollution source (urban, industrial, or agricultural origin, among others), CECs ending in water may be pharmaceuticals, pesticides, industrial chemicals, plasticizers, flame retardants, illegal drugs, tire wear degradation compounds, etc.

For the sake of the safe reuse of wastewater, European regulators have established minimum biological and chemical requirements [7]. However, the protection against CECs is relatively vague [8,9], and relies on the assessment of the risk that the priority substances included in Directive 2013/39/EU [10] and/or the river basin-specific pollutants, as laid down in the Water Framework Directive (WFD) [11], may pose to human and ecosystem health. Therefore, the current legislation only limits the presence of a few organic pollutants, keeping other CECs potentially present in the water on a monitoring status at best or, normally, into oblivion. To reach this minimum water quality, water reclamation plants (WRPs) include a tertiary treatment within their water treatment train to improve the removal of nutrients, pathogens, or suspended solids [2]. Tertiary treatments commonly used are based on advanced oxidation processes (ozonation, UV-based, H2O2, etc.), chlorine disinfection, or advanced biological treatments (membrane bioreactors (MBR), moving bed bioreactors, etc.), among others [2,12,13]. They all provide diverse removal efficiencies for CECs, specific for each contaminant and treatment. Despite these purification efforts, the irrigation systems based on reclaimed water lead to the transfer and accumulation of CECs into irrigated soils and crops. This has been observed in both lab-controlled and field studies over the past few years [14–18]. Irrigation with reclaimed water has also been identified as a relevant source of organic CECs in aquifers underneath agricultural fields [19,20]. Moreover, since antibiotics are among the CECs spread in the environment by treated wastewater, the use of reclaimed water may also promote the emergence of antibiotic-resistant bacteria and genes, a

worldwide threat and a key issue from the One Health perspective [21]. Moreover, although scarcely addressed to date, wastewater reuse may result in occupational exposure to CECs that could lead to adverse health effects, as previously observed for occupational exposure to pesticides [22]. All these aspects should be considered in the ecotoxicological and human risk management of wastewater reuse.

Although legislation is yet to be improved in terms of organic pollution, there is an unquestionable need to characterize the CEC footprint in reclaimed water-based irrigation systems. Many different analytical procedures have been developed in the last decades to monitor CECs in aqueous environmental matrices. Most of them target groups of chemicals preselected based on their physical-chemical properties (chemical class or polarity), their legal status, or their social and toxicological relevance [23–26]. Nevertheless, these approaches reveal only a very narrow piece of the CEC footprint in the aqueous environment, considering the huge variety of anthropogenic organic compounds that are present in wastewater. Wide-scope screening of CECs using ultra-performance liquid chromatography coupled with high-resolution mass spectrometry (UPLC-HRMS) is contributing to overcoming this issue. The recent technological advances in the sorbents packing the chromatographic columns and the use of wide-scope ionization sources (electrospray) allow the characterization of CECs in water in a holistic way [27]. The use of hybrid HRMS analyzers that provide a mass accuracy below 5 ppm and a resolution over 10,000 at full width at half maximum (FWHM) (at m/z 200) enables the annotation of the elemental composition of any detected feature and the elucidation of its molecular structure through the evaluation of fragmentation and isotopic patterns with high precision. One of the main advantages of using HRMS over low-resolution technologies relays on its capability to perform suspect/non-target screening and retrospective analysis without the need for reference standards [27,28]. The suspect screening approach requires the elaboration of curated suspect lists with the compounds of interest. These lists should contain spectral information to compare with the data collected. In 2015, the NORMAN network (www.norman-network.com) initiated the collection of suspect lists created for a wide-scope screening of CECs in the environment. To date, and thanks to this effort, 99 separate suspect lists (covering in total more than 100,000 compounds) are freely available in the NORMAN Suspect List Exchange database [29,30]. In

addition, the use of retention time prediction models like the retention time index (RTI) increases the identification confidence during high-throughput suspect screening and reduces the number of false positives considerably [31].

The huge diversity of organic chemicals (incl. CECs and their transformation products) that may be present in reclaimed water makes their routine monitoring an unmanageable task. Therefore, the development and implementation of effective monitoring programs for CECs require the prioritization of the most relevant chemicals, in terms of occurrence and ecotoxicology. Current prioritization methods, like the one developed by the NORMAN network [32], are mostly based on monitoring data and effect-based analysis. This approach requires a huge collaborative effort of many laboratories to generate sufficient background knowledge. This means the creation of public databases that contain information on CEC occurrence and ecotoxicological risk assessment (ERA), usually expressed as Predicted No Effect Concentrations (PNEC). On a European or national scale, this is an optimum way to support policymakers for CEC regulation. The Slovak Republic, for example, performed a 10-year monitoring and ERA program in more than 400 sites to select the most dangerous substances in this country, in compliance with the WFD [33]. However, such procedures remain out of reach, in terms of cost in human and material resources, for defining site-specific pollutants. At a small geographical scale, simpler and cost-effective approaches have to be applied to, firstly, achieve a comprehensive and reliable identification of the contaminants present in the water resources and, secondly, prioritize the most relevant ones to support decision makers on their way to prevent the potential threats for the public and environmental health in that specific area.

In this context, the objectives of this study were i) to develop a simple and low-cost HRMS-based wide-scope screening and prioritization approach to comprehensively select the most relevant pollutants present in any given site, ii) to apply this approach in a reclaimed water-based irrigation system, and iii) to contribute to the existing collaborative open-access databases to increase the knowledge on CEC occurrence and support policy-making on this field.

2. Materials and methods

2.1. Reagents and chemicals

All the solvents used were UPLC-MS grade. Acetonitrile (ACN) and water for UPLC-HRMS analysis were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Methanol (MeOH), ethyl acetate (EtAc), and water for sample preparation, formic acid (purity, >98%) and ammonium acetate were obtained from Merck (Darmstadt, Germany). Isotopically labeled standards diflufenican-d3, bentazone-d7, linuron-d6, MCPA-d3, benzophenone-d10, imidacloprid-d4, and terbuthylazine-d5 were purchased from either Merck (Darmstadt, Germany) or Toronto Research Chemicals (Toronto, ON, Canada). Regenerated cellulose (RC) membrane syringe filters (0.2 µm pore size) were supplied by Sartorius Stedim Lab (Stonehouse, UK).

2.2. Case study area and sample collection

The study area was located in the Baix Llobregat Agrarian Park (Catalonia), an agricultural area that covers more than 3,400 hectares and produces over 35,000 tonnes/year of food, mainly horticulture [34]. This area is partially irrigated with reclaimed water from the nearby WRP of Gavà-Viladecans, which is distributed through a network of irrigation channels (see map in Fig. 1). This WRP, which mainly works as a wastewater treatment plant, receives 64,000 m3/day of wastewater of urban and industrial origin, and has a capacity for 300,000 people. Following conventional activated sludge treatment, part of the treated wastewater is further processed by MBR for its reclamation [35].

Two sampling campaigns were conducted: summer 2021 (28th July) and winter 2022 (12th January), and four locations were sampled in each campaign (Figure 1, GPS coordinates are shown in Table S1). WRP influent (A) and effluent (B) were collected as 24-h composite samples by personnel of the WWTP in polyethylene containers. One liter of these samples was transferred to amber PET bottles at the WWTP and transported to the analytical laboratory for analysis. Grab water samples were collected in amber PET bottles at the location where the reclaimed water is discharged to the net of irrigation channels (C) and a point of the irrigation network where the water (mix of reclaimed and surface water) is directly used for

irrigation (D) (Figure 1). Blank samples were prepared by treating and processing UPLC-grade water aliquots like wastewater samples.

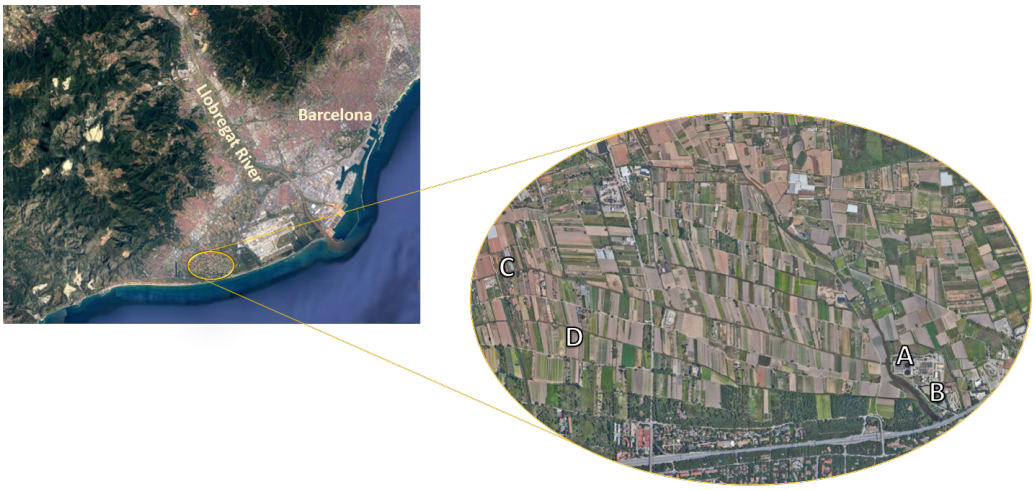

Figure 1. Study area next to the Llobregat River delta in Barcelona, Spain, and sampling locations. A) WRP influent, B) WRP effluent, C) point of release of the WRP effluent to the irrigation channels, D) point at the irrigation channels where water is pumped for field irrigation.

2.3. Sample pre-treatment

For a non-selective extraction of CECs from water, a simple and low-cost method based on sample lyophilization and subsequent re-dissolution of the extract in a series of solvents was developed. Firstly, 500 mL of the water sample was spiked with a mixture of isotopically labeled standard compounds (final extract concentration of 50 µg/L) and frozen before its lyophilization. The freeze-drying method allows sample pre-concentration for high-throughput analysis and reduces the loss of compounds during the extraction step [36,37]. After freeze-drying, the water sample was sequentially reconstituted in 15 mL of MeOH and 15 mL of EtAc. Then, the obtained organic extract was centrifuged at 4,000 rpm for 5 min. The supernatant was evaporated under a soft stream of nitrogen to an approximate volume of 1 mL and reconstituted with MeOH

up to a final volume of 5 mL. Immediately before UPLC-HRMS analysis, an aliquot of 2 mL of this extract was evaporated to 500 µL, diluted with 500 µL of UPLC-grade water, and filtrated through RC filters (0.2 µm pore size) (x200-fold concentrated sample). Standard solutions containing the RTI calibrants were prepared by diluting methanolic concentrated mixtures (final concentration of 1 µg/mL) (Tables S2 and S3) with UPLC-grade water in a proportion 1:1 (v:v). Matrix-matched calibration curves to be used for compound semi-quantification were constructed by dissolving appropriate amounts of a methanolic concentrated mixture containing the calibrants for the ionization efficiency model (Table S4) in a pool of all sample extracts.

2.4. UPLC-HRMS analysis

An Acquity UPLC system from Waters (Milford, MA, USA) coupled to a hybrid quadrupole-orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for UPLC-HRMS analysis. For the chromatographic separation, a reversed-phase column Purospher® STAR RP-18 end-capped Hibar® 150×2.1 mm, 2 µm (Merck, Darmstadt, Germany) was employed. The injection volume was set to 10 µL and the flow rate to 0.3 mL/min. A generic chromatographic gradient was used to achieve an optimal distribution of the wide polarity range of the CECs potentially present in the investigated samples and, hence, high-quality spectral information. This gradient started with 95 % of the aqueous mobile phase. After 1 min, a linear organic gradient was initiated and kept for 17 min until reaching 97 % of the organic mobile phase. This organic proportion was maintained for 2.5 min and, then, initial conditions were restored in 1 min. Finally, re-equilibration of the chromatographic column was done by maintaining the initial conditions for 3.5 min. In total, the duration of the analytical run was 25 min. HRMS analysis was performed both in positive and negative ionization modes and, depending on this condition, the composition of the mobile phase used for the chromatographic separation changed. For positive ionization, water and ACN, both with 0.1 % formic acid, were used. For negative ionization, water and ACN, both with 5 mM ammonium acetate were used.

The HRMS analysis was performed using a heated electrospray ionization (HESI) source operated in either the negative or positive mode. Ion source conditions were: spray voltage, +3000 V in positive mode,

-2500 V in negative mode; capillary temperature, 350 °C; sheath gas, 40 arbitrary units (AU); auxiliary gas, 10 AU; spare gas, 2 AU; probe heater temperature, 300 °C. Nitrogen gas (>99.98%) was used as the sheath, auxiliary and spare gases. HRMS data was obtained using two acquisition modes. Firstly, data-dependent acquisition (DDA) was performed with a full scan (FWHM resolution of 70,000 at 200 m/z) and a DDA MS2 scan (FWHM resolution of 17,500 at 200 m/z). The full scan ranged from 66.7 to 1000 m/z, which covers the great majority of CECs. Then, DDA MS2 scan events were recorded for the n = 5 most intense ions (> 105 counts) detected in each scan. A normalized collision-induced dissociation energy of 35 eV (m/z 500) was established for the MS2 analysis, with 0.1 s of ion accumulation. Secondly, data-independent acquisition (DIA) was also performed with similar conditions to DDA. In this case, the MS2 scans were acquired through an all-ion fragmentation mode with a collision-induced dissociation energy of 0 eV (full scan) and 35 eV (all-ion fragmentation mode). The resolution was set at 70,000 FWHM for full scan and 35,000 FWHM for all-ion fragmentation.

For the first campaign, triplicates were analyzed for each sampling point, in positive and negative modes. After their analysis, no appreciable differences in the detected compounds (false positives or negatives) were observed and, thus, replicates were avoided in the following campaign to reduce the time-consuming efforts derived from sample processing.

2.5. Post-acquisition data processing procedure

Data were processed through the NORMAN Network integrated software Digital Sample Freezing Platform (DSFP, https://dsfp.norman-data.eu/), which was designed for archiving LC-HRMS chromatograms for retrospective evaluation of polar and semi-polar CECs in different environmental matrices [38]. For each sample, DDA and DIA data were uploaded and split into the different collision energies applied to accurately compare the data with the spectral libraries. The DSFP performs the peak picking (mass error < 5 ppm), the componentization of the adducts, isotopes, and in-source fragment peaks for each feature, and the calculation of an experimental RTI for each annotated component. The RTI approach is intended for the harmonization of the chromatographic retention times in any instrumentation and chromatographic conditions. A mixture of selected compounds (Tables S3 and S4) with known RTIs was

prepared in MeOH:water (v/v, 1:1) and analyzed in the same sequence as samples under equal LC-HRMS conditions described in section 2.4. Then, the elucidation of the elution pattern of the detected compounds is performed via a Quantitative Structure-Retention Relationship (QSRR) model that generates a calibration curve for each of the electrospray ionization modes (Figures S1 and S2). Then, an RTI is obtained and, consequently, a retention time can be predicted for a given chemical in a given instrumentation and chromatographic conditions [31].

A suspect screening was performed using the mzCloud database [39] included in the Suspect List Exchange database from the NORMAN Network (https://www.norman-network.com/nds/SLE/). The mzCloud database includes a highly curated spectral library for thousands of CECs, mostly based on HRMS-orbitrap analysis and covering several MS conditions (broad range of collision energies) and, thus, gathering a high reproducibility. The DSFP returned the results of this suspect screening as a list of candidates, for which it included the chemical name, structural data, absolute maximum intensity of the observed signals, mass error, experimental retention time and RTI plausibility, and the number of coincident fragments. Then, a manual evaluation of the obtained results was performed to avoid possible false positives, and to complete the gaps in the identification evidence when needed (isotopic fit, adducts, RTI, spectral fragmentation pattern, etc.). For this purpose, experimental spectral information was revised via Xcalibur v. 4.1 software (Thermo Fisher Scientific, San Jose, CA, USA).

2.6. Semi-quantification approach

Following a novel workflow recently developed by Aalizadeh et al. [40], the concentrations of the CECs detected in the direct irrigation point (D) were semi-quantified to conduct an ecotoxicological risk assessment and CEC prioritization. Some of the compounds found in the WRP influent were removed during water treatment or on their way through the irrigation channels. Therefore, the first filter for CEC prioritization was the survival of the chemical until the irrigation location. The semi-quantification approach used is based on the ionization efficiency (logIE) of the CECs during the analysis. Firstly, matrix-matched calibration curves were constructed within the range 30 to 1000 µg/L for a series of analytes calibrants (Table S4) and the reference compound (dichlorvos), using imidacloprid-d4 as the internal standard at a final concentration of 100 µg/L. The selection

of calibrants was based on their experimental logIE (Table S4). Then a quantitative structure-activity relationship (QSAR) model was constructed using the slope ratio between each calibrant and dichlorvos to predict the logIE of a given compound in the sample (Figure S3). The predicted logIE allows obtaining a calibration curve slope for each compound of interest to estimate its concentration. Calculations were done following the free online application at http://trams.chem.uoa.gr/semiquantification/.

2.7. Prioritization methodology

The ecotoxicological risk of each detected compound was assessed using the corresponding PNEC value. For compounds regulated in Europe under the WFD, the PNEC value corresponded to their lowest Environmental Quality Standard (EQS) in surface waters. In the case of not regulated compounds, the PNEC value was obtained from experimental ecotoxicity data or predicted by QSAR models, when experimental data were not available. This search was performed using the NORMAN (https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php) and INERIS (https://substances.ineris.fr/fr/) databases.

Then, risk quotients (RQ) were calculated by dividing the highest semi-quantified concentration of the compound by its lowest PNEC value, thus, evaluating the worst-case scenario. The RQ value was also used to categorize the risk and prioritize thereby the most environmentally relevant CECs (Table S5). When the RQ value is below 1, no ecotoxicological risk is expected, and when the RQ value is above 1, then, an ecotoxicological risk for the aquatic ecosystem may exist. Moreover, taking into consideration the large number of chemicals present in the water samples, an evaluation of the ecotoxicological risk of the mixture of compounds present in each sample was also performed, following the concentration addition model described by Backhaus and Faust [41].

3. Results

3.1. CECs footprint characterization

Pharmaceuticals, pesticides, or industrial chemicals are some of the enormously diverse types of CECs that can be present in water bodies, together with the rest of the elements that constitute the water (salts, nutrients, natural organic matter, dissolved oxygen, and other gases, microbiota, etc.). CECs are not an extraordinary water component anymore but a permanent actor in all aquatic environmental compartments. However, their occurrence in water bodies strongly depends on nearby land uses and the culture and lifestyle of surrounding populations and, consequently, anthropogenic contamination sources can vary both spatially and temporally. As an example, consumption of antibiotics or antidepressants can increase during fall and winter [42,43], while the usage of the insect repellent DEET presents a higher prevalence of use in summer [44]. DEET levels in water also revealed a different usage of these substances among territories [44]. Therefore, although an EU-wide generic regulation is crucial to fight against chemical pollution (as it is done for priority substances through the EU WFD [11] and its daughter directives (2013/39/EU, 2006/118/EC, 2020/2184)), river basin and site-specific pollutants must be taken into consideration when regulating and controlling pollution sources and CEC occurrence at a local or regional level. In this context, non-target screening (NTS) approaches emerge as a very powerful tool for the identification of site-specific pollution in a holistical and cost-effective way.

For the characterization of the organic contamination present in the reclaimed water-based irrigation system under study, a wide-scope suspect screening approach was implemented at four different points of the reclaimed water cycle (Figure 1), including the WRP influent (A) and effluent (B), the reclaimed water at the point of its release to the irrigation channels (C), and the reclaimed water mixed with surface water in the irrigation channels network at an irrigation location (D). These locations were sampled in two different seasons (summer and winter) to characterize the contamination footprint in the reclaimed and irrigation water as holistically as possible and understand CEC dynamics and occurrence in the field.

After processing the raw data with the DSFP, a total of 2,773 and 2,257 compounds were tentatively identified in the summer and winter samples, respectively. The elimination of "naturally occurring" compounds and compounds with less than three fragments matching the spectral database reduced the list of candidates to 723 and 580 for summer and winter, respectively. Then, a manual evaluation of the remaining data, summed to a rational search of typical CECs found in the literature for these water matrices, resulted in the final tentative identification of 162 CECs in total (131 compounds in summer and 148 in winter) with a confidence level of 2, following Schymanski's scale [45]. This means that the exact mass, the isotopic fit, and the fragmentation pattern matched with the spectral libraries (mzCloud, Massbank), and a plausible retention time was obtained following the RTI procedure (Table 1).

Table 1. List of compounds identified with the suspect screening approach, including their exact mass, *m/z*, formula, ionization mode, retention time, chemical category, and presence in each sampling campaign.

Nº	Compound	Exact mass	*m/z*	Formula	Ion. mode	RT	Category	Campaign
1	1,2-Benzisothiazol-3(2H)-one	151.0092	152.0170	C7H5NOS	+	6.56	Industrial chemical	S
2	1,5-Naphthalenediamine	158.0844	159.0922	C10H10N2	+	4.7	Other	S
3	10,11-Dihydro-10,11-dihydroxycarbamazepine	270.1004	271.1077	C15H14N2O3	+	6.77	Pharmaceutical	S/W
4	10,11-Dihydro-10-Hydroxycarbazepine	254.1055	255.1128	C15H14N2O2	+	7.38	Pharmaceutical	S/W
5	2-(3,4-Dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile	290.1994	291.2072	C17H26N2O2	+	8.04	Pharmaceutical	S/W
6	2(3H)-Benzothiazolone	151.0092	152.0170	C7H5NOS	+	8.56	Other	S
7	2-(Methylthio)benzothiazole	181.0020	182.0098	C8H7NS2	+	13.45	Pesticide	S
8	2-Ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)	277.1830	278.1908	C20H23N	+	9.7	Pharmaceutical	W
9	2-Ethylhexyl diphenyl phosphate	362.1647	363.1725	C20H27O4P	+	17.99	Industrial chemical	S
10	2-Mercaptobenzothiazole	166.9863	167.9941	C7H5NS2	+	10.03	Industrial chemical/ Pesticide	S/W
11	3,3,5,5-Tetramethyl-1-pyrroline N-oxide	141.1154	142.1231	C8H15NO	+	7.82	Other	W
12	3-Hydroxycotinine	192.0898	193.0977	C10H12N2O2	+	1.85	Drug of abuse	S/W
13	4-Androstene-3,17-dione	286.1932	287.2011	C19H26O2	+	12.44	Other	S/W
14	4-Formylaminoantipyrine	231.1007	232.1086	C12H13N3O2	+	5.18	Pharmaceutical	S/W
15	4'-Hydroxy-diclofenac/5-Hydroxy-diclofenac	311.0115	312.0193	C14H11Cl2NO3	+	11.25	Pharmaceutical	S/W
16	4-Indolecarbaldehyde	145.0527	146.0605	C9H7NO	+	7.97	Other	W
17	4-Phenylbutyric acid	164.0837	165.0915	C10H12O2	+	10.72	Pharmaceutical	S/W
18	5-Hydroxyomeprazole	361.1096	362.1174	C17H19N3O4S	+	6.62	Pharmaceutical	W
19	6-Methylthioguanine	181.0422	182.0500	C6H7N5S	+	4.21	Other	S
20	8-Hydroxy-mirtazapine	281.1528	282.1606	C17H19N3O	+	5.83	Pharmaceutical	S
21	8-Hydroxyquinoline	145.0527	146.0605	C9H7NO	+	6.79	Industrial chemical	S/W
22	Acephylline	238.0702	237.0629	C9H10N4O4	-	3.27	Other	W
23	Acetaminophen	151.0633	152.0711	C8H9NO2	+	4.36	Pharmaceutical	S/W
24	Acetaminophen sulfate	231.0201	232.0279	C8H9NO5S	+	3.69	Pharmaceutical	S/W
25	Acetyl sulfamethoxazole	295.0626	296.0704	C12H13N3O4S	+	8.11	Antibiotic	S/W

Table 1 continued

Nº	Compound	Exact mass	*m/z*	Formula	Ion. mode	RT	Category	Campaign
26	Amisulpride	369.1722	370.1800	C17H27N3O4S	+	5.95	Pharmaceutical	S/W
27	Amitriptyline	277.1831	278.1909	C20H23N	+	10.16	Pharmaceutical	S/W
28	Amphetamine	135.1048	136.1126	C9H13N	+	4.02	Drug of abuse	S/W
29	Ampyrone/ 4-aminoantipyrine	203.1058	204.1136	C11H13N3O	+	4.26	Pharmaceutical	S/W
30	Atenolol	266.1630	267.1708	C14H22N2O3	+	4.01	Pharmaceutical	S/W
31	Azithromycin	748.5085	749.5163	C38H72N2O12	+	6.88	Antibiotic	S/W
32	Azoxystrobin	403.1168	404.1246	C22H17N3O5	+	13.01	Pesticide	S/W
33	Azoxystrobin acid	389.1011	390.1090	C21H15N3O5	+	11.19	Pesticide	W
34	Benzothiazole	135.0142	136.0208	C7H5NS	+	9.79	Industrial chemical	S
35	Benzoylecgonine	289.1314	290.1392	C16H19NO4	+	6.05	Drug of abuse	S/W
36	Bezafibrate	361.1080	362.1159	C19H20ClNO4	+	11.55	Pharmaceutical	S/W
37	Bisoprolol	325.2253	326.2331	C18H31NO4	+	7.61	Pharmaceutical	S/W
38	Boldenone	286.1932	287.2011	C19H26O2	+	10.75	Other	S/W
39	Boscalid	342.0326	343.0404	C18H12Cl2N2O	+	13.24	Pesticide	W
40	Bupropion	239.1076	240.1155	C13H18ClNO	+	7.71	Pharmaceutical	W
41	Butyrophenone	148.0888	149.0966	C10H12O	+	13.61	Pharmaceutical	W
42	Caffeine	194.0803	195.0882	C8H10N4O2	+	5.18	Other	S/W
43	Caprolactam	113.0840	114.0910	C6H11NO	+	4.79	Industrial chemical	S/W
44	Carbamazepine	236.0949	237.1027	C15H12N2O	+	9.56	Pharmaceutical	S/W
45	Carbamazepine-10,11-epoxide	252.0899	253.0977	C15H12N2O2	+	8.53	Pharmaceutical	S/W
46	Carbendazim	191.0694	192.0773	C9H9N3O2	+	4.95	Pesticide	W
47	Carboxy-ibuprofen	236.1048	235.0974	C13H16O4	-	2.8	Pharmaceutical	W
48	Cetirizine	388.1553	389.1632	C21H25ClN2O3	+	10.04	Pharmaceutical	S/W
49	Chlorpheniramine	274.1236	275.1315	C16H19ClN2	+	7.18	Pharmaceutical	W
50	Ciprofloxacin	331.1332	332.1410	C17H18FN3O3	+	5.91	Antibiotic	S/W
51	Citalopram	324.1637	325.1716	C20H21FN2O	+	9.03	Pharmaceutical	S/W
52	Clarithromycin	747.4768	748.4846	C38H69NO13	+	9.75	Antibiotic	S/W
53	Clindamycin	424.1798	425.1876	C18H33ClN2O5S	+	7.3	Antibiotic	W
54	Clopidogrel	321.0590	322.0668	C16H16ClNO2S	+	13.02	Pharmaceutical	W
55	Clopidogrel carboxylic acid	307.0433	308.0512	C15H14ClNO2S	+	6.88	Pharmaceutical	S/W
56	Cocaine	303.1470	304.1548	C17H21NO4	+	7.37	Drug of abuse	S/W
57	Cotinine	176.0949	177.1022	C10H12N2O	+	1.86	Drug of abuse	S/W
58	Coumarin	146.0367	147.0446	C9H6O2	+	9.03	Pharmaceutical	W
59	Cyprodinil	225.1266	226.1344	C14H15N3	+	12.15	Pesticide	W
60	DEET	191.1310	192.1388	C12H17NO	+	10.91	Pesticide	S/W
61	Desacetyl-diltiazem	372.1507	373.1585	C20H24N2O3S	+	8.1	Pharmaceutical	S/W
62	Desmethyl-citalopram	310.1481	311.1559	C19H19FN2O	+	8.9	Pharmaceutical	S/W
63	Desmethyl-diazepam	270.0559	271.0638	C15H11ClN2O	+	10.4	Pharmaceutical	S/W
64	Dextromethorphan	271.1936	272.2014	C18H25NO	+	8.55	Pharmaceutical	S/W
65	Dextrorphan/Levorphanol	257.1780	258.1858	C17H23NO	+	6.57	Drug of abuse	S/W
66	Diazepam	284.0716	285.0794	C16H13ClN2O	+	11.86	Pharmaceutical	S/W
67	Diazinon	304.1010	305.1088	C12H21N2O3PS	+	15.35	Pesticide	S/W
68	Dibutyl phthalate	278.1518	279.1596	C16H22O4	+	16.39	Industrial chemical	W
69	Diclofenac	295.0167	296.0245	C14H11Cl2NO2	+	13.35	Pharmaceutical	S/W

Table 1 continued

Nº	Compound	Exact mass	*m/z*	Formula	Ion. mode	RT	Category	Campaign
70	Dicyclohexylamine	181.1830	182.1910	C12H23N	+	6.97	Industrial chemical	S
71	Dicyclohexylurea	224.1889	225.1967	C13H24N2O	+	11.54	Industrial chemical	S
72	Diethyl phthalate	222.0892	223.0970	C12H14O4	+	12.36	Industrial chemical	W
73	Diltiazem	414.1613	415.1691	C22H26N2O4S	+	9.09	Pharmaceutical	S/W
74	Diphenhydramine	255.1623	256.1701	C17H21NO	+	8.93	Pharmaceutical	S/W
75	Diphenylamine	169.0892	170.0970	C12H11N	+	14.07	Pesticide	S
76	Diuron	232.0170	233.0248	C9H10Cl2N2O	+	11.19	Pesticide	S/W
77	Ecgonine methyl ester	199.1208	200.1281	C10H17NO3	+	1.81	Drug of abuse	S/W
78	Emtricitabine	247.0426	248.0505	C8H10FN3O3S	+	3.85	Pharmaceutical	S/W
79	Ephedrine	165.1153	166.1232	C10H15NO	+	4.65	Drug of abuse	W
80	Epoxiconazole	329.0731	330.0809	C17H13ClFN3O	+	13.11	Pesticide	W
81	Erucamide	337.3345	338.3423	C22H43NO	+	20.23	Industrial chemical	S
82	Fenofibric acid	318.0658	319.0737	C17H15ClO4	+	13.31	Pharmaceutical	S/W
83	Flecainide	414.1378	415.1456	C17H20F6N2O3	+	9.17	Pharmaceutical	S/W
84	Fluconazole	306.1040	307.1118	C13H12F2N6O	+	6.63	Pharmaceutical/ Pesticide	S/W
85	Flufenamic acid	281.0663	282.0741	C14H10F3NO2	+	14.39	Pharmaceutical	S/W
86	Gabapentin	171.1259	172.1337	C9H17NO2	+	4.77	Pharmaceutical	S/W
87	Galaxolidone	272.1776	273.1854	C18H24O2	+	16.48	Other	S/W
88	Hydroxy-bupropion	255.1026	256.1104	C13H18ClNO2	+	6.76	Pharmaceutical	S/W
89	Ibuprofen	206.1306	207.1385	C13H18O2	+	13.63	Pharmaceutical	W
90	Imidacloprid	255.0523	256.0601	C9H10ClN5O2	+	7.39	Pesticide	W
91	Isoproturon	206.1419	207.1497	C12H18N2O	+	10.95	Pesticide	S/W
92	Ketamine	237.0920	238.0998	C13H16ClNO	+	6.1	Drug of abuse	S/W
93	Ketoprofen	254.0942	255.1021	C16H14O3	+	11.42	Pharmaceutical	S/W
94	Lamotrigine	255.0078	256.0156	C9H7Cl2N5	+	6.73	Pharmaceutical	S/W
95	Lamotrigine 2-N-glucuronide	432.0477	432.0477	C15H16Cl2N5O6 +	+	5.04	Pharmaceutical	S/W
96	Lauryl diethanolamide	287.2460	288.2539	C16H33NO3	+	13.1	Industrial chemical	S/W
97	Levamisole	204.0721	205.0799	C11H12N2S	+	5.23	Pharmaceutical	S/W
98	Levofloxacin/ofloxacin	361.1437	362.1516	C18H20FN3O4	+	5.83	Antibiotic	S/W
99	Lidocaine	234.1732	235.1810	C14H22N2O	+	6.09	Pharmaceutical	S/W
100	MDMA	193.1102	194.1181	C11H15NO2	+	5.77	Drug of abuse	S/W
101	Mebendazole	295.0956	296.1035	C16H13N3O3	+	9.13	Pharmaceutical	S/W
102	Memantine	179.1674	180.1752	C12H21N	+	7.68	Pharmaceutical	S/W
103	Metformin	129.1014	130.1092	C4H11N5	+	1.22	Pharmaceutical	S/W
104	Methadone	309.2092	310.2170	C21H27NO	+	10.48	Pharmaceutical	S/W
105	Metoclopramide	299.1400	300.1478	C14H22ClN3O2	+	6.34	Pharmaceutical	S/W
106	Metoprolol	267.1834	268.1912	C15H25NO3	+	6.64	Pharmaceutical	S/W
107	Metoprolol acid	267.1470	268.1548	C14H21NO4	+	5.1	Pharmaceutical	S/W
108	Metribuzin-desamino	199.0779	200.0858	C8H13N3OS	+	8.53	Pesticide	S
109	Mirtazapine	265.1579	266.1657	C17H19N3	+	6.29	Pharmaceutical	W
110	Mycophenolic acid	320.1259	321.1338	C17H20O6	+	11.01	Antibiotic	S/W
111	N,N'-diphenylguanidine	211.1110	212.1188	C13H13N3	+	6.48	Industrial chemical	S/W
112	N-Acetyl-5-aminosalicylic acid	195.0531	196.0609	C9H9NO4	+	5.36	Pharmaceutical	S/W
113	N-Acetylaminoantipyrine	245.1164	246.1242	C13H15N3O2	+	5.15	Pharmaceutical	S/W

Table 1 continued

Nº	Compound	Exact mass	*m/z*	Formula	Ion. mode	RT	Category	Campaign
114	Naproxen	230.0942	229.0864	C14H14O3	-	7.12	Pharmaceutical	S/W
115	Nicotine	162.1157	163.1235	C10H14N2	+	1.84	Drug of abuse	S/W
116	N-Phenyl-1-naphthylamine	219.1048	220.1126	C16H13N	+	15.81	Industrial chemical	S/W
117	O-Desmethyl-tramadol	249.1728	250.1807	C15H23NO2	+	5.33	Pharmaceutical	S/W
118	O-Desmethyl-venlafaxine	263.1885	264.1963	C16H25NO2	+	6.11	Pharmaceutical	S/W
119	Omeprazole sulfone	361.1096	362.1174	C17H19N3O4S	+	7.45	Pharmaceutical	W
120	Oxazepam	286.0509	287.0587	C15H11ClN2O2	+	9.89	Pharmaceutical	S/W
121	Oxybenzone	228.0786	229.0864	C14H12O3	+	14.44	Other	S/W
122	Pentaethylene glycol (PEG)	238.1416	239.1494	C10H22O6	+	4.11	Industrial chemical	S/W
123	Perfluorobutanesulfonic acid	299.9502	298.9429	C4H1F9O3S1	-	9.01	Industrial chemical	S/W
124	Perfluorobutanoic acid	213.9864	212.9792	C4H1F7O2	-	5.81	Industrial chemical	S/W
125	Perfluoroheptanoic acid	363.9768	362.9696	C7H1F13O2	-	9.4	Industrial chemical	W
126	Perfluorohexanoic acid	313.9800	312.9728	C6H1F11O2	-	8.5	Industrial chemical	S/W
127	Perfluorooctanoic acid	413.9737	412.9664	C8H1F15O2	-	10.25	Industrial chemical	S/W
128	Perfluoropentanoic acid	263.9833	262.9760	C5H1F9O2	-	7.4	Industrial chemical	S/W
129	Pregabalin	159.1259	160.1337	C8H17NO2	+	4.76	Pharmaceutical	S/W
130	Propranolol	259.1572	260.1650	C16H21NO2	+	8.34	Pharmaceutical	S/W
131	Quetiapine	383.1667	384.1745	C21H25N3O2S	+	8.11	Pharmaceutical	W
132	Ritalinic acid	219.1259	220.1337	C13H17NO2	+	5.89	Drug of abuse	S/W
133	R-Methyl-benzotriazole (R=1, 4, 5)	133.0640	134.0718	C7H7N3	+	7.37	Industrial chemical	S/W
134	R-Methyl-benzotriazole (R=1, 4, 5)	133.0640	134.0718	C7H7N3	+	7.46	Industrial chemical	W
135	Rosuvastatin	481.1683	482.1761	C22H28FN3O6S	+	10.75	Pharmaceutical	S
136	Secbumeton	225.1589	226.1667	C10H19N5O	+	8.27	Pesticide	S/W
137	Sertraline	305.0738	306.0816	C17H17Cl2N	+	10.52	Pharmaceutical	S/W
138	Sitagliptin	407.1180	408.1259	C16H15F6N5O	+	7.24	Pharmaceutical	S/W
139	Sotalol	272.1194	273.1272	C12H20N2O3S	+	4.16	Pharmaceutical	S/W
140	Sulfamethoxazole	253.0521	254.0599	C10H11N3O3S	+	7.87	Antibiotic	S/W
141	Sulpiride	341.1409	342.1487	C15H23N3O4S	+	4.55	Pharmaceutical	S/W
142	Tapentadol	221.1779	222.1857	C14H23NO	+	6.88	Pharmaceutical	S/W
143	Tebuconazole	307.1451	308.1529	C16H22ClN3O	+	13.33	Pesticide	W
144	Temazepam	300.0665	301.0743	C16H13ClN2O2	+	10.97	Pharmaceutical	S/W
145	Terbutryn	241.1361	242.1439	C10H19N5S	+	10.46	Pesticide	S/W
146	Testosterone propionate	344.2351	345.2429	C22H32O3	+	13.82	Drug of abuse	S/W
147	Tetradecylamine	213.2456	214.2534	C14H31N	+	13.32	Industrial chemical	S/W
148	Tetrakis(2-hydroxypropyl)ethylenediamine	292.2362	293.2440	C14H32N2O4	+	1.85	Industrial chemical	S/W
149	Theobromine	180.0647	181.0725	C7H8N4O2	+	3.92	Other	S/W
150	Theophylline	180.0647	181.0725	C7H8N4O2	+	4.38	Other	S/W
151	Tramadol	263.1885	264.1963	C16H25NO2	+	6.71	Pharmaceutical	S/W
152	Tri(chloropropyl) phosphate	326.0008	327.0086	C9H18Cl3O4P	+	12.5	Industrial chemical	S/W
153	Tributyl citrate	360.2148	361.2226	C18H32O7	+	16.14	Industrial chemical	S
154	Tributyl phosphate	266.1647	267.1725	C12H27O4P	+	14.93	Industrial chemical	S/W
155	Triethanolamine	149.1052	150.1130	C6H15NO3	+	1.24	Industrial chemical	S/W
156	Triethylene glycol monobutyl ether	206.1518	207.1596	C10H22O4	+	7.54	Industrial chemical	S/W
157	Triisobutyl phosphate	266.1647	267.1725	C12H27O4P	+	14.97	Industrial chemical	W

Table 1 continued

Nº	Compound	Exact mass	*m/z*	Formula	Ion. mode	RT	Category	Campaign
158	Trimethoprim	290.1378	291.1457	C14H18N4O3	+	5.64	Antibiotic	S/W
159	Triphenyl phosphate	326.0708	327.0786	C18H15O4P	+	15.06	Industrial chemical	W
160	Tris(2-butoxyethyl) phosphate	398.2433	399.2511	C18H39O7P	+	15.6	Industrial chemical	S/W
161	Venlafaxine	277.2042	278.2120	C17H27NO2	+	7.66	Pharmaceutical	S/W
162	Vildagliptin	303.1946	304.2025	C17H25N3O2	+	4.47	Pharmaceutical	S/W

S: summer; W: winter

The chromatographic areas obtained for each compound in both campaigns are provided as supporting information in Tables S6 (winter) and S7 (summer). MS/MS fragments matching with database information for each compound are also provided as supporting information in Tables S8 (winter) and S9 (summer).

Pharmaceuticals, pesticides, and industrial chemicals were the most frequently detected compounds. Pharmaceuticals represented more than 50 % of the total amount of CECs present in the samples. This is in line with the wastewater origin, i.e., wastewater coming from the towns of Gavà, Viladecans, and Begues, in Catalonia, comprising a population of more than a hundred thousand people [35]. Antidepressants, anxiolytics, antibiotics, and non-steroidal anti-inflammatories (NSAIDs) were the most common pharmaceuticals detected, but antihypertensive and antihistaminic drugs, or medicines prescribed for diabetes or epilepsy were also identified. For instance, carbamazepine, one of the most recalcitrant CECs present in the environment, was detected in all the samples along with their three main metabolites (10,11-dihydro-10,11-dihydroxycarbamazepine, 10,11-dihydro-10-hydroxycarbazepine, and carbamazepine-10,11-epoxide), showing negligible degradation in the WRP or the irrigation channels, in line with previous studies that showed poor performance of MBR systems in the elimination of this drug [46]. Additionally, in the WRP influent in both campaigns, eight different antibiotics, namely azithromycin, ciprofloxacin, clarithromycin, clindamycin, ofloxacin/levofloxacin, sulfamethoxazole, trimethoprim, and the metabolite acetyl-sulfamethoxazole, were detected. Then, after water reclamation and circulation through the irrigation channels, five of them (ciprofloxacin, clindamycin, ofloxacin/levofloxacin, sulfamethoxazole, trimethoprim) were found to be present at the irrigation point (Tables S6 and S7). This widespread presence of antibiotics may be contributing to the well-known increase of antimicrobial resistance of

the environmental microbiota and their subsequent associated problems [42].

As for pesticides, 16 compounds, including two metabolites, were identified in the investigated samples. Pesticides such as azoxystrobin and its metabolite azoxystrobin acid, boscalid, carbendazim, and metribuzin-desamino presented their highest peak area at the irrigation point, showing that, probably, the source of these contaminants is not the WRP but pesticide application in the surroundings. As expected, some of these pesticides were only detected in one campaign or presented remarkable differences between campaigns, probably reflecting their pattern of use. DEET, diuron, and the metabolite metribuzin-desamino were mainly found in the summer period, while boscalid, carbendazim, cyprodinil, epoxiconazole, and tebuconazole were mainly present in winter. Apart from these, 8-hydroxyquinoline, diazinon, imidacloprid, isoproturon, secbumeton, and terbutryn were also detected in, at least, one sample (Tables S6 and S7). The agrochemicals identified are predominantly used as fungicides, herbicides, and, to a lesser extent, insecticides.

The WRP also treats pre-processed wastewater from diverse industrial activities located in the area and, hence, another main chemical group found in the water was industrial chemicals. Most of these CECs have multiple sources and, thus, it becomes a difficult task to identify their origin. The detected compounds included perfluoroalkyl substances (PFAS), organophosphates, phthalates, and benzotriazole derivatives, among others. Regarding their anthropogenic usage, these contaminants are mainly used as plasticizers, flame retardants, surfactants, coating and packaging, food additives, and adhesives. Special attention should be given to the chemicals related to tire wear and road runoff that were detected in the samples, which include compounds such as tolyltriazole (4- and 5-methyl-benzotriazole), 1,2-benzisothiazol-3(2H)-one, 2(3H)-benzothiazolone, 2-(methylthio)benzothiazole, 2-mercaptobenzothiazole, benzothiazole, N,N’-diphenylguanidine, dicyclohexylamine, or dicyclohexylurea. Six different PFAS were widespread and persistently present in both campaigns. These compounds are widely used in many manufacturing processes and are commonly reported in the aquatic environment, mostly coming from the discharge from wastewater treatment plants [47].

