TESIS DE LA UNIVERSIDAD DE ZARAGOZA
2023
27

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Studies of emerging contaminants in environmental freshwater ecosystems: a focus on Pyrenees aquatic environment

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ISSN 2254-7606

Prensas de la Universidad
Universidad Zaragoza

ISSN 2254-7606

Universidad Zaragoza
1542

Tesis Doctoral

STUDIES OF EMERGING CONTAMINANTS IN ENVIRONMENTAL FRESHWATER ECOSYSTEMS: A FOCUS ON PYRENEES AQUATIC ENVIRONMENT

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UNIVERSIDAD DE ZARAGOZA
Escuela de Doctorado

Programa de Doctorado en Ciencia Analítica en Química

2022

THÈSE

UNIVERSITE DE PAU ET DES PAYS DE L'ADOUR
École doctorale des Sciences Exactes et leurs Applications
(ED 211 SEA)

Présentée et soutenue le 12 Décembre 2022
par **SEBASTIANO GOZZO**

pour obtenir le grade de docteur
de l'Université de Pau et des Pays de l'Adour
Spécialité :Chimie Analytique

STUDIES OF EMERGING CONTAMINANTS IN ENVIRONMENTAL FRESHWATER ECOSYSTEMS: A FOCUS ON PYRENEES AQUATIC ENVIRONMENT

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D. Eduardo Bolea Morales, Profesor Titular de la Universidad de Zaragoza y Dña. Joanna Szpunar, Ingénieur de Recherche à l'Université de Pau et des Pays de l'Adour,

CERTIFICAN

que la presente Memoria:

"STUDIES OF EMERGING POLLUTANTS IN FRESHWATER ECOSYSTEMS: A FOCUS ON THE AQUATIC ENVIRONMENT OF THE PYRENEES",

ha sido realizada bajo su dirección por D. Sebastiano Gozzo de acuerdo al convenio de cotutela firmado entre la Universidad de Zaragoza y la Université de Pau et Des Pays De l'Adour, para optar al título de Doctor en Ciencia Analítica en Química.

Y asimismo,

AUTORIZAN

la presentación de dicha Memoria para que sea defendida ante el Tribunal correspondiente.

Zaragoza, 30 de septiembre de 2022

Firmado: Eduardo Bolea

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INTRODUCCIÓN

La contaminación de las aguas en el medioambiente por los denominados "contaminantes emergentes (ECs)" presenta hoy en día una considerable amenaza a nivel global, debido principalmente a los efectos adversos desconocidos sobre los ecosistemas y la propia salud humana. Según la OMS, la disminución de la calidad del agua se considera uno de los problemas ambientales más importantes en la actualidad, a la par de la contaminación del aire y el cambio climático. Los ECs son, en un sentido amplio, cualquier producto químico sintético o natural, o cualquier microorganismo que no se controle o regule comúnmente en el medio ambiente con efectos potencialmente adversos conocidos o presuntos para la salud humana y ecológica. Los ECs pueden originarse en diferentes puntos de fuentes de contaminación, como plantas de tratamiento de aguas residuales (EDAR) de áreas urbanas, procedentes de hogares y hospitales, y de áreas industriales, difundiéndose a través de la deposición atmosférica o de cultivos, ganadería y acuicultura. No existe solo la necesidad de identificar, detectar y cuantificar estos contaminantes, sino también la de evaluar sus posibles efectos nocivos, obteniendo de esta manera una imagen más nítida de la evaluación de sus riesgos. Atendiendo a la bibliografía publicada, destaca el alto potencial de la espectrometría de masas (MS) y la Espectrometría de Masas (MS) de alta resolución (HR) junto con la cromatografía de gases (GC) y/o la cromatografía líquida (LC) para superar estos desafíos.

Los antibióticos pueden considerarse como uno de los mayores descubrimientos científicos del siglo XX, además de ser los fármacos más exitosos jamás desarrollados para mejorar la salud humana. Su popularidad ha tenido como consecuencia la creciente liberación en el medio ambiente, convirtiéndose en uno de los grupos que constituyen los contaminantes emergentes debido a sus riesgos potenciales para la salud pública y el medio ambiente. Ya a fines de la década de los 90 y la década de los 2000, la OMS convocó una serie de grupos consultivos, talleres de expertos y reuniones de consenso para evaluar la creciente amenaza para la salud pública que supone la resistencia a los antimicrobianos como resultado del uso excesivo de antibióticos y su liberación al medio ambiente. Una de las principales causas de la contaminación por antibióticos es la ganadería intensiva y la excreción a través de las heces y la orina durante el pastoreo libre de los animales, seguida de la dispersión del estiércol en la tierra y la contaminación por escorrentía. Una vez liberados en el medio ambiente, los antibióticos pasan por diferentes procesos, como la dilución o concentración debido al secado, la dilución en aguas superficiales después de la descarga de las EDAR, la sorción a partículas en suspensión y su degradación.

Por otro lado, los coloides naturales son componentes ubicuos e importantes del sistema biogeoquímico de la Tierra. Estos se forman a partir de diferentes procesos naturales como la ceniza volcánica, la meteorización de las rocas y la descomposición biológica, abarcando una amplia gama de composiciones y conformaciones, siendo muy heterogéneos en tamaño, forma y estructura. Como parte del ecosistema, el conocimiento preciso de los procesos en los que intervienen y su destino final es de gran importancia para la comprensión global de los sistemas acuáticos.

Finalmente, los microplásticos (MP) y su fracción más pequeña, los nanoplásticos (NPT), son un problema emergente y también se han considerado en los últimos años como nuevos ECs.

Aunque la presencia de microplásticos en aguas marinas y dulces está bien documentada, la detección y cuantificación de NPT supone un gran desafío debido a su pequeño tamaño. La aparición de NPT en un entorno acuático real se documentó por primera vez en 2017 en la región subtropical del Atlántico Norte. Es de prever que los NPT sean más peligrosos que los plásticos de mayor tamaño, ya que pueden atravesar barreras fisiológicas más fácilmente y trasladarse a diferentes órganos en organismos acuáticos. Además, no se puede descartar su papel como potenciales portadores de otros contaminantes (metales, contaminantes orgánicos...), por lo que no solo constituyen una amenaza para la vida silvestre acuática, sino para todo el ecosistema.

Se hace evidente, por tanto, la necesidad de más estudios encaminados a la determinación y cuantificación de estos nanomateriales, así como de la evaluación de sus efectos de ecotoxicidad e interacciones con otros contaminantes. Respecto a estos últimos, hay que tener en cuenta que las propiedades de los coloides y NPTs (tamaño, forma, etc.) les confieren características y comportamiento únicos. Además, diferentes factores, como el pH, la salinidad, la dureza del agua, la fuerza iónica, la temperatura y la concentración, pueden influir en su comportamiento y destino final en ambientes acuáticos, así como en su interacción con los contaminantes concurrentes. Por lo tanto, la evaluación de la interacción de coloides y NPT con contaminantes orgánicos, como los antibióticos, y el papel potencial de los primeros como portadores, es un área emergente en la investigación ambiental.

OBJETIVOS

Esta tesis contribuye a los estudios de contaminantes emergentes en ecosistemas de agua dulce, centrados en el medio acuático de los Pirineos y, en particular, en la región POCTEFA. Esta zona ha sido elegida por las considerables actividades agrícolas, incluida la ganadería intensiva, que se realizan en ella y que pueden constituir potencialmente una fuente importante de antibióticos veterinarios. Los objetivos incluyen el desarrollo de metodología analítica, evaluaciones ambientales de la contaminación y sus fuentes y, finalmente, probar distintas hipótesis toxicológicas relacionadas con los efectos de las especies detectadas sobre el zooplancton presente agua dulce.

Este objetivo general implica el desarrollo de los siguientes objetivos específicos:

(i) Realizar un seguimiento de la presencia de antibióticos en aguas superficiales y residuales para proponer objetivos para una posterior evaluación detallada de los mismos.

(ii) Presentar una evaluación cuantitativa a largo plazo de la presencia de antibióticos y sus productos de degradación en aguas superficiales y residuales mediante un método basado en LC-MS optimizado, previa preconcentración mediante extracción en fase sólida (SPE).

(iii) Investigar la posible interacción de los antibióticos con coloides naturales presentes en aguas superficiales y residuales mediante técnicas de fraccionamiento en flujo mediante campo de flujo asimétrico acoplado a espectrometría de masas con plasma ICP (A4F-ICP MS).

(iv) Revelar los efectos de la presencia separada y conjunta de enrofloxacina, el antibiótico más abundante encontrado en el área estudiada, y los nanoplásticos, sobre diferentes parámetros del ciclo vital y sobre la comunidad bacteriana en el tracto intestinal de la *Daphnia magna*, organismo modelo habitualmente utilizado en estudios ecotoxicológicos.

CONCLUSIONES

Esta Tesis Doctoral ha contribuido mejorar el conocimiento sobre la presencia, interacción y efectos tóxicos conjuntos de contaminantes emergentes seleccionados en entornos naturales de agua dulce. En particular esta tesis ha permitido:

1) Desarrollar una metodología basada en SPE-LC-MS/MS. El método ha permitido la determinación de un total de 21 antibióticos, así como la detección de sus metabolitos en aguas seleccionadas a lo largo del territorio POCTEFA.

2) Estudiar la presencia de antibióticos en las aguas superficiales y residuales de la cuenca del Ebro en un estudio de larga duración (4 años). Los resultados han mostrado la presencia de enrofloxacina en casi todas las muestras de aguas superficiales, especialmente en áreas cercanas al desarrollo de actividades de agricultura intensiva. La fluoroquinolona, junto con azitromicina y trimetoprima, también se encontraron en instalaciones de EDAR, especialmente en áreas cercanas a grandes núcleos urbanos. En general, la cantidad de antibióticos encontrada fue mayor que en estudios previos realizados en la zona.

3) Realizar el análisis mediante AF4-UV-Vis-ICP-MS de la fracción coloidal de diferentes muestras de aguas superficiales y EDARs, que ha revelado la presencia de partículas de tamaño superior a los 100 nm, caracterizadas por una elevada componente de dispersión en su espectro UV-Vis y señal de Al asociada a las mismas. Además, se ha utilizado una metodología basada en el uso de ultrafiltración tangencial y UV-Vis para estudiar las interacciones entre los coloides naturales presentes y la enrofloxacina. Mientras que las muestras con una gran fracción de materia orgánica no mostraron interacciones significativas, se observó que hasta un 67% de la enrofloxacina adicionada se asociaba a la fracción coloidal en muestras de aguas naturales con elevada fracción coloidal.

4) Investigar los efectos combinados de los NPTs y los antibióticos en distintos parámetros del ciclo vital de los organismos de agua dulce y la diversidad metabolómica y taxonómica de su comunidad microbiana intestinal. El efecto interactivo de ambos contaminantes emergentes en todos los parámetros estudiados fue diferente en presencia y ausencia de NPTs.

INTRODUCTION

La pollution de l'eau par les contaminants émergents (CE) représente une menace mondiale grave, en raison des effets néfastes inconnus sur l'écosystème et sur la santé humaine. La baisse de la qualité de l'eau est considérée comme l'un des problèmes environnementaux les plus importants de nos jours, au même titre que la pollution de l'air et le changement climatique, selon l'OMS. Les CE sont au sens large tout produit chimique synthétique ou naturel ou tout micro-organisme qui n'est pas couramment surveillé ou réglementé dans l'environnement et qui a des effets nocifs potentiellement connus ou soupçonnés sur l'environnement et la santé humaine. Les CE peuvent provenir de différents points de pollution, tels que les stations d'épuration des eaux usées (STEP) des zones urbaines, où elles proviennent des ménages et des hôpitaux, et des zones industrielles, diffuses par les dépôts atmosphériques ou par les cultures, le bétail et l'aquaculture. Il est non seulement nécessaire d'identifier, de détecter et de quantifier ces contaminants, mais aussi d'évaluer leurs effets nocifs potentiels, obtenant ainsi une image plus claire de l'évaluation des risques. La littérature a mis en évidence le potentiel élevé de la spectrométrie de masse (MS) et de la MS à haute résolution (HR) couplée à la chromatographie en phase gazeuse (GC) et / ou à la chromatographie liquide (LC) pour surmonter ces défis.

Les antibiotiques peuvent être considérés comme l'une des plus grandes découvertes scientifiques du 20ème siècle et les médicaments les plus réussis jamais développés pour améliorer la santé humaine. Leur popularité a entraîné leur rejet croissant dans l'environnement où ils sont devenus des contaminants émergents en raison de leurs risques potentiels pour la santé publique et l'environnement. Déjà à la fin des années 1990 et 2000, l'OMS a organisé une série de groupes consultatifs, d'ateliers d'experts et de réunions de consensus pour évaluer la menace croissante pour la santé publique de la résistance aux antimicrobiens résultant de la surutilisation des antibiotiques et de leur rejet dans l'environnement. L'une des principales causes de la pollution par les antibiotiques est l'agriculture intensive et l'excrétion par les fèces et l'urine pendant le pâturage libre des animaux, suivie de la propagation du fumier sur les terres et de la contamination par ruissellement. Une fois libérés dans l'environnement, les antibiotiques subissent différents processus, tels que la dilution ou la concentration due à l'assaisonnement, la dilution dans les eaux de surface après le rejet STEP, la sorption sur les particules en suspension et la dégradation.

D'autre part, les colloïdes naturels sont des composants omniprésents et importants du système biogéochimique terrestre. Ils sont formés à partir de différents processus naturels tels que les cendres volcaniques, l'altération des roches et la désintégration biologique, couvrant un large éventail de compositions et de conformations, étant très hétérogènes en taille, forme, structure. En tant que partie intégrante de l'écosystème, une connaissance précise de leur sort est d'une grande importance pour la compréhension globale des systèmes aquatiques.

Enfin, les microplastiques (MP) et leur homologue plus petit, les nanoplastiques (NPT), sont un problème émergent et ont également été considérés ces dernières années comme de nouveaux CE. Bien que la présence de microplastiques dans les eaux marines et d'eau douce soit bien documentée, la détection et la quantification des NPT sont plus difficiles en raison de leur petite taille. La présence de NPT dans un environnement aquatique réel a été démontrée pour la première fois en 2017 dans le gyre subtropical de l'Atlantique Nord. On s'attend à ce que les NPT soient plus dangereux que les plastiques plus gros, car ils peuvent passer plus facilement les barrières physiologiques et être

transférés à différents organes dans les organismes aquatiques. De plus, leur rôle potentiel de vecteurs d'autres polluants (métaux, contaminants organiques...) ne peut être écarté. Par conséquent, les NPT ne constituent pas seulement une menace pour la faune aquatique, mais pour l'ensemble de l'écosystème.

Les lacunes dans les connaissances de tous ces nanomatériaux concernent actuellement leur détermination et leur quantification ainsi que l'évaluation de leurs effets d'écotoxicité et de leurs interactions avec d'autres polluants. En ce qui concerne ces derniers, les propriétés des colloïdes et des NPT (taille, forme, etc.) leur confèrent des caractéristiques et un comportement uniques. De plus, différents facteurs, tels que le pH, la salinité, la dureté de l'eau, la force ionique, la température et la concentration, peuvent influencer leur comportement et leur devenir dans le milieu aquatique, ainsi que leur interaction avec les polluants concomitants. Par conséquent, l'évaluation de l'interaction des colloïdes et des NPT avec les polluants organiques, comme les antibiotiques, et le rôle potentiel des premiers en tant que porteurs est un domaine émergent de la recherche environnementale.

OBJECTIFS

Ce projet de thèse contribue à l'étude des contaminants émergents dans les écosystèmes environnementaux d'eau douce en mettant l'accent sur le milieu aquatique des Pyrénées et, en particulier, sur la région POCTEFA. La zone d'étude a été choisie en raison des activités agricoles considérables, y compris l'agriculture intensive, qui peut potentiellement être une source importante d'antibiotiques vétérinaires. Les objectifs comprenaient des développements dans la méthodologie analytique, des évaluations environnementales de la pollution et de ses sources et, enfin, l'essai d'hypothèses toxicologiques liées aux effets des espèces détectées sur le zooplancton d'eau douce.

Les objectifs détaillés étaient les suivants :

(i) effectuer une surveillance exploratoire de la présence d'antibiotiques dans les eaux de surface et les eaux usées afin de proposer des cibles pour une évaluation détaillée ultérieure

(ii) présenter une évaluation quantitative à long terme de la présence d'antibiotiques et de leurs produits de dégradation dans les eaux de surface et les eaux usées par une méthode LC-MS optimisée suite à une préconcentration SPE

(iii) étudier l'interaction possible des antibiotiques avec les colloïdes naturels dans les eaux de surface et les eaux usées par technologie A4F-ICP MS de pointe

(iv) révéler les effets de la présence séparée et conjointe d'enrofloxacine, l'antibiotique le plus abondant trouvé dans la zone étudiée, et de nanoplastiques sur les paramètres du cycle biologique et la communauté bactérienne dans le tractus intestinal de Daphnia magna, un organisme modèle dans les études écotoxicologiques.

CONCLUSIONS

Cette thèse de doctorat a contribué à acquérir des connaissances sur la présence, l'interaction et les effets toxiques conjoints de contaminants émergents sélectionnés dans les environnements d'eau douce:

1) Une méthodologie basée sur la SPE-LC-MS/MS a été développée. La méthode a permis la détermination de 21 antibiotiques ainsi que la détection de leurs métabolites sur le territoire POCTEFA.
2) La présence d'antibiotiques dans les eaux de surface et les eaux usées du bassin de l'Èbre a été étudiée dans le cadre d'une enquête à long terme (4 ans). Les résultats ont montré la présence omniprésente d'enrofloxacine dans presque tous les échantillons des eaux de surface, en particulier dans les zones proches de l'agriculture intensive. Cet antibiotique fluoroquinolone a également été trouvé, avec l'azithromycine et le triméthoprime dans les stations d'épuration, en particulier dans les zones proches des grands noyaux urbains. En général, la quantité d'antibiotiques trouvée était plus élevée que dans les études précédentes menées dans la région.
3) L'analyse par AF4-UV-Vis-ICP-MS de la fraction colloïdale à partir de différents échantillons d'eaux de surface et de stations d'épuration a révélé des particules de plus de 100 nm, avec une grande composante de dispersion et un signal Al associée. Une méthodologie basée sur l'utilisation de l'ultrafiltration tangentielle et des UV-Vis a également été utilisée pour étudier les interactions entre les colloïdes et l'enrofloxacine. Bien que les échantillons avec une grande fraction de matière organique n'aient pas montré d'interactions significatives, jusqu'à 67% de l'enrofloxacine ajoutée a été observée comme étant associée à la fraction colloïdale dans les échantillons d'eaux naturelles avec une grande fraction colloïdale.
4) Les effets combinés des NPT et des antibiotiques sur les paramètres du cycle biologique des organismes d'eau douce et la diversité métabolomique et taxonomique de leur communauté microbienne intestinale ont été étudiés. L'effet interactif des deux contaminants émergents sur tous les paramètres étudiés était différent en présence et en absence de NPT.

A. LITERATURE PART

A.1. Emerging pollutants of freshwater systems

Environmental water pollution by *Emerging Contaminants* (ECs) represents a serious global threat, due to the unknown adverse effects on the ecosystem and on human health. Declining water quality is considered as one of the most significant environmental problems nowadays on a par with air pollution and climate change according to WHO [1].

Following UNESCO definition, ECs are in a broad sense any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored or regulated in the environment with potentially known or suspected adverse ecological and human health effects [2]. They consist of mainly chemicals found in pharmaceuticals, personal care products (PPCPs), pesticides, industrial and household products, metals, surfactants, industrial additives and solvents. Many of them are used and released continuously into the environment even in very low quantities and some may cause chronic toxicity, endocrine disruption in humans and aquatic wildlife and the development of bacterial pathogen resistance [2]. Microplastics (MPs) and their smaller counterpart, nanoplastics (NPTs), are an emerging issue and have been considered in the recent years as new ECs as well [3,4]. Globally, over 190 million organic compounds are registered in the chemical abstract service (CAS, https://www.cas.org/cas-data/cas-registry) database.

A list of ECs is periodically drawn up within the *Network of Reference Laboratories, Research Centres and Related Organisations for Monitoring of Emerging Environmental Substances* (NORMAN, (www.norman-network.net) and includes:

- *biocides* (BIOCID): Biocidal product active substance, Biocidal product co-formulant, biocide product active substance, disinfectants, pesticides
- *drinking water chemicals* (DW) disinfection by-products
- *drugs of abuse* (DDA), designer drugs, opiates, opioids and metabolites, sympathomimetics, synthetic cannabinoids and psychoactive compounds
- *Flame retardants* (FRET), brominated diphenyl ethers, brominated flame retardants, chlorine containing flame retardants, organophosphorus, phosphorous containing flame retardants
- *food additives* (FOODA), antioxidants, artificial sweeteners, foam stabilisers, humectants, other
- *food contact chemicals* (FOODC)
- *human metabolites* (HUME)
- *human neurotoxins* (HUTOX)
- *indoor environment substances* (INDOOR)
- *industrial chemicals* (IND) including: antifouling agents, benzothiazoles, complexing agents, corrosion inhibitors, gasoline additives, lubricants, phenolic antioxidants, phenols, polyaromatic hydrocarbons, polymer stabilisers, precursors synthesis of pigments, intermediates for dyes, pigments, tert-butyl phenols and others.
- *metals and their compounds* (MET)
- *natural toxins* (NATOX), algal toxins, cyanobacteria, mycotoxins, phytotoxins, other
- *per-and polyfluoroalkyl substances* (PFAS)

- *persistent, mobile and toxic substances* (PMT),
- *personal care products* (PCP), antimicrobial agents, antioxidants, fragrances, insect repellents, moth repellents, parabens, siloxanes, sunscreen agents.
- *pharmaceuticals*, among which the most important there are anti-inflammatory, antibiotics, NSAIDs, steroids and hormones
- *plant protection products* (PPP) (bactericides and fungicides)
- *REACH chemicals* (REACH)
- *smoke compounds* (SMOKE)
- *surfactants* (SURF, antifoaming agents, detergents, other

In addition, in the recent years, due to the extensive use of plastic and the widespread of plastic pollutants, it has been decided to include the emerging global issue of microplastic under their activities [5, 6]. Considering that almost all of these products are indispensable in human daily life, their quantity consumed may increase with the world population growth [7].

Stefanakis and Becker [8] distinguish three groups of ECs including industrial additives of recent diffusion in the environment, pharmaceuticals, which could be longer occurred in the environment, either have gained interest in the recent years or whose presence in the environment was known but their toxicological effects have been identified lately (hormones). In addition, thousands of still unknown compounds may occur in the environment which can pose a serious hazard to ecosystems and human health.

ECs can originate from different points of pollution sources, such as wastewater treatment plant (WWTPs) from urban areas, where they originate from household and hospitals, and industrial area, diffuse through atmospheric deposition or from crop, livestock and aquaculture [9]. According to literature, there is no single treatment that can effectively remove all ECs in a single step [10], so that these pollutants ended up in rivers and their transport in the environment is affected by different processes such as biological and photodegradation, and sorption to sediments, even if their fate in the environment remains relatively unknown. WWTPs, biotic and abiotic degradation transform ECs into by-products whose consequences are still not adequately known and may have a greater potential impact and risks to human health and ecosystem; these compounds have unknown complex structures and may occur at very low concentration; besides, the lack of reference standards makes more difficult their identification and quantification. ECs and their transformation products are not only bioactive, but they can also bioaccumulate and be persistent.

Despite of the progress that has been made in the field of analytical chemistry, which allowed the detection of these contaminants even at trace levels, there are several challenges which need to be further addressed [11]:

- Implementation of detection and quantification systems for those compounds whose inclusion in multi-residue multiclass methods remains trammelled (e.g., highly volatile compounds, highly polar/ionic compounds, surfactants)
- Elucidation of the structure of organic contaminants occurring at trace levels, and of transformation products by biotic and abiotic processes.

There is not only the need to identify, detect and quantify these contaminants, but as well to evaluate their potential harmful effects, thus obtaining a clearer picture of the risk assessment. Literature highlighted the high potential of mass spectrometry (MS) and high resolution (HR) MS coupled to gas chromatography (GC) and/or liquid chromatography (LC) to overcome the challenges mentioned above [12].

Regardless of the progress of ecotoxicology with acquiring knowledge on acute toxic effects, there is an increasing demand to move on and overcome the challenge not so much of the effects of individual pollutants but of their mixtures and their transformation products, which are a relevant part of the problem that only recently has started to be addressed. Moreover, majority of the studies focus on acute effects which are unlikely to turn out at environmental concentrations; studies on chronic effects would be more pertinent, as organisms are likely being exposed over a long period; In addition, the ecotoxicological effects of some groups of ECs are still unknown, while studies on nanomaterials have started to be addressed only recently [13].

So that a multidisciplinary approach and integrated research is essential to improve water quality; it involves disciplines such as analytical chemistry, ecotoxicology and engineering (for prevention and elimination), with further scientific investigations on ECs occurrence, fate and effects on environment which would help to establish appropriate regulations and monitoring strategies.

A.1.1. Antibiotics pollution of freshwater environments

Antibiotics can be considered one of the greatest scientific discoveries of the 20th century and most successful ever developed drugs for improving human health. Since Alexander Fleming discovered penicillin, over 160 new antibiotics of different origin (such as natural, synthetic and semi-synthetic), different chemical categories and having different action mechanism (the inhibition of cell wall synthesis, alteration of cell membranes, protein synthesis inhibition, synthesis of nucleic acids inhibition and metabolic or anti-competitive antagonism) have been developed. Antibiotics are drugs able to kill bacteria or inhibit their growth and division. However, their popularity resulted in their growing release in the environment where they have become emerging contaminants due to their potential risks to public health and to the environment [14]. Already in the late 1990s and 2000s, the WHO convened a series of consultative groups, expert workshops, and consensus meetings to assess the growing public health threat of antimicrobial resistance resulting from the overuse of antibiotics and their release to the environment [15]. Indeed, antibiotics are detectable in surface waters, including rivers, lakes and seas [16] in the ng L^{-1} up to µg L^{-1} range, exceeding in some cases the Predicted No Effect Environmental Concentration [17].

One of main causes of antibiotics pollution is intensive farming and the excretion through faeces and urine during the free grazing of animals [18], followed by manure spreading on land [19], and contamination via runoff [20,21]. Hospitals are considered another source of emission of antibiotics, since a high concentration of them are usually found in their effluents [22]. Once released in the environment, antibiotics undergo different processes, such as dilution or concentration due to seasoning, dilution in surface water after WWTPs discharging, sorption to suspended particles, and degradation. The monitoring of the water environmental contamination levels by antibiotics is of high importance to improve knowledge

on their source pathways, transport, fate, and toxicity. Consequently, studies of their transformation products are necessary to assess the risk of antibiotics and improve regulations.

The main classes of antibiotics are listed below:

Aminoglycosides are broad spectrum antibiotics of natural or semisynthetic origin formed by a glycosidic and an amino group. they are among the first antibiotics that have found widespread use in clinical routine. They act through inhibition of protein synthesis.

Amphenicols are broad-spectrum antibiotics with a phenylpropanoid structure. They are mainly used to prevent and treat animal diseases. Their mechanism of action is based on the inhibition of protein synthesis.

β-lactams constitute a large family of antibiotics, comprising numerous molecules structurally characterized by the β-lactam nucleus. They are among the most clinically important antibiotics in both human and veterinary medicine, and act inhibiting cell wall synthesis.

Diaminopyrimidines are a class of organic and synthetic compounds that include two amino groups and a pyrimidine ring. Trimethoprim is the main representative molecule of this class; it finds wide use in combination with sulfamethoxazole for their synergistic effects to treat human and animal bacterial infections. Diaminopyrimidines prevent purine synthesis inhibiting dihydrofolate reductase.

Glycopeptides are a class of organic compounds structurally characterized by a carbohydrate bound to a peptide. They are used to treat bacterial infections in human medicine. The glycopeptide antibiotics work by the inhibition of cell wall synthesis.

Lincosamides consist of a relatively small group of antibiotics with a chemical structure made of amino acid and sugar moieties. They are first-choice bacteriostatic antibiotics used in veterinary microbiology and find applications as well as to threat protozoa and bacterial infections in human. Lincosamides inhibit protein synthesis.

Macrolides are antibiotics with a broad spectrum of activity against many gram-positive bacteria, widely used in both human and animal medicine. They are composed of a macrocyclic lactone of different ring sizes, to which one or more deoxy-sugar or amino sugar residues are attached. As well as lincosamides, macrolides work inhibiting protein synthesis.

Quinolones constitute of a large group of broad-spectrum antibiotics that share a bicyclic core structure related to the substance 4-quinolone. They are widely used, as they find applications in human and veterinary medicine to treat bacterial infections, as well as in animal husbandry. Fluoroquinolones are the main representatives, which contain a fluorine atom in their chemical structure and are effective against both Gram-negative and Gram-positive bacteria. They work inhibiting DNA replication.

Sulfonamides are one of the oldest antibiotics still in use. Structurally containing a sulfonamide group, sulfonamides have multiple applications such as antibacterial, antifungal, antiparasitic, antioxidant and

antitumor one. Sulfonamides act as inhibitors of p-aminobenzoic acid in the folic acid metabolism cycle, inhibiting bacteria multiplication.

Tetracycline are a large group of antibiotics widely used for veterinary, human and agricultural purposes. Tetracyclines are structurally represented by a fused tetracyclic linear basic nucleus (rings A, B, C and D) with several functional groups attached to it. They are inhibitor of protein synthesis.

So as above-described, nowadays antibiotics have found different applications, the main one being preventing and treating human, animals and plants infections. Moreover, certain types of antibiotics are still used in animal farms and fisheries worldwide as growth promoters and, despite the current restriction of the European Commission, the antibiotics registered for prophylactic and metaphylactic administration in livestock pose an additional challenge for regulators. All these applications made antibiotics to be released in large amounts and take several routes into natural ecosystems. These pollutants have been detected in WWTPs, sewage, surface as well as groundwaters.

WWTPs are the major hot spots of contamination; they receive antibiotics from multiple contamination sources, such as hospitals, households (including the incorrect disposal of unused antibiotics), urban run-offs, slaughterhouses and chemical manufactures. Different studies showed that urban treatment facilities are not 100% efficient in removing antibiotics, which implies that if they are not completely eliminated during the purification process, they go through the sewer system reaching the environment surface waters and, to a lesser extent, ground waters and sediments. Their concentrations in surface waters are usually lower than those measured directly in wastewater and sewage treatment plants, due to attenuation processes such as dilution, sorption and (bio)degradation.

Furthermore, surface and groundwaters receive antibiotics from additional sources, such as aquacultures, livestock and poultry. Crops are an additional source of contamination as water from WWTPs are used for irrigation purpose. Once in soil, antibiotics can enter the aquatic environment indirectly via surface runoffs to surface waters. The environmental residual concentrations of antibiotics are due not only to their continuous release into the environment, but also to their intrinsic high persistence.

The occurrence of antibiotics in the aquatic environment is of concern due to the correlations with the development and spread of antibiotics resistance genes (ARGs), which represents one of the main threats to the health sector of the 21st century.

Additionally, once in the environment, antibiotic residues inevitable interact with the biota at different trophic levels. Consequently, antibiotics can have a negative impact on environmental health, especially on microbial community structure and functioning. Literature showed as the occurrence of antibiotics can have different causes, such as reduce microbial biodiversity, influence the growth and enzyme activities of bacterial communities and affect ecological functions such as biomass production and nutrient transformation.

As antibiotics environmental pollution is related to their massive use, it is directly connected to their consumption patterns. The global consumption of antibiotics in humans and in food-producing animals

decreased 23% and 43% respectively from 2011 to 2020. A different trend was observed for broad-spectrum antibiotics, whose consumption has increased, turning out 3.5 times higher than the consumption of low-spectrum antibiotics that should generally be used as first-line therapy. Despite this positive trend, in 2018, in 29 EU/EEA countries, 4264 tons of antibiotics were used in humans and 6358 tons in food-producing animals. It means that their consumption is still high.

Among the most widely used antibiotics are fluoroquinolones (FQs), which are broad-spectrum synthetic antibiotics commonly used in human and veterinary medicine [23,24] and in agriculture and aquaculture [25]. Among FQs, enrofloxacin is broadly used to prevent and treat a broad spectrum of Gram-positive and -negative bacterial infections in livestock and which causes concern due to the spread of antibiotic resistance [26] and is listed among the compounds that can be considered of high ecotoxicological concern [26]. It is usually detected in the effluent of municipal sewage plants and related aquatic environments in the range of ng and µg L−1 [27,28] or even in extreme cases in mg L−1 [29]. Other examples concern their concentrations in surface waters (up to 248 ng L-1, [30]), in groundwater [31], and up to 7.7 mg kg-1 in sediment [32].

Antimicrobial resistance (AMR) occurs when bacteria, viruses, fungi, and parasites change over time and no longer respond to medicines, making common infections harder to treat and increasing the risk of disease spread, severe illness and death. The main factor accelerating the threat of AMR worldwide is overuse and misuse of medicines in humans, livestock and agriculture. WHO has declared that AMR is one of the top 10 global public health threats facing humanity.

The cost of AMR to the economy is significant. In addition to death and disability, prolonged illness results in longer hospital stays, the need for more expensive medicines and financial challenges for those impacted. Without effective antimicrobials, the success of modern medicine in treating infections, including during major surgery and cancer chemotherapy, would be at increased risk. 2014 - WHO stated that increasing antimicrobial resistance is a threat to the effectiveness of a large number of drugs. In 2015 a global cooperative project of WHO, FAO (Food and Agriculture Organization), and the World Organisation for Animal Health (OIE) is launched to combat AMR. The World Antimicrobial Awareness Week celebrated annually since 2015, aims to increase awareness of global antimicrobial resistance and to encourage best practices among the general public, health workers and policy makers to avoid the further emergence and spread of drug-resistant infections. In September 2016 the United Nations General Assembly dedicated a session to the AMR to reiterate the commitment of its members to this global problem.

On the European level, EU/EEA countries have made important progress in recent years in the development and implementation of national action plans on antimicrobial resistance involving multidisciplinary groups. Furthermore, an OECD analysis of the Action Plans of nine EU/EEA countries revealed that, consistent with the WHO Global Action Plan (WHO-GAP), the National Plans emphasize policies to optimize the use of antibiotics.

Efforts must be focused on actions such as: improving hygiene in health care facilities, adopting antimicrobial stewardship programs, increasing the use of rapid diagnostic tests, postponing antibiotic

prescriptions and public awareness, as well as supporting ongoing efforts to strengthen surveillance of antimicrobial resistance in bacteria from humans, animals and the environment.

In human medicine, Long Term Care Facility (LTCF) provide care for older people whose conditions often require antibiotic treatments to prevent infections. In these facilities, the use of antibiotics is on average 5% (2016), of which 75% of prescriptions are inappropriate in terms of the need for therapy, duration and choice of antibiotic. Furthermore, between 54% and 96% of these prescriptions are done without diagnostic confirmation.

As in other care settings, inappropriate use of antibiotics is associated with a high rate of multi-resistant organisms, yet very few countries have reported having national antimicrobial resistance action plans that specifically mention LTCF.

The EU regulation 2019/6 on veterinary medicines provides for a series of measures to limit the use of antimicrobials in farmed animals and aquaculture by 50% by 2030 in a One Health perspective. Some of these measures are:

- mandatory data collection on the sales of veterinary antimicrobials and the use of antimicrobials for animal species a ban on the preventive use of antibiotics in groups of animals
- ban on the use in animals of antimicrobials designated for the treatment of certain conditions in humans
- use alternatives to antimicrobials that have been shown to improve animal health and therefore reduce the occurrence of infections and diseases and therefore the need to use antimicrobials: vaccines, probiotics, prebiotics, bacteriophages and organic acids
- investing in effective cost-saving interventions, such as antimicrobial stewardship programs, IPC initiatives involving education, training and feedback to health professionals, increased biosecurity.

A.1.2. Natural colloids and nanoplastics in aquatic environment

Natural colloids and NPTs are both defined as materials with at least 1 dimension between 1 nm and 1 µm. Although they all belong to the nanoscale range, they cannot be considered as a single homogeneous group. Basically, they have different properties, due to the different materials, shape, size, capping and consequently surface charge, which not only differentiate them from the larger counterpart, but also between them and which give rise to a specific behavior, which must be carefully evaluated.

Natural colloids are ubiquitous and important components of the Earth biogeochemical system. They are formed from different natural processes such as volcanic ash, weathering of rocks and biological decay, covering a wide range of compositions and conformations, being highly heterogeneous in size, shape, structure. As a part of the ecosystem, precise knowledge on their fate is of high importance for the global understanding of aquatic systems.

Environmental colloids have been simplified and modelled in terms of two major colloidal components, namely: inorganic colloids and natural organic matter (NOM). Inorganic colloids are mainly composed of

aluminum phyllosilicates (*e.g*. clay, mica, chlorite) and oxides and hydrous oxides of iron (*e.g*. haematite and magnetite), manganese (*e.g*. pyrolusite) and silicon (*e.g*. silicates). Calcium carbonate is found more as a particulate form (> 1 µm). NOM can be divided into humic substances (humic and fulvic acids), which are usually present as fibrillar material; and macromolecules, such as proteins, nucleic acids, polysaccharides and small molecules such as sugars and amino sugars. As it can be seen in Table A.1, Humic substances are relatively small, compared to inorganic colloids, which are in the range of 10-1000 nm. However, colloids should not be seen as a single unit but as aggregates forming larger structures.

