PHARMACEUTICALS AND ENDOCRINE DISRUPTORS: ACCUMULATION IN AQUATIC BIOTA AND ENVIRONMENTAL EFFECTS

Belinda Huerta Buitrago

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EXPERIMENTAL SCIENCES AND SUSTAINABILITY PhD PROGRAMME

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CERTIFIQUEN:

Que el treball titulat "Pharmaceuticals and endocrine disruptors: accumulation in aquatic biota and environmental effects" que presenta Belinda Huerta Buitrago per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció I que compleix els requeriments per poder optar a la Menció Internacional.

Dr. Damiá Barcelò Culleres

Dra. Sara Rodriguez-Mozaz

El doctorand
Belinda Huerta Buitrago

Girona, Octubre 2015

“Trust your heart if the seas catch fire,

live by love though the stars walk backward”

– E.E. Cummings

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SUMMARY

Occurrence, fate, and adverse effects of emerging contaminants in the aquatic environment have become a matter of concern, since these compounds have been persistently detected in wastewater treatment plant (WWTP) effluents, surface water, seawater and groundwater all over the globe. The incessant release of emerging contaminants into freshwater ecosystems could affect a broad variety of organisms: from bacterial communities to fish, as well as aquatic plants and insect larvae. The impact of these pollutants can be evaluated by measuring the bioaccumulation of the target compounds in the organisms or/and by assessing the toxicological effects.

The present study aims to investigate the potential bioaccumulation and biomagnification of two groups of emerging contaminants − pharmaceuticals and endocrine disruptors − in aquatic biota, including fish, invertebrates and river biofilm in the natural environment. A second aim of this thesis is to evaluate the impacts of exposure to some of these contaminants in the organisms, and the possible relation between bioaccumulation and toxicological effects.

CHAPTER 1 and 2: Introduction and Objectives.

CHAPTER 3: Development of analytical methods for the determination of pharmaceuticals and endocrine disruptors in fish, invertebrates and biofilm.

This chapter aims to develop and validate analytical methods to measure pharmaceuticals and endocrine disruptors in biological matrices: fish tissues, invertebrates and biofilm. This is a fundamental step to have reliable tools to assess the bioaccumulation of these compounds in the environment. Three different methodologies were developed for the three biological matrices considered and were reported accordingly in 3 manuscripts.

The first article included in this chapter describes the analytical methodology developed for the analysis of twenty pharmaceuticals from seven commonly used therapeutic families in fish tissues (whole body, but also liver and muscle). This method is based on pressurized liquid extraction (PLE) and

purification of the extract by gel permeation chromatography (GPC) prior determination by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS). The method was applied to fish tissues of eleven fish species from four heavily impacted Mediterranean rivers. Nine compounds from five therapeutic families were measured at concentrations higher than MDLs. Highest levels were found in trout liver, with a maximum concentration of 18 ng g^{-1} of carbamazepine, whereas the most ubiquitous compound was diclofenac.

The second paper describes the methodology developed for the simultaneous analysis of 41 pharmaceuticals and 21 endocrine disruptors in freshwater macroinvertebrates, based on extraction by sonication, purification via removal of phospholipids, and analysis by ultra-performance liquid chromatography coupled to a mass spectrometer in tandem (UPLC-MS/MS). In addition, bioaccumulation of these contaminants in three macroinvertebrate taxa inhabiting a waste water treatment plant-impacted river was assessed. Two non-steroidal anti-inflammatory drugs, diclofenac and ibuprofen, and four endocrine disruptors – estrone, bisphenol A, TBEP, and nonylphenol – were detected in at least one macroinvertebrate taxa in concentrations up to 183 ng g^{-1}.

The third paper in this chapter focuses on river biofilm, and describes the development of an efficient analytical methodology for the analysis of 44 pharmaceuticals and 13 endocrine disrupting compounds, and evaluated persistence, distribution, and bioaccumulation of these contaminants in natural biofilms inhabiting a WWTP-impacted river. The method is based on pressurized liquid extraction, purification by solid-phase extraction, and analysis by ultra-performance liquid chromatography coupled to a mass spectrometer (UPLC–MS/MS) in tandem. A total of five endocrine disruptors and seven pharmaceuticals were detected in field samples at concentrations up to 200 ng g^{-1}, with diclofenac and methyl paraben as the most concentrated compounds.

CHAPTER 4: Determination of pharmaceuticals and endocrine disruptors in biofilm and invertebrates: trophic transfer.

Trophic transfer will be assessed by uptake of emerging pollutants – 25 pharmaceuticals and 12 endocrine disruptors – in aquatic organisms, in particular biofilm and three aquatic macroinvertebrate taxa with different trophic positions and feeding strategies (*Ancylus fluviatilis, Hydropsyche sp., Phagocata vitta*). Observed bioaccumulation factors (BAFs) were compared to those predicted by a previously-developed empirical model based on octanol–water distribution coefficients (D_{ow}).Two compounds (the anti-inflammatory drug diclofenac, and the flame retardant TBEP) were detected in water, biofilm and (at least) one macroinvertebrate taxa. TBEP was the only compound present in all taxa and showed magnification across trophic levels.

CHAPTER 5: Environmental effects of the occurrence of antibiotics and psychiatric drugs: antibiotic resistance and behavioural alterations.

This chapter aims to evaluate some of the impacts that the exposure to pharmaceuticals could have in aquatic organisms. In the first article, the correlation between the occurrence of antibiotics and the promotion of antibiotic resistance in the bacterial communities of water reservoirs was investigated. With that purpose, the concentration of antibiotics and prevalence of antibiotic resistance genes (ARGs) was determined. Consequently, the potential role of ARGs in shaping bacterial communities was evaluated, and the bacterial groups most probably carrying and disseminating ARGs were identified. Results showed significant correlation between the presence of ARG conferring resistance to macrolides and the composition of bacterial communities, suggesting that antibiotic pollution and the spreading of ARG might play a role in the conformation of bacterial communities in reservoirs. Results also pointed out the bacterial groups *Actinobacteria* and *Firmicutes* as the ones probably carrying and disseminating ARGs.

In the second part of the chapter, the aim was to measure the bioaccumulation of a psychiatric drug in plasma and in different tissues of fish individuals under controlled conditions, and to determine the correlation between that bioaccumulation and the consequential behavioural alterations observed in the fish. Results showed that accumulation in the brain was significantly higher than in the other tissues and plasma. Exposure of fish to 1 and 5 μg L^{-1} resulted in plasma concentrations of oxazepam below the

H_TPC, whereas exposure to 25 μg L^{-1} produced plasma concentrations within the H_TPC range in many individuals. In general, no behavioural effects were observed at any exposure concentration, including the one that produced H_TPCs in fish plasma. A decreased exploratory activity was observed for the group exposed to 5 μg L^{-1} (oxazepam plasma concentrations were approximately 3-fold lower than the H_TPCs). This behaviour may be independent by the drug exposure, due to drug plasma concentrations $<H_TPC$, lack of dose-response, and high inter-individual and inter-tank behavioural variability.

RESUMEN (castellano)

La presencia, destino y efectos adversos de los contaminantes emergentes en el medio acuático se han convertido en un tema preocupante, puesto que esto compuestos se han venido detectando de forma persistente en los efluentes de las Estaciones de Depuración de Aguas Residuales (EDAR) así como en agua superficial, marina y subterránea en todo el planeta. La constante presencia de contaminantes emergentes en el medio acuático continental podría afectar a una gran variedad de organismos: desde comunidades bacterianas hasta peces, así como plantas acuáticas y larvas de insectos. El impacto de estos contaminantes en los organismos y comunidades acuáticas se puede evaluar mediante la determinación de sus efectos tóxicos así como de su bioacumulación en dichos organismos.

El primer objetivo de esta tesis es investigar la potencial bioacumulación y biomagnificación de dos grupos de contaminantes emergentes −fármacos y disruptores endocrinos− en biota acuática, incluyendo peces, invertebrados y biofilm. Un segundo objetivo es evaluar los posibles impactos que la exposición a estos contaminantes puede tener en los organismos, y establecer la posible correlación entre bioacumulación y efectos toxicológicos.

CAPÍTULO 1 y 2: Introducción y Objetivos.

CAPÍTULO 3: Desarrollo de métodos analíticos para la determinación de fármacos y disruptores endocrinos en peces, invertebrados y biofilm.

Este capítulo tiene como objetivo desarrollar y validar métodos analíticos para medir una gran variedad de fármacos y disruptores endocrinos en matrices biológicas: tejidos de peces, invertebrados y biofilm. Disponer de herramientas analíticas de confianza es un paso fundamental para poder determinar la bioacumulación de estos compuestos en biota. Se desarrollaron por lo tanto tres metodologías diferentes para estas matrices, que se recogen en los tres manuscritos incluidos en este capítulo.

El primer artículo incluido en este capítulo describe la metodología analítica desarrollada para el análisis de 20 fármacos pertenecientes a siete familias terapéuticas en tejidos de peces (hígado y musculo,

así como el pez homogeneizado). Este método está basado en una extracción con líquidos presurizados (PLE de las siglas en inglés Pressurized Liquid Extraction) seguida de una purificación del extracto mediante cromatografía de exclusión molecular (GPC, siglas en ingles de Gel Permeation Chromatography) previa a su determinación mediante cromatografía liquida de alto rendimiento en tándem con espectrometría de masas (UPLC–MS/MS). El método se aplicó a los tejidos de once especies de peces procedentes de cuatro ríos mediterráneos altamente contaminados. Nueve compuestos pertenecientes a cinco familias terapéuticas se encontraron a concentraciones superiores a los límites de detección. Los niveles más altos se detectaron en el hígado de trucha, con concentraciones máximas de 18 ng g^{-1} de carbamazepina, mientras que el compuesto más ubicuo fue el diclofenaco.

El segundo artículo describe la metodología desarrollada para el análisis simultáneo de 41 fármacos y 21 disruptores endocrinos en invertebrados acuáticos. Esta metodología está basada en extracción mediante ultrasonidos, purificación vía eliminación de fosfolípidos mediante una placa OSTRO^{TM}, y análisis mediante cromatografía liquida de alto rendimiento en tándem con espectrometría de masas (UPLC–MS/MS). Esta metodología permitió investigar la bioacumulación de estos contaminantes en 3 grupos taxonómicos de macroinvertebrados que habitaban un rio impactado por una EDAR. Dos anti-inflamatorios no esteroideos – diclofenaco e ibuprofeno–, y cuatro disruptores endocrinos – estrona, bisfenol A, TBEP y nonilfenol – fueron detectados en al menos un grupo taxonómico en concentraciones máximas de hasta 183 ng g^{-1}.

El tercer artículo en este capítulo se centra finalmente en el biofilm (perifiton), y describe el desarrollo de una metodología para el análisis de 44 fármacos y 13 disruptores endocrinos; además, se evaluó la persistencia, distribución y bioacumulación de estos contaminantes en biofilm natural procedente de un rio impactado por una EDAR. El método se compone de una extracción con líquidos presurizados, una purificación mediante extracción en fase sólida y el análisis mediante cromatografía líquida de alto rendimiento en tándem con espectrometría de masas (UPLC–MS/MS). Un total de cinco disruptores endocrinos y siete fármacos fueron detectados en las muestras de biofilm del río impactado a niveles de hasta 200 ng g^{-1}, siendo el diclofenaco y metilparabeno los compuestos encontrados a mayor concentración.

CAPÍTULO 4: Determinación de fármacos y disruptores endocrinos en biofilm e invertebrados: trasferencia trófica.

Este capítulo pretende evaluar el papel de la transferencia trófica en la acumulación de contaminantes emergentes seleccionados – 25 fármacos y 12 disruptores endocrinos – por los organismos acuáticos. Para ello se estudió la presencia de estos contaminantes en biofilm y tres clases taxonómicas de macroinvertebrados, con diferentes posiciones y estrategias alimentarias (*Ancylus fluviatilis, Hydropsychesp., Phagocata vitta*). Los factores de bioacumulación (BAFs) observados se compararon con aquellos predichos por un modelos empírico desarrollado previamente y que se basa en los coeficientes de distribución octanol-agua (D_{ow}). Dos compuestos (el anti-inflamatorio diclofenaco y el retardante de llama TBEP) se detectaron en agua, biofilm, y al menos una clase de macroinvertebrados. TBEP fue el único compuesto presente en todos los grupos que presento magnificación en los diferentes niveles tróficos.

CAPÍTULO 5: Efectos ambientales de la presencia de antibióticos y drogas psiquiátricas: Resistencia antibiótica y alteraciones del comportamiento.

El objetivo de este capítulo es evaluar algunos de los impactos que la exposición a fármacos pueda tener en los organismos y comunidades en el medio acuático. En el primer artículo, se investigó la correlación entre la presencia de antibióticos y el incremento de la resistencia antibiótica en las comunidades bacterianas en varios embalses de agua. Con ese propósito, se determinó la concentración de antibióticos y la prevalencia de los genes de resistencia antibiótica (ARGs) en muestra de agua y sedimento. A continuación, se evaluó el papel de los ARGs en la conformación de las comunidades bacterianas, y se identificaron los grupos bacterianos que más probablemente llevan y diseminan ARGs. Los resultados obtenidos mostraron una correlación significativa entre la presencia de los ARGs que proporcionan resistencia a los macrólidos y la composición de las comunidades bacterianas. Esto sugiere que la contaminación por antibióticos y la difusión de ARGs podrían estar influyendo en la conformación de las comunidades bacterianas en los embalses de agua. Los resultados también permitieron identificar a los grupos de bacterias *Actinobacteria* y *Firmicutes* como aquellos con más probabilidad de llevar y diseminar ARGs en los embalses estudiados.

En la segunda parte de este capítulo, el objetivo era medir la bioacumulación de una droga psiquiátrica en diferentes tejidos de peces en un estudio de exposición en laboratorio, y determinar la correlación entre la bioacumulación y las consecuentes alteraciones del comportamiento observadas en los peces. Los resultados mostraron que la acumulación en el cerebro era significativamente mayor que en el resto de tejidos y que en el plasma. Los peces expuestos a concentraciones en agua de 1 y 5 μg L^{-1} presentaban concentraciones en plasma hasta 3 veces por debajo de la “concentración plasmática humana terapéutica” (H_TPC), mientras que en alguno de los peces expuestos a concentraciones de 25 μg L^{-1} sí que se observaron concentraciones plasmáticas en el rango de la H_TPC. En general no se observaron efectos en el comportamiento a ninguna de las concentraciones, incluida la que produjo la H_TPC en los peces. Sin embargo, sí que se observó cierta disminución en la actividad exploratoria de los peces expuestos a la concentración más baja en agua 5 μg L^{-1}, donde la concentración de oxazepam en plasma era menor que la H_TPC. Sin embargo, la variabilidad en el comportamiento entre individuos del mismo o distintos tanques expuestos a la misma concentración, y el hecho de que la concentración de oxazepam en plasma es menor que la H_TPC, sugiere que los efectos observados son independientes de la exposición al fármaco.

