ANALYSIS AND IMPACT OF ANTIBIOTICS IN MARINE ORGANISMS. LABORATORY EXPERIMENTS AND FIELD STUDIES

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Analysis and impact of antibiotics in marine organisms. Laboratory experiments and field studies

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Doctoral Thesis

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DECLAREM:

Que el treball titulat "Analysis and impact of antibiotics in marine organisms. Laboratory experiments and field studies", que presenta Albert Serra Compte per a l'obtenció del títol de doctor/a, ha estat realitzat sota la nostra direcció.

I, perquè així consti i tingui els efectes oportuns, signo aquest document.

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List of publications

This doctoral thesis is a compendium of research articles. The publications presented are listed below:

i. Albert Serra-Compte; Diana Álvarez-Muñoz; Sara Rodríguez-Mozaz; Damià Barceló. 2017. *Multi-residue method for the determination of antibiotics and some of their metabolites in seafood*. Food and Chemical Toxicology. 104. 3-13. (IF: 4.7, Q1).

Candidate contribution in: Experimental design, sample treatment, LC-MS analysis, data processing and manuscript writing.

ii. Albert Serra-Compte; Mariël G. Pikkemaat; Alexander Elferink; David Almeida; Jorge Diogène; Juan António Campillo; Marta Llorca; Diana Álvarez-Muñoz; Damià Barceló; Sara Rodríguez-Mozaz. 2021. *Combining an effect-based methodology with chemical analysis for antibiotics determination in wastewater and receiving freshwater and marine environment.* Environmental Pollution. 271. 116313. (IF: 6,8, Q1).

Candidate contribution in: Experimental design, sample collection and treatment, LC-MS analysis, microbial growth inhibition test analysis, data processing and manuscript writing.

iii. Albert Serra-Compte; Ana Luisa Maulvault; Carolina Camacho; Diana Álvarez-Muñoz; Damià Barceló; Sara Rodríguez-Mozaz; António Marques. 2018. *Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (Mytilus galloprovincialis)*. Environmental Pollution. 236. 824-834 (IF: 6.8, Q1).

Candidate contribution in: Sample treatment, LC-MS analysis, data processing and manuscript writing.

iv. Albert Serra-Compte; Diana Álvarez-Muñoz; Montserrat Solé; Núria Cáceres; Damià Barceló; Sara Rodriguez-Mozaz. 2019. *Comprehensive study of sulfamethoxazole effects in marine mussels: bioconcentration, enzymatic activities and metabolomics*. Environmental Research. 173. 12-22 (IF: 5.7, Q1).

Candidate contribution in: Experimental design, exposure experiments implementation, sample treatment, LC-MS analysis, LC-HRMS analysis for metabolomics, metabolites identification, data processing, support on enzymatic activities characterization and manuscript writing.

v. Albert Serra-Compte; Alexandre Sánchez-Melsió; Diana Álvarez-Muñoz; Damià Barceló; José Luis Balcázar; Sara Rodriguez-Mozaz. 2019. *Exposure to a Subinhibitory Sulfonamide Concentration Promotes the Spread of Antibiotic Resistance in Marine Blue Mussels (Mytilus edulis*). Environmental Science and Technology Letters. 6, 211−215 (IF: 7.7, Q1).

Candidate contribution in: Experimental design, exposure experiments implementation, sample treatment, LC-MS analysis, support in DNA extraction and q-PCR analysis and manuscript writing.

Additional Scientific Publications

Albert Serra-Compte; Natàlia Corcoll; Belina Huerta; Sara Rodríguez-Mozaz; Sergi Sabater; Damià Barceló; Diana Álvarez-Muñoz. 2018. Fluvial biofilms exposed to desiccation and pharmaceutical pollution: New insights using metabolomics. Science of the total environment. 618. 1382-1388.

Ana Luísa Maulvault; Lúcia H.M.L.M. Santos; Carolina Camacho; Patrícia Anacleto; Vera Barbosa; Ricardo Alves; Pedro Pousão Ferreira; **Albert Serra-Compte**; Damià Barceló; Sara Rodriguez-Mozaz; Rui Rosa; Mário Diniz; António Marques. 2018. Antidepressants in a changing ocean: Venlafaxine uptake and elimination in juvenile fish (Argyrosomus regius) exposed to warming and acidification conditions. Chemosphere. 209. 286-297.

Diana Álvarez-Muñoz, Sara Rodríguez-Mozaz, Silke Jacobs, **Albert Serra-Compte**, Nuria Cáceres, Isabelle Sioen, Wim Verbeke, Vera Barbosa, Federico Ferrari, Margarita Fernández-Tejedor, Sara Cunha, Kit Granby, Johan Robbens, Michiel Kotterman, Antonio Marques, Damià Barceló. 2018. Pharmaceuticals and endocrine disruptors in raw and cooked seafood from European market: concentrations and human exposure levels. Environment International. 119:570-581.

Ana Previšić, Marko Rožman, Jordi-René Mor, Vicenç Acuña, **Albert Serra-Compte**, Mira Petrović, Sergi Sabater. 2020. Aquatic macroinvertebrates under stress: Bioaccumulation of emerging contaminants and metabolomics implications. Science of the total environment, 704. 135333

Julio C.López-Doval; **Albert Serra-Compte**; Sara Rodríguez-Mozaz; Damià Barceló; Sergi Sabater. Diet quality and NSAIDs promote changes in formation of prostaglandins by an aquatic invertebrate. 2020. Chemoshpere. 257. 126892

Sara Rodríguez-Mozaz; **Albert Serra-Compte**; Ruben Gil-Solsona; Diana Álvarez-Muñoz Environmental metabolomics and xenometabolomics for the assessment of exposure to contaminant mixtures. 2020. Chapter in the Book: Environmental Metabolomics: Applications in Field and Laboratory Studies to Understand from Exposome to Metabolome. 283-310. Editor(s): Diana Álvarez-Muñoz, Marinella Farré, Elsevier.

Adrián Jaén-Gil , María-José Farré 1, Alexandre Sànchez-Melsió , **Albert Serra-Compte**, Damià Barceló, Sara Rodríguez-Mozaz. Effect-Based Identification of Hazardous Antibiotic Transformation Products after Water Chlorination. 2020.. Environmental Science and Technology. 54, 14, 9062–9073

List of Acronyms

Acronym	Meaning
AChe	Acetylcholinesterase
ARGs	Antibiotic Resistance Genes
BFAs	Bioaccumulation Factors
CAT	Catalase activity
DDD	Defined Daily Doses
dSPE	dispersive Solid Phase Extraction
EDCs	Endocrine Disrupting Compounds
EEA	European Economic Area
ERA	Environmental Risk Assessment
ELISA	Enzyme-linked Immunosorbent Assay
EU	European Union
FD	Fluorescence Detectors
FIA	Fluoroimmunoassay
GPC	Gel Permeation Chromatography
GST	Glutathione-S-transferase
HGT	Horizontal Gene Transfer
HPLC	High-Performance Liquid Chromatography
LC	Liquid Chromatography
LODs	Limits of Detection
NMR	Nuclear Magnetic Resonance
MRL	Maximum Residue Limits
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry

PhACs	Pharmaceuticals
PCA	Principal Component Analyses
PLE	Pressurized Liquid Extraction
PLS	Partial Last Squares
PNEC	Predicted Non-effect Concentration
QuEChERS	Quick Easy Cheap Effective Rugged and Safe
QSARs	Quantitative Structure-Activity Relationships
SPE	Solid-Phase Extraction
SPME	Solid Phase Microextraction
UV	Ultraviolet
VGT	Vertical Gene Transfer
WHO	World Health Organization
WWTP	Wastewater Treatment Plant

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Summary

Antibiotic residues have been detected in the aquatic environment worldwide including rivers, lakes, groundwater and seawater. The accumulation of antibiotics in aquatic organisms has also been reported in several studies, which may provoke ecotoxicological effects to these organisms and alter the functioning of the ecosystems. Besides, antibiotics bioaccumulation can be of concern for the human health when accumulating in animals used for human consumption, such as seafood. Furthermore, antibiotic pollution can contribute to the development and spread of antibiotic resistance in the environment, an issue of high concern due to the risk for the human health.

The main aim of this thesis was to investigate the fate and environmental impact of antibiotics residues in the environment with a special emphasis in the marine ecosystem. Antibiotic bioaccumulation in marine organisms was assessed under both, controlled conditions, and through the analysis of aquatic organisms from aquaculture and natural environments. Exposure experiments were also used to characterize ecotoxicological effects of antibiotics and to evaluate their contribution to the spread of antibiotic resistance genes.

The **first part** is dedicated to the development of analytical methodologies to determine the occurrence of antibiotic residues in marine organisms and water samples. Two different methodologies were explored in this part. On one hand, an analytical method based on QuEChERS extraction followed by Liquid Chromatography coupled to Mass Spectrometry (LC-MS/MS) analysis was developed for the identification and quantification of 23 antibiotics in fish, mussel and clam samples. On the other hand, a methodology based on microbial growth inhibition was optimized for the screening of four antibiotic families in organism's biofluids and water samples. Both methodologies were applied for the determination of antibiotic residues in organism and water samples from a monitoring campaign.

The **second part** evaluates the environmental impact of antibiotic pollution on marine organisms. Three different sets of exposure experiments were performed in order to assess antibiotics bioaccumulation, ecotoxicological effects and spread of antibiotic resistance in marine mussels. The first study assessed alterations on antibiotics bioaccumulation due to climate change expected conditions to the marine environment such as water warming and acidification. The second study comprehensively evaluated the ecotoxicological effects of sulfamethoxazole antibiotic exposure to marine mussels through the characterization of

enzymatic activities, and observation of metabolism changes applying a non-targeted metabolomics approach. Finally, the third study determined the effects of antibiotic water pollution on the abundance of antibiotic resistance genes occurring in the bacteria located in mussel's gastrointestinal tract.

Resumen

La contaminación por antibióticos se ha detectado en diversos ambientes acuáticos en todo el mundo, incluyendo ríos, lagos, agua subterránea y ambientes marinos. Se ha detectado también la acumulación de estos antibióticos en organismos acuáticos; esta bioacumulación puede provocar efectos ecotoxicológicos a los organismos expuestos y alterar el funcionamiento de los ecosistemas. Además, la bioacumulación de antibióticos puede comprometer la salud humana, cuando los antibióticos se acumulan en organismos utilizados para el consumo humano, como, por ejemplo, pez y marisco. Asimismo, la contaminación por antibióticos puede contribuir al desarrollo y diseminación de resistencia a los antibióticos en el medio ambiente, un tema especialmente preocupante debido al riesgo asociado para la salud humana.

El principal objetivo de esta tesis fue investigar el destino y el impacto que tiene la presencia de antibióticos en el medio ambiente, con especial atención al medio marino. La bioacumulación de antibióticos en organismos acuáticos se estudió tanto en condiciones controladas, como a través del análisis de organismos acuáticos provenientes de acuicultura y de ambientes naturales. Los experimentos en condiciones controlados fueron utilizados también para caracterizar los efectos ecotoxicologicos de los antibióticos y su contribución a la diseminación de genes de resistencia a los antibióticos.

La **primera parte** está focalizada en el desarrollo de metodologías analíticas para determinar la presencia de antibióticos en organismos marinos y en muestras de agua. En esta parte, se evaluaron dos tipos de metodologías. Por un lado, se desarrolló un método analítico basado en la extracción de compuestos con QuEChERs seguido del análisis mediante cromatografía de líquidos acoplada a espectrometría de masas para la identificación y cuantificación de 23 antibióticos en muestras de pez, mejillón y almejas. Por otro lado, se optimizó un método basado en la inhibición del crecimiento bacteriano para el cribado de cuatro familias de antibióticos en fluidos de organismos acuáticos y en muestras de agua. Ambas metodologías fueron aplicadas para la determinación de antibióticos en organismos y muestras de agua en campañas de muestreo.

La **segunda parte** se centra en la evaluación del impacto ambiental de la contaminación de antibióticos en los organismos marinos. Se realizaron tres sets de experimentos de exposición para evaluar la biaocumulación de antibióticos, los efectos ecotoxicológicos y la diseminación de genes de resistencia a los antibióticos. En el primer estudio se evaluaron cambios en la

bioacumulación de antibióticos debido a las condiciones esperadas de cambio climático en el ambiente marino, tales como calentamiento y acidificación del agua. En el segundo estudio se determinó de forma exhaustiva los efectos ecotoxicologicos de la exposición del antibiótico sulfametoxazol a mejillones marinos mediante la caracterización de actividades enzimáticas y la evaluación de cambios en el metabolismo aplicando técnicas de metabolómica no dirigida. Finalmente, en el tercer estudio se determinaron los efectos de la contaminación del agua por antibióticos sobre la diseminación de genes de resistencia a los antibióticos presentes en las bacterias del tracto digestivo de los mejillones.

Resum

La contaminació per antibiòtics s'ha detectat en diversos ambients aquàtics de tot el mon, incloent rius, llacs, aigua subterrània i ambients marins. S'ha detectat també l'acumulació d'aquests antibiòtics en organismes aquàtics; aquesta bioacumulació pot provocar efectes ecotoxicològics als organismes exposats i alterar el funcionament dels ecosistemes. A més, la bioacumulació d'antibiòtics pot comprometre la salut humana quan els antibiòtics s´acumulen en organismes utilitzats pel consum humà, com per exemple, peix i marisc. Tanmateix, la contaminació per antibiòtics pot contribuir al desenvolupament i la disseminació de resistència als antibiòtics al medi ambient, un tema especialment preocupant degut al risc associat per la salut humana.

El principal objectiu d'aquesta tesi fou investigar el destí i l'impacte que té la presencia d'antibiòtics al medi ambient, amb especial atenció al medi marí. La bioacumulació d'antibiòtics en organismes aquàtics va ser estudiada tant en condicions controlades com a través d'anàlisis d'organismes aquàtics provinents d'aqüicultura i ambients naturals. Els experiments en condicions controlades foren utilitzats també per caracteritzar els efectes ecotoxicològis dels antibiòtics, així com també la seva contribució a la disseminació dels gens que confereixen resistència als antibiòtics.

La **primera part** està focalitzada en el desenvolupament de metodologies analítiques per determinar la presència d'antibiòtics en organismes marins i en mostres d'aigua. Dins aquesta part es van avaluar dos tipus de mètodes. Per una banda, es va desenvolupar un mètode analític basat en la extracció de compostos amb QuEChERS seguit de l'anàlisi mitjançant cromatografia de líquids acoblada a espectrometria de masses per la identificació i quantificació de 23 antibiòtics en mostres de peix, musclo i cloïssa. Per altra banda, es va optimitzar un mètode basat en la inhibició del creixement bacterià pel cribratge de quatre famílies d'antibiòtics en fluids d'organismes marins i mostres d'aigua. Ambdues metodologies foren aplicades per la determinació d'antibiòtics en organismes i en mostres d'aigua en campanyes de mostreig.

La **segona part** es centra en la avaluació de l'impacte ambiental de la contaminació d'antibiòtics en els organismes marins. Es van realitzar tres sets d'experiments d'exposició per avaluar la bioacumulació d'antibiòtics, els efectes ecotoxicològics i la disseminació dels gens de resistència als antibiòtics. En el primer estudi es van avaluar alteracions de la bioacumulació d'antibiòtics degut a les condicions esperades de canvi climàtic en el medi marí, tals com escalfament i

acidificació de l'aigua. En el segon estudi es van determinar de forma exhaustiva els efectes ecotoxicològics de l'exposició de l'antibiòtic sulfametoxazol a musclos marins mitjançant la caracterització d'activitats enzimàtiques i la avaluació de canvis en el metabolisme aplicant tècniques de metabolòmica no dirigida. Finalment, en el tercer estudi es van determinar els efectes de la contaminació de l'aigua per antibiòtics sobre la disseminació de gens de resistència als antibiòtics presents a les bactèries del tracte digestiu dels musclos.

Chapter 1 - General introduction

1.1 Marine environment: resources and threats

Marine environment covers more than 70% of the total earth surface, being the world largest ecosystem, providing habitat for an extremely rich biodiversity[1]. Marine ecosystem contributes to the global earth functioning in terms of nutrient cycling, climate regulation and absorption of CO_2 among others[1]. It provides an enormous variety of ecosystem services including food resources, extraction of raw materials, components for pharmaceuticals and cosmetics products. It is also an important water source that can contribute to combat global water scarcity[2,3]. Furthermore, marine environment and especially coastal areas are important for recreational activities such as cruises, fishery and water sports, as well as, an aesthetic value[1].

In order to use marine environment services and to provide marine bequest for future generations, it is crucial to maintain it in good health. This has become a complex task due to different threats that marine ecosystem faces. Some of the most important marine threats include human overexploitation, climate change, invasive species and pollution[4–6]. Marine environment conditions are changing due to the anthropogenically induced global climate change. Climate change provokes alterations in weather conditions such as water warming and acidification. It can also affect water circulation and stratification, as well as alter the nutrient input and oxygen content[7,8]. All these environmental modifications are altering habitat conditions, provoking deep changes in marine biodiversity and species distribution[9]. Variations in marine species distribution, in addition to non-intentional species move from their non-native habitat, can lead to the increase of invasive species, compromising the functioning of the ecosystems[9]. Besides, overexploitation of marine biota for human consumption may lead to a depletion of resources and provoke species risk for extinction[10].

In addition to the above-mentioned marine threats, chemical pollution can negatively affect the ecology and the ecosystem services that the marine environment provides. Industrial, urban, hospital and agricultural human activities, among others, produce a large number of chemicals that end up being released into the marine ecosystem. Thousands of different type of substances such as pesticides, flame retardants, pharmaceuticals including antibiotics, personal care products, illicit drugs, polycyclic aromatic hydrocarbons, surfactants, metals, micro and nano plastics, etc. occur in the marine ecosystem[11] and are distributed into the different environmental compartments such as water, sediment and biota. Despite these contaminants are usually found at low concentrations (ng/L in water)[11], they are of concern due to the ecotoxicological effects that may provoke on the exposed organisms[12]. Within chemical contamination, antibiotic pollution is of high concern due to its potential risk for the natural

ecosystems and its contribution to the development and spread of antimicrobial resistance. The One Health approach adopted by the European Commission[13], linking environmental and human health in terms of antibiotic resistance, highlighted the need of research regarding antibiotic and antimicrobial resistance pollution in the environment to face the challenge that this phenomenon represents for the human and animal health[13]. In this regard, this thesis focuses on assessing antibiotic pollution occurrence and its effect on the marine ecosystem, with a special emphasis on aquatic organisms.

1.2 Antibiotics

Antibiotics comprise natural, synthetic and semi-synthetic compounds that can inhibit or abolish the growth of bacteria[14] without being toxic for the host, so they are used to combat human and veterinary infections. Antibiotics can be grouped according to their chemical structure in different antibiotic families; some of the most commonly used ones are summarized in table 1. Besides, the mode of action and bacterial targets of the different antibiotic families, such as cell membrane, DNA replication and protein synthesis, are also presented in table 1.

Table 1. Examples of antibiotic families, their modes of action, and some of the most representative antibiotics from each family. Adapted from Gothwal et al. 2014[15]

Antibiotic family	**Mode of action**	**Representative antibiotic**
Sulfonamides	Inhibition of bacterial beta lactamases	Sulfamethoxazole, sulfamethazine, sulfadiazine, sulfapyridine
Macrolides	Inhibition of protein synthesis	Clarithormycin, erythromycin, tylosine, spiramycin
Tetracyclines	Inhibition of protein synthesis	Tetracycline, oxytetracycline, doxycycline, chlortetracycline
Fluoro(quinolones)	Inhibition of DNA replication	Oxolinic acid, flumequine, cirpofloxacin, norfloxacin, ofloxacin, enrofloxacin

Lincosamides	Inhibit protein synthesis	Clindamycin, lincomycin
Beta-lactams	Inhibition of cell wall synthesis	Amoxicillin, oxacillin, piperacillin
Aminoglycosides	Inhibition of protein synthesis	gentamycin

Concerning antibiotic consumption, the European Centre for Disease Prevention and Control publishes an annual report of antibiotic consumption in the European Union (EU)[16]. The annual report from 2012 showed an increase in antibiotic consumption in the previous decade. However, the latest report, which considers data until 2018 highlighted that the global consumption of antibiotics within EU countries remained stable between 2009 and 2018. Despite this stabilization on antibiotic consumption at European level, some differences between countries at community level were reported. The latest report showed a significant decrease in antibiotic consumption in 11 countries compared with previous years. However, an increase was detected in four countries within the EU region[16]. These differences between countries antibiotic consumption tendency may be attributed to several factors, including the country specific regulations regarding antibiotic consumption limitations established. A comparison of antibiotic consumption within different countries from the European region is shown in figure 1. Antibiotic consumption is reported as Defined Daily Doses (DDD) per 1000 inhabitants per day, which refers to "the assumed average maintenance dose per day for a drug used for its main indication in adults"[17].

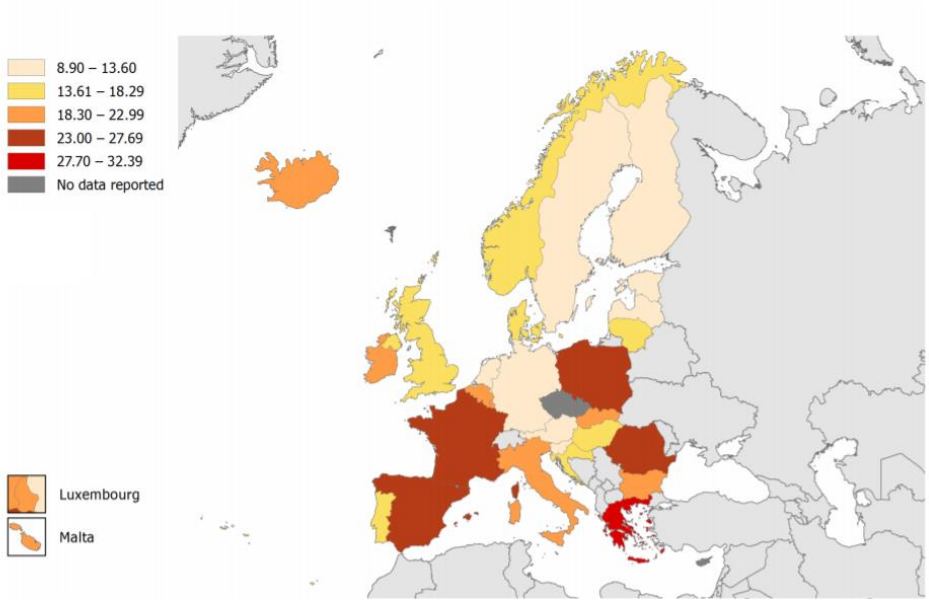

Figure 1. Antibiotic consumption in the EU/EEA countries in 2018 (expressed as DDD per 1000 inhabitants per day, figure from ESAC-Net, 2018[16].

When occurring in the environment, antibiotics are considered micropollutants, which refers to those contaminants present in the aquatic environment in concentrations above the natural background due to anthropogenic activities. However, their concentrations remain at trace level[18]. Micropollutants can provoke known or suspected effects to the exposed organisms, as it will be discussed further down in this chapter. Furthermore, they can be of concern to human health, when polluted organisms are used as a food source[19]. In the European Union, a list was created within the Water Directive Framework, for those substances that should be carefully monitored due to their potential risk for the environment and human health, the "Watch list"[20]. The updated Watch List from 2020 included the antibiotics sulfamethoxazole, trimethoprim, amoxicillin and ciprofloxacin[20], highlighting the concern regarding the presence of these compounds in the environment. In addition to antibiotics, other micropollutants have been also included in the watch list such as the anti-depressant pharmaceutical venlafaxine and its metabolite O-desmethylvenlafaxine, among others.

1.2.1 Sulfamethoxazole as a model antibiotic

Sulfamethoxazole is one of the most consumed antibiotics worldwide; it is extensively used in human medicine to treat urinary infections, sinusitis or toxoplasmosis, among others[21]. Besides, it is also used for veterinary purposes to treat different infections in dogs, cats, horses[22] as well as in aquaculture for fish or shrimp production[23]. Sulfamethoxazole is commonly administrated in combination with trimethoprim as both antibiotics have a synergistic effect by inhibiting

successive steps in the folate synthesis pathway. Actually, its association with trimethoprim is considered an essential medicine by the World Health Organization (WHO)[24]. Sulfamethoxazole was one of the first antibiotics systematically used and commercialized, in the 1960's decade[25]. As a result of its clinical importance, its long-term commercialization and worldwide distribution, sulfamethoxazole can serve as a model compound in order to investigate antibiotic environmental distribution, fate and impact to wildlife and human health. Recently, different studies pinpointed sulfamethoxazole as one of the most frequently detected antibiotics in the marine environment with potential ecological risk[26]. For instance, Li et al. 2020[27], determined sulfamethoxazole and other two antibiotics (sulfamethoxypyridazine and cinoxacin) with ecotoxicological risk in coastal areas from the East China Sea and recommended them for prioritization in antibiotics monitoring and management. For these reasons, sulfamethoxazole is the main compound targeted in this thesis, used as a transversal compound in all the studies included in this thesis.

1.3 Analytical methodologies for antibiotic determination

1.3.1 Sample preparation

Sample preparation (extraction and purification) is a key step for assessing contaminant occurrence in environmental samples. It is especially important when analyzing antibiotics due to their low concentrations in natural ecosystems. Besides, this is also tricky when dealing with a broad range of antibiotics, where many compounds with different physic-chemical properties are targeted. In this case, analytical methodologies covering a broad range of compounds, called multi-residue methods, are usually employed[28] and both matrix characteristics and physic-chemical properties of the target compound should be taken into consideration[29].

For water samples analysis, sample filtration followed by Solid Phase Extraction (SPE) is usually employed as a pretreatment procedure. This process allows the elimination of the biggest particles that can occur in environmental water through its filtration for instance at 0.45 µm[30]. Then, in a second step, SPE is applied for both, water sample cleaning and pre-concentration. The water volume concentrated using SPE can be adjusted according to the expected target contaminants concentrations in the analyzed water body[30], allowing a compromise between analyte preconcentration and sample matrix interferences.

For antibiotic analysis in more complex matrices such as biota, an exhaustive sample extraction is required, due to the high amount of potential interferences in these matrices. Different extraction methodologies have been applied in multi-residue methods for the analysis of multiclass antibiotics in biota samples. Some of the most used include sonication (ultra-

sonication bath and ultra-sonication probe), centrifugation, Quick Easy Cheap Effective Rugged and Safe (QuEChERS) and Pressurized Liquid Extraction (PLE)[28,31–33]. In order to select the most appropriate extraction procedure the type of matrix, the target compounds and the amount of sample available need to be taken into consideration. For example, ultra-sonication probe is especially recommended when a small amount of sample is available and it requires a low quantity of solvent[34]. On the contrary, PLE generally requires a higher amount of sample but this methodology could provide better compound extraction when dealing with samples containing high fat concentration[31]. After sample extraction, a purification step is generally applied in order to further decrease matrix interferences on antibiotic detection. One of the most applied clean-up methodologies for environmental samples is SPE[30]. Besides, dispersive Solid Phase Extraction (dSPE) is also used and commonly applied after QuEChERs extraction[35] and Gel Permeation Chromatography (GPC) is used for lipid removal when dealing with samples with a high fat content[33].

1.3.2 Antibiotics detection

Analytical methodologies for antibiotic residues assessment in environmental samples can be classified into two main groups: screening methodologies and target analysis. Screening methodologies detect the presence of a single antibiotic or antibiotic family, providing qualitative and/or semiquantitative results. Ideal screening methodologies should be easy to use, fast, with good selectivity, low cost, low rate or negligent false negative samples, low false positive and high throughput[36]. Some of the most used screening methodologies for antibiotic residue assessment in different samples (mainly applied in food samples analysis such as meat or milk) include microbiological tests, immunoassays and biosensors[37]. Microbiological methods are based on the specific interaction between antibiotic residues present in the sample and a susceptible organism (generally bacteria). These methods can be classified according to their mode of detection being some of the most used ones luminescence and growth inhibition[38,39]. The main advantage of these methods is that they can detect any antibiotic or antibiotic metabolite with antibacterial activity in a given sample. Immunoassay techniques are based on the specific reaction between the antibiotic and an antibody designed to recognize it. Some examples of immunoassays applied for antibiotic screening include enzyme-linked immunosorbent assay (ELISA)[40] and fluoroimmunoassay (FIA)[41], providing high specificity and sensitivity. On the other hand, biosensors consist in a biological recognition element (antibodies, enzymes, proteins, nucleic acids, etc.) coupled to a signal transduction element[42]. The most used

biosensor for antibiotic screening includes antibodies coupled to an optical detector[43]. The main advantage of biosensors is that they can operate fully automatically[37].

Target analysis allows the specific identification of the antibiotics present in a sample as well as their accurate quantification. These methodologies provide good specificity and sensitivity. The most commonly applied methodology for antibiotic analysis is High-Performance Liquid Chromatography (HPLC) coupled to Mass Spectrometry (MS) or tandem mass spectrometry (MS/MS). Other detectors have been also used to a lesser extent such as Ultraviolet (UV) or Fluorescence Detectors (FD)[44]. Important efforts have been made for developing multi-residue methods for the detection of a broad-spectrum of antibiotics in a single run, either for water or biota samples[45]. However, it remains a complex task due to the different physic-chemical properties of the antibiotics co-occurring in a certain sample. Traditionally, target methodologies for the detection of antibiotics have been focused on the detection of parent compounds, when the analytical standards are available. However, the increasing concern about antibiotics transformation products and their presence in the environment has promoted their inclusion in multi-residue methods. Unfortunately, analytical standards for antibiotic transformation products are not always available, or even some transformation products have not been identified yet, making their inclusion in target methodologies not feasible in all cases. To overcome these drawbacks, suspect screening and non-targeted methodologies based on Liquid Chromatography coupled to High Resolution Mass Spectrometry (LC-HRMS) are gaining importance to monitor a broader range of known, suspected and unknown antibiotics in the environment[46].

1.4 Sources and occurrence of antibiotics in the environment

Although antibiotics occurrence in seawater has recently gained increasing attention, less information is available when compared to freshwater systems such as rivers, streams or lakes. Main sources of antibiotic pollution comprise industrial, urban (including hospitals) and veterinary waste. Besides, not only the administered antibiotics can be found in the waste but also some metabolites produced and excreted by the body[14].

The main source of antibiotics pollution to the marine environment has been pinpointed to be the discharges of Wastewater Treatment Plants (WWTP), which collect sewage from urban and hospital effluents. Conventional WWTP primary and secondary treatments only partly remove antibiotics and other emerging contaminants[47]. As an example, β-lactams have been reported to be eliminated in secondary treatments up to 90% of its initial concentration[48]. On the

contrary, macrolide's elimination has been reported to be significantly lower 30-50%[48]. Significant differences were observed in the elimination of sulfonamides depending on the plant conditions with eliminations ranging from 30 to 90%[48]. The effectiveness of tertiary treatments such as chlorination, UV irradiation and sand filtration in the elimination of antibiotics have also been studied. Despite most studies highlighted chlorination as the most effective treatment for antibiotics elimination compared to UV irradiation and sand filtration, there is a huge discrepancy between the different reported studies[47]. WWTPs can discharge their effluents directly into the marine coastal areas or into rivers, which end up into the sea.

Antibiotic accumulation in WWTPs sludge may also contribute to pollute the environment if the sludge is used as soil fertilizer[50]. In the same manner, the utilization of antibiotics for veterinary purposes can also be a source of environmental pollution, e.g. when antibiotic polluted manure is used as fertilizer in agriculture. Antibiotics present in the soil can further contaminate surface groundwater through surface run-off and infiltration[51]. The use of antibiotics in aquaculture practices can also contribute to water contamination. Some antibiotics used in aquaculture are oxytetracycline, florfenicol, sulfadimethoxine, sulfamethoxazole and sulfamerazine among others[52]. However, due to the increasing concern regarding antibiotic pollution, antibiotics are being replaced by vaccines.

Coastal areas receive a direct impact of the antibiotic sources above mentioned, being the most affected regions within the marine environment[53]. However, several factors may affect the occurrence and concentrations of antibiotics such as natural attenuation of antibiotic concentration from the point source to the surrounding water. This was observed in most studies due to dilution effects and hydrodynamics in coastal areas[54]. Furthermore, seasonal or temporal variation of antibiotics occurrence has also been studied, observing a higher concentration in touristic coastal areas during summer due to the increase of population[55,56].

Antibiotics monitoring campaigns have been performed in the marine environment, mainly in coastal areas in order to elucidate the occurrence of these pollutants[53]. Table 2 summarizes the most frequently detected antibiotics in seawater, marine sediment and marine biota and the range of concentrations for each antibiotic.

Table 2. Antibiotic concentration in marine environment (seawater, sediment and biota). The number of studies shows the amount of scientific publications reporting the occurrence of a given antibiotic. Bibliographic information extracted from the book chapter, Rodríguez-Mozaz et al. 2017[57].

Antibiotic family	Antibiotics	Water			Sediment			Biota		
		Min-max Concentration (µg/L)	Number of studies*	References	Min-max Concentration (ng/g)	Number of studies*	References	Min-max Concentration (ng/g)	Number of studies*	References
Penicilines	Ampicilllin	-	0		-	0		4,9-10	1	58
	Amoxicilin	14×10^{-2}-$76{,}0 \times 10^{-2}$	1	59	-	0		-	0	
Macrolides	Erythromycin	2.0×10^{-5}-$2{,}2 \times 10^{-2}$	7	54,60–64	0,24-51,5	3	64–66	0,1-87	5	58,67–70
	Erythromycin-H2O	$6{,}3 \times 10^{-4}$-1,9	8	54,59,69,71–76	3,4 - 65,3	3	69,77,78	-	0	
	Azithromycin	$6{,}0 \times 10^{-5}$-0,2	5	54,64,72,73,79	1,3-1,6	-	0	1,2-13,3	2	31,80
	Clarithromycin	$7{,}0 \times 10^{-5}$-0,1	12	30,54,63,64,72–74,79,81	0,07-3	2	64,76	-	0	
	Roxithromycin	$9{,}0 \times 10^{-5}$-0,6	8	54,59,60,62,63,72,73	0,7-13,5	2	66,77	-	0	
Tetracyclines	Tetracycline	$7{,}0 \times 10^{-3}$-2,3	4	59,62,71,76	0,2-73	4	66,76,77,82	1,73-13,1	2	82,83
	Oxytetracycline	$3{,}0 \times 10^{-3}$-15,2	3	59,62,76	1,7-176	2	66,76	1,25-178	5	36,41,82–84
	Chlortetracycline	-	0		0,8-194	2	66,76	-	0	
	Doxycycline	-	0		1,3-1,5	1	82	1,2	1	82
Quinolones	Norfloxacin	$3{,}0 \times 10^{-4}$-6,8	6	54,59,60,62,71,76	1,0-69,3	3	66,76,77	1,9-370	3	58,85,86
	Ofloxacin	$2{,}0 \times 10^{-4}$-5,1	6	54,59,60,62,76,87	1,0-458	3	66,76,77	1,2-242	3	70,86,88
	Enrofloxacin	$1{,}8 \times 10^{-3}$-$5{,}7 \times 10^{-2}$	3	54,76,89	0,3-4,8	3	66,76,77	1,3-30,6	2	70,85
	Ciprofloxacin	$2{,}0 \times 10^{-3}$-0,4	4	54,62,76,79	0,2-42,9	2	66,76	7,3-208	3	58,85,86
	Flumequine	-	0		-	0		2,9-25	1	58
Sulfonamides	Sulfamethoxazole	$5{,}0 \times 10^{-4}$-0,8	22	54,59,62–64,69,72,73,78,79,81,87–92	0,7-1,1	2	66,69	0,54-20,1	2	82,86
	Sulfadiazine	$2{,}0 \times 10^{-5}$-$4{,}1 \times 10^{-2}$	5	54,62,72,73,78	0,1-1,7	3	66,76,82	2,1-20	3	58,82,86
	Sulfamethizole	$1{,}6 \times 10^{-2}$	1	69	0,4-1,3	1	82	0,2	1	69

	Sulfathiazole	2,0 x10^{-5}-6,3 x10^{-3}	2	[73,89]	0,1-1,9	1	[82]	0,2	1	[82]
	Sulfamerazine	1,8 x10^{-2}	1	[89]	0,4-3,7	2	[66,82]	3.3-5,8	2	[82,86]
	Sulfamethazine	1,0 x10^{-4}-0,1	5	[54,59,62,76,79]	0,3-4,8	3	[66,77,82]	1,0-24	2	[70,82]
	Sulfadimethoxine	1,0 x10^{-5}-1,0 x10^{-3}	4	[72,73,89,92]	-	0		-	0	
	Sulfapyridine	1,2 x10^{-2}-0,2	1	[89]	0,7-9,1	1	[66]	-	0	
	Sulfamethoxypyridazine	-	0		-	0		1,64-1	1	[82]
Cefalosporinas	Cefalexin	2,0-493	3	[59,71,76]	-	0		-	0	
	Trimethoprim	0,03-870	17	[54,61,62,64,69,71–73,76,79,87–89,91,93,94]	18,2	1	[69]	4,0-15	2	[58,94]
	Metronidazole	4-13,4	2	[61,87]	-	0		-	0	
	Chloramphenicol	-	0		1,0-2,3	1	[82]	1,8	1	[82]

*More than one monitoring campaign may be included in the same reference.

1.4.1 Antibiotics occurrence in seawater

Seawater has been the most analyzed marine compartment. Most of the quantified antibiotics in seawater were found at concentrations below 1 ng/L, though achieving 15 µg/L on some occasions[76]. The antibiotic most commonly detected was sulfamethoxazole, which was reported in 22 studies with levels above its quantification limit. The second most frequently detected antibiotic in seawater was trimethoprim (17 studies) followed by clarithromycin (12 studies) (Table 2). According to the values reported in table 2, the highest concentration was determined for the tetracycline family, concretely oxytetracycline (15,2 µg/L), but also tetracycline antibiotic was detected at high concentrations (above 2,0 µg/L)[76]. Tetracyclines are followed by the quinolones norfloxacin and ofloxacin, which were detected at concentrations of 6,8 and 5,1 µg/L, respectively[62]. The highest concentration for a macrolide antibiotic was for the metabolite of erythromycin (erythromycin-H2O) with 1,9 µg/L[59]. Sulfonamides were detected in concentrations up to 0,8 µg/L (sulfamethoxazole)[90], whereas penicillin's up to $8{,}9 \times 10^{-2}$ µg/L (ampicillin)[76], table 2.

1.4.2 Antibiotics occurrence in marine sediment

Considerably less information is available regarding antibiotics occurrence in marine sediments compared to seawater. Antibiotics that are discharged into the marine environment can absorb on suspended solids and deposit on marine sediment in those places with low flow. Table 2 summarizes those antibiotic concentrations found in marine sediment as well as the number of studies reporting antibiotic occurrence in sediments. Most of the antibiotics detected at levels above their quantification limits had concentrations below 1 ng/g dry weight. The most recurrent antibiotic in marine sediments was tetracycline reported in four different studies, followed by erythromycin, erythromycin-H20, norfloxacin, ofloxacin, enrofloxacin, sulfadiazine and sulfamethazine (all reported in 3 studies), Table 2. As the case of water analysis, some punctual high concentrations of antibiotics have been detected in marine sediment. According to table 2 the highest concentration of an antibiotic found in marine sediment corresponded to ofloxacin (quinolone antibiotic) with a concentration up to 458 ng/g[66], followed by chlortetracycline (194 ng/g)[76] and oxytetracycline (176 ng/g)[76], both belonging to the tetracycline family. The highest concentration reported for a macrolide antibiotic was for erythromycin-H_2O, 65,3 ng/g[76] and for a sulfonamide antibiotic was for sulfapyridine, 9,1 ng/g[66] (Table 2).

1.4.3 Antibiotics occurrence in marine biota

Bioaccumulation of antibiotics in marine organisms is a crucial step in order to evaluate its potential risk for the aquatic environment and to human health. Organisms exposed to environmental contaminants may incorporate them through water or diet, and may thus be transferred through the food chain[95]. Antibiotic accumulation in different marine organisms has been reported mainly in fish and mollusks, including those used for human consumption[75,78,94,96–99]. The antibiotics most frequently detected in marine biota were oxytetracycline and erythromycin in 5 different studies each (Table 2). The levels reported were in general below 1 ng/g dry weight (dw). However, monitoring campaigns reported quinolones concentration up to 370 ng/g dw for norfloxacin[68], 242 ng/g dw, for ofloxacin[68] and 208 ng/g dw for ciprofloxacin[68]. The tetracycline antibiotic oxytetracycline was also reported with concentrations higher than 100 ng/g dw in marine biota[83]. Macrolides, sulfonamides and penicillin were reported in all cases in concentrations below 100 ng/g dw (table 2) being the highest concentration reported for erythromycin, 87 ng/g dw (macrolide)[67], sulfamethazine, 24 ng/g dw (sulfonamide)[70] and ampicillin, 10 ng/g (penicillin)[58].

There is an increasing concern regarding antibiotic occurrence in the marine biota, especially in aquatic organisms used as human food sources. The presence of antibiotics in seafood has potential harmful effects for human health such as allergy or toxicity[96] and it is likely contributing to the spread of antibiotic resistance in aquaculture environments[100]. Thus, authorities have established Maximum Residue Limits (MRL) for some antibiotics in foodstuff from animal origin, including seafood[101]. Table 3 summarizes the antibiotics for which MRL have established by the European Union in food producing animals. A total of 23 antibiotics have been included in the legislation with MRL ranging from 30 up to 500 ng/g wet weight (Table 3). The regulated antibiotics in seafood include antibiotic representatives from the most commonly used families such as penicillins, macrolides, β-lactams, sulfonamides, fluoro(quinolones) and tetracyclines, table 3.

Table 3 List of antibiotics/anti-infectious in alphabetic order included in the EU legislation (Commission regulation (EU) No 37/2010) and the established MRL (ng/g, wet weight) for foodstuff from animal origin including seafood[101].

Antibiotic	**Concentration ng/g**	**Antibiotic**	**Concentration ng/g**
Amoxicillin	50	Flumequine	200
Ampicillin	50	Lincomycin	100
Benzylpenicillin	50	Neomycin	500

Chlortetracycline	100	Oxacillin	300
Cloxacillin	300	Oxalinic acid	100
Colistin	150	Oxytetracycline	100
Danofloxacin	100	Sarafloxacin	30*
Dicloxacillin	300	Tetracycline	100
Difloxacin	300	Thiamphenicol	50
Enrofloxacin	100	Tilmicosin	50
Erythromycin	200	Tylosin	100
Sulfonamides	100		

*Only Salmonidae species

1.5 Evaluation of environmental impact

1.5.1 Classical ecotoxicological approaches

In order to evaluate the effects of antibiotic pollution in aquatic organisms, different ecotoxicological approaches have been used. Ecotoxicology studies have evolved in accordance with the development of new technologies. Traditionally, the ecotoxicology studies used to characterize the response of organisms to chemical exposure have focused on organism level, evaluating parameters such as survival, reproductive function or nutrient cycling[102,103]. The response of the organism was measured at different concentrations of the chemical or chemical mixture in order to obtain a dose-effect relationship. Later on, the ecotoxicological studies focused on understanding sub-organism level effects due to chemical exposure. In this sense, the potential effects of a contaminant were determined at organ or cellular level. The use of batteries of enzymatic activities was developed for different model species and used to test a wide range of physiological responses[104]. Some of the most commonly monitored parameters using enzymatic activities include oxidative stress, through the characterization of specific enzymes capable to determine the occurrence of reactive oxygen species within an organism, which may cause tissue damage. Catalase activity (CAT), Glutathione Peroxidase (GPX) and Glutathione Reductase (GR) are some of the most used enzymatic activities to evaluate oxidative stress in different organisms. Neurotoxic effects are also commonly monitored characterizing the activity of acetylcholinesterase (AChE) enzyme, the impairment of AChE activity is indicative of an impeded neurotransmission function. Besides, the capacity of organisms to metabolize toxic substances within the body can be monitored characterizing the functioning of phase II metabolic enzymes such as glutathione-S-transferase (GST) and Carboxylesterases (Cbe)[105].

Antibiotics ecotoxicological effects at the organism level have been mainly evaluated in natural bacterial communities and microorganisms as they are the main target of antibiotic activity[106,107]. These studies have evaluated the effects of antibiotics exposure on bacterial community composition and metabolism[106,107]. Results reported highly different responses

depending on the combination antibiotic-exposed organism. For instance, Valitatlo et al. 2017[69] pinpointed cyanobacteria and ammonium oxidizing bacteria to be the most sensitive microorganisms to antibiotics (despite other microorganisms can be also affected). The study[69] considered literature information regarding the effect concentration of different antibiotics where the 50% of its maximum effect is observed (EC_{50}) in different microorganisms. The evaluation of antibiotics effects at organism level has also been performed with aquatic biota such as crustaceans, mollusks or fish. The results obtained in these studies indicated that these organisms are less sensitive to antibiotics than bacteria. Isidori et al. 2005[108] evaluated the ecotoxicological effects of six antibiotics, erythromycin, oxytetracycline, sulfamethoxazole, ofloxacin, lincomycin and clarithromycin on different aquatic organisms including bacteria, algae, rotifers, microcrustaceans and fish. The reported results determined that algae were the most sensitive organisms to antibiotics exposure, whereas rotifers, crustaceans and fish were only affected when exposed to high doses of antibiotics. They reported lethal or effect concentrations affecting the 50% of the tested organism populations $L(E)C_{50}$, between 10 to 1000 mg/L[108]. In line with this, Park et al. 2008[109], determined LC_{50} of *O. latipes* fish when exposed to high concentrations of antibiotics (neomycin, oxytetracycline and chlortetracycline), 80-110 mg/L during 96h.

Sub-lethal effects of antibiotic contamination have been previously reported in aquatic organisms. Rainbow trout fish presented a significant increase of CAT activity (related to oxidative stress) in gills when exposed to erythromycin antibiotic at 100 µg/L[110]. Histological alterations were observed in rainbow trout fish due to oxytetracycline exposure at 5 µg/L[111]. Histological damage in gills and alterations in the enzymatic activities CAT in liver and GST in gills were observed in fish species *Gambusia holbrooki,* when exposed to tetracycline at concentrations between 0.005 µg/L to 0.5 µg/L[112]. These results showed that although lethal effects of antibiotics are hardly expected in aquatic organisms like fish in the natural environment, sub-lethal alterations can be observed at much lower concentrations, in some cases very close to environmentally relevant concentrations.

1.5.2 Metabolomics approach

In recent years the omics approaches have gained importance in ecotoxicological studies[113]. Omics disciplines include genomics (the study of organism's genome using DNA analysis), transcriptomics (the study of organism's transcriptome using mRNA analysis), proteomics (the study of organism's proteome using protein analysis) and metabolomics (the study of organism's metabolome, using low molecular weight molecules, metabolites) (Figure 2). Metabolomics

focuses on the analysis of low molecular weight molecules, which are representative of multiple factors related to an organism (or community) physiological state[114]. In other words, metabolomics allows characterizing the biochemistry of an organism at a defined moment[114].

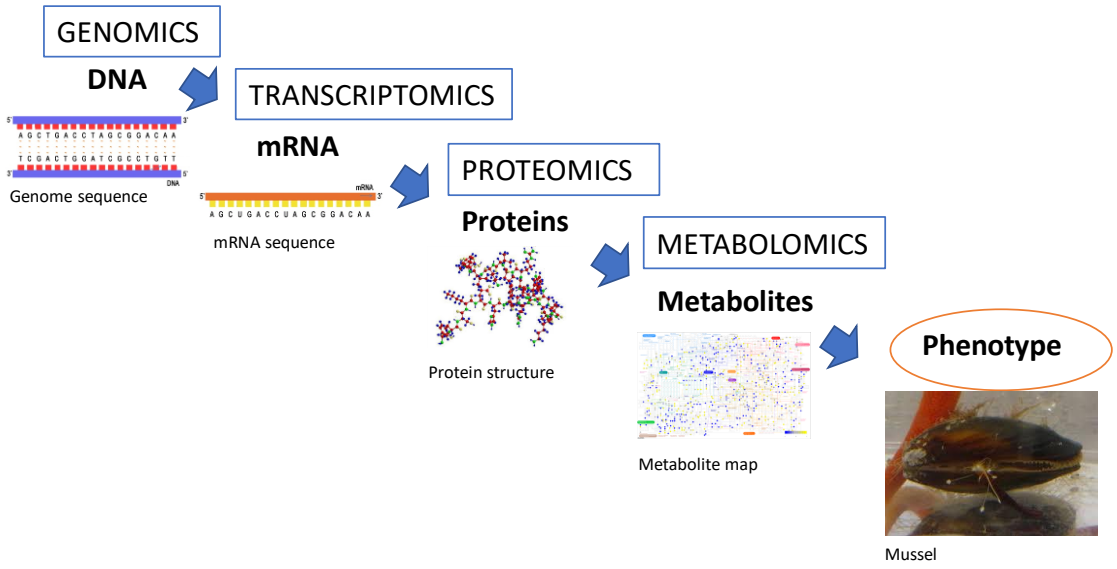

Figure 2. Omics scheme adapted from Shiratake et al. 2016[115].

In ecotoxicological studies, metabolomics has lately been used to characterize alterations in metabolite concentrations and metabolic pathways in exposed organisms due to stress conditions[113]. Two main approaches are followed in metabolomics studies; they are called target and non-target metabolomics. Target metabolomics refers to the measurement and commonly to the quantification of a selected group of metabolites. It requires prior knowledge of the identity of the metabolites of interest. On the other hand, non-target metabolomics aims to characterize the whole metabolome. It focuses on the identification of metabolites and in some cases on their quantification (by running standard calibration curves when they are available for the tentatively identified metabolites) related to the stress factor studied.

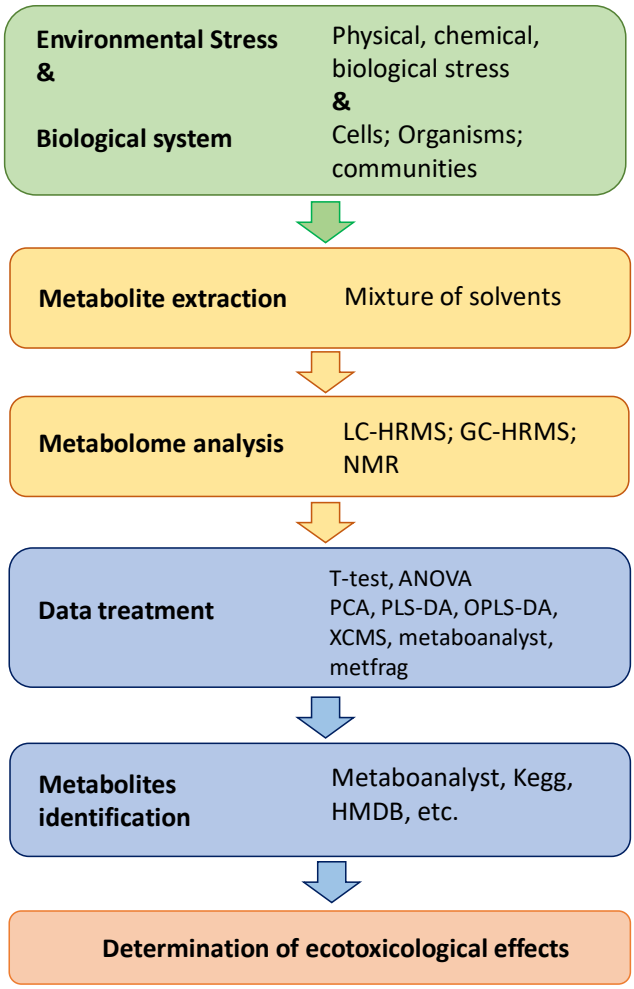

Figure 3. Non-target metabolomics workflow; adapted from Naz et al. 2014[116].

Metabolomics general procedure applies to a biological system under study exposed to a selected stress factor or combination of stress factors. Once the biological system has been exposed to the stress conditions, different biological targets (whole organism extract, tissues or biofluids) can be considered for their analysis depending on the objectives of the study. Figure 3 summarizes the most important steps for the non-targeted metabolomics approach[116]. Metabolite extraction is a key step in metabolomics analysis. The extraction procedure must be "as general as possible" in order to extract the higher number of metabolites possible comprising compounds of different physical-chemical properties[116,117]. Usually, a mixture of solvents (with different polarity) is mandatory in order to extract both polar and nonpolar metabolites[118–120]. However, a target extraction can be performed if the focus of the study is a specific group of metabolites[117]. After metabolites extraction, the samples can be analyzed with different techniques, nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) are the most commonly used analytical platforms for metabolomics

approaches[117,121]. The combination of the biological system analysis with the advanced analytical techniques (HRMS or NMR) produces a high amount of data that need to be processed[116] especially when non-target metabolomics is performed. Advanced statistical treatments are needed to process the data and evaluate the ecotoxicological effects. Multivariate analyses such as principal component analysis (PCA) or partial last squares (PLS) regression analysis are some of the most employed approaches[122]. Statistical data treatment allows identifying those metabolites or groups of metabolites, which levels significantly change due to stress factors. However, identification, confirmation and, in some cases, quantification of these metabolites is a complex task[122]. Commercial software like SIEVE or Compound Discoverer, online tools such as Metaboanalyst[123,124] or Metfrag[125], and metabolites databases like Human metabolomics database[126] and Kyoto Encyclopedia of Genes and Genomes[127] are some of the most used techniques for confirmation purposes. Once metabolites have been identified, the final step is to link these metabolites with the metabolic pathways where they are involved, which may be altered due to the external stressor, and ultimately to pathologies.

Metabolomics has been scarcely used to characterize aquatic organisms' response to antibiotic pollution. In fact, to the best of authors knowledge, only one study applied metabolomics to characterize antibiotics effects to aquatic organisms[128]. Sotto et al. 2017[128] applied different ecotoxicological approaches, including metabolomics, to characterize zebrafish response to antibiotics (clarithromycin, florfenicol and sulfamethazine) exposure. The ecotoxicological studies included fish embryo test, color preference experiment, and non-target metabolomics. Metabolomics results highlighted choline, guanosine and ADP as the most affected metabolites in exposed fish, being glycerophospholipid metabolism (related to cell membrane assembly and functioning) the most altered pathway. In this study metabolomics showed to be the most sensitive approach compared to the rest of the ecotoxicological approaches used in that study, to characterize the effects of antibiotics showing disruptions on metabolites and metabolic pathways[128], which demonstrates its applicability in ecotoxicological studies.

1.5.3 Antibiotic resistance

Antibiotic resistance is reported when an antibiotic loses its capacity to act against a target bacterium and then, the bacteria continue multiplying in presence of a therapeutic concentration of the antibiotic[129]. Antibiotic resistance can occur in the environment as a natural selection process when bacteria carrying antibiotic resistance genes (ARGs) are selected among others. However, the misuse and overuse of antibiotics due to human activities can promote and/or accelerate this situation[130]. The antibiotic resistance phenomenon was documented

since the early beginning of human antibiotic discovering and utilization. In fact, Alexander Fleming highlighted the potential resistance to penicillin if used for a too short time period or used in concentrations below its therapeutic concentration[131]. Figure 4 shows a schematic representation of antibiotic resistance bacteria selection when an antibiotic occurs in the environment.