Table A.1. Different dissolved organic matters (DOM) types and main characteristics

DOM type	Molecular weight, kDa	Most common funtional groups	Charge (4<pH<10)	Solubility in water
Humic acids	2-5	Aliphatic and aromatic COOH, OH, and OH_3 aliphatic CO	negative	Well at high pH
Fulvic acids	0.5-2	Aliphatic and aromatic COOH, OH, and OH_3 aliphatic CO	negative	Well soluble
Carbohydrates	0.18-3000	OH, CO, COOH	Side-group dependent	Side-group dependent
Proteins	10 a few 1000	NH_2, COOH, OH, SH	Side-group dependent	Side-group dependent
Fatty acids	0.25-0.85	COOH	negative	Chain length dependent
Amino acids	< 0.2	CNH_2, COOH	Side-group dependent	Well soluble

On the other hand, NPTs have received increasing attention recently. This term refers to plastic debris of mixed composition and shape, having colloidal behaviour and with sizes ranging between 1 nm and 1 µm [33]. Once in the environment, the enormous amount of plastic waste discharged into surface waters, can be degraded under the effect of several factors (*e.g*. weathering, solar radiation, mechanical forces and biotic factors) and fragmented into small debris like microplastics and NPTs [34]. NPTs are composed of synthetic or semisynthetic materials, such as polyethylene – PE, polyvinyl chloride – PVC, polystyrene – PS, polyhydroxybutyrate – PHB, polylactic acid – PLA, polyethylene terephthalate – PET, polyacrylonitrile – PAN, poly(methyl methacrylate) – PMMA.

Although the presence of microplastics in marine and freshwaters is well documented [35-36], the detection and quantification of NPTs is more challenging due to their small size. The occurrence of NPTs in a real aquatic environment was demonstrated for the first time in 2017 in the North Atlantic subtropical gyre [37]. Authors examined four fractions of the debris collected (meso-, large micro-, small micro-, and nanoplastics). The nano-fraction was obtained by isolating the colloidal fraction of seawater and detected by dynamic light scattering (DLS). More recently, the presence of NPTs was also detected on the beach exposed to the North Atlantic Gyre [38]. Authors determined the composition and size characterization of NPTs by using transmission electron microscopy (TEM), DLS and pyrolysis gas chromatography MS (PyGCMS).

NPTs are expected to be more hazardous than larger plastics since they can pass physiological barriers more easily and be translocated to different organs in aquatic organisms [39]. Furthermore, their potential role as carriers of other pollutants (metals, organic contaminants...) may not be discarded [40]. Therefore, NPTs are not only a threat to the aquatic wildlife, but to the entire ecosystem.

A.1.3. Interaction of colloids and nanoplastics with organic pollutants in aquatic environment - focus on antibiotics

In environmental aquatic system, colloids and NPTs present colloidal properties, which have an impact on: (i) the ability to penetrate cell membranes, (i) their tendency to aggregate, and (iii) a high surface area to volume ratio resulting in a high ability to absorb and release chemicals. Despite an acquired knowledge on the fate and role of colloids in environmental water, the study of such a dynamic system has proven to be complex. In addition, the release of colloids and NPTs increases such complexity, as they might have similar role, but different behavior which needs to be fully investigated.

Knowledge gaps of all these nanomaterials currently concern their determination and quantification as well as the assessment of their ecotoxicity effects and interactions with other pollutants. Regarding the latter, the properties of the colloids and NPTs (size, shape etc) confer them unique characteristics and behavior. Additionally, different factors, such as pH, salinity, water hardness, ionic strength, temperature, and concentration, can influence their behavior and fate in the aquatic environment, as well as their interaction with co-occurring pollutants. Therefore, the assessment of the interaction of colloids and NPTs with organic pollutants, like antibiotics, and the potential role of the former as carriers is an emerging area in environmental research.

A.1.3.1. Colloids-antibiotics interactions

Colloids can also be defined as organic or inorganic entities small enough to be dominated by aggregation processes and to remain in the water column due to Brownian motion (diffusion) over reasonable timescales (> hours-days), but large enough to have supramolecular structure and properties, *e.g.* electrical surface charge and possibility of conformational changes. If different surface chemistries, sizes and other properties are considered, it is easy to see that the range is vast and that they have many different properties which will substantially impact their environmental behavior. These parameters will determine their ability, for instance, to enter the cell membranes and interact with different living organisms present in natural terrestrial and aquatic systems. Moreover, these properties could be altered once they interact with antibiotics.

On the other hand, colloids are present in almost all surface waters. Due to their high mobility and surface area, they play a crucial role in element cycling, pollutant transport and they interact with different living organisms. Despite some acquired knowledge on ecotoxicology of antibiotics and to lesser extent of colloids, little is known about this interaction and their ecotoxicology effects.

Despite it is known that more in-depth research is needed to understand how colloids interact with organic pollutants, some possible sorption mechanisms can be proposed. Figure A.1 shows the different sorption mechanism that can take place between colloids and dissolved organic matter [41].

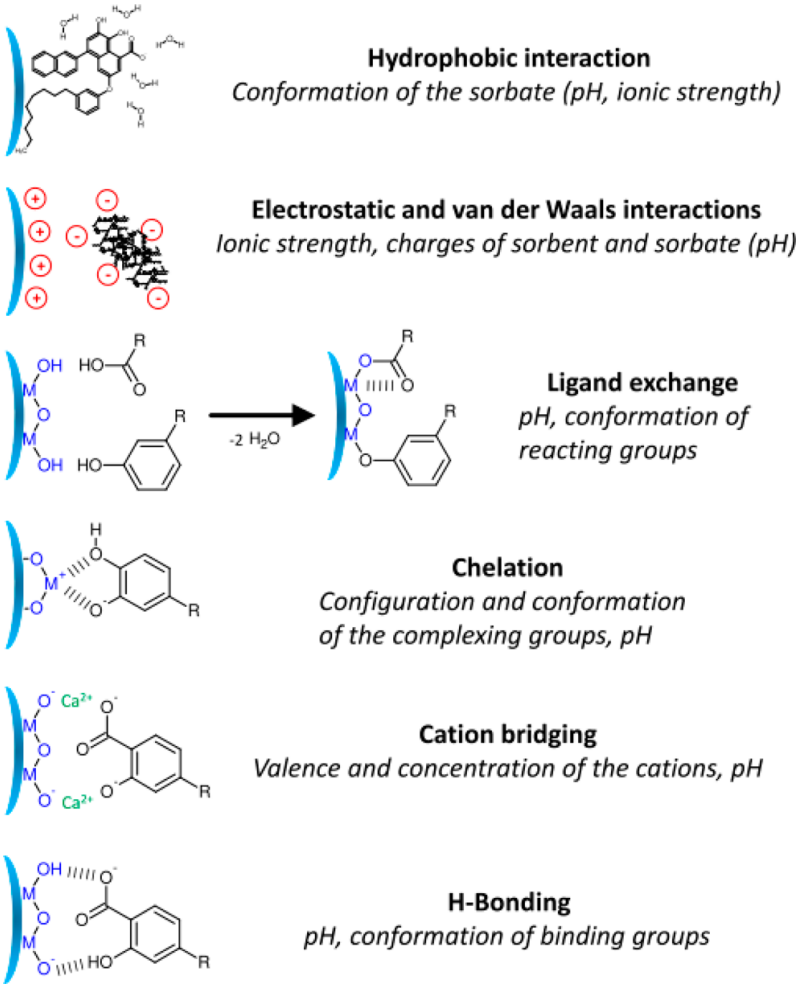


Figure A.1. Different sorption mechanisms of DOM on the surfaces of colloids with the most important parameters written in italic. From [41]

As can be seen in Figure A.1, colloids can interact with organic pollutants via p–p and electrostatic interactions, Van der Waals force, hydrophobic effect, covalent and hydrogen bond. Although Al_2O_3, Fe_2O_3 and Fe_3O_4 present a point of zero charge (PZC) around pH 8, colloids in general are found, most of the time, associated with NOMs, which confer negative charges. SiO_2 is negatively charged at environmental pH while aluminosilicates are often found as plates with positively charged edges and negatively charged faces at environmental pH. p–p interactions depend on the aromaticity degree of antibiotics and NOM sorbed to NPs; however, the presence of a high electronegative atom such as oxygen and changes in solution pH can either decrease the p electron density in the heterocyclic group or influence p–p electron donor acceptor interaction.

Antibiotics contain basic and acid functional groups, at environmental pH exist in ionized form, so that electrostatic and van der Waals interactions should be taken in consideration. For instance, due to the presence of several ionizable groups and the ability to form cationic, anionic or zwitterionic species, fluoroquinolones have a high tendency for sorption in soils and sediments. Figure A.2 shows the molecular structure of some examples of the main classes of antibiotics, as well as some parameters like logP and pK_a.

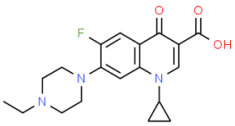
As antibiotics are ionizable compounds and colloids occur in the environment surrounded by NOM, electrostatic interactions might represent the main mechanism, either of these chemicals should have opposite charges in their functional groups at the given experimental and natural conditions. Antibiotics and colloids containing functional groups like –COOH, –OH, –F, $–NH_2$, –NH– might further interact by forming a bond with electronegative atoms such as N and O.

On the other hand, several physicochemical (such as molecular structure, charge, hydrophobicity, molecular weight and conformation) and environmental (such as pH, ionic strength, temperature, NOM concentration and aggregation state) factors may influence sorbent and sorbate in these mechanisms. Two factors that play a fundamental role in this interaction are pH and ionic strength.

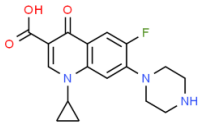
Ionic strength. Concentration and type of ions alter the interaction of colloids and organic pollutants and in fact influences their stability. Divalent cations as Mg^{2+} and Ca^{2+} may increase the adsorption of various micropollutants, probably through cation bridging and surface charge screening, while nitrate, carbonate and phosphate, may increase electrostatic repulsion. The ionic composition of surface waters is typically dominated by four major cations (Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) and four major anions (HCO_3^{-}, CO_3^{-}, SO_4^{2-}, and Cl^{-}) with ionic forms of N^{-3}, PO^{-4} Fe^{2+}, and other trace elements at lower concentrations.

pH. The pH of the solution is a major and sensitive factor that controls the adsorption of organic compounds into colloids and ultimately alters their interaction, stability and transport in the aquatic environment. Hydrophobicity, aggregation, electrostatic attraction/repulsion of organic compounds are influenced by the variation in pH. If solubility declines with either increasing or decreasing pH, then adsorption of the organic compounds into the colloids falls due to the decrease in hydrophobicity.

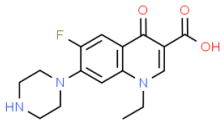
Quinolones



Enrofloxacin



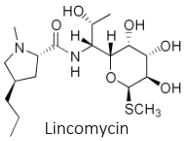
Ciprofloxacin



Norfloxacin

Antibiotic	Solubility (mg L^{-1})	logP		pK_a	
Enrofloxacin	> 1000	0.58	1.15	5.69	6.68
Ciprofloxacin	> 1000	0.28		6.09	
Norfloxacin	178 000	-1.03		5.77	8.68

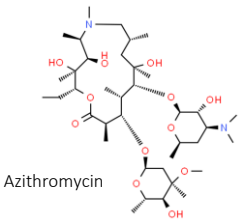
Lincosamides



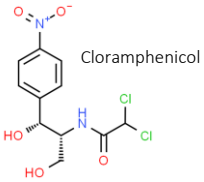
Lincomycin

Antibiotic	Solubility (mg L^{-1})	logP		pK_a	
Lincomycin	29300	0.56	-0.32	12.37	7.97

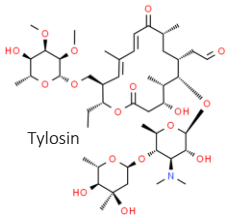
Macrolides



Azithromycin



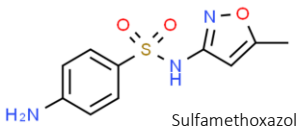
Cloramphenicol



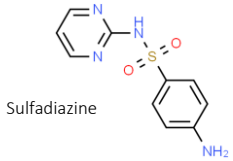
Tylosin

Antibiotic	Solubility (mg L^{-1})	logP		pK_a	
Azithromycin	514	3.03		8.5	
Cloramphenicol	217	3.14		12.46	8.38
Tylosin	211	1.46	2.32	12.45	7.2

Sulfonamides



Sulfamethoxazole



Sulfadiazine

Antibiotic	Solubility (mg L^{-1})	logP		pK_a	
Sulfamethoxazole	610	0.89		1.97	6.16
Sulfadiazine	77	-0.99	0.25/ 0.39	6.36	8.38

Diaminopyrimidines

Antibiotic	Solubility (mg L^{-1})	logP		pK_a	
Trimethropine	400	0.91		7.12	

Figure A.2. Molecular structures of selected examples of antibiotics with some parameters like solubility, logP and pK_a

A.1.3.2. Nanoplastics-antibiotics interactions

The ability of NPTs, due to their high surface area to volume ratio, to adsorb, concentrate and act as vector of toxic pollutants can modify the environmental impact of the latter. In fact, its most combined toxic effects are not simple addition of individual toxicity, but synergistic or antagonistic. For instance, NPTs may decrease the toxicity of other pollutants by first adsorbing and then agglomerating them to form larger particles, reducing the ease of uptake by the biota [42-44]. On the other hand, it has also been revealed that their presence may result in the enhancement of toxicity resulting from on-surface preconcentration (so called "Trojan horse effect" [43,45]. Although numerous ecotoxicological studies suggest that realistic environmental concentrations of micro- and nanoplastics may not induce significant detrimental effects on marine biota nor compromise their survival [46,47], co-exposure of NPs and other associated contaminants/stressors could exacerbate their effects [42].

Antibiotics are of concern since, in recent years, the extensive and their irregular use has gradually induced multifaceted adverse impacts such as enrichment and dissemination of multi drug-resistant bacteria, antibiotic-resistant bacteria (ARB), and antibiotic-resistant genes (ARGs) in the aquatic environment [48,49]. Moreover, their antibacterial impacts are not strain-specific, so, the pathogenic bacteria are killed, while some useful bacteria for organisms health are also targeted, which may cause several negative effect to organisms such as intestinal flora imbalance [50,51]. Different classes of antibiotics have been shown to be toxic towards organisms in different trophic levels, such as algae, bacteria, crustaceans, fish [52].

Several studies published so far have investigated the combined effect of NPTs and antibiotics on cyanobacteria [53], algae [54], bivalvia [55] and fish [44,45,56,57] but not the effect on other aquatic organisms, including planktonic animals such as *Daphnia magna*, being a model organism in ecotoxicological studies and a keystone species in fishless ponds food webs. While the combined effect of the stressors would depend on the organism (*i.e.* may be entirely different for different organisms), it is important to build experimental datasets to quantify and predict the factors and mechanisms responsible for the pattern under different contexts and using a range of different organisms. Moreover, the endpoint of already published studies focused mainly on the effects on molecular level, such as integrated biomarker response and antioxidant indexes, genes expression analysis and histological examinations rather than higher levels of biological organization, such as the combined effect on the life history traits and gut microbiota.

A.2. Research strategies used in environmental studies of emerging pollutants

For many emerging pollutants, the present understanding of their concentrations, transformation and transport in the different environmental compartments is scarce and/or contradictory. It can be expected that they are undergoing a significant change in their environmental cycle and their impact on the biota and potential biological and human health threats needs to be further explored.

A.2.1. Monitoring

Emerging pollutants are very often not included in governmental regulations and therefore they are not subject of compulsory monitoring. Monitoring can concern the presence of the target species, their metabolites (degradation products) and also involve additional parameters such as, *e.g.,* the presence or absence of bioindicators signalling environmental changes. Toxicological information is predominant for most pollutants on high concentrations exposure and short-term effects, while little is known regarding long term and low-level exposure at which they occur in the environment. Furthermore, the identification of future dangerous pollutants is an additional concern. Reliable regulation assessments rely on the

development of analytical methodologies able to provide information at low pollutant concentrations. To get a fully understanding of their potential effects, it is significant that pollutants are measured and monitored at their emissions sources, within the environmental compartments as well as in living organisms. In this context, analytical chemistry plays a first and relevant role as allows establishing the presence and the concentration of pollutants in the environment, characterizing their sources and determining their pathways into the environment, *e.g.*, transformations.

Under these circumstances, analytical chemistry can give high-quality information through two ways: target analysis of compounds of wide-scope monitoring methods and development of fast and efficient screening methods for the determination of non-target or unknown compounds. These improvements have come from the hand of mass spectrometry (MS) mainly coupled to liquid chromatography (LC) which extends its applicability to more polar compounds and metabolites.

Under these scenarios target compounds are identified through their retention time, high resolution and/or MS/MS spectrum within an error of 5 ppm, while untargeted compounds are identified through two ways:

- database (search library), high resolution MS, isotopic profile, MS/MS spectrum and relevant intensity signals.

- unexpected (as transformation products), structure elucidation with the structure by linking high resolution spectrum, isotopic profile, MS/MS spectrum and relevant intensity signals.

A.2.2. Model toxicological experiments of aquatic environment contaminants

Aquatic toxicology studies the adverse effects of chemicals on aquatic organisms. In aquatic toxicity tests, groups of selected organisms are exposed to test materials (water or sediment samples) under defined conditions to determine potential adverse effects. A number of standardized toxicity test protocols have been developed for determining toxicity of chemicals to aquatic species. The observed effects may include, among others, damage to the reproductive, immune, endocrine and/or nervous systems, and even death. Ecotoxicological tests allow to define a cause-effect relationship, even if generally the results obtained are valid only for the experimental conditions used and do not allow to extend the conclusions to other species or to complex natural systems, since they cannot take into account the complex interactions between biota and the environment.

The experiments are carried out at different levels including:

- behaviour of individual species in simulated environmental conditions (e.g., temperature, light, presence of colloids etc.)

- interactions of two or more species

- effect on the biota using models of phyto- and/or zooplakton or higher animals (fish)

- effects on the populations of microorganisms

A toxicity test is based on the principle according to which, by exposing a living organism to a toxic agent, the response is a direct function of the dose taken and indirect of the level of exposure; therefore, in general they are described by dose – response relationships and activity – effect curves. Toxicity tests can be divided into two categories: acute and chronic. The former describes any detrimental effects arising from the exposure of an organism to a pharmaceutical hazard, usually over a span of no more than 15 days [1]; the latter describes detrimental effects resulting from the long-term exposure (≥ 15 days to years) of an organism to the pharmaceutical hazard [2].

The assays can be conducted in the laboratory, that is, under operator-controlled conditions, using a single species or several different species, in independent experiments. The exposure can be static (the medium containing the substance is prepared at the beginning of the experiment and remains so until the end); When the medium is renewed, the test is semi-static, while is continuous if it's continuously renewed.

In order to fully characterize the cause-effect relationship, ecotoxicology use curves are obtained, which take into account the concentration used, the organisms exposed to the poison and the control (organisms that are not exposed to).

In this case, it is possible to identify two toxicity thresholds:

• NOEL (No Observed Effect Level), the highest exposure level in which no adverse effects are yet observed;

• LOEL (Lowest Observed Effect Level), the lowest effective level.

The effects also depend on the type of exposure: single (single exposure to the potentially toxic substance), repeated (multiple exposures in successive times) or chronic (constant exposure).

Although a variety of other non-standardized toxicity test methods are used in ecotoxicological research, emphasis is placed on standardized protocols (e.g., provided by the U.S. EPA), because these are the tests most commonly used in regulatory applications according to official procedures accepted at European and international level:

• acute toxicity on fish; Fish acute toxicity tests are used to assess potential risk to fish species and for other ecological regulatory needs associated with surface water contaminants. To meet EPA data requirements the test is typically conducted in three different fish species: a cold-water freshwater species, a warm-water freshwater species, and a marine/estuarine species.

• acute toxicity on bioluminescent bacteria; In comparison to other ecotoxicological tests, the use of luminescent bacteria reports final toxicity values in a short time (minutes). Bioluminescent bacteria can offer different advantages of an easy, rapid, sensitive, reproducible and cost-effective test

• acute toxicity on *Daphnia magna* and other crustaceans; One of the most internationally used bioassays for toxicity screening of chemicals and for toxicity monitoring of effluents and contaminated waters. Standard methods have been developed for this assay that were gradually endorsed by national and international organisations dealing with toxicity testing procedures, in view of its application within a regulatory framework.

• inhibition of algal growth; The freshwater algae and cyanobacteria growth inhibition test (OECD test guideline 201, [3]) is frequently used to assess the ecotoxicity of chemicals or particles. A central issue is the measurement of algal growth by quantifying algal biomass over time. Chlorophyll fluorescence measurements are recommended for the testing of particles. The analysis of in vivo fluorescence is the simplest and fastest approach, but is only suitable if there is no interference with the materials

• short-term test on fish embryos; The method uses zebrafish embryos and determines the concentration at which 50% of the embryos do not survive (*i.e*. is lethal) after being exposed to a chemical for 96 hours. Determination of the lethal concentration (50%; LC50) is a standardised approach to compare the acute toxicity of chemicals or other substances in a particular context (in this case water). The method aims to reduce tests that are carried out in juvenile or adult fish.

• growth test on juvenile stages of fish; This Test Guideline is designed to assess the effects of prolonged exposure to chemicals on the growth of juvenile fish.

• reproduction test on Daphnia magna and other crustaceans; The primary objective of the test is to assess the effect of chemicals on the reproductive output of Daphnia magna. To this end, young female Daphnia (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced is assessed.

• continuous flow bioaccumulation test on fish.

The most common procedures refer to acronyms such as APAT IRSA-CNR; ASTM, ISO, USEPA, OECD

A.2.3. Aquatic organisms used in studies of environmental pollution

A.2.3.1. Bioindicators

Bioindicators are living organisms such as plants, planktons, animals, and microbes, which are utilized to screen the health of the natural ecosystem in the environment or to determine the environmental risk assessment. They are used for assessing environmental health and biogeographic changes taking place in the environment. Microorganisms are often used as health indicators of aquatic and terrestrial ecosystems. Due to their abundance, they are easy to test and readily available. Microorganisms have a rapid rate of growth and react to even low levels of contaminants and other physicochemical and biological changes.

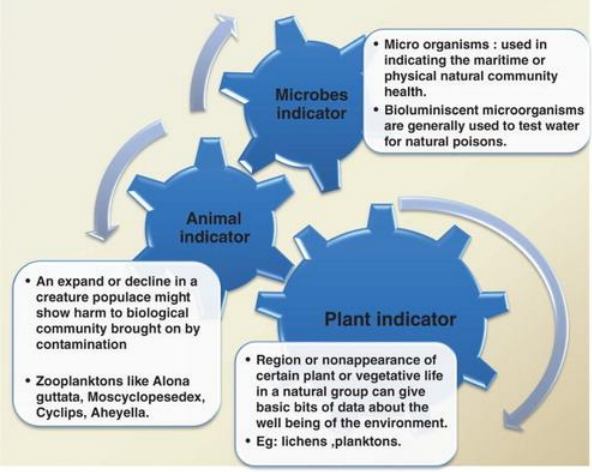


Figure A.3. Type of bioindicators. From *Trishala K. Parmar, Deepak Rawtani & Y. K. Agrawal (2016) Bioindicators: the natural indicator of environmental pollution, Frontiers in Life Science, 9:2, 110-118*

A.2.3.2. Model organisms

Model organisms are often used in biological, medical and environmental research with new ones appearing all the time. They can be plants, microbes or animals (*e.g.,* insects, fish, zooplankton and rodents), all of which are widely studied and have a well-documented genetic makeup. These organisms grow relatively quickly and have short generation times, meaning that they rapidly produce offspring. They also are usually inexpensive to work with and are easily accessible, making them convenient for experimentation.

Bioluminescent bacteria

The strain used is generally *Vibrio fischeri* and is applied for the evaluation of the acute toxicity of discharges, waters of various types, sediments, pure substances. The duration of the tests is about 15/30 minutes. There are also easy-to-apply kits on the market. *Vibrio fischeri* are nonpathogenic, gram-negative marine, luminescent bacteria which are sensitive to a wide range of toxicants.

Crustaceans Toxicity

Tests with crustaceans are carried out on a wide range of matrices, including marine waters. The duration of the tests is usually 24/48 hours, maximum 96, although there are also chronic methods lasting seven days. The most widespread and used species is *Daphnia magna*, which is quite simple to breed, it provides data of quality and the methods are standardized and validated internationally.

Daphnia magna is one of the most commonly used model organisms to assess toxicity of wide range of pharmaceuticals. Currently, daphnia toxicity tests based on immobilisation and lethality standardised by OECD, acute immobilisation test and reproduction test, are mainly used in toxicological studies. Detailed analysis of *Daphnia* biology allows distinguishing the swimming behaviour and physiological endpoints such as swimming speed, distance travelled, hopping frequency, heart rate, ingestion rate, feeding rate, oxygen consumption, thoracic limb activity which could be also useful in assessment of toxic effects. The advantage of behavioural and physiological parameters is the possibility to observe sublethal effects induced by lower concentrations of pharmaceuticals which would not be possible by using OECD tests. Additionally, toxic effects of tested drugs could be assessed using enzymatic and non-enzymatic biomarkers of *Daphnia* toxicity.

Fish

These essays are generally the least widespread and most problematic to implement, for a series of technical and practical reasons. In fact, it is not always easy for the chosen subjects to tolerate well the aquarium conditions in which they are raised; furthermore, it is essential that the fish are all healthy and of uniform size. To all this we must also add the problems of space, feeding and maintenance that must be foreseen. The acute type methods provide for the use of tests generally at 96 hours, while the chronic type tests vary depending on the species used.

The zebrafish (*Danio rerio*) is a fresh water fish that originates from Southeast Asia and is the premier nonmammalian vertebrate for genetics studies. This teleost was first described in the 1800s as an inhabitant of the Ganges River in India. One reason for the popularity of this species is that it is easy to keep and breed in captivity. In fact, in the aquarium hobby it is considered a beginner fish. Indeed, it can be found in most aquarium pet stores, and is rather cheap to buy. It tolerates a wide range of water conditions and temperatures, and accepts all sorts of dried and live fish foods. It is small, highly social, so a large number of zebrafish can be housed in small tanks, and is extremely prolific.

The determination of effects arising from environmental pollutants at the lowest trophic level usually involves the exposure of freshwater algae cyanobacteria, or higher plants, such as *Lemna gibba* (duckweed) to the pollutant of interest.

Algae Tests

This type of ecotoxicity tests mainly consider the inhibition of algal growth as an endpoint. The main reference is constituted by the UNI EN ISO methods. Some examples are: Green algae (P. *subcapitata, D. subspicatus*); Diatoms (*N. pelliculosa*); Cyanobacteria (*A. flos-aquae, S. leopoliensis*).

Vegetables

Tests on vegetables evaluate the effects of toxic substances on germination and root elongation. Equally widespread are phytotoxicity tests which consider, for example, the variation in pot growth of certain species. The times and methods vary depending on the plant used.

A.3. State-of-the-art methodological approaches in the assessment of the antibiotic pollution, nanoparticle characterization and their effects on the aquatic environment

A.3.1. Determination of antibiotics

Occurrence and risk assessment of antibiotics in surface waters have been the object of many recent studies [60-63]. LC – MS/MS using a triple quadrupole analyser has been the most currently applied methods [64-67]. Due to diverse chemical structures of antibiotics, the analytes are ionised using electrospray (ESI) both in the positive (ESI+) and the negative (ESI−) mode. The studies have mostly concerned a single class of antibiotics, for instance fluoroquinolones [68] or sulfonamide antibiotics [69] with similar physico-chemical properties. 5 antibiotics (amoxicillin, clarithromycin, erythromycin, ofloxacin, sulfamethoxazole) could be determined among 40 emerging contaminants [67], 46 antimicrobial drug residues in pond water [70]. The analyses targeted usually the marketed compounds without addressing their metabolites because of the lack of standards [69]. Non-targeted analyses of antibiotics or their degradation products in water by high resolution accurate mass spectrometry (HRAM) using TOF or Orbitrap analysers have been scarce [70,71]. A recent review [72] collected the information on LC-based procedures used for the determination of antibiotics and their metabolites in water samples of different origin (Table A.2).

Table A.2. LC-based procedures employed for determination of antibiotics and their metabolites in water samples of different origin [72].

Analytes/group of analytes	Type and volume of sample	Sample preparation	LC conditions		Detection		LOD/LOQ [ng L^{-1}]	Ref.
			Column	Mobile phase	Type	Ionization		
Sulfomethaxazole	Water (25 mL)[a]	Filtration through a 0.45 µm polyvinylidene fluoride membrane filters, dilution in methanol:water (10:90, v/v) and SPE using Oasis® HLB cartridges (60 mg, 3 ml)	Acquity BEH C18 (50 mm × 2.1 mm i.d., 1.7 µm)	ACN and 0.1% FA in water (gradient)	QqLIT (MRM mode)	ESI+	n/a	[73]
Clarithromycin, fluoxetine, norfluoxetine, carbamazepine	Wastewater (100 mL) and surface water (250 mL)	Samples were acidified to pH 3 with sulphuric acid, SPE using Oasis® HLB Cartridges (150 mg, 6 mL)	Kinetex™ XB-C18 (100 mm × 2.1 mm i.d., 1.7 µm)	EtOH/water 50/50, v/v (gradient of flow rate)	QqQ (MRM mode)	ESI+	0.01–0.20/–	[74]
Phenicol antibiotics: thiamphenicol, florfenicol	Water[a]	SPE using Oasis® HLB cartridges (200 mg, 6 mL)	BEH C18 (50 mm × 2.1 mm i.d., 1.7 µm)/C18 column (150 mm × 2.1 mm i.d. 5 µm)	ACN/water 10/90, v/v (isocratic) and ACN/water + 0.05% FA (gradient)	DAD/QqQ (MRM mode)	ESI+	n/a	[75]
Amoxicillin, Azithromycin, Benzylpenicillin, Ciprofloxacin,	Surface water (500 mL)	Filtration through a 1.0 µm GF/B Whatmann glass fiber and a 0.45 µm Whatmann nylon filter, pH adjustment to	Phenomenex Luna C18(2) (150 × 2.0 mm i.d., 3 µm)	MeOH and water/0.1% FA (gradient)	HRMS (SCAN and MRM mode)	ESI+	<50/–	[76]

Analytes/group of analytes	Type and volume of sample	Sample preparation	LC conditions		Detection		LOD/LOQ [ng L^{-1}]	Ref.
			Column	Mobile phase	Type	Ionization		
Metronidazole, Sulfamethoxazol, Trimethoprim		7 with NaOH and 5% (w/v) Na_2EDTA and SPE using Oasis® HLB cartridges (200 mg, 6 mL)						
Chloramphenicol, ciprofloxacin, levofloxacin, metronidazole, nalidixic acid, sulfadoxin, sulfamethazine, sulfamethoxazole, trimethoprim	Wastewater, surface and ground water (100 mL)	pH adjustment with 5 M NaOH and 10% FA , filtration through a 1.0 µm GF/B Whatman glass fiber filter and then a 0.45 µm Whatman nylon membrane and SPE using Oasis® HLB (200 mg, 6 mL) cartridges	Phenomenex Luna C18 (150 × 2.0 mm i.d., 3 µm)	MeOH and water both with 0.1% FA (ESI+)/ACN and water (ESI−) (gradient)	HRMS (MRM mode)	ESI+/ESI−	<50/–	[77]
Tetracycline (TC) and metabolites 4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC), and anhydrotetracycline (ATC)	Wastewater and surface water (150 mL)	Filtration through Whatman glass microfiber filter (0.7 µm), pH adjustment to 3 with HCl and SPE using Oasis® HLB (500 mg, 6 mL) cartridges followed by Oasis MAX (60 mg, 3 mL) cartridges	ACQUITY UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7 µm)	ACN and water/0.1% FA (gradient)	Q–TRAP (MRM mode)	ESI+	–/920 (TC), 990 (ETC), 1320 (EATC), and 1950 (ATC)	[78]
Penidline, quinolone, tetracycline, sulfonamide antibiotics	Wastewater (100 mL effluent or 50 mL influent)	pH adjustment with 5 M NaOH and 10% FA solution, filtration through a 1.0 µm GF/B Whatman glass fiber filter and then through a 0.45 µm Whatman nylon membrane and SPE using Oasis® HLB (200 mg, 6 mL) cartridges	Phenomenex Luna C18 (150 × 2.0 mm i.d., 3 µm)	MeOH and water both with 0.1% FA (ESI+)/ACN and water (ESI−) (gradient)	HRMS (MRM mode)	ESI+/ESI−	–/–	[79]

Analytes/group of analytes	Type and volume of sample	Sample preparation	LC conditions		Detection		LOD/LOQ [ng L^{-1}]	Ref.
			Column	Mobile phase	Type	Ionization		
Organic pollutants including antibiotics (screening procedure)	Surface water, ground water and effluent wastewater (100 mL)	Sample centrifugation and SPE using Oasis® HLB (60 mg, 3 mL)	Acquity UPLC BEH C18 (100 × 2.1 mm i.d., 1.7 µm)	Water/0.01% FA and MeOH/0.01% FA (gradient)	Q–TOF–MS (SCAN and MRM mode)	ESI+/ESI−	0.1–1/0.1–1 (LOI/SDL)	[80]
Pharmaceuticals and fungicides including sulfonamides tetracyclines, macrolides, penicillines anticiotics (screening procedure and confirmation by UPLC-MS/MS)	Surface and groundwater (100 mL)	pH adjustment to 3 with AA and SPE using Strata-X (200 mg, 6 mL) cartridges	Acquity U-HPLC C18 (100 × 2.1 mm i.d., 1.8 µm)	Water/2 mM ammonium formate/160 µL FA and MeOH/2 mM ammonium formate/160 µL FA (pH 3.5) (gradient)	Exactive Orbitrap MS (SCAN and MRM mode)	ESI+/ESI−	10–50/–	[81]
Sulfapyridine (SULF), p-nitroanisole (PNA) and pyridine (PYR) (degradation study)	Water (3–5 mL)[a]	SPE using Oasis® HLB (60 mg, 3 mL)	SULF: Symmetry C18, (150 × 4.6 mm i.d., 3.5 µm) PNA and PYR: Zorbax HILIC plus (100 × 2.1 mm i.d., 3.5 µm)	Water (A) and ACN/0.05% FA (10 mM, pH 3) (B) SULF: 70:30 A:B PNA and PYR: 80:20 A:B (isocratic)	UV-DAD	–	–	[82]
Macrolide antibiotics (azithromycin, erythromycin, clarithromycin and roxithromycin) and metabolites	Wastewater and surface water (1–250 mL)	Filtration through glass-fiber filters, pH adjustment with FA to 7–7.5 and SPE using Oasis® HLB (200 mg/6 mL) and Strata SAX (100 mg/3 mL)	ACE C18 PFP (150 × 3 mm i.d., 3 µm)	Water/0.1% FA and ACN (gradient)	QqQ (MRM mode)	ESI+	2–30/–	[83]
Metronidazole, sulfamethoxazole, trimethoprim,	Wastewater (100 mL)	Filtration through filter membrane (0.45 µm), pH	X Terra MS C18 (250 mm × 4.6 mm i.d., 5 µm)	Water and ACN/AA pH 3 (ACN and ammonium acetate (60:40) for spiramyc	QqQ (MRM mode)	ESI+	0.0001–0.12/0.0003–0.4 (µg/L)	[84]

Analytes/group of analytes	Type and volume of sample	Sample preparation	LC conditions		Detection		LOD/LOQ [ng L^{-1}]	Ref.
			Column	Mobile phase	Type	Ionization		
ceftazidime, ciprofloxacin, ofloxacin, spiramycin		adjustment with FA to 3.0, and SPE using Waters Sep-Pak C18						

AA acetic acid; ACN acetonitrile; DAD diode array detector ESI electrospray ionization; FA formic acid; HR high resolution; LOD limit of detection LOI limit of identification; LOQ limit o quantification; MeOH methanol; MRM multiply reaction monitoring mode; MS mass spectrometry; n/a not available; QqLIT quadrupole-linear hybrid ion trap MS; QQQ triple quadrupole MS; Q–TOF quadrupole time of flight; Q–TRAP quadrupole ion trap; SDL screening detection limit; SPE solid phase extraction.

A.3.1.1. Sample preparation - preconcentration

Due to generally low concentrations of antibiotics in environmental waters, most of published works involve a preconcentration step. Solid phase extraction (SPE), shown schematically in Figure A.4, is a technique used for rapid, selective sample preparation (purification and/or) preconcentration prior to the chromatographic analysis. In SPE, analytes from a liquid sample are isolated by adsorbing onto a solid stationary phase. It can serve a double purpose: (1) matrix removal resulting from difference affinity of the analytes and the interferents to the stationary phase and (2) preconcentration when a large sample volume is introduced onto the sorbent and then the analytes are eluted by a significantly lower volume of the solvent. In addition, an additional preconcentration step (e.g., by solvent evaporation) can be introduced.

The SPE procedure typically consists of four steps (Figure A.4):

1: Conditioning. The sorbent material is activated by passing a suitable solvent followed by another one that is similar to that in the sample. in this step the wet the dry solid stationary phase is wetted, air and impurities removed and the functional groups are activated.

2: Loading (sample addition). The sample is passed through the sorbent material, by gravity, by pumping or by vacuum aspiration. Samples volumes can vary from 1 mL to 1 L depending on the system and material used. This step allows the preconcentration of analytes on the sorbent and matrix removal.

3: Washing. This optional step allows the washing of the sorbent with a low elution strength solvent (usually water), to eliminate matrix impurities, while retaining the targeted analytes. In order to avoid the presence of water in the final extract, a drying step is often used and usually carried out under vacuum.

4: Elution. The target analytes are eluted from the sorbent material with an appropriate solvent at a suitable flow rate. The nature of the solvent and its volume are chosen based on the chemistry of targeted components and the analytical method to be used.

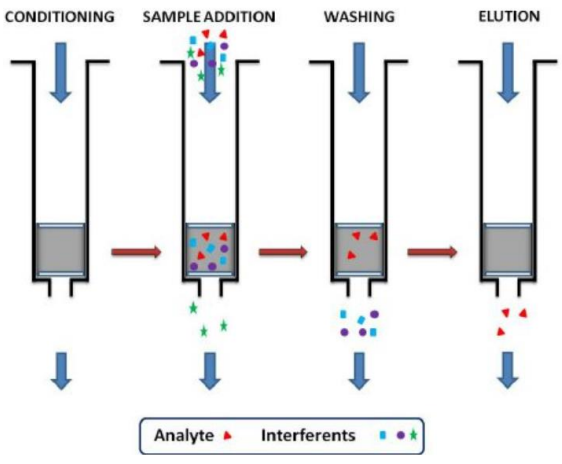


Figure A.4. Steps in SPE. Extracted from [85]

The choice of sorbent material in SPE depends on the sample matrix and chemical characteristics of the target compounds. The most popular sorbents are silica and octadecyl bound to silica materials (C18-

silica). Others include polystyrenedivinylbenzene, divinylbenzene-vinylpyrrolidone copolymers and graphitized carbon black (GCB) sorbents.

