# ANALYSIS, OCCURRENCE, FATE AND BEHAVIOUR OF EMERGING MICROPOLLUTANTS IN WASTEWATER AND THE RECEIVING ENVIRONMENT

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Universitat de Girona

*Doctoral Thesis*

# Analysis, occurrence, fate and behaviour of emerging micropollutants in wastewater and the receiving environment.

Annex 1-2

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Thesis supervision certificate

Prof. Mira Petrovic, research professor at the Catalan Institute for Water Research (ICRA) and the Catalan Institution for Research and Advanced Studies (ICREA), and Dr. Gianluigi Buttiglieri, research scientist at ICRA

DECLARE:

That the doctoral thesis entitled "**Analysis, occurrence, fate and behaviour of emerging micropollutants in wastewater and the receiving environment**" presented by **Lucia Gusmaroli** to obtain a doctoral degree from the University of Girona has been completed under our supervision and fulfils the requirements for the attainment of the international mention.

For all intents and purposes, I hereby sign this document.

Prof. Mira Petrovic                                          Dr. Gianluigi Buttiglieri

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# List of publications

The results of this PhD thesis, presented as chapters herein, have been published or submitted to scientific journals as follows:

Gusmaroli, L.; Insa, S.; Petrovic, M. (2018). **Development of an online SPE-UHPLC-MS/MS method for the multiresidue analysis of the 17 compounds from the EU "Watch list"**. Analytical and Bioanalytical Chemistry 410:4165–4176. (IF$_{2018}$: 3.286)

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Gusmaroli, L.; Buttiglieri, G.; Petrovic, M. (2019). **The EU watch list compounds in the Ebro delta region: Assessment of sources, river transport, and seasonal variations.** Environmental Pollution 253: 606–615. (IF$_{2018}$: 5.714)

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Gusmaroli, L.; Mendoza, E.; Petrovic, M.; Buttiglieri, G. (2020). **How do WWTPs operational parameters affect the removal rates of EU Watch list compounds?** Science of the Total Environment (IF$_{2018}$: 5.589)

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# List of acronyms

| | |
|---|---|
| a.u. | Arbitrary Units |
| ACN | Acetonitrile |
| BHT | 3,5-Di-tert-butyl-4-hydroxytoluene |
| CAS | Conventional Activated Sludge |
| CE | Collision Energy |
| CECs | Contaminants of Emerging Concern |
| CORG | Organic Carbon Content |
| CSO | Combined Sewer Overflow |
| E1 | Estrone |
| E2 | 17β- Estradiol |
| EDAR | Estació de Depuració d'aigües residuals |
| EDCs | Endocrine Disrupting Compounds |
| EDTA | Ethylenediaminetetraacetic Acid |
| EE2 | 17α-Ethynylestradiol |
| EHMC | 2-ethyl-hexyl-4-trimethoxycinnamate |
| EQS | Environmental Quality Standards |
| ESI | Electro-Spray  Ionization |
| GC | Gas Chromatography |
| GC-MS | Gas Chromatography- Mass Spectrometry |
| $H_c$ | Henry's law constant |
| HPLC | High-Performance Liquid Chromatography |
| HRMS | High-Resolution Mass Spectrometry |
| IDL | Instrumental Detection Limit |
| IS | Internal Standard |
| JRC | Joint Research Centre |
| $K_{biol}$ | Biodegradation rate constant |
| $K_d$ | Solid-water partitioning coefficient |
| $K_{ow}$ | Octanol-water partitioning coefficient |
| LC | Liquid Chromatography |
| LC-MS/MS | Liquid Chromatography Tandem Mass Spectrometry |
| LOD | Limit Of Detection |
| LOQ | Limit Of Quantification |
| MDL | Method Detection Limit |
| MEC | Measured Environmental Concentration |
| MeOH | Methanol |
| MLSS | Mixed Liquor Suspended Solids |
| MLVSS | Mixed Liquor Volatile Suspended Solids |
| MQL | Method Quantification Limit |
| MS | Mass Spectrometry |
| MS/MS | Tandem Mass Spectrometry |
| NBS | Nature-Based Solutions |
| $NH_4^+$ | Ammonium ion |

| | |
|---|---|
| NI | Negative Ionization |
| $NO_3^-$ | Nitrate ion |
| PE | Population Equivalents |
| PhACs | Pharmaceutically Active Compounds |
| PI | Positive Ionization |
| PNEC | Predicted No-Effect Concentration |
| PPCPs | Personal Care Products |
| PVDF | Polyvinylidene Fluoride |
| QSAR | Quantitative structure–activity relationship |
| $R^2$ | Coefficient of Determination |
| RQ | Risk Quotient |
| RSD | Relative Standard Deviation |
| RT | Retention Time |
| S/N | Signal-to-Noise Ratio |
| SC | Sampling Campaign |
| SPE | Solid-Phase Extraction |
| SRM | Selected Reaction Monitoring |
| TN | Total Nitrogen |
| TOC | Total Organic Carbon |
| TOF | Time-Of-Flight |
| TPs | Transformation Products |
| TSQ | Triple Stage Quadrupole |
| TSS | Total Suspended Solids |
| UHPLC | Ultra-High Performance Liquid Chromatography |
| UHPLC-MS/MS | Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry |
| UWWTD | Urban Waste Water Treatment Directive |
| WFD | Water Framework Directive |
| WWE | Wastewater Effluent |
| WWI | Wastewater Influent |
| WWTP | Wastewater Treatment Plant |

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# Summary

The presence of xenobiotics - such as pharmaceutically active compounds, endocrine disrupting compounds and pesticides - in the aquatic environment has risen great environmental concern due to their high toxicity even at low concentrations. These so-called micropollutants have been detected in all aquatic compartments and are continuously entering the environment through via point and non-point sources. In particular, the removal of xenobiotics by means of wastewater treatment plants (WWTPs) is known to be incomplete for several compounds, and more efforts are needed in this context.

To tackle this problem, the European Union is updating its legislation. The Directive 2013/39/EU set environmental quality standards in freshwater for 76 priority substances, mainly heavy metals, traditional pesticides and industrial chemicals. With regards of the contaminants of emerging concern, 17 candidates for inclusion in the priority substances list have been included in a Watch list (EU Decision 2015/495). These compounds must be monitored by all member states to collect data on their occurrence in freshwater. Risk assessment will be performed as a decision-making tool to determine whether they pose a risk to or via the aquatic environment.

This thesis aims at filling knowledge gaps on micropollutants and in particular on the Watch list compounds at three levels simultaneously: analytical, monitoring and removal possibilities. Firstly, an analytical methodology based on online SPE-UHPLC-MS/MS for the simultaneous determination of the Watch list compounds was developed. The proposed method offers advantages over already available methods, such as versatility (the whole set of compounds was grouped in one method for the first time), shorter time of analysis, robustness and sensitivity, all in compliance of the EU requirements, with the only exception of EE2, and was validated in both freshwater and, for the first time, wastewater (effluent and influent).

Then, this novel analytical methodology was applied for the monitoring of the Watch list compounds in the Ebro Delta, in the north of Spain, an area of great environmental and economic significance. The spatiotemporal monitoring was carried out in three sampling campaigns and 14 sampling locations including the two main WWTPs of the area (WWTP

Amposta, equipped with conventional activated sludge as secondary treatment, and WWTP Sant Carles de la Ràpita, which also features sand filters as tertiary treatment), the main stretch of the Ebro river and channels. Results evidenced that the extent of contamination in freshwater is not negligible, with total concentrations up to 2.39 µg/L. Pesticides, particularly imidacloprid and oxadiazon, were ubiquitous in freshwater and were strictly related to non-point sources like agricultural activities. Pharmaceuticals were the class accounting for most contamination, azithromycin and diclofenac being the compounds with the highest loads. The two investigated WWTPs were not efficient in removing the Watch list compounds; the highest average removal rates were recorded for diclofenac (47%). The concentration levels of the investigated chemicals were above the predicted no-effect concentration values in in most samples and risk quotients evidenced high risk in most sampling sites.

Parallelly, 8 among the Watch list compounds were selected for (bio)degradation studies in batch tests, with the dual objective of exploring their removal in conventional activated sludge systems - assessing biodegradation and the extent of sorption - and evaluating the importance of WWTPs operational parameters with the aim of enhancing removal rates. Four factors were considered at two different levels: pH, temperature, biomass concentration and redox conditions. The conditions leading to the maximum overall removal were aerobic conditions, high temperature (25 °C), high concentration of biomass (5 g/L) and high pH (7.5) and the parameters with the highest impact on removal rates were biomass concentration and redox conditions. The highest removal rates were obtained for E2, which was always transformed by 96%. For compounds like E1, erythromycin and methiocarb, results swept from zero to high or complete removals, highlighting the importance of the choice of operational parameters. Results show that sorption is a relevant removal pathway for some compounds, especially the hormones (and in particular EE2) and diclofenac.

Overall, the results presented herein show that the Watch list compounds are, at present, only partially removed during conventional treatment, with room for improvement. It is noteworthy that, the Watch list compounds may actually represent a threat for the environment at the concentration at which they occur, as confirmed by the results of the

case study. Despite the advances made during these years, several issues remain open, including analytical difficulties, inconsistency of data and significant legislation gaps.

# Resum

La presència de xenobiòtics, com ara productes farmacèutics, disruptors endocrins i pesticides, en el medi ambient aquàtic ha engendrat una gran preocupació ambiental degut a la seva elevada toxicitat, fins i tot a baixes concentracions. Aquests compostos, anomenats microcontaminants, s'han detectat en tots els compartiments aquàtics i entren al medi de manera continuada a través de fonts puntuals i no puntuals. En particular, se sap que l'eliminació de xenobiòtics en les plantes de tractament d'aigües residuals (EDAR) és incompleta i calen més esforços en aquest context.

Per a solucionar aquest problema, la Unió Europea està actualitzant la seva legislació. La Directiva 2013/39 / UE va establir normes de qualitat ambiental en aigua dolça per a 76 substàncies prioritàries, principalment metalls pesants, pesticides tradicionals i productes químics industrials. Pel que fa als contaminants emergents, 17 compostos candidats a ser afegits a la llista de substàncies prioritàries han estat inclosos en una llista de vigilància (Watch list) (Decisió UE 2015/495). Aquests compostos han de ser monitoritzats per tots els estats membres per a recopilar dades sobre la seva presència en aigua dolça. Es durà a terme l'anàlisi de risc com a eina de presa de decisions per determinar si representen un risc per o a través del medi aquàtic.

Aquesta tesi té com a objectiu principal omplir els buits de coneixement sobre microcontaminants i, en particular, sobre els compostos de la Watch list a tres nivells: anàlisi, monitorització i eliminació. En primer lloc, s'ha desenvolupat una metodologia analítica basada en SPE en línia -UHPLC-MS / MS (extracció en fase sòlida en línia, cromatografia liquida de resolució ultra alta acoblada a espectrometria de masses en tàndem) per a la determinació simultània dels compostos de la Watch list. El mètode proposat ofereix avantatges sobre els mètodes ja disponibles, com la versatilitat (ja que per primera vegada s'han agrupat tots els compostos en un únic mètode), un temps d'anàlisi més curt, robustesa i sensibilitat, tot en compliment dels requisits de la UE, amb l'única excepció de EE2, i s'ha validat tant en aigua dolça com, per primera vegada, en aigües residuals (efluents i influents).

A seguir, el mètode s'ha aplicat per a la monitorització dels compostos de la Watch list al Delta de l'Ebre, al nord d'Espanya, una àrea de gran importància ambiental i econòmica.

L'estudi s'ha dut a terme en tres campanyes i 14 punts de mostreig, incloent les dues principals EDARs de la zona (EDAR Amposta, equipada amb tractament secundari de fangs actius convencionals, i EDAR Sant Carles de la Ràpita, que a més compta amb tractament terciari amb filtres de sorra), el tram principal del riu Ebre i canals. Els resultats demostren que l'abast de la contaminació en l'aigua dolça no és menyspreable, amb concentracions totals de fins a 2.39 mg / L. Els pesticides, particularment imidacloprid i oxadiazon, s'han detectat en totes les mostres d'aigua dolça i la seva presència s'ha relacionat a l'agricultura. Els fàrmacs són la classe que aporta la major part de la contaminació, sent l'azitromicina i el diclofenac els compostos a concentracions més elevades. Les dues EDAR investigades no són eficients en l'eliminació dels compostos de la Watch list; els màxims valors mitjans s'han registrat pel diclofenac (47%). Els nivells de concentració dels compostos investigats se situen per sobre dels valors de concentració prevista sense efecte (PNEC) en la majoria de les mostres i els quocients de risc (RQ) han evidenciat risc elevat en la majoria dels llocs de mostreig.

Paral·lelament, 8 dels compostos de la Watch list s'han estudiat en experiments de (bio)degradació, amb el doble objectiu d'explorar la seva eliminació en sistemes de fangs actius convencionals, avaluant tant la biodegradació com l'abast de la sorció, i avaluar la importància dels paràmetres operatius de les EDAR amb l'objectiu de millorar les taxes d'eliminació. S'han considerat quatre factors a dos nivells diferents: pH, temperatura, concentració de biomassa i condicions redox. La màxima eliminació es verifica en condicions aeròbiques, alta temperatura (25 ° C), major concentració de biomassa (5 g/L) i pH elevat (7,5) i els paràmetres amb major impacte en les taxes d'eliminació són concentració de biomassa i condicions redox. Els nivells més alts d'eliminació s'han obtingut per a E2, que sempre s'ha transformat per sobre del 96%. Per a compostos com E1, eritromicina i methiocarb, els resultats varien entre zero fins eliminació completa, destacant la importància de l'elecció dels paràmetres operatius. Els resultats mostren que la sorció és una via d'eliminació rellevant per a alguns compostos, especialment les hormones (en particular EE2) i el diclofenac.

En general, els resultats presentats en aquest document mostren que els compostos de la Watch list, actualment, només s'eliminen parcialment durant el tractament convencional, amb marge de millora. Cal destacar que els compostos de la Watch list

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poden representar una amenaça per al medi ambient a la concentració a la qual ocorren, com confirmat pels resultats de la monitorització. Malgrat els avenços aconseguits durant aquests anys, diverses qüestions romanen obertes, entre les que s'inclouen dificultats d'anàlisi, incoherència de dades i buits significatius en la legislació.

# Resumen

La presencia de xenobióticos, tales como los productos farmacéuticos, los compuestos de disrupción endocrina y los pesticidas, en el medio ambiente acuático ha generado una gran preocupación ambiental debido a su alta toxicidad incluso a bajas concentraciones. Estos compuestos, llamados microcontaminantes, se han detectado en todos los compartimentos acuáticos y entran continuamente al medio ambiente a través de fuentes puntuales y no puntuales. En particular, se sabe que la eliminación de xenobióticos en las plantas de tratamiento de aguas residuales (EDAR) es incompleta para varios compuestos y se necesitan más esfuerzos en este contexto.

Para solucionar este problema, la Unión Europea está actualizando su legislación. La Directiva 2013/39 / UE establece normas de calidad ambiental en agua dulce para 76 sustancias prioritarias, principalmente metales pesados, pesticidas tradicionales y productos químicos industriales. Con respecto a los contaminantes emergentes, 17 compuestos candidatos a ser añadidos a la lista de sustancias prioritarias se han incluido en una lista de vigilancia (Watch list) (Decisión UE 2015/495). Estos compuestos deben ser monitoreados por todos los estados miembros para recopilar datos sobre su presencia en agua dulce. La evaluación del riesgo ambiental se realizará para determinar si representan un riesgo para (o a través de) el medio ambiente acuático.

Esta tesis tiene como objetivo principal llenar los vacíos de conocimiento sobre microcontaminantes y, en particular, sobre los compuestos de la Watch list a tres niveles: análisis, monitoreo y eliminación. En primer lugar, ha desarrollado una metodología analítica basada en SPE en línea -UHPLC-MS/MS (extracción en fase sólida en línea, cromatografía liquida de resolución ultra alta acoplada a espectrometría de masas en tándem) para la determinación simultánea de los compuestos de la Watch list. El método propuesto ofrece ventajas sobre los métodos ya disponibles, como la versatilidad (ya que por primera vez se han agrupado todos los compuestos en un único), un tiempo de análisis más corto, robustez y sensibilidad, todo en cumplimiento de los requisitos de la UE, con la única excepción de EE2, y se ha validado tanto en agua dulce como, por primera vez, en aguas residuales (efluentes e influentes).

A seguir, el método se ha aplicado para la monitorización de los compuestos de la Watch list en el Delta del Ebro, en el norte de España, un área de gran importancia ambiental y económica. El estudio se ha llevado a cabo en tres campañas y 14 puntos de muestreo, incluyendo las dos principales EDARs de la zona (EDAR Amposta, equipada con tractamento secondario de lodos activados convencionales, y EDAR Sant Carles de la Ràpita, que además cuenta con tractamento terciario con filtros de arena), el tramo principal del río Ebro y canales. Los resultados demuestran que el alcance de la contaminación en el agua dulce no es despreciable, con concentraciones totales de hasta 2.39 μg/L. Los pesticidas, particularmente imidacloprid y oxadiazon, se han detectado en todas las muestras de agua dulce y su presencia está relacionada con la agricultura. Los fármacos son la clase que aporta la mayoría de la contaminación, siendo la azitromicina y el diclofenaco los compuestos a concentraciones más elevadas. Las dos EDAR investigadas no son eficientes en la eliminación de los compuestos de la Watch list; los máximos valores medios se han registrado para diclofenaco (47%). Los niveles de concentración de los productos químicos investigados se sitúan por encima de los valores de concentración prevista sin efecto (PNEC) en la mayoría de las muestras y los cocientes de riesgo (RQ) han evidenciado riesgo elevado en la mayoría de los sitios de muestreo.

Paralelamente, 8 de los compuestos de la Watch list se han estudiado en experimentos de (bio)degradación, con el doble objetivo de explorar su eliminación en sistemas de lodos activados convencionales, evaluando tanto la biodegradación como el alcance de la sorción, y evaluar la importancia de los parámetros operativos de las EDAR con el objetivo de mejorar las tasas de eliminación. Se han considerado cuatro factores a dos niveles diferentes: pH, temperatura, concentración de biomasa y condiciones redox. La máxima eliminación se verifica en condiciones aeróbicas, alta temperatura (25 ° C), mayor concentración de biomasa (5 g / L) y pH elevado (7,5) y los parámetros con mayor impacto en las tasas de eliminación son concentración de biomasa y condiciones redox. Los niveles más altos de eliminación se han obtenido para E2, que siempre se ha transformado por encima del 96%. Para compuestos como E1, eritromicina y methiocarb, los resultados varían entre cero hasta eliminación completa, destacando la importancia de la elección de los parámetros operativos. Los resultados muestran que la sorción es una vía de

eliminación relevante para algunos compuestos, especialmente las hormonas (en particular EE2) y el diclofenaco.

En general, los resultados presentados en este documento muestran que los compuestos de la Watch list, actualmente, solo se eliminan parcialmente durante el tratamiento convencional, con margen de mejora. Cabe destacar que los compuestos de la Watch list pueden representar una amenaza para el medio ambiente en la concentración a la que ocurren, como confirmado por los resultados del monitoreo. A pesar de los avances logrados durante estos años, varias cuestiones permanecen abiertas, entre las que se incluyen dificultades de análisis, incoherencia de datos y huecos significativos en la legislación.

# GENERAL INTRODUCTION

## 1.1. Micropollutants

Despite the great awareness risen this year on the dramatic conditions of the environment, public concern about water pollution is not a new theme. In the past, heavy metals and persistent organic pollutants, such as polycyclic aromatic hydrocarbons, some traditional pesticides, dioxins and others were under the attention of scientists, policy makers and environmental activists (Petrovic et al., 2008). Nowadays, these compounds do not represent an environmental hazard anymore in developed countries, thanks to the adoption of adequate measures that allowed to phase out emissions (Petrovic et al., 2008). However, due to the recent advances in analytical chemistry, other compounds are found to be threatening the aquatic environment, being therefore called contaminants of emerging concern (Alvarino et al., 2018). Such compounds are usually found in the aquatic environment at ultratrace to trace levels, typically in the ng/L to μg/L range, and for this reason they are often addressed to as emerging micropollutants (Stamm et al., 2016). This category includes emerging chemicals from different classes and with diverse properties, such as pharmaceuticals (PhACs), personal care products (PCPs), endocrine disrupting compounds (EDCs), pesticides and perfluoroalkyl substances. The main classes of micropollutants along with representative compounds are listed in Table 1. Micropollutants have been found in all aquatic compartments (Petrovic, 2014), including drinking and groundwater (Luo et al., 2014). Despite the low concentrations, they are continuously discharged into the environment, mainly via wastewater effluents. Since even compounds with short half-lives are entering the environment at a continuous rate, micropollutants are therefore called pseudo-persistent (Buttiglieri and Knepper, 2008).

General Introduction

Table 1. Main classes of micropollutants along with representative compounds.

| Class | Representative compounds |
|---|---|
| Biocides | Quaternary ammonium compounds, isothiazolinones, phenols |
| Disinfection by-products | Trihalomethanes, haloacetic acids, nitrosamines |
| Drugs of abuse | Amphetamines, methamphetamines, cocaine, ketamine, heroin |
| Flame retardants | Brominated flame retardants, organo-phosphate flame retardants, dechlorane plus |
| Nanomaterials | Metallic nanoparticles (nAg, nZnO, nCuO), fullerenes, carbon nanotubes, nanoplastics |
| Perfluorinated compounds | Perfluorooctane sulfonate, perfluoropentanoic acid, perfluorooctanoic acid |
| Personal care products | Fragrances (Galaxolide, tonalide, musk xylol), UV filters (benzophenone-3, EHMC) |
| Pharmaceuticals | Antibiotics (macrolides, tetracyclines, amoxicillin), anti-inflammatory (ibuprofen, naproxen, diclofenac), antidepressants |
| Plant protection products | Glyphosate, neonicotinoids pesticides, carbammate pesticides |
| Plasticizers | Bisphenol A, phthalates (di-butyl phthalate, di-ethylhexyl phthalate) |
| Surfactants | alkylbenzene sulfonates, alkylphenol ethoxylates, linear alkylbenzene sulfonates, fatty alcohol sulfates |
| Microplastics | Polyethylene and polypropylene beads, acrylic, polyamide and nylon fibers |

Among these emerging micropollutants, this PhD thesis will mainly address pharmaceuticals, pesticides, endocrine disrupting compounds and UV filters. PhACs are a group of chemicals that have medicinal properties and include both prescription and over-the-counter therapeutic drugs, in addition to veterinary drugs. Pharmaceuticals are biologically active, generally highly soluble in water, which in turn makes them mobile in

water systems, and not readily biodegradable (Kümmerer, 2008). They are produced and used for their (more or less) specific biological activity and can be classified according to their purpose and/or systemically, as in the case of the anatomical therapeutic chemical classification. They have a broad spectrum of physicochemical and biological properties, though they are generally marked by ionic nature. The most investigated pharmaceutical compounds are usually small molecules, typically in a range of molecular weight from 200 to 1000 Dalton. Besides the active ingredients, adjuvants – including possible EDCs – and sometimes pigments or dyes are added to the formulations. Compared to other chemicals, pharmaceuticals are complex molecules with particular properties. They often present acidic or basic features and can be present in their neutral, cationic, anionic, zwitterionic form under environmental conditions, which results in a complex environmental behaviour (Kümmerer, 2008). Since they are designed to have specific pharmacologic and physiologic activities at low concentrations, they are intrinsically potent and can have unintended effects on wildlife (Halling-Sørensen et al., 1998). It has been estimated that the production of pharmaceuticals amounted to 260,000 million € in 2018 and in Europe alone (European Federation of Pharmaceutical Industries and Associations, 2018). Despite the population numbers in developed countries is either stable or decreasing, the production and sale of these chemicals have been growing in the last years, which means that there has been an increase in per capita consumption of PhACs (Alvarino et al., 2018; aus der Beek et al., 2016). The aging of society is one of the main factors behind this phenomenon, but other changes in trends and social habits also play a role, such as an easier access to contraception, the extensive use of antibiotics and the introduction of new antiviral medicines (Alvarino et al., 2018). Moreover, it is estimated that the consumption of PhACs will increase further due to the population growth, increasing investments in the health sector, advances in research and development and expanded global market availability (aus der Beek et al., 2016; Mandaric et al., 2016).

Among pharmaceuticals, a class of compounds that constitutes a great cause of concern on its own is that of antibiotics. Their overuse and misuse, in recent years, has led to an increase of antibiotic resistant bacteria (Levy and Marshall, 2004).

EDCs, according to a working definition by the European Commission, are exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations (COM, 1999). They are comprised of natural and synthetic hormones as well as other chemicals (pesticides, dioxins, flame retardants, additives used in plastic manufacturing, etc.).

Pesticides are a class of artificial substances employed to fight pests and to improve agricultural production. It has been estimated that a range of over 1000 compounds is available for applications to agricultural crops in order to control undesirable moulds, insects or weeds (Ortelli et al., 2004). In 2017, more than 4 million tonnes pesticides were used worldwide, of which 1,7 million in China alone (Food and Agriculture Organization of the United Nations, 2019). In the same year, in Europe, the use of pesticides amounted to 11.6% of the worldwide figure, equivalent to more than 476,000 tonnes (Food and Agriculture Organization of the United Nations, 2019). The agricultural use of pesticides plays a crucial in role in providing high-quality fruits and vegetables in large scale and at low costs (Goto et al., 2003). These chemicals are usually persistent, and they are long-term toxic agents prone to accumulating in certain organs of living beings. Basically, the properties that make them effective against plagues turn them into polluting agents (Claver et al., 2006).

UV filters represent a vast group of anthropogenic chemicals used as a protection against the harmful effects of the UV solar radiation. They are present in numerous personal care products such as sunscreens, shampoos and lotions. They are also applied in a wide range of industrial manufacturing as additives in polymeric materials where some sort of protection against the sun rays is needed, such as food-packaging materials, fabrics, protective coatings for vehicles, photography devices and many more (Molins-Delgado et al., 2015).

### 1.1.1. Sources and pathways of contamination

Micropollutants enter the environment through different pathways, and mainly via point and non-point sources. Point sources are defined as single identifiable source of pollution from which pollutants are discharged (Hill, 1997) and include the discharge of wastewater

treatment plants (WWTPs) effluents, the outlets of industrial plants and hospitals. Instead, non-point sources are relevant in the context of livestock (for example the release of veterinary drugs with manure) and agriculture (consider the spread of pesticides with runoff), as well as urban runoff and stormwater in combined sewage overflow (CSO) (see Figure 1) (Servais and Passerat, 2009).
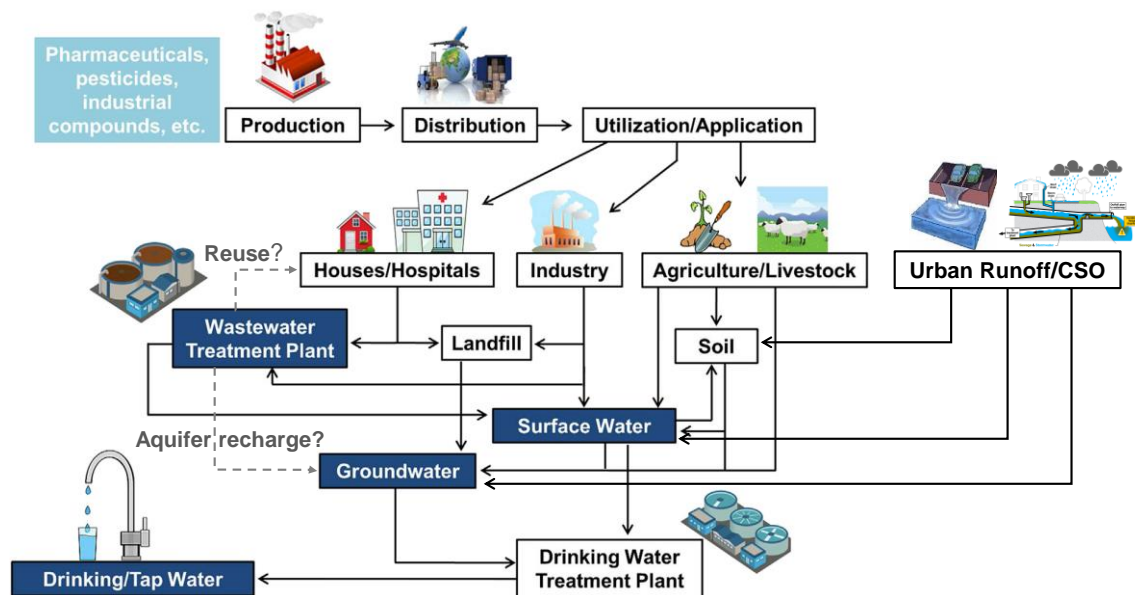


**Figure 1.** Micropollutants entrance pathways into the environment. (Adapted from (Barbosa et al., 2016)).

The entrance and distribution of trace organic contaminants in the aquatic medium is determined by numerous factors: production, prescription, sales and consumption rates in the case of pharmaceuticals; the seasonality of application of pesticides in agriculture; legislation and wealth of the region; human or animal metabolism and excretion rates; presence of tourist or recreational facilities; distribution of WWTPs on the territory and their efficiency in removing micropollutants; climate and precipitations; soil properties; chemical stability, physicochemical properties of the pollutants and so on (Baker and Kasprzyk-Hordern, 2013; Göbel et al., 2005; Luo et al., 2014; Ma et al., 2017; Mandaric et al., 2018).

As a result, the occurrence of micropollutants in the different aquatic compartments (wastewater, freshwater and groundwater) is marked by great spatial and temporal

variations (Luo et al., 2014). Indeed, when micropollutants enter the environment, they undergo several processes that impact their occurrence patterns (Ma et al., 2017). These mechanisms reduce their concentration in what is known as natural attenuation. The most relevant processes are physical ones, such as dilution and dispersion, while the main biotic and abiotic transformations include biodegradation, oxidation, hydrolysis, photolysis, volatilization and sorption to dissolved organic matter and sediments (Mandaric et al., 2018).

### 1.1.2. Emerging micropollutants in wastewater

Once administered, PhACs undergo changes in their chemical structure within the human or animal body, either by enzymes mainly in the liver and kidneys in the case of human metabolism or by microorganisms in animal guts. Metabolites are the molecules resulting from this process. The degree of metabolization varies from drug to drug. Some are metabolized to a great extent before excretion, while others are only moderately or poorly metabolized, if at all. For instance, contrast media are excreted completely in their parent form (Golan et al., 2012). Metabolites may become more or less water soluble and active than the parent compounds, that is, they differ from a toxicological and pharmacological point of view. Many PhACs are excreted as conjugates that can be then hydrolysed, thus releasing the parent compounds at a later stage (Evgenidou et al., 2015). This way, conjugates act as reservoirs of drugs from which micropollutants are released into wastewater or even the receiving environment (Bendz et al., 2005). After excretion, unaltered pharmaceuticals and metabolites can undergo further changes in their structure, resulting in transformation products (TPs), both in WWTPs and in the receiving environment (Deblonde et al., 2011; Verlicchi et al., 2012). Thus, the term "metabolite" is used for compounds originated from changes in humans and animals, whereas "transformation products" are defined as those chemicals generated from biotic or abiotic processes in the environment (Ferrando-Climent et al., 2012).

Natural hormones and contraceptives undergo various transformations in the human liver. They are usually oxidised, hydroxylated, deoxidated and methylated and eventually excreted under the form of inactive polar conjugates (with glucuronic acid or sulphate) (Ternes et al., 1999). During sewage transport and in WWTPs, due to the presence of microorganisms, such as *Escherichia coli*, that present glucuronidase and sulphatase activity, a separation of the glucuronic acid and sulphate moieties occur. This cleavage

brings hormones back to their parent compound. Some hormones, such as E2, undergo a rapid degradation, while others, such as the contraceptive EE2, are more persistent and are likely to be discharged in the receiving water bodies (Ternes et al., 1999).

Pesticides, if persistent, can travel long distances. That is why modern pesticides are theoretically designed to decompose within a relatively short time after application without forming persistent degradation products. These pollutants enter WWTPs mainly through surface runoff from treated sites, but also from the cleaning of pesticide spraying equipment or containers on the farm instead of washing it at the field edge and from inadequate disposal of unused pesticides (Monteith et al., 1995). Moreover, if the pesticides are also used in nonindustrial agricultural applications such as pet flea treatment, horticulture, and household pest control products, they are likely to end up in urban sewage (Sadaria et al., 2016).

While UV filters are certainly entering the environment through direct inputs as consequence of recreational water activities, monitoring studies revealed that most contamination from UV filters derives from wastewater (Gago-Ferrero et al., 2013; Ramos et al., 2016). In fact, they enter the urban sewage system after being rubbed off by towels, washed off during showering, or even from renal excretion after percutaneous or oral uptake, through lipsticks (Li et al., 2007). Studies have shown that their degradation in WWTPs is incomplete, usually below 50% (Molins-Delgado et al., 2018).

### 1.1.3. Removal and transformation mechanisms during wastewater treatment

Activated sludge is defined as the biomass originated by the growth of microorganisms in WWTPs in aeration tanks in the presence of dissolved oxygen. It is called "activated" due to the large amounts of bacteria and other microorganisms in such biomass living on the incoming sewage. Oxygen is necessary for the biomass to live, develop and multiply, therefore wastewater and activated sludge are mixed and aerated with free or bond oxygen forms (e.g. nitrate), with the aim of reducing the dissolved organic content of sewage (Buttiglieri and Knepper, 2008).

Conventional activated sludge (CAS) WWTPs represent the most common treatment for major urban areas (Johnson and Sumpter, 2001). A CAS-WWTP usually includes two lines: the water treatment line, where pollutants are removed from the liquid phase and

sediments with high water content are generated, and the sludge line, where separated activated sludge, produced in the water line, is treated to make it suitable for its final disposal (Buttiglieri and Knepper, 2008).

CAS systems are efficient in decreasing the concentrations of the main organic and inorganic constituents (biological oxygen demand, nitrogen and phosphorus) that may otherwise pollute the receiving waters and lead to eutrophication. However, at the same time, they are known to play a key role in the release of micropollutants into the environment (Gros et al., 2012; Kolpin et al., 2004). Moreover, even if parent compounds are not detected after treatment, TPs and metabolites may still be of concern due to their potential stability or toxicity (Onesios et al., 2009).

The fate of micropollutants in wastewater treatment depends on their physicochemical properties such as solubility, volatility, biodegradability and polarity, their tendency to adsorb and absorb to suspended solids as well as on WWTPs operational parameters. Both could vary greatly among compounds and WWTPs, which explains the huge differences in micropollutants removals described in literature. The main transformation mechanisms are depicted in Figure 2.
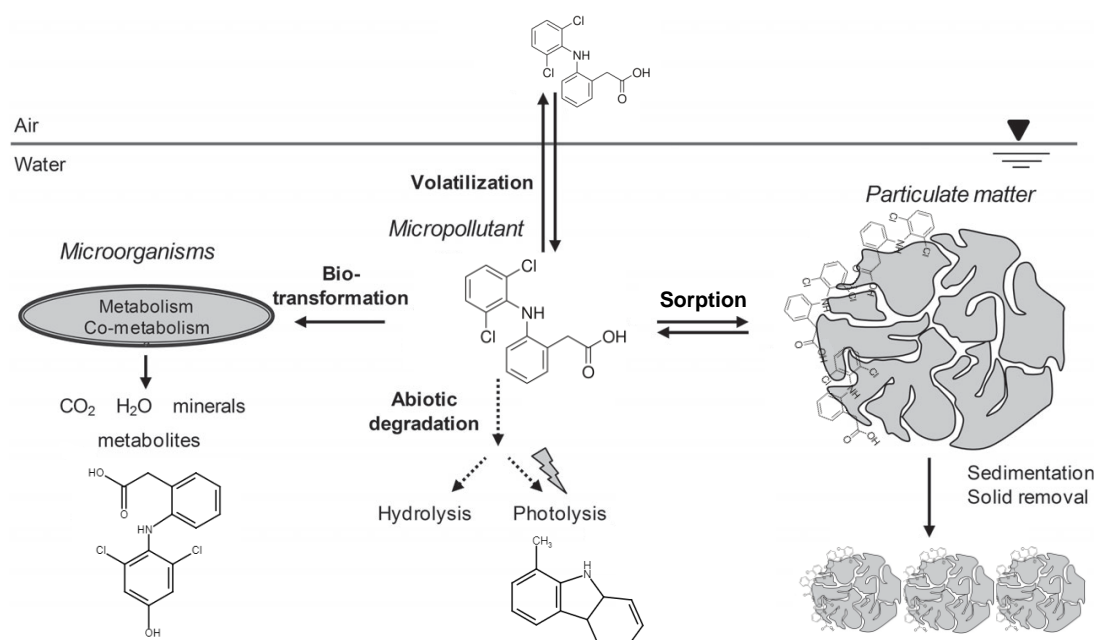


**Figure 2.** Main removal mechanisms of micropollutants in conventional WWTPs (example of the analgesic pharmaceutical diclofenac). Adapted from (Margot et al., 2015).

**Volatilization**, or stripping, consists of the transition from the liquid to the gas phase and usually takes place on the surface of the reactor, at the interface between the two phases, under aeration. The extent of volatilization of micropollutants is described by the Henry's law constant ($H_c$), which express the rate between the fraction dissolved in water and that dissolved in air. Pollutants such as chlorinated hydrocarbons, aromatics, etc. are prone to air stripping, whereas compounds with $H_c$ lower than $10^{-4}$ and a $H_c/K_{ow}$ fraction lower than $10^{-9}$ exhibit a low volatilisation potential (Rogers, 1996). In light of the physicochemical properties of the investigated compounds as well as according to literature data, volatilization is relatively negligible for the chemicals studied in the present thesis (Reif et al., 2008; Schröder et al., 2016; Ting and Praveena, 2017).

**Sorption** implies the transfer of micropollutants from the liquid to the solid phase and is important because it represents a removal pathway with the excess sludge (Hamid and Eskicioglu, 2012). Several factors play a role in sorption, including pH, redox potential, stereo chemical structure and chemical properties of both the sorbent and the compound (Kümmerer, 2009). The extent of sorption is measured by $K_d$, the solid-water distribution coefficient. Sorption can occur through two main mechanisms: absorption, which consists of hydrophobic interactions whose intensity is measured by $K_{ow}$, and adsorption, characterized by the dissociation constant, $pK_a$, which takes place by means of electrostatic interactions and is inherently related to the substance tendency to be ionized in the aqueous phase (Rogers, 1996). During absorption, hydrophobic pollutants interact with suspended solids, extracellular polymeric substances or the lipophilic cell membrane of microorganisms (Margot et al., 2015), whereas in adsorption the positively charged groups of pollutants interact with the negatively charged biomass surface (Ternes et al., 2004). For compounds with log $K_d$ values lower than 2.48, sorption onto secondary sludge is considered negligible (Joss et al., 2005).