Resum (català)

La presència, destí i efectes adversos dels contaminants emergents en el medi aquàtic s'han convertit en un tema de preocupació general ja que, aquests compostos, s'han estat detectant de manera persistent en els efluents de les Estacions Depuradores d'Aigües Residuals (EDAR) així com en aigües superficials, marines i subterrànies de tot el planeta. La constant presència de contaminants emergents en el medi aquàtic continental podria afectar a una gran varietat d'organismes: des de comunitats bacterianes fins a peixos, així com a plantes aquàtiques i larves d'insectes. L'impacte d'aquests contaminants en els organismes i comunitats aquàtiques es pot avaluar mitjançant la determinació dels seus efectes tòxics així com la bioacumulació en els organismes esmentats.

El primer objectiu d'aquesta tesi doctoral és investigar la potencial bioacumulació i biomagnificació de dos grups de contaminants emergents –fàrmacs i disruptors endocrins– en la biota aquàtica incloent peixos, invertebrats i biofilm. El segon objectiu és avaluar els possibles impactes que l'exposició a aquests contaminants poden tenir en els organismes i establir la possible correlació entre bioacumulació i efectes toxicològics.

CAPÍTOL 1 i 2: Introducció i Objectius.

CAPÍTOL 3: Desenvolupament de mètodes analítics per a la determinació de fàrmacs i disruptors endocrins en peixos, invertebrats i biofilm

Aquest capítol té com a objectiu desenvolupar i validar mètodes analítics per mesurar una gran varietat de fàrmacs i disruptors endocrins en matrius biològiques (teixits de peixos, invertebrats i biofilm). Disposar d'eines analítiques de confiança és un pas fonamental per poder determinar la bioacumulació d'aquests compostos. Per aquest motiu es van desenvolupar tres metodologies analítiques per a aquestes matrius i que es recullen en els tres manuscrits inclosos en aquest capítol.

El primer article descriu la metodologia desenvolupada per a l'anàlisi de 20 fàrmacs pertanyents a set famílies terapèutiques d'ús comú en teixits de peixos (fetge i múscul, així com en l'homogenat de tot el peix). Aquest mètode està basat en una extracció mitjançant líquids pressuritzats (PLE de les sigles en anglès Pressurized Liquid Extraction), seguida d'una purificació de l'extracte mitjançant cromatografia d'exclusió de mida (GPC, sigles en anglès Gel Permeation Chromatography) i, finalment, la seva determinació a través de cromatografia líquida d'alt rendiment acoblada a espectrometria de masses en tàndem (UPLC-MS/MS). Aquest mètode es va aplicar als teixits d'onze espècies de peixos procedents de quatre rius mediterranis altament contaminats. Nou compostos, pertanyents a cinc famílies terapèutiques, es van trobar a concentracions superiors als límits de detecció. Els nivells més alts es van detectar en el fetge de la truita, amb concentracions màximes de fins a 18 ng g^{-1} de carbamazepina. No obstant, el compost més obliqua va ser el diclofenac.

El segon article descriu la metodologia desenvolupada per a l'anàlisi simultani de 41 fàrmacs i 21 disruptors endocrins en invertebrats aquàtics. Aquesta metodologia està basada en l'extracció mitjançant ultrasons, purificació via eliminació de fosfolípids i, finalment, anàlisi per UPLC-MS/MS. Aquesta metodologia va permetre investigar la bioacumulació d'aquests contaminants en 3 grups taxonòmics de macroinvertebrats que habitaven un riu impactat per l'efluent d'una EDAR. Dos anti-inflamatoris no esteroïdals - diclofenac i ibuprofè - i quatre disruptors endocrins – estrona, bisfenol A, TBEP i nonilfenol- van ser detectats en almenys un grup taxonòmic a concentracions màximes de fins a 183 ng g^{-1}.

El tercer article d'aquest capítol se centraen el biofilm (perífiton) i descriu el desenvolupament d'una metodologia per a l'anàlisi de 44 fàrmacs i 13 disruptors endocrins; a més a més, es va avaluar la persistència, distribució i bioacumulació d'aquests contaminants en el biofilm natural procedent d'un riu impactat per una EDAR. El mètode consisteix en una extracció per líquids pressuritzats, una purificació mitjançant extracció en fase sòlida i l'anàlisi a través de UPLC-MS/MS. Un total de cinc disruptors endocrins i set fàrmacs van ser detectats en les mostres de biofilm del riu impactat a concentracions màximes de fins a 200 ng g^{-1}, essent el diclofenac i el metilparabè els compostos detectats a majors concentracions.

CAPÍTOL 4: Determinació de fàrmacs i disruptors endocrins en biolfim i invertebrats: transferència tròfica.

En aquest capítol es vol avaluar el paper de la transferència tròfica en l'acumulació dels contaminants emergents seleccionats -25 fàrmacs i 12 disruptors endocrins- per els organismes aquàtics. Per aquest motiu s'ha estudiat la presència d'aquests contaminants en biofilm i tres classes taxonòmiques de macroinvertebrats amb diferents posicions i estratègies alimentàries (*Ancylus fluviatilis, Hydropsychesp., Phagocata vitta*). Els factors de bioacumulació (BAFs) observats es van comparar amb aquells previstos per un model empíric desenvolupat prèviament i que es basa en els coeficients de distribució octanol-aigua (D_{ow}). Dos compostos (l'anti-inflamatori diclofenac i el retardant de flama TBEP) es van detectar en aigua, biofilm i, almenys, en una classe de macroinvertebrats. El TBEP va ser l'únic compost present en tots els grups que va presentar magnificació en els diferents nivells tròfics.

CAPÍTOL 5: Efectes ambientals de la presència d'antibiòtics i drogues psiquiàtriques: resistència antibiòtica i alteracions del comportament.

L'objectiu d'aquest capítol és avaluar alguns dels impactes que l'exposició a fàrmacs pugui tenir en els organismes i comunitats del medi aquàtic. En el primer article es va investigar la correlació entre la presència d'antibiòtics i l'increment de la resistència antibiòtica en les comunitats bacterianes de varis reservoris d'aigua. Amb aquest propòsit, es va determinar la concentració d'antibiòtics i la prevalença d'gens de resistència antibiòtica ARGs en mostres d'aigua i sediment. Seguidament, es va determinar el paper dels ARGs en la conformació de les comunitats bacterianes i es van identificar els grups bacterians que més probablement són portadors d'ARGs. Els resultats obtinguts van mostrar una correlació significativa entre la presència dels ARGs que confereixen resistència a macròlids i la composició de les comunitats bacterianes. Això suggereix que la contaminació per antibiòtics i la disseminació d'ARGs podria estar influint en la conformació de les comunitats bacterianes dels embassaments d'aigua. Els resultats també van permetre identificar als grups de bacteris *Actinobacteria* i *Firmicutes* com com a aquells que més probablement transporten i disseminen els ARGs en els embassaments estudiats.

En la segona part d'aquest capítol, l'objectiu va ser mesurar la bioacumulació d'un medicament psiquiàtric en diferents teixits de peixos en un estudi d'exposició al laboratori, sota condicions controlades, i determinar la correlació entre la bioacumulació i les conseqüents alteracions en el comportament observats en els peixos. Els resultats van mostrar que l'acumulació en el cervell era significativament més elevada que en la resta de teixits, inclòs el plasma. Els peixos exposats a concentracions en aigua d'1 i 5 µg L^{-1} presentaven concentracions en plasma fins a 3 vegades por sota la "concentració plasmàtica humana terapèutica" (H_TPC), mentre que sí que s'observava concentracions plasmàtiques en el rang de la H_TPC en alguns dels individus exposats a concentracions de 25 µg L^{-1} . No obstant, sí que es va observar certa disminució en l'activitat exploratòria en el grup exposat a 5 µg L^{-1} , on la concentració d'oxazepam en plasma era més baixa que la H_TPC. Tot i així, la variabilitat en el comportament entre individus del mateix o diferents tancs i la concentració d'oxazepam en plasma més baixa que la H_TPC suggereixen que els efectes observats són independents a l'exposició al fàrmac.

LIST OF PUBLICATIONS

List of publications in peer-reviewed journals derived from this doctoral thesis and included as chapters in the present thesis:*

Huerta B, Jakimska A, Gros Calvo M, Rodríguez-Mozaz S, Barceló D. Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. 2013. Journal of Chromatography A. 1288, pp. 62 - 72.

Impact Factor: 4.258. 1st Quartile in Analytical Chemistry.

Huerta B, Jakimska A, Llorca M, Ruhí A, Margoutidis G, Acuña V, Sabater S, Rodríguez-Mozaz S, Barceló D. 2014. Development of an extraction and purification method for the determination of multi-class pharmaceuticals and endocrine disruptors in freshwater invertebrates. Talanta. 132, pp. 373 - 381.

Impact Factor: 3.511. 1st Quartile in Analytical Chemistry.

Huerta B, Rodríguez-Mozaz S, Nannou C, Nakis L, Ruhí A, Acuña V, Sabater S, Barceló D. 2015. Determination of a broad spectrum of pharmaceuticals and endocrine disruptors in biofilm from a Waste Water Treatment Plant-impacted river. Science of Total Environment, doi: 10.1016/j.scitotenv.2015.05.049.

Impact Factor: 3.163. 1st Quartile in Environmental Sciences

Huerta B, Marti E, Gros Calvo M, López P, Pompeo M, Armengol J, Barceló D, Balcázar JL, Rodríguez-Mozaz S, Marcé R. 2013. Exploring the links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs. Science of Total Environment. 456-457, pp. 161 - 170.

Impact Factor: 3.163. 1st Quartile in Environmental Sciences

Ruhí A, Acuña V, Barceló D, **Huerta B**, Mor, JR, Rodríguez-Mozaz S, Sabater S. 2015. Bioaccumulation and trophic magnification of pharmaceuticals and endocrine disruptors in a Mediterranean river food web. Science of Total Environment

Impact Factor: 3.163. 1st Quartile in Environmental Sciences

Huerta B, Margiotta-Casaluci L, Rodríguez-Mozaz S, Winter M, Barceló D, Sumper J. 2015. Anti-anxiety drugs and fish behaviour: establishing the link between internal concentrations of oxazepam and behavioural effects. *Submitted.*

**Supplementary material of each article is included in this thesis as part of Chapter 8*

Additional publications related to this thesis:

***Huerta B**, Rodríguez-Mozaz S, Barceló D. 2012. Pharmaceuticals in biota in the aquatic environment: analytical methods and environmental implications. Analytical and Bioanalytical Chemistry. 404(9):2611-24.

Impact Factor: 3.578. 1st Quartile in Analytical Chemistry.

Jakimska A, **Huerta B**, Barganska Z, Kot-Wasik A, Rodríguez-Mozaz S, Barceló D. 2013. Development of a liquid chromatography–tandem mass spectrometry procedure for determination of endocrine disrupting compounds in fish from Mediterranean rivers. Journal of Chromatography A. 1306, pp. 44 - 58.

Impact Factor: 4.258. 1st Quartile in Analytical Chemistry.

Corcoll N, Acuña V, Barceló D, Casellas M, Guasch H, **Huerta B**, Petrovic M, Ponsati L, Rodríguez-Mozaz S, Sabater S. 2014. Pollution-induced community tolerance to non-steroidal anti-inflammatory drugs (NSAIDs) in fluvial biofilm communities affected by WWTP effluents. Chemosphere 112, 185-193.

Impact Factor: 3.499. 1st Quartile in EnvironmentalSciences.

Marti E, **Huerta B**, Rodriguez-Mozaz S, Barcelo D, Jofre J, Balzacar JL. 2014. Characterization of ciprofloxacin-resistant isolates from a wastewater treatment plant and its receiving river. Water Research 61, 67-76.

Impact Factor: 5.323. 1st Quartile in Environmental Sciences

Platikanov S, Rodriguez-Mozaz S, **Huerta B**, Barcelo D, Cros J, Batle M, Poch G, Tauler R. 2014. Chemometrics quality assessment of wastewater treatment plant effluents using physicochemical parameters and UV absorption measurements. Journal of Environmental Management 140, 33-44.
Impact Factor: 3.188. 1st Quartile in Environmental Sciences

Rodriguez-Mozaz S, Chamorro S, Marti E, **Huerta B**, Gros M, Sanchez-Melsio A, Borrego C, Barcelo D, Balzacar JL. 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Research 69, 234-242.
Impact Factor: 5.323. 1st Quartile in Environmental Sciences

Corcoll N, Casellas M, **Huerta B**, Guasch H, Acuña V, Rodríguez-Mozaz S, Serra-Campte A, Barceló D, Sabater S. 2015. Effects of flow intermittency and pharmaceutical exposure on the structure and metabolism of stream biofilms. Science of Total Environment. 503, pp. 159 - 170.
Impact Factor: 3.163. 1st Quartile in Environmental Sciences

Alvarez-Muñoz D, **Huerta B**, Fernandez 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.
Impact Factor: 3.511. 1st Quartile in Analytical Chemistry.

Margiotta-Casaluci L, Owen S, **Huerta B**, Rodríguez-Mozaz S, Kugathas S, Barceló D, Rand-Weaver M, Sumpter J. Internal exposure dynamics drive the Adverse Outcome Pathways of synthetic glucocorticoids in fish. *Submitted*.

Moreno-González R, Rodríguez-Mozaz S, **Huerta B**, Barceló D, León V. Do pharmaceuticals bioaccumulate in marine molluscs and fishes from a coastal lagoon? *Submitted*.

*****Part of this publication has been included in the General Introduction.

LIST OF ABBREVIATIONS

APCI: atmospheric pressure chemical ionization

API: atmospheric pressure ionization

ARGs: antibiotic resistance genes

BFAs: bioaccumulation factors

EDCs: endocrine disrupting compounds

EMA: European medicines agency

ESI: electrospray ionization

FPM: fish plasma model

GC: gas chromatography

GPC: gel-permeation chromatography

H_TPC: Human therapeutic plasma concentration

K_{OW}: octanol–water partitioning coefficient

LC: liquid chromatography

LIT: linear ion trap

LODs: limits of detection

MAE: microwave-assisted extraction

MAME: microwave-assisted micellar extraction

MAX: mixed-mode anion-exchange

MCX: mixed-mode cation-exchange

MS: mass spectrometry

PhACs: pharmaceuticals

PLE: pressurized liquid extraction

SPE: solid-phase extraction

SPME: solid-phase micro-extraction

UPLC: ultra-performance liquid chromatography

UV: ultra violet

WHO: world health organization

WWTP: wastewater treatment plant

Chapter 1

General Introduction

I. PHARMACEUTICALS AND ENDOCRINE DISRUPTORS: SOURCES AND OCCURRENCE IN AQUATIC ECOSYSTEMS

The occurrence, fate, and adverse effects of emerging contaminants in the aquatic environment has become a matter of concern in the last two decades [1–3]. The term “emerging contaminants” refers to those compounds present in various environmental compartments on a global scale, generally not yet subjected to regulation for the protection of human health or the environment and their risks to human health and the environment may be unknown [4]. This definition includes: a) new chemicals that were not previously known or that just recently appeared in the scientific literature; b) compounds with environmental contamination issues that previously were not fully comprehended; and c) legacy contaminants with new information about their environmental and human health risks [5].