The sorbents can be packed into microcolumns, disposable syringes or cartridges or in the form of disks (Fig. A.5). The SPE process can be automated and coupled to analytical systems, such as liquid chromatography. The on-line approach minimizes sample losses, increases reproducibility but does not allow the use of extracts resulting from the SPE in different analyses.

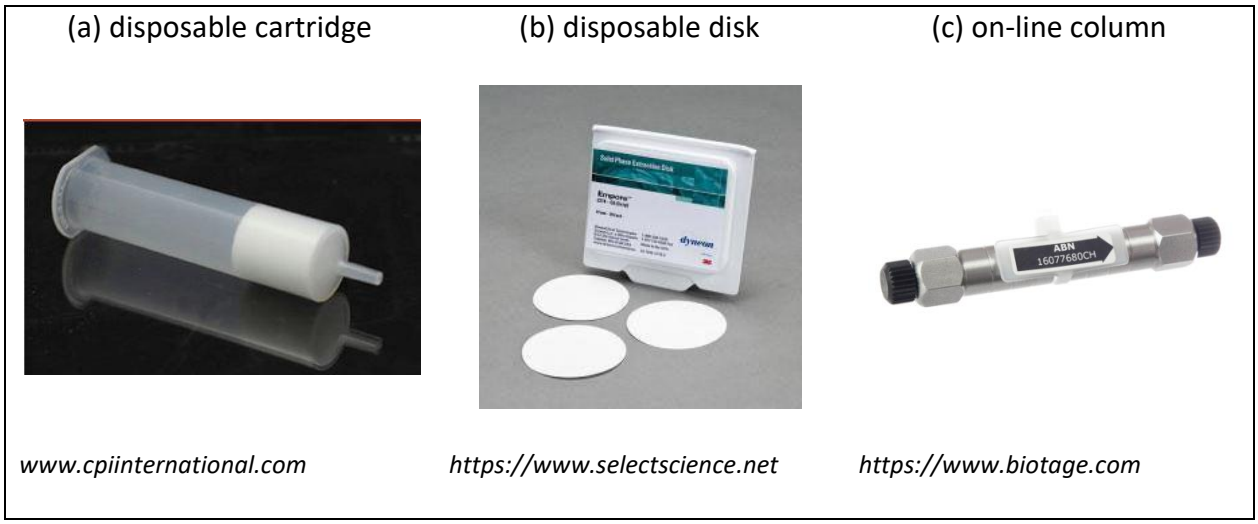


Figure A.5. Examples of available SPE formats.

The sorbent most frequently used in SPE extraction of antibiotics is HLB sorbent (hydrophilic-lipophilic balance sorbent), which is selective and effective in the isolation of polar compounds; in order to increase extraction efficiency, it is suggested that pH be changed through acidification (typically to pH around 3) [72].

A.3.1.2. Liquid chromatography - mass spectrometry (LC-MS)

Mass spectrometry is an analytical technique used to quantify known species (targeted analysis) and/or identify unknown ones (untargeted, exploratory analysis) within a sample by elucidating their structure. The complete analytical process involves the conversion of the analytes into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

A mass spectrometer generates multiple ions from the sample components and then it separates them according to their specific mass-to-charge ratio (m/z), and records the relative abundance of each ion type.

A mass spectrometer consists of the following major components:

- **Ion Source** used to produce gaseous ions from the sample components
- **Analyzer** used to sort the ions according to their mass-to-charge ratio.
- **Detector** used to detect the ions and record the relative abundance of each of the resolved ionic species.

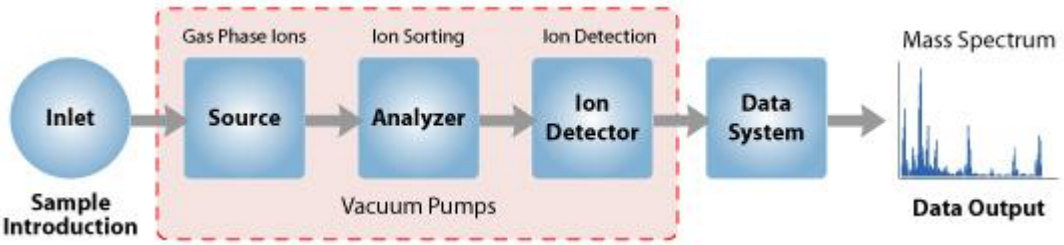


Figure A.6. General scheme of a mass spectrometer [86]

The processes performed by a mass spectrometer in order to accomplish the measurement are the following:

- Producing ions from the sample components in the ionization source.
- Separation of these ions according to their mass-to-charge ratio in the mass analyzer.
- Possibly, fragmenting the selected ions and analyzing the fragments in a second analyzer.
- Detecting the ions emerging from the last analyzer and measuring their abundance with the detector able to convert the ions into electrical signals.
- Processing the signals from the detector that are transmitted to the computer.

A key part of a mass spectrometer is the mass analyzer; different types of mass analyzers, either low or high resolution, can be used. The most popular ones are: quadrupole, time of the flight and Orbitrap.

Quadrupole mass analyzer (Figure A.7) consists of four rods arranged in a tubular like structure. Where a voltage (radiofrequency (RF) and direct-current (DC) components) is applied on the opposite pairs of poles that are connected electrically. At a specific amplitude of voltage, ions of a particular m/z follow a stable typical trajectory through the rods and reach the detector. A mass spectrum is therefore produced by varying the RF and DC voltages in a systematic way to bring ions of increasing or decreasing m/z ratios to the detector. It is considered as an ideal detector for chromatography since it is capable of fast scanning and uses low voltages. However, it is classified as a low-resolution device, unable to provide the elemental composition of an ion [87].

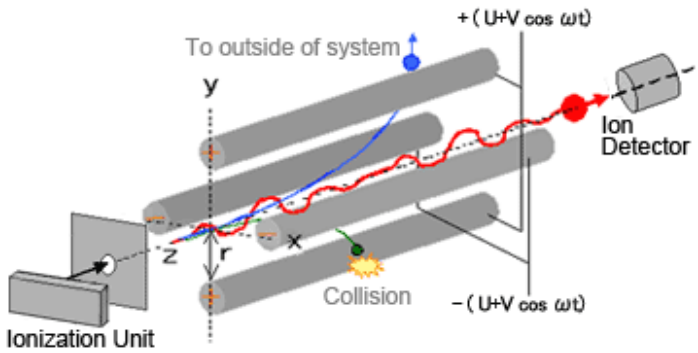


Figure A.7. Diagram of a quadrupole mass analyzer. Source: [88]

The operation of a **time-of-flight** (ToF) mass analyzer is based on the separation of ions according to the time it takes them to travel through a flight tube with known length and reach the detector. The trajectory of an ion through a ToF mass analyzer depends on its momentum and kinetic energy due to an applied pulsed acceleration voltage and m/z ratio of the ion. Based on classical physics, ions with lower m/z will

travel the fastest and arrive at the detector first while ions with larger m/z will travel the slowest and arrive at the detector last. A scheme of the tie-of-flight mass analyzer is shown in Figure A.8.

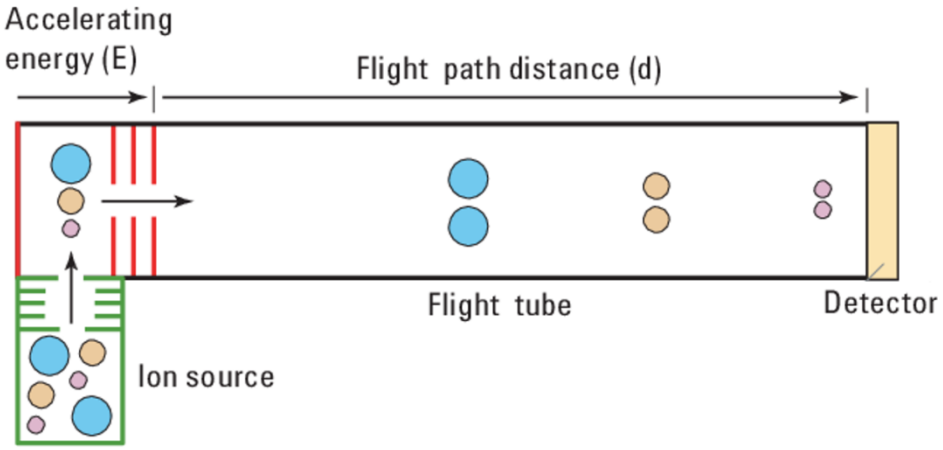


Fig. A.8. A scheme of the time-of-flight mass analyzer. Source: [89]

Orbitrap consists of an outer barrel-like electrode and a coaxial inner spindle-like electrode that traps ions in an orbital motion around the spindle (Figure A.9). The image current from the trapped ions is detected and converted to a mass spectrum using the Fourier transform of the frequency signal [90].

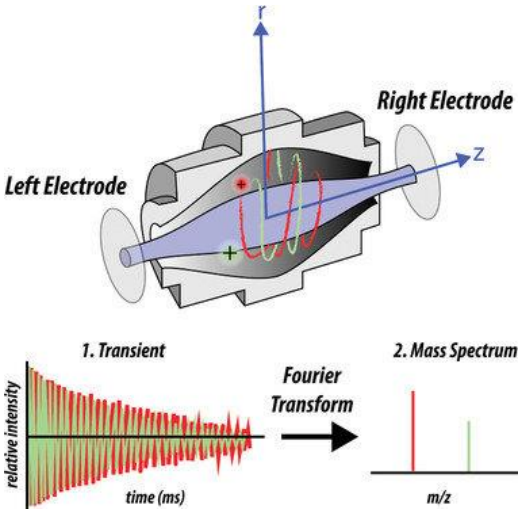


Fig. A.9. A scheme of the Orbitrap FT mass analyzer. In the Orbitrap, ions oscillate around a central spindle-like electrode while also oscillating in the axial dimension. Axial oscillation is detected by electrodes to yield a transient, which is transformed into the resulting mass spectrum by the Fourier transformation. The transient is the frequency of ion oscillation in the axial dimension and will be unique for each *m/z*; all *m/z*'s are detected simultaneously. From [91].

Orbitrap is a high resolution high mass accuracy mass analyzer allowing the determination of the molecular weight to several decimal places. That accuracy allows the determination of molecular formulas. ESI Orbitrap MS allows analyses even in the absence of standards, by comparing to databases, with a higher

selectivity, higher accuracy and the exploration ability of new species. In fact, the resolution can reach up to 1000000 and mass accuracy down to less than 1 ppm (parts per million) under favorable conditions.

In **tandem mass spectrometry**, also known as MS/MS or MS^2, two or more mass analyzers are coupled together using an additional reaction (fragmentation) step to increase their abilities to analyse chemical samples. The basic idea of MS/MS is a selection of a m/z of a given ion formed in the ion source, and subject this ion to fragmentation, usually by collision with inert gas and detection of the product ions. This is a powerful way of confirming the identity of the compounds and determining the structure of unknown species. The most popular configurations of tandem mass spectrometry include: (i) triple quadrupole (QqQ), (ii) quadrupole–time of flight (Q-TOF) and (iii) hybrid quadrupole-Orbitrap mass spectrometer.

(i) Triple quadrupole mass spectrometer uses the first and third quadrupoles as mass filters. In the second quadrupole, the fragmentation of analytes ions takes place through collision with gas. The triple quadrupole is the most frequently used mass spectrometer for MS/MS, mainly because of the relatively low cost and ease of operation.

(ii) Q-TOF mass spectrometer combines quadrupole and TOF mass analyzers, which results in high mass accuracy for product ions and accurate quantitation capability. In this mass spectrometry method fragmentat ions *m/z* ratio is determined through a time-of-flight measurement.

(iii) In a hybrid quadrupole-Orbitrap system (Fig. A.10) used in this project, a quadrupole filters the ions according to their m/z ratios. Then the ions are transferred into the C-trap, where they are accumulated and then injected into the Orbitrap analyzer to obtain mass spectra.

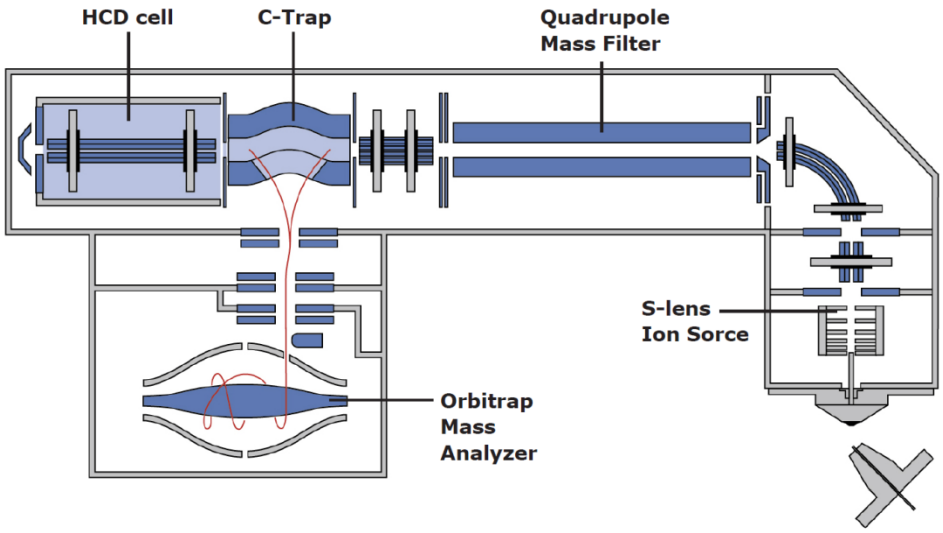


Figure A.10. A scheme of a quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Q Exactive Plus model used in this project)

Mass spectrometry targeted measurement approaches include selective reaction monitoring (SRM) and parallel reaction monitoring (PRM) presented schematically in Figure A.11.

Selective Reaction Monitoring (SRM, also known as Multiple Reaction Monitoring-MRM) is a highly specific and sensitive mass spectrometry technique that can efficiently quantify compounds in complex mixtures.

It uses a triple quadrupole MS that firstly targets the ion corresponding to the compound of interest with subsequent fragmentation of that target ion to produce a range of daughter ions. One (or more) of these fragments can be selected for quantitation purposes. Only analytes that meet these both criteria, *i.e.* specific parent ion and specific daughter ions corresponding to the mass of the molecule of interest are isolated within the mass spectrometer. By ignoring all other ions that enter the mass spectrometer the method achieves sensitivity, while maintaining exquisite accuracy.

Parallel reaction monitoring (PRM) is an ion monitoring technique based on high-resolution and high-precision mass spectrometry. PRM is based on Q-Orbitrap as the representative quadrupole-high resolution mass spectrum platform. Unlike the SRM, which performs one transition at a time, the PRM performs a full scan of each transition by a precursor ion, that is, parallel monitoring of all fragments from the precursor ion. At first step, the PRM uses the quadrupole (Q1) to select the precursor ion, which is then fragmented in the collision cell (Q2); finally, Orbitrap replaces Q3, scans all product ions with high resolution and high accuracy. Therefore, PRM technology not only offers the SRM/MRM target quantitative analysis capabilities, but also have the qualitative ability. The mass accuracy can go down to ppm level, which eliminates the background interferences and false positives better than SRM/MRM, and effectively improves the sensitivity in complex matrices. Moreover, full scan of product ions, without the need to select the ion pairs and optimize the fragmentation energy, makes it easier to develop the analysis. The last but not least, a linear range is increased to 5-6 orders of magnitude.

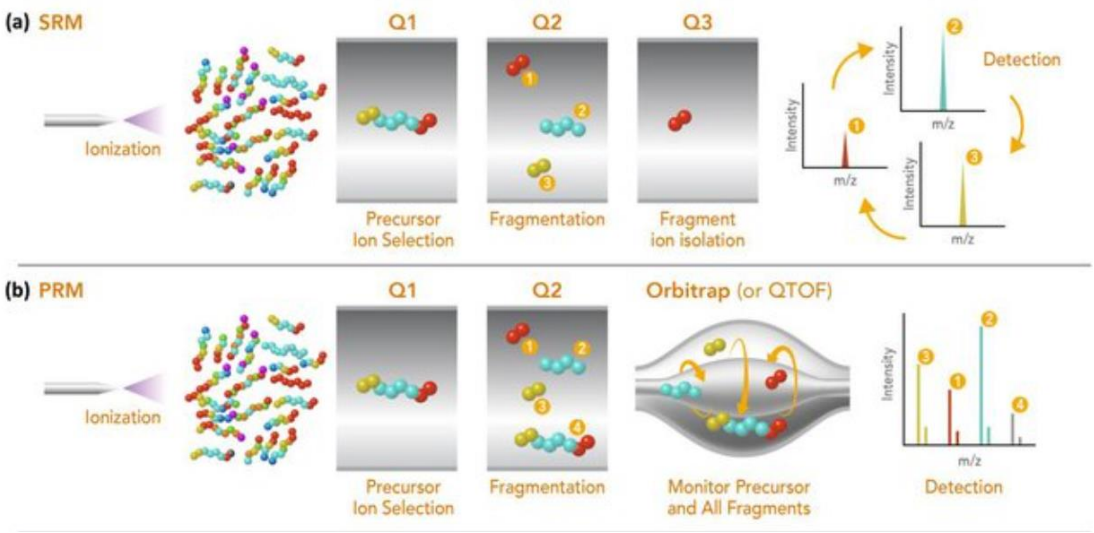


Figure A.11. A schematic representation of targeted measurement approaches: a) SRM and b) PRM. In SRM, each product ion transition (fragment) is monitored individually (normally 1 to 5) and then combined for quantitation. In PRM, all product ion transitions and possible product ions are analyzed in concert with high resolution and mass accuracy. Q1 and Q3 refer to the first and third mass-resolving quadrupoles and Q2 to the quadrupole (or cell) where fragmentation is performed. From [92].

Liquid chromatography–mass spectrometry (LC–MS) combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry discussed above. Liquid chromatography is a method of physical separation in which the components of a liquid sample are

partitioned between two immiscible phases, *i.e.,* stationary and mobile. The use of liquid chromatography as a sample introduction mode greatly enhances the performance of MS by separating multiple sample components and thus simplifying the ionization of analytes by reducing its possible suppression by the matrix. At the same time, a chromatographic retention time is an additional measure of the species identification. The most popular liquid chromatographic separation mode used in the analysis of antibiotics is reversed-phase, which makes use of a nonpolar (hydrophobic) stationary phase and a polar mobile phase. In most applications, the mobile phase is a mixture of water and other polar solvents (*e.g.,* methanol, acetonitrile), and the stationary phase is prepared by attaching long-chain alkyl groups (most often C18) to the external and internal surfaces of irregularly or spherically shaped porous silica particles.

LC-MS can be used to analyze biochemical, organic, and inorganic compounds commonly found in complex environmental and biological samples; it is applied in a wide range of fields including environmental monitoring, biotechnology, food processing, pharmaceutical and agrochemical industries as well as in clinical applications.

A.3.2. Characterization of nanoparticles

A.3.2.1. Fractionation of nanoparticles by field flow fractionation (FFF)

FFF is a family of chromatography-like techniques, in which species are separated due to their interaction with a field applied perpendicular over the cross section of a thin, flat channel (see Figure A.11). In asymmetrical flow field flow fractionation (AF4), a crossflow perpendicular to the carrier flow is applied. This technique can separate nanoparticles and colloids from 2-3 nm up to several microns based on their diffusion coefficient. Using either FFF theory or calibration with size standards, the technique can determine particle size.

In AF4, the bottom wall of the channel consists of a semi permeable ultrafiltration membrane (accumulation wall), allowing the cross flow to pass through the channel, but retaining particles larger than the membrane pore size (commonly 5-10 kDa are used) in the channel. Figure A.12 shows the experimental setup of a AF4 channel.

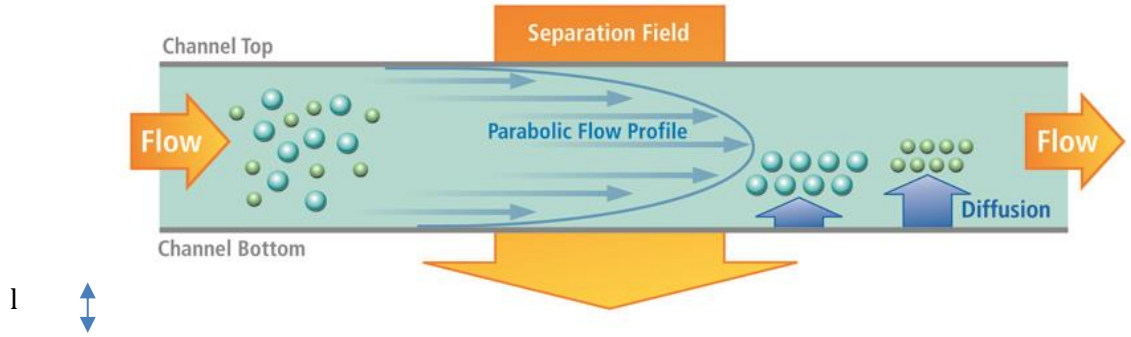


Figure A.11. Separation scheme of Field Flow Fractionation techniques.

The sample is eluted along the channel by a laminar longitudinal channel flow, perpendicular to the cross flow. Although the particles are in constant Brownian motion in all directions, the cross flow will shift their average cross-sectional position closer to the accumulation wall. Due to the opposed movements of field transport (proportional to the crossflow applied), with an induced velocity U and the diffusion coefficient of the particles, *D*, an equilibrium position is reached (l), defined as the distance to the accumulation wall (see figure A.11).

In a polydisperse sample, populations of particles with different diffusion coefficients will thus have different values of l. Due to the geometry of the channel, the longitudinal channel flow will be laminar, with a parabolic flow profile. Since the velocity of the parabolic flow vectors decreases toward the walls of the channel, the transport velocity of particles along the channel will increase with l. Thus, particle retention is a function of diffusion coefficient, and particles with different diffusion coefficients will be separated. Those species not retained by the crossflow applied will elute at the beginning of the fractogram (void peak).

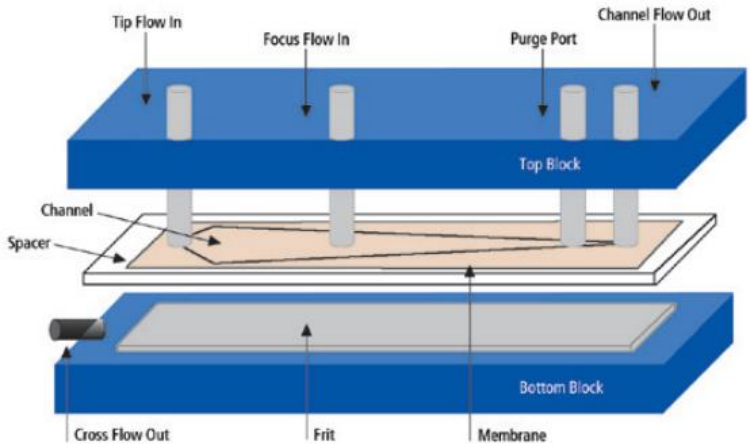


Figure A.12. Schematic of the asymmetrical flow FFF (AF4) channel. From: [93]

Although AF4 can provide a high-resolution separation of nanoparticles, the separation can be compromised through several processes such as (i) particle aggregation, (ii) particle membrane interaction, (iii) pre-elution due to sample overloading or inter-particle electrostatic repulsion, and (iv) steric inversion (particles large enough have a negligible diffusion coefficient compared to the flow applied and elute in the so-called steric mode). Such processes should be monitored and minimized in the method optimisation step. Method optimisation is an essential step in AF4 experimentation in order to achieve a good separation and reduce sample perturbation. Method optimisation should account for (i) the choice of the carrier solution, (ii) the choice of the accumulation wall membrane and (iii) the choice of the applied field (cross flow).

Ideally the carrier solution should mimic the physicochemical properties of the (nano)particle/colloidal suspension (*e.g.* pH, ionic strength and chemical composition) to minimize (nano)particle perturbation such as surface charge, double layer thickness, particle aggregation/disaggregation and dissolution. However,

usually a simple solution of electrolyte at ionic strength and pH condition close to the real sample is selected. Additionally, in some cases a surfactant is used to maximize sample recovery, and a bactericide (*e.g*. sodium azide) is used to prevent bacterial growth, although changes from the *in situ* conditions are more likely. In all cases the carrier solution should be selected to prevent particle aggregation, particle–membrane interaction, particle dissolution and bacterial growth, as well as to maximize particle recovery (fraction eluted respect to what is injected). The accumulation wall membrane should be selected to retain all nanoparticles in the channel, minimizing particle–membrane interaction to maximize nanoparticle recovery. The most widely used membranes in AF4 are regenerated cellulose and polyethersulfonate (PES) with a molecular cut-off in the range of 1000–10,000 Da.

To minimize nanoparticle losses, in particular those in the size range of 1 nm such as humic substances, the smallest membrane cut-off should be used, although pressure issues inside the channel limit that, and larger pore sizes (such as 5 kDa) have to be used instead. To minimize particle–membrane interaction and maximize sample recovery, a charged membrane (same charge as the particles) should be used.

The cross flow is the key factor that determine AF4 resolution and quality of separation. The cross flow should be optimised to achieve the best possible separation while keeping sample losses and void-peak–sample-peak overlap to minimum. Additionally, the cross flow should be selected according to the size of the (nano)particles under consideration. For instance, a high cross flow should be applied for the fractionation of small nanoparticles whereas a low cross flow should be applied for the fractionation of large nanoparticles or particles. For a heterogeneous sample with a wide size distribution, a gradient flow could be applied to reduce sample analysis time and minimize steric inversion effect. Alternatively, a range of cross flows should be applied for heterogeneous samples, in parallel to using an independent size detector technique to assess any fractionation abnormalities if concentration ranges are adequate (in general, light scattering techniques show low sensitivities, especially for the smallest particles). The quality of separation can be evaluated by sample recovery, fractogram reproducibility, absence or minimal height of the void peak or the absence or minimal overlap between the void peak and the fractionated (nano)particles peak.

By combining the high-resolution separation of FFF with the sensitivity and specificity of ICP-MS (see next section), a powerful approach for nanoparticles/colloids characterization is created. In addition to ICP-MS, DLS and/or UV/Vis spectrophotometry can be used in order to either verify ICP-MS results or for complementary information.

A.3.2.2. ICP-MS detection

ICP-MS (inductively coupled plasma mass spectrometry) is an elemental analysis technique, with low detection limits and multi-element capabilities, being the most frequently utilized inorganic mass spectrometric technique nowadays for fast multi-element determination in the trace and ultratrace concentration range [94].

An ICP-MS instrument consists of the ion source (the ICP), a mass spectrometer (MS) – usually a scanning quadrupole mass filter, and a detector. The ICP is at atmospheric pressure, while the MS and detector operate in a vacuum chamber, so an ICP-MS instrument requires a vacuum pump, a vacuum interface, and

electrostatic ion "lenses" to focus the ions through the system. State-of-the-art ICP-MS systems typically contain some device or mechanism to resolve spectral interferences. Four main processes take part in the analyte measurement, they are: sample introduction and aerosol generation, ionization by the plasma source, mass discrimination, and the detection. A basic scheme of an ICP MS instrument is presented in Figure A.13.

ICP-MS is typically used to analyze liquid samples, and it can easily be coupled to chromatographic separation devices, such as an HPLC, IC, GC, CE, or FFF, to provide information on the different chemical forms (species) of each element in a sample. Also, being a mass spectrometric technique, ICP-MS measures the individual isotopes of an element, so it can be used for applications where isotopic abundances or isotope ratios are of interest.

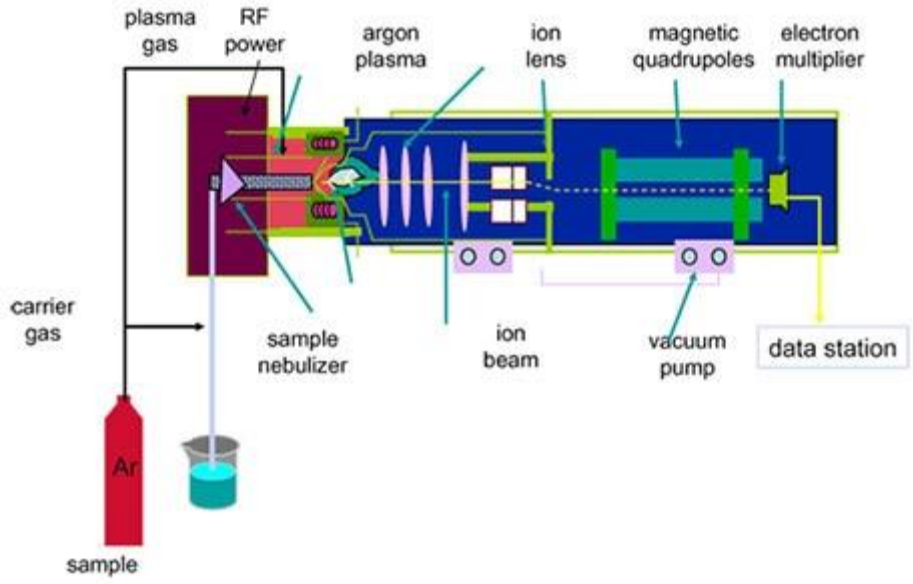


Figure A.13. Scheme of an ICP MS instrument. Source: [95]

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B. GOALS AND OBJECTIVES

This PhD project contributes to the studies of emerging contaminants in environmental freshwater ecosystems with a focus on the Pyrénées aquatic environment and, in particular, on the POCTEFA region. The study area was chosen because of the considerable agricultural activities including intensive farming that can potentially be a significant source of veterinary antibiotics. The goals included developments in analytical methodology, environmental assessments of the pollution and its sources and, finally, testing toxicological hypotheses related to the effects of the detected species on freshwater zooplankton.

The detailed objectives were:

(i) to carry out exploratory monitoring of the presence of antibiotics in surface and wastewaters to propose targets for subsequent detailed evaluation.

(ii) to present a long term quantitative assessment of the presence of antibiotics and their degradation products in surface and wastewaters by optimized LC-MS method following a SPE preconcentration.

(iii) to investigate possible interaction of the antibiotics with natural colloids in surface and waste waters by state-of-the-art A4F-ICP MS.

(iv) to reveal effects of the separate and joint presence of enrofloxacin, the most abundant antibiotic found in the studied area, and nanoplastics on life history parameters and the bacterial community in the intestinal tract of *Daphnia magna*, a model organism in ecotoxicological studies.

C. EXPERIMENTAL PART

C.1. Sampling of environmental waters

Sampling was carried out according to the EPA 1694 Method [1], which is recommended for the analysis of pharmaceuticals and personal hygiene products. Water samples were collected in 1L amber glass bottles, filled to overflowing to avoid the presence of air, and closed with a polypropylene cap and a polytetrafluoroethylene gasket.

C.2. Standards and chemicals

Antibiotics standards were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), except of amoxicillin diketopiperazine and penicilloic acid (LGC, Molsheim, France). All the compounds were of high purity grade (≥ 98%), except of florfenicol (≥ 90%). The solvents (HPLC grade methanol and acetonitrile), formic acid (≥ 95%), ammonium formate (≥ 99%), ammonium acetate and ammonium bicarbonate (≥ 99%) were purchased from Sigma-Aldrich.

Stock antibiotic standard solutions were prepared by dissolving a weighed amount of antibiotics in methanol, except of florfenicol which was dissolved in ethanol, and fluoroquinolones dissolved in 0.2% (*v/v*) hydrochloric acid in 50% (v/v) methanol. Working solutions were prepared by dilution with 50 % methanol. Special precautions were taken for oxytetracycline, which was stored in the dark to avoid photodegradation [2]. Working solutions were prepared each month, while stock solutions were renewed every three months. In particular, enrofloxacin stock solution for toxicological studies was prepared daily at 100 µg mL^{-1}, by dissolving weighted amount in milliQ water followed by sonication. No organic solvent and buffer were used to increase the solubility of enrofloxacin to avoid modifying water parameters.

PS nanoplastics were synthesized, avoiding any additives, especially surfactants, bactericide species, and trace metals potentially present in commercial standards. The synthesis and characteristics of polystyrene models were detailed elsewhere [3]. A suspension of spherical particles (PS22) with surface functionalized by carboxylic groups was used throughout this study. It presents a number-average diameter of 420±20 nm (determined by scanning electron microscopy), a low polydispersity (polydispersity index (PDI) of 0.009), zeta potential of −46 mV at pH 7, particle surface functionality of 45 COOH groups per nm^2.

Ultrapure water (18 MU cm of resistivity) was obtained from a Milli-Q purification device (Millipore Co., Bedford, MA, USA). AF4 mobile phase consisted of ultrapure water at pH 8 (adjusted by addition of 0.1 M NaOH). Mobile phase was filtered through nylon filters of 0.2 µm pore size (Pall Corp. Hampshire, UK).

Natural colloids size calibration through AF4 was made using monodisperse suspensions of silicon dioxide microparticles size standards of 0.150, 0.5, 1, 2, and 4 µm covering both normal and steric modes. All the standards were purchased from Sigma Aldrich (Sigma Aldrich Chemie, Buchs, Switzerland). Solutions of 30 mg L^{-1} were prepared by dilution in the AF4 carrier solution.

In the case of kaolin added to one of the samples to study the effect of colloidal inorganic alluminosilicates, the material used was a solid powder provided by Laboratorios Enosán S.L. (Zaragoza, Spain), with a particle size up to 100 mm. Suspensions prepared in ultrapure water from the fraction below 1 µm were added. To isolate this fraction, the original product was suspended in ultrapure water at a starting concentration of 1000 mg L^{-1} of kaolin. After 1 hour of decantation, 15 mL of the supernatant were withdrawn from the suspension and finally centrifuged during 5 min at 1200 rpm. Kaolinite ($Al_2Si_2O_5(OH_4)$) was the main crystalline structure according to its analysis by X-ray diffraction. The density of the clay is 2.6 g cm^{-3} and the mass fractions of Al and Si are 18.0 and 21.7, respectively.

C.3. Instrumentation

C.3.1. Sample preparation equipment

A solid-phase extraction (SPE) vacuum system (CPI, Amsterdam, the Netherlands) was used to extract, clean up and pre-concentrate antibiotics from water samples. A further 10-fold concentration was achieved by solvent evaporation using a Concentrator FSC400D, dri-block from TECHNE (Fisher Scientific, Illkirch, France).

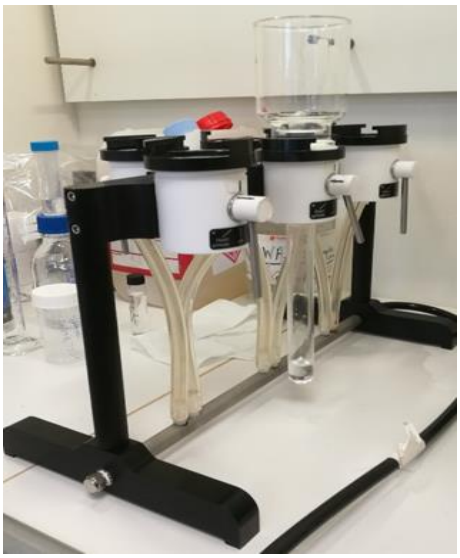

Figure C.1. POCTEFA territory and surface sampling points

C.3.2. Liquid chromatography-MS

The experimental setup used is presented in Figure C.2. An Ultimate 3000 RSLC chromatographic system (ThermoFisher, Dreieich, Germany) was used for the separation of antibiotics. A C18 (Accucore 100 x 2.1 mm, 2.5µm) column was used. The detection was done by a Q Exactive Plus (ThermoFisher) high resolution mass spectrometer fitted with an IonMax ionization source and an HESI II probe. It was operated at: sheath gas flow rate 50, auxiliary gas flow rate 20, sweep gas flow rate 1, capillary temperature (°C) 380, S-lens RF level 50 and aux gas heater temperature (400 °C).

Figure C.2. The experimental setup used.

C.3.3. FFF - ICP MS

The system used was an AF2000 (Postnova Analytics, Landsberg,Germany). A channel of reduced dimensions was utilised, with the following dimensions: 14 cm in length, from 2 to 0.5 cm in width; and a spacer with 350 mm of thickness. Table C.1 summarizes the flow conditions applied. A polyether sulfone (PES) membrane with a cut-off of 5 kDa (Postnova Analytics) was used as accumulation wall. Optimal conditions for the separation of colloids in the range from 15 nm to 1 µm was selected from reference [4].

Table C.1. Injection and elution steps during a measurement in AF4. Detector flow 0.8 mL min^{-1}.

Step	Time (min)		Cross flow (mL min^{-1})
Injection/focusing	14 + 1 (1mL loop) 4 + 1 (100 µL loop)	Injection flow 0.2 mL min^{-1}	1
Elution	10	Constant	0.1
	3	Linear decay	0.1 to 0
	2	Constant	0

Sample loops of 100 µL and 1 mL were used through the entire experimental study. The eluent is directed from the channel through an UV–vis diode array detector (Shimadzu, Duisburg, Germany) recording the signal from 200 to 800 nm. A complete spectrum of the eluent was registered every 2 s. For the element detection, the AF4 system was coupled to an ICP-mass spectrometer (ELAN DRC-e, PerkinElmer, Germany).

The outflow from the system was delivered directly to the nebulizer of the spectrometer; a glass concentric Slurry nebulizer with a cyclonic spray chamber (Glass Expansion, Melbourne, Australia) was used. The ICP-MS readings consisted of 20 sweeps at a dwell time of 50 ms. Default values were used for the rest of the instrumental parameters.

C.3.4. pH and conductivity measurements

pH values were measured in a pH meter (Crison, Barcelona, Spain) with a glass membrane electrode as working electrode and a Ag/AgCl reference electrode. Before measurements, pH meter was calibrated with pH 4 and 7 buffer solutions at room temperature. Conductivity was measured in a conductometer (Hach sension7, Hach, Iowa, USA) provided with a platinum high conductivity cell. The cell was previously calibrated with a KCl solution. Water samples were measured without any previous treatment.