**Biodegradation** is the predominant removal mechanism for several organic micropollutants, especially PCPs, EDCs, plasticizers, and surfactants (Garcia-Becerra and Ortiz, 2018). Microbial biodegradation processes include diverse and often complementary mechanisms that transform the parent compounds and may eventually lead to complete mineralization. Partial degradation via biotransformation or detoxification can result in the formation of less toxic metabolites, but, in a few cases, in

the generation of TPs with toxicity or persistence higher than the initial compound (Tran et al., 2013a) and, eventually, to mineralization. Biodegradation of micropollutants can take place via different mechanisms. In WWTPs it usually occurs through co-metabolism, in which micropollutants are degraded by enzymes generated for the degradation of other primary substrate degradation and are not used as a primary source of carbon and energy for microbial growth or via mixed substrate growth (Luo et al., 2014). Compounds can be classified on the basis of their biodegradation rate constant ($k_{biol}$) into very highly ($k_{biol}$ > 5 L/gSS d), highly (1< $k_{biol}$ <5 L/gSS d), moderately (0.5< $k_{biol}$ <1 L/gSS d) and hardly ($k_{biol}$ <0.5 L/gSS d) biodegradable (Suarez et al., 2010).

### 1.1.4. Environmental impacts

The growing interest in this topic from the general public is evidenced by the increasing coverage given by mass media all around the world. Considering that the concentrations of micropollutants in water are extremely low, especially when compared to conventional macropollutants, they do not represent a cause of concern for their chemical impact, but for other potential problems such as toxicity, teratogenicity, genotoxicity, bioaccumulation and estrogenicity caused to micro and macroorganisms exposed to these emissions (Alvarino et al., 2018). In fact, many trace organic contaminants are highly toxic for both humans and the wildlife. Although for most of them the lowest observed effect concentrations are much higher than the environmental concentrations at which they are usually detected, some of them exert chronic toxicity even at the concentration levels of wastewater effluents (Richardson and Ternes, 2014). Moreover, these compounds are often present as complex mixtures, which could represent a higher hazard owing to synergistic effects. The cocktail effect is also difficult to properly evaluate in risk assessment and models (Houtman, 2010; Schwarzenbach et al., 2006).

The presence of PhACs in the aquatic ecosystems may bring about environmental and public health issues. Besides acute and chronic toxicity, genotoxicity (Ragugnetti et al., 2011) and endocrine disruption (Schultz et al., 2011) have been documented. Moreover, some PhACs including antidepressants and antibiotics are known to bioaccumulate in aquatic organisms, especially fish (Ramirez et al., 2009). This, in turn, is likely to cause trophic transfer (Lagesson et al., 2016) and in some cases even biomagnification through the food web (Xie et al., 2017).

The discharge of antibiotics in the environment may have a direct impact on bacterial communities found in the ecosystems, particularly freshwater. In addition, the exposure of autochthonous bacteria to lower dosages of antibiotics, such are those found in wastewater effluents, has been proven to enhance the development of resistance (Rodríguez-Rojas et al., 2013). Besides the increasing phenomenon of antibiotic resistance among bacteria directly exposed to the contaminated waters, the genetic code for resistance can be transferred to other bacteria in the case it is located on the R-plasmids (Khachatourians, 1998; Lindsey et al., 2001). Resistant bacteria can then reach humans via the food web and lead to severe consequences in debilitated and immunocompromised individuals, whom might not react positively to antibiotic treatment due to the bacterial multiresistance (Schwartz, 2003). During medical treatment, bacteria from the gastrointestinal tract are exposed to high concentrations of antibiotics and may develop resistance therein, before being excreted and released into the environment through wastewater or other pathways (Servais and Passerat, 2009).

EDCs are often widely dispersed in the environment and they have actually been detected in all regions of the world. Even those compounds that are rapidly degraded in the environment or within the human body can have detrimental effects if exposure occurs in critical developmental periods (Casals-Casas and Desvergne, 2011). Hormones were reported to provoke endocrine disrupting effects in surface waters at concentrations as low as ng/L (Desbrow et al., 1998; Routledge et al., 1998). Effects include altered sexual development, induction of plasma vitellogenin, intersex in fish living close to the outfalls and changes in mating behaviour (Hamid and Eskicioglu, 2012; Servos et al., 2005).

A wide range of pesticides, especially those polar and highly water soluble, was detected in waters at ng/L levels (Fenoll et al., 2011; Navarro et al., 2010). Some of these are known to bioaccumulate and biomagnify and, moreover, they may exert both vertebrate and non-vertebrate toxicity, therefore affecting non-target organisms (Moganti et al., 2008). As a matter of fact, the presence of certain classes of pesticides - such as the family of neonicotinoids - in surface waters could be linked to the so-called pollinator colony collapse disorder, causing bees poisoning. Moreover, the neonicotinoids are also likely to negatively affect aquatic invertebrates and ecosystem health (Schaafsma et al., 2015).

In the last decade, UV filters have become contaminants of emerging concern, drawing worldwide attention, mainly because of their ubiquitous occurrence in the environment at global level, their pseudo-persistence and ecotoxicity (Brausch and Rand, 2011; Caliman and Gavrilescu, 2009). Research shows that their degradation in WWTPs is incomplete, usually below 50% (Li et al., 2007). Since most UV filters are lipophilic, they have the tendency to accumulate in sediments and sewage sludge and to bioaccumulate in living organisms. In addition, they have been proved to have estrogenic activity. In fact, in vitro and in vivo tests showed that UV filters affect the reproductive cycle as well as the development of both aquatic and terrestrial organisms (Klammer et al., 2007). The transformation products of UV filters also represent a threat for the environment as they may even exert increased endocrine disrupting effects compared to the parent compounds (Molins-Delgado et al., 2018).

## 1.2. Analytical methods for micropollutants monitoring

A multitude of analytical methods for the analysis of contaminants of emerging concern has been proposed in the past two decades. Given the low concentrations at which these compounds occur in the environment, particularly compared to conventional macropollutants, recent development and continual improvement of advanced instruments and analytical methodologies have evolved to great levels of sensitivity. Over time, a gradual shift from single-class specific analytical methods to multi-residue methods for the simultaneous analysis of over 100 compounds has taken place (Petrovic et al., 2010). Moreover, the development of the so-called hyphenated techniques, featuring either gas chromatography (GC) or liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS), has pushed the method detection limits (MDLs) from micrograms to nano and even picograms per litre (Barceló and Petrovic, 2007). The need for analyte derivatization makes GC-MS a less attractive alternative compared to LC-MS/MS, which is now the technique of choice in the analysis of many micropollutants in water samples (Petrović et al., 2005; Vazquez-Roig et al., 2013; Watabe et al., 2006). In recent years, one of the hottest trends is represented by high-resolution mass spectrometry (HRMS) with LC for the identification of unknown contaminants, particularly TPs (Richardson and Ternes, 2018). In fact, there is a growing interest in the application of Orbitrap and time-of-flight (TOF) mass spectrometers for suspect screening

and non-target analysis. The majority of methods available nowadays focus only on target compounds, and rarely include metabolites and TPs, mainly because most of them are yet to be discovered and because analytical standards are not commercially available or are too expensive (Mandaric et al., 2016). Instead, suspect screening is based on pre-defined lists of suspect compounds followed by tentative confirmation based on accurate mass acquisition (Dürig et al., 2019) and non-target analysis represent a holistic methodology, in which no preselection is performed (Gago-Ferrero et al., 2015). This approach is based on the premises that the compounds usually targeted represent but a small portion of the complex mixtures present in wastewater, and that any kind of selection is potentially biased (Gago-Ferrero et al., 2015). Indeed, potentially, very relevant compounds are systematically neglected for a number of reasons, such as the Matthew effect, the lack of adequate analytical methods and analytical methodology limitations (e.g. poor extraction efficiency, limit of detection (LOD) not sufficiently low to allow the detection in environmental matrices, matrix effect, lack of reference standards etc.) (Petrovic, 2014). HRMS instruments like TOF and Orbitrap mass spectrometers give the possibility of acquiring high-resolution full-scan mass spectra, therefore allowing retrospective data analysis for compounds not included in the first data processing and, potentially, to characterise the whole dissolved organic matter present in (waste)water (Mandaric et al., 2016; Verkh et al., 2018). However, in spite of the great scientific relevance of these new approaches, the identification of unknowns is still a very difficult, time-consuming and expensive task with yet no guarantee of success and, in addition, it is marked by a general lack of mass spectral libraries (Gago-Ferrero et al., 2015; Mandaric et al., 2016). So, for now, albeit with some limitations, low resolution-MS target analysis with selected reaction monitoring (SRM) is still a better approach for the quantitative determination of micropollutants in environmental analysis over HRMS methods (Petrovic, 2014).

Parallelly, in light of the complexity of certain environmental matrices, extraction methods have acquired great importance. The current methodologies have allowed the determination of various classes of micropollutants in matrices from ultrapure water to wastewater (Čelić et al., 2017; Gago-Ferrero et al., 2013; Gros et al., 2012; A Masiá et al., 2013), but also in sewage sludge and sediment samples (Jelić et al., 2009), soil (Vazquez-

Roig et al., 2010), seafood (Serra-Compte et al., 2017) and crops (Martínez-Piernas et al., 2018). However, sample preparation and pre-treatment represent the major bottlenecks and sources of errors in the analysis of micropollutants. Off-line solid-phase extraction (SPE) is the most common technique for the preconcentration of water samples (Richardson and Ternes, 2014), but it comes with a number of drawbacks, especially being a time-consuming and costly task (Bones et al., 2006). Therefore, trends are shifting towards the adoption of other solutions, such as solid-phase microextraction and on-line preconcentration systems (Richardson and Ternes, 2018). Online-SPE allows the direct injection of samples onto a LC column, where concentration and clean-up steps take place, thus reducing sample manipulation and allowing a shorter time of analysis (Hernández et al., 2005).

## 1.3. Legal framework

At European level, action was taken in 2000 with the publication of *Directive 2000/60/EC*, commonly known as the Water Framework Directive (WFD) and it still remains the main European legislation aimed at the protection of water resources and the aquatic environment. According to the WFD, river basins must be managed so that the quality and quantity of water does not affect the ecological services of any specific water body. Ultimately, the primary objective of the WFD is the achievement of a good chemical status of surface and ground water across the European Union (EC, 2000). The term ecological status refers to biological elements and is supported by chemical and physicochemical elements, as well as hydro-morphological elements. Chemical status, instead, is intended in relation to specific pollutants (Buttiglieri and Knepper, 2008).

To reach this goal, the discharge of pollutants into the environment had to be decreased (EC, 2000). The release of pollutants is limited and controlled by means of different pieces of legislation. Examples of that are the *REACH Regulation*, which aims at controlling the addition of chemicals in the industrial production in order to limit the contamination of water bodies (EC, 2006); the *Directive on Plant Protection Products* (EEC, 1991), which focuses on pollutants originating from agriculture, and the *Directive on Industrial Pollution Prevention*, which regulates the discharge of chemicals from industrial activities (EC, 1996). Nevertheless, the monitoring of certain substances in water bodies is necessary to keep track of the actual pollution of surface waters, in order to assess the

real status of water bodies, which could be compromised by the accumulation of contaminants coming from different sources. For this reason, the WFD stated that a list of priority substances (and, among them, the so-called priority hazardous substances) had to be selected amongst the pollutants which could represent a threat to or via the aquatic environment. The general purpose was to cease the release of priority hazardous substances into the European basins and to limit the discharge of priority pollutants. This came into practise with a subsequent directive first published on 16 December 2008, where the first 33 priority substances were listed along with their EQS, that is, concentration limits that cannot be exceeded. The priority pollutants were chosen based on risk assessment studies, taking into account their intrinsic properties in relation to aquatic and human ecotoxicity, the extent of contamination of European waters and the spread of their application at European level (EC, 2008). The Water Framework Directive also established that the list of priority pollutants should be reviewed at the latest every 4 years. Its latest version, published in 2013, includes traditional pesticides (atrazine, diuron, simazine, DDT, cyclodienes), polycyclic aromatic hydrocarbons, brominated and chlorinated compounds, dioxins and heavy metals, among others (EC, 2013). Moreover, The *Directive 2013/39/EU* sets a new mechanism of gathering monitoring information about the concentration of contaminants in the aquatic environment, especially targeting those emerging pollutants for which the occurrence data are insufficient for risk assessment (EC, 2013). Hence, the directive poses the bases for the creation of a Watch list of a limited number of compounds in order to gather high quality data with the aim of supporting the prioritization process.

### 1.3.1. The EU Watch list

The first version of the Watch list appeared in the *Commission Implementing Decision (EU) 2015/495*, published in March 2015, including 17 compounds from different classes, along with their maximum acceptable detection limits and a suggested analytical method (EC, 2015). The Watch list should include 10 compounds or groups of compounds in its first version, then this number should be increased up to 14 in the following updates and the overall temporal framework for monitoring should not exceed four years. Moreover, it is meant to be dynamic and should be updated every two years (EC, 2013). The
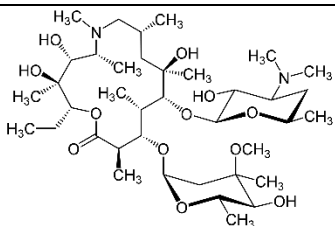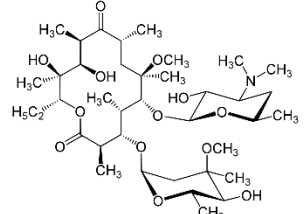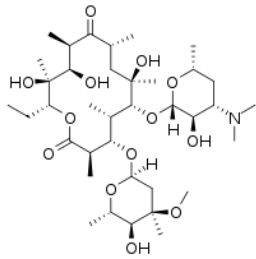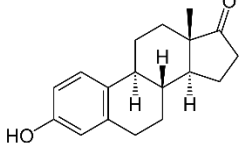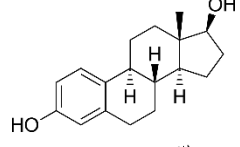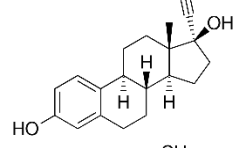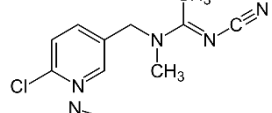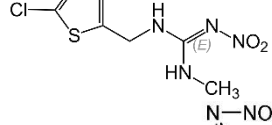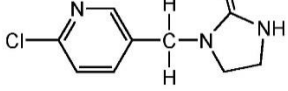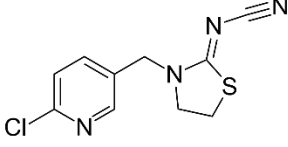
compounds of *Decision 2015/495* are listed in Table 2, while their physicochemical parameters are shown in Table 3.

All EU Member States have the obligation to monitor each substance of the Watch list in at least one monitoring station (or more on the basis of criteria detailed in the Decision text) over at least a 12-month period and they must report the results to the Commission following a fixed timeline. It is worth mentioning that according to the Decision the monitoring has to be carried out in freshwater only.

At the time of writing, the compounds of Decision 2015/495 are undergoing their fourth and last year of monitoring. An updated version of the Watch list was published in June 2018 and featured the inclusion of two antibiotics and a pesticide and the removal of five compounds from the previous version, although most compounds remain unaltered due to the lack of monitoring data (EC, 2018a). The compounds of *Decision 2018/840* are displayed in Table 2. This doctoral thesis, however, focuses on Decision 2015/495 only and, from now on, the denomination Watch list will refer to the 2015 version unless otherwise specified.

Table 2. The compounds of Decisions 2015/495 and 2018/840: compound structure, class, CAS number and relevant version of the Watch list.

| | Structure | Class | CAS | Watch list |
|---|---|---|---|---|
| Azithromycin |  | Macrolide antibiotic | 83905-01-5 | 2015 and 2018 |
| Clarithromycin |  | Macrolide antibiotic | 81103-11-9 | 2015 and 2018 |
| Erythromycin |  | Macrolide antibiotic | 114-07-8 | 2015 and 2018 |
| E1 |  | Steroid hormone | 53-16-7 | 2015 and 2018 |
| E2 |  | Steroid hormone | 50-28-2 | 2015 and 2018 |
| EE2 |  | Synthetic hormone | 57-63-6 | 2015 and 2018 |
| Acetamiprid |  | Neonicotinoid pesticide | 135410-20-7 | 2015 and 2018 |
| Clothianidin |  | Neonicotinoid pesticide | 210880-92-5 | 2015 and 2018 |
| Imidacloprid |  | Neonicotinoid pesticide | 105827-78-9 | 2015 and 2018 |
| Thiacloprid |  | Neonicotinoid pesticide | 111988-49-9 | 2015 and 2018 |

| | Structure | Class | CAS | Watch list |
|---|---|---|---|---|
| Thiamethoxam |  | Neonicotinoid pesticide | 153719-23-4 | 2015 and 2018 |
| Methiocarb |  | Pesticide | 2032-65-7 | 2015 and 2018 |
| Oxadiazon |  | Herbicide | 19666-30-9 | 2015 |
| Triallate |  | Herbicide | 2303-17-5 | 2015 |
| BHT |  | Antioxidant | 128-37-0 | 2015 |
| EHMC |  | UV filter | 5466-77-3 | 2015 |
| Diclofenac |  | PhAC | 15307-86-5 | 2015 |
| Metaflumizone |  | Insecticide | 139968-49-3 | 2018 |
| Amoxicillin |  | Antibiotic | 26787-78-0 | 2018 |
| Ciprofloxacin |  | Antibiotic | 85721-33-1 | 2018 |

**Table 3.** Physicochemical properties of compounds of *Decision 2015/495*. n.a. corresponds to no available data.

| | log Kow | log $K_d$ | Henry LC (atm m³/mol) | pK$_a$ |
|---|---|---|---|---|
| Azithromycin | 4.02[a] | 2.55-2.66[b] | 5.3E-29[c] | 8.74[a] |
| Clarithromycin | 3.16[a] | 2.48-2.60[b] | n.a. | 8.99[a] |
| Erythromycin | 2.48[d]; 3.06[a] | 1.9[d]; 2.2[e] | 2.2E-27[f] | 8.6[g]; 8.8[a] |
| Diclofenac | 4[g]-4.51[h] | 1.2[i] | 4.73E-12[c] | 4.15[j]-4.51[d] |
| E1 | 2.25[d]-3.69[k] | 2.39-2.65[l] | 3.8E-10[f]-6.2E-12[m] | 9.9[g]-10.34[n] |
| E2 | 3.1[o]-4.13[k] | 2.37[l]-2.84[p] | 3.64E-11[c]-6.22E-12[m] | 10[g]-10.46[n] |
| EE2 | 3.67[q]-4.15[r] | 2.5[i]-2.84[l] | 3.75E-12[m] | 10.2[g]-10.7[p] |
| Acetamiprid | 0.8[g] | 1.32[s] | 6.9E-8[c] | 0.7[t]; 0.9[g] |
| Clothianidin | 0.7[t]; 0.91[s] | 1.2[s] | 2.9E-16[t] | 2.4[g]; 11.09[t] |
| Imidacloprid | 8.5[u] | 1.2[s] | 1.65E-15[c] | 0.5[g];11.12[v] |
| Thiacloprid | 0.73[w]-1.3[g] | n.a. | 1.08E-14[c] | n.a. |
| Thiamethoxam | -0.13[t] | 0.37[s] | 4.63E-15[c] | 2.3[g] |
| Methiocarb | 2.92[q];3.1[g] | n.a. | 1.18E-09[x] | 12.2[g] |
| Oxadiazon | 4.8[q];3.9-4.9[w] | n.a. | 7E-8[f] | n.a. |
| Triallate | 4.6[t] | n.a. | 1.2E-5[c] | n.a. |
| BHT | 5.1[y] | n.a. | 2.49E-3[c] | 12.23[z] |
| EHMC | 5.8[g] | n.a. | 8.5E-6[c] | n.a. |

[a] (McFarland et al., 1997); [b] (Cordy et al., 2004); [c] ("US Environmental Protection Agency, Estimation Program Interface (EPI) Suite," n.d.); [d] (Besha et al., 2017); [e] (Jones et al., 2002); [f] ("Syracuse Research Corporation (SRC)," n.d.); [g] (Rubirola et al., 2017); [h] (Avdeef et al., 1998); [i] (Ternes et al., 2004); [j] (Sangster, 1994); [k] (Zorita et al., 2009); [l] (Andersen et al., 2004); [m] (Lai et al., 2002); [n] (Hurwitz and Liu, 1977); [o] (Holthaus et al., 2002); [p] (Clara et al., 2004); [q] (Hansch et al., 1995); [r] (Lai et al., 2000); [s] (Sadaria et al., 2016); [t] (MacBean, n.d.); [u] (Tomlin, n.d.); [v] (Chamberlain et al., 1996); [w] (Krzeminski et al., 2019); [x] ("EPA DSSTox Database," n.d.); [y] ("European Chemicals Agency," n.d.); [z] (Serjeant and Dempsey, 1979)

In order to analyse the evolution of the interest of the scientific community in the Watch list compounds, a bibliometric study has been carried out on Scopus: searching in Title-abstract-keywords for the string "watch list", from 2015 (search string: "PUBYEAR > 2014") to present (October 2019), a total of 145 publications was found. As seen in Figure 3a, a search among publications with the search terms "Watch list" together with the classes of compounds (pharmaceuticals, hormones, pesticides, antibiotics, UV filters and antioxidants) evidenced that pharmaceuticals are the most investigated group of pollutants, followed by hormones and pesticides, for which less than half the number of publications was found (Figure 3a, orange bars). While the lack of publications mentioning antibiotics is justified by the fact that this category actually overlaps with that of pharmaceuticals, for UV filters and antioxidants it reflects an actual absence of

information on the topics. However, it is noteworthy that the publications dealing with the Watch list are totally negligible compared to the huge amounts of literature produced on the investigated classes of compounds covering any field of knowledge (Figure 3a, blue bars, note the logarithmic scale of the X axis).

Diclofenac is the most studied compound, followed by the three hormones, the neonicotinoid imidacloprid and the three antibiotics. This result matches its removal from the Watch list in the 2018 update, as enough high-quality monitoring data were collected in the 2015-2018 period (Loos et al., 2018). Curiously, as highlighted elsewhere (Alvarino et al., 2018), E2 received more attention than EE2 and E1, although EE2 is known to be a more potent endocrine disruptor than the natural hormones (Thorpe et al., 2003). As expected, EHMC and BHT are the least mentioned chemicals, with only 2 studies featuring them in their title, abstract or keywords. Although few research efforts have been devoted to the study of these two compounds, BHT was not included in Decision 2018/840 since the data collected by EU member states are enough to carry out environmental risk assessment. EHMC was also removed from the list due to its high tendency to sorb to sediment. Since the monitoring in sediment samples has not been implemented yet, it has temporarily been excluded from the Watch list.

The bibliometric search evidenced that many research articles deal with only a part of the Watch list compounds, probably because of technical difficulties in the analysis in achieving such low LODs for compounds with very different properties, with the same analytical method. In fact, Figure 3c reveals that publications featuring the search term "analysis" are more than three times those about removal of occurrence and, as expected, occurrence and removal of the Watch list compounds were investigated only once the first analytical methodologies were established and are now growing. This confirms that the topic is relatively new and that, although few of these pollutants have been studied thoroughly in recent years, such as diclofenac and the hormones, there was a general lack of analytical methodologies that allowed the quantification of all of them at the required concentration levels. Moreover, it appears that most of the Watch list compounds are gaining the attention of the scientific community following their inclusion in Decision 2015/495.

a



b



c
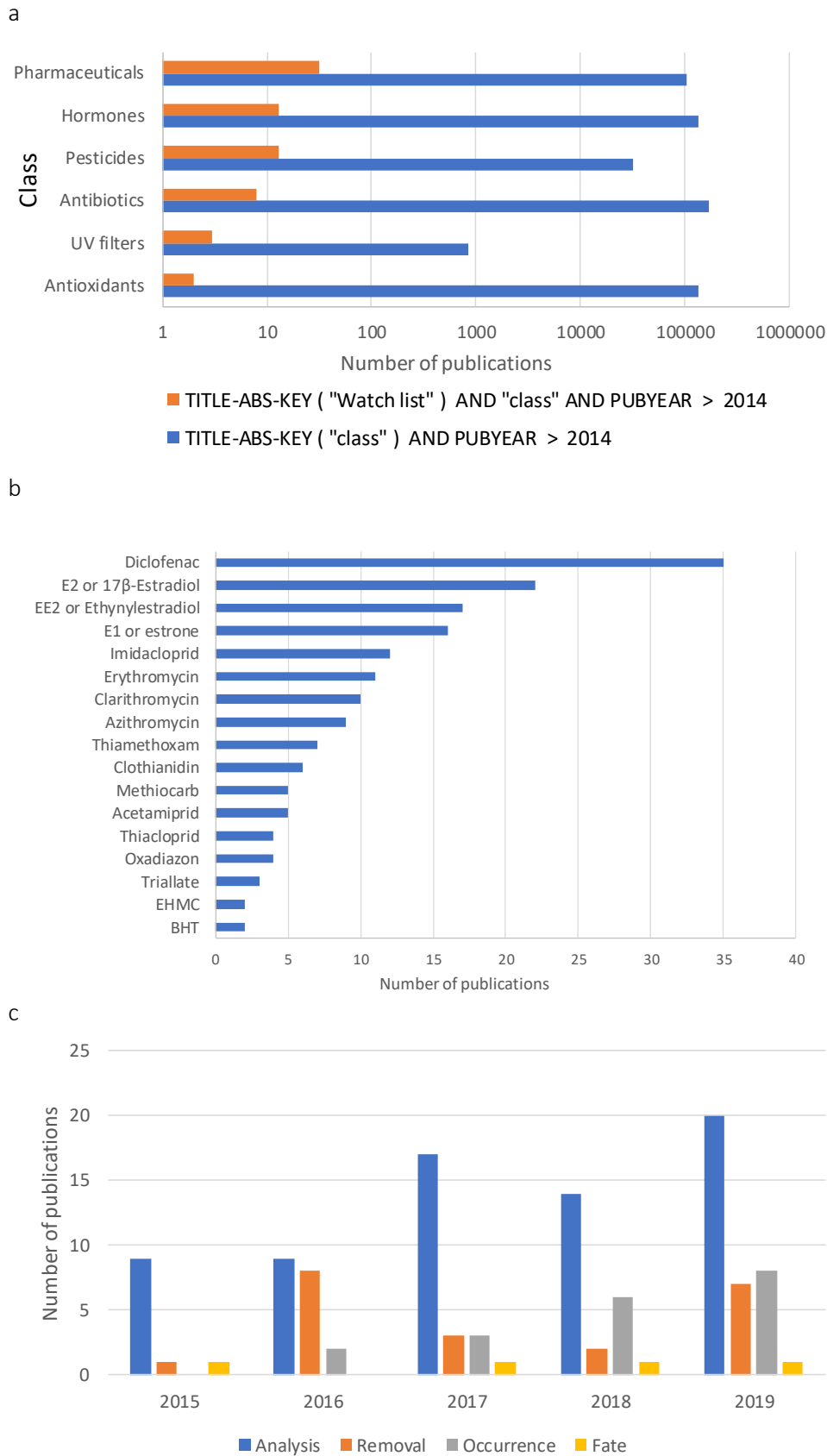


**Figure 3.** Output of a bibliometric search carried out with the following search terms: a: class of compounds (blue bars) versus "Watch list" AND class of compounds (orange bars); b: "Watch list" AND name of the chemical and c: "Watch list" AND topic of study over the years (title, abstract or keywords only, starting from 2015)

### 1.3.1.1. Occurrence and risk of the Watch list compounds

E1 and E2 are among the main human oestrogens. E2 has the highest biological activity among natural oestrogens and, on average, is excreted between 0.5 and 5 µg/day. (de Mes et al., 2005; Nie et al., 2009; Ternes et al., 1999). EE2 is a synthetically-produced hormone with estrogenic activity higher than that of E1 and E2 and is more persistent than natural oestrogens in WWTPs (de Mes et al., 2005; Li, 2014). E1, E2 and EE2 act as endocrine disrupting compounds on aquatic organisms: studies show that, for example, chronic exposure to EE2 as low as 5-6 ng/L induced feminization of male fish and altered oogenesis in female fish (Kidd et al., 2007). Diclofenac is one of the most frequently and abundantly found non-steroidal anti-inflammatory drug, with concentrations in water up to 4.4 µg/L (Barbosa et al., 2016), due to its widespread use and to the low/inconsistent removal rates recorded in WWTPs (Verlicchi et al., 2012). The three macrolide antibiotics (azithromycin, clarithromycin and erythromycin) are commonly employed to treat severe infections in humans, animals and in aquaculture (Lange et al., 2006; Xekoukoulotakis et al., 2010). They have been detected at ng/L to µg/L ranges in various aquatic matrices (López-Serna et al., 2013, 2012; Silva et al., 2011) and are marked by incomplete removal during wastewater treatment (López-Serna et al., 2012; Verlicchi et al., 2012). Moreover, they are causing concern on the development of antibiotic resistance in bacteria (Xekoukoulotakis et al., 2010). The neonicotinoid insecticides, often referred to as neonics, have taken over the use of more traditional compounds, such as organophosphate and carbamate, in the past years (Hladik et al., 2014). They are likely to negatively affect aquatic invertebrates and ecosystem health (Schaafsma et al., 2015), threaten bees wellbeing, as well as having adverse effects on pollinators (Spivak et al., 2013). They are highly soluble in water and persistent, especially clothianidin (Hladik et al., 2014). Nevertheless, there is a general lack of scientific literature on their occurrence, with most studies considering imidacloprid only. After the publication of the Watch list 2015/495, a neonicotinoids ban entered into force. Indeed, the *Commission Implementing Regulations (EU) 2018/783-5* of 29 May 2018 banned the use of imidacloprid, clothianidin and thiamethoxam, respectively. Risk assessment revealed high acute risks for bees from plant protection products containing such chemicals and therefore all outdoor uses have been prohibited, limiting the use of these pesticides to permanent greenhouses and only when the resulting crop stays within a permanent

greenhouse during its entire life cycle. The sale and use of seeds treated with these chemicals have also been restricted with the same conditions (EC, 2018b, 2018c, 2018d). On the contrary, the European Food Safety Authority has established that acetamiprid entails a low risk to bees. Thiacloprid has also been under examination due to its alleged endocrine disrupting activity. Its approval will expire in April 2020 and a renewal might be refused by the European commission (EC, n.d.). Methiocarb is one of the most widely used carbamate pesticides, exhibiting high toxicity (Qiang et al., 2014) and, on the whole, its occurrence in surface water, as well as its removal in WWTPs has not been investigated exhaustively. Oxadiazon and triallate are two herbicides also marked by scarce literature data. To the authors' knowledge, the removal of such compounds in WWTPs has not been reported yet. Both substances tend to adsorb to soils, but leaching may occur, leading to their release into the aquatic medium (Barbosa et al., 2016). The environmental risk associated to the antioxidant BHT is caused by its degradation into BHT-CHO, known to provoke cellular and DNA damage in mice and rats (Fries and Püttmann, 2004). Its presence in freshwater has been reported up to 620 ng/L (Bendz et al., 2005) and it has also been detected in groundwater at high levels (Fries and Püttmann, 2004). No studies on its removal from wastewater are known to the authors. EHMC, finally, is an organic UV filter known to cause estrogenic effects as well as non-estrogenic hormonal issues on biota and humans (Ramos et al., 2016). It has the tendency to partition into sediments and suspended particulate matter and it has been found at µg/kg levels in macroinvertebrates and fish samples (Kaiser et al., 2012). Nevertheless, it has been detected in water matrices too (Barbosa et al., 2016) but little information is available in freshwater and, especially, in wastewater. Literature data on the occurrence of the Watch list compounds in freshwater and wastewater are summarised in Table 4 and Table 5 respectively, while the reported removal rates are displayed in Table 6.

**Table 4.** Occurrence of the Watch list compounds in freshwater as reported in literature. n.a. corresponds to no available data.

| Compound | Range (ng/L) | Reference |
|---|---|---|
| Azithromycin | <LOD-90.8 | (Hoa et al., 2011; Sousa et al., 2019; Tong et al., 2014) |
| Clarithromycin | <LOD-778 | (Al Aukidy et al., 2012; Gracia-Lor et al., 2011; Hoa et al., 2011; Silva et al., 2011; Sousa et al., 2019; Tong et al., 2014) |
| Erythromycin | <LOD-2246 | (Gracia-Lor et al., 2011; Hoa et al., 2011; Silva et al., 2011; Sousa et al., 2019) |
| Diclofenac | <LOD-3224 | (Li, 2014; Silva et al., 2011; Sousa et al., 2019; Spongberg et al., 2011; Stasinakis et al., 2012) |
| E1 | <LOD-69.1 | (Bolong et al., 2009; Luo et al., 2014; Sousa et al., 2019; Vulliet and Cren-Olivé, 2011) |
| E2 | 0.2-10.1 | (Bolong et al., 2009; Luo et al., 2014; Vulliet and Cren-Olivé, 2011) |
| EE2 | 0.2-1.9 | (Bolong et al., 2009; Luo et al., 2014; Vulliet and Cren-Olivé, 2011) |
| Acetamiprid | 20-380 | (Sánchez-Bayo and Hyne, 2014) |
| Clothianidin | <LOD-420 | (Hladik et al., 2014; Li, 2014; Sánchez-Bayo and Hyne, 2014; Sousa et al., 2019; Székács et al., 2015) |
| Imidacloprid | <LOD-480 | (Ccanccapa et al., 2016; Gonzalez-Rey et al., 2015; Hladik et al., 2014; Ana Masiá et al., 2013; Papadakis et al., 2015; Sánchez-Bayo and Hyne, 2014; Sousa et al., 2019) |
| Thiacloprid | <LOD-400 | (Rubirola et al., 2017; Sánchez-Bayo and Hyne, 2014; Sousa et al., 2019) |
| Thiamethoxam | <LOD-1580 | (Chau et al., 2015; da Rocha et al., 2015; Hladik et al., 2014; Papadakis et al., 2015; Sánchez-Bayo and Hyne, 2014; Sousa et al., 2019; Székács et al., 2015) |
| Methiocarb | <LOD-391.44 | (Campo et al., 2013; Ana Masiá et al., 2013; Rubirola et al., 2017) |
| Oxadiazon | <LOD-1440 | (Furtula et al., 2006; Sousa et al., 2019) |
| Triallate | <LOD-513 | (Rubirola et al., 2017; Sousa et al., 2019) |
| BHT | <LOD-1115 | (Bendz et al., 2005; Benotti et al., 2009; Fries and Püttmann, 2004, 2002; Kolpin and Meyer, 2002; Liu et al., 2015b; Sousa et al., 2019) |
| EHMC | <LOD-7552 | (Amine et al., 2012; Capriotti et al., 2014; Gómez et al., 2009; Rodil et al., 2009; Sousa et al., 2019) |

Table 5. Occurrence of the Watch list compounds in CAS WWTP (influent and effluent) as reported in literature. n.a. corresponds to no available data.

| Compound | Influent | | Effluent | |
|---|---|---|---|---|
| | Range (ng/L) | Reference | Range (ng/L) | Reference |
| Azithromycin | <LOD-6810 | (Botero-Coy et al., 2018; Collado et al., 2014; Göbel et al., 2005; Loganathan et al., 2009; Pereira et al., 2015; Rivera-Utrilla et al., 2013; Senta et al., 2013) | <LOD-1220 | (Al Aukidy et al., 2012; Birošová et al., 2014; Botero-Coy et al., 2018; Collado et al., 2014; Gibs et al., 2013; Göbel et al., 2005; Loganathan et al., 2009; Pereira et al., 2015; Rivera-Utrilla et al., 2013; Senta et al., 2013) |
| Clarithromycin | <LOD-8000 | (Birošová et al., 2014; Collado et al., 2014; Guerra et al., 2014; Loganathan et al., 2009; Margot et al., 2013; Tran et al., 2018) | 8-1890 | (Al Aukidy et al., 2012; Birošová et al., 2014; Castiglioni et al., 2005; Collado et al., 2014; Lara-Martín et al., 2014; Moreira et al., 2015; Prieto-Rodriguez et al., 2012) |
| Erythromycin | <LOD-2310 | (Botero-Coy et al., 2018; Collado et al., 2014; Göbel et al., 2005; Gros et al., 2006; Papageorgiou et al., 2016; Rosal et al., 2010; S. Yang and Carlson, 2004) | 6-760 | (Birošová et al., 2014; Castiglioni et al., 2005; Collado et al., 2014; Gibs et al., 2013; Moreira et al., 2015; Prieto-Rodriguez et al., 2012; Rosal et al., 2010; Yan et al., 2014) |
| Diclofenac | <LOD-4114 | (Clara et al., 2005; Collado et al., 2014; Gros et al., 2006; Margot et al., 2013; Pereira et al., 2015; Rivera-Utrilla et al., 2013; Sari et al., 2014) | <LOD-4425 | (Al Aukidy et al., 2012; Collado et al., 2014; Lara-Martín et al., 2014; Pereira et al., 2015; Prieto-Rodriguez et al., 2012; Rivera-Utrilla et al., 2013) |
| E1 | 11.6-224 | (Baronti et al., 2000; Ekpeghere et al., 2018; Margot et al., 2013; Zhou et al., 2012) | <LOD-220 | (Baronti et al., 2000; Behera et al., 2011; Bolong et al., 2009; Castiglioni et al., 2005; Nie et al., 2012; Zorita et al., 2009) |
| E2 | 3.7-140 | (Baronti et al., 2000; Ekpeghere et al., 2018; Margot et al., 2013; Zhou et al., 2012) | <LOD-110.4 | (Baronti et al., 2000; Behera et al., 2011; Bolong et al., 2009; Castiglioni et al., 2005; Moreira et al., 2015; Nie et al., 2012; Zorita et al., 2009) |

| Compound | Influent | | Effluent | |
|---|---|---|---|---|
| | Range (ng/L) | Reference | Range (ng/L) | Reference |
| EE2 | <LOD-330 | (Baronti et al., 2000; Ekpeghere et al., 2018; Margot et al., 2013; Zhou et al., 2012) | <LOD-391.4 | (Baronti et al., 2000; Behera et al., 2011; Bolong et al., 2009; Castiglioni et al., 2005; Moreira et al., 2015; Nie et al., 2012; Zorita et al., 2009) |
| Acetamiprid | 3.7 | (Sadaria et al., 2016) | n.a. | - |
| Clothianidin | 149.7 | (Sadaria et al., 2016) | n.a. | - |
| Imidacloprid | 54.7 | (Sadaria et al., 2016) | 48.6 | (Sadaria et al., 2016) |
| Thiacloprid | <LOD | (Rubirola et al., 2017; Sadaria et al., 2016) | n.a. | - |
| Thiamethoxam | <LOD | (Sadaria et al., 2016) | n.a. | - |
| Methiocarb | n.a. | - | <LOD-105.31 | (Campo et al., 2013; Ana Masiá et al., 2013) |
| Oxadiazon | n.a. | - | n.a. | - |
| Triallate | n.a. | - | n.a. | - |
| BHT | 263-2420 | (Fries and Püttmann, 2004; Liu et al., 2015b) | 2510 | (Liu et al., 2015b) |
| EHMC | <LOD-1732 | (Ekpeghere et al., 2016; Magi et al., 2013; Negreira et al., 2010; Rodil et al., 2009; Tsui et al., 2014) | <LOD-579 | (Gómez et al., 2009; Langford et al., 2015; Li et al., 2007; Negreira et al., 2010; Tsui et al., 2014) |

Table 6. Removals of the Watch list compounds in CAS treatment as reported in literature. n.a. corresponds to no available data.

| Compound | Removal (%) | Reference |
|---|---|---|
| Azithromycin | 0-100 | (Göbel et al., 2005; Loganathan et al., 2009; Margot et al., 2013; Pereira et al., 2015) |
| Clarithromycin | 37 | (Margot et al., 2013) |
| Erythromycin | <0-100 | (Göbel et al., 2005; Gros et al., 2006; Rosal et al., 2010; S Yang and Carlson, 2004) |
| Diclofenac | <0-100 | (Clara et al., 2005; Luo et al., 2014; Margot et al., 2013; Pereira et al., 2015; Sari et al., 2014) |
| E1 | 12-94 | (Baronti et al., 2000; Margot et al., 2013; Zhou et al., 2012) |
| E2 | 83-96 | (Baronti et al., 2000; Margot et al., 2013; Zhou et al., 2012) |
| EE2 | 18-94 | (Baronti et al., 2000; Margot et al., 2013; Zhou et al., 2012) |
| Acetamiprid | 18 | (Sadaria et al., 2016) |
| Clothianidin | 13 | (Sadaria et al., 2016) |
| Imidacloprid | 11 | (Sadaria et al., 2016) |
| Thiacloprid | n.a. | - |
| Thiamethoxam | n.a. | - |
| Methiocarb | n.a. | - |
| Oxadiazon | n.a. | - |
| Triallate | n.a. | - |
| BHT | 25.9-95.4 | (Fries and Püttmann, 2004; Liu et al., 2015b) |
| EHMC | 30-55 | (Tsui et al., 2014) |

# OBJECTIVES

The ultimate objective of this doctoral thesis is an advance in the knowledge on micropollutants, focussing on the EU regulatory framework, and precisely on the compounds addressed in the EU Decision 2015/495, through a multidisciplinary approach combining the development of a novel analytical methodology, sampling campaigns and study of removal possibilities.