Emerging contaminants include many types of contaminants such as polar pesticides, pharmaceuticals and personal care products, fragrances, plasticizers, hormones, flame retardants, nanoparticles, perfluoroalkyl compounds, chlorinated paraffins, siloxanes, algal toxins, various trace elements including rare earths and radionuclides, etc. This dissertation is addressing the environmental fate of two of these groups: pharmaceutical active compounds (PhACs) and endocrine disrupting compounds (EDCs). While the classification of PhACs obviously refers to their use as therapeutic agents and their common chemical structures, this is not so clear in the case of EDCs. These compounds are classified based on the effects they have in the hormonal system, and this definition includes many different chemicals, including some pharmaceuticals (i.e. 17-α-ethynil-estradiol). At the same time, other chemicals associated with the use of EDCs are also considered in this thesis.

Pharmaceuticals

Thousands of substances are used as pharmaceuticals, including in analgesics, antibiotics, anti-hypertensives, contraceptives, lipid regulators, antidepressants, and cytotoxic drugs [2]. Pharmaceuticals have been detected at relatively low concentrations in wastewater treatment plant (WWTP) effluents, surface water, seawater and groundwater all over the globe [6–9]. As an example,

Table 1 shows a summary of the concentration of some PhACs in WWTP influents, effluents, rivers and groundwater detected in some countries in the last five years.

Table 1 Average concentrations (ng L^{-1}) of various pharmaceuticals published in the literature between 2009 and 2014

Family	**Compound**	**WWTP Influent (ng L^{-1})**	**WWTP Effluent (ng L^{-1})**	**Surface Water (ng L^{-1})**	**Groundwater (ng L^{-1})**
Analgesics and anti-inflammatories	Acetaminophen	243[a]	16720[a]	338[a]	nd[f]*
	Codeine	800[a]	142[a]	19[a]	nd[f]
	Diclofenac	436[a]	376[a]	52[a]	225[f]
	Ibuprofen	3812[a]	nd[a]	380[a]	61[f]
	Naproxen	4240[a]	150[a]	156[a]	0.4[f]
	Salicylic acid	295[b]	30[b]	70[b]	110[f]
	Phenazone	nd[a]	49[a]	12[a]	24[f]
Antibiotics	Azythromycin	205[a]	1032[e]	4.6[d]	16[f]
	Ciprofloxacin	252[a]	67[c]	28[e]	17[f]
	Clarithromycin	459[a]	238[e]	89[e]	11[f]
	Erythromycin	63[a]	677[e]	174[e]	29[f]
	Ofloxacin	309[a]	277[e]	9[d]	30[f]
	Sulfamethoxazole	768[a]	140[e]	0.5[d]	13[f]
	Tetracycline	nd[a]	171[e]	29[e]	4[f]
Antihistaminics	Cimetidine	166[a]	32[e]	17[e]	4[f]
	Loratadine	nd[a]	7[e]	0.4[d]	nd[f]
	Ranitidine	592[a]	120[c]	18[d]	1[f]
Antihypertensives	Losartan	366[a]	75[a]	34[a]	-
	Irbesartan	1096[a]	1526[a]	139[a]	-
	Valsartan	3696[a]	574[a]	734[a]	-
Beta-blockers	Atenolol	2644[a]	118[e]	63[e]	nd[f]
	Metoprolol	nd[a]	97[e]	5[d]	191[f]
	Propanolol	172[a]	33[c]	6[d]	nd[f]
Diuretics	Furosemide	960[a]	1120[e]	174[e]	147[f]
	Hydrochloro-thiazide	1632[a]	1100[c]	8[e]	262[f]
	Torasemide	48[a]	32[a]	12[a]	-
Lipid regulators	Bezafibrate	53[b]	17[b]	4[b]	7[f]
	Gemfibrozil	924[a]	420[c]	2[e]	209[f]
	Atorvastatin	182[a]	3[e]	2[e]	3[f]
	Pravastatin	152[a]	nd[e]	8[d]	2[f]
Psychiatric drugs	Carbamazepine	95[a]	97[c]	5.9[d]	115[f]
	Diazepam	16[a]	19[e]	6.5[e]	18[f]
	Fluoxetine	23[a]	8.7[c]	4.7[d]	nd[f]
	Lorazepam	143[a]	115[e]	4[d]	15[f]
	Paroxetine	nd[a]	7[e]	1[d]	0.4[f]
	Venlafaxine	528[a]	376[a]	43[a]	-

* nd: not detected

(a)[10]
(b) [11]
(c) [12]
(d) [13]
(e) [14]
(f) [15]

After the drug administration, the unchanged parent compound and, in many cases, active and inactive metabolites are excreted and released into the municipal sewage systems (see Figure 1). As conventional WWTPs are not specifically designed to remove these compounds, many of them go through wastewater treatment plants either as unchanged compounds or as transformation products [16]. Veterinary drug usage for treating domestic animals, livestock, and aquaculture is another route of entrance, whether through direct deposition on land via faeces or through runoff to receiving waters: solid waste, such as sludge, containing pharmaceutical residues can result in leaching into the soil where sludge is applied, or lead to runoff to receiving waters [1]. Agriculture and aquaculture also consume large quantities of specific pharmaceutical compounds, such as antibiotics and hormones for growth promotion, therapeutic treatment, or disease prevention [17].

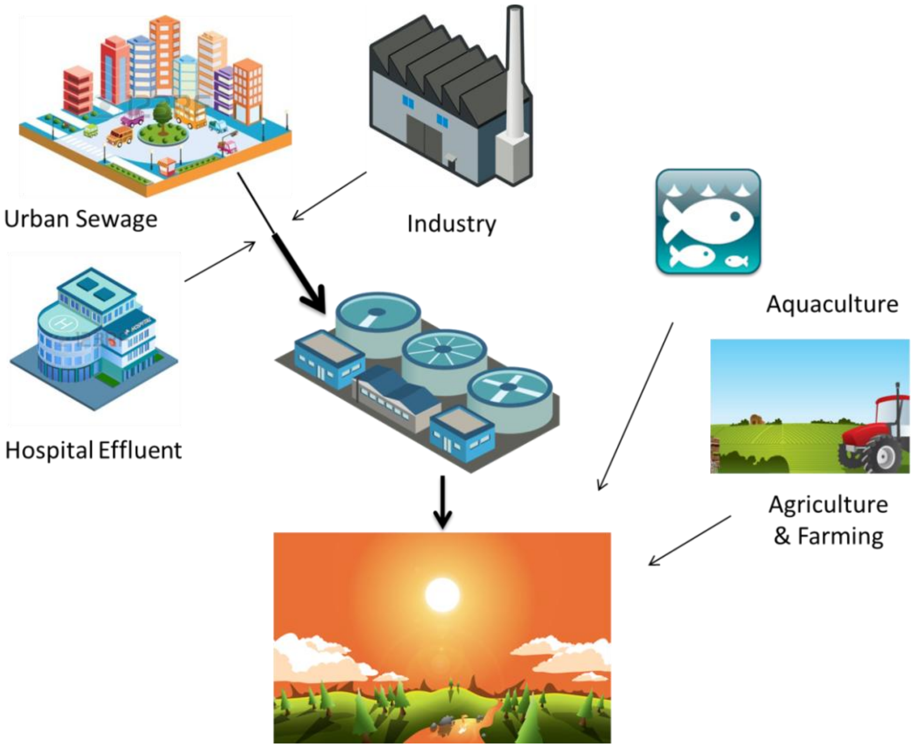

Figure 1. Sources of emerging contaminants in the aquatic environment

Even though at relatively low levels, pharmaceuticals can be found in the natural ecosystems: their generally swift degradation rates, are exceeded by their introduction rates in the aquatic environment and thus, they are considered pseudo persistent contaminants [18]. One of the main concerns related to the presence of pharmaceutical compounds in the environment is that they are biologically active (they are specifically designed to target specific metabolic and molecular pathways in humans and animals), resulting in unexpected effects in non-target aquatic organisms [19,20]. Effects on wildlife of chronic exposure to low levels and to mixtures of compounds, in some cases with the same mode of action, are still uncertain [2].

Endocrine Disruptors

According to the International Program on Chemical Safety [21], "an endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations", which means that these kind of chemicals may interfere with normal hormone action, and in doing so they can alter endocrine function to a point where it leads to adverse effects on human and wildlife health [22]. This definition comprises those compounds that are able to mimic or antagonize the effects of endogenous hormones, such as oestrogens and androgens, or to disrupt synthesis and metabolisms of endogenous hormonal receptors, and this definition includes a wide spectrum of chemicals with very different properties, sources and fates in the environment, including natural and synthetic hormones, surfactants, pesticides, antibacterials, personal care products, cosmetics, plastics, textiles and construction materials [23].

Despite the differences between chemical properties and physiological effects in wildlife, EDCs share some features with PhACs. As in the case of PhACs, EDCs are also released into the environment through wastewater treatment discharges and surface non-point runoff, but they also come from atmospheric deposition of particulates and aerosols, urban and industrial use of cleaners containing surfactants, or agrochemicals containing surfactants [23]. Table 2 presents a list of some of these EDCs and related compounds, and their concentration in influents, effluents, surface water and groundwater reported in the last five years. The range of concentrations is very variable: from the low ng L^{-1} to several μg L^{-1}.

Table 2 Average concentration (ng L^{-1}), unless otherwise indicated, of endocrine disruptors published in the literature between 2009 and 2014. The data were compiled from(a)[24], (b) [25] (median values), (c) [26] (median values), (d) [27], (e) [28], (f) [29], and (g) [30] (median values).

Group	**Compound**	**WWTP Influent (ng L^{-1})**	**WWTP Effluent (ng L^{-1})**	**Surface Water (ng L^{-1})**	**Groundwater (ng L^{-1})**
Natural and synthetic estrogens and conjugates	Estradiol	5^a	5^a	1^a	0.7^d
	Estrone	15^a	5^a	7^b	0.7^d
	Estriol	26^c	nd^{a*}	0.5^a	-
	Ethinylestradiol	2.5^c	nd^a	8^b	1.2^d
Antimicrobials/disinfectants	Triclorocarban	nd^a	nd^a	10^b	-
	Triclosan	23^a	8^a	100^b	$1\text{-}345^f$
Preservatives	Methylparaben	67^a	22^a	8^a	20^e
	Ethylparaben	222^a	2^a	13^b	-
	Propylparaben	209^a	7^a	30^b	-
	Benzylparaben	nd^a	nd^a	0.25^a	-
Plasticizer	Bisphenol A	267^a	104^a	106^b	79^e
Alkylphenolic compounds	Nonylphenol	128^a	34^a	850^b	83^e
	Octylphenol	339^a	23^a	125^b	1^e
	Nonylphenolmonocarboxylate	772^a	1947^a	1342^b	263^e
Anticorrosives	1H-Benzotriazole	864^a	555^a	659^b	24^e
	Tolytriazole	4174^a	3449^a	718^b	23.4^g
Organophosphorus flame retardants	Tris (butoxyethyl) phosphate	1989^a	1345^a	2563^b	-
	Tris (chloroisopropyl) phosphate	1209^a	3417^a	831^b	-
	Tris (2-chloroetyhl) phosphate	302^a	549^a	1048^b	-

* nd: not detected

In the living organisms, natural hormones are biologically active at very low concentrations, which suggests that some unwanted effects may occur when aquatic organisms are exposed to natural and synthetic hormones at environmental concentrations [31]. Many other EDCs are generally believed to have low binding affinity for the oestrogenic receptors when compared to natural and synthetic hormones [32], and thus are less potent as endocrine disruptors. However, their environmental concentrations often reach concentrations in the μg L^{-1} range, so they constitute an issue of concern.

Literature has extensively reported environmental concentrations PhACs and EDCs in inert compartments: water, sediments, sludge, soil, etc. In fact, these studies have led to regulatory measures for some of these compounds in the European Union [33], such as the anti-inflammatory diclofenac or the synthetic hormones EE2, which have been included in the so called ‘watch list’ of priority substances under the Water Framework Directive for the “specific purpose of facilitating the determination of appropriate measures to address the risk posed by these substances [34]. In the US, the Drinking Water Contaminant Candidate List also contains several PhACs and EDCs, including antibiotics, and hormones [35]. Other PhACs, including some antibiotics, anti-inflammatories and psychiatric drugs, have been classified as high priority pharmaceuticals to the water cycle by the GWRC, Global Water Research Coalition [36].

A vital step in the impact assessment of emerging contaminants in aquatic ecosystems is to discern whether these compounds are also present in biological tissues. For instance, the European Medicines Agency (EMA) stipulates that an initial screening phase of the risk assessment process involves identifying the environmental exposure of pharmaceuticals based on their potential for bioaccumulation and persistence in the environment [37]. For that reason, the development and validation of analytical methods for extraction and determination of PhACs and EDCs in aquatic organisms is very pertinent. Analytical methodologies to detect these contaminants at trace levels in biological matrices have developed significantly in the last years. Next section a will expand on this matter whereas chapter 3of this dissertation gathers the research performed to this respect in the frame of the thesis.

II. ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF PHARMACEUTICALS AND ENDOCRINE DISRUPTORS IN BIOTA

Sample Extraction

For the analysis of environmental samples, the target analytes must be previously isolated and concentrated from the matrix. Sample preparation is particularly critical when biota samples are involved due to the low concentration of analytes and to the higher complexity of these matrices, especially rich in undesirable components that could interfere with the analysis (lipids, proteins, and pigments). Considering the aforementioned problems, along with the widely varying physicochemical properties (e.g., polarity, solubility, and stability) between groups of pharmaceuticals and EDCs (see

Table 3), one of the main challenges for the development of a good analytical method is to obtain efficient extraction for the target compounds [38,39], as it usually requires extensive and lengthy sample preparation [40].