C.4. Procedures

C.4.1. Initial sample preparation and storage of water samples

In the case of the samples for colloidal studies, no filtration was carried out after collection. Samples were stored in the fridge at 4^0C until its analysis.

C.4.2. Antibiotics analysis

C.4.2.1. Solid phase extraction

The experimental conditions for SPE purification/preconcentration were adapted from previous reports [5,6]. In brief, a 250-mL water sample was loaded at 5 mL min^{-1} onto an Oasis HLB cartridge (diameter 47 mm, Waters, Guyancourt, France) preconditioned with 32 mL MeOH, and rinsed with 12 mL water and then with 12 mL water at pH 2 ± 0.5. The pH was determined by means of a Mettler Toledo InLab Expert Pro-ISM (Viroflay, France). The cartridge was dried for 5 min and eluted with 25 mL MeOH at 5 mL min^{-1}. A 1-mL aliquot of the resulting eluate was brought to dryness at 60° C and reconstituted with 100 µL of 20% MeOH.

C.4.2.2. Quantitative analysis by LC-HR MS/MS

The antibiotics were eluted in gradient mode. Eluent A was 0.004 mM ammonium acetate/0.004 M ammonium formate in water. Eluent B was 0.004 mM ammonium acetate/0.004 M ammonium formate in a mixture containing 30% MeOH, 30% ACN, and 40% water). pH of eluent A was adjusted with 0.3% of formic acid, while that of eluent B with 0.015 M of ammonium bicarbonate. These mobile phases allowed the sprayer voltage to be kept at less than 50 kV over the chromatographic run. The elution gradient was: from 3% to 100% B in 16.5 min, 100% B for 8.5 min and back to 3% B within 3 min. Injection volume was 20 µl and flow rate 0.3 mL min^{-1}. Temperature was set at 35° C.

MS data acquisition was performed in positive mode using parallel reaction monitoring (PRM). m/z isolation window was 0.5 Da, resolution 17,500, and AGC target 1e5. The precursor ions and two product ions per compounds that were monitored. They are listed, together with the collision energies used in **Table C.2.**

Quantification was carried out using an 8-point matrix-matched calibration curve ($R \geq 0.998$).

Table C.2. MS detection parameters used in quantitative analysis of antibiotic species

CLASS	ANTIBIOTIC	CAS	RT	Parent ion (m/z) $[M+H]^+$	CE	Product ion 1	Product ion 2	LoD (ng L^{-1})	LoQ (ng L^{-1})
ß-Lactamase	Amoxicillin	26787-78-0	8.99	366.11	10	349.08	208.04	0.152	0.500
	Ampicillin	69-53-4	5.93	350.12	20	192.05	106.07	0.021	0.071
	Diketopiperazine	94659-47-9	9.36	366.11	10	207.08	160.04	0.300	0.100
	Peniciloic acid	210289-72-8	5.50	384.12	10	367.09	323.11	0.015	0.05
Diaminopyrimidine	Trimethoprim	738-70-5	9,06	291,15	50	261,0979	230,1158	0,026	0,085
Fluoroquinolone	Ciprofloxacin	85721-33-1	9.91	332.14	65	249.07	231.06	0.028	0.094
	Enrofloxacin	93106-60-6	10.47	360.17	35	316.18	245.11	0.041	0.135
	Moxifloxacin	354812-41-2	12.64	402.18	40	382.18	261.10	0.011	0.035
	Norfloxacin	70458-96-7	9.66	320.14	35	276.15	233.11	0.015	0.050
Lincosamide	Lincomycin	154-21-2	8.44	407.22	25	359.22	126.13	0.025	0.082
Macrolide	Azithromycin	83905-01-5	13.99	749.51	25	591.42	158.12	0.02	0.067
	Clarithromycin	81103-11-9	20.91	734.12	20	590.39	158.12	0.018	0.059
	Clarithromycin N-oxide	118074-07-0	20.87	764.48	28	606.38	123.08	0.012	0.038
Sulfonamide	Dapsone	80-08-0	9.94	249.07	35	156.01	108.04	0.029	0.095
	Sulfacetamide	144-80-9	5.69	215.05	35	156.01	156.04	0.049	0.163
	Sulfadiazine	68-35-9	6.71	251.06	30	156.01	108.04	0.015	0.050
	Sulfadoxine	2447-57-6	11.31	311.08	40	156.01	108.04	0.012	0.038
	Sulfamerazine	127-79-7	8.14	265.07	35	190.03	156.01	0.023	0.075
	Sulfapyridine	144-83-2	6.82	250.06	35	184.09	156.01	0.015	0.05
	Sulfamethoxazole	723-46-6	11.19	254.05	40	156.01	108.04	0.008	0.025
	Sulfamethoxine	122-11-2	10.73	281.07	30	156.01	126.07	0.058	0.197
Tetracycline	Oxytetracycline	79-57-2	9.75	461.15	20	426.12	381.06	0.028	0.094

C.4.2.3. Detection and identification of antibiotics degradation products by LC-HR MS/MS

The HPLC column mentioned above was used. Eluent A was 0.1% formic acid and eluent B was 0.1 formic acid in MeOH:ACN (1:1, v/v). The elution gradient was: from 10% to 95% B within 9.5 min, 95% B for 2 min, and back to 10% B within 1 min. The column was reconditioned with 10% B for 3 min. Injection volume was 20 µl, flow rate of 0.3 ml min^{-1}, and temperature 35°. Sheath gas flow rate was 50, auxiliary gas flow rate 20, sweep gas flow rate 1, capillary temperature 380 °C, S-lens RF level 50 and auxiliary gas heater temperature 400 °C.

MS and MS2 data acquisition was performed in positive move in full MS-ddMS2 (top5, with an exclusion list extracted from the last blank before analysing the samples). The settings were: full MS-SIM HR 70,000, AGC target 1e6, scan range 100-800 m/z, dd-MS2 resolution 35000, AGC target 1e5, loop count 5, m/z isolation window 0.5, (N)CE 15 and 45. All fragmentation information could be obtained using one full scan and without sample re-injection in MS2 mode.

The compounds were identified using Compound Discoverer software. For the investigation of the transformation products a list was assembled from the literature [7-10]. For the confirmation by CD an already existing ThermoFischer Scientific Workflow applied with the addition of Fragment Ion Search (FISh) processing was used [11]

C.4.3. Characterization of natural colloids

Samples were homogenized before the analysis by AF4 for 10 min in a rotary tumbler at 28 rpm. After that, 20 mL were transferred to a falcon tube to settle for 1 h and afterwards 15 mL of sample from the upper part were transferred to a 50 mL falcon tube, so large fractions (>1 µm) were removed by sedimentation. Finally, 1 mL were injected in the AF4 instrument using the conditions described in the previous section C.3.3.

C.4.4. Studies of enrofloxacin interactions with the colloidal fraction in samples.

C.4.4.1. Preparation of enrofloxacin standard stock solution

Enrofloxacin was dissolved in phosphate buffer (PB) (pH 7.4) as suggested elsewhere [12].

C.4.4.2. Measurement procedures

C.4.4.2.1. Enrofloxacin ultrafiltration recovery study

Ultrafiltration tubes with a membrane cutoff of 5KDa (Microsep Advance, Pall Corp. Hampshire, UK) were used along the interaction studies. Filter tubes were centrifuged in a centrifuge (Thermo Fisher Sci. MS, USA, model Multifuge X1R) at 3000 rpm at 22 ºC for 5 min.

C.4.4.2.2. Interaction studies with samples

All samples were allowed to settle to remove those particles larger than 1µm. Initially, samples were homogenized previously for 10 min in a rotary tumbler at 28 rpm. After that, 20 mL were transferred to a falcon tube to settle for 1 h. 15 mL of sample from the upper size were transferred to a 50 mL falcon tube. Then, a variable concentration of enrofloxacin (from 50 to 500 µg L^{-1} were studied) were added to the samples and left at room temperature for 24 hours (as in [13]). Afterwards, samples were ultrafiltered following the conditions described in C.4.4.2.1. The ultrafiltrate was analyzed by a UV/Vis spectrophotometer (Jasco V-730, Jasco Inc. Easton, USA) to quantify the enrofloxacin concentration remaining in solution.

Same procedure was followed for sample control to measure the absorbance contribution of the sample after ultrafiltration, without any addition.

Enrofloxacin quantification was made by standard addition. Different enrofloxacin concentrations (50, 250 and 500 µg L^{-1}) were added to the samples after ultrafiltration. (Standard addition slope: 0.078 ± 0.003; direct calibration: 0.092 ± 0.004)

The percentage of enrofloxacin associated to the colloidal fraction was calculated by the following expression:

$$\% \text{ of enrofloxacin associated to colloids} = 1 - \frac{[enrofloxacin]_{ultrafiltrate}}{[enrofloxacin]_{added}} * 100$$

All the experiments were prepared in triplicate (n=3).

C.5.4. Ecotoxicological experiments

The experiments were performed at the Hydrobiological field station of the University of Warsaw in Pilchy (https://pilchy.biol.uw.edu.pl/). The analyses of the samples obtained during experiments to assess community level metabolic fingerprinting and the life history parameters of *Daphnia* were performed at the station during and after the end of the experiments. Other analyses were performed in the laboratories of the Department of Hydrobiology, Faculty of Biology at the Biological and Chemical Research Centre, University of Warsaw (samples to assess the taxonomic diversity of bacterial communities).

Three replicates of the experiments were performed, each using different clone of *D. magna* (MB01, MN and MD). Clone MB was sampled from lake Binnen (54°19’29”N ; 10°37’39”E, Germany), clone MN from pond Nový Rybnik (50°13'27.8"N ; 14°4'3.1"E, Czech Republic), and clone MD from the pond Domin (49°00'21.3"N ; 14°26'29.1"E, Czech Republic). *Daphnia* were cultured in 5-L containers, 25 individuals per container, at room temperature and natural photoperiod. Daily food supply was added ad libidum in the amount of 1.6 mg C × L^{-1} of unicellular green algae, *Chlamydomonas klinobasis* (strain SAG 56) from a stationary phase, chemostat culture grown in WC medium (Guillard, 1975). Algal concentration was assessed using a portable fluorometer (AquaFluor handheld fluorometer, Turner Designs®, USA).

C.5.4.1. Experimental protocol

The system was located in a room with a constant foto-photoperiod (16L:8D) and consisted of 12 glass containers (L = 25, W = 25, H = 40 cm, large enough to minimize the scale-effects) filled with 9 L media placed in a water bath (L = 150 cm, W = 50 cm, H = 50 cm, V = 200 L) with submersible water-heater (Aquael Neoheather 150 W) and water pumps (Aquael Circulator 500) to maintain stable temperature. The water bath had opaque walls with mounted warm white (3000 K) LED lamps (2 1.2 m strips, 5.76 Watts, manufacturer ID: FSLEDWW1200-EF, Green Lighting®) inside.

The experiments were performed between May and July 2021 in 12 variants being the combination of the 3 concentrations of the enrofloxacin (0, E_l = 10 and E_h = 100 ng × L^{-1}) and 4 densities of PS-NPs (0, $N_l = 1\times10^3$, $N_m = 1\times10^6$ and $N_h = 1\times10^9$ particles × L^{-1}). The $1 \times 10^9 \times L^{-1}$ concentration corresponds to the range of bacteria abundance in lake samples. The temperature was fixed at 23 ± 0.3 °C, and the media

were supplied daily with the same amount of algal food (*Ch. klinobasis*) set at slightly limiting concentration of 0.6 C mg × L^{-1}. The organic carbon content was calculated from the calibration curve relating organic carbon concentration to level of absorbance at 800 nm. Relatively high temperature and low amount of food in the experiments were chosen to increase *Daphnia* filtration rate. LED lamps inside the water bath provided relatively homogenous throughout the water column and low light intensity (1.0 ± 0.4 μmol × m^{-2} × s^{-1}) measured by Li-Cor 189 quantum sensor measuring radiance (LiCor Biosciences). Low light intensity was used since, according to the literature, photodegradation is the major cause of deactivation of fluoroquinolones, including enrofloxacin, in the environment [14].

At the beginning of each experiment, we added to each of the 12 containers (one container per variant) the tap water filtered through 0.45 μm pore membrane filters and aerated for 24 h to reach an oxygen concentration up to 8.00 ± 0.08 mg × L^{-1} (pH = 7.4, μS cm^{-1} = 373 ± 0.7). Next, the physicochemical parameters (temperature, conductivity, oxygen concentration) were determined using a multiparametric YSI 6000 probe (Yellow Spring, YSI Inc./ Xylem Inc. USA), and NPs, enrofloxacin, algal food were added in the following order. Finally, 90 newborn (0-24 h) *Daphnia* were collected and distributed into each of the containers to a final density of 10 ind. × L^{-1} in each variant. Every six hours the media were gently mixed. The fresh media were prepared and replaced every 24-hours. During media preparation, *Daphnia* were gently removed from each of the containers using a strainer with plankton net and placed temporarily in 250-mL glass containers with the respective media. The fresh media were prepared in the same order as the initial media. The experiments lasted 5 days, when at least 50% of individuals started to produce eggs in each of the variants. Individuals were gently removed from each of the containers using a strainer with plankton net and placed temporarily in 250-mL plastic containers with Milli-Q water.

C.5.4.2. Life history parameters

All the individuals collected in each of the variants were photographed from the lateral side under dissecting microscope connected with the camera and computer. Using NIS program (Nikon Nis Elements) to analyse the photographs, the length and height of each *Daphnia* were measured in the photographs*. The* length was measured from the top of the eye to the base of the tail spine, the height was measured for the greatest dimension of the body across the length of the body. Based on these measurements, the body volume of each individual was calculated assuming an ellipsoidal shape for each individual according to the formula $4/3\pi \times 1/2a \times 1/2b \times 1/2c$, where *a* stands for the length, *b* the height and *c* the width (assuming that the body width is equal to the body height, [15]). Moreover, for each of the ovigerous females the eggs were counted and the volume of an egg (as the mean for at least 2 eggs in the clutch) was calculated using the same formula as that employed for body volume evaluation. Finally, the clutch volume was calculated as the number of eggs multiplied by the mean egg volume for each ovigerous females. Additionally, in the first replicate of the experiments, photographed individuals were transferred in new plastic containers with Milli-Q water to remove non-symbiotic bacteria from their guts. Then two groups of randomly selected individuals from each variant were used to determine either metabolic (25 individuals) or taxonomic (10 individuals) diversity of bacterial community in *Daphnia* guts.

C.5.4.3. Metabolomic diversity of gut microbiota

The extracted *Daphnia* guts were homogenized in 1 ml of Milli-Q water and transferred into 15 ml of their respective medium which were earlier filtered through 0.45 μm Nylon filters and autoclaved. These samples were used for the analysis of metabolomic diversity of the gut microbiota by The Biolog EcoPlate method [16,17]. EcoPlate (Biolog, USA) is used to measure the ability of the bacterial community to utilize different carbon substrates. An EcoPlate is a 96-well microplate composed of triplicates of control wells (containing no additional carbon source) and 31 wells containing various carbon sources (**Table C.3**).

Table C.3. Different carbon sources used by the Biolog EcoPlate method to measure the ability of the gut bacterial community of *Daphnia* to utilize carbon substrates

carbohydrates	D-cellobiose
	α-D-lactose
	β-methyl-D-glucoside
	D-xylose
	Erythritol
	D-mannitol
	N-acetyl-D-glucosamine
	D-galactonic acid γ-lactone
phosphorylated carbons	glucose-1-phosphate
	D,L-α-glycerol phosphate
amines	phenylethylamine
	putrescine
carboxylic acids	D-glucosaminic acid
	D-galacturonic acid
	γ-hydroxybutyric acid
	itaconic acid
	α-ketobutyric acid
	D-malic acid
	pyruvic acid methyl ester
	2-hydroxy benzoic acid
	4-hydroxy benzoic acid
complex carbon	Tween 40
	Tween 80
	α-cyclodextrin
	glycogen
amino acids	L-arginine
	L-asparagine
	L-phenylalanine
	L-serine
	L-threonine
	glycyl-L-glutamic acid

In total 12 plates were used, one for each variant. Each well of a single plate, except the control well (filled with Milli-Q water), was filled with 150 μL aliquote of homogenised and diluted content of *Daphnia* guts from one variant. The plates were incubated in darkness at a temperature of 22 °C for

72 hours. Due to reduction of tetrazolium chloride by electrons derived mainly from oxidation chain the coulor in wells increased proportionally to respiration rate. The absorbance was measured every 4 hours at 590 nm wavelength using a Biotek Synergy H1 plate reader (Biotek Corporation, USA). For analysis, we used the maximal colour development rates (V_{max}). For the calculation of V_{max} values, Gen 5 software (Biotec Corporation, USA) was used. The slope for every four consecutive reads was identified. V_{max} was calculated using a linear regression model by determining the maximum slope during the 72-hour incubation time. The maximal rate of colour development, V_{max} (mOD × min^{-1}), was treated as indicator of intensity of respiration of a single carbon source by daphnia's gut microbial communities. To determine the influence of each additional carbon source on the respiration rate, the difference in V_{max} between carbon-containing wells and control wells was calculated (delta). When delta was lower than zero or equal to zero, we assumed that the source was not utilized effectively by the microorganism community, and we set this value of V_{max} to zero. For the analyses of community-level physiological fingerprinting, we used the relative values of the respiration rate for each carbon source, calculated as the percentage share of each carbon source in the sum of V_{max} for the whole plate. Overall microbial activity in each microplate was expressed as mean V_{max} for plate, it was calculated as the average of all wells, including control. It is equivalent for commonly used average well colour development.

C.5.4.4. Taxonomic diversity of gut microbiota

The *D. magna* digestive tracts were collected in 1.5 mL Eppendorf and stored, at – 20° C. Afterwards DNA extraction was performed by spin-column-based method using the GeneMATRIX DNA Purification kit (EurX, Gdańsk, Poland), according to manufacturer procedure. Total DNA was assessed for quality and quantity by absorbance measurement in a Synergy H1 microplate reader (Gen5 software, BioTek, USA) equipped with Take3 microvolume plate. The samples were then stored at -20°C for further analysis. The phylogenetic analysis of the bacterial community was performed using Illumina sequencing [18]. For the sequencing, the 16S rRNA genes, V3–V4 hypervariable regions (amplicons of approximately 459 bp) were selected. PCR amplification was carried out using Q5 Hot Start High-Fidelity 2X Master Mix using reaction conditions as recommended by the manufacturer (95 °C for 3 minutes, 25 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds and, after the last cycle, 72 °C for 5 minutes) with region-specific (341F and 785R, [19]) primers that include the Illumina flowcell adapter sequences. The primer sequence was as follows: forward primer: 5' CGGGNGGCWGCAG 3', reverse primer: 5' GACTACHVGGGTATCTAATCC 3'. The Illumina overhang adapter sequences added to the locus-specific-sequences were as follows: forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG (locus-specific sequence), reverse overhang: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG (locus-specific sequence). The amplicons were sequenced using an MiSeq (Illumina) platform on a single run using the MiSeq Reagent Kit v2 (Illumina, San Diego, California, USA) and the paired-end method (2 × 300 bp) according to the standard protocols by Genomed (Warsaw, Poland).

Demultiplexing and trimming of Illumina adapter sequences (cutadapt software;[20]) was performed. Quality inspection, visualisation and assessment of raw FASTQ files was performed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and MultiQC [21] The

sequences were processed using the DADA2 plugin within QIIME 2 [22]. Sequences were trimmed at 270 nt, and first 8 nt were truncated. Alpha rarefaction plots confirmed that the amount of remaining sequences is sufficient to detect microbial diversity present. Taxonomies were assigned to the resulting amplicon sequence variants (ASV) with q2-feature-classifier plugin using pre-trained Naive Bayes classifier based on 16S rRNA silva 138 SILVA SSU gene database at 99% similarity. Phylogenetic and non-phylogenetic core diversity metrics were calculated using Core-diversity-metrics pipeline. Data for this purpose were rarefied to a sampling-depth equal to lowest frequency among samples (23500 reads).

C.5.4.5. Statistical analysis

The statistical analysis was performed using R platform (v.4.2.0) by setting the level of significance at $\alpha = 0.05$ for all of the statistics. In order to test the effect of NPs and enrofloxacin on the life history parameters: body length, body volume, clutch size, egg volume and clutch volume, two-way between-subjects ANOVAs with the *post-hoc* HSD Tukey test were conducted. The analyses were conducted in R (version 4.1.2) using the *'anova'* function from the *'car'* package. For all six measures, the assumptions for the traditional parametric ANOVA were not met, since the data for all six population parameters were not normally distributed and included many outliers (defined using boxplots). To normalize distributions, the data were winsorized (within conditions, using the *'Winsorize'* function from the *'DescTools'* package).

To determine the combined effect of the stressors on metabolic rate of the gut microbiota, we used Aligned Rank Transform for Nonparametric Factorial two-way ANOVA (ART ANOVA; ARTool package v.0.11.1,[23]) with the "art" function (ARTool package 0.11.1, [24]), which allowed to fit model despite non-normality and heteroscedasticity of initial data distribution. To verify that the ART procedure was correctly applied and is appropriate for this dataset the "summary" function was used. To tests of differences in pairwise combinations of levels between factors in interactions the ART-C method (multifactor contrast test) were conducted by using the "art.con" function (ARTool, [25]).

To group the experimental variants according to the microorganism metabolic and phylogenetic differences, Bray-Curtis-based NMDS (non-parametric multidimensional scaling) was performed (PAST3 software, [26]). For the analyses of community-level physiological fingerprinting, we used the relative values of the respiration rate for each carbon source, calculated as the percentage share of each carbon source in the sum of V_{max} for the whole plate. For taxonomic NMDS the relative abundance of ASV at the family level was used. Statistica 13 software (StatSoft, TIBCO Software Inc., USA) was used for the NMDS analysis and data visualization.

Additionally, to reveal the correlation between two Bray-Curtis similarity-based matrices of relative metabolic and relative phylogenetic data, Mantel correlation between taxonomic and metabolic profiles was performed (PAST3 software).

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D. RESULTS AND DISCUSSION

D.1. Long-Term Study of Antibiotic Presence in Ebro River Basin (Spain): Identification of the Emission Sources

D.1.1. Study Area

The Ebro River basin is located in the northeast of Spain (Figure D.1.1). The extension is 85,000 km^2 flowing out into the Mediterranean Sea, in the Province of Tarragona. In the Iberian peninsula, the Ebro ranks second in length after the Tajo River and second in discharge volume and drainage basin after the Duero River. It is the longest river entirely within Spain. The importance of studying this basin lies in the fact that it encompasses more than twenty urban areas, including large areas such as Pamplona, Zaragoza and Logroño. Moreover, one of the main economic activities of most of these areas is animal farming. The area studied included 20 surface waters sampling points corresponding to 17 rivers from the Ebro river basin (Spain), which are listed in Table D.1.1 and shown in Figure D.1.1. Selection criteria for surface waters sampling points were: (i) their proximity to poultry and pig intensive farms (Figures D.1.2-D.1.3), the selection was carried out in collaboration with the Ebro Hydrographic Confederation; (ii) the proximity to WWTPs, taking a sample upstream from the WWTP discharge and another one downstream, the exact locations were chosen in collaboration with the Navarra de Infraestructuras Locales S.A (NILSA, Tudela, Spain).

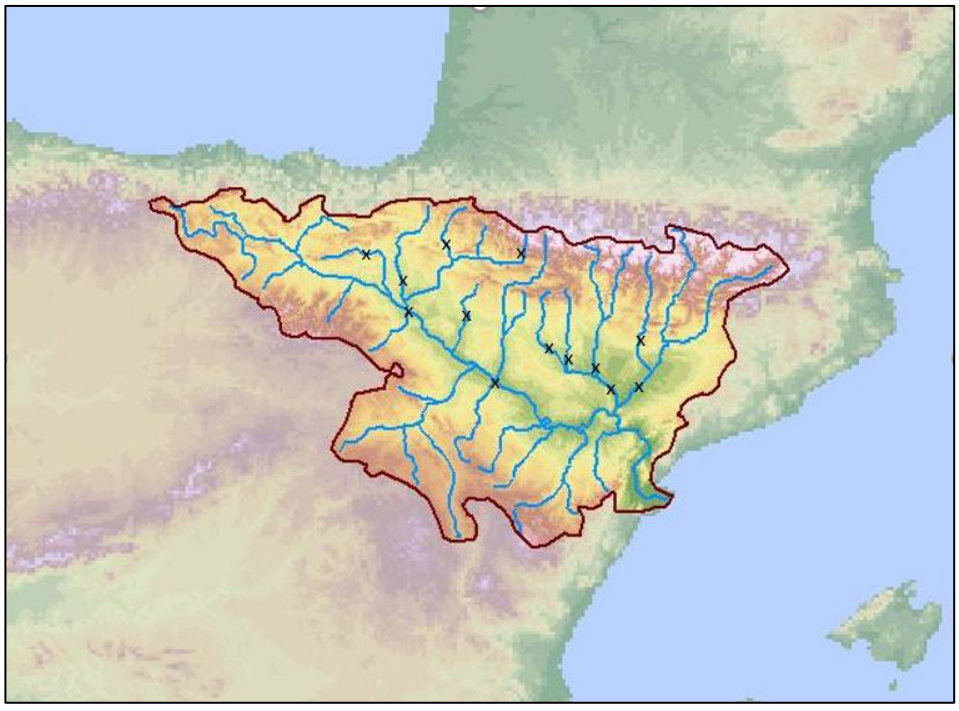


Figure D.1.1. Surface waters sampling points in the Ebro River basin (North of Spain)

Table D.1.1. List of surface water sampling points

River	Location	Sub-basin	Pressure	
			livestock	WWTP
Segre River	Torres de Segre	Segre	High	Null
Noguera Ribagorzana River	Corbins	Segre	High	Null
Clamor Amarga River	Zaidín	Cinca	High	High
Cinca River	Fraga	Cinca	High	Null
Alcanadre River	Sariñena	Alcanadre	High	Null
Flumen River	Albalatillo	Alcanadre	High	Null
Gállego River	San Mateo de Gállego	Gallego	Low	Null
Arba de Ríquel River	Ejea de los Caballeros	Ebro	High	Low
Aragon Subordan River	Javierregay	Aragón	Low	Null
Aragon River	Caparroso	Aragón	High	Null
Irantzu River	Estella	Ega	Medium	Null
Arakil River	Irañeta	Arga	High	Null
Queiles River	Novallas	Arga	High	High
Alhama River	Alfaro	Alhama	High	Null
Ega River	Estella	Ega	Low	Medium
Ega River	Downstream Estella	Ega	Low	High
Ega River	Upstream Pamplona	Arga	Null	Low
Arga River	Downstream Pamplona	Arga	Null	High
Ega River	Upstream Tudela	Ebro	Low	Low
Ebro River	Downstream Tudela	Ebro	Low	Medium

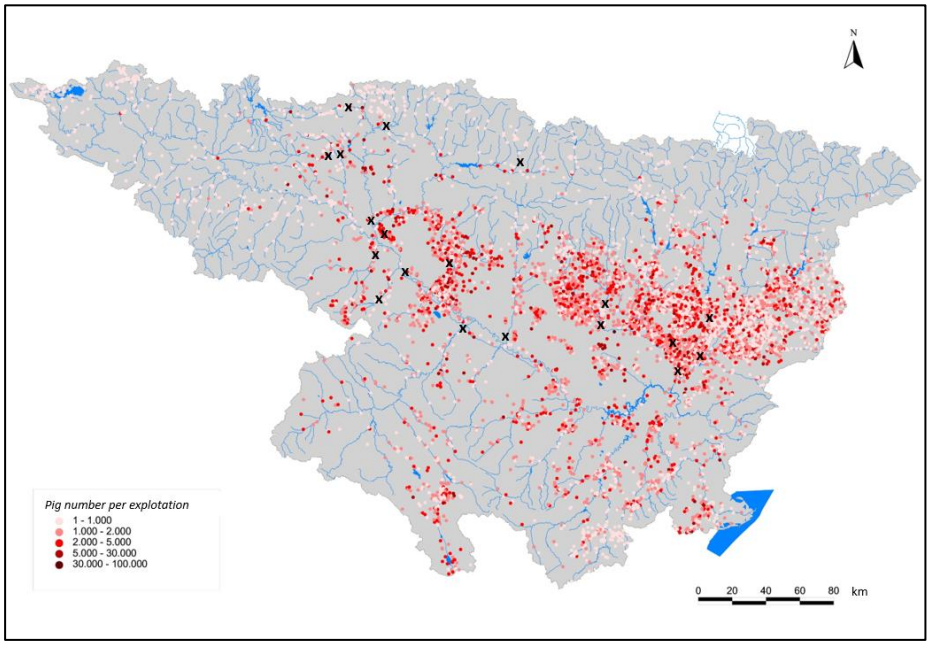


Figure D.1.2. Distribution of number of pigs per farm in the Ebro River basin and sampling points (Source: Ebro Hydrographic Confederation, 2016)

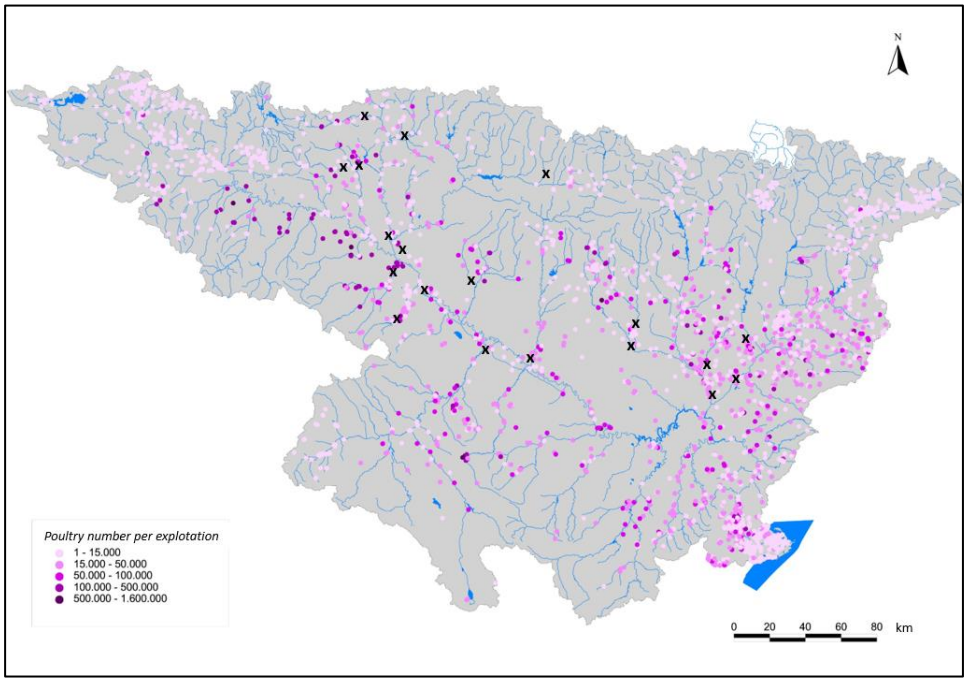


Figure D.1.3. Distribution of number of poultry per farm in the Ebro river basin and sampling points (Source:Ebro Hydrographic Confederation, 2016)

The affluents and effluents of up to three WWTPs, one hospital and three slaughterhouse effluents were monitored along this project. The characteristics of the water treatment protocols used by the studied WWTPs are given in Table D.1.2. In summary, a total of 30 sampling points was examined. Out of these, 2/3 corresponded to surface waters and 1/3 to wastewaters.

Table D.1.2. Main characteristics of studied WWTPs

#WWTP	Equivalent inhabitants	Inlet flow (m^3/day)	Water treatment line						
			Grit and grease separator	Activated Sludge	Decanter (1st stage)	Trickling filter (1st stage)	Trickling filter (2nd stage)	Decanter (2nd stage)	Moving bed biofilm reactor
WWTP1	695.232	129.600	x	x	x				
WWTP2	82.500	22.150	x		x	x	x	x	
WWTP3	51.336	7.500	x		x			x	x

D.1.2. Antibiotic Selection

The first selection criterion was a revision of the literature, determining which antibiotics show the most significant sales and use in Spain. It should be mentioned that several previous studies have been carried out on the surface waters of the Ebro River basin related to monitoring selected emerging pollutants, such as microplastics [1] or pharmaceuticals [2,3], and sulfonamide residues [4]. Several authors have also studied the presence of pharmaceuticals in wastewater-treatment plants located in the Ebro River [3], including some antibiotics [2,3]. According to the most recent studies, trimethoprims, macrolides, sulfonamides and fluoroquinolones are four of the most detected antibiotic groups in Spanish and European rivers and wastewaters [5]. The literature reports the concentrations of antibiotics up to µg/L for: sulfonamides [3], trimethoprim [6,7] fluoroquinolones [8] and macrolides [2,3,9], which all represent a potentially significant risk for the environment. The European Medicines Agency (EMA) annually publishes a report on the sales and use of veterinary antibiotics within the framework of the European Surveillance Survey of the Consumption of Veterinary Antimicrobial Medicines (ESVAC). According to the last ESVAC report, sales of tetracycline, penicillin, and sulfonamides represented almost 70% of all antibiotics sold in Europe [10]. The first step to establish a target antibiotic for quantitative analysis is a qualitative screening, which was carried out in the spring of 2018. Its results were grouped by antibiotic families, due to the great variety of antibiotics detected. As revealed in Figure D.1.4, fluoroquinolones were the most detected species with enrofloxacin present in 70% of the samples. The second group of antibiotics that was more frequently detected is the family of sulfonamides (present in 30% of the samples). Sulfadiazine was detected in more than 70% of the samples. Finally, trimethoprim and azithromycin were present in 60% and 55% of the samples, respectively. As a result of the screening data, sulfadiazine (sulfonamide), enrofloxacin (fluoroquinolone), trimethoprim (trimethoprim) and azithromycin (macrolide) were selected as target antibiotics for quantitative analysis. Table D.1.3 shows the group and CAS numbers as well as physicochemical properties of the target antibiotics, (acid dissociation constant (pKa), molecular weight and molecular structure.

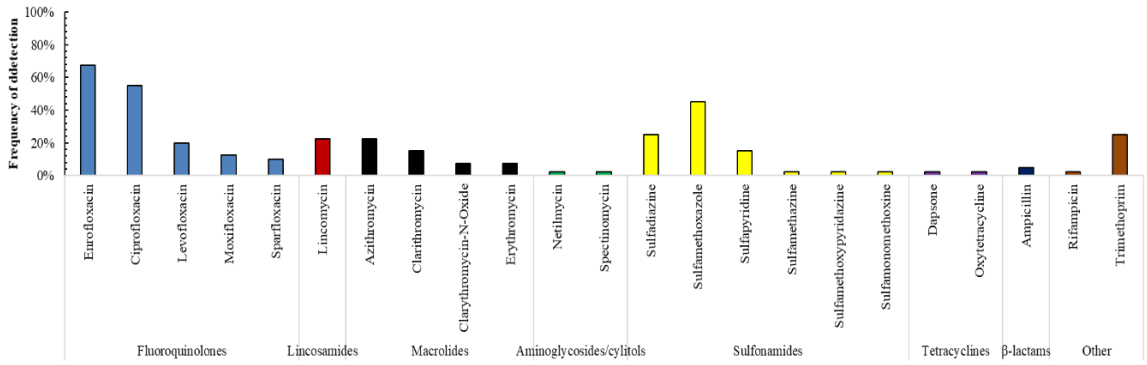
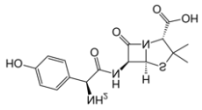
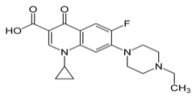
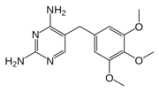
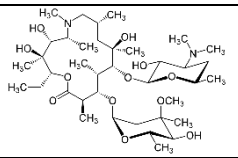


Figure D.1.4. Frequency of occurrence of most detected antibiotics in the screening (2018)

Table D.1.3. Physicochemical properties of target antibiotics

Group	Antibiotic	CAS	Molecular weight (g/mol)	Molecular structure
Sulfonamide	Sulfadiazine	26787-78-0	365.4	
Trimethoprim	Trimethoprim	93106-60-6	359.4	
Fluoroquinolone	Enrofloxacin	738-70-5	290.3	
Macrolide	Azithromycin	83905-01-5	749.0	

D.1.3. Results

D.1.3.1. Antibiotics in surface waters

The overall results obtained for the concentrations of the target antibiotics in all surface-water-sampling points (2018–2021) are shown in Figure D.1.5a. For a more detailed interpretation of the results, the data were processed by grouping the surface-water-sampling points into the different sub-basins that form the Ebro River basin (Figure D.1.5b). It should be noticed that the boxplots of antibiotic concentrations have been elaborated by the concentration results shown in Tables S1–S4 from the different sampling campaigns. Unusual values are not represented in the boxplot graphs. In order to complement the statistical analysis of this research, Tables S1–S4 list quantitative antibiotic-concentration results obtained during the 6 sampling campaigns that were carried out. Figure S1 shows the river flows of the six sampling campaigns. According to Figure D.1.5a, enrofloxacin and sulfadiazine were frequently detected in concentrations from 20–180 ng L^{-1} in the surface-water-sampling points.