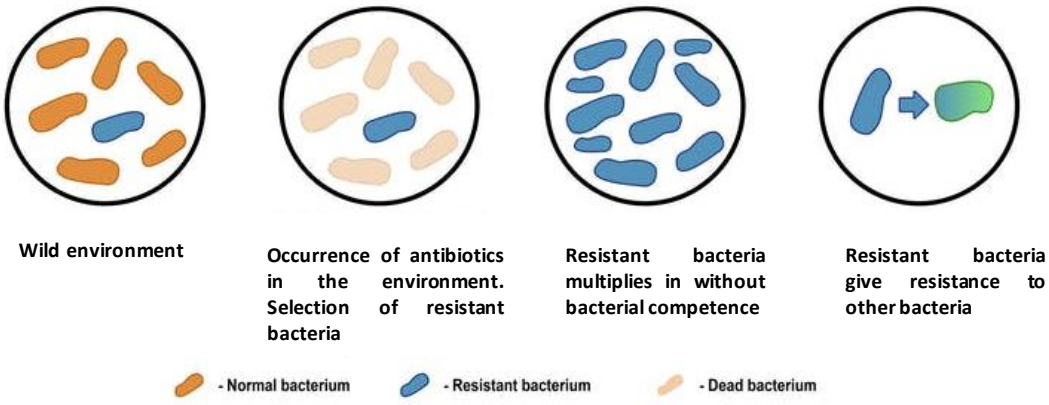

Figure 4. Scheme of selection process of resistant bacteria in an antibiotic polluted environment. Adapted from shutterstock[132].

Bacteria can have intrinsic resistance to antibiotics, then, antibiotic presence can exert selective pressure to favor the multiplication of those bacteria carrying out genes that confer resistance to the exposed antibiotic/s (Figure 4). Antibiotic resistance genes can provide resistance to bacteria with different mechanisms such as inactivating antibiotics, decreasing antibiotic permeability or changing the target site. Resistant bacteria can synthetize enzymes capable to modify the antibiotic molecule. For instance, the β-lactamases enzymes provide resistance against beta-lactamase antibiotics through the hydrolysis of the beta-lactam ring avoiding the capacity of the molecule to bind to the cellular receptor, provoking the antibiotic inefficiency[133]. On the other hand, resistant bacteria can reduce intracellular antibiotic accumulation by regulating protein channels avoiding antibiotic penetration by increasing the expression of efflux pumps to promote antibiotic expulsion from the cell[134]. Besides, bacteria can modify the antibiotic target site within the bacteria cell (through spontaneous mutation) avoiding the activity of the antibiotic[135].

Bacteria can acquire resistance to antibiotics by incorporating resistant genes through different mechanisms. i) Vertical Gene Transfer (VGT) refers to the ability of bacteria to transfer resistance to its progeny through DNA replication. This mechanism allows transferring resistance to other bacteria from the same species. ii) Horizontal Gene Transfer (HGT), refers to the ability

of bacteria to incorporate genetic material from other bacteria coming from the same specie or even from other species. There are different mechanisms of HGT such as the incorporation of DNA present in the surrounding environment (i.e. due to the lysis of another bacteria cell), named transformation mechanism. Conjugation refers to the direct DNA transference between two bacteria cells through a physical bridge between the bacteria. Besides, bacteriophages (viruses that infect bacteria), can play an important role in antibiotic resistance dissemination through transduction which is the mechanism where one bacterium acquires DNA material from another through the infection of a bacteriophage [136–138]. This high capacity of bacteria to modify their DNA facilitates bacterial antimicrobial resistance acquisition. This spread of antimicrobial resistance is of special concern when pathogenic bacteria acquire it as it compromises the effectiveness of the antibiotics used.

Due to the above mentioned concern regarding antibiotic resistance of pathogenic bacteria, ARGs have been lately considered contaminants of emerging concern in the environment[139]. Different ARGs have been pointed out for conferring resistance to the most used antibiotics and antibiotic families (Table 4). Antibiotic resistance genes spread in those environments combining bacterial and antibiotics occurrence and therefore the selective pressure favors the multiplication of the bacteria carrying ARGs. Urban sewers containing bacteria and antibiotics excreted, hospital environments and farms are reported as some of the most important environments where ARGs spread occur[140]. Then, ARGs can enter into the aquatic environment through the discharge of untreated wastewater (collecting ARGs from urban and hospital effluents), but also from treated effluents[141], which indicates that conventional WWTPs cannot completely eliminate ARGs. Besides, the application of animal manure to fertilize the soil can provoke the spread of ARGs into the terrestrial environment[51]. Then, due to runoff processes, these ARGs can be transferred to surface and groundwater. Therefore, marine ecosystem can be polluted with these contaminants through river contributions, through the direct discharge of WWTPs effluent or through antibiotic use in aquaculture [142,143]. Consequently, the occurrence of ARGs is monitored in the above mentioned ARGs sources as well as in water, sediment and biota of natural environments in order to understand their environmental fate and relevance.

Table 4. Examples of ARGs and the corresponding antibiotic or antibiotic family that they confer resistance to.

Antibiotic class	**ARGs (number of variants)**	**Related antibiotic**
Penicillins	blaamp (111), blaCTX (124), blaOXA (2014), blaSHV (202), blaTEM (150)	Amoxicillin, amplicillin and other penicillins
Macrolides	car (2), cfr (2), erm (30), ole (2), srm (1), tlr (2)	Erythromycin and other macolides
Fluoroquinolones	qnr (60), qep (2), nor	All quinolones
Aminoglycosides	aac (67), aad (28), aph (32), str (2)	All aminoglycosides
Sulfonamides	sul (3)	All sulfonamides
Tetracyclines	tet (44)	All tetracyclines

Monitoring studies have highlighted the widespread distribution of ARGs in fertilized soils, surface water (rivers and lakes), groundwater and seawater[93,144–147]. Genes conferring resistance to macrolides (MLSb) beta-lactams (*bla*$_{GES}$, *bla*$_{OXA}$, and *bla*$_{VEB}$), sulfonamides (*sul1*), tetracyclines (*tetM* and *tetQ*), aminoglycosides (*aadA* and *strB*) and multidrug resistance (*qacEdelta1* and *qacH*), have been extensively detected in different environmental compartments along Europe[146,147]. In accordance with the detection of ARGs, increasing evidence of bacteria resistant to commonly used antibiotics has been observed. For instance, antibiotic resistant bacteria, such as *Acinetobacter* and *Aeromonas* sulfamethoxazole-resistant genera were found in aquaculture environment[148]; whereas multiple-antibiotic resistant bacteria were found in surface waters (rivers) and groundwater[149].

The European Center for Disease Control and Prevention calculates that 33000 people die every year from an infection caused by a bacterium resistant to antibiotics[150]. As an example, figure 5 shows the percentage of resistant *E. coli* to third-generation cephalosporins, fluoroquinolones and aminoglycosides in European counties EU/EEA. As a consequence of the current situation and the forecasted future, a huge concern is growing in the scientific community, decision makers and population [150]. This concern is based on the fact that if bacteria continue acquiring resistance, together with the difficulties to develop new antibiotics, the treatment of infectious diseases will be compromised, with the subsequent risk for human and animal health.

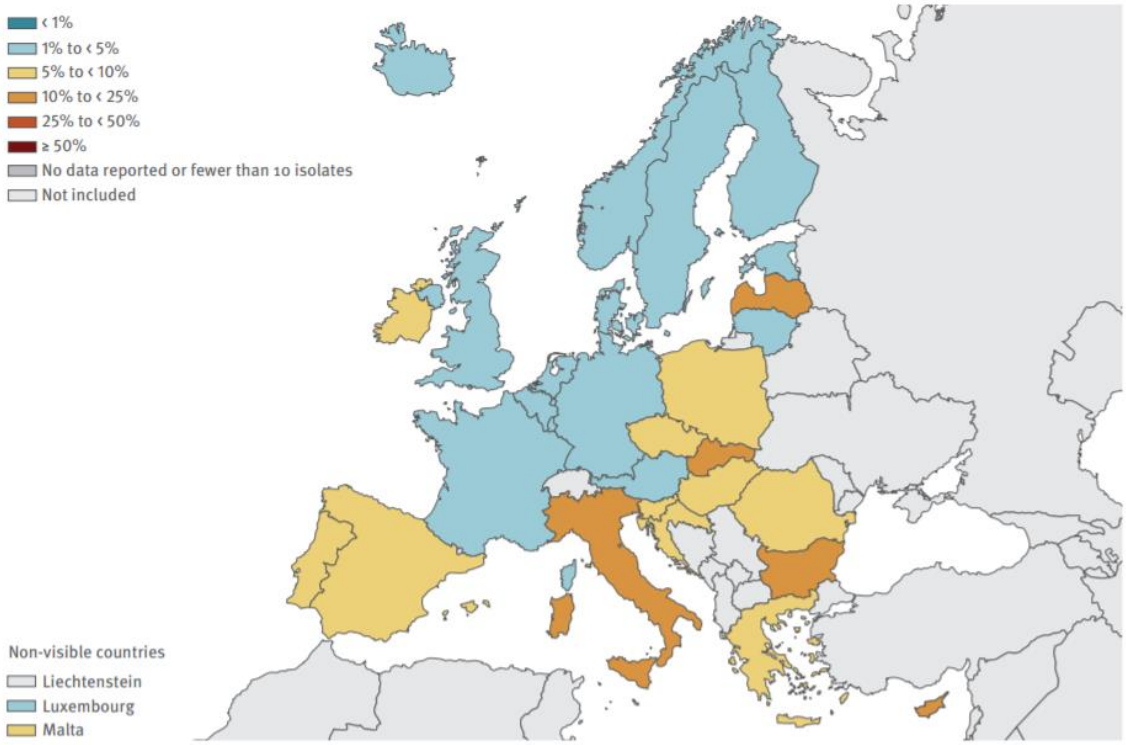

Figure 5. Percentage of resistant *E. coli* to third-generation cephalosporins, fluoroquinolones and aminoglycosides in European counties EU/EEA, year 2017, map source European Comision[113]

In order to combat the spread of ARGs, strategies to reduce the amount of antibiotic occurring in the environment are being investigated. The effectivity of the waste water treatment plants has been studied [151,152], as well as different tertiary treatments. Ozonation, photo-fenton or UV-based elimination have been tested, demonstrating different efficiencies in removing ARGs. For instance, the efficiency of different UV-based treatments was compared to eliminate ARGs[153] but its effectivity was highly dependent on the water matrix treated. Furthermore, other treatments such as constructed wetlands [154], or elimination of ARGs by algae and fungi have also been tested[155]. Despite the high effort done in testing different strategies to reduce the occurrence of ARGs in the environment, further research is needed in order to understand the mechanisms of ARGs inactivation and controlling HGT. Besides, tests with real water and real conditions will provide further understanding in the strategy for fighting against the worldwide antimicrobial spread.

1.5.4 Environmental risk assessment

In order to estimate the impact of contaminants including antibiotics in the environment, environmental risk assessment (ERA) has gained importance[156]. Environmental risk is assessed by comparing the environmental concentration of a given compound (or the predicted

environmental concentration) by the worst case predicted non-effect concentration (PNEC)[156,157]. PNECs are determined by standard toxicity tests. When no information regarding ecotoxicological effects of a compound is available, Quantitative Structure-Activity Relationships (QSARs) models can be used to predict compound properties such as toxicity or bioaccumulation potential, among others[158,159]. QSAR models use compound parameters such as chemical structure, molecular size or octanol-water partition coefficient to estimate the desired compound properties[159]. Environment risk assessment facilitates determining the risk potential of a novel or currently used pharmaceutical in the environment, as well as, allows prioritizing the compounds with a high-risk potential in the environment. This approach has been widely applied to investigate the environmental risk of emerging pollutants, including pharmaceutical compounds and antibiotics. In a recent study, the environmental risk was assessed for 593 pharmaceutical compounds in China[160]. The three compounds that showed a higher potential risk for the environment (HQ>10) were macrolide antibiotics namely, erythromycin, azithromycin and clarithromycin[160]. These results highlighted antibiotics as an important group of contaminants with a need for prioritization in environmental monitoring and management.

Recently, a specific ERA for antibiotics has been postulated considering the contribution of antibiotic pollution to the development of antibiotic resistance[161]. In this case, the lowest concentration of a given antibiotic to promote resistance is considered in combination with the ecotoxicological PNEC. Based on the worst-scenario approach the lowest of both PNECs (ecotoxicological or antibiotic resistance promotion) is considered for the ERA calculation of a given antibiotic[162]. This approach has been recently applied to evaluate the risk posed by antibiotics discharged through WWTP effluents into freshwater bodies of several countries in Europe[163]. In this study, ciprofloxacin, azithromycin and cefalexin were the antibiotics posing the highest risk. Besides, Du et al. 2019[164] determined the antibiotic risk assessment, considering both ecotoxicological and antimicrobial resistance risks, in a coastal area influenced by mariculture in the Bohai Sea (China). They determined low ecotoxicological risks of individual antibiotics, despite the effects of antibiotic mixture was highlighted as a matter of concern. Furthermore, enrofloxacin antibiotic showed concentrations high enough to promote antimicrobial resistance in the studied area[164]. These results highlighted the need of considering both, antibiotic ecotoxicological effects and the contribution to the spread of antimicrobial resistance, to broadly address the risks of antibiotic pollution to the aquatic ecosystems.

Chapter 2 - Objectives

The main goal of this thesis was to evaluate the accumulation of antibiotic residues in marine biota including wild and aquaculture organisms such as fish, mussel and clam. The second core objective of this thesis was to determine the environmental impact of antibiotic contamination in these marine organisms.

To accomplish these objectives, the following specific goals were defined:

i. To develop and validate analytical methodologies for the determination of antibiotic residues in different matrices including seawater, fish, mussel and clam.

ii. To study antibiotics occurrence in wild and aquaculture marine organisms, through the utilization of the methodologies developed.

iii. To evaluate the bioaccumulation of antibiotics – and other emerging contaminants – in marine organisms using exposure experiments with different environmental conditions such as climate change scenarios (water warming and acidification).

iv. To assess the effects of antibiotic exposure in marine mussel through classical (enzymatic activities) and novel (metabolomics) ecotoxicological approaches.

v. To determine the contribution of antibiotic pollution to the spread of antibiotic resistance within the bacteria located in the mussel's gastrointestinal tract.

According to these objectives and based on the published articles, the content of this thesis is structured in two more chapters:

Chapter 3: Analytical methodologies antibiotics determination

Article 1. Multi-residue method for the determination of antibiotics and some of their metabolites in seafood

Article 2. Combining an effect-based methodology with chemical analysis for environmental risk assessment of antibiotics

Chapter 4: Environmental impact of antibiotics

Article 3. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*).

Article 4. Comprehensive study of sulfamethoxazole effects in marine mussels: bioconcentration, enzymatic activities and metabolomics.

Article 5. Subinhibitory sulfonamide concentration promotes the spread of antibiotic resistance in marine blue mussels (*Mytilus edulis*).

Chapter 3 - Analytical methodologies for antibiotics determination

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Multi-residue method for the determination of antibiotics and some of their metabolites in seafood

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ABSTRACT

The presence of antibiotics in seafood for human consumption may pose a risk for consumers. A methodology for the analysis of antibiotics in seafood based on QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction, followed by detection and quantification using liquid chromatography coupled to mass spectrometry was developed. The analytical method was evaluated for the determination of 23 antibiotics (including parent compounds and some metabolites) in fish, mussels and clams. Recoveries ranged between 30% and 70% for most of the compounds and method detection and quantification limits (MDLs and MQLs) were between 0.01 and 0.31 ng/g dry weigh (dw) and 0.02–1.03 ng/g (dw) respectively. Real seafood samples were analysed using this method. Nine antibiotics were found at levels above MDLs; however none of them exceed the maximum residue limits (MRL) established by the authorities. Tetracycline was the most ubiquitous compound, presenting also the highest concentration: 5.63 ng/g (dw) in fish from Netherlands. In addition, an alternative technique based on microbial growth inhibition was explored as semiquantitative detection method of antibiotics in seafood. This methodology could be applied as a fast screening technique for the detection of macrolides and β-lactams in seafood but further research is needed for other antibiotics families.

1. Introduction

Antibiotics usage in human and veterinary medicine has become a common therapeutic practice (Manzetti and Ghisi, 2014). This high antibiotic consumption, resulted in a gradual accumulation of antibiotics in the water bodies, being wastewater discharges, agricultural runoff and aquaculture the most important sources of this type of contamination into the environment (Loos et al., 2013; Nödler et al., 2014). It is well known that antibiotics pose a significant risk to environment, even at low concentrations (Kümmerer, 2009). For example antibiotics like bacitracin, flumequine, lincomycin and aminosidine showed to be harmful to aquatic organisms such as *Artemia* (Migliore et al., 1997), or metronidazole which showed a toxic effect to *Chlorella* spp and *Selenastrum capricornutum* (Lanzky and Halting-Sørensen, 1997). In addition, the occurrence of antibiotics in the natural aquatic systems may pose a risk for the wild organisms due to their bioaccumulative potential as for instance roxithormycin that showed a bioaccumulation factor higher than 600 L/Kg in different aquatic organisms (Xie et al., 2015). Furthermore, the bioaccumulation factor of some antibiotics in fish has been reported to be higher than 3000 L/Kg (Gao et al., 2012) in agreement with this, Chen et al. (2014) reported a bioaccumulation factor of 6488 L/Kg for trimethoprim in fish (*Lutjanus russelli*). Residues of these drugs can remain in fish tissues with the consequent potential risk of exposure for fish consumers (Cabello, 2006); especially when antibiotics are accumulated in seafood species highly consumed by the population. The use of antibiotics in food producing animals may provoke undesirable effects on consumer's health. If antibiotics are present at high enough concentrations in food producing animals then they may cause allergies or development of antibiotic resistant bacteria

Abbreviations: ACN, acetonitrile; CAFOs, confined animal feeding operations; dSPE, dispersive solid phase extraction; dw, dry weight; EU, european union; IS, internal standards; MDLs, method detection limits; MQLs, method quantification limits; MRLs, maximum residue limits; QuEChERS, quick, easy, cheap, effective, rugged, and safe; SPE, solid phase extraction; UHPLC-MS/MS, ultra high pressure liquid chromatography- tandem mass spectrometry; US, ultrasonic extraction; ww, wet weight.

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(Alderman and Hastings, 1998; Cañada-Cañada et al., 2009) causing treatment resistant illness, which can be a human health problem when treating infections (Heuer et al., 2009).

In order to protect human health and avoid the potential risks above mentioned, regulatory authorities like the European Union (EU) establishes Maximum Residue Limits (MRLs) for some pharmaceutical compounds, including antibiotics, in different foodstuffs from animal origin like fish and others seafood species (EU No 37/2010). Seafood for human consumption produced in aquaculture are likely to contain antibiotic residues since many antibiotics are commonly used in confined animal feeding operations (CAFOs) and aquaculture activities in order to treat or prevent bacterial infections (Stolker and Brinkman, 2005). Therefore, information regarding the presence of antibiotics in seafood is crucial for evaluating the fate, environmental effects, and human health risks of these substances. Most of the analytical methods developed so far have focused on one (Samanidou et al., 2008) or few (Evaggelopoulou and Samanidou, 2013) antibiotic families. Moreover, most of them were specific for one organism class like fish (Cháfer-Pericás et al., 2010a) or shrimps (Villar-Pulido et al., 2011). Analytical methods able to detect a broad spectrum of antibiotics are still scarce (Dasenaki and Thomaidis, 2010; Fedorova et al., 2013; Li et al., 2012). The limited number of analytical methods covering the detection of antibiotics belonging to several chemical families may be explained by the difficulty of the simultaneous extraction of antibiotics with different physicochemical properties. The extraction procedure technique and the solvents used are key issues for the simultaneous extraction of different antibiotics. Usually a compromise should be made between the extraction conditions and good performance of the method in terms of recovery, sensitivity, reproducibility, etc. Furthermore, most of the methods developed focus on pharmaceutical compounds administered to humans or animals, but few of them include antibiotics metabolites (Fernandez-Torres et al., 2011). The inclusion of antibiotics metabolites in multi-residue analytical methods is of great interest since they can be accumulated even at higher degree than the antibiotics themselves (Gros et al., 2013), and can be as bioactive or even more than the corresponding parent compound. As example, García-Galán et al. (2012) found that acetylated metabolites of some sulfonamides can be more toxic than the parent compound. According to this paper a risk classification ranked N_4-acetylsulfapyridine metabolite as toxic, whereas its parent compound, sulfapyridine, was classified as harmful (European Commission, 2002). However, other studies suggested that metabolites of antibiotics like sulfonamides may reduce their toxicity in microalgae (Eguchi et al., 2004).

Most of the methods mentioned above for the analysis of antibiotics in seafood are based on detection with LC-MS/MS (i. e. Dasenaki and Thomaidis, 2010; Fedorova et al., 2013). However, alternative detection methodologies like immunoassay techniques or microbial growth inhibition tests have been tested for the analysis of antibiotics in seafood, but its applicability is still scarce. Immunoassays were applied for the detection of oxytetracycline (Cháfer-Pericás et al., 2010c) and sulfonamides (Cháfer-Pericás et al., 2010b) in fish samples. Some of them are commercially available, such as ELISA test kits for the specific detection of antibiotics like tetracyclines, β-lactams or chloramphenicol in seafood and meat (Randoxfood, 2016). A microbial growth inhibition test was applied for the analysis of three antibiotic families including quinolones, sulfonamides and tetracyclines in shrimps (Dang et al., 2010); whereas Barker, (1994) applied this methodology for the specific analysis of quinolones in fish. Some kits based on microbial growth inhibition are also commercially available i.e. PremiTest Antibiotic Test (Nelsonjameson, 2016), which provides a qualitative detection of a broad spectrum of antibiotics. Microbial growth inhibition tests are not as sensitive as LC-MS/MS methods and do not allow to distinguish between individual compounds. This type of test is rather intended as a screening methodology for the preliminary detection of some antibiotic residues and its metabolites with a similar mode of action in different types of food from animal origin. Furthermore, the application of this screening technique does not require the use of complex instrumentation. This would reduce the cost of the analysis and facilitate the implementation of this technique as routine method for the analysis of seafood in laboratories or aquaculture facilities.

The aim of this paper was to develop a fast methodology based on ultra high pressure liquid chromatography-triple quadrupole mass spectrometry (UHPLC-MS/MS) for the detection of antibiotics (from different chemical families), and some of their major metabolites, in several seafood matrices, especially in highly consumed species. Different extraction and clean-up procedures were tested in order to obtain a simple and fast method covering the maximum number of antibiotics possible. The method allowed the detection and identification of 23 individual compounds (including four of their major metabolites). After that, the method was applied for the analysis of real seafood samples of highly consumed species collected from aquaculture and natural environments. In addition, a method based on the inhibition of susceptible bacterium in the presence of antimicrobial residues was tested as an alternative technique for the detection of antibiotic families such as tetracyclines, quinolones, macrolides/β-lactams, amino-glycosides and sulfonamides.

2. Material and methods

2.1. Chemical and reagents

A list with the antibiotics included in the analysis based on UHPLC-MS/MS detection is presented on the supplementary material (Table S1). Antibiotic standards were of high purity grade (>90%). All antibiotic standards were purchased from Sigma-Aldrich except N-acetylsulfadiazine, N-acetylsulfamerazine and N-acetylsulfamethazine that were obtained from Toronto Research Chemicals (TRC), clarithromycin was purchased from Fluka and clindamycin from European Pharmacopeia (EP). Isotopically labelled compounds used as internal standards, azithromycin-d3, ampicilin-d5, erythromyicin-d13, ibuprofen-d3, lincomycin-d3 and sulfamethoxazole-d4 were obtained from TRC whereas ronidazole-d3, ofloxacin-d3 and ciprofloxacin-d8 were purchased from Sigma-Aldrich.

The cartridges used for solid phase extraction OASIS HLB (200 mg, 6 mL), the QuEChERS extract tubes (AOAC method), and the QuEChERS for dispersive solid phase extraction (dSPE) (15 mL, fatty acids tubes) were obtained from Water Corporation (Milford, MA, U.S.A.). PVDF filters (0.45 μm pore) were purchased from Merck Millipore Corporation (Darmstadt, Germany). HPLC grade methanol, water and acetonitrile were purchased from Merck (Darmstadt, Germany), whereas formic acid (98% purity), EDTA 0.01 mol/L, hydrochloric acid 0.1 mol/L and sodium hydroxide 1 mol/L were obtained from Sharlab (Barcelona, Spain).

Stock standards and isotopically labelled internal standards were prepared in methanol at a concentration of 1000 mg/L and stored at −20 °C. Working standard solutions containing all antibiotics and isotopically labelled internal standards (1 mg/L) were prepared in methanol/water (50/50, v/v) before each analytical run.

2.2. Sample collection and pre-treatment

Clams (*Chamelea gallina*) were the organisms selected to perform the different extraction procedures in order to find out

which one was the most suitable one for antibiotics. This organism has low fat content minimizing the co-extraction of undesirable compounds (mainly fats) that possibly will interfere in the detection of the analytes (Huerta et al., 2013). In addition, C. *gallina* are abundant and easy to capture. They were collected from the Ebro Delta, Tarragona, Spain, between November and December 2013.

The sample pre-treatment consisted in removing clam's shell and a pool with 50 individual organisms was prepared with the edible content. After homogenization, samples were freeze-dried, grounded in a mortar and kept at −20 °C until its analysis. Freeze-drying of the samples was aimed at the preservation of antibiotics in the samples, as the water content in non-dried samples may degrade the compounds. Furthermore, as antibiotics are not volatile compounds, the freeze-drying process should not affect the final amount of antibiotics present in the samples. A previous experiment regarding stability of pharmaceuticals after freeze drying was carried out and showed no loss of compounds after freeze-drying process (data not shown). Once the extraction procedure was optimized, the method based on detection and quantification of analytes using UHPLC-MS/MS was validated for the analysis of antibiotics in clams, mussels (*Mytilus galloprovincialis*), and fish (*Platichthys flesus*). Mussels were collected from the Ebro Delta, Tarragona, Spain, whereas fish was taken from the Scheldt estuary, Netherlands. Mussels were pre-treated in the same way than clams, whereas for fish samples the skin was removed and only muscle tissue was further freeze-dried, grounded in a mortar and kept at −20 °C for the analysis.

Once the method was optimized and validated, it was applied for the analysis of real samples. Eight samples were taken from aquaculture facilities (five mussels and three fish). The *Mytilus galloprovincialis* from Spain, *Mytilus galloprovincialis* from Italy, *Mytilus* spp from Netherlands, *Pangasius* spp from Vietnam, *Salmo salar* from Scotland, and *Salmo salar* from Norway were bought from local supermarkets. Whereas the two *Mytilus* spp from Greece were directly sampled in the aquaculture facility, pooled, homogenized and snap frozen before the transport. After this all the samples were freeze-dried and kept at 20 °C until their analysis. All aquaculture samples were commercialized in European countries (*Pangasius* spp was imported). Four samples (three mussels and one fish) were collected from natural environments: *Mytilus galloprovincialis* from the bay of Saint-brieuc, France, *Mytilus galloprovincialis* from Po Delta, Italy, *Mytilus* spp from Tagus estuary, Portugal, and *Platichthys flesus* from The Scheldt estuary, Netherlands.

For the microbial growth inhibition test evaluation, the mussel sample *Mytilus galloprovincialis* collected from the bay of Saint-brieuc, France, was selected.

2.3. Extraction and clean-up procedure optimization

Four different extraction and clean-up procedures were tested and a recovery study was performed in order to evaluate the efficiency of each extraction procedure. Approximately 0.5 g of freeze-dried clam tissue were weighted and placed in a glass tube. Samples were then spiked with a mixture of antibiotics and some metabolites at a final concentration of 50 ng/g (dw); half of the MRLs established by the authorities for those compounds included in the method and regulated by the authorities (sulphonamides, tetracycline, tilmicosin, tylosin and lincomycin) (European Commission, 2010). All compounds added to the spiking mix and their corresponding internal standards are listed in the supplementary information (Table S2). Besides, control samples were also analysed in order to determine the background levels of the target compounds. Both spiked and control samples were analysed in triplicate. The detection and quantification of the target compounds were done with UHPLC-MS/MS. Recoveries were then calculated by comparing the concentrations measured in the sample after the analytical procedure with the initial spiked concentration. The concentrations measured in the sample were determined by using internal sample calibration. The internal standard curve was made in clam extract in a range of 0.01–50 ng/g (dw).

Two extraction techniques were used; QuEChERS and ultrasonic bath, and four different extraction procedures were tested. Two of them based on QuEChERS (i and ii) whereas the other two were based on ultrasonic bath (iii and iv). The methods were performed as follows:

(i) QuEChERS extraction only: spiked samples were placed in a 50 mL polypropylene tube, 2 mL of HPLC water and 10 mL of ACN:MeOH (75:25, v/v) were added and shaken in a rotator shaker for 15 min. Then, the extraction salts (magnesium sulphate 6 g and sodium acetate 1.5 g) were added and the mix was shaken again for 15 min in a rotator shaker. The samples were centrifuged 5 min at 10.000 rpm. Four mL of the extract were taken out, evaporated to dryness, and reconstituted in 1 mL of MeOH. Then, the samples were filtered through PVDF filters of 0.45 μm and kept at −20 °C until its analysis.

(ii) QuEChERS extraction followed by dispersive solid phase extraction (dSPE): spiked samples were placed in a 50 mL polypropylene tube. 5 mL of HPLC water were added and vortexed for 30 s followed by the addition of 10 mL of acetonitrile (ACN) with the subsequent vortex for 1 min. Then, the QuEChERS extraction salts composed by magnesium sulphate 6 g and sodium acetate 1.5 g were added and the mix was hand shacked for 1 min. Samples were centrifuged 5 min at 10.000 rpm. The ACN layer was transferred to a tube containing the dispersive sorbents (primary secondary amine (PSA) 149.9 mg; octadecyl (C18) 149.9 mg and magnesium sulphate 900.2 mg) in order to carry out a dSPE. The sample was vortex for 1 min and centrifuged 10 min at 5000 rpm. Finally, 6 mL of the extract were evaporated to dryness, reconstituted in 1 mL of methanol (MeOH) and kept at −20 °C until its analysis.

(iii) Ultrasonic extraction (US) with ACN:water followed by solid phase extraction (SPE): spiked samples were placed in a 50 mL polypropylene tube, 5 mL of ACN:H_2O (3:1) were added; the mixture was vortexed 1 min and sonicated for 15 min. After that, the samples were centrifuged for 10 min at 3500 rpm and the supernatant was collected. This process was repeated another time. Later on, SPE was performed as follows: 240 μL of EDTA was added to each sample, and the pH was adjusted to 2.5 using hydrochloric acid. The cartridges (Oasis HLB 200 mg, 6 ml) were conditioned with 5 mL of MeOH followed by 5 mL of HPLC water at pH 2.5. After sample loading the cartridges were rinsed with 5 mL of HPLC water and dried under a gentle stream of nitrogen for 5 min. Finally, samples were eluted with 6 mL of methanol, dried down under nitrogen, reconstituted in 1 mL of MeOH and kept at −20 °C until its analysis.

(iv) Ultrasonic extraction (US) with NaOH y NaCl followed by solid phase extraction (SPE): spiked samples were placed in a 50 mL polypropylene tube, 5 mL of 0.1 M sodium hydroxide (NaOH) and 0.1 g of sodium chloride (NaCl) were added to each sample. The mixture was vortexed 1 min and sonicated for 15 min. After that, the samples were centrifuged for 10 min at 3500 rpm and the supernatant was collected. This process was repeated two times. Then, solid phase extraction was performed as follows: Oasis HLB (200 mg 6 mL)

cartridges were conditioned with 6 mL of methanol followed by 6 mL of HPLC water. After sample loading, cartridges were rinsed with 6 mL of HPLC water. Finally, samples were eluted with 6 mL of methanol, dried down under nitrogen, reconstituted in 1 mL of MeOH and kept at −20 °C until its analysis.

All purified samples were evaporated, re-dissolved in 1 mL of methanol-water (50:50) and 10 μL of internal standard (IS) mixture 1 mg/L (Table S2) was added to each extract before UHPLC-MS/MS analysis.

2.4. Instrumental analysis

The sample extracts were analysed using an ultra high pressure liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometry (UHPLC-QqLIT) following the method of (Gros et al., 2013). The chromatographic separations were performed using a Water Acquity Ultra-Performance™ liquid chromatography system, equipped with two binary pumps (Milford, MA, USA), using an Acquity HSS T_3 column (50 mm × 2.1 mm i.d., 1.8 μm particle size) with a precolumn Acquity UPLC HSS T3 1.8 μm particle size. The chromatographic separation conditions were: solvent (A) Acetonitrile, solvent (B) HPLC grade water acidified with 0.1% of formic acid. The flow rate was 0.5 mL/min and the gradient elution was: initial conditions 5% A; 0–3 min 5–70% A; 3.0–5.0 min, 100% A; 5.0–5.1 return to initial conditions and from 5.1 to 6.0 equilibrium of the column. The sample volume injected was 5 μL. The UHPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. All the compounds were analysed under positive electrospray ionization except for chloramphenicol that was analysed under negative ionization. Chloramphenicol was analysed with the same instrument describe above following the method developed by Gros et al. (2012) and using an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size). The chromatographic separation conditions were: solvent (A) Acetonitrile, solvent (B) 5 mM ammonium acetate/ammonia (pH 8). The flow rate was 0.6 mL/min and the gradient elution was: 0–1.5min, 0–60% A; 1.5–2.0min, 100% A; 2.0–3.0, 100% A; 3.20 return to the initial conditions; 3.20–3.70 equilibration of the column. The sample volume injected was 5 μL. Blank samples (MeOH and MeOH:H_2O 50:50) were run every 3 samples on the sample queue both between standards, spiked and non-spiked in order to detect any possible carryover effect. Two selected reaction monitoring (SRM) transitions were monitored for each antibiotic. The first transition was used for antibiotics quantification and for the calculation of the validation parameters, whereas the second transition was used for confirmation of the identity. The relative abundance of the two transitions was compared with those in the standards and the difference was within ±20% in all cases.

2.5. Statistical analysis

For the determination of significant differences between the different extraction procedures tested, one way ANOVAs were performed using R software (i386 3.1.0) comparing the different recoveries obtained for each compound in each extraction procedure. The normality and homogeneity of the data was tested before ANOVAs by using Shapiro-Wilk test and Levene's test respectively. For those compounds that the data showed no normality or homogeneity, a kruskal-Wallis test was performed using the same software. Results are presented in supporting information, Tables S4 and S5.

2.6. Microbial growth inhibition test

Once the extraction procedure was optimized a microbial growth inhibition test was performed using Water-Scan plates supplied by RIKILT (Wageningen University, Netherlands) as alternative detection technique. The test system contains five plates, one for each antibiotic family considered: tetracyclines, quinolones, macrolides/β-lactams, amino-glycosides and sulfonamides. The preparation of the Water-Scan plates, including the test organisms, the agar mediums and the supplements was done following the method of (Pikkemaat et al., 2008). The test requires samples to be in liquid phase and, therefore, a prior extraction procedure of seafood samples was mandatory. In this sense, the extraction method showing the best performance among the four previously tested was employed (QuEChERS extraction only, full details in Section 3.1). However, this extraction procedure was not suitable for a further analysis with the microbial growth inhibition test, probably due to interferences with the extraction salts used (data not shown). Therefore, an alternative extraction procedure based on ultrasonic extraction (US) and solid phase purification (SPE) was applied. Full details of the extraction procedure and plates preparation are given in supporting information. Three samples were analysed with the microbial growth inhibition test: a procedure blank (sample treated with the same extracting procedure but without biological matrix), a control sample (mussel sample previously analysed with QuEChERS extraction and UHPLC-MS/MS that did not show the presence of any antibiotics), and the same control sample extract spiked with 100 μg/L of oxytetracycline, 200 μg/L of flumequine, 100 μg/L of erythromycin, and 100 μg/L of sulfamethoxazole, the spiking values have been chosen as they are in the range of the MRLs established by the regulatory authorities (European Commission, 2010). In addition, a solvent blank (1:1) methanol:demineralised water, and demineralised water only, were used as negative controls. A positive control was also used in each plate for tetracyclines 100 μg/L of oxytetracycline; for quinolones 200 μg/L of flumequine; for macrolides/β-lactam 100 μg/L of amoxicillin; for sulfonamides 100 μg/L of sulfamethoxazole and for aminoglycosides 100 μg/L of neomycine.

3. Results and discussion

3.1. Extraction procedure optimization

Initially the following antibiotics families were targeted for their inclusion in the multi-residue method: macrolides, tetracyclines, fluoro(quinolones), lincosamides, sulfonamides, nitroimidazoles, dihydrofolate reductase inhibitors and amphenicols (Table S2) but due to the recoveries obtained with the extraction methods tested some of them had to be removed. This is the case of (fluoro)quinolones, which presented very poor recoveries for the methods i and ii (Table S3). The method based on ultrasonic bath using NaOH as extraction solvent and NaCl (method iv) achieved the highest recoveries for this group of antibiotics. However, this method was discarded due to the bad recoveries obtained for macrolides antibiotics group (Fig. 1). All the antibiotics included in the method (except for metronidazole-OH and chloramphenicol that were added in the spiking mixture in a further stage of the extraction method development) and the recoveries obtained for each procedure tested are shown in Fig. 1. Table S4 provides the standard deviation and statistical differences between the different treatments. Within the different extraction and clean-up procedures tested, the method based on ultrasonic bath using ACN:H_2O (3:1) as extraction solvent (method iii) was discarded due to the low recoveries for most of the compounds analysed (Fig. 1). The two methods based on QuEChERS showed similar recoveries for the

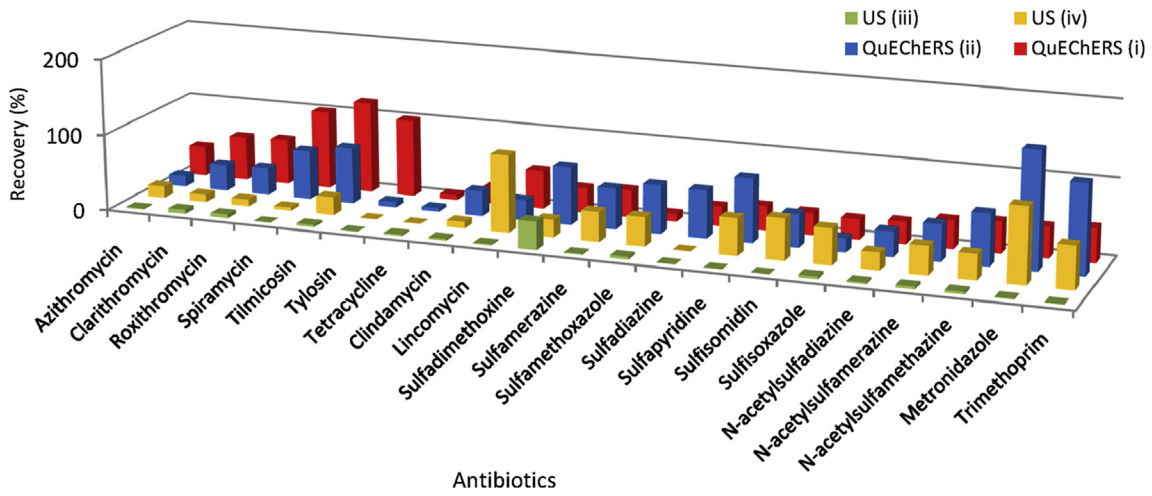

Fig. 1. Comparison of extraction efficiencies (%) obatined for each extraction procedure: QuEChERS (i), QuEChERS (ii) US (iii) and US (iv). Mean of 3 replicates (n = 3). Metronidazole-OH and Chloramphenicol are not represented because these compounds were included in a later stage of the method development.

majority of the compounds except for macrolides where QuEChERS extraction using ACN:MeOH (75:25 v/v) presented higher percentages of recoveries. Besides, this method was able to extract a higher number of antibiotic families and also presented good reproducibility with smaller standard deviation (Fig. 1, Table S4).

However, tetracyclines were not extracted with this procedure and due to their frequent use in aquaculture (De la Cruz et al., 2013; Rico et al., 2013) a decrease of pH in the extraction solvent was tested in order to improve their extraction. This has been previously reported to increase the recoveries in certain compounds (Lopes et al., 2012). Different amounts of formic acid (FA) were added to the extraction solvent: ACN:MeOH (0.1% FA) and ACN:MeOH (1% FA), and the results obtained are shown in Fig. 2. Table S5 provides the standard deviation for each compound and the statistical differences between the treatments. No significant increase in the extraction recoveries were found when adding 0.1% of FA to the extraction solvent. However, when 1% of formic acid was added tetracycline antibiotic was extracted with an acceptable recovery (35.4%). Besides, lincosamides, sulfonamides, nitroimidazoles, dihydrofolate reductase inhibitors and amphenicols were still satisfactory extracted with the addition of 1% formic acid. Although macrolides recoveries decreased due to the addition of formic acid (ranging from 38.6% to 119.6% without acid and from 37.4% to 60.15% with the addition of 1% formic acid), their recoveries were still satisfactory (Fig. 2, Table S5).

After all the test performed the method showing the best performance was QuEChERS only (i) with the addition of 1% of formic acid in the extraction solvent. No further clean-up procedure was needed, but some evaporation steps were performed under a gentle stream of nitrogen at room temperature. These concentration steps didn't affect the stability of the compounds and neither the recoveries. An additional filtration was carried out before running the samples on the mass spectrometry. Consequently, the final method developed is simple, effective and fast, only one extraction with QuEChERS followed by evaporation and filtration of the sample was undertaken. In addition the cost of sample analysis was also considerably reduced. The total time of analysis was less than 3 h allowing simultaneously analysis of 30 samples per day.

3.2. Method performance evaluation

The performance of the final method was evaluated for clams (*Chamalea gallina*), mussels (*Mytilus galloprovincialis*), and fish (*Platichtys flesus*). The recoveries obtained for the three seafood

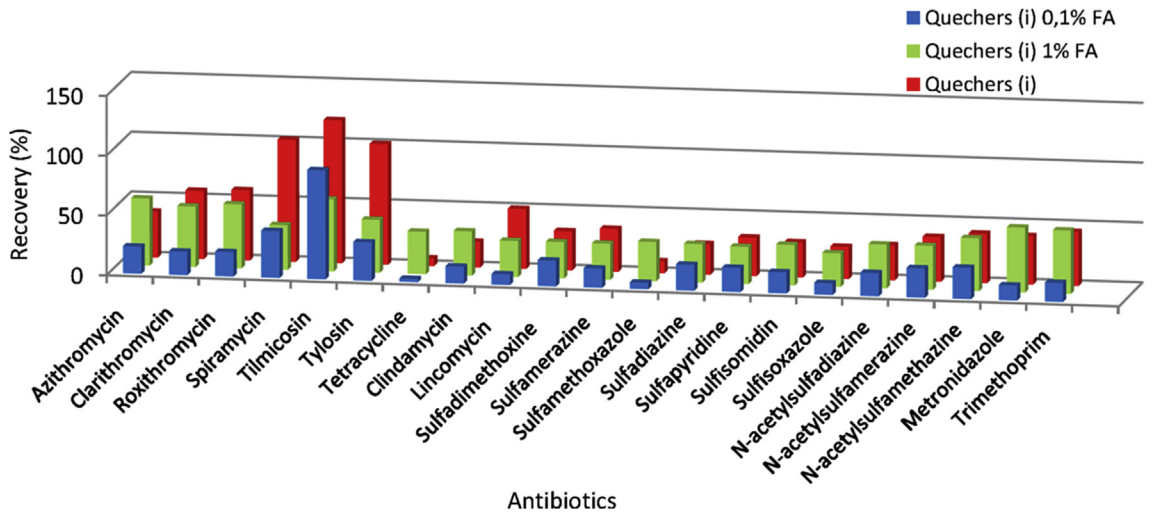

Fig. 2. Extraction efficiency (%) obtained with the method developed by using QuEChERS (method i, without FA) and with the addition of formic acid in the extraction solvent at 0.1% and 1%. Mean of 3 replicates (n = 3).

species are presented in Table 1. Twenty-three different compounds belonging to seven chemical families were analysed using this methodology. Recoveries for most of the compounds ranged between near 30% and 70%. Concretely, for clams it varies between 28% for sulfisoxazole and 60% for tilmicosin, for mussels between 29% for sulfisoxazole and 59% for tilmicosin and for fish between 28% for chloramphenicol to 70% for tilmicosin. In other methods referred in the literature for the analysis of antibiotics in seafood the recoveries were higher than the ones reported in the present work ranging from 50% to 104% (Dasenaki and Thomaidis, 2010; Evaggelopoulou and Samanidou, 2013). However, as mentioned above, most of them focused on one or two families of antibiotics with similar physic-chemical properties which facilitate the development of a more specific methodology than in multi-residue methods. Next to this, when applying multi-residues methods in biota samples, recoveries are usually considered acceptable when they are over 30% due to the analytical challenge of developing a method for diverse compounds with different lipophilicity and pKa (Huerta et al., 2013). The method developed covers antibiotics commonly used in aquaculture as macrolides, sulfonamides and tetracyclines (Cañada-Cañada et al., 2009) and four of their major metabolites (N-acetylsulfadiazine, N-acetylsulfamerazine, N-acetylsulfamethazine and metronidazole-OH). Besides, the banned substance chloramphenicol was also included. Despite the fact that chloramphenicol is not authorised for its use in food-producing animals in the European Union (EFSA, 2014) some residues are still detected in seafood (EFSA, 2014) due to illegal practices.

Method detection limits (MDLs) and method quantification limits (MQLs) were calculated for *C. gallina* (clam), *M. galloprovincialis* (mussel) and *P. flesus* (fish). Results are shown in Table 2. MDLs and MQLs both determined in spiked samples were calculated using the first SRM considering the minimum amount of analyte with a signal-to-noise ratio of 3 and 10 respectively. MDLs ranged between 0.02 and 0.31 ng/g (dw), 0.01–0.29 ng/g (dw) and 0.01–0.20 ng/g (dw), whereas MQLs ranged between 0.06 and 1.03 ng/g (dw) 0.05–0.97 ng/g (dw) and 0.02–0.66 ng/g (dw) for clam, mussel and fish respectively (Table 2). The method detection and quantification limits obtained in the present work were lower than those previously reported for the analysis of antibiotics in seafood by other authors (Dasenaki and Thomaidis, 2010; Dickson, 2014), and in the same range that those calculated by Fedorova et al. (2013).

Calibration curves were generated using linear regression analysis ($r^2 \geq 0.990$ see Table S6), they were prepared in the corresponding seafood extract (clam, mussel and fish) and used for the quantification of their corresponding matrix samples. The preparation of the standard curves in seafood matrix is of great interest as matrix effects may strongly influence the compounds analysis using UHPLC-MS/MS especially when dealing with complex matrices like biota (Álvarez-Muñoz et al., 2015; Gros et al., 2009). Therefore, the matrix effect on the MS signal was evaluated for each compound in each matrix comparing the peak areas of the calibration curve prepared in the seafood extract and those prepared in solvent (MeOH:H_2O 50:50) both spiked at 5, 10, 25 and 50 ng/mL. The percentages of reduction or enhancement are presented in Fig. S2. The majority of the compounds presented ion suppression. Only 5 compounds out of the 23 included in the method presented ion enhancement, three macrolides (azithromycin, spiramycin and tilmicosin), one tetracycline (tetracycline), and chloramphenicol (only in fish matrix). Ion enhancement in some antibiotics (e. g. azithromycin) has been previously reported in seafood matrices (Álvarez-Muñoz et al., 2015). The "internal sample calibration approach", calibration curve made up in the matrix with addition of isotopically labelled internal standards, was used to minimize matrix interferences and to avoid any under or over estimation during quantification. This approach has been previously demonstrated to be effective when analyzing target compounds in complex samples such as biota (Huerta et al., 2013; Stüber and Reemtsma, 2004).

Accuracy of the whole method for each seafood matrix was

Table 1
Mean percentage recoveries (%) and standard deviation (n = 3) of the target compounds in *Chamalea gallina*, *Mytilus galloprovincialis* and *Platichthys flesus* spiked at 50 ng/g dry weight.

Therapeutic family	Antibiotic	Recovery (%) ± SD		
		C. gallina	*M. galloprovincialis*	*P. flesus*
Macrolides	Azithromycin	56 ± 3	55 ± 0	52 ± 4
	Clarithromycin	51 ± 4	46 ± 3	43 ± 2
	Roxithromycin	54 ± 1	50 ± 2	47 ± 2
	Spiramycin	37 ± 5	38 ± 3	47 ± 11
	Tilmicosin	60 ± 2	60 ± 2	71 ± 5
	Tylosin	44 ± 6	51 ± 2	59 ± 7
Tetracyclines	Tetracycline	35 ± 9	33 ± 4	48 ± 6
Lincosamides	Clindamycin	37 ± 1	37 ± 5	41 ± 4
	Lincomycin	30 ± 2	31 ± 3	32 ± 6
Sulfonamides	Sulfadimethoxine	30 ± 7	34 ± 3	53 ± 1
	Sulfamerazine	30 ± 3	29 ± 3	40 ± 1
	Sulfamethoxazole	33 ± 8	30 ± 2	31 ± 2
	Sulfadiazine	32 ± 8	40 ± 4	45 ± 4
	Sulfapyridine	31 ± 13	34 ± 7	32 ± 7
	Sulfisomidin	34 ± 9	30 ± 4	33 ± 2
	Sulfisoxazole	28 ± 4	29 ± 3	33 ± 1
(Metabolite)	N-acetylsulfadiazine	37 ± 4	38 ± 8	38 ± 4
(Metabolite)	N-acetylsulfamerazine	37 ± 3	39 ± 4	43 ± 3
(Metabolite)	N-acetylsulfamethazine	44 ± 3	40 ± 3	42 ± 2
Nitroimidazoles	Metronidazole	54 ± 11	45 ± 4	48 ± 2
(Metabolite)	Metronidazole-OH	40 ± 4	39 ± 6	32 ± 3
Dihydrofolate reductase inhibitors	Trimethoprim	53 ± 12	50 ± 5	41 ± 2
Amphenicols	Chloramphenicol	28 ± 2	32 ± 2	28 ± 1

Table 2
Method detection limits (MDLs) and method quantification limits (MQLs) of the target compounds in clam (*C. gallina*), mussel (*M. galloprovincialis*) and fish (*P. flesus*).

Antibiotic	MDLs (ng/g dw)			MQLs (ng/g dw)		
	Chamalea gallina	*Mytilus galloprovincialis*	*Platichthys flesus*	*Chamalea gallina*	*Mytilus galloprovincialis*	*Platichthys flesus*
Azithromycin	0.06	0.03	0.05	0.18	0.10	0.17
Clarithromycin	0.05	0.04	0.07	0.16	0.15	0.23
Roxithromycin	0.20	0.17	0.13	0.67	0.56	0.43
Spiramycin	0.18	0.03	0.01	0.59	0.09	0.03
Tilmicosin	0.02	0.02	0.06	0.07	0.05	0.20
Tylosin	0.05	0.05	0.03	0.17	0.17	0.10
Tetracycline	0.10	0.05	0.13	0.33	0.15	0.45
Clindamycin	0.05	0.07	0.03	0.16	0.23	0.08
Lincomycin	0.13	0.03	0.09	0.42	0.09	0.29
Sulfadimethoxine	0.18	0.12	0.01	0.61	0.40	0.02
Sulfamerazine	0.08	0.14	0.06	0.26	0.46	0.21
Sulfamethoxazole	0.21	0.25	0.04	0.69	0.84	0.12
Sulfadiazine	0.10	0.18	0.08	0.34	0.60	0.26
Sulfapyridine	0.09	0.25	0.14	0.30	0.83	0.47
Sulfisomidin	0.31	0.29	0.06	1.03	0.97	0.19
Sulfisoxazole	0.07	0.08	0.03	0.24	0.25	0.09
N-acetylsulfadiazine	0.02	0.10	0.03	0.06	0.34	0.11
N-acetylsulfamerazine	0.05	0.02	0.13	0.17	0.05	0.44
N-acetylsulfamethazine	0.07	0.03	0.20	0.25	0.10	0.66
Metronidazole	0.07	0.01	0.06	0.24	0.05	0.19
Metronidazole-OH	0.07	0.10	0.06	0.22	0.32	0.20
Trimethoprim	0.15	0.07	0.02	0.51	0.24	0.08
Chloramphenicol	0.09	0.18	0.04	0.31	0.61	0.13

calculated intra-day from five repeated injections of a sample spiked at 50 ng/g and extracted, and inter-day from three injections of this sample on three different days (Table 3). Accuracy was calculated according to Bogialli et al., 2003 as the deviation of the measured mean concentration from the spiked concentration, expressed in percentage, and for most of the cases the values were lower than 20%. The instrumental precision was calculated intra-day (repeatability) and inter-day (reproducibility) as the relative standard deviation of the measured concentration (Table 3). Both values were lower than 20% for the majority of the compounds, indicating good repeatability and reproducibility, demonstrating the effectiveness of the method for quantification purposes.

3.3. Method application to farmed and wild seafood samples

The method developed was applied to seafood samples (fish and mussel) taken from different aquaculture and natural environments. Antibiotics concentrations found in the different organisms analysed are represented in Table 4. Six out of the twelve samples analysed showed the presence of at least one antibiotic with concentrations above MDLs, including three samples from aquaculture facilities and another three from natural environments. Nine

Table 3
Accuracy and precision of the target compounds in clam (C. *gallina*), mussel (M. *galloprovincialis*) and fish (P. *flesus*).