This translates into the following specific objectives:

- To develop an analytical methodology for the detection of all the 17 compounds of the Watch list by means of online-SPE-UHPLC-MS/MS in fulfilment of the requirements detailed in the EU Decision.
- To validate the analytical methodology in freshwater, as required by the EU Decision, but also in influent and effluent wastewater.
- To assess the extent of contamination caused by the Watch list compounds, the sources of pollution, river transport, the impacts of WWTPs and seasonal variations by means of sampling campaigns in the relevant case study of the Ebro Delta Area (NE, Spain).
- To compare the data obtained in the case study with the predicted no-effect concentrations (PNECs) and to calculate risk quotients (RQs) for each sampling site.
- To gain insights into fate and behaviour of the Watch list compounds in CAS systems to evaluate the removal, the biodegradability of selected chemicals and the role of sorption at controlled conditions by means of a series of batch tests.
- To determine the most relevant parameters affecting the (bio)degradation of the Watch list compounds among four operational parameters: temperature, pH, biomass concentration and redox conditions, in ranges usually found in temperate climate conventional WWTPs.

# RESULTS

<div align="right">

CHAPTER 1

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# Development of an online SPE-UHPLC-MS/MS method for the multiresidue analysis of the 17 compounds from the EU "Watch list"
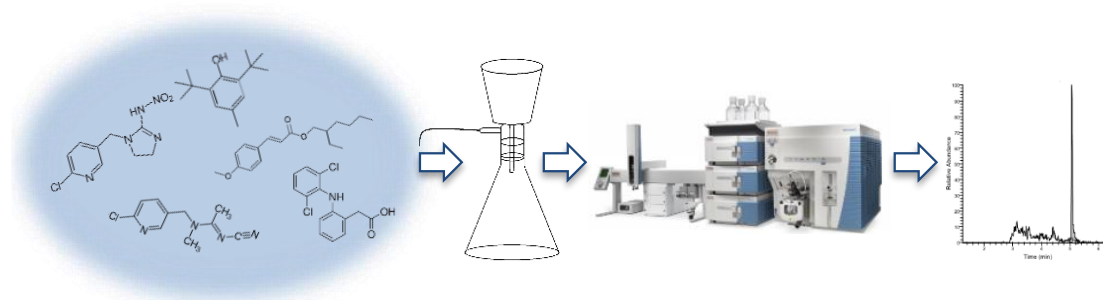
# Development of an online SPE-UHPLC-MS/MS method for the multiresidue analysis of the 17 compounds from the EU "Watch list"

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## Abstract

During the last decades, the quality of the aquatic ecosystems has been threatened by increasing levels of pollutions, caused by the discharge of man-made chemicals, both via accidental release of pollutants as well as a consequence of the constant outflow of inadequately treated wastewater effluents. For this reason, the European Union is updating its legislations with the aim of limiting the release of emerging contaminants. The Commission Implementing Decision (EU) 2015/495 published in March 2015 drafts a "Watch list" of compounds to be monitored Europe-wide. In this study, a methodology based on online solid-phase extraction (SPE) ultra-high-performance liquid chromatography coupled to a triple-quadrupole mass spectrometer (UHPLC-MS/MS) was developed for the simultaneous determination of the 17 compounds listed therein. The proposed method offers advantages over already available methods, such as versatility (all 17 compounds can be analyzed simultaneously), shorter time required for the analysis, robustness and sensitivity. The employment of online sample preparation minimized sample manipulation and reduced dramatically the sample volume needed and time required, making thus the analysis fast and reliable. The method was successfully validated in surface water, influent and effluent wastewater. Limits of detection ranged from sub- to low-ng/L levels, in compliance with the EU limits, with the only exception of EE2.

## Keywords

EU Watch list; online preconcentration; ultra-high-performance liquid-chromatography; online SPE; UHPLC-MS/MS; water samples

## 1. Introduction

### 1.1. European legislation

The *Directive 2000/60/EC*, commonly known as the Water Framework Directive (WFD), has, as a main objective, the achievement of a good chemical status of surface and ground water across the European Union. To reach this goal, the discharge of contaminants into the environment have to be decreased [1]. Nowadays, the release of pollutants is limited and controlled by means of different pieces of legislation. Some are focused on prevention, such as the REACH regulation, which aims at controlling the addition of chemicals in the industrial production in order to limit the contamination of water bodies [2]; the Directive on Plant Protection Products, which focuses on pollutants related to agriculture [3], and the Directive 2010/75/EU on industrial emissions, which regulates the discharge of chemicals from industrial activities [4]. At the end side, the Urban Waste Water Treatment Directive (UWWTD) is crucial to prevent the contamination of receiving waters, by requiring appropriate treatment of wastewater prior to discharge [5]. Nevertheless, the monitoring of certain substances in water bodies is necessary to keep track of the actual pollution of surface waters. This would allow the assessment of the real status of water bodies, which could be compromised by the accumulation of contaminants originating from different sources. For this reason, the WFD proposed the creation of a list of priority substances. This came into practise with a subsequent directive first published on 16 December 2008, where the first 33 priority substances were listed along with their Environmental Quality Standards (EQS), that is, concentration limits that shall not be exceeded. These substances were chosen based on risk assessment studies, taking into account their intrinsic properties in relation to aquatic and human ecotoxicity, the extent of contamination of European waters and the spread of their application at European level [6]. The Directive 2013/39/EU, which includes the latest version of the list expanding the number of priority substances to 45, also sets a new mechanism of gathering occurrence data of contaminants in the aquatic

environment, especially targeting those chemicals for which the existing information is insufficient for risk assessment. The above mentioned directive therefore prepared the ground for the creation of a Watch list of a limited number of compounds to be monitored in a specific temporal framework, in order to gather high quality data with the aim of supporting the prioritization process [7]. The first version of the Watch list appeared in the Commission Implementing Decision (EU) 2015/495, published in March 2015. The Watch list includes 17 compounds from different chemical families, paired with the detection limits and the suggested analytical method [8].The compounds indicated in the Watch list include three endocrine disrupting compounds, pharmaceuticals (a painkiller and three antibiotics from the macrolides family), an antioxidant agent, a UV filter and various pesticides, mainly neonicotinoids. Since the analysis of such compounds will have to be carried out Europe-wide on a routine basis, a comprehensive multi-residue analytical method represents a useful tool for shortening the time and costs of analysis.

## 1.2. State of the art

To date, different methods have been published for the detection of some of the Watch list compounds in waters, such as pesticides [9–11], antibiotics [11,12], pharmaceuticals [14], UV filters [15] and antioxidants [16]. Recently, a method based on on-line solid phase extraction (SPE), using disposable cartridges, was published for the analysis of 24 WFD priority substances including the majority of the Watch list compounds [17]. However, to facilitate the water authorities, we believe that a robust methodology which allows the analysis of all 17 compounds, as developed here, would be a useful tool. Moreover, the proposed method is faster and employs a long-lasting chromatographic column for preconcentration instead of disposable cartridges, thus becoming a greener alternative. Some of the Watch list compounds have traditionally been analysed by gas chromatography-mass spectrometry (GC-MS), especially BHT [17,18], but the need for analytes derivatization makes GC-MS a less attractive alternative compared to liquid chromatography tandem mass spectrometry (LC-MS/MS) [19,20]. LC-MS/MS is now the technique of choice in the analysis of micropollutants in water samples [22]. The bottleneck in the analysis of emerging pollutants is often represented by sample preparation and pretreatment. So far, the most common technique for water samples preconcentration is off line SPE [22,23]. Such practise is costly and particularly time

consuming, as it often involves several steps before a final extract suitable for instrumental analysis is obtained [25]. Moreover, it implies large volumes of toxic solvents, as well as large sample volumes, which might not be always available [26–29]. Also, the possibility of contamination is high and loss of analytes during evaporation and/or their degradation during preconcentration are not uncommon [28, 29]. The most recent trends are shifting towards the employment of online sample preconcentration for the analysis of trace emerging contaminants in water [32, 33].The direct injection of samples onto a LC column, where concentration and clean-up steps take place, allows to reduce the sample volume (typically 1 to 5 mL), it decreases dramatically sample manipulation and the use of organic solvents, it shortens the time of analysis and brings to an increase in precision [34].

The aim of this work is the development and optimization of a new, fast and robust analytical methodology, using dual column LC switching system coupled to MS, for the analysis of the 17 compounds indicated in the EU Watch list. The preconcentration of the analytes is achieved by means of an automated Equan™ Direct Injection Technology coupled to an ultra-high-performance liquid-chromatograph triple-quadrupole mass spectrometer (UHPLC-MS/MS) for separation and detection. The multi-reside methodology has been validated in different water matrices, namely freshwater, wastewater effluent and wastewater influent offering advantages over existing methods in terms of versatility and sensitivity.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Clarithromycin, Erythromycin, Azithromycin, 2-Ethylhexyl 4-Methoxycinnamate (EHMC), Imidacloprid, Methiocarb, Thiacloprid, Thiamethoxam, Clothianidin, Acetamiprid, Oxadiazon, Triallate, Estrone (E1), β-Estradiol (E2), 17α-Ethynylestradiol (EE2), Butylated hydroxytoluene (BHT), Diclofenac (sodium salt) were obtained from Sigma Aldrich (St. Louis, MO, USA). Isotopically labelled compounds, used as internal standards (IS), were chosen according to the chemical properties and retention times of the analytes. Erythromycin-($N,N$-dimethyl-$^{13}C_2$), Methiocarb-($N$-methyl-$d_3$), Thiamethoxam-d3, Imidacloprid-d4, Oxybenzone-(Phenyl-d5), Clothianidin-d3, BHT-d21, β-Estradiol-$d_2$ (E2-$d_2$), Estrone-2,4,16,16-$d_4$ (E1-$d_4$) were purchased from Sigma Aldrich (St. Louis, MO, USA),

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while Ethynylestradiol-d4 (EE2-d$_4$), Diclofenac-d4 and Azithromycin-d3 were supplied by TRC (Toronto, Canada). The purity grade of standards was always above 95%.

Individual stock solutions were prepared on a weight basis at a concentration of 1 mg/mL in methanol and stored at -20°C in the dark, in order to preserve the compounds from possible photodegradation [14, 35]. Antibiotics solutions were renewed each 3 months [14], while the solutions of deuterated antibiotics and the rest of compounds were renewed each 6 months. Intermediate mixed solutions containing all the analytes and all the labelled compounds respectively were prepared in methanol monthly. Aqueous working standard solutions were renewed before every analytical run. To make the detection of BHT possible, its concentration in the aqueous mixed solution was 10-fold that of the other compounds, while the concentration of BHT-d21 in the IS mix was 20-fold the other deuterated standards.

Durapore Hydrophilic PVDF filters (47 mm 0.45 µm) were purchased from Merck Millipore (Bedford, MA, USA). Extra pure Formic acid and EDTA disodium salt 0.1 M were acquired from Scharlau (Barcelona, Spain). Ammonium acetate for HPLC (VWR, Radnor, PA, USA), Ammonium formate 99% pure (Acros Organics), Ammonium fluoride for LC-MS (Sigma Aldrich, St. Louis, MO, USA) were tested as mobile phase modifiers. LC/MS grade solvents (water, methanol and acetonitrile) were supplied by Fisher (Optima™ LC/MS).

## 2.2. Sample collection and preparation

The method was validated and optimized in various matrices, namely LC/MS grade water, freshwater, wastewater (wastewater treatment plant (WWTP) influent and effluent). The matrices mentioned above are representative of increasing complexity, being wastewater influents rich in dissolved organic matter. Freshwater samples were collected from the Ebro river in proximity of the river delta, in the province of Tarragona (Catalonia, NE of Spain). 24-hour time-integrated samples of influent and effluent wastewater were collected from a WWTP located in the Ebro river delta area (Amposta, 27.500 population equivalents (PE)). Samples were collected in amber PET bottles and transported in ice to the research facilities, where they were frozen upon arrival. Before injection into the online SPE-UPHLC-MS/MS system, the water samples used for the method optimization were filtered with Durapore PVDF filters (0.45 µm), then transferred into 10 mL amber SPE vials from Supelco and spiked with the appropriate volume of a working aqueous

solution containing the analytes and with a mixture of internal standards in order to obtain an IS concentration of 50 ng/L (1 µg/L in the case of BHT-d21). It was observed that the background concentrations of certain compounds (Azithromycin, Clarithromycin, Diclofenac and Erythromycin) in WWTP effluent and influent was too high to allow the calibration. Since cleaner matrices were not available, wastewater influent and effluent were diluted at a 1:100 ratio before spiking with the analytes to allow the estimation of the validation parameters for such compounds.

## 2.3. Instrumental analysis

Online preconcentration was carried out by means of an Equan MAX™ fully automated system consisting of a PAL autosampler (CTC Analysis) and two pumps: a loading pump (AccelaTM 600) and an eluting pump (AccelaTM 1250), both from Thermo Fisher Scientific. Two LC columns were used, one for analytes preconcentration and the other for chromatographic separation. The LC columns were connected via a three-valve switching device unit with a six-port valve. The loading and elution of the two columns was controlled by the switching of a divert valve. The injection volume was set at 2 mL for all matrices. A Hypersil GOLD aQ (20 x 2.1 mm, 12 µm particle size, Thermo Fisher Scientific) column was employed for the preconcentration of the analytes. The samples were loaded at a flow rate of 1750 µL/min and then the column was washed and conditioned with methanol and water during the chromatographic run. In the following step the analytes were eluted from the chromatographic column at flow rate of 400 µL/min. Due to the differences in the analytes properties, two different LC chromatographic setups were adopted according to the polarity: the compounds analysed in positive ionization (PI) were separated by means of a Kinetex Biphenyl (1.7 µm particle size, 100 x 2.1 mm i.d.), while a Kinetex EVO C18 (1.7 µm particle size, 100 mm × 2.1 mm i.d.) was employed for the analysis in negative ionization (NI). Both chromatographic columns were purchased from Phenomenex. The detection was performed using a TSQ Vantage mass spectrometer (Thermo Fisher Scientific), equipped with an electrospray turbo spray ionization (ESI) source. Two Selected Reaction Monitoring (SRM) transitions were recorded for each compound: one for quantification and the other for confirmation. Time-specific SRM windows were set for each retention time in order to enhance the sensitivity. The whole system was controlled via the Xcalibur

2.2 software (Thermo Fisher Scientific), while quantification of the analytes was performed with TraceFinder EFS 3.1 (Thermo Fisher Scientific).

### 2.3.1. Chromatographic conditions

Since the compounds displayed different properties and, as a consequence, chromatographic behaviour, the method was split in two and the compounds were analysed in separate chromatographic runs according to their polarity. EHMC, Erythromycin, Clarithromycin, Azithromycin, Methiocarb, Imidacloprid, Thiacloprid, Thiamethoxam, Clothianidin, Acetamiprid, Oxadiazon and Triallate were analysed under PI mode. The mobile phase used for the chromatographic separation consisted of MeOH (A) and water with 0.1% formic acid (B). The optimized initial gradient conditions were 60% A, which was kept during the first 1.75 min, then it was brought to 75% A in 0.13 min approximately and then it was increased to 100% A in 2.12 min. Isocratic conditions were held during 1.75 min after which the system was brought to initial gradient conditions. The total duration of the chromatographic run was 8 minutes.

EE2, E2, E1, Diclofenac and BHT were analysed under NI mode. As organic mobile phase, a mixture of MeOH (A) and ACN (B) was used during the initial stages of the gradient, while the compounds eluted when the percentage of ACN reached almost 100%. The aqueous mobile phase consisted of LC/MS grade water with ammonium fluoride 1 mM (C). The chosen gradient consisted of: 15% A and 15% B and were held during the first 2 minutes. 100% B was reached in 4 minutes. Isocratic conditions were held for 1.50 minutes. The initial conditions were restored in 1 minute and then column equilibration was allowed. Each chromatographic run lasted 10.5 minutes.

Figure 1 shows the chromatogram obtained for a selection of compounds spiked at 5 ng/L in a river sample. An example of the chromatographic behaviour of one of the analytes in all the studied matrices is displayed in Figure 2.
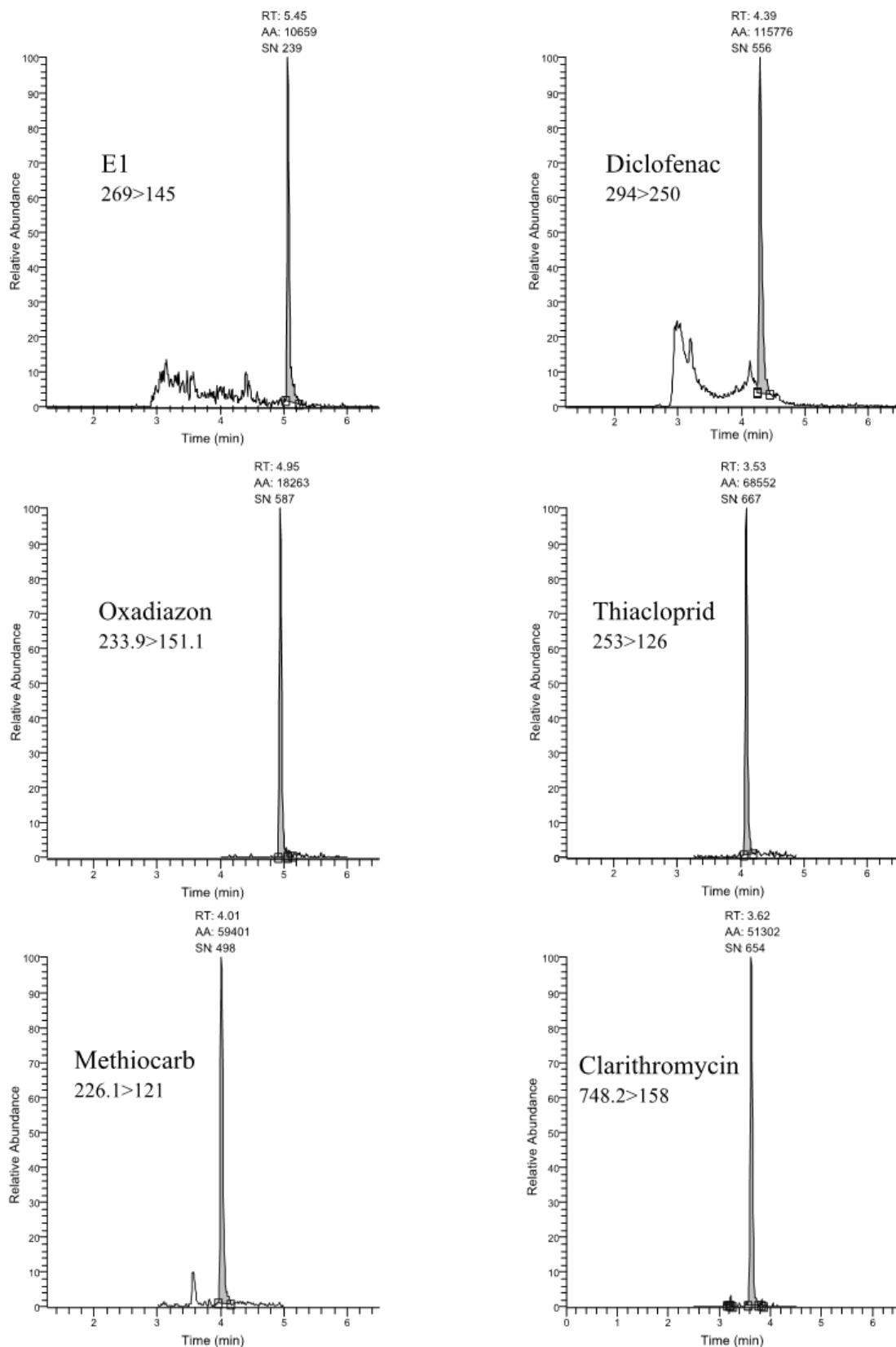
**Figure 1.** SRM chromatograms of a selection of compounds obtained from a freshwater sample spiked at 5 ng/L.
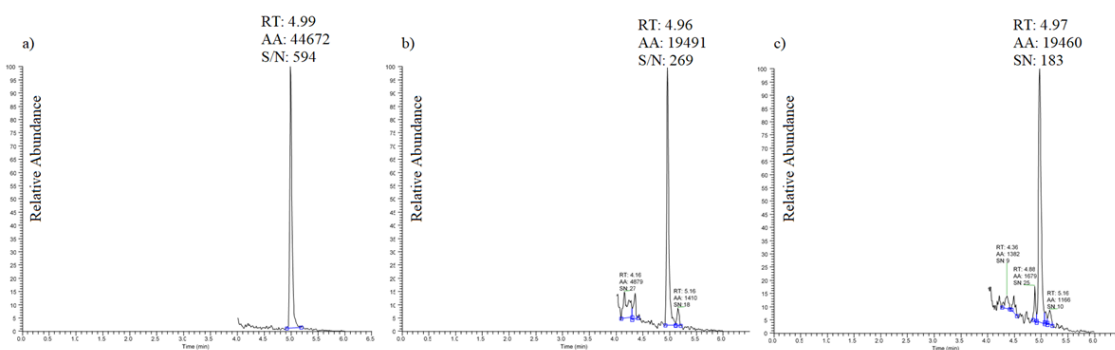
**Figure 2.** SRM chromatograms of triallate obtained under PI conditions at a spiked concentration of 25 ng/L in a) freshwater, b) wastewater effluent, c) wastewater influent.

## 3. Results and discussion

### 3.1. Mass spectrometry optimization

The optimization of the MS experimental conditions was carried out by direct infusion in order to explore the behaviour of the compounds under different ionization conditions and to gather information regarding the fragmentation of the parent ion. The optimized source conditions for the analysis in PI mode were: capillary temperature 300 °C, vaporizer temperature 350°C, sheath gas pressure 40 ($N_2$ arbitrary anits (a.u.)), auxiliary gas pressure 15 ($N_2$ a.u.), ion sweep gas pressure 0 ($N_2$ a.u.), spray voltage 3000 V. For NI mode, the chosen conditions were: capillary temperature 300 °C, vaporizer temperature 350°C, sheath gas pressure 20 ($N_2$ a.u.), auxiliary gas pressure 20 ($N_2$ a.u.), ion sweep gas pressure 2 ($N_2$ a.u.), spray voltage 2500 V. The analyses were carried out in SRM mode, using two transitions for each analyte, normally corresponding to the two most abundant fragments. The first transition was used for quantitation while the second served for confirmation purposes. When possible, [M+H]$^+$ and [M-H]$^-$ for PI and NI respectively were selected as precursor ions. The optimized parameters for SRM analysis are detailed in Table 1. To minimize uncertainty in the identification, the maximum difference allowed between the chromatographic retention time (RT) of the compound in the sample and in the calibration curve was ±2% and the ratio between the two SRM transitions could not exceed a difference of ±20% between the standard solutions and the actual samples, in accordance with the Council Directive 96/23/EC implementation of 2002 [36]. For the analyses, time-specific SRM windows were set in function of the chromatographic RTs of target compounds in order to improve sensitivity.

**Table 1.** Acquisition parameters: corresponding internal standard (IS), ESI polarity, S-Lens, precursor ions, SRM transitions and collision energies (CE).

| Compound | IS | Polarity | Precursor ion | S-Lens | SRM 1 | CE (EV) | SRM 2 | CE (EV) |
|---|---|---|---|---|---|---|---|---|
| EHMC | Oxybenzone-phenyl-d5 | + | 291.2 | 57 | 161 | 16 | 179 | 5 |
| Erythromycin | Erythromycin-(N,N-dimethyl-$^{13}$C2) | + | 734.1 | 152 | 158 | 39 | 576 | 19 |
| Clarithromycin | Erythromycin-(N,N-dimethyl-$^{13}$C2) | + | 748.2 | 156 | 158 | 41 | 116 | 41 |
| Azithromycin | Erythromycin-(N,N-dimethyl-$^{13}$C2) | + | 376.7 | 109 | 83.1 | 19 | 158.1 | 21 |
| Methiocarb | Methiocarb-N-methyl-d3 | + | 226.1 | 67 | 121 | 16 | 169 | 5 |
| Imidacloprid | Imidacloprid-d4 | + | 256.1 | 80 | 209 | 16 | 175 | 17 |
| Thiacloprid | Imidacloprid-d4 | + | 253 | 79 | 126 | 32 | 90 | 35 |
| Thiamethoxam | Thiamethoxam-d3 | + | 292 | 73 | 211 | 10 | 181 | 20 |
| Clothianidin | Clothianidin-d3 | + | 250 | 69 | 169 | 11 | 132 | 14 |
| Acetamiprid | Imidacloprid-d4 | + | 223. 1 | 67 | 90 | 32 | 126 | 12 |
| Oxadiazon | Oxybenzone-phenyl-d5 | + | 233.9 | 106 | 151.1 | 26 | 110.1 | 18 |
| Triallate | Oxybenzone-phenyl-d5 | + | 304 | 101 | 143 | 25 | 86 | 15 |
| EE2 | EE2-d4 | - | 295 | 126 | 145 | 41 | 159 | 33 |
| E2 | Estradiol-d2 | - | 271 | 148 | 183.2 | 42 | 145.2 | 43 |
| E1 | Estrone-d4 | - | 269 | 120 | 145 | 38 | 143 | 54 |
| Diclofenac | Diclofenac-d4 | - | 294 | 65 | 250 | 14 | 214 | 22 |
| BHT | BHT-d21 | - | 219.1 | 105 | 203.3 | 30 | 163.2 | 32 |

## 3.2. Sample loading and preconcentration

A Hypersil GOLD aQ column was used for preconcentration of the analytes in the Equan™ system and the samples were loaded onto the preconcentration column at a flow rate of 1750 µL/min. During the loading step, the mobile phase initially used for the preconcentration process consisted of water and 2% methanol. Additionally, tests were also conducted employing 100% water to check whether methanol could elute the compounds retained on the column, thus leading to analytes loss. The employment of mere water resulted in worse sensitivity towards polar compounds and no significant improvement in the signals intensities of the other analytes. The addition of 0.1% formic acid to the aqueous phase in positive ESI and of ammonium fluoride 1 mM in NI ESI was also assessed. The introduction of formic acid led to narrower peaks and improved signal-to-noise (S/N) ratios. A slight decrease in the response of

antibiotics was noticed, but the results were overall satisfactory. On the contrary, the employment of ammonium fluoride in NI proved unsuitable since it resulted in lower peak areas and worse peak shapes, especially in the case of diclofenac. In the end, 0.1% formic acid was adopted for analyses in PI while no modifier was added to the solvents in NI mode.

The optimization also included an evaluation of the transfer time, that is to say, the time during which the sample is passed from the injection loop to the Equan$^{TM}$ column. 70, 75, 80 and 90 seconds were assessed. A transfer time of 75 seconds was chosen since it assured the complete transfer of the sample from the injection loop onto the column. Longer transfer times might lead to the loss of analytes since they might run through the column and be flushed to the waste.

## 3.3. Chromatographic separation

The initial stage of the chromatographic separation optimization was carried out in offline mode in order to test the chromatographic profile of the analytes. As mentioned before, the analytes showed great differences in their properties that made the choice of univocal chromatographic conditions impossible. For this reason, two separate methods were developed according to the polarity of the ESI source.

### 3.3.1. PI mode

Different UHPLC columns were tested. A Kinetex EVO C18 (1.7 µm particle size, 100 mm × 2.1 mm i.d.) and a Kinetex Biphenyl (1.7 µm particle size, 100 x 2.1 mm i.d.), both from Phenomenex, were assessed in PI mode. The latter yielded better results, since its internal composition makes it suitable for the analysis of hydrophobic, aromatic and polar compounds. In particular, when the Kinetex Biphenyl was used, better peaks shapes and higher areas were observed, especially in the case of antibiotics.

As organic mobile phase, various percentages of methanol and acetonitrile were tested. In the end, methanol was chosen over acetonitrile. Despite decreasing the retention times of the analytes, the employment of acetonitrile resulted in a dramatic drop in sensitivity. The aqueous phase contained 0.1% formic acid, for improved peak shape and resolution. The usage of acidic media to increase the ionization efficiency, especially for the analysis of antibiotics, is well known and widely applied [31,38].The absence of

carryover was tested injecting vials of unspiked LC-MS/MS grade water. Once the gradient was determined, time-specific SRM acquisition windows were set. An injection volume of 2 mL, as reported in similar analytical methods [37] was considered adequate since it allowed the detection of the Watch List compounds in compliance with the Decision. Therefore, no further optimization of the injection volume was considered.

### 3.3.2. NI mode

For the analysis in NI mode, the same columns tested in PI mode were assessed. The Kinetex EVO C18 (1.7 μm particle size, 100 mm × 2.1 mm i.d.) was chosen since it allowed a better chromatographic separation. The choice of the mobile phase composition and gradient conditions in NI mode proved challenging due to the presence of an interference in the same retention time frame as EE2 which caused coelution. As in PI mode, methanol and acetonitrile were evaluated as organic phase. Several combinations of solvents and various gradients were tested. In particular, the challenge was represented by a dual need: separating the estrogens from the interferences coming from the real matrices and still being able to achieve low limits of detection as stated in the EU Decision. Methanol is particularly convenient when it comes to sensitivity, while the usage of acetonitrile resulted in narrower peaks and it allowed a better chromatographic separation of the hormones, despite causing a slight drop in sensitivity. Eventually, a mixture of methanol and acetonitrile was employed during the initial and final stages of the gradient, in order to combine the high sensitivity obtained with methanol with the separation efficiency of acetonitrile, especially regarding the detection of the hormones. During isocratic conditions, the mobile phase consisted of 100% acetonitrile.

The ionization of diclofenac and especially BHT was poor in the absence of a mobile phase additive. To the best of our knowledge, only one study reports the detection of BHT by LC-MS/MS [16], since it is traditionally analysed by GC-MS. To make the analysis of such compound possible by LC-MS/MS, the BHT concentration in the working standard solutions was always 10 times the concentration of the other compounds, but still at concentration levels in compliance with the requirements of Decision (EU) 2015/495. Moreover, to overcome the difficulties, a study on different mobile phase modifiers was carried out. The effects of ammonium acetate (5 mM), ammonium formate (5 mM) and ammonium fluoride (1 mM) on ionization were assessed, since ammonium is known to

improve deprotonation in the gas phase, ammonium salts are often employed as additives in NI mode [39,40]. The results were compared to those obtained with aqueous phase without any additive. Ammonium acetate gave fair results in the ionization of diclofenac, but the estrogens and BHT peaks areas were low. Ammonium formate proved unsatisfactory in terms of signal intensity and signal to noise ratio for the detection of estrogens and the sensitivity towards both diclofenac and BHT was poor. Ammonium fluoride yielded the best results, allowing the desired sensitivity towards BHT and diclofenac, as well a good detection of the estrogens. This is due to the fact that the fluoride anion acts as a strong base during ionization and enhances the deprotonation significantly [41]. The effect of ammonium fluoride at different concentrations was explored carrying out experiments at 0.1 mM, 1 mM and 10 mM. It was shown that a concentration of 1 mM yields the best response.

The sample injection volume was also optimized, in particular 2 and 5 mL were tested. It was concluded that an increased sample volume did not correspond to a higher sensitivity. In the end, an injection sample of 2 mL was chosen since it permits multiple injections from the same vial and it might prove useful in situations of scarce sample availability.

### 3.3.2.1. pH adjustment and Na$_2$EDTA addition

Experiments were carried out adjusting the pH of freshwater samples to 9 and 11. Previous studies suggest that a basic pH could enhance the extraction efficiency of hormones [42].The samples were then filtered and spiked with a mixed solution of analytes as well as with the internal standards. The results obtained at different pH values were compared to those resulting from the original samples. At pH 9 no significant change was produced, while at pH 11 a slight improvement in the hormones signals was noticed, but the signals of diclofenac and BHT were negatively affected. In the end, it was concluded that no pH adjustment should be made in order to make the simultaneous detection of all the investigated compounds possible.

EDTA sodium salt is often added to natural samples with the aim of chelating the metal ions that are present in the waters and avoiding interferences [43–45]. An appropriate volume of EDTA was added to real matrix samples to achieve a final concentration of 0.1% [43] and the results were compared to analyses carried out in the absence of such

additive. Results showed that EDTA led to general signal suppression and did not bring any advantage in separation. It was concluded that no EDTA should be added to the samples.

## 3.4. Method performance evaluation

To evaluate the effects of the matrix on the ionization, the signals of target compounds in freshly-spiked matrix extracts and standards solutions are traditionally compared [45–47]. Nevertheless, when operating with an online-SPE system, it becomes impossible to separate the effects that the matrix exerts on the extraction recovery from those on the ionization efficiency.

Therefore, these two factors are determined jointly by calculating process efficiency, which sums up the two contributions and represents a useful parameter to assess method performance. Such parameter was calculated for each analyte in the three matrices investigated in this study (river water, wastewater effluent and wastewater influent) as indicated in the following equation.

$$\% \, Process \, Efficiency = \left[\frac{(A_s - A_{US})}{A_0}\right] \times 100\%$$

Where $A_S$ is the analyte peak area in the spiked sample, $A_{US}$ is the analyte peak area in the unspiked matrix and $A_0$ is the analyte peak area in LC-MS/MS grade water.

This parameter was calculated at a spiked concentration of 100 ng/L. Results relative to river water and wastewater effluent are displayed in Figure 3.