Tables S4–S6 present the ANOVA and Turkey's HSD test results in high-livestock pressure sampling points. Significant differences (p-value < 0.05) were found between the enrofloxacin concentration and the rest of antibiotic concentrations at points that represent high-livestock-pressure sampling points. This result, coupled to the high concentration detected of this veterinary-use antibiotic, in comparison with the rest of the selected antibiotics, points to the fact that the fluoroquinolone was present in higher concentration than the rest of the antibiotics in rivers near intensive-farming areas. In addition, the sampling point ASE_19 can be considered as a reference point, because it is the only one that presents low wastewater pressure and low livestock pressure. As a result, the ANOVA test was used for the concentration of the different drugs at this point and others with medium or high livestock pressure. Significant differences (p-value < 0.05) were found for sulfadiazine concentration. The levels of drugs at this point were lower than the rest of the points in this study, except for enrofloxacin in the spring of 2019, which presented an unusual concentration (Table S2)

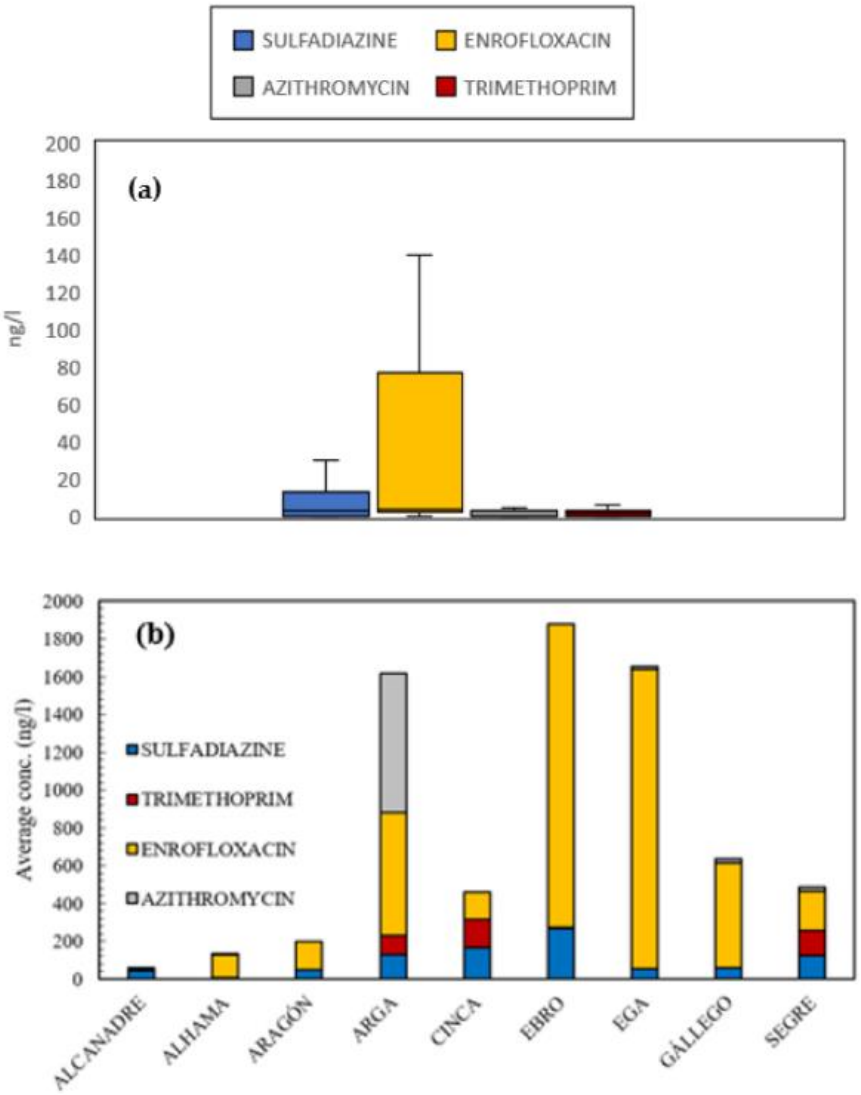


Figure D.1.5. (a) Boxplots of selected antibiotics and (b) average concentration (ng L^{-1}) of target antibiotics among all surface-water-sampling points in the Ebro River basin area (2018–2021).

ANOVA and Turkey's HSD test results in high-WWTP-pressure sampling points showed that a significant difference between the points exposed to this kind of pressure and the concentration of individual antibiotics in rivers does not exist. However, as revealed by Figure D.1.5b, the areas that present the highest total concentrations of antibiotics are Arga, Ebro and Ega, which present a medium-high WWTP pressure. In the Alcanadre River sub-basin, 46 ng L^{-1} average concentration of azithromycin appeared in Flumen River (Table S3). Moreover, the presence of enrofloxacin, trimethoprim and amoxicillin was also detected in concentrations close to the quantification limit; this might be associated with the presence of pig farms and low-flow rivers. In terms of detection frequency, sulfadiazine appeared in 40% of the samples, enrofloxacin in 20% of the surface-water-sampling points; these antibiotics can be associated with the presence of pig farms. In fact, trimethoprim was present only in 10% of the samples, and azithromycin was not detected in this area. The Aragón River sub-basin presented an average concentration of 147 ng L^{-1} of enrofloxacin. Downstream, as it passes through the town of Caparroso, the Aragón River area has a significant presence of pigs, poultry and rabbit farms and, as a result, 40% of the total samples contained fluoroquinolone (Table S2) and 30% of the surface-water-sampling points were polluted by sulfadiazine (Table S1). Regarding the Arga River sub-basin, the four target antibiotics were detected. The presence of sulfadiazine and enrofloxacin was detected in the concentration range of 100 to 130 ng L^{-1} (Tables S1 and S2). However, azithromycin and enrofloxacin appeared in average concentrations of up to 739 ng L^{-1}. It should be noted that this region is marked by the presence of an urban area (Pamplona). Concerning the detection frequency of target antibiotics, sulfadiazine and trimethoprim were found in 55% of the samples. Moreover, enrofloxacin was present in more than 50% of the river samples. These results might suggest that urban areas show a greater variety of antibiotics. In the Cinca River sub-basin, where there is a notable presence of pig and poultry farms, average concentrations close to 150 ng L^{-1} of enrofloxacin, sulfadiazine and trimethoprim were detected. Enrofloxacin and sulfadiazine appeared in 60% of the samples, while trimethoprim was detected only in 10% of them. This behavior confirms that enrofloxacin mainly appears in rivers where diffuse pollution from intensive farming of pig and poultry and agriculture-activity occurs. In the Ebro sub-basin, a high average concentration of enrofloxacin (1604 ng/L) was detected. It should be noted that the detection frequency of this fluoroquinolone antibiotic was about 75%. Sulfadiazine presented the average concentration of 270 ng L^{-1}; it appeared in 40% of the samples. It is interesting that all the target antibiotics appeared in this subbasin, which is very close to urban areas such as Logroño or Zaragoza, so it is marked by both urban areas and intensive farming, in which pig farms predominate. Consequently, these results point to the fact that a greater number of antibiotics were detected near urban areas. Moreover, this trend could also suggest that enrofloxacin and sulfadiazine can be associated with farming. Enrofloxacin was also detected in the French rivers Seine, Marne and Oise, presenting a maximum concentration of 100 ng L^{-1} [11]. The presence of 249 ng L^{-1} of this substance was also reported in the Polish rivers Gościcina and Reda, which are also associated with livestock pressure [8]. This antibiotic was also detected in the Mondego River (Portugal), in the Lllobregat River (Spain) and in the Ebro River (Spain), presenting concentrations of 76–178 ng L^{-1} [12]. In the Gállego sub-basin, sulfadiazine and trimethoprim presented average concentrations of up to 60 ng L^{-1}. On the other hand, enrofloxacin significantly exceeded 700 ng L^{-1}. This behavior can be attributed to the fact that the sampling point (at San Mateo de Gállego) is located downstream of several pig farms, as well as receiving the contribution of other rivers that discharge upstream, in areas where there is also an important farming presence (Huesca). Regarding the sub-basin of the Ega River, enrofloxacin once again presented high average concentrations, exceeding 1600 ng L^{-1} which can be linked to poultry and pig farming predominating in this area. Sulfadiazine was also detected at an average level close to 60 ng L^{-1}. In the Alhama River sub-basin, an average concentration of 125 ng L^{-1} of enrofloxacin appeared,

which can be associated with the presence, in this case, of poultry farming. There are a smaller number of poultry farms in this area than in others such as Segre or Ebro; however, in the areas where poultry farming predominates, antibiotic concentration is lower than in rivers that are located near pig farming. This behavior might suggest that pig-intensive farming presents a higher antibiotic load than poultry farming. Finally, in the Segre River sub-basin, which is subject to high farming pressure due to the presence of a large number of pig farms, all the studied antibiotics appeared. Enrofloxacin was again the antibiotic that presented the highest average concentration (205 ng L^{-1}) and detection frequency (80%). This behavior confirms that the vast majority of rivers near pig farms tend to be polluted by enrofloxacin. In fact, sulfadiazine chronicity was around 40% and trimethoprim was detected in 30% of the samples, but their average concentrations were relatively low: 121 ng L^{-1} and 140 ng L^{-1}, respectively. Furthermore, azithromycin appeared only in 10% of the samples. This decrease could confirm that the macrolide is only present near large urban areas in the Ebro River basin. However, other authors report concentrations of this macrolide antibiotic up to 1000 ng L^{-1} in rivers of Spain and France [13,14]. Regarding the river-flow effect, despite the existence of a significant difference between the average flows on rainier days and a consequent dilution of the species (Figure S1), the concentration of the selected antibiotics remained quite similar in drier and rainier seasons. A relevant fluctuation of the levels of drugs in river water between the sampling campaigns was observed. This could be due to the different flows that have been observed during these campaigns, which are listed in Figure S1. Although the ANOVA test confirms that there are not significant differences between the antibiotic levels and river flow, the antibiotics enrofloxacin and sulfadiazine tended to present higher concentrations in the rainier seasons. The data showed an increase in the levels of these antibiotics in autumn of 2020, which could be due to the initial stage of the pandemic of COVID-19, when the use of antibiotics and their subsequent emission into surface waters was augmented, as other authors suggest [15,16].

D.1.3.2. Antibiotics in waste waters

Tables S8 and S9 present the ANOVA and Turkey's HSD test of antibiotic concentrations in wastewater-sampling points. Significant differences (p-value < 0.05) were found between the azithromycin concentration and the rest of the selected antibiotics This result, coupled to the high detected concentration of this macrolide antibiotic, in comparison with the rest of the selected antibiotic, points to the fact that azithromycin was present in higher concentration than the other antibiotics in WWTPs. Concerning the average concentration results obtained for antibiotics in wastewater, which are shown in Figure D.1.6a, the macrolide azithromycin presented the highest average levels. Regarding Figure D.1.6b, the presence of this antibiotic was especially high in the WWT1, which is the one that presented the highest number of equivalent inhabitants, where the average azithromycin concentration exceeded 5000 ng L^{-1}. Other authors have reported the presence of this macrolide antibiotic in WWTPs in the range 20–2800 ng L^{-1} [5,17,18]. The total average concentrations of all the studied antibiotics reached 8000 and 5000 ng L^{-1} in the affluent and in the effluent, respectively.

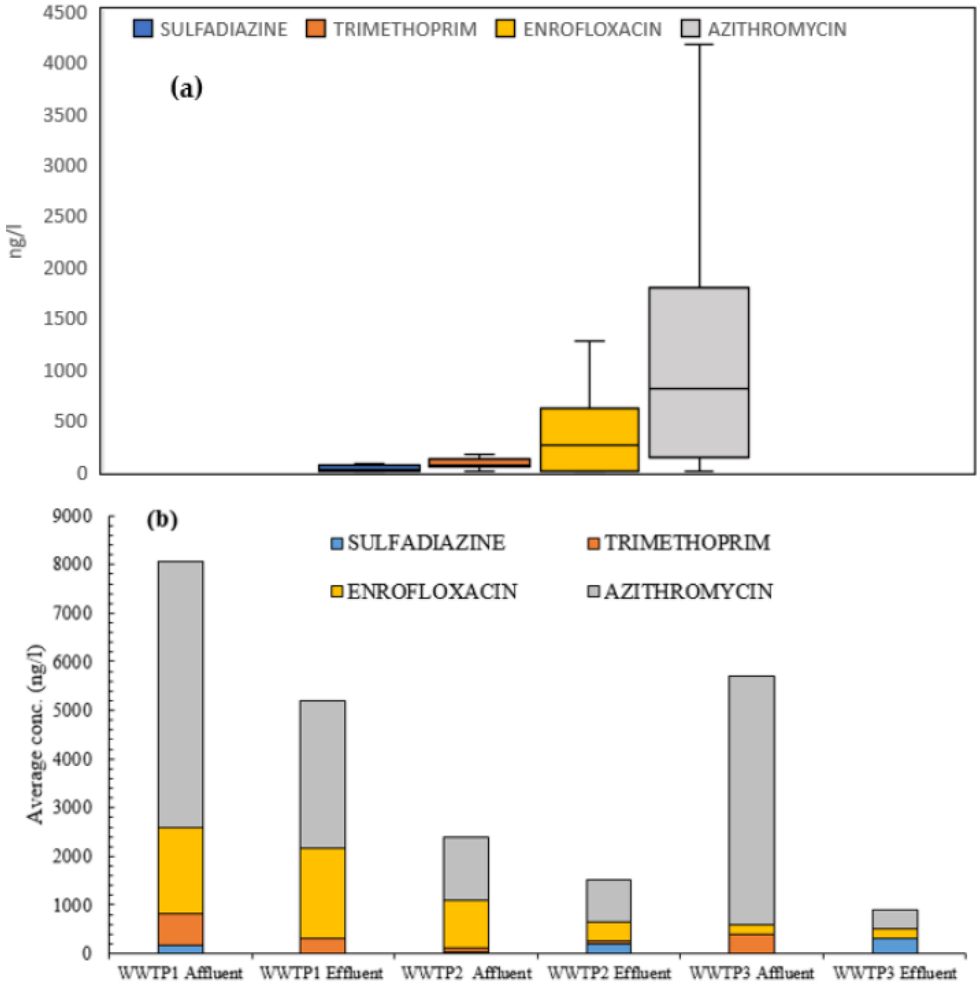


Figure D.1.6. (a) Boxplots of selected antibiotics and (b) the average concentration of the target antibiotics in selected WWTPs (2018–2021) located in the Ebro River basin area.

According to Figure D.1.6a, after azithromycin, enrofloxacin appeared in high concentration in the studied WWTPs, presenting average levels of 1300 ng L^{-1}. Enrofloxacin has been detected in 15 WWTPs of Croatia at a similar concentration [19]. Additionally, this antibiotic has also been found both in Slovakia in several WWTPs effluents [20] and in five WWTPs located in the Spanish territory [21]. The maximum level of azithromycin was reported at the entrance of WWTP1 and was up to 21,000 ng L^{-1}. These results are significantly superior to the ones reported in literature for other WWTPs located in Ebro River basin ten years before [3,22,23]. This increase might point to an incipient consumption of antibiotics, which is consistent with the reports published by the European Medicines Agency [10]. Sulfadiazine was also detected in the WWTP samples but in lower concentrations, reaching 300 ng L^{-1}. Comparing these outcomes with the literature, other authors detected the presence of this antibiotic in concentrations up to 846 ng L^{-1} in affluent and effluents from the Volos WWTP (Greece) [24,25]. The presence of sulfadiazine has also been evidenced in 22 treatment plants in Spain, with a concentration range of 49–1240 ng L^{-1} and 8–286 ng L^{-1} in in the affluents and effluents, respectively, which are similar to the concentrations found in this study [4]. The presence of trimethoprim in WWTPs was especially widespread in this study, appearing in the entirety of the effluents and showing an average concentration near 400 ng L^{-1}. However, concentrations of this substance up to 1866 ng L^{-1} have been reported in several WWTPs in Greece [26]. As revealed in Figure 6b, azithromycin was also present in

smaller urban areas, such as WWTP3. The average concentration of this antibiotic in WWTP3 was higher than 5000 ng L^{-1}. Other authors have reported the same concentration in studies on wastewater quality [27,28]. This behavior is probably associated with the fact that both sampling points are subject to high urban and industrial pressure. The results of the average antibiotic concentrations for the slaughterhouse and hospital effluents are shown in Figure D.1.7. According to Figure D.1.7a, azithromycin, again, was the antibiotic that presented the highest average concentration (2000 ng L^{-1}), especially in the hospital effluent, reaching 5000 ng L^{-1} (Figure D.1.7b). It should be noted that this antibiotic was used to treat symptoms of COVID-19 in 2020 and 2021 [29]. In addition, the trimethoprim level was also relatively high in the hospital effluent (>1500 ng L^{-1}). These results confirmed that the presence of azithromycin and trimethoprim is commonly due to human medicine, whereas to a lesser extent, they could also be found in poultry and rabbit slaughterhouses. In the literature, azithromycin presence in European hospital effluents varies in the range 1–10 µg L^{-1} [21]. The sulfonamide antibiotic sulfadiazine was found in the hospital effluent in low concentration up to 80 ng/L. Sulfadiazine concentrations reported in the literature for hospital effluents in Valencia, Spain range from 9–137 ng L^{-1} [30]. According to the literature, trimethoprim has been detected in hospital effluents, reaching concentrations up to 1800 ng L^{-1} [17]. However, in our study, this antibiotic appeared only in the hospital effluent at a significant concentration (1368 ng L^{-1}) and in the poultry slaughterhouse at a concentration of 390 ng L^{-1}. Compared to the rest of the studied slaughterhouses, only the duck slaughterhouse, where concentrations exceeding 1500 ng L^{-1} were detected, presented significant concentrations of azithromycin. Regarding enrofloxacin and sulfadiazine, the highest average concentrations were observed in the rabbit slaughterhouse (970 and 1835 ng L^{-1}, respectively).

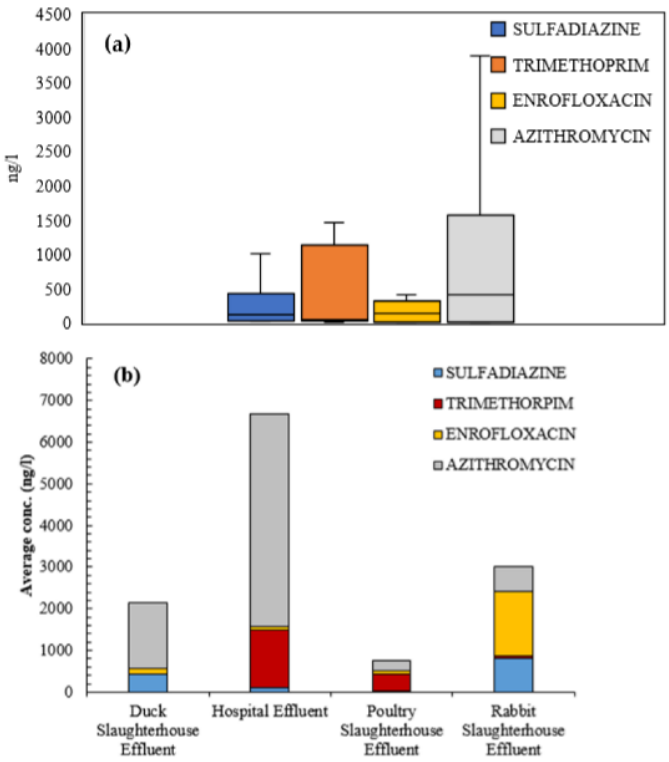


Figure D.1.7. (a) Boxplots and (b) average concentration (ng/L) of target antibiotics in a hospital and three slaughterhouse effluents.

D.1.4. Conclusions

The results of this research work show a long-term survey of the presence of antibiotics among surface waters and wastewaters in the Ebro basin (northeast of Spain) for four years (2018–2021). The choice of the target antibiotics was made based on a multispecies screening campaign carried out in the spring of 2018 and supported by the information on the sales and use of veterinary antimicrobials in Spain. Despite the European and national measures taken to restrict the use of antibiotics and exposure to these substances [27,28,31], the collected data demonstrated that:

• Enrofloxacin and sulfadiazine were present in almost all surface-water control points, which denotes high, direct exposure to these substances, especially in areas that are close to intensive farming. In fact, this fluoroquinolone antibiotic appears at very high concentrations in rivers of the Ebro basin near intensive farming, such as the Segre, Gallego or Cinca Rivers. Significant differences were found between the areas exposed to high livestock pressure and the concentration of enrofloxacin.

• Azithromycin was detected at very high concentrations in WWTPs. Complementarily, trimethoprim and enrofloxacin were detected in wastewaters of the Ebro River basin, especially in areas near large urban cores (>100,000 equivalent inhabitants).

• According to previous studies carried out in Ebro River basin in 2012 and 2010 [3,12], another important finding of this research is an increasing quantitative presence of antibiotics. Consequently, comprehensive studies of antibiotic assessment in Spanish rivers, wastewater, tap water, seawater and groundwater should be continued in order to establish water-quality standards for legislative guidance

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SUPPLEMENTARY DATA

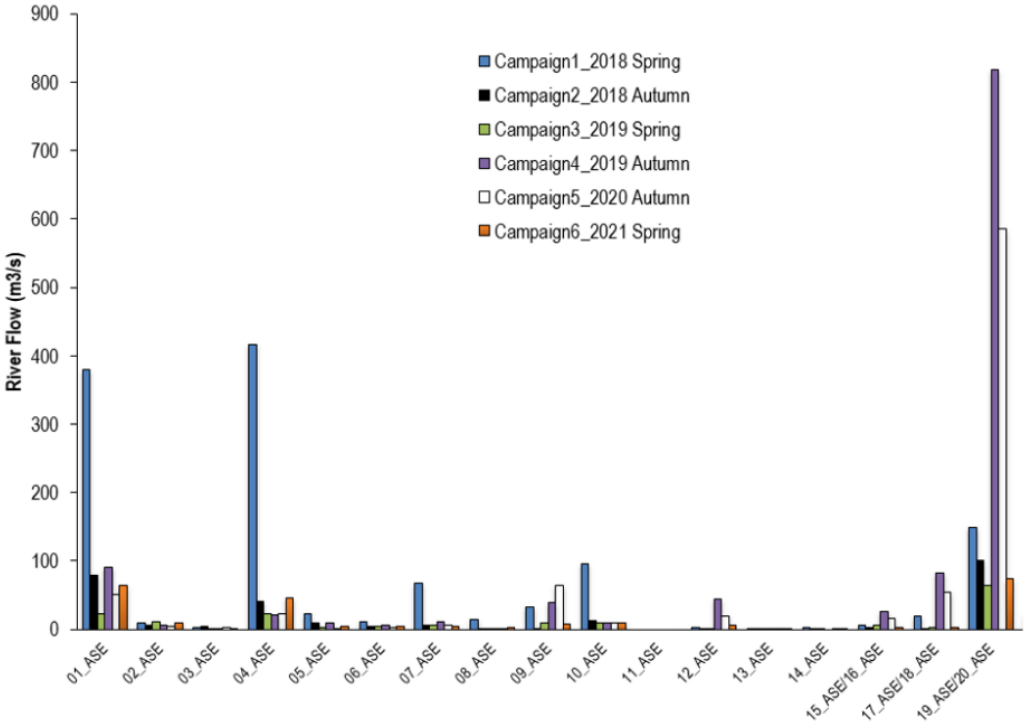

Figure S1. River flow during the six sampling campaigns 2018–2021.

Table S1. Sulfadiazine quantitative concentration results in surface control points (ng/L). D = detected (LOD < D < LOQ), n/d = not detected (<LOD).

RIVER	SUB-BASIN	LOCATION	SULFADIAZINE (ng/L)					
			Spring 2018	Autumn 2018	Spring 2019	Autumn 2019	Autumn 2020	Spring 2021
Segre River	Segre	Torres de Segre	n/d	D	D	5	120	9
Noguera Ribagorzana River	Segre	Corbins	D	D	D	28	214	118
Clamor Amarga River	Cinca	Zaidín	300	19	D	3	227	16
Cinca River	Cinca	Fraga	n/d	D	D	3	325	4
Alcanadre River	Alcanadre	Sariñena	n/d	n/d	D	8	n/d	1
Flumen River	Alcanadre	Albalatillo	D	40	D	2	95	30
Gállego River	Gallego	San Mateo de Gállego	n/d	D	D	34	85	1
Arba de Ríquel River	Ebro	Ejea de los Caballeros	D	n/d	n/d	18	521	3
Aragon Subordan River	Aragón	Javierregay	n/d	n/d	n/d	n/d	50	n/d
Aragon River	Aragón	Caparroso	n/d	n/d	n/d	1	12	n/d
Irantzu River	Ega	Estella	n/d	21	n/d	4	144	27
Arakil River	Arga	Irañeta	n/d	23	D	20	13	2
Queiles River	Arga	Novallas	D	n/d	n/d	1.5	12	2
Alhama River	Alhama	Alfaro	n/d	n/d	n/d	2	13	3
Ega River	Ega	Estella	D	n/d	n/d	2	20	14
Ega River	Ega	Estella	n/d	n/d	D	n/d	11	n/d
Arga River	Arga	Arazuri	n/d	D	258	1.6	n/d	n/d
Arga River	Arga	Ororbia	D	16	D	2	n/d	7
Ebro River	Ebro	Fontellas	D	D	D	12	n/d	n/d
Ebro River	Ebro	Tudela	n/d	n/d	D	77	n/d	n/d

It should be taken in account that spring 2020 sampling campaign was postponed to autumn 2020 as a result of COVID-19.

Table S2. Enrofloxacin quantitative concentration results (ng/L) in surface control points (ng/L). D = detected (LOD < D < LOQ), n/d = not detected (<LOD).

RIVER	SUB-BASIN	LOCATION	ENROFLOXACIN (ng/L)					
			Spring 2018	Autumn 2018	Spring 2019	Autumn 2019	Autumn 2020	Spring 2021
Segre River	Segre	Torres de Segre	240	140	D	6	106	5
Noguera Ribagorzana River	Segre	Corbins	250	D	523	17	31	966
Clamor Amarga River	Cinca	Zaidín	350	D	D	32	41	5
Cinca River	Cinca	Fraga	380	D	D	5	28	2
Alcanadre River	Alcanadre	Sariñena	n/d	D	n/d	n/d	11	2
Flumen River	Alcanadre	Albalatillo	D	D	D	n/d	7	13
Gállego River	Gallego	San Mateo de Gállego	1,560	D	D	11	88	3
Arba de Ríquel River	Ebro	Ejea de los Caballeros	4,390	D	D	314	107	4
Aragon Subordan River	Aragón	Javierregay	240	n/d	n/d	n/d	8	2
Aragon River	Aragón	Caparroso	280	D	D	n/d	15	3
Irantzu River	Ega	Estella	240	n/d	n/d	n/d	11	15
Arakil River	Arga	Irañeta	260	n/d	n/d	n/d	n/d	7
Queiles River	Arga	Novallas	250	D	D	2	n/d	2
Alhama River	Alhama	Alfaro	350	D	n/d	3	18	7
Ega River	Ega	Estella	250	2,920	n/d	n/d	n/d	9
Ega River	Ega	Estella	240	330	n/d	1.5	15	2
Arga River	Arga	Arazuri	590	210	D	n/d	4	3
Arga River	Arga	Ororbia	650	D	D	n/d	n/d	13
Ebro River	Ebro	Fontellas	D	D	3,033	n/d	42	11
Ebro River	Ebro	Tudela	240	D	672	200	39	4

It should be taken in account that spring 2020 sampling campaign was postponed to autumn 2020 as a result of COVID-19.

Table S3. Azithromycin quantitative concentration results (ng/L) in surface control points (ng/L). D = detected (LOD < D < LOQ), n/d = not detected (<LOD).

RIVER	SUB-BASIN	LOCATION	AZITHROMYCIN (ng/L)					
			Spring 2018	Autumn 2018	Spring 2019	Autumn 2019	Autumn 2020	Spring 2021
Segre River	Segre	Torres de Segre	n/d	n/d	D	n/d	22	2
Noguera Ribagorzana River	Segre	Corbins	n/d	n/d	n/d	n/d	n/d	4
Clamor Amarga River	Cinca	Zaidín	n/d	n/d	n/d	n/d	n/d	4
Cinca River	Cinca	Fraga	n/d	n/d	D	n/d	n/d	n/d
Alcanadre River	Alcanadre	Sariñena	n/d	n/d	D	n/d	n/d	n/d
Flumen River	Alcanadre	Albalatillo	n/d	n/d	D	n/d	n/d	1
Gállego River	Gallego	San Mateo de Gállego	n/d	n/d	n/d	n/d	n/d	1
Arba de Ríquel River	Ebro	Ejea de los Caballeros	n/d	n/d	n/d	n/d	n/d	n/d
Aragon Subordan River	Aragón	Javierregay	n/d	n/d	n/d	n/d	n/d	n/d
Aragon River	Aragón	Caparroso	n/d	n/d	n/d	n/d	n/d	n/d
Irantzu River	Ega	Estella	n/d	n/d	D	n/d	n/d	1
Arakil River	Arga	Irañeta	n/d	n/d	n/d	n/d	n/d	n/d
Queiles River	Arga	Novallas	n/d	n/d	D	22	n/d	1
Alhama River	Alhama	Alfaro	n/d	n/d	n/d	4	n/d	5
Ega River	Ega	Estella	n/d	n/d	n/d	3	n/d	1
Ega River	Ega	Estella	n/d	n/d	D	n/d	12	2
Arga River	Arga	Arazuri	n/d	n/d	D	n/d	n/d	1
Arga River	Arga	Ororbia	n/d	739	D	n/d	n/d	0
Ebro River	Ebro	Fontellas	n/d	n/d	n/d	n/d	n/d	5
Ebro River	Ebro	Tudela	n/d	n/d	n/d	n/d	n/d	1

It should be taken in account that spring 2020 sampling campaign was postponed to autumn 2020 as a result of COVID-19.

Table S4. Trimethoprim quantitative concentration results (ng/L) in surface control points. D = detected (LOD<D<LOQ), n/d = not detected (<LOD).

RIVER	SUB-BASIN	LOCATION	TRIMETHOPRIM (ng/L)					
			Spring 2018	Autumn 2018	Spring 2019	Autumn 2019	Autumn 2020	Spring 2021
Segre River	Segre	Torres de Segre	n/d	D	D	n/d	138	**12**
Noguera Ribagorzana River	Segre	Corbins	n/d	n/d	D	30	246	106
Clamor Amarga River	Cinca	Zaidín	D	n/d	D	n/d	153	4
Cinca River	Cinca	Fraga	n/d	D	D	n/d	n/d	1
Alcanadre River	Alcanadre	Sariñena	n/d	n/d	D	5	n/d	n/d
Flumen River	Alcanadre	Albalatillo	D	n/d	D	n/d	n/d	1
Gállego River	Gallego	San Mateo de Gállego	n/d	D	D	6	n/d	n/d
Arba de Ríquel River	Ebro	Ejea de los Caballeros	n/d	D	n/d	4	n/d	n/d
Aragon Subordan River	Aragón	Javierregay	n/d	n/d	n/d	n/d	n/d	1
Aragon River	Aragón	Caparroso	n/d	n/d	n/d	n/d	n/d	1
Irantzu River	Ega	Estella	n/d	D	D	n/d	n/d	1
Arakil River	Arga	Irañeta	n/d	D	n/d	n/d	n/d	6
Queiles River	Arga	Novallas	D	D	D	3	n/d	4
Alhama River	Alhama	Alfaro	D	n/d	D	n/d	n/d	2
Ega River	Ega	Estella	D	n/d	n/d	n/d	n/d	1
Ega River	Ega	Estella	n/d	D	D	n/d	n/d	1
Arga River	Arga	Arazuri	n/d	D	D	n/d	n/d	1
Arga River	Arga	Ororbia	10	D	189		n/d	16
Ebro River	Ebro	Fontellas	D	D	n/d	n/d	n/d	96
Ebro River	Ebro	Tudela	n/d	D	n/d	4	n/d	23

It should be taken in account that spring 2020 sampling campaign was postponed to autumn 2020 as a result of COVID-19.

Table S5. One-way ANOVA test of antibiotics concentration in high-livestock-pressure points.

Sources of Variation	Sum of Squares	Degree of Freedom	Mean Square	Factor F	*p*-Value
Between antibiotics	88,463	3	294,876,667	F = 4.41669	0.009 *
Within antibiotics	2,670,569,091	40	66,764,227		
Total	3,555,199,091	43			

* significant at $p < 0.05$.

Table S6. Tukey's honestly significant difference test results in high-livestock pressure points.

Pairwise Comparisons		HSD.05 = 93.3888 HSD.01 = 115.6699	Q.05 = 3.790 Q.01 = 4.695
SDZ:ENR	M1 = 25.91 M2 = 114.09	88.18	Q = 3.58 (*p*-value = 0.070) **
SDZ:TMT	M1 = 25.91 M3 = 10.73	15.18	Q = 0.62 (*p*-value = 0.971)
SDZ:AZI	M1 = 25.91 M4 = 1.09	24.82	Q = 1.01 (*p*-value = 0.892)
ENR:TMT	M2 = 114.09 M3 = 10.73	103.36	Q = 4.20 (*p*-value = 0.025) **
ENR:AZI	M2 = 114.09 M4 = 1.09	113.00	Q = 4.59 (*p*-value = 0.012) **
AZI:TMT	M3 = 10.73 M4 = 1.09	9.64	Q = 0.39 (*p*-value = 0.992)

** significant at $p < 0.05$. M = mean, Q = Studentized range distribution statistic, HSD = honestly significant difference

Table S7. One-way ANOVA test of sulfadiazine concentration between the reference point ASE19 and the points exposed to high and medium livestock pressure.

	Sum of Squares	Degree of Freedom	Mean Square	Factor F	*p*-Value
Between-treatments	82,990,139	1	82,990,139	F = 5.24636	0.032 *
Within-treatments	348,009,444	22	15,818,611		
Total	430,999,583	23			

* significant at $p < 0.05$.

Table S8. One-way ANOVA test of antibiotics concentration in WWTPs.

Sources of Variation	Sum of Squares	Degree of Freedom	Mean Square	Factor F	*p*-Value
Between antibiotics	353,593,698,419	3	11,786,456,614	F = 9.49363	0.009 *
Within antibiotics	434,529,413,889	35	12,415,126,111		
Total	788,123,112,308	38			

* significant at $p < 0.05$.

Table S9. Tukey's honestly significant difference test results in WWTPs.

Pairwise Comparisons		HSD.05 = 1,362.4039 HSD.01 = 1,692.9315	Q.05 = 3.8140 Q.01 = 4.7393
SDZ:ENR	$M_1 = 181.20$ $M_2 = 367.50$	186.30	Q = 0.52 ($p = 0.98259$)
SDZ:TMT	$M_1 = 181.20$ $M_3 = 784.11$	602.91	Q = 1.69 ($p = 0.63495$)
SDZ:AZI	$M_1 = 181.20$ $M_4 = 2,557.40$	2,376.20	Q = 6.65 ($p = 0.00022$) *
ENR:TMT	$M_2 = 367.50$ $M_3 = 784.11$	416.61	Q = 1.17 ($p = 0.84239$)
ENR:AZI	$M_2 = 367.50$ $M_4 = 2,557.40$	2,189.90	Q = 6.13 ($p = 0.00065$) *
AZI:TMT	$M_3 = 784.11$ $M_4 = 2,557.40$	1,773.29	Q = 4.96 ($p = 0.00656$) *

* significant at $p < 0.05$. M = mean, Q = Studentized range distribution statistic, HSD= honestly significant difference.

D.2. Screening for antibiotics and their degradation products in surface and wastewaters of the POCTEFA territory

D.2.1. Study area

POCTEFA is the acronym of the Interreg V-A Spain-France-Andorra Program. It is a European cross-border cooperation program created to promote the sustainable development of the border territories of the three countries. POCTEFA constitutes the Community financial support intended to strengthen the economic and social integration of this border area. POCTEFA co-finances cross-border cooperation projects designed and managed by actors located on both sides of the Pyrenees and coastal areas that participate in the Program by preserving the intelligent, sustainable and inclusive growth of the territory. The program promotes the sustainable development of the border territory of Spain, France and Andorra through cross-border cooperation. It contributes to reducing the differences in the development of the territory and to joining forces in order to achieve the sustainable development of the region and the cohesion of the regions that make it up. The aim of the program is to contribute to improving the quality of life of the inhabitants of the region [www.poctefa.eu]. The POCTEFA territory, shown in Figure D.2.1 includes the provinces of Navarra, Huesca, Zaragoza, and Lleida in Spain and the departments of Pyrénées Atlantiques, Hautes Pyrénées, Pyrénées Orientales, Haute Garonne and Ariege in France. The POCTEFA territory covers an area of 115 583 km^2 and is populated by more than 15 million inhabitants [1]. Significant agricultural, intensive farming and industrial pressure characterize the region.

Figure D.2.1 Map of POCTEFA taeeritory

A clear difference can be observed between the Spanish and the French parts of the POCTEFA territory regarding the consumption of veterinary antibiotics [2-4]. Between 2010 and 2018, antibiotics sales patterns in Spain varied with the most popular being penicillins and tetracyclines followed by aminoglycosides, lincosamides, macrolides and sulfonamides. The information is difficult to interpret due to different strategies of data collection, nevertheless a significant decline was observed in 2014, likely due to the adoption of the first "Spanish National Plan against Antibiotic Resistance" [5]. More complete data exist for France, where the sales of veterinary antibiotics have been monitored since

1999 by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) and its predecessors [6]. In 2015, France was the second largest consumer of veterinary pharmaceuticals worldwide, and the largest in Europe [13]. The level of exposure of animals to antibiotics, all routes and species combined, has decreased by 41.3% (from 1999 to 2019). In 2019, the overall exposure fell by 10.9% compared to the previous year and by 45.3% compared to 2011. Between 2018 and 2019 the change in exposure in France varied according to the species: -9.9% for cattle, -16.4% for pigs, -12.8% for poultry, and +1.5% for rabbits. Animals have been treated primarily with tetracyclines, penicillins, aminoglycosides, macrolides and polymyxins, followed by sulfonamides. The large decrease in antimicrobials used in animals in France is the result of collective action by all stakeholders to implement the French Action Plan 'EcoAntibio' 2012-2017. Another important source of antibiotics is human primary and hospital care. This use of antibiotics in Spain is among the highest in Europe while the consumption in France is 30% higher than the mean European rate [7].

The impact of antibiotics on the environment is not a simple function of the consumed global amount. There is a difference in potency and doses between different drugs; new generation antibiotics are generally more efficient and require the administration of smaller doses of active ingredient. An emerging issue is the consideration of the transformation products of the originally administered antibiotics, via biotic or abiotic processes [8-9]. As antibiotics belong to different groups of compounds and have different structures, elemental compositions and physicochemical properties, there no general rules governing their transformations [10]. Degradation products can show higher stability and toxicity than their parent compounds and possibly contribute to the development of antibiotics resistance gene [11-12].

Figure D.2.2. POCTEFA territory and surface sampling points

D.2.1. Quantitative analysis of antibiotics: method development and validation

Reversed-phase HPLC using double-gradient (pH and organic solvent) has been an established approach to the separation of antibiotics [13-14]. The use of electrospray ionization imposes the use of volatile buffers and reduce concentration of the salts which might increase sprayer voltage leading to rim emission or corona discharge.