Antibiotic	*Chamalea gallina*				*Mytilus galloprovincialis*				*Platichthys flesus*			
	Intra-day		Inter-day		Intra-day		Inter-day		Intra-day		Inter-day	
	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy
Azithromycin	3.7	−1.4	6.3	3.0	3.2	−1.3	1.9	−2.6	1.7	−1.3	2.9	2.5
Clarithromycin	5.0	−1.7	8.1	−0.3	3.5	2.8	11.1	19.1	3.5	−0.3	14.9	−6.8
Roxithromycin	4.9	7.1	9.5	10.6	2.0	−0.6	12.7	13.7	2.8	0.0	12.5	−6.8
Spiramycin	4.8	2.1	1.9	11.0	2.7	3.6	14.6	−9.5	5.1	−15.3	20.4	5.8
Tilmicosin	2.4	−0.7	5.1	3.0	3.1	−3.3	3.6	−4.5	2.5	2.7	2.6	3.7
Tylosin	5.5	6.9	9.7	13.5	3.2	−2.9	6.5	2.9	3.1	−0.5	0.6	−1.7
Tetracycline	3.6	−6.9	12.3	−14.7	6.6	−5.5	7.4	−10.2	7.4	3.6	12.5	−1.8
Clindamycin	2.5	3.2	7.6	1.2	9.7	1.2	19.3	8.1	3.6	1.2	0.8	5.1
Lincomycin	3.7	−3.8	4.7	0.5	6.8	0.1	11.2	2.2	9.5	9.8	5.2	14.0
Sulfadimethoxine	8.4	17.4	6.9	7.9	4.2	3.6	10.0	−6.5	10.0	3.9	17.6	−3.3
Sulfamerazine	3.9	10.1	20.3	−3.0	4.0	−2.1	5.3	−6.8	8.6	19.0	8.0	13.9
Sulfamethoxazole	5.3	15.7	14.6	−0.1	5.3	−4.0	2.3	−10.5	5.8	14.8	11.0	13.8
Sulfadiazine	2.5	10.1	17.0	−1.4	5.4	−2.1	7.1	−9.6	8.1	10.2	16.9	5.3
Sulfapyridine	4.8	20.2	1.9	10.2	9.7	−0.2	11.0	−13.9	9.5	12.3	9.8	13.9
Sulfisomidin	3.4	12.6	7.6	−1.4	7.5	−5.5	5.5	−12.0	8.8	−1.7	16.0	−10.7
Sulfisoxazole	3.0	17.0	167	8.0	2.3	−1.2	5.1	−9.9	8.7	8.8	13.7	2.8
N-acetylsulfadiazine	1.,2	1.5	7.7	−18.0	13.6	3.7	15.8	−5.9	8.3	2.5	19.9	−0.8
N-acetylsulfamerazine	9.4	1.2	11.2	−9.9	7.3	−4.1	7.0	−14.7	8.2	−3.2	14.7	−4.8
N-acetylsulfamethazine	5.5	−1.2	4.1	−8.6	7.4	0.3	7.0	−13.3	5.7	7.5	18.8	14.1
Metronidazole	6.4	0.0	16.1	−15.9	5.9	6.5	6.8	5.8	4.1	−3.4	18.9	−6.3
Metronidazole-OH	4.1	−1.5	16.1	−11.3	9.5	−0.3	8.6	−1.9	8.0	1.9	11.3	−2.0
Trimethoprim	2.0	2.3	11.6	−8.8	7.1	11.6	11.3	−7.6	8.7	−0.3	10.6	−2.0
Chloramphenicol	16.7	−8.4	12.9	8.7	4.7	0.1	5.7	−1.4	12.1	0.0	3.4	−0.01

different antibiotics out of the 23 included in the method were detected with levels above MDLs. These compounds belong to three different antibiotic families: macrolides, tetracyclines and sulfonamides (Table 4). Among these nine compounds, seven were detected in aquaculture samples, three of them (Clarithromycin, sulfadimethoxine and sulfamethoxazole) at levels below MQLs, and the other four (roxithromycin, tilmicosin, tylosin and tetracycline) above MQLs in at least three out of the eight species analysed. Their quantifiable levels ranged from 0.19 ng/g (dw) of tylosin in salmon from Scotland, up to 4.96 ng/g (dw) of tetracycline in the same sample. In the seafood samples collected from natural environments, only 4 antibiotics were found at levels above MDLs, and among them only 2 were above MQLs. Concretely, azithromycin and tetracycline with levels ranging from 0.77 ng/g (dw) in *Mytilus* spp from Tagus estuary to 5.63 ng/g (dw) in *Platichtys flesus* from Scheldt estuary. These results showed that samples coming from aquacultures have a higher amount of antibiotics than those coming from natural environments. These results are in line with previous studies which reported that seafood from aquacultures have higher presence of man-made chemicals such as antibiotics than the wild organisms (Cole et al., 2009). Unfortunately, water sample from the same location where seafood samples were taken was not available for analysis so the concentration of the contaminants in the surrounding media was not measured, and therefore, their bioaccumulation factor could not be calculated.

Regarding the occurrence of antibiotics in the samples analysed, macrolides was the most frequently detected group with at least one antibiotic from this family detected in six out of the twelve samples analysed. Macrolides are potent antimicrobials used in veterinary practices against a wide bacteria range, furthermore, they are some of the most effective medicine against diseases produced by *Mycoplasmas*, and therefore, they are commonly used in food-producing animals in order to treat or prevent bacterial infections (Cañada-Cañada et al., 2009; Horie et al., 2003). In the particular case of azithromycin, it was only detected in environmental samples. This antibiotic is commonly indicated for human treatment but is rarely used in aquaculture, which may explain that this compound was not found in any aquaculture sample. Similar azithromycin concentrations in mussels from natural environments (Ebro delta, Spain) have been previously reported in the same concentration range (Álvarez-Muñoz et al., 2015). In the case of sulfonamides sulfadimethoxine, sulfamethoxazole and sulfisoxazole were detected but none of them showed levels above MQLs. Sulfonamides are synthetic antimicrobials widely used in fish cultures (Huet et al., 2010). However, its occurrence in edible tissues of seafood has been rarely reported (Baran et al., 2011). Indeed, only in few commercial seafood samples the presence of sulfonamides have been reported with levels between non-detected to 20 ng/g (dw) (Done and Halden, 2014; Fedorova et al., 2013). Despite the fact that some sulfonamides metabolites were included in the analytical method (N-acetylsulfadiazine, N-acetylsulfamerazine and N-acetylsulfamethazine), none of them were detected above MDLs in the samples, probably due to the low concentrations of the parent compounds detected, being all of them below MQLs. Tetracycline was the most ubiquitous compound being present in four out of the twelve samples analysed. It was also the antibiotic which presented the highest concentrations in natural environments, 5.63 ng/g (dw) in *Platichthys flesus* from Netherlands, and also in aquaculture samples 4.96 ng/g (dw) in *Salmo salar* from Scotland (Table 4). Tetracycline antibiotic is commonly used in aquacultures as it is a broad-spectrum antibiotic, and it is also used for promoting growth in the farming industry (Cañada-Cañada et al., 2009). Similar values of tetracycline antibiotic (from non-detected

Table 4
List of antibiotics which were found in at least one sample with values above MDLs in different fish and mussel species collected from aquacultures and natural environments around Europe. Concentrations are represented in ng/g (dw) mean of 3 replicates and standard deviation (n = 3 ± sd).

Therapeutic family	Antibiotic	Aquaculture samples (ng/g dw) ± SD						Environmental samples (ng/g dw) ± SD					
		Pangasius spp (Vietnam)	*Salmo salar* (Scotland)	*Salmo salar* (Norway)	*Mytilus galloprovincialis* (Spain)	*Mytilus galloprovincialis* (Italy)	*Mytilus galloprovincialis* (Netherlands)	*Mytilus* spp (Greece)	*Mytilus* spp (Greece)	*Mytilus galloprovincialis* (Po delta)	*Mytilus* spp. (Tagus estuary)	*Platichthys flesus* (Scheldt)	*Mytilus Gallorpovincialis* (Bay Saint-Brieuc)
Macrolides	Azithromycin	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	2.13 ± 0.09	0.77 ± 0.04	< MDL	< MDL
	Clarithromycin	< MDL	< MQL	< MQL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Roxithromycin	1.12 ± 0.14	< MDL	0.48 ± 0.05	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL	< MDL
	Tilmicosin	< MDL	0.23 ± 0.10	0.42 ± 0.08	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Tylosin	< MDL	0.19 ± 0.05	0.24 ± 0.02	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
Tetracyclines	Tetracycline	2.38 ± 1.56	4.96 ± 0.50	3.36 ± 0.28	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	5.63 ± 0.41	< MDL
Sulfonamides	Sulfadimethoxine	< MDL	< MQL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Sulfamethoxazole	< MQL	< MQL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Sulfisoxazole	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL	< MDL

to 13.1 ng/g (dw) were detected in fish (*Sparus aurata*) collected from marine farms from Cartagena, Spain (Cháfer-Pericás et al., 2011); whereas Na et al., 2013 analysed different marine species in coastal waters from China and found tetracycline antibiotic at concentrations around 1.73 ng/g wet weight (ww).

Despite the fact that some antibiotics residues were found in seafood samples their levels were far away from the Maximum Residue Limits established by the authorities being between 100 and 600 ng/g (ww) for the compounds detected in the analysed samples (EU No 37/2010). Furthermore, the banned substance chloramphenicol, which can provoke serious toxic effects in humans, was not detected in any sample. Therefore it is very unlikely that antibiotics present in seafood could cause an adverse effect in consumers due to the single intake of seafood. However, other dietary and non-dietary sources needs to be taken into consideration in order to assess their potential risk and identify if the levels ingested are below the acceptable daily intake advice by authorities (Australian Government Department of Health - Office of Chemical Safety, 2016). Besides, the risk for individual allergic people should be taken into consideration. Furthermore, the additive toxic effect of antibiotics together with other contaminants also present in seafood like mercury, polychlorinated biphenols (PCBs) and dioxins is not known yet, as well as the effect of chronic exposure to low concentrations of this cocktail of pollutants (Cole et al., 2009; Jones et al., 2004).

3.4. Microbial growth inhibition test

A detection technique based on the microbial growth inhibition was preliminarily evaluated for the screening of antibiotics in seafood samples. The response in the microbial growth inhibition test was checked for a procedure blank sample, a positive and negative control sample, a clean sample (with no presence of any antibiotics) and a spiked sample (fortified with 100 μg/L of oxytetracycline, 200 μg/L of flumequine, 100 μg/L of erythromycin, and 100 μg/L of sulfamethoxazole). Positive controls showed inhibition in all corresponding plates, and negative controls presented no inhibition zone in any plate analysed (Fig. 3) and therefore the performance of the test was considered correct. Regarding the samples analysed, the procedure blank sample did not show inhibition in the plate for any antibiotic family. However, the clean sample showed inhibition for almost all antibiotic families and some interference due to the biological matrix was postulated. Furthermore amino-glycosides plate showed inhibition although no amino-glycoside compound was added to the spiking mix, which may indicate some cross-reactive interferences. Only for macrolides/β-lactams plate a clear differentiation between the clean sample and the spiked one was observed. Therefore, the application of the microbial growth inhibition test was only feasible for a qualitative identification of macrolides/β-lactams. Application of the microbial growth inhibition test to other antibiotic families will need further investigation in order to improve the extraction procedure and to assure the removal of matrix interferences.

4. Conclusions

A methodology for the analysis of antibiotics in seafood based on QuEChERS extraction followed by detection using UHPLC-MS/MS was developed. The method allowed the simultaneous analysis of twenty-three antibiotics belonging to seven different therapeutic families, and including four major metabolites. The performance of the method was good for the analysis of antibiotics in seafood (fish, mussels and clams) in terms of recoveries, accuracy, precision, MDL and MQL, proving the effectiveness of this methodology for a fast routine analysis of these compounds. The method was applied for the analysis of antibiotics in seafood species from aquacultures and natural environments and a total of nine antibiotics were detected with levels above MDLs in six out of the twelve samples analysed. Aquaculture samples presented higher amount of antibiotics than those samples coming from natural environments, however no toxic effect for consumers is expected as all concentrations detected were lower than the MRLs established.

An alternative detection technique based on microbial growth inhibition for the detection of antibiotics in seafood was also tested. The method allowed a rapid and simple detection of macrolides and β-lactams antibiotics in seafood. However, some drawbacks of this methodology were observed (matrix interferences and cross-reactivity) when analyzing other antibiotics families in seafood.

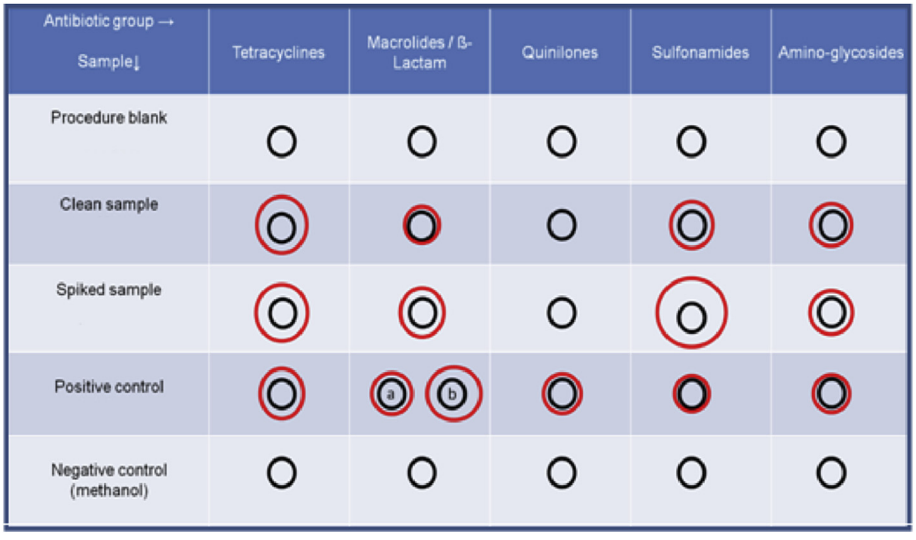

Fig. 3. Schematic representation of the results for the three samples analysed extracted using ultrasonication followed by solid phase purification, the results for the positive and negative controls are also presented. The black circle represents the well to which samples were added, the red circle represents the inhibition zone (no growth of bacteria). a) It corresponds to macrolides positive control, and b) to β-lactams positive control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Based on these limitations, further experiments will be needed in order to improve the response of the test for seafood samples.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2016.11.031.

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Combining an effect-based methodology with chemical analysis for antibiotics determination in wastewater and receiving freshwater and marine environment☆

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ABSTRACT

Two different methodologies were combined to evaluate the risks that antibiotics can pose in the environment; i) an effect-based methodology based on microbial growth inhibition and ii) an analytical method based on liquid-chromatography coupled to mass spectrometry (LC-MS). The first approach was adapted and validated for the screening of four antibiotic families, specifically macrolides/β-lactams, quinolones, sulfonamides and tetracyclines. The LC-MS method was applied for the identification and quantification of target antibiotics; then, the obtained results were combined with ecotoxicological data from literature to determine the environmental risk. The two methodologies were used for the analysis of antibiotics in water samples (wastewater, river water and seawater) and biofluids (fish plasma and mollusk hemolymph) in two monitoring campaigns undertaken in the Ebro Delta and Mar Menor Lagoon (both in the Mediterranean coast of Spain). Both approaches highlighted macrolides (azithromycin) and quinolones (ciprofloxacin and ofloxacin) as the main antibiotics in wastewater treatment plant (WWTP) effluents with potential risk for the environment. However, no risk for the aquatic life was identified in the river, lagoon and seawater as antibiotic levels were much lower than those in WWTP effluents. Fish from Ebro River were the organisms presenting the highest antibiotic concentration when compared with bivalves (mussels) from the Mediterranean Sea and gastropods (marine snails) from the Mar Menor Lagoon. The effect-based methodology successfully determined antibiotic risk in wastewater, but its applicability was less clear in environmental waters such as seawater, due to its high detection limits. Improving sample preconcentration could increase the method sensibility. Overall, combination of both methodologies provides comprehensive insights in antibiotic occurrence and risk associated in areas under study.

1. Introduction

The presence of antibiotics in the aquatic environment is an issue of increasing concern. The highest concentrations are usually detected in wastewater, up to few μg/L, (Manzetti and Ghisi, 2014), whereas lower levels, below 0.001 μg/L, are commonly measured in

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surface and groundwater (Manzetti and Ghisi, 2014). Natural attenuation processes such as dilution, sorption to sediment or to suspended solids, chemical and biological degradation, contribute to the reduction of antibiotics concentrations from Waste Water Treatment Plants (WWTP) effluents to the receiving water bodies (Celic et al., 2019; Manzetti and Ghisi, 2014). However, the continuous discharge of these contaminants makes them pseudo-persistent in the aquatic environment (Carvalho and Santos, 2016). As a result, some of the most consumed antibiotics for human or veterinary purposes like tetracyclines, quinolones, β-lactams, macrolides and lincosamides, among others, have been detected in several water bodies worldwide ranging from ng/L up to several μg/L (Chen et al., 2014; Kümmerer, 2009; Rodriguez-Mozaz et al., 2017).

Since antibiotics are used to kill or inhibit pathogenic bacteria, their presence in natural environments may pose a risk for the aquatic communities (Kümmerer, 2009), including non-targeted organisms. Primary producers and decomposers may be vulnerable to these contaminants, compromising the essential ecological functions that these organisms perform in the natural ecosystem, such as the biogeochemical cycling and organic contaminant degradation (Grenni et al., 2018). In addition, the continuous exposure to antibiotics allows them to bioaccumulate, as well as, provoke ecotoxicological effects, altering organisms functions and metabolism in invertebrates or fish (Le Bris and Pouliquen, 2004; Serra-Compte et al., 2019a). Antibiotics can also promote the spread of antibiotic resistant genes (ARGs) in free-living bacteria from the different aquatic environments, including rivers, lakes and coastal areas (Martínez, 2008). Besides, some studies have described the increase of ARGs copies in the bacteria located in gastrointestinal tracts of shrimp (Su et al., 2017), and mussel (Serra-Compte et al., 2019b) as a result of their exposure to antibiotics.

In order to evaluate the risk that antibiotics pose to the environment, several studies have determined antibiotics concentration threshold i.e. predicted non effect concentration (PNEC) based on ecotoxicological parameters, such as survival or reproduction impairment (Park and Choi, 2008; Santos et al., 2013). Recently, a PNEC was developed considering the capacity of antibiotics to promote antimicrobial resistance spread (Bengtsson-Palme and Larsson, 2016; Tell et al., 2019). This approach determined the lowest concentration of an antibiotic in the environment capable to promote antibiotic resistance dissemination. The combination of both, ecotoxicological PNEC and PNEC related to antibiotic resistance promotion was postulated as a comprehensive approach to establish a final PNEC for antibiotics in the environment (Tell et al., 2019).

In addition to the effects that antibiotic pollution may provoke to the exposed organisms, it may be of concern in terms of human health. The presence of antibiotics in seafood may pose a risk for consumers such as allergy and toxicity (Cabello, 2006). To reduce this risk, authorities have established measures to control the occurrence of these contaminants in the natural environment and in the foodstuff from animal origin. For instance, the use of antibiotics as growth promoters in livestock has been forbidden in the European Union since 2006 (Carvalho and Santos, 2016). Besides, Maximum Residue Limits (MRLs) have been established by the authorities for some antibiotics in foodstuff from animal origin (European Commission, 2010). Recently, the European Union (EU) included four antibiotics in the latest watch list revision (EU, 2018) highlighting the increasing concern of antibiotic occurrence in the environment.

Monitoring antibiotic occurrence in the water bodies and organisms is the first step to evaluate the risk of these contaminants for the environment and human health. In this regard, effect-based techniques for screening chemical pollution in the environment have gained importance as they provide a powerful tool for water quality monitoring without the necessity of analyzing hundreds of chemical contaminants potentially present in the sample (Doyle et al., 2015). Effect-based methodologies for antibiotics screening, like microbial growth inhibition tests (Pikkemaat et al., 2008), can provide a wide view of antibiotic pollution in a given sample, as not only the antibiotics, but also their active transformation products and metabolites can be detected. Besides, microbial growth inhibition are cost-effective tests when compared with immunological or receptor-based assays but they do not provide single compound identification nor quantification, also the required analysis time is usually longer than immunoassays. (Cháfer-Pericás et al., 2010; Pikkemaat, 2009). Few methodologies based on microbial growth inhibition have been developed, they were applied to food control in livestock production (Gondová et al., 2014; Pikkemaat et al., 2008), in seafood like shrimps (Dang et al., 2010) and in rainbow trout (Barker, 1994). The use of biota biofluids (such as mussel hemolymph) instead of organism's tissues (like mussels soft tissue) extract also allows simplifying the extraction protocol and reducing the potential loss of antibiotics during the extraction procedure. Furthermore, matrix complexity, which may interfere with their detection in the microbial inhibition test, is lower in biofluids than in biota extracts (Serra-Compte et al., 2017). The microbial growth inhibition test has been applied to screen antibiotics in environmental samples such as sediment and water (Huerta et al., 2011). However, it has not yet been used for monitoring of biota samples in natural aquatic ecosystems, nor to the monitoring of wastewater samples.

In this work, a screening method based on microbial growth inhibition was adapted for the detection of a broad range of antibiotics in biota biofluids (mollusks hemolymph and fish plasma) and in water sample extracts; namely WWTP influents and effluents, freshwater and seawater. The screening method was applied for the screening of antibiotics in biological and water samples from two monitoring campaigns undertaken in two areas of ecological and human interest located in the Mediterranean coast of Spain: river Ebro delta and Mar Menor Lagoon. In addition, a chemical analysis based on liquid-chromatography coupled to mass-spectrometry (LC-MS) was used for the identification and quantification of the target antibiotics.

2. Material and methods

2.1. Chemicals and reagents

Antibiotic standards were of high purity grade (>90%), purchased from Sigma-Aldrich (St Louis, MO, USA) (Table S1, list of antibiotics). Stock standards were prepared in methanol at a concentration of 1000 mg/L and stored at −20 °C. The cartridges OASIS HLB (200 mg, 6 mL) were used for solid phase extraction. HPLC grade methanol, water and acetonitrile were purchased from Merck (Darmstadt, Germany), EDTA 0.01 mol/L, was obtained from Scharlab (Barcelona, Spain).

2.2. Study areas and sample collection

The Ebro delta is located in NE Spain and has a surface area of approximately 320 Km^2. Most of its surface is used for agriculture, mainly rice crops. The Ebro delta is composed of a wide variety of environments such as natural lagoons, wetlands, marshes and it includes two coastal bays (Alfacs and Fangar). Further information regarding the Ebro delta area can be found elsewhere e.g. (Čelic et al., 2019). A sampling campaign of water and biota samples was performed in June 2018 in dry weather conditions. Twenty-

four hours composite water samples were obtained from wastewater, whereas grab samples were collected from freshwater and marine environments. For freshwater analysis, water samples were taken from three different sampling sites at the Ebro river (FW1, FW2, FW3), Fig. 1A. Wastewater influent and effluent samples were obtained from two different wastewater treatment plants, WWTP1, WWTP2, Fig. 1A. WWTP1 has a primary and secondary treatment with activated sludge, with a capacity of 27.500 inhabitant equivalent, and it discharges directly into the Ebro river. WWTP2 has a primary, secondary and tertiary treatment, consisting in activated sludge followed by a sand filter. Its maximum capacity is 28.921 inhabitant equivalents, and it discharges into the Mediterranean Sea (Alfacs Bay). Seawater samples were collected from eight different sampling sites, four of them located in Fangar bay (SW1, SW2, SW3, SW4), and the other four in Alfacs bay (SW5, SW6, SW7, SW8) at locations ranging between 4 and 10 Km approximately from the WWTP2 facility (Fig. 1A). Fish and mussels were sampled for biofluid extraction in sampling sites located close to those selected for water. Freshwater fish were taken from 2 sampling sites located at the Ebro river, marine fish and mussels were sampled from the Mediterranean sea concretely, fish from 2 sites located at Alfacs bay (Fig. 1A) and mussels from aquaculture structures at 2 sampling sites at Alfacs bay and another 2 at the Fangar bay (Fig. 1A).

Mar Menor Lagoon is located in the South East of Spain. It is a hypersaline restricted lagoon, covering an area of 135 km^2. Water

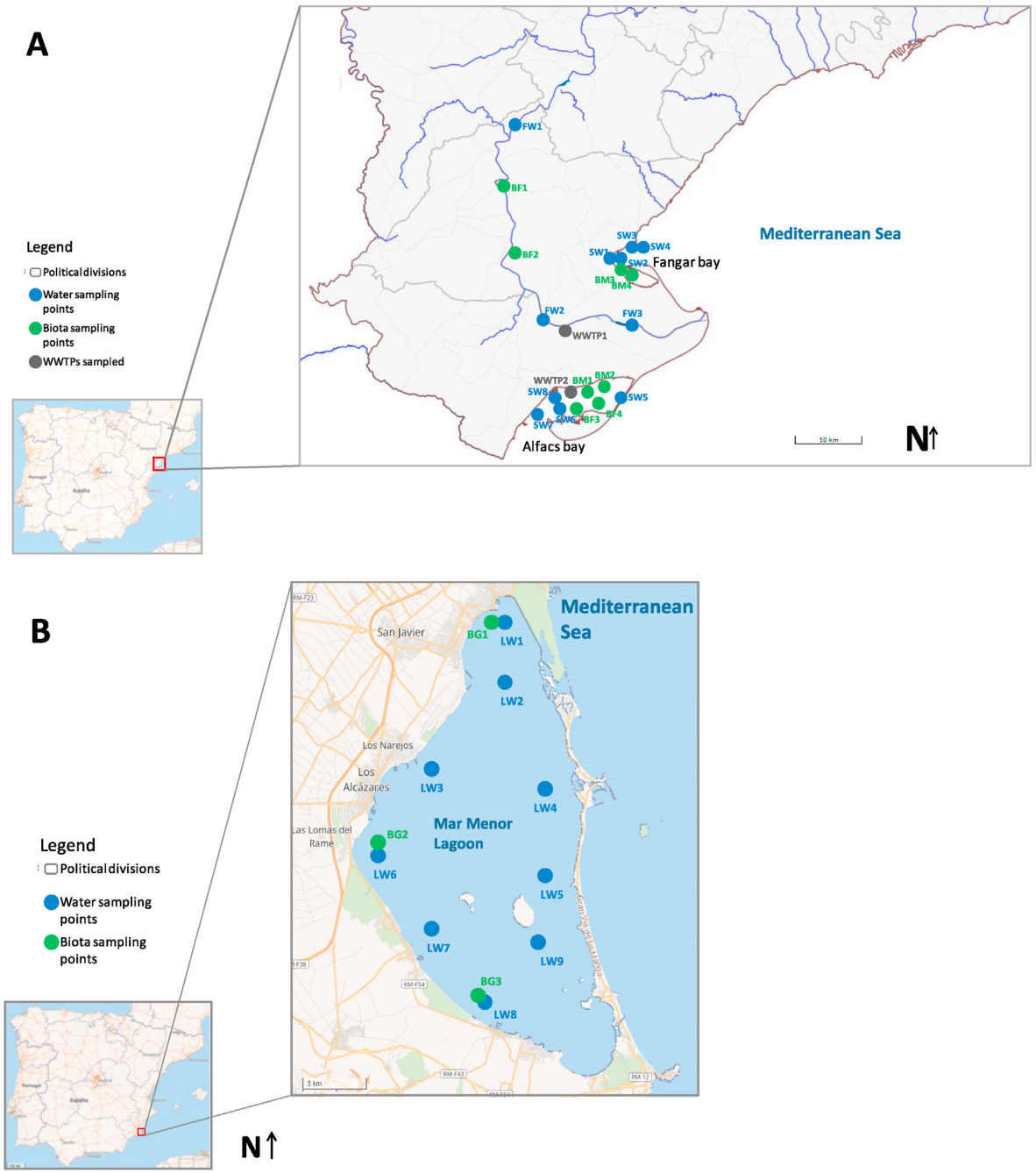

Fig. 1. Sampling sites in A) the Ebro Delta area and B) Mar Menor Lagoon.

was collected from the lagoon in nine sampling sites, (LW1, LW2, LW3, LW4, LW5, LW6, LW7, LW8, LW9), (Fig. 1B), whereas biota, gastropod (*Hexaplex trunculus*), was taken in three of them (BG1, BG2, BG3), (Fig. 1B).

2.3. Sample pre-treatment

Sample pre-treatment for the different matrices and for the two methodologies applied (microbial and chemical analysis) are summarized in Fig. S1. For water analysis, 1 L of seawater or freshwater was pre-concentrated using solid phase extraction (SPE) following the methodology developed by Gros et al. (2013) (except for WWTP influent and effluent where 300 mL were used). Briefly, water samples were filtered through 1 μm glass fiber filters and 0.45 μm nylon membrane filter prior SPE extraction. SPE cartridges were conditioned with 6 mL of methanol, followed by 6 mL of HPLC water at pH 2.5. Then, the pH of water samples was adjusted at 2.5 and passed through the cartridges, prior addition of an appropriate amount of EDTA. Then, cartridges were rinsed with 6 mL of water at pH 2.5 and dried under air for 5 min. Samples were eluted with 6 mL of methanol, dried down under nitrogen and reconstituted in 1 mL of methanol:water (30:70) before their analysis with the microbial growth inhibition test. For chemical analysis, an aliquot (50 μL) of the same extract was further dried down and reconstituted with 100 μL methanol:water 50:50 (dilution 1:2), to reduce matrix interferences. Acceptable extraction recoveries were obtained for most of the tested antibiotics. Despite lower recoveries were achieved in biota samples compared to water; they were similar than previously reported values for pharmaceuticals extraction in biota matrices (Fernandez-Torres et al., 2011; Huerta et al., 2013). The obtained recoveries were used for correction of contaminants concentration in the different matrices (Table S2).

Mussels (*Mytilus galloprovincialis*) collected in the study sites from the Mediterranean Sea were transported under refrigerated conditions to the laboratory. The same day of mussel sampling, hemolymph was extracted from the mussel's adductor muscle, and collected in vials containing heparin. Then, samples were centrifuged at 3000 rpm during 10 min and immediately frozen. A similar protocol was followed for gastropod hemolymph extraction from the Mar Menor Lagoon. Hemolymph was extracted from the foot muscle and collected in vials without heparin. Samples were centrifuged at 1000 g for 10 min, then, the supernatant was collected and frozen until analysis. Fish blood extracted (at each sampling site) was transferred to vials containing heparin, immediately centrifuged at 3000 rpm during 10 min, plasma (≈3 mL) was collected and frozen until analysis. Both, mollusk hemolymph and fish plasma were kept at −70 °C until their analysis. Biota biofluids extracts were analyzed in the microbial growth inhibition test whereas a dilution with methanol (1:2) followed by centrifugation (10 min at 5000 rpm) was necessary previously to their analysis in LC-MS.

2.4. Chemical analysis − LC-MS

The obtained extracts from water and biota biofluids samples (as explained in section 2.3) were analyzed in triplicate by liquid chromatography coupled to mass spectrometry using ultra high-pressure liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometry (UHPLC-QqLIT) following the method of Gros et al. (2013) for the target analysis of 27 antibiotics. Chromatographic separation was done with an Acquity HSS T3 column 5 (50 mm × 2.1 mm i.d., 1.8 μm particle size), solvent (A) Acetonitrile, solvent (B) HPLC grade water acidified with 0.1% of formic acid. Further details of the method can be found elsewhere (Gros et al., 2013). Further information regarding chemical analysis, limits of quantification and detection can be found in Table S2.

2.5. Microbial growth inhibition test

The test comprises four plates for the specific analysis of each of the four antibiotic families namely, sulfonamides, tetracyclines, fluoro(quinolones) and macrolides/β-lactams. The microorganisms used: *Kocuria rhizophila* (formerly known as *Micrococcus luteus*) ATCC 9341 (macrolides/β-lactams); *Bacillus cereus* ATCC 17788 (tetracyclines); *Yersinia ruckeri* NCIM 13282 (quinolones); *Bacillus pumilus* CN 607 (sulfonamides), were kept at −70 °C, until the analysis. The culture media were, plate count agar from Difco, BD diagnostic systems (Breda, Netherlands) and DST-agar and Isosensitest agar purchased from Oxoid (Basingstoke, UK). The characteristics of the test plates are specified in Table 1. Plates preparation was adapted from (Pikkemaat et al., 2008). Briefly, after sterilization, media were cooled down and the synergistic antibiotics to increase method sensitivity were added to the corresponding plate namely, tylosine (macrolides/β-lactams), chloramphenicol (tetracyclines), cloxacilline (quinolones) and trimethoprim (sulfonamides) (Table 1). When agar temperature was below 48 °C, bacteria were inoculated into the liquid agar which was poured to form a 2.5 mm thick layer except for sulfonamides that was 3 mm. Fourteen-millimeter diameter holes were made in the agar after its solidification. Two hundred fifty microliters of sample extract (sample extraction explanation can be found in section 2.3) was applied into the punched holes in the agar and 50 μL of the corresponding buffer were added prior incubation at 30–37 °C for 16/18 h. After overnight incubation, plates were observed. A positive result consists of a bacterial growth inhibition area around the punched hole. An example of the developed plate can be seen in Fig. S2. The diameter of the inhibition areas was measured with a precision of 0.1 mm using a Vernier caliper.

2.6. Microbial growth inhibition test adaptation

Microbial method optimization was carried out with blank sample extracts (for sample extraction, see section 2.3) (seawater,

Table 1
Microbial growth inhibition test parameters.

Antibiotic family	Agar medium	pH	Synergistic antibiotic	Bacteria	Supplement buffer	Incubation conditions
Macrolides/β-lactams	Iso-sensitest agar	8.0	7.5 μg/L tylosine	*M. luteus ATCC 9341*	1M phosphate buffer pH 8.0 + 0.01 μg/mL tylosine/0.5 M phosphate pH 7.5[a]	30 °C/16–18 h
Tetracyclines	Iso-sensitest agar	6.0	625 μg/L chloramphenicol	*B. cereus ATCC 17788*	1M phosphate buffer pH 6.0	30 °C/16–18 h
Quinolones	2/3 PCA +1 M 5% fosfat buffer pH 6.5	6.5	8000 μg/L cloxicilline	*Y. ruckeri NCIM 13282*	1M phosphate buffer pH 6.5	30 °C/16–18 h
Sulphonamides	DST agar	7.0	7 μg/L trimethoprim	*B. pumilus CN 607*	1.5M phosphate buffer pH 8 + 0.01 μg/mL TMP	37 °C/16–18 h

[a] 0.5 M phosphate pH 7.5 phosphate buffer was used in water samples.

freshwater, mollusk hemolymph and fish plasma) spiked with known concentrations of the tested antibiotics (ranging from 1 to 200 μg/L). Prior spiking, blank samples were analyzed with a method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS) (Gros et al., 2013; Serra-Compte et al., 2017) showing no presence of antibiotics. The screening biological method was adapted for the detection of the 17 antibiotics presented in Table S1. These antibiotics were selected according to their reported presence and potential impact to the aquatic ecosystem and human health based on their MRL in foodstuff from animal origin (European Commission, 2010; Rodriguez-Mozaz et al., 2015, 2017; Santos et al., 2013). The detection limit, defined as the minimum concentration of each antibiotic showing a clear inhibition area (>1 mm around the punched hole), was established for the different matrices tested and for each of the 17 antibiotics considered. The detection limit was calculated by correcting the lowest spiked concentration showing a clear inhibition area with the percentage of recovery, as well as by the total sample volume preconcentrated (1 L freshwater and seawater, 300 mL wastewater and 1 mL biota biofluids). Besides, a positive control of spiked water (100 μg/L) with oxytetracycline, enrofloxacin, erythromycin and sulfamethoxazole was applied in a hole of each of the corresponding plates: tetracycline, fluoro (quinolones), macrolides/β-lactams and sulfonamides, respectively; and a negative control by analyzing a blank sample (seawater, freshwater, mollusk hemolymph and/or fish plasma depending on the analysis undertaken) without antibiotic presence.

Once the method was optimized it was validated in terms of accuracy, sensitivity and specificity according to Dang et al., (2010). Sets of 20 blank samples and 20 spiked samples were analyzed for the different matrix types and the 17 antibiotics reported in Table S1. Spiking was done for each antibiotic at its corresponding detection limit. Accuracy was defined as the number of correct results (when no false positive or negatives results were reported) given by the methodology considering the total number of analyzed samples and expressed as percentage. Sensitivity was defined as the number of positive samples correctly given by the methodology considering the total number of positive samples (also expressed in percentage). Specificity was defined as the number of negative samples correctly given by the methodology taking into account the total number of negative samples analyzed (Dang et al., 2010). Furthermore, method ruggedness was evaluated through its implementation in two different laboratories (namely, Wageningen Food Safety Research, Netherlands, and ICRA, Spain), hence, different batches of tests, different days, and spikes from different standard solutions, as well as, different instrumentation were applied (Pikkemaat, 2009). Due to the low availability of fish plasma and the difficulty to obtain wastewater without antibiotics, the method was validated for freshwater, seawater and mollusk hemolymph.

2.7. Antibiotics risk assessment

Antibiotics risk was evaluated by calculating a hazard quotient (HQ) for each compound according to the European Community (EC) guidelines (European Commission, 2003). HQs were calculated as follows:

$$HQ = \text{Antibiotic concentration} / \text{Predicted No Effect Concentration (PNEC)}$$

Antibiotic concentration refers to the measured concentration of antibiotics in the environment (LC-MS methodology). PNECs were calculated for each antibiotic following the approach of Tell et al., (2019), which combines ecotoxicological PNEC and MIC-PNEC (related to antimicrobial resistance spread). Ecotoxicological PNECs were obtained from the reported literature (when information was not available from literature the ECOSAR software was used), presented as the lowest EC50 or LC50 and applying an assessment factor of 1000 (European Commission, 2003). MIC-PNECs were also obtained from the literature (Bengston-Palme et al., 2016). The final PNEC was determined for each antibiotic as the lowest one reported when comparing ecotoxicological PNEC and MIC-PNEC (ecotoxicological, MIC and final PNECs for the tested antibiotics are reported at Table S1). Antibiotics with a HQ above 1 are considered a potential risk for the environment, (European Commission, 2003). In order to assess the environmental risk of antibiotics mixtures, the sum of calculated HQ was performed per each water sample, as previously reported in the literature (Backhaus, 2016).

3. Results and discussion

3.1. Microbial growth inhibition test performance

The microbial growth inhibition test conditions indicated in Table 1 were used to screen antibiotics in all the matrices tested; the only difference was the buffer used in the macrolides/β-lactams plate. Therefore, in the macrolides/β-lactams plate, a buffer without tylosin and with a slightly lower pH (which reduced the sensitivity of the analysis in the macrolides/β-lactams plate) allowed avoiding false positive in water analysis.

The detection limits of the plates were established by using the final method conditions and analyzing different sets of blank samples (freshwater, seawater, wastewater, mussel hemolymph and fish plasma). The detection limits in the plates (Table 2) were similar for freshwater and seawater ranging between 0.01 μg/L and 0.29 μg/L. Overall, for water samples the analysis of tetracyclines, quinolones and macrolides/β-lactams allowed lower detection limits when compared to sulfonamides (Table 2). Regarding the biota biofluids, mollusk hemolymph and fish plasma, similar results were obtained for both matrices, ranging from 10 μg/L up to 100 μg/L. Despite the high differences even within the same antibiotic family, tetracyclines were detected with the lowest detection limits whereas sulfonamides the highest (Table 2).

Microbial growth inhibition test showed good performance in terms of accuracy and sensitivity being higher than 95% for all the tested antibiotics, results are presented at supporting information, Table S3. Specificity was 100% for all the antibiotics as no false positive were detected in any analysis (data not shown). Besides, no differences in methodology results were obtained when performed in different laboratories. Consequently, the method was validated in terms of accuracy, sensitivity and specificity as the error was 5% or lower in all cases (Commission Decision, 2002; Dang et al., 2010), and showed robust results.

3.2. Antibiotic occurrence and risk assessment in wastewater

Wastewater samples can contain high concentrations of antibiotics coming from different urban or farming activities. In this

Table 2
Antibiotic list with predicted non effect concentration and microbial growth inhibition test detection limits in different matrices.

Antibiotic family	Compound	PNEC (μg/L)	Detection limits (μg/L)				
			Freshwater	Seawater	Wastewater	Fish plasma	Mussel hemolymph
Tetracyclines	Oxytetracycline	0.31	0.08	0.12	0.27	100	100
	Chlortetracycline	5.00	0.25	0.02	0.83	10	10
	Tetracycline	1.00	0.06	0.08	0.20	50	50
	Doxycycline	0.30	0.02	0.02	0.07	10	10
Quinolones	Ofloxacin	0.02	0.11	0.10	0.37	100	100
	Enrofloxacin	0.06	0.05	0.04	0.17	25	25
	Ciprofloxacin	0.05	0.04	0.04	0.13	10	50
	Norfloxacin	0.50	0.07	0.11	0.23	100	150
Macrolides	Tylosin	1.00	0.11	0.29	0.37	100	100
	Tilmicosin	0.52	0.11	0.06	0.37	100	50
	Erythromycin	0.20	0.06	0.06	0.20	50	25
	Azithromycin	0.01	0.01	0.01	0.03	25	25
	Spiramycin	0.50	0.13	0.18	0.43	100	100
Sulfonamides	Sulfamethazine	4.00	0.16	0.25	0.53	100	100
	Sulfadiazine	10.33	0.24	0.29	0.80	150	50
	Sulfamethoxazole	0.03	0.16	0.10	0.53	100	50
	Sulfapyridine	6.20	0.17	0.16	0.57	100	100

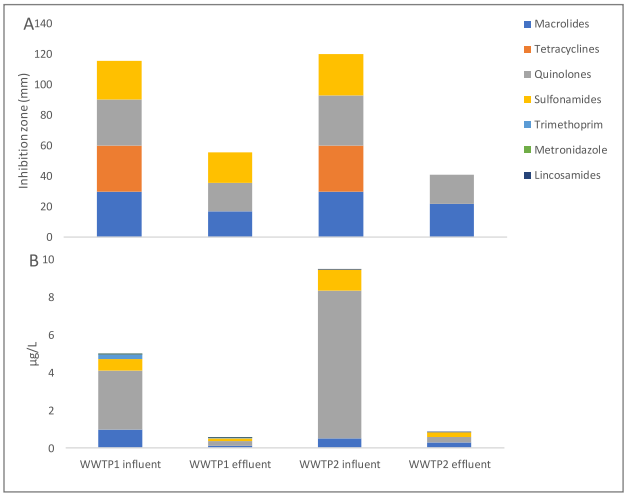

Fig. 2. Antibiotics occurrence in wastewater (influent and effluent). A) Antibiotic families detected with the microbial growth inhibition test (macrolides and tetracyclines area in both influent samples are approximate inhibition area); B) antibiotic families quantified with LC-MS/MS methodology.

study, two WWTPs were considered in the area of the Ebro Delta, receiving effluents from the surrounding towns. Results of antibiotics determination in wastewater are shown in Fig. 2 (Fig. 2A microbial test results; Fig. 2B wastewater characterization with LC-MS analysis) and Table 3 and at supporting information, Table S4 microbial test inhibition areas and Table S5 quantification of antibiotics with LC-MS. Both methodologies (chemical and microbial analysis) showed the occurrence of quinolones, macrolides and sulfonamides antibiotics in WWTP influent samples. The antibiotic detected with the highest concentration, determined with LC-MS analysis, was ciprofloxacin, at 2.1 and 5.9 μg/L in the influent of WWTP1 and WWTP2, respectively. The only mismatch between both methodologies in influent samples was found for tetracyclines because they showed an inhibition area in the microbial test, but tetracyclines were not detected with LC-MS analysis. The inhibition observed in the tetracycline plates test can be attributed to other substances, such as soaps or disinfectants, which occur in WWTP influents and are able to inhibit the growth of *B. cereus* (Monarca et al., 2000). The occurrence of these substances with bactericidal properties in untreated wastewater may also provoke the irregular inhibition zone observed in macrolides plates, despite macrolide antibiotics occurred in WWTP influent samples.

WWTP significantly reduced antibiotic concentrations and antibiotic activity when comparing influent and effluent samples (Fig. 2). However, in few cases higher concentrations of antibiotics were found in the effluent when compared with influent, as it was observed for azithromycin antibiotic. Previous studies reported this behavior for some contaminants, including macrolide antibiotics

Table 3
Summary of antibiotic concentration and antibiotic risk from the different water matrices analyzed. Antibiotic concentration refers to the sum of individual antibiotics measured from a same antibiotic family; the highest concentration of the different sites is presented. + refers that antibiotic risk was identified. − no antibiotic risk identified.

Antibiotic family	Wastewater effluent[a]			Freshwater[b]			Seawater[c]		
	Antibiotic concentration (μg/L)	Antibiotic risk (LC-MS)	Microbial inhibition	Antibiotic concentration (μg/L)	Antibiotic risk (LC-MS)	Microbial inhibition	Antibiotic concentration (μg/L)	Antibiotic risk (LC-MS)	Microbial inhibition
Macrolides	0,30	+	+	0,00	−	−	0,03	−	−
Tetracyclines	0,00	−	+	0,08	−	+	0,00	−	−
Quinolones	0,27	+	+	0,00	−	−	0,00	−	−
Sulfonamides	0,27	−	+	0,01	−	+	0,02	−	−
Trimethoprim	0,03	−	n.p.	0,01	−	n.p.	0,00	−	n.p.
Metronidazole	0,00	−	n.p.	0,00	−	n.p.	0,00	−	n.p.
Lincosamides	0,04	−	n.p.	0,01	−	n.p.	0,00	−	n.p.

n.p. = no specific microbial inhibition plate.
[a] Highest antibiotc concentration from the two WWTP effluents measured.
[b] Highest antibiotic concentration from the three freshwater sites monitored.
[c] Highest antibiotic concentration from the 16 seawater and lagoon sites monitored.

(Gros et al., 2010), which was attributed to the conversion of glucuronide metabolites to the parent compound. Effluent samples of the two analyzed WWTPs were dominated by quinolones and macrolides families according to both methodologies (Fig. 2). Sulfonamides were present in both effluents according to LC-MS analysis but in higher concentration in WWTP2. However, the microbial growth inhibition test only showed inhibition in the sulfonamides plate at the effluent of WWTP1. This can be explained by the presence of other antibiotics in the WWTP1 effluent that inhibited the activity of this plate, such as, trimethoprim (not occurring in the effluent of WWTP2). These results indicated that the interaction between sulfonamides (sulfamethoxazole) and trimethoprim provoked a higher antibacterial activity when compared with the activity of sulfonamides alone (WHO, 2019). This demonstrated the potential of the microbial test in identifying synergistic activity between antibiotics.

The occurrence of antibiotics in WWTP effluents can pose a risk for the receiving environments. Effluent samples from WWTP1 and WWTP2 presented HQ > 1 for individual antibiotics, such as azithromycin, ciprofloxacin and ofloxacin (Fig. 3) and showed inhibition in the corresponding plates of the microbial test (macrolides and quinolones) (Fig. 2). In previous studies that targeted several WWTPs located at the Ebro River area, macrolides (azithromycin), sulfonamides (sulfamethoxazole), quinolones (ofloxacin and ciprofloxacin) and trimethoprim were the main antibiotics discharged by the WWTPs effluents to the receiving environment (Celic et al., 2019; Gros et al., 2007). Garcia-Galán (García-galán et al., 2011) also reported a HQ value higher than 1 for sulfamethoxazole in the effluent of another WWTP located in the area of Ebro Delta.

3.3. Antibiotic occurrence and risk assessment in freshwater

Freshwater samples were characterized from the lower reach of the Ebro River. Results of water samples from the Ebro River are shown in Fig. 4 (4A microbial test; 4B LC-MS analysis) and Table 3 and at supporting information, Table S4 shows the measured inhibition area values with microbial test and Table S5 quantification of antibiotics with LC-MS analysis. Both methodologies pointed out the sites FW1 and FW3 as the most antibiotic polluted ones in the Ebro River (Fig. 4); whereas, FW2 site presented lower concentration of antibiotics according to LC-MS and no inhibition in the test plates. Inhibition in tetracyclines plate in sites FW1 and FW3 could

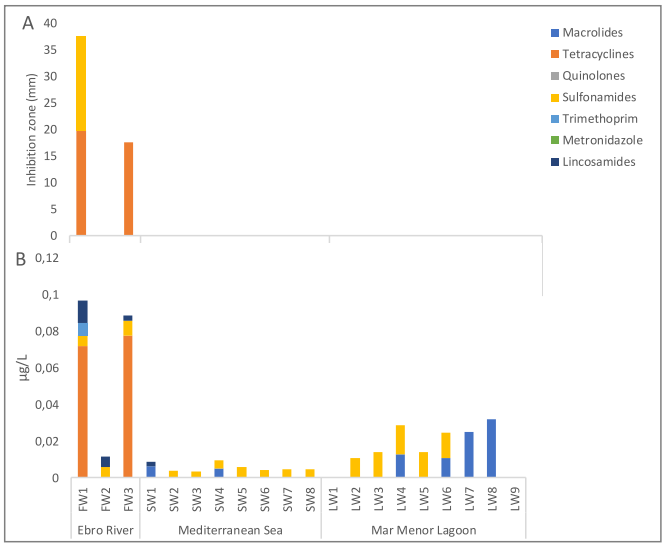

Fig. 4. Antibiotics occurrence in surface water (freshwater, Ebro River; seawater, (Mediterranean Sea and Mar Menor Lagoon). A) antibiotic families detected with SPE+microbial growth inhibition test; B) antibiotic families quantified with SPE+LC-MS methodology.

be attributed to doxycycline occurrence quantified with LC-MS method at levels of 0.07 and 0.08 µg/L in FW1 and FW3 samples, respectively. Inhibition in sulfonamides plate in a sample from FW1 could be due to simultaneous occurrence of sulfonamides and trimethoprim antibiotics, as it was observed for WWTP samples. The synergistic interaction between these two antibiotics was shown in the plates. Lincosamides were also quantified with LC-MS analysis in all river samples (FW1, FW2 and FW3) but at lower concentrations compared to tetracyclines, Fig. 4B.

Samples taken in the river water FW1 showed some of the highest antibiotic's concentrations, despite it is located upstream of the discharge of both WWTPs. The same was observed in previous studies in this area and was attributed to the anthropogenic and agricultural activities from towns located near to this sampling site (Čelic et al., 2019). Furthermore, the antibiotics with the highest concentrations in FW1 were tetracyclines, not found in the effluent

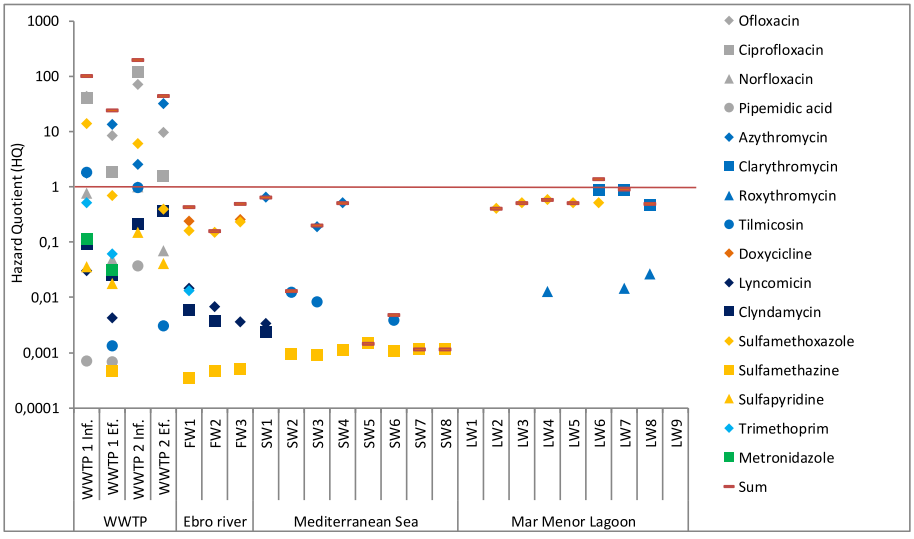

Fig. 3. Hazard quotients (HQ) representation for the antibiotic quantified in water samples with LC-MS. Individual antibiotic HQ and the sum per water sample is presented.

of the WWTP (Fig. 4). Therefore, non-point sources or WWTP discharges located upstream, but not considered in the present work, may explain the occurrence of these compounds in this sampling site of Ebro river. Lower concentration of antibiotics was observed in the FW2 sampling site, probably due to dilution effects from upstream site (FW1) and the absence of WWTP discharge in this river section (Fig. 4). FW3 sampling site, located downstream of the WWTP1 presented a higher amount of antibiotics compared to the FW2. FW3 showed antibiotic occurrence mainly for sulfonamides and lincosamides, also present in WWTP1 effluent, so these antibiotics may be related to the input of WWTP effluents. The contribution of WWTP to pharmaceuticals including antibiotics occurrence in the area of Ebro River was previously observed, mainly for macrolides and sulfonamide antibiotics (Silva et al., 2011). However, the antibiotics detected at the highest concentration in FW3 site where tetracyclines, not occurring in WWTP1 effluent. Therefore, as the case of FW1 site, other sources of antibiotics such as livestock production should be considered. Despite tetracyclines were the antibiotics detected at the highest concentration in river water, they posed no risk for the ecosystem according to the calculated HQ (Fig. 3), and no risk was determined for the rest of the antibiotics quantified in river water nor for the sum of HQ per sample (Fig. 3).

3.4. Antibiotic occurrence and risk assessment in seawater

Two different types of marine environments were considered in the study. The Mediterranean Sea area located in the Ebro Delta, receiving the Ebro River discharge (Fig. 1), and the Mar Menor Lagoon, a costal saltwater lagoon located in the south-east of Spain near the Mediterranean Sea (Fig. 1).

Regarding seawater in the Mediterranean Sea area, the microbial growth inhibition test showed no inhibition in any of the analyzed samples (Fig. 4A, Table 3, Table S4), whereas, chemical analysis with LC-MS reported antibiotic concentration (mainly for sulfonamides, macrolides and lincosamides) in all the samples at low concentrations (all of them were detected at concentrations of few ng/L) (Fig. 4B, Table 3, Table S5). These differences between the outcome of the two methodologies can be attributed to higher sensitivity of LC-MS when compared with the microbial inhibition test. Sulfonamides were the most widespread antibiotics in seawater present in all samples except for site SW1 (Fig. 4). They were found at concentrations ranging from 3 to 6 ng/L and no differences were observed between the different locations, probably due to dilution effects. The reported antibiotic concentrations in sea water presented no risk for the ecosystem according to the calculated individual antibiotic HQ and the sum of HQ per sample, Fig. 3; similar concentrations in Mediterranean Sea water (low ng/L levels) were observed for emerging contaminants including some antibiotic (Brumovsky et al., 2017). Despite the lack of reported risk, the chronic exposure of wildlife to biological active substances needs further research to discard any potential negative implications.

Similar results to Mediterranean Sea water were obtained when characterizing the Mar Menor Lagoon. The microbial growth inhibition test did not report inhibition in any of the test samples (Fig. 4A). Chemical analysis showed occurrence of antibiotics in 7 out of the 9 samples analyzed (Fig. 4B). Sulfonamides were the most widespread antibiotic family in the Mar Menor Lagoon, although macrolides were detected in four out of the nine samples analyzed. Previous studies determined the main antibiotic inputs to Mar Menor Lagoon including the presence of sulfamethoxazole and clarithromycin (Moreno-González et al., 2014), two of the main antibiotics determined in the present work. However, the concentrations determined in the present work, ranging from 6 to 16 ng/L, were lower than the ones obtained in previous studies (Moreno-González et al., 2014) which can be related with the improvement of this environment through the reduction of WWTP discharges. Furthermore, the studied area is strongly affected by tourism, which may provoke seasonal variations on the impact of emerging contaminants, as previously observed in other environments (Mandaric et al., 2017). The low concentrations of antibiotics presented no risk for the ecosystem according to the individual antibiotic HQ. Only one sample (LW6) showed a HQ higher than 1 when summing the individual antibiotic risks of sulfamethoxazole and clarithromycin.

3.5. Antibiotic occurrence in biota biofluids

In this study, different biota classes were characterized, namely, fish samples from the Ebro River and the Mediterranean Sea, marine mussels from the Mediterranean Sea and gastropods from the Mar Menor Lagoon. Analysis was performed in the organisms biofluids (fish plasma and mollusk hemolymph). The microbial test showed inhibition in the sulfonamide's plates in two plasma samples from Ebro fish (Fig. 5A, Table S6). Chemical analysis reported antibiotic concentration of tetracyclines, macrolides, lincosamides and trimethoprim in four fish samples (Ebro River) and quinolones in one mussel sample from Mediterranean Sea (Fig. 5B, Table S7). No antibiotic occurrence was detected in gastropod from the Mar Menor Lagoon, neither with chemical analysis nor with the microbial test.