The urban origin of the wastewater also resulted in the presence of drugs of abuse in the reclaimed water irrigation system. Twelve different compounds were identified within this category, including five metabolites. Nicotine and its two major metabolites (cotinine and 3-hydroxycotinine) were detected in both campaigns, although only cotinine persisted after water reclamation. Similarly, cocaine and its metabolites benzoylecgonine and ecgonine methyl ester were also present in the investigated waters, but ecgonine methyl ester was the only one surviving the regeneration treatment. MDMA, amphetamine, ketamine, and ephedrine were also found in either one or both campaigns.

Finally, a myriad of CECs without a grouping category was detected, including compounds such as caffeine, theophylline, and theobromine, coming from the coffee, tea, and cacao metabolism, respectively, the UV filter oxybenzone (included in the third watch list from the European Commission [48]), or galaxolidone, a metabolite of the personal care product galaxolide, among others.

3.2. CECs dynamics

MBRs combine biological treatments and membrane-based separation techniques. In terms of CEC removal, their efficiency is highly variable, providing removals from 0 to 93.5 % for the same compound, depending on the case study [49]. This efficiency depends on many factors, including the compound physical-chemical properties, biological conditions, specific microbial communities, MBR configuration, etc. The main removal processes that CECs experience in MBR systems are biodegradation, adsorption, and filtration but, when removal is not complete, CECs reach the MBR effluents [50,51]. Figure 2 summarizes the dynamics of the organic CECs detected in each sample and campaign (based on the data from Tables S6-S7) in terms of the number of CECs detected and the total area. As can be seen in Figure 2, considering the sum of the chromatographic peak area of each analyte, the CEC load is partially removed during water reclamation (73 % and 80 % of removal in summer and winter, respectively). There is also a clear decrease in the CEC load during the water residence time in the irrigation channels, from the discharge point to the irrigation point, in summer (30 %). The irrigation system under study consists of several surface channels where CECs can still be bio- and/or photodegraded, or even the transformation products can be transformed back into their parent

compounds. Both degradation processes are enhanced during summer compared to winter because the water temperature in the irrigation channels is higher (30 °C approximately) and daylight time is longer. The use of this parameter (sum of areas) to evaluate CEC dynamics is useful but has to be read with caution since the analytical technique used (UPLC-ESI-HRMS) is subject to matrix effects that may vary among the investigated samples, and hence, affect the observed area of each CEC in each matrix.

Although based on the observed chromatographic peak areas, considerable mitigation of CEC loads in the reclaimed water irrigation system under study could be inferred, the total number of CECs present in the water used for irrigation was remarkably high. Out of the 123 and 130 compounds detected in the influent sample in summer and winter, respectively, 73 (summer) and 109 (winter) were still present in the water used for irrigation (Figure 2B). Figure 3 shows the distribution of contaminants in the four sampling points in each campaign, highlighting the number of compounds that were common in two or more samples, or were only detected in one sample. As can be seen, 25 (summer) and 24 (winter) compounds were completely degraded throughout the WRP treatment, including CECs such as nicotine or acetaminophen (specific compounds can be found in Tables S6 and S7). Most of these compounds showed different behaviors between campaigns, demonstrating that many factors may affect the WRP removal efficiency. The incoming concentration level of the contaminants may also be decisive in the elimination rates. On the othe hand, the water composition in terms of CECs from the WRP effluent to the discharge point into the channels was very similar. This could be expected as the water is pumped through an underground tubing and it is not subjected to external agents. Finally, up to 61 compounds in summer and 93 compounds in winter were found in all samples, revealing the recalcitrance of most of the CECs identified. Moreover, this is in line with the fact that warmer conditions may enhance the bio- and photodegradation processes along the surface irrigation channels. Lastly, we can depict from Figure 3 that 98 % of the CECs detected came directly from the WRP, becoming the main source of contamination of the irrigation system. The only exceptions to this were azoxystrobin acid, oxybenzone, and triisobutyl phosphate in winter, and 2-ethylhexyl diphenyl phosphate in summer.

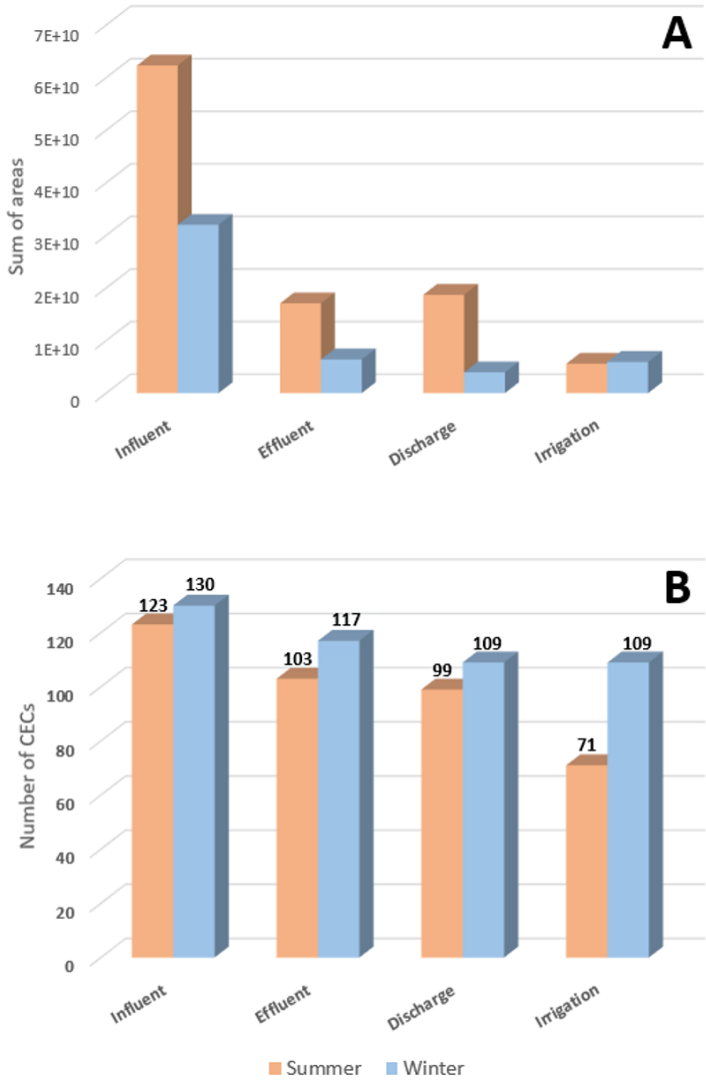

Figure 2. CEC dynamics in the study area, including removal efficiency in the WRP and irrigation channels. A) Sum of the chromatographic peak areas of the CECs detected in each sample and sampling campaign, and B) number of CECs detected in each sample and sampling campaign.

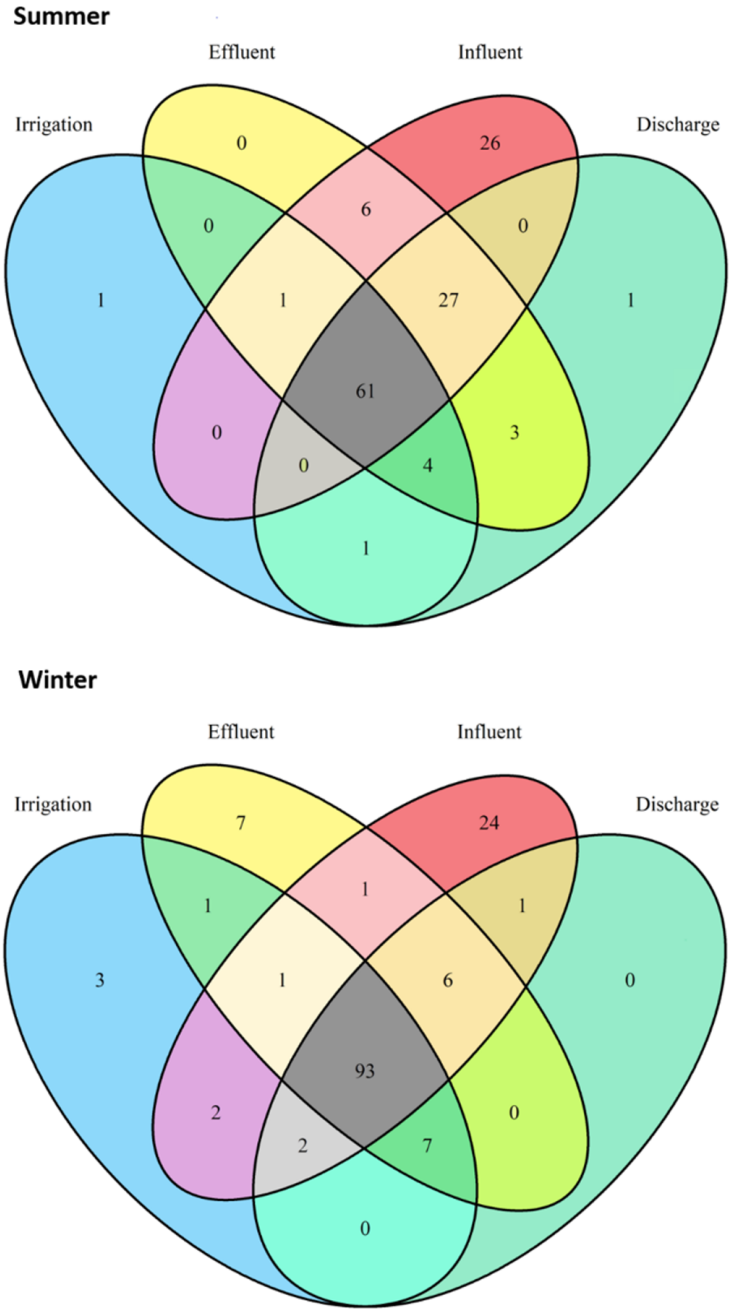

Figure 3. Van Venn diagrams showing the CEC diversity in the investigated samples in summer and winter.

3.3. Semi-quantification, ecotoxicological risk assessment, and prioritization

Once the CEC contamination footprint and its dynamics through the reclaimed water-based irrigation system were characterized, a prioritization of the most relevant compounds in terms of ecotoxicity and occurrence was performed. For this purpose, only those compounds that persisted until the irrigation point, and hence, recalcitrant to degradation during water reclamation and circulation along the irrigation channels, were considered. An RQ value was calculated for each CEC detected in the water used for irrigation, based on their lowest PNEC values and semi-quantified concentrations (see section 2.6) [40]. The highest value obtained for each CEC was selected as the measured environmental concentration (MEC) to assess the ecotoxicological risk in the worst-case scenario. The semi-quantified concentrations of the CECs found in the water used for irrigation in winter and summer, the MEC and PNEC values used for RQ calculation, the RQ value, and the risk category for each CEC are shown in Table 2.

CEC concentrations ranged from 0.3 ng/L for 2-(3,4-Dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile in summer to 97 µg/L for caprolactam (a compound used for fabrication of nylon) in winter. Most of the compounds presented concentrations below 1 µg/L. Although it is remarkable the higher concentrations found, in general, in winter as compared to summer, the concentrations measured were in agreement with the typical concentrations observed for these CECs in effluent or surface waters (ranging in the ng/L level). Some of the highest concentrations found in the samples corresponded to compounds included in any of the four watch lists established in the field of water policy under Directives 2008/105/EC and 2013/39/EU from the European Commission [10,48,52–54], pointing to their environmental relevance. These were the antidepressant venlafaxine and its metabolite O-desmethyl-venlafaxine (423 and 1,070 ng/L, respectively), the sunscreen agent oxybenzone (4,180 ng/L), metformin (5,230 ng/L) and, in a lesser extent, sulfamethoxazole (133 ng/L), ofloxacin/levofloxacin (8,4 ng/L), diclofenac (12 ng/L), azoxystrobin and its metabolite azoxystrobin acid (41 and 35 ng/L, respectively), clindamycin (14 ng/L), fluconazole (21 ng/L), ciprofloxacin (3.6 ng/L), trimethoprim (6.4 ng/L) or tebuconazole (1,9 ng/L). Moreover, the pesticides diuron and isoproturon, two priority

Table 2. Compounds detected at the irrigation point in summer and winter, semi-quantified levels and potential ecotoxicological risk

Nº	Compound	Concentration (ng/L)			Risk Quotient (RQ)		
		Winter	Summer	Max Conc.	PNEC (ng/L)	RQ	Risk category
1	O-Desmethyl Venlafaxine	1,070	44.0	1,070	6.1	175	HR
2	Venlafaxine	423	14.0	423	6.1	69.3	MR
3	Galaxolidone	1,730	72.0	1,730	100	17.3	MR
4	Theophylline/ Paraxanthine	672	n.d.	672	100	6.72	LR
5	Oxybenzone	4,180	155	4,180	670	6.24	LR
6	N-Phenyl-1-naphthylamine	242	106	242	60	4.03	LR
7	2-Ethylhexyl diphenyl phosphate	n.d.	60.0	60.0	18	3.31	LR
8	Terbutryn	176	91.0	176	65	2.70	LR
9	Carbamazepine	82.0	37.0	82.0	50	1.63	LR
10	N,N'-diphenylguanidine	1,280	1.80	1,280	860	1.49	LR
11	Caprolactam	97,400	43.0	97,400	67,400	1.45	LR
12	Caffeine	1,690	n.d.	1,690	1,200	1.41	LR
13	Sulfamethoxazole	133	17.0	133	100	1.33	LR
14	Temazepam	85.0	31.0	85.0	71	1.20	LR
15	Tris(2-butoxyethyl) phosphate	102	37.0	102	140	0.728	NR
16	MDMA	118	n.d.	118	216	0.546	NR
17	Secbumeton	26.0	2.90	26.0	48	0.545	NR
18	Flecainide	260	56.0	260	640	0.407	NR
19	Ofloxacin/levofloxacin	8.40	2.10	8.40	21	0.401	NR
20	Sitagliptin	363	20.0	363	920	0.394	NR
21	Butyrophenone	683	n.d.	683	1,750	0.390	NR
22	Diuron	n.d.	27.0	27.0	70	0.383	NR
23	Carbendazim	156	n.d.	156	150	0.356	NR
24	2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)	23.0	n.d.	23.0	85	0.276	NR
25	Sulpiride	1,120	65.0	1,120	4,090	0.274	NR
26	10,11-Dihydro-10,11-dihydroxycarbamazepine	511	203	511	1,910	0.268	NR
27	Clopidogrel carboxylic acid	154	17.0	154	650	0.238	NR
28	Diclofenac	12.0	n.d.	12.0	50	0.230	NR
29	Lidocaine	1,070	120	1,070	4,670	0.229	NR
30	Oxazepam	82.0	14.0	82.0	370	0.221	NR
31	Amisulpride	312	n.d.	312	1,430	0.218	NR
32	Azoxystrobin	41.0	6.70	41.0	200	0.205	NR
33	Perfluorooctanoic acid	21.0	34.0	34.0	180	0.190	NR
34	Azoxystrobin acid	35.0	n.d.	35.0	210	0.166	NR
35	Diazinon	1.60	0.60	1.60	10	0.163	NR
36	Clindamycin	14.0	n.d.	14.0	100	0.139	NR
37	Propranolol	52.0	n.d.	52.0	410	0.128	NR
38	Tapentadol	313	14.0	313	2,470	0.127	NR
39	Tramadol	967	36.0	967	8,650	0.112	NR
40	Diltiazem	25.0	n.d.	25.0	230	0.108	NR

Table 2 continued

Nº	Compound	Concentration (ng/L)			Risk Quotient (RQ)		
		Winter	Summer	Max Conc.	PNEC (ng/L)	Winter	Summer
41	Amitriptyline	14.0	n.d.	14.0	140	0.098	NR
42	Diphenylamine	n.d.	116	116	1,200	0.097	NR
43	Isoproturon	28.0	7.00	28.0	320	0.088	NR
44	Fluconazole	21.0	15.0	21.0	250	0.083	NR
45	Cetirizine	32.0	7.20	32.0	410	0.077	NR
46	Desacetyl diltiazem	27.0	n.d.	27.0	360	0.076	NR
47	O-desmethyltramadol	640	37.0	640	9,120	0.070	NR
48	Dextrorphan/levorphanol	76.0	2.00	76.0	1,360	0.056	NR
49	Gabapentin	564	94.0	564	10,000	0.056	NR
50	Desmethylcitalopram	26.0	n.d.	26.0	500	0.053	NR
51	Perfluorobutanesulfonic acid	102	215	215	4,080	0.053	NR
52	Desmethyldiazepam	22.0	9.00	22.0	430	0.051	NR
53	10,11-Dihydro-10-Hydroxycarbazepine	199	54.0	199	4,030	0.050	NR
54	2-(Methylthio)benzothiazole	n.d.	53.0	53.0	1,140	0.046	NR
55	Ciprofloxacin	n.d.	3.60	3.60	89	0.041	NR
56	Methadone	33.0	0.59	33.0	840	0.039	NR
57	Bisoprolol	113	n.d.	113	3,180	0.036	NR
58	Metformin	5,230	66.0	5,230	156,000	0.034	NR
59	2-Mercaptobenzothiazole	24.0	n.d.	24.0	760	0.032	NR
60	8-Hydroxyquinoline	16.0	64.0	64.0	1,990	0.032	NR
61	Levamisole	55.0	2.40	55.0	1,810	0.031	NR
62	Flufenamic acid	11.0	0.40	11.0	400	0.028	NR
63	Memantine	52.0	9.40	52.0	1,840	0.028	NR
64	Perfluoroheptanoic acid	13.0	n.d.	13.0	500	0.026	NR
65	3,3,5,5-Tetramethyl-1-pyrroline N-oxide	342	n.d.	342	13,800	0.025	NR
66	Lamotrigine	248	107	248	10,000	0.025	NR
67	Tri(chloropropyl) phosphate	n.d.	171	171	7,390	0.023	NR
68	Dicyclohexylamine	n.d.	74.0	74.0	1,600	0.022	NR
69	Diazepam	6.0	3.50	6.00	290	0.021	NR
70	Amphetamine	498	n.d.	498	24,800	0.020	NR
71	Diethyl phthalate	319	n.d.	319	16,000	0.02	NR
72	N-Acetylaminoantipyrine	1,810	525	1,810	100,000	0.018	NR
73	Cocaine	39.0	n.d.	39.0	2,280	0.017	NR
74	Sotalol	100	n.d.	100	6,520	0.015	NR
75	Ketamine	77.0	12.0	77.0	5,710	0.014	NR
76	Triethanolamine	155	n.d.	155	11,000	0.014	NR
77	Trimethoprim	6.40	n.d.	6.40	500	0.013	NR
78	Ephedrine	42.0	n.d.	42.0	3,620	0.012	NR
79	Mebendazole	2.00	n.d.	2.00	160	0.012	NR
80	Metoprolol acid	622	127	622	49,900	0.012	NR
81	Perfluoropentanoic acid	23.0	47.0	47.0	3,910	0.012	NR

Table 2 continued

Nº	Compound	Concentration (ng/L)			Risk Quotient (RQ)		
		Winter	Summer	Max Conc.	PNEC (ng/L)	Winter	Summer
82	Tributyl phosphate	769	182	769	66,000	0.012	NR
83	2-(3,4-dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile	14.0	0.30	14.0	1,570	0.009	NR
84	Acephylline	106	n.d.	106	13,200	0.008	NR
85	Diphenhydramine	8.10	n.d.	8.10	990	0.008	NR
86	Sertraline	0.70	n.d.	0.70	91	0.008	NR
87	Tebuconazole	1.90	n.d.	1.90	240	0.008	NR
88	Dicyclohexylurea	n.d.	42.0	42.0	5,910	0.007	NR
89	Vildagliptin	73.0	13.0	73.0	9,750	0.007	NR
90	Ketoprofen	12.0	n.d.	12.0	2,100	0.006	NR
91	Theobromine	636	n.d.	636	100,000	0.006	NR
92	Bupropion	22.0	n.d.	22.0	4,400	0.005	NR
93	Cotinine	46.0	n.d.	46.0	10,000	0.005	NR
94	Pentaethylene glycol (PEG)	1,000	n.d.	1,000	202,000	0.005	NR
95	Ritalinic acid	72.0	n.d.	72.0	14,200	0.005	NR
96	2(3H)-Benzothiazolone	n.d.	54.0	54.0	14,000	0.004	NR
97	Boscalid	43.0	n.d.	43.0	11,600	0.004	NR
98	Clopidogrel	12.0	n.d.	12.0	3,210	0.004	NR
99	4-Formylaminoantipyrine	2,870	1,600	2,870	1,000,000	0.003	NR
100	4-Indolecarbaldehyde	20.0	n.d.	20.0	6,080	0.003	NR
101	DEET	n.d.	277	277	88,000	0.003	NR
102	Dextromethorphan	9.10	n.d.	9.10	3,320	0.003	NR
103	Metoprolol	25.0	n.d.	25.0	8,600	0.003	NR
104	3-Hydroxycotinine	36.0	n.d.	36.0	20,600	0.002	NR
105	Carbamazepine-10,11-epoxide	4.80	n.d.	4.80	2,570	0.002	NR
106	Citalopram	36.0	0.60	36.0	16,000	0.002	NR
107	Emtricitabine	46.0	15.0	46.0	23,800	0.002	NR
108	Ampyrone/ 4-aminoantipyrine	24.0	n.d.	24.0	32,500	0.001	NR
109	Atenolol	98.0	n.d.	98.0	150,000	0.001	NR
110	Bezafibrate	2.00	n.d.	2.00	2,300	0.001	NR
111	Chlorpheniramine	1.60	n.d.	1.60	1,560	0.001	NR
112	Ecgonine methyl ester	46.0	n.d.	46.0	88,800	0.001	NR
113	Fenofibric acid	2.00	n.d.	2.00	2,350	0.001	NR
114	Metribuzin-desamino	n.d.	28.0	28.0	46,800	0.001	NR
115	Mirtazapine	1.40	n.d.	1.40	1,000	0.001	NR
116	Pregabalin	43.0	21.0	43.0	66,100	0.001	NR
117	Perfluorobutanoic acid	9.50	10	10.0	27,800	0.0004	NR
118	Hydroxy-bupropion	n.d.	1.90	1.90	11,800	0.0002	NR
119	Perfluorohexanoic acid	n.d.	30.0	30.0	140,000	0.0002	NR
120	Benzoylecgonine	269	n.d.	269	6,840,000	0.00004	NR
121	1,2-benzisothiazol-3(2H)-one	n.d.	1.10	1.10	379,000	0.000003	NR

n.d.: not detected; HR: high risk; MR: medium risk; LR: Low risk; NR: no risk.

substances established in the European Commission WFD [11], were detected at maximum concentrations of 27 and 28 ng/L, respectively.

CEC concentrations ranged from 0.3 ng/L for 2-(3,4-Dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile in summer to 97 µg/L for caprolactam (a compound used for fabrication of nylon) in winter. Most of the compounds presented concentrations below 1 µg/L. Although it is remarkable the higher concentrations found, in general, in winter as compared to summer, the concentrations measured were in agreement with the typical concentrations observed for these CECs in effluent or surface waters (ranging in the ng/L level). Some of the highest concentrations found in the samples corresponded to compounds included in any of the four watch lists established in the field of water policy under Directives 2008/105/EC and 2013/39/EU from the European Commission [10,48,52–54], pointing to their environmental relevance. These were the antidepressant venlafaxine and its metabolite O-desmethyl-venlafaxine (423 and 1,070 ng/L, respectively), the sunscreen agent oxybenzone (4,180 ng/L), metformin (5,230 ng/L) and, in a lesser extent, sulfamethoxazole (133 ng/L), ofloxacin/levofloxacin (8,4 ng/L), diclofenac (12 ng/L), azoxystrobin and its metabolite azoxystrobin acid (41 and 35 ng/L, respectively), clindamycin (14 ng/L), fluconazole (21 ng/L), ciprofloxacin (3.6 ng/L), trimethoprim (6.4 ng/L) or tebuconazole (1,9 ng/L). Moreover, the pesticides diuron and isoproturon, two priority substances established in the European Commission WFD [11], were detected at maximum concentrations of 27 and 28 ng/L, respectively.

Regarding their ecotoxicological risk, 14 out of 121 compounds showed an individual RQ over 1 and, therefore, presented a concentration potentially toxic for the aquatic environment. From these, O-desmethyl-venlafaxine would pose the highest risk with an RQ value of 175. Then, venlafaxine and galaxolidone (a metabolite of the personal care product galaxolide) presented a medium risk ($10 < RQ < 100$), while the rest showed low risk ($1 < RQ < 10$). This last category included industrial chemicals (2-ethylhexyl diphenyl phosphate, N-phenyl-1-naphthylamine, and caprolactam), pharmaceuticals (carbamazepine, sulfamethoxazole, and temazepam), tire wear compounds (N,N'-diphenylguanidine), and caffeine and its metabolite theophylline. The remaining CECs detected in water used for irrigation did not pose a risk per se to exposed organisms. However,

due to the large number of chemicals that may reach crops through irrigation with reclaimed water, an evaluation of the "cocktail effect" of the mixture in the water was performed. Previous studies have demonstrated that low doses of a mixture of contaminants at concentrations under the No Observed Effect Concentration (NOEC) can have toxic effects on aquatic organisms [55], pointing out the possible additive or synergistic properties of the mixtures. Following a concentration addition model [41], in which all CECs are supposed to have a similar mode and site of action on aquatic organisms, an approximation to the worst-case scenario is given and it should only be understood as that. The RQmix was 298 and 20 for the winter and summer campaigns, respectively, representing an environmental risk in both cases, being 10 times higher in winter than in summer.

Due to the difficulty that may represent the semi-quantification approach, we attempted to evaluate a simpler prioritization procedure to avoid this step. This procedure was based on a scoring system that takes into consideration the occurrence (based on the chromatographic peak areas and the ubiquity in both campaigns) and the ecotoxicity of contaminants (based on the PNEC values). Four points were distributed along five 20 % percentile groups that were generated according to the maximum absolute value of the chromatographic peak area. One point was distributed depending on the presence of each compound in one or two campaigns and, finally, five points were distributed along five 20 % percentile groups according to the PNEC value of each compound. With this scoring system, the maximum final score would be ten points (Table S10). As for the semi-quantification-based prioritization, only the contaminants present at the irrigation point were evaluated using their maximum chromatographic peak area value for the scoring. Table S11 shows the score of each compound broken down among the three evaluated parameters and the total score. Then, the priority list obtained through this method was compared to that obtained using the semi-quantification-based approach. For example, there was a 76 % of similarity between the lists when comparing either the 25 compounds or the 50 compounds at the top of both lists (Table S12). This approach, despite the stronger deficiencies in terms of accuracy and precision compared to the semi-quantification-based prioritization, could also be used as an approximation for the prioritization of CECs, mostly at a local level.

Nevertheless, with its limitations, the semi-quantification-based procedure allowed us to cost-effectively select the most relevant compounds in terms of ecotoxicity in the area of study, also considering the potential spatial and temporal variabilities on CEC occurrence. In contrast to target and quantitative methodologies, for which a preselection of analytes and costly and time-consuming analyses need to be done to study a reasonable variety of contaminants, this prioritization based on a suspect screening analysis allows taking into consideration a much higher number of contaminants and avoid the loss of any possible candidate for further environmental monitoring programs. Regulators and public administrations may use this approach and the resulting selection of contaminants of special concern to establish monitoring programs and evaluate possible attenuation measures when possible.

4. Conclusion

A cost-effective site-specific prioritization of the most relevant CECs present in an agricultural area irrigated with reclaimed water has been performed. The selection of the compounds to be further controlled was based on their occurrence and their potential ecotoxicological risk. In contrast to the typical targeted analysis, a wide-scope methodology was applied to holistically characterize the CEC footprint in the water, and its seasonal and spatial variability in the reclaimed water-based irrigation system. Although this approach presents some limitations in terms of the analysis because highly polar and apolar compounds were not covered, and compounds can be missing in the suspect lists used (e.g. transformation products), this procedure expands with no doubt the chemical space covered by target methods. In total, 162 CECs were identified with a confidence level of 2 and their dynamics throughout the reclaimed water-based irrigation system were evaluated. It has been observed that the incomplete removal during water reclamation and their residence time in the irrigation channels leads to the accumulation of more than a hundred compounds (in winter) in the water used for crop irrigation. Considering the worst-case scenario in terms of ecotoxicity, this reclaimed water-based irrigation system would pose a high risk for the aquatic environment that may also arrive to crops. However, further research is needed to confirm this point. The next step, in fact, in the frame of the H2020 project (MAGO) in which this work has been carried out, is to develop target methodologies

for analysis of the prioritized compounds in irrigation water and lettuce, and study potential temporal and spatial variations in their concentration levels and the risk associated to them.

Finally, it may be worth emphasizing that the methodology developed can be widely implemented in any other locations, opening the door to detecting local contaminants that could be missed with the national or European regulations, rationally designing monitoring and attenuation programs, and supporting legislators in their way to manage the water contamination issue and water reuse.

Acknowledgments

This work was supported by the project MAGO (Project number 2022) funded by the PRIMA-MED Programme within EU Horizon2020, by the grant CEX2018–000794-S funded by MCIN/AEI (10.13039/501100011033) and by the Government of Catalonia (2021 SGR 00753). CP acknowledges grant RYC2020-028901-I funded by MCIN/AEI/10.13039/501100011033 and "ESF investing in your future". The staff from the WRP of Gavà-Viladecans is acknowledged for the provision of the wastewater samples. Pablo Gago, Reza Aalizadeh and the Trace Analysis and Mass Spectrometry group for their support using the RTI platform and for the provision of the analytical standards needed for the RTI calculation.

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Prioritization of the most relevant organic contaminants in a reclaimed water irrigation system through an LC-MS/HRMS-based suspect screening workflow

Supporting Information

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Number of pages: 33

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Table S1. GPS coordinates for the sampling points

Sampling Point		Latitude	Longitude
A	Influent WRP	41.276142	2.040351
B	Effluent WRP	41.276142	2.040351
C	First surface discharge	41.278750	1.997611
D	Irrigation Point	41.278384	2.009450

Table S2. Calibrants used in the RTI model in the positive ionization mode (ESI+), and corresponding SMILES and experimental retention time (RT).

Calibrants	SMILES	Experimental RT (min)
Amitrole	C1=NNC(=N1)N	1.17
Guanylurea	C(=NC(=O)N)(N)N	1.18
Histamine	C1=C(NC=N1)CCN	n.d.
Chlormequat	C[N+](C)(C)CCCl	1.25
Methamidophos	COP(=O)(N)SC	2.77
Vancomycin	CC1C(C(CC(O1)OC2C(C(C(OC2OC3=C4C=C5C=C3OC6=C(C=C(C=C6)C(C(C(=O)NC(C(=O)NC5C(=O)NC7C8=CC(=C(C=C8)O)C9=C(C=C(C=C9O)O)C(NC(=O)C(C(C1=CC(=C(O4)C=C1)Cl)O)NC7=O)C(=O)O)CC(=O)N)NC(=O)C(CC(C)C)NC)O)Cl)CO)O)O)(C)N)O	4.34
Trichlorfon	COP(=O)(C(C(Cl)(Cl)Cl)O)OC	6.69
Cefoperazone	CCN1CCN(C(=O)C1=O)C(=O)NC(C2=CC=C(C=C2)O)C(=O)NC3C4N(C3=O)C(=C(CS4)CSC5=NN=NN5C)C(=O)O	7.35
Butocarboxim	CC(C(=NOC(=O)NC)C)SC	8.50
Dichlorvos	COP(=O)(OC)OC=C(Cl)Cl	9.69
Tylosin	CCC1C(C=C(C=CC(=O)C(CC(C(C(CC(=O)O1)O)C)OC2C(C(C(O2)C)OC3CC(C(C(O3)C)O)(C)O)N(C)C)O)CC=O)C)C)COC4C(C(C(C(O4)C)O)OC)OC	n.d.
Rifaximin	CC1C=CC=C(C(=O)NC2=C(C3=C(C4=C(C(=C3O)C)OC(C4=O)(OC=CC(C(C(C(C(C1O)C)O)C)OC(=O)C)C)OC)C)C5=C2N6C=CC(=CC6=N5)C)O)C	12.35
Spinosad_A	CCC1CCCC(C(C(=O)C2=CC3C4CC(CC4C=CC3C2CC(=O)O1)OC5C(C(C(C(O5)C)OC)OC)OC)C)OC6CCC(C(O6)C)N(C)C	12.85
TCMTB	C1=CC=C2C(=C1)N=C(S2)SCSC#N	13.57
Emamectin	CCC(C)C1C(C=CC2(O1)CC3CC(O2)CC=C(C(C(C=CC=C4COC5C4(C(C=C(C5O)C)C(=O)O3)O)C)OC6CC(C(C(O6)C)OC7CC(C(C(O7)C)NC)OC)OC)C)C	14.28
Avermectin	CCC(C)C1C(C=CC2(O1)CC3CC(O2)CC=C(C(C(C=CC=C4COC5C4(C(C=C(C5O)C)C(=O)O3)O)C)OC6CC(C(C(O6)C)OC7CC(C(C(O7)C)O)OC)OC)C)C	17.79
Nigericin	CC1CCC(OC1C(C)C(=O)O)CC2CC(C(C3(O2)C(CC(O3)(C)C4CCC(O4)(C)C5C(CC(O5)C6C(CC(C(O6)(CO)O)C)C)C)C)C)OC	n.d.
Ivermectin	CCC(C)C1C(CCC2(O1)CC3CC(O2)CC=C(C(C(C=CC=C4COC5C4(C(C=C(C5O)C)C(=O)O3)O)C)OC6CC(C(C(O6)C)OC7CC(C(C(O7)C)O)OC)OC)C)C	20.05

n.d.: not detected

Table S3. Calibrants used in the RTI model in the negative ionization mode (ESI+), and corresponding SMILES and experimental retention time (RT).

Calibrants	SMILES	Experimental RT (min)
Amitrole	C1=NNC(=N1)N	1.27
Benzoic acid	C1=CC=C(C=C1)C(=O)O	n.d.
Acephate	CC(=O)NP(=O)(OC)SC	3.52
Salicylic acid	C1=CC=C(C(=C1)C(=O)O)O	4.39
Tepraloxydim	CCC(=NOCC=CCl)C1=C(CC(CC1=O)C2CCOCC2)O	5.26
Simazine-2-Hydroxy	CCNC1=NC(=NC(=O)N1)NCC	5.61
Valproic acid	CCCC(CCC)C(=O)O	6.41
Bromoxynil	C1=C(C=C(C(=C1Br)O)Br)C#N	6.66
MCPA	CC1=C(C=CC(=C1)Cl)OCC(=O)O	6.69
Flamprop	CC(C(=O)O)N(C1=CC(=C(C=C1)F)Cl)C(=O)C2=CC=CC=C2	7.89
Dinoterb	CC(C)(C)C1=C(C(=CC(=C1)[N+](=O)[O-])[N+](=O)[O-])O	9.66
Phenytoin	C1=CC=C(C=C1)C2(C(=O)NC(=O)N2)C3=CC=CC=C3	9.70
Inabenfide	C1=CC=C(C=C1)C(C2=C(C=CC(=C2)Cl)NC(=O)C3=CC=NC=C3)O	11.74
Benodanil	C1=CC=C(C=C1)NC(=O)C2=CC=CC=C2I	12.23
Coumaphos	CCOP(=S)(OCC)OC1=CC2=C(C=C1)C(=C(C(=O)O2)Cl)C	15.32
Triclosan	C1=CC(=C(C=C1Cl)O)OC2=C(C=C(C=C2)Cl)Cl	15.48
Abamectin	CCC(C)C1C(C=CC2(O1)CC3CC(O2)CC=C(C(C(C=CC=C4COC5C4(C(C=C(C5O)C)C(=O)O3)O)C)OC6CC(C(C(O6)C)OC7CC(C(C(O7)C)O)OC)OC)C)C.CC1C=CC=C2COC3C2(C(C=C(C3O)C)C(=O)OC4CC(CC=C(C1OC5CC(C(C(O5)C)OC6CC(C(C(O6)C)O)OC)OC)C)OC7(C4)C=CC(C(O7)C(C)C)C)O	17.76
Salinomycin	CCC(C1CCC(C(O1)C(C)C(C(C)C(=O)C(CC)C2C(CC(C3(O2)C=CC(C4(O3)CCC(O4)(C)C5CCC(C(O5)C)(CC)O)O)C)C)O)C)C(=O)O	18.18

n.d.: not detected

Table S4. Calibrants used to harmonize ionization efficiency in the semi-quantification process, and their corresponding SMILES and calibration slopes.

Compound	SMILES	Slope
Tylosin	CCC1C(C=C(C=CC(=O)C(CC(C(C(C(CC(=O)O1)O)C)OC2C(C(C(O2)C)OC3CC(C(C(O3)C)O)(C)O)N(C)C)O)CC=O)C)C)COC4C(C(C(C(O4)C)O)OC)OC	7.0186
Cefoperazone	CCN1CCN(C(=O)C1=O)C(=O)NC(C2=CC=C(C=C2)O)C(=O)NC3C4N(C3=O)C(=C(CS4)CSC5=NN=NN5C)C(=O)O	0.3752
Rifaximin	CC1C=CC=C(C(=O)NC2=C(C3=C(C4=C(C(=C3O)C)OC(C4=O)(OC=CC(C(C(C(C(C1O)C)O)C)OC(=O)C)C)OC)C)C5=C2N6C=CC(=CC6=N5)C)O)C	8.6585
Theophylline	CN1C2=C(C(=O)N(C1=O)C)NC=N2	10.606
Spinosad A	CCC1CCCC(C(C(=O)C2=CC3C4CC(CC4C=CC3C2CC(=O)O1)OC5C(C(C(C(O5)C)OC)OC)OC)C)OC6CCC(C(O6)C)N(C)C	29.7311
Caffeine	CN1C=NC2=C1C(=O)N(C(=O)N2C)C	16.2092
Trichlorfon	COP(=O)(C(C(Cl)(Cl)Cl)O)OC	2.7366
Imidacloprid	C1CN(C(=N[N+](=O)[O-])N1)CC2=CN=C(C=C2)Cl	12.0581
Dichlorvos	COP(=O)(OC)OC=C(Cl)Cl	11.774
DEA	CC(C)NC1=NC(=NC(=N1)N)Cl	65.4132
Sulfamethoxazole	CC1=CC(=NO1)NS(=O)(=O)C2=CC=C(C=C2)N	20.8841
Acetaminophen	CC(=O)NC1=CC=C(C=C1)O	13.3848
Atrazine	CCNC1=NC(=NC(=N1)Cl)NC(C)C	147.9813
Simazine	CCNC1=NC(=NC(=N1)Cl)NCC	111.2744
Acetamiprid	CC(=NC#N)N(C)CC1=CN=C(C=C1)Cl	21.9081
Thiacloprid	C1CSC(=NC#N)N1CC2=CN=C(C=C2)Cl	13.7316
Carbamazepine	C1=CC=C2C(=C1)C=CC3=CC=CC=C3N2C(=O)N	41.4815
2-amino-benzothiazole	C1=CC=C2C(=C1)N=C(S2)N	75.1253
DEET	CCN(CC)C(=O)C1=CC=CC(=C1)C	151.151

Table S5. Individual and mixture ecotoxicological risk ranking categories based on the value of the risk quotient (RQ)

Risk Quotient (RQ)	Categories
RQ < 1	No risk
1 < RQ < 10	Low risk
10 < RQ < 100	Medium risk
RQ > 100	High risk

Table S6. Compounds detected in the winter campaign and their area in each water sample.