The LC coupling to high-resolution hybrid quadrupole-Orbitrap mass spectrometry allowed the identification and quantification of compounds in one chromatographic run. Parallel reaction monitoring (PRM) scan mode strategy was used; it consists of the isolation of a targeted precursor in Q1, and then all generated MS/MS fragment ions are recorded in parallel with characteristics of full scan, accurate mass and high-resolution. A baseline separation of the 21 antibiotics and 3 degradation products (amoxicillin diketopiperazine and penicilloic acid as well as clarithromycin N-oxide), for which commercial standards could be purchased, has been achieved as shown in Figure D.2.3.

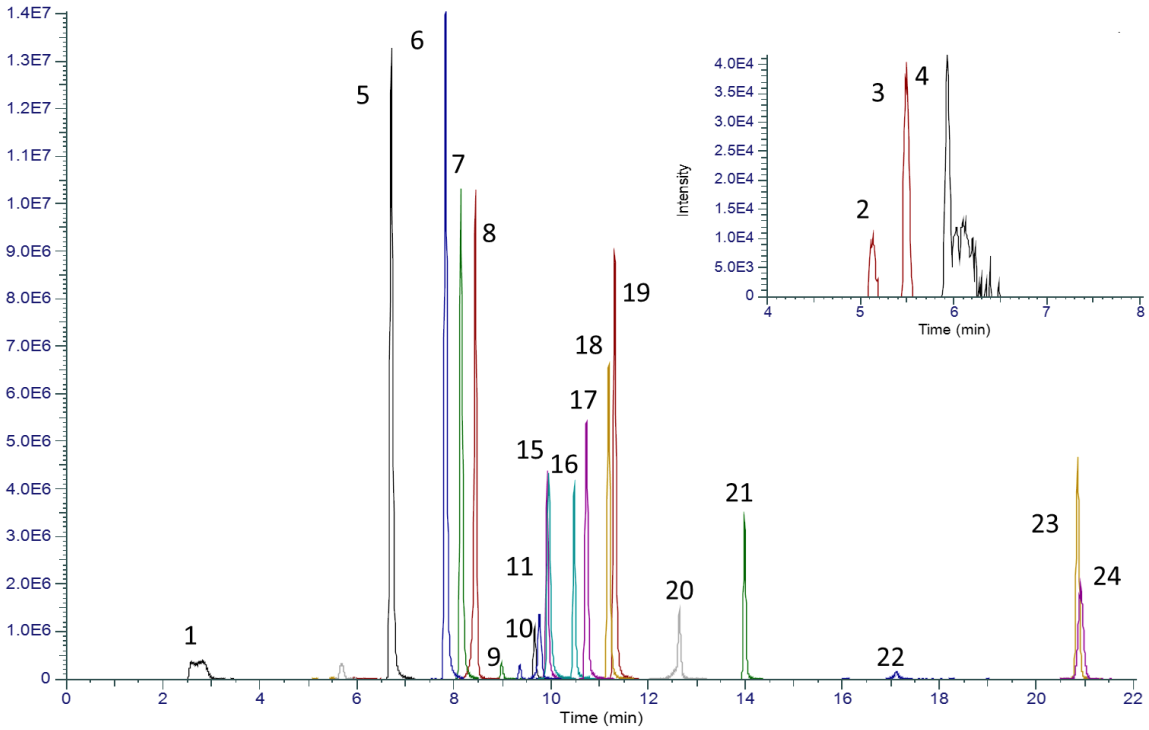

Figure D.2.3. LC-MS chromatogram obtained for of standards (5 ppb): 1 - florfenicol amine, 2 - peniciloic acid, 3 – sulfacetamide, 4 – amoxicillin, 5 –sulfadiazine, 6 – sulfapyridine, 7 – sulfamerazine, 8 – lincomycin, 9 – ampicillin, 10 – trimethoprim, 11 – amoxicillin diketopiperazine, 12 –norfloxacin, 13 – oxytetracycline, 14 – ciprofloxacin, 15 – dapsone, 16 – enrofloxacin, 17 – sulfamethoxine, 18 – sulfamethoxazole, 19 – sulfadoxine, 20 - moxifloxacin, 21 – azithromycin, 22 – erythromcin, 23 - clarithromycin n-oxide, 24 – clarithromycin

The relatively low levels of the antibiotics found in fresh water samples required a SPE protocol to extract and preconcentrate them; the procedure served also to clean-up (more polluted) waste water samples. Indeed, a problem encountered in the development of targeted analytical methods for species over a wide range of properties is that a balance must be kept between the ability to preconcentrate them without simultaneously extracting too many other compounds that result in heavy matrix. The SPE extraction and preconcetration was optimised by assessing the effect of several variables including sample pH, type of the cartridge, cleaning step and elution solvents as well as the

evaluation of different preconcentration factors on possible interferences. Finally, the method used was based on the EPA1694 with some modification, in particular no EDTA was used.

A matrix-matched calibration was used to perform the quantification; a calibration curve was constructed with 8 points using a least-square linear regression ($R \geq 0{,}998$). Recovery was measured by spiking 3 different concentrations (0.01, 0.05 and 0.1 ng L^{-1}) in water matrix and the analysis of the resulted solutions. For estimation of the method recovery (R%) three samples of each kind of water matrix were fortified at 500 ng L^{-1}, and were subjected to the entire analytical procedure mentioned before. The instrumental repeatability of the LC–QqLIT–MS equipment was calculated through six consecutive injections of a standard antibiotic mixture solution corresponding to a concentration of 500 ng L^{-1}. Three blank samples for each matrix were also evaluated to avoid overestimations in the calculation of the recovery. Instrumental detection limits (ILODs) were experimentally calculated from the injection of the standard solution with a concentration corresponding to the lowest used to build the calibration curves (in this study, 0.05 ng L^{-1}). Method LODs and LOQs were experimentally calculated from the analysis of spiked water samples on the basis of a signal to noise ratio of 3 and 10, respectively. Recoveries higher than 50% were obtained for all the antibiotics, except for clarithromycin and clarithromycin N-oxide ($40<R\%<50$). The highest, fully quantitative, recoveries were obtained for trimethoprim and azithromycin. In general, β-lactamases showed lower recoveries than fluoroquinolones, which exhibited lower recovery than sulfonamides. Instrumental repeatability was calculated through six consecutive injections of standard antibiotic spiked in matrix water. LoD and LoQ (given in Table 1) were calculated using the standard deviation of the lowest point (analysed 20 times), divided by the slope and multiplied respectively for 3 and 10.

Figure D.2.4 shows an example chromatogram for hospital (Figure D.2.4a) and (Figure D.2.4b) bird slaughterhouse samples. It can be seen how the chromatograms are quite different. The hospital effluent presents a broad variety of antibiotics detected from different families (more than 15 peaks). However, the slaughterhouse effluent presented less than 10 antibiotics, which are individually in higher concentrations, although the total antibiotic concentration of the hospital effluent where about 5 times higher than the slaughterhouse effluent. Moreover, in the case of the slaughterhouse they correspond to specifically veterinary antibiotics (*e.g*. enrofloxacin).

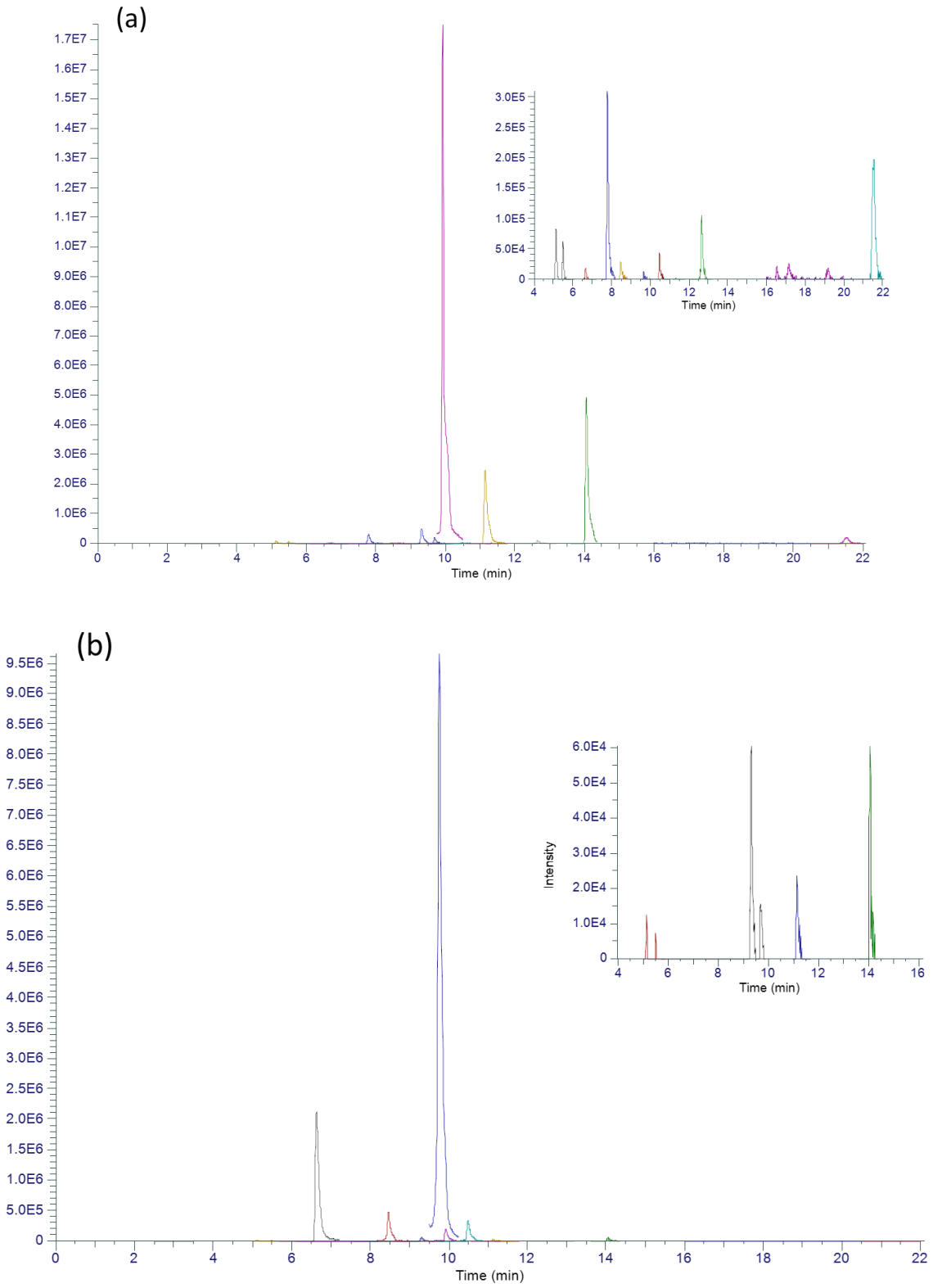

Figure D.2.4. LC-MS example chromatograms obtained for (a) hospital and (b) bird slaughterhouse samples.

D.2.2 Quantification of antibiotics in surface waters

The frequency of detection and concentration range of target antibiotic obtained for surface waters are summarized in Table D.2.1. The results are discussed in the context of the data reported for the quantification of antibiotics in European surface waters are summarized in Table D.2.2.

Table D.2.1. Frequency of detection and concentration range of target antibiotic in surface waters

Class	Antibiotic	Frequency of detection (%)	Concentration range (ng L^{-1})
ß-Lactamase	amoxicillin	4 %	LOQ-8.0
	ampicillin	14 %	15,7-79.6
	diketopiperazine	<LOQ	<LOQ
	penicilloic acid	<LOQ	<LOQ
Fluoroquinolone	ciprofloxacin	29 %	LOQ-33.6
	enrofloxacin	89 %	11.8-970.0
	moxifloxacin	7 %	1.4-9.8
	norfloxacin	4 %	LOQ-3.2
Lincosamide	lincomycin	29 %	0.9-70.7
Macrolides	azithromycin	21 %	1.7-67.0
	erithromycin	<LOQ	<LOQ
	clarithromycin	7 %	3.8-7.0
	clarithromycin n-oxide	4 %	LOQ-4.0
Sulfonamide	dapsone	4 %	1.1-30.7
	sulfadiazine	61 %	0.4-48.3
	sulfamerazine	4%	LOQ-8.6
	sulfamethoxazole	68 %	LOQ-63.9
	sulfadaxone	<LOQ	<LOQ
	sulfapyridine	11%	1.4-48.8
Tetracycline	oxytetracycline	4 %	LOQ-7.8
Diaminopyrimidine	trimethoprim	64 %	2.5-106.0

Table D.2.2. Frequency of detection concentration range of antibiotics in comparison with the maximum reported in European surface waters

Class	Antibiotic	Frequency of detection (%)	Concentration range (ng/l)	C_{max} (ng L^{-1})*
ß-lactamase	amoxicillin	4	LOQ - 8.0	522 (UK) [15]
	ampicillin	14	15.7 - 79.6	26 (Germany) [16]
	diketopiperazine	n.d	< LOQ	not given
	penicilloic acid	n.d	< LOQ	not given
fluoroquinolone	ciprofloxacin	29	LOQ - 33.6	9,660 (France) [17]
	enrofloxacin	89	11.8 - 970	210 (Portugal) [18]
	moxifloxacin	7	1.4 - 9.8	210 (Spain) [19]
	norfloxacin	4	LOQ - 3.2	160 (France) [20]
lincosamide	lincomycin	29	0.9 - 70.7	250 (Italy) [21]
macrolides	azithromycin	21	1.7 - 67.0	1,600(Croatia) [22]
	erythromycin	n.d	< LOQ	1,700 (Germany) [23]
	clarithromycin	7	3.8 - 7.0	2,330 (France) [17]
	clarithromycin N-oxide	4	LOQ - 4.0	not given
sulfonamide	dapsone	4	1.1 - 30.7	not given
	sulfadiazine	61	0.4 - 48.3	2,400 (Croatia) [22]
	sulfamerazine	4	LOQ - 8.6	11,000 (Croatia) [22]
	sulfamethoxazole	68	LOQ - 63.9	11,000 (Spain) [24]
	sulfapyridine	11	1.4 - 48.8	12,000 (Spain) [25,26]
tetracycline	oxytetracycline	4	LOQ - 7.8	not given
diaminopyrimidine	trimethoprim	64	2.5 - 106	11,000 (Croatia) [22]

* Maximum concentration reported of target antibiotic in surface waters Europe for the target antibiotics

Target antibiotics have been detected in all the surface water samples with the exception of erythromycin, diketopiperazine and penicilloic acid. There is, however, no single antibiotic which would be detected in all the samples which contrasts with the omnipresence of some antibiotics observed elsewhere [27]. The frequency of detection of antibiotics in this study ranges from 4% to 89%, The highest was observed for enrofloxacin (89 %), sulfamethoxazole (68 %), trimethoprim (64 %), sulfadiazine (61%) and ciprofloxacin (29 %). This observation is similar to that made in Portuguese surface waters [28,29], where sulfamethoxazole was most frequently (92%) detected, followed by ciprofloxacin (75%).

It is interesting to compare the concentrations found in this study with the maximum concentrations (Table D.2.1) reported for antibiotics in surface waters in Europe (Table D.2.2). The most striking is the case of enrofloxacin of which the maximum concentration measured in this study reaches 970 ng/l which is almost 5 times higher than the maximum concentration of this antibiotic reported so far in Europe (212 ng/l in Portugal) [18]. The exposure to this antibiotic in the POCTEFA territory is corroborated with its highest frequency of detection. A similar observation was made from ampicillin for which the highest concentration found in this study (79.6 ng L^{-1}) is ca. 3 times higher than reported elsewhere in Europe (26 ng L^{-1}) [16].

On the other hand, the concentrations found for the other antibiotics (trimethoprim, sulfadiazine, sulfamethoxazole, azithromycin) are much lower than the concentrations reported elsewhere for these compounds. For example, for trimethoprim and sulfamethoxazole, which were the next most frequently detected antibiotics, the maximum concentrations are 106 ng L^{-1} for trimethoprim and 63.9 ng L^{-1} for sulfamethoxazole, while in Europe the maximum concentration detected for these antibiotics were ten times higher: 11,000 ng L^{-1} in Croatia [22] for trimethoprim and 11,000 ng L^{-1} in Spain [22]. This observation is similar for sulfadiazine, azithromycin and ciprofloxacin, of which the concentrations measured ranged from 33.6 ng L^{-1} to 67.0 ng L^{-1}, while their maximum concentrations reported in Europe were an order of magnitude higher. Lincomycin showed a concentration of 70.7 ng L^{-1} whereas the maximum concentration in European surface waters was 248.9 ng L^{-1}.

The ANOVA test was implemented to determine the existence of significant differences between Spanish and French rivers, a p-value of 0.05 was selected according to the literature [30]. Regarding the results, which are summarized in Table S1, significant differences ($p<0.05$) has been found between the concentration of target antibiotic in French and Spanish rivers. The total concentration of the target antibiotics in French rivers reaches about 10 ng L^{-1} in the Adour river, near Bayonne (23_ASA/G in Figure D.2.2.), while the total concentration of the studied antibiotics in a sampling point in Spanish exceeds 1000 ng L^{-1}. This fact could be due to fact that the use of antibiotics in Spain is the highest in the EU/EEA [31-32], or to the later adoption of the plan against antibiotic resistance in Spain than in France.

These observations are consistent with the data reported on the occurrence of antibiotics in Spain and France which were summarized in Table S2. The concentrations of antibiotics measured in Spain are generally higher than in France with the exceptions of ciprofloxacin and clarithromycin. This conclusion should be taken with caution as there have been many more studies of the presence of antibiotics in Spain (ca. 171) than in France (ca. 30) [33]. The maximum total concentration of target antibiotics measured in a singular sampling point reaches 1247.6 ng L^{-1}, which is similar to the concentration reported in Chinese rivers for these antibiotics [27].

D.2.3. Concentrations of antibiotics measured near to wastewaters treatment plants

The results (concentration range and frequency of detection) obtained for the quantification of antibiotics in waste waters are summarized in Table D.2.3.

Target compounds have been detected in all the selected water samples with the exceptions of clirothormycin n-oxide, sulfamerazine, diketopiperazine and Penicilloic acid. They present a high frequency of detection from 8% to 92%. The highest frequency of detection has been observed for the antibiotics: azithromycin (92 %), sulfamethoxazole (92%), trimethoprim (75%), ciprofloxacin (75%), enrofloxacin (61%) and clarithromycin (50%). Comparing these results with the obtained in the previous section, it can be seen as ciprofloxacin, enrofloxacin, sulfamethoxazole and trimethoprim are the antibiotics which appears in highest chronicity in both, surface waters and wastewaters effluents. Among the antibiotics investigated, ciprofloxacin was predominant in WWPT influent and effluents. Sulfamethoxazole was also very abundant in the influents (> 83%) and effluents (> 79%).

Table D.2.3. Concentrations of antibiotics found in waste waters

Class	Antibiotic	Frequency of detection (%)	Concentration range (ng L^{-1})
ß-lactamase	amoxicillin	8 %	7.0-15.0
	ampicillin	25 %	LOQ-26.0
	diketopiperazine	<LOQ	<LOQ
	penicilloic acid.	<LOQ	<LOQ
fluoroquinolone	ciprofloxacin	75 %	3.8-172.7
	enrofloxacin	61 %	48.3
	moxifloxacin	42 %	2.6-46.9
	norfloxacin	25 %	LOQ-3.2
lincosamide	lincomycin	50 %	LOQ-26.0
macrolides	azithromycin	92 %	1.8-144,0
	erithromycin	8 %	LOQ-43.0
	clarithromycin	50 %	6.6-62.7
	clarithromycin N-oxide	n/d	n/d
sulfonamide	dapsone	8 %	LOQ-30.7
	sulfadiazine	42 %	
	sulfamerazine	n/d	n/d
	sulfamethoxazole	92%	5.9-256.0
	sulfapyridine	42 %	2.8-12.6
tetracycline	oxytetracycline	8%	535-2670
Diaminopyrimidine	Trimethoprim	75 %	1.4-122.9

In the present research, oxytetracycline (2670 ng L^{-1}) azithromycin (144 ng L^{-1}), ciprofloxacin (176 ng L^{-1}) and sulfamethoxazole (256 ng L^{-1}), appeared in the highest concentrations. Other authors detected also the presence of ciprofloxacin as the antibiotic predominant in Greece [21], presenting concentrations up to 591 ng L^{-1}. This work also suggest that sulfamethoxazole was very abundant in wastewater, (>83%) and effluents (>9%), however, this sulfonamide antibiotic was found in relatively low concentrations (< 137.9 ng L^{-1}) in influents and (< 43 ng L^{-1}) effluents [34]. Other studies have evaluated occurrence of pharmaceuticals in the effluent of wastewater treatment plants in Italy, reporting again that although sulfamethoxazole and ciprofloxacin presented a frequency of detection of 100%, these compounds appeared in relatively low concentrations effluents [35], more precisely, they reported concentrations of ciprofloxacin ranging from 10-500 ng L^{-1} [36] and from 35-185 ng L^{-1} of sulfamethoxazole.

Regarding the load of antibiotics, in this research, azithromycin, trimethoprim and oxytetracycline showed the maximum concentrations, ranging from 144 ng L^{-1} for azithromycin to 2670 ng L^{-1} for oxytetracycline. Although this antibiotic was only detected in 8% of the wastewaters samples, this concentration can be due to a punctual emission.

Table D.2.4. Summary of the results in recent study of 7 WWTPs in the European scenario [64]

Group	Antibiotic	C_{max} (ng L^{-1})	Country
Fluoroquinolones	**Ciprofloxacin**	**1435,5**	**Portugal**
	Enrofloxacin	176,4	Spain
Macrolides	azithromycin	1577	Portugal
	clarithromycin	337	Ireland
Sulfonamides	sulfamethoxazole	176,6	Spain
Diaminopyrimidine	Trimethoprim	330	Finland

Table D.2.4 shows a brief summary of a European study, which reports antibiotic concentration in WWTPs effluent of seven European countries (Portugal, Spain, Ireland, Cyprus, Germany, Finland, and Norway). The compounds with the highest loads in the countries studied in this report were macrolides and fluoroquinolones [37]. As a result, ciprofloxacin was selected as marker of antibiotic pollution and were suggested to be used for widespread temporal and geographical characterization of environmental water or WWTP effluents by other authors [37].

D.2.4 Analysis for antibiotics degradation products

β-Lactams are structurally characterized by the β-lactam nucleus, which is susceptible to cleavage, e.g., by high temperatures, light, extremes in pH, metal ions, oxidizing and reducing agents [38] as well as enzymatic and biological degradation. Consequently, low environmental exposure levels of β-lactams are expected, despite their high consumption [1]. Although the hydrolysis of β-lactam ring results in a loss of the antibiotic activity, the identification of their transformation products and the study of their occurrence, fate, efficiency, and persistent in the environment are essential for proper risk assessments [39-40]. Two degradation products of amoxicillin (penicilloic acid and diketopiperazine), for which commercial standards are available, could be quantified.

The metabolites for which standards were unavailable were searched for, either in a targeted way based on the literature information or using exploratory workflows. The species found, together with their formulas and masses are summarized in Table D.2.5; they have been previously reported in environmental waters [10,41,42,43].

Table D.2.5. The antibiotic degradation products detected

CLASS	Antibiotic species	Degradation product	RT	Formula	Exact mass	Δ ppm	Ref.
diaminopyrimidine	trimethoprim	4-desmethyl-TMP	4,16	$C_{13}H_{17}N_4O_3$	277,130	-0,25	[42]
fluoroquinolone	enrofloxacin	enrofloxacin 5Bwt	5,80	$C_{17}H_{21}FN_3O_3$	334,157	-1,37	[44]
macrolides	azithromycin	azithromycin double cleavage	5,90	$C_{22}H_{44}N0_7$	434,312	-0,38	[43]
sulphonamide	sulfadiazine	N-acetyl sulfadiazine	4,98	$C_{12}H_{13}N_4O_3S$	293,071	-0,73	[10,41]
	sulfamethoxazole	N-acetyl sulfamethoxazole	6,63	$C_{12}H_{14}N_3O_4S$	296,070	-0,6	[10]

The results of frequency of detection of antibiotics degradation products in surface and waste waters are summarized in Table D.2.6.

Table D.2.6. Frequency of detection of antibiotics degradation products in surface and waste waters

class	antibiotic species	degradation product	frequency of detection (%)	
			surface water	wastewater
□-lactamates	amoxicillin	penicilloic acid	<LOD	<LOD
		diketopiperazine	<LOD	<LOD
macrolides	azithromycin	azithromycin double cleavage	57%	50%
diaminopyrimidines	trimethoprim	4-desmethyl-TMP	7%	50%
fluoroquinolones	enrofloxacin	enrofloxacin 5Bwt	14%	58%
sulphonamides	sulfadiazine	N-acetyl sulfadiazine	11%	8%
	sulfamethoxazole	N-acetyl sulfamethoxazole	11%	67%

Note that even that some antibiotics were hardly detected in surface waters, their metabolites were. For example, azithromycin, showed a detection frequency of 21%, while its degradation product with double cleavage was detected almost three times more often (57%). This corroborates earlier indications [10,45] that azithromycin is metabolized in the aquatic environment.

The detection frequency of the metabolites of sulfamethoxazole (N-acetyl-sulfamethoxazole) and enrofloxacin (enrofloxacin-5Bwt) in wastewaters follows that of their parent compounds. Note that enrofloxacin-5Bwt shows a frequent presence in wastewaters (58%), but is less frequently detected in surface river waters (14%). This can be attributed to the degradation of fluoroquinolones in the biological processes applied in wastewater treatment and/or to the degradation of enrofloxacin in the environment [46-48].

The amoxicillin β-lactamate metabolites, penicilloic acid and diketopiperazine, for which standards exist and which were monitored in a targeted way, were not detected in any sample.

D.2.5 Conclusions

The developed SPE-LC-MS/MS method allowed the determination of 21 antibiotics and detection their metabolites in the POCTEFA territory. There are significant differences between the concentration of antibiotics in surface waters of France and Spain, reflecting they larger use of antibiotics in Spain. In surface waters, enrofloxacin, sulfamethoxazole and trimethoprim appear at highest concentrations and show the highest frequencies of detection. In wastewaters, oxytetracycline, azithromycin, ciprofloxacin, and sulfamethoxazole, appeared in the highest concentrations. Ciprofloxacin, enrofloxacin, sulfamethoxazole and trimethoprim are the antibiotics which appears in highest chronicity in both, surface waters and wastewaters effluents. Some degradation products of antibiotics, *e.g.* azithromycin, present higher frequency of detection than their parent's compounds suggesting a degradation occurring in WWTPs and in the environment.

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Supplementary material

Table S1. ANOVA Test between Spanish and French rivers

Sources of variation	Sum of squares	Degree of freedom	Mean Square	Factor F	p-value
Between rivers	21.642.135	1	21.642.135	F= 5.27929	0.024
Within rivers	344.353.214	84	4.099.443		
Total	365.995.349	85			

Table S2. Antibiotics maximum concentration in Spain and France

CLASS	ANTIBIOTIC	Cmax Spain (ng/l)	Reference	Cmax France	Reference
ß-Lactamase	Amoxicillin	n/d		68	[49]
	Ampicillin	n/d		n/d	
Fluoroquinolone	Ciprofloxacin	740	[50]	9660	17
	Enrofloxacin	178	[57]	n/d	
	Moxifloxacin	205	[50]	n/d	
	Norfloxacin	n/d	n/d	163	[20]
Lincosamide	Lincomycin	47	[50]	n/d	
Macrolides	Azithromycin	28	[51]	n/d	
	Erithromycin	70	[52]	4	[49]
	Clarithromycin	91	[50]	2330	[17]
Sulfonamide	Sulfadiazine	2312	[53]	n/d	
	Sulfamerazine	n/d		n/d	
	Sulfamethoxazole	11000	[53]	544	[20]
	Sulfapyridine	12000	[54]	1	[55]
Diaminopyrimidine	Trimethoprim	252	[56]	20	[55]

D.3. Interactions of antibiotics with colloids in surface and waste waters

D.3.1. Presence of colloids in natural waters. Characterization of the different fractions detected.

D.3.1.1. Measurement of pH and estimation of ionic strength of samples collected during the campaign 4 (2019 autumn)

pH and ionic strength of waters can exert a strong influence on interactions between natural colloids and antibiotics, as discussed in section A.1.3.1. pH and conductivity values of samples (from different natural and waste waters from POCTEFA territory. See Figure D.2.2. for reference index of the location of each sample) collected are shown in Table D.3.1. Samples were measured once they arrived at the lab (January 2020), although values shown correspond to the measurements made once normal activity was restored after the lockdown caused by COVID-19, in May 2020. In general, pH and conductivity values kept constant for most of the samples analyzed (just samples 37 and 40 gave significant differences on pH values, probably due to the degradation of the organic matter (at relatively high concentration in these two samples, slaughterhouse effluent and a hospital effluent, respectively).

Table D.3.1. Average pH and conductivity values of water samples measured in May 2020 (n=3). RSD < 1% and < 3% for pH and conductivity measurements, respectively.

Sample	Type	pH	Conductivity (µS cm^{-1})
4	RIVER	7.90	1570
5	RIVER	8.16	387
6	RIVER	8.18	1280
24	RIVER	7.58	210
25	RIVER	7.99	197
29	WWTP2 IN	6.98	2070
30	WWTP2 OUT	6.75	1450
31	WWTP1 IN	6.91	930
32	WWTP1 OUT	7.35	580
37	Slaughterhouse	6.27	1647
38	Slaughterhouse	7.12	1707
40	Hospital	7.98	1157

In general, pH values are between 6.5 and 8.5, which are considered normal values for natural waters [1]. The highest conductivity, associated to the highest ionic strength and therefore with a high mineral content, corresponds to sample 29 (a sample collected upstream from WWTP2), together with those collected from a slaughterhouse effluent (37 and 38). The lowest conductivity corresponds to natural river waters (samples 24 and 25).

D.3.1.2. Characterization of the colloidal fraction in samples by AF4-UV-Vis-ICP-MS

D.3.1.2.1. AF4 Channel size calibration

As discussed in section A.3.2.1., particle size determination in AF4 is possible using theoretical expression that relate retention times of the particles eluted with their diffusion coefficients (and hence with their hydrodynamic diameter according to Stokes' law) or using size standards that behave during the separation in a similar way that the species to be characterized. In this case, since the study is focused on the colloidal fraction in natural waters, and inorganic colloids are mainly composed of aluminum phyllosilicates and silicates, the separation channel was calibrated against silicon dioxide standards of known size (0.15, 0.5, 1, 2 and 4 µm). These standards are commercially available and characterized by transmission electronic microscopy (TEM). For calibration, a sample loop of 100 µL were used, whereas rest of conditions are those shown in table C.1. The UV-Vis detector was set at a wavelength of 254 nm to monitor the signal. The fractograms obtained for the 5 standards are shown in Figure D.3.1.

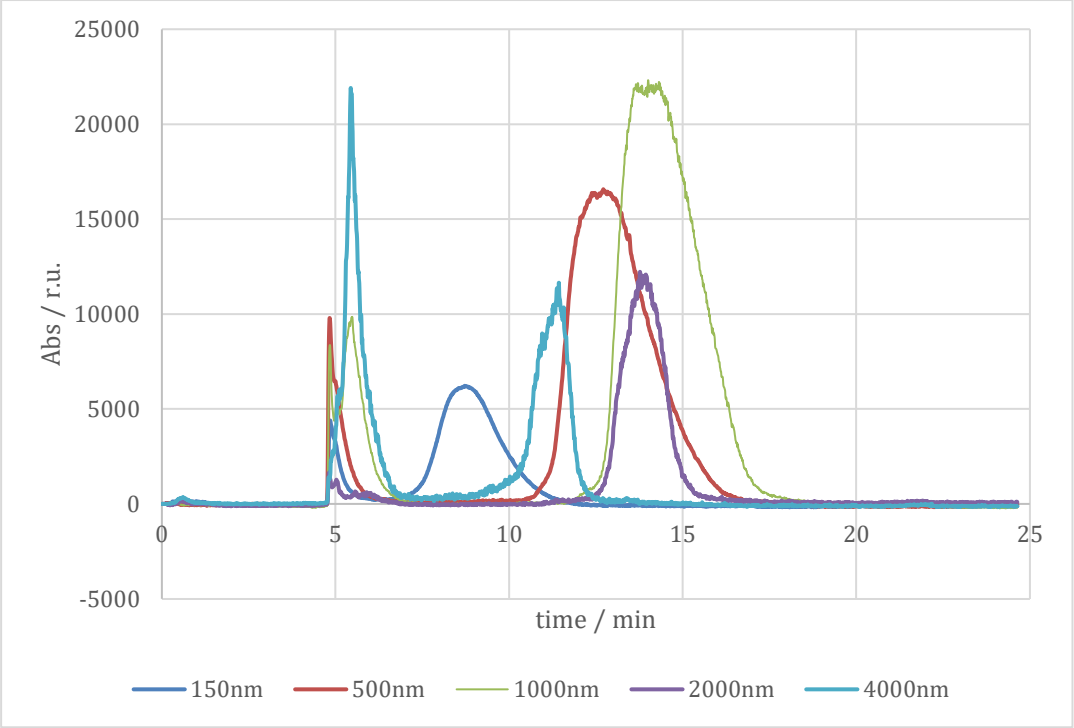

Figure D.3.1. UV-Vis at 254 nm fractograms of different SiO_2 size standards suspensions. Injected volume: 100 µL. Concentration for all standard suspensions 30 mg/L.

As it can be observed, the elution order does not follow the size order of the standards, meaning that the inversion point of the channel has been reached passing from the elution in normal mode to the elution in steric mode. By plotting the elution time t, (calculated as t_r-t_0, in min, where t_0 is the time of the void peak, which corresponds to the elution of species not retained) against the size of the standards (d, in nm) the calibrations curves of both elution modes can be estimated. Results are shown in Figure D.3.2. Size standard of 1 µm shows a mixed behaviour so its elution time does not fit into neither the normal nor steric calibration curves.

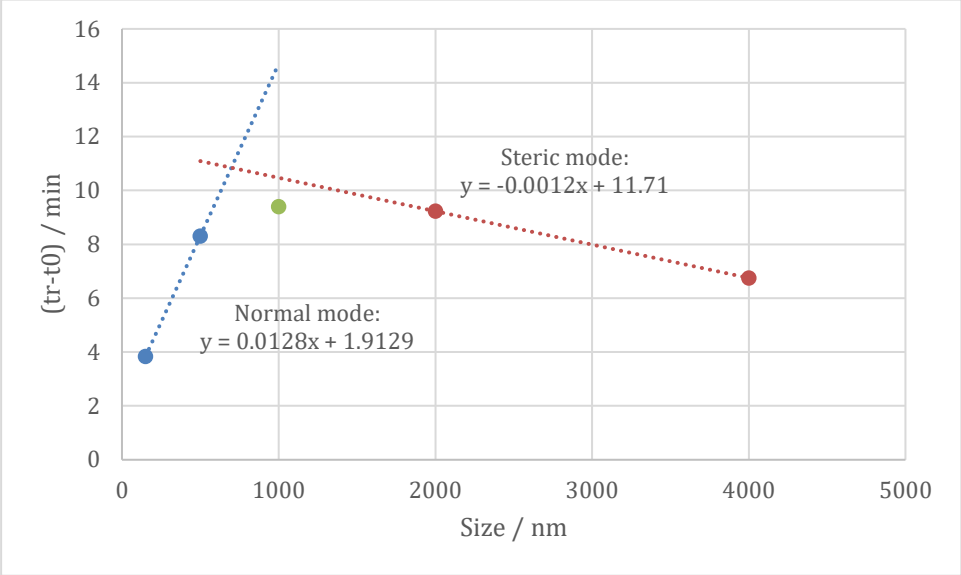

Figure D.3.2. Calibration curves for normal and steric elution modes using different SiO_2 size standards suspensions. Injected volume: 100 µL. Concentration for all standard suspensions 30 mg/L.

Through these equations, the inversion point of the channel could be calculated as 0.7 µm. This means that if species larger than 0.7 µm are injected, they will elute in inverse order (larger first) respect to normal mode (smaller first). If the size range of the particles in a sample is unknown or covers both elution modes, particles of different size could coelute, so a previous separation (*i.e.* by filtration) step is required to assess the size of a peak in the fractogram.

D.3.1.2.2. Analysis of samples by AF4-UV-Vis-ICP-MS

All the samples were analyzed by AF4-UV-Vis-ICP-MS following the procedure described in section C.3.3. UV-Vis absorbance at 254 nm was used for general purpose to detect both organic and colloidal species eluted from the channel, whereas ICP-MS was used as an elemental detector to characterize basically those aluminum containing colloids present in samples. Other elements, such as Mg, Si, Ca, Ti, Mn, Fe or Pb, were also monitored, showing similar profiles as aluminum or showing no significant signal at all for all these elements. Figures D.3.3 and D.3.4 shows the fractograms obtained for samples 6 and 24 (natural waters), respectively.

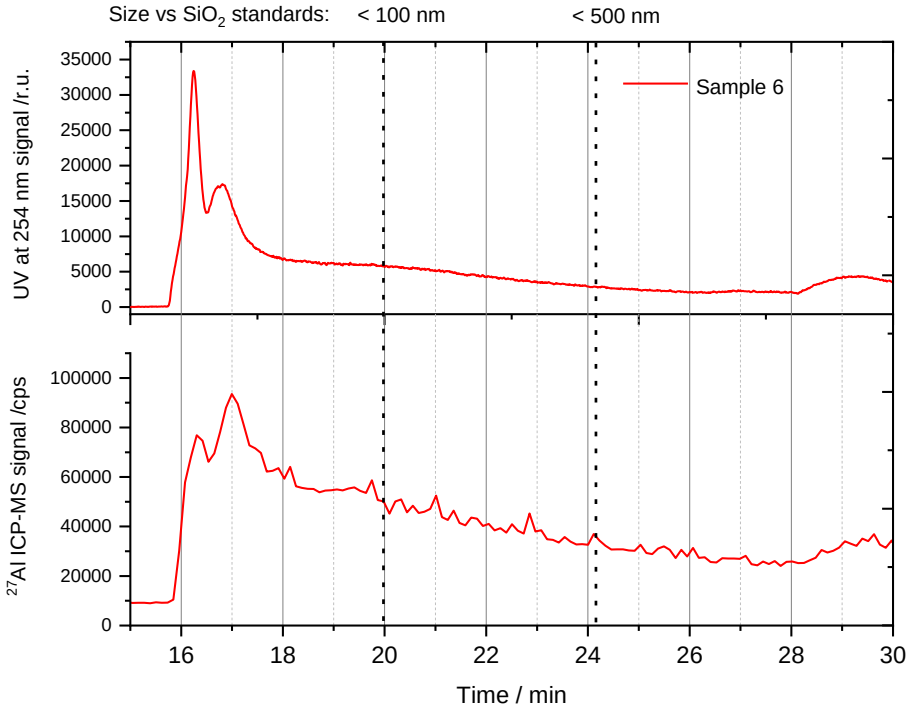

Figure D.3.3. UV-Vis at 254 nm and ^{27}Al ICP-MS fractograms of sample 6 (natural water). Dash lines correspond to size calibration vs. SiO_2 size standards. Injected volume: 1 mL.