Method development for multiclass compound determination in biota is difficult, as it requires a compromise in the selection of the experimental conditions, which usually are not the best for all the analytes studied. The more differences between compounds, the greater the difficulty in finding a single extraction procedure for all analytes with acceptable recoveries [39]. Consequently, most of the methods reported focused on single compounds or a family of compounds [41–47], with only a few exceptions in the last years [48–53]. However, in view of the scope of this thesis, the evaluation of the fate of these contaminants with multi-residue methods as broad as possible was deemed necessary; in particular for those organisms less studied (invertebrates, biofilm).

Table 3 Physico-chemical properties of selected PhACs and EDCs extracted from the literature (Sources: ChemAxon and Drugbank)

Compound	**Molecular mass (g/mol)**	**Log Kow**	**Log Dow (pH 7.4)**	**pKa**	**Solubility (mg/L)**
Pharmaceuticals					
Diclofenac	296	4.3	1.4	4.0	2
Erythromycin	734	2.6	1.2	8.4	2000
Hydrochlorothiazide	297	-0.6	-0.6	9.1	722
Ibuprofen	206	3.8	1.7	4.9	21
Propanolol	259	2.6	0.0	9.7	62
Sertraline	306	5.2	2.4	9.9	0.0001
Venlafaxine	277	2.7	0.8	8.9	0.2
Endocrine Disruptors					
1H-benzotriazole	119	1.2	1.3	8.6	20000
Bisphenol A	228	4.0	4.0	9.8	200
Ethynilestradiol (EE2)	296	3.9	3.9	10.3	11
Methylparaben	152	1.7	1.7	8.5	2500
Nonylphenol	220	5.7	5.7	10.3	5
Tris (2-butoxyethyl) phosphate	398	3.9	3.9	-9.9	1000
Triclosan	290	4.7	5.1	-3.8	10

Biota samples can be liquid (such as bile or plasma) or solid (whole body, muscle, liver, etc.), which require very different analytical approaches. In the case of non-solid biological matrices, the main objective is to pre-concentrate the analytes previously removing unwanted molecules, such as proteins and lipids. The most common method applied is the use of solid-phase extraction (SPE) cartridges, with sorbents such as cartridges Oasis HLB or mixed-mode ion-exchange sorbent (MCX, MAX, etc., specific for basic or acidic compounds) or Strata-X, as they have shown an efficient performance during the extraction of a wide range of pharmaceuticals [54–59]. Alternative techniques include centrifugation step with an organic solvent [60,61], Empore^{TM} mixed phase disks [62],andsolid-phase micro-extraction (SPME) [63]. In this thesis, a novel procedure for the analysis of a psychiatric drug in fish plasma was selected, the Ostro^{TM} plate (Waters, Milford, USA), due to the excellent extraction recoveries, negligible matrix effects and user-friendly application (Chapter 5).

Extraction of pharmaceuticals from solid matrices usually requires extensive procedures, such as ultra-sonication [42,53,59,64–67], microwave-assisted extraction (MAE) [68–70], microwave-assisted micellar extraction (MAME) [71,72], centrifugation [56,57,73–75], QuEChERS[50] and rotary extraction [41,48,76–78] (see Figure 2). A very attractive technique is SPME, which has been applied to the analysis of fish muscle of *in vivo* studies. The procedure consists in inserting a fibre in the fish muscle to adsorb the target analytes. After a short exposure, the fibre is extracted from the organism and desorbed through agitation in an organic solvent. In the majority of the cases, results were comparable with those obtained by a solid–liquid extraction to determine the extractable concentrations of target analytes in fish muscle, although its application has been limited to a few studies [63,79,80]. An increasing trend is the use of pressurized liquid extraction (PLE) for the determination of contaminants in solid samples [43,46,49,52,81,82]. This technique involves extraction with conventional solvents at high pressure (100–140 bar) and temperatures (80–180 °C), without reaching their critical point, to increase the extraction of pollutants from solid samples [83,84].

During the development of this thesis, several of these extraction methods were applied. For instance, PLE was used for the extraction of fish tissues, as it allowed a pre-purification step during the extraction, which facilitated the lipid removal [82]. It was also applied for the extraction of biofilm samples after obtaining better repeatability in the recoveries when compared to ultra-sonication [85]. However, a sonication probe applied to small amounts of solvent was deemed appropriate for the extraction of small invertebrates, when sample size was a limiting factor [86].

Sample Purification

Direct analysis after extraction is not always possible for the quantitative determination of target compounds in biota samples without further purification to clean the extracts, as they may contain matrix co-eluents, which could spoil or hinder an accurate detection. The great majority of the clean-up procedures are based on SPE, as it is relatively swift, requires small quantities of solvent, and can absorb compounds with very different physicochemical properties. Florisil, alumina, or silica gel columns have been used especially in the case of fatty samples [41,46,67,78,87,88], and it has been sparingly applied for purification of PhACs in fish tissues [61]. More often, polymeric sorbents mixed with polymeric and cation-exchange sorbents are applied to the analysis of PhACs in environmental matrices. SPE Oasis HLB cartridges have been frequently applied to pre-concentrate the target analytes and reduce the presence of co-eluents, as they are able to extract acidic, neutral, and basic compounds with high efficiencies in algae, invertebrates, and fish [43,49,66,72,89,90].

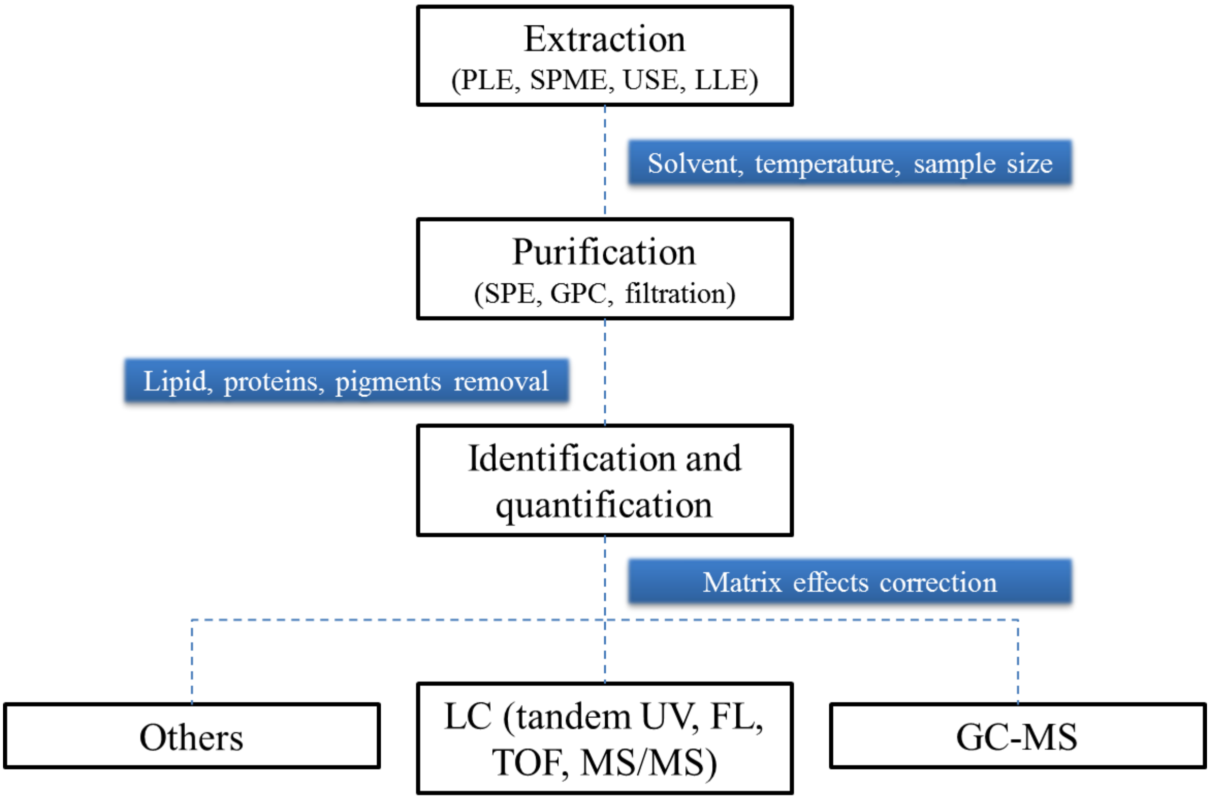

Figure 2 Basic analytic steps in methodologies for determination of pharmaceuticals in biota and most relevant factors affecting each step

Removal of lipids from the biota extract is a crucial step, as the fat content often constitutes between 5% and 50% of the samples [82]. Gel-permeation chromatography (GPC) (also known as size exclusion chromatography) has the advantage of good separation of large molecules from the small molecules with a minimal volume of eluate, and the column usually can be used over several months with no detriment on clean-up capacity [84]. This technique has been used in tandem with other clean-up strategies, such as SPE [43,69,78], liquid–liquid extraction with hexane to eliminate non-polar matrix residues, and freezing the sample extracts in acetonitrile to precipitate remaining lipids such as cholesterol [91].

During this thesis, several purification methods were tested according to the requirements of each biological matrix. In the case of the analysis of fish tissues (with values of lipids content varying between 10 and 50%), GPC with an EnviroPrep (Agilent) column was compared to SPE with Florisil and Oasis HLB cartridges, and GPC purification step was selected as clean-up method, as it provided satisfactory results for most of the target compounds and reduced considerably interferences during analysis [92]. In contrast, for the analysis of biofilm, a matrix with lower lipid content (< 10%), SPE was selected, as it had a lower solvent consumption and preparation time [85] (Chapter 3). Finally, for the analysis of fish plasma samples and small invertebrates, proteins and phospholipids removal was of utmost importance, so the Ostro^{TM} plate was selected [86] (Chapter 3 &5).

Sample Separation and Detection

Analytic techniques to detect PhACs and EDCs at trace quantities in environmental matrices have advanced significantly in the last few years. Most of the analytic methods in biota matrices found in the literature are based either on liquid chromatography (LC) [48,49,51–53,58,65,75,76,82,93–95], or gas chromatography (GC) [41,61,69,74,77,81,91,96] in combination with mass spectrometry (MS) or mass spectrophotometry detection, but alternative techniques have also been described and applied, for instance, immunoassays [73] and biosensors [97].

Gas Chromatography

Gas chromatography–tandem mass spectrometry (GC–MS) has been mostly limited to compounds that are volatile enough to be transferred into the gas phase or that can be derivatised to volatile species [74,91]. This derivatisation step may be an arduous process in complex sample matrices like biota, although it has the advantage of being less susceptible to the matrix effects than other techniques. GC has been adeptly applied for the detection of psychiatric drugs [41,81], anti-inflammatories [61,74,96], surfactants [47,69,77], flame retardants [46,78], and hormones [69,70,74].

Liquid Chromatography

LC has grown to be a fundamental separation method for the determination of polar and thermo-labile compounds. This separation technique has the advantage over GC of avoiding the derivatisation step previously mentioned. On the contrary it can be seriously affected by matrix effects derived from interfering compounds extracted from the biological samples, particularly when applied in combination with mass spectrometry detection [98].

Most analysis of PhACs and EDCs from biota samples has been conducted in reversed-phase columns, such as C8 and C18 [55,56,71,75,76,99,100], although the use of normal-phase chromatography has also been reported [45,77]. Recently, nano-liquid chromatography, a novel chromatographic technique which uses capillary columns of small internal diameter, was successfully applied to the analysis of biota samples of small size [50,101]. Acetonitrile and methanol [43,57,102] have often been used in mobile phases for chromatographic separation of pharmaceuticals, often accompanied by modifiers, such as formic acid [102,103] or ammonium hydroxide [58] to stabilize the pH and obtain a better peak shape and reproducibility and to increase the ionization efficiency when an MS is used as detection method.

LC in tandem with spectrophotometric detection, such as diode-array detection, ultra-violet (UV) absorbance detection, and fluorescence detection, has been used for the detection of surfactants in algae and fish tissues [45]. Studies have reported the determination of quinolones in fish, crustaceans, and bryophytes with fluorescence detection, achieving limits of detection (LODs) between 1 and 7 ng g^{-1} [164,87,104,105]. These applications are, however, limited to compounds with specific physicochemical characteristic, such as the presence of chromophores or fluorescent properties.

Even though LC tandem diode-array, UV absorbance, or fluorescence detection may be a cost-effective technology, these techniques have been progressively replaced by mass spectrometric detection in the determination of emerging contaminants in environmental matrices, as it provides high selectivity, specificity, and sensitivity [2,98,106].

MS methods applied to environmental matrices comprise diverse technologies, such as single-quadrupole MS [61,77,96,107], tandem MS (MS/MS), triple quadrupole MS/MS [43], and time-of-flight MS [54,55,108]. LC-MS/MS is preferred to LC–MS for the measurement of PhACs and EDCs in biota samples, as the fragmentation ions allow increasing the specificity of the analysis. Consequently, most of the analytical methods developed for the analysis of pharmaceuticals in biota are based on LC–MS/MS [43,48,49,53,56,58,59,71,76,82,90,93,109–112].

The most commonly used ionization method for LC interfaces is atmospheric pressure ionization (API), which includes electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). ESI appears to be the most employed mode of ionization in pharmaceutical determination, since it is particularly suitable for both polar and nonpolar analytes and for thermo-labile substances. However, it is known to be more susceptible to signal suppression than APCI [98]. Unfortunately, only a few of these compounds can be efficiently ionized by APCI [43], and most of them have to be analysed by ESI. During the development of this thesis, ultra-performance liquid chromatography (UPLC) in tandem with a triple quadrupole/linear ion trap (LIT) MS was applied, for all matrices, and a high resolution Orbitrap MS was used to accurately identify diclofenac in invertebrates.

Matrix Effects

As mentioned in the previous section, a downside of LC–MS and LC–MS/MS is that co-extracted matrix components tend to interfere with API interfaces. Matrix effects are usually caused by co-eluted matrix components that have common ions with target analytes, as a competition occurs between matrix co-eluents and analyte ions for gas-phase emission during the ionization. The matrix effects result in suppression or enhancement of the signal of the target analyte during the ionization process, which can acutely compromise the accuracy of quantitative data and affect the LODs in real samples. This is particularly acute at low analyte concentration, which is a common situation when analysing emerging contaminants in biota [113,114]. Chu and Metcalfe [43] reported signal suppression between 19% and 39% when analysing paroxetine, fluoxetine, and norfluoxetine in whole fish, whereas Dussault et al. [113] reported effects ranging from 14% signal suppression to 25% signal enhancement in the analysis of selected pharmaceuticals in invertebrates.