As expected, more complex matrices yielded lower process efficiency. Matrix effect was particularly prominent in wastewater influent (data not shown). Nevertheless, the employment of internal standards and matrix match calibration permits a mitigation of matrix effect, allowing to achieve good recovery values. Several parameters such as linearity, detection limits, repeatability and reproducibility were calculated to assess the method performances. The linear response of target compounds usually ranged from 0.5 ng/L to 200 ng/L with $R^2$ values always above 0.99. It must be remarked once more that BHT was spiked at a concentration one order of magnitude higher to allow its detection. Instrumental detection limits (IDL) ranged from 0.01 to 8.9 ng/L and were calculated from

signal to noise ratios (S/N × 3) of low concentration calibration standards in ultrapure water (Table 2).
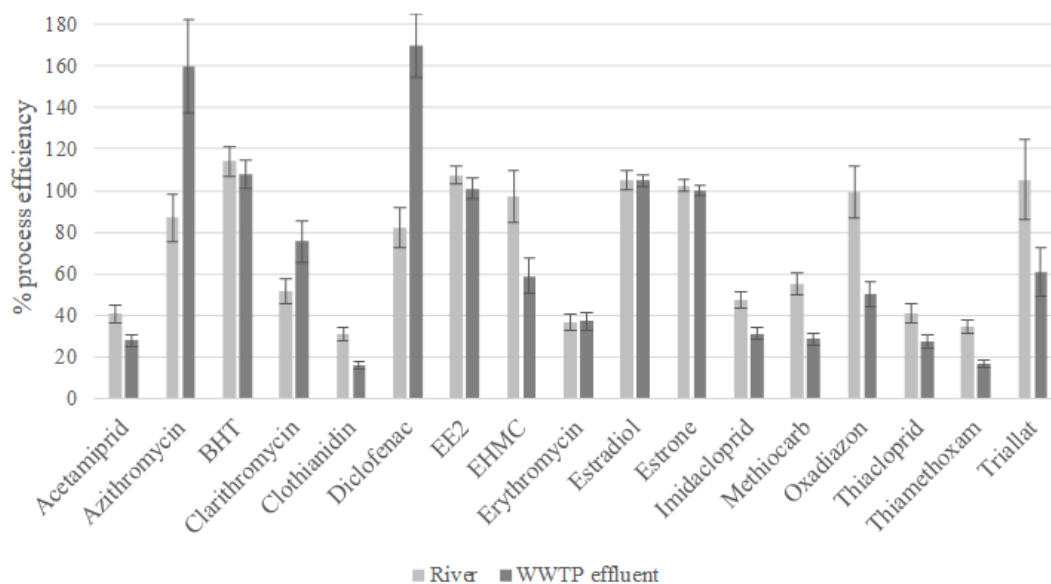


**Figure 3.** Process efficiency in river water and WWTP effluent, calculated at a spiked concentration of 100 ng/L.

**Table 2.** Analytical method validation parameters for target compounds in LC-MS/MS grade water: retention times (RTs), instrumental detection limits (IDLs), repeatability, reproducibility and recovery. Repeatability, reproducibility and recovery were calculated for a spiked concentration of 100 ng/L (1 µg/L in the case of BHT).

| Compound | RT (min) | IDL (ng/L) | Repeatibility %RSD (n=7) | Reproducibility %RDS (n=3) | % Recovery (n=3) | | %RSD |
|---|---|---|---|---|---|---|---|
| Acetamiprid | 3.06 | 0.05 | 1.5 | 1.8 | 99.1 | ± | 3.5 |
| Azithromycin | 2.23 | 0.07 | 3.7 | 5.3 | 101 | ± | 2.5 |
| BHT | 7.05 | 8.88 | 2.6 | 2.8 | 98.2 | ± | 0.4 |
| Clarithromycin | 3.59 | 0.01 | 1.1 | 2.3 | 100 | ± | 3.2 |
| Clothianidin | 2.44 | 0.02 | 1.2 | 1.7 | 100 | ± | 2.2 |
| Diclofenac | 4.38 | 0.12 | 3.6 | 4.1 | 97.1 | ± | 1.1 |
| EE2 | 5.39 | 0.18 | 6.0 | 6.9 | 101 | ± | 0.9 |
| EHMC | 5.43 | 0.16 | 2.8 | 6.4 | 92.8 | ± | 4.0 |
| Erythromycin | 3.08 | 0.01 | 0.9 | 1.6 | 97.6 | ± | 1.2 |
| Estradiol | 5.27 | 0.08 | 3.4 | 3.4 | 104 | ± | 1.5 |
| Estrone | 5.46 | 0.03 | 3.4 | 3.9 | 98.0 | ± | 0.4 |
| Imidacloprid | 2.89 | 0.10 | 1.4 | 1.8 | 99.9 | ± | 0.9 |
| Methiocarb | 4.00 | 0.07 | 1.3 | 1.4 | 99.6 | ± | 4.0 |
| Oxadiazon | 5.03 | 0.01 | 1.0 | 2.7 | 99.8 | ± | 1.2 |
| Thiacloprid | 3.33 | 0.01 | 1.7 | 3.6 | 102 | ± | 3.5 |
| Thiamethoxam | 2.59 | 0.08 | 1.8 | 2.8 | 98.2 | ± | 4.8 |
| Triallat | 5.13 | 0.02 | 4.1 | 4.9 | 99.0 | ± | 4.8 |

Method limits of detection (LODs) and limits of quantification (LOQs) were determined as the minimum detectable amount of analyte with a S/N of 3 and 10, respectively, in the spiked matrices. These ranged from 0.05 ng/L to 9.7 ng/L in freshwater (Table 3) and they increased when working with more complex matrices (Table 4). The LODs obtained in freshwater comply with the maximum LOD indicated in the EU Decision 2015/495, and in most cases they are much lower than the indicated values. The only compound which does not fulfill the requirements is EE2, which yielded a LOD of 0.34 ng/L in river water instead of the 0.035 ng/L indicated as maximum [8]. Such a low LOD can be reached under specific conditions favouring ionization and detection of this compound, such as pH 11, as described in M. Celic et al.(2017) [42]. The above-mentioned method is specific for hormones and the operational conditions described herein could not be adopted in this case because they were not compatible with the detection of the other compounds of the Watch list, that, as noted before, exhibit considerably different properties. Another possibility to lower the LOD of EE2 is the preconcentration of larger sample volumes using an offline procedure. Yet, the method LOD presented in this method are similar or lower than those already reported in literature for the investigated compounds [9, 16, 17, 37, 43, 48].

Repeatability in ultrapure water was determined from seven injections of a 100 ng/L standard mixed solution during the same day and reproducibility in three consecutive days. RSD for both repeatability and reproducibility was below 7% for all analytes (Table 2). Repeatability and reproducibility in freshwater are shown in Table 3. The obtained values fell below 20% at low level (5 ng/L of spiked concentration) and below 14% at high level (100 ng/L of spiked concentration), being less than 10% in most cases.

The investigated matrices, as well as LC-MS/MS grade water, were spiked at 100 ng/L and the concentrations obtained by internal standards was compared to the initial spiked concentration; method recovery and precision were therefore calculated. Results are displayed in Table 2, Table 3 and Table 4. The obtained recovery values were excellent, ranging between 95 and 110% for all matrices. Method precision, calculated as relative standard deviation (RSD%) was also adequate, with most values below 10% and the maximum around 15%.

**Table 3.** Analytical method performance parameters in freshwater: method detection and quantification limits (LOD and LOQ), repeatability, reproducibility (calculated at low and high spiked concentrations) and recovery (calculated for a spiked concentration of 100 ng/L). When indicated with N.A., the assessment of repeatability and reproducibility was not possible since the point fell out of the calibration curve.

| Compound | LOD (ng/L) | LOQ (ng/L) | Repeatibility (%RSD) | | Reproducibility (%RDS) | | % Recovery (n=3) | %RSD | |
|---|---|---|---|---|---|---|---|---|---|
| | | | (5 ng/L) | (100 ng/L) | (5 ng/L) | (100 ng/L) | | | |
| Acetamiprid | 0.35 | 1.2 | 7.3 | 5.1 | 7.4 | 5.3 | 99.7 | ± | 1.2 |
| Azithromycin | 0.24 | 0.80 | 4.1 | 3.9 | 7.2 | 13 | 102 | ± | 2.6 |
| BHT | 9.7 | 32 | 7.0 | 4.4 | N.A. | 5.0 | 101 | ± | 7.8 |
| Clarithromycin | 0.060 | 0.20 | 8.0 | 3.2 | 15 | 3.6 | 99.2 | ± | 2.9 |
| Clothianidin | 0.16 | 0.52 | 17 | 5.7 | 17 | 6.0 | 100 | ± | 4.3 |
| Diclofenac | 0.33 | 1.6 | N.A. | 11 | N.A. | 11 | 103 | ± | 6.6 |
| EE2 | 0.48 | 1.6 | 8.2 | 6.1 | 8.6 | 6.1 | 92.1 | ± | 2.7 |
| EHMC | 0.16 | 0.54 | 7.3 | 7.3 | 8.0 | 7.8 | 102 | ± | 15 |
| Erythromycin | 0.18 | 0.59 | 5.7 | 2.3 | 7.3 | 2.5 | 100 | ± | 1.6 |
| Estradiol | 0.15 | 0.51 | 7.6 | 3.8 | 11 | 4.2 | 96.5 | ± | 5.4 |
| Estrone | 0.10 | 0.32 | 4.5 | 3.5 | 4.7 | 4.1 | 98.5 | ± | 0.0 |
| Imidacloprid | 0.17 | 0.58 | 2.7 | 2.3 | 18 | 3.7 | 101 | ± | 2.8 |
| Methiocarb | 0.23 | 0.77 | 3.6 | 2.7 | 8.2 | 2.9 | 99.4 | ± | 2.7 |
| Oxadiazon | 0.050 | 0.17 | 8.1 | 1.7 | 8.3 | 6.1 | 99.8 | ± | 0.2 |
| Thiacloprid | 0.064 | 0.21 | 6.8 | 4.6 | 7.2 | 4.8 | 101 | ± | 1.9 |
| Thiamethoxam | 0.18 | 0.59 | 8.2 | 2.7 | 8.8 | 2.9 | 99.9 | ± | 1.5 |
| Triallat | 0.12 | 0.39 | 4.8 | 4.5 | 5.3 | 5.0 | 101 | ± | 1.5 |

**Table 4.** Analytical method performance parameters in wastewater effluent and influent: method detection and quantification limits (LOD and LOQ) and recovery (calculated for a spiked concentration of 100 ng/L). The numbers marked with an asterisk (*) indicate the results obtained in a diluted matrix due to an excessive background concentration. When indicated with N.A., the calculation of the validation parameters was not possible due to the low S/N ratios obtained in WWTP influent.

| Compound | LODs (ng/L) WWE | WWI | LOQs (ng/L) WWE | WWI | % Recovery (%RSD) (n=3) WWE | | WWI | |
|---|---|---|---|---|---|---|---|---|
| Acetamiprid | 0.59 | 0.53 | 2.0 | 1.8 | 100 | ± 5.1 | 98.1 | ± 2.0 |
| Azithromycin | 0.13* | 0.15* | 0.42* | 0.49* | 102 | ± 5.4 | 99.8 | ± 5.6 |
| BHT | 12 | 12 | 41 | 41 | 100 | ± 0.6 | 104 | ± 2.6 |
| Clarithromycin | 0.012 | 0.060* | 0.041 | 0.20* | 90.3 | ± 5.7 | 102 | ± 3.1 |
| Clothianidin | 0.77 | 2.0 | 2.6 | 6.5 | 99.3 | ± 2.5 | 101 | ± 5.4 |
| Diclofenac | 0.58* | 0.56* | 1.9* | 1.9* | 110* | ± 2.6 | 99.7* | ± 5.5 |
| EE2 | 2.3 | N.A. | 7.6 | N.A. | 98.8 | ± 2.8 | N.A. | |
| EHMC | 0.34 | 1.0 | 1.1 | 3.3 | 101 | ± 2.8 | 99.1 | ± 5.0 |
| Erythromycin | 0.10 | 0.29 | 0.33 | 0.98 | 94.5 | ± 2.1 | 98.6 | ± 7.0 |
| Estradiol | 0.46 | N.A. | 1.5 | N.A. | 99.2 | ± 3.3 | N.A. | |
| Estrone | 0.15 | 0.60 | 0.48 | 2.0 | 103 | ± 1.4 | 100 | ± 9.7 |
| Imidacloprid | 0.069 | 0.12 | 0.23 | 0.39 | 101 | ± 8.8 | 99.2 | ± 0.8 |
| Methiocarb | 0.55 | 0.51 | 1.8 | 1.7 | 99.8 | ± 3.8 | 98.8 | ± 1.0 |
| Oxadiazon | 0.17 | 0.079 | 0.58 | 0.26 | 101 | ± 4.0 | 97.3 | ± 4.0 |
| Thiacloprid | 0.18 | 0.23 | 0.61 | 0.75 | 102 | ± 3.7 | 102 | ± 0.6 |
| Thiamethoxam | 0.70 | 0.42 | 2.3 | 1.4 | 100 | ± 5.1 | 96.6 | ± 13 |
| Triallat | 0.28 | 0.34 | 0.93 | 1.1 | 99.6 | ± 2.0 | 99.8 | ± 4.2 |

### 3.5. Application to environmental samples

The developed method was applied to evaluate the concentration of the Watchlist compounds in three matrices: surface waters, WWTP effluent and WWTP influent. The obtained results are summarized in Table 5. The concentrations of the Watch list compounds in freshwater matrix were in all cases below 10 ng/L. Only the three antibiotics (azithromycin, clarithromycin, erythromycin) and diclofenac were detected in all the three matrices. BHT, EE2, Estradiol, Methiocarb and Triallate were not detected in any of the investigated matrices.

**Table 5.** Watch list compounds concentrations and standard deviation, expressed in ng/L, detected in river water, WWTP effluent and WWTP influent.

| Compound | Concentration (ng/L) | | |
|---|---|---|---|
| | River | Effluent | Influent |
| Acetamiprid | < LOQ | 2.2 ± 0.2 | < LOD |
| Azithromycin | 1.6 ± 0.3 | 109 ± 14 | 190 ± 3.5 |
| BHT | < LOD | < LOD | < LOQ |
| Clarithromycin | 1.5 ± 0.0 | 291 ± 12 | 162 ± 12 |
| Clothianidin | 1.2 ± 0.3 | < LOD | 8.3 ± 0.4 |
| Diclofenac | 7.9 ± 0.8 | 461 ± 7 | 1310 ± 30 |
| EE2 | < LOD | < LOD | < LOD |
| EHMC | 4.7 ± 0.4 | < LOD | 8.4 ± 2.7 |
| Erythromycin | 1.0 ± 0.0 | 47.6 ± 0.2 | 16.9 ± 0.1 |
| Estradiol | < LOQ | < LOD | < LOD |
| Estrone | < LOD | < LOD | 12.7 ± 1.0 |
| Imidacloprid | < LOQ | 11.9 ± 0.7 | 8.6 ± 0.0 |
| Methiocarb | < LOD | < LOQ | < LOD |
| Oxadiazon | 0.9 ± 0.1 | < LOD | < LOD |
| Thiacloprid | < LOQ | 1.7 ± 0.1 | < LOD |
| Thiamethoxam | < LOD | 17.6 ± 0.2 | 6.4 ± 1.6 |
| Triallat | < LOD | < LOD | < LOD |

Nine of the target compounds were found in WWTP influent, eight of them in WWTP effluent and only seven were identified in river water. Diclofenac was the compound with the highest registered concentration in all the three matrices, reaching μg/L levels in WWTP influent, in consistency with literature [49]. Its concentration in WWTP effluent was 461 ng/L, indicating a removal rate around 65%. Dilution played an important role in freshwater, since diclofenac concentration in the Ebro river fell in the low ng/L range. The antibiotics azithromycin and clarithromycin were also present at hundreds ng/L in wastewater. The results are in the same concentration range as described in other studies

[49]. Erythromycin was found at lower concentration levels, probably due to the conversion to erythromycin-H2O in water [50, 51]. Moreover, its concentration in WWTP effluent is higher than in the influent. A negative removal rate of such compound is also reported in literature [49].

## 4. Conclusions

The online SPE-UHPLC-MS/MS methodology described herein represents a new, fast and efficient procedure for the preconcentration and determination of the 17 target compounds listed in the Commission Implementing Decision (EU) 2015/495. The obtained results show high sensitivity, with limits of detection in the sub- and low-ng/L ranges, complying with the EU requirements in all cases with the exception of EE2, for which analytical issues are still open. The method allows the simultaneous analysis of compounds pertaining to different classes with dissimilar structures and properties, some of which have traditionally been analysed with other techniques (i.e. BHT). Pretreatment operations are minimized thanks to the employment of online-SPE, being filtration the only step prior to analysis, and analysis is carried out in 8 and 10.5 minutes in PI and NI ESI, respectively. The method was successfully applied for the analysis of samples collected in the Ebro river delta region (Catalonia, Spain), including WWTPs and the receiving environment.

## Acknowledgements

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**References**

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2. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/4

3. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC

4. Directive 2010/75/EU of the European Parliament and of the Council of 24 November 2010 on industrial emissions (integrated pollution prevention and control).

5. Commission Directive 98/15/EC of 27 February 1998 amending Council Directive 91/271/EEC with respect to certain requirements established in Annex I thereof

6. Directive 2008/105/EC of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/ECC, 86/280/ECC and amending Directive 2000/60/EC

7. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy

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CHAPTER 2

# The EU Watch list compounds in the Ebro delta region: assessment of sources, river transport, and seasonal variations

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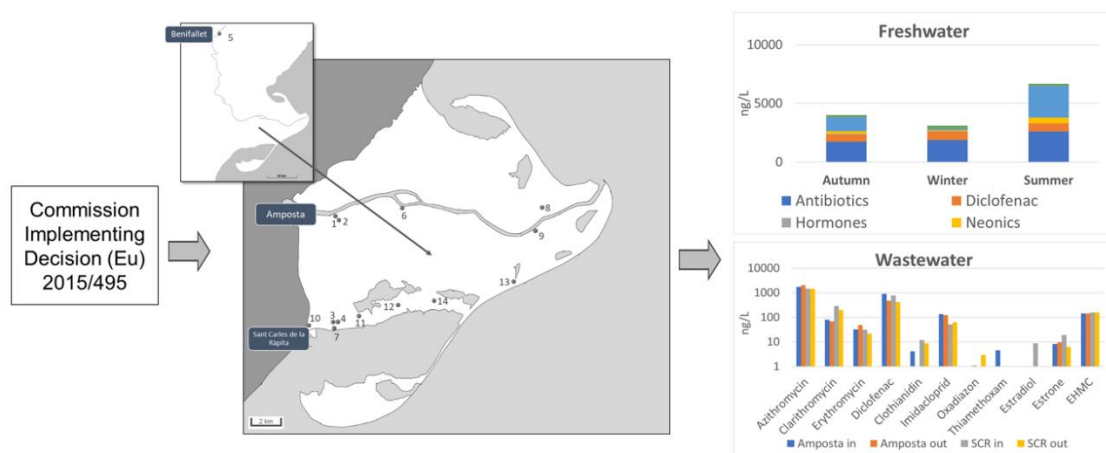
# The EU Watch list compounds in the Ebro delta region: assessment of sources, river transport, and seasonal variations

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**Abstract**

The presence of xenobiotics in the aquatic environment has drawn scientific concern due to possible detrimental effects on the ecosystems. With EU Decision 2015/495, a first Watch list of compounds that could potentially represent a threat for the environment was created, with the objective of gathering high quality monitoring data and support their prioritization. Literature data are still very scarce and the presence of many of the compounds has not been investigated thoroughly. In this study, all the 17 compounds of the EU Watch list 2015/495 were monitored in 14 sampling locations, comprised of freshwater and, for the first time, wastewater. The study was carried out in the Ebro delta, in the north east of Spain, a representative and crucial area not only for its environmental and naturalistic significance, but also for Spain's productivity, especially as regards rice agriculture. Results show that contamination originates both from wastewater treatment plants (WWTPs) and agricultural activities. High levels of pharmaceuticals were detected in wastewater, with azithromycin and diclofenac present at mean concentrations of 1.65 µg/L and 636 ng/L respectively. In freshwater samples, besides antibiotics and diclofenac, substantial contamination by pesticides was reported, with oxadiazon reaching up to 591 ng/L and imidacloprid being present in 93% of samples. Moreover, the study provided insight into the origin of the selected contaminants. The removal of the studied micropollutants in WWTPs was low to moderate. The assessment of risk quotients, calculated based on the available PNECs,

demonstrated that the concentrations recorded for these compounds may pose a significant risk in most sampling sites.

**Capsule**

All the 17 compounds of the Watch list 2015/495 were spatially and temporally monitored in freshwater and wastewater in the Ebro delta area, in Spain.

**Keywords**

EU Watch list; wastewater; freshwater; UHPLC-MS/MS; spatiotemporal monitoring

## 1. Introduction

Since the adoption of the Water Framework Directive, the EU has taken measures to tackle pollution of freshwater ecosystems (Directive 2000/60/EC) through a system of structured prioritization. In March 2015, a Watch list of contaminants of emerging concern (CECs) was published (Decision (EU) 2015/495) (Table 1). The compounds listed in it have to be monitored Europe-wide in order to gather data on their occurrence. Risk assessment will be carried out on the collected data and then the Watch list compounds might be included in the priority pollutants list, adopting the consequent environmental quality standards (Directive 2013/39/EU). The compounds listed in Decision 2015/495 are now undergoing monitoring for the fourth consecutive year. In June 2018, the second updated version of the EU Watch list made its appearance (Decision 2018/840). Oxadiazon, Triallate, BHT, EHMC and diclofenac were removed from the list, while the two antibiotics amoxicillin and ciprofloxacin and the pesticide metaflumizone were added. However, since most compounds of Decision 2015/495 are still characterised by insufficient available monitoring data to carry out appropriate risk assessment, they have been included in the Decision 2018/840 as well.

The compounds listed in Decision 2015/495 include: three hormones (estrone, E1; β-estradiol, E2 and 17α-ethynylestradiol, EE2), three macrolide antibiotics (azithromycin, clarithromycin and erythromycin), the anti-inflammatory diclofenac, five pesticides from the neonicotinoid family (acetamiprid, thiacloprid, clothianidin, imidacloprid and thiamethoxam), a carbamate pesticide (methiocarb), two herbicides (oxadiazon and triallate), an antioxidant (butylated hydroxytoluene, BHT) and a UV filter (2-ethylhexyl 4-methoxycinnamate, EHMC). Detailed information on the investigated compounds,

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including their ecotoxicity, can be found in the section "The compounds of emerging concern included in Decision 2015/495" in Supplementary Material.

The Ebro delta, located in north east of Spain, was chosen as study area. The last stretch of the Ebro river consists of an ecosystem of crucial importance for both the environment and the Spanish economy. Known as the third largest wetland area in the western Mediterranean region, the delta has a surface area of 320 km$^2$. It is a natural park since 1984 and 7736 ha of it were included in the Ramsar Convention list in 1993. Moreover, the Fangar and Àlfacs bays have been recently included in the Catalan list of sensitive areas (Resolución TES/757/2019).

The Ebro Delta area is comprised of the last tract of the Ebro river, as well as drainage and irrigation channels, beaches, natural lagoons, salt pans, bays and marshes that are home to several wildlife species (Čelić et al., 2019), including a great variety of permanent and migratory birds that attract thousands of tourists per year (Prado et al., 2019). Nevertheless, it is also a highly modified human area, since 65% of the salt marsh-estuarine ecosystems have been turned into rice farming in the last 150 years (Lloret et al., 2004; Prado et al., 2019). The area is lush with paddies, which account for 22 thousand hectares, and produces 45 million kilos of rice per year of 14 different varieties. What makes it a favourable area for biological productivity is the fact that, unlike most Mediterranean rivers, the Ebro estuarine area collects the runoffs from snow-covered mountains, the Pyrenees, wastewater from large urban areas, as well as agriculture and industries, resulting in high nutrients concentrations (Lloret et al., 2004).

**Table 1.** Compounds listed in Decision 2015/495, CAS numbers, class and maximum acceptable method detection limits in freshwater as indicated in the EU document.

| Name of substance | CAS number | Class | Maximum acceptable MDL in freshwater (ng/l) |
|---|---|---|---|
| Azithromycin | 83905-01-5 | Macrolide antibiotic | 90 |
| Clarithromycin | 81103-11-9 | Macrolide antibiotic | 90 |
| Erythromycin | 114-07-8 | Macrolide antibiotic | 90 |
| Diclofenac | 15307-86-5 | Antinflammatory | 10 |
| Estrone (E1) | 53-16-7 | Hormone | 0.4 |
| 17-Beta-estradiol (E2) | 50-28-2 | Hormone | 0.4 |
| 17-Alpha-ethinylestradiol (EE2) | 57-63-6 | Hormone | 0.035 |
| Acetamiprid | 135410-20-7/ 160430-64-8 | Neonicotinoid pesticide | 9 |
| Clothianidin | 210880-92-5 | Neonicotinoid pesticide | 9 |
| Imidacloprid | 105827-78-9/ 138261-41-3 | Neonicotinoid pesticide | 9 |
| Thiacloprid | 111988-49-9 | Neonicotinoid pesticide | 9 |
| Thiamethoxam | 153719-23-4 | Neonicotinoid pesticide | 9 |
| Methiocarb | 2032-65-7 | Pesticide | 10 |
| Oxadiazon | 19666-30-9 | Herbicide | 88 |
| Triallate | 2303-17-5 | Herbicide | 670 |
| 2,6-Ditert-butyl-4-methylphenol (BHT) | 128-37-0 | Antioxidant | 3160 |
| 2-Ethylhexyl 4-methoxycinnamate (EHMC) | 5466-77-3 | UV-filter | 6000 |

The main sources of contamination in the Ebro river basin are represented by WWTPs and – in the case of pesticides – agriculture (Claver et al., 2006; Gros et al., 2007). To our knowledge, different studies have focussed on the Ebro delta, especially on the occurrence of pesticides (Hildebrandt et al., 2007; Kuster et al., 2008; Feo et al., 2010; Navarro et al., 2010; Sánchez-Avila et al., 2012), but also pharmaceutically active compounds (Čelić et al., 2019; Gros et al., 2007). Nevertheless, the Watch list compounds have not been investigated yet. The objective of this study was to monitor for the first time the occurrence of all the compounds included in Decision 2015/495 in the Ebro delta, assessing river transport, the impact of two of the most important WWTPs and possible seasonal variations. Insights into the origins and extent of contamination might be helpful for policy makers to define the most adequate mitigation strategies.

## 2. Material and methods

### 2.1. Chemicals and reagents

Clarithromycin, erythromycin, azithromycin, EHMC, imidacloprid, methiocarb, thiacloprid, thiamethoxam, clothianidin, acetamiprid, oxadiazon, triallate, E1, E2, EE2, BHT, diclofenac (sodium salt) were purchased from Sigma Aldrich (St. Louis, MO, USA). The isotopically labelled compounds, used as internal standards (IS), erythromycin-(*N,N*-

*dimethyl*-$^{13}C_2$), methiocarb-(*N-methyl*-d$_3$), thiamethoxam-d3, imidacloprid-d4, oxybenzone-(Phenyl-d5), clothianidin-d3, BHT-d21, β-estradiol-d$_2$ (E2-d$_2$), estrone-2,4,16,16-d$_4$ (E1-d$_4$) were provided by Sigma Aldrich (St. Louis, MO, USA); ethynylestradiol-d4 (EE2-d$_4$), diclofenac-d4 and azithromycin-d3 were supplied by TRC (Toronto, Canada). Individual stock solutions were prepared on a weight basis at a concentration of 1 mg/mL in methanol (MeOH) and stored at -20 ˚C in the dark, to preserve the compounds from possible photodegradation (Capriotti et al., 2014). Intermediate mixed solutions containing all analytes and all the internal standards respectively were prepared in MeOH monthly and then appropriately diluted in water. The concentration of BHT in the aqueous mixed solutions was 10-fold the other compounds, while the concentration of BHT-d21 in the IS mix was 20-fold the other deuterated standards in order to enhance its detectability (500 ng/L and 1 µg/L in the case of BHT and BHT-d21, respectively).

Durapore Hydrophilic PVDF filters (47 mm, 0.45 µm) were provided by Merck Millipore (Bedford, MA, USA). Extra pure formic acid was purchased from Scharlau (Barcelona, Spain) and ammonium fluoride for LC-MS was purchased from Sigma Aldrich (St. Louis, MO, USA). LC/MS grade solvents (water, methanol and acetonitrile) were acquired from Fisher (Optima™ LC/MS).

## 2.2. Area of study

The study was carried out in the Ebro delta area, focussing on the last kilometres of the Ebro River and its tributary channels, in the province of Tarragona, Catalonia (NE Spain) (Figure 1). As mentioned in the introduction, most of the land is devoted to agricultural activities, especially rice farming, nevertheless the Ebro Delta Natural Park still integrates around 30% of the remaining wetland surface (Prado et al., 2019). Moreover, the area is home to thirteen aquaculture facilities, producing especially mussels and oysters, located in the proximities of the Àlfacs Bay. This makes the Ebro delta a crucial spot for Spanish aquaculture, accounting for 20% of the Spanish continental production (Čelić et al., 2019). As for industrial pollution, in the area there are chemical industries and a nuclear power plant (Čelić et al., 2019). There are several WWTPs, located in the proximities of the main towns, such as Sant Carles de la Ràpita, Amposta, l'Ampolla, Deltebre, l'Aldea, Sant Jaume d'Enveja, Camarles and els Muntells. Among these, the ones serving the

highest population equivalents (PE) are those in Amposta and Sant Carles de la Ràpita and can therefore been considered as the main point-sources of contamination (Čelić et al., 2019). WWTP Amposta is designed for 27,500 PE and is equipped with primary and secondary treatment with conventional activated sludge (Consorci de Polítiques Ambientals de les Terres de l'Ebre); WWTP Sant Carles de la Ràpita, besides primary and secondary treatment, includes sand filters as tertiary treatment and is designed for 28,921 PE (Consorci de Polítiques Ambientals de les Terres de l'Ebre).
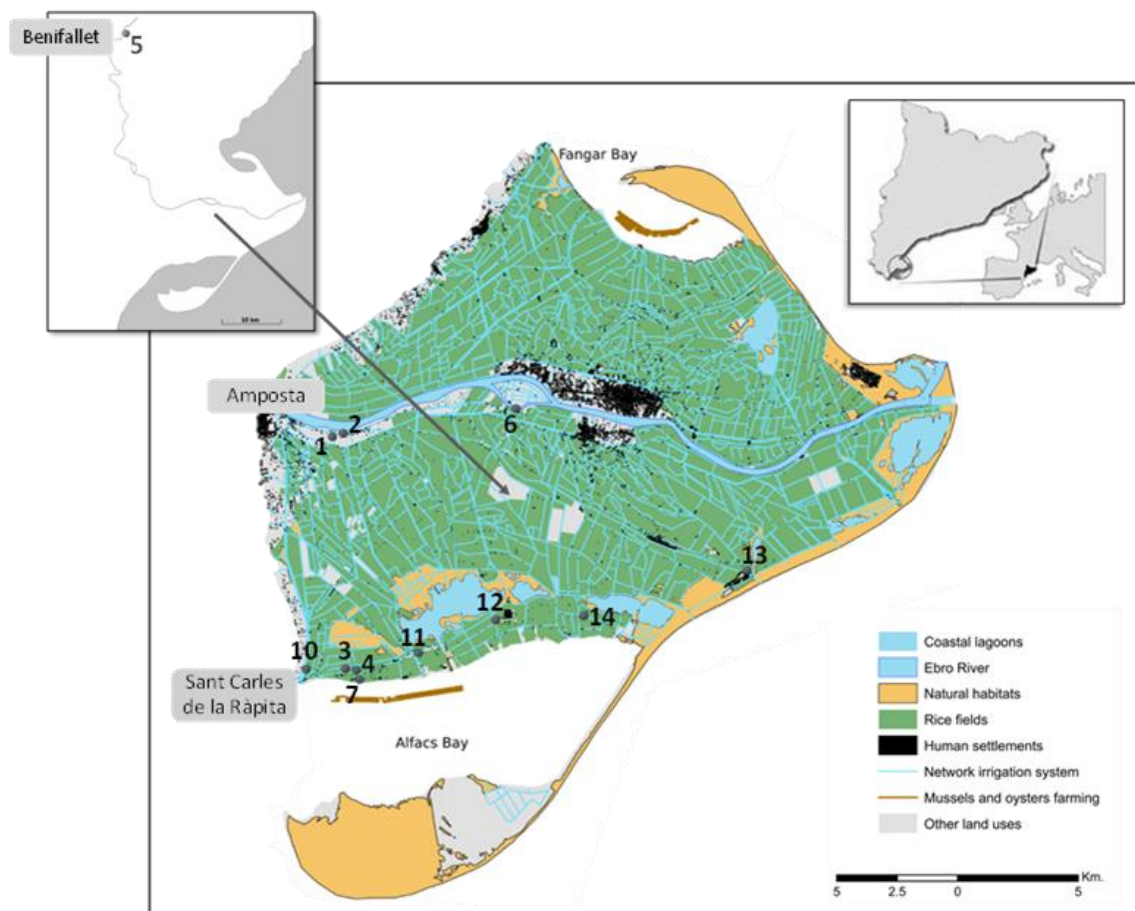


**Figure 1.** Map of the area of study, adapted from Prado et al. 2019. The main square shows the area where most samples were collected. Each sampling sites is marked by a point and its corresponding number. The square at the right top corner shows the position of Catalonia in Europe and, enclosed in the circle, the Ebro delta region. The square to the left shows a zoomed-out view where the upstream control point, next to the municipality of Benifallet, is visible.

The climate of the Ebro Delta is typically Mediterranean, with temperatures in the range of 14-22°C. Rainfall is concentrated between autumn and spring (200-300 mm) and summer is characterized by drought (<50 mm) (Pignotti et al., 2017).

The area is crossed by a network of channel and irrigation ditches. Among them, the main, and oldest, ones are those to the left and right side of the Ebro, dug between the second half of the XIX century and the early decades of the XX century. Their waters come from the Ebro river, both extracted from the Xerta dam. The left channel is 35 km long and was dug for irrigation purposes, while the right channel was originally built for navigation purposes, including a 10-km branch that connects the municipalities of Amposta and Sant Carles de la Ràpita, called "Maritime channel". The right channel is 52 km long, it has a higher flow regime compared to the left channel and irrigates a larger surface of land (Confederación Hidrográfica del Ebro). Drainage channels that flow into pumping stations are also worth mentioning, including Sèquia Gran, since they assure that the water flows across the network and is eventually discharged into the sea.

## 2.3. Sample collection and preparation

Three sampling campaigns were carried out in order to consider possible seasonal variations. These took place in October-November 2015 (SC1), February 2016 (SC2) and June-July 2016 (SC3). The sampling area is shown in Figure 1 and information on the sampling sites are displayed in Table S1. Two samples were collected from the Ebro river: a site located in Benifallet, upstream the town of Amposta, which was considered as a control site and one located downstream the discharge from WWTP Amposta (sampling sites 5 and 6 respectively). The other freshwater sampling sites were seven drainage and irrigation channels. Sampling sites marked as 8 and 9 were taken from the so called left and right channels, respectively. Wastewater samples consisted of influent and effluent wastewaters from WWTPs Amposta and Sant Carles de la Ràpita. The emissary of WWTP Sant Carles de la Ràpita, which is a wastewater effluent-dominated stream discharging into the Àlfacs Bay was also sampled. Wastewater samples were 24-hour time-integrated samples, whereas the others were grab samples. The sampling volume for each point was approximately 100 mL.

Samples were collected in PET bottles previously rinsed with ultrapure water, transported in refrigerated containers and frozen at -20 °C upon arrival at the research facilities. Physicochemical parameters of samples, such as temperature, pH, oxygen, conductivity, salinity and flow, were measured in situ by using hand-held probes (WTW, Weilheim, Germany) and are displayed in Table S2.

Prior to analysis, the samples were filtered with Durapore PVDF filters (0.45 μm), then transferred into 10 mL amber vials (Supelco) and spiked with a mixture of internal standards in order to obtain an IS concentration of 50 ng/L (1 μg/L in the case of BHT-d21).

## 2.4. Instrumental analysis

The Watch list compounds were analysed by means of online solid-phase extraction (SPE) ultra-high-performance liquid chromatography coupled to a triple-quadrupole mass spectrometer (UHPLC-MS/MS) following the method described by Gusmaroli et al., 2018. Briefly, the samples were loaded onto a Hypersil GOLD aQ (20 x 2.1 mm, 12 μm particle size, Thermo Fisher Scientific) column at a flow rate of 1750 μL/min for preconcentration and then the analytes were eluted from the chromatographic column at flow rate of 400 μL/min. Twelve compounds were analysed under positive ionization mode by means of a Kinetex Biphenyl (1.7 μm particle size, 100 x 2.1 mm i.d., Phenomenex). The mobile phase consisted of MeOH (A) and water with 0.1% formic acid (B) and each chromatographic run lasted 8 minutes. The gradient started with 60% A, which was kept during the first 1.75 min, then it was brought to 75% A in 0.13 min approximately and finally increased to 100% A in 2.12 min. Isocratic conditions were held during 1.75 min after which the system was brought to initial gradient conditions. The hormones, diclofenac and BHT were analysed under negative ionization (NI) mode. As organic mobile phase, a mixture of MeOH (A) and acetonitrile (ACN) (B) was used during the initial stages of the gradient, though the compounds eluted when the percentage of ACN reached almost 100%. The aqueous mobile phase consisted of LC/MS grade water with ammonium fluoride 1 mM (C). The gradient consisted of 15% A and 15% B and was held during the first 2 min. One hundred percent B was reached in 4 min. Isocratic conditions were held for 1.50 min. The initial conditions were restored in 1 min and then column equilibration was allowed. The time of analysis in NI mode lasted 10.5 minutes.

Detection was performed by means of a TSQ Vantage mass spectrometer (Thermo Fisher Scientific), equipped with an electrospray turbo spray ionization source. Two Selected Reaction Monitoring (SRM) transitions were recorded for each compound (see Table S3). Moreover, time specific SRM windows were set for each retention time in order to enhance sensitivity. The obtained detection limits in freshwater were compliant with

those detailed in Decision 2015/495, with the sole exception of EE2 and can be consulted in Table S4.

## 2.5. Quality assurance and quality control

Quality parameters of the analytical method for each matrix included: recoveries and method precision, limits of detection (MDLs) and quantification (MQLs). Results are displayed in Table S4 in SM. Quantification, based on peak areas, was performed by means of internal standard calibration at 9 calibration levels, covering the range from 0.5 to 500 ppt. Calibration curves were injected at the beginning and at the end of each sequence. Moreover, to keep track of possible contamination and sensitivity drifts, HPLC-grade water spiked with a mixture of the analytes at known concentrations as well instrumental blanks of MeOH: $H_2O$ (50:50) were run every 10 samples. $R^2$ values were above 0.99 for all investigated compounds. Method and instrumental blanks were measured to check for any background levels. Recoveries were determined in triplicate by spiking a portion of the matrix with a known concentration of the analytes (100 ng/L) and comparing the obtained concentration with the amount of analytes actually present in it. To be noted that operating with an online preconcentration system makes it impossible to distinguish the effects of the matrix from the efficiency of extraction. However, the employment of internal standards and matrix match calibration permits a mitigation of matrix effect, allowing to achieve good recovery values. For each compound its corresponding isotopically labelled analogue was used, except for those substances whose corresponding deuterated compound was not available. In this case, the most similar labelled substance, in terms of chemical structure and chromatographic retention time, was used as IS (see Table S3). To minimize uncertainty in the identification, the maximum difference allowed between the chromatographic retention time (RT) of the compound in the sample and in the calibration curve was ± 2% and the ratio between the two SRM transitions could not exceed a difference of ± 20% between the standard solutions and the actual samples, in accordance with the Council Directive 96/23/EC implementation of 2002 (European Commission, 2002).

## 2.6. Data analysis

Data analysis was performed with Excel (Microsoft Office) and SPSS (IBM). In all statistical analyses, not detected compounds (<MDL) and concentrations falling below the method

limits of quantification (<MQL) were replaced with MDL/2 and MQL/2 respectively. Since the data were not normally distributed, two nonparametric methods, the Mann-Whitney U and the Kruskal-Wallis H test, were employed to assess the statistical differences in the Watch list compounds concentrations between each sampling season and between sampling points. The significance level for all applied analysis was at level $p < 0.05$. All tests were performed at 95% confidence level.