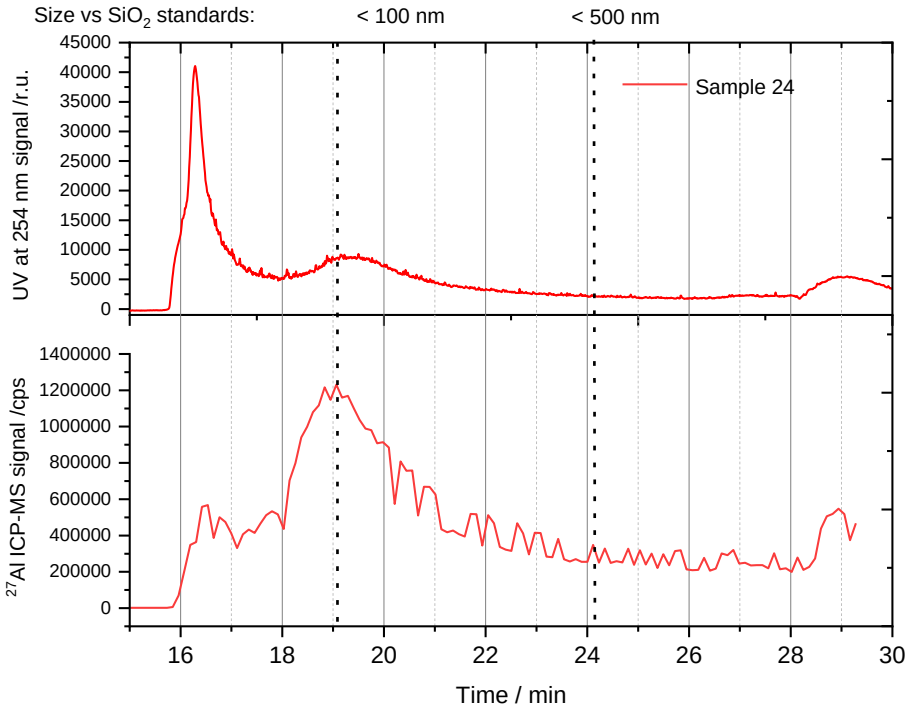

Figure D.3.4. UV-Vis at 254 nm and ^{27}Al ICP-MS fractograms of sample 24 (natural water). Dash lines correspond to size calibration vs. SiO_2 size standards. Injected volume: 1 mL.

UV-Vis at 254 nm fractograms show two peaks, one eluted at the beginning of the fractogram, overlapped with the void peak (t_r=16.2 min), and a second peak eluted at different times for sample 6 (t_r =16.8 min) and 24 (t_r =19.3 min). ICP-MS ^{27}Al isotope signal shows a profile with a maximum overlapped with this second peak. Channel calibration vs. SiO_2 standards show that these peaks may correspond to species with an equivalent size lower or close to 100 nm, although the Al signal covers the entire range calibrated, up to 500 nm and beyond. Given that the inversion point was stablished around 0.7 µm (see previous section D.3.1.2.1), it is possible that some of these species were eluted in steric mode, with sizes larger than 500 nm. To confirm that, samples were filtrated through a 0.45 µm pore size membrane. Results obtained for sample 6 are shown in Figure D.3.5.

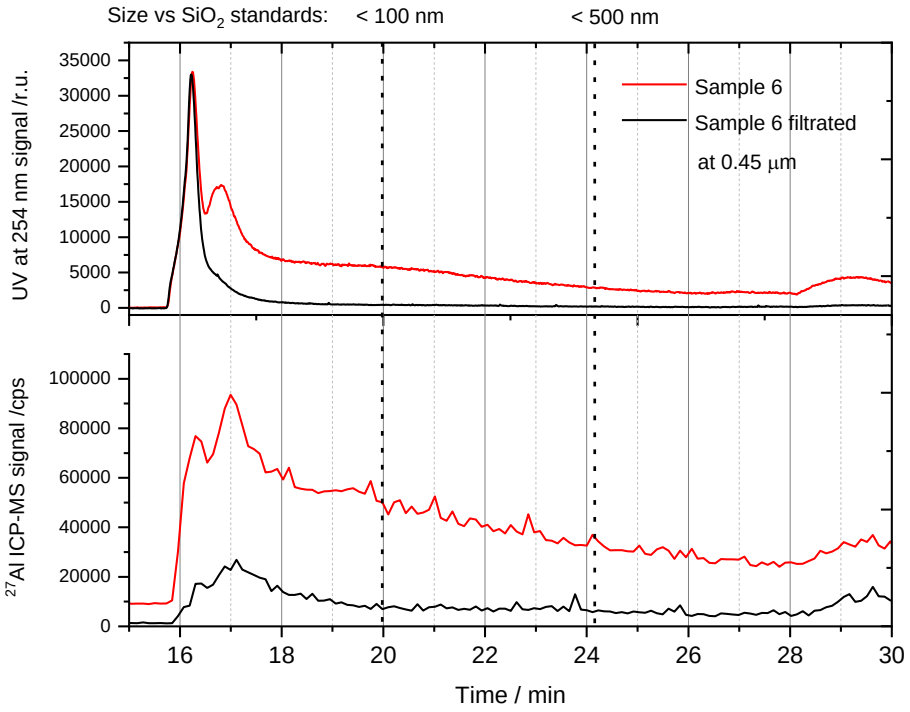

Figure D.3.5. UV-Vis at 254 nm and ^{27}Al ICP-MS fractograms of sample 6 (natural water) without treatment (red line) and after filtration at 0.45 µm (black line). Dash lines correspond to size calibration vs. SiO_2 size standards. Injected volume: 1 mL.

It can be observed that the second peak in UV-Vis fractogram disappears, and Al signal diminishes, which suggest that most of the Al-containing species in this sample correspond to particles/colloids larger than approximately 0.45 µm. However, it is also possible that undesired interactions between some species of lower size with the filter membrane occur, so the presence of colloids <0.45 µm cannot be discarded.

Respect to the nature of these two fractions, additional information was obtained through the UV-Vis spectra measured at the two maxima of the fractogram. Figure D.3.6 shows UV-Vis spectra for sample 6 at t_r =16.2 min and t_r =16.8 min.

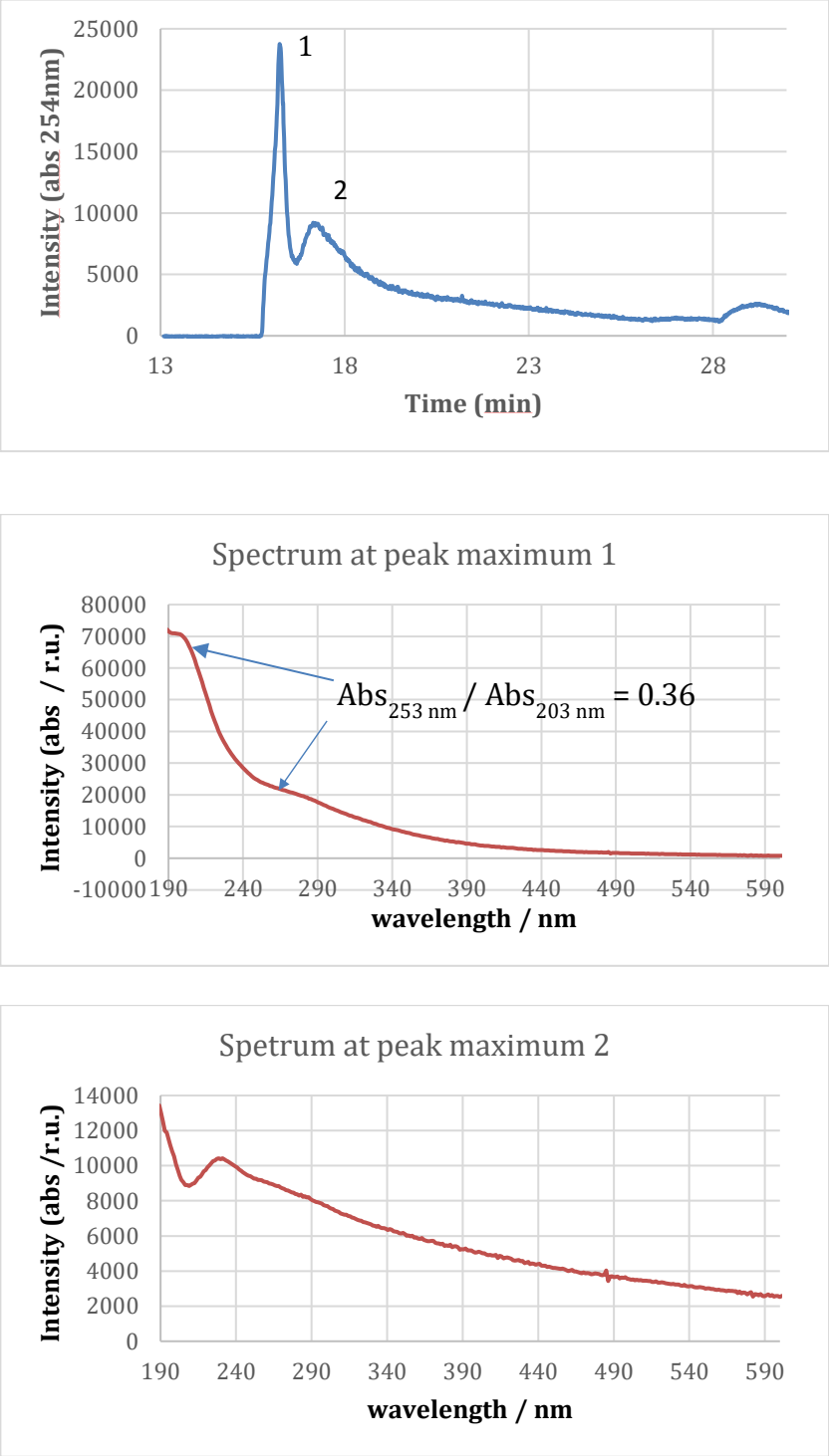

Figure D.3.6. UV-Vis at 254 nm fractogram of sample 6 (natural water) (blue line) and UV-Vis spectra (red line) at peak maxima 1 and 2. Injected volume: 1 mL.

It can be observed that, in the case of the first peak, a ratio $Abs_{253\ nm}$ / $Abs_{203\ nm}$ close to 0.4 (0.36) is obtained, which likely corresponds to natural organic matter (such as humic substances). This ratio has been widely used as an indicator for humic acid chemical structural changes [2] and indicates a variation in the proportion of polar groups linked to aromatic moieties. A low UV absorbance 253/203

nm ratio (<0.7) corresponds to substances of high molecular weights. Given the pore size of the membrane used in AF4 (5 kDa), only large molecules are eluted, so ratios < 0.7 are expected. On the other hand, the spectrum of the second peak shows a large dispersion component (proportional to the size, and more significant when dispersant size is similar or larger than the wavelength of the radiation), in agreement with the presence of a large fraction (likely larger than 0.45 µm in size). The chemical nature (Al signal) points out to an inorganic colloidal fraction present in natural waters, mainly as clays (aluminum silicates) or silica [1]. Although colloids of oxides and hydrous oxides of iron are also common in natural waters, no significant Fe signal was obtained in these samples, so its presence could not be confirmed.

The rest of the samples of natural waters (from 4 to 25) showed a similar profile, with a small colloidal fraction characterized by a small peak eluted at times larger than 16.5 min, with a significant Al signal, being much smaller than that shown by samples 6 or 24. For this reason, samples 6 and 24 were selected as a model of a natural water with a high colloidal fraction. Although Al was not quantified, the areas of the peaks in the fractograms were measured and compared. Since all the samples were measured under the same conditions with similar sensitivity, direct comparison was possible as an approach. Samples from WWTPs and from effluents of hospitals and slaughterhouses showed a large first peak (UV-Vis at 254 nm fractograms of samples 31 and 32 are shown in Figure D.3.7 compared to fractogram of sample 24), which corresponds to a large organic matter fraction. These samples were selected in the next section as a model to study the antibiotic interactions with the organic matter in waters.

D.3.2. Interaction of antibiotics with the colloidal fraction in samples. Studies of enrofloxacin as antibiotic model.

Enrofloxacin was selected as model for interaction studies with the colloidal fraction because of its abundance in the samples analyzed (as discussed along section D.1.), its solubility in water and its absorbance maximum at 270 nm, which allows its quantification by UV-Vis molecular absorbance spectroscopy. The amount of enrofloxacin from the stock solution added to the samples was calculated so a final concentration of 500 µg L^{-1} was obtained. Although this concentration is quite high respect to the concentrations found in the natural waters analyzed, that concentration gives enough absorbance signal variations in the samples respect to uncertainties associated to the methodology used to obtain significant results. The use of lower concentrations, such as 200 µg L^{-1}, was also tested, with no significant variation on results, although uncertainties increased. As previously discussed, sample 24 was selected as model of a natural water with a high colloidal fraction to study potential interactions with antibiotics under real conditions.

These interaction studies were carried out following the procedure described in C.4.4. Basically, enrofloxacin was added to the samples, allow to interact for 24 h, and subsequent separation of the remaining free enrofloxacin from the enrofloxacin associated to the colloidal fraction by ultrafiltration.

Firstly, the recovery of enrofloxacin in the ultrafiltration membranes used along the interaction studies was calculated following the procedure described in C.4.4.2.2. A value close to 100% (92 ± 3%) was obtained. This result confirms the low interaction of the enrofloxacin with the membrane material selected for colloidal fraction retention. In any case, enrofloxacin concentration found in the ultrafiltrates was corrected in further studies with this value.

As previously stated, only samples 6 and 24 (natural river waters) were selected, as samples with a significant colloidal fraction, considering the aluminum signal observed in the fractograms (Figures D.3.3 and D.3.4) respect to the rest of samples analyzed (larger in sample 24).
In the case of sample 6 no significant interaction was observed, whereas sample 24 showed a significant interaction, with a 67 ± 1% (see Table D.3.3) of the total enrofloxacin added associated to the fraction retained in the ultrafiltrate, which suggest a potential interaction of the enrofloxacin with the colloidal fraction in sample 24, much larger than in sample 6.

The effect of contact time of enrofloxacin with the sample was also studied. Two different conditions were used: 1) sample was ultrafiltrated immediately after the addition of enrofloxacin, so no contact time was considered, and 2) ultrafiltration after 24 h, being the rest of conditions the same as previously described. Results (Table D.3.3) shown that there is a significant variation on the percentage of enrofloxacin retained depending on the time enrofloxacin is allowed to interact with the sample components (no time or 24 h).

Table D.3.3. Percentage of enrofloxacin retained in ultrafiltration filters of 5 kDa and associated to the retained fraction under different contact time with sample 24. Calculations based on Abs at 270 nm, following the procedure described in the experimental section.

Time / h	% enrofloxacin retained (associated to colloidal fraction)
0 h	30 ± 8 %
24 h	67 ± 1 %

Therefore, the interaction of enrofloxacin with colloids in natural waters is a dynamic process, so it is expected that it reaches a steady state after it is release into a natural system, being associated to large fractions detected in this kind of samples (natural waters with a relatively high colloidal fraction).

D.3.2.1. Interaction of antibiotics in samples with relatively high natural organic matter content.

Samples studied so far show two defined fractions, one constituted mainly by natural organic matter (NOM), and another one formed by colloids of inorganic nature, characterized by their elemental composition (Al signal in ICP-MS), different elution times (longer time and subsequently larger sizes) and UV-Vis spectra (dominated by the dispersion component). Antibiotic interaction studies in these samples are expected to be driven by the colloidal fraction, minimizing the potential influence of the organic matter fraction.
The evaluation of antibiotic interactions (enrofloxacin) with organic matter was carried out with WWTP1 waters (samples 31 and 32, at the entrance and the exit of the plant respectively), since this is the predominant component in this kind of samples, as can be seen in Figure D.3.7. When compared to samples of natural waters (such as sample 24), it is evident that the organic matter content is much larger at the entrance of the treatment plant than at the exit, where this component is similar to that observed in the natural water (even smaller if a direct relationship between absorption at 254 nm and organic matter concentration is assumed and no other factors are considered).

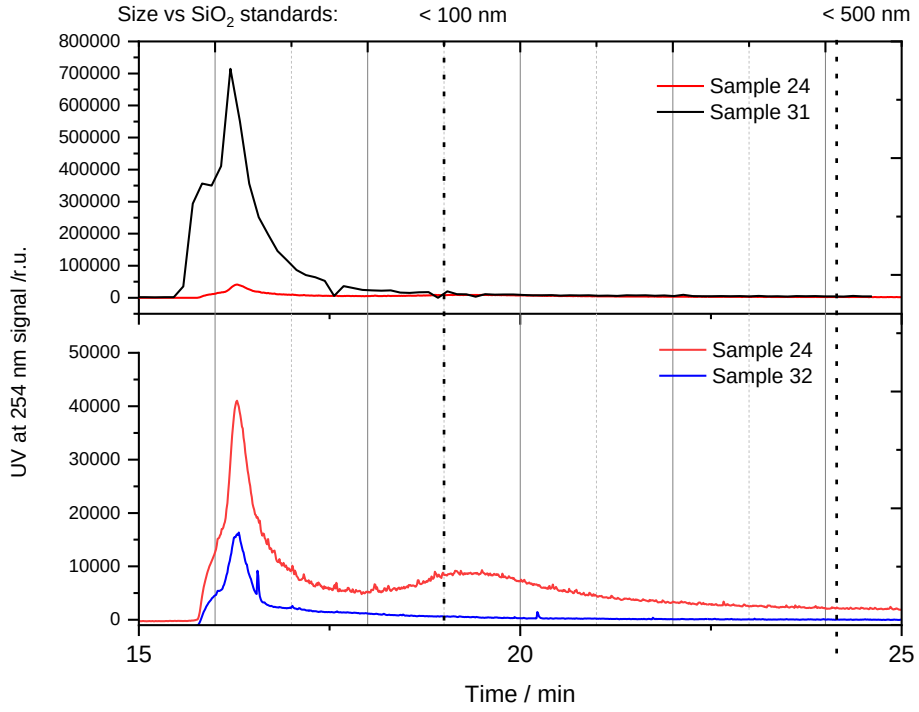

Figure D.3.7. UV-Vis at 254 nm of sample 24 (natural water, red line) and sample 31 (WWTP1-IN, black line) and 32 (WWTP1-OUT, blue line). Dash lines correspond to size calibration vs. SiO_2 size standards. Injected volume: 1 mL.

The quantification of enrofloxacin along these studies were made following the procedure optimized and described in C.4.2.2, based on LC-HR-MS/MS. Enrofloxacin addition was set so final added concentration was 100 µg L^{-1}. Enrofloxacin recoveries higher than 90% were achieved following this procedure. Two different sample pretreatments based on filtration through 0.45 µm membrane were followed to isolate the interaction with the organic matter fraction. In one set of experiments, addition of enrofloxacin was made before filtration, so large fractions in the sample (which could coelute with the organic matter in AF4) were considered. In the second set, addition was made after filtration, so only the organic matter could interact with the enrofloxacin added. Separation of free enrofloxacin was made by tangential ultrafiltration under the conditions described in C.4.4.2.2. Results are shown in Table D.3.4.

Table D.3.4. Percentage of enrofloxacin retained in ultrafiltration filters of 5 kDa and associated to the retained fraction (colloids + organic matter or organic matter) in two different samples (at the entrance and exit of a WWTP2). Calculations based on quantification by LC-MS/MS, following the procedure described in the experimental section.

Sample		% enrofloxacin retained
WWTP1 IN	All fractions	8 ± 5 %
WWTP1 OUT	"	2 ± 9 %
WWTP1 IN	Only organic matter	10 ± 4 %
WWTP1 OUT	"	2 ± 2 %

The large uncertainty obtained, together with the relatively small percentage of enrofloxacin retained, make differences observed not statistically significant. In any case, it seems that organic matter does not interact significantly with enrofloxacin, neither the colloidal fraction (if any) in the samples studied.

D.3.2.2. Interaction of antibiotics with natural aluminium silicate (kaolin) added to samples with low natural organic matter content.

Given the differences observed between sample 24 (a natural water with a significant colloidal fraction of inorganic nature eluted at 19-20 min (see figure D.3.4) and sample 32 (WWTP1 OUT with only one fraction detected of organic matter eluted at the beginning of the fractogram (at 16.3 min) (Figure D.3.7)) on interaction with enrofloxacin, an addition of a colloidal natural aluminosilicate (kaolin) to sample 32 was made to confirm that inorganic colloids are responsible of the interactions observed along the previous studies. A suspension of the fraction < 1µm isolated (see section C.2 of chemicals for details) from a natural kaolin was added to the sample 32 at three levels of concentration: low (5 mg L^{-1}), medium (45 mg L^{-1}) and high (337 mg L^{-1}). Since no significant interaction was observed between the organic matter present in sample 32 and enrofloxacin (Table D.3.4), it is assumed that the fraction of enrofloxacin retained along this experiment is associated to the aluminosilicate added. Results are shown in Table D.3.5.

Table D.3.5. Percentage of enrofloxacin retained in ultrafiltration filters of 5 kDa and associated to the retained fraction as a function of the concentration of natural kaolin added. Calculations based on Abs at 270 nm, following the procedure described in the experimental section.

Sample 32 Concentration of kaolin added / mg/L	% enrofloxacin retained (associated to colloidal fraction)
5	35 ± 3 %
45	43 ± 3 %
337	54 ± 3 %

These results confirm that there is an interaction between enrofloxacin and the retained fraction, which corresponds to the natural aluminosilicate added. In addition, the percentage retained increases as long as the concentration of kaolin added is higher. Although a slight variation on pH and conductivity was observed after the addition of the kaolin (from 7.6 at 5 mg L^{-1} to 7.8 at 337 mg L^{-1}; and from 493 µS cm^{-1} at 5 mg L^{-1} to 533 µS cm^{-1} at 337 mg L^{-1}) which may change the net charges of the species involved and the kind of interactions stablished, the systematic increment observed points out a potential interactions with enrofloxacin.

D.3.3. Conclusions

The colloidal fraction from different samples of natural river waters and WWTPs have been characterized by AF4-UV-Vis-ICP-MS. Separation conditions used allowed the separation of this fraction from natural organic matter also present in the samples. Colloidal fractions detected were larger than 100 nm in size (vs. SiO_2 standards), characterized by a UV-Vis spectrum with a large light dispersion component and Al signal in ICP-MS.

To study the interactions between antibiotics and colloids in water samples, a methodology based on the addition of enrofloxacin, used as a model, and subsequent separation of the non-associated enrofloxacin by tangential ultrafiltration is proposed. Given the weak nature of these interactions, mild separation conditions were used to avoid altering equilibria as much as possible.

These studies have revealed that natural waters samples with a large colloidal fraction (high Al ICP-MS signal) showed significant interactions (up to 67% of enrofloxacin added remained associated to the colloidal fraction). The addition of a natural aluminosilicate, such as kaolin (a natural nanoclay) to a sample with no colloids also produced interactions, more significant at higher nanoclay concentration.

On the contrary, in samples with a large organic matter fraction, such as the samples at the entrance of WWTP (sample 31), no significant interactions were observed.

References

1. Particulate matter and Aquatic contaminants. Salem S. Rao (Ed.) Lewis Publishers (1993)
2. Reiller P, Amekraz B, Moulin C. Sorption of Aldrich humic acid onto hematite: Insights into fractionation phenomena by electrospray ionization with quadrupole time-of-flight mass spectrometry. Environ Sci Technol. (2006), 40(7):2235–41.

D.4. Single and combined effects of enrofloxacin and polystyrene nanoplastics on the life histories and microbiome of Daphnia magna

The aim of this part of the project was to study the single and combined effects of polystyrene NPTs and enrofloxacin on selected life history traits of *D. magna* as well as the metabolic and taxonomic diversity of bacterial community in their intestinal tract. The questions to be answered included

- does the presence each of the stressors result in decreasing body size and reproductive parameters of *Daphnia*, is the effect of enrofloxacin different in the presence and absence of NPTs?
- does the presence of NPTs result in increase and the presence of enrofloxacin results in decrease the metabolic rate of the gut microbiota of *Daphnia*, is there an interaction in the effects of both stressors?
- is the metabolic fingerprint (measured as the relative use of different carbon sources) different in the presence of each of the stressors alone and combined?
- does the stressors affect the taxonomic diversity in the gut microbiota of *Daphnia*, is there an interaction in the effect of both of them?

D.4.1. The effect of the stressors on the life history parameters

The significant main effect on body length was only found for enrofloxacin and its interaction with NPTs (Table D.4.1). The effect of enrofloxacin was negative and was apparent at its high concentration in relation to the control and in the presence of low density of NPTs (*post-hoc*, Table S1, Supplementary Material, Fig. D.4.1). The effect of NPTs was also negative and was apparent: (1) at low and medium density treatment in the presence of low concentration of enrofloxacin, and (2) between its medium and low density treatments in the presence of high enrofloxacin concentrations (*post-hoc*, Table S1, Supplementary Material, Fig. D.4.1.). The presence of NPTs resulted in increasing the negative effect of enrofloxacin (Table D.4.1, Fig. D.4.1).

Table D.4.1. The main effects of the two-way between-subjects ANOVA for both stressors PS- NPs, enrofloxacin and their interaction on the life history parameters of *Daphnia*. Statistically significant differences are marked with bold.

	Body length	Body volume	Clutch size	Egg volume	Clutch volume
Factor or interaction	$P, F_{df,\ Error\ df}$	$P, F_{df,\ Error\ df}$	$P, F_{df,\ Error\ df}$	$P, F_{df,\ Error\ df}$	$P, F_{df,\ Error\ df}$
Np	0.060 $2.47_{3,\ 2195}$	0.057 $2.47_{3,\ 2195}$	**< 0.001 $20.4_{3,\ 1753}$**	**< 0.001 $18.1_{3,\ 2519}$**	**< 0.001 $46.0_{3,\ 1361}$**
Enr.	**0.002, $6.37_{2,\ 2195}$**	**< 0.001 $10.3_{2,\ 2188}$**	**< 0.001 $21.4_{2,\ 1753}$**	**< 0.001 $32.5_{2,\ 2519}$**	**< 0.001 $71.3_{2,\ 1361}$**
Np × Enr.	**0.001, $5.92_{6,\ 2195}$**	**< 0.001 $4.18_{6,\ 2188}$**	**< 0.001 $6.23_{6,\ 1753}$**	**< 0.001 $4.29_{6,\ 2519}$**	**< 0.001 $10.4_{6,\ 1361}$**

The significant main effect on body volume was only found for enrofloxacin and its interaction with NPTs (Table D.4.1). The effect of enrofloxacin was negative and was apparent at its high concentration in relation to the control and in the presence of low density of NPTs (*post-hoc*, Table S2, Supplementary Material, Fig. D.4.1). The effect of NPTs was also negative and was apparent at their low and medium densities at low enrofloxacin concentration (*post-hoc*, Table S2, Supplementary Material, Fig. D.41.). The presence of NPsT resulted in increasing the negative effect of enrofloxacin (Table D.4.2, Fig. D.4.1).

The clutch size was affected by NPTs and ENR and there was an interaction between the stressors (Table D.4.1). The effect of enrofloxacin was negative and was apparent at both concentrations in relation to the control (*post-hoc*, Table S3, Supplementary Material, Fig. D.4.1). The effect of NPTs was

also negative and was apparent at each of the three densities in relation to the control and between their medium and low density in the presence of low enrofloxacin concentration (*post-hoc*, Table S3, Supplementary Material, Fig. D.4.1). However, the combined effect of the stressors was only slightly greater than each of the stressors alone (Table D.4.1, Fig. D.4.3c).

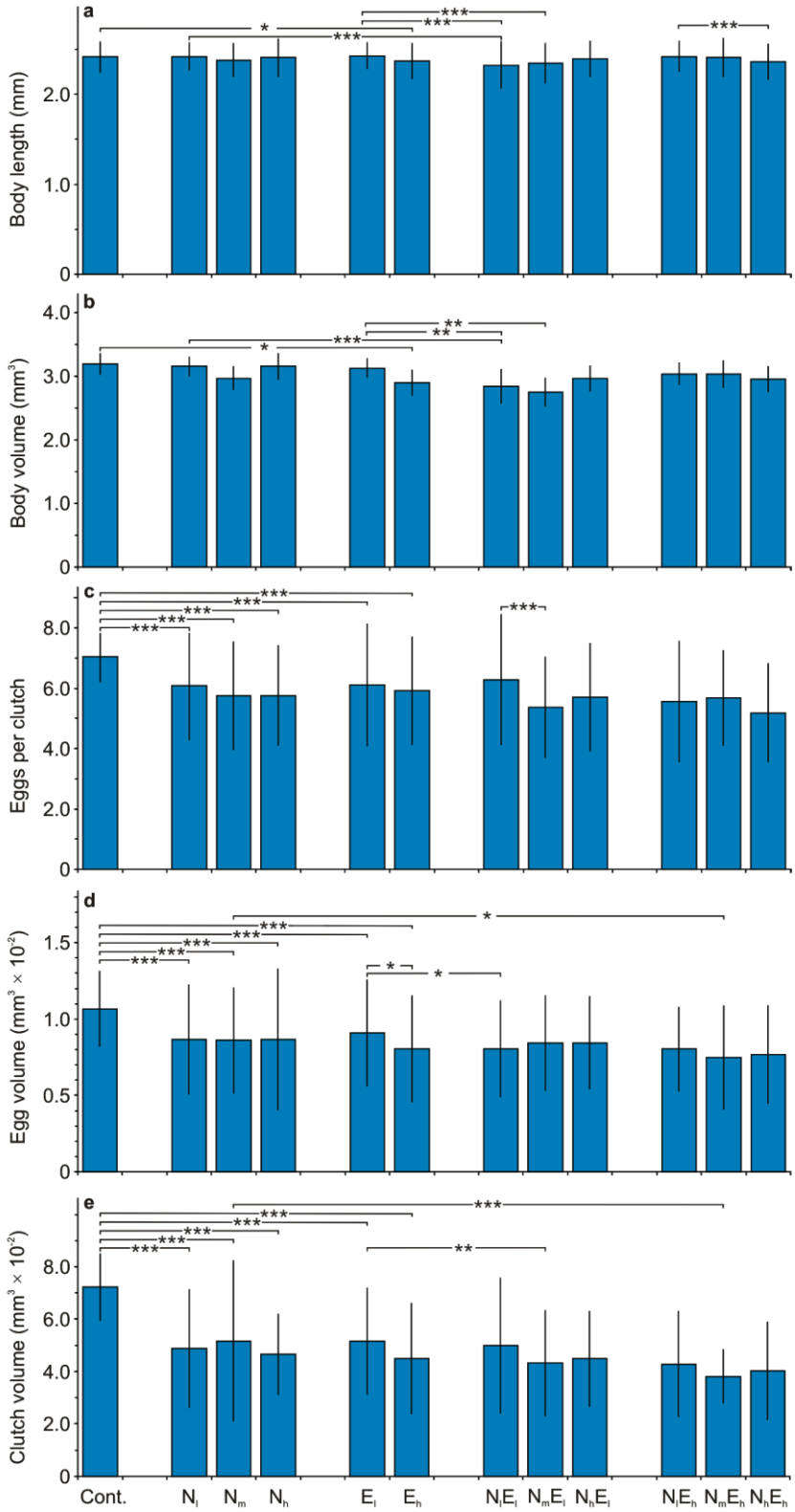

Figure D.4.1. Mean value (± 1SD) of: a) body length, b) body volume, c) clutch size (the number of eggs per ovigerous female), d) egg volume, and e) clutch volume (the currency of the total investment for reproduction) of 5 day old *D. magna* in each of the 12 variants of the combination of: (1) zero, low, medium and high density of polystyrene NPs (N_l = 10^3, N_m = 10^6, and N_h = 10^9 particles L^{-1}, respectively) and (2) zero, low and high concentration of enrofloxacin (E_l = 10 and E_h = 100 ng L^{-1}, respectively). Statistical significance is accepted at $*p < 0.05$, $**p < 0.005$ or $***p < 0.0005$.

The egg volume was affected by both NPTs and ENR and there was an interaction between the stressors (Table D.4.1). The effect of enrofloxacin was negative and was apparent: (1) at both concentrations in relation to the control, (2) at high concentration in the presence of the medium density of NPTs, and (3) between the treatments with low and high concentration of enrofloxacin (*post-hoc*, Table S4, Supplementary Material, Fig. D.4.1). The effect of NPTs was also negative and was apparent at each of the three densities in relation to the control and at its low density in the presence of low enrofloxacin concentration (*post-hoc*, Table S4, Supplementary Material, Fig. D.4.1). However, the combined effect of the stressors was only slightly greater than each of the stressors alone (Table D.4.1, Fig. D.4.3d).

The clutch volume as the common currency of the reproductive investment was affected by both NPTs and ENR and there was an interaction between the stressors (Table D.4.1). The effect of enrofloxacin was negative and was apparent at both concentrations in relation to the control and at high concentration in the presence of the medium density of NPTs (*post-hoc*, Table S5, Supplementary Material, Fig. D.4.1). The effect of NPTs was also negative and was apparent at each of the three densities in relation to the control and at its medium density in the presence of low enrofloxacin concentration (*post-hoc*, Table S5, Supplementary Material, Fig. D.4.1). However, the combined effect of the stressors was only slightly greater than each of the stressors alone (Table D.4.1, Fig. D.4.3e).

The results confirmed our hypothesis as the presence of both stressors resulted in decreasing of *Daphnia* body size and their reproductive parameters, including an average egg volume in the brood cavity and the number of eggs in the clutch of individuals during the first reproduction, which in turn also resulted in decreasing the clutch volume being a common currency of the reproductive investment.

In the case of NPTs, these results may have been caused by clogging of the filtration appendages and the gut, what in turn results in decreasing filtration and assimilation rate, which were observed in several earlier studies (e.g. [1]). These findings are consistent with a great number of earlier studies in which the presence of NPs suspended in water alters life history traits of various animals, and that these alterations are manifold [2], including a reduction in the body size of adult and juvenile individuals, reproduction (i.e. decreased numbers and body size of neonates), individual growth rate and survival of freshwater zooplankton (e.g. [3]), saline lake zooplankton (e.g. [4]), cnidarians [5], and fishes (e.g. [6-7]) although other studies did not find any effect of acute exposure on life history parameters, including negligible effects on the survival rate and development of *Danio rerio* (e.g. [8]), survival and individual growth rate of *Gammarus pulex* [9].

In the case of enrofloxacin, the decrease in all measured life history parameters is consistent with numerous earlier studies performed on fish [7-10], and *Daphnia* [11]. For example, several studies revealed that parental exposure of marine fish (*Oryzias melastigma*) to dietary sulfamethazine (4.62 mg × g^{-1}) may negatively affect the growth performance in adults [7-10]. Another study revealed the negative effect of tetracycline (1 μg × L^{-1}) on reproduction and survival of *D. magna* [11]. However, it is should be pointed out that not all studies found the effect of antibiotics on life history parameters.

For example, Nunes et al. [12] showed that ecologically relevant ciprofloxacin concentrations (0.005-0.195 mg × L^{-1}), did not cause significant impacts concerning individual growth rate and reproductive parameters of *D. magna*, and Ma et al. [13] did not find the effect of tetracycline (10 mg × L^{-1}) on the growth rate of soil annelid *Enchytraeus crypticus* (e.g. [13]). Moreover, in a single study, it has been revealed that at very low ciprofloxacin concentrations (10 μg L^{-1}), that is at lower concentration used in our study, the growth, and fecundity of *D. magna* were even higher than in control animals [14].

In our study, the negative effect of enrofloxacin on the body size was stronger and on the reproductive parameters was weaker in the presence of NPTs, which also confirmed our first hypothesis and may suggest that *Daphnia* in the presence of cummulative stress redirect more resources to reproduction at the expense of somatic growth. We are aware of only two earlier studies in which the combined effect of NPs and antibiotics on the life history parameters was determined [7,13]. While the first study revealed that the effect of tetracycline on the dry weight of *E. crypticus* after 7 days exposure was stronger in the presence than absence of polystyrene [13], the second study revealed that the adverse impact of the mixture of polystyrene NPs and sulfamethazine on the dry weight in the *O. melastigma* was more modest than that of NPTs alone [7].

D.4.2. Metabolomic diversity of gut microbiota

Mean respiration rate of gut microbiota expresed as V_{max} values was greater in the presence of NPTs and was lower in the presence of enrofloxacin in relation to the control (Table D.4.2, Fig. D.4.2). The effect of NPTs was significant: (1) in its low and medium density in relation to the control, (2) in its low and medium density in the presence of low concentration of enrofloxacin, and (3) between its high and low density in high enrofloxacin concentration (Table S6, Supplementary Material, Fig. D.4.2). The effect of enrofloxacin was significant: (1) in its low and high concentrations in low density of NPs, (2) in its low and high concentrations in medium density of NPTs, (3) in its high concentration in high density of NPTs, (4) and between the treatment with its high and low concentrations in high density of NPTs (Table S6, Supplementary Material, Fig. D.4.2). There was a significant interaction between the stressors (Table D.4.2), which was apparent in greater effect of enrofloxacin in the presence than absence of NPTs (Table S6, Supplementary Material, Fig. D.4.