Different approaches have been used to correct these variations on ionization efficiency. Some authors have obtained good results when quantifying with external sample calibration (spiked standards in pure solvent), probably because the analysis was reduced to a small number of compounds and the extraction methods applied were very selective [96,99,115]. Standard addition, where the standard is added directly to the aliquots of analysed sample, is the most effective approach for compensating matrix effects, although it can be difficult and time-consuming when a large number of samples are considered. A standard addition method was used to evaluate the influence of matrix effects on the analysis of psychiatric drugs (and their corresponding labelled compounds) in fish tissues by Chu and Metcalfe [43]. Since results showed that the internal standards were subjected to the same recoveries and matrix effects as the target compounds, the reduced response of the analyte was compensated by the internal standard response. In fact, the most popular approach consists in the addition of internal standards (isotopically labelled compounds), which can correct variations during instrumental detection [43,58,76,81,103]. Ideally, there should be one internal standard for each analyte that elutes from the chromatographic column at the same time as the native compounds, as there is a gradual decrease in the matrix effect with increasing retention time [116]. However, this option is not always possible, as they are quite expensive and not available in all cases [60,89]. For this reason, other corrective measures are becoming increasingly used, such as matrix-matched calibration, where the calibration standards are prepared in blank or “clean” matrix and subjected to the extraction procedure that is used for the samples [48,55,56,68,74,107]. This also has some drawbacks, as uncontaminated matrix for the preparation of a matrix-matched calibration is usually difficult to obtain and the generation of a calibration curve for each type of sample is unfeasible when analysing samples of very different origin [117]. An alternative strategy has been applied, namely, internal sample calibration [118,119], where the quantification uses a calibration curve prepared with spiked sample extracts and internal standard addition. The later strategy was selected as the most effective to correct the matrix effects during method development performed during this thesis, as the characteristics of the organisms involved, even within the same species, were extremely different [82,85,86].

III. BIOACCUMULATION OF PHARMACEUTICALS AND ENDOCRINE DISRUPTORS IN AQUATIC BIOTA

The incessant release of emerging contaminants into freshwater ecosystems could affect a broad variety of organisms: from bacterial communities to fish, as well as aquatic plants and insect larvae. The impact of these pollutants can be evaluated by measuring the bioaccumulation of the target compounds in the organisms [37], or/and by assessing the toxicological effects, which will be examined in the next section.

Biological factors such as life cycle, reproductive strategy, feeding type, and habitat preference significantly influence the sensitivity of the species to contaminants [120–122]. Ubiquity and the significant role of fish as a carrier of energy from lower to higher trophic levels make them potentially more susceptible to pollutants bioaccumulation and thus, they have been more commonly studied. Nevertheless, other organisms, such as invertebrates and algae, also play a critical role in the natural flow of energy and nutrients in aquatic systems. They also possess the capacity to integrate rapid environmental variations, which validates their position as indicator species of environmental change, so they should be taken into consideration for the monitoring of PhACs and EDCs in biota. However, bioaccumulation in aquatic invertebrates in natural ecosystems has been less frequently investigated, although this trend is starting to change and more studies about invertebrates and algae have been published in the last five years [45,47,49,51,70,78,90,93,104,123,124].

Conventionally, the main factor to predict or to explain bioaccumulation of a compound is the octanol-water partitioning coefficient (K_{ow}), a measure of hydrophobicity that drives sorption and accumulation. It is generally accepted that substances with log K_{ow} values higher than or equal to 3 have the potential to bioaccumulate in biological tissues. Many EDCs have moderate to high log K_{ow} values (see Table 3), so they often end up sorbed to sediments or suspended organic material, or in the lipid compartment of wild organisms. Therefore, log K_{ow} of EDCs and tissue lipid content of organisms provide a strong basis for estimating tissue/water partitioning [125]. However, this may not be fully applicable to pharmaceuticals, which are in general quite polar compounds [126,127] (see Table 3), and in many cases ionisable, so the environmental pH is going to determine their ionic form. Therefore, in both cases but particularly when assessing bioaccumulation of pharmaceuticals, one must take into consideration other issues, such as rates of metabolism in various organisms and the accumulation behaviour of the metabolites, hydrogen bonding and van der Waals forces, and the uptake and depuration kinetics [128,129].

Previous Reports of Accumulation in Wildlife

Table 4 presents a summary of studies performed in wild freshwater biota in the last ten years, excluding the ones related to this thesis. First studies of PhACs in freshwater fish were published by Brooks et al., [41] and Ramirez et al. [48,100], in an effluent-dominated stream in Texas, USA reporting concentrations of psychiatric drugs and other PhACs in fish brain tissue and whole body homogenate, respectively . Simultaneously, the presence of psychiatric drugs in fish samples collected from Hamilton Harbour, Canada were published by Chu and Metcalfe [43]. In all these studies, concentrations were in the low ng g^{-1} range.

In Europe, initially most of the data on the presence of pharmaceuticals, mainly antibiotics, in aquatic organisms emerged from aquaculture and food control studies closely related to human consumption and regulation demands [64,107,130].For instance, around 100 ng g^{-1} of antibiotics were reported in fish muscle in Spain by Berrada et al.,[107] and Cháfer-Pericás et al.,[131]. However, in the last few years, the interest in this subject have exponentially increased, and many reports have been published in France [123], Italy [132], United Kingdom [90], Czech Republic [95,124], as well as other countries like China [52,59] or Japan [53]. Nevertheless, almost 70% of the published studies refer to freshwater fish, followed by invertebrates (~15%), mussels and biofilm (~15% together). Similar tendency can be observed in the analysis of marine biota, where fish samples correspond to more than 50% of the studies, followed closely by molluscs, and with crustaceans and macroalgae in very small proportion.

Table 4.- Occurrence of PhACs and EDCs in freshwater biota. Data compiled from studies published in the literature between 2005 and 2015

Organism	Matrix	Therapeutic Class	Compounds	Concentration ($ng\ g^{-1}$ or $ng\ L^{-1}$)	Country	Reference
Fish	Homogenate	Psychiatric drugs	Paroxetine, Fluoxetine & Norfluoxetine	nd*- 1	Canada	43
		Hormones	17α-Ethinylestradiol	1- 2	Canada	133
			Estrone, 17β-Estradiol 17α-Ethynylestradiol	< 10	China	69
		Surfactants	4-tert-Octylphenol, 4-Cumylphenol, 4-Nonlyphenol	< 80	China	69
	Muscle	Analgesics/Anti-inflammatories	Diclofenac	< 1.2	China	52
		Antibiotic	Roxithromycin, Erythromycin	< 8	China	52
		Antihistamine	Diphenhydramine	0.6 - 1	USA	48
		Antihypertensive	Atenolol, Metoprolol, Propanolol, Diltiazem	0.1 - 0.3	USA	48
		Psychiatric drugs	Carbamazepine	< 0.2	China	52
			Carbamazepine, Fluoxetine, Norfluoxetine, Sertraline	0.8 - 5.1	USA	48
			Fluoxetine, Sertraline, Norfluoxetine and Desmethylsertraline	0.1-2	USA	41
	Liver	Antibiotics	Roxithromycin, Erythromycin	< 40	China	52
		Analgesics/Anti-inflammatories	Diclofenac	< 9	China	52
		Psychiatric drugs	Fluoxetine, Sertraline, Norfluoxetine and Desmethylsertraline	1-12	USA	41
			Carbamazepine, Sertraline, Norsertraline	< 15	Japan	53
			Carbamazepine	< 2	China	52
	Brain	Antibiotics	Roxithromycin, Erythromycin	< 28	China	52
		Analgesics/Anti-inflammatories	Diclofenac	< 4	China	52
		Psychiatric drugs	Fluoxetine, Sertraline, Norfluoxetine and	1-30	USA	41

			Desmethylsertraline			
			Fluoxetine, Norfluoxetine, Sertraline, Norsertraline, Paroxetine, Citalopram, Fluvoxamine, Duloxetine, Venlafaxine, and Bupropion	nd - 6	USA	[42]
			Carbamazepine, Sertraline, Norsertraline	< 10	Japan	[53]
	Gills	Antibiotics	Roxithromycin, Erythromycin	< 25	China	[52]
	Plasma	Antihistamine	Diphenhydramine	< 25	USA	[134]
		Analgesics/Anti-inflammatories	Diclofenac, Ibuprofen	< 23	Japan	[53]
		Hormones	17α-Ethinylestradiol	< 3	USA	[62]
		Psychiatric drugs	Carbamazepine, Norfluoxetine, Sertraline, Desmethylsertraline	nd - 14	USA	[134]
			Carbamazepine, Sertraline, Norsertraline	< 1	Japan	[53]
			Citalopram, Fluvoxamine, Paroxetine, Sertraline, Venlafaxine	< 4	USA	[62]
		Antibacterial	Triclosan	< 0.3	USA	[135]
Invertebrates	Homogenate	Analgesics/Anti-inflammatories	Diclofenac	nd - 33	USA	[134]
			Diclofenac	not quantifiable	UK	[90]
		Antibiotics	Trimetoprim		UK	[90]

			Azythromycin, Clarithromycin	nd - 85	Czech Republic	[124]
		Antihistamine	Diphenhydramine	< 7	USA	[134]
		Psychiatric drugs	Fluoxetine	nd - 79	USA	[93]
			Carbamazepine, Sertraline, Fluoxetine, Desmethylsertraline	nd - 140	USA	[134]
			Citalopram, Sertraline	< 5	Czech Republic	[124]
			Carbamazepine	< 2	France	[123]
			Carbamazepine, Diazepam	< 9	UK	[90]
		Surfactants	4-tert-octylphenol, 4-n-octylphenol, 4-n-nonylphenol, nonylphenol	< 1300	Spain	[136]
Periphyton	Homogenate	Analgesics/Anti-inflammatories	Diclofenac	< 4	USA	[134]
		Antihistamine	Diphenhydramine	18	USA	[134]
		Psychiatric drugs	Carbamazepine	1.3	USA	[134]
		Antibacterials	Triclosan, Triclocarban	50 - 400	USA	[137]

*nd: non-detected

In the case of EDCs, first reports of EDCs go back to the nineties, with the detection of alkylphenols in algae and fish in the µg g^{-1} range [45], relatively high concentrations when compared to PhACS. Although the concentration of alkylphenols in freshwaters decreased drastically in Europe after the restrictions or ban in many countries [142] in the late nineties, especially after the implementation of the WFD in 2002, they are still being used in substantial amounts in institutional and industrial applications because of lower production costs, and are still detected in the aquatic environment [143].

More recent studies include the detection of hormones in fish at very low concentrations [69,70,133], antibacterials such as triclosan [137–140], and surfactants [69,77,136,141] in fish, algae, invertebrates, and even dolphin plasma.

Accumulation in Different Biological Tissues

Many of the studies about bioaccumulation in fish so far have focused on muscle tissue or whole body concentrations [49,69,93,102], in particular in the early studies. However, several studies [41,53,59,76,95,144] have reported concentration range of some compounds higher in fish liver and brain tissues than in muscle or whole body. In fact fish under controlled exposure have shown antidepressants concentrations and the analgesic diclofenac exhibited a 25-fold increase in tissues such as liver and brain versus muscle [96,145]. This suggests that bioaccumulation studies should consider the most probable target organ, in particular in the case of pharmaceuticals, where well-studied pharmacokinetics and pharmacodynamics may provide useful information non-available for other contaminants. Higher bioconcentration factors could be related to their therapeutic mode of action, that is, a psychiatric drug like sertraline should be expected to be present at higher levels in the brain [41].

Accumulation of PhACs in plasma has acquired a significant interest lately. The validation of the Fish Plasma Model (FPM) [146,147] for pharmaceuticals in particular has raised a lot of interest. FPM estimates fish plasma concentrations of drugs based on physicochemical properties (lipophilicity) and the environmental concentration, and compares these concentrations with therapeutic human plasma concentrations. When the fish plasma concentration is in the same range as the human therapeutic plasma concentration, the compound is assumed to have potential adverse effects in aquatic organisms. This assumption is based on the hypothesis that the main targets (receptors and enzymes) of the drug are similar between humans and fish [148]. A recent study showed that fluoxetine, an antidepressant, was able to alter fish behaviour at the human therapeutic plasma concentration [149]. However, more research is needed regarding the implications of internal concentrations and its effect in the aquatic organisms exposed to these compounds in the environment. This issue will be further discussed in section IV. This thesis discusses the accumulation of pharmaceuticals in whole body homogenate and liver samples in wild biota (Chapter 3), but also includes differential accumulation of a psychiatric drug, oxazepam, in brain, liver, muscle and plasma after an exposure experiment under controlled conditions (Chapter 5).

Biomagnification in the Trophic Chain

The issue of bioaccumulation in different trophic levels is also addressed in this thesis. A few studies have investigated the increase in concentration of PhACs and EDCs at successive trophic levels in the food chain, i.e., biomagnification. One example is the study by Vernouillet et al.,[110], who demonstrated carbamazepine bioaccumulation of under controlled conditions at sub-lethal levels in two levels of a trophic chain, but not in the highest. The same exercise was performed with the surfactant nonylphenol, but only traces could be found in the second level (a crustacean fed with NP-treated algae) possibly indicating an efficient NP metabolization [150], also in agreement with another study where no trophic magnification for nonylphenol and some related compounds was observed in the field [77]. More recently, Du et al.,[134] examined the occurrence of diphenhydramine and carbamazepine in various aquatic species (periphyton, invertebrates and fish) in an effluent-dependent stream, and observed the opposite effect: trophic dilution. Another study showed that although algae could accumulate the β-blocker propranolol, significant bioaccumulation did not occur in the upper trophic levels, indicating the inability of this PhAC to transfer through aquatic food chain [151].

In this thesis, a study in a small river heavily impacted by the discharge of a nearby WWTP confirmed the results obtained by Du et al., and Ding et al., as no biomagnification of PhACs were observed. However one flame retardant TBEP, with endocrine disrupting activity, was measured in increasing concentrations along three trophic levels (Chapter 4).

IV. ENVIRONMENTAL EFFECTS OF PHARMACEUTICALS AND ENDOCRINE DISRUPTORS IN AQUATIC ECOSYSTEMS

Several reviews have been published on the assessment of ecotoxicity and environmental risk of PhACs [6,19,106,152–157] and EDCs [158–164]. Many examples of unintended side effects due to exposure to PhACs and EDCs are well-known, such as the feminization of male fish in UK Rivers attributed to the oestrogen derivate ethinylestradiol in combination with other hormones. Another example is the toxicity of the anti-inflammatory drug diclofenac, which caused the death of millions of vultures in Asia [165–167]. Other effects observed after controlled exposure ranged from behavioural modifications [168] to histopathological changes [96], reproductive alterations [93], mortality [121], and modifications of gene expression and proteomic changes [54,66,169,170]. All these data support the hypothesis that severe effects may occur when organisms are exposed to environmentally relevant concentrations of these contaminants [60].