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
10,11-Dihydro-10,11-dihydroxycarbamazepine		28,802,236	33,566,120	51,333,621	23,935,101
10,11-Dihydro-10-Hydroxycarbazepine		24,933,483	52,809,579	63,229,838	43,776,113
2-(3,4-Dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile		4,381,333	10,502,821	9,906,463	5,485,307
2-Ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)		9,985,065	23,913,163	31,454,033	26,287,915
2-Mercaptobenzothiazole	871,480	3,177,200	3,890,011	5,165,838	2,070,890
3,3,5,5-Tetramethyl-1-pyrroline N-oxide		26,859,935	38,645,652	323,515,205	551,028,316
3-Hydroxycotinine		1,877,722	2,752,381	2,621,400	17,236,118
4'-Hydroxydiclofenac					6,089,163
4-Androstene-3,17-dione		-	-	-	77,110,913
4-Formylaminoantipyrine		175,584,323	299,236,807	511,525,481	153,697,176
4-Indolecarbaldehyde	222,304	2,123,078	9,221,892	5,701,027	28,412,659
4-Phenylbutyric acid		-	-	-	33,847,981
5-Hydroxyomeprazole		-	2,231,351	3,688,848	6,931,047
8-Hydroxyquinoline	69,096	1,981,849	1,239,311	1,365,183	42,933,129
Acephylline		1,626,513	1,699,435	3,363,678	150,188
Acetaminophen		-	-	-	3,606,214,471
Acetaminophen sulfate					181,938,708
Acetyl sulfamethoxazole					19,571,136
Amisulpride		27,028,566	32,515,543	61,171,839	16,402,012
Amitriptyline		3,412,679	22,633,319	13,044,835	16,783,038
Amphetamine	2,546,909	50,181,091	19,634,493	26,052,515	121,473,752
Ampyrone/ 4-aminoantipyrine		1,834,271	4,672,515	26,193,761	59,818,639
Atenolol		6,113,266	11,091,006	18,422,944	57,699,520
Azithromycin				909,560.00	
Azoxystrobin		6,755,362	205,953	314,829	374,328
Azoxystrobin acid		5,501,093	-	-	-
Benzoylecgonine		10,332,351	-	-	129,574,213
Bezafibrate		291,604	454,797	491,114	5,013,108
Bisoprolol		12,978,670	19,432,304	23,736,639	33,176,998
Boldenone		-	-	-	60,154,066
Boscalid		5,790,429	66,974	105,456	-
Bupropion		2,840,306	4,841,521	3,813,620	1,481,301

Table S6 continued

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
Butyrophenone	1,580,852	39,887,861	26,614,913	47,605,092	6,533,394
Caffeine		34,632,858	1,029,111	1,266,623	1,233,948,117
Caprolactam	28,197,270	2,631,402,710	236,750,939	234,777,778	195,242,444
Carbamazepine	298,001	18,439,369	44,572,422	31,713,778	16,487,527
Carbamazepine-10,11-epoxide		1,089,705	4,460,027	41,018,817	15,025,874
Carbendazim		10,085,701	1,610,915	1,819,522	5,519,371
Carboxy-ibuprofen					7,944,030
Cetirizine		9,286,820	39,569,660	27,748,873	32,211,946
Chlorpheniramine		399,516	4,122,959	4,748,106	-
Ciprofloxacin		-	296,886	7,139,247	14,214,891
Citalopram		10,412,011	56,933,934	41,440,923	41,766,110
Clarithromycin				1,416,465.00	
Clindamycin		787,911		3,190,349.00	
Clopidogrel		1,225,882	7,063,010	7,164,165	2,889,293
Clopidogrel carboxylic acid		12,734,076	15,282,108	21,677,900	7,680,546
Cocaine	110,787	2,106,619	-	1,012,461	112,417,140
Cotinine	69,293	6,101,028	1,195,333	1,031,565	64,595,751
Coumarin		-	414,870	-	36,008,492
Cyprodinil		-	-	76,515	46,151,878
DEET	8,485,815	30,734,296	32,267,346	43,034,198	265,149,494
Desacetyl diltiazem		2,582,415	8,018,496	9,630,096	2,852,343
Desmethylcitalopram		7,316,943	27,924,962	21,894,533	17,904,849
Desmethyldiazepam		3,827,689	10,027,743	7,218,687	4,974,750
Dextromethorphan		4,557,018	16,534,719	10,769,300	6,059,018
dextrorphan/levorphanol		34,690,035	42,548,985	107,405,045	37,456,824
Diazepam		1,068,872	1,707,557	2,376,854	1,168,373
Diazinon		333,316	475,452	1,349,985	1,318,161
Dibutyl phthalate	18,042,016	44,817,763	41,446,794	87,899,239	35,302,504
Diclofenac		2,317,957	12,278,978	23,368,196	13,550,597
Diethyl phthalate	8,556,187	29,607,075	29,173,902	36,165,725	77,487,160
Diltiazem		1,370,584	4,125,310	4,386,467	5,622,543
Diphenhydramine		1,143,231	8,788,560	6,907,068	4,765,788

Table S6 continued

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
Diuron				4,530,668.00	
Ecgonine methyl ester		3,378,260	5,423,467	5,384,177	7,481,055
Emtricitabine		1,732,701	839,195	4,596,483	5,495,139
Ephedrine		4,272,290	12,502,940	16,822,804	48,894,748
Epoxiconazole		-	3,460,534	4,281,339	122,307
Fenofibric acid		160,665	2,221,117	2,191,615	43,589,568
Flecainide		51,695,391	111,976,548	73,739,033	78,249,003
Fluconazole		3,489,715	4,649,614	8,178,337	2,758,996
Flufenamic acid		1,932,496	5,980,904	15,507,235	4,621,538
Gabapentin		39,346,796	37,901,862	72,850,065	457,100,720
Galaxolidone	1,278,044	148,815,289	217,463,268	257,869,604	101,497,021
Hydroxy-bupropion					3,939,358
Ibuprofen		-	-	-	2,466,589
Imidacloprid				4,090,139.00	
Isoproturon		5,881,582	6,051,649	6,640,211	7,559,292
Ketamine		11,177,334	15,730,056	24,722,700	6,296,912
Ketoprofen		433,119	6,891,024	8,424,571	42,790,663
Lamotrigine	40,662	36,078,747	47,214,397	67,600,595	11,329,320
Lamotrigine 2-N-glucuronide					1,003,666
Lauryl diethanolamide	8,785,485	15,204,060	11,964,749	11,552,279	2,670,872,257
Levamisole		10,583,716	23,467,684	38,985,559	7,579,722
Levofloxacin/ofloxacin		674,276	4,580,973	12,114,013	10,143,758
Lidocaine		66,526,748	66,544,115	90,657,902	33,252,288
MDMA		12,243,616	11,946,960	18,088,081	5,898,410
Mebendazole		230,739	1,044,795	932,917	9,423,980
Memantine		12,005,828	21,577,450	20,771,071	18,283,092
Metformin		198,713,057	13,107,946	15,242,462	1,128,019,508
Methadone		8,418,179	20,092,707	20,179,267	8,945,427
Metoclopramide		-	2,779,834	7,282,234	1,482,540
Metoprolol		2,487,618	2,875,558	4,319,653	2,065,162
Metoprolol acid		38,524,508	39,131,354	58,080,433	13,820,458
Mirtazapine		520,239	3,428,278	6,393,503	2,302,115

Table S6 continued

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
Mycophenolic acid		-	-	-	54,097,106
N,N'-Diphenylguanidine	337,666	147,344,113	38,551,527	77,911,650	40,636,189
N-Acetyl-5-aminosalicylic acid		-	-	-	59,411,235
N-Acetylaminoantipyrine		121,008,666	156,205,169	268,655,500	502,486,674
Naproxen		-	-	-	2,381,368
Nicotine		-	-	-	90,319,120
N-Phenyl-1-naphthylamine	10,110,039	57,321,224	56,063,410	117,208,575	4,869,026
O-Desmethyl Venlafaxine		280,376,328	265,772,408	534,563,953	167,066,027
O-desmethyltramadol		184,847,728	161,948,712	373,755,687	94,706,883
Omeprazole sulfone		-	1,570,559	2,816,011	1,255,802
Oxazepam		4,432,088	7,715,176	8,059,645	6,236,707
Oxybenzone	89,267,904	423,078,174	78,864,335	115,773,003	17,894,347
Pentaethylene glycol (PEG)	7,958,492	82,843,782	26,003,007	59,434,458	1,420,018,883
Perfluorobutanesulfonic acid (PFBS)	411,539	5,119,992	3,710,469	6,772,305	1,360,360
Perfluorobutanoic acid (PFBA)	81,903	1,148,274	624,487	1,052,114	718,794
Perfluoroheptanoic acid (PFHpA)	127,467	1,226,857	633,071	1,356,920	176,094
Perfluorohexanoic acid (PFHxA)	1,356,104	1,356,104	1,991,227	6,259,050	1,522,888
Perfluorooctanoic acid (PFOA)	68,074	2,115,278	841,397	967,575	520,828
Perfluoropentanoic acid (PFPeA)		1,379,211	1,027,568	1,964,029	205,337
Pregabalin		3,278,291	1,424,433	3,027,276	148,874,629
Propranolol		4,834,741	19,393,365	17,066,150	7,241,523
Quetiapine		-	-	-	3,590,722
R-Methyl-benzotriazole (R=1, 4, 5)			340,406,757	360,541,484	125,863,115
R-Methyl-benzotriazole (R=1, 4, 5)				323,956,720	
Ritalinic acid		6,432,319	4,397,258	7,416,885	14,982,791
Secbumeton		2,576,470	8,010,429	4,162,586	88,215
Sertraline		313,098	1,651,038	1,986,788	8,905,255
Sitagliptin		39,352,462	67,541,917	114,218,139	56,483,851
Sotalol		4,156,110	5,862,502	9,996,212	3,346,857
Sulfamethoxazole		5,206,590	5,772,014	10,380,316	16,441,035
Sulpiride		57,801,159	53,558,905	81,264,144	13,119,337
Tapentadol		100,940,372	111,568,461	270,317,932	98,090,255

Table S6 continued

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
Tebuconazole		531,948	614,900	868,677	-
Temazepam		4,651,440	9,226,487	8,763,810	5,293,719
Terbutryn	146,627	21,702,033	30,713,604	47,394,253	53,749,219
Testosterone propionate		-	-	-	37,607,007
Tetradecylamine	7,636,971	20,571,457	17,854,000	21,926,063	14,619,996,349
Tetrakis(2-hydroxypropyl)ethylenediamine		-	-	-	21,982,019
Theobromine		12,001,253	3,147,484	6,900,279	378,510,747
Theophylline/ Paraxanthine		13,738,154	1,777,135	-	326,542,019
Tramadol		309,180,190	381,509,621	560,585,832	157,888,451
Tri(chloropropyl) phosphate	2,520,094	18,971,233	20,657,976	31,809,710	85,956,630
Tributyl phosphate	15,890,995	63,944,436	65,512,766	75,424,227	29,316,906
Triethanolamine	1,573,674	8,977,992	2,227,076	2,824,968	298,674,507
Triethylene glycol monobutyl ether	1,042,465	2,336,948	1,001,677	2,061,741	437,488,081
Triisobutyl phosphate	4,953,077	51,714,297	12,165,553	18,384,292	6,217,829
Trimethoprim	44,780	1,011,207	3,792,529	8,493,457	11,843,418
Triphenyl phosphate	956,138	2,479,712	1,509,219	2,929,440	10,768,324
Tris(2-butoxyethyl) phosphate	224,981	11,002,007	10,611,995	7,740,892	216,715,060
Venlafaxine	221,857	123,110,873	260,409,983	232,418,941	119,510,457
Vildagliptin		7,036,946	9,118,227	14,785,127	4,899,123

Table S7. Compounds detected in the summer campaign and their area in each water sample.

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
1,2-Benzisothiazol-3(2H)-one		704,246	3,545,607	5,217,069	99,157,476
1,5-Naphthalenediamine		-	-	-	7,262,825
10,11-Dihydro-10,11-dihydroxycarbamazepine		136,189,705	121,576,004	133,233,311	47,103,354
10,11-Dihydro-10-Hydroxycarbazepine		78,736,372	125,718,413	122,662,927	81,524,615
2-(3,4-Dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile		980,702	3,325,804	32,580,821	13,111,395
2(3H)-Benzothiazolone		51,977,226	28,114,643	10,278,039	73,216,345
2-(Methylthio)benzothiazole	3,036,258	66,770,897	77,869,422	7,544,591	17,177,678
2-Ethylhexyl diphenyl phosphate	11,618,981	103,943,268	7,771,437	5,706,774	189,432
2-Mercaptobenzothiazole		-	-	-	66,034,497
3-Hydroxycotinine		-	-	-	169,753,693
4-Androstene-3,17-dione		-	-	-	142,929,816
4-Formylaminoantipyrine		1,145,860,802	1,191,591,497	1,488,014,498	454,837,010
4'-Hydroxydiclofenac/5-Hydroxydiclofenac		-	23,333,243	17,629,507	15,017,990
4-Phenylbutyric acid		-	-	-	88,250,015
6-Methylthioguanine		-	-	9,105,063	20,083,174
8-Hydroxy Mirtazapine		-	-	1,314,019	6,679,767
8-Hydroxyquinoline		90,276,617	-	6,616,901	484,577,393
Acetaminophen		-	-	-	8,332,637,735
Acetaminophen sulfate		-	-	-	69,669,379
Acetyl sulfamethoxazole		-	-	236,280	24,366,860
Amisulpiride		-	201,442,592	239,647,655	76,790,775
Amitriptyline		-	71,328,179	53,404,581	23,962,951
Amphetamine	60,124,059	65,531,138	437,352,812	117,677,539	506,453,391
Ampyrone/ 4-aminoantipyrine		-	38,749,862	59,909,524	277,139,574
Atenolol		-	-	32,935,878	180,079,727
Azithromycin		-	5,324,438	4,472,447	-
Azoxystrobin		12,797,978	6,913,954	7,601,419	4,539,617
Benzothiazole	17,157,905	25,354,727	23,231,145	252,285,850	55,231,119
Benzoylecgonine		-	-	-	553,014,848
Bezafibrate		-	968,451	1,609,895	11,308,149
Bisoprolol		-	53,389,871	62,272,462	69,542,141
Boldenone		-	-	-	82,641,226
Caffeine		-	-	-	4,192,368,457
Caprolactam	691,330,512	704,713,517	2,978,548,464	516,134,058	1,156,643,965
Carbamazepine		96,308,554	130,569,553	126,862,957	47,831,638
Carbamazepine-10,11-epoxide		-	91,105,243	85,626,038	398,856,756
Cetirizine		24,853,418	50,919,639	70,653,265	22,978,273

Table S7 continued

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
Ciprofloxacin		4,592,809	953,486	32,335,623	49,507,096
Citalopram		1,922,927	158,613,548	169,277,156	80,015,579
Clarithromycin		-	1,408,453	1,301,563	8,091,501
Clopidogrel carboxylic acid		16,689,100	56,247,348	74,596,220	23,071,005
Cocaine		-	-	-	392,572,728
Cotinine		-	-	-	319,768,193
DEET	83,485,122	464,493,357	1,138,242,141	688,838,202	4,580,483,685
Desacetyl diltiazem		-	36,698,748	39,108,738	10,616,105
Desmethyl-citalopram		-	66,701,670	77,637,767	29,066,695
Desmethyldiazepam		18,239,787	25,961,091	28,991,441	8,034,835
Dextromethorphan		-	19,707,844	16,683,602	3,426,631
Dextrorphan		10,766,046	44,380,180	61,720,642	19,005,426
Diazepam		7,250,467	10,453,862	11,305,512	2,208,866
Diazinon		1,427,457	3,135,782	5,031,091	-
Diclofenac		-	70,581,364	66,804,866	25,587,729
Dicyclohexylamine	10,583,688	205,177,872	242,890,624	77,645,484	129,230,358
Dicyclohexylurea	1,702,039	63,585,647	111,113,240	106,352,974	496,994,326
Diltiazem		-	29,950,505	33,141,965	30,582,732
Diphenhydramine		-	35,733,344	33,668,864	-
Diphenylamine		127,885,222	235,113,027	22,694,209	29,150,408
Diuron		27,151,237	75,945,061	63,429,446	12,064,084
Ecgonine methyl ester		-	-	-	56,914,466
Emtricitabine		6,479,965	7,517,833	10,026,286	17,743,407
Fenofibric acid		-	9,781,715	7,468,368	54,672,236
Flecainide		130,718,339	512,372,618	477,465,644	181,176,759
Fluconazole		30,126,100	19,575,304	25,559,883	9,401,484
Flufenamic acid		814,649	75,165,673	82,440,437	5,123,015
Gabapentin		76,346,190	237,595,680	261,276,676	1,201,211,545
Galaxolidone	22,551,886	93,837,255	2,143,333,231	1,957,618,908	160,827,836
Hydroxy-bupropion		4,702,311	5,646,123	34,333,988	8,823,470
Isoproturon		17,125,234	49,493,681	43,858,862	18,232,609
Ketamine		20,565,183	30,140,369	42,808,973	10,246,345
Ketoprofen		-	17,525,207	16,950,062	67,926,058
Lamotrigine		181,268,490	154,059,137	159,227,803	28,333,355
Lamotrigine 2-N-glucuronide		-	-	241,743	3,501,092
Lauryl diethanolamide	30,185,464	20,438,615	18,117,515	18,824,597	2,102,788,546
Levamisole		5,408,098	56,287,716	77,119,993	14,538,148
Levofloxacin/ofloxacin		1,940,291	20,841,698	23,088,832	16,377,390

Table S7 continued

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
Lidocaine		86,993,060	282,460,679	326,429,343	125,558,123
MDMA		-	60,415,654	56,125,387	30,085,837
Mebendazole		-	4,935,073	5,161,978	9,086,032
Memantine		25,193,965	43,912,192	41,556,054	9,525,650
Metformin	1,252,183	30,613,164	434,378,392	597,964,284	4,731,065,645
Methadone		1,772,911	76,323,622	65,294,813	22,427,236
Metoclopramide		-	13,226,941	17,562,221	6,500,771
Metoprolol		-	8,359,143	11,511,432	5,433,326
Metoprolol acid		91,662,578	186,616,061	210,219,213	43,270,304
Metribuzin-desamino		22,688,523	738,323	696,812	-
Mycophenolic acid		-	-	-	47,336,661
N,N'-Diphenylguanidine	647,989	3,098,197	67,372,702	184,521,388	168,107,447
N-Acetyl-5-aminosalicylic acid		-	-	-	78,997,186
N-Acetylaminoantipyrine		408,373,817	299,846,763	375,737,292	1,254,883,869
Naproxen		-	1,756,495	1,229,059	12,748,751
Nicotine		-	-	-	563,684,919
N-Phenyl-1-naphthylamine	2,003,929	242,277,493	16,167,278	2,304,658	5,204,983
O-Desmethyl Venlafaxine		133,642,119	1,202,096,879	1,535,081,093	561,323,005
O-Desmethyltramadol		123,773,298	679,456,713	1,032,712,812	206,222,094
Oxazepam		8,653,874	28,568,948	30,398,013	10,842,116
Oxybenzone	25,180,952	169,725,868	151,796,906	5,376,206	23,977,678
Pentaethylene glycol (PEG)	209,293,814	28,673,469	100,984,159	33,310,584	3,721,047,990
Perfluorobutanesulfonic acid (PFBS)	845,554	116,627,211	69,417,414	56,321,320	26,000,550
Perfluorobutanoic acid (PFBA)	-	13,404,718	7,606,668	4,920,534	2,417,047
Perfluorooctanoic acid (PFOA)	2,685,822	41,864,582	16,572,818	8,628,298	3,790,582
Perfluoropentanoic acid (PFPeA)	162,182	33,147,293	17,061,039	11,678,099	2,411,174
Pregabalin		18,777,672	16,423,049	16,398,326	313,591,229
Propranolol		-	53,524,909	52,588,968	15,392,764
R-Methyl-benzotriazole (R = 1, 4, 5)	1,600,427	-	329,378,879	574,494,598	556,676,311
Ritalinic acid		-	-	-	30,173,631
Rosuvastatin		-	-	-	14,785,224
Secbumeton		3,354,509	58,404,039	10,083,790	-
Sertraline		-	9,637,472	18,324,202	-
Sitagliptin		25,203,221	206,749,882	242,182,707	157,247,661
Sotalol		-	19,971,959	24,192,917	11,852,352
Sulfamethoxazole		7,548,802	17,443,696	12,035,469	33,402,309
Sulpiride		39,277,469	190,700,686	245,106,954	94,966,466
Tapentadol		54,083,495	576,773,782	815,701,883	281,334,608

Table S7 continued

Compound	Blank	Irrigation point	Discharge	Effluent	Influent
Temazepam		19,773,671	32,814,438	37,026,134	10,556,989
Terbutryn		130,363,394	126,316,583	135,069,915	56,513,841
Testosterone propionate		-	-	-	22,001,843
Tetradecylamine	313,533,485	130,660,744	120,493,636	95,773,432	16,976,077,798
Tetrakis(2-hydroxypropyl)ethylenediamine		-	28,091,742	36,635,444	152,795,591
Theobromine		-	-	-	833,782,289
Theophylline/ Paraxanthine		-	-	-	868,649,990
Tramadol		133,642,119	1,202,096,879	1,535,081,093	561,323,005
Tri(chloropropyl) phosphate		134,058,966	224,482,952	162,444,404	201,778,582
Tributyl citrate	1,701,878	2,108,156	1,549,308	2,531,716	33,543,227
Tributyl phosphate	45,518,968	132,693,070	214,062,622	136,244,557	26,542,521
Triethanolamine		-	3,396,025	8,755,350	1,043,183,529
Triethylene glycol monobutyl ether		-	-	-	683,827,002
Trimethoprim		-	3,747,854	5,576,792	22,214,798
Tris(2-butoxyethyl) phosphate	2,740,762	48,237,538	383,925,225	135,976,808	805,793,003
Venlafaxine		46,535,508	790,273,016	819,910,795	237,922,829
Vildagliptin		14,665,719	28,443,872	39,845,515	19,918,909

Table S8. MS/MS fragments for each compound detected in the winter campaign.

Compound	Fragments
10,11-Dihydro-10,11-dihydroxycarbamazepine	180.0805/210.0910/254.0808/208.0755/192.0810/182.0962/167.0723
10,11-Dihydro-10-Hydroxycarbazepine	194.0963/192.0807/193.0872/195.0997
2-(3,4-Dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile	260.1643/248.1516/218.1174/233.1534/177.0908/165.0909/151.0753/142.1590/122.0963/96.0807/70.0651
2-Ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)	249.1510/234.1274/186.1270/201.1501/172.1116/200.1429
2-Mercaptobenzothiazole	136.0215/135.0137/124.0215/109.0106/104.0495/92.0495
3,3,5,5-Tetramethyl-1-pyrroline N-oxide	86.0599/68.0495
3-Hydroxycotinine	134.0600/80.0495/149.0709/162.0555
4'-Hydroxydiclofenac/5-OH-DCF	266.0130/231.0442/230.0367/196.0760
4-Androstene-3,17-dione	269.1902/251.1792/241.2356/211.1480/185.1326/159.1173/123.0804/109.0648/97.0648
4-Formylaminoantipyrine	214.0973/204.1131/187.0864/173.0709/159.0916/146.0600/104.0494/83.0603/56.0495
4-Indolecarbaldehyde	118.0649/91.0542
4-Phenylbutyric acid	147.0804/137.0961/123.0804/119.0855/105.0699/93.0699/91.0542/81.0698/67.0544
5-Hydroxyomeprazole	214.0529/196.0426/184.0967/168.0479/152.0705/149.0708/106.0653
8-Hydroxyquinoline	128.0493/118.0650/91.0542
Acephylline	193.0732/179.0579/136.0516/108.0563
Acetaminophen	110.0599/134.0601/109.0524/93.0334
Acetaminophen sulfate	152.0706/134.0598/110.0600/93.0334/74.0601
Acetyl sulfamethoxazole	198.0220/188.0819/162.0662/160.0869/136.0757/134.0600/108.0443/93.0573
Amisulpride	242.0478/196.0058/214.0171/155.1178/112.1119
Amitriptyline	233.1323/218.1089/205.1012/193.1012/191.0854/179.0853/155.0855/117.0698/105.0698/91.0542/84.0807/58.0654
Amphetamine	91.0541
Ampyrone/ 4-aminoantipyrine	189.0899/187.0865/173.0714/159.0918/146.0601/130.0653/118.0652/111.0552/94.0651/83.0604/56.0498
Atenolol	249.1594/225.1231/208.0966/190.0861/178.0861/164.0703/162.0912/152.0704/145.0647/133.0648/121.0646/116.1068/98.0963/74.0600/72.0808
Azithromycin	573.4109/158.1175/116.1069/83.0491/72.0808
Azoxystrobin	372.0975/344.1026/329.0790/316.1083/216.0657/172.0387/134.0600
Azoxystrobin acid	372.0976/344.1030/329.0794/216.0654/134.0600/143.0607
Benzoylecgonine	272.1277/168.1017/150.0913/140.1067/124.1120/105.0334/100.0757/82.0651
Bezafibrate	318.2998/276.0779/161.0960/138.9944/121.0646
Bisoprolol	222.1482/204.1381/162.0912/147.0804/133.0646/116.1069/107.0490/98.0964/91.0541/74.0600/56.0498
Boldenone	203.0700/173.0959/135.1167/121.0646/93.0699
Boscalid	307.0633/271.0865/272.0944/139.9897/112.0397/96.0444
Bupropion	184.0523/166.0416/133.0759/139.0305/131.0729/57.0701/167.0256
Butyrophenone	131.0854/129.0697/131.0854/107.0489/93.0698/91.0541/79.0542/71.0492/57.0334
Caffeine	138.0661/110.0711/69.0448
Caprolactam	55.0546/69.0699/79.0541/96.0886/97.0646

Table S8 continued

Compound	Fragments
Carbamazepine	194.0963/192.0806/220.0752/193.0868
Carbamazepine-10,11-epoxide	208.0754/210.0910/180.0804/182.0963/236.0703/192.0808/181.0857
Carbendazim	160.0504/133.0643/132.0556/105.0447/92.0494
Carboxy-ibuprofen	191.1081/73.0296/117.0195
Cetirizine	201.0465/166.0777
Chlorpheniramine	167.0729/230.0729/232.0701/202.0417
Ciprofloxacin	288.1503/268.1440/245.1082/231.0924/205.0768/204.0687/203.0609/70.0651/58.0652
Citalopram	307.1602/280.1133/262.1026/247.0792/234.0712/221.0631/184.0762/166.065/156.0809/116.0494/109.0448
Clarithromycin	158.1175
Clindamycin	126.1278
Clopidogrel	184.0524/212.0473/152.0262/155.0258/125.0153
Clopidogrel carboxylic acid	198.0315/170.0367/169.0049/152.0260/141.0100/125.0151
Cocaine	272.1282/182.1174/150.0912/105.0334/91.0541/82.0651/108.0807/154.0867
Cotinine	146.0598/98.0599/80.0494/81.0527
Coumarin	103.0541/91.0541/105.0697
Cyprodinil	209.1071/210.1020/185.1073/133.0759/108.0808/93.0572
DEET	119.0492/109.0646/100.0757/72.0444/91.0542
Desacetyl diltiazem	178.0321/223.0902/72.0808/328.0994/197.1107/150.0372
Desmethylcitalopram	293.1445/262.1024/247.0792/234.0711/221.0632/166.0649/156.0805/116.0495/109.0447/69.0448
Desmethyldiazepam	243.0682/208.0991/165.0214/140.0260/226.0413/
Dextromethorphan	215.1430/147.0803/213.1272/159.0801/171.0802/121.0646
Dextrorphan/levorphanol	201.1272/199.1116
Diazepam	228.0574/154.0418/257.0839/222.1151/193.0886
Diazinon	169.0795/153.1024/114.9613/84.0444
Dibutyl phthalate	149.0233/57.0702/121.0284/167.0336/150.0266
Diclofenac	215.0493/250.0182/214.0418/180.0800/179.0730
Diethyl phthalate	149.0231/150.0264/177.0543/178.0580/167.0336
Diltiazem	178.0320/371.2054/310.0890/191.0402/150.0371/72.0808/137.0597
Diphenhydramine	167.0854/165.0696/152.0618
Diuron	72.0444
Ecgonine methyl ester	182.1174/82.0651/108.0807/150.0913/168.1014
Emtricitabine	130.0411/101.0055
Ephedrine	149.1154/148.1119/133.0885/117.0698/70.0651/56.0497
Epoxiconazole	121.0448/123.0241/141.0100/70.0400
Fenofibric acid	233.0361/138.9944/121.0830
Flecainide	315.1070/301.0291/287.0486/232.0968/178.0317/98.0964/81.0699
Fluconazole	238.0784/220.0679/169.0458/141.0510/127.0353/70.0400
Flufenamic acid	264.0631/244.0568/195.0679/167.0729
Gabapentin	154.1224/137.0960/119.0854/109.1011/95.0854/55.0181/67.0543/156.1097

Table S8 continued

Compound	Fragments
Galaxolidone	255.1742/240.1508/227.1790/203.1063/175.1118/157.1014
Hydroxy-bupropion	238.0990/167.0494/184.0523/139.0307/131.0728/166.0416
Ibuprofen	161.1325/119.0855/105.0698/91.0543/133.1011
Imidacloprid	209.0588/175.0978/128.0261/84.0556
Isoproturon	165.1022/134.0964/72.0444
Ketamine	220.0884/207.0570/189.0463/179.0619/163.0306/152.0259/125.0153/67.0543
Ketoprofen	209.0958/194.0724/177.0543/131.0490/109.1010/105.0334/95.0490
Lamotrigine	229.0041/210.9821/186.9824/185.9871/166.9871/165.0211/158.9760
Lamotrigine 2-N-glucuronide	256.0151
Lauryl diethanolamide	227.2003/106.0861/88.0756/70.0652/57.0701
Levamisole	178.0683/146.0964/88.0215/118.0649/188.0523
Levofloxacin/ofloxacin	318.1611/344.1431/261.1033/221.0712/247.0878/233.0711/58.0654/72.0808
Lidocaine	86.0964/87.0997/58.0654
MDMA	163.0751/151.0753/135.0439/133.0648/105.0697/58.0653
Mebendazole	264.0767/105.0335
Memantine	163.1479/107.0853/121.1010/
Metformin	113.0822/88.0869/85.0508/71.0604/60.0558
Methadone	265.1583/223.1114/219.1166/195.1167/159.1167/129.0698/117.0696/105.0333/91.0541/57.0337
Metoclopramide	227.0581/184.0159/222.0347
Metoprolol	250.01426/226.1434/218.1538/191.1064/176.1064/159.0804/133.0647/121.0648/116.1069/98.0964/74.0600/56.0497
Metoprolol acid	226.1070/191.0700/165.0543/145.0646/116.1068/98.0963/74.0600/72.0807/56.0497
Mirtazapine	235.1225/209.1071/195.0916/110.0600/72.0808
Mycophenolic acid	275.1275/285.1119/207.0651/159.0437/177.0545/195.0651
N,N'-Diphenylguanidine	195.0914/119.0602/94.0650/95.0683/92.0493
N-Acetyl-5-aminosalicylic acid	178.0499/152.0706/136.0392/108.0442/110.0600/80.0493
N-Acetylaminoantipyrine	228.1128/204.1131/104.0494/83.0603/187.0864/159.0916/146.0600/94.0650/56.0498
Naproxen	170.0742/158.0378/185.0977
Nicotine	132.0807/130.0651/120.0807/116.0527/106.0560/84.0808/80.0494
N-Phenyl-1-naphthylamine	205.0886/143.0729/142.0651/128.0619/115.0540/92.0494/65.0388
O-Desmethyl Venlafaxine	246.1852/201.1273/173.0957/159.0804/133.0647/107.0491/58.0654
O-desmethyltramadol	58.0651/232.1690
Omeprazole sulfone	214.0532/195.0226/184.0971/168.1019/166.0863/149.0710/150.0913/120.0807/93.0698
Oxazepam	269.0476/241.0526/231.0683/216.0994/166.0055/128.0262/104.0495
Oxybenzone	151.0388/105.0334/95.0491/152.0421
Pentaethylene glycol (PEG)	89.0596/107.0702/133.0858/151.0964/177.1127
Perfluorobutanesulfonic acid (PFBS)	168.9897/118.9927/98.9562/82.9612/79.9576

Table S8 continued

Compound	Fragments
Perfluorobutanoic acid (PFBA)	168.9894
Perfluoroheptanoic acid (PFHpA)	168.9897/118.9930
Perfluorohexanoic acid (PFHxA)	118.9925
Perfluorooctanoic acid (PFOA)	168.9897/118.9930
Perfluoropentanoic acid (PFPeA)	218.9862
Pregabalin	143.1066/142.1225/125.0961/124.1119/107.0854/97.1011/83.0854/69.0698/55.0545
Propranolol	218.1174/183.0804/157.0647/155.0853/116.1068/98.0963/74.0600
Quetiapine	253.0791/279.0950/221.1072/247.1225/210.0370/158.1175
R-Methyl-benzotriazole (R=1, 4, 5)	106.0650/79.0542/77.0382
R-Methyl-benzotriazole (R=1, 4, 5)	106.0650/79.0542/77.0382
Ritalinic acid	174.1275/84.0807
Secbumeton	170.1035/142.0723/114.0660/100.0502/86.0345/85.0757/57.0703
Sertraline	275.0389/240.0707/196.9918/158.9763/129.0698/91.0542
Sitagliptin	391.0984/235.0798/193.0694/174.0523/127.0353
Sotalol	255.1158/213.0690/176.1308/133.0759/106.0649
Sulfamethoxazole	156.0112/108.0442/92.0494/68.0495/110.0599/188.0817/99.0552
Sulpiride	214.0164/112.1119/98.0963/84.0807/58.0653
Tapentadol	135.0804/121.0647/107.0490/177.1272
Tebuconazole	70.0399/125.0153
Temazepam	283.0632/255.0680/228.0570/216.0572/193.0883/180.0211/107.0853
Terbutryn	186.0808/158.0492/171.0573/138.0774/116.0275/102.0377/91.0324
Testosterone propionate	97.0648/109.0648/271.2054/253.1947/175.1481/123.0804/213.1633/79.0541/81.0698/83.0491
Tetradecylamine	141.0003/85.1011/71.0856/57.0701
Tetrakis(2-hydroxypropyl)ethylenediamine	160.1330/142.1226/102.0913/84.0807
Theobromine	137.0821/138.0661/110.0712
Theophylline/ Paraxanthine	124.0504/142.0610
Tramadol	246.1850/58.0654
Tri(chloropropyl) phosphate	98.9841/174.9920/116.9946/77.0152
Tributyl phosphate	116.9945/98.9841/57.0701/155.0471
Triethanolamine	132.1018/114.0912/88.0756/70.0652
Triethylene glycol monobutyl ether	133.0858/101.0960/89.0596/73.0648/57.0701
Tributyl phosphate	98.9842/116.9971/57.0698
Trimethoprim	275.1139/261.0980/245.1034/230.1160/181.0859/123.0664
Triphenyl phosphate	309.0676/251.0465/233.0359/228.0930/215.0253/202.0777/171.0802/153.0698/152.0621/105.0446/95.0490/77.0382
Tris(2-butoxyethyl) phosphate	199.0729/143.0105/124.9997/101.0690/83.0854/57.0701
Venlafaxine	260.2007/215.1430/187.1118/173.0962/159.0802/147.0803/121.0646/107.0854/58.0654
Vildagliptin	154.0973/151.1117/97.0760/70.0652/93.0698/107.0854/133.1012

Table S9. MS/MS fragments for each compound detected in the summer campaign.

Compound	Fragments
1,2-Benzisothiazol-3(2H)-one	152.0162/109.0105
1,5-Naphthalenediamine	143.0729/130.0651/115.0542
10,11-Dihydro-10,11-dihydroxycarbamazepine	180.0804/182.0960/210.0908/236.0697/254.0805
10,11-Dihydro-10-Hydroxycarbazepine	194.0964/237.1021/192.0806/196.1391
2-(3,4-Dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile	260.1642/248.1514/218.1170/177.0907/165.0907/151.0751/122.0961/96.0806/70.0651
2(3H)-Benzothiazolone	124.0214/109.0106/92.0493
2-(Methylthio)benzothiazole	182.0089/166.9855
2-Ethylhexyl diphenyl phosphate	251.0454/153.0690/233.0350/175.0145/95.0486
2-Mercaptobenzothiazole	135.0130/109.0101/136.0208/124.0209
3-Hydroxycotinine	193.0970/134.0599/80.0494
4-Androstene-3,17-dione	287.2004/251.1792/229.1582/211.1481/185.1325/173.1325/159.1167/123.0803/109.0647/97.0647
4-Formylaminoantipyrine	204.1127/159.0913/146.0599/130.0651/128.0494/104.0491/83.06032
4'-Hydroxydiclofenac/5-Hydroxydiclofenac	230.0355/231.0433
4-Phenylbutyric acid	165.0909/147.0801/137.0960/122.0724/119.0851/109.1010/95.0854/91.0542/67.0543
6-Methylthioguanine	134.0464/167.0252/80.0238/107.0347
8-Hydroxy Mirtazapine	211.0855/282.1586
8-Hydroxyquinoline	128.0490/129.0441/95.0486/117.0567
Acetaminophen	152.0703/110.0594/92.0489/93.0329
Acetaminophen sulfate	152.0706/134.0598/110.0600/93.0334/74.0601
Acetyl sulfamethoxazole	198.0220/188.0819/162.0662/160.0869/136.0757/134.0600/108.0443/93.0573
Amisulpiride	242.0478/340.1790/112.1119/196.0058
Amitriptyline	233.1320/278.1898/91.0540/105.0696/117.0696/191.0851/155.0852/205.1008/218.1084
Amphetamine	92.05737/91.05401
Ampyrone/ 4-aminoantipyrine	204.1144/189.0896/187.0863/173.0707/159.0915/146.0600/111.0550/94.0650/85.0759/83.0603/56.0497
Atenolol	267.1699/190.0860/145.0646/116.1068/74.0600/72.0808/225.1230/208.0966/178.0860/162.0912/98.0963/56.0497
Azithromycin	591.4185/158.1167/116.1063/83.0487
Azoxystrobin	372.0960/344.1013/316.1065
Benzothiazole	65.0388/95.0489/105.0443/109.0100
Benzoylecgonine	168.1017/105.0333/82.0650/150.0911/119.0490
Bezafibrate	69.0336/87.0439/121.0648/138.9943/161.0958/189.0911/207.1015/276.0786/316.1099
Bisoprolol	56.0498/58.0654/72.0808/74.0600/91.0542/98.0964/116.1069/133.0647/147.0803/162.0911/204.1385/222.1491/326.2325
Boldenone	121.0647/135.1167/147.0804/149.1324/161.1324/173.0961/179.1430/187.1116/269.1895/
Caffeine	138.0654/110.0707/83.0599/123.0421/69.0444
Caprolactam	55.0546/69.0699/79.0541/96.0886/97.0646/
Carbamazepine	237.1020/194.0962/192.0806
Carbamazepine-10,11-epoxide	208.0755/210.0911/236.0704/180.0806/182.0964/253.0972

Table S9 continued

Compound	Fragments
Cetirizine	165.0696/166.0776/187.1073/201.0464
Ciprofloxacin	288.1507/268.1440/245.1081/224.0693/191.0612/170.0651
Citalopram	58.0653/109.0446/116.0492/156.0806/166.0648/234.0708/247.0786/262.1021/325.1703
Clarithromycin	158.1167/116.1063
Clopidogrel carboxylic acid	198.0305/170.0360/169.0047/152.0257/141.0100/140.0257/125.0152/113.0149/111.0262/
Cocaine	182.1174/82.0650/105.0334/150.0912/122.0961
Cotinine	177.1020/80.0494/98.0599/146.0597
DEET	72.0443/91.0539/100.0754/119.0488/192.1378
Desacetyl diltiazem	373.1566/328.1001/223.0899/197.1107/178.0319/150.0372/72.0807
Desmethyl-citalopram	311.1542/293.1446/280.1127/262.1024/234.0711/221.0631/166.0651/116.0493/109.0447
Desmethyldiazepam	243.0682/208.0991/165.0214/140.0260
Dextromethorphan	272.2004/241.1581/215.1427/213.1273/171.0799/159.0801/147.0803/121.0644
Dextrorphan	258.1852/199.1117/201.1273/157.0647/133.0647/145.0647
Diazepam	257.0827/222.1140/193.0876/154.0410
Diazinon	169.0786/153.1014/114.9607/96.9502
Diclofenac	278.0124/250.0175/215.0175
Dicyclohexylamine	55.0544/83.0854/100.1119/182.1901
Dicyclohexylurea	225.1956/143.1176/100.1118/83.0854/61.0398
Diltiazem	178.0318/150.0370/137.00595/72.0807/174.0370/310.0891/191.0398
Diphenhydramine	167.0847/152.0612/167.0847/165.0690
Diphenylamine	93.0568/92.0493/153.0702/170.0961
Diuron	149.9744/185.9509/121.9797
Ecgonine methyl ester	200.1278/182.1174/168.1016/156.1015/150.0912/108.0805/100.0755/82.0650
Emtricitabine	130.0410/113.0143/101.0055/73.0106/61.0110
Erucamide	338.3417/321.3152/303.3046
Fenofibric acid	233.0361/138.9944/121.0830
Flecainide	415.1447/398.1180/315.1074/301.0290/232.0966/98.0963/81.0698
Fluconazole	238.0774/220.0774/169.0451/139.0347/70.0400
Flufenamic acid	244.0566/216.0623/195.0678/167.0733/57.0701
Gabapentin	172.1329/155.1258/154.1224/137.0959/119.0855/109.1009/95.0854/93.0698/67.0543/55.0181
Galaxolidone	256.1777/255.1735/245.1536/240.1505/227.1791/225.1275/212.1555/211.1116/210.1038/203.1064/197.1317/185.0963/183.1168/182.1090/175.1111/171.1166/170.1090/169.1005/168.0935/157.1010/156.0938/155.0855/142.0778/141.0693/16.361/3876107/129.0696/128.0616/117.0696/115.0540/255.1737/240.1507/227.1792/225.1274/197.1318/185.0962/183.1168/171.1166/169.1007/157.1010/156.0936/143.0856/142.0778/129.0697/128.0617/117.0696
Hydroxy-bupropion	238.0990/220.0884/167.0494/184.0523/139.0307/131.0728/166.0416
Isoproturon	165.1022/134.0964/72.0444

Table S9 continued

Compound	Fragments
Ketamine	238.0991/220.0884/207.0568/189.0463/179.0619/165.0098/163.0306/152.0259/141.0104/125.0153/67.0542
Ketoprofen	255.1015/210.0990/209.0957/194.0724/177.0542/131.0490/105.0333/95.0490
Lamotrigine	256.0143/210.9813/166.0284/212.9784
Lamotrigine 2-N-glucuronide	256.0151/
Lauryl diethanolamide	106.0861/88.0756/227.2003/70.0652/270.2422/288.2520
Levamisole	205.0792/178.0683/188.0516/146.0962/88.0214/118.0646
Levofloxacin/ofloxacin	362.1511/319.1645/318.1609/261.1030/247.0874/233.0711/221.0719/205.0404/70.0651/58.0651
Lidocaine	235.1805/87.0996/86.0963/58.0654
MDMA	194.1173/163.0751/135.1165/133.0646/105.0697/58.0653
Mebendazole	296.1014/264.0754
Memantine	180.1744/163.1479/121.1010/107.0852/97.9911/93.0696/81.0697/67.0541
Metformin	130.1080/113.0716/88.0865/85.0505/71.0599
Methadone	105.0333/265.1582/223.1114/219.1164/159.1165/195.1166/187.1116
Metoclopramide	227.0570/184.0150/212.0336/183.0310
Metoprolol	268.1902/250.1435/226.1432/191.1067/159.0804/121.0645/116.1067/98.0963/86.0962/74.0600/72.0807/56.0497
Metoprolol acid	268.1536/226.1069/191.0700/165.0544/145.0645/116.1067/98.0963/74.06/72.0807/56.0497
Metribuzin-desamino	200.0848/172.0899/106.9917/89.0164/57.0701/116.0274
Mycophenolic acid	191.0349/275.1288/319.1183/179.0349/287.0924/192.0425/205.0504/203.0347/207.0660
N,N'-Diphenylguanidine	92.0495/94.0650/95.0489/119.0602/195.0915/212.1178
N-Acetyl-5-aminosalicylic acid	178.0499/152.0706/136.0392/108.0442/110.0600/80.0493
N-Acetylaminoantipyrine	228.1127/204.1128/159.0915/104.0493
Naproxen	170.0683/169.0650
Nicotine	132.0806/130.0650/120.0806/106.0649/84.0804/80.0494
N-Phenyl-1-naphthylamine	143.0722/128.0614/115.0536/92.0489
O-Desmethyl Venlafaxine	58.0653/107.0490/201.1270/246.1850/264.1957
O-Desmethyltramadol	58.0653/232.1684
Oxazepam	241.0522/269.0471/231.0680/287.0576/104.0492/128.0260/166.0052
Oxybenzone	229.0859/151.0389/105.0334/77.0386
Pentaethylene glycol (PEG)	89.0596/133.0857/151.0964/107.0701
Perfluorobutanesulfonic acid (PFBS)	79.9574/82.9609/84.9906/98.9559/118.9926/168.9892
Perfluorobutanoic acid (PFBA)	168.9892/212.0748
Perfluorooctanoic acid (PFOA)	168.9894/218.9859
Perfluoropentanoic acid (PFPeA)	218.9859
Pregabalin	143.1066/142.1225/125.0959/124.1119/107.0854/97.1010/83.0854/69.0699/59.0494/55.0545

Table S9 continued

Compound	Fragments
Propranolol	260.1640/218.1176/183.0802/157.0645/155.0852/129.0692/116.1067/98.0962/86.0962/74.0600/58.0651/56.0497
R-Methyl-benzotriazole (R = 1, 4, 5)	134.071/79.0541/106.49/121.9663
Ritalinic acid	220.1330/174.1275/84.0807
Rosuvastatin	258.1391/300.1495/272.1547/270.1389
Secbumeton	170.1039/198.1350/152.118/142.0723/128.0818/114.0661/100.0504/96.0556
Sertraline	91.0542/129.0698/158.9754/196.9918/275.0375
Sitagliptin	174.0524/193.0695/235.0799/391.0984/408.1237
Sotalol	255.1156/213.0688/176.1305/134.0838/133.0760/106.0649
Sulfamethoxazole	254.0589/188.0815/160.0867/156.0111/147.0789/108.0442/99.0551/92.0493/68.0495
Sulpiride	342.1480/214.0166/112.1119/98.0964/84.0807/58.0654
Tapentadol	222.1850/177.1270/135.0802/121.0646/107.0490
Temazepam	255.0670/177.0205/180.0201
Terbutryn	186.0806/242.1430/171.057/158.0496/144.0591/138.0774/116.0278
Testosterone propionate	345.2425/271.2054/213.1640/189.1638/175.1480/145.1010/123.0803/109.0647/97.0647/83.0491/69.0701
Tetradecylamine	214.2525/85.1012/71.0855/57.0676
Tetrakis(2-hydroxypropyl)ethylenediamine	160.1324/142.1219/102.0908/84.0803/98.0959
Theobromine	163.0606/122.0581/108.0551/69.0444
Theophylline/ Paraxanthine	124.0499/69.0444/96.0551/125.0536
Tramadol	264.1950/246.1850/58.0653
Tri(chloropropyl) phosphate	98.9841/174.9920/116.9946/77.0152
Tributyl citrate	129.0180/185.0807/157.0130/139.0025/111.0072/89.0595/68.9971/57.0701
Tributyl phosphate	116.9945/98.9841/57.0701/155.0471
Triethanolamine	150.1124/132.1018/114.0912/106.0860/88.0756/70.0652
Triethylene glycol monobutyl ether	89.0596/73.0648/83.0854/90.0631/101.0959/107.0699/133.0561
Trimethoprim	291.1475/275.1137/261.0982/245.1033/230.1162/123.0664
Tris(2-butoxyethyl) phosphate	55.0544 /57.0701 / 98.9840 /101.0959/83.0854/ 124.9995/ 143.0102/ 59.0493
Venlafaxine	58.0654/121.0647/147.0804/159.0802/173.0962/183.1183/193.1850/215.1430
Vildagliptin	304.2010/154.0972/151.1116/127.0863/107.0853/97.0759/93.0697/70.0651/59.0494

Table S10. Scoring system for the alternative prioritization procedure based on the chromatographic peak areas and PNEC values.