## 3. Results and discussion

### 3.1. Occurrence in freshwater

The individual concentrations of the Watch list compounds detected in freshwater (Ebro river and channel samples), collected during the three sampling seasons, are displayed in Tables S5, S6 and S7. In the upstream point, located in Benifallet, the analytes were detected at non-negligible concentrations, up to 326 ng/L, with a maximum total concentration of 627 ng/L, registered in SC3. It is worth mentioning that, despite being located upstream WWTP Amposta, Benifallet is located in the final part of the Ebro: over a total of 930 km, it is only approximately 65 km far from the outlet into the Mediterranean Sea. Recent studies carried out in the same area have also reported the presence of pharmaceuticals (Čelić et al., 2019) and perfluoroalkyl substances (Pignotti et al., 2017), suggesting that the contamination in the river delta originates not only from the nearby sources of pollution, but can also be transported from upstream sites.

The most contaminated site in all three sampling campaigns was the emissary of Sant Carles de la Ràpita, which can be explained considering that it collects the outflows of WWTP Sant Carles de la Ràpita and discharges them into the Àlfacs bay, being therefore a wastewater-dominated stream. The highest contamination was detected in SC3, with a total concentration of 2.39 µg/L, antibiotics and diclofenac being the most abundant classes of pollutants. In particular, azithromycin and diclofenac were the most abundant compounds, accounting for 70% of pollution in that site.

The most frequently detected compounds in freshwater samples were oxadiazon and imidacloprid (93% of detection), followed by clarithromycin (see Table S9). Extremely limited reference is currently available in terms of monitoring studies of Watch list compounds. Sousa et al. (2019) found that the most frequent contaminants were

diclofenac, azithromycin and EHMC in the Ave and Sousa rivers in Portugal (Sousa et al., 2019).

Oxadiazon is known to cause adverse effects on non-target organisms (EFSA, 2010). Its presence is strictly related to rice agriculture, since it is one of the most common herbicides to control unwanted weed, used both as a pre-emergent or early post-emergent herbicide (Mesléard et al., 2016). Oxadiazon was detected in the current study at high concentrations (up to 591 ng/L in spring, in a canal sample). High oxadiazon concentrations and frequency of detection have as well been reported in a monitoring study carried out in rivers in Portugal, in basins where rice is one of the main crops (Silva et al., 2015). On the contrary, it was found only in one sample and below MQL in the monitoring carried out by Sousa et al., 2019.

Differently from oxadiazon, imidacloprid, despite being ubiquitous, exhibited lower concentrations, its mean values ranging from 7.66 to 40.5 ng/L in the three sampling campaigns. Imidacloprid was detected with less frequency but, in a few cases, at higher concentrations in a study carried out in La Rioja, a Spanish region renowned for its vineyards, located in an upper section of the Ebro river (Herrero-Hernández et al., 2013). It is worth noting that the MDL for imidacloprid in the aforementioned study was 19 ng/L, much higher than several observations registered in the current monitoring.

Clarithromycin was detected in 86% of freshwater samples, in the range of <MDL to 293 ng/L and a mean concentration of 38.8 ng/L. Its concentration in the Ebro river was remarkably higher than in the surrounding irrigation channels, suggesting that its presence is related to urban wastewaters and its incomplete removal in WWTPs (Verlicchi et al., 2012). Its widespread presence in the Ebro basin is also documented in López-Serna et al. (2012), where it was detected in 100% of samples, though at lower levels, the mean concentration in the Ebro river being 12.1 ng/L. Another study reports a maximum mean of 4.86 ng/L in the Ebro delta (Silva et al., 2011).

The Kruskal-Wallis H test was performed to assess if the target compounds concentrations in freshwater varied significantly among the sampling campaigns. Results show that the occurrence of E1, imidacloprid and oxadiazon was statistically different among seasons. In fact, for the three compounds, concentrations in winter were much

lower and they were detected with less frequency. This could be ascribed to dilution due to higher precipitations and, in turn, flows typically recorded in winter (Servei Meteorològic de Catalunya, 2016, 2015) and, in the case of the two pesticides, to the fact that fields are yet to be sown at that time of the year (Silva et al., 2015).

The occurrence of pesticides was previously monitored in the Ebro basin, including two sampling points located in the same area considered in this study (Ccanccapa et al., 2016). The study reports that methiocarb was hardly ever present, with detection frequencies ranging between 0 and 16% and its concentrations ranged between sub to low ng/L, in agreement with the data obtained in this monitoring. On the contrary, the detection frequencies and concentrations reported for imidacloprid were significantly lower, with means below 2 ng/L (Ccanccapa et al., 2016).
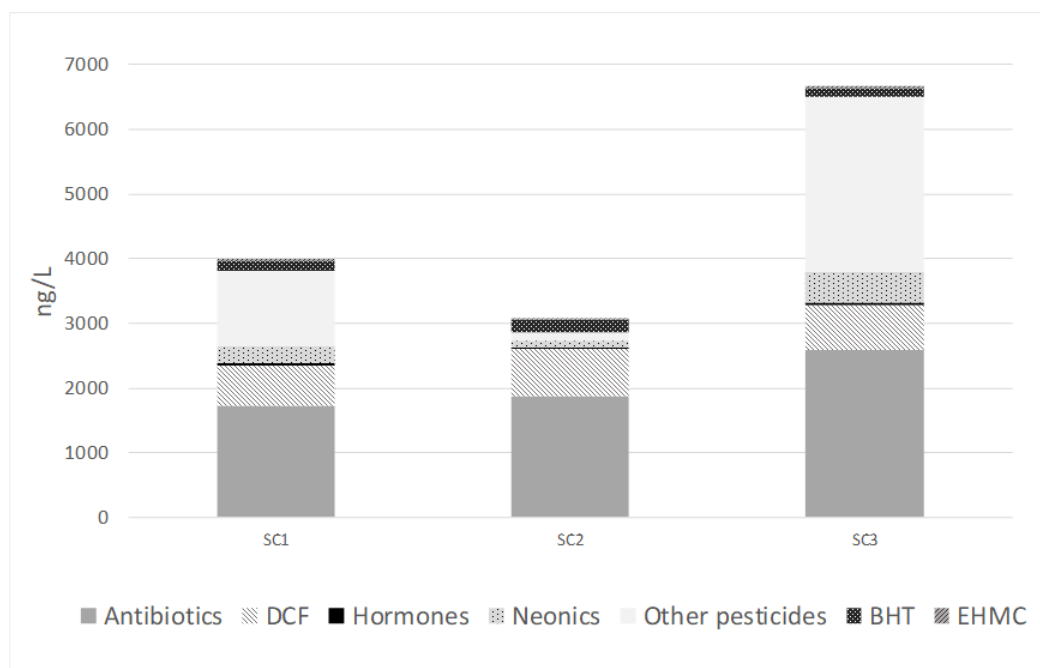


**Figure 2.** Total concentrations by groups in freshwater samples for each sampling campaign. SC1: October 2015; SC2: February 2016; SC3: May-June 2016.

Azithromycin was the compound with the highest mean concentration in freshwater samples (171 ng/L), followed by oxadiazon (138 ng/L) and diclofenac (70.9 ng/L). These results partially differ from the Watch list monitoring in Portugal, where EHMC, imidacloprid and diclofenac exhibited the highest concentrations (Sousa et al., 2019). In general, pharmaceuticals (antibiotics and diclofenac) were the most abundant groups in

river water, while pesticides accounted for most contamination of channels. This pattern is clearly visible in Figure 3.

The hormones was the group of pollutants that exhibited the lowest concentrations in freshwater, the maximum being 6.90 ng/L for E1, consistently with concentration levels reported in literature (Gorga et al., 2014).

Kruskal-Wallis non-parametric H test was also performed to assess seasonal variations between campaigns in channel samples. Results showed that differences in concentrations between seasons were significant for imidacloprid, clothianidin, oxadiazon and E1. In fact, their concentration in SC2 was substantially lower and in many cases the compounds were not detected. The seasonal variation of pesticides concentrations can be ascribed to the seasonality of agricultural activities.

The highest levels of contaminations were found in SC3, carried out in summer (see Figure 2) in agreement with results obtained in other studies in the same area, for micropollutants other than the ones studied in the present work (Čelić et al., 2019; Navarro et al., 2010). In this season, a major increase in pesticide contamination was observed, both for neonicotinoids and other pesticides, especially oxadiazon. This seasonal pattern, known as "spring flush", is correlated with the application of such products during planting and growth of crops and it has been previously described in literature (Buttiglieri et al., 2009; Hladik et al., 2014).
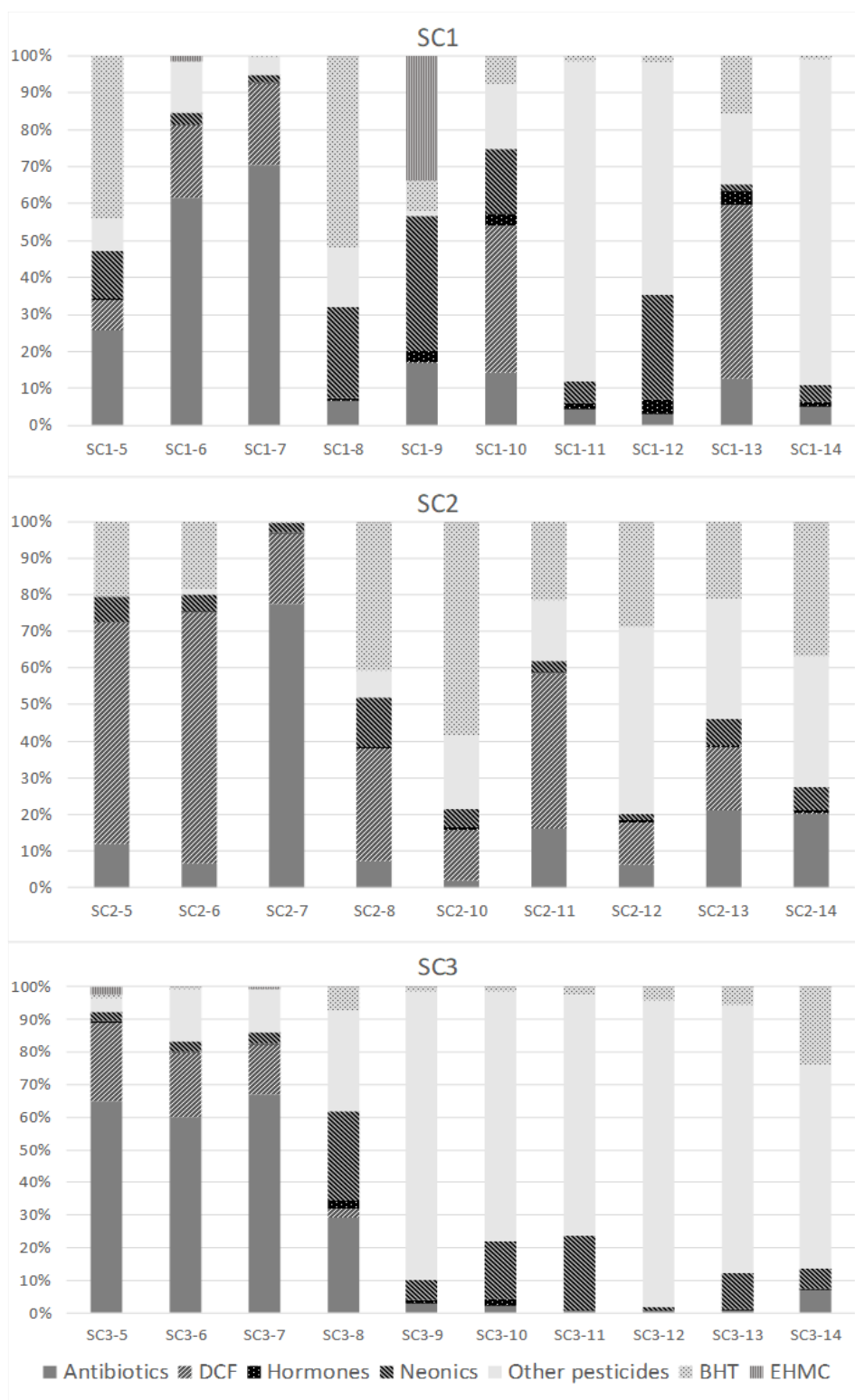
**Figure 3.** Composition profile of the Watch list compounds in freshwater in the three sampling campaigns. "SC" refers to the sampling campaign (SC1: October 2015; SC2: February 2016; SC3: May-June 2016), while the number indicates the sampling site.

### 3.2. Occurrence in wastewater

WWTPs are highly efficient in influent wastewater purification in terms of organic matter, suspended solids and/or nutrient but they are not specifically designed to remove organic micropollutants and are known to be point source of contamination (Buttiglieri and Knepper, 2008; Carballa et al., 2017). According to the EU decision, the Watch list substances are intended for monitoring in freshwater only. Nevertheless, it is crucial to establish a link between their presence and fate in wastewater and their occurrence in freshwater. Not only will this help pinpoint the sources of contamination and the entry pathways of the Watch list compounds into the environment, but information on the presence of these CECs in wastewater will also give insights on WWTPs performances. This, in turn, may facilitate the assessment of effluents quality for wastewater reuse. With the objective of assessing the occurrence of the Watch list compounds in the Ebro delta, consequently, influent and effluent waters of two of the main WWTPs in the area were sampled and analysed. The results are summarized in Figure 4 and Table S8.

Of the 17 investigated compounds, 11 were quantified in influent and 9 in effluent samples. Only 5 compounds exhibited a detection frequency of 100% (azithromycin, clarithromycin, diclofenac, imidacloprid and EHMC), while methiocarb, thiacloprid, acetamiprid, EE2, BHT and triallate were never above the MQL in wastewater (see Table S9). Limited occurrence data are found in literature for some of the contaminants studied herein. To the best of the authors' knowledge, methiocarb was only targeted in one study, in wastewater effluents, and it was not detected (Rubirola et al., 2017). Thiacloprid and acetamiprid were also addressed in one research paper only: the former was not found, in consistency with this study, and the latter detected at 3.7 ng/L (Sadaria et al., 2016). The occurrence of EE2 has been investigated in a variety of works and in a broad range of concentrations (Krzeminski et al., 2018), including below the MDL (Ekpeghere et al., 2018). On the contrary, the few available monitoring studies of BHT report concentrations of 22 to 258 ng/L in Germany (Fries and Püttmann, 2004) and of 2.42 µg/L in China (Liu et al., 2015). No data on the occurrence of triallate is available and it is here reported for the first time in wastewater.
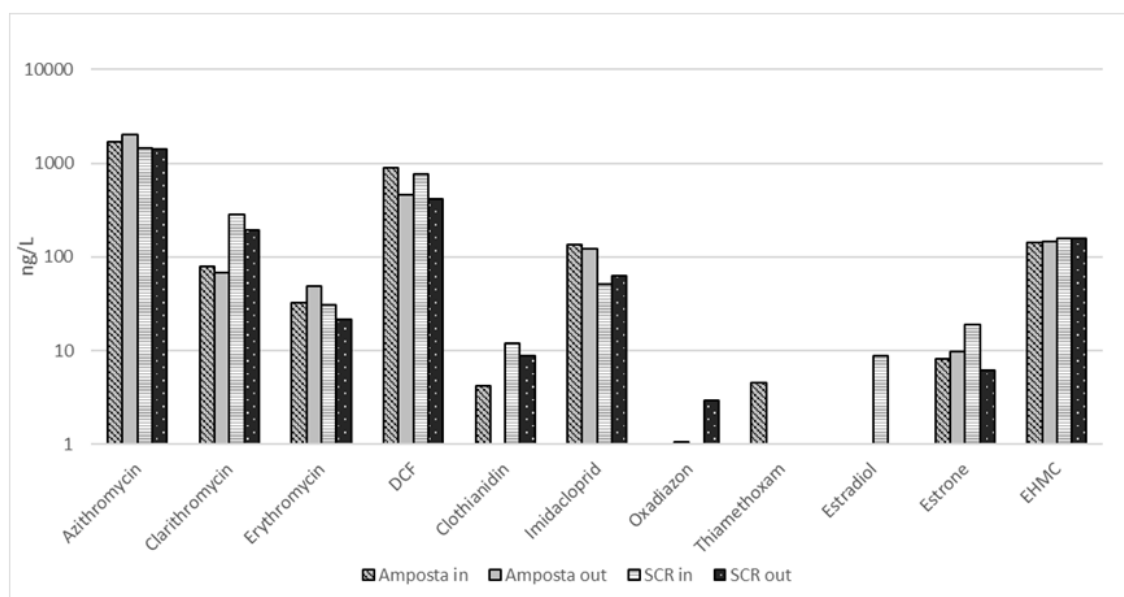
**Figure 4.** Concentrations of the investigated compounds in wastewater. Note the logarithmic scale on the Y axis.

Total concentrations ranged from 5.67 ng/L to 2.67 μg/L in influent wastewaters and between 3.04 ng/L to 3.47 μg/L in effluents. The highest contamination was recorded in the influent of WWTP Amposta in SC3, with a total Watch list compounds concentration of 4.32 μg/L. Azithromycin was the compound detected at the highest concentrations, both in influents and effluents, exhibiting a mean of 1.65 μg/L, followed by diclofenac (636 ng/L), in agreement with levels found in literature (Birošová et al., 2014; Farré et al., 2016; Verlicchi et al., 2012). Just like in freshwater, the group of pharmaceuticals was the most abundant.

Interestingly, oxadiazon, which was detected at high levels and frequencies in freshwater, as presented in the previous paragraph, was found at concentrations below 10 ng/L in wastewater samples. Thus, the group of non-neonicotinoids pesticides was the least abundant class of pollutants in wastewater. Similarly, some of the neonicotinoids were present in wastewaters at lower levels than in freshwater. Even when their concentrations in WWTPs were higher than in freshwater, the difference was unlikely to depend on just dilution and natural attenuation phenomena. These results suggest that contamination by oxadiazon and neonicotinoids is hardly generated in urban contexts and that such compounds enter freshwater ecosystems mainly through other pathways (e.g. runoff) (Anderson et al., 2015). On the contrary, EHMC was almost exclusively

present in wastewater, hinting that after its release into the environment it undergoes natural attenuation.

When comparing the influents among the three sampling campaigns (Kruskal-Wallis H test), no significant differences were observed. The same was detected in the assessment of seasonal variations among effluents, both results consistent with a study carried out in the same area (Čelić et al., 2019). The concentrations in the influents of the two WWTPs were also compared between them and it appeared that only EHMC concentrations differ, marginally, between the two (Mann-Whitney U test, p=0.05). Data show, in fact, that EHMC occurred at slightly higher levels in WWTP Sant Carles de la Ràpita. For these reasons, Figure 4 shows influent and effluent concentrations of the two WWTPs as means between the three sampling campaigns.

The difference in the Watch list compounds concentrations between influent and effluent was assessed by means of the Mann-Whitney U test. Erythromycin in Amposta and thiamethoxam and oxadiazon in WWTP Sant Carles de la Ràpita showed significantly differences after the treatment. In fact, erythromycin shows a negative removal in WWTP Amposta (-52%). A high variability in erythromycin removal rates is reported in literature and negative removals are not uncommon (Krzeminski et al., 2018; Verlicchi et al., 2012), probably due to its conversion to erythromycin-$H_2O$ (Dolar et al., 2012; Hirsch et al., 1999). In WWTP Sant Carles de la Ràpita, instead, it showed a removal of 31%. The pesticide thiamethoxam was detected only in one sample (Amposta influent, SC1) and the herbicide oxadiazon only on two occasions in effluent samples and always below 10 ng/L. Regarding the rest of compounds, the removal rates were generally low. Azithromycin, known for its low removal (Barbosa et al., 2016; Verlicchi et al., 2012), was found at same or slightly higher concentrations in effluents (1.8 and -17% removal in WWTPs Sant Carles de la Ràpita and Amposta respectively). Clarithromycin was characterized by low to moderate removal rates, between 15 and 32%, similar (Margot et al., 2013), or even somewhat higher, to literature data (Collado et al., 2014; Göbel et al., 2007). Diclofenac exhibited a removal rate around 47% in both WWTPs, in good agreement with previous works (Verlicchi et al., 2012). Imidacloprid removal amounted to 11% in WWTP Amposta, in consistency with another study (Sadaria et al., 2016), while its concentration increased by 22% after treatment in Sant Carles de la Ràpita. EHMC,

unlike in other studies (Biel-Maeso et al., 2019; Tsui et al., 2014), was not removed. The rest of compounds were detected at low concentrations. Further studies are needed to unveil the behaviour of such contaminants in wastewater.

In light of the generally poor removals obtained, the differences in concentrations between the sampling points located upstream and downstream WWTP Amposta (points 5 and 6) were checked and no significant changes were detected (Mann-Whitney U test). This means that WWTP Amposta does not negatively affect the quality of the receiving waters for the compounds evaluated in the present work. In the case of WWTP Sant Carles de la Ràpita, instead, the comparison between the control site and the emissary (points 5 and 7) reveals that azithromycin, clarithromycin, diclofenac, acetamiprid and imidacloprid are significantly higher after the discharge. This is not surprising, as Sant Carles de la Ràpita emissary consists of a highly impacted wastewater dominated drainage canal that transports the outflows of the WWTP to the sea. As said before, it was also the most contaminated site among freshwater samples. The low removals, in addition to the extremely limited scientific literature available for some of the investigated CECs, highlight the need for further studies on the fate and behaviour of the Watch list compounds in wastewater.

### 3.3. Ratio of environmental concentrations with predicted no-effect concentrations

In Decision 2015/495, the maximum acceptable MDL (as shown in Table 1) were chosen on the basis of Predicted No-Effect Concentrations (PNECs) (European Commission, 2015). In the technical report in preparation of the Watch list, the available ecotoxicological data are discussed and the calculations of PNECs are detailed for each compound, each one including an appropriate assessment factor (Carvalho et al., 2015). Over the course of the years, the PNEC values have been updated and the new values are lower for some of the compounds (Loos et al., 2018) (see Table S10).

In the current study, concentrations were often largely above PNEC values in several cases. Indeed, considering all compounds and sampling sites, PNECs were exceeded in 23% and 12% of cases in wastewater and freshwater, respectively. Imidacloprid concentrations exceeded the PNEC in 63% of samples, followed by diclofenac (51%) and azithromycin (49%). Risk quotients (RQ) are usually calculated by dividing the measured

environmental concentration by the PNEC. A RQ below 0.1 is conventionally considered as low, a quotient between 0.1 and 1 suggesting a medium risk and above 1 proposing a high risk (Sousa et al., 2018). Data are displayed in Tables S11-13 and mean RQs calculated as a mean of all three sampling campaign can be seen in figure S1. Following this classification and considering the sum of the RQs for all the compounds, each of the sampling point proved to be subject to high risk with the exception of three channel samples in the second sampling campaign (SC2-10, SC2-12, SC2-14). The highest values for freshwater were recorded for the emissary of Sant Carles de la Ràpita (sampling site 07), with RQ up to 169, azithromycin and EE2 accounting for the highest scores. These results suggest that the Watch list compounds may actually pose a risk to and through the environment in the Ebro delta region. It is therefore crucial to take measures to limit the presence of these compounds in the environment.

## 4. Conclusions

A monitoring of the 17 compounds included in the Watch list 2015/495 was carried out in the final stretch of the Ebro river, an area chosen as representative both for its economic and environmental relevance. The Watch list compounds have been reported for the first time both in wastewater and natural water. Information on the occurrence of the compounds of the Watch list 2015/495 are of EU relevance and, especially for certain compounds, are scarcely and sparsely available in literature.

Results show that the group of pharmaceuticals (macrolide antibiotics, in particular azithromycin and diclofenac) were the most abundant compounds. Pesticides, in particular oxadiazon and imidacloprid, were ubiquitous in freshwater samples and their occurrence is connected to the seasonality of agriculture. Seasonal variations were noticed in freshwater for some of the investigated compounds, whereas no significant differences were shown in wastewater. The most polluted site was found to be the emissary of WWTP Sant Carles de la Ràpita, where the Watch list contaminants amounted to 2.39 μg/L, but pollution was encountered even in the control site, likely to be originated upstream. The studied compounds proved to be recalcitrant or poorly degraded in WWTPs, the highest removal rates found for diclofenac, around 47%. The Watch list compounds concentrations were largely above PNEC values in most freshwater

and wastewater samples and the assessment of RQs proved that in the vast majority of sampling sites are subject to high risk (RQ >1).

## 5. Acknowledgements

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CHAPTER 3

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# How do WWTPs operational parameters affect the removal rates of EU Watch list compounds?

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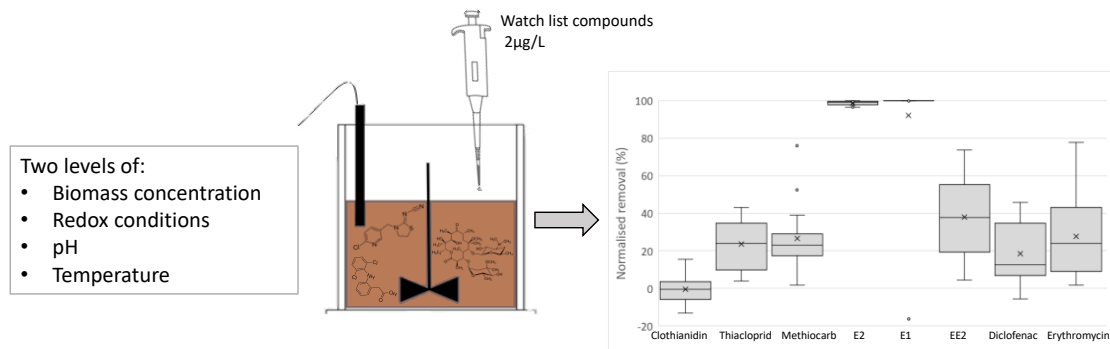
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# How do WWTPs operational parameters affect the removal rates of EU Watch list compounds?

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## Abstract

This work aims at achieving a better understanding of the mechanisms and the operative conditions regulating the removal of a set of relevant micropollutants in conventional activated sludge (CAS) systems to maximize their removal and, if possible, biodegradation. Eight compounds from the EU Watch list (clothianidin, thiacloprid, methiocarb, E1, E2, EE2, diclofenac and erythromycin) were spiked at 2 µg/L in CAS systems and their behaviour was studied in 6-hour batch tests. The role of sorption was also investigated. Information on the removal of the pesticides clothianidin, thiacloprid and methiocarb is here presented for the first time to the best of the authors' knowledge. With the aim of enhancing the removal of the selected compounds in wastewater treatment, four parameters were explored: biomass concentration, temperature, pH and redox conditions. For each parameter, a low and a high value were chosen, based on the ranges usually applied in wastewater treatment plants (WWTPs). Results show that biomass concentration is the most relevant parameter among the ones investigated, followed by the redox conditions. The operational conditions that maximised removal rates were: 5 g/L of biomass, aerobic conditions, 25 °C and pH 7.5. High variability in removal rates was observed for compounds such as E1, erythromycin and methiocarb.

The pesticides clothianidin and thiacloprid did not prove to be easily degradable. The highest removal rates were recorded for the hormones, particularly E2, with a transformation rate of at least 96% under all conditions. Sorption proved to be a relevant removal route for EE2, for which the highest sorption rates were recorded, and diclofenac, where the adsorption mechanisms was hypothesised for its prevalence at lower pH values.

**Keywords**

Watch list; Micropollutants; Conventional activated sludge; Biodegradation; Sorption

## 1. Introduction

The occurrence and fate of organic micropollutants, such as endocrine disrupting compounds (EDCs), pharmaceuticals and pesticides, has raised great environmental concern during the last decades (Daughton and Ternes, 1999). While the majority of these emerging contaminants is still unregulated in most countries, legal frameworks have been recently implemented in order to phase out the release into the environment of those compounds that threat the aquatic ecosystems or to regulate their presence in water bodies (EC, 2013, 2000). The Directive 2013/39/EU sets concentration limits for a number of chemicals with prejudicial environmental effects, the so-called priority pollutants, but also sets a new decision-making tool for future prioritisation exercises through the Watch list system (Commission Implementing Decision 2015/495 (EC, 2015) and 2018/840 (EC, 2018)). The Watch list, in force since March 2015, includes compounds that could pose a risk to or via the aquatic environment, but for which high-quality monitoring data are not enough to carry out appropriate risk assessment (EC, 2015). The compounds included in the Watch list should be monitored in all EU Member States for a maximum of 4 years after which they might be regulated. In June 2018 a new version of the Watch list was published. Five compounds (oxadiazon, triallate, BHT, EHMC and diclofenac) were removed from the 2018 Watch list, three compounds were added (two antibiotics and a pesticide) while the majority of compounds remained unaltered due to the lack of occurrence data for risk assessment (EC, 2018). According to the EU Decision, the Watch list substances are intended for monitoring in freshwater only. Nonetheless, WWTPs, even though essential to contain anthropogenic contamination, are among the

most relevant point sources of contamination. Therefore, unravelling the fate of emerging pollutants and, possibly, enhancing their removal when passing through WWTP are crucial, ultimately ending out in substantial improvement of water quality and environmental standards (Gusmaroli et al., 2019).

Conventional activated sludge wastewater treatment plants (CAS-WWTPs) represent the most common treatment for major urban areas. They are highly efficient in influent wastewater purification in terms of organic matter, suspended solids and/or nutrient but they are not specifically designed to remove micropollutants (Buttiglieri and Knepper, 2008; Carballa et al., 2017). Advanced treatment processes could be employed, but limitations are posed due to maintenance and operational costs (Luo et al., 2014; Schröder et al., 2016). Improving the biodegradation process can be, therefore, a suitable solution due to its lower cost and its potential for complete micropollutants removal. Nonetheless, information is largely missing for many emerging micropollutants in terms of operative parameters affecting their removal in CAS systems.

The main mechanisms that might play a role in micropollutants removal are biodegradation, sorption onto sludge, air stripping and photo-transformation. For many emerging organic micropollutants, including the pollutants selected in this study, both air stripping and photo-transformation can be neglected (Sipma et al., 2010). Air stripping depends indeed on the Henry's law constant ($H_c$): with $H_c$ lower than $10^{-4}$ and the fraction $H_c/K_{ow}$ lower than $10^{-9}$, there is a low volatilisation potential (Rogers, 1996). For hydrophobic compounds, the main removal mechanism is usually sorption, while hydrophilic compounds are more prone to biodegradation (Alturki et al., 2010). The extent of sorption is measured by the solid–water distribution coefficients ($K_d$), defined as the ratio between the concentrations of a substance in the solid and in the aqueous phase at equilibrium. This parameter, in turn, includes two mechanisms: absorption, which consists of hydrophobic interactions – whose extent is measured with $K_{ow}$ – and adsorption, regulated by electrostatic interactions, characterized by pKa. Compounds with a log $K_{ow}$ below 2.5 exhibit a low sorption potential, between 2.5 and 4.0 a medium sorption potential and higher than 4.0 a high sorption potential (Rogers, 1996). Reviewing literature, it becomes evident that fewer studies deal with absorption than adsorption. Moreover, several works use the term sorption, or even adsorption, to refer to both

mechanisms, without making distinction between the two processes. Hence, in some cases, it is difficult to tell the two mechanisms apart and to compare the results with previous works. On the whole, sorption onto activated sewage sludge might be less relevant for many micropollutants, as shown by their relatively low sorption coefficients ($K_d$) (Sipma et al., 2010).

The compounds chosen for this study were clothianidin, thiacloprid, methiocarb, erythromycin, diclofenac, estrone (E1), estradiol (E2) and ethinylestradiol (EE2). These were originally included in Decision 2015/495, which is currently undergoing its fourth year of monitoring, and, out of the eight compounds, seven of them have been reconfirmed in the 2018 Watch list too, with the only exception of diclofenac (EC, 2018). The chosen compounds are representative of different classes: clothianidin, thiacloprid and methiocarb are pesticides, erythromycin is an antibiotic, diclofenac is a NSAID pharmaceutical, E1 and E2 are natural hormones whereas EE2 is a synthetical hormone. In addition, these compounds exhibit different biodegradability, from recalcitrant to highly biodegradable. Some of these compounds, such as clothianidin, thiacloprid and methiocarb, lack information regarding their behaviour and removal mechanisms in WWTPs, while others, like erythromycin, diclofenac and EE2, are marked by huge variability in terms of occurrence and removal in full-scale WWTPs (Barbosa et al., 2016).

In order to shed light on the behaviour and fate of the selected contaminants in CAS systems, batch experiments were conducted and the extent of micropollutants biodegradation and sorption was assessed. Four operational parameters were evaluated: temperature, pH, mixed liquor suspended solid (MLSS) concentration and redox conditions, in ranges usually found in temperate climate conventional WWTPs.

## 2. Materials and methods

### 2.1. Reagents and solutions preparation

Erythromycin, methiocarb, thiacloprid, clothianidin, E1, E2, EE2 and diclofenac (sodium salt) were purchased from Sigma Aldrich (St. Louis, MO, USA). Individual stock solutions were prepared on a weight basis by dissolving 10 mg of the desired compound in 10 mL of methanol (stock 1, concentration of 1000 mg/L). Once prepared, they were stored at -20 ˚C in the dark, to preserve the compounds from possible photodegradation (Capriotti

et al., 2014). Due to the well-known limited stability of antibiotics solutions, the erythromycin stock solution was renewed each 3 months (Gros et al., 2013), while for the rest of compounds stocks were renewed each 6 months. Then, 100 µL of each compound was added to a vial containing a few drops of methanol and the mix was evaporated under a gentle nitrogen stream until complete dryness. The solution was then reconstituted in 10 mL of water (MIX 1, 10 mg/L) and placed into an ultrasonic bath for 15 minutes to ensure complete dissolution. This mix was then further diluted in 10 mL of water to obtain the final spiking solution (MIX 2, 0.5 mg/L). Lastly, 1 mL of MIX2 was spiked in the jacketed reactors (see paragraph 2.4) to obtain the experiments design initial concentration of 2 µg/L.

## 2.2. Biomass source and preparation

The biomass was regularly withdrawn from Celrà WWTP (Catalonia, Spain). Celrà WWTP has a capacity of 18,900 PE by design, a load of 10,009 PE ("iAgua - EDAR de Celrà," n.d.), a hydraulic retention time of 48 h and a sludge retention time of 20-22 days (Collado et al., 2014). Celrà WWTP has 80% flow industrial contribution from a nearby industrialized area with several pharmaceutical industries, which have their own wastewater treatment process before discharging their effluents to the WWTP of study (Collado et al., 2014). Moreover, the WWTP is equipped for phosphorous and nitrogen removal ("iAgua - EDAR de Celrà," n.d.).

Before the beginning of each test, the biomass was aerated for one hour in order to minimise the amount of rapidly degradable organic matter still present. The initial mixed liquor suspended solids (MLSS) content of the sludge, usually related to biomass content, was determined according to Standard Methods (APHA, 2012). To determine the role of adsorption to sludge in micropollutants removal, experiments with inactivated biomass were also carried out. Inactivated biomass was obtained by autoclavation at 120°C for 20 minutes before each experiment.

## 2.3. Experimental design

The list of experiments is presented in Table 1. Four parameters were explored: temperature, MLSS, redox conditions and pH. A lower and higher levels were defined for each parameter: 12 and 25°C for temperature, 1 and 5 g/L for MLSS, anoxic and aerobic for redox conditions and 6.5 and 7.5 for the pH. Such ranges were chosen to include typical WWTP operational paramters in temperate climates, and in accordance with Celrà WWTP. All experiments were performed in duplicate.

**Table 1** – List of experimental conditions.

| Exp. | Temp (°C) | MLSS (g/L) | Redox | pH |
|---|---|---|---|---|
| 1 | 12 | 1 | anoxic | 6.5 |
| 2 | 25 | 1 | anoxic | 6.5 |
| 3 | 12 | 5 | anoxic | 6.5 |
| 4 | 25 | 5 | anoxic | 6.5 |
| 5 | 12 | 1 | oxic | 6.5 |
| 6 | 25 | 1 | oxic | 6.5 |
| 7 | 12 | 5 | oxic | 6.5 |
| 8 | 25 | 5 | oxic | 6.5 |
| 9 | 12 | 1 | anoxic | 7.5 |
| 10 | 25 | 1 | anoxic | 7.5 |
| 11 | 12 | 5 | anoxic | 7.5 |
| 12 | 25 | 5 | anoxic | 7.5 |
| 13 | 12 | 1 | oxic | 7.5 |
| 14 | 25 | 1 | oxic | 7.5 |
| 15 | 12 | 5 | oxic | 7.5 |
| 16 | 25 | 5 | oxic | 7.5 |

## 2.4. Experimental setup, sampling procedure and analyses

The experiments were carried out in jacketed reactors connected to a thermostatic bath to maintain the temperature constant throughout the experiment. The batch tests were conducted under continuous stirring (200 revolutions per minute) by means of magnetic stirrers. The biomass was diluted with tap water in order to achieve the desired concentration and a total working volume of 250 mL. To mimic real influent wastewater conditions 0.25 mL of trace elements solution (Kampschreur et al., 2007), 10 mL of buffer/inorganic solution, 1 mL of organic solution and 1 mL of either nitrate or ammonia solutions were added before the start of each test, following a procedure adapted from Collado et al., 2013 and Kassotaki et al., 2019. The composition of such solutions can be consulted in SM. pH was set to either 6.5 or 7.5 by addition of acid (HCl) or base (NaOH) at 0.5 M and monitored throughout the batch tests with a portable pH-meter, making adjustments when necessary. Aerobic conditions (>2.5 mg $O_2$/L) were ensured by continuous, gentle, air supply through an air diffuser, while anoxic conditions were achieved by dosing $NaNO_3$ (see Table S4 in SM).

To check the background concentration of micropollutants and to evaluate the matrix effect, samples were collected before starting the experiments. Typically, 4 mL approximately were taken from the reactor with a syringe and immediately filtered through syringe-driven filter units (Millex Syringe PVDF 33mm, pore size 0.45 μm, by Merck Millipore) in order to separate the liquid phase from the sludge, thus interrupting the reaction. The samples were stored in 2-mL vials and immediately frozen until analysis.

At time zero, micropollutants were spiked into the system (design initial concentration: 2 μg/L) and, after allowing 30 seconds for mixing, time-zero samples were taken following the procedure described above. Micropollutants were also sampled at elapsed time 10 minutes, 1 hour, 2 hours and 6 hours. The analyses were carried out by measuring the concentration of micropollutants in the liquid phase by means of online SPE-UHPLC-MS/MS according to the method described in Gusmaroli et al., 2018. Shortly, the samples were loaded onto a Hypersil GOLD aQ (20 x 2.1 mm, 12 μm particle size, Thermo Fisher Scientific) column at a flow rate of 1750 μL/min for preconcentration and then the analytes were eluted from the chromatographic column at flow rate of 400 μL/min.