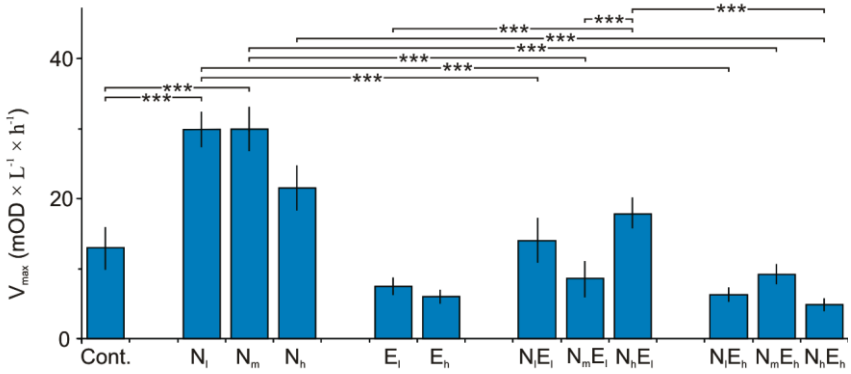

Fig. D.4.2. Mean respiration rate expresed as V_{max} values of 31 carbon sources by *Daphnia* gut microbiota from the control variant (Cont.), and from variants of the combination of two variables: (1) NPTs in low, medium and high concentration (N_l, N_m, N_h, respectively) and (2) enrofloxacin in low and high concentration (E_l and E_h, respectively). Bars show standard errors (SE).

Table D.4.2. The main effects of the two-way between-subjects ANOVA for both stressors PS- NPs, enrofloxacin and their interaction on the respiration rate of gut microbiota of *Daphnia* expressed as V_{max} values. Statistically significant differences effects are marked in bold.

Factor	*df*	*Df. res*	*F*-value	*Pr*(>*F*)
PS	**3**	**360**	**16.062**	**<0.0001**
ENR	**2**	**360**	**81.844**	**<0.0001**
PS × ENR	**6**	**360**	**13.375**	**<0.0001**

The analysis of the percentage share of respiration rate of different carbon sources by the gut microbiota of *Daphnia* revealed that the presence of each of the stressors resulted in relative increase of the usage of carboxylic acids and amino acids and in relative decrease of the usage of phosphorylated carbons and complex carbon sources in relation to the control (Fig. D.4.3).

Bray–Curtis-based NMDS analysis revealed the two distinct groups of variants that is, variants with high concentration of enrofloxacin (in the presence and absence of NPTs) and variants with low and medium density of NPTs, which suggests different effect of each of the stressors on the metabolic profile of the gut microbial community (Fig. D.4.4). In the first group apparent was a relatively low usage of complex carbon sources, phosphorylated carbon and amines in relation to the majority of remaining variants (Fig. D.4.3). In the second group apparent was a relatively even usage of different carbon sources with a relatively low usage of complex carbon sources and a relatively high usage of amines in relation to the majority of remaining variants (Fig. D.4.3).

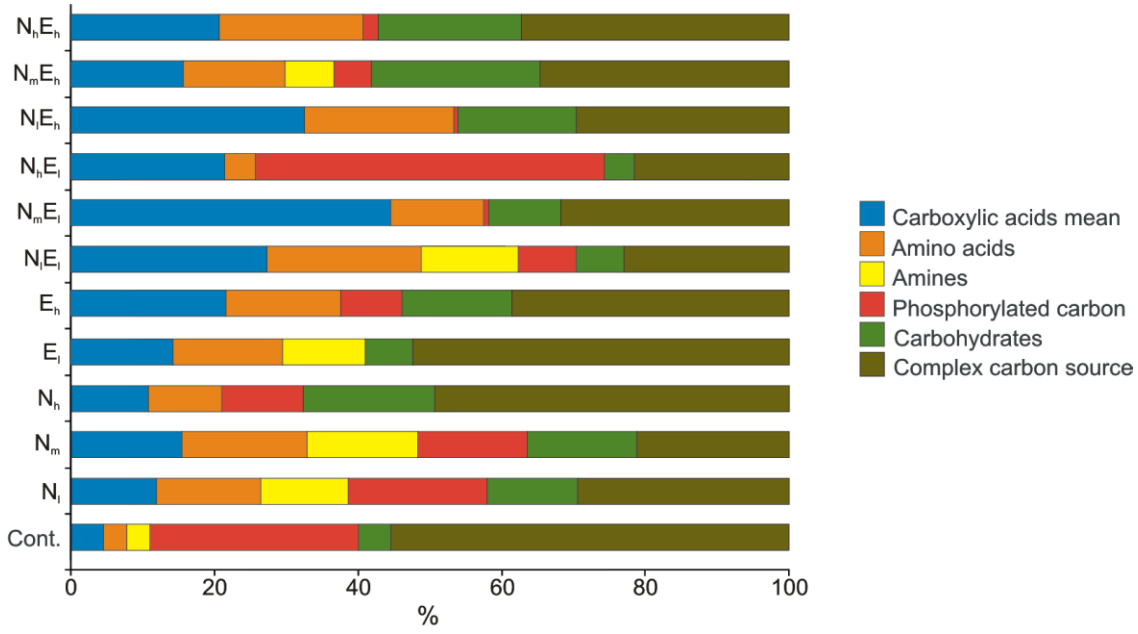

Figure D.4.3. Percentage share of respiration rate (V_{max}) of different carbon sources by the gut microbiota of *Daphnia* from the control variant (Cont.), and from variants of the combination of two variables: (1) NPTs in low, medium and high concentration (N_l, N_m, N_h, respectively) and (2) enrofloxacin in low and high concentration (E_l and E_h, respectively).

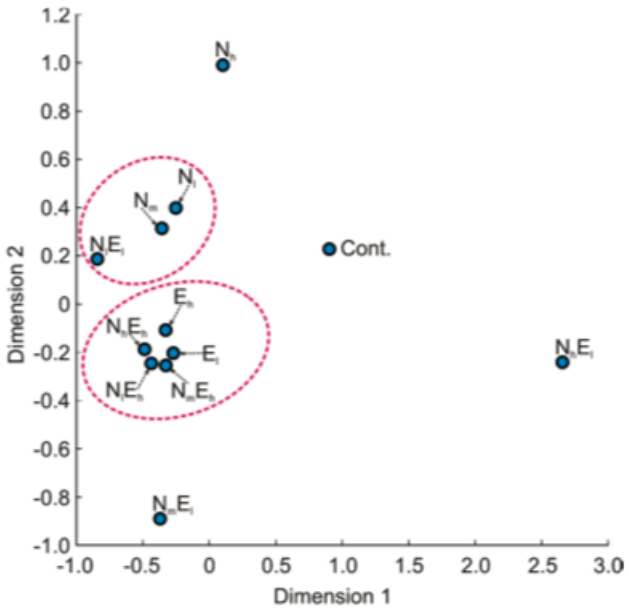

Figure. D.4.4. Bray–Curtis-based NMDS analysis of the relative respiration of 31 different carbon sources by *D. magna* gut microbiota from the control (Cont.), and from variants of the combination of two variables: (1) NPTs in low, medium and high concentration (N_l, N_m, N_h, respectively) and (2) enrofloxacin in low and high concentration (E_l and E_h, respectively).

The results also confirmed our second hypothesis since the presence of NPs results in increase and the presence of enrofloxacin results in decrease of the metabolic rate of the gut microbiota of *Daphnia,* what may be the result of both decreased abundance and modified functional metabolic fingerprint of bacteria. The decrease of the metabolic rate in the presence of enrofloxacin agrees with existing data on the effect of antibiotics on bacteria metabolism, since there is identified link between antibiotic-induced cellular respiration and bactericidal lethality. Antibiotics perturb the metabolic state of bacteria and the metabolic state of bacteria impacts antibiotic efficacy [15]. The results also confirmed our second hypothesis concerning interaction in the effect of both stressors. Our results are of a great interest since in the literature there is lack of data on the presence of NPTs and joint effect of the presence of antibiotics and NPTs on the metabolic rate of the gut microbiota.

The results also confirmed our third hypothesis as the presence of enrofloxacin affected metabolic fingerprint measured as the relative use of different carbon sources in relation to the control. That is, the functional structure of the bacteria had changed. The presence of each of the stressors resulted in relative increase of the usage of carboxylic acids and amino acids and in relative decrease of the usage of phosphorylated carbons and complex carbon sources in relation to the control, what suggest that both stressors forced for the use of easily digestible carbon sources [16]. Moreover, each of the stressors had specific fingerprint on the daphnia's gut microbial community, what suggest that the modifications caused by each of the stressors were different. This result is in accordance with the present literature for marine fish as He et al. [7] has shown that gut microbiota in medaka in the presence of sulfamethazine (SMZ) changes its function in such a way that carbohydrate metabolism was significantly decreased. Similar results have been shown by Zhang et al. [17], who also revealed that carbohydrate metabolism was weakened in the low concentration of SMZ (0.5 mg × g^{-1}

provided in fish food) treated females of marine medaka, but also showed that lipid and amino acids metabolism were enhanced. In contrary, in the murine model other antibiotic – amoxicillin has been shown to elevate expression of carbohydrates utilization genes in the form of starch in *Bacteroides thetaiotaomicron* [18].

The results also confirmed our third hypothesis in case of NPs as their presence affected metabolic fingerprint measured as the relative use of different carbon sources in relation to the control. The presence of polystyrene NPTs on carbohydrate metabolism by gut microbiota of marine medaka fish was already shown to be significantly altered [7]. However, Zhang et al. [17] did not show any significant effect of the presence of NPTs on the predicted metabolic pathways of the gut microbiota in the same fish species. A negative picture of the impact of NPTs on metabolic fingerprint emerges from research on freshwater biofilm done with Biolog EcoPlate method, which revealed that microbial metabolic functional diversity was reduced in the presence of NPTs (1, 5 and 10 mg × L^{-1}). The total carbon metabolic functions remained constant with elevated NP concentrations, but some specific carbon sources (*e.g*. esters) changed in their utilization ability [19]. Moreover, the interaction again was also apparent in different metabolic fingerprint in the presence of each of the stressors alone and combined, what suggest existence of the interaction between the two stressors. The simultaneous presence of SMZ and NPs also significantly altered carbohydrate metabolism as revealed by He et al. [7]. However, it should be pointed out, that the results obtained by He et al. [7] and Zhang et al. [13] concerning the effects of antibiotics and NPTs on change in gut microbial metabolism fingerprint were obtained by the bio-informatics prediction of the function of gut microbiota basing on the SILVA database. Our results come from direct measurement of microbial metabolic fingerprint by Biolog EcoPlate method.

D.4.3. Taxonomic diversity of gut microbiota

Taxonomic diversity of the gut microbiota was rather small, mainly composing of the 3 taxa at the phylum level, that is *Achinobacteriota*, *Fimicutes* and *Proteobacteria* with an apparent predominance of the last two taxa (Fig. D.4.5). The presence of each of the stressors resulted in relative increase of *Firmicutes* over the remaining taxa in all of the variants in relation to the control (Fig. D.4.5). This effect was also apparent with increasing of the relative abundance of *Firmicutes* with increasing concentrations of NPs and enrofloxacin (Fig. D.4.5). Despite the apparent effect of both stressors alone on the relative taxonomic abundance, the effect was not strengthned in the variants with both stressors combined (Fig. D.4.5). NMDS of the values of Bray–Curtis index did not show any apparent group of similar variants (Fig. D.4.6). However, it revealed that the variants with the combined effect of both stressors and with high concentrations of each of the stressors alone are further away from the control than the other variants (*i.e*. with low concentrations of each of the stressors alone and combined).

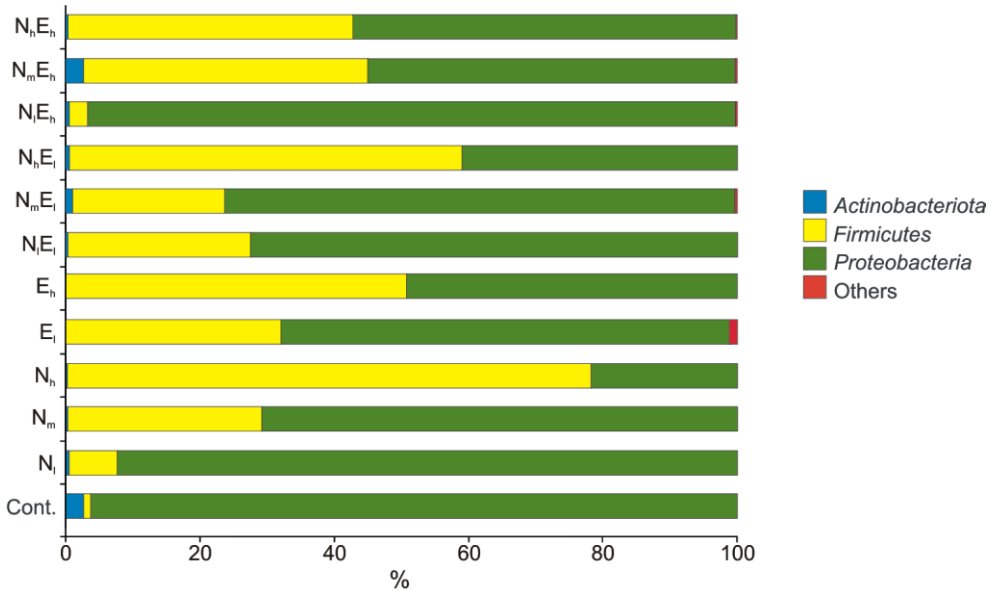

Figure. D.4.5. The relative abundances of the top 3 dominant bacteria taxa at the phylum level present in the gut of *D. magna* from the control variant (Cont.), and from variants of the combination of two variables: (1) NPTs in low, medium and high concentration (N_l, N_m, N_h, respectively) and (2) enrofloxacin in low and high concentration (E_l and E_h, respectively).

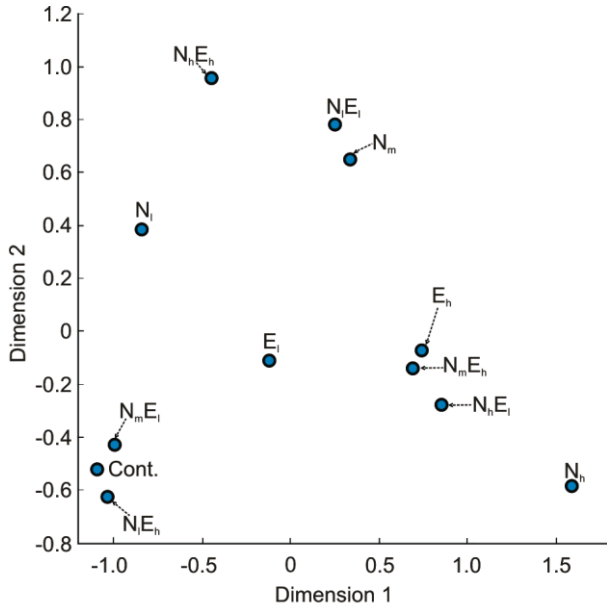

Figure D.4.6. The graphical results of the Bray-Curtis-based NMDS analysis of sequence data, binned by taxonomic assignment to family. The figure shows the relative distances between the gut microbiota of *D. magna* from control (Cont.) variant, and from variants of the combination of two variables: (1) NTPs in low, medium and high concentration (N_l, N_m, N_h, respectively) and (2) enrofloxacin in low and high concentration (E_l and E_h, respectively).

The taxonomic profile at the family level vs. EcoPlate data (Bray – Curtis matrices Mantel Correlation) showed permutation N = 9999, correlation R = 0.1531 with *P* = 0.2052. As the Mantel correlation was

not significant, it was concluded that no statistically significant correlation between taxonomic and metabolic profile existed.

Finally, the results also confirmed the fourth hypothesis, as the taxonomic diversity was affected by the presence of each of the two stressors alone, which was apparent in the relative increase of *Fimicutes* in relation to *Actinobacteriota* and *Proteobacteria* at the phylum level. Despite this, in all of the experimental variants the most dominant taxa at the phylum level was *Proteobacteria*, which is consistent with the previous studies on the microbiome of other animals, including soil fauna [13,20,21]. On the one hand, the relative increase of *Firmicutes* may have a positive effect on *Daphnia*, as it resulted in increasing the diversity of microbial composition, which is often equated with the improvement of the host's health [22]. Moreover, it has been revealed that *Firmicutes* produce short-chain fatty acids, which could be utilized for lipid or glucose *de novo* synthesis and an additional energy source for the host [7,23,24]. Therefore, from the perspective of host energy input, *Firmicutes* in the gut may play positive role. On the other hand, in other studies it has been revealed that increased proportion of *Firmicutes* to other bacteria phyla is an indicator of metabolic disorders in animals [25,26], which in turn, may explain the reduction in body size and reproductive potential of *Daphnia* by each of the stressors alone in our study.

In the case of NPTs, the relative increase of *Fimicutes* in the gut microbiota may be due to the fact that NPTs can provide them with a better matrix to growth in relation to other bacteria taxa. The results are consistent with several recent studies in which it was found that the presence of NPs may affect both a community of free living bacteria [27], and the microbiom of *E. crypticus* [13], a marin mollusk *Mytilus galloprovincialis* [28] and various fish species, including *O. melastigma* [29] and *D. rerio* [30,31], which may induce dysbiosis and inflammation in their intestine. For example, it has been revealed that the dietary NPs (1 mg × g^{-1}) affected the relative proportions of *Microbacteriaceae*, *Streptococcaceae*, *Enterobacteriaceae* and *Rhodocyclaceae* in the whole body microbial community of *E. crypticus* [13]. All of these taxa has been recognised as potentially negatively (in the case of *Enterobacteriaceae*; [32]) or positively (in the case of remaining taxa; [33,34]) affecting the host. However, our study is in contradiction to the results obtained by He et al. [7], who found a decrease in the relative abundance of *Firmicutes* in males exposed to (3.45 mg × g^{-1}) dietary polystyrene NPs. Additionally, it is worth attention that in our study the taxonomic diversity of bacteria increased in the presence of NPs, as the relative abundance of different taxa was more even compared to the controls, which is consistent with the earlier studies for the diversity of microbial communities in the gut of *D. rerio* (e.g. [30]).

The increase of the relative abundance of *Firmicutes* in the presence of enrofloxacin is consistent with several previous studies, which revealed that antibiotics may disturb the taxonomic diversity of gut microbiota of animals, including mouse [35] soil organisms [13,26], fish [13] and *Daphnia* [11]. For example, it has been revealed that tetracycline exposure (1 μg × L^{-1}) resulted in increase the relative abundance of *Pseudomonaceae* in intestinal microbial community of *D. magna* [11]. In other study it has been revealed that the dietary tetracycline (0.01 mg × g^{-1}) affected the relative proportions of *Microbacteriaceae*, *Streptococcaceae*, *Enterobacteriaceae* and *Rhodocyclaceae* in the whole body microbial community of *E. crypticus* [13].

Moreover, the fourth hypothesis was also confirmed as we identified the interaction between the two stressors on the community structure of bacteria, which was apparent in the lower effect of enrofloxacin alone than combined. We are aware of only the two earlier studies in which the combined effect of NPTs and antibiotics was investigated [7,13]. In the first study, it has been revealed that the combined exposure of tetracycline and polystyrene NPTs negatively affected the abundance of bacteria belonging to several families, including *Microbacteriaceae* and *Streptococcaceae*, and *Enterobacteriaceae* in the whole body microbiome of *E. crypticus*, and additionally, that higher ratios of *Planococcaceae* to *Chitinophagaceae* and *Bacillaceae* to *Chitinophagaceae* was observed in relation to the tratments with both stressors alone [13]. Moreover, it has been revealed that after terminating exposure, the microbiome was not permanently changed but reversibly impacted [13]. In the second study, it has been revealed that parental exposure of polystyrene NPTs and sulfamethazine had lower effect on the gut microbial communities in the offspring of marine fish (*O. melastigma*), that each of the stressors alone [7].

D.4.4. Conclusion

In conclusion, despite the growing interest in research into the effects of NPTs and antibiotics on the biology of organisms, knowledge on the subject is still fragmentary. Future studies concerning the issue should primarily focus on integrating the results from different levels of biological organisation on the combined effect of both stressors on different taxa. Our study seems to be the first one in which was investigated the combined effect of NPTs and antibiotics on the life history parameters of freshwater organisms, and metabolomic and taxonomic diversity of their intestinal microbial community.

The results of our work revealed the interactive effect of both stressors (NPTs and enrofloxacin) on all of the measured life history parameters of *D. magna* as well as all of metabolic and taxonomic diversity parameters of bacterial community in their intestinal tract, that is, the effect of enrofloxacin on these parameters was different in the presence and absence of NPTs.

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Table S1. The results of HSD *post-hoc* Tukey test (difference, lower and upper limit of the confidence interval, and *P*) performed after the two-way between-subjects ANOVA for all relevant comparisons between nanoplastic and enrofloxacin treatments on the body length of *Daphnia*. Statistically significant differences are marked with bold.

Comparison	Diff	Lwr	Upr	*P*
N_l × Control	-0.0136	-0.0726	0.0453	0.9998
N_m × Control	-0.0484	-0.1082	0.0114	0.2550
N_h × Control	-0.0199	-0.0774	0.0375	0.9930
E_l × Control	-0.0025	-0.0607	0.0556	1.0000
E_h × Control	**-0.0547**	**-0.1122**	**0.0027**	**0.0097**
N_m × N_l	-0.0347	-0.0944	0.0249	0.7574
N_h × N_l	-0.0062	-0.0636	0.0510	1.0000
N_lE_l × N_l	**-0.0933**	**-0.1511**	**-0.0355**	**< 0.0001**
N_lE_h × N_l	0.0085	-0.0528	0.0699	0.9999
N_h × N_m	0.0284	-0.0297	0.0866	0.9098
N_mE_l × N_m	-0.0288	-0.0885	0.0309	0.9170
N_mE_h × N_m	0.0325	-0.0248	0.0899	0.7866
N_hE_l × N_h	-0.0104	-0.0674	0.0464	0.9999
N_hE_h × N_h	-0.0429	-0.1005	0.0146	0.3793
N_lE_l × E_l	**-0.1044**	**-0.1615**	**-0.0474**	**< 0.0001**
N_mE_l × E_l	**-0.0746**	**-0.1327**	**-0.0166**	**0.0015**
N_hE_l × E_l	-0.0279	-0.0855	0.0297	0.9149
E_l × E_h	-0.0522	-0.1087	0.0043	0.1030
N_mE_l × N_lE_l	0.0298	-0.0280	0.0876	0.8747
N_hE_l × N_lE_l	0.0765	0.0191	0.1339	0.0788
N_lE_h × N_lE_l	0.1018	0.0414	0.1622	0.6666
N_hE_l × N_mE_l	0.0467	-0.0116	0.1052	0.2699
N_mE_h × N_mE_l	0.0613	0.0048	0.1178	0.2244
N_hE_h × N_hE_l	-0.0324	-0.0911	0.0262	0.8127

Table S2. The results of HSD *post-hoc* Tukey test (difference, lower and upper limit of the confidence interval, and *P*) performed after the two-way between-subjects ANOVA for all relevant comparisons between nanoplastic and enrofloxacin treatments on the body volume of *Daphnia*. Statistically significant differences are marked with bold.

Comparison	Diff	Lwr	Upr	*P*
N_l × Control	-0.0535	-0.2852	0.1780	0.9998
N_m × Control	-0.1814	-0.4165	0.0535	0.3238
N_h × Control	-0.0576	-0.2834	0.1681	0.9995
E_l × Control	-0.0932	-0.3217	0.1353	0.9746
E_h × Control	**-0.3031**	**-0.5289**	**-0.0773**	**0.0072**
N_m × N_l	-0.1279	-0.3623	0.1064	0.8261
N_h × N_l	-0.0040	-0.2291	0.2210	1.0000
N_lE_l × N_l	**-0.3160**	**-0.5430**	**-0.0889**	**0.0003**
N_lE_h × N_l	-0.1166	-0.3576	0.1244	0.9154
N_h × N_m	0.12385	-0.1047	0.3524	0.8330
N_mE_l × N_m	-0.1868	-0.4212	0.0475	0.2755
N_mE_h × N_m	-0.0169	-0.2439	0.2101	1.0000
N_hE_l × N_h	-0.1755	-0.3990	0.0479	0.2979
N_hE_h × N_h	-0.1913	-0.4174	0.0348	0.1945
N_lE_l × E_l	**-0.2763**	**-0.5003**	**-0.0524**	**0.0032**
N_mE_l × E_l	**-0.2751**	**-0.5030**	**-0.0472**	**0.0046**
N_hE_l × E_l	-0.1399	-0.3662	0.0863	0.6776
E_l × E_h	-0.2099	-0.4318	0.0120	0.0843
N_mE_l × N_lE_l	0.0012	-0.2258	0.2282	1.0000
N_hE_l × N_lE_l	0.1364	-0.0890	0.3618	0.7075
N_lE_h × N_lE_l	0.1993	-0.0378	0.4366	0.2030
N_hE_l × N_mE_l	0.1351	-0.0942	0.3646	0.7418
N_mE_h × N_mE_l	0.1699	-0.0536	0.3934	0.3487
N_hE_h × N_hE_l	-0.0158	-0.2462	0.2146	1.0000

Table S3. The results of HSD *post-hoc* Tukey test (difference, lower and upper limit of the confidence interval, and *P*) performed after the two-way between-subjects ANOVA for all relevant comparisons between nanoplastic and enrofloxacin treatments on the clutch size of *Daphnia*. Statistically significant differences are marked with bold.

Comparison	Diff	Lwr	Upr	*P*
N_l × Control	**-0.9960**	**-1.6290**	**-0.3629**	**< 0.0001**
N_m × Control	**-1.3316**	**-1.9786**	**-0.6846**	**< 0.0001**
N_h × Control	**-1.2602**	**-1.8774**	**-0.6430**	**< 0.0001**
E_l × Control	**-0.9655**	**-1.5932**	**-0.3377**	**< 0.0001**
E_h × Control	**-1.1638**	**-1.7847**	**-0.5429**	**< 0.0001**
N_m × N_l	-0.3355	-0.9905	0.3194	0.8788
N_h × N_l	-0.2641	-0.8897	0.3613	0.9669
N_lE_l × N_l	0.2658	-0.3855	0.9171	0.9744
N_lE_h × N_l	-0.5186	-1.1883	0.1509	0.3187
N_h × N_m	0.0714	-0.5683	0.7111	1.0000
N_mE_l × N_m	-0.4193	-1.1064	0.2677	0.6954
N_mE_h × N_m	-0.0549	-0.6895	0.5796	1.0000
N_hE_l × N_h	-0.1271	-0.7619	0.5075	0.9999
N_hE_h × N_h	-0.5891	-1.2395	0.0612	0.1200
N_lE_l × E_l	0.2352	-0.4109	0.8815	0.9896
N_mE_l × E_l	-0.7854	-1.4545	-0.1164	0.0070
N_hE_l × E_l	-0.4219	-1.0669	0.2231	0.5935
E_l × E_h	-0.1983	-0.8223	0.4255	0.9967
N_mE_l × N_lE_l	**-1.0207**	**-1.7044**	**-0.3371**	**< 0.0001**
N_hE_l × N_lE_l	-0.6571	-1.3173	0.0030	0.0523
N_lE_h × N_lE_l	-0.7845	-1.4638	-0.1051	0.0089
N_hE_l × N_mE_l	0.3635	-0.3189	1.0461	0.8478
N_mE_h × N_mE_l	0.3644	-0.2897	1.0185	0.8054
N_hE_h × N_hE_l	-0.4619	-1.1360	0.2120	0.5184
N_lE_h × E_h	-0.3508	-1.0090	0.3073	0.8472
N_mE_h × E_h	-0.2227	-0.8306	0.3852	0.9891
N_hE_h × E_h	-0.6854	-1.3393	-0.0316	0.0302
N_mE_h × N_lE_h	0.1281	-0.5215	0.7778	0.9999
N_hE_h × N_lE_h	-0.3346	-1.0274	0.3581	0.9161
N_hE_h × N_mE_h	-0.4627	-1.1081	0.1825	0.4435

Table S4. The results of HSD *post-hoc* Tukey test (difference, lower and upper limit of the confidence interval, and *P*) performed after the two-way between-subjects ANOVA for all relevant comparisons between nanoplastic and enrofloxacin treatments on the egg volume of *Daphnia*. Statistically significant differences are marked with bold

Comparison	Diff	Lwr	Upr	*P*
N_l × Control	**-0.0016**	**-0.0026**	**-0.0006**	**< 0.0001**
N_m × Control	**-0.0017**	**-0.0028**	**-0.0007**	**< 0.0001**
N_h × Control	**-0.0018**	**-0.0028**	**-0.0009**	**< 0.0001**
E_l × Control	**-0.0012**	**-0.0022**	**-0.0003**	**0.0012**
E_h × Control	**-0.0023**	**-0.0032**	**-0.0013**	**< 0.0001**
N_m × N_l	-0.0001	-0.0011	0.0009	1.0000
N_h × N_l	-0.0001	-0.0011	0.0008	0.9999
N_lE_l × N_l	-0.0006	-0.0017	0.0003	0.5666
N_lE_h × N_l	-0.0006	-0.0016	0.0004	0.7735
N_h × N_m	-0.0001	-0.0011	0.0009	1.0000
N_mE_l × N_m	-0.0001	-0.0012	0.0009	0.9999
N_mE_h × N_m	**-0.0011**	**-0.0021**	**-0.0001**	**0.0170**
N_hE_l × N_h	-0.0001	-0.0010	0.0009	1.0000
N_hE_h × N_h	-0.0008	-0.0018	0.0001	0.2171
N_lE_l × E_l	**-0.0011**	**-0.0021**	**0.0001**	**0.0170**
N_mE_l × E_l	-0.0006	-0.0016	0.0003	0.5892
N_hE_l × E_l	-0.0006	-0.0016	0.0004	0.7080
E_l × E_h	**-0.0010**	**-0.0020**	**0.0001**	**0.0170**
N_mE_l × N_lE_l	0.0004	-0.0005	0.0014	0.9687
N_hE_l × N_lE_l	0.0004	-0.0005	0.0015	0.9310
N_lE_h × N_lE_l	0.0001	-0.0009	0.0011	1.0000
N_hE_l × N_mE_l	0.0001	-0.0009	0.0010	1.0000
N_mE_h × N_mE_l	-0.0009	-0.0019	0.0001	0.0624
N_hE_h × N_hE_l	-0.0008	-0.0018	0.0002	0.3361
N_lE_h × E_h	0.0001	-0.0009	0.0010	1.0000
N_mE_h × E_h	-0.0005	-0.0014	0.0003	0.7382
N_hE_h × E_h	-0.0003	-0.0013	0.0006	0.9936
N_mE_h × N_lE_h	-0.0005	-0.0015	0.0004	0.7327
N_hE_h × N_lE_h	-0.0003	-0.0014	0.0006	0.9896
N_hE_h × N_mE_h	0.0002	-0.0007	0.0011	0.9999

Table S5. The results of HSD *post-hoc* Tukey test (difference, lower and upper limit of the confidence interval, and *P*) performed after the two-way between-subjects ANOVA for all relevant comparisons between nanoplastic and enrofloxacin treatments on the clutch volume of *Daphnia*. Statistically significant differences are marked with bold.

Comparison	Diff	Lwr	Upr	*P*
N_l × Control	**-0.0232**	**-0.0312**	**-0.0151**	**< 0.0001**
N_m × Control	**-0.0214**	**-0.0296**	**-0.0131**	**< 0.0001**
N_h × Control	**-0.0250**	**-0.0329**	**-0.0172**	**< 0.0001**
E_l × Control	**-0.0201**	**-0.0280**	**-0.0121**	**< 0.0001**
E_h × Control	**-0.0278**	**-0.0356**	**-0.0200**	**< 0.0001**
N_m × N_l	0.0018	-0.0065	0.0101	0.9999
N_h × N_l	-0.0018	-0.0098	0.0060	0.9998
N_lE_l × N_l	0.0008	-0.0073	0.0089	1.0000
N_lE_h × N_l	-0.0066	-0.0150	0.0016	0.2635
N_h × N_m	-0.0036	-0.0118	0.0044	0.9476
N_mE_l × N_m	-0.0088	-0.0174	-0.0001	0.0404
N_mE_h × N_m	**-0.0122**	**-0.0202**	**-0.0043**	**< 0.0001**
N_hE_l × N_h	-0.0026	-0.0107	0.0053	0.9949
N_hE_h × N_h	-0.0074	-0.0154	0.0006	0.1069
N_lE_l × E_l	-0.0022	-0.0103	0.0057	0.9987
N_mE_l × E_l	**-0.0100**	**-0.0183**	**-0.0017**	**0.0042**
N_hE_l × E_l	-0.0076	-0.0157	0.0004	0.0866
E_l × E_h	-0.0077	-0.0155	0.0001	0.0550
N_mE_l × N_lE_l	-0.0078	-0.0162	0.0006	0.1007
N_hE_l × N_lE_l	-0.0053	-0.0135	0.0028	0.5999
N_lE_h × N_lE_l	-0.0075	-0.0158	0.0008	0.1252
N_hE_l × N_mE_l	0.0024	-0.0060	0.0109	0.9987
N_mE_h × N_mE_l	-0.0034	-0.0115	0.0045	0.9610
N_hE_h × N_hE_l	-0.0047	-0.0130	0.0036	0.7888
N_lE_h × E_h	-0.0020	-0.0101	0.0060	0.9995
N_mE_h × E_h	-0.0058	-0.0133	0.0016	0.3106
N_hE_h × E_h	-0.0046	-0.0126	0.0033	0.7603
N_mE_h × N_lE_h	-0.0037	-0.0117	0.0041	0.9263
N_hE_h × N_lE_h	-0.0025	-0.0109	0.0058	0.9978
N_hE_h × N_mE_h	0.0012	-0.0066	0.0090	0.9999

Table S6. Planned contrasts of estimated marginal means for linear model to test the effect of of the presence of the polystyrene nanoparticles (in the absence, low, medium and high concentration) and the presence of the enrofloxacin (in the absence, low and high concentration) on the on the respiration rate of gut microbiota of *Daphnia* expresed as V_{max} values. Statistically significant differences effect are marked with bold (E – estimate, SE – standard error, df – degree of freedom, *T* – T-ratio, $P_{corr.}$ – *p*-value corrected).

contrast	estimate	SE	df	t.ratio	p.value
N_l × control	**130.0645**	**22**	**360**	**5.909**	**< 0.0001**
N_m × control	**118.3226**	**22**	**360**	**5.375**	**< 0.0001**
N_h × control	64.4193	22	360	2.927	0.1361
E_l × control	37.1290	22	360	-1.687	0.8733
E_h × control	-48.3548	22	360	-2.197	0.5531
N_l × N_m	11.7419	22	360	0.533	1.0000
N_h × N_l	-65.6452	22	360	-2.982	0.1181
N_lE_l × N_l	**-124.5807**	**22**	**360**	**-5.660**	**< 0.0001**
N_lE_h × N_l	**-178.3548**	**22**	**360**	**-8.103**	**< 0.0001**
N_h × N_m	-53.9032	22	360	-2.449	0.3762
N_mE_l × N_m	**-162.7097**	**22**	**360**	**-7.392**	**< 0.0001**
N_mE_h × N_m	**-136.1129**	**22**	**360**	**-6.184**	**< 0.0001**
N_hE_l × N_h	-10.8064	22	360	-0.491	1.0000
N_hE_h × N_h	**-139.9000**	**22**	**360**	**-6.356**	**< 0.0001**
N_lE_l × E_l	42.6129	22	360	1.936	0.7359
N_mE_l × E_l	-7.2581	22	360	-0.330	1.0000
N_hE_l × E_l	**90.7419**	**22**	**360**	**4.122**	**0.0027**
E_h × E_l	-11.2258	22	360	-0.510	1.0000
N_lE_l × N_mE_l	49.8710	22	360	2.266	0.5032
N_hE_l × N_lE_l	48.1290	22	360	2.186	0.5606
N_lE_h × N_lE_l	-53.7742	22	360	-2.443	0.3800
N_hE_l × N_mE_l	**98.0000**	**22**	**360**	**4.452**	**0.0007**
N_mE_h × N_mE_l	26.5968	22	360	1.208	0.9881
N_hE_h × N_hE_l	**-129.1129**	**22**	**360**	**-5.866**	**< 0.0001**
N_lE_h × E_h	0.0645	22	360	0.003	1.0000
N_mE_h × E_h	30.5645	22	360	1.389	0.9652
N_hE_h × E_h	-27.1452	22	360	-1.233	0.9860
N_lE_h × N_mE_h	-30.5000	22	360	-1.386	0.9657
N_hE_h × N_mE_h	-57.7097	22	360	-2.622	0.2715

E. GENERAL CONCLUSIONS

This Doctoral Thesis has contributed to gain knowledge about the presence, interaction, and joint toxic effects of selected emerging contaminants in freshwater environments:

1. A methodology based on SPE-LC-MS/MS was developed. The method allowed the determination of 21 antibiotics as well as the detection of their metabolites in the POCTEFA territory.
2. The presence of antibiotics among surface waters and waste waters in the Ebro basin was studied in a long term survey (4 years). The results showed the ubiquitous presence of enrofloxacin in almost all samples from surface waters showed, especially in areas close to intensive farming. This fluoroquinolone antibiotic was also found, together with azithromycin and trimethoprim in WWTPs, especially in areas near large urban cores. Generally, the amount of antibiotics found was higher than in previous studies carried out in the area.
3. The analysis by AF4-UV-Vis-ICP-MS of the colloidal fraction from different samples of surface waters and WWTPs revealed particles larger than 100 nm in size, with a large dispersion component and Al signal associated. A methodology based on the use of tangential ultrafiltration and UV-Vis was also used to investigate the interactions between colloids and enrofloxacin. While samples with a large organic matter fraction did not show significant interactions, up to 67% of enrofloxacin added was observed to be associated to the colloidal fraction in natural waters samples with a large colloidal fraction.
4. The combined effects of NPTs and antibiotics on the life history parameters of freshwater organisms, and metabolomic and taxonomic diversity of their intestinal microbial community was investigated. The interactive effect of both emerging contaminants on all the parameters studied was different in the presence and absence of NPTs.

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