Chromatographic peak area			**PNEC**			**Ubiquity**	
Percentil	Area threshold	Score	Percentil	PNEC value threshold (µg/L)	Score	Frequency of occurrence	Score
20%	2,181,764	**0.8**	20%	0.21	**5**	1 campaign	**0.5**
40%	7,433,214	**1.6**	40%	0.99	**4**		
60%	24,420,439	**2.4**	60%	3.56	**3**	2 campaigns	**1**
80%	91,927,261	**3.2**	80%	14.92	**2**		
100%	2,603,205,440	**4**	100%	6,840	**1**		

Table S11. Score system for the prioritization process based on the peak areas and PNEC values of the compounds detected at the irrigation point in both campaigns (summer and winter)

Compound	Areas	Score Areas	Score campaigns	PNEC (µg/L)	Score PNEC	Total Score
1,2-benzisothiazol-3(2H)-one	704,246	0.8	0.5	379	1	2.3
10,11-Dihydro-10,11-dihydroxycarbamazepine	136,189,705	4	1	1.91	3	8
10,11-Dihydro-10-Hydroxycarbazepine	78,736,372	3.2	1	4.03	2	6.2
2-(3,4-dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile	4,381,333	1.6	1	1.57	3	5.6
2(3H)-Benzothiazolone	51,977,226	3.2	0.5	14	2	5.7
2-(Methylthio)benzothiazole	63,734,639	3.2	0.5	1.14	3	6.7
2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)	9,985,065	2.4	0.5	0.085	5	7.9
2-Ethylhexyl diphenyl phosphate	92,324,287	3.2	0.5	0.018	5	8.7
2-Mercaptobenzothiazole	2,305,720	0.8	0.5	0.76	4	5.3
3,3,5,5-Tetramethyl-1-pyrroline N-oxide	26,859,935	3.2	0.5	13.8	2	5.7
3-Hydroxycotinine	1,877,722	0.8	0.5	20.6	1	2.3
4-Formylaminoantipyrine	1,145,860,802	4	1	1000	1	6
4-indolecarbaldehyde	1,900,774	0.8	0.5	6.08	2	3.3
8-Hydroxyquinoline	90,276,617	3.2	1	1.99	3	7.2
Acephylline	1,626,513	0.8	0.5	13.2	2	3.3
Amisulpride	27,028,566	3.2	0.5	1.43	3	6.7
Amitriptyline	3,412,679	1.6	0.5	0.14	5	7.1
Amphetamine	47,634,182	3.2	0.5	24.8	1	4.7
Ampyrone/ 4-aminoantipyrine	1,834,271	0.8	0.5	32.5	1	2.3
Atenolol	6,113,266	1.6	0.5	150	1	3.1
Azoxystrobin	12,797,978	2.4	1	0.2	5	8.4
Azoxystrobin acid	5,501,093	1.6	0.5	0.21	4	6.1
Benzoylecgonine	10,332,351	2.4	0.5	6840	1	3.9
Bezafibrate	291,604	0.8	0.5	2.3	3	4.3

Table S11 continued

Compound	Areas	Score Areas	Score campaigns	PNEC (µg/L)	Score PNEC	Total Score
Bisoprolol	12,978,670	2.4	0.5	3.18	3	5.9
Boscalid	5,790,429	1.6	0.5	11.6	2	4.1
Bupropion	2,840,306	1.6	0.5	4.4	2	4.1
Butyrophenone	38,307,009	3.2	0.5	1.75	3	6.7
Caffeine	34,632,858	3.2	0.5	1.2	3	6.7
Caprolactam	2,603,205,440	4	1	67.40	1	6
Carbamazepine	96,308,554	4	1	0.05	5	10
Carbamazepine-10,11-epoxide	1,089,705	0.8	0.5	2.57	3	4.3
Carbendazim	10,085,701	2.4	0.5	0.15	5	7.9
Cetirizine	24,853,418	2.4	1	0.41	4	7.4
Chlorpheniramine	399,516	0.8	0.5	1.56	3	4.3
Ciprofloxacin	4,592,809	1.6	0.5	0.089	5	7.1
Citalopram	10,412,011	2.4	1	16	1	4.4
Clindamycin	787,911	0.8	0.5	0.1	5	6.3
Clopidogrel	1,225,882	0.8	0.5	3.21	3	4.3
Clopidogrel carboxylic acid	16,689,100	2.4	1	0.65	4	7.4
Cocaine	1,995,832	0.8	0.5	2.28	3	4.3
Cotinine	6,031,735	1.6	0.5	10	2	4.1
DEET	381,008,235	4	0.5	88	1	5.5
Desacetyl diltiazem	2,582,415	1.6	0.5	0.36	4	6.1
desmethylcitalopram	7,316,943	1.6	0.5	0.5	4	6.1
Desmethyldiazepam	18,239,787	2.4	1	0.43	4	7.4
Dextromethorphan	4,557,018	1.6	0.5	3.32	3	5.1
Dextrorphan/levorphanol	10,766,046	2.4	0.5	1.36	3	5.9

Table S11 continued

Compound	Areas	Score Areas	Score campaigns	PNEC (µg/L)	Score PNEC	Total Score
Diazepam	7,250,467	1.6	1	0.29	4	6.6
Diazinon	1,427,457	0.8	1	0.01	5	6.8
Diclofenac	2,317,957	1.6	0.5	0.05	5	7.1
Dicyclohexylamine	194,594,184	4	0.5	1.60	3	7.5
Dicyclohexylurea	61,883,608	3.2	0.5	5.91	2	5.7
Diethyl phthalate	21,050,888	2.4	0.5	16	1	3.9
Diltiazem	1,370,584	0.8	0.5	0.23	4	5.3
Diphenhydramine	1,143,231	0.8	0.5	0.99	3	4.3
Diphenylamine	127,885,222	4	0.5	1.2	3	7.5
Diuron	27,151,237	3.2	0.5	0.07	5	8.7
Ecgonine methyl ester	3,378,260	1.6	0.5	88.8	1	3.1
Emtricitabine	6,479,965	1.6	1	23.8	1	3.6
Ephedrine	4,272,290	1.6	0.5	3.62	2	4.1
Fenofibric acid	160,665	0.8	0.5	2.35	3	4.3
Flecainide	130,718,339	4	1	0.64	4	9
Fluconazole	30,126,100	3.2	1	0.25	4	8.2
Flufenamic acid	1,932,496	0.8	1	0.4	4	5.8
Gabapentin	76,346,190	3.2	1	10	2	6.2
Galaxolidone	147,537,245	4	1	0.1	5	10
Hydroxy-bupropion	4,702,311	1.6	0.5	11.8	2	4.1
Isoproturon	17,125,234	2.4	1	0.32	4	7.4
Ketamine	20,565,183	2.4	0.5	5.71	2	4.9
Ketoprofen	433,119	0.8	0.5	2.1	3	4.3
Lamotrigine	181,268,490	4	1	10	2	7

Table S11 continued

Compound	Areas	Score Areas	Score campaigns	PNEC (µg/L)	Score PNEC	Total Score
Levamisole	10,583,716	2.4	1	1.81	3	6.4
Lidocaine	86,993,060	3.2	1	4.67	2	6.2
MDMA	12,243,616	2.4	0.5	0.216	4	6.9
Mebendazole	230,739	0.8	0.5	0.16	5	6.3
Memantine	25,193,965	3.2	1	1.84	3	7.2
Metformin	198,713,057	4	1	156	1	6
Methadone	8,418,179	2.4	1	0.84	4	7.4
Metoprolol	2,487,618	1.6	0.5	8.6	2	4.1
Metoprolol acid	91,662,578	3.2	1	49.9	1	5.2
Metribuzin-desamino	22,688,523	2.4	0.5	46.8	1	3.9
Mirtazapine	520,239	0.8	0.5	1	3	4.3
N,N'-Diphenylguanidine	147,006,447	4	1	0.86	4	9
N-Acetylaminoantipyrine	408,373,817	4	1	100	1	6
N-Phenyl-1-naphthylamine	240,273,564	4	0.5	0.06	5	9.5
O-Desmethyl Venlafaxine	280,376,328	4	1	0.0061	5	10
O-desmethyltramadol	184,847,728	4	1	9.12	2	7
Ofloxacin/levofloxacin	1,940,291	0.8	1	0.021	5	6.8
Oxazepam	8,653,874	2.4	1	0.37	4	7.4
Oxybenzone	333,810,270	4	1	0.67	4	9
Pentaethylene glycol (PEG)	74,885,290	3.2	0.5	202	1	4.7
Perfluorobutanesulfonic acid	115,781,657	4	1	4.08	2	7
Perfluorobutanoic acid (PFBA)	13,404,718	2.4	1	27.80	1	4.4
Perfluoroheptanoic acid (PFHpA)	1,099,390	0.8	0.5	0.50	4	5.3
Perfluorohexanoic acid (PFHxA)	25,876,100	3.2	0.5	140.00	1	4.7

Table S11 continued

Compound	Areas	Score Areas	Score campaigns	PNEC (µg/L)	Score PNEC	Total Score
Perfluorooctanoic acid (PFOA)	39,178,760	3.2	1	0.18	5	9.2
Perfluoropentanoic acid (PFPeA)	32,985,111	3.2	1	3.91	2	6.2
Pregabalin	18,777,672	2.4	1	66.1	1	4.4
Propranolol	4,834,741	1.6	0.5	0.41	4	6.1
Ritalinic acid	6,432,319	1.6	0.5	14.2	1	3.1
Secbumeton	3,354,509	1.6	1	0.048	5	7.6
Sertraline	313,098	0.8	0.5	0.091	5	6.3
Sitagliptin	39,352,462	3.2	1	0.92	4	8.2
Sotalol	4,156,110	1.6	0.5	6.52	2	4.1
Sulfamethoxazole	7,548,802	1.6	1	0.1	5	7.6
Sulpiride	57,801,159	3.2	1	4.09	2	6.2
Tapentadol	100,940,372	4	1	2.47	3	8
Tebuconazole	531,948	0.8	0.5	0.24	4	5.3
Temazepam	19,773,671	2.4	1	0.071	5	8.4
Terbutryn	130,363,394	4	1	0.065	5	10
Theobromine	12,001,253	2.4	0.5	100	1	3.9
Theophylline/ Paraxanthine	13,738,154	2.4	0.5	0.1	5	7.9
Tramadol	309,180,190	4	1	8.65	2	7
Tri(chloropropyl) phosphate	134,058,966	4	0.5	7.39	2	6.5
Tributyl phosphate	132,693,070	4	0.5	66	1	5.5
Triethanolamine	7,404,318	1.6	0.5	93.7	1	3.1
Trimethoprim	966,427	0.8	0.5	0.5	4	5.3
Tris(2-butoxyethyl) phosphate	45,496,776	3.2	1	0.14	5	9.2
Venlafaxine	122,889,016	4	1	0.0061	5	10
Vildagliptin	14,665,719	2.4	1	9.75	2	5.4

Table S12. Comparison of the 50 priority compounds of both prioritization methods.

Order	Priority list (peak areas)	Priority list (semi-quantification)
1	**Carbamazepine***	**O-Desmethyl Venlafaxine**
2	**Galaxolidone**	**Venlafaxine**
3	**O-Desmethyl Venlafaxine**	**Galaxolidone**
4	**Terbutryn**	**Theophylline/ Paraxanthine**
5	**Venlafaxine**	**Oxybenzone**
6	**N-Phenyl-1-naphthylamine**	**N-Phenyl-1-naphthylamine**
7	**Perfluorooctanoic acid (PFOA)**	**2-Ethylhexyl diphenyl phosphate**
8	**Tris(2-butoxyethyl) phosphate**	**Terbutryn**
9	**Flecainide**	**Carbamazepine**
10	**N,N'-Diphenylguanidine**	**N,N'-Diphenylguanidine**
11	**Oxybenzone**	Caprolactam
12	**2-Ethylhexyl diphenyl phosphate**	**Caffeine**
13	**Diuron**	**Sulfamethoxazole**
14	Azoxystrobin	**Temazepam**
15	**Temazepam**	**Tris(2-butoxyethyl) phosphate**
16	**Fluconazole**	**MDMA**
17	**Sitagliptin**	**Secbumeton**
18	**10,11-Dihydro-10,11-dihydroxycarbamazepine**	**Flecainide**
19	**Tapentadol**	**Ofloxacin/levofloxacin**
20	**2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)**	**Sitagliptin**
21	**Carbendazim**	**Butyrophenone**
22	**Theophylline/ Paraxanthine**	**Diuron**
23	**Secbumeton**	**Carbendazim**
24	**Sulfamethoxazole**	**2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)**
25	Dicyclohexylamine	Sulpiride
26	Diphenylamine	**10,11-Dihydro-10,11-dihydroxycarbamazepine**
27	**Cetirizine**	**Clopidogrel carboxylic acid**
28	**Clopidogrel carboxylic acid**	**Diclofenac**
29	Desmethyldiazepam	Lidocaine
30	**Isoproturon**	**Oxazepam**
31	Methadone	**Amisulpride**
32	**Oxazepam**	Azoxystrobin
33	8-Hydroxyquinoline	**Perfluorooctanoic acid (PFOA)**
34	Memantine	Azoxystrobin acid
35	**Amitriptyline**	**Diazinon**
36	Ciprofloxacin	Clindamycin
37	**Diclofenac**	Propranolol
38	Lamotrigine	**Tapentadol**
39	**O-desmethyltramadol**	**Tramadol**
40	Perfluorobutanesulfonic acid (PFBS)	Diltiazem
41	**Tramadol**	**Amitriptyline**
42	**MDMA**	Diphenylamine
43	**Diazinon**	**Isoproturon**
44	**Ofloxacin/levofloxacin**	**Fluconazole**
45	2-(Methylthio)benzothiazole	**Cetirizine**
46	**Amisulpride**	Desacetyl diltiazem
47	**Butyrophenone**	**O-desmethyltramadol**
48	**Caffeine**	Dextrorphan/levorphanol
49	Diazepam	Gabapentin
50	Tri(chloropropyl) phosphate	Desmethylcitalopram

*Compounds in bold are present in both lists.

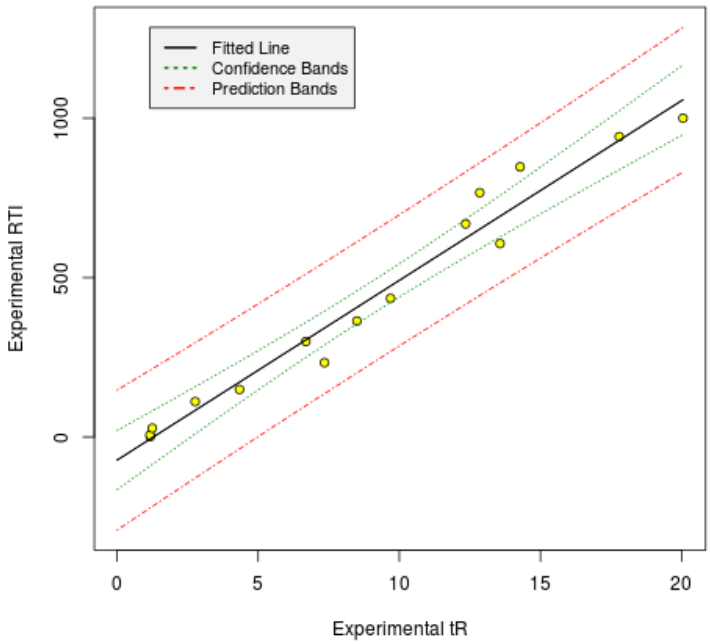

Figure S1. RTI (ESI+) calibration curve obtained through the RTI platform developed by the University of Athens (http://rti.chem.uoa.gr/. Compounds in table S3.

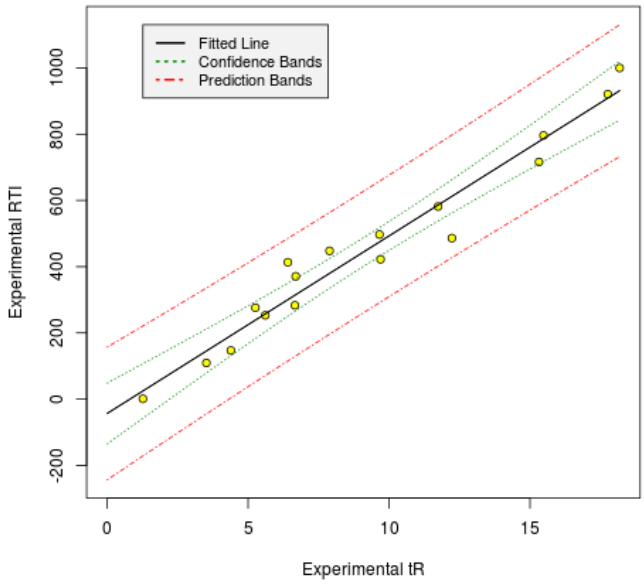

Figure S2. RTI (ESI-) calibration curve obtained through the RTI platform developed by the University of Athens (http://rti.chem.uoa.gr/). Compounds in table S4.

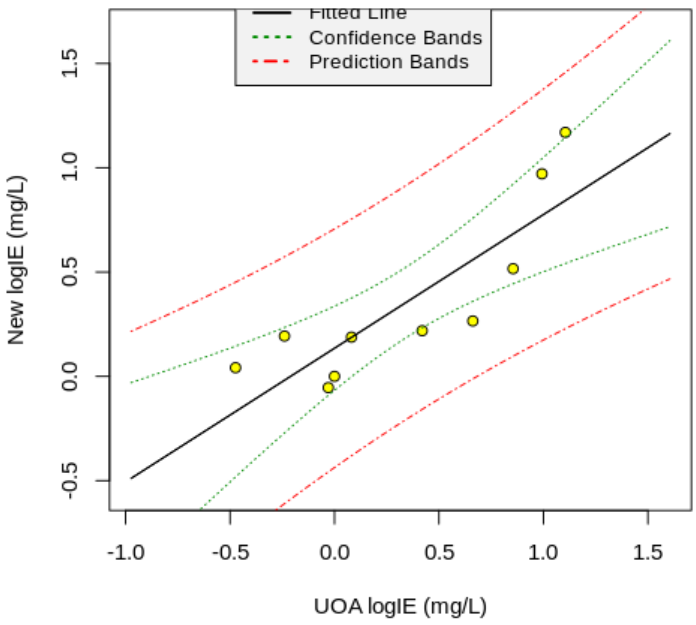

Figure S3. Harmonization of logIE after obtaining the slopes for each calibrant of the semi-quantification process. Calculations and graphical report wereobtained through the online application developed by the University of Athens (UoA) (http://trams.chem.uoa.gr/semiquantification/)

3.3. Attenuation strategies for the removal of pesticide residues from water

As stated in the introduction, CECs are poorly removed from WWTPs. This includes pesticides that, summed to the overspread in crop fields, enter the environment via wastewater (Köck-Schulmeyer et al., 2013). Therefore, there is an increasing demand on developing new attenuation strategies for these compounds that could also be applied in agricultural areas. At this point, bioremediation techniques appear to be an interesting alternative to traditional wastewater treatment processes. Non-biological treatments have already been tested, as the use of nanofiltration, reverse osmosis or electron-Fenton oxidation, among others, for pesticides elimination showing a highly cost-effective performance (Musbah et al., 2013; Plakas & Karabelas, 2012; Zhao et al., 2012).

Compared to physical and chemical-based treatments, bioremediation approaches present some advantages in terms of sustainability and cost that make them a suitable alternative. Moreover, recalcitrant compounds may behave differently against the complex enzymatic systems of some of the microorganisms normally used in bioremediation strategies. This is the case of the fungus Trametes versicolor, a white-rot fungi able to degrade lignin, a highly stable polymer, due to the combination of intra- and extracellular enzymes that non-specifically oxidize the molecule. On the other hand, microalgae-based treatment systems also present numerous benefits showing a high rate nutrients and pollutants removal (via bio- and photodegradation), together with some operational advantages (Muñoz & Guieysse, 2006).

In this context, the works described in the scientific publications #4 and #5 aimed at determining the capability of two bioremediation approaches, the white-rot fungi Trametes versicolor and microalgae, respectively, on degrading some of the most ecotoxicologically relevant pesticides and shed light in the biodegradation processes identifying the TPs formed during the treatments and the enzymes involved in the pesticide transformation.

These works were performed as part of the BECAS project in collaboration with the Chemical, Biological and Environmental Engineering

Department from the Autonomous University of Barcelona, who were responsible for optimizing the operational parameters of the bioremediation systems and their scale-up from batch laboratory experiments to a pilot plant. The BECAS project (Spanish State Research Agency and European Regional Development Fund (ERDF), CTM2016-75587-C2-2-R) aimed at determining the presence of pesticides in water and soils and studying their biodegradation through new bioremediation processes.

3.3.1. Scientific publication #4

Remediation of bentazone contaminated water by Trametes versicolor: Characterization, identification of transformation products, and implementation in a trickle-bed reactor under non-sterile conditions

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Journal of Hazardous Materials, 409 (2021), 124476

(DOI: https://doi.org/10.1016/j.jhazmat.2020.124476)

Journal of Hazardous Materials 409 (2021) 124476

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Remediation of bentazone contaminated water by *Trametes versicolor*: Characterization, identification of transformation products, and implementation in a trickle-bed reactor under non-sterile conditions

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ARTICLE INFO

Editor: Dr. R. Debora

Keywords:
Pesticide
Bioremediation
Fungal reactor
Biodegradation products
Suspect screening

ABSTRACT

Bentazone, an herbicide widely applied in rice and cereal crops, is widespread in the aquatic environment. This study evaluated the capacity of *Trametes versicolor* to remove bentazone from water. The fungus was able to completely remove bentazone after three days at Erlenmeyer-scale incubation. Both laccase and cytochrome P450 enzymatic systems were involved in bentazone degradation. A total of 19 transformation products (TPs) were identified to be formed during the process. The reactions involved in their formation included hydroxylations, oxidations, methylations, N-nitrosation, and dimerization. A laccase mediated radical mechanism was proposed for TP formation. In light of the results obtained at the Erlenmeyer scale, a trickle-bed reactor with *T. versicolor* immobilized on pine wood chips was set up to evaluate its stability during bentazone removal under non-sterile conditions. After 30 days of sequencing batch operation, an average bentazone removal of 48% was obtained, with a considerable contribution of adsorption onto the lignocellulosic support material. Bacterial contamination, which is the bottleneck in the implementation of fungal bioreactors, was successfully addressed by this particular system according to its maintained performance. This research is a pioneering step forward to the implementation of fungal bioremediation on a real scale.

1. Introduction

Pesticides are the most predominant chemical substances used in agriculture to prevent and control pests. Although their use has substantial benefits in crop yields and food storage, the environmental damage and human health risks that may result from pesticide application cannot be ignored (Bernardes et al., 2015). Herbicides constitute one of the pesticide groups most frequently used in the EU during the last decade (Eurostat, 2019). Among all currently used herbicides, bentazone (3-isopropyl-1 H-2,1,3-benzothiadiazine-4(3 H)-one-2,2-dioxide) is mainly applied against dicotyledonous weeds in cereals, beans, alfalfa, and other crops worldwide. Different studies have shown that bentazone has a very low rate of mineralization in the environment (EFSA, 2015), low sorption to soil (soil adsorption coefficient K_{oc} = 55.3), and relatively high solubility in water (7112 mg L^{-1}) (PPDB, 2020). These properties favor leaching (moderate Groundwater Ubiquity Score (GUS) index = 1.95) (PPDB, 2020) and run-off of the pesticide from the soil and, thus, explain its ubiquity in groundwater (Lopez et al., 2015; Hakoun et al., 2017; Malaguerra et al., 2012) and surface water (Laganà et al., 2002; Kuster et al., 2008; Köck-Schulmeyer et al., 2013; Loos et al., 2010; Papadakis et al., 2018). Note that the soil type, pH, and the amount and type of organic material in the soil play also an important role in pesticide mobility, and hence, its fate in the environment. Although bentazone is not highly bioaccumulative, it has also been detected in aquatic organisms (Álvarez-Muñoz et al., 2019). Concerns on the environmental occurrence of bentazone led to consider its

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https://doi.org/10.1016/j.jhazmat.2020.124476
Received 30 June 2020; Received in revised form 10 October 2020; Accepted 2 November 2020
Available online 4 November 2020

inclusion in the list of priority substances in surface water under the EU Water Framework Directive (EC, 2008).

Under aerobic conditions, bentazone can partially be degraded in soils by microbiota, resulting in the major formation of 8-hydroxy-bentazone, and other transformation products (TPs), namely, 6-hydroxy-bentazone, N-methyl-bentazone, and 2-amino-N-propan-2-ylbenzamide (AIBA) (Knauber et al., 2000). In the soil upper layers, it is also susceptible to photolysis, transforming into 8-hydroxy-bentazone, among other photoproducts (EFSA, 2015). A considerable higher resistance of bentazone to hydrolytic processes (half-lives of 46-99 days) compared with photolytic ones (half-lives of 2.3–7.5 h) has been reported elsewhere (Song et al., 2019). This, together with the aforementioned physical-chemical properties, makes bentazone highly recalcitrant in groundwater (Broholm et al., 2001). This is confirmed by a numerical model that predicted the presence of bentazone in the aquifer after 20 years since its last field application (Aisopou et al., 2015). Thus, the development of innovative technologies able to reduce or even eliminate bentazone concentrations in the environment is justified and urgent.

Different strategies have been explored for the removal of bentazone from water. Mir et al., (2014) and Berberidou et al., (2017) proposed the use of advanced oxidation processes (AOPs) such as TiO_2 heterogeneous photocatalysis. The pathways involved in the photocatalytic decomposition of bentazone could be elucidated after identifying 21 phototransformation products formed during the process (e.g., hydroxyl and/or keto derivatives, and dimers) (Berberidou et al., 2017). Compared with chemical and physical treatments, bioremediation appears as a more environmentally friendly and cheaper solution than AOPs. However, not every bioremediation technique may be suitable for all the contaminants and environmental matrices (Azubuike et al., 2016). For instance, while bentazone persisted in wastewater effluent treated in a pilot-scale membrane bioreactor (MBR) (González et al., 2006), a significant removal was observed in groundwater using different sand filters at full-plant scale (Hedegaard and Albrechtsen, 2014). Such a promising result was only observed in one of the three investigated drinking waterworks, which also presented high levels of methane in the groundwater. This finding spurred additional studies that proved the co-metabolic transformation of bentazone into hydroxyl-bentazone by methanotrophic bacteria, in the presence of methane (Hedegaard et al., 2018). Biodegradation of bentazone in biological rapid sand filtration occurred mainly through three biotransformation pathways: oxidation of the isopropyl moiety to the corresponding carboxylic acid, oxidation of the aromatic ring leading to ring cleavage and subsequent decarboxylation, and N-methylation followed by oxidation to a carboxylic acid (Hedegaard et al., 2019).

White-rot fungi (WRF) are basidiomycetes well-known for their ability to aerobically degrade lignin and various xenobiotics. This is possible thanks to their collaborative group of enzymes including extracellular laccases and peroxidases, and the intracellular cytochrome P450 system (Olicón-Hernández et al., 2017; Magan et al., 2010). Thus, using the non-specific oxidizing enzymes of WRF seemed advantageous for the elimination of recalcitrant pollutants such as bentazone (Harms et al., 2011). In this regard, *Trametes versicolor,* one of the most common WRF species, has been demonstrated to biodegrade a wide number of recalcitrant pollutants including ibuprofen, carbamazepine, atenolol, propranolol, clofibric acid, different estrogens, and even the widespread antimicrobial triclosan (Hundt et al., 2000; Jelic et al., 2012; Marco-Urrea et al., 2009, 2010).

The objectives of this work were to determine the ability of the fungus *T. versicolor* to degrade bentazone and to characterize the degradation process in terms of the enzymatic systems responsible for the abatement and the main degradation pathways leading to the identified transformation products (TPs) formed during the process. Furthermore, the stability of a trickle bed reactor (TBR) with immobilized *T. versicolor* to remove bentazone under non-sterile conditions was evaluated.

2. Materials and methods

2.1. Fungus and culture conditions

Trametes versicolor ATCC® 42530™ was acquired from American Type Culture Collection (Manassas, VA) and maintained by subculturing every 30 days on 2% (w/v) malt extract petri dishes (pH 4.5) at 25 °C. Blended mycelial suspension and fungal pellets were prepared using malt extract medium (20 g L^{-1}, pH 4.5) according to a previously described method (Blánquez et al., 2004). Briefly, four agar plugs of 1 cm^2 area from the petri dish grown with the fungi were transferred into 500 mL Erlenmeyer flasks containing 150 mL fresh medium. Then, cultures were incubated at 25 °C under continuous shaking with an orbital shaker (135 rpm). After 5–7 days of incubation, the harvested dense mycelial biomass was blended using an X10/20 homogenizer (Ystral GmbH, Germany), thereby obtaining the mycelial suspension inoculum. Pellets of *T. versicolor* were prepared in 1 L Erlenmeyer flask by inoculating 1 mL of mycelial suspension into 250 mL of fresh medium. After 5–7 days of incubation under shaking condition (135 rpm) at 25 °C, the mycelia pellets were collected and washed with sterile distilled water.

The defined medium used in the Erlenmeyer-scale degradation experiments consisted of 8 g of glucose L^{-1}, 3.3 g of ammonium tartrate L^{-1}, 1.68 g of dimethyl succinate L^{-1}, 10 mL of micronutrients L^{-1}, and 100 mL of macronutrients L^{-1} (Kirk et al., 1978). The pH of the defined medium was adjusted to 4.5 with 1 M HCl or 1 M NaOH. As for TBR experiments, the harvested mycelial suspension was used to inoculate autoclaved pine wood (*Pinus* sp.) chips as reported by Torán et al., (2017). Inoculated pine wood chips were statically incubated for 4 weeks at 25 °C before use.

2.2. Chemicals, reagents, and agricultural wastewater

Analytical standards (purity > 99%) of bentazone and its deuterated analog bentazone-d_7, dimethyl succinate, commercial laccase purified from *T. versicolor* (20 AU mg^{-1}), the laccase mediator 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (98% pure), and the cytochrome P450 inhibitor 1-aminobenzotriazole (ABT) (98% pure) were purchased from Sigma-Aldrich (Barcelona, Spain). Commercial herbicide KAOS-B (bentazone, 48%) was obtained from SAPEC AGRO (Barcelona, Spain). Formic acid (purity, > 98%) and ammonium acetate were provided by Merck (Darmstadt, Germany). Chromatographic grade acetonitrile (ACN) used for liquid chromatography (LC)-UV analysis was purchased from Carlo Erba Reagents S.A.S. (Val de Reuil Cedex, France). Water and ACN used for ultra-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) analysis were LC-MS-grade and supplied by Merck (Darmstadt, Germany) or Thermo Fisher Scientific (USA). A stock solution of high-purity bentazone (5 mg L^{-1}) was prepared in ethanol and stored at –20 °C for LC-UV determination of bentazone concentrations. Stock solutions of high-purity bentazone and its deuterated analog were prepared in methanol at a concentration of 10 mg L^{-1} for TPs analysis.

The agricultural wastewater used in the TBR was directly collected from an irrigation channel in Gavà agricultural fields, located in the lower Llobregat River basin (Catalonia, NE Spain), and stored at 4 °C until use. The characteristics of this water are provided as supporting information (SI) in Table S1.

2.3. Degradation experiments in Erlenmeyer flasks

Degradation experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL of fresh defined medium spiked with the commercial bentazone solution at a final concentration of 10 mg L^{-1} of bentazone. This concentration is about 50 times higher than that reported in previous studies in surface waters impacted by rice-growing activities (0.13–0.18 mg L^{-1}) (Kuster et al., 2008; Barbieri et al.,

2020), but it was selected to facilitate the analytical assessment of the system performance. Briefly, pellets were transferred into each flask as inoculum, achieving a concentration of approximately 2.5 g d.w. L^{-1}. Afterward, the cultures were incubated at 25 °C under continuous shaking (135 rpm) for 7 days. Abiotic (non-inoculated) controls, as well as heat-killed culture (121 °C, 30 min) controls, both containing the pesticide were also prepared. All experiments were run in triplicate. Aliquot samples were taken at specific intervals of time during incubation (t = 0, 3, and 7 days) to measure bentazone and glucose concentrations, and laccase activity.

2.4. Experiments to evaluate the enzymatic system involved in bentazone degradation

To investigate the role of the different enzymatic systems of *T. versicolor* during bentazone biodegradation, experiments with purified laccase and adding a cytochrome P450 inhibitor were performed.

Laccase-mediated degradation experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL dimethyl succinate solution (1.68 g L^{-1}, pH 4.5) at a final enzyme activity of 1000 AU L^{-1}, and a pesticide concentration of 10 mg L^{-1}. The laccase mediator ABTS was added to a final concentration of 0.8 mM (Marco-Urrea et al., 2009), to evaluate its effect on bentazone degradation. Abiotic (no laccase) and flasks without the addition of the mediator were also prepared as experimental controls. The flasks were incubated using an orbital shaker (135 rpm) at 25 °C for 24 h. At designated incubation times, 1 mL aliquots were collected and mixed with 100 μL of 1 M HCl to stop the reaction to measure bentazone concentration. After 24 h, 1 mL of the culture was withdrawn from the laccase control to measure the enzymatic activity.

Experiments designed to evaluate the effect of inhibiting the cytochrome P450 system on bentazone degradation (10 mg L^{-1}) were also performed in Erlenmeyer flasks containing 2.5 g d.w. L^{-1} of fungal pellets. 1-aminobenzotriazole was added as an inhibitor, to a final concentration of 5 mM (Marco-Urrea et al., 2009). Then, pellet cultures were incubated for 42 h at 25 °C under continuous shaking (135 rpm). An experimental control that consisted of the pellet culture incubated in the absence of an inhibitor was run in parallel. Each experimental condition was conducted in triplicate. Aliquot samples were collected at designated times during the culture incubation for the analysis of bentazone concentration.

2.5. Degradation experiments for transformation products identification

Biodegradation experiments for the identification of TPs were carried out in 500 mL Erlenmeyer flasks, with 100 mL of fresh defined medium spiked with analytical-grade bentazone at a final concentration of 1 mg L^{-1}, and a concentration of fungal pellets of 2.5 g d.w. L^{-1}. Cultures were incubated in the dark at 25 °C under agitation (135 rpm) for 7 days. Abiotic (non-inoculated) and heat-killed culture (121 °C during 30 min) solutions, both containing 1 mg L^{-1} of bentazone were used as experimental controls. A solution containing fungal pellets but no bentazone was used also as a control to detect potential artifacts formed during fungal degradation. 4 mL samples were taken at 0 h, 6 h, 11 h, 24 h, 3 d, and 7 d, centrifuged at room temperature at 17,700g for 4 min, and stored at −20 °C until UPLC-HRMS analysis. All experimental conditions were run in triplicate.

2.6. Degradation experiments in a TBR bioreactor

A cylindrical TBR filled with pre-inoculated pine wood chips was set up (Fig. 1). One liter of agricultural wastewater (Table S1) fortified with the commercial bentazone solution to a final concentration of 10 mg L^{-1} of bentazone was loaded into the packing bed at the top of the reactor through a rotary distributor, and then collected in the reservoir tank placed at the bottom. The tank was equipped with a magnetic stirrer and

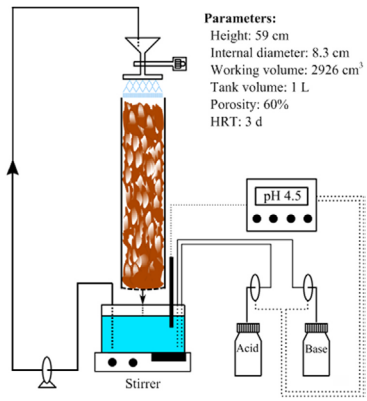

Fig. 1. Schematic representation of the experimental TBR set-up. HRT, hydraulic retention time.

a pH controller, which assisted in keeping the pH of the solution at 4.5 by adding 1 M HCl or 1 M NaOH during the duration of the experiment. To assess the role that adsorption by the lignocellulosic support plays, an identical reactor filled with non-inoculated pine wood chips was set as a control. Multiple runs were operated in sequencing batch reactor (SBR) mode with a 3-day cycle at room temperature. Wastewater was renovated after each batch experiment and a recycling ratio (RR) of 300 was adopted. Samples were taken from the tank at designated time intervals to measure bentazone concentration, laccase activity, heterotrophic plate counts (HPC), and chemical oxygen demand (COD).

2.7. Analytical methods

2.7.1. Laccase activity

Laccase activity was measured through the oxidation of 2,6-dymetoxyphenol (DMP) by the enzyme in the absence of a cofactor, using a modified version of the method for the manganese peroxidase system (Wariishi et al., 1992). Activity units per liter (AU L^{-1}) are defined as the amount of DMP in μM oxidized per minute. The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1}.

2.7.2. Bentazone concentration

Samples were firstly filtered through a Millipore Millex-GV PVDF membrane (0.22 μm). Then the residual bentazone concentration was determined using an HPLC Ultimate 3000 (Dionex, USA) equipped with a UV detector. Chromatographic analysis was achieved with a C18 reversed-phase column (Phenomenex®, Kinetex® EVO C18 100 Å, 4.6 mm × 150 mm, 5 μm), kept at 30 ºC, and a mobile phase consisting of water, containing 0.01% formic acid, (v/v) (A) and acetonitrile (B) at a constant flow rate of 0.8 mL min^{-1}. The organic gradient for chromatographic separation was as follows: 35% of B from 0 min to 5 min, then a linear increase of B to 45% from 5 min to 15 min, return to initial conditions in 1 min, and maintenance of initial conditions for 2 more min. The injection volume was 40 μL. The detection wavelength was set at 254 nm. The limit of detection was 0.5 mg L^{-1}.

2.7.3. Analyses for agricultural wastewater characterization

The absorbance at 650 nm was determined by a UNICAM 8625 UV/VIS spectrometer, and the conductivity was monitored by a CRISON MicroCM 2100 conductometer. The total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to the standard methods 2540 D and 2540 E, respectively (Baird et al., 2017). The total organic carbon (TOC) was determined using an Analytik Jena multi

N/C 2100 S/1 analyzer. The HPC results were reported as the logarithm of colony-forming units (CFU) per mL [lg (CFU mL^{-1})] using the spread-plate method with a plate count agar (PCA) following the standard method 9215 (Baird et al., 2017). The N-NH_4^+ concentration and the COD were analyzed using commercial kits LCK 303 and LCK 314 or LCK 114, respectively (Hach Lange, Germany). Chloride, sulfate, nitrite, and nitrate anions were measured by ion chromatography using a Dionex ICS-2000 equipped with Dionex IonPac AS18-HC column (250 mm × 4 mm) which was eluted at 1 mL min^{-1} with a 13 mM KOH aqueous solution.

2.7.4. Identification of TPs

The analysis of the biotransformation products formed during bentazone degradation was done with a UPLC system Acquity (Waters, Milford, MA, USA) coupled to a hybrid quadrupole-Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Sample components were separated on a chromatographic column Purospher® STAR RP-18 endcapped Hibar® (150 ×2.1 mm, 2 μm) (Merck, Darmstadt, Germany), using an organic gradient of a mobile phase consisting of water with 20 mM of ammonium acetate (A) and acetonitrile (B) at a constant flow rate of 0.3 mL min^{-1}. After 1 min of isocratic conditions (5% of acetonitrile), the proportion of the organic component increased linearly to 20% in 2 min, to 80% in the next 3 min, and 100% in one more min. After 2 min of static conditions, a fast gradient restored the mobile phase to initial conditions (in 0.5 min), which were maintained during 4 min for column re-equilibration. The injection volume was set to 10 μL.

The HRMS analysis was performed using a heated electrospray ionization (HESI) source operated in the negative ion mode. Ion source conditions were: capillary voltage, − 2500 V; temperature, 350 °C; sheath gas flow rate, 40 arbitrary units; auxiliary gas, 10 arbitrary units; vaporizer temperature, 400 °C. Nitrogen (>99.98%) was employed as the sheath, auxiliary, and sweep gas. Accurate mass measurements were done in data-dependent acquisition mode. First, a full MS scan was conducted using a full width at half maximum (FWHM) resolution of 70,000 (at *m/z* 200). The *m/z* range covered expanded from 70 to 600 to include also phase II metabolites. Then, data-dependent MS/MS scan events were recorded for the n = 5 most intense ions (>10^5 counts) detected in each scan with an FWHM resolution of 17,500 (at *m/z* 200) and using a normalized collision-induced dissociation energy of 40. MS data acquisition was done using Xcalibur v4.1.

Data processing was conducted with Compound Discoverer v.3.1 (Thermo Fisher Scientific, San Jose, CA, USA). This software was used for peak alignment and deconvolution (with a retention time maximum shift of 2 min and a mass tolerance of 5 ppm), feature grouping, and elemental composition prediction. A handmade MS library of suspect compounds, that included bentazone TPs published in the peer-reviewed literature (Berberidou et al., 2017; EFSA, 2015; Knauber et al., 2000; Song et al., 2019) and those obtained with the EAWAG-BBD Pathway Prediction tool (EAWAG-BBD), and different chemical compound databases (ChemSpider, mzCloud, mzVault) were used to assign potential compound identities. Then, the *m/z* list initially generated was manually revised to search for potential TP candidates (i.e., those present only in experimental reactors at t = 3 and 7 days and absent in control (t=0) and blank samples), and their MS^2 spectra were examined for structure identification. Fragment rationalization and structure proposal of the TPs identified were supported by the software ChemDraw Professional v18.1 (PerkinElmer Informatics).

2.7.5. Data analysis

The degradation efficiency of the different investigated systems was evaluated through the percentage of bentazone remaining in the solution, according to the equation:

$$\text{Degradation percentage} = \frac{C_0 - C_t}{C_0} \times 100\%$$

where C_0 is the initial bentazone concentration at t_0 and C_t corresponds to the residual bentazone concentration in the culture at a given time t. The mean and standard deviation (SD) of triplicate measurements were calculated. The statistical significance of the changes observed, with a level of confidence of 95% ($\alpha = 0.05$), was determined using SPSS v22.0.

3. Results

3.1. Degradation of bentazone by T. versicolor

The capability of *T. versicolor* to degrade bentazone was firstly evaluated under sterile conditions at Erlenmeyer-scale. *T. versicolor* completely removed 10 mg L^{-1} of bentazone within 3 days (Fig. 2). Comparing bentazone concentration decay in the abiotic and the heat-killed controls, 9% of the bentazone removal could be ascribed to adsorption of the compound onto the biomass. A maximum laccase activity (9.12 AU L^{-1}) was also achieved after 3 days and then reduced during incubation. Concerning glucose, it was almost completely utilized after 3 days.

3.2. The role of laccase and cytochrome P450 enzymatic systems in the degradation of bentazone

Both laccase and cytochrome P450 systems have been reported to be involved in the degradation of recalcitrant environmental pollutants by filamentous fungi (Olicón-Hernández et al., 2017; Yang et al., 2017). Laccase, an extracellular enzyme, is a good indicator of fungal activity during the degradation process. The cytochrome P450 system, an intracellular enzymatic system, has been reported to catalyze the first detoxification step towards a wide range of toxic compounds in mammals and therefore, it could also play a key role in the degradation process of bentazone.

The participation of the laccase enzymatic system in bentazone degradation was investigated with *in vitro* experiments using commercial laccase. Results showed that the laccase system could degrade bentazone completely in the presence of the ABTS mediator after 1 h of treatment (Table S2). On the contrary, when ABTS was not present in the solution, only 11% of the initial bentazone concentration was degraded in the same period and this figure did not improve in 24 h of treatment. However, laccase was still active at the end of the incubation period (100.38 AU L^{-1}). Thus, our findings indicate that laccase is involved in the biotransformation process.

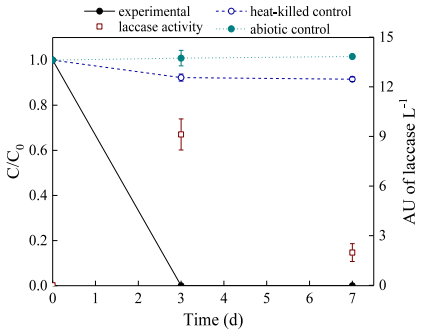

Fig. 2. Time-course of bentazone degradation by *T. versicolor* (y-axis on the left) and laccase activity during the process (y-axis on the right). C represents the residual concentration of bentazone in the sample (mg L^{-1}), and C_0 corresponds to the concentration of bentazone at the beginning of the experiment (10 mg L^{-1}). Values are the mean and standard deviation of three replicates.