The two applied methodologies reported different results in biota biofluids analysis. None of the antibiotic concentrations quantified with LC-MS was high enough to provoke inhibition to the test plates. Namely, the sensitivity of the microbial test (LODs between 10 and 150 μg/L) was not enough to detect the presence of these compounds in the biological samples (concentrations between 0.1 and 5.8 μg/L). Besides, the two fish plasma samples that showed inhibition with the microbial inhibition test presented low or no quantifiable levels of antibiotics, Fig. 5. No matrix interferences would be expected as no inhibition was seen in the other characterized fish plasma samples. The occurrence of other antibiotics in fish plasma not targeted with the LC-MS methodology or the presence of antibiotic active metabolites, may explain the observed inhibition.

The reported concentrations of antibiotics in biota fluids measured by LC-MS, could be related with the antibiotic occurrence in water samples. Tetracyclines, lincosamides and trimethoprim detected in fish plasma samples from the Ebro River were also detected in the water samples closest to the fish sampling point. However, other antibiotics like macrolides and quinolones found in biota biofluids were not detected in environmental water samples, although they were highly detected in WWTP effluents. Quinolones persistence time in surface water is low due to its rapid photodegradation, hence, they are more frequently detected in sediment and biota, rather than in water, which may explain its detection in biota tissues but not in surrounding water (Li et al., 2012). Besides, the bioaccumulation measured of macrolides and quinolones may correspond to other time frame, as bioaccumulation of contaminants in aquatic organisms represent long time series rather than an occasional sampling time.

3.6. Combining chemical and microbial methodologies

The combination of different methodologies for the determination of antibiotics in environmental samples can facilitate the implementation of antibiotics monitoring in the environment.

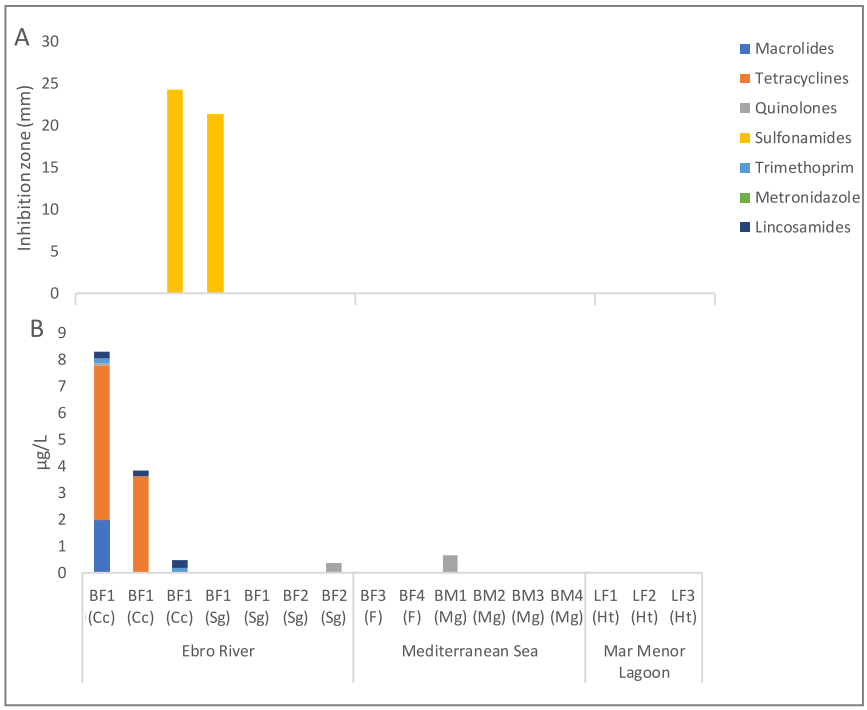

Fig. 5. Antibiotics occurrence in biota biofluids for each sampling site (localization codes according to Fig. 1). A) Antibiotic families detected with the microbial growth inhibition test; B) antibiotic families quantified with LC-MS methodology. In brackets letters indicate organism species, Cc, *Cyprinus carpio; Sg, Silurus glanis; Mg, Mytilus galloprovincialis; Ht, Hexaplex trunculus*.

Besides, further insights regarding the risks posed by antibiotics may be spotlighted.

All water samples that showed a potential antibiotic risk based on their HQ calculated with LC-MS results also exhibited inhibition with the microbial growth inhibition test. Therefore, the method can be used to screen those water samples with potential antibiotic risk. Then, antibiotic identification and quantification can be carried out with chemical analysis only in those samples with potential risk. This combination could provide a significant decrease of analytical costs and facilitate its implementation and application to a broader range of institutions and/or companies for routine analysis of antibiotics risk such as WWTPs, hospital and livestock production effluents. In fact, the microbial inhibition test is routinely applied for the screening of antibiotics in livestock samples for food quality control (Pikkemaat et al., 2008). Besides, the application of both methodologies provided further insights regarding antibiotic risk in the aquatic environment, allowing to determine antibiotic occurrence (with LC-MS) and potential antibiotic synergistic effects (microbial test), However, the environmental water samples presenting low levels of antibiotics concentrations were not highlighted as positive with the microbial inhibition test. Other approaches used to evaluate antibiotic risk based on LC-MS/MS analysis followed by antibiotic risk calculation, can provide lower limits of detection but they lack on identifying synergies between compounds (Yan et al., 2013). Recently applied methods such as suspect screening or non-target analysis for environmental contaminants prioritization allow the identification of a broader range of contaminants in a single run including compounds of different classes (pharmaceuticals, pesticides, herbicides, etc.), and they are not limited by compounds with analytical standards availability (Čelic et al., 2021). Therefore, comprehensive risk assessment can be obtained with these methodologies, but requiring complex instrumentation and exhaustive data treatment.

4. Conclusions

In this work an effect-based methodology based on microbial growth inhibition test was adapted for its application in different environmental matrices (water and biota biofluids). The optimized screening method was combined with LC-MS for antibiotics risk assessment in the Ebro Delta area and the Mar Menor Lagoon. According to the reported antibiotic occurrence, the different water samples characterized can be ordered as follows (decreasing order) WWTP influent > WWTP effluent > river water > Lagoon water > seawater mainly related to dilution effects. Biota samples (fish) from the Ebro river showed significant higher concentrations compared with mussels (Mediterranean Sea) and gastropods (Mar Menor Lagoon). The combination of screening methods followed by chemical analysis can provide a reduction of antibiotics analysis costs, facilitating its implementation for environmental monitoring. Besides, the antibiotics identification and quantification capacity of LC-MS can be complemented with the potential of the microbial test to determine synergistic effects between antibiotics. However, the high effect-based methodology detection limits difficulted its applicability in surface waters, such as seawater. Further improvement of water preconcentration step could increase the effect-based methodology sensibility to screen antibiotics when occur at low concentrations. The application of combined approaches such as this would be beneficial in order better understand and evaluate the risk of antibiotics in the environment and the potential hazard consequences for the environment and the human health.

Credit author statement

Albert Serra-Compte, Conceptualization; Investigation; Writing - Original Draft. Mariël G. Pikkemaat: Conceptualization; Methodology; Supervision. Alexander Elferink, Investigation; Methodology. David Almeida: Investigation; Resources. Jorge Diogène: Investigation; Resources. Juan Antonio Campillo: Investigation; Resources. Marta Llorca: Investigation; Resources. Diana Álvarez-Muñoz: Conceptualization; supervision; Writing - Review & Editing. Damià Barceló: Conceptualization; supervision; Writing - Review & Editinz. Sara Rodríguez-Mozaz: Conceptualization; supervision; Writing - Review & Editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116313.

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Chapter 4 – Antibiotics environmental impact

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Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*)☆

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ABSTRACT

Warming and acidification are expected impacts of climate change to the marine environment. Besides, organisms that live in coastal areas, such as bivalves, can also be exposed to anthropogenic pollutants like pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs). In this study, the effects of warming and acidification on the bioconcentration, metabolization and depuration of five PhACs (sotalol, sulfamethoxazole, venlafaxine, carbamazepine and citalopram) and two EDCs (methylparaben and triclosan) were investigated in the mussel species (*Mytilus galloprovincialis*), under controlled conditions. Mussels were exposed to warming and acidification, as well as to the mixture of contaminants up to 15.7 $\mu g\,L^{-1}$ during 20 days; followed by 20 days of depuration. All contaminants bioconcentrated in mussels with levels ranging from 1.8 $\mu g\,kg^{-1}$ dry weight (dw) for methylparaben to 12889.4 $\mu g\,kg^{-1}$ dw for citalopram. Warming increased the bioconcentration factor (BCF) of sulfamethoxazole and sotalol, whereas acidification increased the BCF of sulfamethoxazole, sotalol and methylparaben. In contrast, acidification decreased triclosan levels, while both stressors decreased venlafaxine and citalopram BCFs. Warming and acidification facilitated the elimination of some of the tested compounds (i.e. sotalol from 50% in control to 60% and 68% of elimination in acidification and warming respectively). However, acidification decreased mussels' capacity to metabolize contaminants (i.e. venlafaxine). This work provides a first insight in the understanding of aquatic organisms' response to emerging contaminants pollution under warming and acidification scenarios.

1. Introduction

The effects that climate change may have on the environment are a topic of increasing concern. The release of carbon dioxide to the atmosphere, mainly attributed to human activities, has contributed to global warming (IPCC, 2014; Pinguelli-Rosa and Kahn-Ribeiro, 2001). In addition, the carbon dioxide deposition in water bodies promotes seawater acidification (Sabine et al., 2004). Warming and acidification are two major threats to the marine environment. The forecasted rise of few Celsius degrees in seawater temperature, accompanied by a decrease of few tenths in seawater pH, may provoke huge changes in aquatic organisms' lifestyle in the future (IPCC, 2014; Wernberg et al., 2011). Several studies described adverse effects in marine organisms submitted to warming and acidification, including reduction of calcification rates, changes in metabolism functioning and increase of oxidative stress, among others (Duarte et al., 2014; Ko et al., 2014; Kroeker et al., 2014, 2013, 2010; Lesser, 2016; Poore et al., 2013; Rosa et al., 2012).

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In addition to the direct effects on organisms' physiology, climate change impacts are also expected to influence the behavior of chemical contaminants in aquatic systems (Schiedek et al., 2007). Thus, warming and acidification may alter the way that organisms interact with contaminants present in the environment and in their potential to accumulate them. Previous studies revealed changes in contaminants accumulation, like metals, in bivalve species under warming and acidification (López et al., 2010; Maulvault et al., 2016). However, to the best of our knowledge, there is no information available about the influence of climate change on the accumulation, metabolization and depuration of emerging contaminants like pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) in marine organisms. PhACs may pose a risk for aquatic communities since they are designed to be pharmacologically active in organisms, even at very low concentrations. Different studies reported the presence of these compounds in water bodies and its accumulation in freshwater and marine biota worldwide (Álvarez-Muñoz et al., 2015; Li, 2014; Llorca et al., 2016; Rodriguez-Mozaz et al., 2017, 2016; Serra-Compte et al., 2017)). In addition, adverse effects in aquatic organisms due to an exposure of PhACs have been reported (Corcoll et al., 2015; Cortez et al., 2012; Godoy et al., 2015; Minguez et al., 2016; Santos et al., 2010; Serra-Compte et al., 2018). On the other hand, EDCs are substances that can mimic the activity of endogenous compounds, altering the normal functioning of an organism (Tijani et al., 2013). EDCs have been found in marine bivalves in concentrations ranging from below MDL up to 39.4 ng g^{-1} dw (Vandermeersch et al., 2015). Some of the most frequently detected EDCs in marine bivalves from the Mediterranean zone are caffeine, TCEP, TBEP, methylparaben, ethylparaben, propylparaben, triclosan and bisphenol A; and they were proposed as priority contaminants for future studies (Álvarez-Muñoz et al., 2015; Huerta et al., 2015). Alterations in organisms' molecular and gene expression (Park and Kwak, 2010), changes in the immunological system (Casanova-Nakayama et al., 2011) or an increased frequency of gonadal regression and atresia in mussels (*Mytilus trossulus*) (Smolarz et al., 2017) have been described in different organisms due to EDCs exposure. These contaminants (PhACs and EDCs) reach to the marine environment mainly through waste water treatment plant (WWTP) effluents as they are not completely removed in WWTPs (Kostich et al., 2014). Therefore, coastal areas receiving an input of WWTP effluents (mainly through rivers discharge), are some of the most impacted marine aquatic ecosystem, concerning wastewater derived contaminants. Organisms living in these areas, like bivalves, are thus exposed to chemical pollution (i.e. PhACs and EDCs, among others) and are also particularly vulnerable to changes in environmental conditions (e.g. temperature, pH). Bivalves are filter feeding organisms thus easily accumulating contaminants (Ismail et al., 2014); therefore, they are used as sentinel organisms to monitor chemical pollution in coastal areas (Hellou and Law, 2003; OSPAR, 2016). In addition, they have an important role in the ecosystem by filtering toxins and bacteria from the surrounding water, and serve as food source for many species, including humans (Zippay and Helmuth, 2012).

Understanding the bioaccumulation of emerging contaminants in marine organisms under expected climate change conditions becomes of great interest, not only from an ecological perspective but also in relation to seafood consumption safety. In this work, an *in vivo* exposure experiment of mussels (*Mytilus galloprovincialis*) to five PhACs (sotalol, sulfamethoxazole, venlafaxine, carbamazepine and citalopram) and two EDCs (methylparaben and triclosan) under water warming and acidification scenarios was carried out in order to evaluate the effects of temperature and pH on the bioconcentration and depuration of these compounds. In addition, the formation of the main metabolites of sulfamethoxazole, venlafaxine and carbamazepine was also investigated. Finally, linear quantitative structure-activity relationship (QSAR) models were evaluated for the prediction of PhACs and EDCs accumulation in bivalves; the predicted values were compared with those obtained experimentally providing further information about the mechanisms of emerging contaminants accumulation in biota.

2. Material and methods

2.1. Chemicals and reagents

Pharmaceutical standards were of high purity grade (>90%). All pharmaceutical standards (listed in Table 1) were purchased from Sigma-Aldrich, whereas the metabolites N-desmethylvenlafaxine, O-desmethylvenlafaxine, NN-didesmethylvenlafaxine, NO-didesmethylvenlafaxine, NN-didesmethyl-O-desmethylvenlfaxine, carbamazepine-10,11epoxy, carbamazepine-2-hydroxy, N-acetylsulfamethoxazole and desamino-sulfamethoxazole were obtained from Toronto Research Chemicals (TRC). HPLC grade methanol, water and acetonitrile were purchased from Merck (Darmstadt, Germany). The QuEChERS extract tubes (AOAC method), and the QuEChERS for dispersive solid phase extraction

Table 1
List of target compounds analysed. Precursor ion, retention time (RT), and the two MRM transitions used for compound identification.

Therapeutic family	Compound	Precursor ion	RT (min)	Q3	Q3
Psychiatric drugs	Venlafaxine	278 $[M+H]^+$	2.75	58	260
	Citalopram	325 $[M+H]^+$	2.90	109	262
	Carbamazepine	237 $[M+H]^+$	3.19	193	194
(metabolite)	O-desmethylvenlafaxine	264 $[M+H]^+$	2.14	134	198
(metabolite)	NN-didesmethyl-O-desmethylvenlafaxine	235 $[M+H]^+$	2.15	159	218
(metabolite)	NO-didesmethylvenlafaxine	250 $[M+H]^+$	2.16	43	214
(metabolite)	NN-didesmethylvenlafaxine	250 $[M+H]^+$	2.77	214	232
(metabolite)	N-desmethylvenlafaxine	263 $[M+H]^+$	2.76	215	246
(metabolite)	Carbamazepine-2-hydroxy	252 $[M+H]^+$	2.71	208	210
(metabolite)	Carbamazepine-10,11-epoxide	252 $[M+H]^+$	2.72	180	236
Antibiotics	Sulfamethoxazole	254 $[M+H]^+$	1.98	92	156
(metabolite)	N-acetylsulfamethoxazole	296 $[M+H]^+$	2.38	134	198
(metabolite)	Desamino-sulfamethoxazole	238 $[M+H]^+$	2.66	77	131
Beta-blocker	Sotalol	273 $[M+H]^+$	1.10	255	133
Endocrine disrupting compounds	Methylparaben	151 $[M-H]^-$	1.30	92	136
	Triclosan	286 $[M-H]^-$	3.50	34	–

(dSPE) (15 mL, fatty acids tubes) were obtained from Waters Corporation (Milford, MA, U.S.A.). PVDF filters (0.22 μm pore) were purchased from Merck Millipore Corporation (Darmstadt, Germany). Isotopically-labelled internal standards (carbamazepine-d10, citalopram-d4, venlafaxine-d6, sulfamethoxazole-d4, atenolol-d7, triclosan-d3 and methylparaben-d4) were purchased from Sigma-Aldrich. Stock standards and isotopically labelled internal standards were prepared in methanol at a concentration of 1000 mg L^{-1} and stored at -20 °C. Working standard solutions (1 mg L^{-1}) were prepared in methanol:water (10:90 v/v) before each analytical run.

2.2. Experimental design

Mussels were collected in Tagus Estuary (Portugal) during summer season. Bivalves were transported at 18 °C in transport cooling chambers to the aquaculture facilities of Laboratório Maritimo da Guia (Cascais, Portugal). Then, 50 animals per tank were randomly distributed in ten shaped glass tanks (100 L) of a recirculation aquaculture system (RAS), each with independent functioning (i.e. protein skimmers, chemical and biological filtration, UV disinfection, aeration and light control). Temperature was set and adjusted using an automatic seawater refrigeration system (±0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as, submerged digital thermostats (200 W, V2Therm, TMCIberia, Portugal). Seawater pH was set and maintained using individual pH probes (GHL, Germany) connected to a computerized pH control system (±0.1 pH units; Profilux 3.1N, GHL, Germany), which monitored seawater pH in each tank every 2 s, and adjusted them whenever needed, via submerged air stones, by injecting CO_2 (Air Liquide, Portugal; to decrease pH) or by CO_2-filtered aeration (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom). Mussels were acclimated for a period of 7 day at 18 ± 0.5 °C and pH 8.00 ± 0.05 units (mean water temperature and pH of Tagus Estuary during summer season; set as control temperature and pH conditions). Water physical-chemical parameters were monitored on a weekly basis during all experimental days (40 days); they are presented in Table S1. Ammonia (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were daily checked (Tropic Marin, USA), and kept below detectable levels, with the exception of nitrates, which were kept below 2 mg L^{-1}. Specimens were kept under the following conditions: dissolved oxygen >5 mg L^{-1}, salinity = 35 ± 1‰ and photoperiod of 12 h light and 12 h dark (12L:12D). Mussels were fed three times per day (15 mg of powdered feed L^{-1}) with a commercial microalgae mix suitable for bivalve molluscs, composed by 2.000×10^6 cells mL^{-1} of the following species: *Isochrysis* spp., *Tetraselmis* spp, *Nannochloropsis* spp, *Thalassiosira* spp. (Microalgae Mix 18%, Acuinuga, Spain). Besides, on a daily basis, 25% of water was renewed. Low mortality (1%) was only observed in the first 2 days after arrival to the laboratory (i.e. during the first days of acclimation) whereas no mortality was observed during the 40 days of trial. After the acclimation period, temperature was slowly raised (1 °C per day) until 22.0 °C ± 0.5 in warming treatments, whereas pH was slowly decreased (0.1 units per day) until 7.60 ± 0.05 in water acidification treatments; to rise 1 °C of water temperature and reduce 0.1 pH units per day was done in order to acclimate the animals to the experimental conditions, thus avoiding the physiological stress induced by drastic temperature and pH variations. The increase of 4 °C in water temperature and a decrease of pH in 0.4 units were selected on the basis of the expected effects of climate change in seawater temperature and pH in the marine ecosystem (IPCC, 2014; McNeil and Sasse, 2016). The four resulting treatments (in duplicate tanks) exposed to the contaminants were: control (Cont.) (reference temperature and pH conditions, Tº 18 °C, pH 8.00 units); water warming (WW) (Tº 22 °C, pH 8.00 units); water acidification (WA) (Tº 18 °C, pH 7.60 units) and water warming plus acidification (WW + WA) (Tº 22 °C, pH 7.60 units). In addition, two tanks were not spiked with the mixture of contaminants (Non-spiked tanks, NST) at Tº 18 °C and pH 8.00 units. A schematic representation of the experimental design is shown at supporting information (Fig. S1). The experiment lasted 40 days. In the first 20 days, bivalves were exposed to the mixture of contaminants, whereas in the last 20 days spiking was stopped and a depuration period was conducted. During the contaminant exposure phase (20 days), a volume of each contaminant stock solution (contaminants stock solution prepared on < 5 mL of solvent, methanol, chloroform, or acid nitric, according to contaminant's chemical properties, and the final volume adjusted with seawater to 500 mL) was added to the water of each contaminated treatment. In Non-spiked tanks the equivalent amount of solvent was also added to ensure that no carrier solvent lethal toxicity occurred. The contaminants were added via water on a daily basis with detected concentrations in the spiked tanks ranging from below method detection limit for triclosan and methylparaben up to 15.7 μg L^{-1} for carbamazepine. The studied compounds were selected as representative of different chemical families and on the basis of their occurrence in the natural environment (Álvarez-Muñoz et al., 2015; Nödler et al., 2014; Rodriguez-Mozaz et al. 2017). In order to evaluate the putative loss of contaminants due to adsorption in the system, a previous trial was performed by adding the contaminants mixture to the tanks without mussels; also, microalgae feed was added to the system too (data not shown). Contaminant loss in the system differed depending on the compound in a range of 22% loss of its initial concentration for carbamazepine to a loss of 97% for methylparaben after 24 h of spiking. Therefore, the spiked concentrations in the final experiment were slightly above those reported in the environment, about one order of magnitude (Nödler et al., 2014), in order to compensate the loss of contaminants due to adsorption process in the system. Water and bivalves were sampled at days, 0, 2, 10, 20 (exposure) 22, 30 and 40 (depuration). Two tanks were used per treatment. Six animals (n = 3 per replicate treatment) were randomly sampled. Then, edible tissues were collected, pooled (i.e. n = 2 pools per treatment, per sampling day) and immediately frozen at -80 °C. Then, it was freeze-dried and kept at -80 °C until further analysis.

2.3. Sample extraction and UPLC-MS/MS analysis

As mentioned before, mussel samples were collected and pooled at each sampling time. Each of the two experimental replicates was extracted in duplicate: 0.5 g of dry tissue were weighted prior to extraction with QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), followed by a clean-up using dispersive solid phase extraction (dSPE), applying a methodology adapted from Jakimska et al. (2013). Full details of the extraction and clean-up procedure are given as supporting information. Regarding water samples, they were processed by evaporating 1 mL of sample (in triplicate) and reconstituting each in 1 mL of MeOH:H_2O (10:90 v/v). The analysis of biota extracts and water samples was done by means of ultra-performance liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometry (UPLC-QqLIT), following the method of Gros et al. (2012) for PhACs, whereas the analysis of EDCs was done in the same equipment, following the method of Jakimska et al. (2013). Finally, an adaptation of the Gros et al. (2012) method was used for the target analysis of the main metabolites of sulfamethoxazole, venlafaxine and carbamazepine (listed in Table 1). Explanation of the UPLC-MS/MS conditions for the analysis of PhACs, EDCs and metabolites is given as supporting information.

2.4. Linear quantitative structure-activity relationship (QSAR) estimation

Two existing QSAR models for the theoretical determination of compounds bioconcentration factors based on Octanol-Water partition coefficient (Log K_{ow}) were applied. The first model was developed by Veith et al. (1979) for the bioconcentration factor prediction of 55 chemicals in fathead minnows (*Pimephales promelas*). In contrast, Mackay (1982) developed a model by applying the same dataset than Veith et al. (1979) but with the inclusion of 13 additional compounds. The developed models are the following:

Log BCF = 0.85 Log K_{ow} - 0.70 (Veith et al., 1979)

Log BCF = 1 Log K_{ow} - 1.32 (Mackay, 1982)

2.5. Data analysis

The bioconcentration factors (expressed in L kg^{-1}) were calculated with the following equation:

$$\text{Bioconcentration factor (L kg}^{-1}) = C_{biota}/C_{water},$$

where C_{biota} is the contaminants concentration in mussels (μg Kg^{-1} dw) at the end of the exposure phase (day 20), whereas C_{water} is the contaminants concentration in water (μg L^{-1}) at the same sampling time (day 20).

The percentage of contaminants elimination during the depuration phase was calculated according to the following equation:

$$\text{Percentage of elimination (\%)} = 100 - [(C_{end}/C_{initial}) * 100],$$

where C_{end} is the concentration of contaminants in bivalves (μg kg^{-1} dw) at the end of the experiment (day 40), whereas $C_{initial}$ is the concentration of contaminants in bivalves (μg Kg^{-1} dw) at the end of the exposure phase (day 20).

Log K_{ow} of each compound was calculated using the online tool ChemAxon (Chemicalize); whereas, Log D_{ow} was calculated for each compound at each studied pH, using the same online tool as log K_{ow} (ChemAxon, Chemicalize).

For the determination of significant differences in contaminants bioconcentration, BCFs and contaminants depuration percentage within the different treatments, one way ANOVAs were performed. The normality and variances homogeneity of the data was tested before ANOVAs by using Shapiro-Wilk and Levene's test, respectively. For those compounds that data showed no normality or homogeneity, a Kruskal-Wallis test was performed. Additionally, Tukey's post hoc test or Conover test (for non-parametric data), were applied. All statistical analysis was performed using R software (3.1.0) with a significance level of p-value < 0.05.

3. Results

3.1. Concentration of PhACs and EDCs in water

Results showed that levels of contaminants in water samples from non-spiked tanks (NST) were below method detection limits (MDL) in all cases (Table S2). Regarding the exposure treatments, levels of venlafaxine, carbamazepine and citalopram in water were kept constant along the exposure phase. The average concentration considering exposure treatments was 10.7 ± 1.6 μg L^{-1}, 12.0 ± 1.9 μg L^{-1} and 5.1 ± 0.9 μg L^{-1} for venlafaxine, carbamazepine and citalopram, respectively, with a decrease in their concentrations up to 2.9 ± 0.7 μg L^{-1}, 4.9 ± 1.2 μg L^{-1} and 0.7 ± 0.2 μg L^{-1}, respectively, after 20 days of depuration (Table S2). Sotalol and sulfamethoxazole were less stable in water and their concentrations highly differed between treatments, ranging from 1.3 to 9.7 μg L^{-1} and 1.0–13.0 μg L^{-1} respectively along the exposure phase. After 20 days of depuration these two compounds were only detected in few samples with values above method quantification limits (MQL). Methylparaben and triclosan were only detected in few samples at day 2 of exposure with concentrations above MDL (Table S2).

3.2. Bioconcentration of PhACs and EDCs in marine mussels

Bivalves from non-spiked tanks (NST) showed levels below MQL for the majority of the compounds. Only citalopram and methylparaben presented levels above MQL, 2.3 ± 1.7 μg kg^{-1} dry weight (dw) and 3.2 ± 2.3 μg kg^{-1} dw respectively, (mean concentrations over the 40 days of the experiment) (Table S3), probably due to contamination of mussels at the sampling site in Tagus estuary as has been reported previously (Álvarez-Muñoz et al., 2015). Regarding the levels of contaminants found in mussels from the exposure treatments, all compounds showed bioconcentration in bivalves, though at different degree. The concentration of the studied compounds measured in mussels for the exposure treatments during experiment days are shown in Fig. 1, whereas the specific values obtained for all treatments including those in non-spiked tanks are reported in Table S3. Citalopram was detected at the highest concentration after 20 days of exposure in control treatment (12889.4 μg kg^{-1} dw), followed by venlafaxine (5419.5 μg kg^{-1} dw). Triclosan and carbamazepine presented their highest value after two days of exposure, 1106.4 μg kg^{-1} dw and 453.2 μg kg^{-1} dw in the warming treatment. Sotalol and sulfamethoxazole showed their highest concentrations after 20 days of exposure in control mussels, i.e. 182.6 μg kg^{-1} dw and 81.3 μg kg^{-1} dw, respectively. Finally, methylparaben reached its highest concentration after two days of exposure, 45.4 μg kg^{-1} dw in mussels maintained under acidification conditions.

The majority of the compounds showed their highest concentrations in bivalves during the exposure phase under control conditions, with the exception of triclosan and carbamazepine (highest concentration in water warming conditions) and methylparaben (highest concentration in water acidification conditions). However, contaminants concentrations in water differed between treatments, especially sulfamethoxazole and sotalol levels; these differences can be corrected by calculating the bioconcentration factors (BCFs). The BCFs of each compound at the end of the exposure phase (day 20) for each treatment are presented in Fig. 2. Citalopram and venlafaxine presented the highest BCFs, followed by sotalol and carbamazepine, whereas sulfamethoxazole showed the lowest BCFs. In the case of EDCs, triclosan and methylparaben, only an estimation of the BCF could be calculated, as their concentrations in water were below MDL or MQL in most cases, so half of MDL and MQL were respectively used as water concentration for these compounds (Table S1). Triclosan showed a higher BFC compared to methylparaben, (Table 2).

Warming and acidification affected the BFCs of PhACs and EDCs in mussels in different ways depending on the compound. Warming significantly increased BCFs of sotalol and sulfamethoxazole, whereas they decreased for venlafaxine and citalopram, compared to the control treatment (Fig. 2). In contrast, no significant differences were observed for triclosan, carbamazepine and methylparaben. Acidification significantly increased the BCF of methylparaben, sotalol and sulfamethoxazole when compared to the control treatment, and decreased venlafaxine, citalopram and triclosan BFCs, whereas carbamazepine concentrations were not

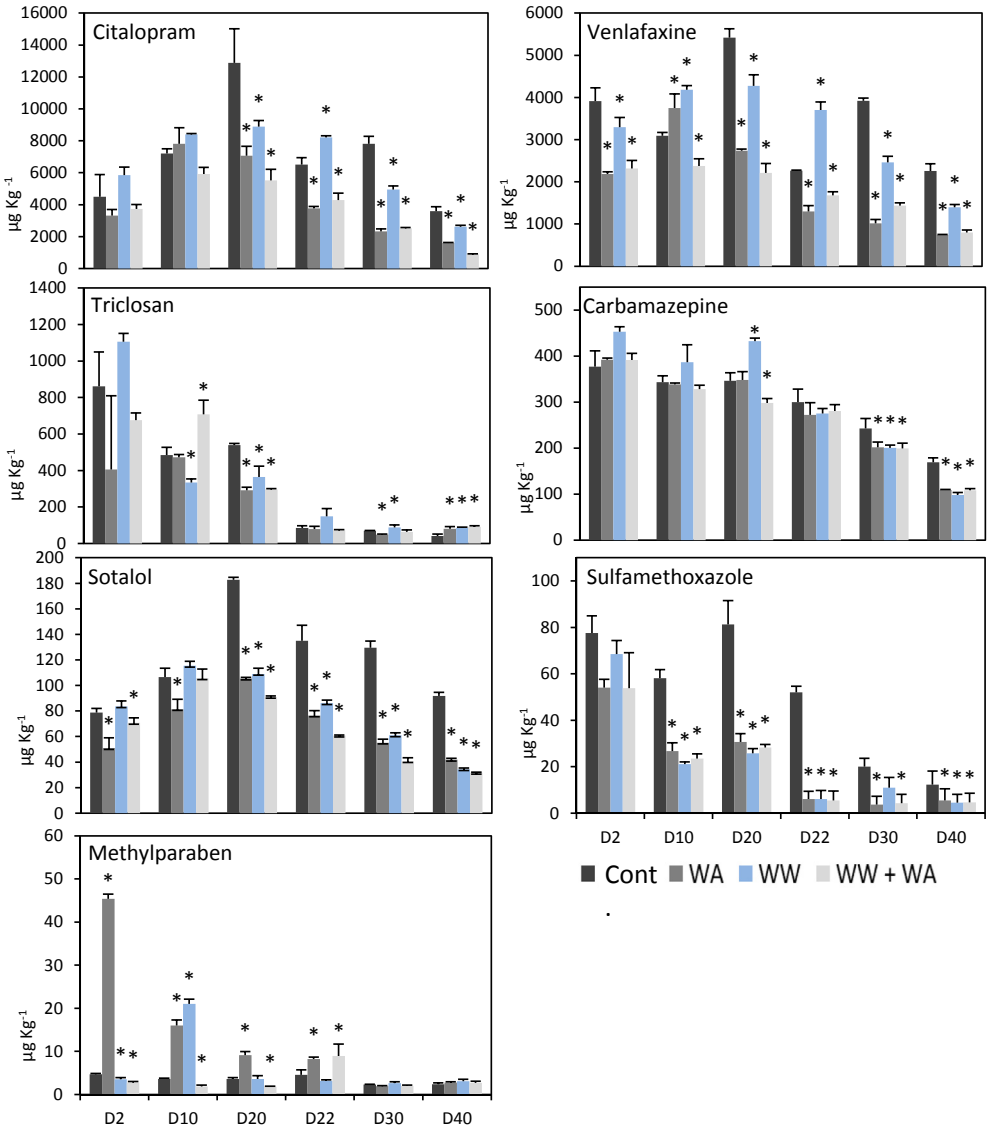

Fig. 1. Contaminants concentration in biota (µg kg^{-1}, dw) for the different treatments, Cont. (control); WW (warming); WA (acidification) (WW + WA) warming plus acidification. (*) indicates significant differences between treatments (WA, WW or WW + WA) and control. P-value < 0.05, (n = 4).

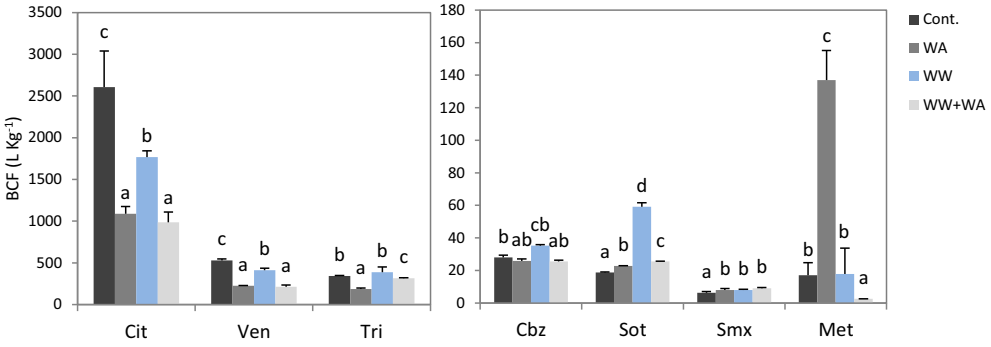

Fig. 2. Experimental bioconcentration factors (L kg^{-1}) for each compound, Cit, citalopram; Ven, venlafaxine; Tri, triclosan; Cbz, carbamazepine; Sot, sotalol; Smx, sulfamethoxazole and Met, methylparaben and each treatment; Cont. (control); WW (warming); WA (acidification) (WW + WA) warming plus acidification, after 20 days of exposure. Different letters indicate statistical differences for each compound between the treatments (p-value < 0.05; n = 4).

affected by acidification. The combined effects of warming and acidification significantly increased the BCF for sulfamethoxazole and sotalol, compared to the control treatment, but decreased the BFC of venlafaxine, citalopram, methylparaben and triclosan.

Table 2
Bioconcentration factors (L kg^{-1}) obtained experimentally (within the different treatments) and those predicted with the QSAR models developed by Mackay (1982) and Veith et al. (1979). In addition, the Log K_{ow} and Log D_{ow} (at pH 7.6 units and pH 8.0 units) values for each compound are reported. Log K_{ow} and Log D_{ow} values were obtained using ChemAxon (Chemicalize) online tool.

Parameters	Compounds						
	Citalopram	Venlafaxine	Triclosan	Carbamazepine	Sotalol	Sulfamethoxazole	Methylparaben
Experimental BCF	985.6–2606.2	213.3–528.1	185.9–313.8	25.8–35.3	18.8–59.2	6.2–9.0	2.5–137.3
BCF Mackay	275.4	26.3	4570.9	28.2	0.02	0.3	2.2
BCF Veith	313.3	42.6	3411.9	45.1	0.1	0.9	5.2
Log K_{ow}	3.8	2.7	5	2.8	−0.4	0.8	1.7
Log D_{ow} (pH 7.6)	1.6	1.4	4.7	2.8	−2.1	−0.1	1.6
Log D_{ow} (pH 8.0)	2	1.8	4.5	2.8	−1.6	−0.1	1.6
Major species pH 7.6	Positive	Positive	Neutral	Neutral	Positive	Negative	Neutral
Major species pH 8.0	Positive	Positive	Negative	Neutral	Positive	Negative	Neutral

Acidification seems to be the dominant factor when both conditions (warming and acidification) were combined, as BCFs were closer to those shown when mussels were exposed to acidification only, with the exception of the EDCs triclosan and methylparaben (Fig. 2).

3.2.1. Application of QSAR models for BCF prediction

A comparison between BCFs obtained experimentally and those predicted with QSAR models based on contaminants Log K_{ow} is presented at Table 2. The BCF for most of the compounds was underestimated when applying QSAR models, with the exception of triclosan and methylparaben that were overestimated (considering all treatments). However, BCFs obtained experimentally for carbamazepine were closer to values provided by QSAR models, being the experimental values in the range of 25–35 L kg^{-1} and those predicted with the models between, 28–45 L kg^{-1} (Table 2).

3.3. Metabolization of PhACs

The main metabolites of sulfamethoxazole, carbamazepine and venlafaxine were investigated in mussel and water samples (the target metabolites are listed at Table 1). These compounds were selected since they are reported in pharmacokinetics and drug metabolism studies and metabolites are available as analytical standards. The target metabolites of sulfamethoxazole and carbamazepine were not detected in any biota samples. Regarding venlafaxine metabolization, the three main metabolites selected (N-desmethylvenlafaxine, O-desmethylvenlafaxine and NO-didesmethylvenlafaxine) were detected in mussel samples. Their concentrations, as well as that of the parent compound venlafaxine in biota are shown in Fig. 3 and Table S4, whereas statistical differences between treatments are reported in Table S5. All metabolites showed its highest value during the depuration phase, between day 20 and day 40 (Fig. 3; Table S4). N-desmethylvenlafaxine presented its highest concentration at the end of the experiment (day 40) in the control treatment, followed by O-desmethylvenlafaxine at the end of the exposure phase (day 20) in the control treatment. Finally, NO-didesmethylvenlafaxine reached its highest value, after 10 days of depuration (day 30) in the warming treatment (Fig. 3; Table S4). The concentration of all venlafaxine metabolites detected in biota, decreased in the acidification treatments for the majority of the sampling times, whereas warming effects on venlafaxine metabolites concentration were less clear compared to the control treatment (Table S5). In agreement with the results found in the BCF study, acidification seems to be the dominant factor regarding venlafaxine metabolites concentration in mussels when both stressors are combined.

The occurrence of metabolites into water was also investigated (results are presented in Table S6). Only N-desmethylvenlafaxine and O-desmethylvenlafaxine were detected in water, being their

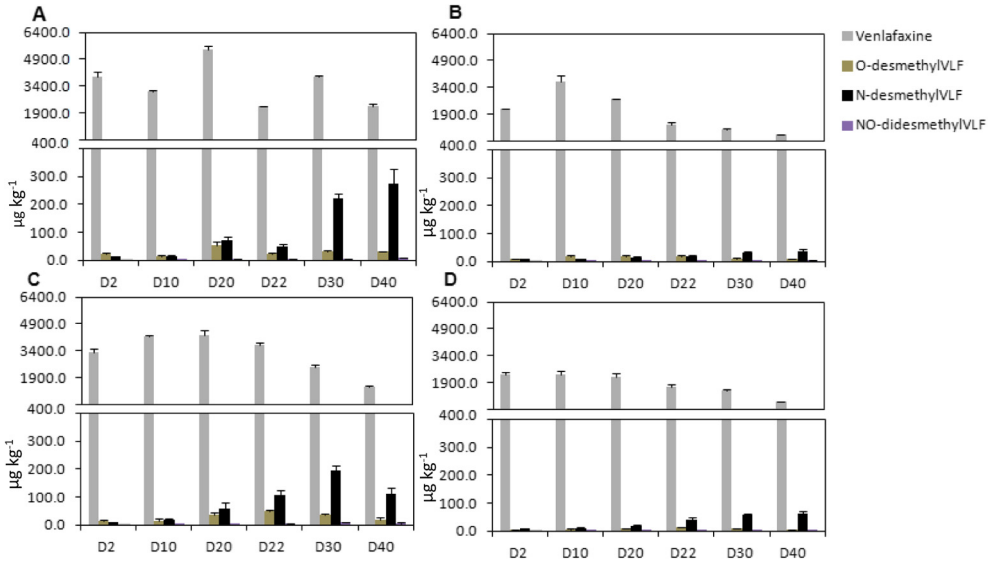

Fig. 3. Venlafaxine and its metabolites concentrations in biota (µg kg^{-1}, dw) in each treatment: A, control; B, acidification; C, warming; D, warming plus acidification. Number of replicates, n = 4. The statistical analysis between the different treatments is reported in Table S4.

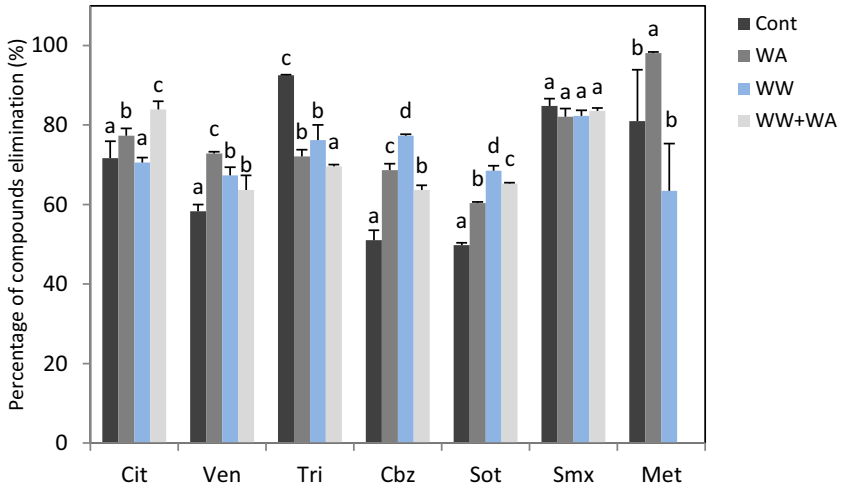

Fig. 4. Percentage of depuration of each compound, Cit, citalopram; Ven, venlafaxine; Tri, triclosan; Cbz, carbamazepine; Sot, sotalol; Smx, sulfamethoxazole and Met, methylparaben and each treatment; Cont. (control); WW (warming); WA (acidification) (WW + WA) warming plus acidification, after 20 days of depuration. Different letters indicate statistical differences for each compound between the treatments, (p-value < 0.05; n = 4).

highest concentrations registered after 10 days of depuration (day 30) in the control treatment and after 20 days of exposure (day 20) in the warming treatment, respectively (Table S6). Their concentrations in water followed the same tendency than the ones found in bivalves, being higher in control and warming treatments and lower in those tanks with acidified water. In addition, metabolites highest concentrations in water were reached between days 20 and 40 of the experiment, when mussels revealed the highest concentrations of these metabolites. NO-didesmethylvenlafaxine was not detected in any water sample nor were carbamazepine and sulfamethoxazole metabolites.

3.4. Depuration of PhACs and EDCs

The percentage of depuration of each compound is presented in Fig. 4. During the depuration phase contaminants spiking was stopped, but the remaining concentration from the exposure phase was still present for most of PhACs during this phase in water, though at lower concentration (Table S2). Depuration percentage (mean of all treatments) after 20 days was higher than 60% for all compounds. Overall, compounds showing the highest depuration were methylparaben and sulfamethoxazole, both showing to eliminate more than 80% of the concentration found at day 20 (end of exposure). Triclosan and citalopram revealed elimination percentages above 70%, whereas venlafaxine, carbamazepine and sotalol showed elimination values above 60%. Warming and acidification significantly increase the depuration percentage for sotalol, venlafaxine and carbamazepine, whereas lower values were observed for triclosan. Citalopram and methylparaben depuration decreased only when exposed to acidification while sulfamethoxazole did not show significant changes in its depuration percentage under the tested conditions (Fig. 4).

4. Discussion

Bioconcentration is an important parameter for the assessment of contaminants toxicity to non-target organisms. Bioconcentration factor (BCF), which relates the concentration of a certain contaminant in biota with its corresponding concentration in water, is the most used parameter to assess contaminants uptake by organisms. Regulatory authorities established a BCF higher than 1000 L kg^{-1} wet weight (ww) (EPA, 2012), or > 2000 L kg^{-1} (ww) (EU, 2011) threshold for considering a compound to be bioaccumulative in organisms. Taking these limits into account, none of the tested contaminants could be considered bioaccumulative in mussels, when converting our data expressed in dry weight to wet weight, by dividing the experimental BCF for the conversion factor (i.e. 5.5) usually reported for bivalves (Dahlgaard et al., 2001). These results are in line with other studies that reported BCFs in the same range for similar compounds in aquatic organisms. Indeed, Boillot et al. (2015) reported a BCF of 3.9 and 4.5 L kg^{-1} dw for carbamazepine and its metabolite 10-hydroxy-10,11-dihydro-carbamazepine respectively, in mussels; Valdés et al. (2016) observed a BCF of 9.0 L kg^{-1} for carbamazepine in the fish species *Jenynsia multidentata*, those values are slightly lower than those reported in the present work for carbamazepine (between 25.8 and 35.3 L kg^{-1}). Nallani et al. (2011) reported low BCFs for ibuprofen, ranging from 0.1 to 1.4 L kg^{-1} in the two fish species *Pimephales promelas* and *Ictalurus punctatu.*

Contaminants physical-chemical properties may determine the way that they accumulate in organisms. The Octanol-Water Partition Coefficient (Log K_{ow}) is the most used parameter to predict the bioconcentration potential of contaminants with a Log K_{ow} up to 8 units. For these compounds, the higher their Log K_{ow} is, the more likely they bioconcentrate in organisms (EU, 2011). This general tendency is followed for most of the compounds analysed. For instance, sotalol and sulfamethoxazole have low Log K_{ow} values (-0.4 and 0.8 respectively) and their BCFs are the lowest detected. On the contrary, triclosan, citalopram and venlafaxine have higher Log K_{ow} values (5.0, 3.8 and 2.7, respectively) and their BCFs are the highest detected (Table 2). However, when applying QSAR models based on contaminants Log K_{ow} for BCF prediction, only carbamazepine, which is non-ionisable, was correctly predicted with the models. These results are in agreement with previous studies, which highlighted that QSAR models can be successfully applied for non-ionisable compounds, but when dealing with ionisable compounds its prediction capacity is limited (Fu et al., 2009). Contaminants ionization may affect their capacity to accumulate in organisms (Du et al., 2015). Ionized species are expected to have lower bioaccumulation in organisms as they are less hydrophobic than the neutral ones and the lipid bilayer is impermeable to the ionized species, preventing them entering into the cells (Erickson et al., 2006; Nakamura et al., 2008). However, in the present study, ionized contaminants presented a higher BCF than the

predicted with the theoretical models, especially for those compounds positively ionized (citalopram, venlafaxine and sotalol). Positively ionized contaminants have shown a higher adsorption (adhesion of pollutants to the biomass surface) than the neutral or the negatively ionized species in activated sludge biosolids and soil (Franco and Trapp, 2008; Stevens-Garmon et al., 2011). This phenomenon has also been observed in biological matrices (biofilm) (Torresi et al., 2017), and despite it is expected to be lower than in the case of soil, adsorption might occur in mussels too (Filipkowska et al., 2005). The results obtained here, showed that both, adsorption of contaminants and absorption (accumulation of contaminants into the cells) are occurring when mussels are exposed to contaminants. While ionization of contaminants prevents them from entering into the cells, it would also facilitate its adhesion to the biological matrix (mussel's tissue) especially for those compounds positively ionized. Therefore, the final result when analysing all mussels' soft tissue (as it was done in the present work) reveals a higher bioaccumulation for the positively ionized species and higher than theoretical one (Table 2). Further studies are needed in this direction, for example comparing the real bioaccumulation in the organism through the analysis of biofluids with the concentration in the whole organism or comparing the bioaccumulation in different mussel's organs as they may have different affinity for the contaminants.

Changes in the environmental conditions (i.e. water acidification) may alter the physical-chemical properties of contaminants altering their bioaccumulation capacity in organisms. In the present work, the comparison between the Log D_{ow} values of each compound at pH 8.0 units and pH 7.6 units may explain the increase or decrease of their bioconcentration capacity under acidification (Table 2). For compounds in which Log D_{ow} was reduced with acidification, such as venlafaxine and citalopram, their BCFs decreased under this environmental condition. Carbamazepine presented no variation in their Log D_{ow} between the two studied pH values, and its BCF didn't change under water acidification. However, this rule doesn't apply always. For instance, triclosan Log D_{ow} increased under acidification but its BCF decreased. In addition, sotalol Log D_{ow} decreased due to acidification, but its BCF increased, and methylparaben and sulfamethoxazole Log D_{ow} didn't change under water acidification, but their BCF significantly changed. Compounds' Log K_{ow} and Log D_{ow} only partially explained the bioconcentration capacity of contaminants in mussels and their variations in BCFs when environmental conditions change (i.e. water acidification); highlighting the above mentioned that other mechanisms may play important roles in the electrochemical affinity between the compound and the interacting matrix (mussels tissue in the present work) (Huerta et al., 2015; Yamamoto et al., 2009).

Changes in environmental conditions may not only affect the physical-chemical properties of contaminants, they can also alter the physiological status of the organisms (Cherkasov et al., 2007; Nardi et al., 2017) which may influence contaminants bioconcentration. It has been shown that an increase in water temperature, within a certain species thermal tolerance range, increases aquatic organisms metabolic activity, i.e. feeding adsorption in mussels (Navarro et al., 2016). The increase in mussels feeding adsorption may lead to a higher uptake of contaminants (Heugens et al., 2003). Results showed that two out of the seven contaminants considered (sotalol and sulfamethoxazole) significantly increased its BCF under warming conditions comparing to the control treatment. Triclosan, carbamazepine and methylparaben slightly increased its BCF under warming conditions too. However, venlafaxine and citalopram BCF decreased under this condition. Therefore, a direct relationship between warming and higher bioconcentration cannot be established for all compounds. In line with this, an increase on cadmium accumulation in mussels (*Mytilus galloprovincialis*) under warming conditions was not found by Nardi et al. (2017), pinpointing that the bioconcentration capacity of each contaminant under water warming should be evaluated separately. On the other hand, acidification can also affect aquatic organisms functioning. Kroeker et al. (2010) showed that calcifying and sessile organisms like mussels are sensible to acidification. Higher energy demand for homeostatic processes in bivalves have been shown to provoke a depletion of growth, fitness and metabolic rates under acidification in marine organisms (Kroeker et al., 2014, 2010). Thus, it can be hypothesized that the reduction of mussel's biological activities may promote a decrease of contaminants uptake under acidification. In this sense, a decrease of contaminants accumulation under acidification was mainly observed for citalopram, venlafaxine and triclosan. Carbamazepine also slightly decreased its BCF under acidification.

In addition to parent compounds, the presence of metabolites in environmental compartments has become a topic of increasing interest. In the present work, the main metabolites of sulfamethoxazole, carbamazepine and venlafaxine were investigated. Since the metabolites of sulfamethoxazole and carbamazepine were not detected (limits of detection in biota ranging from 0.1 to 0.7 ng g^{-1}) their metabolization by mussels under the tested exposure concentrations cannot be confirmed. To the best of our knowledge no study has been published yet reporting sulfamethoxazole metabolization by mussels. In this sense, more research focused on this issue would be required in order to confirm our hypothesis of non-metabolization capacity by mussels. Regarding carbamazepine metabolization, Boillot et al. (2015) detected two carbamazepine metabolites in an *in vivo* exposure of mussels. They detected acridine and carbamazepine-10,11-epoxide metabolites when exposing bivalves at 100 μg L^{-1} of carbamazepine in water. However, in the same study, when mussels were exposed to 10 μg L^{-1}, no metabolization of carbamazepine was detected with levels above MQL, indicating that the exposure concentration in the present work (12.0 ± 1.9 μg L^{-1}) might not be sufficient to produce or detect carbamazepine metabolites.

On the contrary, metabolization of venlafaxine was confirmed, as three main metabolites were detected in mussels (N-desmethylvenlafaxine, O-desmethylvenlafaxine and NO-didesmethylvenlafaxine). Due to the conservation of metabolic pathways within organisms, PhACs (like venlafaxine) can be metabolised by non-target organisms like mussels. Cytochromes P450 monooxygenase enzymes (CYPs) have been detected in mussels digestive tract, which is thought to be the main organ involved in the detoxification of xenobiotic substances in bivalves (Livingstone and Farrar, 1984). Although little is known about CYP system in bivalves, different CYP enzymes (i. e. CYP4) and CYP-like genes (CYP1 and CYP3) have been detected in these organisms (Snyder, 1998; Zanette et al., 2013). CYP3 enzymes are involved in the metabolization of venlafaxine to N-desmethylvenlafaxine in humans (Magalhães et al., 2014), which may also be the main venlafaxine metabolization pathway in mussels as N-desmethylvenlafaxine was the main metabolite detected; indicating a different venlafaxine metabolization than humans, where O-desmethylvenlafaxine is the major metabolite (Magalhães et al., 2014). Despite metabolization of pharmaceuticals (i. e. venlafaxine) may occur in the system due to abiotic factors (light, temperature, etc.) or other biotic factors (i. e. microorganisms), metabolization of venlafaxine by mussels has been described by other authors such as Martínez Bueno et al. (2014), who also found N-desmethylvenlafaxine as the most frequently detected venlafaxine metabolite in mussels.

Changes in the environmental conditions, like water warming, may alter the metabolization of xenobiotic compounds by

organisms. Kim et al. (2010) found that under warming (from 15 °C to 25 °C) the metabolization of the pharmaceutical acetaminophen increased in aquatic organisms, which was explained by the increase of organism's metabolism activity under this condition. In the present work, no clear increase in the venlafaxine metabolites concentration was found due to warming, probability due to the fact that the raise of temperature (from 18 °C to 22 °C) was not sufficient to increase venlafaxine metabolization in mussels. However, when mussels were exposed to acidification, the concentration of all venlafaxine metabolites significantly decreased (Fig. 3; Table S5). These results are in line with the reduction of the parent compound (venlafaxine) accumulation under acidification. In addition, the percentage of venlafaxine metabolization was calculated by dividing the sum of all venlafaxine metabolites concentrations by the concentration of the parent compound for each treatment in each sampling time (Fig. S2). These results suggested not only a reduction of venlafaxine accumulation, but also a decrease of its metabolization under acidification conditions. Venlafaxine and its metabolite, O-desmethylvenlafaxine have a similar pharmacological activity, whereas other metabolites like N-desmethylvenlafaxine and NO-didesmethylvenlafaxine are less active (in humans) (Magalhães et al., 2014). Therefore, the decrease of mussels' capacity to metabolize venlafaxine to less-active metabolites, like N-desmethylvenlafaxine and NO-didesmethylvenlafaxine, under acidification may alter the effects of this contaminant in mussels.