Clothianidin, erythromycin, methiocarb and thiacloprid were analysed under positive ionization mode by means of a Kinetex Biphenyl (1.7 µm particle size, 100 x 2.1 mm i.d., Phenomenex). The mobile phase consisted of MeOH and water with 0.1% formic acid and separation was achieved in 8 minutes. The hormones and diclofenac were analysed under negative ionization (NI) mode. As organic mobile phase, a mixture of MeOH and acetonitrile (ACN) was used during the initial stages of the gradient, though the compounds eluted when the percentage of ACN reached almost 100%. The aqueous mobile phase consisted of LC/MS grade water with ammonium fluoride 1 mM. The time of analysis in NI mode lasted 10.5 minutes. Detection was performed by means of a TSQ Vantage mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray turbo spray ionization source, recording two Selected Reaction Monitoring (SRM) transitions for each compound. To obtain information on process efficiency in the preparation of the spiking mix, the concentration of the investigated compounds in the spiking mixture was checked for each experiment. Recoveries of the spiking mix ranged from 55 to 117% for all compounds. The results are presented as normalized concentrations in order to homogenize the concentration levels and allow inter-day comparisons. Normalisation was obtained by dividing each concentration by the concentration value recorded at time 0. The resulting value was then multiplied by 100, thus rescaling all data to a 0-100% scale. Removals were calculated as the difference between initial and final normalised concentrations. Compounds exhibiting removal rates below 25% were classified as poorly degradable, between 25 and 65% were considered medium and above than 65% were classified as highly degradable. The analyses were conducted only on the liquid phase, therefore the term removal is intended as the combination of biological and physical phenomena, whereas sorption refers to the decrease in concentration observed in the experiments carried out with inactivated biomass. Thus, biodegradation was calculated as the difference between the total removal and sorption.

Besides the selected micropollutants, other analyses were carried out at the beginning and at the end of each test: mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS), total organic carbon (TOC), total nitrogen (TN) and ammonia, nitrite and nitrate concentrations. TOC samples were filtered through syringe-

driven nylon filter units with pore size 0.45 µm and samples for nitrogen species analyses were filtered through 0.22 µm nylon filters (Millex Syringe, 33 mm, by Merck Millipore). TOC and TN were analysed with a TOC/TN analyser, while the other nitrogen species were analysed by ion chromatography (APHA, 2012).

## 3. Results and discussion

### 3.1. Effect of experimental conditions on the overall removal

If we approach the removal of all the micropollutants considered in this study, that is to say, considering the whole set, the highest removals were obtained during experiment 16, in which all the parameters were at the higher level: aerobic conditions, 25°C, 5 g/L of biomass and pH 7.5. Please note that removal, described in this section and in section 3.2, is intended as the combined effect of biodegradation and sorption to sludge. Figure 1 shows the average removals, for all compounds, attained in each experiment. Detailed results for each test are shown in Figure S1 of Supplementary Materials.

The most relevant factor affecting micropollutants removal is MLSS concentration, which yields higher rates at high concentrations, as expected. The second most relevant operational parameter is the redox condition, as working under aerobic conditions enhances the removal. The third one is temperature, which seems to give better results at high level (25°C) followed by pH, a parameter that, at least apparently, has little effect on the overall micropollutants removal in the considered range and the chosen compounds. The analysis of nutrients confirmed that temperature helps accelerating the kinetics of the process as those tests at 25 °C present higher increase/decrease of nutrients (Table S5 in SM). Moreover, the depletion of $NH_4^+$ that is converted to $NO_3^-$ highlighted that nitrification takes place in aerobic conditions, and more sharply in experiments 8 and 16, at 5 g/L MLSS and 25 °C.
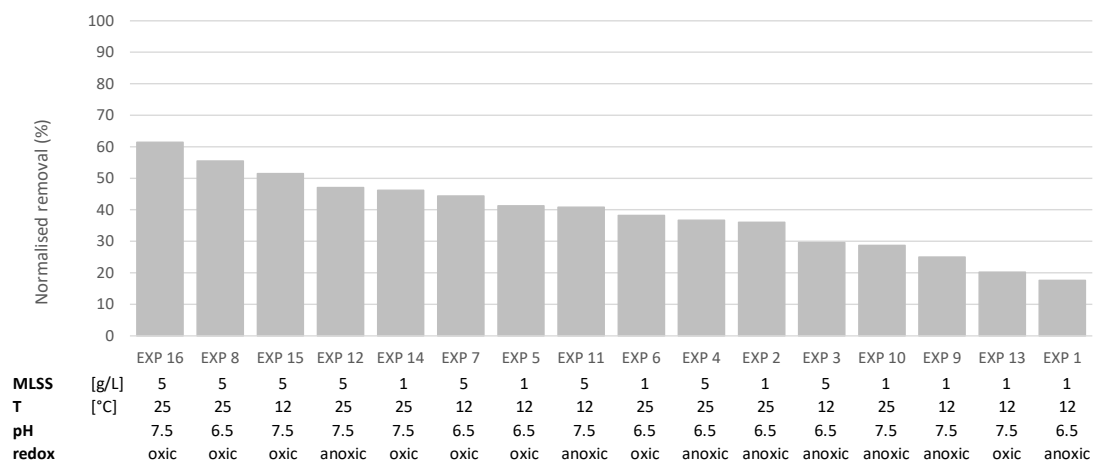
**Figure 1 -** Normalised average removal of all compounds. Data were normalised by dividing the concentrations obtained at each sampling time by the concentration of time-0 samples. The obtained values were then multiplied by 100 and are here presented as percentages.

## 3.2. Effect of the experimental conditions on the removal of single compounds
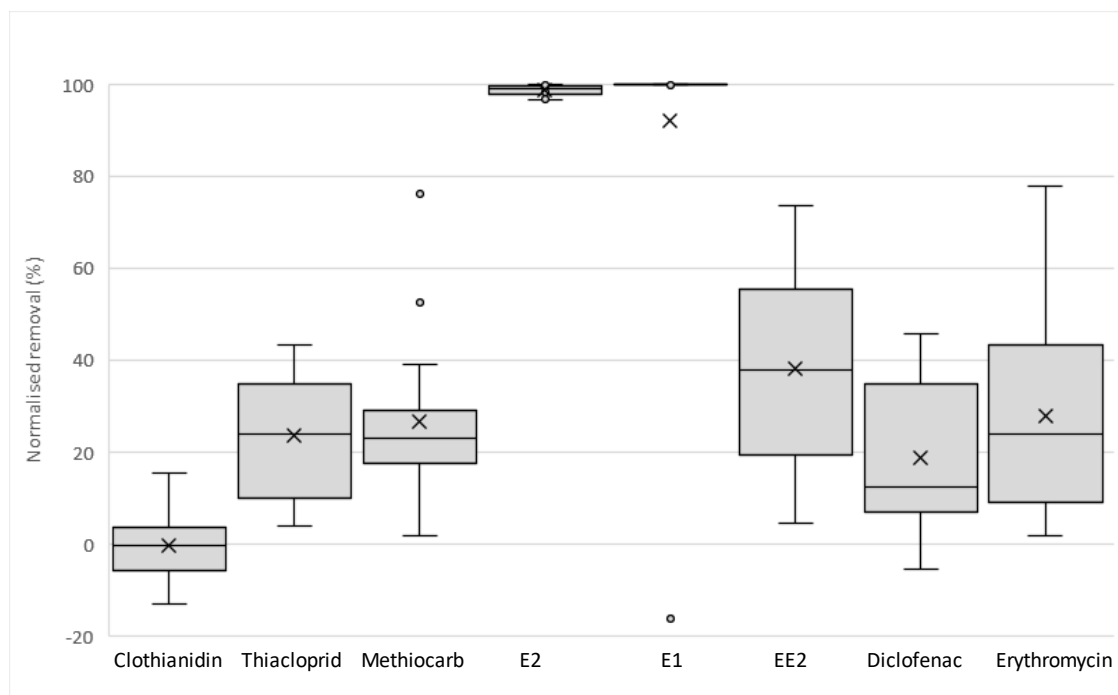


**Figure 2 -** Total removal by compound
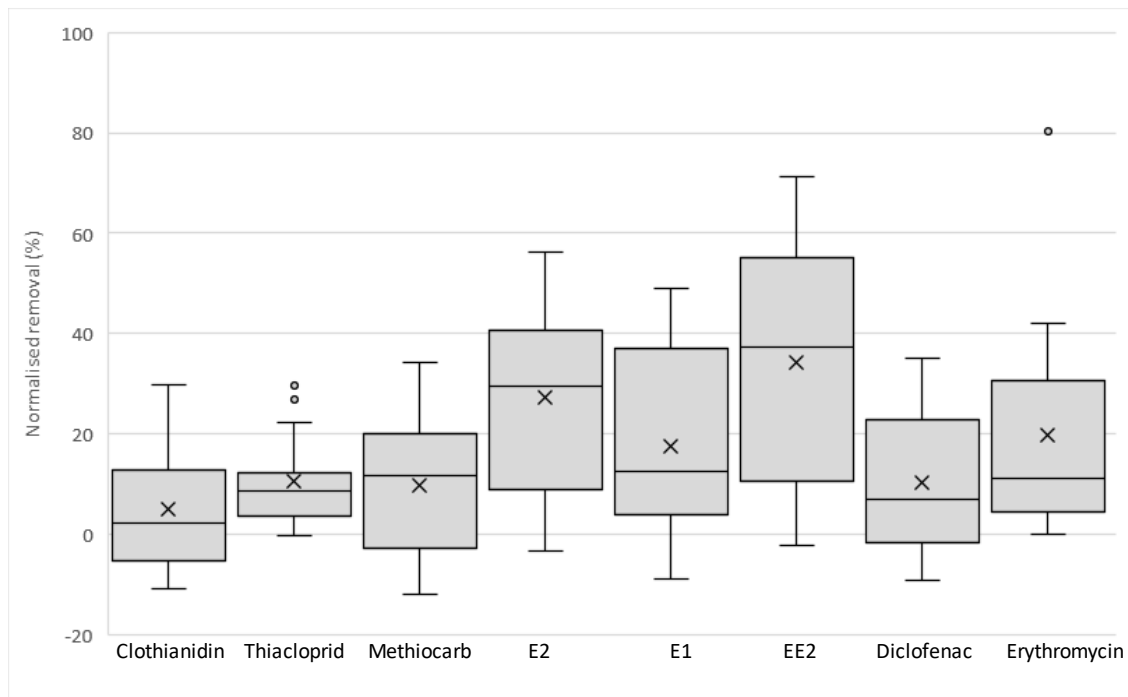
**Figure 3** – Sorption by compound

With regards to the removal by compound, Figure 2 displays the statistical distribution. For compounds such as E1, erythromycin and methiocarb, the ranges sweep from zero to very high or complete removals, highlighting the importance of the choice of the right operational parameters, since even minute variations produce a significantly different response.

To the best of the authors' knowledge, literature data concerning the occurrence of clothianidin, thiacloprid and methiocarb in WWTPs are extremely limited in number and no publications exploring their fate and behaviour are available. The lowest removal was observed for clothianidin with a null to low removal (up to 15%, Figure 2 under all experimental conditions, in consistency with the extremely rare literature data concerning its occurrence in a real-scale WWTP (Sadaria et al., 2016).

Thiacloprid removal ranged between 3.8 and 43.2% (Figure 2) Among the investigated compounds, thiacloprid is the only one that showed higher removals when operating at the lower level of temperature. This could hint that adsorption is a significant removal pathway, as it is an exothermic process, but the removal rates are so low that it is hard to tell the mechanisms apart. The lowest removal occurred at 25°C, under anoxic conditions, at 1 g/L MLSS and pH 7.5, while the highest degradation rate was obtained under anoxic conditions, at 12°C, high biomass concentration and pH 7.5. Information on

thiacloprid biodegradation is completely absent in literature and its occurrence in WWTPs has been studied in an extremely limited number of works. To the best of the authors' knowledge, it was never detected in wastewater (Gusmaroli et al., 2019; Rubirola et al., 2017; Sadaria et al., 2016).

The removal of methiocarb spanned through a wide range. At its highest, it was removed up to 76%, under aerobic conditions, at 5 g/L MLSS, pH 7.5 and low temperature (Figure 2). The second and third best removals were achieved when operating under aerobic conditions, high levels of MLSS and temperature and at pH 7.5 and 6.5. Nevertheless, its removal was unsatisfactory in most cases, the lowest recorded value equalling 1.9% when operating under anoxic conditions at low concentrations of biomass. The factor accounting for most differences in methiocarb degradation, in the tested conditions, is MLSS. Overall, the removal of this compound does not present a clear pattern and no comparisons are possible due to the absence of references in the literature. Its occurrence has been poorly investigated too: in two recent studies, it was not detected in wastewater (Gusmaroli et al., 2019; Rubirola et al., 2017) and, when detected, its concentration ranged between 1.26 and 105.31 ng/L. A negative removal from wastewater of over 7000% was observed (Campo et al., 2013), a value probably resulting from sampling limitations (neither sludge retention time nor hydraulic retention time were taken into consideration) (Barbosa et al., 2016). More studies are needed to shed light on the factors that enhance the removal and biodegradability of clothianidin, thiacloprid and methiocarb.

Among the investigated micropollutants, the compound with the highest biotransformation rate was E2, with removals always above 96% (Figure 2). In particular, a rapid removal of E2 ranging from 60 to 95% occurs in the first 10 minutes, the former at 12 °C, 1 g/L, pH 6.5 and under aerobic conditions and the latter at 25 °C, 5 g/L, aerobic conditions and pH 6.5. This is due to the quick oxidation of E2 into E1, a phenomenon already described in literature (Ternes et al., 1999). Despite this difference in terms of E2 biotransformation rate, anyway, by the first 60 minutes of contact time, at least 93.8% in respect to its initial concentration was biodegraded under all experimental conditions. The easy biotransformation of E2 has been widely reported in literature, observed both in full-scale WWTPs (Baronti et al., 2000; de Mes et al., 2005) and in laboratory

experiments (López-Fernández et al., 2013), and it has been observed in all redox conditions (aerobic, anaerobic and anoxic) (Li et al., 2011).
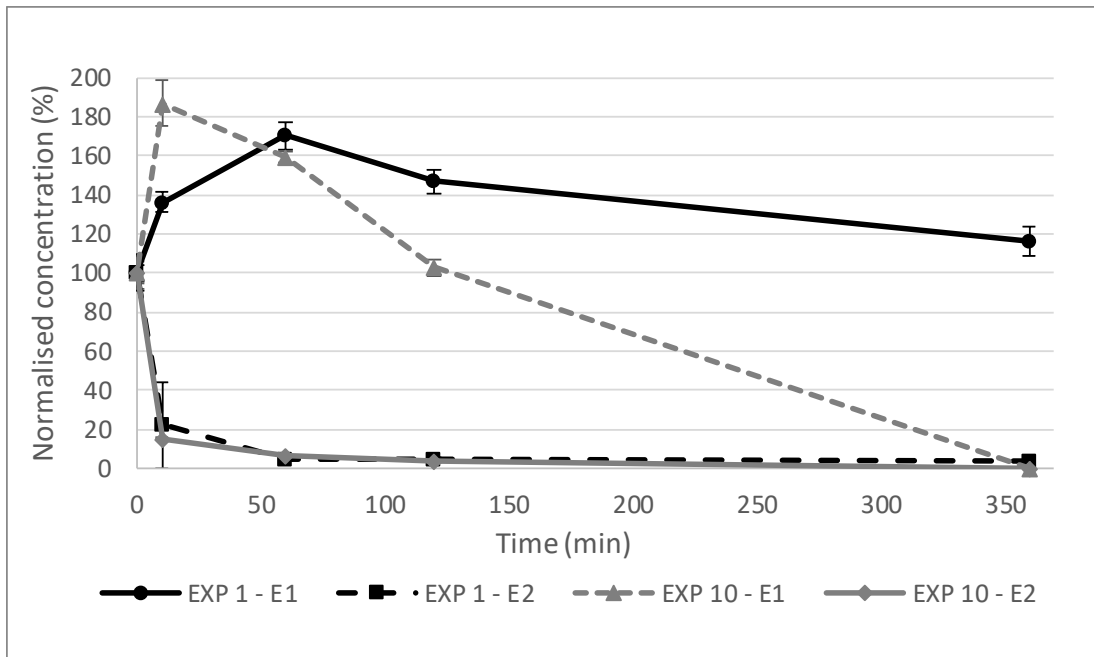


**Figure 4** - E1 and E2 removal profiles in experiments 1 and 10

The compound showing the second highest removal rates was E1. This compound showed medium to high removal rates except when working at 12 °C and at pH 6.5 under anoxic conditions, which led to null to low removals, up to 19.1%. Medium removals, around 40%, were observed at 12 °C, 1 g/L biomass under aerobic conditions. For this compound, an increase up to 190% in respect of its initial concentration was recorded in almost all cases during the first minutes of reaction due to the above-mentioned conversion of E2 into E1 (see Figure 4). Afterwards, the concentration decreases either gradually or abruptly, depending on experimental conditions. High temperature, biomass concentration and pH led to the fastest removals, regardless of redox conditions. Temperature appears to be one of the factors affecting the kinetics of E1 removal the most. In fact, at 25 °C, the removal was always complete (>99%). An improvement in estrogens biodegradability at higher temperatures has been observed in full-scale WWTPs (de Mes et al., 2005) as well as batch tests (Li et al., 2005; Zeng et al., 2009). Studies have demonstrated that E1 eventually even undergoes mineralization in WWTPs, with 70-80% conversion of E2/E1 into $CO_2$ in 24 hours (Layton et al., 2000).

EE2 removals ranged from 5 to 73.6%, proving the least biodegradable amongst the investigated hormones, in consistency with literature (de Mes et al., 2005; Ting and Praveena, 2017). The lowest removals were obtained at low concentrations of biomass and under anoxic conditions. EE2 behaviour, not only in WWTPs but also in aerobic batch experiments, is reportedly inconsistent. Literature data include an observed persistency over a 5-day period (Norpoth et al., 1973) but also, contrarywise, a complete removal achieved by nitrifying sludge after 6 days (Vader et al., 2000). According to previous works, EE2, when biotransformed, is supposedly degraded by co-metabolism, differently from natural estrogens. The lower biodegradability of EE2 could be caused by its ethynyl group, which sterically hinders metabolism and other reactions (Racz and Goel, 2009). The two hydroxyl groups present in EE2 molecular structure would be susceptible to microbial attack; however, the ethinyl group located on the same C atom which possesses the hydroxyl group makes ring cleavage more difficult and, in turn, causes EE2 to be recalcitrant (Zuo et al., 2013). Nitrifying sludge is held accountable for the conversion of EE2 into more hydrophilic metabolites by ammonium monooxygenase and the phenomenon is usually not observed in sludges with low nitrifying activity (Vader et al., 2000). The maximum EE2 removal (>70%) was achieved under aerobic conditions, at high levels of temperature, biomass concentration and almost regardless of pH, in accordance with the above-mentioned literature data. However, surprisingly, similar removals were obtained under anoxic conditions at 12°C, 5 g/L MLSS, pH 7.5 and with a faster degradation profile (60% removed during the first 10 minutes of contact time). This result seems to contradict previous studies in which EE2 was removed at a significant rate only under aerobic conditions (Joss et al., 2004).

Diclofenac was hardly removed, its biodegradation rates ranging from null to moderate, with a maximum removal of 45.7%, obtained under aerobic conditions at 25°C, 5g/L MLSS, pH 6.5. Diclofenac's poor biodegradability during biological wastewater treatment has been previously reported (Lee et al., 2012; Pérez and Barceló, 2008; Quintana et al., 2005; Vieno and Sillanpää, 2014). The microbial processes that play a role in diclofenac biodegradation and biotransformation have been investigated thoroughly, but the picture is still unclear. High variability can be found in literature, with elimination rates of up to about 80% (Yang et al., 2011) and even negative removals (Clara et al., 2005; Zorita

et al., 2009); however, values in the range of 20–50%, similarly to those found in the current study, are more common (Vieno and Sillanpää, 2014). Apparently, aerobic conditions favoured its degradation, while operating at pH 7.5 seems to have a negative effect on its removal. In consistency with this finding, Suárez and co-workers observed no biodegradation under anoxic conditions (Suarez et al., 2010). The dependence on pH suggests that adsorption is a relevant removal route for diclofenac. In fact, the carboxylic acid moiety of diclofenac is negatively charged at neutral pH (the $pK_a$ of diclofenac is 4.15) and therefore the compound repels the negatively charged sludge surface. At lower pH values, on the contrary, the equilibrium shifts towards the electronically neutral form, thus allowing adsorption onto sludge (Vieno and Sillanpää, 2014). This phenomenon has been reported in literature (Ternes et al., 2004). Nevertheless, biodegradation is still considered as its main degradation pathway (Vieno and Sillanpää, 2014).

Erythromycin was removed between 1.7 and 77%. The circumstances under which it was not removed were anoxic conditions, 12°C, 1 g/L MLSS and pH 7.5. On the contrary, the highest removal was obtained under aerobic conditions, at high levels of temperature, pH and MLSS. The most relevant parameter was the redox conditions, followed by MLSS concentration and temperature, the three of which yielded higher removals when operating at the higher level. On the contrary, the contribution of pH was limited and the low level, corresponding to pH 6.5, gave better performances. Erythromycin was generally found to be recalcitrant during biological treatment in several studies conducted in real wastewater effluents (Guerra et al., 2014; Kim et al., 2014; Pasquini et al., 2014; Yang et al., 2011) or even at higher concentrations in effluents than in influents (Gusmaroli et al., 2019; Krzeminski et al., 2019; Verlicchi et al., 2012). Nevertheless, wide ranges of variability, including removals up to 80%, are documented in literature (Verlicchi et al., 2012). In a study carried out by Suarez et al., erythromycin was successfully removed (≈90%) in aerobic reactors, while only 20% removal occurred in anoxic reactors, thus hinting that nitrifying bacteria may have a higher affinity to this antibiotic (Suarez et al., 2010).

## 3.3. Effect of experimental conditions on sorption

Considering the whole set of studied micropollutants, removal by sorption was generally low to medium, ranging from an average of 5% in the case of clothianidin to the above-mentioned 34% for EE2 (see Figure 5). In a few cases, adsorption rates were slightly higher than the total removal. This could be ascribed to the fact that the conventional activated sludge was thermally treated for inactivation and this could somewhat alter its properties. There is evidence that heat-inactivation of biomass increases surface area by 6-25%, probably due to the loosening of the attachment between extracellular polymeric substances and bacteria in the flocs (Racz et al., 2012). After inactivation, the biomass is no longer biologically active and therefore the decrease in micropollutants concentration is entirely ascribable to sorption. Biodegradation was determined as the difference between total removal and sorption.

When considering the factors that have the most impact onto the extent of sorption, the most important operational parameter proved to be the redox conditions. In fact, working under aerobic conditions maximized sorption, as seen elsewhere (Suarez et al., 2010). The second most relevant parameter was the MLSS concentration. As one could easily predict, highest concentrations of biomass (5 g/L MLSS) gave better removals by sorption, since the presence of more substrate offered more surface area for the phenomenon to take place. The dependence of sorption on sludge concentration has also been reported in previous studies (de Mes et al., 2005). These results might prove useful when evaluating the parameters that maximise removal by sorption, as well as for modelling purposes and the formulation of constants.

## 3.4. Effect of the experimental conditions on sorption of single compounds

It is visible from Figure 3 that the highest sorption rates were achieved for the hormones. EE2 was the compound with the highest average sorption rates over the 16 experiments, with a value of 34% in respect to the spiked concentration, and a maximum of 71.2% (experiment 8), the second highest sorption rate among all compounds and batch tests. Except when working at low temperature and concentration of biomass, sorption accounted for the majority or even the entirety of the removal for EE2, as shown in Figure 5, in accordance with literature data (Suarez et al., 2010).
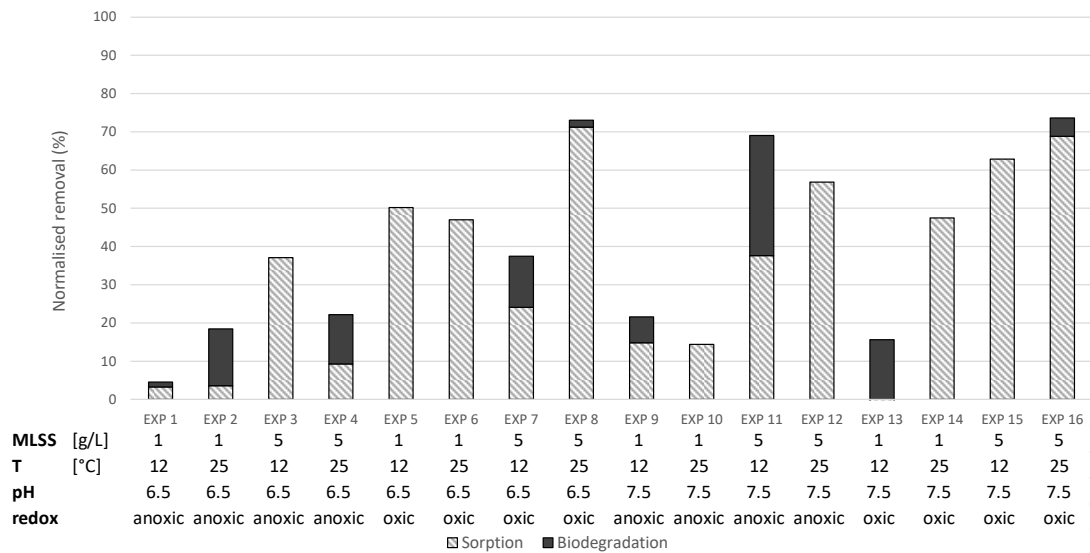
**Figure 5 -** Removal profiles of EE2 throughout the experiments, detailing the contributions of sorption and biodegradation.

Working at higher temperatures increased the sorption of EE2, in contrast with previous findings (Feng et al., 2010). Since adsorption is an exothermic process, this could mean that the process involved is absorption instead, which is known to increase with the increase of temperature. Another factor suggesting such mechanism is the increase of sorption rates with the pH, indicating a lesser relevance of pKa in the sorption process when operating in this range of pH. On the contrary, its $K_{ow}$ value of 3.67 - 4.15 (Hansch, C., Leo, A., Hoekman, 1995; Lai et al., 2000) indicates a medium-high absorption potential. However, further studies are necessary to confirm this hypothesis and, as mentioned in section 1, sometimes confusion arises from the misuse of terminology. E2 and E1 were sorbed 27.3% and 17.4% on average, respectively. However, as shown in Figure 6, biodegradation accounts for most of E2 removal. The charts for the rest of compounds are displayed in SI.

| | | EXP 1 | EXP 2 | EXP 3 | EXP 4 | EXP 5 | EXP 6 | EXP 7 | EXP 8 | EXP 9 | EXP 10 | EXP 11 | EXP 12 | EXP 13 | EXP 14 | EXP 15 | EXP 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MLSS | [g/L] | 1 | 1 | 5 | 5 | 1 | 1 | 5 | 5 | 1 | 1 | 5 | 5 | 1 | 1 | 5 | 5 |
| T | [°C] | 12 | 25 | 12 | 25 | 12 | 25 | 12 | 25 | 12 | 25 | 12 | 25 | 12 | 25 | 12 | 25 |
| pH | | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 |
| redox | | anoxic | anoxic | anoxic | anoxic | oxic | oxic | oxic | oxic | anoxic | anoxic | anoxic | anoxic | oxic | oxic | oxic | oxic |
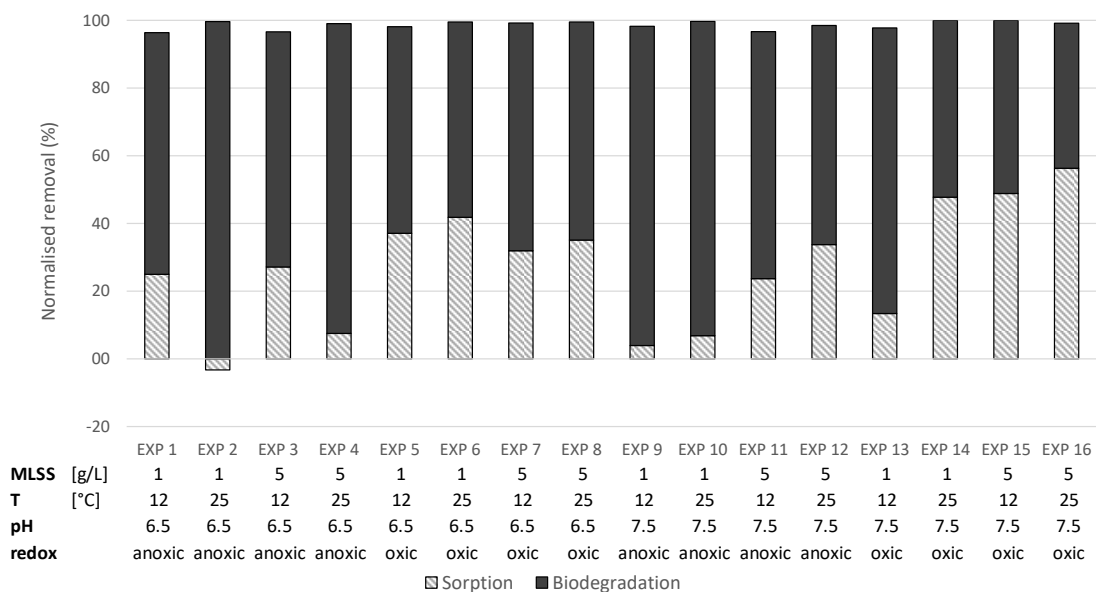
**Figure 6 -** Removal profiles of E2 throughout the experiments, detailing the contributions of sorption and biodegradation.

It is noteworthy that the same order in sorption rates, that is EE2>E2>E1, was observed by Andersen and co-workers as well (Andersen et al., 2005) and reflects the decrease of their average $K_{ow}$ values (E2 $K_{ow}$: $3.1 - 4.13$ (Holthaus et al., 2002; Zorita et al., 2009); E1 $K_{ow}$: 2.25 - 3.69 (Besha et al., 2017; Zorita et al., 2009)). Both for E2 and E1, the sorbed fraction was comprised between 30 and 50% of the total removal in almost all experiments. Previous studies dealing with sorption of hormones onto conventional activated sludge gave diverse and sometimes contradictory results (Hamid and Eskicioglu, 2012; Silva et al., 2012).

As for diclofenac, as commented in paragraph 3.2, adsorption was indeed relevant at pH 6.5, probably due to electrostatic interaction between protonated diclofenac and the negative sludge surface. In batch tests conducted under aerobic conditions at low pH, where the highest removal rates were attained, the contribution of sorption was between 48% and 101.9% of the whole removal rates (see **Figure 7**). This mechanism was also hypothesised in other studies carried out in the same pH range (Ternes et al., 2004; Urase et al., 2005).

**Figure 7** - Removal profiles of diclofenac throughout the experiments, detailing the contributions of sorption and biodegradation.

The single highest sorption was recorded for erythromycin, 80.4%, accounting for approximately the whole removal attained in experiment 16 (25°C, 5 g/L MLSS, pH 7.5 and under aerobic conditions, Figure 3. Nevertheless, its average sorption rate was 19.4%. Medium sorption rates were obtained when operating at pH 6.5 and aerobic conditions, accounting for 100% of the removals achieved in those expe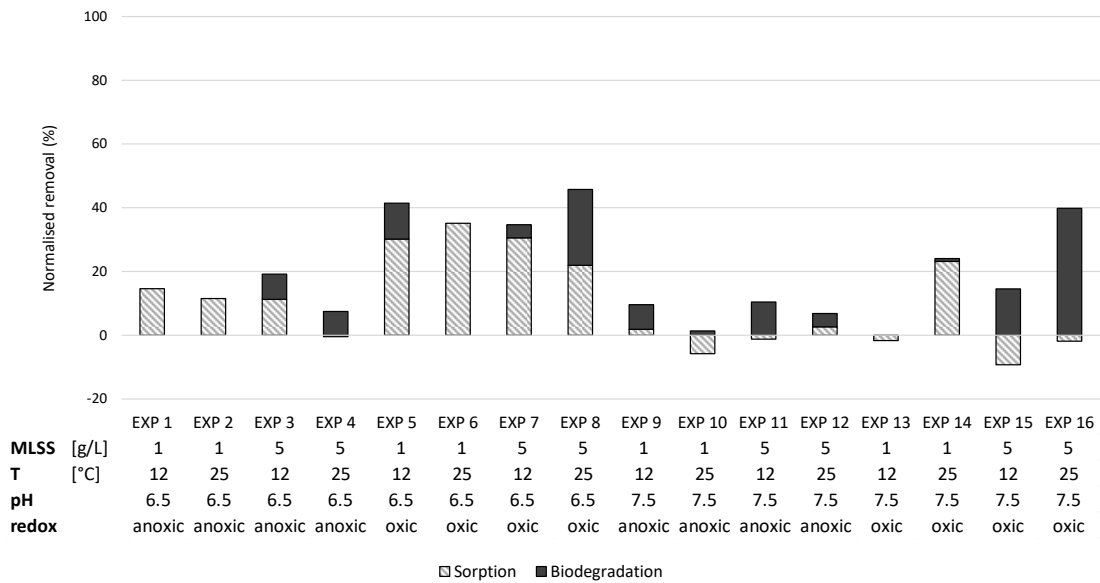riments. Although the overall removal rates increased at pH 7.5, sorption at this pH was generally negligible or low, with the exception of experiments 15 and 16 (5 g/L of MLSS, aerobic conditions and pH 7.5), where sorption rates of 36.1 and 80.4% were obtained, respectively, and accounted for the totality of the removal. Erythromycin has a low $K_{ow}$ value and the relevant sorption mechanism is therefore supposed to be adsorption. Since its pKa is 8.9 (McFarland et al., 1997), erythromycin is predominantly in its protonated form at both working pH values (Wunder et al., 2011), prone to electrostatic interactions with the negative charges on the sludge. At higher pH, the protonated fraction starts decreasing, thus explaining the decrease in sorption rates. On the whole, the sorption behaviour pattern of erythromycin is marked by inconsistent literature data. For example, no biotransformation or sorption were reported from a WWTP in France (Pasquini et al., 2014). On the contrary, in a study conducted in nitrifying and denitrifying reactors, erythromycin removal was always complete, more than 80% of which under aerobic

conditions and less than 10% under anoxic conditions due to sorption (Suarez et al., 2010).

Clothianidin, thiacloprid and methiocarb were marked both by negligible sorption rates, ranging from 5.1 to 10.5%, and lack of literature data. In the only study assessing clothianidin in a real-scale WWTP known to the authors, it was reportedly not sorbed to primary sludge nor removed during secondary treatment (Sadaria et al., 2016).

## 4. Conclusions

Batch tests under different operational conditions were carried out in order to shed light on the fate and behaviour of a set of eight micropollutants chosen from the EU Watch list (Decision 2015/495 and 2018/840). Total removal as well as the role of sorption were assessed. The chosen compounds are marked by inconsistency in literature or, as in the case of pesticides, by lack of information on their biodegradation and sorption in conventional activated sludge. Considering the totality of compounds, the maximum removal was attained under aerobic conditions at high temperature (25 °C), high concentration of biomass (5 g/L) and high pH (7.5). The parameters with the most influence on total removal proved to be the MLSS concentration and redox conditions. The highest removal rates were recorded for E2, with a removal of at least 96% attained in all experiments. Clothianidin and thiacloprid proved recalcitrant. For compounds such as E1, erythromycin and methiocarb, the ranges sweep from zero to very high or complete removals, highlighting the importance of the choice of the right operational parameters, since even minute variations produce a significantly different response. EE2 exhibited the highest sorption rates (34% on average under all conditions), followed by E2 and E1, which were nonetheless removed primarily via biodegradation. Diclofenac was removed at a maximum rate of 45.7% and mostly through adsorption, for which a strong dependence on pH was noticed. These results highlight the importance of the choice of operational parameters during wastewater, which have indeed a great impact on micropollutants removal. More studies, including the evaluation of constants and modelling, are needed to shed light on the mechanisms involved.

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GENERAL DISCUSSION

## Analytical challenges

In the present thesis, a novel methodology for the analysis of the 17 compounds of Decision 2015/495 was developed for the first time for both river and wastewater matrices. The Decision text included indicative methods of analysis and it specified that they should not entail excessive costs. Moreover, it set the PNECs of each substance as maximum method detection limit (EC, 2015). Although the Watch list compounds exhibit very different properties,  it is believed that an analytical methodology grouping all 17 compounds represents a valuable tool to facilitate the job of water utilities and researchers. BHT, EHMC and certain pesticides have traditionally been analysed by GC-MS, but the idea was set aside because we were pursuing a fast methodology that involved as little sample manipulation as possible. In the end, a methodology based on online-SPE ultra-high-performance liquid chromatography coupled to a triple-quadrupole mass spectrometer (UHPLC-MS/MS) was chosen. The online-SPE system allowed to save time and reduce errors, as it eliminated all pre-treatment steps but filtration. Above all, bypassing offline SPE means reducing dramatically sample volume, avoiding the usage of large volumes of toxic solvents, reducing the possibility of analyte loss during evaporation or their degradation during preconcentration. Moreover, using a chromatographic column for preconcentration represents a greener and cheaper alternative, as it lasts for hundreds of injections. Several methods have addressed certain classes of the Watch list compounds in the past years. Some have focused on pharmaceuticals (Gros et al., 2012), antibiotics (Gros et al., 2006; Senta et al., 2008), hormones (Čelić et al., 2017), pesticides (Fenoll et al., 2011; A Masiá et al., 2013; Masià et al., 2013), UV filters (Gago-Ferrero et al., 2011) and antioxidants (Liu et al., 2015a). In 2017, a methodology based on automated online SPE-LC-MS/MS for the determination of 24 compounds of EU relevance, including the majority of the Watch list compounds, in drinking water, freshwater and effluent wastewater was published (Rubirola et al., 2017). The methodology described therein employed disposable trace enrichment cartridges onto which 10 mL samples were loaded and then directly eluted into the LC system, allowing a total time of 30 mins per analysis per polarity (Rubirola et al., 2017). In spite of the undeniable advantages in terms of reduced sample manipulation and time consumption over the traditional online SPE offered by the above-mentioned methodology, sample preconcentration onto a LC column, as proposed in this thesis, overcomes the production

of waste (e.g. the spent cartridges) and allows a further improvement in time of analysis and sample volume injected, in this case as low as 2 mL per sample per polarity. At present, besides the case study presented in Chapter 2, there is only one paper dealing with the monitoring the whole set of the Watch list compounds. In that case, off-line SPE was performed, followed by either UHPLC-MS/MS or GC-MS analysis (Sousa et al., 2019).