In this thesis, two aspects in terms of effect in biota related to the presence of pharmaceuticals were studied in depth: i) the promotion of antibiotic resistance genes due to the presence of antibiotic compounds in a field study; and ii) behavioural alterations in fish due to exposure to psychoactive drugs under controlled exposure (Chapter 5).

Antibiotic Resistance in aquatic ecosystem

Microorganisms are greatly affected by the presence of pharmaceutical residues in aquatic systems. The impact of antibiotics on the promotion of antimicrobial resistance could have severe consequences for the structure and function of aquatic ecosystems, as well as human health implications [106]. Antibiotic resistance occurs when one microorganism is able to multiply in the presence of an antibiotic, which is natural process. But the abuse and misuse of antimicrobial agents has led to the proliferation of drug-resistant microorganisms, which is considered by the World Health Organization (WHO) as one of the main threats for public health [171].

The introduction of antibiotics into the environment, including discharges of wastewater effluent contaminated with high concentrations of these compounds, may promote the increase of antibiotic resistance genes (ARGs) and other genetic elements and spread of antibiotic resistance in environmental bacterial communities [171–174]. In this thesis, a tentative attempt was made to assess whether the occurrence of antibiotic compounds and ARGs have an impact in the composition of microbial communities and the promotion of antibiotic resistance in three water supply reservoirs.

Behavioural Alterations in Fish

Due to similarities of many of their physiological processes with those of mammals, fish are the most likely vertebrate organisms to be affected by pharmaceuticals in the aquatic environment. The worst case scenario occurs in the case of long-term or chronic exposure and early life stages as the capacity to metabolize pollutants is lower [2]. Recent studies have shown that environmentally relevant concentrations of psychoactive drugs can affect fish behavioural traits, being ecologically and evolutionarily relevant as a way to predict how individuals respond to environmental changes [103,149,168].

Considering that fish usually respond to stressful situations with very specific behaviours − for instance when they are introduced into a novel environment they dive to the bottom until they feel safe enough to start exploring. One way to assess if their behaviour is altered by continuous exposure to an anxiolytic drug is by measuring if the time it takes for them to explore the new environment is reduced, with the assumption that the drug has the same mode of action than in humans. Under this hypothesis, the last article of this thesis examines the effect in fathead minnow (*Pimephales promelas*) behaviour when exposed to an anxiolytic drug at three concentrations and how this relates to fish plasma (and other tissues) concentrations.

Chapter 2

Objectives

The main goal of this thesis is to investigate the potential bioaccumulation and biomagnification of two groups of emerging contaminants − pharmaceuticals and endocrine disruptors − in freshwater biota, including fish, invertebrates and river biofilm in the natural environment. A second objective is to evaluate the impacts of exposure to these contaminants in the organisms, and the possible correlation between bioaccumulation and toxicological effects.

To accomplish this main goal, the following specific objectives were defined:

- To develop and validate analytical methods to measure up to 61 pharmaceuticals (from 12 therapeutic families) and 22 endocrine disruptors (including 5 groups, such as hormones, flame retardants, parabens, antibacterials and plasticizers; and related compounds such as caffeine) in biological matrices: biofilm, invertebrates, fish tissues,(Chapter 3) and fish plasma (Chapter 5).

- To assess the bioaccumulation and potential biomagnification of these compounds in different trophic levels (biofilm, invertebrates, fish) in the natural environment (Chapter 4).

- To evaluate the impacts of exposure to antibiotics and antibiotic resistance genes (*bla*TEM, *ermB*, *qnrS*, and *sulI*) in the promotion of antibiotic resistance in the bacterial communities of water reservoirs (Chapter 5).

- To determine the bioaccumulation of a psychiatric drug in different tissues and plasma of fish individuals under controlled experimental conditions, and the correlation between that bioaccumulation and the consequential behavioural alterations observed in the fish (Chapter 5).

Chapter 3

Analytical development for the analysis of pharmaceuticals and endocrine disruptors in fish, invertebrates and biofilm

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Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

A new sensitive method based on pressurized liquid extraction (PLE) and purification by gel permeation chromatography (GPC) prior to ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) was developed for the determination in fish homogenate, liver and muscle of twenty pharmaceuticals compounds and metabolites from seven commonly used therapeutic families. An extensive matrix effect evaluation was performed in order to select the best approach when analyzing such complex matrices. Limits of detection (MDLs) for the target compounds were in the range of 0.03–0.50 ng/g for fish homogenate, 0.01–0.42 ng/g for fish muscle, and 0.08–0.98 ng/g for fish liver. The method was applied to fish tissues of eleven fish species from four heavily impacted Mediterranean rivers. Nine compounds from five therapeutic families were measured at concentrations higher than MDLs. Highest levels were found in trout liver, with a maximum concentration of 18 ng/g for carbamazepine, whereas the most ubiquitous compound was diclofenac.

1. Introduction

In recent years, the occurrence, fate, and adverse effects of pharmaceutical residues in aquatic organisms have become a noteworthy issue. In Europe, the legislative proposal for amending the list of priority substances that represent a significant risk to or via the aquatic environment was presented by the European Commission on 31 January 2012, and included for the first time the pharmaceutical substances 17-α-ethinylestradiol (EE2), 17-ß-estradiol (E2) and diclofenac [1]. The U.S. Environmental Protection Agency (EPA) has also included some pharmaceutical substances in the Drinking Water Contaminant Candidate List, such as the antibiotic erythromycin and the estrogenic hormones 17-α-estradiol, estriol and estrone [2]. In addition, many reports have highlighted the potential of pharmaceuticals and/or their metabolites to accumulate in tissues of aquatic organisms, such as crustaceans, molluscs and fish, as a consequence of their chronic exposure in aquatic ecosystems [3–5]. In general, pharmaceutically active compounds (PhACs) are highly hydrophilic, and their bioaccumulation potential might be considered irrelevant, particularly when compared to other pollutants, such as pesticides and persistent organic compounds (POPs). These conventional pollutants have been reported in a vast number of studies to bioaccumulate in different organisms because of their lipophilicity and tendency to bind to organic matter [6–10] and are considered in many priority pollutants lists [11,12]. However, some studies have indicated that the bioaccumulation of PhACs is not only determined by chemical lipophilicity, and other processes should also be considered, such as active transport through biological membranes or uptake and depuration kinetics [13–15].

Analytical techniques used for the detection of PhACs presence at (ultra)trace quantities in environmental matrices have advanced significantly in the last few years and have been summarized in recent reviews [16–24]. Even though an increasing number of analytical procedures have been reported for several therapeutic families in biota in the last years, they are still sparse, probably due to the challenges associated with the complexity of the biological matrices [25]. Groups of PhACs analyzed in biota so far include psychiatric drugs [5,26–29], synthetic hormones [30], and antibiotics [3,29,31,32]. Exhaustive sample preparation followed by sensitive detection techniques is required in these cases, due to the very low concentration of analytes in biological matrices [33]. Both, ultrasonication and pressurized liquid extraction (PLE) have been often used for the extraction of PhACs in aquatic organisms, such as crustaceans, mussels, algae, and fish [27,28,30,34–37]. Regarding the crucial purification step of the sample extract, different

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clean-up procedures have been used: solid-phase extraction (SPE) in Florisil columns [38], Oasis HLB cartridges [39] or techniques like gel-permeation chromatography (GPC) [30,40]. Last step in the analytical process includes the identification and determination of PhACs, usually based on liquid (LC) or gas chromatography (GC) in combination with mass spectrometry (MS) detection, because it provides high selectivity, specificity, and sensitivity required for this type of studies [5,27,29].

This work describes the development, optimization and validation of a method for the determination of 20 multi-class pharmaceuticals and metabolites in fish (Table 1), selected according to their detection frequency in water and sediment in Mediterranean rivers [41,42] as well as to their potential negative effects in aquatic organisms [43–46]. Selected compounds included analgesics and non-steroidal anti-inflammatories (NSAIDs), diuretics, psychiatric drugs, anti-histaminics, antihelmintics, β2-adrenergic receptor agonists and β-blockers. The method developed is based on an extraction step using pressurized liquid extraction (PLE) followed by gel permeation chromatography (GPC) clean-up and ultra performance liquid chromatography–triple quadrupole mass spectrometry (UPLC–MS/MS) for the detection of target compounds. Critical steps in method development involved (i) the selection and optimization of the most appropriate sample pre-treatment step that allowed the simultaneous extraction of selected compounds from fish homogenates, liver and muscle tissues, (ii) lipid removal from the extract to reduce matrix interferences during analysis and (iii) comparison of several strategies to correct the observed matrix effects.

The method was subsequently applied to assess the occurrence of target compounds in fish samples collected from different sites of four Mediterranean rivers in Spain: Ebro, Llobregat, Júcar and Guadalquivir, all subjected to intensive anthropogenic activity. Nine pharmaceutical compounds belonging to five different therapeutic families were detected for the first time in fish from Mediterranean rivers.

2. Materials and methods

2.1. Standards and reagents

High purity grade (>95%) pharmaceutical standards diclofenac, codeine, carbamazepine, citalopram, diazepam, lorazepam, atenolol, sotalol, propanolol, nadolol, carazolol, hydrochlorothiazide, clopidogrel, salbutamol and levamisole were acquire from Sigma–Aldrich. Sertraline and velafaxine were purchased from the European Pharmacopeia (EP). Metabolites 2-hydroxycarbamazepine (2-HydroxyCBZ) and 10, 11-epoxycarbamazepine (10, 11-EpoxyCBZ) were purchased from Toronto Research Chemicals (TRC). Metropolol was obtained from the US Pharmacopeia (USP). Isotopically labeled compounds, used as internal standards, ibuprofen-d_3, diazepam-d_5, ronidazole-d_3 and fluoxetine-d_5 were acquired from Sigma–Aldrich. Atenolol-d_7, carbamazepine-d_{10}, hydrochlorothiazide-d_2, and citalopram-d_4 were purchased from CDN isotopes. Venlafaxine-d_6 was from TRC. Antypirine-d_3 and cimetidine-d_3, used as surrogate standards, were purchased from CDN isotopes. Individual stock standards, isotopically labeled internal standards and surrogate standards were prepared in methanol at a concentration of 1000 mg/L. Stock solutions and 20 mg/L mixtures in methanol were stored at -20 °C. Working standard solutions (1 mg/L) of all pharmaceuticals, mixtures of isotopically labeled internal standards and surrogate were prepared in methanol/water (10:90, v/v) before each analytical run.

2.2. Sample collection and preservation

Fish individuals belonging to 11 different species (*Barbus graellsii*, *Micropterus salmoides*, *Cyprinus carpio*, *Salmo trutta*, *Silurus glanis*, *Anguilla anguilla*, *Lepomis gibbosus*, *Gobio gobio*, *Luciobarbus sclateri*, *Aburnus alburnus*, and *Pseudochondrostoma willkommii*) were collected in five sampling points in each of the four rivers selected (Ebro, Llobregat, Júcar and Guadalquivir) during the summer of 2010. Whole individuals of each class ($n=3$) were homogenized using a meat grinder, composited into a single sample, freeze-dried and kept at −20 °C until analysis. Fish samples (*S. trutta*) from La Llosa del Cavall reservoir (Llobregat River Basin) were also taken during June of 2011. Liver and muscle tissues were dissected from fish individuals and composited separately. Lipid content was evaluated for fish homogenate of several species, as well as for liver and muscle tissues of Salmo trutta, according to the method developed by Spiric et al. [47].

2.3. Sample extraction optimization

In order to optimize the most suitable extraction method, the following extraction techniques (Fig. 1) were tested for fish homogenate: ultrasonic extraction (USE) (Bandelin), QuEChERS (Agilent Technologies) and pressurized liquid extraction (PLE) with ASE 350® (Thermo Scientific Dionex). Fish homogenate samples were spiked with a mixture of the analytes and subsequently subjected to the chosen extraction methodologies described next to obtain the best recovery results for the target compounds. Ultrasonic extraction was adapted from Schultz et al. [28], using a mixture of 0.1 M aqueous acetic acid/methanol (1:1) as extraction solvent. The extraction included 3 cycles (15 min each) and the supernatant was collected after each cycle and centrifugation at 3500 rpm for 5 min. The second methodology was based on QuEChERS, which involved micro-scale extraction and purification of the extract using dispersive solid-phase extraction (d-SPE). After vortexing with one portion of water for 0.5 min, two portions of ACN were added for the extraction and vortexed again for 1 min. Magnesium sulfate (6 g) and sodium acetate (1.2 g) were used as extraction salts. The mixture was shaken intensively for 1 min and centrifuged (11,000 rpm, 5 min, 4 °C) for the separation of the organic and aqueous phases. An aliquot of the organic phase was purified by dispersive SPE employing sorbent mixture of PSA (0.4 g), C18 (0.4 g) and magnesium sulfate (1.2 g) sorbents for the removal of interfering compounds. Sample preparation using pressurized PLE was finally applied, adapting the method described by Chu et al. [27]. Approximately 1 g of fish homogenate was mixed with hydromatrix (diatomaceous earth, ASE prep DE, Dionex) and placed in a 22 mL stainless steel extraction cell containing a glass-fiber filter (27 mm diameter, type D28, Dionex) in the cell inlet and outlet. The PLE conditions were as follows: oven temperature, 50 °C; pressure, 1500 psi; 5 min heat-up time; three static cycles; static time, 5 min. Additional purification steps were added during PLE method optimization. Three preliminary purification methods were evaluated: (a) addition of approximately 2 g of neutral aluminum oxide (70–230 mesh, Merck) at the bottom of the extraction cell to function as a lipid retainer [37,48]; (b) freezing-lipid technique, by means of redissolving the extract in acetonitrile, which has low solubility for lipids, and storing the extract in the freezer at −20 °C for 30 min. Most of the lipids precipitated and the extract was immediately passed through paper filter to remove them [49]; and (c) a fractionation method by extraction with hexane (1 cycle) to remove nonpolar lipids (defatting step) followed by a second extraction of the sample with methanol (3 cycles) to recover the target analytes [50]. After selecting neutral alumina as the most efficient pre-cleanup step during PLE extraction, other parameters were tested such as the sample size (0.5, 1 and 2 g of fish

Table 1
Chemical structures and precursor ions of selected pharmaceuticals (calculated with MarvinSketch software).