Metabolization of contaminants and excretion of the unchanged parent compound into the environment are two strategies of mussels to eliminate toxic compounds. The decrease of venlafaxine metabolization under acidification was not associated with a reduction of venlafaxine depuration under this condition. The percentage of venlafaxine metabolization was below 10% in most cases (Fig. S2), indicating that the main pathway for this compound depuration is the excretion of the unchanged parent compound. Indeed, the reduction of venlafaxine metabolization didn't affect its final depuration percentage. In agreement, no metabolization of sulfamethoxazole was detected, whereas this compound was highly depurated. As it was mentioned above, warming may increase the mussels' metabolic activity, enhancing contaminants depuration (Kim et al., 2010; Noyes et al., 2009). Such pattern was observed mainly for sotalol, venlafaxine and carbamazepine. On the other hand, mussels exposed to acidification showed an increase in its excretion rate in order to eliminate ammonium, which was postulated to be an important mechanism for acid excretion (Lindinger et al., 1984; Michaelidis et al., 2005). Therefore, this increase in the excretion may provoke the higher elimination percentage observed for the majority of the tested compounds (citalopram, venlafaxine, carbamazepine, sotalol and methylparaben) under this condition.

Acidification seems to be the dominant factor when both stressors act together regarding contaminants BCFs and for the venlafaxine metabolites concentration, as their values under warming plus acidification treatment are closer to those shown when mussels were exposed to acidification only. This may be explained by different factors. As mentioned above, acidification may alter contaminants physical-chemical properties, changing its capacity to accumulate in organisms; whereas a low increase in water temperature (+4 °C) is not expected to affect or counteract these changes when in combination with acidification. In addition, acidification seems to alter more the physiological functioning of calcifying organisms than water warming (Duarte et al., 2014; Findlay et al., 2010); and dominate the final response of the organisms when in combination with water warming, as previously seen for the growth rate of mussel's *Mytilus chilensis* (Duarte et al., 2014) and the growth rate of barnacle *Semibalanus balanoides* (Findlay et al., 2010). Despite, in some other cases, the effects of water acidification in bivalves can be compensated by low levels of temperature increase (Byrne, 2011).

5. Conclusions

The expected warming and acidification of seawater altered the bioconcentration of the selected PhACs and EDCs in mussels (*Mytillus galloprovincialis*). Several factors may influence the bioconcentration of contaminants under these conditions, such as changes in the physical-chemical properties of contaminants and/or the biological status of the organism. Thus, warming and acidification induced different responses on contaminants bioconcentration in mussels under the tested conditions, making difficult to forecast contaminants behavior (increase or decrease of its bioconcentration) under these conditions. The results obtained revealed that acidification was the dominant factor when both stressors were combined, regarding contaminants bioconcentration. In most cases, depuration percentages were enhanced by both warming and acidification. The analysis of contaminants' metabolization provided further understanding of the effect of warming and acidification in marine mussels, and evidenced that acidification may hinder mussel's capacity to metabolize some contaminants, such as venlafaxine.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.02.018.

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Comprehensive study of sulfamethoxazole effects in marine mussels: Bioconcentration, enzymatic activities and metabolomics

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ABSTRACT

Antibiotics accumulation in aquatic organisms may be of great concern from an ecological point of view but also from a human perspective, especially when they are accumulated in edible animals like marine mussels. In this work, mussels (*Mytilus galloprovincialis)* were exposed to sulfamethoxazole antibiotic (SMX) at 10 μg/L during 96 h, followed by 24 h of depuration. The experiment was carried out at summer and winter conditions. SMX showed a bioconcentration factor in mussel of 1.5 L/kg (dry weight) and 69% of the compound was eliminated from the organism in 24 h. The metabolomics approach revealed alterations in amino acids levels (aspartate, phenylalanine, valine and tryptophan) pinpointing disturbances in osmotic regulation and energy metabolism. Besides, the levels of some nucleotides (guanosine and inosine) and a carboxylic acid were also affected. However, SMX exposed mussels did not show any significant alteration in the enzymatic activities related to the xenobiotic metabolism and oxidative stress. Moreover, some of the changes observed in mussel's metabolites suggested alterations in mussel's organoleptic characteristics that can affect its quality as seafood commodity. Overall, our results showed that SMX exposure to marine mussels may have ecological implications by provoking sub-lethal effects to exposed organisms. Nevertheless, no risk for consumers derived from mussel ingestion is expected due to the low bioconcentration capacity of SMX and fast depuration in this seafood type.

1. Introduction

Antibiotics are worldwide contaminants of emerging concern. They reach the aquatic environment mainly through waste water treatment plant effluents, among other sources (Fatta-Kassinos et al., 2011). These compounds can pose a risk for the aquatic community chronically exposed to them. Besides, there is a growing concern about the contribution of antibiotic pollution to the development of antibiotic resistant bacteria (Grenni et al., 2018; Kümmerer, 2009). Sulfamethoxazole (SMX) is a bacteriostatic antibiotic, effective against both, gram negative and gram positive bacteria. It is extensively used due to its bactericidal broad spectrum and low cost (Carvalho and Santos, 2016). Sulfamethoxazole reaches the coastal areas mainly through river discharges and due to its use in aquaculture (Shimizu et al., 2013; Zhang et al., 2012). Its presence in coastal waters and particularly estuaries, harbors and lagoons has been widely studied with levels ranging from low ng/L up to few μg/L (Rodriguez-Mozaz et al., 2017). Although SMX is not expected to kill eukaryotic organisms at environmental concentrations, it may produce sub-lethal effects and alter the normal functioning of the organisms in the aquatic ecosystem. To this respect, the analysis of enzymatic activities related to xenometabolism and oxidative stress have been commonly used for the characterization of organisms response to different stress factors, including chemical pollution (Vidal-Liñán, 2015). Furthermore, the analysis of organisms metabolome (through a metabolomics approach) has been used in many fields including ecotoxicology, for the evaluation of sub-lethal alterations in organisms exposed to different contaminants, revealing new ecotoxicological effects and postulating biomarkers of exposure (Álvarez-Muñoz et al., 2014; Serra-Compte et al., 2018a). Metabolomics is also applied in food science (foodomics) for the characterization of food properties like nutritional value, savor, taste and odor within others, and its changes under different conditions or treatments (Cevallos-Cevallos and Reyes-De-Corcuera, 2012).

Among aquatic organisms, marine mussels are highly valuable for ecological studies and also as food source for human consumption. Their characteristics as sessile and filter-feeding organisms make them

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prone to bioaccumulate contaminants, including antibiotics, present in their surrounding environment (Álvarez-Muñoz et al., 2015a, 2015b). Therefore, they are extensively used as sentinels for chemical pollution monitoring in natural environments (Hellou and Law, 2003; OSPAR, 2016). Despite several studies showed that changes in environmental conditions (i. e. water temperature) or the annual reproductive cycle of mussels, may influence their response to chemical pollution (Costa et al., 2008; González-Fernández et al., 2016; Maulvault et al., 2016; Serra-Compte et al., 2018b) few studies considered seasonality when assessing bioconcentration of contaminants on these organisms (Claudi and Mackie, 1994; Costa et al., 2008; Galvao et al., 2015). On the other hand, the presence of antibiotics in marine mussels may pose a risk for consumers such as allergy and toxicity (Cabello, 2006). Therefore, maximum residue limits (MRL) for some antibiotics in foodstuff from animal origin have been established by the authorities (including SMX, 100 ng/g, wet weight, European Commission, 2010).

Little is known about the ecotoxicological implications of antibiotics exposure to the aquatic organisms. Furthermore, the evaluation of antibiotics effects in foodstuff from animal origin, such as mussels, is also of great interest from a human perspective. We thereby hypothesize that SMX will induce enzymatic responses and modulate the metabolome profile of marine mussels. In this work, an in-vivo exposure of marine mussels to the antibiotic SMX was carried out during 96 h at summer (water temperature 16.0 °C) and winter (water temperature 12.5 °C) conditions. This was undertaken in order to study potential differences in the response of mussel to chemical pollution due to seasonality and consequently to different state of maturity. The ecotoxicological effects were evaluated using metabolomics and through the analysis of enzymatic activities involved in xenometabolism and oxidative stress responses such as carboxylesterases (CbE), glutathione S-transferase (GSTs), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO) levels. The main objectives of this work were: i) to study bioaccumulation, metabolization and depuration of SMX in commercial mussels, ii) to assess the response of mussels to SMX through changes in xenometabolism and oxidative stress enzymatic activities, and iii) to evaluate alterations in mussel's metabolome using a non-targeted metabolomics approach and postulate potential biomarkers of exposure. This is the first time that a metabolomics approach has been addressed to characterize the response of marine mussels to antibiotics pollution.

2. Material and methods

2.1. Standards and reagents

The cartridges used for solid phase extraction, OASIS HLB (200 mg, 6 mL), were obtained from Water Corporation (Milford, MA, U.S.A.). HPLC grade methanol and water were purchased from Merck (Darmstadt, Germany). Sulfamethoxazole standard as well as the isotopically-labelled internal standard sulfamethoxazole-d4 were purchased from Sigma-Aldrich (St Louis, MO, USA). Sulfamethoxazole metabolites, N-acetyl sulfamethoxazole, desamino-sulfamethoxazole and glucoronide sulfamethoxazole were purchased from Toronto Research Chemicals (TRC) (Ontario, Canada). All analytical standards were HPLC suitable and were prepared in methanol at a concentration of 1000 mg/L and stored at -20 °C. Working standard solutions (1 mg/L) were prepared in methanol before each analytical run.

2.2. Experimental design

Mussels and seawater were obtained from a bivalve's supply plant (Girona, Spain) in two different seasons, summer and winter. Bivalves and seawater were transported to the facilities of Catalan Institute for Water Research (Girona, Spain). The experiment lasted ten days divided in three different periods; an acclimation period (5 days), where mussels were kept in the laboratory experimental conditions but without addition of SMX. Then, half of the mussels were exposed to SMX during 4 days (exposure period of 96 h), while the other half remained in control conditions (without SMX). This short term exposure allowed the evaluation of early ecotoxicological responses of mussels due to antibiotics pollution. After the exposure period, a depuration period without addition of SMX was carried out (during 24 h) at a commercial bivalve's supply plant. Initially, 300 individuals were acclimated in a 500 L tank of a water recirculation system, equipped with protein skimmers, biological filtration, temperature control and aeration. After the acclimation period, the 300 individuals were randomly distributed in two separate tanks, 150 individuals per tank (500 L) with the same equipment described above. The experiment was carried out two times in order to have two experimental replicates. Specimens were kept under the following conditions: temperature: 16.0 ± 0.7 °C during the summer trial and 12.5 ± 0.8 °C during the winter trial. The temperature was set at the same temperature than the one registered in the Mediterranean Sea (Girona coast) during the two sampling trials (June and February). During both trials the pH was 8.0 ± 0.1 units, dissolved oxygen (DO) > 80% DO/L, salinity 40.4 ± 0.6‰ and photoperiod of 12 h light and 12 h dark (12 L:12D). Ammonia (NH_3/NH_4^+) was kept below 0.2 mg/L and nitrates (NO_3^-) below 1 mg/L, nitrites were in all analysis below quantification levels (0.004 mg/L). The utilization of water filtration systems (protein skimmer and biological filters) and the large amount of water per tank (500 L), guaranteed the optimal conditions for mussels through the exposure phase (96 h) without the need of water renewal. Besides, low mortality, below 2%, was observed considering the whole experiment (acclimation, exposure and depuration). Mussels were fed two times per day with a commercial microalgae mix suitable for bivalve molluscs, composed by 2000 x 10^6 cells/mL of the following species: *Isochrysis* spp., *Tetraselmis* spp, *Nannochloropsis* spp, *Thalassiosira* spp. (Acuinuga, Spain). Sulfamethoxazole was added at a final concentration of 10 µg/L via water in the exposure tanks. Sulfamethoxazole exposure concentration was higher than the one commonly found in the environment, especially in coastal areas (Rodriguez-Mozaz et al., 2017), but lower than the predicted non effect concentration (PNEC) for many aquatic organisms (Nguyen Dang Giang et al., 2015), in order to evaluate sublethal effects of this compound in mussels. Every day, 20% of the initial concentration of SMX was added to the exposure tanks in order to maintain it constant, and compensate the loss due to degradation and/or adsorption of the chemical in the system. This percentage of loss was measured in a previous experiment carried out for charactering the whole system (aquarium, filter, skimmer, etc.) without presence of organisms. The system was spiked at the target concentration and the compound loss was evaluated in seawater resulting in a loss of 20% of SMX after 24 h (data not shown). In non-exposure tanks, the equivalent amount of solvent (methanol) was also added. The total amount of solvent added to the tanks represented a 0.05% of the total water volume. After 96 h of exposure, mussels were transported to the bivalve's supply plant (approximately 1 h drive in refrigerated conditions) for a depuration period of 24 h under real commercial conditions. A depuration period of 24 h in the bivalve's supply facilities is the usual procedure that commercial mussels follow before being sold in the markets. The depuration system comprises 30 water aquariums distributed in columns of three. The total water capacity is 17 m^3. Seawater was continuously renewed, 6000 L/h in every column of aquariums. Organisms were maintained at the same temperature than in the exposure period as seawater was directly collected from the sea by the bivalve's supply plant. The system is equipped with decanting pit, skimmer, ozonation and temperature control (Innovaqua S. L.). Therefore, the contribution of this depuration period to the elimination of contaminants, like SMX, that may be accumulated in mussels during farming was evaluated.

For SMX bioaccumulation study, seawater and mussel's soft tissue were sampled at 0, 1, 3, 6, 9, 24, 48, 72 and 96 h of the exposure phase and after 6, 12 and 24 h of depuration phase (from both control and

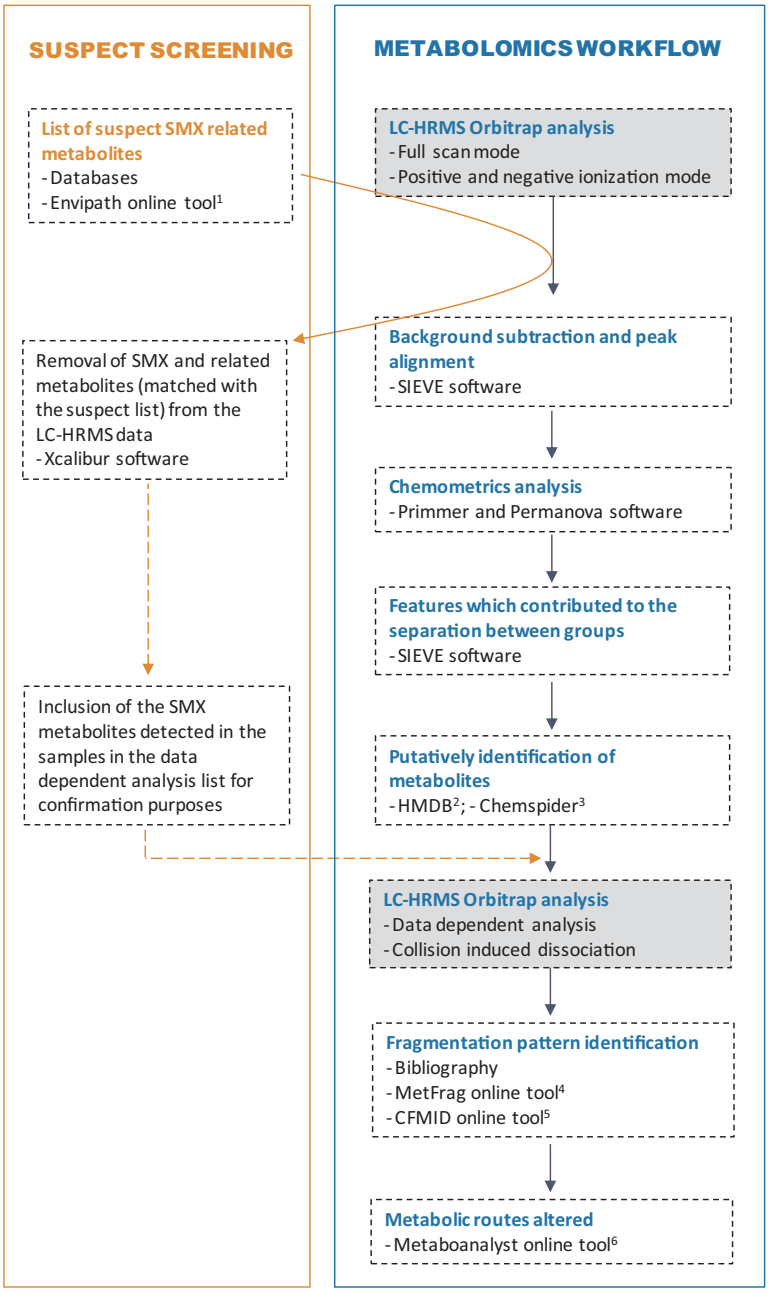

Fig. 1. Metabolomics and suspect screening of SMX related metabolites workflow. [1]https://envipath.org; [2]https://www.hmdb.ca; [3]http://chemspider.com; [4]https://msbi.ipb-halle.de/MetFragBeta; [5]http://cfmid.wishtartlab.com/predict; [6]http://www.metaboanalyst.ca.

exposure tanks) whereas haemolymph samples were withdrawn from mussel's adductor muscle at 0, 24, 48, 72 and 96 h of the exposure phase and after 24 h depuration. Haemolymph for the metabolomics study, and mussel's digestive gland and gills for enzymatic activity measures, were taken after 96 h of exposure. In all cases mussels were processed individually in order to cope with the biological variation between organisms.

2.3. Target analysis of SMX and its metabolites in mussel's soft tissue, haemolymph and seawater

For SMX accumulation in mussel's soft tissue and haemolymph, four organisms were sampled at each sampling time and treatment (control and SMX exposed mussels). Mussel's soft tissue of each individual separately was taken, snap frozen, freeze-dried and kept at -20 °C until its analysis. Freeze-dried samples were weighted and extracted using pressurized liquid extraction followed by a clean-up using solid phase extraction, applying a method developed by Álvarez-Muñoz et al. (2015a, 2015b). Then, the target analysis of SMX and its related metabolites (N-acetyl-sulfamethoxazole, desamino-sulfamethoxazole and glucoronide sulfamethoxazole) was performed using ultra-performance liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometry (UPLC-QqLIT) following the same method (Álvarez-Muñoz et al., 2015a, 2015b). These metabolites were selected because they are some of the most common SMX metabolites in humans and analytical standards are available. Mussel's haemolymph was extracted from the mussel's adductor muscle and diluted with an anticoagulant (Alsever's solution) 1:1; then, it was snap frozen by immersion in liquid nitrogen and kept at -80 °C until its analysis. Haemolymph was further diluted 1:1 with methanol before injection in the UPLC-QqLIT. Two

replicates of water samples were taken from each tank at each sampling time and they were frozen at -20 °C until its analysis. Water samples were directly analyzed using the method above indicated based on UPLC-QqLIT. Detailed explanation of sample preparation and UPLC conditions for the target analysis of SMX and its related metabolites in water, haemolymph and mussel's tissue are given as supporting information, as well as the performance of the method, detection limits and recoveries (Table S1).

2.4. Enzymatic activities analysis

Six individuals per treatment were sampled after 96 h of exposure. Mussel's digestive gland and gills were weighted and immediately frozen by immersion in liquid nitrogen and kept at -80 ºC until its analysis. Later on, mussel's digestive gland and gills of each organism were homogenized at a 1:5 (w:v) ratio in 100 mM phosphate buffer pH 7.4 containing 150 mM KCl, 1 mM ethylenediaminotetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) in the case of digestive gland and 50 mM phosphate buffer pH 7.4 containing 1 mM EDTA in the case of gills using a polytron blender. Then, the extracts were centrifuged at 10,000*g* for 30 min and the supernatant obtained was used for enzymatic activities analysis. The different assays related with oxidative stress were glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) and lipid peroxidation (LPO); whereas enzymatic activities related with xenometabolism were glutathione-S-transferase (GSTs) and carboxylesterases (CbE) using different substrates, *p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl butyrate (pNPB), α-naphthyl acetate (αNA) and α-naphthyl butyrate (αNB) were carried out in triplicate at 25 °C in a TECAN Infinite M200 microplate reader. The methodologies for enzymatic determinations, as well as total protein content can be found elsewhere (Dallarés et al., 2018; Solé et al., 2018).

2.5. Non-targeted metabolomics analysis and suspect screening of SMX related metabolites

Ten individuals were sampled for each treatment after 96 h of exposure for metabolomics and SMX related metabolites analysis. Haemolymph samples were pre-treated separately as explained for the bioaccumulation analysis (Section 2.3). The workflow of the non-target metabolomics analysis and the suspect screening analysis for SMX related metabolites is shown in Fig. 1 and a detailed explanation of the methodology is also provided in supporting information. Briefly, the analysis of mussel's haemolymph samples was performed by High-Performance Liquid chromatography–High-Resolution Mass Spectrometry (HPLC-HRMS) using a LC-LTQ-OrbitrapVelos™ from Thermo Fisher Scientific, equipped with electrospray ionization (ESI) operating both in positive and negative mode. For the metabolomics approach, data files generated in the Orbitrap were processed using the Thermo Scientific SIEVE 2.0 software which does background subtraction, component detection, peak alignment and differential analysis, Fig. 1. After, a list of candidate structures was built up by searching the exact molecular weight in open source databases such as Human Metabolome database, Pubchem, Chemspider and METLIN. Regarding the suspect screening approach, used for the detection of SMX related metabolites; a list of suspected SMX related metabolites was built based on databases research and prediction tools for SMX degradation under biological processes (Table S2, list of the SMX suspected metabolites). These SMX related metabolites were searched in the chromatogram generated in the LC-LTQ-Orbitrap, Fig. 1. Explanation of HPLC-HRMS conditions as well as the quality controls used is specified in detail in supporting information.

For confirmation purposes, a second injection in the LC-LTQ-Orbitrap using collision induced dissociation (CID) was performed, by using data-dependent analysis through the MS fragmentation of the significant metabolites previously identified based on their exact mass at 3 normalized collision energies (20, 30 and 35 eV).

2.6. Data analysis

The bioconcentration factors (in L/kg) were calculated using the following formula:

$$\text{Bioconcentration factor } (L/\text{kg dw}) = C_{biota} / C_{water},$$

where C_{biota} is the SMX concentration in mussels (μg/kg dw), whereas C_{water} is the SMX concentration in water (μg/L). Bioconcentration factors were calculated for each sampling time during the exposure days.

One-way ANOVA test, followed by Tukey`s post hoc test were performed for the determination of significant differences in SMX bioconcentration in mussel's soft tissue and SMX accumulation in haemolymph along the exposure and depuration period. The normality and variances homogeneity of the data was tested before ANOVAs by using Shapiro-Wilk and Levene's test, respectively. For those compounds that data showed no normality or homogeneity, a Kruskal-Wallis test was performed followed by a Conover post hoc test. These statistical analyses were performed using R software (3.1.0) with a significance level of p-value < 0.05. Principal component analysis (PCA) was undertaken to identify the discriminatory variables for the enzymatic activities belonging to oxidative stress and mussel's xenometabolism, for gills and digestive gland. PCA was also used for the profiling of the metabolome by using all the detected features as variables (after excluding SMX and related metabolites when detected). All PCA were performed using PRIMMER and PERMANOVA software (6.0). Finally, significant differences in metabolites levels comparing control and SMX exposed mussels were determined with a *t*-test using SIEVE software with a significance level of p-value ≤ 0.05. The fold change and the standard error (SE) of the identified metabolites were calculated according to Motulsky (Motulsky, 1995).

3. Results

3.1. SMX and its related metabolites occurrence in seawater and mussels

3.1.1. SMX concentration in seawater

SMX concentration in water during the exposure and the depuration phase in both trials is shown in supporting information, Fig. S1. Sulfamethoxazole levels in both trials (summer and winter) were equivalent being the mean concentration during the exposure phase 8.8 ± 0.9 μg/L (mean of summer and winter trials). The daily addition of a 20% of the initial SMX concentration, allowed a constant SMX concentration along the exposure phase. Sulfamethoxazole was not detected in water from the depuration tanks, with levels always below method detection limit (MDL) (Fig. S1), nor was detected in water from control tanks (data not shown).

3.1.2. SMX concentration in mussels

SMX was not detected with levels below MDL in any sample from control tanks (data not shown). Since no significant differences were observed between summer and winter experiments on SMX concentration accumulated in mussels for most of sampling times (Fig. S2), the mean SMX values of the two trials per sampling time was used for further discussion (Fig. 2). Sulfamethoxazole concentration in mussels increased during the exposure phase up to 13.2 ± 0.7 ng/g dw, after 96 h of exposure. After 6 h of depuration the concentration of SMX in mussels reached its highest value being 16.8 ± 1.1 ng/g dw, whereas at the end of the depuration phase (24 h of depuration) SMX concentration in mussels decreased to 4.1 ± 1.1 ng/g (69% of elimination when comparing the concentration at the end of the exposure phase with the SMX concentration at the end of the depuration phase).

The correlation between SMX bioconcentration factor (BCF) (mean of summer and winter trials) and exposure time is presented in Fig. 3. The highest BCF of 1.5 ± 0.1 L/kg was achieved after 96 h of exposure. Sulfamethoxazole uptake by mussels followed a logarithmic pattern with an $R^2 = 0.93$ (Fig. 3). A stabilization of the BCF was reached after

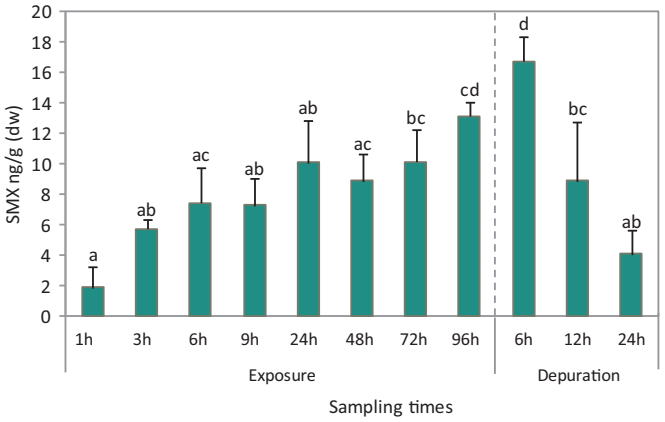

Fig. 2. Sulfamethoxazole concentration in mussel's soft tissue from exposed tanks (ng/g, dw) along the exposure and depuration sampling times. Mean concentration of summer and winter trials are presented. Different lowercase letters represent significant differences between the experiment sampling times, significance p value < 0.05. ANOVA test.

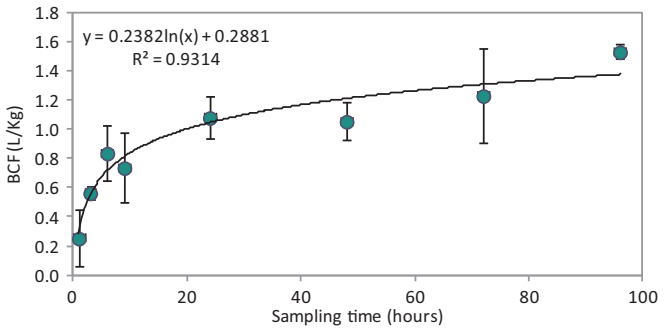

Fig. 3. Sulfamethoxazole bioconcentration factors (L/kg dw) determined in mussel's soft tissue, mean BCF of summer and winter trials, along the experiment (exposure phase).

24 h of exposure which suggests that the steady state (ss) was achieved after this period. Although a slight increase in the BCF was observed after 96 h of exposure it was not statistically different from the BCFss (ANOVA test p-value < 0.05).

The concentration of SMX in mussel's haemolymph is shown in Fig. 4. Similarly to observations in mussel's soft tissue, no significant differences were seen between the two trials conducted along the exposure phase (Fig. S3). The highest concentration was found at 48 h of exposure, 3.39 ± 0.48 µg/L. Sulfamethoxazole was not detected in any mussel's haemolymph after 24 h of depuration.

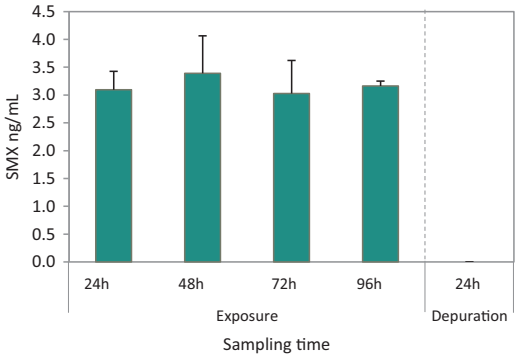

Fig. 4. Sulfamethoxazole concentration in mussel's haemolymph (ng/mL) along the exposure and depuration sampling times. Average concentration of summer and winter trials are presented. No significant variations were observed between the experiment sampling times after ANOVA test significance p-value < 0.05.

3.1.3. Target and suspect screening of SMX related metabolites

An analysis of SMX related metabolites with available commercial analytical standards, N-acetyl sulfamethoxazole, desamino-sulfamethoxazole and glucuronide sulfamethoxazole, was targeted in mussel's soft tissue, haemolymph and in seawater of the tanks. None of the target metabolites was detected in any of the samples analyzed. Furthermore, a suspect screening approach was performed to investigate the occurrence of other metabolites whose analytical standards are not available in mussel's haemolymph (Table S2 shows the list of suspect SMX related metabolites). As in the case of the target analysis none of the searched SMX metabolites was found in mussel's haemolymph.

3.2. Xenobiotic and oxidative stress related enzymatic activities

In order to assess the overall effects of SMX exposure on mussel's xenometabolism and oxidative stress, principal component analysis was undertaken considering all the parameters analyzed at the two trials (Fig. 5). Fig. 5a and b show the xenobiotic and oxidative stress related activities measured in gills, whereas Fig. 5c and Fig. 5d present the xenobiotic and oxidative stress activities in digestive gland. None of the PCAs showed any clear separation of the groups due to SMX exposure, indicating no significant effects of SMX exposure on mussel's xenobiotic metabolism and oxidative stress. The individual comparisons between control and SMX exposed mussels for each enzymatic activity are shown in supporting information Figs. S4 and S5 and showed no significant changes for any of the enzymes studied due to SMX exposure in gills or digestive gland.

3.3. Metabolomics approach

3.3.1. Chemometric analysis

Liquid chromatography coupled to mass spectrometry (LC-LTQ-Orbitrap) analysis led to the detection of 1123 features in summer and 1324 in winter trials (579 features were common in both seasons) considering both positive and negative ionization modes. The dataset including all features detected in summer and winter (1868 features) was used for the PCA (Fig. S6). A clear separation was observed along the first axis (95% of the variation) between those samples from summer compared to samples from winter; showing that mussels metabolome highly differed between the two studied seasons. In order to assess the putative effects of SMX on mussel's metabolome, samples from summer and winter trials were analyzed separately, Fig. 6 (6A summer, 6B winter). Samples from winter showed a separation between the control and SMX exposed treatment along the second axis (20% of the total variability), Fig. 6B. However, no separation between control and SMX exposed samples was observed in mussels from summer, Fig. 6A.

In order to identify compound classes of interest, a Van Krevelen diagram was plotted out using the molecular features obtained from winter trial (Fig. 7) where a clear separation between the groups was observed in the PCA. The Van Krevelen diagram (plotting the ratio H/C versus O/C) regions have been associated to different classes of compounds (Alañón et al., 2015; Minor et al., 2014). In the present work, the majority of the features detected fell into the lipids and amino acids and peptides regions, followed by the condensed hydrocarbons and nucleic acid regions. Only few of them were placed into the amino sugars and carbohydrates regions (Fig. 7). When control and SMX exposed mussels were compared a higher amount of features in the SMX exposed treatment were observed in the amino acids and peptides region, but the abundance of lipids seemed to remain constant between the two treatments (Fig. 7).

For the screening of features of interest regarding SMX exposure, the significance of each feature that contributed to the separation between

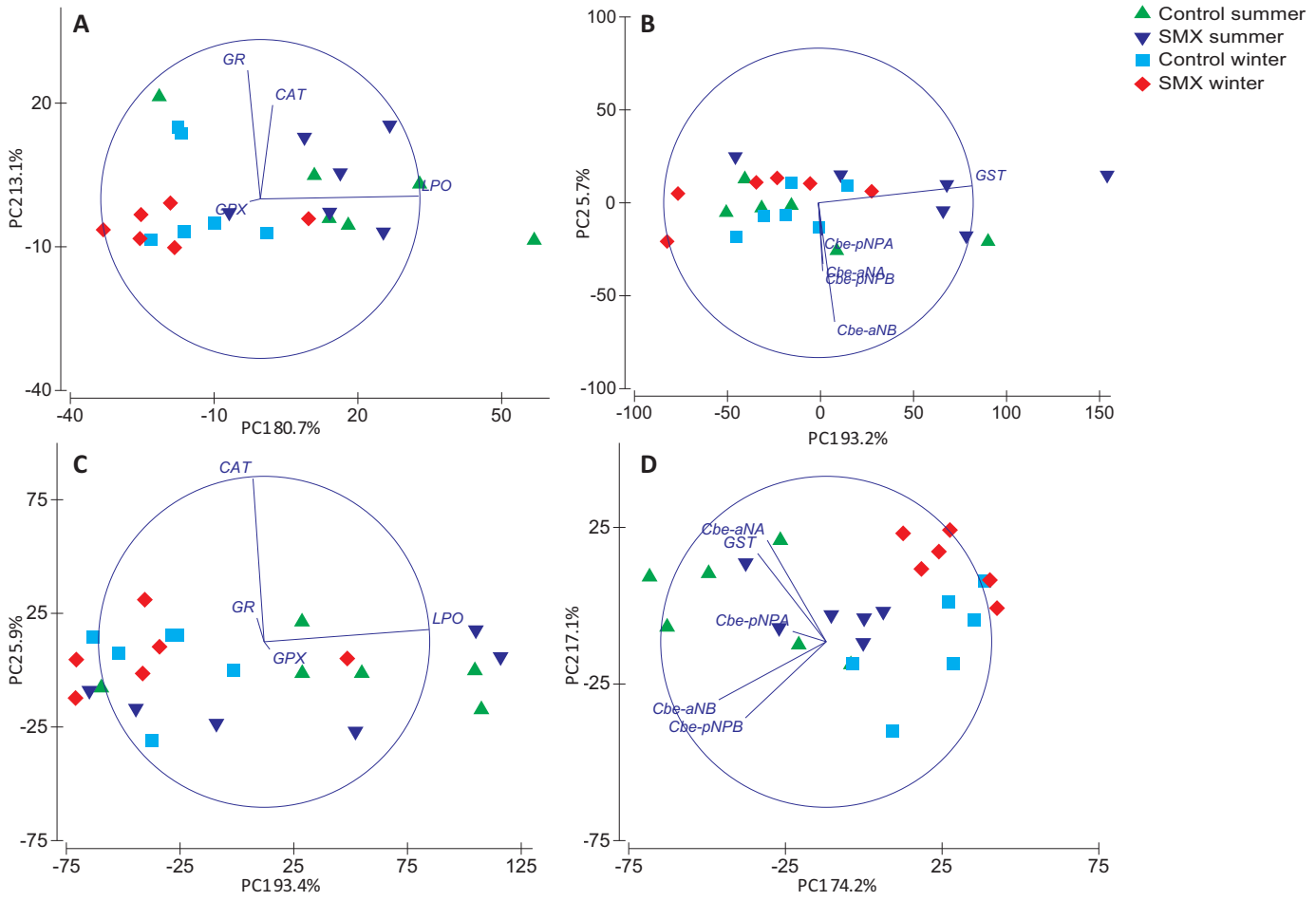

Fig. 5. PCA scores plot for the enzymatic activities analyzed. Plots A and B correspond to mussel's gills: A are enzymatic activities related with oxidative stress (CAT, GR, GPX and LPO) and B enzymatic activities related with xenometabolism (GSTs and CbE with different substrates). Plots C and D correspond to mussel's digestive gland: C are enzymatic activities related with oxidative stress and D enzymatic activities related with xenometabolism. The percentages of explained variation for the first two components (PC1 and PC2) are displayed on the relative axes.

the control and the SMX exposed treatment during winter trial, was evaluated by assessing their p-value ≤ 0.05 (T-test). This resulted in the detection of 378 significant features, which were considered for further identification.

3.3.2. Metabolites identification

For identification purposes, the 378 significant features (between control and SMX exposed treatments) from winter trial, were searched in the databases (i. e. Human Metabolomics Database, Chemspider,

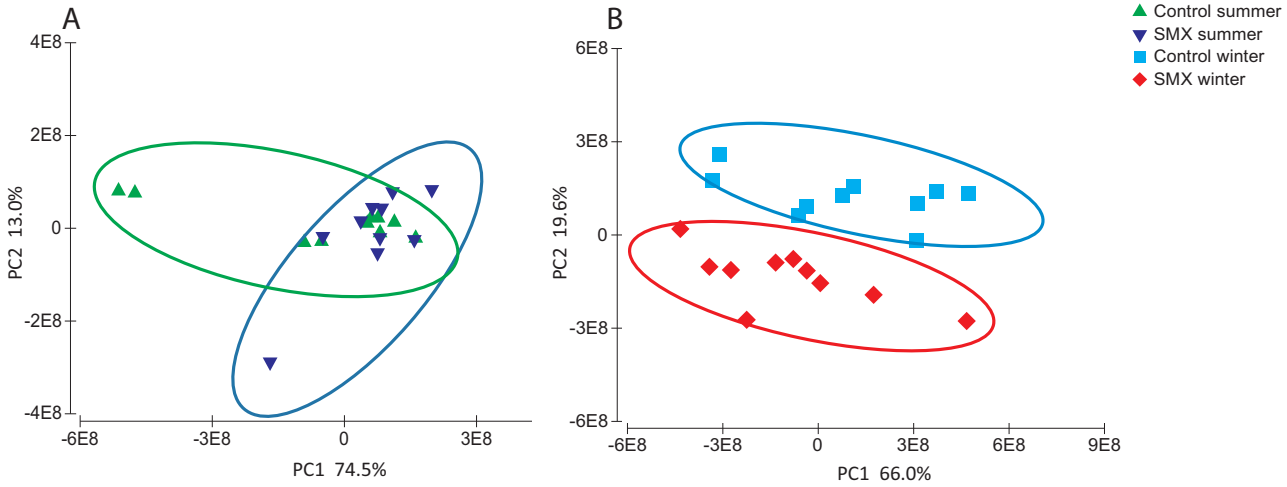

Fig. 6. PCA score plots of the metabolomic profiles of mussels. Graphic A, summer trial, graphic B winter trial. Mussel's haemolymph were profiled by LC-LTQ-Orbitrap, with all features detected in +ESI and −ESI mode. The percentages of explained variation for the first two components (PC1 and PC2) are displayed on the relative axes.

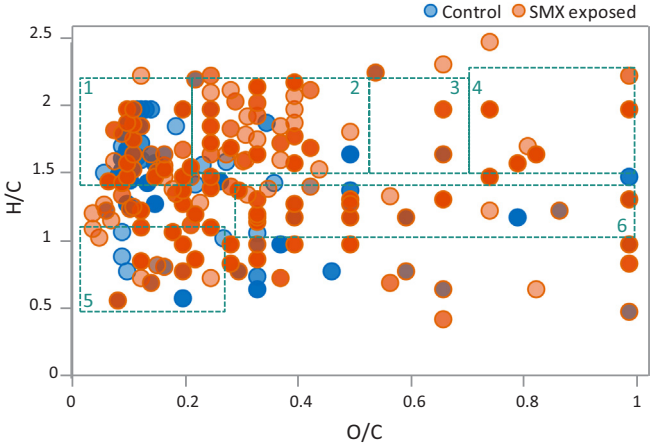

Fig. 7. Van Krevelen diagram of molecular features from the metabolomics analysis in mussels from winter season. The rectangles represent the different compound class areas. 1, lipids; 2 amino acids and peptides; 3, amino sugars; 4, carbohydrates; 5, condensed hydrocarbons; 6, nucleic acids.

etc.) on the basis of their molecular exact mass. Features whose exact mass differed less than 5 ppm from the molecular mass of suspect compounds were prioritized and a list of 26 putative metabolites was built up and considered for confirmation. A second injection of the extracts was thus performed in the data-dependent acquisition mode at different collision energies and the main fragments obtained were also identified through their exact mass with an error below 5 ppm and used for confirmation of the 26 suspects. This approach for a tentative confirmation of metabolites identities is commonly used in metabolomics studies (Serra-Compte et al., 2018a; Villalobos et al., 2013). Seven metabolites were identified: aspartate, benzoate, phenylalanine, valine, guanosine, tryptophan and inosine (Table S3 presents their exact mass, retention time, CID generated data and the identification of the main observed fragment ions). Compounds were detected in negative electrospray ionization mode (−ESI) as the $[M-H]^-$ ion, or as the $[M+H]^+$ in positive electrospray ionization mode (+ESI). The most common fragments observed were the $[M-H-18]^-$ fragment, corresponding to the loss of a water molecule from the carboxyl group and the $[M-H-44]^-$ fragment corresponding to the loss of a carbon dioxide molecule. These fragments have been previously reported for similar compounds (Serra-Compte et al., 2018a; Villalobos et al., 2013). Explanation of all fragments of the identified compounds is presented in supplementary information, Table S3. After the identification of the metabolites they were also searched in samples from summer trial in order to evaluate if they exhibited any change (increase or decrease), and compare their response in the two seasons studied.

3.3.3. *Endogenous metabolites altered due to SMX exposure*

The metabolites identified in mussels and their significant change due to SMX exposure (increase or decrease) are presented in Fig. 8. The most altered group of compounds were amino acids (four out of the seven compounds identified), followed by nucleosides (two altered compounds) and one carboxylic acid (Table S3). All the metabolites identified (except aspartate) increased their levels in winter trial due to SMX exposure. Guanosine was the metabolite which presented the highest fold change (x4) within the markers identified. Among the seven metabolites identified in winter trial, aspartate and benzoate also changed significantly during summer trial. Therefore, they can be proposed as biomarkers of SMX exposure in marine mussels. Aspartate showed the same response in both trials (a decrease) whereas benzoate level decreased in summer and increased in winter.

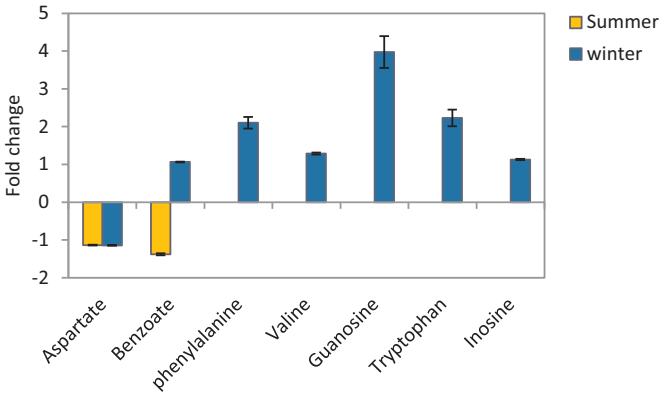

Fig. 8. Apparent fold change for the metabolites identified with significant changes between control and SMX exposed mussels.

4. Discussion

4.1. *SMX bioconcentration, depuration and risk assessment*

Assessing contaminants bioaccumulation in marine organisms is the first step to evaluate their potential risk for the environment and also for human health when accumulating in foodstuff from animal origin. Contaminants accumulation capacity in marine organisms may be determined by both, contaminants physical-chemical properties and the characteristics of the exposed organism. The low bioconcentration capacity of SMX in mussel's soft tissue (up to 1.5 L/kg, dw) may be related to its physical-chemical properties; in particular, the low octanol-water partition coefficient LogKow 0.8 units for SMX (Log Dow -0.1 at pH 8). Similar BCFs (between 0 and 10 L/kg) have been previously reported for SMX in mussels both laboratory and field studies (Klosterhaus et al., 2013; Serra-Compte et al., 2018b).

An increase in SMX concentration in mussel's tissue was observed after 6 h of depuration reaching up to 16.8 ± 1.1 ng/g dw (Fig. 2). Since SMX was not detected in the water from the depuration tanks, this increase in bioconcentration may suggest that mussels accumulated SMX from the contaminated seawater kept inside the valves while they were transported to the depuration plant. After 12 h of depuration, SMX concentration decreased, although the variability was high (Fig. 2). This may be attributed to biological differences between organisms facing anoxia and valve closure during transportation. After 24 h of depuration the concentration of SMX was significantly reduced in mussel's soft tissue (69% of elimination) with a value of 4.1 ± 1.1 ng/g dw.

Despite SMX was not completely eliminated from the mussel's edible tissue after 24 h of depuration, it was completely eliminated from the haemolymph after the same period. This may be explained because ionizable compounds such as SMX, that are transported in the circulatory system, may be removed from the haemolymph stream and bioaccumulated in the organism, by entering inside the cells or by adsorption to the organism's tissues.

Apart from the accumulation of the parent compound in organisms, the presence of its related metabolites in biota is becoming a topic of great interest, as some antibiotic metabolites can be biologically active and it also provides information about the metabolization degree of the compounds. In this study, two different approaches were used for the analysis of SMX related metabolites in mussels. A target analysis of some of the most common SMX metabolites described in humans, for which commercial analytical standards are available, and a suspect screening analysis based on databases research and theoretical prediction of SMX biotransformation products. None of the two approaches allowed for the detection of SMX related metabolites in mussels. This suggests that mussels might not be able to metabolize this compound at the tested conditions. Despite the fact that mussels are able to

metabolize other organic compounds, including pharmaceuticals (Boillot et al., 2015; Dallarés et al., 2019), and that SMX is highly metabolized in humans (around 60% is excreted as metabolites, mainly as N4-acetylated metabolite form), the set of enzymes responsible of the xenobiotic metabolism in mussel is more restricted than in humans, and to the best of the authors knowledge there is no evidence in the literature of SMX metabolization by bivalves. This suggests that metabolization of pharmaceuticals by mussels may be not only limited but also compound dependent. In line with this, in previous studies carried out with marine mussels exposed to pharmaceutical compounds, SMX and carbamazepine metabolites were not detected while venlafaxine was metabolized since metabolites were detected (Serra-Compte et al., 2018b), as well as, metabolites were detected when exposed to the antiviral Tamiflu (Dallarés et al., 2019).

Regarding the putative risk for consumers, the highest SMX concentration detected in mussels was 16.8 ± 1.1 ng/g dw after 6 h of depuration, or 3.05 ng/g ww, when converting to wet weight by applying a conversion factor of 5.5, commonly used for the conversion of shell-free dry weight to wet weight in bivalves (Ricciardi and Bourget, 1998). The SMX concentration measured in mussels was much lower than the MRL established at 100 ng/g ww by the authorities for sulfonamides residues in foodstuff from animal origin (European Commission, 2010). As this experiment was performed at SMX concentrations higher than the ones normally found in the marine environment, no risk for consumers is foreseen in commercially available marine mussels. Besides, a depuration period of 24 h under existent commercial practices, significantly reduced SMX levels in edible mussels even more. Nevertheless, as sulfonamides antibiotics may be ingested through other food sources apart from seafood, such as meat or eggs (Mehtabuddin et al., 2012), an aggregated assessment would be recommended. On the other hand, as even higher levels of SMX were measured after mussels transportation to the bivalve's supply plant compared to the end of the exposure phase of the experiment, the analysis of mussels collected in the markets final destination (after its transportation) and not only at the aquaculture facility, is recommended to correctly assess the potential risk for shellfish consumers.

4.2. Metabolomics and enzymatic activities analysis

Two different approaches were used in the present work in order to characterize the ecotoxicological effects of SMX exposure to marine mussels, the analysis of enzymatic activities related to mussel's xenobiotic metabolism and oxidative stress, and a non-targeted metabolomics approach in order to detect changes in mussel's endogenous metabolites. None of the enzymatic activities possibly related to mussel's xenobiotic metabolism were significantly altered due to SMX exposure. Carboxylesterases are key enzymes involved in phase I metabolism of xenobiotics (Xu et al., 2016), whereas glutathione S-transferase is involved in phase II metabolism. Alteration of these activities in mussel due to an exposure of contaminants may be indicative of a detoxification mechanism for different contaminants including pesticides and pharmaceuticals (Dallarés et al., 2019; Solé and Sanchez-Hernandez, 2018). The no alteration of enzymatic activities related to xenometabolism activity in the present work supports the hypothesis of no metabolization of SMX by mussels at the tested conditions, as previously discussed in Section 4.1.

Regarding oxidative stress related enzymatic activities, no changes were observed in mussels due to SMX exposure. In agreement to our findings, other authors found slight alterations in mussel's oxidative stress when they were exposed to antibiotics. As example, (Matozzo et al., 2016) observed slight alterations in mussels GSTs but not in LPO when mussels were exposed to high concentrations (100, 200 and 400 µg/L) of the antibiotic amoxicillin, after seven days of exposure. Considering other pharmaceutical compounds such as gemfibrozil and diclofenac (Schmidt et al., 2011) found alterations in mussel's enzymatic activities like GSTs when exposed at 1 µg/L and 100 µg/L during 24 h and 96 h. On the other hand, Oliveira et al. (2017), did not observe an oxidative stress induction, when Mediterranean mussels were exposed to carbamazepine at concentrations up to 9 µg/L, for 96 h (acute) and 28 days (chronic) exposures. These results pinpoint low induction of oxidative stress enzymes in mussels due to pharmaceuticals and especially to antibiotics after short term exposures.

The main ecotoxicological alterations in mussels due to SMX exposure were encountered through the metabolomics approach. Mussel's metabolome seems to respond differently to SMX pollution in a season-dependent manner, being more responsive in winter than in summer. These differences may be attributed to the different physiological status of the organisms, since a clear difference in mussel's metabolome was observed between organisms from summer and winter (Fig. S6). Furthermore, enzymatic activities also differed between the two studied seasons, especially those related with oxidative stress in gills and those related with oxidative stress and xenometabolism in digestive gland (Fig. 5a, c and d). This highlights the different physiological state of mussels between the two different seasons. Previous studies showed different sensitivity of mussels to pesticides (molluscicidals) depending on the season. This was related to an increased filtration rate in mussels during summer season (which may in turn increase contaminants accumulation) (Costa et al., 2008), or to mussels physiological status, mainly due to the spawning/resting cycle (Claudi and Mackie, 1994). In the present work, the bioaccumulation of SMX was similar in the two seasons. Therefore, the differences observed in the mussel's response to SMX exposure are most likely due to different physiological status of the organisms (mainly related to reproduction) rather than an increase in filtration rate. Higher sensitivity to pollutants after mussels spawning due to physiological fatigue has been previously reported (Claudi and Mackie, 1994; González-Fernández et al., 2016) and in the case of *Mytilus galloprovincialis* the spawning occurs mainly at the end of winter, January-February (Da Ros et al., 1985), when the winter trial was conducted in our study. Therefore, mussel's metabolome seems to be more affected due to SMX exposure in winter experiments compared to summer experiments, though some of the identified metabolites were also altered during summer (Fig. 8).

The Van Krevelen diagram showed that the highest differences between control and SMX exposed mussels in winter trial were in the amino acids and peptides region; in concordance with these results, four out of the seven compounds identified in this study were amino acids. Apart from them, two nucleotides and one carboxylic acid were identified. The identified compounds are involved in different metabolic pathways that may be altered due to SMX exposure. Using MetaboAnalyst 4.0, the metabolic pathways potentially affected based on the identified compounds are presented in Fig. 9. MetaboAnalyst asses a p-value of each altered pathway based on the number of metabolites identified belonging to the same pathway and the role of each metabolite in a specific pathway. Taking a threshold limit of $p < 0.05$, eight metabolic pathways were significantly altered in mussels due to SMX exposure based on the metabolites identified (Table S4). Aminoacyl-tRNA biosintyesis (phenylalanine, aspartate, tryptophan and valine are involved in this pathway), nitrogen metabolism (phenylalanine, tryptophan and aspartate are involved in this pathway) and pantathenate and CoA biosynthesis (valine and aspartate are involved in this pathway) were the most altered pathways in mussels due to SMX exposure. The rest of the altered metabolic pathways are involved in the biosynthesis of the identified metabolites, mainly amino acids, such as phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism and glycine, serine and threonine metabolism. Besides, alteration in purine metabolism pathway was also significant (Fig. 9).

Amino acids have important roles in mussel's metabolism such as osmoregulation, energy metabolism and synthesis of proteins. Alteration in mussel's amino acids levels when exposed to different environmental contaminants has been previously observed (Bonnefille et al., 2017; Sanchís et al., 2018) and they seem to be one of the

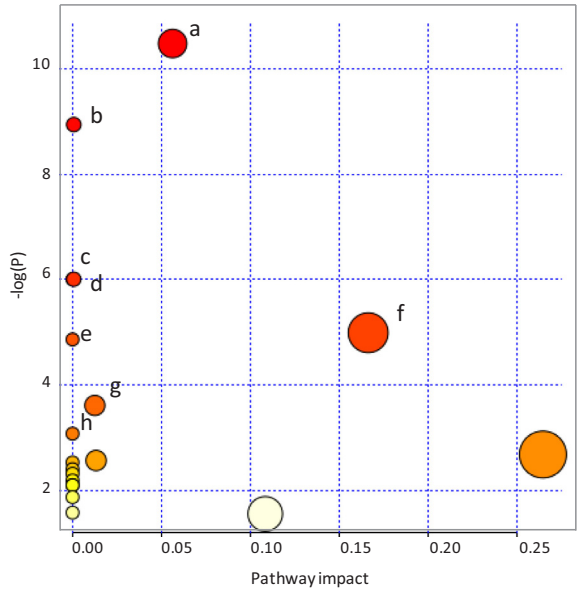

Fig. 9. Metabolic pathways matched based on the metabolites identified, using MetaboAnalyst 4.0. The color and size of each circle was based on p-value and pathway impact value, respectively. Only metabolic pathways with a p-value < 0.05 are identified in the figure. (a) Aminoacyl-tRNA biosynthesis; (b) Nitrogen metabolism; (c) Pantothenate and CoA biosynthesis; (d) Phenylalanine, tyrosine and tryptophan biosynthesis; (e) Glycine, serine and threonine metabolism; (f) Phenylalanine metabolism; (g) Purine metabolism metabolism; (h) Cyanomino acid metabolism.

primary response of mussels to different stressors. Alterations in free amino acids levels in marine mussels have been related to an imbalance of osmoregulation. Kwon et al. (2012) and Liu et al., 2014 found an increase in free amino acids such as valine and phenylalanine in mussels due to heavy metals exposure and *Vibrio harveyi* induction respectively, in both cases it was related to alterations in osmoregulation. Besides, alterations in mussel's nitrogen metabolism has also been related to changes in osmotic regulation when mussels were exposed to stress conditions, such as different concentrations of water salinity (Livingstone et al., 1979). These previous findings support the hypothesis of a possible alteration in the osmotic regulation of mussels due to SMX exposure. Oxidation of amino acids to produce energy was observed in mussels exposed to stressors (*Vibrio harveyi* exposure), as well as, changes in other molecules involved in the energy metabolism such as ATP or glucose (Liu et al., 2014). In the present work, besides the changes found in amino acids, a significant alteration in pantothenate and CoA biosynthesis was detected, which may also suggest disturbances in mussel's energy metabolism. Finally, amino acids are the main constituents of more complex molecules such as proteins. Changes in amino acids levels may lead to changes in protein synthesis (through the aminoacyl-tRNA biosintyesis), and this may have further implications for the metabolism of the exposed organisms (Song et al., 2016).