On the whole, although the method proposed in this thesis represents a valuable analytical tool, analytical challenges are still open. The MDLs indicated in the Decision, corresponding to PNEC values, varied from 6 µg/L in the case of EHMC to 0.035 ng/L for EE2 (EC, 2015). Overall, 10 out of 17 compounds had 10 ng/L or less as a maximum acceptable MDL, which calls for a good method sensitivity. This adds to the difficulties in finding ionization and chromatographic conditions suitable for compounds displaying diverse properties, as thoroughly remarked in Chapter 1. However, the MDLs obtained in freshwater comply with Decision 2015/495, and in most cases, they are much lower than the indicated values. In fact, over the course of the years, some PNEC values were updated to lower concentrations (Loos et al., 2018) and the methodology proposed herein still complies with the updated MDLs. The only compound which does not fulfil the requirements is EE2, which yielded a MDL of 0.34 ng/L in river water instead of the 0.035 ng/L PNEC. Such a low value could be reached, in a method for the detection of hormones developed by Celic et al., 2017, under specific conditions that enhance the ionization of this compound, such as pH 11 (Čelić et al., 2017). Another possibility to lower the MDL of EE2 is the preconcentration of larger sample volumes using an offline procedure, which however brings about the inconveniences associated with offline SPE. The 2018 Joint Research Centre (JRC) document also reports unsatisfactory data quality for EE2 because 12 member states could not achieve the required MDLs. This is also the reason for the inclusion of EE2 in the Watch list of Decision 2018/840 (Loos et al., 2018). A member state which succeeded in achieving the low MDL for EE2 reportedly extracted only 400 mL of water by liquid-liquid extraction and used a GC-MS-MS instrument of the latest generation (following the EPA Method 1698 for derivatization with trimethylsilyl-ether). Another member state employed SPE-GC-MS-MS without disclosing further details, while another combined the extraction of 1 L of water by SPE with Oasis HLB cartridges with LC-MS-MS analysis (Loos et al., 2018). Although it is undoubtfully possible

to reach all the required MDLs, these are currently incompatible with the employment of a single multi-residue method that allows the simultaneous determination of the Watch list compounds in a fast, efficient, trustful and cheap way.

## Assessment of WWTPs efficiency: is there room for improvement?

After the method was developed and validated, it was applied to investigate the presence of the Watch list compounds in the Ebro delta to evaluate the extent of their occurrence (Chapter 2 of Results). The area was chosen for its natural significance and economical importance. The Ebro is the second longest river in the Iberian Peninsula and it streams through southern Catalonia before discharging into the Mediterranean Sea. The Ebro delta is a fragile ecosystem where the main stretch of the river flows through a highly cultivated area, however rich in marshes, irrigation channels and lagoons. This makes it a unique area for fauna conservation, especially migratory birds. However, biodiversity might be jeopardised by anthropogenic contamination. Three sampling campaigns were performed to assess seasonal variations. Besides the Ebro and tributary channels and irrigation ditches, the influent and effluent wastewaters of the two main WWTPs of the area were also monitored. WWTP Amposta is equipped with secondary treatment, while WWTP Sant Carles de la Ràpita also features sand filters as tertiary treatment. pH values were 7.7, 8.0 and 8.2 in SC1, SC2 and SC3, respectively, and the mean temperatures were 21.2, 13.2 and 25.5 °C in the three sampling campaigns, respectively. The single values are displayed in Table S2 in Chapter 2 of the Annex.

Besides, a set of batch tests were carried out to explore the (bio)degradability in CAS of 8 compounds of Decision 2015/495 under different operational conditions (Chapter 3). The combinations of two levels of pH, biomass concentration, redox conditions and temperature were explored with the aim of finding the most relevant factors affecting removal and the optimum conditions for maximised degradation. The chosen chemicals were: clothianidin, thiacloprid, methiocarb, erythromycin, diclofenac, E1, E2 and EE2.

In this section, the findings of Chapter 2 and 3 will be discussed and compared, in order to assess whether the studied compounds represent a threat for the environment and if they can be removed by means of conventional treatments.

General discussion

In Chapter 2, the analyses of influent and effluent wastewaters of the two WWTPs revealed that some compounds were never present above the method quantification limits (MQLs), including thiacloprid, methiocarb and EE2, which makes impossible the comparison with the other work for these chemicals. However, diclofenac was quantified in 100% of samples and at the second highest mean concentration (636 ng/L), whereas the concentration and occurrence frequency for the rest of compounds can be consulted in Table S8 and S9 in Chapter 2 of the Annex. The concentration levels of erythromycin in WWTPs influents were less than 55 ng/L in all samples, therefore much lower than time-zero concentrations of the batch tests. It was negatively removed in WWTP Amposta, with a mean increase in concentration of 52%, probably due to its conversion into erythromycin-$H_2O$ (Dolar et al., 2012; Hirsch et al., 1999). In WWTP Sant Carles de la Ràpita, instead, its removal was poor (4.9%) in SC1, negative in SC2 (-9.2%) and almost complete in SC3 (99.8%) with the highest temperature among the sampling campaigns. A dependence of erythromycin degradation on temperature was observed in Chapter 3, which could at least partially explain these results. However, erythromycin is marked by huge variations in removal values, as evidenced in several studies (Kim et al., 2014; Krzeminski et al., 2019; Pasquini et al., 2014; Verlicchi et al., 2012). The average removal efficiency in real WWTPs (Chapter 2) for Diclofenac was 47%, with values ranging from 11.7% to 64.1%, actually similar to the ones found in Chapter 3 (from null to 45.7%). Clothianidin was only quantified in WWTP Sant Carles in the second sampling campaign, where a 25% removal was observed. The maximum removal attained in the batch tests was slightly lower (15.2%), but it must be remarked that clothianidin concentrations in real wastewater samples were too low to be compared with the 2 μg/L spike of batch tests. E2 was present only in the influent of WWTP Sant Carles in the third sampling campaign, at a concentration of 25 ng/L, and it was fully degraded, in line with the findings of Chapter 3. The production of few ng/L of E1 was observed in 4 out of 6 samples, probably ascribable to the oxidation of E2 (Johnson and Sumpter, 2001), but removals of 58.0% and 100% in Amposta during SC2 and Sant Carles in SC3 were reported, respectively. Taking into account the temperatures registered in each sampling campaign, these results are in consistency with the ranges observed in the (bio)degradation study at 12 and 25°C, respectively.

Overall, the data obtained in Chapter 2 are compatible with the findings of Chapter 3. As remarked in the discussion of the monitoring study, the removal of the Watch list compounds in the two investigated WWTPs was generally unsatisfactory. This, jointly with the pollution generated upstream the studied river stretch and the contamination from pesticides, likely caused by runoff, led to fairly high concentrations of the Watch list compounds in freshwater. Risk quotients were calculated dividing the measured environmental concentrations by PNECs. Results, displayed in Tables S11-S13 in Supplementary Material of Chapter 2 of Annex, show total RQs, calculated as the sum of the RQ values of each compound, between 40 and 207 for wastewater samples and up to 169 in freshwater sampling sites. Moreover, all freshwater sites were subject to high risk (RQ>1) with the only exception of three locations during SC2. Given that the operational parameters adopted in experiment 16 (aerobic conditions, 5 g/L of mixed liquor volatile suspended solids (MLVSS), 25°C and pH 7.5) were found to maximise (bio)degradation and that these conditions are easily attainable in any wastewater treatment plant, it is thought that WWTPs efficiency towards the elimination of the Watch list compounds could improve applying different operative conditions. In fact, the concentrations of wastewater effluents detailed in Chapter 2 were recalculated for the chemicals studied in Chapter 3, using the removal rates of experiment 16, which were found to be the conditions leading to the highest overall removal. For each of the compounds investigated in Chapter 3, all WWTP influent concentrations were multiplied by the removal rate obtained in experiment 16 of Chapter 3, thus obtaining the hypothetically removed fraction. The hypothetical effluent concentration was calculated as the difference between the concentration in the influent and the hypothetically removed fraction. Results show a decrease in RQs by 7 points in total (see Table 7), with improvement for all samples but those collected in SC3, where the higher temperature or pH could have been even more beneficial for micropollutants removal. Further studies are needed to confirm these hypotheses, including an in-depth risk assessment and the exploration of the degradation potential for the whole set of compounds.

**Table 7.** Comparison between RQs calculated from measured effluent concentrations (Chapter 2) and hypothetical RQs calculated for concentrations hypothesised on the basis of the removal efficiencies obtained in Chapter 3.

| | SC1-02 measured conc (ng/L) | RQ | SC1-04 measured conc (ng/L) | RQ | SC1-02 hypothetical conc (ng/L) | hypothetical RQ | SC1-04 hypothetical conc (ng/L) | hypothetical RQ |
|---|---|---|---|---|---|---|---|---|
| Erythromycin | 40.5 | 0.203 | 34.7 | 0.174 | 6.5 | 0.0327 | 8.1 | 0.0407 |
| DCF | 614 | 12.3 | 484.1 | 9.68 | 526.5 | 10.5 | 410.7 | 8.21 |
| E1 | 10.1 | 2.81 | 8.8 | 2.44 | <LOD | n.a. | <LOD | n.a. |
| E2 | <LOD | n.a. | <LOD | n.a. | <LOD | n.a. | <LOD | n.a. |
| Clothianidin | 3.32 | 0.0255 | <LOD | n.a. | <LOD | n.a. | <LOD | n.a. |
| TOTAL RQ | | 15.3 | | 12.3 | ✔ | 10.6 | ✔ | 8.3 |

| | SC2-02 measured conc (ng/L) | RQ | SC2-04 measured conc (ng/L) | RQ | SC2-02 hypothetical conc (ng/L) | hypothetical RQ | SC2-04 hypothetical conc (ng/L) | hypothetical RQ |
|---|---|---|---|---|---|---|---|---|
| Erythromycin | 52.4 | 0.262 | 29.7 | 0.149 | 6.49 | 0.032 | 6.07 | 0.0303 |
| DCF | 231.1 | 4.62 | 200.3 | 4.01 | 161 | 3.227 | 136 | 2.73 |
| E1 | 9.4 | 2.61 | 9.5 | 2.64 | <LOD | n.a. | <LOD | n.a. |
| E2 | <LOD | n.a. | <LOD | n.a. | <LOD | n.a. | <LOD | n.a. |
| Clothianidin | <LOD | n.a. | 25.4425 | 0.196 | <LOD | n.a. | 31.9 | 0.245 |
| TOTAL RQ | | 7.49 | | 6.99 | ✔ | 3.26 | ✔ | 3.01 |

| | SC3-02 measured conc (ng/L) | RQ | SC3-04 measured conc (ng/L) | RQ | SC3-02 hypothetical conc (ng/L) | hypothetical RQ | SC3-04 hypothetical conc (ng/L) | hypothetical RQ |
|---|---|---|---|---|---|---|---|---|
| Erythromycin | 53.7 | 0.268 | <LOD | n.a. | 8.52 | 0.0426 | 6.5 | 0.0324 |
| DCF | 552 | 11.0 | 560.7 | 11.2 | 925 | 18.5 | 842.8 | 16.9 |
| E1 | 9.60 | 2.67 | <LOD | n.a. | <LOD | n.a. | <LOD | n.a. |
| E2 | <LOD | n.a. | <LOD | n.a. | <LOD | n.a. | <LOD | n.a. |
| Clothianidin | <LOD | n.a. | <LOD | n.a. | 7.74 | 0.0596 | <LOD | n.a. |
| TOTAL RQ | | 14.0 | | 11.2 | ✖ | 18.6 | ✖ | 16.9 |

| | |
|---|---|
| Sum of measured RQs | 67.3 |
| Sum of hypothetical RQs | 60.6 |

## Legislation gaps

As mentioned in section 1.3.1, the Watch list compounds are currently undergoing monitoring in freshwater only. These data are going to be used for risk assessment, which will be the basis of future prioritization. However, the absence of a systematic monitoring of these pollutants in wastewater at EU level means that a crucial piece of information is being disregarded. First of all, it is of primary importance to establish a link between the presence and fate of contaminants of emerging concern in wastewater and their occurrence in freshwater. Since WWTPs are known to play a key role as point sources of contamination, this will help pinpoint the entry pathways of the Watch list compounds into the environment. Moreover, information on the occurrence of these pollutants in wastewater will also give insights on WWTPs performances.

For example, in Switzerland, the Water Protection Ordinance set, in 2016, that WWTPs with certain characteristics (population equivalents (PE), nearness to lakes, discharge of effluents into a wastewater dominated watercourse…) should be upgraded with

advanced treatment (ozonation or powdered activated carbon) by 2035 in order to ensure the removal of at least 80% of micropollutants loads. To keep track of WWTPs efficiency, 12 compounds, including clarithromycin and diclofenac, that are usually not biologically removed but are degradable in both the proposed technologies, were chosen as indicators. Each canton has to choose 5 for monitoring through sampling campaigns (8-24 samples per year depending on the size of each WWTP) and for each sampling the arithmetic mean of the individual removal efficiency of the 5 selected compounds has to be 80%.

Another important reason for analysing contaminants of emerging concern in wastewater is the assessment of effluents quality for wastewater reuse and/or direct or indirect potable reuse. The European Commission put forward a proposal for a regulation setting EU standards for reclaimed water in May 2018. This proposal is based on scientific evidence gathered in a JRC report (JRC et al., 2017) and is strongly related to the 2015 circular economy action plan (European Commission, 2015), the seventh environment action programme (The European Parliament and the Council of the EU, 2013), and, globally, to the UN's sustainable development goals. The aim is to reduce water stress by promoting the use of treated wastewater in agriculture proposing a regulation that sets minimum quality standards. According to the European Environment Agency, agriculture irrigation accounts for approximately 50% of the water consumed in the EU. At present, Cyprus, France, Italy, Greece and Spain have legislation setting requirements for wastewater reuse; Portugal has nonregulatory standards on reused-water quality. It is believed that the adoption of a unified regulation for all member states would encourage the free circulation of produce irrigated with reclaimed water (JRC et al., 2017). The proposed requirements include microbiological parameters (presence of pathogens: *E. coli*, *Legionella spp*. and intestinal nematodes) and physicochemical parameters (biochemical oxygen demand, total suspended solids and turbidity), but micropollutants are not mentioned. Another limitation lies in the fact that the proposed legislation would consider irrigation as the only application, therefore disregarding possible utilizations in potable reuse, direct or indirect and/or in nature-based solutions (NBS), now among the prioritized solutions at EU level. Quality standards would be set according to the fit-for purpose approach, depending on crop category and irrigation method. A risk management plan would also be established (JRC et al., 2017). It is important to highlight

that this proposal considers solely reclaimed wastewater, that is, water complying with the quality standards detailed in the Urban Wastewater Treatment Directive, excluding other possible sources (e.g. harvested rainwater, greywater, etc). Knowing which contaminants are present in wastewater, and at which concentrations, might allow a broader range of reuse perspectives.

The fact that micropollutants in wastewater have not been targeted by any European legislation yet is a significant omission. On the one hand, it will delay the adoption of a common regulatory framework in the EU for water reuse/greywater/harvested rainwater or hinder its potential. This could prevent from implementing new technologies, practises and solutions (e.g. NBS); on the other hand, the lack of regulations on micropollutants might bring to underestimate the risk posed by such compounds.

Other countries outside Europe, such as California (California State Water Resources Control Board, 2013), Australia (Australian Government, 2018) and Singapore (PUB, 2018) have implemented cutting edge policies on water reuse, including specific regulations on greywater and direct or indirect potable reuse. In some cases, micropollutants are also monitored. The legislation of these countries might pave the way for implementation at EU levels.

# FUTURE PERSPECTIVES

The topic of organic micropollutants is a huge puzzle from which several pieces are still missing. Although it has been thoroughly explored for about twenty years, several challenges are still open.

In regard to analytical chemistry, the next few years will likely see the rise of non-target approaches. With a more widespread use of HR instrumentation and an improvement of methodologies to enhance the reliability of detection, that is, identifying analytes at high levels of confidence, suspect and non-target screening will take over the traditional target approaches. This would be particularly useful, as the portion of micropollutants that we are currently addressing is only an infinitesimal fraction of the chemicals that are likely to be present in the environment. The Chemical Abstract Service contains over 100 million entries, the REACH legislation in the EU reports that Europe produces or imports around 140 000 chemicals, and its US analogue, the Toxic Substances Control Act, includes about 85 000 compounds (Hollender et al., 2017). It has been estimated that between 30 000 and 70 000 chemicals, including pharmaceuticals, pesticides and PCPs, are used in household contexts alone (Schwarzenbach et al., 2006). In light of this, it is evident that the 76 priority substances and the 17 Watch list candidates at EU level and the 126 priority pollutants addressed by the USA Clean Water Act are merely a drop in the ocean. Although several thousands of chemicals have been detected in the environment, it is clear that such a number of analytes cannot be targeted in the traditional way, let alone the TPs conundrum.

The monitoring of the Watch list compounds in the aquatic environment evidenced that they represent a potential threat to the environment, as their concentration levels were potentially perilous in many a sampling location in the studied area. Tracking the occurrence of relevant micropollutants in the water cycle, especially in fragile ecosystems and not only in freshwater is, at present, of paramount importance. Moreover, as largely explained in the general discussion, assessing the occurrence of contaminants in wastewater is the best way to receive precise feedback on WWTPs efficiency. The sooner this matter is included in the EU regulatory framework, the sooner we will be able to promote the implementation of cutting-edge water policies.

Future perspectives

The (bio)degradation study in CAS has evidenced that the Watch list compounds are generally removed at unsatisfactory rates, with the exception of E2 and E1. However, further studies are needed to disclose their fate in wastewater, including all 17 chemicals. Although the application of tertiary treatment might be needed in some cases to ensure good effluent quality, the enhancement of biodegradation during secondary treatment could be a key technology to develop (Garcia-Becerra and Ortiz, 2018), especially knowing that removal rates can vary dramatically by slightly toggling the operational conditions in WWTPs.

To further improve micropollutants biodegradation, several path can be followed. An example is treatment with specific microbial species or communities, as proposed in recent studies (Kassotaki et al., 2018; Tran et al., 2013b). Exploring the behaviour of heterogeneous microbial communities and studying microbial consortia in engineered systems, including determining the nature of the interactions among microorganisms, that can be either positive or antagonistic, and their combined treatment capabilities might bring about significant improvements.

In addition, or as an alternative, tertiary treatments can be considered. Advanced oxidation processes and adsorption onto activated carbon have been identified as technologies with potential for large-scale applications in terms of efficiency, cost and energy consumption (Margot et al., 2015). Other possibilities that can be taken into account, and whose micropollutants removal capabilities as well as their associated risks should be evaluated, include NBS as an alternative to conventional engineering solutions, decentralised treatment systems and hybrid systems.

As mentioned before, the topic of transformation products needs more attention. Some TPs are known to be more abundant in the aquatic environment than their parent compounds and most TPs present in water have not even been identified yet (Boxall, 2009; Escher and Fenner, 2011). Considering that these chemicals may be more persistent and/or toxic than their parent compounds (Jaén-gil et al., 2018), it becomes evident that the issue cannot be ignored. The environmental risk related to the presence of TPs adds further complexity to the unknown cocktail of chemicals that humans and aquatic ecosystems are exposed to (Escher and Fenner, 2011). It is therefore of

paramount importance to i) dedicate more efforts to the development of reliable and simple analytical methodologies, ii) unravel their formation pathways, iii) disclose their toxicity and include them in risk assessment.

An important tool is represented by modelling. Quantitative structure–activity relationship (QSAR) models aim to link removal rates to micropollutants functional groups. At present, there is an extensive body of studies addressing several aspects including biodegradation products, the estimation of micropollutants physicochemical properties and degradation half-lives, the calculation of persistence metric and the joint persistence (Garcia-Becerra and Ortiz, 2018). However, QSAR models to investigate the biodegradability of heterogenous mixes of micropollutants and metabolites/TPs have yet to be developed. In addition, there is a need for models focussing on kinetics and metabolic pathways, which in turn might suggest how to approach these concepts experimentally. For now, it is crucial to monitor micropollutants in the water cycle.

Other fields that might lead to advances in the matter of micropollutants are ecotoxicology and ecological risk assessment, which demonstrate the potential of chemicals to cause environmental damage to organisms and affect the functions of ecosystems. Understanding how physicochemical properties and biological toxicity of micropollutants change in complex mixtures is also important to elucidate. Besides, alternative control strategies such as segregation of sources; development of more environmentally friendly substances; improvement of drug disposal; more considerate prescription of pharmaceuticals, antibiotics and hormones as well as rising users' awareness to avoid overuse of substances; promotion of best management practices must be developed.

The data collected in this thesis highlight the importance of a multidisciplinary approach, where analysis, monitoring and removal studies are key aspects.

# CONCLUSIONS

A summary of the main conclusions of each chapter of this thesis is presented below.

Chapter I:

**Development of an online SPE-UHPLC-MS/MS method for the multiresidue analysis of the 17 compounds from the EU "Watch List".**

- A methodology based on online-SPE-UHPLC-MS/MS for the preconcentration and determination of the 17 compounds listed in the EU Decision 2015/495 was developed.

- The method was validated in freshwater, as required by the EU Decision, but also in influent and effluent wastewater. The analysis in PI and NI mode lasted 8 and 10.5 minutes, respectively.

- The MDL obtained in freshwater were in the sub- and low- ng/L ranges, complying with the EU requirements in all cases, with the only exception of EE2.

Chapter II:

**The EU Watch list compounds in the Ebro Delta region: assessment of sources, river transport, and seasonal variations**

- The novel method was applied in a spatiotemporal monitoring carried out in the Ebro Delta in South Catalonia, Spain. The study included 14 sampling sites comprising of the two main WWTPs of the area, the main stretch of the Ebro river and several channels and irrigation ditches.

- For the first time triallate was reported in wastewater and the whole set of Watch list compounds were reported both in freshwater and wastewater.

- Pharmaceuticals, and in particular azithromycin and diclofenac, were the most abundant in all matrices. Pesticides, especially oxadiazon and imidacloprid, were ubiquitous in freshwater. Seasonal variations were detected in freshwater for some compounds (like pesticides) but not in wastewater.

- The most polluted site was the emissary of Sant Carles de la Ràpita, a wastewater dominated stream, with a total concentration of 2.39 μg/L.

However, pollution was encountered also in the control site, located upstream the WWTP of Amposta.

- The two investigated WWTPs were generally not able to eliminate the Watch list compounds at satisfactory rates. The highest removal efficiencies were recorded for diclofenac, with an average of 47% removal.

- The Watch list compounds occurred at concentrations largely above PNEC values in most freshwater and wastewater samples. The assessment of total RQ values evidenced that the majority of sites are subject to high risk (total RQ>1).

Chapter III:

How do WWTPs operational parameters affect the removal rates of EU Watch list compounds?

- The fate and behaviour of 8 Watch list compounds was investigated in a set of conventional activated sludge batch tests under different operational conditions.

- The maximum overall removal was attained under aerobic conditions, at high temperature (25 °C), high concentration of biomass (5 g/L) and high pH (7.5). The parameters that affect removal the most were MLVSS and redox conditions.

- E2 was the compound with the highest removal rate (always above 96%). Clothianidin and thiacloprid proved recalcitrant, while for compounds such as E1, erythromycin and methiocarb, results ranged from zero to high or complete removals, according to the operational parameters.

- The compound with the highest sorption rates proved to be EE2 (average 34%) under all conditions, followed by E2 and E1, which were however primarily biodegraded. Sorption proved to be a relevant removal route for many a compound, including diclofenac, which was removed at a maximum rate of 45.7% and mostly via adsorption, which a strong dependence on pH.

- The choice of operational parameters in wastewater treatment proved crucial, as even minute variations have a great impact on micropollutants removal.

ANNEX

# SUPPLEMENTARY MATERIAL OF CHAPTER 2

## The EU Watch list compounds in the Ebro delta region: assessment of sources, river transport, and seasonal variations

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**Table S1.** Sampling sites information

| Sampling site ID number | Sample name | Type of water | Coordinates (WGS84) | |
|---|---|---|---|---|
| | | | X | Y |
| 1 | Amposta WWTP IN | influent | 0.608608 | 40.704057 |
| 2 | Amposta WWTP OUT | effluent | 0.608608 | 40.704057 |
| 3 | Sant Carles de la Ràpita WWTP IN | influent | 0.622121 | 40.627036 |
| 4 | Sant Carles de la Ràpita WWTP OUT | effluent | 0.622121 | 40.627036 |
| 5 | Upstream of Amposta (Benifallet) | river | 0.518484 | 40.976154 |
| 6 | Downstream of Amposta | river | 0.689317 | 40.713142 |
| 7 | Sant Carles de la Ràpita emissary | river | 0.622754 | 40.622579 |
| 8 | Left channel* | channel | 0.809254 | 40.706929 |
| 9 | Right channel | channel | 0.806554 | 40.696697 |
| 10 | Maritime channel | channel | 0.598582 | 40.622214 |
| 11 | Drainage channel 1 (Sèquia gran) | channel | 0.647442 | 40.631728 |
| 12 | Drainage channel 2 (Desaigüe de la fortalesa) | channel | 0.681305 | 40.642791 |
| 13 | Drainage channel 3 (Desaigüe del riuet) | channel | 0.789064 | 40.661281 |
| 14 | Drainage channel 4 (Desaigüe de les magdalenes) | channel | 0.717146 | 40.646098 |

*not available in SC2

## Table S2. Physicochemical parameters for each sampling point.

| Sample name | 1st sampling campaign | | | | | | | 2nd sampling campaign | | | | | | | 3rd sampling campaign | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T | pH | $O_2$ | Conductivity | Salinity | Flow* | Corg* | T | pH | $O_2$ | Conductivity | Salinity | Flow* | Corg* | T | pH | $O_2$ | Conductivity | Salinity | Flow* | Corg* |
| | (°C) | | (mg/L) | (µS/cm) | (ppt) | (m³/s) | (%) | (°C) | | (mg/L) | (µS/cm) | (ppt) | (m³/s) | (%) | (°C) | | (mg/L) | (µS/cm) | (ppt) | (m³/s) | (%) |
| Amposta WWTP IN | 21.7 | 7.8 | 9 | 1362 | 0.7 | N/D | x | 11.9 | 8.2 | 8.6 | 1290 | 0.6 | N/D | x | 23.5 | 7.9 | 9.6 | 682 | 0.3 | N/D | x |
| Amposta WWTP OUT | 20.3 | 7.6 | 7.8 | 2690 | 1.4 | N/D | x | 12.6 | 7.7 | 9.6 | 1344 | 0.6 | N/D | x | 24.7 | 8.5 | N/A | 1433 | 0.7 | N/D | x |
| Sant Carles de la Ràpita WWTP IN | 20.9 | 7.4 | 7.4 | 20046 | 11.9 | N/D | x | 14.6 | 7.8 | 12.9 | 3837 | 2.6 | N/D | x | 28 | 7.7 | N/A | 3940 | 2.1 | N/D | x |
| Sant Carles de la Ràpita WWTP OUT | 21.8 | 7.8 | 8 | 1190 | 0.6 | N/D | x | 13.7 | 8.4 | 9.1 | 1332 | 0.6 | N/D | x | 25.8 | 8.7 | N/A | 671 | 0.3 | N/D | x |
| Upstream of Amposta (Benifallet) | 21.9 | 7.9 | 9.7 | 1189 | 0.6 | 161 | 3.07 | N/A | N/A | N/A | N/A | 1.2 | 447 | 2.36 | 25.9 | 8.5 | N/A | 672 | 0.3 | 166 | 0.59 |
| Downstream of Amposta | 21.3 | 7.5 | 12.9 | 2933 | 1.5 | 161 | 2.45 | 19 | 7.6 | 15.1 | 2791 | 1.7 | 447 | 1.87 | 23.6 | 7.7 | 12.8 | 2151 | 1.1 | 166 | 1.02 |
| Sant Carles de la Ràpita emissary | 17.1 | 7.1 | 5.1 | 2100 | 1.1 | N/D | 3.23 | 15.3 | 7.8 | 10.9 | 2765 | 1.8 | N/D | 3.94 | 23.7 | 7.6 | 7.1 | 2595 | 1.4 | N/D | 4.8 |
| Left channel* | 17 | 7.3 | 6.6 | 1800 | 0.9 | 19 | 1.63 | 20.1 | 7.9 | 20.9 | 27049 | 18.5 | 19 | 5.57 | 22.7 | 7.5 | 6.4 | 3801 | 2.1 | 19 | 5.9 |
| Right channel | 14.9 | 7.2 | 6.2 | 1500 | 0.8 | 25 | 1.71 | 12 | 7.3 | 10.4 | 8762 | 6.7 | 0 | 0 | 24.6 | 7.8 | 7.4 | 1451 | 0.7 | 30 | 2.1 |
| Maritime channel | 14.9 | 7.4 | 4.2 | 1476 | 0.7 | N/D | 3.24 | 16.8 | 8.2 | 25.6 | 13499 | 9.5 | N/D | 4.71 | 25.2 | 7.6 | 13.3 | 17502 | 10.3 | N/D | 3.09 |
| Drainage channel 1 | 17.3 | 7.6 | 7.4 | 35971 | 22.7 | N/D | 4.27 | N/A | N/A | N/A | N/A | 29.5 | N/D | 5.59 | 31.6 | 8.2 | 7.7 | 65759 | 38.4 | N/D | 4.85 |
| Drainage channel 2 | N/A | N/A | N/A | N/A | 30.3 | N/D | 3.2 | 10.7 | 7.9 | 11.2 | 37256 | 33.5 | N/D | 3.61 | 28.1 | 8 | 6.4 | 59303 | 37.1 | N/D | 1.45 |
| Drainage channel 3 | 19.2 | 7.8 | 7 | 45332 | 29.3 | N/D | 4.28 | 12.2 | 7.9 | 10.5 | 39462 | 34.3 | N/D | 5.78 | 28 | 8 | 6.1 | 59344 | 37.2 | N/D | 3.27 |
| Drainage channel 4 | N/A | N/A | N/A | N/A | 29.8 | N/D | 2.63 | 12.6 | 7.8 | 10.1 | 40346 | 34.7 | N/D | 3.84 | 27.5 | 8 | 6.5 | 59939 | 37.9 | N/D | 2.9 |

N/A: not analysed

N/D: not available

*Mean values obtained by SAIH Ebro

x: unavailable sampling point

**Table S3.** Acquisition parameters: target compounds, corresponding internal standard (IS), ESI polarity, precursor ions, SRM transitions and collision energies (CE). Taken from Gusmaroli et al., 2018.

| Target compound | IS | Polarity | Precursor ion | SRM 1 | CE (EV) | SRM 2 | CE (EV) |
|---|---|---|---|---|---|---|---|
| EHMC | Oxybenzone-phenyl-$d_5$ | + | 291.2 | 161 | 16 | 179 | 5 |
| Erythromycin | Erythromycin-(N,N-dimethyl-$^{13}C_2$) | + | 734.1 | 158 | 39 | 576 | 19 |
| Clarithromycin | Erythromycin-(N,N-dimethyl-$^{13}C_2$) | + | 748.2 | 158 | 41 | 116 | 41 |
| Azithromycin | Erythromycin-(N,N-dimethyl-$^{13}C_2$) | + | 376.7 | 83.1 | 19 | 158.1 | 21 |
| Methiocarb | Methiocarb-N-methyl-$d_3$ | + | 226.1 | 121 | 16 | 169 | 5 |
| Imidacloprid | Imidacloprid-$d_4$ | + | 256.1 | 209 | 16 | 175 | 17 |
| Thiacloprid | Imidacloprid-$d_4$ | + | 253 | 126 | 32 | 90 | 35 |
| Thiamethoxam | Thiamethoxam-$d_3$ | + | 292 | 211 | 10 | 181 | 20 |
| Clothianidin | Clothianidin-$d_3$ | + | 250 | 169 | 11 | 132 | 14 |
| Acetamiprid | Imidacloprid-$d_4$ | + | 223. 1 | 90 | 32 | 126 | 12 |
| Oxadiazon | Oxybenzone-phenyl-$d_5$ | + | 233.9 | 151.1 | 26 | 110.1 | 18 |
| Triallate | Oxybenzone-phenyl-$d_5$ | + | 304 | 143 | 25 | 86 | 15 |
| EE2 | EE2-$d_4$ | - | 295 | 145 | 41 | 159 | 33 |
| E2 | Estradiol-$d_2$ | - | 271 | 183.2 | 42 | 145.2 | 43 |
| E1 | Estrone-$d_4$ | - | 269 | 145 | 38 | 143 | 54 |
| Diclofenac | Diclofenac-$d_4$ | - | 294 | 250 | 14 | 214 | 22 |
| BHT | BHT-$d_{21}$ | - | 219.1 | 203.3 | 30 | 163.2 | 32 |

**Table S4.** Analytical method performance parameters: method detection and quantification limits (MDL and MQL), recovery (calculated in triplicate for a spiked concentration of 100 ng/L) and precision in all matrices.

| Compound | MDLs (ng/L) | | | MQLs (ng/L) | | | % Recovery (%RSD, 100 ng/L) (n=3) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | River | Effluent | Influent | River | Effluent | Influent | River | | Effluent | | Influent | |
| Acetamiprid | 0.35 | 0.59 | 0.53 | 1.15 | 1.97 | 1.78 | 99.7 | 1.2 | 99.9 | 5.1 | 98.1 | 2.0 |
| Azithromycin | 0.24 | 0.13 | 0.15 | 0.80 | 0.42 | 0.49 | 102 | 2.6 | 102 | 5.4 | 99.8 | 5.6 |
| BHT | 9.73 | 12.3 | 12.3 | 32.4 | 41.1 | 41.1 | 101 | 7.8 | 100 | 0.6 | 104 | 2.6 |
| Clarithromycin | 0.06 | 0.01 | 0.06 | 0.20 | 0.04 | 0.20 | 99.2 | 2.9 | 90.3 | 5.7 | 102 | 3.1 |
| Clothianidin | 0.16 | 0.77 | 1.95 | 0.52 | 2.56 | 6.50 | 100 | 4.3 | 99.3 | 2.5 | 102 | 5.4 |
| Diclofenac | 0.33 | 0.58 | 0.56 | 1.58 | 1.94 | 1.88 | 103 | 6.6 | 110 | 2.6 | 99.7 | 5.5 |
| EE2 | 0.34 | 2.28 | 0.69 | 1.13 | 7.60 | 2.30 | 92.1 | 2.7 | 98.8 | 2.8 | 107 | 6.1 |
| EHMC | 0.16 | 0.34 | 1.00 | 0.54 | 1.13 | 3.32 | 102 | 15 | 101 | 2.8 | 99.1 | 5.0 |
| Erythromycin | 0.18 | 0.10 | 0.29 | 0.59 | 0.33 | 0.98 | 100 | 1.6 | 94.5 | 2.1 | 98.6 | 7.0 |
| Estradiol | 0.15 | 0.46 | 0.47 | 0.51 | 1.55 | 1.56 | 96.5 | 5.4 | 99.2 | 3.3 | 98.8 | 3.5 |
| Estrone | 0.08 | 0.15 | 0.60 | 0.26 | 0.48 | 1.99 | 98.5 | 0.0 | 103 | 1.4 | 100 | 9.7 |
| Imidacloprid | 0.17 | 0.07 | 0.12 | 0.58 | 0.23 | 0.39 | 101 | 2.8 | 101 | 8.8 | 99.2 | 0.8 |
| Methiocarb | 0.23 | 0.55 | 0.51 | 0.77 | 1.82 | 1.68 | 99.4 | 2.7 | 99.8 | 3.8 | 98.8 | 1.0 |
| Oxadiazon | 0.05 | 0.17 | 0.08 | 0.17 | 0.58 | 0.26 | 99.8 | 0.2 | 101 | 4.0 | 97.3 | 4.0 |
| Thiacloprid | 0.06 | 0.18 | 0.23 | 0.21 | 0.61 | 0.75 | 101 | 1.9 | 102 | 3.7 | 102 | 0.6 |
| Thiamethoxam | 0.18 | 0.70 | 0.42 | 0.59 | 2.32 | 1.40 | 99.9 | 1.5 | 100 | 5.1 | 96.6 | 13 |
| Triallate | 0.12 | 0.28 | 0.34 | 0.39 | 0.93 | 1.15 | 101 | 1.5 | 99.6 | 2.0 | 99.8 | 4.2 |