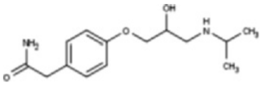
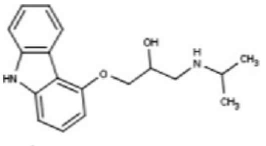
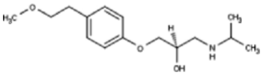
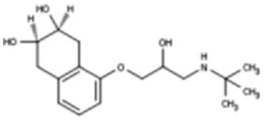
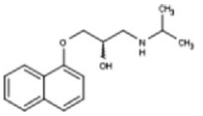
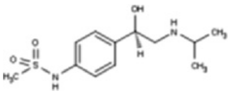
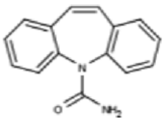
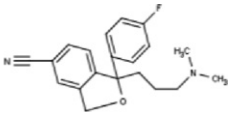
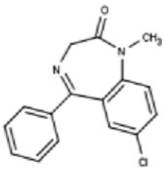
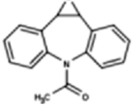
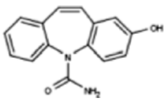
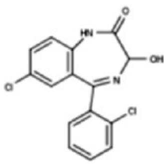
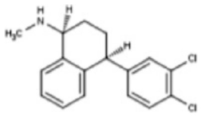
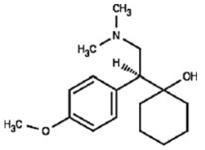
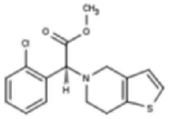
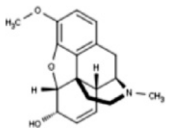
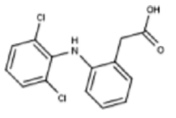
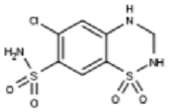
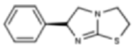
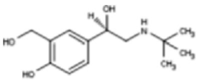
Compound	Therapeutic family	Molecular structure	Precursor ion	Internal standard	pKa	log *P*
Atenolol	β-Blockers		267 $[M+H]^+$	Atenolol-d$_7$	pKa$_1$: 9.67 pKa$_2$: 14.08 pKa$_3$: 15.95	0.43
Carazolol	β-Blockers		299 $[M+H]^+$	Atenolol-d$_7$	pKa$_1$: 9.67 pKa$_2$: 14.03 pKa$_3$: 15.00	2.71
Metropolol	β-Blockers		268 $[M+H]^+$	Atenolol-d$_7$	pKa$_1$: 9.67 pKa$_2$: 14.09	1.76
Nadolol	β-Blockers		310 $[M+H]^+$	Atenolol-d$_7$	pKa$_1$: 9.76 pKa$_2$: 13.59 pKa$_3$: 14.22	0.87
Propanolol	β-Blockers		260 $[M+H]^+$	Atenolol-d$_7$	pKa$_1$: 9.67 pKa$_2$: 14.09	2.58
Sotalol	β-Blockers		273 $[M+H]^+$	Atenolol-d$_7$	pKa$_1$: 9.43 pKa$_2$: 10.07 pKa$_3$: 14.10	0.05
Carbamazepine	Psychiatric drugs		237 $[M+H]^+$	Carbamazepine-d$_{10}$	pKa: 13.94	2.77
Citalopram	Psychiatric drugs		325 $[M+H]^+$	Citalopram-d$_4$	pKa: 9.78	3.76
Diazepam	Psychiatric drugs		285 $[M+H]^+$	Diazepam-d$_5$	pKa: 2.92	3.08
10,11-EpoxyCBZ	Psychiatric drugs		253 $[M+H]^+$	Carbamazepine-d$_{10}$	pKa: 19.65	2.31
2-HydroxyCBZ	Psychiatric drugs		253 $[M+H]^+$	Carbamazepine-d$_{10}$	pKa$_1$: 9.15 pKa$_2$: 15.96	2.46
Lorazepam	Psychiatric drugs		321 $[M+H]^+$	Diazepam-d$_5$	pKa$_1$: 10.61 pKa$_2$: 12.46	2.53
Sertraline	Psychiatric drugs		307 $[M+H]^+$	Fluoxetine-d$_5$	pKa: 9.85	5.15

Table 1 (*Continued*)

Compound	Therapeutic family	Molecular structure	Precursor ion	Internal standard	pKa	log *P*
Venlafaxine	Psychiatric drugs		278 $[M+H]^+$	Venlafaxine-d_6	pKa_1: 8.91 pKa_2: 14.42	2.74
Clopidrogel	Antiplatelet agent		322 $[M+H]^+$	Diazepam-d_5	pKa: 5.14	4.03
Codeine	Analgesics/anti-inflammatories		300 $[M+H]^+$	Carbamazepine-d_{10}	pKa_1: 9.19 pKa_2: 13.78	1.34
Diclofenac	Analgesics/anti-inflammatories		294 $[M\text{-}H]^-$	Ibuprofen-d_3	pKa_1: 4.00 pKa_2: 16.40	4.26
Hydrochlorothiazide	Diuretic		296 $[M-H]^-$	Hydrochlorothiazide-d_2	pKa_1: 9.09 pKa_2: 9.83 pKa_3: 11.31	−0.58
Levamisole	Antihelmintics		205 $[M+H]^+$	Ronidazole-d_3	pKa: 6.98	2.36
Salbutamol	To treat asthma		240 $[M+H]^+$	Atenolol-d_7	pKa_1: 9.40 pKa_2: 10.12 pKa_3: 14.18	0.88

homogenate), extraction solvent (methanol, acetonitrile, water), number of extraction cycles (3 or 4 extraction cycles), and extraction temperature (50, 70, 80 and 90 °C), with the aim to obtain the maximum extraction efficiency with the minimum presence of interfering compounds. Presented results led to the selection of an extraction protocol method based on PLE, with methanol as extraction solvent, 4 extraction cycles of five minutes each at 50 °C for a sample size of 1 g of fish homogenate, 1 g of muscle tissue and 0.5 g of liver tissue. Final extracts were evaporated to dryness under a stream of nitrogen and redissolved in the corresponding organic solvent for the purification step.

2.4. Sample purification optimization

Fish homogenate extracts were spiked with a mixture of the target analytes and a comparison between three purification techniques (Fig. 1) was performed: (a) SPE Florisil cartridges (1 g, 6 ml cartridges); (b) Gel permeation chromatography (GPC) in an Agilent 1260 Infinity high pressure liquid chromatography system in tandem with a diode array detector (HPLC-DAD); (c) SPE Oasis HLB (200 mg, 6 ml cartridges) followed by GPC. For the clean-up method based on SPE Florisil, fish extracts were redissolved in 10 ml of acetonitrile prior SPE, which was performed in a J.T.Baker® system. Florisil cartridges were conditioned with 5 ml hexane followed by 5 ml acetonitrile. Extract was passed through the cartridge and collected for evaporation to dryness. For the purification method based on the use of GPC, 250 µL of 1 ml extracts in methanol were passed through an EnviroPrep, 300 mm × 21.2 mm (10 µm pore size) column coupled to a PLgel Guard column (50 mm × 7.5 mm) (Agilent Technologies). Purified fractions containing target compounds (between minute 13.5 to 26.5) were collected and evaporated to dryness. Finally, for the third clean-up protocol tested fish extracts were redissolved in 250 ml of HPLC-grade water and loaded in Oasis HLB cartridges for SPE. Oasis HLB cartridges were previously conditioned with 6 ml methanol followed by 6 ml HPLC-grade water. After the sample was loaded at 2 mL/min, analytes were eluted with 6 ml of methanol. Eluates were evaporated to final volume of 1 mL of methanol and then injected in GPC for further purification. Final extracts were evaporated to dryness and reconstituted with 1 ml methanol/water (10:90, v/v), and 50 µL of a 1 mg/L mixture containing the internal standards were added.

2.5. Liquid chromatography and mass spectrometry analysis

The chromatographic tandem mass spectrometry method applied for the analysis of fish extracts was adapted from Gros et al. [51]. Briefly, chromatographic separations were carried out with a Waters Acquity Ultra-PerformanceTM liquid chromatography system, using an Acquity HSS T3 colum (50 mm × 2.1 mm i.d., 1.8 µm particle size) for the compounds analyzed in positive electrospray ionization (PI) mode and an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size) for the ones analyzed under negative electrospray ionization (NI) mode. For the analysis in PI mode, separation conditions were as follows: solvent (A) methanol, solvent (B) 10 mM formic acid/ammonium formate (pH 3.2) at a flow rate of 0.5 mL/min. The analysis in NI mode was performed by using acetonitrile (A) and (B) 5 mM ammonium acetate/ammonia (pH = 8) at a flow rate of 0.6 mL/min. The sample volume injected was 5 µL in both cases. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems). Source-dependent parameters for compounds analyzed under PI were: curtain gas (CUR), 30 psi; nitrogen collision gas (CAD) medium; source

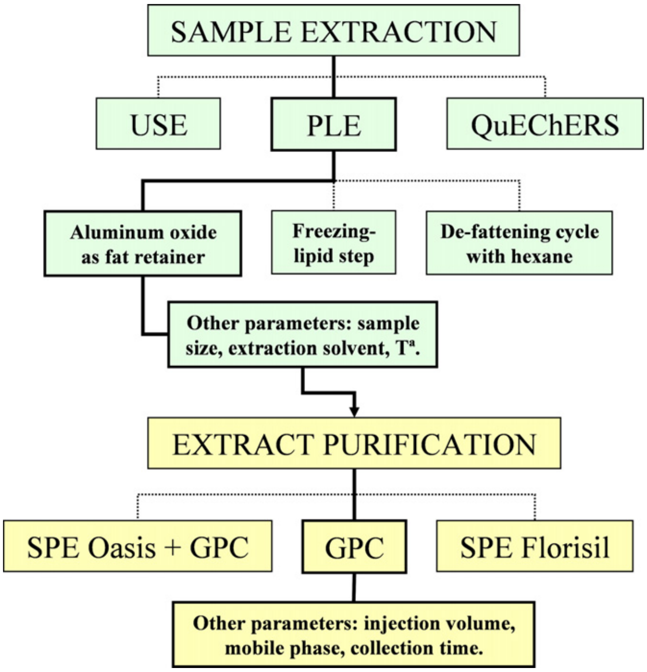


Fig. 1. Sample pretreatment scheme tested during method optimization.

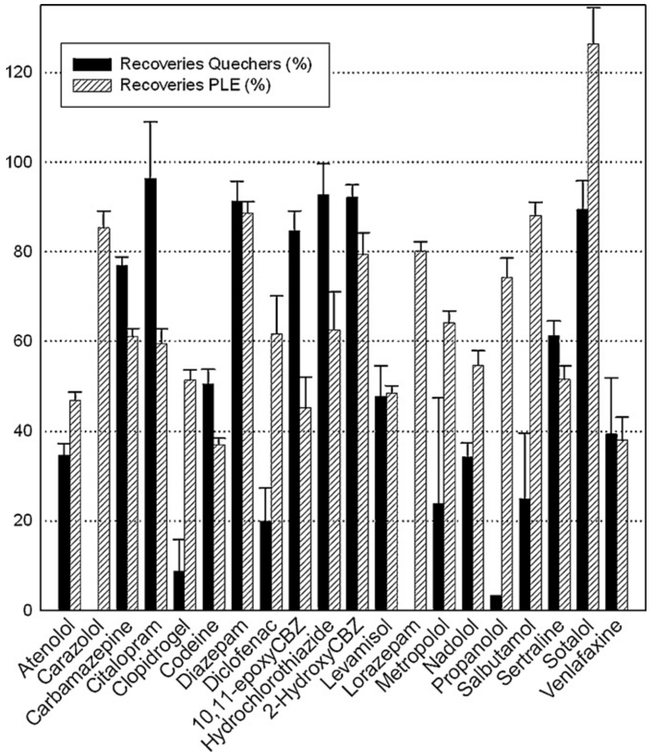


Fig. 2. Comparison of extraction efficiencies (%) between QuEChERS and PLE for the target analytes ($n = 3$).

temperature (TEM) was 650 °C; ion spray voltage was 5500 V; ion source gases GS1 and GS2 were set 60 and 50 psi, respectively. For compounds analyzed under NI, such parameters were: curtain gas (CUR), 30 psi; nitrogen collision gas (CAD) medium; source temperature (TEM) was 650 °C; ion spray voltage was −3500 V; ion source gases GS1 and GS2 were set 60 and 70 psi. Two SRM transitions between the precursor ion and the two most abundant fragment ions were monitored for each compound. The first transition was used for quantification purposes, whereas the second one was used to confirm the identity of the target compounds. Besides the monitoring of the SRM transitions, the relative abundance of the two SRM transitions in the sample were compared with those in the standards, and the relative abundances in the samples must be within ±20% of the two SRM ratios in the analytical standards [51].

3. Results and discussion

3.1. Extraction and purification of the analytes from fish tissues

After the comparison of the three preliminary extraction techniques described in the previous section (USE, QuEChERS and PLE), USE was discarded as a viable option, as only five out of twenty compounds were efficiently recovered. Results obtained with QuEChERS, which had before been successfully applied to the analysis of polar compounds in biological matrices, such as pesticides in food matrices [52] and antibiotics in bovine liver [53], were quite satisfactory, with recoveries surpassing 40% for most of compounds (Fig. 2). However, PLE was finally selected as the most appropriate extraction technique, due to the overall better recoveries (Fig. 2) of relevant compounds (diclofenac, propanolol), the lower relative standard deviation (RSD) (%), and the significantly lesser matrix interferences observed during analysis when compared to QuEChERS. PLE allows the use of conventional solvents at high temperature and pressure to improve the extraction of pollutants from solid samples, but it may still co-extract a high proportion of matrix interferences [33,54]. Therefore, the possibility of a simultaneous purification step (pre clean-up) during PLE extraction to reduce the coextraction of other matrix components was assessed. An intra-cell clean-up with neutral alumina (2 g) was selected among the tested methods as the best sorbent, since the extraction efficiency for target compounds did not decrease while the lipid content in the extracts was reduced (results not shown). Further refinement of the PLE procedure was accomplished by optimizing several critical parameters, such as extraction solvent and its volume, cycle time and number of extraction cycles, extraction temperature and amount of biomass. Among all these parameters, extraction solvent appeared to be the most relevant. At 50 °C, methanol provided the highest recoveries when compared to other solvents (acetonitrile), solvent mixtures (methanol/water, 1:1; methanol/acetonitrile, 1:1) or solvent with additive (acidified methanol) (Fig. S1). The rest of the parameters did not significantly improve the recoveries. Sample size was set at 1 g for fish homogenate and muscle tissues, whereas 0.5 g was selected in the case of liver tissue to minimize the lipid content in the extract and thus the potential interferences during analysis. Temperature was set at 50 °C to avoid analyte degradation, as pharmaceuticals are often thermolabile [20]. Four cycles of 5 min each was established as extraction time and 100% flush volume and 90 s of nitrogen purge were the final parameters selected.