In addition to the above mentioned metabolites, the metabolomics approach allowed the detection of two nucleotides altered due to SMX, inosine and guanosine. Changes in the nucleotide metabolism has been detected previously in mussels due to different occasions (i. e. wild and food limitation conditions), and nucleotides were pinpointed as a biomarker of health status in mussels (Roznere et al., 2014). We here observed that nucleotides may also be indicative of SMX pollution in mussels, but alterations were only observed under winter conditions.

Changes in mussel's metabolites profile can also be discussed from a gastronomic perspective, as changes in endogenous metabolites levels may influence the quality of mussels for its human consumption. Mussels are a highly valuable food source of proteins, lipids, and carbohydrates which have shown to be very beneficial for human health (Grienke et al., 2014). Besides, the concentration of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) is well recognized as health beneficial and nutritional (Fuentes et al., 2009). In the present work, no changes in lipids concentrations were found; pinpointing that the nutritional characteristics of mussels were not affected by SMX exposure. However, as shown in the Van Krevelen diagram (Fig. 7) the amino acids and peptides region was highly affected by SMX exposure, and this was confirmed after compounds identification where some amino acids were altered due to SMX exposure. Alterations in amino acids levels may affect organoleptic aspects of mussels such as taste, odor, aroma and flavor (Fuentes et al., 2009). Furthermore, the amino acid aspartate, that decreased due to SMX exposure, is one of the most important taste-active compounds in mussels (Cha et al., 1998). These changes in the amino acids profile and especially aspartate, can influence in the mussel's characteristics as food product (Cha et al., 1998; Fuentes et al., 2009). Besides, alterations in amino acids levels may also influence the final protein content or the protein profile of mussels. It is well known that parameters such as seawater conditions, food availability and the gametogenesis cycle influence the meat characteristics of mussels (Fernández et al., 2015; Orban et al., 2002). Here it is suggested that the exposure of mussels to environmental contaminants, such as antibiotics, may also affect the meat characteristics, influencing mussel's commercial quality and organoleptic properties. However, further research is needed in order to confirm this hypothesis and complemented with food sensory tests would be recommended.

5. Conclusions

Sulfamethoxazole showed low bioconcentration capacity in mussels exposed via water under laboratory controlled conditions. Twenty four hours of depuration under real commercial conditions allowed a significant reduction (69%) of SMX concentration. No SMX related metabolites were detected, suggesting that mussels may not metabolize this compound at the tested conditions. In line with these findings, SMX exposure did not provoke any change in the enzymes related to xenometabolism nor in those related to oxidative stress. However, the metabolomics analysis did reveal alteration is mussel's metabolome mainly during winter conditions. Amino acids were the most altered group of compounds, which may be related to mussel's osmotic regulation and energy metabolism. The amino acid aspartate and the carboxylic acid benzoate were altered under both conditions and they can be postulated as biomarkers of SMX exposure. On the other hand, due to the low accumulation tendency observed for SMX in mussels, no risk for consumers is expected (according to the MRL established by European authorities). However, changes in mussel's organoleptic characteristics are suggested which may affect their commercial quality.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2019.03.021.

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Exposure to a Subinhibitory Sulfonamide Concentration Promotes the Spread of Antibiotic Resistance in Marine Blue Mussels (*Mytilus edulis*)

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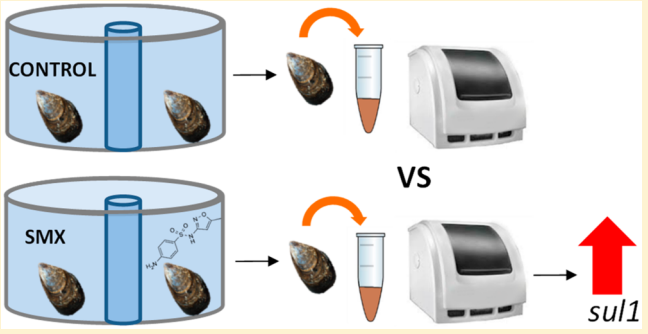
Supporting Information

ABSTRACT: Although antibiotic resistance has become a significant and growing threat to public and environmental health, the occurrence and prevalence of this phenomenon in seafood have not been extensively explored. This study aims to evaluate the impact of subinhibitory antibiotic concentrations on the spread of antibiotic resistance in mussels. Marine blue mussels were exposed to 100 μg/L sulfamethoxazole (SMX); then, the presence of genes conferring resistance to sulfonamides (*sul1* and *sul2*) and the class 1 integron-integrase gene (*intI1*) and the bacterial community composition associated with the gastrointestinal tract were investigated. Results showed that all analyzed genes were present in mussels, even in those not exposed to SMX. Moreover, exposure to SMX caused a significant increase in the absolute copy number of *sul1* in mussels, although no significant changes were observed for *sul2* and *intI1* genes. Because the bacterial community composition was not affected by SMX exposure, the increase for *sul1* may be attributed to its spread within mussel's microbiome due to the pressure exerted by SMX pollution. Overall, our results showed the presence of antibiotic resistance genes (ARGs) in blue mussels and highlighted the contribution of anthropogenic pollution to the spread of ARGs in aquatic organisms.



1. INTRODUCTION

Antibiotic resistance has been classified as one of the greatest global health threats of the 21st century.[1] The main reason for the rapid spread of this phenomenon is the overuse and misuse of antibiotics.[2] In fact, an increasing prevalence of antibiotic resistance has been found within a wide range of environments, such as wastewater treatment plant (WWTP) effluents, surface water and groundwater, river and lake sediments, and coastal areas.[3−9]

The occurrence and prevalence of antibiotic resistance genes (ARGs) have been extensively reported in coastal environments;[10−12] however, the surrounding biota has received less attention. Genes conferring resistance to chloramphenicol and sulfonamides (*cmlA* and *sul1*, respectively) have been detected in farmed shrimps, showing that ARGs can occur in bacteria living within the seafood intestinal tract.[13] Among the organisms for which there is a high level of interest in aquaculture, marine blue mussels are one of the most consumed seafoods worldwide. Mussels for human consumption are produced in coastal areas, which may be chronically exposed to environmental pollutants. The accumulation of antibiotics in marine mussels has been previously reported.[14,15] For instance, the antibiotic sulfamethoxazole (SMX) has been found in different compartments (water, sediments, and biota) in the marine environment.[15] In fact, SMX has been detected in commercial aquatic organisms, at concentrations of ≤245.91 ng/g in *Pangasius* from Thailand.[16] Moreover, authorities have established maximum residue limits (MRLs) for some antibiotics in foodstuff of an animal origin, including marine blue mussels (i.e., 100 ng of SMX/g of wet weight).[17] Therefore, the analysis of ARGs in frequently consumed marine mussel species as well as the putative contribution of antibiotic pollution to the spread of ARGs in mussels is of great concern in terms of seafood safety.

Given this, the main aim of this work was to evaluate the contribution of antibiotic contamination to the selection of ARGs in bacterial communities located in the mussel

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gastrointestinal tract. To address this objective, mussels were exposed to 100 μg/L SMX, a concentration lower than its minimum inhibitory concentrations (MICs), i.e., 1−16 μg/mL.[18,19] After the exposure period, the effects of SMX pollution were evaluated by considering the abundance of genes conferring resistance to sulfonamides (*sul1* and *sul2*) and the class 1 integron-integrase gene (*intI1*) as well as the bacterial community composition associated with the mussel's gastrointestinal tract.

2. MATERIALS AND METHODS

2.1. Experimental Design. Blue mussels and seawater were obtained from a bivalve's supply plant (Mediterranean coast, Girona, Spain) and transported to the Catalan Institute for Water Research facilities. Mussels were randomly distributed in four tanks, 5 L each (two tanks per each treatment; namely, control and exposure experiment), with a maximum density of one organism per liter of seawater. Animals were acclimatized for 5 days under the following conditions: temperature of 13.5 ± 0.2 °C, which was the same as that registered in the Mediterranean Sea (Girona coast) during sampling (November 2017); pH of 8.0 ± 0.1; dissolved oxygen (DO) level of >90% DO/L; salinity of 43.5 ± 0.7‰; and photoperiod of 12 h light and 12 h dark (12L:12D). Ammonia, nitrite, and nitrate levels were evaluated in the water sampled from the tanks using ionic chromatography. The concentrations of ammonia and nitrates were kept below 0.5 and 1 mg/L, respectively, during the whole experiment, acclimation and exposure, whereas the concentrations of nitrites were below quantification levels (0.004 mg/L) in all cases. After the acclimatization period, mussels from the exposure treatment were exposed to SMX via water at a nominal concentration of 100 μg/L. The exposure period lasted 96 h, which has been shown to be enough to reach the steady state in marine mussels.[20] The mussels from the control treatment were held under the same conditions but without the addition of SMX; only an equivalent amount of solvent (methanol) was added to control water. The amount of solvent added to the tanks was <0.05% of the total water volume. During the entire experimental period, seawater was renewed daily at 80% of the total volume, and the corresponding amount of SMX (and methanol in the control tanks) was added after each water renewal. Low mortality was observed (<1%) only during the acclimatization period. During the whole experiment, mussels were fed two times daily with a commercial algal mix suitable for bivalves (Acuinuga, Spain). The amount of algal mix added to each tank corresponded to 1% of the estimated wet weight of mussels placed in a tank. To evaluate the effects of SMX exposure, organisms were sampled only after the exposure period (96 h). Six individuals from control tanks and six from exposure tanks were sampled (n = 6), and their gastrointestinal tracts were collected and kept at −20 °C until the DNA was extracted. From the same individuals, hemolymph was extracted, immediately frozen by immersion in liquid nitrogen, and kept at −70 °C until SMX analysis. Finally, water samples (n = 4 per treatment) were collected during the exposure period (0, 24, 72, and 96 h) and kept at −20 °C until SMX analysis.

2.2. DNA Extraction and Quantification of Genes. Mussel gastrointestinal tracts were weighed. DNA was then extracted from each individual separately using a commercial kit (DNeasy Power Soil Kit, Qiagen) and quantified using NanoDrop 2000 (Thermo Scientific, Wilmington, DE). The abundance of 16S rRNA, *intI1*, *sul1*, and *sul2* genes was quantified using real-time polymerase chain reaction (qPCR) assays following the conditions previously described.[21] Previous studies have demonstrated that *sul1* and *sul2* are the most abundant sulfonamide resistance genes in different environments, including marine areas;[22] therefore, they were selected to be analyzed in this work. The copy numbers of ARGs were normalized to grams of sample (wet weight) to obtain the absolute copy number. Experimental details regarding the quantification of genes are described in the Supporting Information.

2.3. Sequence Analysis and Phylogenetic Classification. Genomic DNA samples were submitted to BMR Genomics (Padua, Italy) for 16S rRNA gene high-throughput sequencing; their hypervariable (V3 and V4) regions were amplified using universal primers[23] and sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA) using 2 × 300 bp paired-end reads. Sequences were then quality trimmed using the MOTHUR software package[24] and aligned using the SILVA reference database.[25] Subsequently, sequence libraries were randomly subsampled to contain the same number of sequences (8008) for α- and β-diversity analyses. The Shannon diversity index (H') and the Chao1 richness estimator were calculated for α-diversity comparisons, whereas the Yue & Clayton index, which measures community structure (number of shared genera and their relative abundances), was calculated for β-diversity comparisons. Analysis of molecular variance (AMOVA) was used to test statistically significant differences between the bacterial communities based on the Yue & Clayton index. Sequences were assigned to operational taxonomic units (OTUs) based on a 97% sequence similarity. The Ribosomal Database Project (RDP) pipeline and Classifier function were used to assign identities at a confidence threshold of 80%.[26]

2.4. SMX Analysis in Seawater and the Hemolymph from Mussels. The SMX concentration in seawater and hemolymph (diluted 1:1 with methanol) was analyzed by ultra-high-performance liquid chromatography coupled to quadrupole linear ion trap tandem mass spectrometry (UPLC−QqLIT), using a previously described adapted method.[27] Experimental details regarding UPLC−QqLIT parameters are described in the Supporting Information.

2.5. Data Analysis. A t test was used to compare the ARG concentrations between mussels exposed to SMX and not exposed (control); differences were considered significant at the $p < 0.05$ level. All statistical analyses were performed using R software (version 3.1.0).

3. RESULTS AND DISCUSSION

3.1. Occurrence of SMX in Seawater and Accumulation in the Hemolymph of Mussels. SMX was not detected in any control seawater sample or hemolymph samples from control mussels (data not shown). The SMX concentrations in seawater from SMX exposure treatments were similar throughout the exposure phase (Figure S1); the concentration after exposure for 96 h was 125.5 ± 9.2 μg/L, whereas the concentration in the hemolymph of the mussels was 81.3 ± 18.2 μg/L, after the same exposure time.

3.2. Quantification of ARGs. ARGs (*sul1* and *sul2*), *intI1*, and the 16S rRNA genes were quantified by qPCR in the gastrointestinal tract of marine mussels. High R^2 values of >0.992 and high efficiencies of >90% (except that of *sul2*, which was 80%) were obtained, indicating the validity of these

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quantifications. The total copy numbers of the 16S rRNA gene were consistent in all samples [no differences were observed between control and SMX-exposed mussels ($p > 0.05$)]. The concentrations of *intI1*, *sul1*, and *sul2* genes normalized to grams of sample (mussel gastrointestinal tract) are shown in Figure 1, whereas the concentrations normalized to 16S rRNA

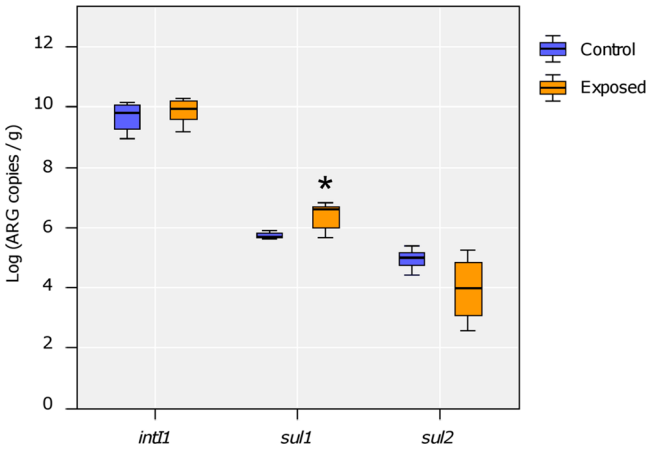

Figure 1. Absolute concentrations of ARGs in gastrointestinal tract samples from mussels exposed to SMX and not exposed (control). The asterisk indicates significant differences ($p < 0.05$).

copies and nanograms of DNA are shown in Figures S2 and S3, respectively. Similar results were obtained for all analyzed genes using the three different normalizations (Figure 1 and Figures S2 and S3).

3.3. Abundance of *sul1*, *sul2*, and *intI1* Genes in Marine Mussels Not Exposed to SMX. The analysis of ARGs in control mussels revealed the occurrence of *sul1*, *sul2*, and *intI1* genes in these organisms, despite the absence of antibiotic pressure in water. The *intI1* gene presented the highest concentration with a mean value of 6.8×10^9 copies/g, followed by *sul1* (5.5×10^5 copies/g) and *sul2* (1.1×10^5 copies/g) (Figure 1 and Table S1). These values are in the same range as the ARG concentrations (including *sul1* and *sul2* among others) found in the intestinal tract of aquaculture shrimps, ranging from 1.26×10^4 to 1.74×10^7 copies/g.[13] The presence of ARGs in control mussels (not exposed to SMX) demonstrates that mussels can act as reservoirs of ARGs (e.g., *sul1*, *sul2*, and *intI1* genes); to the best of our knowledge, this is the first time that *sul1*, *sul2*, and *intI1* genes have been detected in marine blue mussels. The presence of the *intI1* gene has already been reported in bacteria isolated from other aquatic organisms, such as fish (rainbow trout), in Australia.[28] The *sul1* and *sul2* genes have also been detected in aquatic organisms such as fish[29,30] and shrimps.[13] Our results confirm *sul1* as a predominant sulfonamide resistance gene in mussels when compared to *sul2*. Moreover, the occurrence of *sul1*, *sul2*, and *intI1* genes in marine blue mussels highlighted the importance of monitoring ARG in these organisms to evaluate the potential risks not only for the environment but also for humans after the ingestion of organisms carrying these ARGs. The ingested bacteria may occasionally survive the human gastric barrier[31] and then come into contact with the human microbiota. In fact, the human intestinal microbiome has been postulated to be a reservoir of ARGs.[32] This resistance can then be transferred through horizontal gene transfer (HGT) to other endogenous human bacteria, including potential human pathogens.[33]

3.4. Impact of Sulfamethoxazole on the Abundance of ARGs and the Bacterial Community Composition. The addition of SMX to the water in which mussels were growing led to an increase for the *intI1* gene of ~1 order of magnitude (from 6.8×10^9 copies/g in control mussels to 6.0×10^{10} copies/g in SMX-exposed mussels) (Figure 1). However, this increase was not statistically significant ($p >$

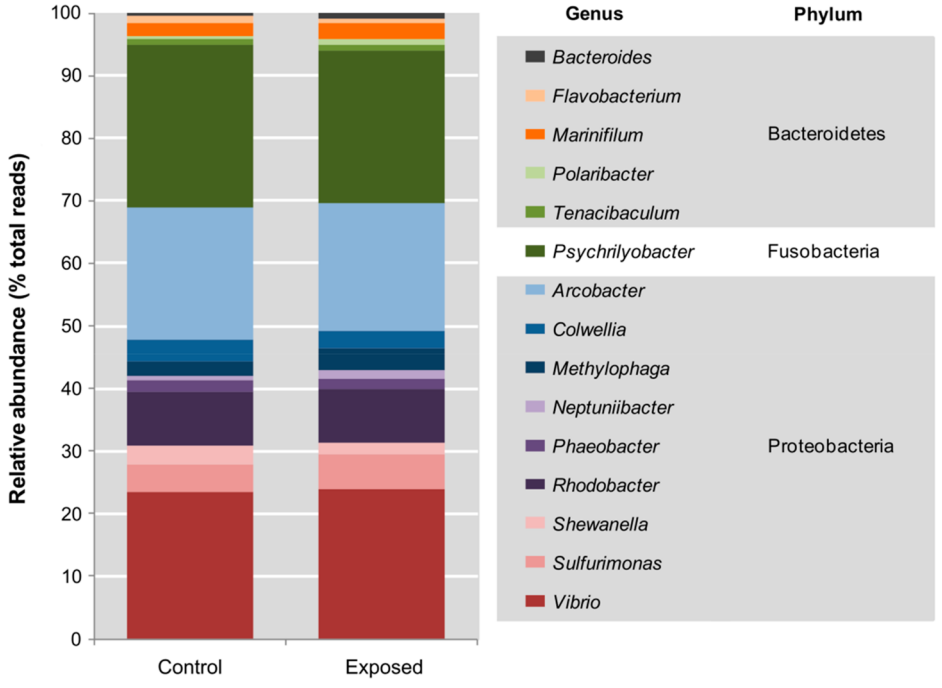

Figure 2. Relative abundance of major bacterial genera found in gastrointestinal tract samples from mussels exposed to SMX and not exposed (control). Average values for control and exposed communities are shown ($n = 6$). Only values of >0.5% are shown.

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0.05), probably due to the high biological variability between organisms. On the other hand, SMX exposure caused a significant increase ($p < 0.05$) in the absolute copy number of *sul1* in exposed mussels when compared to those not exposed to SMX (from 5.5×10^5 copies/g in control mussels to 3.5×10^6 copies/g in SMX-exposed mussels) (Figure 1 and Table S1). With regard to the *sul2* gene, it did not show significant changes ($p > 0.05$) when mussels were exposed to SMX compared with control mussels (Figure 1): 1.1×10^5 and 5.4×10^4 copies/g in control and SMX-exposed mussels, respectively (Figure 1 and Table S1).

The bacterial community composition was investigated in control and SMX-exposed mussels. Figure 2 shows the most abundant phyla and genera observed in both control and SMX-exposed communities. Shannon diversity and Chao richness indices were calculated for both treatments, and the values are listed in Table 1.

Table 1. Measures of α Diversity in Gastrointestinal Tract Samples from Mussels Exposed to SMX and Not Exposed (control)[a]

group	no. of reads	total no. of OTUs	Shannon diversity index	Chao richness estimator
control	8008	226 ± 27	2.64 ± 0.09	589 ± 104
exposed	8008	233 ± 27	2.49 ± 0.26	689 ± 175

[a]Average values ± the standard deviation are shown ($n = 6$). OTUs were defined at a threshold of 97% similarity.

No significant differences ($p > 0.05$) were observed in bacterial diversity and richness between control and SMX-exposed mussels. Moreover, the AMOVA test (as implemented by MOTHUR) did not show significant differences ($p > 0.05$) in the bacterial community structure of both treatments. These results confirm that a SMX exposure under its MIC did not cause alterations in the microbiota associated with the mussel gastrointestinal tract. However, despite the fact that no alterations in the bacterial community composition were seen, an increase in the abundance of *sul1* was observed, as indicated above. This increase could be attributed to gene mobilization through HGT. In fact, previous studies have shown that the exposure to antibiotics at sublethal concentrations can promote HGT.[34] Moreover, the increase (despite not being statistically significant) observed in the absolute copy number of *intI1* in SMX-exposed mussels may support gene mobilization through HGT. The *intI1* gene is a gene capture platform, with which *sul1* has been reported to be linked. The *intI1* gene is also located in mobile genetic elements (MGEs), which can be mobilized under anthropogenic pollution with pesticides, heavy metals, pharmaceuticals, personal care products, etc.[35] In contrast, *sul2*, not reported to be located in MGEs, did not change after SMX exposure. Therefore, the location of ARGs on MGEs is shown to be a key factor influencing their spread in different environments,[36] including the bacteria living within the mussel gastrointestinal tract.

Our results demonstrated the presence of sulfonamide resistance genes, *sul1* and *sul2*, in marine blue mussels. The occurrence of all investigated resistance genes in control mussels (without the presence of an antibiotic in water) highlighted the fact that mussels can act as reservoirs of ARGs in the marine environment. Furthermore, SMX exposure caused a significant increase in the absolute copy number of *sul1* in the gastrointestinal tract of marine mussels, suggesting that the presence of antibiotics in the marine environment where mussels grow is a key factor influencing the abundance of ARGs in these organisms. These results may have further implications regarding the spread of ARGs, as exposure to a single antibiotic can promote the development and spread of unrelated ARGs via plasmids and transposons.[37] Given this, monitoring of antibiotics in the aquatic environment and the implementation of strategies for reducing their amounts, especially in seafood-producing areas, become crucial for mitigating the occurrence and prevalence of ARGs and the associated risks for human and animal health.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.9b00112.

Additional information about DNA extraction and SMX analysis in seawater and the hemolymph of mussels and results of SMX quantification in seawater (Figure S1) and quantification of ARGs (Figures S2 and S3 and Table S1) (PDF)

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Notes

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Chapter 5 – General Discussion

5.1 Antibiotic detection methodologies

The development of analytical methodologies capable to determine the antibiotic occurrence in a given sample is crucial to assess their potential environmental risk. Due to the wide amount of antibiotics commercialized and used, analytical strategies should focus on determining a broad range of compounds in a single run. In this thesis, two different approaches for antibiotics analysis in aquatic biota and water samples were optimized (chapter 3). On one hand, a multi-residue method based on LC-MS/MS allowed to detect and quantify up to 23 antibiotics including some metabolites belonging to seven different antibiotic families in aquatic organisms (fish, mussel and clam) (manuscript 1). On the other hand, a screening methodology based on microbial growth inhibition was optimized for the screening of 17 antibiotics in aquatic biota biofluids and water samples.

The optimization of both methodologies, and specifically the sample pre-treatment step, highlighted the complexity of extracting a broad range of compounds in a single methodology[165]. Sample pre-treatment and an exhaustive sample clean-up are required for multi-residue analysis of antibiotics based on LC-MS/MS, allowing the extraction of a high number of compounds with the required instrumentation[28]. The optimized pre-treatment for biota samples (after testing four different strategies) was based on QuEChERS extraction with an acidified solvent. It allowed the extraction of a broad range of antibiotics providing a good preconcentration for LC-MS/MS analysis which obtained acceptable recoveries with good repeatability and reproducibility of the results and providing low detection and quantification limits. In the case of biota, for the analysis of antibiotics in the organism's tissue extract with the microbial growth inhibition test, the co-extraction of matrix interferences was high (manuscript 1). In contrast, for the analysis of antibiotics in biofluids (manuscript 2) matrix interferences were low allowing good accuracy, sensitivity and specificity in the microbial test for this matrix.

Both methodologies (LC-MS/MS based methodology and microbial growth inhibition test) have the potential to characterize the degree of antibiotic pollution in a given sample. However, their different characteristics make them feasible to be applied for different purposes. The microbial growth inhibition test is a screening tool, allowing the detection of a wide range of antibiotics (i.e. for a given antibiotic family). Besides, they are cost-effective methods compared with other analytical methodologies such as biosensors, immunological tests, or conventional methodologies such as LC-MS/MS[39]. However, screening methods neither provide information about the identity of the antibiotics occurring in a sample nor the concentration of these antibiotics. Moreover, the detection limits are generally higher than those achieved by LC-MS/MS based methodologies and are not as specific as those.

Microbial inhibition tests have been applied for the screening of antibiotic residues in foodstuff from animal origin, such as milk or meat[166,167]. Due to the high number of samples to be analyzed in food producing industries, screening techniques are used as a first step to identify the samples with potential occurrence of antibiotics. Then, the presence of antibiotics can be confirmed with more specific methodologies such as LC-MS/MS, and their concentrations can also be calculated.

In this thesis, the microbial growth inhibition test was applied for the screening of antibiotics in environmental samples including biota biofluids and water samples (manuscript 2). The test demonstrated its applicability for the screening of water samples such as river water, seawater and WWTP effluents. Therefore, the method could be implemented as a routine analysis for the screening of antibiotic discharge in WWTP. On the contrary, the method had some limitations when analyzing biota biofluids, mainly due to its high limits of detection. This limitation can prevent its application for antibiotics determination in aquatic organisms, as they are generally found at low concentrations.

Chemical analyses based on LC-MS/MS have been extensively applied for the analysis of contaminants, including antibiotics, in a wide range of matrices. As above mentioned, these techniques provide high specificity and the capacity to quantify the target compounds. However, they require complex instrumentation and specialized personnel. Chemical analyses are generally applied when there is a need to identify and quantify the antibiotics occurring in the target samples. These methods can be used for an accurate environmental assessment of the occurrence and risk of antibiotics[168]. Besides, chemical analyses are also applied for the quantification of contaminants in samples from food-producing animals, to evaluate the compliance with the Maximum Residue Limit (MRL) established by the authorities[169].

In this thesis, the methodology based on LC-MS/MS was applied for both, the assessment of antibiotic residues in foodstuff from animal origin, including seafood (manuscript 1), and the identification of antibiotic occurrence in environmental matrices (manuscript 2). The assessment of antibiotic residues in seafood allowed to specifically evaluate the occurrence and the concentration of the selected antibiotics and compare the obtained results with the MRL established. Concerning environmental analysis of antibiotics, LC-MS/MS methodology determined the occurrence of antibiotics in the different matrices analyzed (biota biofluids, river water, seawater and wastewater), even when antibiotics occurred at very low concentrations. This technique allowed to precisely determine the antibiotic risk for the analyzed environmental samples.

5.2 Antibiotic (and other contaminants) bioaccumulation

Antibiotic occurrence in the aquatic environment may affect the exposed living organisms. The evaluation of contaminants bioaccumulation allows confirming organism's exposure to the identified bioaccumulated pollutants. Besides, bioaccumulation may be used as a first step for the determination of potential ecotoxicological risk. Previous studies have demonstrated that pharmaceutical compounds including antibiotics can accumulate in aquatic organisms via water or food ingestion[95,170]. Different organisms such as primary producers, crustaceans, mollusks and fish have been studied in order to determine contaminants bioaccumulation[171,172]. Primary producer's bioaccumulation analysis can be useful for the characterization of short-term pollution episodes, whereas the analysis of fish will represent a longer time series[173,174]. Sessile organisms' analysis will represent the local degree of contamination, but mobile organisms will show a wider geospatial distribution[175]. In this thesis, the main organism used for bioaccumulation studies was the marine mussel (*Mytilus galloprovincialis and Mytilus edulis)*. Mussel is a sessile filter-feeding organism, it is prone to bioaccumulate contaminants[175] and it is considered as a bioindicator of water contamination. It is widely distributed along the coastal areas and extensively used as human food source. Consequently, contaminants bioaccumulation analysis in mussel can provide information regarding both, environmental and human health risks.

In order to investigate the bioaccumulation of antibiotics (and other emerging contaminants) in aquatic organisms, two approaches were followed (Chapter 4). First, exposure experiments were carried out under controlled conditions to specifically evaluate the bioaccumulation of the selected contaminants and second, antibiotics occurrence in aquatic organisms was determined in field experiments including wild and aquaculture organisms.

5.2.1 Contaminants bioaccumulation in exposure experiments

In manuscript 3, contaminants accumulation was assessed through exposure experiments of marine mussel to a mixture of 7 contaminants including pharmaceutical compounds, antibiotics and endocrine disrupting compounds. In this work, two major expected consequences of climate change to the marine environment, water warming and acidification were investigated to determine their effects on contaminants bioaccumulation. The results of the exposure experiment showed that SMX presented a higher bioconcentration factor (BCF) under water warming and water acidification separately and also to both factors jointly (when compared to control conditions). Sotalol BCF also increased due to water warming and methylparaben because of water acidification. However, other contaminants showed an opposite trend to the

exposed conditions. Acidification decreased the levels of triclosan and venlafaxine whereas citalopram BCFs decreased due to the combination of water warming and acidification. Water warming and acidification may alter the physic-chemical properties of the contaminants occurring in the environment and the metabolism of the living organisms, which may imply changes in the relationship between organisms-exposed contaminants[176]. Overall, it has been shown that changes in the environmental conditions may affect the bioaccumulation of contaminants, which should be considered to evaluate the chemical contamination risks for the *ocean of tomorrow* [176].

In order to globally understand the bioaccumulation potential of contaminants, the capacity of organisms to metabolize and excrete bioaccumulated compounds should be investigated. The metabolization of three contaminants, sulfamethoxazole (SMX), venlafaxine and carbamazepine was assessed through target analysis in manuscript 3. Only venlafaxine metabolites (O-desmethylvenlafaxine, N-desmethylvenlafaxine and N,O-desmethylvenlafaxine) were detected in mussel tissues after the exposure period. These results showed the capacity of mussel to metabolize contaminants. Venlafaxine metabolization was also observed in other aquatic organisms such as fish (*Argyrosomus regius).*N-desmethylvenlafaxine was the main venlafaxine metabolite in this fish[177] as it was also observed in our study for mussel. In addition to metabolites identification, their pharmacological activity should be considered to evaluate the potential ecotoxicological risk of their accumulation. In the case above mentioned, O-desmethylvenlafaxine has a similar pharmacological activity than venlafaxine; whereas N-desmethylvenlafaxine and NO-didesmethylvenlafaxine have been reported to be less active [178]. No metabolization was observed for SMX nor for carbamazepine which indicates that metabolization may be contaminant and/or species dependent. SMX metabolization was also assessed under controlled conditions in manuscript 4 through target and suspect screening analysis. Neither in manuscript 3 nor in 4, SMX metabolites were detected and hence its metabolization in marine mussel could not be proven under the exposure conditions. In line with this, no metabolization of SMX was postulated in fish rainbow trout in previous studies[179]. Similarly, no previous studies were found regarding carbamazepine metabolization by mussels, but by fish (*J. multidentate)* through the detection of two of its metabolites (carbamazepine-10,11-epoxide – CBZ-EP and 2-hydroxycarbamazepine – 2-OH-CBZ)[180]. Metabolization of contaminants contributes to their elimination from the organism. In the present study, only metabolization of venlafaxine was observed under the studied conditions. However, contaminants might be metabolized under different pathways than the known ones, or metabolites may be present at concentrations below the detection limits of the applied

methodologies. The current literature information regarding contaminants metabolization by mussels is scarce. Therefore, further studies, for instance, applying non-targeted approaches, may provide wider information regarding contaminants metabolization by mussels.

The capacity of mussels to excrete the bioaccumulated contaminants was investigated by transferring them to a clean environment (free of contaminant) after the exposure phase. After the depuration step, the concentrations of all tested contaminants (SMX, sotalol, venlafaxine, citalopram, carbamazepine, triclosan and methylparaben) decreased more than 60% compared to the concentrations at the end of the exposure phase (manuscript 3). SMX depuration was also assessed in manuscript 4 showing a decrease of its concentration at the end of the depuration phase of 69%. However, in none of the studies performed the contaminant concentration measured at the end of the depuration period was below the detection limit, which indicates that probably a long period of time was required to completely eliminate the contaminants from the organism. This issue plus the chronic exposure to pollutants in organisms living environment may provoke different degrees of bioaccumulation.

5.2.2 Antibiotic bioaccumulation in real samples

Antibiotic occurrence in aquatic organisms from field studies was evaluated in two sampling campaigns (manuscript 2). Organisms were obtained from the Ebro Delta region and from Mar Menor Lagoon (Spanish Mediterranean coast). The accumulation of antibiotics was assessed in fish from the Ebro river and Mediterranean Sea, mussel from the Mediterranean Sea and gastropod from Mar Menor lagoon. The organism showing the highest antibiotics concentrations was fish from the Ebro river; it accumulated tetracyclines, lincosamides and macrolides, in the range of few µg/L in their plasma. The organisms analyzed from the Mediterranean Sea, mussel and fish, presented low concentrations of antibiotics. Only one fish sample and one mussel sample showed occurrence of quinolones. Whereas, gastropod from Mar Menor lagoon presented no antibiotic occurrence. The bioaccumulation of antibiotics in organisms was in accordance with the occurrence of antibiotics in their surrounding environment; being river water significantly more polluted compared to lagoon water or seawater. The link between environmental pollution and antibiotic bioaccumulation by aquatic organisms may be of special concern when organisms are intended for human consumption.

The antibiotic occurrence in commercial seafood can be a direct source of antibiotic intake for the population with potential risk for human health, such as toxicity or allergy[96]. In manuscript 1, antibiotics occurrence in seafood was evaluated through the analysis of 23 antibiotics in

different marine organisms (fish, mussel and clam) used as human food source. Target organisms included aquaculture and wild organisms. The results obtained showed the occurrence of three different antibiotic families in commercial seafood species. Concretely, macrolides, tetracyclines and sulfonamides with concentrations detected above method detection limits (MDL). However, only macrolides and tetracyclines reported quantifiable levels in commercial seafood. Aquaculture samples presented a higher amount of antibiotics with quantifiable levels compared to wild organisms; being four antibiotics detected in aquaculture samples (roxithromycin, tilmicosin, tylosin and tetracycline) and two in wild organisms (azithromycin and tetracycline). These differences in antibiotic occurrence between aquaculture and wild organisms may be related to the antibiotic pollution in their surrounding environment. Aquaculture facilities are generally located on the coast in locations with high anthropogenic activity and hence, more prone to be polluted with antibiotics compared to wild organisms. Furthermore, antibiotics can be applied for veterinary purposes in aquaculture facilities, increasing organism's exposure to these compounds. Despite some antibiotics were detected and quantified in seafood samples none of the concentrations were higher than the MRL established by the EU in foodstuff from animal origin[101]. Therefore, a potential risk derived from seafood consumption is not foreseen although the amount of seafood consumed should be taken into consideration to determine the Tolerable Daily Intake. Besides, other food sources should also be considered to establish a potential human daily intake of antibiotic residues through diet.

5.2.3 Sulfamethoxazole accumulation

As stated in the introduction, SMX was used in this thesis as a transversal antibiotic in all the studies. Therefore, the results obtained for this compound may allow linking the outcomes from the different experiments, including field and exposure experiments. SMX was one of the most frequently detected antibiotics in water samples (manuscript 2), mainly in the Mar Menor lagoon and in the Ebro river (as well as in the discharge of the two WWTPs studied) in the range of few ng/L. In line with this, SMX has been extensively found in different aquatic environments[27,181]. However, SMX was scarcely detected in biota samples. Only two seafood samples from aquaculture, *Pangasius spp and Salmo salar*, showed occurrence of SMX but the levels were below quantification limits (manuscript 1). Besides, SMX was not detected in wild biota samples from the sampling campaigns performed in manuscript 2.

SMX accumulation in marine mussel was evaluated in three studies performed in the laboratory under controlled conditions (manuscripts 3, 4 and 5). The BCF of SMX in mussels was low in all

the studies (below 10 L/Kg). The physical-chemical properties of compounds (such as the Log K_{ow}) may play an important role in their capacity to accumulate in aquatic organisms[182]. In this sense, the low Log Kow for SMX (0.8) indicates the tendency to remain in the water phase, which would prevent its accumulation in organisms at a high degree. Similar results were seen in previous studies linking antibiotics low Log Kow values (Log Kow <2) with scarce accumulation in mussel[96].

5.3 Environmental effects of antibiotic pollution

As it has been shown in the previous section (5.2), antibiotics have the capacity to accumulate in the aquatic biota. Pollutants bioaccumulation capacity can be crucial to attain high contaminant body burden and provoke negative effects to the exposed organisms. In order to investigate the risk of antibiotics pollution to exposed organisms, different approaches were used (chapter 4). Exposure experiments were carried out for the determination of antibiotic ecotoxicological effects through the characterization of conventional and novel ecotoxicological parameters (manuscript 4). Besides, exposure experiments were also used to study the relationship between antibiotic pollution and the spread of antibiotic resistance genes (manuscript 5). Finally, antibiotic risk was assessed in field studies through monitoring campaigns of environmental samples (manuscript 2).

5.3.1 Antibiotics ecotoxicological effects

In order to link the exposure of a specific antibiotic with its potential ecotoxicological effects in target organisms, exposure experiments under controlled conditions were carried out, which eliminated the variability inherent to field conditions. In manuscript 4, marine mussels were exposed to SMX at 10 µg/L for 96h, whereas a control group of mussels was maintained in the same conditions without any antibiotic addition.

Antibiotic ecotoxicological effects were evaluated through the characterization of enzymatic activities in mussel's digestive tract and gills. Enzymatic activities were used to characterize oxidative stress through the analysis of glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) and lipid peroxidation (LPO). Besides, xenometabolism related enzymes were also studied (glutathione-S-transferase (GSTs) and carboxylesterases (CbE)). Results from the enzymatic activities characterization showed no significant differences between control and exposed organisms. Previous studies reported no effects on enzymatic activities due to antibiotics exposure in aquatic organisms[183]. However, other studies did observe changes in

enzymatic activities but when organisms were exposed to high concentrations of antibiotics (100 µg/L)[184].

To further understand the effects of SMX exposure in marine mussel, a novel ecotoxicological approach through non-targeted metabolomics was carried out (manuscript 4). Non-targeted metabolomics has the capacity to evaluate alterations in all the metabolites of an organism in a given moment. This ecotoxicological evaluation has no previous bias on expected effects of a stress factor. Two metabolites (aspartate and benzoate) were altered under both, winter and summer conditions. Under both conditions' aspartate decreased its levels due to SMX exposure, but benzoate showed an opposite trend. Based on that, aspartate was proposed as a biomarker of effect to SMX in mussels. Other metabolites were also altered due to SMX exposure, being amino acids the metabolite group most affected including, phenylalanine, valine, tryptophan and the previously mentioned aspartate. Based on the metabolic pathways involving these metabolites, alterations in mussel's osmotic regulation and energy metabolism were postulated. Despite these effects are not expected to compromise organism's survival, they may have effects on its physiology. Therefore, further research on potential long-term impact of these sub-lethal alterations should be considered to understand the risk for the exposed organisms.

The results obtained from metabolomics characterization represent new evidence of antibiotic ecotoxicological effects in marine organisms. Furthermore, ecotoxicological alterations were not detected with enzymatic activities characterization, pinpointing metabolomics as a more sensitive ecotoxicological approach. Therefore, metabolomics can be used as an early warning ecotoxicological tool. Up to date, few studies used metabolomics to investigate antibiotic effects in exposed organisms. Only clarithromycin, florfenicol and sulfamethazine effects on zebrafish have been previously studied[128]. However, the constant evolution of analytical techniques, in addition to the development of more complete metabolites databases, software, and online resources will help to apply metabolomics in a more routine manner. This will contribute to generate a repository of information regarding ecotoxicological effects through metabolomics, providing further insights on environmental risk assessment.

5.3.2 Antibiotic resistance

One of the aspects rising more concern regarding environmental antibiotic pollution is its suspected contribution to the spread of antibiotic resistance[185,186]. Monitoring campaigns have highlighted the wide distribution of ARGs in water bodies worldwide[141,187]. Despite a lot of information is gathering on ARGs environmental occurrence, its link with aquatic organisms is

still scarce. In manuscript 5, we investigated the contribution of SMX seawater pollution to the spread of ARGs within the bacterial community from the mussel's digestive tract. The experiment was performed under controlled conditions to determine the effects of antibiotics pollution on the ARGs abundance. Results of the study showed the occurrence of ARGs (*intI1, sul1* and *sul2*) in both, control and exposed organisms. This may indicate that mussels can serve as reservoirs of antimicrobial resistance, as it has previously been shown for shrimps[188]. Besides, an increase of *sul1* absolute concentration was observed in mussel due to SMX exposure. Therefore, antibiotic water pollution can contribute to the increase of ARGs abundance in exposed organisms. This can be of special concern when organisms are used as human food source because ARGs may be uptaken by consumers. Further research on ARGs should be done in food products to comprehensively evaluate their risk through ingestion.

5.3.3 Environmental risk assessment of antibiotics

In addition to the above described exposure experiments, antibiotic risk was also assessed in field studies using different approaches (manuscript 2): i) through the calculation of hazard quotients (HQ) based on data of the antibiotic's concentrations in water and ecotoxicological values in non-target organisms and ii) through the measurement of the microbial growth inhibition caused by the environmental samples using a screening specific test.

Target antibiotic analysis was performed in WWTP influent and effluents, river water (Ebro river), Mediterranean Sea and Mar Menor lagoon. As expected, the highest antibiotic concentrations were found in the influent of WWTPs. Although a significant decrease of antibiotics occurrence was observed in WWTPs, some antibiotics were still present in the treated effluent. The most frequently detected antibiotics in WWTP effluents were quinolones, sulfonamides, macrolides and lincosamides, in the range of few µg/L. The same antibiotic families (except quinolones) were prevalent in river water impacted by WWTPs as well as in seawater, although at concentrations below 0.1 µg/L and 0.01 µg/L respectively. The low occurrence of quinolones in environmental water samples may be attributed to photodegradation of these compounds. Quinolones have a half-life time in pure water of 105 and 90 min[189], thus, they have mainly been detected in sediment and biota samples rather than in water[190]. On the contrary, tetracyclines were detected in river water but not in WWTP effluents. Their presence may be related to agricultural activities runoff since tetracyclines are commonly used in livestock activities[191]. Lagoon water from Mar Menor presented mainly occurrence of macrolides and sulfonamides, also at low concentrations (below 0.04 ug/L).

The obtained results regarding antibiotics occurrence in water samples were used for the assessment of their environmental risk based on the calculation of the HQ:

HQ = Measured antibiotic concentration in water (MEC)/Predicted No Effect Concentration (PNEC).

Where MEC is the measured antibiotic concentration in water and PNEC value was determined according to Tell et al. 2019[162]. This approach considers the lowest of two PNECs; one based on ecotoxicological studies and another one corresponding to the lowest concentration of an antibiotic that can promote antibiotic resistance[162]. In our study, both PNECs were obtained from a literature review. A compound is considered to have potential risk for the environment when its HQ > 1.

The HQs were calculated for the antibiotics quantified in the monitoring campaigns of the Ebro Delta and Mar Menor lagoon (manuscript 2). Antibiotics belonging to macrolide and quinolone families (including ciprofloxacin, ofloxacin and azithromycin) presented HQ levels > 1 in the effluents from the two WWTP considered (WWTP1 and WWTP2), which discharge their effluent into the Ebro River and Mediterranean Sea, respectively. However, none of the antibiotic HQ calculated in these water bodies was higher than 1 and therefore no risk for the aquatic life was expected. No risk was observed in the Mar Menor lagoon either based on the HQ calculated from the antibiotics quantified. These results confirm WWTP effluents as a source of antibiotic pollution[47] but the derived risk for the receiving environments was low. Dilution effects, transformation or degradation of antibiotics in the receiving environment may contribute to reduce their concentration, and consequently their risk for the aquatic community[54].

The microbial growth inhibition test was also applied for the determination of antibiotics and their potential risk in the studied sites (manuscript 2). Microbial inhibition test reported the highest antibiotic activity in wastewater samples, mainly for quinolones, macrolides and sulfonamides; whereas, lower antibiotic activity was observed in environmental samples. Only two samples from the Ebro river showed inhibition in the tetracyclines and sulfonamides plates (also detected with LC-MS/MS analysis) but no antibiotic activity was seen for the rest of the samples (Mediterranean Sea and Mar Menor lagoon). The inhibition observed in sulfonamides plates may be related to the join occurrence in these samples of sulfonamides (SMX) and trimethoprim, which have synergistic effects, as previously described (chapter 1).

The combination of both chemical and biological techniques allowed providing further insights into the assessment of environmental risks posed by antibiotics. LC-MS/MS analysis allowed the

calculation of specific HQ, whereas the microbial inhibition test permitted the identification of potential synergistic effects of antibiotics in environmental samples.

Chapter 6 – Conclusions

I. A multi-residue methodology based on QuEChERs extraction followed by LC-MS/MS was developed for the analysis of antibiotics in aquatic biota samples at low concentrations.

II. A methodology based on microbial growth inhibition was optimized for the screening of four antibiotic families (namely, sulfonamides, macrolides, tetracyclines and quinolones) in biota biofluids and water samples allowing to screen them in environmental samples though with higher detection limits than those obtained with conventional methodologies.

III. Wastewater presented high levels of antibiotics whereas river water, seawater and lagoon showed significantly lower antibiotic concentrations. The main antibiotic families detected in these environments were sulfonamides, macrolides and lincosamides. Their reported concentrations posed no risk for the environment according to the corresponding HQ.

IV. Seafood samples presented occurrence of antibiotics, mainly macrolides and tetracyclines, at low levels, posing no risk for consumers according to the MRL established.

V. Climate change simulation (water warming, increase of 4°C and acidification decrease of 0,4 pH units) provoked alterations on antibiotics bioaccumulation (as well as on that of other emerging pollutants) in marine mussel.

VI. Sulfamethoxazole metabolization in marine mussel exposed in controlled conditions was not observed through targeted and suspect screening analysis. However, other contaminants such as venlafaxine were highly metabolized.

VII. Sulfamethoxazole and other emerging contaminants were not fully removed from mussels' tissue after a depuration period (between 1 and 20 days), which indicates that long depuration periods are required for a complete contaminant's elimination.

VIII. Metabolomics showed that SMX exposure affected mussel's amino acids levels, which was related to alterations in their osmoregulation and energy metabolism. However, no changes were observed in mussels xenometabolism nor in oxidative stress.

IX. The exposure of marine mussel to SMX under controlled conditions provoked an increase in *sul1* resistant gene abundance in the bacteria located in mussel's gastrointestinal tract. Therefore, antibiotic water pollution can enhance ARGs abundance in marine organisms.

Concluding remarks and future perspectives

In this thesis, different aspects related to antibiotic pollution in the marine environment have been tackled; including, antibiotic analysis, ecotoxicological effects, bioaccumulation and impact on antibiotic resistance spread. Some future trends can be foreseen concerning each of the aspects addressed in the thesis:

- Antibiotic detection techniques:

So far, the analytical methodologies have been focused on the development of target analysis of a limited number of antibiotics. In this thesis, a multi-residue method based on LC-MS for the analysis of antibiotics in biota samples was developed. Despite the methodology was optimized for a broad range of antibiotics determination, it was restricted to a target list of compounds. Thus, there is a need for analytical methodologies such as the so-called non-target analysis, based on High Resolution Mass Spectrometry (HRMS), which do not require the availability of commercial analytical standards of the suspects. These technics can also provide information regarding antibiotic metabolites occurrence in environmental samples. Metabolites monitoring is of increasing concern as they can be as pharmacologically active as the parent compound and hence, with potential ecotoxicological consequences of their presence. In addition to the implementation of non-target analysis, further development of fast and low cost monitoring methods for routine analysis of antibiotics (such as the effect-based methodology applied in this thesis) would allow monitoring antibiotic presence in a much large number of facilities such as hospital effluents or WWTP discharges, providing better water management.

- Antibiotic ecotoxicological effects

The results reported in this thesis showed low ecotoxicological effects of antibiotics to non-target organisms in the studied sites (river, lagoon and seawater). Besides, laboratory exposure experiments to selected antibiotics showed no alterations on mussels' enzymatic activities that are extensively used endpoints to evaluate ecotoxicological effects in different organisms. Therefore, environmental concentrations of antibiotics do not pose a high ecotoxicological risk. Nevertheless, in order to discard any consequence for the communities exposed to antibiotics it is recommended i) to evaluate long-term effects, as most of the studies reported in the literature consider acute effects. ii) to apply novel ecotoxicological approaches such as, metabolomics, which will reveal new antibiotic effects on marine mussels (as presented in this thesis). Further research in this direction may help to better understand the antibiotic ecotoxicological impact, including the discovery of new biomarkers of exposure and impact.

In order to comprehensively understand ecotoxicological effects in wild organisms. Combination of non-targeted metabolomics with the analysis of xenobiotic compounds in the same biological samples (xenometabolomics approach) will provide a deep understanding of both, chemical exposure and metabolism alterations in the studied samples. These analyses can provide information regarding the toxic effects of a chemical mixture, but lack of determining single compound effects. Besides, reference conditions (namely, organisms used as control group) for environmental metabolomics studies may be hard to determine due to the biological variability of organisms.

- Climate change impact on antibiotics environmental risk

Anthropogenically driven climate change is a topic of concern worldwide for its potential effects on the environment and environmental services. Results of this thesis showed that water warming and acidification (expected effects of climate change) altered the bioaccumulation and metabolization of emerging contaminants in mussels. Further studies with a large number of compounds should be performed under expected climate change conditions to evaluate and characterize contaminant fate and risk. This would allow a better prediction of contamination scenarios and the evolution of regulation, monitoring and mitigation strategies in the future.

- Antibiotic resistance

The occurrence of antibiotic resistance genes and antibiotic resistant bacteria have been demonstrated in a wide range of water bodies worldwide. Furthermore, the correlation between antibiotic pollution and the spread of antibiotic resistance to the environment has also been postulated. However, the aquatic biota compartment has been scarcely investigated. The occurrence of antibiotic resistance genes in bacteria located in aquatic organisms' digestive tract has been observed in this thesis and in few other publications. However, the role of aquatic organisms as reservoirs of antibiotic resistance and their potential contribution to the spread of resistance to other bacteria should be further investigated. Besides, the occurrence of antibiotic resistance to organisms used as human food source needs further research to determine their risk for human health. Further studies evaluating the bioaccesibility of antibiotic resistance and the antibiotic resistance acquisition by human microbiome due to the ingestion of polluted food are urgently needed.

Results of this thesis highlighted the widespread distribution of antibiotics in the water bodies and its accumulation in exposed organisms. Furthermore, potential risk for the environment and especially for human health may be expected. To overcome this situation different measures should be taken, which may include:

i) The reduction of antibiotic utilization. The application of antibiotics for non-therapeutic practices should be severely restricted (or banned). Medical practices such as lowering antibiotic dosage can also contribute to the decrease in antibiotic utilization. Personalized administration of antibiotics dosage will help to balance an effective medical treatment with decreasing antibiotic occurrence in the environment. Similarly, veterinary practices may also be improved for the reduction of antibiotic utilization such as vaccination of animals to prevent infections in livestock production.

ii) The improvement of water treatment technologies to eliminate antibiotic residues in wastewater. As described in this thesis and extensively reported in the literature, WWTPs do not efficiently eliminate these residues and are thus antibiotic input sources into the environment. Cost effective and efficient water treatment technologies for antibiotic elimination should be developed and incorporated in water treatment facilities. Special focus should be paid on those WWTP receiving hospital or livestock production discharge, with potentially high levels of antibiotics. On-site water treatment can also contribute to reduce overall antibiotic occurrence in the water bodies.

iii) Legislation regarding antibiotic occurrence in the environment. As described in chapter 1, no legislation exists regarding antibiotic maximum residue limits for environmental samples. Nevertheless, some antibiotics have been included in the EU "watch list" which highlights the concern of policy-makers. Legislation regarding antibiotic occurrence in the environment may help to boost the implementation of the measures for its reduction.

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Supplementary Information

SUPPORTING INFORMATION

Multi-residue method for the determination of antibiotics and some of their metabolites in seafood

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Material and Methods

Table S1. List of target compounds included in the analysis based on detection with LC-MS-MS. Antibiotics are organised by their therapeutic family, precursor ion, retention time (RT) and the corresponding internal standard are also presented.

Therapeutic family	Antibiotic	Precursor ion	RT (min)	Q3	Q3	logP	pKa
Macrolides	Azithromycin	749 $[M+H]^+$	1.53	591	116	8.91	2.44
	Clarithromycin	748 $[M+H]^+$	2.20	158	590	8.38	3.24
	Roxithromycin	837 $[M+H]^+$	2.23	679	158	9.17[a]	1.7[b]
	Spiramycin	843 $[M+H]^+$	1.49	174	43	7.9[c]	2.75[e]
	Tilmicosin	869 $[M+H]^+$	1.72	88	696	8.18[d]	3.80[d]
	Tylosin	916 $[M+H]^+$	1.99	174	772	3.31[a]	3.27[e]
Tetracyclines	Tetracycline	445 $[M+H]^+$	1.30	410	154	4.67	-0.78
Lyncosamides	Clindamycin	425 $[M+H]^+$	1.58	126	377	7.55[b]	1.76[b]
	Lincomycin	407 $[M+H]^+$	1.02	359	389	7.79[a]	0.56[b]
Sulphonamides	Sulfadimethoxine	310 $[M+H]^+$	2.00	156	65	2.13[a]	1.63[b]
	Sulfamerazine	264 $[M+H]^+$	1.28	156	92	2.06[a]	0.14[b]
	Sulfamethoxazole	253 $[M+H]^+$	1.77	156	92	0.25	0.79
	Sulfadiazine	250 $[M+H]^+$	1.11	156	92	2.01[b]	0.25[b]
	Sulfapyridine	249 $[M+H]^+$	1.02	156	92	2.63[b]	0.84[b]
	Sulfisomidin	278 $[M+H]^+$	0.98	186	65		
	Sulfisoxazole	267 $[M+H]^+$	1.84	156	113	2.17[b]	1.14[b]
(Metabolite)	N-acetylsulfadiazine	293 $[M+H]^+$	1.23	65	134	-	-
(Metabolite)	N-acetylsulfamerazine	307 $[M+H]^+$	1.31	65	134	-	-
(Metabolite)	N-acetylsulfamethazine	321 $[M+H]^+$	1.48	65	134	-	-
Nitroimidazoles	Metronidazole	172 $[M+H]^+$	0.94	82	128	3.09	-0.46
(Metabolite)	Metronidazole-OH	187 $[M+H]^+$	0.82	126	123	1.55	1.28
Dihydrofolate reductase inhibitors	Trimethoprim	291 $[M+H]^+$	1.17	230	261	7.16	1.28
						-2.8[b]	1.15[b]
Amphenicols	Chloramphenicol	321 $[M-H]^-$	1.19	152	194	8.91	2.44

The logP and pKa values were obtained from: [a](Qiang & Adams 2004); [b]http://www.drugbank.ca/ ; [c]https://www.drugs.com/; [d]https://toxnet.nlm.nih.gov/; [e]http://www.lookchem.com/.