To investigate the role of the cytochrome P450 enzymatic system in bentazone degradation, *in vivo* experiments were conducted in the presence of the cytochrome P450 inhibitor, 1-aminobenzotriazole. Bentazone degradation was considerably inhibited when 1-aminobenzotriazole was added into the system (Fig. 3). The initial decrease in bentazone concentration observed at the beginning of the experiments could be attributed to adsorption onto the fungal pellets (in line with the results obtained in the initial degradation experiments, Section 3.1). In any case, it is clear that the cytochrome P450 system also participates in bentazone degradation by *T. versicolor*.

3.3. Identification of TPs and main degradation pathways

The identification of TP candidates in Compound Discoverer-processed HRMS data required a filtration step. For this, different criteria were used including i) the ratio of the *m/z* feature between time zero and the different sampling times, ii) the presence/absence of the molecular ions in the abiotic and heat-killed controls, and iii) the retention time.

Main TPs and corresponding characteristic fragment ions detected using the workflow described in Section 2.7.4. and the aforementioned filtration step are shown in Table S3. Based on the TPs identified, a biodegradation pathway has been proposed (Fig. 4*)*. In total, 19 molecular ions were identified as TPs by UPLC-HRMS; however, a chemical structure could be only tentatively proposed for 8 of them. Their corresponding full MS chromatograms and MS^2 spectra are provided in Figs. S2-S20 as SI, and their time-evolution is shown in Fig. S1. As chemical structures are not confirmed with the analysis of reference standards, they are proposed with a confidence level of 3 in all cases except for TP494, for which no MS^2 data were obtained and, therefore, its identification fits a confidence level of 5 (Schymanski et al., 2014). As shown in Fig. S2 in SI, bentazone (t_R = 5.10 min) gets deprotonated under HESI(-) conditions (*m/z* 239.0496). Upon collision-induced dissociation of the precursor ion, fragment ions were detected at *m/z* 197.0021, *m/z* 175.0877, and *m/z* 132.0329, which correspond to the loss of the isopropyl moiety, the loss of the sulfonyl group, and the combined loss of the aforementioned moieties, respectively.

Although N-methyl-bentazone, a bentazone TP commonly found in the environment (EFSA, 2015; Knauber et al., 2000), was not detected during the degradation experiments, TP268 and TP284a were identified as oxidized forms of N-methyl-bentazone. TP268 appeared after 5 h while TP284a was formed at a later stage, which could indicate that TP284a results from the oxidation of TP268 (Fig. 4*)*. This kind of tertiary carbon oxidation is a reaction frequently attributed to the cytochrome P450 system (Guengerich, 2001). Moreover, cytochrome P450 may also

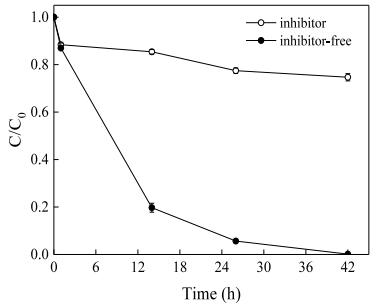

Fig. 3. Influence of the cytochrome P450 inhibitor 1-aminobenzotriazole on the degradation of bentazone by *T. versicolor*. C represents the residual concentration of bentazone in the sample (mg L^{-1}), and C_0 corresponds to the concentration of bentazone at the beginning of the experiment (10 mg L^{-1}). Values are the mean and standard deviation of three replicates.

catalyze the hydroxylation of the aromatic ring, resulting in the formation of TP256 (hydroxyl-bentazone, t_R = 4.62 min). 6-OH and 8-OH-bentazone are typical TPs formed by the soil microbiota metabolism (EFSA, 2015; Knauber et al., 2000); however, with the available structural information, it is not possible to indicate the isomer that corresponds to the TP formed during fungal degradation. Similar to TP284a, TP284b is also proposed to be formed after the carboxylation of bentazone. Due to its fragmentation pattern, carboxylation of TP284b could only be possible at the isopropyl moiety (Fig. 4*)*. The transformation of bentazone into TP285 was tentatively produced by hydroxylation and N-nitrosation of the free secondary amine of the parent compound. Microbiologically induced N-nitrosation has been reported for different species (Ji et al., 1986; Adjei et al., 2006) and resulted in the formation of stable products after secondary amine nitrosation (Chandan et al., 2013). Although the identity of this TP has not been confirmed yet, it is of special concern, because most N-nitroso compounds are classified as carcinogens (Lijinsky, 1999). Main transformation pathways, including oxidation of the isopropyl moiety, N-methylated oxidations, or hydroxylations of the aromatic ring are in line with those reported during microbial biodegradation in sand filters (Hedegaard et al., 2019).

The laccase catalytic mechanism consists of the abstraction of an electron from a substrate to produce free radicals (Magan et al., 2010; Kudanga et al., 2017). TP258 and TP286 seem to be formed from a hydroxylated form of bentazone through this laccase-mediated mechanism (Fig. 4). The formation of a radical in one of the hydroxyl groups of the aromatic ring could derive in the electronic rearrangement to produce an iminoquinone intermediate. Then, a nucleophilic attack of a free $HO^{\bullet}$ and the subsequent intramolecular ring closure results in the formation of TP258 (Fig. 5). Later, the addition of the hydroxyl-methyl group that had previously been eliminated from the iminoquinone intermediate could generate TP286. Regarding TP494, no MS^2 data were obtained; however, laccase induced dimerization of bentazone could be a plausible reaction already observed in nature (Knauber et al., 2000). Most of the remaining TPs were grouped on phase II metabolites, although no logical structure could be proposed for them. Their fragmentation pattern showed a common fragment ion at *m/z* 239.0496, corresponding to the molecular ion of bentazone. HRMS data provided a good insight into the biodegradation pathway carried out by *T. versicolor*; however, further work is needed for the identification of those TPs for which a plausible structure could not be proposed.

3.4. Removal of bentazone in TBR bioreactor

Once proved that *T. versicolor* was able to degrade bentazone, its degrading ability in time under non-sterile conditions was evaluated. For this, a TBR containing wood chips immobilized with the fungus was set up for the degradation of fortified agricultural wastewater, without the addition of other carbon sources. The TBR was operated in the SBR mode because information on its performance can be obtained in a short period, instead of waiting for the stationary-state as in continuous flow treatments. The operation of the TBR in SBR mode presents additional advantages over the purely continuous mode, such as higher removal efficiencies, and less energy consumption. However, the SBR system also presents some limitations such as the lack of automatization and consequently, the requirement of high maintenance during the operation. The bioreactor stability was assessed along with the operation. A 3-day cycle was arbitrarily established, ensuring that bentazone was not completely removed at the end of each batch so that the degradation performance of the TBR could be evaluated.

In previous experiences with TBR (Torán et al., 2017), high adsorption of the investigated pollutants onto the wood chips was observed. The adsorption rate is highly dependent on the physical-chemical properties of the pollutant. Thus, to evaluate the adsorption rate of bentazone on TBR, two reactors were set up: one containing pine wood chips colonized with the fungus (experimental

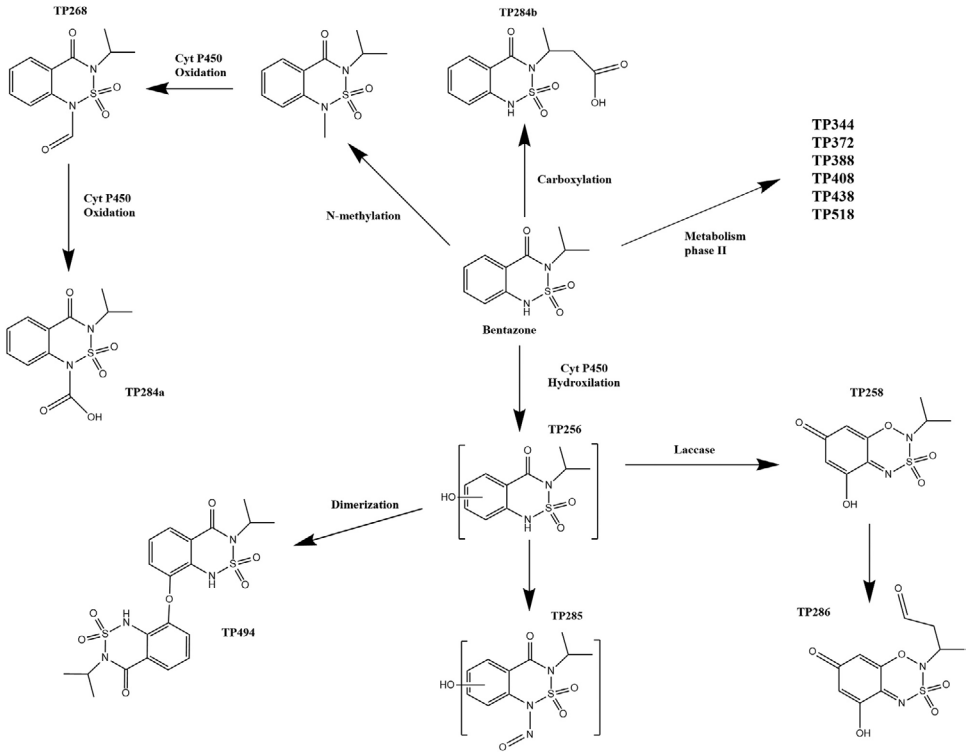

Fig. 4. Bentazone biodegradation by *T. versicolor*.

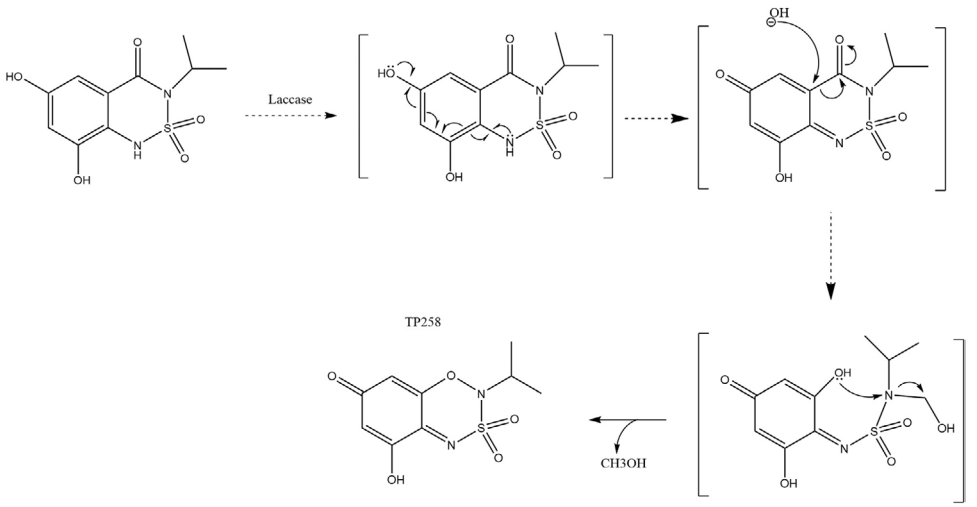

Fig. 5. Proposal of the laccase mediated radical reaction involved in the formation of TP258.

TBR) and another one filled only with the lignocellulosic material (control TBR). The results obtained using a recirculating flow of 70 mL min^{-1} are shown in Fig. 6. The laccase activity, measured throughout the treatment, reached a maximum value of 46.68 AU L^{-1} during the first batch (Fig. 6a). This could be explained by its accumulation within the static incubation. Then, the enzymatic activity kept at a low constant level and showed a decreasing trend, probably ascribed to the fungus aging behavior and biomass washing out.

Bentazone removal in the TBR was moderate (48% on average), and lower than that observed in lab-scale batch reactors. This could be explained by a lower amount of biomass in the TBR (approximately 4.2×10^{-3} g d.w. biomass/g dry wood, equivalent to 0.84 g d.w. L^{-1}) than in the Erlenmeyer reactors (2.5 g d.w. L^{-1}), the short operation time fixed for the TBR and the less available nutrients in the reactor (pine wood chips in the TBR vs the rich defined medium in the lab-scale batch reactors). Adsorption contributed to more than half (58%) of the removal, which is in line with the fact that the employed lignocellulosic materials serve as an effective sorbent for pesticide removal (Beltrán-Flores et al., 2020). The final concentration of bentazone after each batch is shown in Fig. 6b. As can be seen, adsorption decreased throughout the sequential batches, while the removal yield was always higher in the experimental TBR than in the control TBR. This suggests that the immobilized fungus maintained bioactive for 30 days. Thus, this result proves that the TBR tested, besides being very simple in terms of configuration, is highly cost-efficient, since there was neither addition of C nor N sources during the treatment to maintain the fungus activity, a reusable waste is used as support, and requires low operation energy. The highest operation expenses would result from the long retention times (3 days) and pH adjustment of the water.

The experimental reactor was stopped after one month of operation because its removal efficiency dropped to 29%, whereas the value in the control reactor was 11%. Although decreasing bioactivity was observed, the obtained results are still promising, considering that limited biomass was introduced without any other complementary nutrients. Hence, some improvements such as the replacement of the lignocellulosic support materials or scale-up of the reactor could be taken into consideration to increase the amount of immobilized biomass. Furthermore, the contact time between the immobilized fungus and the pesticide was quite low based on empirical calculations and therefore, the contribution from the cytochrome P450 system to bentazone degradation could be restricted.

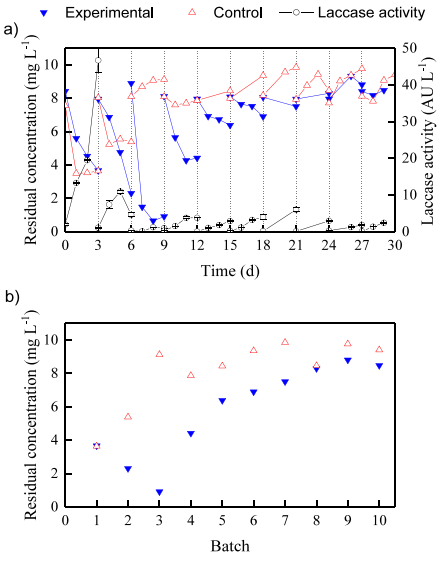

Fig. 6. Removal of bentazone by *T. versicolor* in sequencing batch TBR under non-sterile conditions. a. Time-course of bentazone variation during the treatment; b. The final concentration of bentazone in each batch.

HPC was monitored during the TBR treatment (Table 1). CFU count did not significantly increase during the treatment. It was indeed reduced within the first 4 batches compared to the original value (4.68 log CFU mL^{-1}, Table S1). This could be attributed to nutrient limitation. Thus this approach offers strong potential for dealing with bacterial contamination that represents one of the main barriers in the implementation of fungal reactors to treat wastewater (Torán et al., 2017). The adjustment of the wastewater pH to 4.5 is also useful to favor the fungus bioactivity and limit bacteria growth (Mir-Tutusaus et al., 2018). In this regard, fewer microbial counts were found in the experimental TBR than in the control. A reasonable hypothesis is that the more active the fungus is, the stronger the antagonistic interactions between the fungus and the bacteria may be (Folman et al., 2008). COD was also analyzed during the TBR operation, and it dropped in both experimental and control sets. Although the control reactor showed overall a lower COD than the experimental one, the COD content was still much higher than the original value (31.85 mg L^{-1}, Table S1). This could be explained by the addition of bentazone to the water, the elution of wood particles from the packed bed, and/or the wood rotting by *T. versicolor*, and needs to be addressed in future research.

4. Conclusions

T. versicolor could effectively degrade bentazone, during which both laccase and cytochrome P450 were involved. Up to 19 TPs were captured and identified, indicating that hydroxylations, oxidations, methylations, N-nitrosation, and dimerization played important roles during the detoxification process. The TBR system operated in SBR mode was effective to remove bentazone throughout 30 days of operation, and thus, represents a promising strategy to deal with bentazone contamination at a real scale. However, some improvements should be considered in future research to address the high COD levels resulting in water and the low biomass present in the reactor. The operation of the TBR without supplementing nutrients and at acidic pH values aids in its good performance under non-sterile conditions. While the latter would result in additional expenses during the implementation of the process at real-scale, they could be offset by the no-nutrients requirement. Although the toxicity of the treated water due to the potential formation of recalcitrant bentazone TPs during the process still needs to be addressed; overall, this work points out *T. versicolor* as a suitable candidate towards bentazone degradation and the adopted reactor system shows promise for bentazone bioremediation at a real scale.

CRediT authorship contribution statement

Manuel García Vara: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Kaidi Hu:** Investigation, Formal analysis, Writing - original draft, Writing - review &

Table 1
HPC and COD of the agricultural wastewater throughout the sequencing batch TBR treatment.

Batch	Microbial counts [Log (CFU) mL^{-1}]		COD (mg L^{-1})	
	Experimental	Control	Experimental	Control
2	2.90	6.28	590	433
4	3.90	5.99	438	325
6	6.10	5.80	318	254
8	5.99	6.59	288	225
10	5.87	5.71	248	205

editing. **Cristina Postigo:** Supervision, Writing - review & editing. **Lluc Olmo:** Investigation. **Gloria Caminal:** Supervision, Writing - review & editing. **Montserrat Sarra:** Supervision, Conceptualization, Resources, Writing - review & editing. **Mien López de Alda:** Supervision, Conceptualization, Resources, Writing - review & editing. 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.

Acknowledgments

This work has been supported by the Spanish State Research Agency of the Ministry of Economy and Competitiveness and co-financed by the European Regional Development Fund (ERDF) through the project BECAS (CTM2016–75587-C2–1-R and CTM2016–75587-C2–2-R). It has also received the support of the EU Horizon 2020 Research and Innovation programme through the WATERPROTECT project (727450), the Catalan Government (Generalitat de Catalunya) (Consolidate Research Group 2017-SGR-01404), and the Spanish Ministry of Science and Innovation (Project CEX2018–000794-S). The Department of Chemical, Biological and Environmental Engineering of the Universitat Autònoma de Barcelona is a member of the Xarxa de Referència en Biotecnologia de la Generalitat de Catalunya. K. Hu acknowledges the financial support from the Chinese Scholarship Council.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.124476.

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Supporting Information

Remediation of bentazone contaminated water by *Trametes versicolor*: characterization, identification of transformation products, and implementation in a trickle-bed reactor under non-sterile conditions

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Table S1. Physicochemical characterization of the agricultural wastewater used in the trickle-bed reactor (TBR).

Parameter*	Agricultural wastewater**
pH	7.67 ± 0.04
Absorbance at 655 nm	0.047 ± 0.003
Conductivity (mS cm^{-1})	2.25 ± 0.07
TSS (mg L^{-1})	6.33 ± 1.36
VSS (mg L^{-1})	4.27 ± 1.50
TOC (mg L^{-1})	16.23 ± 0.81
HPC [lg (CFU mL^{-1})]	4.68 ± 4.43
Ammonia	n.d.
COD (mg O_2 L^{-1})	31.85 ± 0.78
Chloride (mg Cl L^{-1})	570.50 ± 3.76
Sulfate (mg SO_4^{-2} L^{-1})	51.24 ± 0.06
Nitrite (mg NO_2^- L^{-1})	2.78 ± 0.06
Nitrate (mg NO_3^- L^{-1})	0.08 ± 0.01

*TSS: total suspended solids; VSS: volatile suspended solids; TOC: Total organic carbon; HPC: heterotrophic plate counts expressed as the logarithm of colony-forming units per mL, COD: chemical oxygen demand.

** Mean value and standard deviation of triplicate measurements are shown.

Table S2. Bentazone degradation by laccase enzymatic system in the presence and absence of ABTS.

Time	C/C_0		
	Abiotic control	Laccase	ABTS
0 h	1	1	1
30 min	0.97 ± 0.039	0.88 ± 0.039	0.068 ± 0.0079
1 h	0.96 ± 0.037	0.89 ± 0.037	0
2 h	0.98 ± 0.041	0.89 ± 0.038	0
4 h	0.98 ± 0.039	0.88 ± 0.039	0
6 h	0.98 ± 0.041	0.91 ± 0.013	0
10 h	0.97 ± 0.042	0.89 ± 0.037	0
24 h	0.98 ± 0.041	0.88 ± 0.024	0

Note: Each value represents the mean of triplicate measurements ± SD.

Table S3. Accurate mass measurements of the parent and fragment ions corresponding to bentazone TPs as determined by UPLC-(HESI-)-HRMS/MS, elemental composition with their theoretical m/z, relative mass error, and ring and double bond equivalents (RDB).

Compound	t_R (min)	*m/z* measured $[M-H]^-$	Elemental composition	*m/z* theoretical $[M-H]^-$	Relative mass error (ppm)	RDB
Bentazone	5.10	*239.0486**	*$C_{10}H_{11}N_2O_3S$*	*239.0496*	*0.651*	*6.5*
		197.0014	$C_7H_5N_2O_3S$	197.0021	-0.707	6.5
		175.0863	$C_{10}H11N_2O$	175.0877	-1.654	6.5
		132.0313	$C_7H_4N_2O$	132.0329	-3.895	7.0
TP256 (OH-BTZ)	4.62	*255.0439**	*$C_{10}H_{11}N_2O_4S$*	*255.0445*	*-2.356*	*6.5*
		197.0015	$C_7H_5N_2O_3S$	197.0021	-0.707	6.5
		191.0814	$C_{10}H_{11}N_2O_2$	191.0826	-5.709	6.5
		132.0314	$C_7H_4N_2O$	132.0329	-3.895	7.0
		79.9557	O_3S	79.9574	-21.173	1.0
TP268	5.32	*267.0440**	*$C_{11}H_{11}N_2O_4S$*	*267.0445*	*2.382*	*7.5*
		224.9967	$C_8H_5N_2O_4S$	224.9970	0.961	7.5
		203.0815	$C_{11}H_{11}N_2O_2$	203.0826	0.127	7.5
		160.0265	$C_8H_4N_2O_2$	160.0278	-1.680	8.0
TP285	5.84	*284.0342**	*$C_{10}H_{10}N_3O_5S$*	*284.0347*	*-1.494*	*7.5*
		241.9869	$C_7H_4N_3O_5S$	241.9872	-3.365	7.5
		220.0719	$C_{10}H_{10}N_3O_3$	220.0728	-4.292	7.5
		197.0014	$C_7H_5N_2O_3S$	197.0021	-0.707	6.5
		177.0169	$C_7H_3N_3O_3$	177.0180	-6.210	8.0
		132.0311	$C_7H_4N_2O$	132.0329	-3.895	7.0
TP284a	2.76	*283.0389**	*$C_{11}H_{11}N_2O_5S$*	*283.0394*	*-1.892*	*7.5*
		240.9915	$C_8H_5N_2O_5S$	240.9919	-3.549	7.5
		239.0485	$C_{10}H_{11}N_2O_3S$	239.0496	-4.502	6.5
		219.0765	$C_{11}H_{11}N_2O_3$	219.0775	-4.681	7.5
		197.0014	$C_7H_5N_2O_3S$	197.0021	-0.707	6.5
		177.0286	$C_8H_5N_2O_3$	177.0300	-11.328	7.5
		176.0210	$C_8H_4N_2O_3$	176.0227	-10.001	8
TP284b	5.67	*283.0388**	*$C_{11}H_{11}N_2O_5S$*	*283.0394*	*-2.104*	*7.5*
		239.0487	$C_{10}H_{11}N_2O_3S$	239.0496	-3.875	6.5
		197.0014	$C_7H_5N_2O_3S$	197.0021	-0.707	6.5
		175.0863	$C_{10}H_{11}N_2O$	175.0877	-1.654	6.5
		133.0393	$C_7H_5N_2O$	133.0402	-10.720	6.5
		132.0313	$C_7H_4N_2O$	132.0329	-12.581	7.0
		59.0115	$C_2H_3O_2$	59.0133	-39.190	1.5
TP438	5.55	*437.0781**				
		239.0486	$C_{10}H_{11}N_2O_3S$	239.0496	0.651	6.5
		197.0012	$C_7H_5N_2O_3S$	197.0021	-0.707	6.5
		175.0862	$C_{10}H11N_2O$	175.0877	-1.654	6.5
TP344	5.96	*343.0748**				
TP372	5.84	*371.0913**				
		239.0487	$C_{10}H_{11}N_2O_3S$	239.0496	0.651	6.5
		197.0015	$C_7H_5N_2O_3S$	197.0021	-0.707	6.5
		175.0863	$C_{10}H11N_2O$	175.0877	-1.654	6.5
		160.0389				
		116.0489				

Table S3. (cont.)

Compound	t_R (min)	*m/z* measured $[M-H]^-$	Elemental composition	*m/z* theoretical $[M-H]^-$	Δm (ppm)	RDB
TP408	5.83	*407.0676**				
		239.0488	$C_{10}H_{11}N_2O_3S$	239.0496	0.651	6.5
		175.0864	$C_{10}H11N_2O$	175.0877	-1.654	6.5
		132.0312	$C_7H_4N_2O$	132.0329	-3.895	7.0
TP435	5.85	*434.0869**				
		241.0442				
		239.0487	$C_{10}H_{11}N_2O_3S$	239.0496	0.651	6.5
		198.9963				
		175.0866	$C_{10}H11N_2O$	175.0877	-1.654	6.5
		116.0490				
		61.9867				
TP432	5.85	*431.1922**				
		239.0488	$C_{10}H_{11}N_2O_3S$	239.0496	0.651	6.5
		175.0863	$C_{10}H11N_2O$	175.0877	-1.654	6.5
		132.0312	$C_7H_4N_2O$	132.0329	-3.895	7.0
		59.0122	$C_2H_3O_2$	59.0133		
TP430	5.92	*429.2486**				
		239.0490	$C_{10}H_{11}N_2O_3S$	239.0496	0.651	6.5
		167.0308				
		59.0116	$C_2H_3O_2$	59.0133	-20.435	1.5
TP258	5.39	*257.0230**	$C_9H_9N_2O_5S$	257.0238	0.745	6.5
		214.9756	$C_6H_3N_2O_5S$	214.9763	-0.551	6.5
		193.0604	$C_9H_9N_2O_3$	193.0619	-0.511	6.5
		151.0134	$C_6H_3N_2O_2$	151.0144	-2.771	6.5
		134.0105	$C_6H_2N_2O_2$	134.0116	-3.797	7.0
TP286	4.64	*285.0182**	$C_{10}H_9N_2O_6S$	285.0187	2.234	7.5
		257.0232	$C_9H_9N_2O_5S$	257.0238	1.912	6.5
		214.9759	$C_6H_3N_2O_5S$	214.9763	1.016	6.5
		193.0610	$C_9H_9N_2O_3$	193.0619	1.301	6.5
		151.0136	$C_6H_3N_2O_2$	151.0144	-1.072	6.5
		150.0055	$C_6H_2N_2O_2$	150.0071	-3.132	7.0
		135.0182	$C_6H_2N_2O_2$	135.0195	-5.213	6.5
		77.9638	NO_2S	77.9650	-7.7483	1.5
TP220	3.15	*219.0070**				
		191.0119				
		163.0168				
		121.9537				
		97.9866				
		77.9637	NO_2S	77.9650	-8.729	1.5
TP388	6.25	*387.1225**				
		239.0489	$C_{10}H_{11}N_2O_3S$	239.0496	0.651	6.5
		141.0154				
		116.9710				
		59.0116	$C_2H_3O_2$	59.0133	-20.604	1.5
TP518	5.85	*517.1489**				
TP192	4.54	*191.0119**				
		163.0168				
		121.9537				
		59.0121	$C_2H_3O_2$	59.0133	-10.606	1.5
TP494	5.85	*493.0856**	$C_{20}H_{21}N_4O_7S_2$	493.0857	-0.165	12.5

*corresponds to the *precursor ion*

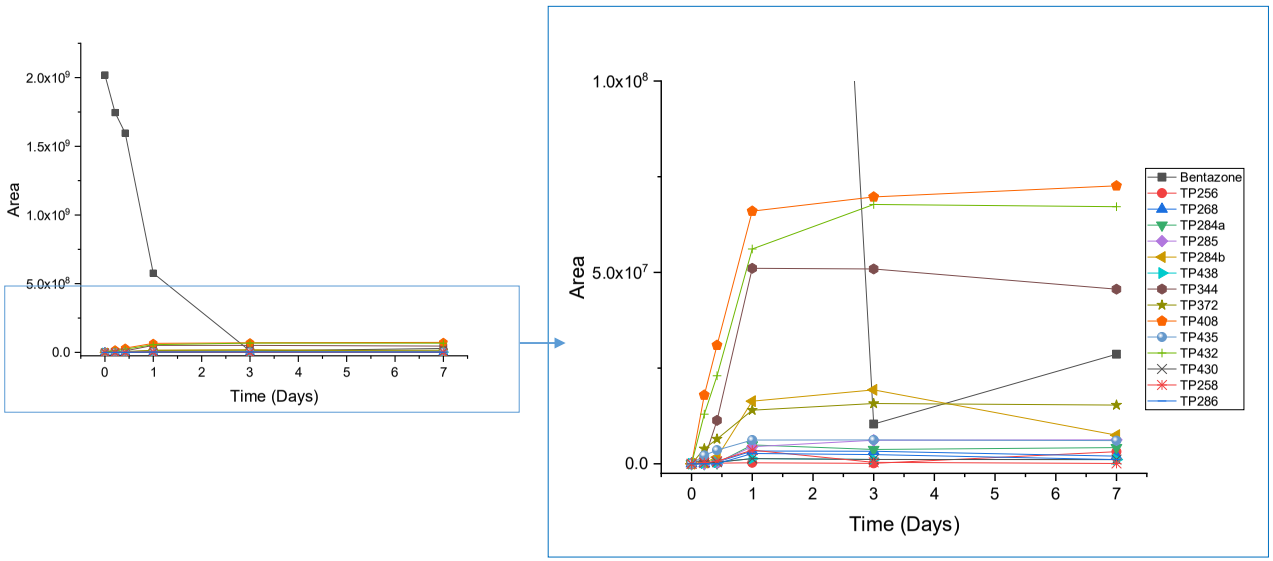


Figure S1. Evolution of bentazone and TPs during *T.versicolor* - mediated biodegradation experiments at lab-scale.

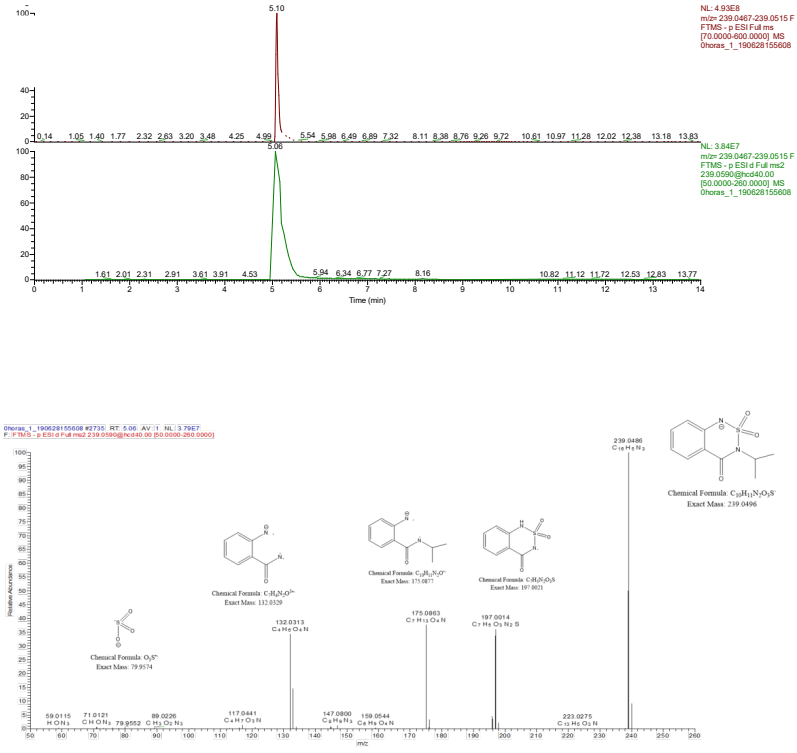


Figure S2. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 239.0496 (bentazone).

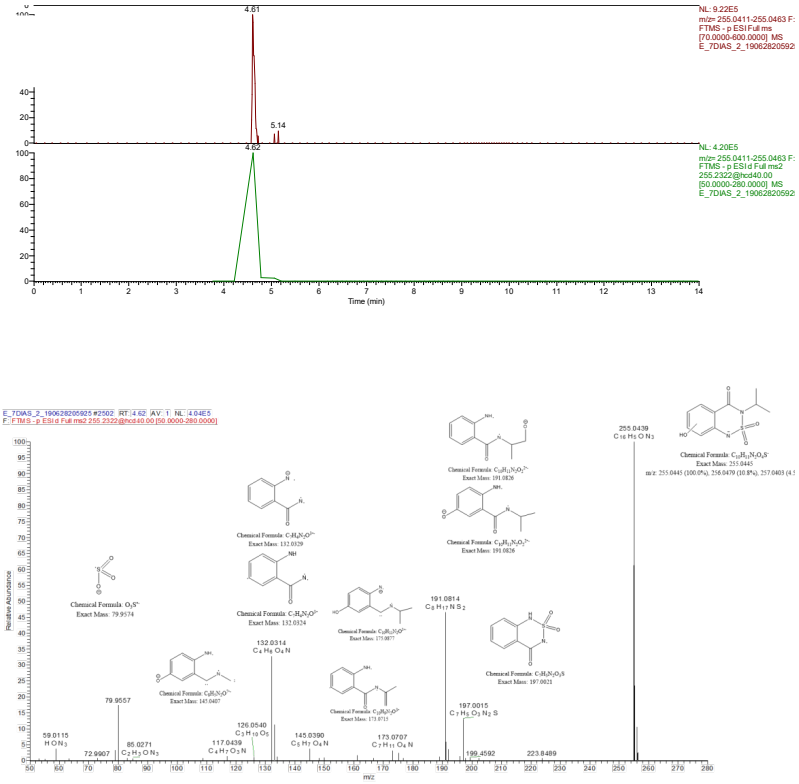


Figure S3. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 255.0445 (TP256, OH-BTZ).

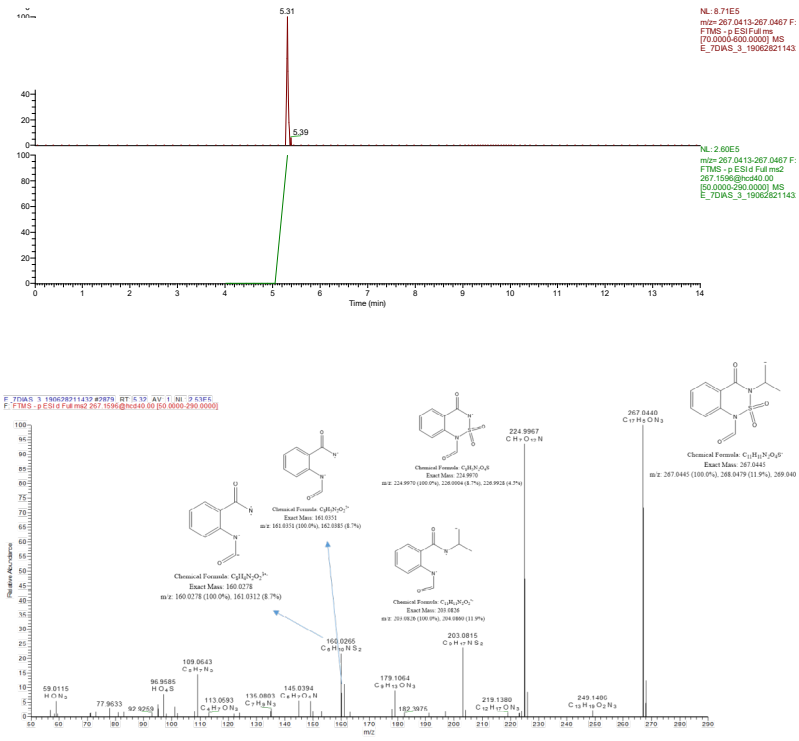


Figure S4. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 267.0445 (TP268).

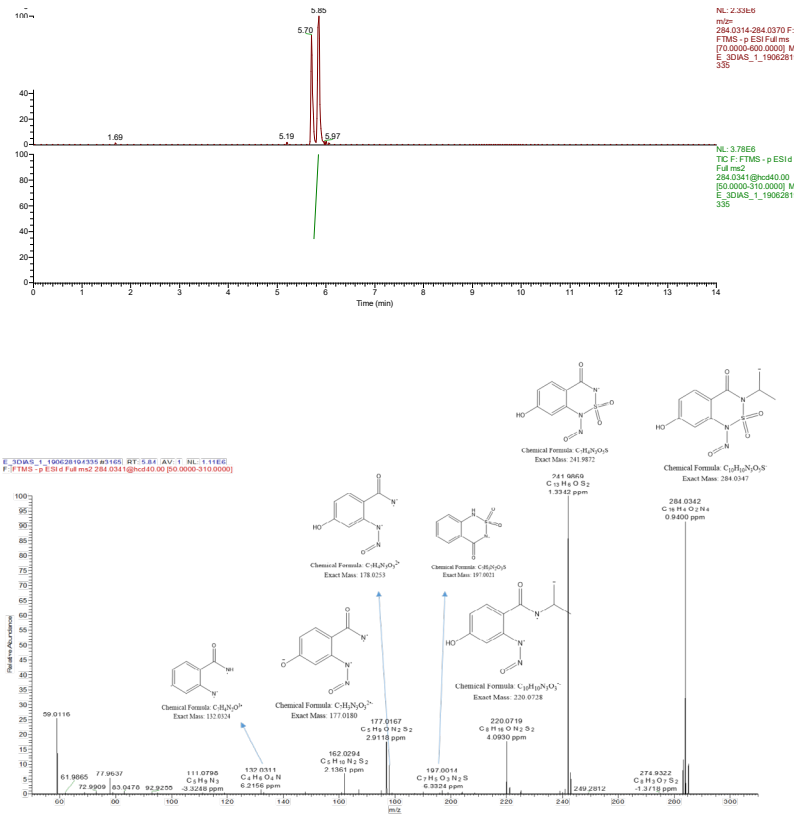


Figure S5. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 284.0347 (TP285).

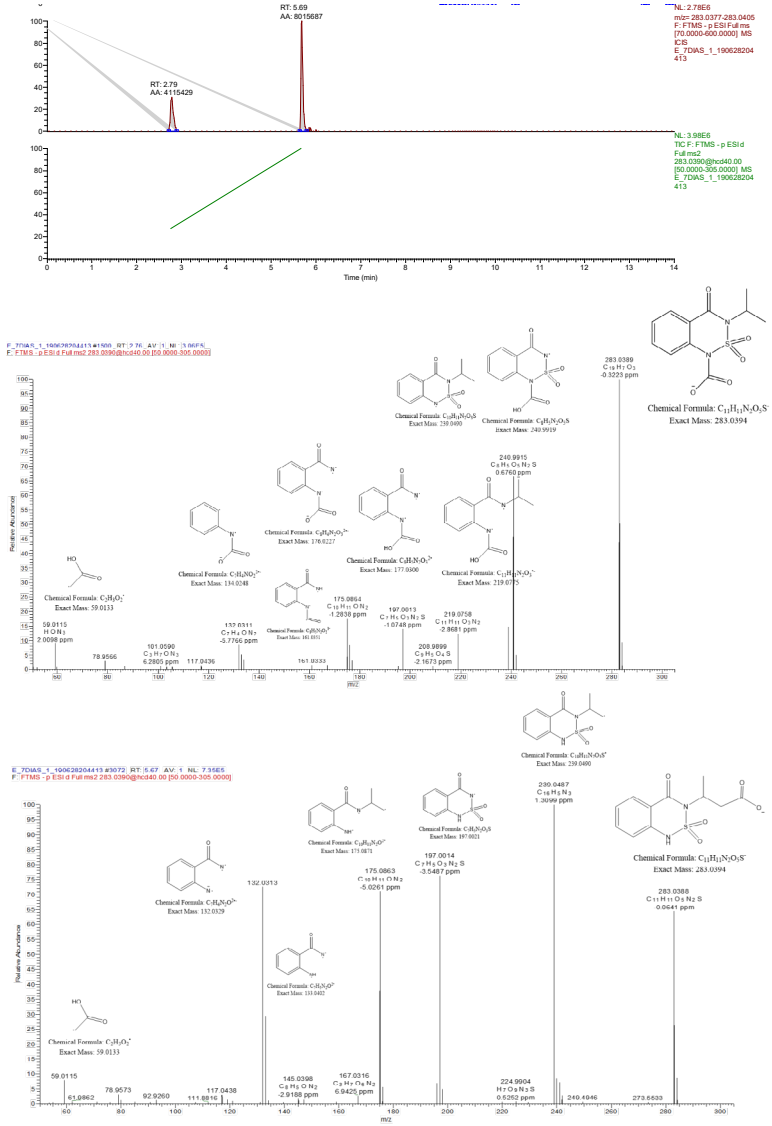


Figure S6. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectra of *m/z* 283.0394 at t_R 2.76min and 5.67 min (TP284a and TP284b, respectively).

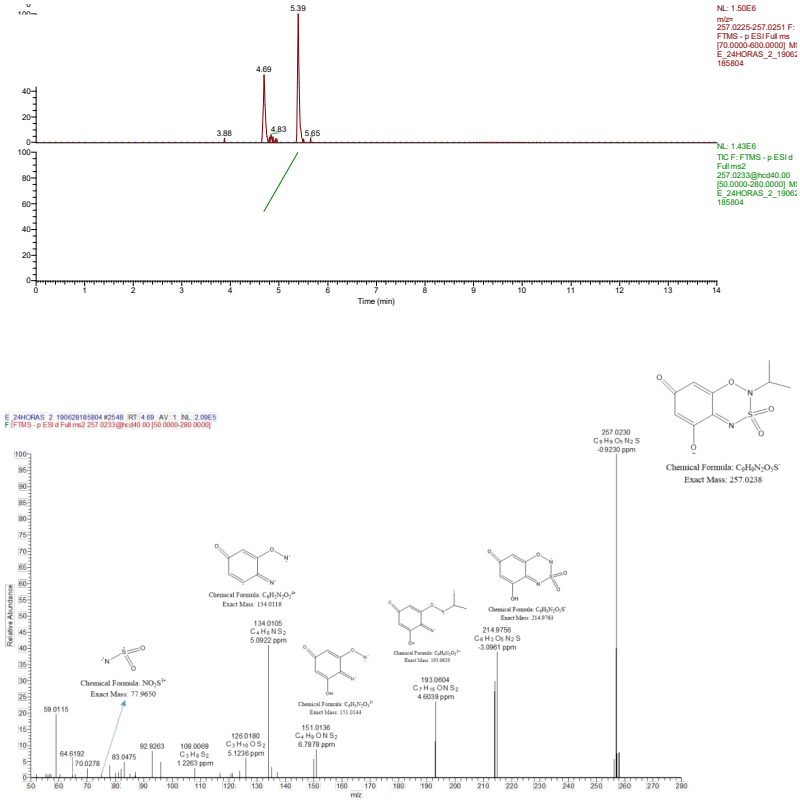


Figure S7. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 257.0238 (TP258).