**Table S5.** Individual concentrations of the Watch list compounds in freshwater in the first sampling campaign

| | SC1-05 | SC1-06 | SC1-07 | SC1-08 | SC1-09 | SC1-10 | SC1-11 | SC1-12 | SC1-13 | SC1-14 |
|---|---|---|---|---|---|---|---|---|---|---|
| Azithromycin | < MDL | 579 ± 14 | 719 ± 85 | < MDL | < MDL | < MDL | 10.2 ± 0.4 | < MDL | 22.6 ± 0.6 | 12.7 ± 0.4 |
| Clarithromycin | 4.00 ± 0.66 | 159 ± 3 | 129 ± 1 | 4.30 ± 0.14 | 7.16 ± 1.1 | 6.30 ± 0.01 | < MDL | 4.99 ± 0.16 | < MDL | 13.5 ± 1.9 |
| Erythromycin | 20.8 ± 2.9 | 3.54 ± 0.76 | 14.8 ± 0.2 | < MDL | 2.94 ± 0.30 | 2.50 ± 0.20 | 2.75 ± 0.33 | 3.21 ± 0.07 | < MDL | < MDL |
| DCF | 7.69 ± 0.84 | 235 ± 26 | 273 ± 5 | < MDL | < MDL | 25.0 ± 0.87 | < MDL | < MDL | 84.5 ± 9.2 | < MDL |
| E1 | < MDL | 1.06 ± 0.03 | 1.76 ± 0.28 | < MDL | 1.19 ± 0.01 | 1.74 ± 0.16 | 2.80 ± 0.23 | 6.82 ± 0.62 | 6.90 ± 0.18 | 4.42 ± 0.04 |
| E2 | < MDL | < MDL | < MDL | < MDL | < MQL | < MDL | < MDL | 1.36 ± 0.05 | < MDL | < MDL |
| EE2 | < MDL | 1.72 ± 0.09 | < MDL | < MDL | < MDL | < MDL | 2.00 ± 0.29 | 2.88 ± 0.35 | < MDL | < MQL |
| Acetamiprid | < MDL | < MQL | 2.00 ± 0.10 | < MDL | < MDL | < MDL | 1.77 ± 0.09 | 57.8 ± 1.8 | < MDL | < MDL |
| Clothianidin | 4.01 ± 0.56 | 3.76 ± 0.21 | < MDL | 6.88 ± 0.96 | 12.6 ± 0.8 | 2.79 ± 0.16 | 6.85 ± 0.97 | 8.46 ± 1.2 | 0.87 ± 0.05 | 9.89 ± 0.56 |
| Imidacloprid | 5.69 ± 0.40 | 33.3 ± 1.3 | 22.3 ± 1.9 | 5.79 ± 0.70 | 6.57 ± 0.75 | 8.00 ± 1.44 | 9.58 ± 1.85 | 11.3 ± 1.1 | 1.02 ± 0.02 | 13.0 ± 2.2 |
| Thiacloprid | 2.50 ± 0.57 | 0.63 ± 0.32 | < MDL | 2.51 ± 0.28 | 1.19 ± 0.03 | < MDL | < MDL | 0.27 ± 0.01 | 0.49 ± 0.02 | 1.37 ± 0.09 |
| Thiamethoxam | < MDL | < MDL | 1.46 ± 0.60 | 1.71 ± 0.05 | 1.48 ± 0.04 | < MDL | < MDL | < MDL | < MQL | 0.66 ± 0.02 |
| Methiocarb | < MDL | < MDL | 1.43 ± 0.04 | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL |
| Oxadiazon | 8.43 ± 0.55 | 161 ± 8 | 56.1 ± 1.6 | 10.5 ± 0.6 | 0.62 ± 0.01 | 10.7 ± 1.6 | 265 ± 3 | 173 ± 10 | 34.5 ± 4.1 | 452 ± 5 |
| Triallat | < MDL | < MDL | < MDL | 0.53 ± 0.01 | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL |
| BHT | 42.3 ± 3.6 | < MDL | < MDL | 35.9 ± 2.7 | < MDL | < MDL | < MDL | < MDL | 28.1 ± 1.2 | < MDL |
| EHMC | < MDL | 18.7 ± 0.4 | < MDL | < MDL | 20.4 ± 1.3 | < MDL | < MDL | < MDL | < MDL | < MDL |

**Table S6.** Individual concentrations of the Watch list compounds in freshwater in the second sampling campaign

| | SC2-05 | SC2-06 | SC2-07 | SC2-08 | SC2-10 | SC2-11 | SC2-12 | SC2-13 | SC2-14 |
|---|---|---|---|---|---|---|---|---|---|
| Azithromycin | < MDL | < MDL | 1493 ± 69 | < MDL | < MDL | 12.4 ± 0.9 | < MDL | 19.5 ± 0.6 | 6.61 ± 0.19 |
| Clarithromycin | 10.1 ± 1.1 | 10.6 ± 1.1 | 269 ± 19 | < MDL | < MDL | 3.45 ± 0.31 | 3.59 ± 0.31 | 6.57 ± 0.46 | 5.26 ± 0.66 |
| Erythromycin | 4.46 ± 0.46 | < MDL | 15.3 ± 2.6 | 0.96 ± 0.14 | < MDL | 0.88 ± 0.11 | < MDL | 0.87 ± 0.01 | < MDL |
| Diclofenac | 74.8 ± 8.1 | 116 ± 13 | 446 ± 49 | 23.4 ± 2.6 | < MDL | 44.0 ± 4.8 | 7.33 ± 0.80 | 21.8 ± 2.4 | < MDL |
| E1 | < MDL | < MDL | 0.92 ± 0.02 | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL |
| E2 | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL |
| EE2 | < MDL | < MDL | 2.56 ± 0.13 | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL |
| Acetamiprid | < MDL | < MDL | 8.74 ± 0.45 | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL |
| Clothianidin | 1.33 ± 0.08 | 1.20 ± 0.07 | 9.22 ± 0.53 | 1.81 ± 0.35 | < MDL | < MDL | < MDL | 0.68 ± 0.04 | < MDL |
| Imidacloprid | 4.19 ± 0.10 | 4.01 ± 0.64 | 46.4 ± 6.3 | 1.80 ± 0.04 | < MDL | 2.11 ± 0.20 | < MDL | 3.41 ± 0.74 | < MDL |
| Thiacloprid | 2.41 ± 0.11 | 2.25 ± 0.10 | < MQL | 0.44 ± 0.02 | < MDL | < MDL | 0.65 ± 0.03 | 3.54 ± 0.46 | 1.98 ± 0.09 |
| Thiamethoxam | < MQL | < MQL | < MDL | < MQL | < MDL | < MDL | < MDL | 1.62 ± 0.04 | 1.36 ± 0.04 |
| Methiocarb | < MDL | < MDL | < MDL | < MDL | < MDL | < MQL | < MDL | < MQL | < MQL |
| Oxadiazon | < MDL | < MDL | 0.67 ± 0.01 | 5.39 ± 0.09 | 9.83 ± 0.83 | 15.4 ± 1.3 | 30.9 ± 2.5 | 41.5 ± 4.1 | 20.2 ± 2.2 |
| Triallate | < MDL | 2.26 ± 0.04 | < MDL | < MDL | < MDL | 1.85 ± 0.03 | 0.71 ± 0.01 | < MDL | 0.68 ± 0.01 |
| BHT | 25.1 ± 1.2 | 30.8 ± 1.9 | < MDL | 30.9 ± 1.4 | < MDL | 22.2 ± 0.3 | 17.9 ± 0.8 | 26.8 ± 1.0 | 21.6 ± 0.42 |
| EHMC | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL | < MDL |

**Table S7.** Individual concentrations of the Watch list compounds in freshwater in the third sampling campaign.

| | SC3-05 | SC3-06 | SC3-07 | SC3-08 | SC3-09 | SC3-10 | SC3-11 | SC3-12 | SC3-13 | SC3-14 |
|---|---|---|---|---|---|---|---|---|---|---|
| Azithromycin | 326 ± 11 | 425 ± 28 | 1306 ± 180 | 13.2 ± 0.7 | 5.17 ± 0.15 | <MDL | <MDL | <MDL | <MDL | 6.90 ± 0.20 |
| Clarithromycin | 72.8 ± 8.7 | 89.7 ± 7.9 | 293 ± 22 | 3.51 ± 0.35 | 3.95 ± 0.13 | 8.03 ± 0.30 | 5.45 ± 0.17 | 4.32 ± 0.61 | 4.28 ± 0.31 | <MDL |
| Erythromycin | 8.10 ± 1.3 | 8.74 ± 0.83 | 4.57 ± 0.68 | 3.36 ± 0.17 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| Diclofenac | 151 ± 10 | 172 ± 5 | 363 ± 40 | 2.14 ± 0.23 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| E1 | 2.12 ± 0.19 | 2.40 ± 0.22 | <MQL | <MDL | 3.35 ± 0.09 | 4.92 ± 0.13 | <MDL | <MDL | <MDL | <MDL |
| E2 | <MDL | <MDL | <MDL | 0.61 ± 0.02 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| EE2 | 1.82 ± 0.16 | <MDL | 2.46 ± 0.12 | <MQL | <MDL | 2.62 ± 0.23 | <MDL | <MDL | <MDL | <MDL |
| Acetamiprid | <MDL | 3.67 ± 0.40 | 3.64 ± 0.30 | <MDL | <MQL | 40.2 ± 2.0 | <MDL | <MDL | <MDL | <MDL |
| Clothianidin | <MDL | <MDL | 4.04 ± 0.23 | 0.40 ± 0.09 | 7.96 ± 0.45 | 6.96 ± 0.40 | <MDL | <MDL | <MDL | <MDL |
| Imidacloprid | 15.8 ± 0.37 | 23.3 ± 0.18 | 75.2 ± 1.1 | 15.6 ± 0.94 | 11.1 ± 1.8 | 15.0 ± 0.4 | 182 ± 12 | 4.06 ± 0.09 | 56.5 ± 3.4 | 5.69 ± 0.13 |
| Thiacloprid | <MDL | <MDL | 1.27 ± 0.06 | 2.76 ± 0.33 | 0.47 ± 0.02 | 1.20 ± 0.16 | 1.23 ± 0.22 | 1.01 ± 0.05 | 0.83 ± 0.04 | <MDL |
| Thiamethoxam | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MQL | 0.60 ± 0.02 | <MDL |
| Methiocarb | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 1.49 ± 0.18 | <MQL | <MDL |
| Oxadiazon | 27.6 ± 1.0 | 139 ± 2 | 310 ± 1 | 21.1 ± 1.5 | 283 ± 0.2 | 277 ± 5 | 592 ± 19 | 562 ± 34 | 421 ± 25 | 62.1 ± 3.7 |
| Triallate | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 0.88 ± 0.02 | <MDL | 0.83 ± 0.01 |
| BHT | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 20.5 ± 0.9 | 25.6 ± 2.3 | 28.7 ± 2.2 | 24.2 ± 0.7 |
| EHMC | 16.2 ± 3.0 | <MDL | 19.0 ± 1.8 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |

**Table S8.** Individual concentrations of the Watch list compounds in wastewater in all sampling campaigns.

| | SC1-01 | SC1-02 | SC1-03 | SC1-04 | SC2-01 | SC2-02 | SC2-03 | SC2-04 | SC3-01 | SC3-02 | SC3-03 | SC3-04 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Azithromycin | 250±18 | 367±24 | 1977±269 | 1615±108 | 2670±195 | 3474±227 | 641±47 | 946±62 | 2217±90 | 2177±342 | 1727±126 | 1707±31 |
| Clarithromycin | 38.8±2.4 | 24.0±2.1 | 85.7±5.3 | 61.9±5.3 | 90.5±5.6 | 86.3±7.4 | 61.6±3.8 | 45.5±3.9 | 107±7 | 92±10 | 707±44 | 478±21 |
| Erythromycin | 29.4±3.0 | 40.5±3.3 | 36.5±4.7 | 34.7±6.1 | 29.1±5.7 | 52.4±2.9 | 27.2±0.6 | 29.7±0.4 | 38.2±1.0 | 53.7±4.6 | 29.1±1.0 | <MDL |
| Diclofenac | 875±35 | 614±26 | 682±25 | 484±20 | 268±11 | 231±10 | 227±9 | 200±8 | 1538±62 | 552±23 | 1401±56 | 561±24 |
| E1 | <MQL | 10.1±0.1 | <MQL | 8.80±0.10 | 22.4±2.0 | 9.40±0.10 | <MQL | 9.50±0.10 | <MQL | 9.60±0.11 | 55.2±4.91 | <MDL |
| E2 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 25.8±0.9 | <MDL |
| EE2 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| Acetamiprid | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MQL | <MDL |
| Clothianidin | <MDL | 3.32±0.12 | <MDL | <MDL | <MQL | 34.1±2.8 | 25.4±1.0 | 8.29±0.68 | <MDL | <MDL | <MDL | <MDL |
| Imidacloprid | 119.7±0.2 | 91±11 | 127±20 | 56.4±2.8 | 34.3±1.4 | 52.2±3.1 | 11.2±0.4 | 17.0±1.3 | 257±30 | 221±25 | 14.6±3.6 | 114±12 |
| Thiacloprid | <MDL | <MQL | <MDL | <MQL | <MQL | <MDL | <MQL | <MQL | <MDL | <MQL | <MDL | <MQL |
| Thiamethoxam | 13.3±1.1 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| Methiocarb | <MDL | <MDL | <MDL | <MDL | <MQL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| Oxadiazon | <MDL | <MDL | <MDL | <MDL | <MDL | 3.04±0.12 | <MDL | <MDL | 5.67±0.22 | <MDL | <MDL | 8.7±2.5 |
| Triallate | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| BHT | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| EHMC | 140±3 | 148±6 | 153±8 | 156±8 | 150±14 | 143±1 | 153±3 | 168±11 | 143±10 | 147±5 | 166±7 | 146±15 |

Table S9. Detection frequencies in freshwater and wastewater samples.

| | Det. Freq. in freshwater (%) | Det. Freq. in wastewater (%) |
|---|---|---|
| Azithromycin | 51.7 | 100 |
| Clarithromycin | 86.2 | 100 |
| Erythromycin | 58.6 | 91.7 |
| Diclofenac | 58.6 | 100 |
| E1 | 48.3 | 58.3 |
| E2 | 10.3 | 8.33 |
| EE2 | 31.0 | 0.00 |
| Acetamiprid | 31.0 | 0.00 |
| Clothianidin | 62.1 | 33.3 |
| Imidacloprid | 93.1 | 100 |
| Thiacloprid | 72.4 | 0.00 |
| Thiamethoxam | 44.8 | 8.33 |
| Methiocarb | 20.7 | 0.00 |
| Oxadiazon | 93.1 | 25.0 |
| Triallate | 24.1 | 0.00 |
| BHT | 51.7 | 0.00 |
| EHMC | 13.8 | 100 |

Table S10. Updated predicted no-effect concentrations (PNECs) for the Watch list compounds, taken from Loos et al., 2018.

| | PNEC (ng/L) |
|---|---|
| Azithromycin | 19 |
| Clarithromycin | 120 |
| Erythromycin | 200 |
| Diclofenac | 50 |
| E1 | 3.6 |
| E2 | 0.4 |
| EE2 | 0.035 |
| Acetamiprid | 50 |
| Clothianidin | 130 |
| Imidacloprid | 8.3 |
| Thiacloprid | 10 |
| Thiamethoxam | 42 |
| Methiocarb | 2 |
| Oxadiazon | 88 |
| Triallat | 410 |
| BHT | 3160 |
| EHMC | 6000 |

**Table S11.** Risk quotients (RQs) for each compound and sampling site in the first sampling campaign. The sums of RQs are displayed in the bottom row. Where indicated with n.a., RQs could not be calculated because the environmental concentration was < MQL.

| | SC1-01 | SC1-02 | SC1-03 | SC1-04 | SC1-05 | SC1-06 | SC1-07 | SC1-08 | SC1-09 | SC1-10 | SC1-11 | SC1-12 | SC1-13 | SC1-14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Azithromycin | 13.1 | 19.3 | 104 | 85.0 | n.a. | 30.5 | 37.9 | n.a. | n.a. | n.a. | 0.539 | n.a. | 1.19 | 0.670 |
| Clarithromycin | 0.324 | 0.200 | 0.714 | 0.516 | 0.0334 | 1.07 | 0.0359 | 0.0596 | 0.0525 | n.a. | 0.0416 | n.a. | n.a. | 0.112 |
| Erythromycin | 0.147 | 0.203 | 0.183 | 0.174 | 0.104 | 0.0177 | 0.0742 | 0.0147 | 0.0125 | 0.0138 | 0.0160 | n.a. | n.a. | n.a. |
| Diclofenac | 17.5 | 12.3 | 13.6 | 9.68 | 0.154 | 4.69 | 5.46 | n.a. | 0.501 | n.a. | n.a. | 1.69 | n.a. | n.a. |
| E1 | n.a. | 2.81 | n.a. | 2.44 | n.a. | 0.294 | 0.488 | 0.331 | 0.483 | 0.776 | 1.89 | 1.92 | n.a. | 1.23 |
| E2 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 3.40 | n.a. |
| EE2 | n.a. | n.a. | n.a. | n.a. | n.a. | 49.1 | n.a. | n.a. | n.a. | n.a. | 57.0 | 82.1 | n.a. | n.a. |
| Acetamiprid | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.0399 | n.a. | n.a. | n.a. | 0.0354 | 1.16 | n.a. | n.a. |
| Clothianidin | n.a. | 0.0255 | n.a. | 0.0309 | 0.0290 | 0.0529 | 0.0969 | 0.0215 | 0.0527 | 0.0215 | 0.0651 | 0.00673 | 0.0760 | n.a. |
| Imidacloprid | 14.4 | 10.9 | 15.3 | 6.80 | 0.686 | 4.01 | 2.68 | 0.698 | 0.791 | 0.963 | 1.15 | 1.36 | 0.12 | 1.56 |
| Thiacloprid | n.a. | n.a. | n.a. | n.a. | 0.0625 | n.a. | 0.250 | 0.251 | 0.119 | n.a. | 0.0271 | 0.0492 | n.a. | 0.14 |
| Thiamethoxam | 0.316 | n.a. | n.a. | n.a. | n.a. | 0.0346 | 0.0346 | 0.0407 | 0.0353 | n.a. | n.a. | 0.0271 | 0.0157 | 0.0157 |
| Methiocarb | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.714 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| Oxadiazon | n.a. | n.a. | n.a. | n.a. | 0.0958 | 1.83 | 0.637 | 0.119 | 0.00699 | 0.122 | 3.01 | 1.97 | 0.392 | 5.14 |
| Triallat | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.00129 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| BHT | n.a. | n.a. | n.a. | n.a. | 0.0134 | n.a. | n.a. | 0.0113 | n.a. | n.a. | n.a. | n.a. | 0.00891 | n.a. |
| EHMC | 0.0233 | 0.0247 | 0.0255 | 0.0260 | n.a. | 0.00311 | n.a. | 0.00341 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| *TOTAL* | *45.9* | *45.7* | *134* | *105* | *1.37* | *91.9* | *49.1* | *1.46* | *1.21* | *2.16* | *62.6* | *92.1* | *5.38* | *8.93* |

**Table S12.** Risk quotients (RQs) for each compound and sampling site in the second sampling campaign. The sums of RQs are displayed in the bottom row. Where indicated with n.a., RQs could not be calculated because the environmental concentration was < MQL.

| | SC2-01 | SC2-02 | SC2-03 | SC2-04 | SC2-05 | SC2-06 | SC2-07 | SC2-08 | SC2-10 | SC2-11 | SC2-12 | SC2-13 | SC2-14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Azithromycin | 141 | 183 | 33.7 | 49.8 | n.a. | n.a. | 78.6 | n.a. | n.a. | 0.654 | n.a. | 1.03 | 0.348 |
| Clarithromycin | 0.754 | 0.719 | 0.514 | 0.379 | 0.0842 | 0.0881 | 2.24 | 0.0365 | n.a. | 0.0288 | 0.0299 | 0.0547 | 0.0438 |
| Erythromycin | 0.146 | 0.262 | 0.136 | 0.149 | 0.0223 | n.a. | 0.0764 | 0.00 | 0.00 | 0.00437 | n.a. | 0.00436 | n.a. |
| Diclofenac | 5.36 | 4.62 | 4.54 | 4.01 | 1.50 | 2.32 | 8.93 | 0.469 | 0.140 | 0.880 | 0.147 | 0.436 | n.a. |
| E1 | 6.22 | 2.61 | n.a. | 2.64 | n.a. | n.a. | 0.256 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| E2 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| EE2 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 73.1 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| Acetamiprid | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.175 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| Clothianidin | n.a. | n.a. | 0.263 | 0.196 | 0.0102 | 0.00923 | 0.0709 | 0.0139 | n.a. | n.a. | n.a. | 0.00522 | n.a. |
| Imidacloprid | 4.13 | 6.29 | 1.34 | 2.04 | 0.505 | 0.483 | 5.59 | 0.827 | 0.217 | 0.254 | n.a. | 0.411 | 0.198 |
| Thiacloprid | n.a. | n.a. | n.a. | n.a. | 0.241 | 0.225 | n.a. | 0.134 | 0.0441 | n.a. | 0.0655 | 0.354 | 0.0323 |
| Thiamethoxam | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.0165 | n.a. | 0.0387 | n.a. |
| Methiocarb | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| Oxadiazon | n.a. | 0.0346 | n.a. | n.a. | n.a. | n.a. | 0.00764 | 0.0613 | 0.112 | 0.175 | 0.351 | 0.472 | 0.230 |
| Triallat | n.a. | n.a. | n.a. | n.a. | n.a. | 0.00552 | n.a. | n.a. | n.a. | 0.00452 | 0.00173 | n.a. | 0.00165 |
| BHT | n.a. | n.a. | n.a. | n.a. | 0.00794 | 0.00975 | n.a. | 0.00979 | 0.00934 | 0.00703 | 0.00566 | 0.00848 | 0.00685 |
| EHMC | 0.0250 | 0.0239 | 0.0255 | 0.0281 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| TOTAL | 157 | 197 | 40.6 | 59.2 | 2.37 | 3.14 | 169 | 1.56 | 0.526 | 2.03 | 0.601 | 2.81 | 0.860 |

**Table S13**. Risk quotients (RQs) for each compound and sampling site in the third sampling campaign. The sums of RQs are displayed in the bottom row. Where indicated with n.a., RQs could not be calculated because the environmental concentration was < MQL.

| | SC3-01 | SC3-02 | SC3-03 | SC3-04 | SC3-05 | SC3-06 | SC3-07 | SC3-08 | SC3-09 | SC3-10 | SC3-11 | SC3-12 | SC3-13 | SC3-14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Azithromycin | 117 | 115 | 90.9 | 89.8 | 17.1 | 22.4 | 68.8 | 0.692 | 0.272 | n.a. | n.a. | n.a. | n.a. | 0.363 |
| Clarithromycin | 0.894 | 0.766 | 5.89 | 3.98 | 0.606 | 0.748 | 2.45 | 0.0293 | 0.0330 | 0.0669 | 0.0454 | 0.0360 | 0.0356 | n.a. |
| Erythromycin | 0.191 | 0.268 | 0.146 | n.a. | 0.0405 | 0.0437 | 0.0229 | 0.0168 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| Diclofenac | 30.8 | 11.0 | 28.0 | 11.2 | 3.02 | 3.45 | 7.27 | 0.04 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| E1 | n.a. | 2.67 | 15.3 | 0.589 | 0.667 | n.a. | 0.929 | n.a. | n.a. | 1.37 | n.a. | n.a. | n.a. | n.a. |
| E2 | n.a. | n.a. | 64.5 | n.a. | n.a. | n.a. | n.a. | 1.51 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| EE2 | n.a. | n.a. | n.a. | n.a. | 51.9 | n.a. | 70.3 | n.a. | 0.804 | 74.7 | n.a. | n.a. | n.a. | n.a. |
| Acetamiprid | n.a. | n.a. | n.a. | n.a. | 0.0733 | 0.0728 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| Clothianidin | 0.0638 | n.a. | n.a. | n.a. | n.a. | 0.0311 | 0.00304 | 0.0612 | 0.0535 | n.a. | n.a. | n.a. | n.a. | n.a. |
| Imidacloprid | 31.0 | 26.6 | 1.75 | 13.7 | 1.90 | 2.81 | 9.07 | 1.88 | 1.33 | 1.81 | 22.0 | 0.489 | 6.81 | 0.686 |
| Thiacloprid | n.a. | n.a. | n.a. | n.a. | n.a. | 0.127 | 0.276 | 0.0471 | 0.120 | n.a. | 0.123 | 0.101 | 0.0831 | n.a. |
| Thiamethoxam | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.0143 | n.a. |
| Methiocarb | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.746 | n.a. | n.a. |
| Oxadiazon | 0.0644 | n.a. | n.a. | 0.0983 | 0.314 | 1.58 | 3.53 | 0.240 | 3.22 | 3.14 | 6.72 | 6.39 | 4.79 | 0.706 |
| Triallat | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.00215 | n.a. | 0.00203 |
| BHT | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | 0.00647 | 0.00810 | 0.00908 | 0.00766 |
| EHMC | 0.0238 | 0.0244 | 0.0277 | 0.0244 | 0.00270 | 0.00316 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| *TOTAL* | *180* | *156* | *207* | *119* | *75.5* | *31.8* | *162* | *4.69* | *5.90* | *82.1* | *28.8* | *7.77* | *11.7* | *1.76* |

**Figure S1**. Risk quotients (RQs) for each sampling point calculated as a mean for the three sampling campaigns.
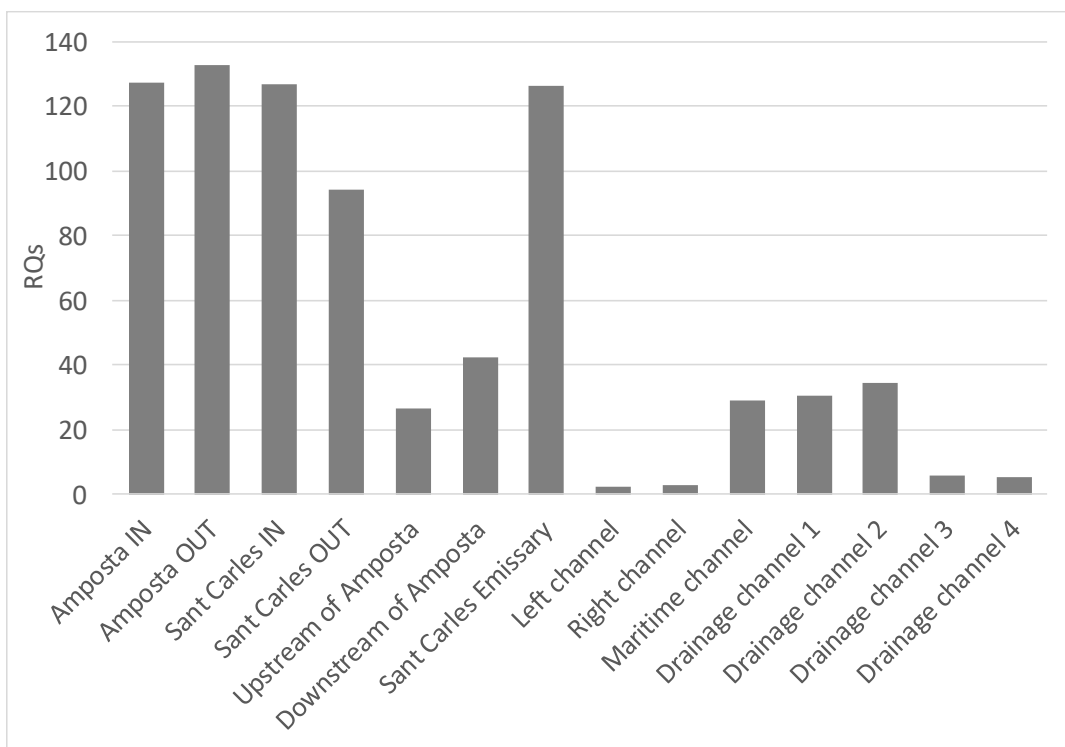
# SUPPLEMENTARY MATERIAL OF CHAPTER 3

## How do WWTPs operational parameters affect the removal rates of EU Watch list compounds?

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Table S1. Composition of the trace elements solution

|  | g/L |
|---|---|
| $FeCl_3 6H_2O$ | 1.5 |
| $H_3BO_3$ | 0.15 |
| $CuSO_4 5H_2O$ | 0.03 |
| KI | 0.18 |
| $MnCl_2 4H_2O$ | 0.12 |
| $Na_2MoO_4 2H_2O$ | 0.06 |
| $ZnSO_4 7H_2O$ | 0.12 |
| $CoCl_2 6H_2O$ | 0.14 |
| EDTA | 10 |

Table S2. Composition of the buffer/inorganic solution

|  | g/L |
|---|---|
| $KH_2PO_4$ | 0,95 |
| $Na_2HPO_4$ | 2,56 |
| $NaHCO_3$ | 28,0 |

Table S3. Composition of the organic solution

|  | g/L/gTSS |
|---|---|
| Na Acetate | 0.643 |
| Propionate | 0.215 |
| Yeast extract | 0.0715 |

Table S4. Nitrogen concentrations as a function of the redox conditions

| Anoxic conditions | 100 mg $N-NO_3$/L as $NaNO_3$ |
|---|---|
| Oxic conditions | 20 mg $N-NH_4$/L as $NH_4Cl$ |
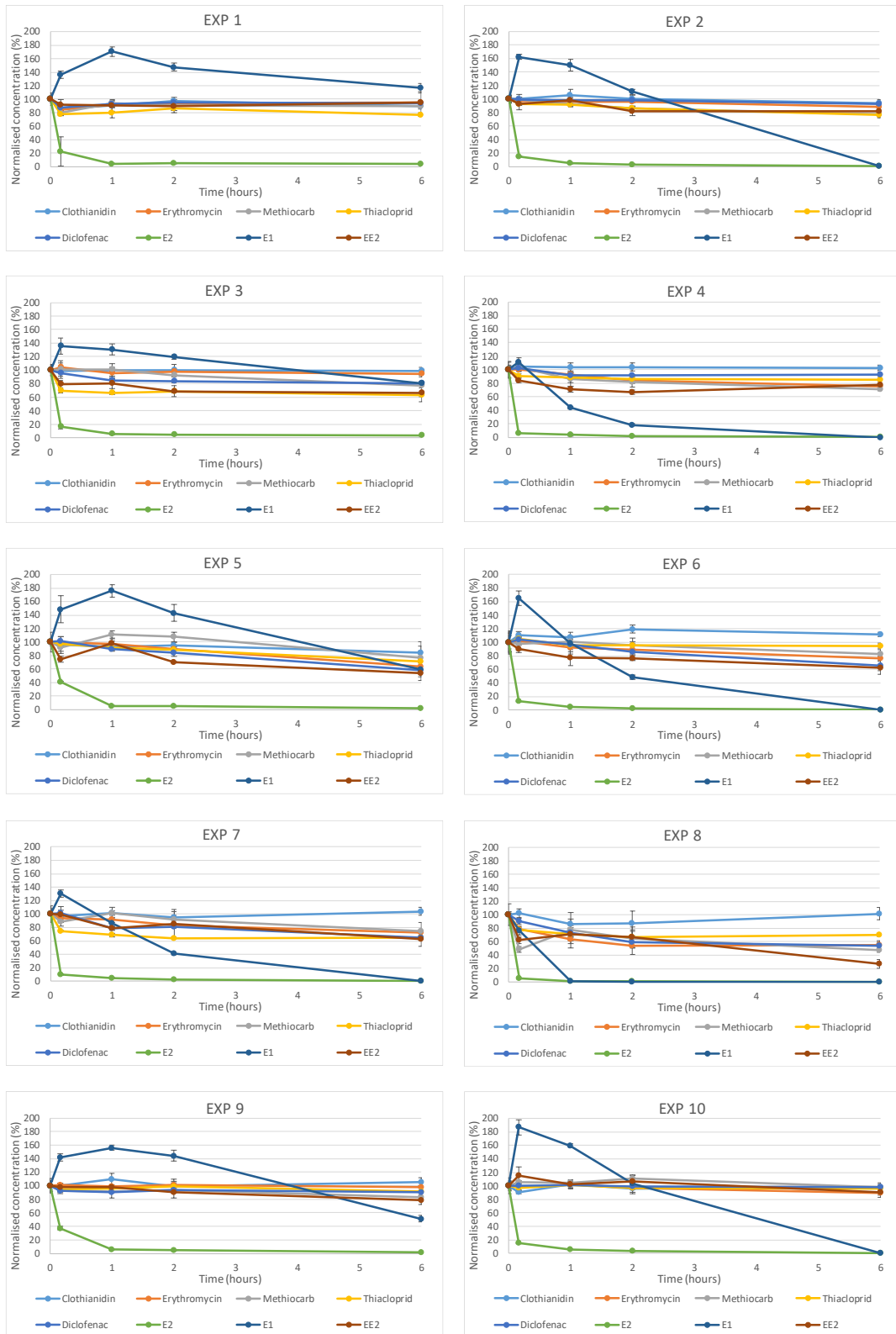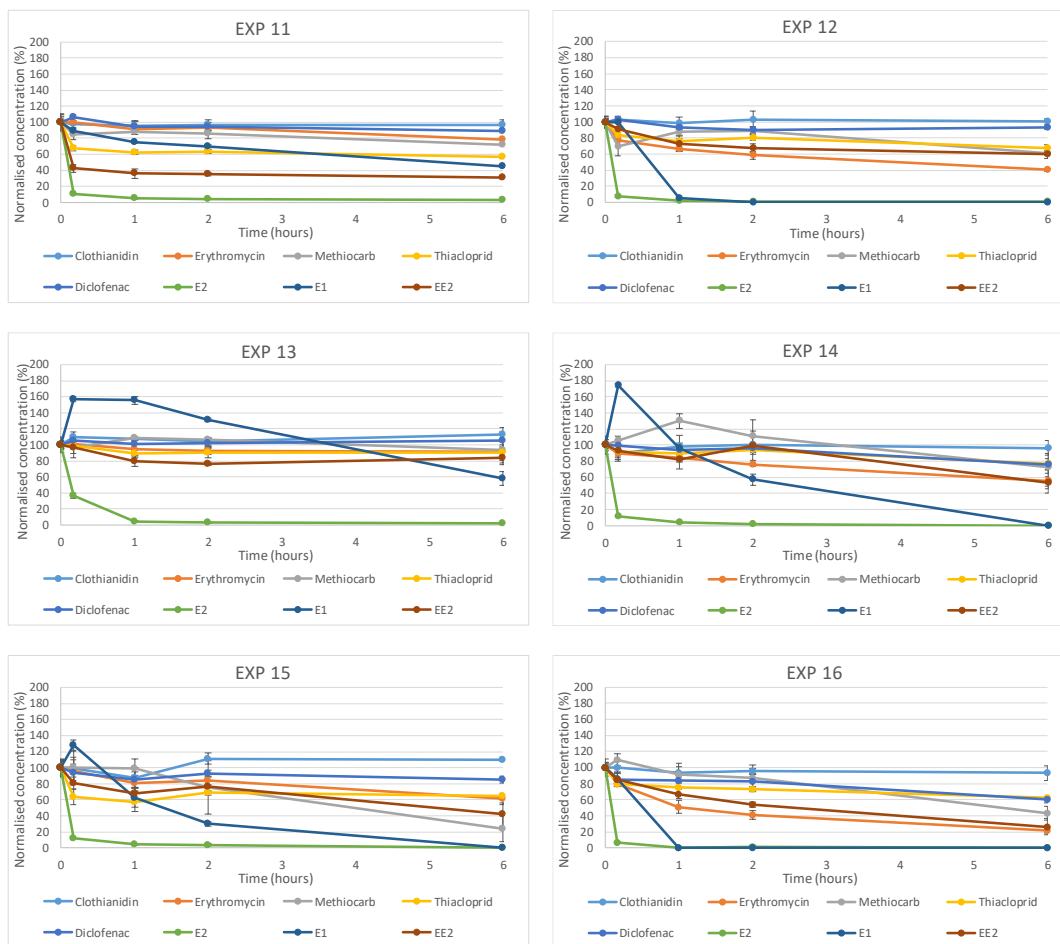
**Figure S1.** Evolution of the investigated compounds concentrations over time under all experimental conditions.

a)



b)



c)



d)



e)
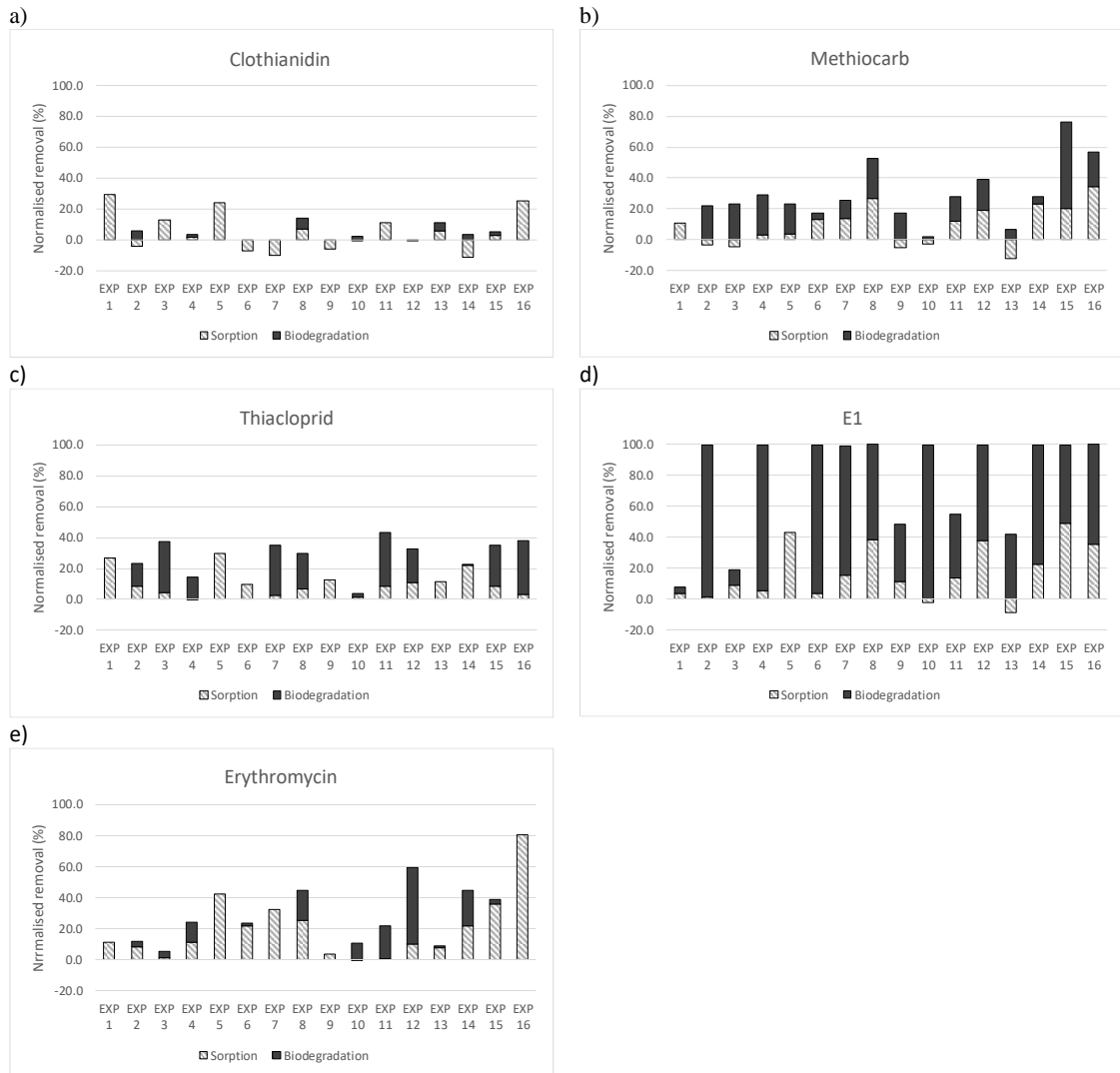


**Figure S2.** Removal profiles of a) clothianidin; b) methiocarb; c) thiacloprid; d) E1; e) erythromycin throughout the experiments, detailing the contributions of sorption and biodegradation

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