Table S2. List of all compounds included in the spiking mix organised by their therapeutic family, also their corresponding internal standard is specified.

Therapeutic family	Antibiotic	Internal Standard
Macrolides	Azithromycin	Azithromycin-d3
	Clarithromycin	Azithromycin-d3
	Roxithromycin	Azithromycin-d3
	Spiramycin	Ampicillin-d5
	Tilmicosin	Erythromycin-d13
	Tylosin	Erythromycin-d13
Tetracyclines	Tetracycline	Sulfamethoxazole-d4
Fluoro(quinolones)	Cinoxacin	Ofloxacin-d3
	Ciprofloxacin	Ciprofloxacin-d8
	Danofloxacin	Ofloxacin-d3
	Enrofloxacin	Ofloxacin-d3
	Marbofloxacin	Ofloxacin-d3
	Norfloxacin	Ofloxacin-d3
	Ofloxacin	Ofloxacin-d3
	Orbifloxacin	Ofloxacin-d3
	Nalidixic acid	Ofloxacin-d3
	Oxolinic acid	Ofloxacin-d3
	Flumequine	Ofloxacin-d3
	Pipemidic acid	Ofloxacin-d3
Lyncosamides	Clindamycin	Lincomycin-d3
	Lincomycin	Lincomycin-d3
Sulphonamides	Sulfadimethoxine	Sulfamethoxazole-d4
	Sulfamerazine	Sulfamethoxazole-d4
	Sulfamethoxazole	Sulfamethoxazole-d4
	Sulfadiazine	Sulfamethoxazole-d4
	Sulfapyridine	Sulfamethoxazole-d4
	Sulfisomidin	Sulfamethoxazole-d4
	Sulfisoxazole	Sulfamethoxazole-d4
(Metabolite)	N-acetylsulfadiazine	Sulfamethoxazole-d4
(Metabolite)	N-acetylsulfamerazine	Sulfamethoxazole-d4
(Metabolite)	N-acetylsulfamethazine	Sulfamethoxazole-d4
Nitroimidazoles	Metronidazole	Ronidazole-d3
(Metabolite)	Metronidazole-OH*	Ronidazole-d3
Dihydrofolate reductase inhibitors	Trimethoprim	Sulfamethoxazole-d4
Amphenicols	Chloramphenicol*	Ibuprofen-d3

*Metronidazole-OH and Chloramphenicol were added to the spiking mix once the final extraction procedure was developed.

Microbial growth inhibition test

An extraction procedure based on ultrasonic extraction, solid phase purification was applied for the further analysis of the samples with the microbial growth inhibition test. For this extraction procedure, ACN:MeOH (75:25, v/v) 1% FA was employed as extraction solvent and ultrasonic bath as extraction technique (adapted from Capone et al., 1996). Briefly, samples were weighted (0.5 g) and placed in a polypropylene tube, 4 mL of ACN:MeOH (75:25, v/v) 1% FA, were added and vortexed for 30s. After that, the samples were sonicated for 3 min and centrifuged at 1500 rpm for 5 min. The supernatant was collected and the process was repeated two more times. The supernatant resulting from the three ultrasonic extractions was mix and centrifuged at 5000 rpm for 10 min. Then, the samples were dried down under nitrogen and reconstituted in 200 mL of HPLC water. After that, clean up with SPE was performed as explained in the manuscript (section 2.3, method iv).

The final extracts were diluted 1:1 with demineralised water before their application in the microbial plates. Then, 250 µl of every diluted sample were transferred to a specific well on each plate. Forty µl of application fluid (a plate-specific buffer solution) were also added to each well. After this, the plates were incubated at 30°C except for the sulfonamides plate which was incubated at 37°C. After 16 to 24 hours of incubation, the tests were photographed and visually inspected for the determination of the inhibition zone, which indicates antimicrobial activity. A positive result is shown as no bacterial growth around the well where the sample was added, and therefore the presence of the antibiotic family in the sample (or a biocidal compound that could inhibit bacterial growth, like triclosan). Besides, a bigger inhibition zone indicated a higher concentration of antibiotic in the sample, or a more toxic effect of the compound for the bacteria (figure S1).

Results and discussion

Table S3. Extraction efficiencies (%) and standard deviation (n=3) for the fluoro(quinolones) analysed with the different extraction procedures tested US (iii), US (iv), QuEChERS (i) and QuEChERS (ii)

Compound	Recovery (%) ± SD			
	QuEChERS (i)	QuEChERS (ii)	US (iii)	US (iv)
Cinoxacin	0 ± 0	8 ± 2	0 ± 0	19 ± 6
Ciprofloxacin	6 ± 4	3 ± 1	37 ±17	25 ± 17
Danofloxacin	8 ± 4	0 ± 0	34 ± 23	0 ± 0
Enrofloxacin	4 ± 0	7 ± 0	8 ± 1	17 ± 9
Marbofloxacin	0 ± 0	5 ± 1	7 ± 2	32 ± 16
Norfloxacin	0 ± 0	6 ± 1	23 ± 16	41 ± 18
Ofloxacin	0 ± 0	6 ± 1	17 ± 7	33 ± 14
Orbifloxacin	5 ± 1	0 ± 0	7 ± 1	26 ± 11
Nalidixic acid	0 ± 0	13 ± 1	10 ± 8	13 ± 8
Oxolinic acid	0 ± 0	12 ± 1	11 ± 4	7 ± 4
Flumequine	0 ± 0	14 ± 2	11 ± 7	31 ± 12
Pipemidic acid	6 ± 1	0 ± 0	0 ± 0	10 ± 7

Table S4. Extraction efficiencies (%) standard deviation (n=3) for all the compounds included in the method with the exception of Metronidazole-OH and Chloramphenicol included later on in the method. Statistical analysis performed with ANOVA or Kruskal-Wallis (depending if the data set was parametric or non parametric) is presented. Different letters indicate significant differences and the p-values are also reported.

Therapeutic family	**Antibiotic / statistical test**	**QuEChERS (i)**	**QuEChERS (ii)**	**US (iii)**	**US (iv)**	**p-value**
Macrolides	Azithromycin	39 ± 26	14 ± 13	1 ± 0	15 ± 6	
	(ANOVA)	a	a	a	a	9.4E-02
	Clarithromycin	57 ± 36	34 ± 13	5 ± 0	10 ± 5	
	(ANOVA)	a	a	a	a	5.0E-02
	Roxithromycin	59 ± 38	36 ± 11	5 ± 0	9 ± 5	
	(ANOVA)	a	a	a	a	5.0E-02
	Spiramycin	102 ± 10	64 ± 46	0 ± 0	4 ± 1	
	(Kruskal-Wallis)	-	-	-	-	2.0E-02
	Tilmicosin	120 ± 3	73 ± 39	3 ± 0	24 ± 8	
	(Kruskal-Wallis)	-	-	-	-	2.4E-02
	Tylosin	101 ± 2	7 ± 5	0 ± 0	0 ± 0	
	(Kruskal-Wallis)	-	-	-	-	2.0E-02

Tetracyclines	Tetracycline	7 ± 1	5 ± 5	2 ± 0	0 ± 0	
	(Kruskal-Wallis)	-	-	-	-	2.2E-02
Lyncosamides	Clindamycin	22 ± 7	33 ± 13	2 ± 0	9 ± 5	
	(Kruskal-Wallis)	-	-	-	-	5.2E-02
	Lincomycin	50 ± 2	27 ± 11	2 ± 0	101 ± 3	
	(Kruskal-Wallis)	-	-	-	-	3.7E-02
Sulfonamides	Sulfadimethoxine	33 ± 7	76 ± 64	37 ± 10	23 ± 8	
	(Kruskal-Wallis)	-	-	-	-	3.8E-01
	Sulfamerazine	36 ± 45	54 ± 46	1 ± 0	39 ± 7	
	(ANOVA)	a	a	a	a	3.4E-01
	Sulfamethoxazole	10 ± 6	64 ± 53	4 ± 0	38 ± 10	
	(Kruskal-Wallis)	-	-	-	-	5.9E-02
	Sulfadiazine	26 ± 9	63 ± 52	0 ± 0	0 ± 0	
	(Kruskal-Wallis)	-	-	-	-	3.7E-02
	Sulfapyridine	32 ± 3	84 ± 73	0 ± 0	49 ± 15	
	(Kruskal-Wallis)	-	-	-	-	5.7E-02
	Sulfisomidin	29 ± 2	44 ± 37	0 ± 0	54 ± 15	
	(Kruskal-Wallis)	-	-	-	-	8.1E-02
	Sulfisoxazole	27 ± 4	18 ± 16	4 ± 0	48 ± 1	
	(ANOVA)	bc	ab	a	c	3.4E-03
	N-acetylsulfadiazine	29 ± 5	33 ± 24	2 ± 1	23 ± 8	
	(Kruskal-Wallis)	-	-	-	-	1.1E-01
	N-acetylsulfamerazine	37 ± 1	48 ± 36	3 ± 0	38 ± 12	
	(Kruskal-Wallis)	-	-	-	-	1.3E-01
	N-acetylsulfamethazine	41 ± 5	68 ± 51	3 ± 0	34 ± 9	
	(Kruskal-Wallis)	-	-	-	-	9.3E-02
Nitromidazoles	Metronidazole	40 ± 6	153 ± 131	1 ± 0	99 ± 8	
	(Kruskal-Wallis)	-	-	-	-	3.3E-02
Dihdrofolate reductase inhibitors	Trimethoprim	45 ± 5	117 ± 95	1 ± 0	56 ± 5	
	(Kruskal-Wallis)	-	-	-	-	5.2E-02

Table S5. Extraction efficiencies (%) and standard deviation (n=3) for QuEChERS extraction procedure (i) and the addition of different amount of formic acid in the extraction solvent (0.1% and 1%) for all the compounds included in the method with the exception of Metronidazole-OH and Chloramphenicol. Statistical analysis performed with ANOVA or Kruskal-Wallis (depending if the data set was parametric or non parametric) is presented. Different letters indicate significant differences and the p-values are also reported.

Therapeutic family	Antibiotic / Statistical test	Quechers (i)	Quechers (i) 0,1% FA	Quechers (i) 1% FA	p-value
Macrolids	Azithromycin	39 ± 26	23 ± 4	56 ± 3	
	(ANOVA)	a	a	a	1.3E-01
	Clarithromycin	57 ± 36	20 ± 2	51 ± 4	
	(ANOVA)	a	a	a	1.6E-01
	Roxithromycin	59 ± 38	20 ± 2	54 ± 1	
	(Kruskal-Wallis)	-	-	-	6.0E-02
	Spiramycin	102 ± 10	39 ± 4	37 ± 5	
	(ANOVA)	b	a	a	3.9E-05
	Tilmicosin	120 ± 3	91 ± 2	60 ± 2	
	(ANOVA)	c	a	b	1.2E-07
	Tylosin	101 ± 2	32 ± 3	44 ± 6	
	(ANOVA)	b	a	a	2.1E-06
Tetracyclines	Tetracycline	6 ± 1	3 ± 0	35 ± 9	
	(ANOVA)	b	a	c	4.0E-07
Lyncosamides	Clindamycin	22 ± 2	14 ± 3	37 ± 1	
	(ANOVA)	b	a	c	4.0E-05
	Lincomycin	50 ± 2	9 ± 1	30 ± 1	
	(Kruskal-Wallis)	-	-	-	2.7E-02
Sulfonamides	Sulfadimethoxine	33 ± 7	22 ± 3	30 ± 7	
	(ANOVA)	a	a	a	1.0E-01
	Sulfamerazine	36 ± 45	16 ± 3	30 ± 3	
	(Kruskal-Wallis)	-	-	-	2.0E-01
	Sulfamethoxazole	10 ± 6	5 ± 7	33 ± 8	
	(ANOVA)	a	a	b	2.0E-02
	Sulfadiazine	26 ± 9	21 ± 2	32 ± 8	
	(ANOVA)	a	a	a	1.4E-01
	Sulfapyridine	32 ± 3	20 ± 2	31 ± 13	
	(ANOVA)	a	a	a	2.0E-01
	Sulfisomidin	29 ± 3	18 ± 3	34 ± 9	
	(ANOVA)	b	a	ab	9.0E-04
	Sulfisoxazole	27 ± 4	10 ± 1	28 ± 4	
	(ANOVA)	b	a	b	1.0E-03

	N-acetylsulfadiazine	29 ± 5	19 ± 5	37 ± 4	
	(ANOVA)	ab	a	b	2.0E-02
	N-acetylsulfamerazine	37 ± 1	24 ± 4	37 ± 3	
	(ANOVA)	b	a	b	3.0E-03
	N-acetylsulfamethazine	4 ± 5	26 ± 4	44 ± 3	
	(ANOVA)	b	a	b	2.0E-02
Nitromidazoles	Metronidazole	40 ± 6	13 ± 1	54 ± 11	
	(ANOVA)	b	a	b	5.8E-05
Dihidrofolate reductase inhibitors	Trimethoprim	45 ± 5	16 ± 2	53 ± 12	
	(ANOVA)	b	a	b	3.3E-05

Table S6. Linearity (regression coefficient) obtained from calibration curves made in the corresponding seafood extract (clam, mussel and fish), concentration range from 0.01- 50 ng/ml.

Antibiotic	**Clam (r^2)**	**Mussel (r^2)**	**Fish (r^2)**
Azithromycin	0.991	0.995	0.998
Clarithromycin	0.991	0.995	0.994
Roxithromycin	0.993	0.995	0.995
Spiramycin	0.999	0.999	0.991
Tilmicosin	0.992	0.997	0.997
Tylosin	0.998	0.999	0.997
Tetracycline	0.998	0.997	0.990
Clindamycin	0.999	0.998	0.999
Lincomycin	0.999	0.999	0.998
N-acetylsulfadiazine	0.999	0.996	0.997
N-acetylsulfamerazine	0.995	0.999	0.994
N-acetylsulfamethazine	0.999	0.999	0.996
Sulfadimethoxine	0.998	0.999	0.999
Sulfamerazine	0.995	0.999	0.999
Sulfamethoxazole	0.999	0.999	0.999
Sulfadiazine	0.998	0.999	0.999
Sulfapyridine	0.994	0.997	0.994
Sulfisomidin	0.999	0.999	0.997
Sulfisoxazole	0.999	0.999	0.999
Metronidazole-HO	0.996	0.997	0.997
Metronidazole	0.999	0.999	0.999
Trimethoprim	0.997	0.999	0.999
Chloramphenicol	0.999	0.999	0.996

Figure S1. Photography of the tetracyclines Water-Scan plate with the nine wells, four of them showed inhibition zone (the three on the left and the one in the center), whereas the others did not show inhibition.

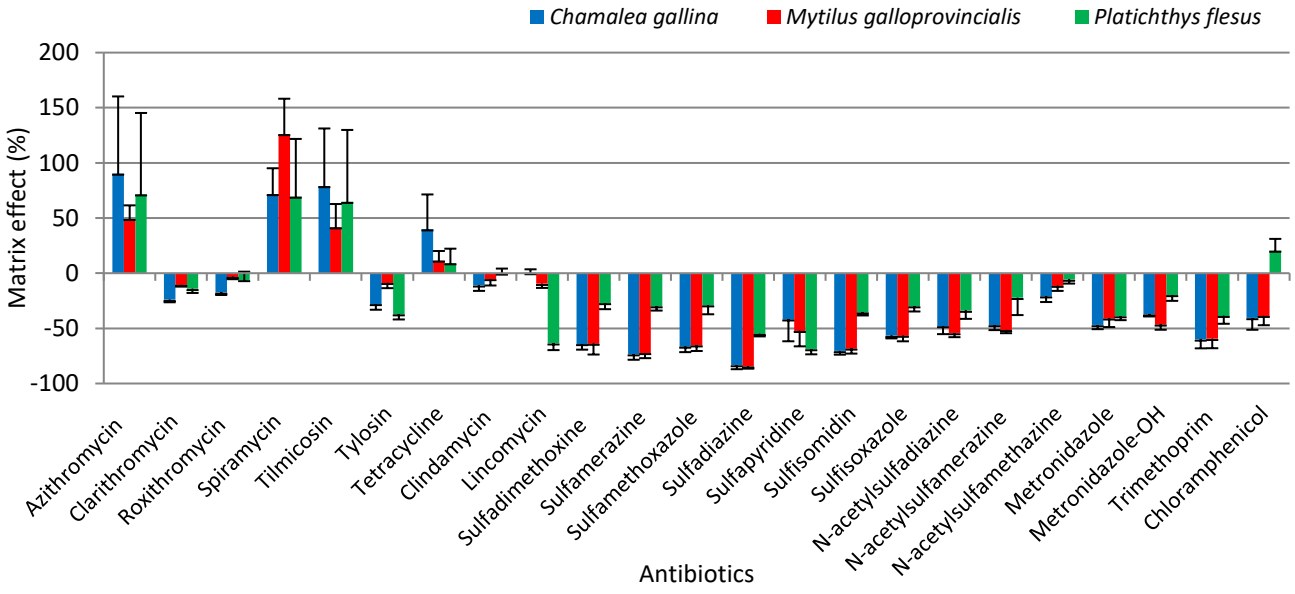

Figure S2. Matrix effect (%) expressed as ion enhancement (+) or supression (-) of the target compounds in clam (*C. gallina*), mussel (*M. galloprovincialis*) and fish (*P. flesus*).

Supporting Information

Combining an effect-based methodology with chemical analysis for antibiotics determination in wastewater and receiving freshwater and marine environment

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Table S1 List of antibiotics and their corresponding predicted-non-effect concentration (PNEC) based on literature review or software prediction.

Antibiotic family	**Antibiotics**	**Included in the microbial inhibition test**	**Ecotoxicological**	**Reference Ecotoxicological PNEC**	**PNEC-MIC**	**Reference PNEC-MIC**	**Final PNEC**
			PNEC (μg/L)		**(μg/L)**		**(μg/L)**
Tetracyclines	Oxytetracycline	Yes	0.31	(Ji et al., 2012)	0.5	(Bengtsson-Palme and Larsson, 2016; Tell et al., 2019)	0.31
	Chlortetracycline	Yes	5	(Ji et al., 2012)	N/A		5
	Tetracycline	Yes	5.4	ECOSAR	1		1
	Doxycycline	Yes	0.3	(Al Aukidy et al., 2014)	2		0.3
Quinolones	Ofloxacin	Yes	0.016	(Santos et al., 2013)	0.5		0.02
	Enrofloxacin	Yes	0.49	(Andrieu et al., 2015)	0.06		0.06
	Ciprofloxacin	Yes	0.05	(Andrieu et al., 2015)	0.06		0.05
	Norfloxacin	Yes	1.03	(Lindberg and Bjo, 2007)	0.5		0.5
	Pipemidic acid	No	7.1	(Choi et al., 2008)	N/A		7.1
	Marbofloxacin	No			N/A		
Macrolides	Tylosine	Yes	1	(Tell et al. 2019)	4		1
	Tilmicosin	Yes	0.52	ECOSAR	1		0.52
	Erythromycin	Yes	0.2	(Ji et al., 2012)	1		0.2
	Azithromycin	Yes	0.0094	(Grill et al., 2016)	0.5		0.01
	Spyramycin	Yes	0.5	(Tell et al. 2019)	0.5		0.5
	Clarithromycin	No	0.012	(Santos et al., 2013)	0.25		0.012
	Roxithromycin	No	4	(Verlicchi et al., 2012)	1		1
Sulfonamides	Sulfamethazine	Yes	4	(Verlicchi et al., 2012)	N/A		4
	Sulfadiazine	Yes	10.3	ECOSAR	N/A		10.33
	Sulfamethoxazole	Yes	0.0268	(Santos et al., 2013)	16		0.03
	Sulfapyridine	Yes	6.2	ECOSAR	N/A		6.2
	Sulfisoxazole	No	5	ECOSAR	N/A		N/A
Lincosamides	Lincomycin	No	0.81	(Tell et al. 2019)	2		0.81
	Clindamycin	No	0.1	(Tell et al. 2019)	1		0.1
Others	Trimethoprim	No	2.6	(Al Aukidy et al., 2014)	o.5		0.5
	Metronidazole	No	1.68	(Santos et al., 2013)	0.13		0.13
	Metronidazole OH	No	2.5	(Daouk et al., 2016)	N/A		2.5

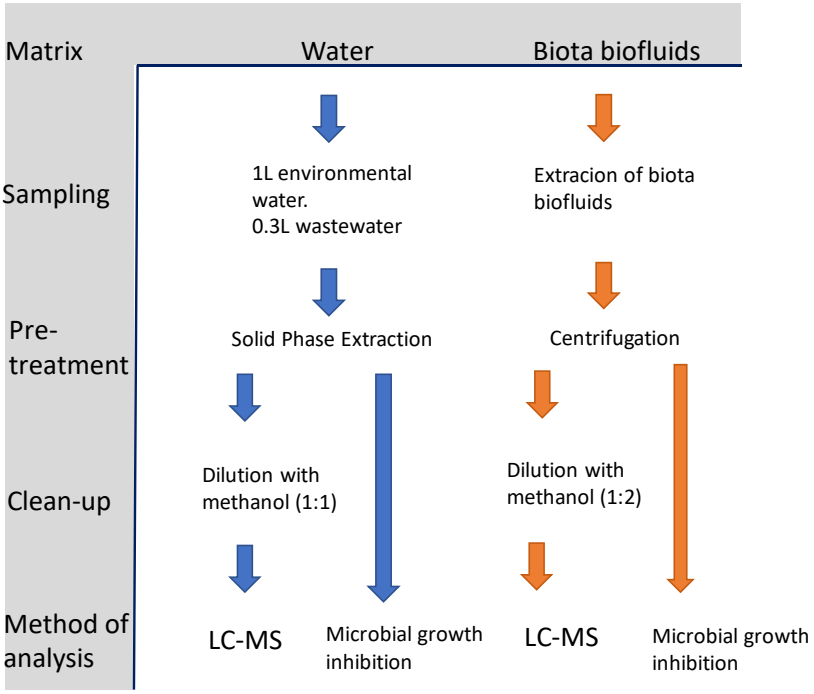

Figure S1. Scheme of the sample pre-treatment steps performed for each matrix and analytical method (microbial or chemical analysis).

Table S2 Analytical performance of the LC-MS/MS method. Method detection limit (MDL), method quantification limit (MQL) and recovery ± standard deviation (%) for the antibiotics analyzed in the different matrices.

Antibiotic family	Antibiotic	Biota biofluids					
		Fish plasma			Hemolymph (mussel and marine snail)		
		MDL (µg/L)	MQL (µg/L)	Recov (%)	MDL (µg/L)	MQL (µg/L)	Recov (%)
Tetracyclines	Oxytetracycline	0.41	1.36	33.8 ± 6.9	0.21	0.7	83.8 ± 6.8
	Chlortetracycline	0.03	0.11	35.5 ± 3.5	0.34	1.13	79.4 ± 0.3
	Tetracycline	0.59	1.98	43.2 ± 5.6	0.9	3	85.1 ± 5.3
	Doxycycline	2.07	6.91	40.1 ± 4.7	0.54	1.83	88.2 ± 3.4
Quinolones	Ofloxacin	0.02	0.05	35.2 ± 4.3	0.2	0.65	63.2 ± 6.1
	Enrofloxacin	1.46	4.86	78.7 ± 8.6	0.22	0.72	95.6 ± 6.4
	Ciprofloxacin	0.04	0.13	31.6 ± 0.8	0.03	0.09	72.5 ± 10.4
	Norfloxacin	0.51	1.69	66.0 ± 3.8	0.03	0.12	103.5 ± 20.3
	Pipemidic acid	0.13	0.43	31.8 ± 3.8	0.56	1.87	100.7 ± 5.2
	Marbofloxacin	0.01	0.04	31.6 ± 5.9	0.1	0.33	70.3 ± 5.1
Macrolides	Tylosine	nm	nm	nm	nm	nm	nm
	Tilmicosin	0.5	1.8	69.6 ± 4.8	0.22	0.75	68.1 ± 5.4
	Erythromycin	nm	nm	nm	nm	nm	nm
	Azithromycin	0.6	2	98.9 ± 5.1	0.23	0.75	65.4 ± 1.7
	Spyramycin	0.2	0.7	74.7 ± 4.6	0.31	0.95	75.2 ± 5.3
	Clarithromycin	0.06	0.19	102.8 ± 18.9	0.15	0.51	79.3 ± 0.3
	Roxithromycin	0.3	1.1	78.4 ± 3.8	0.51	1.79	71.3 ± 2.5
Sulfonamides	Sulfamethazine	0.02	0.06	67.7 ± 6.1	0.2	0.7	53.8 ± 1.8
	Sulfadiazine	0.07	0.23	34.19 ± 6.1	0.1	0.3	58.6 ± 2.1
	Sulfamethoxazole	0.04	0.13	30.2 ± 6.5	0	0.1	59.1 ± 4.0
	Sulfapyridine	0.02	0.06	105.2 ± 13.3	0.2	0.7	55.7 ± 3.4
	Sulfisoxazole	0.03	0.07	44.8 ± 4.1	0.1	0.3	50.6 ± 7.4
Lincosamides	Lincomycin	0.03	0.1	30.3 ± 2.5	0	0.1	112.8 ± 8.9
	Clindamycin	0	0.01	56.2 ± 9.6	0.2	0.7	61.1 ± 4.6
Others	Trimethoprim	0.01	0.03	36.18 ± 3.8	0	0.1	53.5 ± 7.9
	Metronidazole	0.01	0.04	45.32 ± 1.4	0	0.1	54.5 ± 3.3
	Metronidazole OH	0.03	0.1	37.6 ± 0.6	0	0.1	56.4 ± 0.2

Table S2 continuation

Antibiotic family	Antibiotic	Water					
		Freshwater			Seawater		
		MDL (µg/L)	MQL (µg/L)	Recov (%)	MDL (µg/L)	MQL (µg/L)	Recov (%)
Tetracyclines	Oxytetracycline	0.0003	0.0009	128.3 ± 17.9	0.001	0.0032	82.3 ± 8.9
	Chlortetracycline	0.0041	0.0138	99.9 ± 9.5	0.0036	0.0119	117.1 ± 1.7
	Tetracycline	0.002	0.0066	158.3 ± 13.4	0.0013	0.0043	118.7 ± 4.7
	Doxycycline	0.0001	0.0002	124.8 ± 10.5	0.0023	0.0075	124.0 ± 9.2
Quinolones	Ofloxacin	0.0012	0.004	95.0 ± 10.4	0.0032	0.0108	102.7 ± 18.8
	Enrofloxacin	0.0016	0.0055	118.5 ± 6.2	0.0016	0.0052	123.1 ± 12.7
	Ciprofloxacin	0.0001	0.0004	53.8 ± 2.9	0.001	0.0033	70.4 ± 9.6
	Norfloxacin	0.0012	0.004	138.9 ± 11.4	0.0028	0.009	94.1 ± 13.4
	Pipemidic acid	0.0007	0.0024	118.2 ± 8.7	0.0014	0.0045	115.2 ± 8.2
	Marbofloxacin	0.0012	0.004	78.7 ± 10.5	0.001	0.0032	114.3 ± 11.3
Macrolides	Tylosine	nm	nm	nm	nm	nm	nm
	Tilmicosin	0.002	0.0066	92.3 ± 5.8	0.005	0.0167	156.3 ± 6.7
	Erythromycin	nm	nm	nm	nm	nm	nm
	Azithromycin	0.0005	0.0015	127.3 ± 3.1	0.0012	0.0042	108.1 ± 9.0
	Spyramycin	0.003	0.0098	75.4 ± 2.3	0.0003	0.001	56.2 ± 2.0
	Clarithromycin	0.0007	0.0023	114.1 ± 8.9	0.0003	0.001	11.2 ± 2.4
	Roxithromycin	0.0004	0.0015	49.2 ± 6.7	0.0003	0.0009	34.9 ± 2.3
Sulfonamides	Sulfamethazine	0.0004	0.0013	123.1 ± 4.4	0.0003	0.0009	81.4 ± 10.4
	Sulfadiazine	0.0002	0.0007	82.2 ± 5.4	0.0002	0.0006	68.6 ± 17.4
	Sulfamethoxazole	0.0004	0.0013	62.3 ± 3.9	0.0003	0.0009	97.4 ± 21.4
	Sulfapyridine	0.0003	0.0009	89.2 ± 2.3	0.0001	0.0004	95.1 ± 4.1
	Sulfisoxazole	0.0003	0.0011	65.7 ± 7.4	0.0024	0.0078	55.3 ± 4.1
Lincosamides	Lincomycin	0.0005	0.0017	62.3 ± 5.4	0.0009	0.002	98.5 ± 10.1
	Clindamycin	0.0001	0.0002	125.8 ± 7.8	0.0003	0.0009	48.9 ± 5.3
Others	Trimethoprim	0.0008	0.0026	117.2 ± 14.4	0.0003	0.0011	136.1 ± 13.2
	Metronidazole	0.0005	0.0017	121.5 ± 7.5	0.0005	0.0016	88.9 ± 20.1
	Metronidazole OH	0.0005	0.0017	128.6 ± 5.2	0.0006	0.002	86.4 ± 3.1

Nm: not measured

Figure S2. detail of an inhibition plate with the 9 wells. The two upper left wells presenting an inhibition growth area.

Table S3. Microbial inhibition test validation in terms of accuracy and sensitivity for freshwater, seawater and mussel hemolymph.

Antibiotic family	Compound	Validation parameters					
		Freshwater		Seawater		Mussel hemolymph	
		Accuracy	Sensitivity	Accuracy	Sensitivity	Accuracy	Sensitivity
Tetracyclines	Oxytetracycline	100%	100%	100%	100%	100%	100%
	Chlortetracycline	100%	100%	100%	100%	100%	100%
	Tetracycline	100%	100%	100%	100%	100%	100%
	Doxycycline	100%	100%	100%	100%	100%	100%
Quinolones	Ofloxacin	100%	100%	100%	100%	100%	100%
	Enrofloxacin	100%	100%	100%	100%	100%	100%
	Ciprofloxacin	100%	100%	100%	100%	100%	100%
	Norfloxacin	100%	100%	100%	100%	100%	100%
Macrolides	Tylosine	100%	100%	97.5%	95%	100%	100%
	Tilmicosin	100%	100%	100%	100%	100%	100%
	Erythromycin	97.5%	95%	100%	100%	100%	100%
	Azithromycin	100%	100%	100%	100%	100%	100%
	Spiramycin	97.5%	95%	100%	100%	100%	100%
Sulfonamides	Sulfamethazine	100%	100%	100%	100%	100%	100%
	Sulfadiazine	100%	100%	97.5%	95%	97.5%	95%
	Sulfamethoxazole	100%	100%	100%	100%	100%	100%
	Sulfapyridine	100%	100%	100%	100%	100%	100%

Table S4. Inhibition area diameter (mm) in the microbial growth inhibition test of water samples. “High” indicates a high inhibition area with irregular shape; therefore, the diameter was not measured.

Water type	Sampling point	Macrolides	Tetracyclines	Quinolones	Sulfonamides
Wastewater	WWTP1 influent	High	High	30,5 ± 0,7	25,1 ± 0,02
	WWTP1 effluent	16,7 ± 0,2	<MDL	18,8 ± 0,9	19,9 ± 0,3
	WWTP2 influent	High	High	32,8 ± 3,9	27,2 ± 2,6
	WWTP2 effluent	21,7 ± 0,9	<MDL	19,3 ± 0,5	<MDL
Freshwater Ebro River	FW1	<MDL	19,8 ± 0,9	<MDL	17,8 ± 1,0
	FW2	<MDL	<MDL	<MDL	<MDL
	FW3	<MDL	17,6 ± 0,5	<MDL	<MDL
Seawater Mediterranean Sea	SW1	<MDL	<MDL	<MDL	<MDL
	SW2	<MDL	<MDL	<MDL	<MDL
	SW3	<MDL	<MDL	<MDL	<MDL
	SW4	<MDL	<MDL	<MDL	<MDL
	SW5	<MDL	<MDL	<MDL	<MDL

	SW6	<MDL	<MDL	<MDL	<MDL
	SW7	<MDL	<MDL	<MDL	<MDL
	SW8	<MDL	<MDL	<MDL	<MDL
Salt Water Mar Menor Lagoon	LW1	<MDL	<MDL	<MDL	<MDL
	LW2	<MDL	<MDL	<MDL	<MDL
	LW3	<MDL	<MDL	<MDL	<MDL
	LW4	<MDL	<MDL	<MDL	<MDL
	LW5	<MDL	<MDL	<MDL	<MDL
	LW6	<MDL	<MDL	<MDL	<MDL
	LW7	<MDL	<MDL	<MDL	<MDL
	LW8	<MDL	<MDL	<MDL	<MDL
	LW9	<MDL	<MDL	<MDL	<MDL

<MDL: below method detection limit

Table S5. Antibiotics quantification using SPE+LC-MS in Mediterranean seawater, Mar Menor Lagoon water, Ebro river freshwater and WWTP influent and effluent. Values in µg/L ± standard deviation (n=3). MDL (Method detection limit), MQL (method quantification limits)

Antibiotic family	Antibiotic	Waste Water				Mediterranean Sea							
		WWTP1 influent	WWTP1 effluent	WWTP2 influent	WWTP2 effluent	SW1	SW2	SW3	SW4	SW5	SW6	SW7	SW8
Tetracyclines	Tetracycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Doxycycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Chlortetracycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Oxytetracycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Quinolones	Ofloxacin	0.687 ± 0.07	0.137 ± 0.02	1.161 ± 0.05	0.155 ± 0.03	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Ciprofloxacin	2.062 ± 0.19	0.093 ± 0.04	5.875 ± 0.25	0.080 ± 0.03	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Enrofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Norfloxacin	0.387 ± 0.02	0.025 ± 0.004	0.497 ± 0.08	0.035 ± 0.009	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Marbofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Pipemidic acid	0.005 ± 0.0001	0.005 ± 0.002	0.266 ± 0.022	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Macrolides	Azithromycin	0.017 ± 0.002	0.128 ± 0.02	0.024 ± 0.02	0.304 ± 0.06	0.006 ± 0.002	<MDL	<MQL	0.005 ± 0.0009	<MDL	<MDL	<MDL	<MDL
	Clarithromycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Erythromycin	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
	Roxithromycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Spiramycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Tilmicosin	0.951 ± 0.22	<MDL	0.511 ± 0.06	<MDL	<MDL	<MQL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	tylosin	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
Sulfonamides	Sulfadimethoxine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfamethoxazol	0.378 ± 0.04	0.010 ± 0.002	0.165 ± 0.02	0.011 ± 0.003	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	sulfamethazine	<MDL	0.002 ± 0.0004	<MDL	<MDL	<MDL	0.004 ± 0.001	0.004 ± 0.002	0.005 ±	0.006 ± 0.001	0.004 ± 0.0006	0.005 ± 0.0002	0.005 ± 0.001

									0.00009				
	Sulfadiazine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfapyridine	0.221 ± 0.03	0.111 ± 0.09	0.949 ± 0.233	0.258 ± 0.03	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfisoxazole	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Lincosamides	Lincomycin	0.025 ± 0.004	0.003 ± 0.0001	<MDL	<MDL	0.003 ± 0.002	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Clindamycin	0.009 ± 0.001	0.003 ± 0.0002	0.021 ± 0.002	0.037 ± 0.002	<MQL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Others	Trimethoprim	0.256 ± 0.02	0.031 ± 0.0005	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Metronidazole	0.015 ± 0.0007	0.004 ± 0.0005	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Metronidazole OH	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL

Table S5 continuation

Antibiotic family	Antibiotic	Ebro river			Mar Menor Lagoon								
		FW1	FW2	FW3	LW1	LW2	LW3	LW4	LW5	LW6	LW7	LW8	LW9
Tetracyclines	Tetracycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Doxycycline	0.072 ± 0.02	<MDL	0.078 ± 0.006	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Chlortetracycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Oxytetracycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Quinolones	Ofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Ciprofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Enrofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Norfloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Marbofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Pipemidic acid	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL

Macrolides	Azithromycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Clarithromycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.012 ± 0.004	0.011 ± 0.005	0.006 ± 0.0001	
	Erythromycin	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
	Roxithromycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.013 ± 0.0003	<MDL	<MDL	0.014 ± 0.007	0.026 ± 0.04	
	Spiramycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Tilmicosin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	tylosin	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
Sulfonamides	Sulfadimethoxine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfamethoxazol	0.004 ± 0.002	0.004 ± 0.002	0.006 ± 0.0005	<MDL	0.011 ± 0.003	0.014 ± 0.006	0.016 ± 0.0008	0.014 ± 0.007	0.014 ± 0.0004	<MDL	<MDL	<MDL
	sulfamethazine	0.001 ± 0.0004	0.002 ± 0.0002	0.002 ± 0.001	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfadiazine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfapyridine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfisoxazole	<MDL	<MDL	<MDL	<MDL	<MQL	<MQL	<MQL	<MDL	<MDL	<MQL	<MDL	<MQL
Lincosamides	Lincomycin	0.012 ± 0.001	0.005 ± 0.001	0.003 ± 0.0006	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Clindamycin	0.0006 ± 0.00009	0.0004 ± 0.00007	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Others	Trimethoprim	0.007 ± 0.001	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Metronidazole	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Metronidazole OH	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL

Table S6. Microbial growth inhibition test results (inhibition area diameter) for biota biofluids analysis.

Sample site	Sample point	Organism	Macrolides	Tetracyclines	Quinolones	Sulfonamides
Ebro River	BF1	Fish	<MDL	<MDL	<MDL	<MDL
		Fish	<MDL	<MDL	<MDL	<MDL
		Fish	<MDL	<MDL	<MDL	24.33
		Fish	<MDL	<MDL	<MDL	21.32
		Fish	<MDL	<MDL	<MDL	<MDL
	BF2	Fish	<MDL	<MDL	<MDL	<MDL
		Fish	<MDL	<MDL	<MDL	<MDL
Mediterranean Sea	BF3	Fish	<MDL	<MDL	<MDL	<MDL
	BF4	Fish	<MDL	<MDL	<MDL	<MDL
	BM1	Mussel	<MDL	<MDL	<MDL	<MDL
	BM2	Mussel	<MDL	<MDL	<MDL	<MDL
	BM3	Mussel	<MDL	<MDL	<MDL	<MDL
	BM4	Mussel	<MDL	<MDL	<MDL	<MDL
Mar Menor Lagoon	BG1	Marine snail	<MDL	<MDL	<MDL	<MDL
	BG2	Marine snail	<MDL	<MDL	<MDL	<MDL
	BG3	Marine snail	<MDL	<MDL	<MDL	<MDL

<MDL: below method detection limit

Table S7. Antibiotics quantification in biota biofluids (fish plasma and mussel and gastropod hemolymph) using SPE+LC-MS. Values in µg/L ± standard deviation. MDL (Method detection limit), MQL (method quantification limits).

		Ebro river							Mediterranean Sea						Mar Menor Lagoon		
Antibiotic family	Antibiotic	BF1	BF1	BF1	BF1	BF1	BF2	BF2	BF3	BF4	BM1	BM2	BM3	BM4	LF1	LF2	LF3
		Fish	Fish	Fish	Fish	Fish	Fish	Fish	Fish	Fish	Mussel	Mussel	Mussel	Mussel	Gastropod	Gastropod	Gastropod
Tetracyclines	Tetracycline	2.008 ± 0.58	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Doxycycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Chlortetracycline	5.816 ± 2.52	3.615 ± 0.90	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Oxytetracycline	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Quinolones	Ofloxacin	0.056 ± 0.02	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Ciprofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Enrofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.203 ± 0.05	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Norfloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Marbofloxacin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.353 ± 0.19	<MDL	<MDL	0.435 ± 0.11	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Pipemidic acid	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Macrolides	Azithromycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Clarithromycin	1.960 ± 1.43	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Erythromycin	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
	Roxithromycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Spiramycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Tilmicosin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	tylosin	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
Sulfonamides	Sulfadimethoxine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfamethoxazol	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	sulfamethazine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL

	Sulfadiazine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfapyridine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Sulfisoxazole	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Lincosamides	Lincomycin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Clindamycin	0.246 ± 0.03	0.194 ± 0.04	0.293 ± 0.06	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Others	Trimethoprim	0.206 ± 0.03	<MDL	0.1668 ± 0.08	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Metronidazole	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	Metronidazole OH	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL

Nm: not measured

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SUPPORTING INFORMATION

Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*)

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Material and Methods

2.2 Experimental design

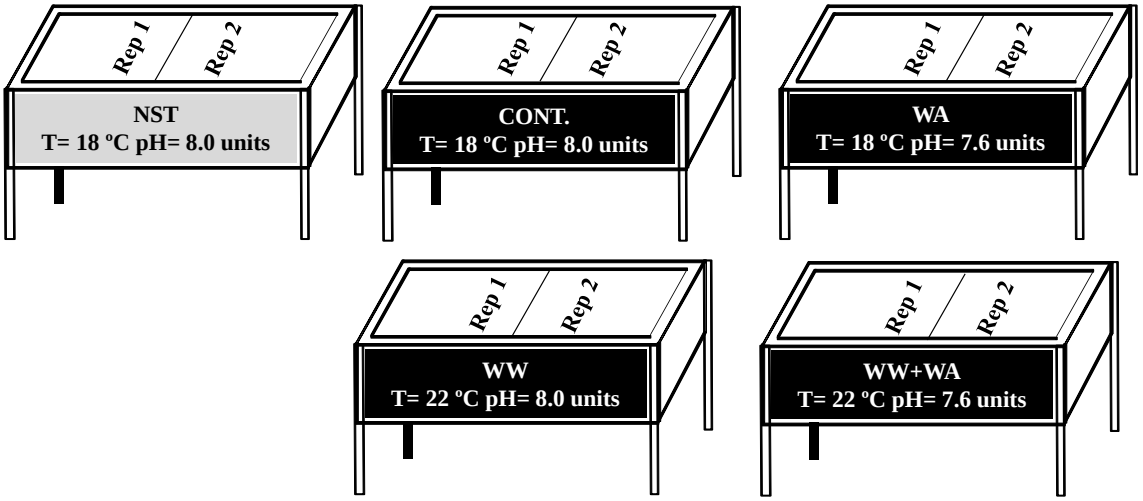

Figure S1 Schematic representation of the experimental design. NST (Non-spiked tanks); Cont. (Control); WA (water acidification); WW (water warming); WW+WA (warming plus acidification). Grey shadowed tanks were not spiked with the mixture of contaminants, whereas in the black shadowed ones the mixture of contaminants was added during the exposure phase.

Table S1 Seawater physical-chemical parameters (mean ± standard deviation) in each treatment. Abbreviations: NST – (non-spiked tanks); CONT - Control; WA – water acidification; WW – water warming; WW+WA - warming plus acidification; TA – total alkalinity; P CO_2 - partial CO_2 pressure; T CO_2 - Total CO_2 concentration; HCO_3^- - bicarbonate; CO_3^{2-} - carbonate ion concentrations; ΩCal - calcite saturation state; ΩAra – aragonite saturation state.

Treatment	**NST**	**CONT**	**WA**	**WW**	**WW+WA**
Water parameters					
Temperature (°C)	18.0 ± 0.2	18.0 ± 0.3	18.0 ± 0.1	22.0 ± 0.1	22.0 ± 0.2
pH	8.01 ± 0.02	8.01 ± 0.03	7.63 ± 0.02	8.02 ± 0.03	7.62 ± 0.02
TA (µmol kg^{-1})	2112.4 ± 111.1	2141.9 ± 100.2	2002.4 ± 120.1	2130.8 ± 100.3	1998.7 ± 90.9
***P* CO_2 (µatm)**	404.5 ± 7.9	410.0 ± 6.8	1090.1 ± 99.8	408.1 ± 5.2	1081.5 ± 80.4
***T* CO_2 (µmol kg^{-1})**	1889.2 ± 39.8	1907.0 ± 21.1	1927.5 ± 43.8	1906.7 ± 23.9	1932.3 ± 38.7
***HCO₃*⁻ (µmol kg^{-1})**	1719.3 ± 100.8	1728.8 ± 99.3	1822.7 ± 110.3	1735.5 ± 92.4	1830.8 ± 75.6
CO_3^{2-} (µmol kg^{-1})	156.7 ± 37.2	165.3 ± 23.4	71.1 ± 13.7	157.8 ± 28.9	66.3 ± 10.8
Ω Ara	2.4 ± 0.4	2.6 ± 0.2	1.1 ± 0.5	2.4 ± 0.3	1.1 ± 0.1
Ω Cal	3.7 ± 0.5	3.9 ± 0.2	1.7 ± 0.4	3.8 ± 0.2	1.6 ± 0.2

2.3 Sample pre-treatment and UPLC-MS/MS analysis

Biota samples were extracted following the procedure of Jakimska et al. (2013): 0.5 g of dry sample was placed in a polypropylene tube. Then, 5 mL of HPLC water were added and vortexed for 30 seconds, and 10 mL of acetonitrile 99.9% (ACN) were added with the subsequent vortex for 1 min. After the extraction salts addition (sodium acetate, 1.5 g and magnesium sulfate, 6.0 g) the mixture was hand shaked for 1 min. The ACN layer was transferred to a tube containing the dispersive sorbents (primary secondary amine (PSA) 149.9 mg; octadecyl (C18) 149.9 mg and magnesium sulphate 900.2 mg) in order to carry out dispersive solid phase extraction (dSPE). The sample was vortexed for 1 min and centrifuged 10 min at 5,000 rpm. Finally, 6 mL of the extract were evaporated to dryness, reconstituted with 1 ml of methanol 99.9% and kept at -20ºC until its analysis. Prior to UPLC-MS/MS analysis, samples were dried and reconstituted with 1 mL of MeOH:H_20 (10:90 v/v), finally they were filtered with PVDF filters 0.2 μm pore size.

Biota and water were analysed under the following conditions in the UPLC-MS/MS. UPLC-MS/MS conditions for pharmaceuticals analysis: sample extracts were analysed using an ultra-high pressure liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometry (UHPLC-QqLIT) following the method of Gros et al. (2012). The chromatographic separations were performed using a Water Acquity Ultra-Performance™ liquid chromatography system, equipped with two binary pumps (Milford, MA, USA), using an Acquity HSS T3 column (50 mm × 2.1 mm i.d., 1.8 μm particle size). The chromatographic separation conditions were: solvent (A) methanol 99.9%, solvent (B) 10 mM formic acid 98-100%/ammonium formate 99.0% (pH 3.2) at a flow rate of 0.5 mL/min. The gradient elution was: initial conditions 5% A; 0–4.5 min, 5–95% A; 4.5–4.6 min, 100% A; 4.6–6.0 min, 100% A; from 6.0 to 6.1 return to initial

conditions; 6.1–6.7, equilibration of the column. The sample volume injected was 5 μL. The UHPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. All compounds were analysed under positive electrospray ionization.

UPLC-MS/MS conditions for EDCs analysis: The same equipment was used following the method of Jakimska et al. (2013). The chromatographic separations were performed using an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) purchased from Waters Corporation. The optimized separation conditions were as follows: solvent (A) methanol 99.9% and (B) water (pH 9, adjusted with ammonia 99.0%) at a flow rate of 0.4 mL/min. The gradient elution was: 0–4 min, 30–100% A; 4–5 min, 100% A; 5–6 min return to initial conditions; 6–7.5 min, equilibration of the column. The column was maintained at 40ºC. The sample volume injected was 5 μL. All the compounds were analysed under negative electrospray ionization.

UPLC-MS/MS conditions for metabolites analysis: An adaptation of the Gros et al. (2012) method was used for the analysis of metabolites. The equipment used was the same as the two previous methods. The chromatographic separations were performed using a Hypersil Gold PFP column (10 mm × 2.1 mm id, 1.9 μm,) purchased from ThermoFisher Scientific Company; Villebon-France. The chromatographic separation conditions were: solvent (A) methanol 99.9%, solvent (B) 10 mM formic acid, 98-100%/ammonium formate 99.0% (pH 3.2) at a flow rate of 0.4 mL/min. The gradient elution was: initial condition,s 5% A; 0–4.5 min, 5–95% A; 4.5–4.6 min, 100% A; 4.6–6.0 min, 100% A; from 6.0 to 6.1 return to initial conditions; 6.1–8.0, equilibration of the column. The sample volume injected was 5 μL. All compounds were analysed under positive electrospray ionization.

Results

Table S2 Mean concentration measured (µg L^{-1}) in water samples along the time 2, 20, 20, 22, 30 and 40 days of exposure (D2,D10,D20,D22,D30 and D40) for each compound, (n=3) ± standard deviation (S.D.). NST means Non-spiked tanks; among the spiked treatments the meanings are: Cont. (Control); WW (warming); WA (acidification) (WW+WA) warming plus acidification. In addition, method detection limits (MDL) and method quantification limits (MQL) are presented for each compound.

Sample	Compound Concentration ± SD						
	Sotalol	Sulfamethoxazole	Venlafaxine	Citalopram	Carbamazepine	Methylparaben	Triclosan
NST D2	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
NST D10	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
NST D20	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
NST D22	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
NST D30	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
NST D40	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
D2 Cont.	7.6 ± 0.4	5.1 ± 0.5	9.2 ± 0.6	4.1 ± 0.4	11.9 ± 0.8	<MDL	<MQL
D10 Cont.	9.3 ± 0.3	12.0 ± 0.2	11.4 ± 0.1	5.3 ± 0.1	12.0 ± 0.2	<MDL	<MQL
D20 Cont.	9.7 ± 0.9	13.0 ± 0.8	10.3 ± 0.3	5.0 ± 0.2	12.4 ± 0.4	<MDL	<MQL
D22 Cont.	6.4 ± 0.3	8.0 ± 0.4	8.9 ± 0.5	3.8 ± 0.4	11.6 ± 0.5	<MDL	<MDL
D30 Cont.	2.1 ± 0.2	2.8 ± 0.3	5.2 ± 0.7	1.5 ± 0.2	8.6 ± 1.0	<MDL	<MDL
D40 Cont.	0.5 ± 0.2	1.1 ± 0.1	3.6 ± 0.2	0.8 ± 0.1	6.6 ± 0.3	<MDL	<MDL
D2 WA	9.2 ± 2.1	8.4 ± 1.5	14.2 ± 2.3	6.3 ± 1.0	15.7 ± 2.7	2.6 ± 0.4	<MQL
D10 WA	2.3 ± 0.3	2.4 ± 0.2	8.9 ± 0.5	4.8 ± 0.3	9.3 ± 0.5	<MDL	<MQL
D20 WA	4.6 ± 0.1	4.0 ± 0.2	12.1 ± 0.5	6.5 ± 0.4	13.5 ± 0.5	<MDL	<MQL
D22 WA	0.5 ± 0.1	<MDL	9.5 ± 0.6	5.0 ±0.3	10.7 ± 0.5	<MDL	<MDL
D30 WA	<MDL	<MDL	6.1 ± 0.3	2.4 ± 0.1	7.8 ± 0.3	<MDL	<MDL
D40 WA	<MDL	<MDL	3.3 ± 0.1	1.0 ± 0.1	4.6 ± 0.1	<MDL	<MDL
D2 WW	8.1 ± 0.5	5.6 ± 0.3	10.4 ± 0.4	3.8 ± 0.1	12.3 ± 0.3	0.2 ± 0.1	<MQL
D10 WW	1.9 ± 0.1	2.7 ± 0.2	11.5 ± 0.5	5.1 ± 0.2	12.4 ± 0.5	<MDL	<MQL
D20 WW	1.8 ± 0.1	3.3 ± 0.1	10.4 ± 0.5	5.0 ± 0.4	12.3 ± 0.4	<MDL	<MDL
D22 WW	<MQL	0.3 ± 0.1	9.2 ± 0.3	3.9 ± 0.3	11.6 ± 0.5	<MDL	<MDL
D30 WW	<MDL	<MDL	5.1 ± 0.3	1.9 ± 0.2	7.9 ± 0.5	<MDL	<MDL
D40 WW	<MDL	<MDL	2.0 ± 0.1	0.6 ± 0.1	3.7 ± 0.2	<MDL	<MDL
D2 WW+WA	7.6 ± 1.2	3.8 ± 0.4	11.2 ± 1.0	4.5 ± 0.4	13.0 ± 0.9	0.2 ± 0.1	<MDL
D10 WW+WA	1.3 ± 0.1	1.0 ± 0.1	8.0 ± 0.5	4.6 ± 0.3	8.2 ± 0.4	<MDL	<MQL
D20 WW+WA	3.6 ± 0.2	3.1 ± 0.2	10.4 ± 0.1	5.6 ± 0.3	11.7 ± 0.2	<MDL	<MDL
D22 WW+WA	0.5 ± 0.1	0.3 ± 0.1	10.9 ± 1.6	5.3 ± 0.7	12.4 ± 1.5	<MDL	<MDL
D30 WW+WA	<MDL	<MDL	6.1 ± 1.0	1.7 ± 0.3	8.5 ± 1.2	<MDL	<MDL
D40 WW+WA	<MDL	<MDL	2.7 ± 0.3	0.5 ± 0.1	4.5 ± 0.5	<MDL	<MDL
MDL (µg L^{-1})	0.02	0.06	0.06	0.01	0.06	0.05	0.94
MQL (µg L^{-1})	0.07	0.21	0.21	0.04	0.21	0.15	3.13

Table S3 Mean concentration measured (µg kg^{-1} dw) in biota sample along the time after 2, 20, 20, 22, 30 and 40 days of exposure (D2,D10,D20,D22,D30 and D40) for each compound, (n=4) ± standard deviation (S.D.). NST means Non-spiked tanks; among the spiked treatments the meanings are: Cont. (Control); WW (warming); WA (acidification) (WW+WA) warming plus acidification. In addition, recovery in percentage, method detection limits (MDL) and method quantification limits (MQL) are presented for each compound.