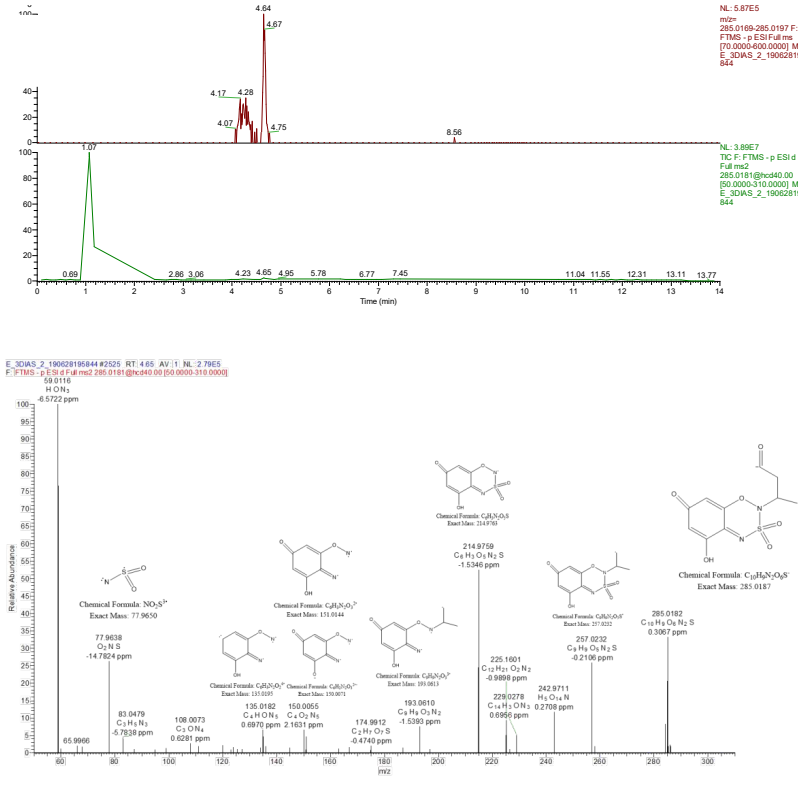


Figure S8. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 285.0187 (TP286).

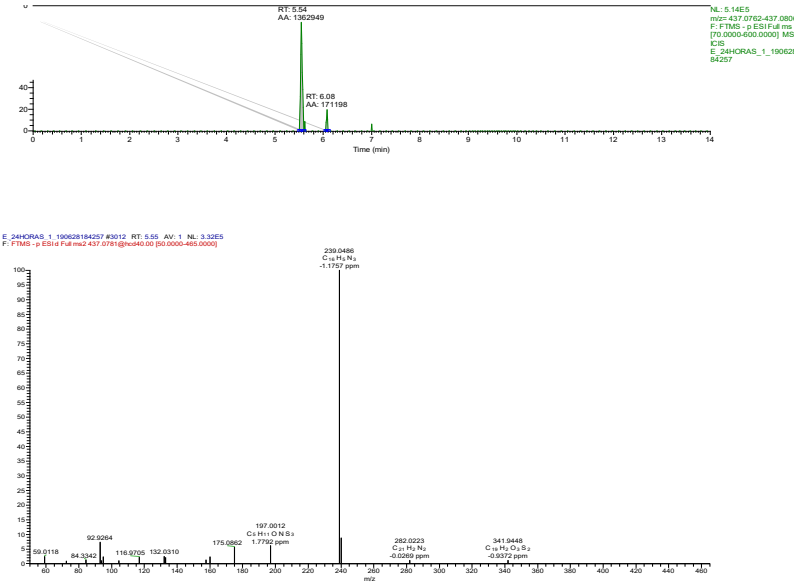
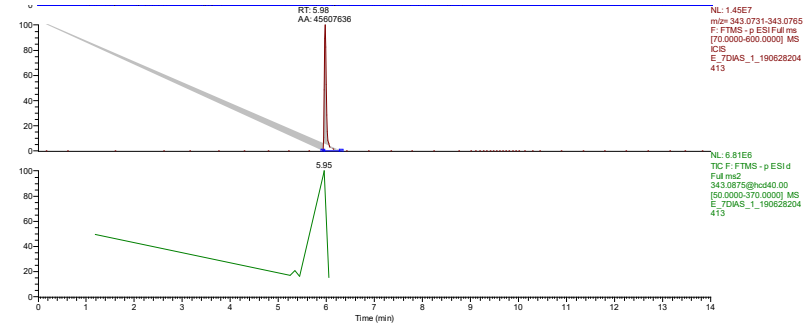


Figure S9. Extracted ion chromatogram and MS^2 spectrum of *m/z* 437.0781 (TP438).



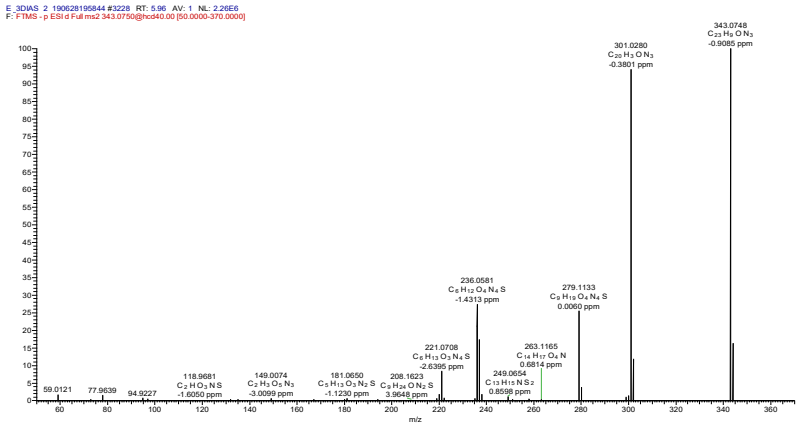


Figure S10. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 343.0748 (TP344).

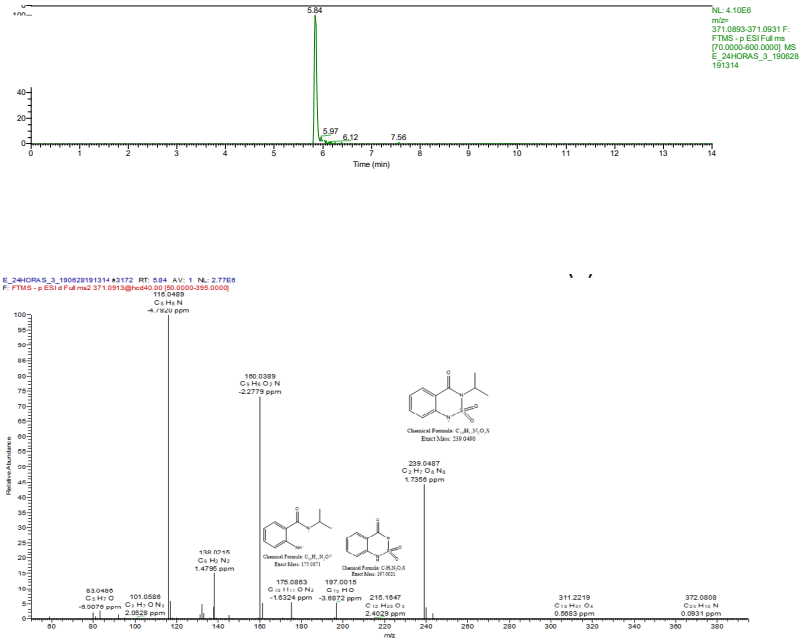


Figure S11. Extracted ion chromatogram and MS^2 spectrum of *m/z* 371.0913 (TP372).

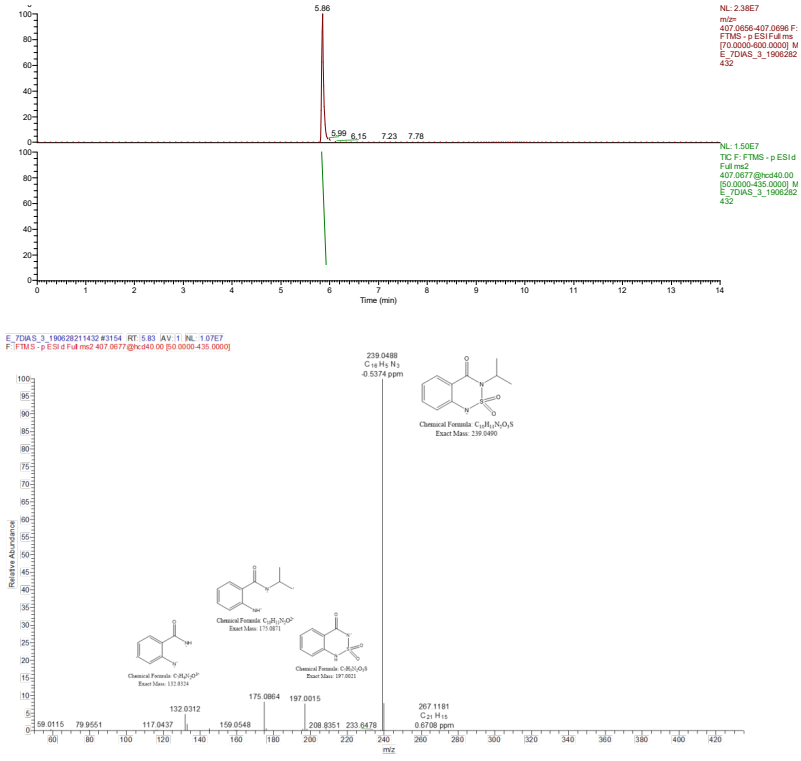


Figure S12. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 407.0676 (TP408).

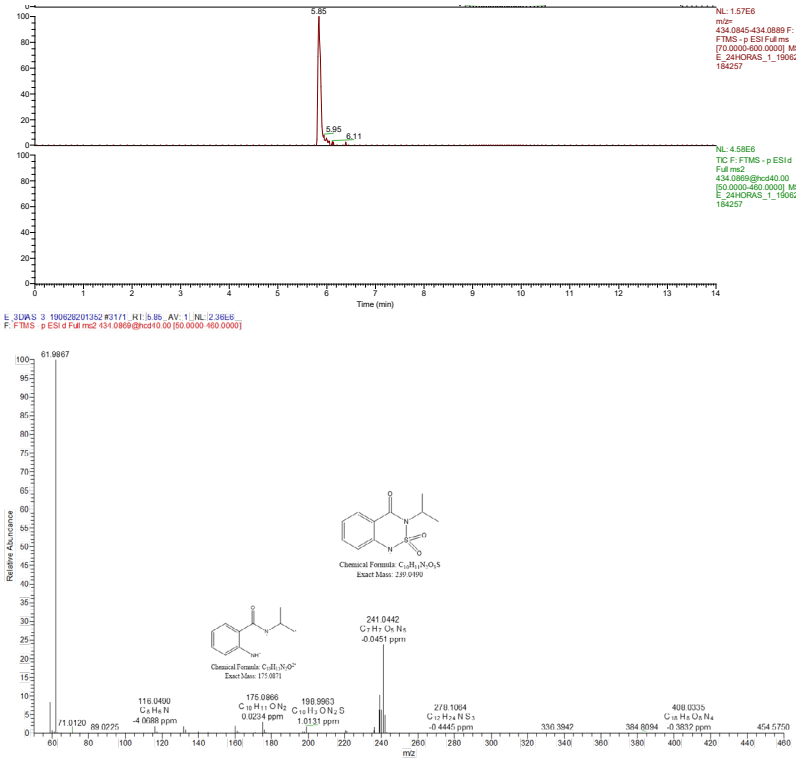


Figure S13. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 434.0869 (TP435).

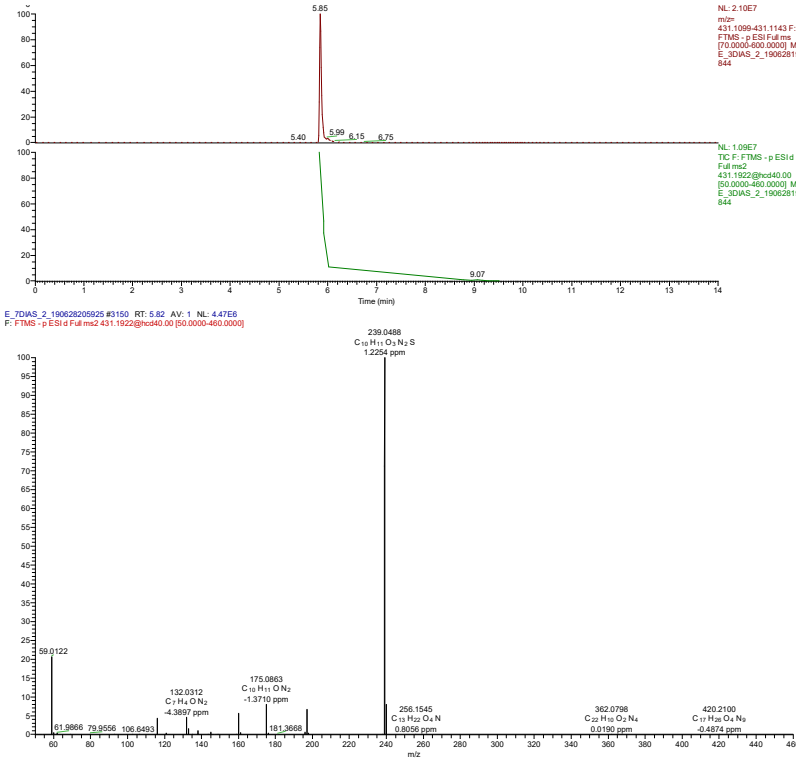


Figure S14. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 431.1922 (TP432).

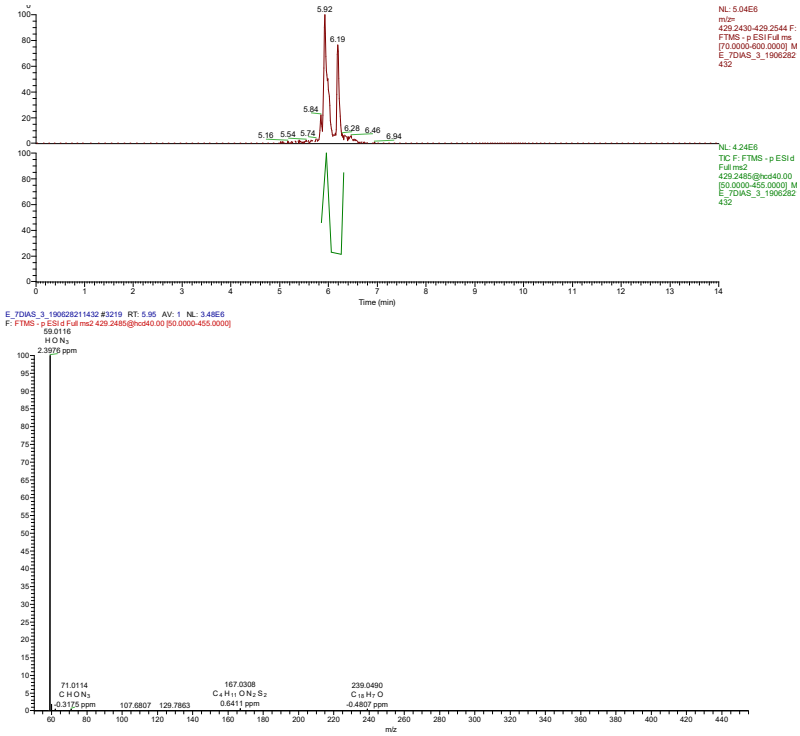


Figure S15. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 429.2486 (TP430).

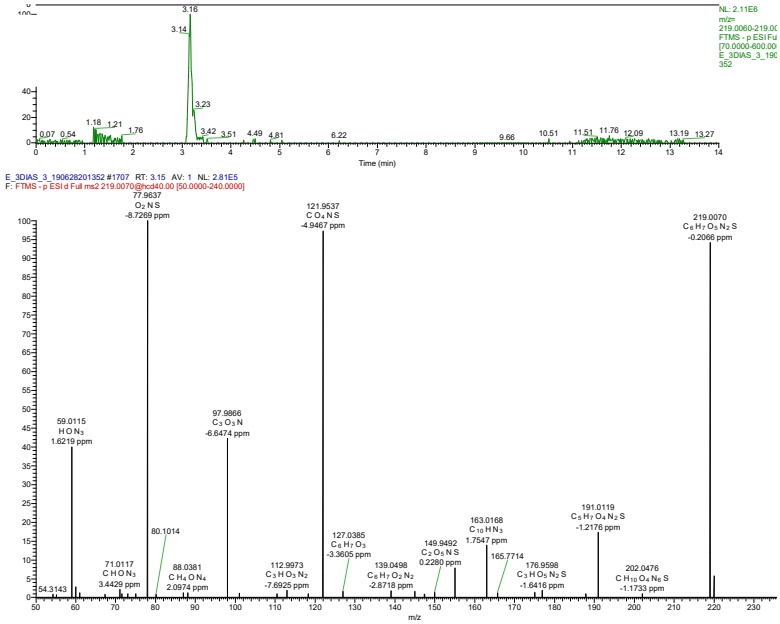


Figure S16. Extracted ion chromatogram and MS^2 spectrum of *m/z* 219.0070 (TP220).

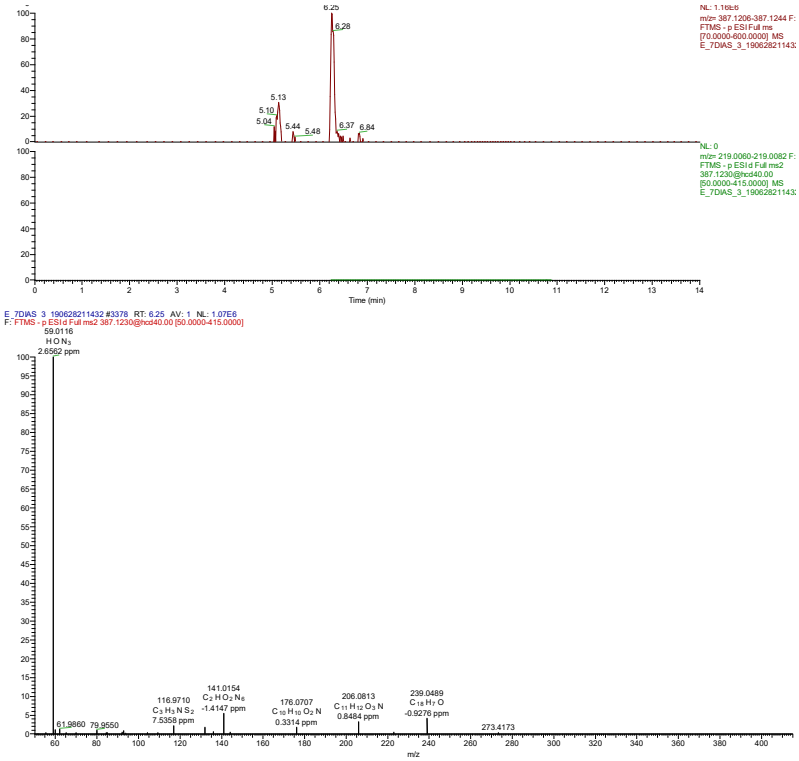


Figure S17. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 387.1225 (TP388).

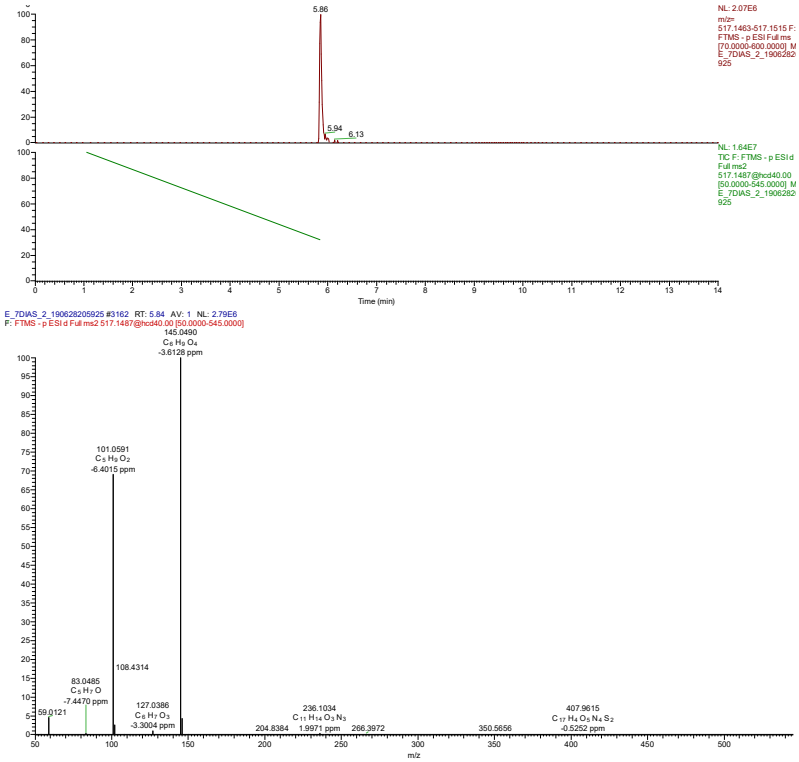


Figure S18. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 517.1489 (TP518).

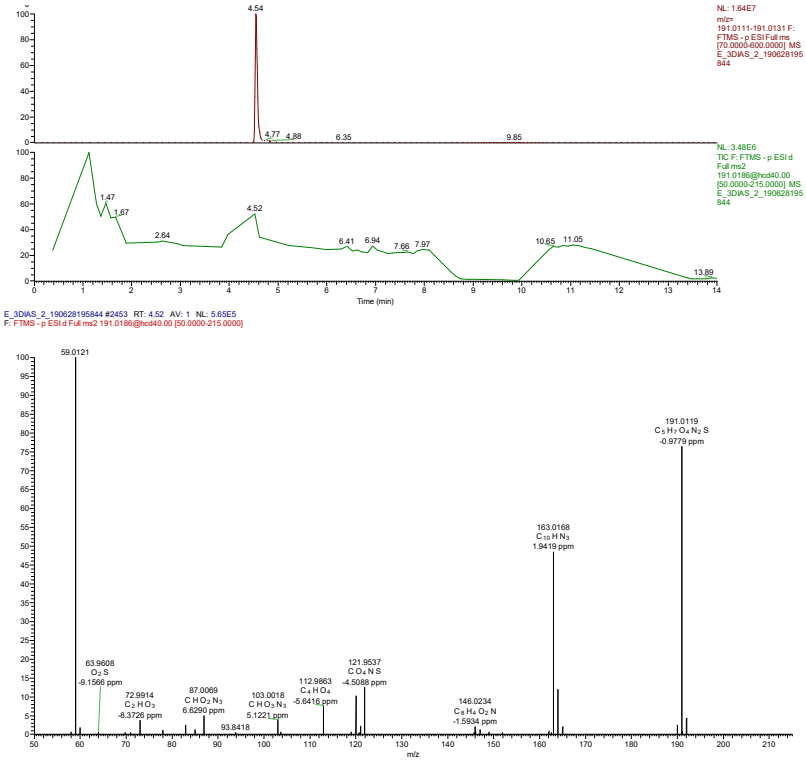


Figure S19. Extracted ion chromatograms (top: full MS, down: full MS^2) and MS^2 spectrum of *m/z* 191.0119 (TP192).

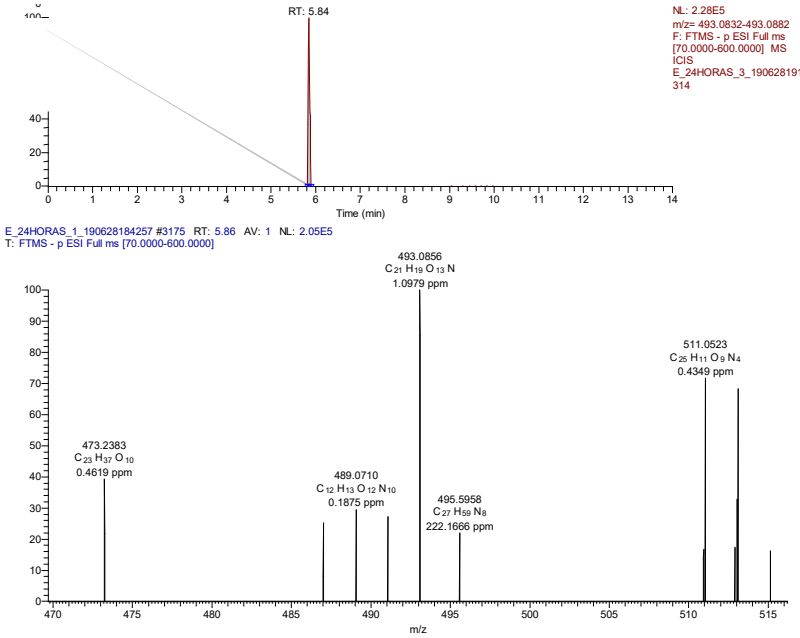


Figure S20. Extracted ion chromatogram and full MS spectrum of *m/z* 493.0856 (TP494).

3.3.2. Scientific publication #5

Evaluation of an outdoor pilot-scale tubular photobioreactor for removal of selected pesticides from water

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Manuel García-Vara
Esther López García
Cristina Postigo
Miren López de Alda
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Science of the Total Environment, 809 (2022), 150040
(DOI: https://doi.org/10.1016/j.scitotenv.2021.150040)

Science of the Total Environment 804 (2022) 150040

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Evaluation of an outdoor pilot-scale tubular photobioreactor for removal of selected pesticides from water

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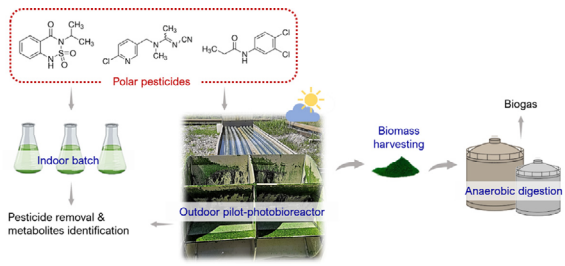
HIGHLIGHTS

- Microalgae-based systems degrade acetamiprid and propanil, but not bentazone.
- Four acetamiprid and 2 propanil transformation products were identified at lab-scale.
- Propanil and acetamiprid were removed in the pilot-PBR by 99% and 71%, respectively.
- Only three acetamiprid transformation products were found in the pilot-PBR.
- Harvested biomass can be used for biofuel production via anaerobic digestion.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 May 2021
Received in revised form 24 August 2021
Accepted 26 August 2021
Available online 1 September 2021

Editor: Yolanda Picó

Keywords:
Photobioreactor
Organic micropollutants
Biodegradation products
Harvesting
Biogas

ABSTRACT

This work assesses the capacity of a microalgae-based system to remove three highly to medium polar pesticides typically found in freshwater: acetamiprid, bentazone, and propanil. Degradation of the pesticides was firstly studied individually at batch lab-scale reactors and abiotic and heated-killed controls were employed to clarify their removal pathways. At lab-scale, propanil and acetamiprid were completely removed after 7 days whereas bentazone was not removed. Four and two transformation products (TPs) were generated in the biodegradation process for acetamiprid and propanil, respectively. Then, the simultaneous removal of the pesticides was assessed in an outdoor pilot photobioreactor, operated with a hydraulic residence time of 8 days. During the steady-state, high removal efficiencies were observed for propanil (99%) and acetamiprid (71%). The results from batch experiments suggest that removal is mainly caused by algal-mediated biodegradation. Acetamiprid TPs raised throughout the operational time in the photobioreactor, while no propanil TP was detected at the pilot-scale. This suggests complete mineralization of propanil or residual formation of its TPs at concentrations below the analytical method detection limit. Aiming at biomass valorization, diverse microalgae harvesting methods were investigated for biomass concentration, and the effect of residual pesticides on the biogas yield was determined by biochemical methane potential tests. Anaerobic digestion was not inhibited by the pesticides as verified by the digestion performance. The results highlight the potential of microalgae-based systems to couple nutrient removal, biomass production, micropollutant biodegradation, and biofuel production.

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https://doi.org/10.1016/j.scitotenv.2021.150040

1. Introduction

Concern about organic micropollutants (OMs) in the environment has risen in recent decades, boosted by the progress in the analytical technologies that allow their detection at very low levels in the different environmental compartments (Gavrilescu et al., 2015; Geissen et al., 2015). OMs include diverse compounds, such as pharmaceuticals, cosmetics, personal care products, pesticides, surfactants, flame retardants, or plasticizers (Barbosa et al., 2016), many of which have been reported to potentially pose adverse effects on ecosystems and human health (Grandclément et al., 2017; Luo et al., 2014).

Many OMs, including pesticides, are not efficiently removed with conventional wastewater treatment. In some cases, the concentration of specific OMs in the wastewater treatment plant (WWTP) effluent can be even higher than in the influent (Grandclément et al., 2017; Köck-Schulmeyer et al., 2013; Sadaria et al., 2016). Between 2011 and 2018, around 360,000 t of pesticides were sold per year in the European Union (EU), where Germany, Spain, France, and Italy are the main agricultural producers, and hence the main users of these products (Eurostat, 2020). Once those pesticides are released into the environment, several physical, chemical, and biological processes can transform them. The resulting transformation products (TPs) may be even more hazardous than the parent compounds (Ccanccapa et al., 2016; Fenner et al., 2013). Pesticide residues have been found in the environment at concentrations ranging from nanogram (ng) to microgram (μg) per liter (Fenner et al., 2013). The European Drinking Water Directive 98/83/EC (EC, 1998) and its revision proposal (EC, 2018) establishes 0.1 μg L^{-1} and 0.5 μg L^{-1} as parametric values for single and total pesticides, respectively, in water intended for human consumption, and the same values are set in the Groundwater Directive 2006/118/EC (EC, 2006).

Current advanced wastewater treatment technologies such as nanofiltration, reverse osmosis, and electro-Fenton oxidation, among others, generate waste and do not guarantee a cost-effective elimination of pesticides (Musbah et al., 2013; Plakas and Karabelas, 2012; Zhao et al., 2012). Microalgae-based treatment systems are attractive due to their feasibility to couple the removal of nutrients and pollutants with the production of biofuels and high-added value bio-products (Muñoz and Guieysse, 2006; Parladé et al., 2018; Subashchandrabose et al., 2011). Moreover, these biological methods for wastewater treatment represent a cost-effective mitigation technology for OMs removal, have lower capital and operational costs, and do not require an external supply of oxygen and additional chemicals (Sutherland and Ralph, 2019). Photosynthetic aeration generates O_2 used by heterotrophic bacteria to mineralize organic compounds while released CO_2 from bacterial respiration can be fixed by photosynthetic microorganisms (Muñoz and Guieysse, 2006). Complex interactions between cyanobacteria, microalgae, and bacteria include cooperative and competitive relations that strongly favor pollutant removal (Liu et al., 2017; Muñoz and Guieysse, 2006; Parladé et al., 2018). Likewise, these interactions contribute to the robustness of these systems, as they present high resistance to environmental fluctuations, predators, and oscillations in nutrient availability (Cuellar-Bermudez et al., 2017; Matamoros et al., 2015). Bioremediation of OMs by microalgae has attracted a lot of attention in recent years, and several studies have already focused on this topic (Nguyen et al., 2020; Sutherland and Ralph, 2019; Tolboom et al., 2019; Vo et al., 2019). Major mechanisms driving OM removal in microalgae-bacteria-based systems are biodegradation, photodegradation, and sorption. While many studies have addressed the removal of pesticides by pure (Jin et al., 2012; Kabra et al., 2014; Kurade et al., 2016; Subramanian et al., 1994; Zhang et al., 2011) and mixed microalgae cultures (El-Bestawy et al., 2007; Smedbol et al., 2018) in batch mode at lab-scale, only very few studies have evaluated the removal of pesticides by microalgae consortia, reporting different removal efficiencies (García-Galán et al., 2020, 2018; Matamoros et al., 2015). The potential of microalgae-bacteria-based systems to remove OMs, including pesticides, from wastewater has been already investigated at pilot-scale using open systems such as high-rate algal ponds (HRAPs) (García-Galán et al., 2019; Matamoros et al., 2015; Villar-Navarro et al., 2018; de Godos et al., 2012; Hom-Diaz et al., 2017b) and closed systems such as tubular photobioreactors (PBRs) (García-Galán et al., 2020, 2018; Hom-Diaz et al., 2017a; Parladé et al., 2018). However, knowledge regarding OM removal in outdoor pilot-scale tubular PBRs is limited and deserves more attention to advance in their potential implementation at full scale. The use of enclosed tubular PBRs for OM removal is interesting due to their advantages in comparison with other photobioreactor configurations, i.e. higher light use efficiency that boosts biomass productivity, better control of operational parameters, better mixing, and lower risk of contamination (Molina Grima et al., 1999; Muñoz and Guieysse, 2006). Moreover, pollutant loss by volatilization is less likely in closed tubular PBRs (Muñoz and Guieysse, 2006). Overheating, biofouling, and difficulty to scale up have been described as the main challenges to overcome in this PBR systems (Mata et al., 2010).

Additionally, from a biorefinery paradigm, the valorization of residual algal biomass used in wastewater treatment is crucial to guarantee the economic feasibility of the process (Chew et al., 2017; Javed et al., 2019). In this sense, anaerobic digestion is among the most straightforward and suitable techniques for energy recovery (Díez-Montero et al., 2020). In this work, we assessed the biochemical methane potential of the harvested algal biomass to generate biogas, and the effect that the potential presence of pesticides and/or TPs retained on the algal biomass may have on the digestion performance.

In this work, three pesticides representative of different chemical classes and modes of action, recently found in surface waters of an important agricultural area at levels that may pose a serious hazard for aquatic non-target organisms (maximum concentrations between 4 and 180 μg L^{-1}) (Barbieri et al., 2020), were selected to investigate the removal potential of microalgae systems. The pesticides under study comprised the neonicotinoid insecticide acetamiprid, included in the second European Watch List (Commission Implementing Decision 2018/840/EC), and the herbicides bentazone (benzothiazinone) and propanil (anilide), widely used in rice and cereal fields. These two herbicides have been also detected in dead shellfish organisms (oysters) and their presence was related to markers of tissue damage during DNA strand breakage (Ochoa et al., 2012). The environmental toxicity of propanil has been reported in various non-target animals, including amphibians, birds, and fishes (Kanawi et al., 2016). Moreover, due to their low mineralization rate and moderate sorption capacity to soil (Arena et al., 2018), both herbicides have been frequently detected in groundwater (Kanawi et al., 2016; Lopez et al., 2015; Malaguerra et al., 2012).

In this context, the main objectives of the present study were to evaluate the feasibility of removal of these three highly to medium polar pesticides (log $K_{ow} < 2.3$) by a microalgae-based system. This was done first at batch lab-scale and individually to determine the degradation mechanisms by microalgae and facilitate TP identification, and afterward in an outdoor pilot-scale PBR to evaluate its performance for simultaneous nutrients and pesticides removal and detect TPs generated in the process. Moreover, this work also aimed at assessing biomass harvesting in the pilot-PBR by sedimentation, flocculation, and coagulation, for its subsequent conversion to bioenergy through anaerobic digestion.

2. Materials and methods

2.1. Chemicals and reagents

Acetamiprid [N-[(6-chloro-3-pyridyl)methyl]-N′-cyano-*N*-methylacetamidine], bentazone [3-isopropyl-1H-2,1,3-benzothiadiazin-4 (3H)-one 2,2-dioxide], propanil [3′,4′-dichloropropionanilide], and their isotopically labelled compounds (acetamiprid-d_3, bentazone-d_6 and propanil-d_5) used as surrogate standards (SS) for quantitative

analysis, were purchased from Sigma-Aldrich (Steinheim, Germany). The physical-chemical properties of these compounds are presented in Table 1. HPLC-grade acetonitrile, methanol, and water used in the chemical analysis were supplied by Merck (Darmstadt, Germany). Mineral salts were obtained from Scharlab (Barcelona), and methanol used to prepare stock solutions of each pesticide was purchased from Fisher Scientific (UK). The flocculant was provided by Derypol, S.A. (Barcelona, Spain).

2.2. Photobioreactor

An outdoor semi-closed and tubular pilot-PBR located on the roof of the Chemical, Biological and Environmental Engineering Department at Universitat Autònoma de Barcelona (Barcelona, Spain) previously employed in other studies (Hom-Diaz et al., 2017a) was used. The PBR has a working volume of 1000 L and consists of eight tubes (length 7 m) connected at each side with two distribution chambers (Fig. S1 in Supplementary Information). The bigger chamber contains a paddle wheel for culture mixing and circulation in the PBR at a speed of 0.13 m s^{-1}. The tubes, placed inside an open cuvette containing tap water to balance temperature changes, are made of transparent low-density polyethylene while the chambers and the PBR structure are made of propylene. The PBR was exposed to rainfall events, sunlight irradiation, and ambient temperature variability.

The PBR was operated under semi-continuous mode and fed once a day (from Monday to Friday) with 175 L of modified Mann and Myers medium at 8 days of hydraulic residence time (HRT). The HRT was the same as the solid retention time (HRT = SRT). The employed medium is typically used to cultivate *Scenedesmus* sp. and *Chlorella* sp. (Escapa et al., 2016; Solimeno et al., 2017).

2.3. Experimental set-up for pesticide removal evaluation

2.3.1. Indoor batch experiments

Indoor batch experiments for individual assessment of the degradation of the three target pesticides by the microalgae system and identification of the TPs that could potentially be formed were performed at lab-scale. The reactors consisted of 250 mL Erlenmeyer-flasks containing 100 mL of microalgae culture taken from the PBR. The initial microalgal biomass concentration is shown in Table 2. The flasks were aerated by orbital shaking (100 rpm) in a constant-temperature chamber (25 ± 1 °C) and exposed to continuous light using cool white fluorescent tubes (light intensity: 35 μmol photon m^{-2} s^{-1}). Three different experimental conditions were assessed in parallel to determine their influence on the removal of the target pesticides. The microalgae reactor contained an active PBR microalgae culture and the target pesticide. The heat-killed reactor contained the target pesticide and death biomass (PBR microalgae culture autoclaved at 121 °C, 20 min), which allowed to determine the influence of pesticide sorption onto the biomass in the overall removal. Additionally, the influence of other losses was assessed using an abiotic reactor containing distilled water and the target pesticide. In all cases, the initial pesticide concentration was 1 mg L^{-1}. Each condition was studied in triplicate, and experiments were run under non-sterile conditions. Microalgal biomass in batch reactors was determined by optical density (OD), correlated with the total suspended solids (TSS) concentration as shown in Eq. (1):

$$\mathrm{TSS}\left(\mathrm{g\,L^{-1}}\right) = 0.7565 * \mathrm{OD_{680}} - 0.0422 \ (r^2 = 0.962) \quad (1)$$

Table 2
Initial microalgal biomass concentration in indoor batch experiments.

Parameter	Acetamiprid	Bentazone	Propanil
Total suspended solids (g L^{-1})	0.72 ± 0.01	0.63 ± 0.02	0.71 ± 0.05
Volatile suspended solids (g L^{-1})	0.57 ± 0.01	0.50 ± 0.02	0.56 ± 0.04
pH	8.1	9.2	8.4

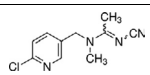
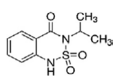
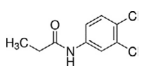
2.3.2. Outdoor pilot-scale PBR experiments

Given the results of the indoor batch experiments, the degradation of acetamiprid and propanil was further investigated in the outdoor pilot-PBR.

A defined volume of a methanolic solution of each pesticide was daily added to the PBR influent (125 L per day) to reach a concentration of 5 μg L^{-1} of acetamiprid and 50 μg L^{-1} of propanil. At the initial time, the concentrations of acetamiprid and propanil in the PBR were 0.875 and 8.75 μg L^{-1}, respectively. These concentrations were selected based on the maximum levels found for them in a previous study conducted in the Ebro River Delta (NE Spain) to assess the pesticides of highest concern in the investigated area (Barbieri et al., 2020). Experiments were run between April and June 2019 (during spring and the beginning of the summer season). The length of daylight during this period was ca. 14 h per day. Water loss due to evaporation was compensated daily by adding the corresponding volume.

Dissolved oxygen concentration (DO), pH, and temperature were measured in situ. A 45 mL sample was taken daily from the PBR effluent and filtered (0.45 μm) to analyze the following parameters: nitrate (N-NO_3^-), nitrite (N-NO_2^-), total organic carbon (TOC), total carbon (TC), inorganic carbon (IC), total nitrogen (TN), and orthophosphate (P-PO_4^{3-}).

Table 1
Physical-chemical properties of the target pesticides.

Pesticide (CAS number)	Chemical structure	Chemical family	Molecular formula	Molecular mass (g mol^{-1})	Log K_{ow}	K_H* (mol m^{-3}.Pa^{-1}) at 25°C	Water solubility (mg L^{-1}) at 20 or 25 °C
Acetamiprid (160430-64-8)		Neonicotinoid	$C_{10}H_{11}ClN_4$	222.7	0.8 [a]	5.3 10^{-8} [a]	2.95 10^3 [a]
Bentazone (25057-89-0)		Thiadiazine	$C_{10}H_{12}N_2O_3S$	240.3	−0.46 [b]	7.2 10^{-5} [b]	570 [b]
Propanil (709-98-8)		Anilide	$C_9H_9Cl_2NO$	218.1	2.29 [c]	4.4 10^{-4} [c]	95 [c]

* K_H= Henry's law constant.
[a] European Commission (2004).
[b] Galhano et al. (2011).
[c] Kanawi et al. (2016).

2.4. Microalgae consortium

The PBR was inoculated with 100 L of a consortium of microorganisms from an operating outdoor hybrid PBR (located in the campus Agròpolis, Viladecans, Barcelona) treating a mixture of wastewater from an agricultural irrigation channel and domestic wastewater from a septic tank as described elsewhere (Díez-Montero et al., 2020). The inoculum was mainly constituted by a mixed microalgae culture dominated by *Chlorella* sp. and *Scenedesmus* sp., while bacteria and protozoa were also present.

For indoor batch experiments, microalgae samples were taken from the pilot-PBR 24 h after feeding, and biomass concentration was determined through total suspended solids (TSS) and volatile suspended solids (VSS). As regards pilot-scale experiments, biomass concentration was determined 3 to 5 times per week. In both cases, photosynthetic microorganisms were characterized according to standard taxonomic literature (Bourrelly, 1966; Komárek and Anagnostidis, 2005; Palmer, 1962) using an optical microscope (Zeiss, AixoCam ERc 5s).

2.5. Microalgae harvesting and biogas production

Biomass from the pilot-PBR effluent was harvested by sedimentation, flocculation, and coagulation. Sedimentation by gravity was assessed in 1 L glass tube during 24 h. A cationic polymer of diallyl dimethyl ammonium chloride (polyDADMAC) free of acrylamide was used as flocculant at a dose of 250 ppm (Avila et al., 2020). Besides, Ferric (III) chloride ($FeCl_3$), a ferric salt coagulant, was used as a coagulant at a dose of 150 ppm. Flocculation and coagulation procedures were performed according to Mir-Tutusaus et al. (2017). Harvesting efficiency was calculated considering the increase in biomass concentration at the bottom of the glass tube according to (Eq. (2)):

$$\text{Harvesting efficiency } (\%) = \frac{(TS_f - TS_i)}{TS_i} * 100 \quad (2)$$

where initial total solids (TS_i) and final total solids (TS_f) is the biomass concentration before and after the harvest, respectively.

Biogas potential from the PBR harvested biomass was determined through biochemical methane potential tests (BMP). Anaerobic batch tests were performed according to Martín-González et al. (2010), taking into account the recommendations from Angelidaki et al. (2009) and Holliger et al. (2016). Digestate from the anaerobic sludge digester of a municipal WWTP (Sabadell, Barcelona) was used as fresh inoculum. The inoculum was pre-incubated at 37 ± 1 °C along 15 days to deplete its organic content. Reactors employed in the BMP tests were 120 mL glass bottles with a working volume of 80 mL. The harvested microalgal biomass, the inoculum, and water were added to the reactors. For the reactor containing microalgae harvested by sedimentation, 18 mL of inoculum was employed, while in the other reactors (flocculation and coagulation reactors), 9 mL of inoculum was added. Therefore, two different blanks (containing only the added amount of inoculum and water) were also prepared. Reactors were filled up to the working volume using tap water. Reactor bottles were purged with N_2 gas to remove oxygen and closed with a gastight butyl rubber septum. Later, they were incubated at 37 ± 1 °C in a controlled temperature chamber. Biogas production and accumulation in the headspace of the bottles was measured over time employing an SMC pressure Switch manometer (1 bar, 5% accuracy) until biogas generation ceased. Bottles were manually shacked before pressure measurement. Blank reactors were used to quantify the background methane production of the inoculum, and reference reactors with crystalline cellulose were used to verify the quality of the inoculum. Each reactor type was investigated in triplicate. The accumulated volumetric biogas production was calculated considering the pressure increase in the headspace volume and expressed in standard temperature and pressure (STP) conditions (273.15 K, 1.0135 bar). Net biogas production represents the gross methane production of the harvested biomass reactors subtracting the background methane production of the inoculum (blank). Periodically, gas samples were taken from the reactors to analyze biogas composition by gas chromatography.