Sample	Compound Concentration ± SD						
	Sotalol	Sulfamethoxazole	Venlafaxine	Citalopram	Carbamazepine	Triclosan	Methylparaben
NST 0	<MDL	<MDL	<MQL	<MQL	<MQL	<MQL	0.5 ± 0.4
NST D2	<MDL	<MDL	<MDL	<MQL	<MQL	<MDL	7.8 ± 0.1
NST D10	<MDL	<MDL	<MDL	3.1 ± 0.2	<MQL	<MDL	3.3 ± 0.9
NST D20	<MDL	<MDL	<MQL	3.7 ± 0.3	<MQL	<MDL	2.8 ± 0.2
NST D22	<MDL	<MDL	<MQL	2.5 ± 0.1	<MQL	<MDL	2.2 ± 0.1
NST D30	<MDL	<MDL	<MDL	4.5 ± 1.2	<MQL	<MDL	1.6 ± 0.4
NST D40	<MDL	<MDL	<MQL	2.4 ± 0.3	<MQL	<MDL	4.4 ± 0.6
D2 Cont.	78.8 ± 3.2	77.5 ± 7.4	3912.0 ± 315.5	4499.6 ± 1384.6	377.3 ± 34.3	861.9 ± 187.7	4.7 ± 0.2
D10 Cont.	106.5 ± 7.0	58.2 ± 3.7	3095.0 ± 74.1	7197.8 ± 302.3	343.7 ± 13.7	484.4 ± 43.0	3.6 ± 0.2
D20 Cont.	182.6 ± 2.1	81.3 ± 10.2	5419.5 ± 209.7	12889.4 ± 2132.4	346.7 ± 17.3	538.8 ± 8.9	3.6 ± 0.4
D22 Cont.	134.9 ± 12.2	52.1 ± 2.6	2264.8 ± 9.4	6507.8 ± 435.7	299.9 ± 28.3	85.4 ±12.3	4.5 ± 1.2
D30 Cont.	129.5 ± 5.2	20.1 ± 3.6	3916.9 ± 69.5	7807.0 ± 465.8	242.9 ± 21.6	69.0 ± 2.6	2.3 ± 0.1
D40 Cont.	91.7 ± 2.8	12.2 ± 5.9	2256.2 ± 173.2	3591.0 ± 273.8	169.4 ± 9.5	40.3 ± 10.7	2.4 ± 0.3
D2 WA	51.0 ± 8.0	54.1 ± 3.5	2183.4 ± 56.0	3307.8 ± 386.9	392.4 ± 3.6	405.7± 403.7	45.4 ± 1.1
D10 WA	81.4 ± 7.9	26.7 ± 3.6	3752.3 ± 334.2	7807.2 ± 1005.5	338.7 ± 3.3	472.4 ± 15.6	16.0 ± 1.3
D20 WA	105.5 ± 0.9	30.7 ± 3.5	2735.5 ± 42.1	7073.1 ± 583.1	348.2 ± 18.0	291.2 ± 17.2	9.1 ± 0.8
D22 WA	76.5 ± 3.9	6.1 ± 3.3	1297.5 ± 137.5	3768.3 ± 117.5	272.4 ± 26.4	79.3 ± 14.8	8.2 ± 0.5
D30 WA	55.2 ± 2.7	3.7 ± 3.5	1013.6 ± 95.6	2324.9 ± 151.4	201.6 ± 11.7	48.0 ± 3.4	1.9 ± 0.1
D40 WA	41.8 ± 1.0	5.4 ± 5.0	742.7 ± 13.0	1601.0 ± 21.0	109.0 ± 1.4	81.0 ± 10.8	2.8 ± 0.2
D2 WW	83.6 ± 4.3	68.5 ± 5.9	3295.9 ± 229.8	5843.3 ± 509.9	453.2 ± 10.9	1106.4 ± 44.7	3.5 ± 0.4
D10 WW	115.5 ± 3.5	21.1 ± 0.9	4181.2 ± 102.3	8396.1 ± 62.2	387.1 ± 37.8	333.5 ± 20.4	21.0 ± 1.1
D20 WW	109.0 ± 4.4	25.8 ± 2.0	4277.3 ± 263.1	8885.2 ± 386.5	432.5 ± 6.7	364.5 ± 58.8	3.6 ± 0.7
D22 WW	86.0 ± 2.5	6.1 ± 3.7	3699.9 ± 194.2	8216.3 ± 106.8	275.4 ± 11.0	149.3 ± 41.8	3.2 ± 0.2
D30 WW	60.9 ± 2.0	11.0 ± 4.4	2458.9 ± 144.8	4947.3 ± 217.2	200.4 ± 6.3	87.9 ± 13.5	2.6 ± 0.3
D40 WW	34.3 ± 1.0	4.6 ± 3.6	1393.4 ± 67.9	2616.6 ± 97.3	98.2 ± 5.6	85.1 ± 4.3	3.1 ± 0.5
D2 WW+WA	70.7 ± 4.0	53.8 ± 15.2	2316.4 ± 188.7	3725.1 ± 283.8	391.8 ± 14.2	676.2 ± 38.6	2.7 ± 0.3
D10 WW+WA	105.7 ± 7.1	23.5 ± 2.1	2376.8 ± 168.2	5917.0 ± 422.5	328.3 ± 8.5	708.4 ± 76.2	1.9 ± 0.3
D20 WW+WA	91.0 ± 0.7	28.3 ± 1.3	2214.0 ± 220.9	5526.1 ± 696.9	298.3 ± 9.6	296.8 ± 4.6	1.8 ± 0.1
D22 WW+WA	60.7 ± 0.5	5.5 ± 4.0	1681.0 ± 82.9	4290.0 ± 427.0	280.6 ± 14.3	70.8 ± 4.7	9.0 ± 2.8
D30 WW+WA	40.6 ± 2.9	4.3 ± 3.8	1436.3 ± 65.8	2518.1 ± 52.9	199.7 ± 11.1	63.5 ± 10.6	2.0 ± 0.2
D40 WW+WA	31.6 ± 0.4	4.7 ± 4.0	798.0 ± 62.5	879.8 ± 38.3	108.3 ± 3.9	90.2 ± 6.5	2.7 ± 0.4
MDL (µg kg^{-1})	0.02	0.02	0.30	0.52	0.13	3.56	0.03
MQL (µg kg^{-1})	0.08	0.07	1.00	1.74	0.43	11.87	0.12
Recovery (%)	65.28	17.92	76.76	77.71	80.05	165.26	43.41

Table S4 Mean concentration measured (µg kg^{-1} dw) of venlafaxine and its metabolites metabolites in each biota sample along the time (after 2, 20, 20, 22, 30 and 40 days of exposure): (n=4) ± standard deviation (S.D.). Cont. (Control); WW (warming); WA (acidification) (WW+WA) warming plus acidification. In addition, recovery in percentage, method detection limits (MDL) and method quantification limits (MQL) are presented for each compound.

Sample	**Compound Concentration ± SD**			
	O-desmethylVLF	**N-desmethylVLF**	**NO-didesmethylVLF**	**Venlafaxine**
D2 Cont.	21.2 ± 4.9	13.3 ± 3.1	<MDL	3912.0 ± 315.5
D10 Cont.	15.2 ± 2.6	13.9 ± 3.4	<MDL	3095.0 ± 74.1
D20 Cont.	55.7 ± 12.0	70.9 ± 14.7	5.4 ± 1.2	5419.5 ± 209.7
D22 Cont.	22.0 ± 4.1	51.3 ± 8.2	3.3 ± 0.3	2264.8 ± 9.4
D30 Cont.	31.2 ± 5.4	221.6 ± 18.1	6.3 ± 0.2	3916.9 ± 69.5
D40 Cont.	30.5 ± 3.3	276.8 ± 51.7	7.8 ± 0.2	2256.2 ± 173.2
D2 WA	9.9 ± 1.9	8.2 ± 2.1	<MDL	2183.4 ± 56.0
D10 WA	19.6 ± 4.1	7.9 ± 2.8	<MDL	3752.3 ± 334.2
D20 WA	20.6 ± 3.7	15.6 ± 4.7	<MDL	2735.5 ± 42.1
D22 WA	18.4 ± 3.2	17.9 ± 5.2	<MDL	1297.5 ± 137.5
D30 WA	10.4 ± 1.8	29.7 ± 5.4	2.1 ± 0.2	1013.6 ± 95.6
D40 WA	8.5 ± 0.8	35.9 ± 7.5	3.8 ± 0.4	742.7 ± 13.0
D2 WW	15.3 ± 3.2	8.4 ± 2.6	<MDL	3295.9 ± 229.8
D10 WW	14.2 ± 8.0	19.0 ± 4.5	<MDL	4181.2 ± 102.3
D20 WW	34.5 ± 10.0	57.3 ± 25.1	<MDL	4277.3 ± 263.1
D22 WW	50.7 ± 3.0	105.4 ± 21.4	5.4 ± 0.4	3699.9 ± 194.2
D30 WW	36.2 ± 3.1	196.6 ± 18.0	8.3 ± 0.2	2458.9 ± 144.8
D40 WW	20.1 ± 6.9	112.9 ± 19.3	7.5 ± 0.6	1393.4 ± 67.9
D2 WW+WA	5.0 ± 2.2	8.6 ± 2.4	<MDL	2316.4 ± 188.7
D10 WW+WA	7.3 ± 1.7	11.7 ± 2.1	<MDL	2376.8 ± 168.2
D20 WW+WA	8.3 ± 1.9	17.7 ± 4.5	<MDL	2214.0 ± 220.9
D22 WW+WA	12.1 ± 1.5	42.8 ± 8.2	<MDL	1681.0 ± 82.9
D30 WW+WA	8.8 ± 2.3	57.8 ± 5.4	2.1 ± 0.2	1436.3 ± 65.8
D40 WW+WA	4.8 ± 0.6	60.9 ± 12.3	2.3 ± 0.1	798.0 ± 62.5
MDL (µg kg^{-1})	0.08	0.09	0.06	0.30
MQL (µg kg^{-1})	0.26	0.29	0.21	1.00
Recovery (%)	79.6	34.79	63.8	76.76

Table S5 Statistical analysis between the different treatments (cont, control; WA, water acidification; WW, water warming; WW+WA, water warming plus acidification) along the experiment days. Different letters indicate statistical significant differences, using one way ANOVA test or Kruskal-Wallis test followed by a Post-Hoc. (-) indicates that no statistical analysis was performed as the compound was detected <MQL. In addition, the p-value for each sampling time is shown.

O-desmethylVLF	Cont.	WA	WW	WW+WA	p-value
D2	c	ab	bc	a	<0.001
D10	a	a	a	a	0.070
D20	c	ab	b	a	<0.001
D22	b	ab	c	a	<0.001
D30	b	a	b	a	<0.001
D40	c	a	b	a	<0.001
N-desmethylVLF	Cont.	WA	WW	WW+WA	
D2	a	a	a	a	0.101
D10	ab	a	b	ab	0.014
D20	b	a	b	a	<0.001
D22	a	b	c	a	0.006
D30	b	a	b	a	<0.001
D40	b	a	c	d	0.004
NO-didesmethylVLF	Cont.	WA	WW	WW+WA	
D2	-	-	-	-	
D10	-	-	-	-	
D20	-	-	-	-	
D22	-	-	-	-	
D30	b	a	c	a	<0.001
D40	c	b	c	a	<0.001

Table S6 Mean concentration measured (μg L^{-1}) of venlafaxine and its metabolites in each water sample along the time (after2, 20, 20, 22, 30 and 40 days of exposure): (n=3) ± standard deviation (S.D.). Cont. (Control); WW (warming); WA (acidification) (WW+WA) warming plus acidification. In addition, method detection limits (MDL) and method quantification limits (MQL) are presented for each compound.

Sample	**Compound Concentration ± SD**			
	O-desmethylVLF	**N-desmethylVLF**	**NO-didesmethylVLF**	**Venlafaxine**
D2 Cont.	<MDL	<MDL	<MDL	9.2 ± 0.6
D10 Cont.	0.20 ± 0.002	<MQL	<MDL	11.4 ± 0.1
D20 Cont.	0.15 ± 0.004	0.34 ± 0.01	<MDL	10.3 ± 0.3
D22 Cont.	0.24 ± 0.005	0.63 ± 0.003	<MDL	8.9 ± 0.5
D30 Cont.	0.28 ± 0.008	0.65 ± 0.01	<MDL	5.2 ± 0.7
D40 Cont.	0.23 ± 0.004	0.46 ± 0.03	<MDL	3.6 ± 0.2
D2 WA	<MDL	<MDL	<MDL	14.2 ± 2.3
D10 WA	<MDL	<MDL	<MDL	8.9 ± 0.5
D20 WA	0.14 ± 0.01	<MQL	<MDL	12.1 ± 0.5
D22 WA	0.18 ± 0.008	0.29 ± 0.008	<MDL	9.5 ± 0.6
D30 WA	0.18 ± 0.005	0.29 ± 0.01	<MDL	6.1 ± 0.3
D40 WA	0.11 ± 0.003	<MQL	<MDL	3.3 ± 0.1
D2 WW	<MDL	<MDL	<MDL	10.4 ± 0.4
D10 WW	<MDL	<MDL	<MDL	11.5 ± 0.5
D20 WW	0.32 ± 0.004	0.26 ± 0.004	<MDL	10.4 ± 0.5
D22 WW	0.31 ± 0.10	0.48 ± 0.03	<MDL	9.2 ± 0.3
D30 WW	0.27 ± 0.01	0.48 ± 0.03	<MDL	5.1 ± 0.3

D40 WW	0.14 ± 0.002	0.38 ± 0.01	<MDL	2.0 ± 0.1
D2 WW+WA	<MDL	<MDL	<MDL	11.2 ± 1.0
D10 WW+WA	<MDL	<MQL	<MDL	8.0 ± 0.5
D20 WW+WA	0.16 ± 0.007	<MQL	<MDL	10.4 ± 0.1
D22 WW+WA	0.18 ± 0.007	0.38 ± 0.01	<MDL	10.9 ± 1.6
D30 WW+WA	0.15 ± 0.005	0.45 ± 0.01	<MDL	6.1 ± 1.0
D40 WW+WA	0.13 ± 0.002	0.45 ± 0.01	<MDL	2.7 ± 0.3
MDL (µg L^{-1})	0.02	0.07	0.05	0.06
MQL (µg L^{-1})	0.06	0.23	0.19	0.21

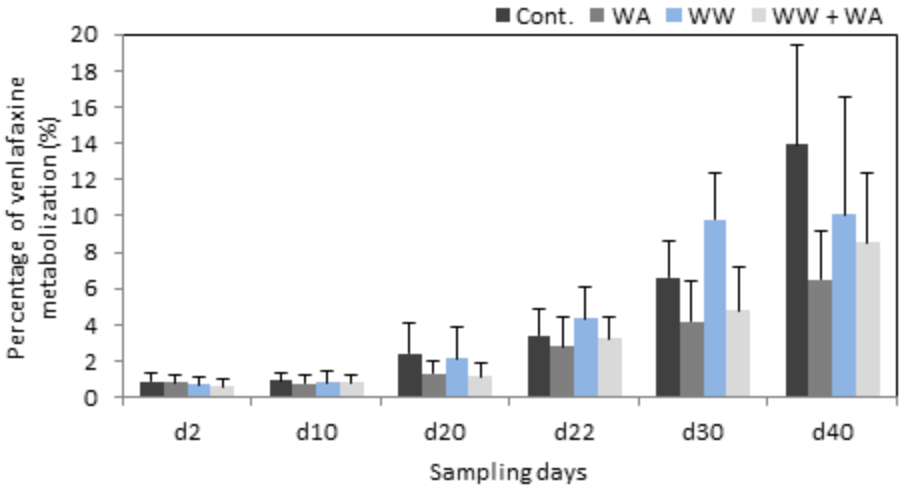

Figure S2 Percentage of venlafaxine metabolization, calculated by dividing the concentration of the sum of all venlafaxine metabolites (N-desmethylvenlafaxine, O-desmethylvenlafaxine and NO-didesmethylvenlafaxine) with the concentrations parent compound (venlafaxine) in biota and in each sampling time and treatment (Cont., Control; WA, acidification; WW, warming; WW+WA, warming plus acidification).

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Supporting information

Comprehensive study of sulfamethoxazole effects in marine mussels: bioconcentration, enzymatic activities and metabolomics

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Material and Methods

Target analysis of SMX and its metabolites in mussel's soft tissue, haemolymph and seawater

SMX analysis in mussel's soft tissue was done following the method of Álvarez-Munoz et al. 2015; 0.5 g of dry tissue were weighted. SMX was extracted using pressurized liquid extraction (PLE). Dry tissue was placed in a 22 mL cell containing 2 g of neutral aluminium oxide, hydromatrix and a glass fiber filter in the top and in the bottom of the cell. PLE conditions were, oven temperature 50 C, pressure 1500 psi, 5 min heat up time, 3 static cycles and 5 min static time. The extraction solvent was methanol/water (1:2, v/v) and the extraction volume was 30 mL. After extraction, extracts were dried down under nitrogen up to 1/3 of its initial volume (to remove the organic solvent). The samples were dissolved up to 200 mL with Milli Q water and 6 mL of Na2EDTA were added. Then a clean-up was done using solid phase extraction (SPE). OASIS HLB (200 mg, 60 mL) cartridges were conditioned with 6 mL of methanol followed by 6 mL of water. After sample loading, cartridges were rinsed with 6 mL of water and dried with air. Elution was done with 6 mL of methanol. Finally, the extracts were evaporated to dryness and re-dissolved in 1 mL of methanol/water (10:90, v/v) prior instrumental analysis. Mussel's haemolymph was extracted from the mussel's adductor muscle and diluted with an anticoagulant (Alsever's solution) 1:1, then, it was snap frozen by immersion in liquid nitrogen and kept at -80 ºC until its analysis; whereas seawater was directly analysed in the instrument.

Mussel extracts, mussel haemolymph and seawater were analyzed by ultra-high performance liquid chromatography coupled to quadrupole linear ion trap tandem mass spectrometry (UPLC–QqLIT). The chromatographic separations were carried out using an Acquity HSS T3 column (50 mm×2.1 mm i.d., 1.8 µm particle size). The chromatographic separation conditions were methanol (A) and 10 mM of formic acid/ammonium formate at pH 3.2 (B) at a flow rate of 0.5 mL/min. The sample volume injected was 5 µL. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole–linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. SMX and its related metabolites were analyzed under positive mode ionization.

Table S1. Method detection limits (MDL) and method quantification limits (MQL) for SMX and its target metabolites.

Compound	Mussel soft tissue			Haemolymph		Seawater	
	MDL ng/g	MQL ng/g	Recovery %	MDL µg/L	MQL µg/L	MDL µg/L	MQL µg/L
Sulfamethoxazole	0.05	0.16	48.22	0.001	0.004	0.04	0.13
N-acetyl-sulfamethoxazole	0.11	0.38	100.23	0.01	0.02	0.02	0.05
Desamino-sulfamethoxazole	0.66	2.21	113.21	0.04	0.14	0.03	0.11
Glucoronide-SMX	0.07	0.24	24.31	0.03	0.10	0.02	0.08

High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (HPLC-HRMS) analysis

Ten individuals were sampled for each treatment after 96 h of exposure for metabolomics and SMX related metabolites analysis. Haemolymph samples were pre-treated as explained for the bioaccumulation analysis (section 2.3) and processed following a workflow for the non-target metabolomics analysis and the suspect screening analysis for SMX related metabolites (figure 1). The analysis of mussel's haemolymph samples was performed by High-Performance Liquid chromatography–High-Resolution Mass Spectrometry (HPLC-HRMS) using a LC-LTQ-OrbitrapVelosTM from Thermo Fisher Scientific, equipped with electrospray ionization (ESI) operating both in positive and negative mode. Haemolymph (10 µl) were separated using a Thermo Hypersil GOLD PFP column (100 mm x 2.1 mm, 1.9 µm particle size). Mobile phase solvents were acetonitrile (A) and water (B) in negative ionization mode, and acetonitrile (A) 0.1% formic acid in water (B) in positive mode. The flow rate was 0.5 mL/min. The linear gradient for positive and negative ionization mode was: 0-1 min, 5% A; 1-16 min 5-100% A; 16-18 min 100% A; at 18 min return to initial conditions 5% A, and 22-24 min equilibration of the column. The samples were acquired using full scan within a mass-to-charge (m/z) range of 50 to 700 m/z at a resolving power of 60,000 FWHM. For positive mode, the ionization voltage was set at 3.5 KV with the sheath gas flow at 40, auxiliary gas flow at 20, S-Lens RF level at 69%, the capillary temperature 350 ºC and the source heater temperature at 300ºC. For negative mode; the ionization voltage was set at 3.0 KV with the sheath gas flow at 35, auxiliary gas flow at 10, S-Lens RF level at 60% and the capillary temperature and the source heater temperature at 450 ºC.

For the suspect screening approach, used for the detection of SMX related metabolites; a list of suspected SMX related metabolites (table S2) was built based on databases research (https://www.drugbank.ca) and prediction tools for SMX degradation under biological

processes (https://envipath.org). These SMX related metabolites were searched in the chromatogram generated in the LC-LTQ-Orbitrap (figure 1).

For the metabolomics approach, composite samples were used to monitor LC-LTQ-Orbitrap performance during batch analyses. Data files generated in the Orbitrap were processed using the Thermo Scientific SIEVE 2.0 which was used for background subtraction, component detection, peak alignment and differential analysis; i.e. the evaluation of metabolites changes between control and exposed treatments. SIEVE 2.0 conditions were the following: selected mass-to-charge range was 50 – 700 Da, from 0.1 to 20 min, with m/z width of 5 ppm. The intensity threshold was set at 100,000; the minimum scans across a peak were 5, the signal-to-noise ratio at 10 and the m/z step size at 5.0. PCA was used for the profiling of the metabolome by using all the detected features as variables (after excluding SMX and related metabolites when detected) and for summer and winter trials separately. After, the significance of the differences between control and SMX exposed mussels in each metabolite was investigated by assessing a p-value with T-test using SIEVE software with a significance level of $\text{p-value} \leq 0.05$. The identity of discriminatory metabolites was determined from their accurate mass by searching in open source databases such as Human Metabolome database, Pubchem, Chemspider and METLIN. For compound identities confirmation a second injection of the samples in the LC-LTQ-Orbitrap was done using data dependent analysis with a list of the exact masses from those metabolites which databases reported a putative identity. Collision induced dissociation was applied at different collision energies (20 eV, 30 eV and 35 eV), the resulting fragments were identified with a mass error below 5 ppm and helped to manually confirm structural elucidation of suspects.

Table S2. List of suspected SMX related metabolites. Precursor masses for the protonated or deprotonated SMX related metabolites were specified as target masses. Sources: https://www.drugbank.ca and https://envipath.org.

Common name	**IUPAC identification**	**Formula**	**Molecular weight**	**Reference**
5-Hydroxy-sulfamethoxazole	4-amino-N-[5-(hydroxymethyl)-1,2-oxazol-3-yl]benzene-1-sulfonamide	$C_{10}H_{11}N_3O_4S$	269.04702	drugbank
4-nitroso-sulfamethoxazole	N-(5-methyl-1,2-oxazol-3-yl)-4-nitrosobenzene-1-sulfonamide	$C_{10}H_9N_3O_4S$	267.03137	drugbank
Sulfamethoxazole GSH conjugate	(2S)-2-amino-4-{[(1R)-1-[(carboxymethyl)carbamoyl]-2-{[({4-[(5-methyl-1,2-oxazol-3-yl)sulfamoyl]phenyl}amino)sulfinyl]sulfanyl}ethyl]carbamoyl}butanoic	$C_{20}H_{26}N_6O_{10}S_3$	606.08725	drugbank

	acid			
N/A	4-amino-2,3-dihydroxy-N-(5-methyl-1,2-oxazol-3-yl)benzene-1-sulfonamide	$C_{10}H_{11}N_3O_5S$	285.04194	EnviPath
N/A	2,3,4,5-tetrahydroxy-N-(5-methyl-1,2-oxazol-3-yl)benzene-1-sulfonamide	$C_{10}H_{10}N_2O_7S$	302.02087	EnviPath
N/A	4-amino-2,3,6-trihydroxy-N-(5-methyl-1,2-oxazol-3-yl)benzene-1-sulfonamide	$C_{10}H_{11}N_3O_6S$	301.03685	EnviPath
N/A	2,3,4-trihydroxy-N-(5-methyl-1,2-oxazol-3-yl)benzene-1-sulfonamide	$C_{10}H_{10}N_2O_6S$	286.02595	EnviPath
N/A	5-[(5-methyl-1,2-oxazol-3-yl)sulfamoyl]-2-oxopent-4-enoate	$C_9H_9N_2O_6S$	273.01868	EnviPath
N/A	3,4-dihydroxy-N-(5-methyl-1,2-oxazol-3-yl)benzene-1-sulfonamide	$C_{10}H_{10}N_2O_5S$	270.03104	EnviPath
N/A	4-aminobenzene-1-sulfonate	$C_6H_6NO_3S$	172.00738	EnviPath
N/A	5-methyl-1,2-oxazol-3-amine	$C_4H_6N_2O$	98.048012	EnviPath
N/A	4-amino-2,3-dihydroxybenzene-1-sulfonate	$C_6H_6NO_5S$	203.99721	EnviPath
N/A	(3-amino-1,2-oxazol-5-yl)methanol	$C_4H_6N_2O_2$	114.04292	EnviPath

N/A, not available.

Results

SMX occurrence in water, mussel haemolymph and tissue

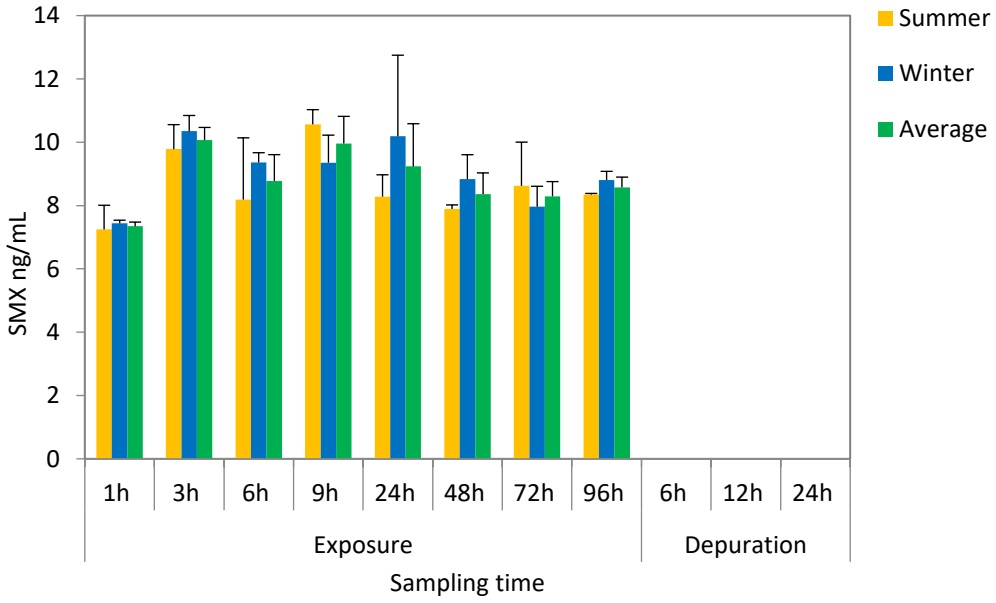

Figure S1. SMX concentration in seawater along the exposure and depuration sampling times. Concentrations for summer (yellow bars), winter (blue bars) and the mean of summer and winter (grey bars) are presented as well as the standard deviation.

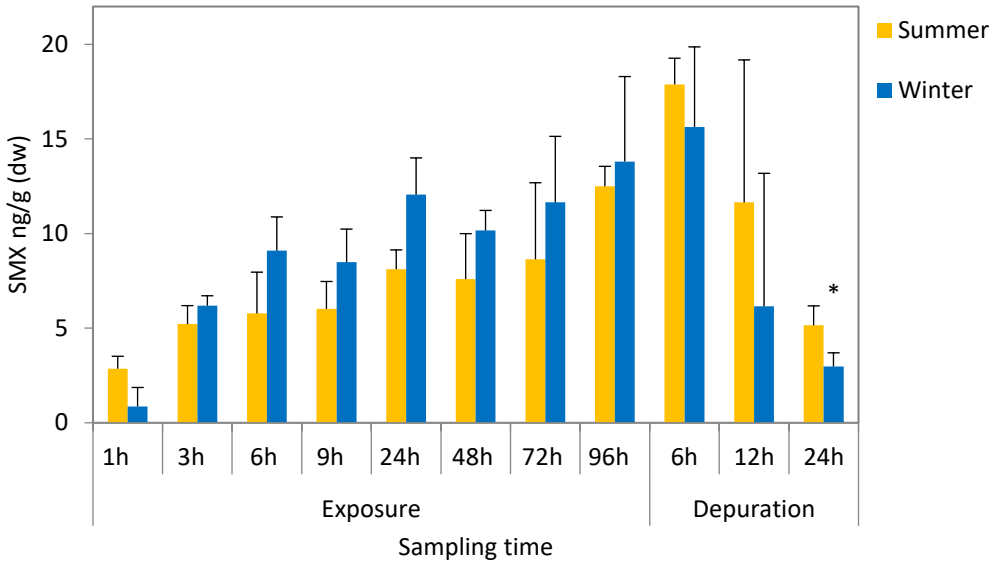

Figure S2. SMX concentration in mussel's soft tissue along the exposure and depuration sampling times in summer (yellow bars) and winter (blue bars) trials. No significant differences were observed between summer and winter trials at any sampling time (p value < 0.05, ANOVA test), except after 24 hours of depuration (*).

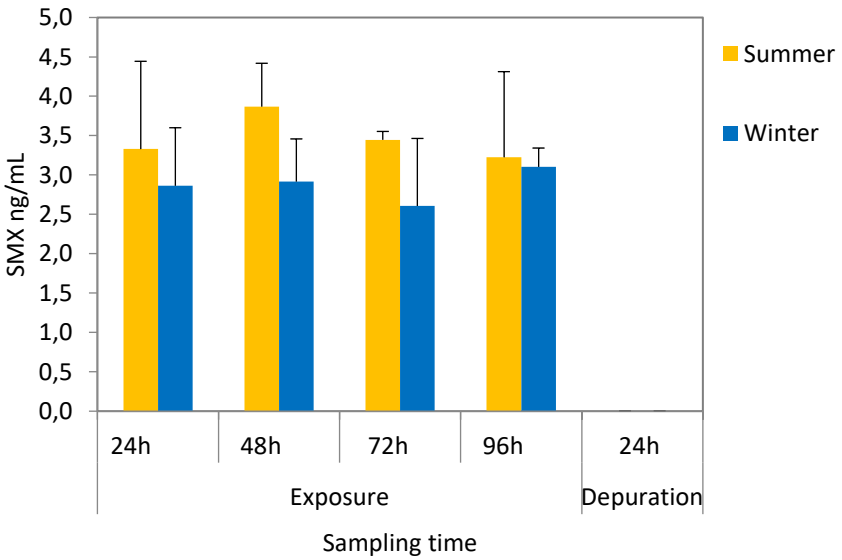

Figure S3. SMX concentration in mussel's haemolymph along the exposure and depuration sampling times. Concentrations for summer (yellow bars) and winter (blue bars) are presented. No significant differences were observed between summer and winter trials at any sampling time using ANOVA test (p value < 0.05).

Enzymatic activities

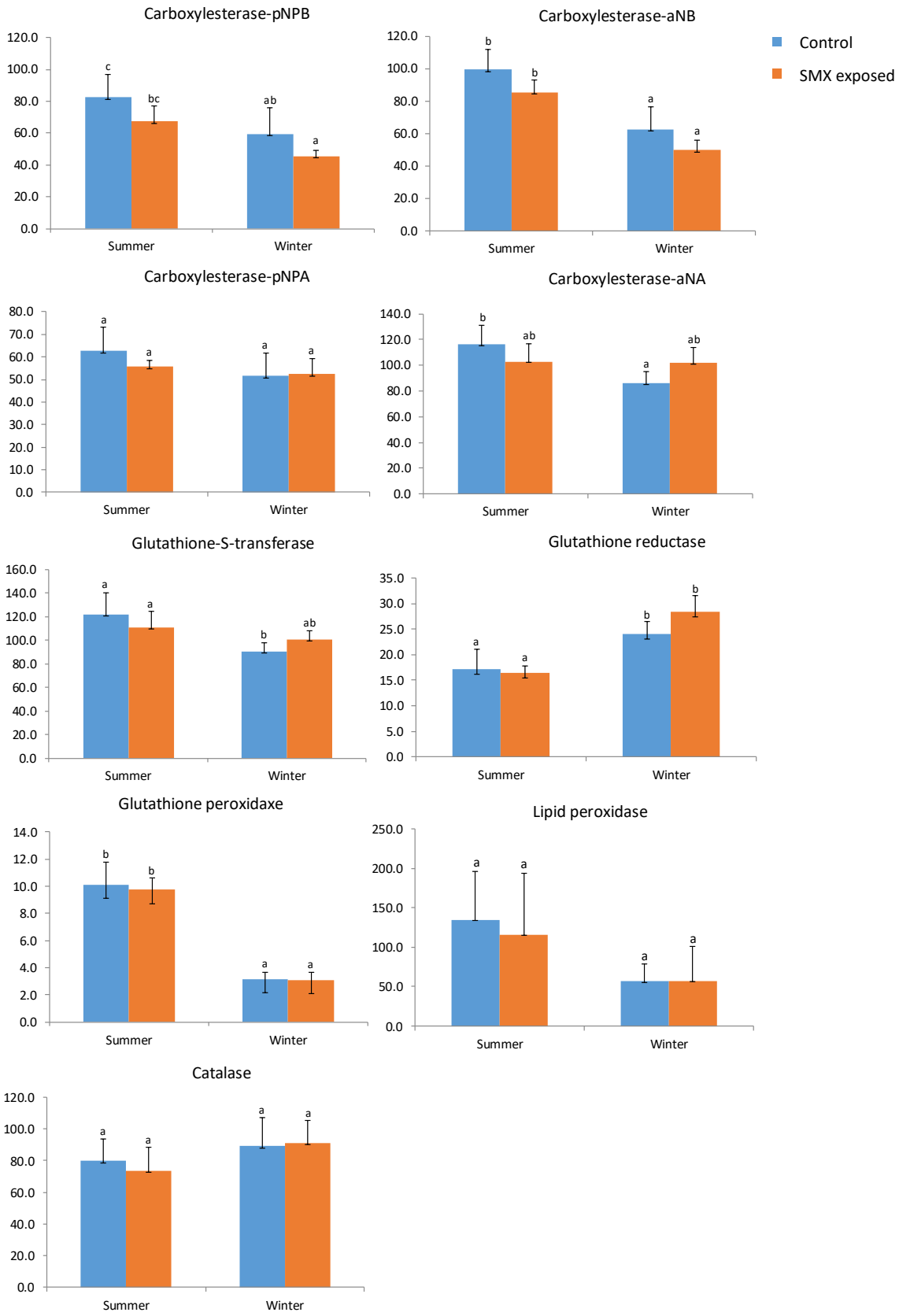

Figure S4 Enzymatic activities characterized in mussel's gills. Carboxylestarases, glutatione-S transferase, glutathione reductase, glutathione peroxidase and catalase are represented in nmol/min/ mg protein, whereas lipid peroxidase is presented in nmol MDA/g (ww). Within each enzymatic activity different letters indicate statistical differences (ANOVA test p-value < 0.05) between the different treatment (control and SMX exposed) and trial (summer and winter).

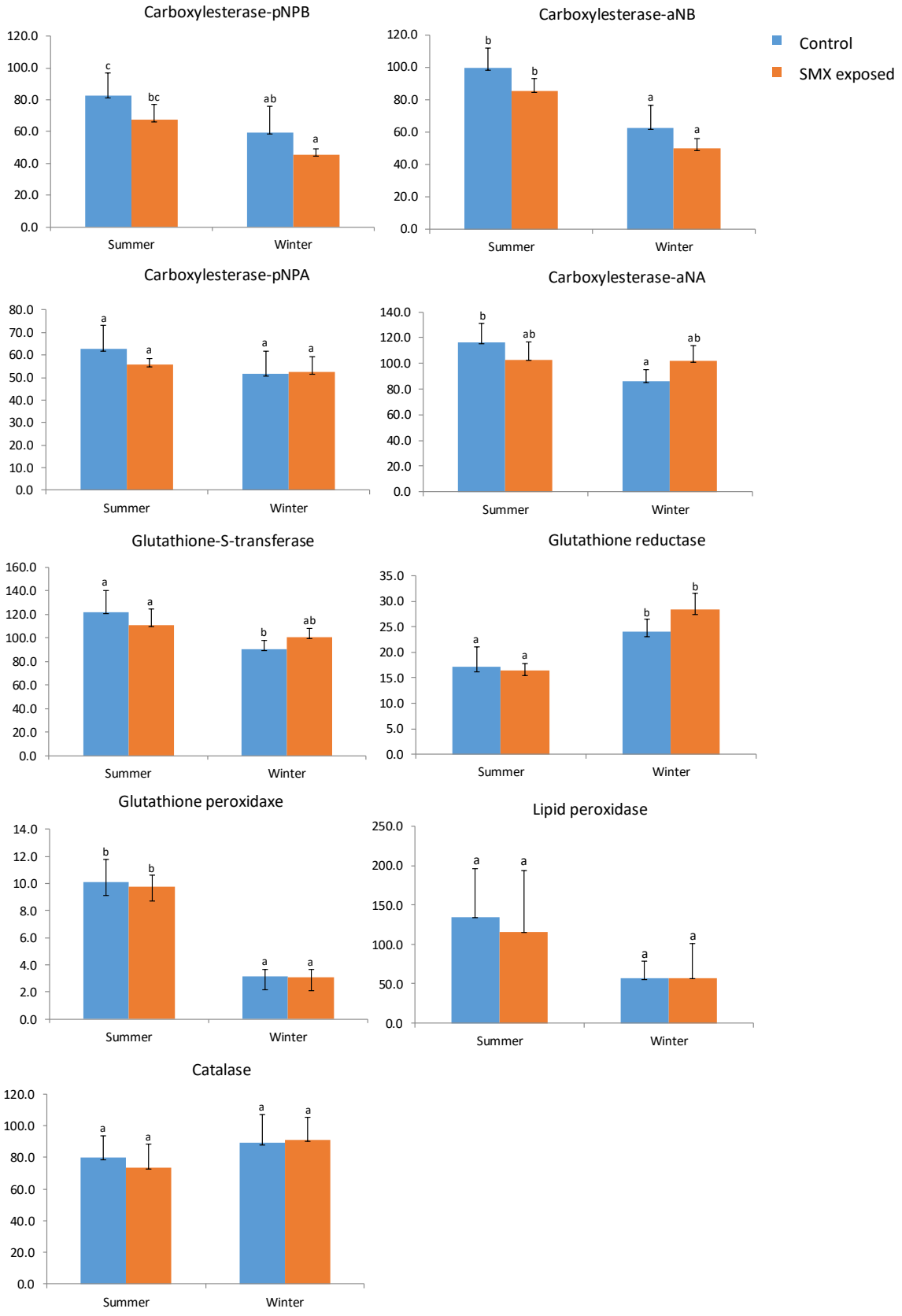

Figure S5 Enzymatic activities characterized in mussel's digestive gland. Carboxylestarases, glutatione-S transferase, glutathione reductase, glutathione peroxidase and catalase are represented in nmol/min/ mg protein, whereas lipid peroxidase is presented in nmol MDA/g (ww). Different letters

indicate statistical differences (ANOVA test, p-value < 0.05) between the different treatment (control and SMX exposed) and trial (summer and winter) within each enzymatic activity.

Metabolomics approach

Metabolomics quality control: Composite samples were used to monitor LC-LTQ-Orbitrap performance during batch analyses. The variation of retention time and mass accuracy was assessed for all the putatively identified compounds. Within each batch of sample analyses, the variation of retention time was ± 0.07 and ± 0.03 min for positive and negative ionization mode respectively, whereas the variation of the mass accuracy was below ± 3 ppm for positive ionization mode and below ± 2 ppm for negative ionization mode, showing no difference in mean response values between batches of samples analysed at the beginning, middle and end of the analytical runs revealing little drift in instrument sensitivity.

Table S3. Metabolites identified in mussels haemolymph after comparing control and SMX exposed mussels in winter trial; their fragmentation pattern obtained with LC-LTQ-Orbitrap collision induced dissociation (CID) analysis is shown. All fragments were confirmed with the metabolite fragment prediction tools CFM-ID (http://cfmid.wishartlab.com/predict) and/or reported in the databases MetFgrag (https://msbi.ipb-halle.de/MetFragBeta).

M/Z value of marker ion	RT (min)	ESI	Experimental molecular formula ion	Putative identity of metabolite	Compound class	Mass of observed fragments	Formula of observed fragment	Fragment explanation	Error observed fragments (ppm)
134.0450	0.54	+	$C_4H_7NO_4$ $[M+H]^+$	Aspartate	Amino acid	73.0284 87.0318 116.0342	$C_3H_5O_2$ $C_3H_5O_2N$ $C_4H_6O_3N$	$[M+H-CH_2NO_2]^+$ $[M+H-CH_2O_2]^+$ $[M+H-HO_2]^+$	0.33 3.45 0.004
118.0866	0.78	+	$C_5H_{12}NO_2$ $[M+H]^+$	Valine	Amino acid	58.0649 59.0727 72.0805	C_3H_8N C_3H_9N $C_4H_{10}N$	$[M+H-C_2H_4O_2]^+$ $[M+H-C2H_3O_2]^+$ $[M+H-CH_2O_2]^+$	-4.23 -3.57 -3.69
284.0995	0.98	+	$C_{10}H_{14}N_5O_5$ $[M+H]^+$	Guanosine	Nucleotide	184.0713 196.0827 218.1011 240.1091 245.1126	$C_7H_{10}O_3N_3$ $C_7H_{10}O_2N_5$ $C_7H_{14}O_4N_4$ $C_9H_{14}O_3N_5$ $C_8H_{15}O_4N_5$	$[M+H-C_3H_4O_2N_2]^+$ $[M+H-C_3H_4O_3]^+$ $[M+H-C3NO]^+$ $[M+H-CO_2]^+$ $[M+H_2-C_2]^+$	-0.39 -0.89 0.71 -0.08 3.04
166.0864	1.24	+	$C_9H_{12}NO_2$ $[M+H]^+$	Phenylala-nine	Amino acid	103.0542 120.0805 131.0487 138.0545 148.0751 149.0592	C_8H_7 $C_8H_{10}N$ C_9H_7O $C_7H_8O_2N$ $C_9H_{10}ON$ $C_9H_9O_2$	$[M+H-CH_5NO_2]^+$ $[M+H-CH_2O_2]^+$ $[M+H-H_5NO]^+$ $[M+H-C2H_3O]^+$ $[M+H-H_2O]^+$ $[M+H-H_3N]^+$	-0.3 -2.2 -3.1 -3.5 -4.1 -3.5
267.0742	1.33	-	$C_{10}H_{11}N_4O_5$ $[M-H]^-$	Inosine	Nucleotide	108.0203 135.0312 149.0469	$C_4H_2ON_3$ $C_5H_3ON_4$ $C_6H_5ON_4$	$[M-H-C_6H_{10}O_4N]^-$ $[M-H-C_5H_9O_4]^-$ $[M-H-C_4H_7O_4]^-$	-0.60 -0.33 -0.90

						177.0417 249.0629	$C_7H_5O_2N_4$ $C_{10}H_9O_4N_4$	$[M-H-C_3H_7O_3]^-$ $[M-H-H_3O]^-$	1.08 2.02
203.0828	2.44	-	$C_{11}H_{11}N_2O_2$ $[M-H]^-$	Trypto-phan	Amino acid	116.0506 130.0659 142.0661 159.0927 186.0560	C_8H_6N C_9H_8N $C_{10}H_8N$ $C_{10}H_{11}N_2$ $C_{11}H_8O_2N$	$[M-H-C_3H_6O_2N]^-$ $[M-H-C_2H_4O_2N]^-$ $[M-H-CH_4O_2N]^-$ $[M-H-CHO_2]^-$ $[M-H-H4N]^-$	-2.00 -2.56 -1.07 -0.64 -0.39
121.0296	4.24	-	$C_7H_5O_2$ $[M-H]^-$	Benzoate	Carboxylic acid	77.0399	C_6H_5	$[M-H-CO_2]^-$	3.46

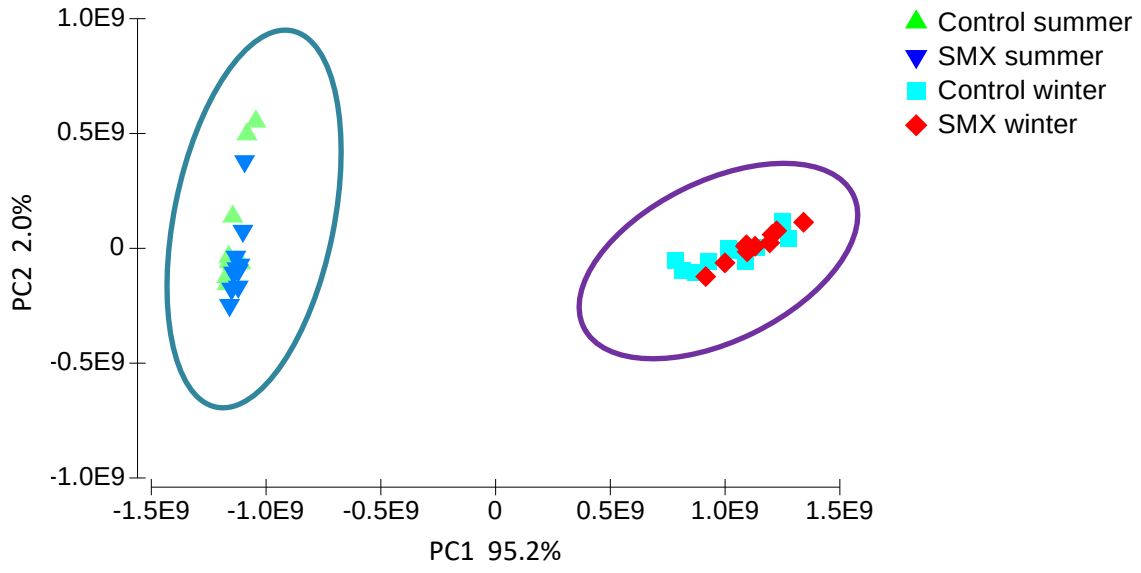

Figure S6 PCA score plots of the metabolomic profiles of mussels. Mussel's haemolymph were profiled by LC-LTQ-Orbitrap, with all markers detected in +ESI and −ESI mode. The percentages of explained variation for the first two components (PC1 and PC2) are displayed on the relative axes.

Discussion

Table S4. List of metabolic pathways identified using MetaboAnalyst 4.0, matching with the identified compounds. Only those pathways with a p-value < 0.05 are explained.

Pathway Name	Identified metabolites/ total metabolites pathway	p	-log(p)
Aminoacyl-tRNA biosynthesis	4/75	2.83E-5	10.47
Nitrogen metabolism	3/39	1.31E-4	8.93
Pantothenate and CoA biosynthesis	2/27	0.002	6.01
Phenylalanine, tyrosine and tryptophan biosynthesis	2/27	0.002	6.01
Phenylalanine metabolism	2/45	0.006	4.99
Glycine, serine and threonine metabolism	2/48	0.007	4.87
Purine metabolism	2/92	0.02	3.62
Cyanoamino acid metabolism	1/16	0.04	3.08

References

Álvarez-Muñoz, D., Huerta, B., Fernandez-Tejedor, M., Rodríguez-Mozaz, S., Barceló, D., 2015. Multi-residue method for the analysis of pharmaceuticals and some of their metabolites in bivalves. Talanta 136, 174–182. https://doi.org/10.1016/j.talanta.2014.12.035

Supporting Information

Exposure to a Subinhibitory Sulfonamide Concentration Promotes the Spread of Antibiotic Resistance in Marine Blue Mussels (*Mytilus edulis*)

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2. Materials and Methods

2.1 DNA extraction and genes quantification

All qPCR assays were developed using the SYBR Green Master Mix (Applied Biosystems) containing 2 µL of the extracted DNA, and each gene was amplified using specific primers. For specificity of the amplified products, dissociation curves were constructed by increasing the temperature from 65 to 95 °C and compared with known quantities of cloned target genes as previously described[1].

2.2 SMX analysis in seawater and mussel's haemolymph

Calibration curves were prepared in the corresponding matrix, diluted seawater and control mussel haemolymph respectively. Internal standard (sulfamethoxazole-d4) was added to all samples and standard calibration curve prior instrumental analysis. An acquity HSS T3 column (50 mm×2.1 mm i.d., 1.8 µm particle size) was used for chromatographic separation; the mobile phase used were (A) methanol and (B) 10 mM of formic acid/ammonium formate at pH 3.2. The UPLC instrument was coupled to a 5500 QTRAP linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. SMX was analyzed under positive ionization mode.

3. Results and Discussion

3.1 SMX occurrence in seawater and accumulation in mussel's haemolymph

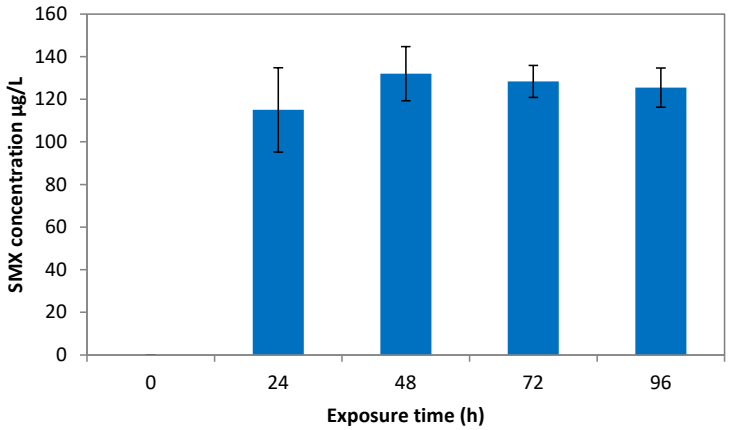

Figure S1. SMX concentration in spiked tanks along the exposure phase.

3.2 Quantification of ARGs

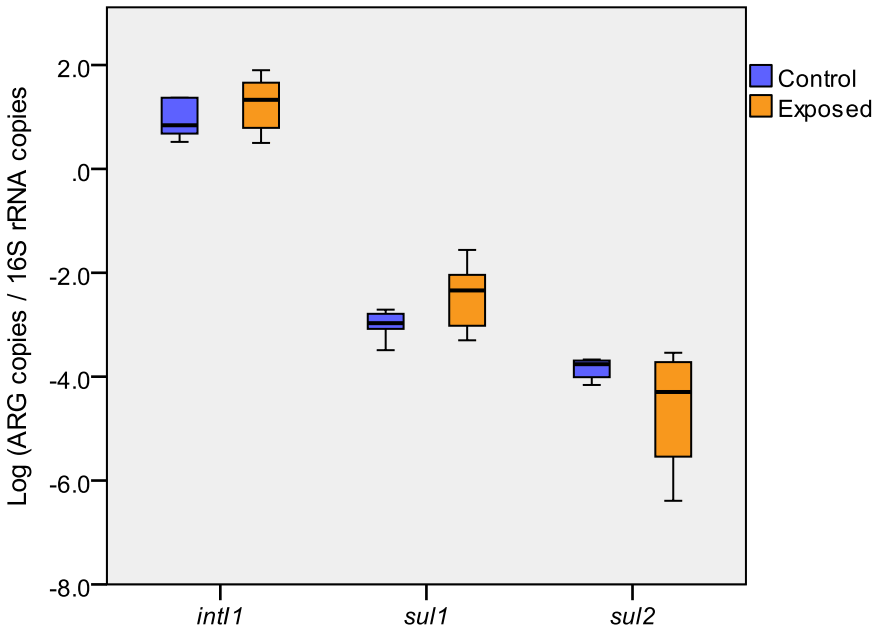

Figure S2. Relative abundance (normalized to 16S rRNA copies) of ARGs in mussel's gastrointestinal tract.

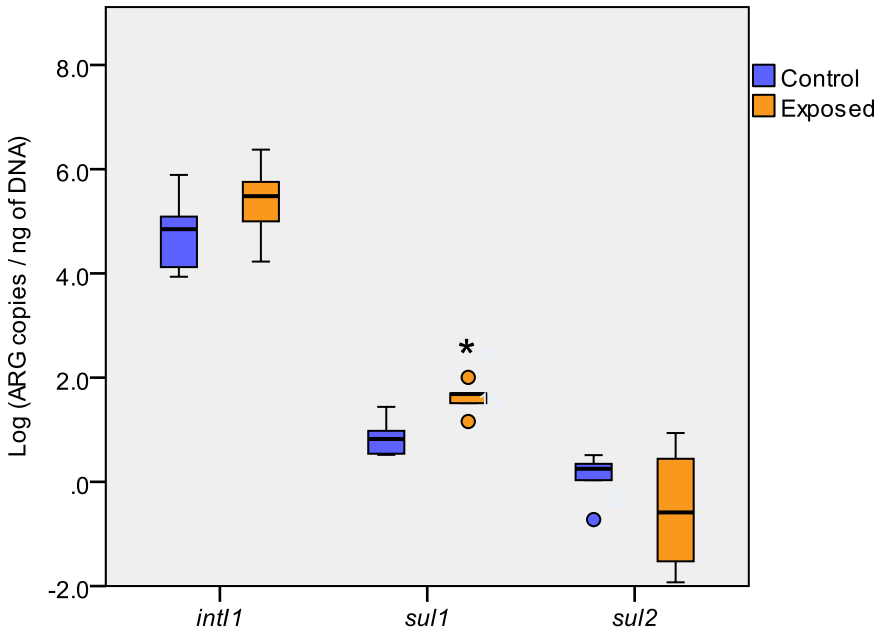

Figure S3. Total concentration (normalized to ng of DNA) of ARGs in mussel's gastrointestinal tract. Asterisks (*) indicates significant differences.

Table S1. Values of *intI1*, *sul1* and *sul2* normalized to gram of sample, ng of DNA and 16S rRNA copies.

ARGs copies normalized to gram of sample			
	intI1	*sul1*	*sul2*
SMX exposed	6.0E+10 ± 1.3E+11	3.5E+06 ± 2.7E+06	5.4E+04 ± 8.8E+04
Control	6.8E+09 ± 5.2E+09	5.5E+05 ± 1.2E+05	1.1E+05 ± 8.2E+04
ARGs copies normalized to ng of DNA			
	intI1	*sul1*	*sul2*
SMX exposed	6.23E+05± 8.84E+05	4.10E+01 ± 3.53E+01	1.61E+00 ± 3.47E+00
Control	1.77E+05 ± 2.96E+05	9.59E+00 ± 9.16E+00	1.42E+00± 1.25E+00
ARGs copies normalized to 16S rRNA copies			
	intI1	*sul1*	*sul2*
SMX exposed	2.99E+01± 9.29E+00	7.08E-03± 7.08E-03	1.08E-04± 1.08E-04
Control	1.14E+01± 9.29E+00	1.15E-03± 5.87E-04	1.52E-04 ± 6.51E-05

References

(1) Rodriguez-Mozaz, S.; Chamorro, S.; Marti, E.; Huerta, B.; Gros, M.; Sànchez-Melsió, A.; Borrego, C. M.; Barceló, D.; Balcázar, J. L. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* **2015**, *69*, 234–242.