2.6. Analytical procedures

2.6.1. Pesticide quantification and TP identification

Monitoring of the pesticide concentration and identification of TPs was done in samples collected at time 0, and after 2 and 7 days of the start of the experiment from all batch reactors. For this, 4 mL samples were taken from the flasks at each established sampling time, and 1.5 mL of the supernatant obtained after centrifugation (10,000 rpm, 4 min) was added to vials containing 75 μL of the isotopically labelled standards at a concentration of 10 μg mL^{-1}. Samples were frozen at −20 °C until their analysis with ultra-high performance liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry (HRMS). Details on the HPLC-HRMS analysis are provided in Text S1 in Supplementary Information. Quantification of the pesticide removal was done using the corresponding deuterated analog as the internal standard. Limits of detection (LODs) and quantification (LOQs) for propanil, acetamiprid, and bentazone were 0.20 and 0.66 μg L^{-1}, 0.22 and 0.74 μg L^{-1} and 2.9 and 9.6 μg L^{-1}, respectively.

For TP discovery, samples collected at times 2 and 7 days were compared with control samples (collected at time 0), and newly formed peaks present in the samples from the experimental reactor and absent in the samples from heat-killed and abiotic reactors were evaluated according to the elemental composition of the molecular and fragment ions, fragment rationalization (assisted by fragment ion search scoring), and isotopic patterns (Text S1 in Supplementary Information).

A volume of 60 mL of sample for pesticides quantification and TP detection was taken from the pilot-scale PBR once per week before the hydraulic steady-state ($n = 5$) was reached, and twice per week once the steady-state was reached ($n = 5$). Sample collection and monitoring at the PBR were performed at the same hour (noon) every sampling day. The sample was centrifuged (7000 rpm, 10 min), and a volume of 40 mL of the supernatant was added to a vial containing the deuterated analogs of the pesticides. Samples were frozen at −20 °C until their analysis with HPLC-HRMS after large-volume injection (400 μL). Details on the HPLC-HRMS analysis are provided in Text S2 in Supplementary Information. Quantification of the pesticide concentration in the samples was done with the internal standard method. The limits of detection and quantitation obtained for both acetamiprid and propanil were 0.017 and 0.05 μg L^{-1}, respectively.

TP identification was done using a suspect screening. For this, a suspect list including the TPs found in the lab-scale batch reactors and other potential TPs that may be formed according to the EAWAG BBD Pathway Prediction System tool was generated. After screening the suspect molecular ions, a comprehensive analysis of the MS^2 spectra was performed for structural elucidation.

2.6.2. Other analyses

On-site measurements included dissolved oxygen (DO), pH, and temperature, determined by a PCE_PHD 1 multimeter (PCE Instruments, Spain). Total solids (TS), volatile solids (VS), total suspended solids (TSS), and volatile suspended solids (VSS) were determined according to Standard Methods (APHA, 1999). Glass fibre filters (GF/C, Whatman, GE Healthcare, USA) were used in the determination of TSS and VSS. Orthophosphate concentration was quantified by the colorimetric LCK 348 kit (Hach, Germany). Nitrate and nitrite anions were analyzed after filtering (45 μm filter, Merck, Germany) with a Dionex ICS-2000 ionic chromatograph (Dionex Corporation, USA). Soluble TC, IC, TOC, and TN content were determined after

filtration (0.45 μm filter) by a multi-N/C 2100S analyzer (Analytikjena AG, Germany). The volumetric organic load and the volumetric load of nutrients (mg L^{-1} d^{-1}) was calculated as shown in Eq. (3):

$$\text{Nutrient volumetric load} = \frac{Q * N}{V} \quad (3)$$

where Q is the flow (L d^{-1}), N is the TOC for the organic load, and the nutrient concentration ($N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$) in the influent (mg L^{-1}), and V (L) is the PBR volume.

Biomass production in the PBR (g VSS L^{-1} d^{-1}) was estimated according to Eq. (4):

$$\text{Biomass production} = \frac{Q * VSS}{V} \quad (4)$$

where Q is the flow (L d^{-1}), VSS is the biomass concentration in the PBR (g L^{-1}) and V (L) is the PBR volume.

Biogas composition (carbon dioxide and methane content) was analyzed using a gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Mississauga, Canada) equipped with a thermal conductivity detector (TCD) and a Supelco Porapack Q column (3 m × 3.2 mm) (Pennsylvania, USA). Helium was the carrier gas (338 KPa), and the oven, injector, and detector temperatures were 70, 150, and 180 °C, respectively. Samples were injected with a 100 μL syringe (VICI PS Syringe A-2, 0.74 mm × 0.13 mm × 50.8 mm).

Acetic, propionic, and butyric acid (volatile fatty acids, VFAs) concentrations were determined with a Dionex 3000 ultimate HPLC system (Barcelona, Spain) equipped with a UV/visible detector (210 nm). The chromatographic separation was performed in an ICE-COREGEL 87H3 column (7.8 × 300 mm, Transgenomic, USA), heated at 40 °C, employing 0.006 mM of H_2SO_4 as a mobile phase at a flow rate of 0.5 mL min^{-1}. Samples were previously centrifuged (10 min, 8000 rpm, Beckman Coulter, Avanti J20 XP, USA), and then filtered through 0.45 μm nylon syringe filters. All samples were analyzed in triplicate.

2.7. QSAR toxicity prediction

An in silico toxicity evaluation was performed for the selected pesticides and their identified transformation products through the Ecological Structure-Activity Relationships (ECOSAR) predictive model (v2.0, US Environmental Protection Agency (EPA)) (US EPA, 2021). In the absence of CAS for the unknown compounds, SMILES, necessary for the toxicity calculation, were generated with ChemDraw Professional v18.1 (PerkinElmer Informatics). Acute toxicity values calculated included LC50 (50% lethal concentration) for fish and daphnia after being exposed to the analyzed chemical for 96 h and 48 h, respectively, and EC50 (50% effective concentration) for green algae to inhibit its growth after 96 h of exposure. Following a conservative approach, the lowest LC50 and EC50 values obtained for each compound were considered.

2.8. Statistical analysis

The experimental data from batch experiments were statistically evaluated using one-way analysis of variance (ANOVA) of repeated measures. A Bonferroni post-hoc test was applied when significant differences were obtained ($p < 0.05$). *P*-values represent Bonferroni corrected significance levels. Data from BMP tests were statistically evaluated employing one-way ANOVA, differences were considered significant at *p* values below 0.05. Statistics were performed with R (version 3.6.3).

3. Results and discussion

3.1. Pesticide degradation and TP identification at indoor batch conditions

The pesticides studied are characterized by low octanol-water partition coefficient (Log K_{ow}), high water solubility, and low adsorption onto solids (Table 1) and, consequently, their presence and that of their TPs was only monitored in the aqueous phase. As previously reported in other studies (Avila et al., 2021; Parladé et al., 2018), to discriminate between potential degradation mechanisms, an abiotic control containing the pesticide in the absence of microalgae was set up to determine the possible influence of photodegradation and volatilization among other abiotic processes on pesticide removal, and a heat-killed control containing autoclaved (121 °C, 20 min) dead biomass was performed to quantify the removal of the target pesticides by sorption.

The relation between the pesticide concentration in the liquid phase at a certain time with the initial pesticide concentration was used to calculate the percentage of pesticide remaining in the solution. Results showed that 57% of acetamiprid remained at day 2 in the batch reactors ($p < 0.01$), and the pesticide was completely removed by the microalgae consortium at day 7 ($p < 0.05$) (Fig. 1). Its low K_{ow} and high solubility in water (Table 1) make biodegradation and photodegradation feasible removal pathways. However, acetamiprid concentration was not altered by abiotic factors. The heat-killed reactor showed slight adsorption (8%) on day 2, and desorption of acetamiprid on the following days, as reported for other OMs in green algae reactors (Bai and Acharya, 2016). Furthermore, a high water solubility ensures a high bioavailability for biodegradation (Blum et al., 2018).

Regarding bentazone, its concentration in the microalgae lab-scale batch reactors remained constant, and thus, no degradation occurred throughout the 7 days (Fig. 1). The same behavior was also detected in the heat-killed reactors. However, in the absence of the microalgae consortium (abiotic control), a slight removal occurred (1.9% and 3% at days 2 and 7, respectively), although it was not statistically significant ($p > 0.05$). Direct photolysis of bentazone has been reported as an important removal pathway of this pesticide in water (Al Housari et al., 2011; Carena et al., 2020; Ferrando and Matamoros, 2020). The low photolysis effect on bentazone removal in the abiotic and heat-killed controls could be attributed to the absence of organic matter (photosensitizers) in solution capable of oxidizing the herbicide in the presence of light (Wei et al., 2020) and the reduced light penetration in the water when the biomass is present, respectively.

Conversely, propanil was completely removed after 2 days ($p < 0.01$) in the microalgae batch reactors (Fig. 1). Since propanil concentration in the abiotic and heat-killed reactors remained constant over the 7 days, the degradation could be exclusively attributed to the microalgae active consortium. Despite direct and indirect photolysis of propanil has been reported elsewhere (Kanawi et al., 2016), it was not observed in the abiotic reactors. However, our findings are in agreement with other studies that report that propanil is not susceptible to chemical hydrolysis (Milan et al., 2012) and does not sorb onto suspended particles and does not volatilize (Kanawi et al., 2016), pointing out biodegradation as the main dissipation route in aquatic systems (Kanawi et al., 2016).

Scarce knowledge is available in the peer-reviewed literature regarding the removal of these pesticides by microalgae-bacteria consortia. For instance, Zhou et al. (2014) reported 54% removal of bentazone employing a green microalgae strain (*C. vulgaris*). John et al. (1982) observed propanil degradation by pure cultures of green algae (such as *Chlamydomonas reinhardtii*, *Tolypothrix tenuis*, and *Ulothrix fimbriata*) and cyanobacteria (such as *Anacystis nidulans*, and *Anabaena cylindrica*). More recently, Ferrando and Matamoros (2020) reported higher removal efficiencies for antibiotics (i.e., sulfacetamide, sulfamethazine, and sulfamethoxazole) than for hydrophilic pesticides (i.e., bromacil, atrazine, diuron,

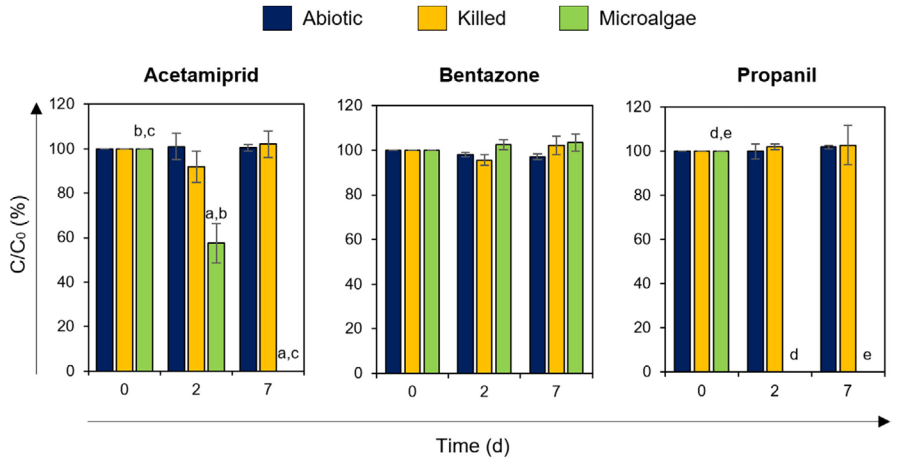


Fig. 1. Removal of acetamiprid, bentazone, and propanil throughout the experimental time under abiotic, heat-killed, and microalgae conditions in lab-scale batch degradation assays, expressed as the percentage of initial amount remaining in solution. Error bars indicate the relative standard deviation of the mean ($n = 3$, except for propanil under killed conditions where $n = 2$). Statistically significant differences when comparing the mean pesticide content in the reactors along time are indicated by letters as follows: a = $p < 0.05$; b, d and e = $p < 0.01$; and c = $p < 0.001$.

bentazone, and mecoprop), and among them a 15% removal of bentazone when using microalgae-bacteria systems in batch (10 incubation days and 8 μg L^{-1}). In the present work, in which initial concentrations were much higher concentrations (1 mg L^{-1}), bentazone resulted practically unaffected while acetamiprid and propanil were completely removed.

Main TPs generated during the degradation of these pesticides by the microalgae consortium are shown in Table 3. As expected, no TPs were found in the bentazone samples, as no degradation occurred. In total six TPs were identified in the investigated samples. Logical tentative structures were proposed for four of them with a confidence level of 3 according to Schymanski scale (Schymanski et al., 2014), since they could not be confirmed with the analysis of pure standard solutions, and with a confidence level of 5 for the other two TPs, because MS^2 data were either missing or did not provide additional evidence of their molecular structure.

The four TPs identified for acetamiprid were N2-carbamoyl-N1-[(6-chloro-3-pyridyl)methyl]-N1-methylacetamidine (TP240), N-((6-chloropyridin-3-yl)methyl)-*N*-methylacetimidamide (TP197), N-[(6-chloropyridin-3-yl)methyl]methylamine (TP156), and 6-chloronicotinic acid (TP157). All of them remained in solution after 7 days of treatment showing an increasing trend by the end of the experiment (Fig. S2 in Supplementary Information). TP240 is believed to be formed after addition of one water molecule to the acetamiprid structure, TP197 after the loss of the cyano group, TP156 after the loss of the N-ethylidenecynamide group, and TP157 after the loss of the group attached to the 2-chloropyridine ring and subsequent carboxylation. The Joint FAO/WHO Meeting on Pesticides Residues (JMPR) identified the previous TPs in the metabolic breakdown of acetamiprid in plants after foliar application (FAO and JMPR, 2005).

In the case of propanil, two TPs, namely, N-(3,4-dichlorophenyl) acetamide (TP203) and 3,4-dichloroaniline (3,4 DCA) (TP161) were identified. Both TPs remained in solution after 7 days of treatment; however, both TPs can be considered as an intermediate by-product due to the decreasing trend observed by the end of the experiment (Fig. S3 in Supplementary Information). It has been reported that the metabolite 3,4 DCA has a longer half-life than its parent compound (Milan et al., 2012). Thus, complete mineralization of propanil could be expected at longer degradation times. The formation of these TPs can be explained after the loss of a methyl group (TP203), and after the loss of a propaldehyde group (TP161).

3.2. Performance of the pilot-PBR, pesticide removal, and TP detection at pilot-scale

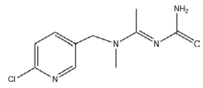
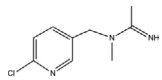
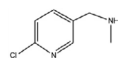
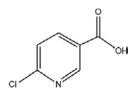
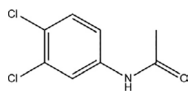
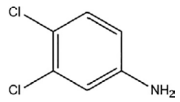
3.2.1. Performance of the pilot-PBR

In light of the efficient degradation of acetamiprid and propanil by the microalgae consortium in the lab-scale batch experiments, the removal of these two pesticides was further studied at pilot-scale in the outdoor PBR.

Biomass evolution in the pilot-PBR was determined by the VSS content of the PBR effluent (Fig. 2). VSS represents the biomass concentration of microorganisms considering a consortium of photoautotrophs (microalgae and cyanobacteria) and heterotrophs (bacteria, protozoa, and other microorganisms), characteristic of these systems (Cuellar-Bermudez et al., 2017; Parladé et al., 2018; Posadas et al., 2014). The VSS/TSS ratio was 80%, in agreement with values typically found in microalgae-based systems (>70%) (García-Galán et al., 2020). At the beginning of the operation, biomass concentration increased faster, reaching 0.7 g VSS L^{-1} after 3 days (Fig. 2). From day 7 to the end of the experiment, the average biomass concentration in the mixed liquor was 0.22 ± 0.12 g VSS L^{-1}, with a production rate of 0.03 ± 0.01 g VSS L^{-1} d^{-1}, similar to the values reported in previous experiments (Hom-Diaz et al., 2017a; Parladé et al., 2018).

Temperature and sunlight irradiation have a direct effect on the photosynthetic activity as they drive microalgae growth, and hence, influence the photosynthetic species composition, and the PBR performance (Hom-Diaz et al., 2017a; Lee et al., 2015). During the PBR operation, the temperature increased gradually in line with the beginning of the summer season and ranged between 12.4 and 30.5 °C (Fig. 2). Thus temperature was close or at the optimum for most microalgae species (15–30 °C) (Singh and Singh, 2015; Sutherland et al., 2015). As shown in Fig. 2, during the first days of the PBR operation, biomass concentration increased in coherence with seasonality (rise in temperature and sunlight irradiation – average solar radiation on the horizontal surface was 4.78, 5.95, and 7.35 kWh m^{-2} d^{-1} in April, May, and June, respectively (NASA, 2021)). Nonetheless, from day 11 to the end of the experiment,

Table 3
Transformation products (TPs) formed during acetamiprid and propanil degradation process at indoor batch experiments.

Pesticide	TPs	t_R (min)	HESI mode	Full scan				MS/MS				Suspect identity (Confidence level)
				m/z	Formula	RDB	Δm (ppm)	*m/z*	Formula	RDB	Δm (ppm)	
Acetamiprid	TP240	5.6	+	241.0862	$C_{10}H_{14}ON_4Cl$	5.5	4.7	224.0595	$C_{10}H_{11}ClN_3O$	6.5	5.1	
								198.0804	$C_9H_{13}ClN_3$	4.5	5.8	
								181.0534	$C_9H_{10}ClN_2$	5.5	6.5	
								157.0538	$C_7H_{10}N_2Cl$	3.5	5.8	
								128.0271	C_6H_7ClN	3.5	7.1	
								126.0115	C_6H_5ClN	4.5	4.7	
	TP197	5.4	+	198.0804	$C_9H_{13}ClN_3$	4.5	5.7	198.0804	$C_9H_{13}ClN_3$	4.5	5.7	
								157.0536	$C_7H_{10}ClN_2$	3.5	5.8	
								128.0270	C_6H_7ClN	3.5	6.5	
								126.0114	C_6H_5ClN	4.5	7.0	
	TP156	4.6	+	157.0536	$C_7H_{10}N_2Cl$	3.5	5.9	157.0536	$C_7H_{10}ClN_2$	3.5	5.9	
								126.0114	C_6H_5ClN	4.5	7.2	
	TP157	7.5	+	158.0013	$C_6H_5O_2NCl$	4.5	6.3	158.0013	$C_6H_5ClN_2O$	4.5	6.3	
Propanil	TP203	8.7	+	203.9991	$C_8H_8ONCl_2$	4.5	6.6					
	TP161	8.5	+	161.9885	$C_6H_6NCl_2$	3.5	7.9	161.9885	$C_6H_6NCl_2$	3.5	7.96	

t_R: chromatographic retention time; HESI, heated-electrospray ionization; Δm, mass measurement error; RDB, ring and double bound equivalents; CL, confidence level according to Schymanski scale (Schymanski et al., 2014).

biomass concentration decreased slowly regardless of temperature increase; meanwhile, biomass colonization of the PBR tubes, walls, and chambers was observed. Thus, reduced biomass productivity could be explained by the biofilm developing in the PBR, limiting light penetration into the mixed liquor and negatively affecting biomass growth. Other studies using tubular PBRs reported that the biofilm is generated during PBR operation, influencing sunlight distribution throughout the entire culture (García-Galán et al., 2020). Similarly, a high microalgae growth in HRAP produces a shading effect inside the reactor reducing light penetration into the system (Wang et al., 2015). Moreover, biofilm development could be correlated with microscopically observed changes in PBR populations (Table 4). Phototrophic biofilms are constituted by an assembly of filamentous cyanobacteria, microalgae, and heterotrophs (Sabater et al., 2002). During period I, the PBR biomass was mainly constituted by unicellular microalgae (*Chlorella* sp. and *Scenedesmus* sp.) while the presence of filamentous microalgae (*Stigeoclonium* sp. and *Ulothrix* sp.) and cyanobacteria (*Phormidium* sp., *Oscillatoria* sp., *Nostoc* sp., and *Tolypothrix* sp.) increased towards the next periods (Table 4). Due to the outdoor operation, variations in the PBR populations could be associated with operational parameters, the aforementioned dynamic changes in environmental conditions, and the predation by grazers (Deruyck et al., 2019). Moreover, the dominance of certain species could be also linked to their tolerance to the pesticides present in the solution. Cyanobacteria *Oscillatoria* sp. and *Phormidium* sp., as well as the green algae *Chlorella* sp., *Scenedesmus*

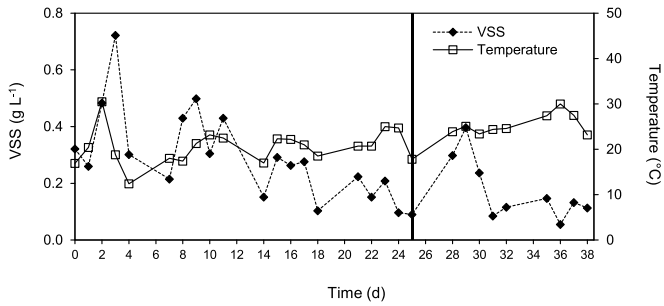


Fig. 2. Evolution of biomass concentration (VSS) and temperature in the pilot-PBR (HRT = 8 d). The vertical black line indicates the beginning of the steady-state.

sp., and *Stigeoclonium* sp., are considered among the most tolerant species to polluted environments (Palmer, 1969). *Chlorella* sp. and *Stigeoclonium* sp. were indeed dominant in the biofilm in the last days of the experiment.

Table 5 outlines the mean values of the parameters analyzed in the influent and the effluent of the pilot-PBR during the steady-state. The pH varied between 8.8 and 9.6 during the operation of the PBR, in agreement with a high photosynthetic activity during high irradiance periods in the spring and summer seasons (Hom-Diaz et al., 2017a). The pH in water is influenced by several factors such as microalgal growth, ammonium nitrification (release of H^+ and decrease of pH), the buffer capacity of the influent, and the excretion of acidic or basic metabolites from organic matter biodegradation (González et al., 2008). The mean DO concentration (8.6 ± 1.4 mg L^{-1}) is associated with the photosynthetic activity during the midday and was consistent with previous values (Hom-Diaz et al., 2017a).

The mean organic loading rate in the influent during the steady-state was 0.9 mg TOC L^{-1} d^{-1}. During this period, the inorganic carbon (IC) was reduced by 12% and transformed to microalgal biomass, increasing the TOC in the effluent by 83% (Table 5), probably due to the fraction of soluble carbon released from the photosynthetically fixed carbon as reported elsewhere (García-Galán et al., 2020, 2018). The N:P ratio of the influent was 28:1 in a molar basis, in line with the optimal N:P ratio reported for *Scenedesmus* sp. (Klausmeier et al., 2004). As shown in Fig. 3, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ were the sole inorganic nitrogen and phosphorous sources bioavailable to microalgae (Monfet and Unc, 2017) in the employed culture media. $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ loading rates in the PBR influent were 4.4 and 0.8 mg L^{-1} d^{-1}, respectively. Nitrate and phosphate removal efficiencies were 24 ± 4% and 94 ± 2%, respectively. Higher values for nitrate removal were reported by other authors, which could be explained by higher microalgal biomass concentration in those studies, conducted at indoor and controlled conditions. For instance, Ferrando and Matamoros (2020) evaluated the removal of 200 mg L^{-1} of $N\text{-}NO_3^-$ from a groundwater sample spiked with hydrophilic pesticides in an indoor continuous reactor with immobilized microalgae, achieving 41% attenuation of $N\text{-}NO_3^-$ at an HRT of 8 days. Likewise, Arias et al. (2018) reported 58% of $N\text{-}NO_3^-$ removal when treating secondary wastewater effluent and digestate in an indoor closed PBR with a mixed microalgae culture operating at an HRT of 8 days. As for orthophosphate, similar performances were attained when using microalgae-bacteria systems. For example, De Godos et al. (2009) reported 80% PO_4^{3-} removal when treating swine slurry in a tubular biofilm PBR constituted by cyanobacteria, microalgae, and bacteria consortium. The higher phosphate uptake observed in the present study could be related to the presence of cyanobacteria with the ability to accumulate phosphate as polyphosphate granules that can also be released to the medium with cell death (Jansson, 1988). Another mechanism influencing $P\text{-}PO_4^{3-}$ removal could be related to its pH-mediated precipitation with cations (Ca^{2+} and Mg^{2+}). This process begins at pH values between 8.9 and 9.5, and depends on the buffer capacity of the water (Craggs et al., 1996; González et al., 2008). Phosphorous could be firstly removed by pH-mediated precipitation, followed by biomass assimilation at lower concentrations (Craggs et al., 1996). Other mechanisms might be related to $P\text{-}PO_4^{3-}$ precipitation within the algal-bacterial biofilm (De Godos et al., 2009), and surface adsorption to biomass (Yao et al., 2011).

Table 4
Qualitative characterization of the main microalgae and cyanobacteria genus in the pilot-PBR. References: (+++) dominant, (++) relative, (+) rare.

Species	Period I (30th April to 12th May)	Period II (13th May to 24th May)	Period III[a] (25th May to 6th June)
Algae			
Chlorella sp.	+++	+++	+++
Scenedesmus sp.	+++	+++	++
Stigeoclonium sp.	+	++	+++
Ulothrix sp.	+	+	++
Cyanobacteria			
Phormidium sp.	++	++	+
Oscillatoria sp.	+	+	++
Nostoc sp.	+	++	++
Tolypothrix sp.	+	+	++

[a] Period III corresponds to the steady-state.

Table 5
Characterization of the influent and effluent of the pilot-PBR during the steady-state (mean ± SD, n = 3 for the influent and *n* = 9 for the effluent).

Parameter	Influent	Effluent
Total organic carbon (mg L^{-1})	7.2 ± 0.7	13.1 ± 2.7
Inorganic carbon (mg L^{-1})	90.1 ± 6.1	80.9 ± 7.4
Total carbon (mg L^{-1})	97.7 ± 4.7	94.0 ± 9.0
Total nitrogen (mg L^{-1})	154.7 ± 30.9	128.3 ± 4.8
$N\text{-}NO_3^-$ (mg L^{-1})	34.8 ± 2.3	26.1 ± 1.5
$P\text{-}PO_4^{3-}$ (mg L^{-1})	6.2 ± 2.3	0.4 ± 0.1
$N\text{-}NO_2^-$ (mg L^{-1})	1.3 ± 0.1	5.1 ± 2.0
Volatile suspended solids (g L^{-1})	n/a	0.2 ± 0.1
Biomass production (g VSS L^{-1} d^{-1})	n/a	0.02 ± 0.01
Temperature (°C)	n/a	24.7 ± 3.3
pH	7.8 ± 0.4	9.1 ± 0.2
Dissolved oxygen (mg L^{-1})	n/a	8.6 ± 1.4

n/a = not applicable.

3.2.2. Pesticide removal and TP detection in the pilot-PBR

The simultaneous removal of the targeted pesticides acetamiprid and propanil by the microalgae-based system in the pilot-scale PBR was evaluated during 38 days of continuous operation (Figs. 4 and 5). Pesticide removal in the pilot-PBR was calculated considering the difference between the concentration of the target pesticide in the influent and the effluent at each sampling time.

Propanil was added to the pilot-PBR at a higher concentration (50 μg L^{-1}) than acetamiprid (5 μg L^{-1}) since it was degraded at a higher rate in the lab-scale batch experiments. Individual concentrations of propanil and acetamiprid were below the threshold ecotoxicity values reported for algae (NORMAN, 2021; University of Hertfordshire, 2020). After 1 h of starting pesticide addition (day 1), propanil was removed by 97% and during the following three weeks its removal efficiency was >99% (Fig. 4). Propanil concentration in the PBR effluent increased slightly when the steady-state was reached (after 25 days) attaining a removal of 97%. During the steady-state propanil was nearly completely removed, achieving a mean removal efficiency of 99%. These results agree with the propanil degradation rate observed in lab-scale experiments (microalgae reactor) where it was completely removed within 2 days. Overall, propanil removal efficiency was enhanced over time which could be associated with biomass acclimation, as reported in previous works (Ferrando and Matamoros, 2020). Milan et al. (2012) studied the dissipation of propanil and 3,4 DCA in rice management systems, indicating a rapid conversion of propanil to 3,4 DCA due to microbial degradation, followed by a slow decrease in its concentration. In the present study, no propanil TPs were detected in the PBR effluent, in line with the decreasing trend of the TP concentrations observed in the lab-scale batch experiments. This finding suggests that propanil and its intermediate by-products could be completely mineralized under continuous operation with an HRT of 8 days. Nevertheless, the presence of residual concentrations of the TPs in the effluent cannot be completely ruled out since these chemicals could be present at levels below the instrumental limit of detection (which cannot be calculated in the absence of standards). Furthermore, based on the results obtained in indoor batch experiments and previously discussed in Section 3.1, biodegradation is proposed as the main attenuation mechanism for propanil removal in the PBR.

On the other hand, acetamiprid concentration in the effluent was reduced to 0.87 μg L^{-1} after 1 h of pesticide addition (day 1) which suggests a removal of 87% (Fig. 4). During the following two weeks,

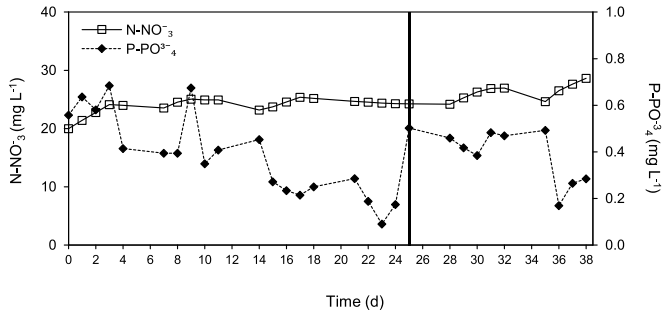


Fig. 3. $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ evolution in the pilot-PBR effluent. The vertical black line indicates the beginning of the steady-state.

acetamiprid removal increased up to 96% (Fig. 4). By the beginning of the hydraulic steady-state (day 25), acetamiprid removal decreased to 62%, increasing its concentration in the effluent (Fig. 4). The mean acetamiprid removal during the steady-state was 71%. According to these results, the performance of the microalgae reactor in batch and continuous operational modes indicates that acetamiprid requires a longer time than propanil for its complete degradation. At lab-scale batch experiments, 42% of the acetamiprid removal was obtained within 2 days while a total removal was accomplished by day 7. Although acetamiprid was added to the PBR at a minor concentration than propanil, its removal was lower. Differences in removal efficiencies for acetamiprid in continuous mode could be attributed to its physical-chemical properties. Acetamiprid is stable to hydrolysis (FAO and JMPR, 2005), and its low Henry's law constant value and log K_{ow} (Table 1) result in negligible volatilization and sorption onto biomass. According to the results obtained in batch reactors, photodegradation did not affect acetamiprid removal, as previously reported (US EPA, 2002). In contrast, its high-water solubility contributes to its availability in the aqueous phase for biodegradation. Thus, the declining biomass concentration in the pilot-PBR associated with the biomass washing effect might be a feasible explanation for the decrease in acetamiprid removal, as it has been previously reported for continuous-feeding operational mode (Ferrando and Matamoros, 2020). Washing effect could be avoided by immobilising microalgae in a membrane PBR as reported

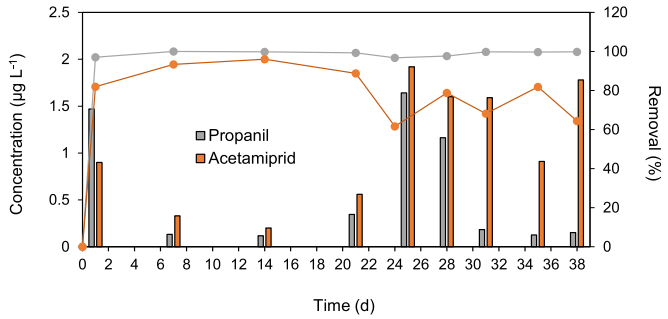


Fig. 4. Evolution of acetamiprid and propanil concentration in the pilot-PBR effluent (bar chart) and their removal efficiency (line chart).

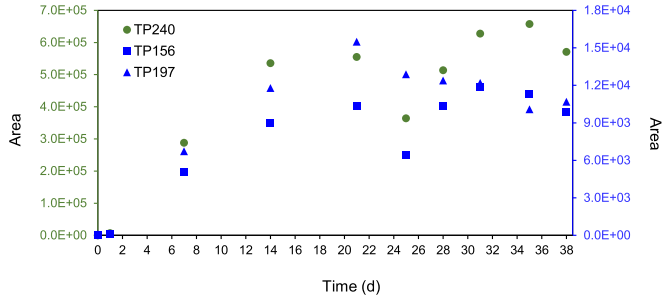


Fig. 5. Transformation products from acetamiprid detected in the pilot-PBR.

by other authors (Derakhshan et al., 2019). Moreover, different removal efficiencies observed in batch and continuous-mode reactors could be associated with the non-controlled environmental conditions in the PBR system such as light irradiance, temperature, precipitation, and presence of grazers, among others. According to our results, these factors may have a greater influence on acetamiprid than on propanil. Only two acetamiprid TPs (TP240 and TP197) were found in the PBR effluent after 7 days of operation (Fig. 5) and their concentration, although with fluctuations, increased throughout the studied period until day 35 in the case of TP240 and day 21 in the case of TP197, to decrease afterward. Their MS^2 spectra fitted those obtained at the indoor batch experiments (Figs. S4 and S5 in Supplementary Information), which confirms their identity. The molecular ion of another acetamiprid TP, namely TP156, was also detected; however, its MS^2 spectrum was not available (Fig. S6 in Supplementary Information) and, therefore, its identity could not be confirmed. Despite acetamiprid was not completely removed at an HRT of 8 days, the three TPs identified were generated right after its interaction with the microalgae-based system.

Data currently available on the removal of pesticides by microalgae-based systems in closed or semi-closed PBRs are still scarce and in most cases obtained under laboratory-controlled conditions. Thus, this study verifies the capacity of these low-cost nature-based systems for the treatment of wastewater containing OMs.

3.2.3. Microalgae harvesting and biogas production

From a biorefinery approach, coupling nutrient removal and OM degradation with biogas production contributes to boosting the economic feasibility of the overall process (Ward et al., 2014). Accordingly, anaerobic digestion was explored as a technique for algal biomass recycling after water treatment.

Microalgae harvesting from the PBR was performed in the period of hydraulic steady-state, through gravity sedimentation, flocculation with an organic flocculant, and coagulation with $FeCl_3$. Coagulation and flocculation were used to increase natural sedimentation efficiency.

Flocculation and coagulation techniques were performed in 30 min (15 min mixing, followed by 15 min sedimentation) showing similar harvesting performances (41–44%) (Table 6). In contrast, gravity sedimentation for 24 h attained 75% biomass concentration. Settleability performance could be ascribed to the dominance of filamentous self-aggregating microalgae and cyanobacteria which contribute to immobilize microalgae cells and constitute an additional surface for bacteria colonization (Craggs et al., 1996). These mixed flocs avoid biomass from washing out, provide diverse removal pathways of nutrients by different microorganisms, enhance the robustness of the system, and overcome difficulties associated with harvesting (Liu et al., 2017).

The potential of biogas production of the harvested biomass by the three different techniques was determined through BMP tests (each trial was identified with the same name of the harvesting technique). As shown in Table 6, biogas production was highest when $FeCl_3$ was employed (69.7 ± 4.8 NmL biogas g VS^{-1}), followed by flocculated biomass (54.8 ± 7.5 NmL biogas g VS^{-1}), and gravity collected biomass (50.0 ± 18.3 NmL biogas g VS^{-1}); however, statistical differences were not significant ($p > 0.05$). The greater average methane content along with the absence of VFAs in the digestate of the sedimentation trial (Table 6) suggests a better conversion of biomass. The use of coagulated biomass might influence the anaerobic process since Fe among other trace metals (such as Ni, Cu, Mo, and Zn) has an important role as cofactors of some enzymes or catalytic centers at active sites (Glass and Orphan, 2012). Indeed, Fe is one of the most required trace elements by methanogenic microorganisms for methane production (Glass and Orphan, 2012). Some authors have also reported improvement in biogas production due to $FeCl_3$ addition (Qin et al., 2019; Song et al., 2001; Yu et al., 2015). The results obtained suggest that neither the coagulant nor the flocculant used was toxic for the methanization.

3.3. QSAR toxicity prediction

The QSAR-predicted toxicity values obtained for the three studied pesticides and their identified TPs by the ECOSAR predictive model are shown in Table 7. Following the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) criteria (Winder et al., 2005), chemicals can be classified according to their aquatic toxicity into 3 categories: category 1, which corresponds to the highest hazard, when the LC50/EC50 values of the chemical for fish, crustacea and/or algae are below 1 mg L^{-1}, category 2, when these values are between 1 and 10 mg L^{-1}, and category 3, which represents the lowest environmental hazard, when the LC50/EC50 values are between 10 and 100 mg L^{-1}. According to this, none of the studied compounds would show high acute aquatic toxicity (all LC50/EC50 values are higher than 1 mg L^{-1}). However, acetamiprid for daphnia and algae, propanil and its TP 203 for algae, and the other propanil TP 161 for both daphnia and algae would be moderately toxic (LC50/EC50 between 1 and 10 mg L^{-1}), while the remaining compounds would in principle exhibit low or negligible aquatic toxicity for the considered organisms.

Comparison of the toxicity values obtained for the pesticides and their corresponding TPs indicates that acetamiprid is biodegraded into less toxic, environmentally safer compounds in all cases. In contrast, the degradation of propanil leads to the formation of a comparatively less toxic compound in the case of TP 203, while TP 161 is predicted to be more harmful than propanil for both fish and daphnia. Nevertheless, considering together both toxicity and abundance of the species at the beginning and at the end of the indoor batch experiments (Figs. S2 and S3 in Supplementary Information), a reduction of the initial toxicity associated to the pesticide by 10 to 50 times can be expected throughout the bioremediation process.

4. Conclusions

This work provides new insights into the removal of pesticides typically found in surface waters. Acetamiprid and propanil were effectively biodegraded by the microalgae consortium in lab-scale batch reactors, whereas bentazone was recalcitrant. The latter can be directly related to the molecular structure of the pesticide and the absence of microorganisms capable of starting its degradation in the biofilm. Two and four TPs were formed during propanil and acetamiprid biodegradation, respectively. Acetamiprid TPs accumulated in solution while propanil TPs further degraded, which suggests its mineralization.

Table 6
Harvesting efficiency and biochemical methane potential (BMP) tests parameters.

Trial	Harvesting		BMP			
	TS after harvesting (g L^{-1})	Harvesting efficiency (%)	Experimental biogas yield (NmL biogas g VS^{-1})	CH_4 content (%)	Propionic acid[a] concentration (mg L^{-1})	VS removal (%)
Sedimentation (S)	5.8 ± 0.0	75	50.0 ± 18.3	73 ± 7	0.0 ± 0.0	18 ± 3
Flocculation (F)	4.6 ± 0.6	41	54.8 ± 7.5	59 ± 27	49.1 ± 8.2	21 ± 7
Coagulation (C)	4.8 ± 0.1	44	69.7 ± 4.8	67 ± 25	51.5 ± 12.5	30 ± 3

[a] Propionic acid was the only volatile fatty acid detected.

Table 7
Predicted aquatic toxicity of acetamiprid, bentazone, propanil, and their degradation products by the ECOSAR program.

Compound	SMILES	LC50 fish 96 h (mg L^{-1})	LC50 daphnia 48 h (mg L^{-1})	EC50 green algae 96 h (mg L^{-1})
Acetamiprid	CC(=NC#N)N(*C*)CC1=CN=C(C=C1)Cl	*18.7*	**2.38**	**1.73**
TP240	CC(N(CC1=CC=C(*Cl*)N=C1)C)=NC(N)=O	667	*64.8*	*80.2*
TP197	CC(N(CC1=CC=C(*Cl*)N=C1)C)=N	2,180	190	290
TP156	CNCC1=CC=C(*Cl*)N=C1	237	*24.1*	*27.2*
TP157	O=C(*O*)C1=CN=C(*Cl*)C=C1	*21.1*	*12.8*	n.a.
Bentazone	CC(*C*)N1C(=O)C2=CC=CC=C2NS1(=O)=O	122	124	*13.1*
Propanil	CCC(=O)NC1=CC(=C(C=C1)Cl)Cl	*12.5*	*10.7*	**2.1**
TP203	CC(NC1=CC=C(*Cl*)C(Cl)=C1)=O	*28.3*	*26.0*	**3.97**
TP161	NC1=CC=C(*Cl*)C(Cl)=C1	*10.1*	**1.35**	**3.32**

Abbreviations: LC50, 50% lethal concentration; EC50, 50% effective concentration; n/a, not available.
Aquatic toxicity values falling within category 2 (1 mg L^{-1} < LC50/EC50 ≤ 10 mg L^{-1}) are highlighted in bold, and those within category 3 (10 mg L^{-1} < LC50/EC50 ≤ 100 mg L^{-1}) in italics.

Experiments conducted in an outdoor semi-continuous pilot-PBR confirmed the results of lab-scale reactors. Propanil and acetamiprid were removed on average 99% and 71%, respectively, in the steady-state. Three acetamiprid TPs were detected during the continuous PBR operation while none was detected for propanil.

Biomass harvesting efficiency through gravity sedimentation exhibited better results (75%) than flocculation (41%) and coagulation (44%). Similar biogas yields (50–69.7 NmL biogas g VS^{-1}) were obtained after the anaerobic digestion of the harvested biomass indicating the absence of toxicity of the employed coagulant and flocculant.

The present work highlights the capacity of this nature-based treatment technology for the simultaneous consumption of nutrients in wastewater, the removal and degradation of pesticides, and the generation of biomass for biofuel production.

CRediT authorship contribution statement

Romina Avila: Investigation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Manuel García-Vara:** Investigation, Writing – original draft, Writing – review & editing. **Ester López-García:** Investigation, Writing – original draft, Writing – review & editing. **Cristina Postigo:** Investigation, Writing – review & editing. **Miren López de Alda:** Funding acquisition, Supervision, Conceptualization, Writing – review & editing. **Teresa Vicent:** Methodology, Supervision, Conceptualization, Writing – review & editing, Funding acquisition. **Paqui Blánquez:** Methodology, Supervision, Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of competing interest

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

Acknowledgments

This work has been supported by the Spanish Ministry of Economy and Competitiveness State Research Agency (CTM2016-75587-C2-1-R and CTM2016-75587-C2-2-R) and co-financed by the European Union through the European Regional Development Fund (ERDF) and the Horizon 2020 research and innovation WATERPROTECT project (727450). This work was also partly supported by the Generalitat de Catalunya (Consolidate Research Groups 2017-SGR-01404 and 2017-SGR-014) and the Ministry of Science and Innovation (Project CEX2018-000794-S).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.150040.

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Evaluation of an outdoor pilot-scale tubular photobioreactor for removal of selected pesticides from water

Supplementary Information

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Text S1. Pesticide quantification and TP identification in indoor batch experiments

Text 2. Pesticide quantification and TP identification in pilot-scale PBR experiments

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Text S1. Pesticide quantification and TP identification in indoor batch experiments

The evaluation of pesticide degradation and the identification of the transformation products (TPs) formed in indoor batch experiments were done after direct injection of the samples into the analytical instrument, an ultra-high performance liquid chromatography (UHPLC) system Acquity (Waters, Milford, MA, USA) coupled to a hybrid quadrupole-Orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific, San Jose, CA, USA), equipped with a heated-electrospray ionization source HESI. Acetamiprid and propanil were analyzed in the positive ionization mode (HESI+) while bentazone was analyzed in the negative ionization mode (HESI-). TPs formed during the degradation process were determined in both positive and negative ionization modes. Chromatographic separation was achieved with a Purospher® STAR RP-18 endcapped Hibar® HR (150 × 2.1 mm, 2 µm) column from Merck (Darmstadt, Germany) and a linear gradient of the organic constituent of the mobile phase. The mobile phase employed for HESI+ analyses consisted of (A) water and (B) methanol, both containing 0.1% of formic acid (flow rate of 0.2 mL min^{-1}), whereas in HESI- a mobile phase of (A) water and (B) acetonitrile (flow rate of 0.3 mL min^{-1}) was used. In both cases, the organic gradient employed was as follows: 5% B from the start to time (t) = 1 min, 20 % B at t = 3 min, 80% B at t = 6 min, 100% B at t = 7 min. Pure organic conditions were maintained until t = 9 min. Finally, initial conditions (5% B) were again achieved at t = 9.5 min and held for 4.5 min for column re-equilibration. The injection volume was 10 µL.

The specific conditions used in the HESI interface were: ion spray voltage, 3.0 kV in HESI+ and -2.5 kV in HESI-; sheath gas flow rate, 40 arbitrary units; auxiliary gas, 10 arbitrary units; capillary temperature, 350 ºC; and vaporizer temperature, 400 ºC. Nitrogen (>99.98%) was employed as sheath, auxiliary, and sweep gas. Accurate mass detection was conducted in data-dependent acquisition (DDA) mode. First, a full scan was acquired over the *m/z* range 70-1,000 at full width at half maximum (FWHM) resolution of 70,000 (at *m/z* 200). Then, data-dependent MS/MS scan events (FWHM resolution of 17,500 at *m/z* 200) were recorded for the five most intense ions (>$10e^5$) detected in each scan, with a normalized collision energy of 40%. Data acquisition was controlled by Xcalibur 2.2 software (Thermo Fisher Scientific).

The software Compound Discover 3.1 from Thermo Fisher Scientific was used to process the HRMS data generated with the LC-Orbitrap MS to identify the TPs formed during batch degradation experiments. Briefly, experimental samples (i.e. those collected at time 2 and 7 days) were compared with control samples (i.e., samples collected at t = 0 days). The software was used for peak alignment and deconvolution using 2 min as maximum retention time shift and 5 ppm of mass tolerance. Then, the different peaks detected were grouped and their elemental composition predicted. In parallel, a search by formula or mass was performed in various MS libraries and compound databases (mzCloud, mzVault, ChemSpider) for the assignment of potential compound identity. The list of potential candidates was subsequently revised to identify TPs that were only present in experimental samples and absent in control samples (heat-killed and abiotic reactors). Once identified, the molecular structures proposed by the software were evaluated according to the elemental composition of the molecular and fragment ions, fragment rationalization (assisted by fragment ion search scoring), and isotopic patterns.

Text 2. Pesticide quantification and TP identification in pilot-scale PBR experiments

Pesticide removal and TP formation in pilot-scale PBR experiments were monitored after direct injection of 400 µL of each sample into an analytical system consisting of a SCIEX Exion LC™ AD chromatograph, that incorporates a Shimadzu FCV-11AL Reservoir Selection Valve and a 0.5 mL injection loop, coupled to a SCIEX X500R QTOF detector, equipped with a Turbo V™ source (Sciex, Framingham, MA). Chromatographic separation was achieved with a Purospher® STAR RP-18 endcapped Hibar® (125 × 4 mm, 5 µm) column from Merck (Darmstadt, Germany) and a mobile phase at a flow rate of 1 mL min^{-1} consisting of (A) water and (B) acetonitrile, with the following organic gradient: 5% B from the start to time (t) = 1 min, 80% B at t = 15 min, 100% B at t = 17 min. Pure organic conditions were maintained for 3 min and, then, in 2 min, initial conditions (5% B) were again achieved and held for 4 min for column re-equilibration.

The specific conditions used in the ion source were a curtain gas of 35 arbitrary units, ion source gas 1 and 2 of 60 and 45 psi, respectively, temperature 700 ºC, with an ion spray voltage of -4500 V (negative mode) and 5500 V (positive mode). Accurate mass detection was achieved through a full TOF-MS scan over the *m/z* range 70-650, using an accumulation time of 0.125 s, a declustering potential of 70 V with a spread of 20 V, and a collision energy of 10 V, followed by an Information Dependent Acquisition (IDA) TOF-MS/MS analysis with a workflow for small molecules, i.e., a maximum of 6 candidate ions, an intensity threshold of 100 counts/s, with dynamic background subtraction and dynamic accumulation, a mass tolerance of 20 mDa and an inclusion list containing the m/z of the target pesticides and the TPs identified in the batch experiments. Data acquisition was controlled by SCIEX OS v.1.5 (Sciex). This software was also used for data treatment.

Figure S1. Pilot outdoor photobioreactor.

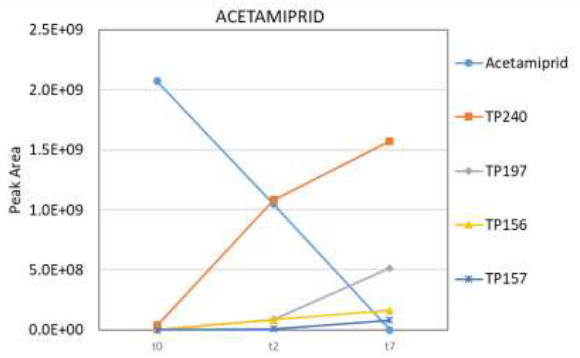

Figure S2. Transformation products identified for acetamiprid degradation by microalgae at indoor batch conditions.

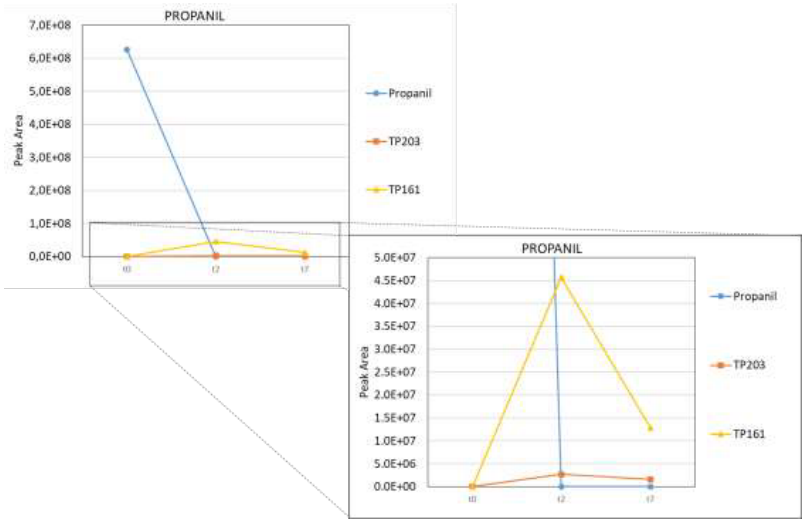

Figure S3. Transformation products identified for propanil degradation by microalgae at indoor batch conditions.

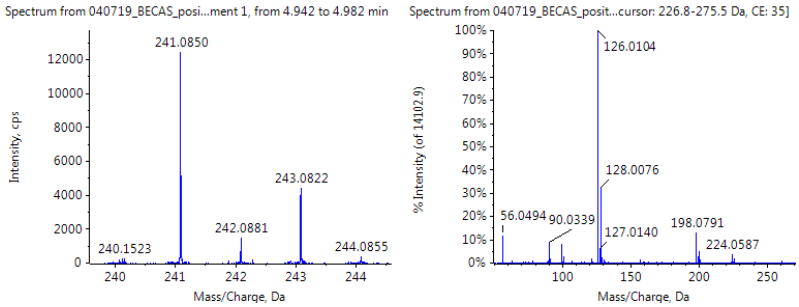

Figure S4. TOF-MS (left) and MS/MS (right) spectra of m/z 241.0850 (TP240 from acetamiprid).

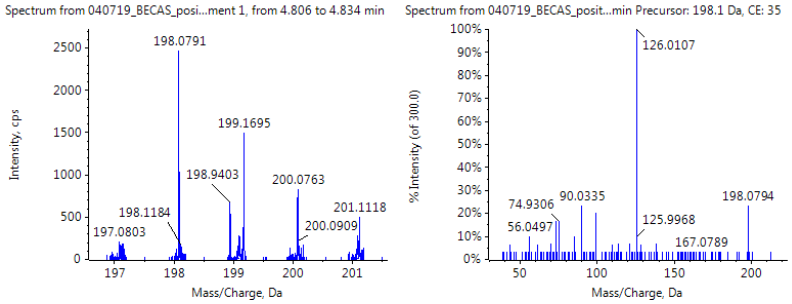

Figure S5. TOF-MS (left) and MS/MS (right) spectra of m/z 198.0791 (TP197 from acetamiprid).

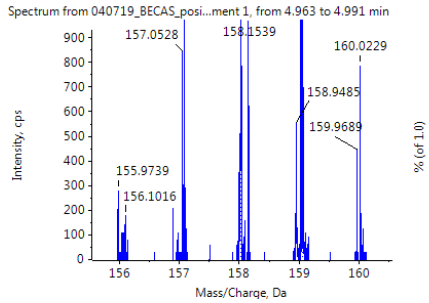

Figure S6. TOF-MS (left) spectra of m/z 157.0528 (TP156 from acetamiprid).

Chapter 4

Discussion

4.1. Analytical methodologies

In this thesis, different analytical methodologies have been developed and implemented for the determination of target pesticides in various food matrices, the wide-scope (suspect) screening of CECs in water, and the identification of unknown transformation products formed during the bioremediation of selected pesticides in water (non-target screening). Target methods were specifically optimized and validated for the determination of 42 pesticides, including a selection of herbicides, insecticides, fungicides, and some of their TPs, based on their usage and toxicity, in five different food commodities. On the other hand, generic and wide-scope analytical procedures were applied for the suspect and non-target screening approaches, as no specific compounds were expected.

Regarding the extraction procedures, since the matrices investigated and the focus of the experiments were very different, diverse specific extraction techniques were applied in each case. For the analysis of pesticides in food commodities (scientific publications #1 and #2) (namely corn, grapes, alfalfa, sunflower seeds, and olives), QuEChERS-based extraction procedures were optimized for each matrix through a rationale design of experiments. Taking into consideration the potential effects of matrix components in the analytical determination with LC-MS, key steps in the QuEChERS extraction method, such as the selection of the salting-out buffer and the clean-up sorbents were optimized to minimize the extraction of matrix components and maximize the recovery of the target pesticides. Moreover, since the acidification of the extraction process could stabilize some pH-labile compounds and enhance both the extraction and the LC-MS signal, the acidification of both the acetonitrile used for the extraction and the final extract were also evaluated in the design of experiments. With these premises, optimal conditions for the extraction of up to 42 pesticides were obtained in a rationale and cost-effective way (Table 4.1.).

Table 4.1. Design of experiments methods optimization results

	Corn	Grapes	Alfalfa	Sunflower seeds	Olives
Proportion of formic acid in the extraction solvent	0.5%	1%	0.5%	1%	1%
Extractive salts	Citrate buffer	Acetate buffer	Citrate buffer	Acetate buffer	Acetate buffer
Clean-up sorbent	900 mg MgSO4, 150 mg PSA, 150 mg C18	900 mg MgSO4, 150 mg PSA, 45 mg GCB	900 mg MgSO4, 150 mg PSA, 45 mg GCB	900 mg MgSO4, 150 mg PSA, 150 mg C18e	1200 mg MgSO4, 400 mg PSA, 400 mg GCB, 400 mg C18e
Proportion of formic acid in the final extract	1%	0.7%	0.7%	0.5%	0.7%

For the suspect screening of CECs in water samples (scientific publication #3), a freeze-drying step, followed by a re-dissolution of the solid residue in organic solvents and centrifugation of the extracts obtained, was used for CEC extraction, in an effort to perform a generic method that minimizes compound losses (common during solid phase or liquid-liquid extraction and clean-up processes). Lyophilisation also allowed pre-concentrating the samples and obtaining better sensitivity despite the higher matrix effects. Finally, for the identification of pesticide TPs formed during the bioremediation treatments (scientific publications #4 and #5), a fast and simple centrifugation of the samples (spiked with the pesticides at sufficiently high concentrations) was enough for subsequent LC-HRMS analysis.

The analytical procedures for pesticide determination in foodstuff were validated in terms of linearity, accuracy, precision, sensitivity and matrix effects and, as expected, results remarkably varied among matrices, showing the high influence of matrix interferences in the analytical performance. Regarding linearity, calibration curves were obtained using the internal standard method, which allowed working linear intervals greater than four orders of magnitude for most compounds with a coefficient of determination over 0.99. In all cases, least squares linear regression models were built with a weighting factor of 1/x to reduce the weight of the high concentration points in the models.

Concerning accuracy, the average relative recoveries obtained with the five methodologies developed were always between 70 and 120%, except for a few cases that slightly deviated from this range. The results were in agreement with the analytical acceptability parameters stated in the SANTE 11312/2021, the European Commission guidance for method

validation and analytical quality control for pesticides analysis in food and feed (European Commission, 2021). Moreover, the precision, calculated as the relative standard deviation (RSD) of the five replicates for each validation level, was consistent, with values below 20% in almost all cases.

In terms of sensitivity, LODs obtained along the development and validation of the five methodologies were in all cases below the established MRLs, with very few exceptions (Figure 4.1). The methods performance, thus, guarantee the consumer security as not detecting a compound would correspond to concentrations at least lower than the permitted values. Only fenthion and fenthion sulfone in corn, bromoxynil in alfalfa, and dichlorvos, fenthion and fenthion sulfone in olives did not reach the regulatory limits, as shown in figure 4.1. Comparing among matrices, the lowest LDets were obtained for alfalfa and grapes, while higher LDet values were obtained in the case of olives, probably due to the comparatively higher matrix interferences in this food commodity (Figure 4.2). LDet observed for pesticide analysis with the developed methodologies ranged over a few orders of magnitude, mostly influenced by the physical-chemical properties of the compound, the matrix effects, and the instrumental efficiency (Figure 4.2). This finding is common in multiresidue analytical methodologies (Maria Vittoria Barbieri et al., 2019; López-Blanco et al., 2016).

Contrariwise, the methodology described in the scientific publication #3 aimed at performing a wide-scope suspect screening approach without any filter or focus on specific chemicals. This objective, thus, influenced the sample treatment strategy implemented, looking for a generic extraction approach that could minimize the loss of contaminants as much as possible. Extraction methodologies for wide-scope LC-HRMS analysis of water are typically based on solid phase extraction (SPE), using different sorbents in line so that compounds with very different physical-chemical properties can be retained (Menger et al., 2020). These may be cation and anion exchange sorbents, nonpolar sorbents as Isolute ENV+, or the commonly used hydrophilic-lipophilic balance (HLB) sorbents (Kern et al., 2009). Nevertheless, the high mass accuracy and high mass resolution of HRMS technologies compared to low-resolution instruments improve the signal-to-noise ratio and distinguish better the ions of interest from the background signals, reducing the need for complex clean-up processes

Figure 4.1. Heat map of the LODs achieved for the target pesticides with the methodologies developed in the framework of this doctoral thesis compared to the EC MRLs thresholds. Green: LOD < MRL; Red: LOD > MRL; Yellow: pesticide not authorised for that food; Grey: no data.

(Leendert et al., 2015). In this context, lyophilisation appeared as a simple alternative to SPE extraction, achieving at once analyte enrichment and extraction in organic solvents. This technique has already been implemented in the analysis of pharmaceuticals, personal care products and pesticides (Sinha et al., 2014; Zhang et al., 2021). The main advantages of lyophilisation are the reduced loss of compounds of interest, its low cost, and easy performance, even though high matrix effects may interfere in the analysis. At this point, in this study, hundreds of compounds with a high chemical diversity were recovered in all samples: from PFAS and polar pharmaceuticals to phthalates, semi-polar pesticides or industrial chemicals.

In the case of the pesticide biodegradation processes (scientific publications #4 and #5), samples were not pre-concentrated due to the sufficiently high concentrations used for the experiments (1 mg L-1). Therefore, samples were simply centrifuged prior to their analysis. However, pre-concentration might had allowed identifying TPs generated at low concentrations. The main strategy followed for LC-HRMS analysis was the

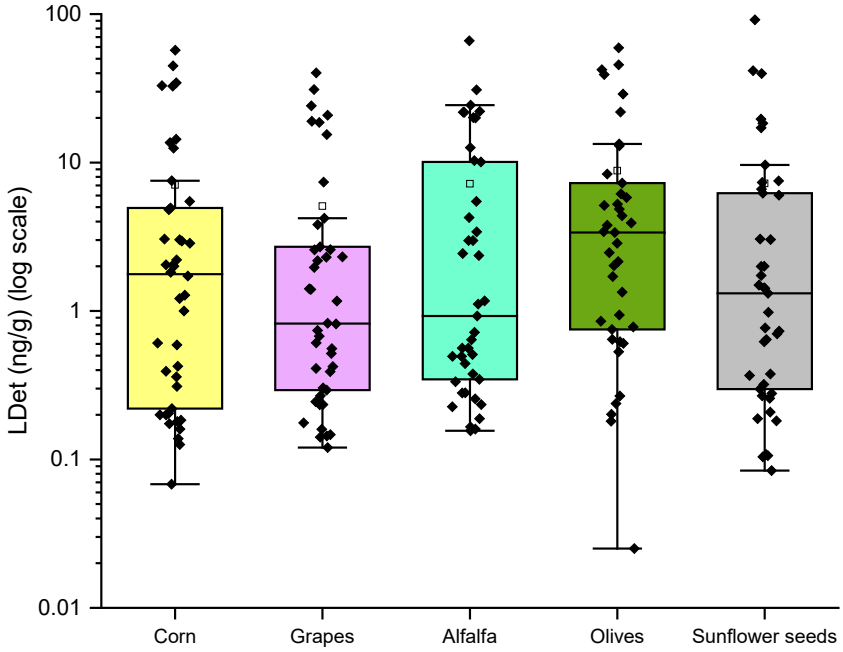

Figure 4.2. Box plot showing the LDet distribution of the 42 analysed pesticides in each validated matrix.

optimization of the chromatographic conditions for the detection of the parent compounds, because, in general, their corresponding TPs could be expected to have similar behaviour. Biodegradation processes normally generate TPs with higher polarity than the parent compounds and smaller sizes. The typical reactions are oxidations such as hydroxylations and carboxylations, methylations, and lytic reactions as hydrolysis. Moreover, it was essential to achieve good chromatographic peaks to guarantee high quality MS2 spectral information to, subsequently, enable the structural elucidation of the TPs formed. A reverse phase C18 chromatographic column was used for the analyses and, therefore, some extremely polar TPs could have been overlooked. Figures 4.3 and 4.4 show the extracted ion chromatograms of the three pesticides investigated.

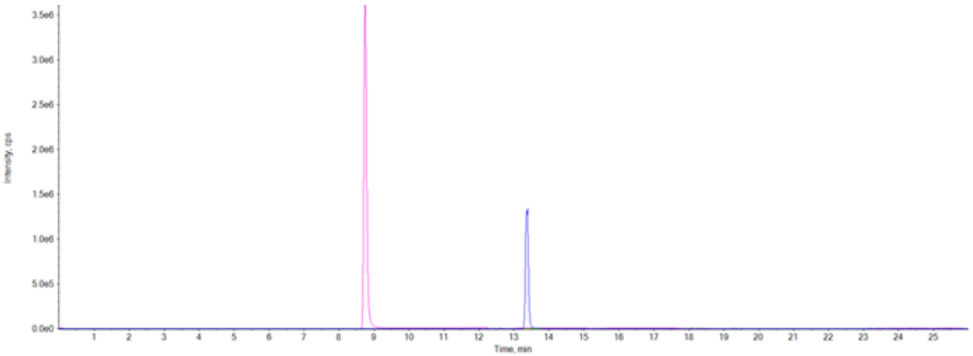

Figure 4.3. Extracted ion chromatogram of acetamiprid molecular ion (pink coloured peak, m/z 223.0745, mass error ≤ 5 ppm) and propanil molecular ion (blue coloured peak, m/z 218.0134, mass error ≤ 5 ppm) from a fortified sample.

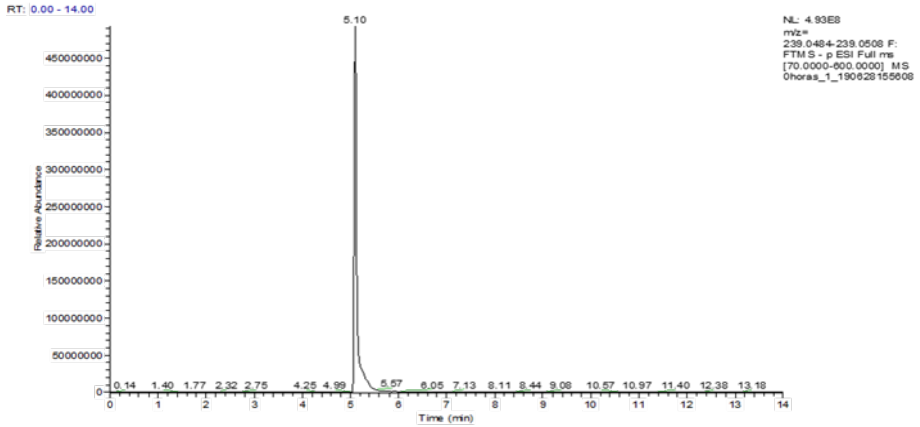

Figure 4.4. Extracted ion chromatogram of bentazone molecular ion (m/z 239.0496, mass error ≤ 5 ppm) from a fortified agricultural wastewater sample.

4.2. Occurrence and (eco)toxicity of the CECs detected in crops and irrigation water

In this section, the presence and distribution of the CECs found in the food matrices and irrigation waters analysed are evaluated (scientific publications #1, #2, and #3).

Firstly, the focus was set on pesticide occurrence due to the environmental problems related to their usage in the field and the potential human exposure through the diet. Once applied in the field, pesticides can undergo different processes such as transfer from the target site to other environmental compartments (adsorption to soil or plants, translocation into crops and entrance in their metabolic routes, leaching and run off to ground and surface waters) or degradation via chemical reactions, microbes metabolism or photolytic processes (Tudi et al., 2021). Nevertheless, from these, adsorption by plants or translocation of pesticides into crops are the main routes that may pose a risk to consumers. Scientific publications #1 and #2 focused the efforts in the development of analytical methods in compliance with the SANTE 12682/2019 guidance (European Commission, 2019) to guarantee consumers' safety. This was accomplished, once the limits of detection of the developed methodologies for the target pesticides achieved values lower than the established MRLs. Figure 4.5 summarizes the pesticides detected in each of the food products analyzed in the different monitoring campaigns performed, the range of concentrations that they presented, and the frequency of detection. As can be seen, most of the pesticides detected were found in more than one sample from the same commodity, showing the general ubiquity of pesticides during their usage. However, concentrations can vary among samples from the same campaign because of an irregular application and/or distribution of the agrochemicals, that may depend on many different factors (scientific publication #1, Figure 2; scientific publication #2, Figure 2).

Corn was found to be the only food commodity free from pesticides. Four different pesticides, namely, acetamiprid, chlothianidin, imidacloprid and thiacloprid, were detected in grapes in the two sampling campaigns performed. These four compounds are insecticides from the chemical group of neonicotinoids. It is well known that grapevines are highly sensitive to infestation by insects that consume leaves, shoots, roots or fruits. Potato

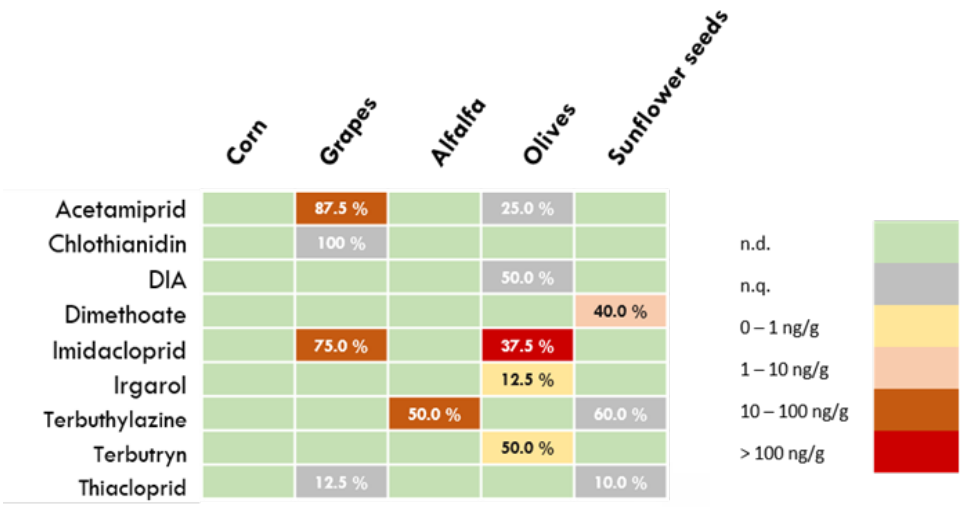

Figure 4.5. Heat map showing the concentration ranges of the pesticides detected in real food samples and the frequency of detection in each matrix.

leafhopper (Empoasca fabae Harris), Japanese beetles (Popillia japonica Newman), and grape berry moth [Paralobesia viteana (Clemens)] are the most typical pests in grapevines for which neonicotinoid insecticides have shown to be effective (Van Timmeren et al., 2012). In this line, olive crops also suffer from insect pests such as the olive fruit fly (Bactrocera oleae) or the black scale (Saissetia oleae), usually controlled by neonicotinoid-based insecticides (Allema et al., 2017). Consequently, acetamiprid and imidacloprid were also detected in olive samples. Although this chemical group acts at the nicotinic acetylcholine receptors from insects irreversibly binding to them and stimulating the peripheral and central nervous system, mammals may also be affected by them at a lower extent (Tomizawa & Casida, 2005). The widespread use, high persistence, and water solubility of neonicotinoid pesticides poses an environmental risk, threatening wildlife and biodiversity, and even human health (Klingelhöfer et al., 2022). Apart from these compounds, herbicides such as deisopropylatrazine (DIA), irgarol, and terbutryn were also found in olive samples. These herbicides are used to control weed growth that may reduce plant growth and olive yields. Terbuthylazine, a triazine-based herbicide used to control weeds in different crop varieties, was found in half of the alfalfa samples and in most of the sunflower seed samples. A few decades ago, terbuthylazine became the most important herbicide for many different crops in European

fields (Heri et al., 2008). In addition to terbuthylazine, two insecticides, dimethoate and thiacloprid were also detected in sunflower seeds with a lower distribution than terbuthylazine. The use of insecticides as dimethoate in sunflower crops has been related to a decrease in seed productivity due to pollinators intoxications (Nderitu et al., 2007). Although insect pests are well controlled, honeybees (Apis mellifera L.), the most important pollinator for these crops, are also affected reducing their population and, therefore, the pollination efficiency. The study by Nderitu et al. (2007) also observed that the period of spraying application was determining production yields (size and quantity of seeds), as pre-flowering spraying was less harmful than during the sunflower blooming.

Corn was found to be the only food commodity free from pesticides. Four different pesticides, namely, acetamiprid, chlothianidin, imidacloprid and thiacloprid, were detected in grapes in the two sampling campaigns performed. These four compounds are insecticides from the chemical group of neonicotinoids. It is well known that grapevines are highly sensitive to infestation by insects that consume leaves, shoots, roots or fruits. Potato leafhopper (Empoasca fabae Harris), Japanese beetles (Popillia japonica Newman), and grape berry moth [Paralobesia viteana (Clemens)] are the most typical pests in grapevines for which neonicotinoid insecticides have shown to be effective (Van Timmeren et al., 2012). In this line, olive crops also suffer from insect pests such as the olive fruit fly (Bactrocera oleae) or the black scale (Saissetia oleae), usually controlled by neonicotinoid-based insecticides (Allema et al., 2017). Consequently, acetamiprid and imidacloprid were also detected in olive samples. Although this chemical group acts at the nicotinic acetylcholine receptors from insects irreversibly binding to them and stimulating the peripheral and central nervous system, mammals may also be affected by them at a lower extent (Tomizawa & Casida, 2005). The widespread use, high persistence, and water solubility of neonicotinoid pesticides poses an environmental risk, threatening wildlife and biodiversity, and even human health (Klingelhöfer et al., 2022). Apart from these compounds, herbicides such as deisopropylatrazine (DIA), irgarol, and terbutryn were also found in olive samples. These herbicides are used to control weed growth that may reduce plant growth and olive yields. Terbuthylazine, a triazine-based herbicide used to control weeds in different crop varieties, was found in half of the alfalfa samples and in most of the sunflower seed samples. A few decades ago, terbuthylazine

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These analyses revealed the presence of a few pesticides from a set of pre-selected agrochemicals that might have been used in the crop fields and, through the development and validation of the described analytical methodologies, concentrations of these compounds were calculated. However, many other compounds may be occurring in the agricultural environments and they could eventually reach crops. These include anthropogenic pollutants like pharmaceuticals, micro and nanoplastics, and industrial chemicals, among others. These substances may end up in soils and crops mainly through the use of treated wastewater, the discharge of treated wastewater into surface waters, that can also be connected with groundwaters, (Moeck et al., 2022; Panthi et al., 2019), and the application of these waters for irrigation. Moreover, the use of sewage sludge, the mixture of organic and inorganic products generated after wastewater treatment, as fertilizer for agriculture has become another source of pollution for crops (Paraíba & Saito, 2005).

In this context, the necessity of tools that allow targeting the right substances in the different matrices in the monitoring programs is clear. Thus, the scientific publication #3 shows a prioritization procedure to select site-specific priority pollutants, applied to identify the most relevant contaminants in a reclaimed water-based irrigation system from a specific agricultural area in Catalonia, Spain. Following the generic analytical methodology previously explained in section 4.1, a characterization of the organic contamination footprint of the water matrices in the irrigation system under study was performed. As can be seen in section 3.1 from

the scientific publication #3, out the 162 CECs identified, only 16 were pesticides and some of them had their origin in the water reclamation plant. This shows that pesticide application in the field to control pests represents just a small piece of the total contamination footprint that may arrive to crops. Pharmaceuticals, industrial chemicals, drugs of abuse, plastic-derived compounds or tire wear and road runoff chemicals, among others, should also be taken into consideration when analysing crop matrices.

Nevertheless, it is not possible to manage a quantitative target methodology for the hundreds of compounds that may be present in environmental samples. Therefore, considering the irrigation water as the main contamination vector for crops, the prioritization approach performed was essential to identify the most abundant and frequently detected and toxicologically relevant compounds in a rational and cost-effective way. For this purpose, the semi-quantification approach carried out allowed us to perform an ecotoxicological risk assessment and, thereby, classify CECs based on their occurrence and ecotoxicity. With this procedure, a selection of priority CECs based on the local occurrence and ecotoxicity was obtained, reducing the limitations of a pre-selection of compounds based on the chemical group and literature data. At this point of the screening procedure the confirmation level of the identified compounds is 2, according to the system described by Schymanski et al. (Schymanski et al., 2014), since level 1 is only achieved through confirmation with the corresponding analytical standards. The next step now is the purchase of such standards and the development of target analytical methods for the monitoring of the selected compounds in the irrigation water and food matrices of interest to guarantee the consumers safety, which will be done in the framework of the MAGO project.

4.3. Attenuation strategies

The previous studies (scientific publications #1, #2, and #3) showed the occurrence of numerous pollutants in the different environmental compartments evaluated. From a few target pesticides in most of the analysed food samples to hundreds of CECs detected in reclaimed water used for irrigation, organic pollutants show a relatively high ubiquity in the agricultural environment. Overall, wastewater treatment

plants have been proven ineffective for CEC removal, leading to their widespread occurrence in surface waters and, hence, in the environment. Specifically, pesticides are poorly removed in WWTPs and there is evidence that urban wastewaters are also a relevant source of pesticides into the environment (Köck-Schulmeyer et al., 2013). CEC removal is both compound and treatment process dependent and, normally, hybrid processes are recommended for effectively tackling the organic pollution issue (Grandclément et al., 2017). Although there is a great concern in the scientific community and public institutions to handle this problem, solutions are still upcoming. The works described in the scientific publications #4 and #5 aimed at providing new alternative bioremediation processes to remove specific organic pollutants from water. Three pesticides, namely bentazone, acetamiprid, and propanil, were selected based on their widespread use in the Catalan region and the high concentrations at which they were found in surface waters of agricultural relevant areas in this territory, that may pose a risk to the aquatic ecosystem (M.V. Barbieri et al., 2020).

As explained in section 1.4, TPs are still hardly studied and their occurrence and toxicity remain unknown. Thus, apart from evaluating the feasibility of pesticides removal of the two bioremediation strategies tested (Trametes versicolor and microalgae-based systems), focus was also put on the characterization of the biodegradation pathways, by identifying the TPs formed during the process.

Firstly, in the case of bentazone degradation by the white-rot fungi Trametes versicolor (scientific publication #4), up to 19 TPs were detected during fungal biodegradation experiments and, for eight of them, a chemical structure could be proposed based on their spectral information. The MS2 spectra was crucial for the determination of the chemical modifications with respect to the parent compound. Reactions involved in these biotransformations were hydroxylations, oxidations, methylations, N-nitrosation, and dimerization, which was in line with the expected reactivity of the cytochrome P450 and laccase enzymatic systems that, actually, characterize the degradation ability of Trametes versicolor. In the case of the microalgae-based biodegradation (scientific publication #5), four TPs were identified for acetamiprid, and two for propanil, including demethylations, oxidations, and hydrolytic reactions.

This first step identifying new TPs and understanding natural biodegradation pathways is critical to fill the knowledge gaps in this field and, in the future, open the door to characterize the environmental contamination footprint on a more holistic way, including the probably huge amount of yet unknown TPs. HRMS instrumentation technologies in combination with potent informatics tools are rapidly improving and, once the spectral libraries include the mass spectra generated through lab-scale experiments such as the obtained in the works held in scientific publications #4 and #5, routine monitoring programs will be able to detect these potential water contaminants (Hollender et al., 2017).

Moreover, there is still very little information about the effects and toxicity of TPs in the different environmental compartments. TPs are usually structurally similar to their parent compounds and, hence, they may retain the biological activity or even increase it. In addition, these compounds can also be more toxic, more persistent and more mobile than the original compounds, increasing their ecotoxicological risks (Müller et al., 2016). To assess the toxicity of these chemicals, the use of QSAR models appears as a green, fast and low-cost tool, alternative to the in vivo and in vitro laboratory assays, ideal for an initial approximation of the ecotoxicity of TPs. The QSAR-predicted LC50 and EC50 values for the three representative aquatic species, viz., fish, daphnia, and green algae of the evaluated compounds and their respective TPs are shown in Table 4.2. 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 (Winder et al., 2005). According to these values, the toxicity data obtained was divided into four levels (i.e., very toxic, toxic, harmful, and not harmful).

Bentazone was predicted as not harmful for fish and daphnia (LC50 > 100 mg/L), and harmful for green algae (EC50 value between 10 and 100 mg/L). Regarding the eight identified TPs, three of them, namely TP284a, TP284b, and TP268 decreased the toxicity with respect to their parent compound, while the rest of TPs evolved in a higher toxicity. The worse value was obtained for TP256, which was very toxic to daphnia, toxic to green algae, and harmful to fish. This TP corresponded to the hydroxylation of the aromatic ring of bentazone. TP285, which was formed after N-nitrosation of the TP256, was harmful for fish and toxic for daphnia

Table 4.2. Predicted toxicity of bentazone, acetamiprid, propanil and their identified TPs by the ECOSAR program*, **

Compound	TP	SMILES	LC50 fish 96h (mg/L)	LC50 daphnia 48h (mg/L)	EC50 green algae 96h (mg/L)
Bentazone		CC(N1C(C2=CC=CC=C2NS1(=O)=O)=O)C	122	124	13.1
	TP284a	O=C(C1=CC=CC=C1N2C(O)=O)N(C(C)C)S2(=O)=O	496	563	41.5
	TP284b	O=C(C1=CC=CC=C1N2)N(C(CC(O)=O)C)S2(=O)=O	5490	6290	450
	TP268	O=C(C1=CC=CC=C1N2C=O)N(C(C)C)S2(=O)=O	447	506	37.8
	TP256	O=C(C1=CC=CC(O)=C1N2)N(C(C)C)S2(=O)=O	***10.4***	***0.976***	***1.78***
	TP494	O=C(C1=CC=CC(OC2=C(NS(N(C(C)C)C3=O)(=O)=O)C3=CC=C2)=C1N4)N(C(C)C)S4(=O)=O	***50.2***	***44.9***	***7.49***
	TP285	O=C(C1=CC=CC(O)=C1N2N=O)N(C(C)C)S2(=O)=O	***13.2***	***1.15***	***2.20***
	TP268	CC(C)N(O1)S(N=C2C1=CC(C=C2O)=O)(=O)=O	***10.2***	***30.4***	***1.01***
	TP286	CC(CC=O)N(O1)S(N=C2C1=CC(C=C2O)=O)(=O)=O	***22.1***	***73.7***	***2.88***
Acetamiprid		CC(=NC#N)N(C)CC1=CN=C(C=C1)Cl	18.7	2.38	1.73
	TP240	CC(N(CC1=CC=C(Cl)N=C1)C)=NC(N)=O	667	64.8	80.2
	TP197	CC(N(CC1=CC=C(Cl)N=C1)C)=N	2180	190	290
	TP156	CNCC1=CC=C(Cl)N=C1	237	24.1	27.2
	TP157	O=C(O)C1=CN=C(Cl)C=C1	21.1	12.8	363
Propanil		CCC(=O)NC1=CC(=C(C=C1)Cl)Cl	12.5	10.7	2.1
	TP203	CC(NC1=CC=C(Cl)C(Cl)=C1)=O	28.3	26.0	3.97
	TP161	NC1=CC=C(Cl)C(Cl)=C1	***10.1***	***1.35***	3.32

Abbreviations: LC50, 50% lethal concentration; EC50, 50% effective concentration.
*Toxicity ranges were highlighted with colours: red: LC50/EC50 ≤ 1, very toxic; orange: 1 < LC50/EC50 ≤ 10, toxic; blue: 10 < LC50/EC50 ≤ 100, harmful; green: LC50/EC50 > 100, not harmful.
**TPs parameters with a higher toxicity than the parent compound were highlighted in bold and italics.

and green algae. Then, the laccase-derived degradation products, TP268 and TP286, were also toxic for green algae, and harmful for fish and daphnia. In general, fungal-based biodegradation of bentazone ended up in the formation of a myriad of TPs with a relatively higher toxicity than their parent compound. Nevertheless, additional research is required to know the real occurrence of these compounds in the environment, as well as other parameters as mobility and persistence to perform a stronger ecotoxicological evaluation. In the case of acetamiprid, the four TPs generated after the microalgae-based degradation procedure had a lower toxicity to fish, daphnia, and green algae than the parent compound. For propanil, TP203 relatively improved all the ecotoxicological parameters,

while TP161 was less toxic to green algae, but posed a higher risk to fish and daphnia than propanil. The identified TPs were not confirmed as for most of TPs no commercial standards are available. However, other tools as the Retention Time Index (Aalizadeh et al., 2021) may be used to increase the identification confidence (explained in section 2.5, scientific publication #3). Then, semi-quantification approaches, as the structurally similar standards approach or the ionization efficiency model (Aalizadeh et al., 2022), could be used to study their occurrence in the environment.

Results obtained with these two bioremediation alternatives show the compound structure dependency of the wastewater treatment technologies and the need of hybridization for a better CEC removal efficiency. While bentazone was not degraded by the microalgae-based system, the white-rot fungi Trametes versicolor showed a good performance, although most of the generated TPs had a higher predicted ecotoxicity. Meanwhile, microalgae consortium was able to biodegrade acetamiprid and propanil, generating four stable TPs in the case of acetamiprid while, apparently, propanil degradation products were mineralized. Nevertheless, these systems offer many different advantages in terms of sustainability, low energy demand and/or low operational costs. Further research is still needed to optimize relevant operational conditions and make these bioremediation strategies implementable at real scale, since the results obtained reflect a promising future.

Chapter 5

Conclusions

5. Conclusions

The main results and conclusions of the work carried out during this doctoral thesis are summarized as follows:

- The analytical methodologies developed and validated for the analysis of 42 medium to highly polar pesticides and their metabolites in five different plant origin foods, namely, corn, grapes, alfalfa, sunflower seeds, and olives, showed high sensitivity, with LODs nearly always below the MRLs established by the European Commission. The QuEChERS extraction procedures were cost-effectively optimized through a fractional factorial design of experiments taking into consideration each matrix idiosyncrasy. Higher LODets were obtained for olives and corn matrices compared to grapes, alfalfa, and sunflower seeds, probably due to stronger matrix interferences. These methods enable an economical, simple, and reliable determination of a wide range of pesticides. Moreover, the use of isotopically-labelled surrogate standards corrects the analyte losses that may occur during sample manipulation as well as the MS signal fluctuations, and thus, facilitates the quantification process.

- To the authors' knowledge, these were the first analytical methodologies described in the peer-reviewed literature for the determination of eleven (sunflower seeds), eighteen (olives), five (corn), thirteen (grapes), and twenty-four (alfalfa) of the target pesticides.

- The analysis of real samples harvested at different locations along the Iberian Peninsula revealed the presence of a few insecticides and herbicides, some of them included in the PAN International List of Highly Hazardous Pesticides (acetamiprid, clothianidin, imidacloprid, and thiacloprid) and/or currently banned for use in the European Union (clothianidin, dimethoate, imidacloprid, terbutryn, thiacloprid, and thiamethoxam).

- The wide-scope LC-HRMS-based screening of CECs in a reclaimed water-based irrigation system revealed the presence of up to 162 organic pollutants in the investigated water samples (influent

and effluent of the water reclamation plant, the treated water at the point of discharge to the net of irrigation channels, and irrigation water (mix of reclaimed and surface water), including pharmaceuticals, industrial chemicals, pesticides, and drugs of abuse, among others. More than a hundred compounds were detected at the water used for irrigation, 98 % of which had their origin in the reclaimed water. Thus, reclaimed water was identified as the main contamination source of CECs in the investigated reclaimed water-based irrigation system.

- A simple and cost-effective prioritization approach developed and implemented in the previously mentioned reclaimed water-based irrigation system allowed to identify the most relevant contaminants present in the irrigation water, in terms of abundance and toxicity. This approach allows the rational design of programs to monitor site-specific priority contaminants in water, otherwise overlooked by national or European regulation, in contrast to the classical preselection of compounds based on their chemical class or usage that may differ from the real contamination footprint at that specific area. Moreover, it contributes to implement the appropriate contamination mitigation measures and supports water managers and policy makers in their way to control water contamination and ensure safety in water reuse practices.

- Bioremediation strategies based on fungi and microalgae revealed as interesting alternatives for CEC removal. As in any other water treatment, CEC removal depends on the compound physical-chemical properties and the treatment itself. While the WRF Trametes versicolor showed a high degradation capability for bentazone, the microalgae-based system was not able to remove it. Microalgae readily degraded propanil and acetamiprid. However, in all cases except for propanil, the degradation processes involved did not end up in the complete mineralization of the pesticide, and TPs were generated during the biodegradation processes. In the case of bentazone degradation by Trametes versicolor, 19 TPs were formed (eight of them tentatively identified). Microalgae biodegradation of propanil and acetamiprid generated two and

four TPs, respectively, which in the case of propanil were also biodegradable.

- QSAR-based ecotoxicological risk assessment of the TPs generated during the biodegradation processes revealed a few TPs, in the case of propanil and bentazone, more toxic for the aquatic environment than the parent compound, while some others showed less toxicity.

- Although further research is needed in this field, there is not a single water treatment technology for the efficient removal of all CECs and, thus, the combination of complementary treatments is recommended to enhance CEC removal. In this context, bioremediation approaches, such as those investigated in the framework of this doctoral thesis, are a good alternative for CEC removal with a promising future to be implemented at real scale in decentralized water treatment systems.

- The identification of CEC TPs and their natural biodegradation pathways is critical to characterize the organic chemicals that may be occurring in water and, contributes to facilitate the comprehensive HRMS analysis of the contamination footprint in the different water bodies. The holistic characterization of the CEC mixtures is key to identify the relevant contaminants in each mixture and implement control and mitigation measures to handle CEC contamination.

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