Regarding clean-up procedures, Fig. 3 presents the recoveries for the target analytes obtained with the three purification techniques tested. Florisil cartridges showed generally low recoveries, and thus it was discarded. SPE with Oasis HLB followed by GPC caused decrease of overall recoveries, particularly in the case of β-blocker compounds and, in consequence, a single GPC purification step was selected as clean-up method, as it provided satisfactory results for most of the target compounds. Several parameters were additionally optimized: mobile phase (dichloromethane (DCM), DCM/MeOH mixtures), flow rate (3, 4 and 5 ml/min) and injection volume (100, 250, 500, 750 μL). The final selected parameters were the following: DCM/MeOH (90:10, v/v) as mobile phase, at 5 mL/min flow rate, injection volume of 250 μL for homogenate samples, 500 μL for liver samples and 750 μL for muscle samples.

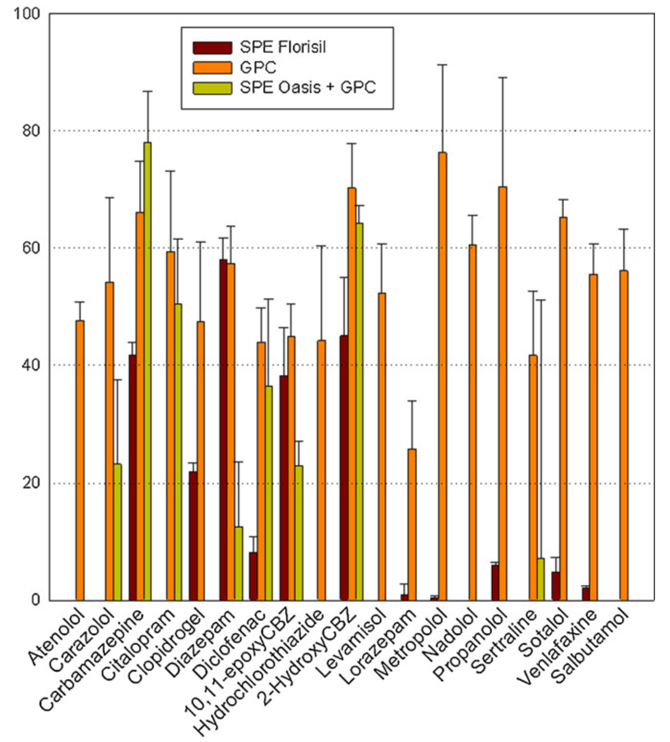


Fig. 3. Comparison of cleanup recoveries (%) between three purification techniques for the selected compounds ($n = 3$).

Collection time interval for the pharmaceuticals was established from min. 13.5 to 26.5 of the 40 min chromatographic cycle.

Final method consisted in PLE extraction using methanol as solvent at 50 °T during 4 cycles of 5 min, followed by an extensive GPC purification, with DCM/MeOH (90:10, v/v) as mobile phase at 5 ml/min flow rate. Total recoveries were determined for the final sample treatment method and for different fish tissues (fish homogenate, liver and muscle) by comparing concentrations obtained after the whole analytical procedure, calculated by internal sample calibration. Results from each matrix are presented for fish homogenate of three representative species (*Cyprinus carpio*, *Barbus graellsii* and *Silurus glanis*) (Table 2) and for liver and muscle tissues (Table 3). Two spiking levels (20 and 100 ng/g) were considered, the lowest level selected according to concentrations found in previous studies found in literature [26,55]. Recoveries were considered acceptable when they were in the range 40–140%, due to (a) the analytical challenge that poses the development of a multi-analyte method for such diverse PhACs (with different lipophilicity and pKa) and (b) to the intensive sample pretreatment demanded in biota matrices [4,5]. Surrogate addition was applied to monitor for recovery differences and problems during the extraction phase of the analysis. Two isotopically labeled compounds were selected: antipyrine-d$_3$ and cimetidine-d$_3$, which presented recoveries of 108.3 ± 1.3 and 78.8 ± 3.0 (%), respectively.

3.2. *Matrix effects*

Ion suppression or enhancement by the presence of coextracted matrix components is a major problem for mass spectrometry detectors when ionization is performed by electrospray interfaces, particularly with complex matrices such as biota [56]. To evaluate matrix effects on the analysis of target compounds in the different fish tissues, peak areas of fish extract (previous subtraction of the analytes peak areas corresponding to the native analytes present in the sample) spiked at 5 ng/g were compared to those of the analytes in the solvent (methanol/water 10:90, v/v) spiked at the same concentration. Calculations were performed in triplicate for fish homogenate, muscle and liver tissues.

Percentage of signal reduction or enhancement for all the compounds is presented in Table S1. The MS signal of the majority of the compounds analyzed in the different biota extracts was suppressed. The highest signal suppression was found in liver tissues (up to 83%), which may be attributed to the higher lipid content of liver (48%) compared to fish homogenate (15%). Matrix effects calculated for various tissues and fish species were contrasted with the lipid content, as presented in Fig. 4 for selected compounds. As expected, ion suppresion (carbamazepine, clopidogrel and venlafaxine) or enhancement (diclofenac) was more obvious as the percentage of lipids increased. Further evaluation of the matrix effects was performed in fish homogenates from three representative fish species considering that lipid percentage for *C. carpio* and *B. graellsii* was approximately 10–15%, while for *Silurus glanis* was

Table 2
Mean percent recoveries ($n = 3$) at two spiking levels of the target compounds in fish homogenates.

	% Recovery ± RSD					
	Spiking level: 20 ng/g			Spiking level: 100 ng/g		
	Barbus graellsii	*Cyprinus carpio*	*Silurus glanis*	*Barbus graellsii*	*Cyprinus carpio*	*Silurus glanis*
Atenolol	46.2 ± 2.0	48.3 ± 6.8	47.7 ± 3.1	47.4 ± 10	60.8 ± 5.3	47.5 ± 8.7
Carazolol	39.7 ± 5.9	27.4 ± 2.9	54.2 ± 14	52.1 ± 9.2	35.3 ± 8.2	38.2 ± 4.7
Carbamazepine	66.2 ± 3.3	71.5 ± 4.2	66.1 ± 8.7	64.1 ± 11	78.7 ± 3.1	74.9 ± 7.0
Citalopram	60.8 ± 4.7	63.9 ± 0.6	59.4 ± 14	107.8 ± 0.4	59.2 ± 23	43.3 ± 18
Clopidrogel	36.9 ± 2.4	31.2 ± 2.0	47.4 ± 14	40.6 ± 11	51.3 ± 2.9	48.4 ± 9.0
Codeine	44.9 ± 7.9	51.8 ± 5.1	42.6 ± 7.5	31.2 ± 4.4	35.3 ± 8.2	36.7 ± 2.3
Diazepam	53.4 ± 3.1	55.8 ± 3.9	57.3 ± 6.3	59.3 ± 11	64.2 ± 4.0	60.1 ± 10
Diclofenac	56.9 ± 12	56.3 ± 24	43.9 ± 5.9	61.3 ± 11	41.6 ± 8.2	44.4 ± 1.0
10,11-epoxyCBZ	45.1 ± 20	33.4 ± 4.3	44.8 ± 5.6	33.9 ± 3.9	34.7 ± 0.6	44.8 ± 4.4
Hydrochlorothiazide	40.3 ± 11	19.3 ± 5.8	44.2 ± 16	68.1 ± 8.1	60.4 ± 12	65.6 ± 11
2-HydroxyCBZ	61.7 ± 21	56.2 ± 2.8	70.2 ± 7.5	64.9 ± 6.2	59.2 ± 2.8	71.4 ± 6.8
Levamisol	44.6 ± 7.6	40.3 ± 4.0	52.4 ± 8.4	45.2 ± 2.5	46.6 ± 1.6	50.8 ± 8.2
Lorazepam	39.6 ± 12	41.8 ± 16	25.8 ± 8.2	58.9 ± 15	35.4 ± 4.5	55.6 ± 5.1
Metropolol	66.9 ± 2.9	48.2 ± 2.4	76.2 ± 15	98.6 ± 19	76.4 ± 4.7	61.7 ± 12
Nadolol	46.9 ± 19	53.0 ± 4.3	60.6 ± 5.0	53.7 ± 9.7	46.2 ± 3.0	37.2 ± 7.3
Propanolol	55.8 ± 8.8	41.2 ± 3.4	70.5 ± 18	97.1 ± 18	62.5 ± 15	42 ± 0.6
Salbutamol	50.5 ± 3.6	27.4 ± 13	56.1 ± 7.1	46.0 ± 9.2	60.5 ± 4.6	55.1 ± 1.4
Sertraline	38.0 ± 11	29.2 ± 9.5	41.6 ± 11	37.3 ± 6.8	54.8 ± 5.1	34.0 ± 8.0
Sotalol	56.1 ± 2.2	66.0 ± 4.3	41.6 ± 11	54.1 ± 8.6	42.8 ± 3.2	34.0 ± 8.0
Venlafaxine	57.0 ± 2.2	48.3 ± 5.9	55.5 ± 5.1	41.8 ± 10	57.2 ± 1.6	55.7 ± 7.2

Table 3
Mean percent recoveries ($n = 3$) in fish liver and muscle tissues (spiking level: 20 & 100 ng/g).

	% Recovery ± RSD			
	Spiking level: 20 ng/g		Spiking level: 100 ng/g	
	Liver	Muscle	Liver	Muscle
Atenolol	47.4 ± 0.6	59.9 ± 2.7	68.4 ± 12	46.9 ± 7.2
Carazolol	57.9 ± 2.7	67.9 ± 8.3	74.8 ± 3.1	85.3 ± 15
Carbamazepine	85.5 ± 3.3	61.2 ± 8.8	102.3 ± 8.7	74.8 ± 4.8
Citalopram	52.3 ± 0.3	33.5 ± 0.6	125.7 ± 12	59.5 ± 13
Clopidrogel	44.0 ± 11	60.3 ± 2.3	45.0 ± 9.5	51.3 ± 9.2
Codeine	38.7 ± 0.3	53.0 ± 0.7	62.3 ± 3.2	50.1 ± 5.3
Diazepam	31.5 ± 3.1	44.2 ± 5.5	110.0 ± 10	88.7 ± 6.6
Diclofenac	38.0 ± 2.8	66.8 ± 5.9	43.9 ± 3.4	63.7 ± 12
10,11-epoxyCBZ	27.5 ± 4.5	27.5 ± 7.3	49.3 ± 3.6	45.2 ± 7.9
Hydrochlorothiazide	68.2 ± 0.9	79.7 ± 7.4	91.9 ± 16	67.2 ± 19
2-HydroxyCBZ	45.1 ± 0.2	49.9 ± 2.6	95.5 ± 4.4	79.3 ± 13
Levamisol	35.0 ± 2.3	84.1 ± 4.1	28.1 ± 13	48.5 ± 15
Lorazepam	54.8 ± 1.8	86.2 ± 8.1	21.3 ± 8.0	42.8 ± 2.2
Metropolol	45.1 ± 0.3	79.7 ± 7.4	91.9 ± 7.9	64.1 ± 5.6
Nadolol	55.8 ± 1.2	60.3 ± 2.3	79.3 ± 5.8	47.3 ± 10
Propanolol	60.8 ± 9.8	84.6 ± 2.1	92.8 ± 5.7	74.3 ± 17
Sertraline	85.0 ± 0.6	92.0 ± 5.7	150.6 ± 8.2	88.1 ± 11
Sotalol	50.0 ± 7.7	87.7 ± 2.1	86.3 ± 13	48.5 ± 12
Venlafaxine	63.1 ± 0.1	68.8 ± 2.7	124.4 ± 6.6	126.4 ± 11.
Salbutamol	47.1 ± 4.2	49.1 ± 3.2	52.9 ± 4.4	38.0 ± 1.2

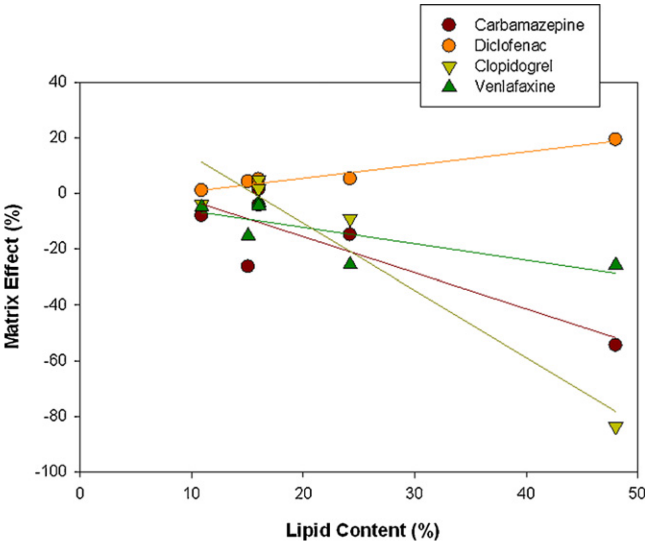


Fig. 4. Matrix effects vs lipid content represented for carbamazepine (fit curve $R^2 = 0.74$), clopidogrel ($R^2 = 0.92$), diclofenac ($R^2 = 0.95$), and venlafaxine ($R^2 = 0.77$).

up to 25%. The results presented in Table S1 show a great similarity in the response in (a) *C. carpio* and (b) *B. graellsii*, and opposite to (c) *S. glanis*, where the majority of the compounds presented greater ion suppression and a very low deviation compared to the other species, which could be a direct consequence of the higher lipid content of *S. glanis*. These results highlighted the relevance of using a suitable approach to compensate for matrix effects during analysis of the same biota matrix, considering the variability in composition that different species might have.

An evaluation of the best approach to compensate for the observed matrix effects was therefore performed. In the first place, standard addition was considered, as it is, by definition, the most effective approach for compensating matrix effects. However, it was difficult and time-consuming due to the great of samples to processes. Other approaches include matrix-matched calibration and internal calibration with isotopically labeled standards. Matrix-matched calibration requires a control matrix similar to the real sample and that does not contain the target analytes, which was not available in this case. Internal standard calibration entails the addition to the extract of isotopically labeled standards that are structurally similar to the target analytes to compare the instrument response during the analysis. Since only ten (out of 20 target compounds) isotopically labeled standards were available to use as internal standard during method optimization, an alternative strategy for the correction of matrix effect were explored, namely internal sample calibration [57,58]. Two calibration curves in the range of 0.1–25 ng/mL were prepared in both, a solvent mixture and fish extract, and internal standards were added to the two of them at the same concentration. Fig. 5 presents the internal standard calibration (solvent curve) and the internal sample calibration (curve prepared in fish extract) for two compounds, carbamazepine and clopidogrel. In the case of carbamazepine (Fig. 5a), whose quantification was corrected with its analogue labeled compound (carbamazapine-d_{10}), both calibration curves were very similar, which indicates that the internal standard was compensating the observed matrix effects. In the case of clopidogrel (Fig. 5b) whose corresponding analogue labeled compound was not available, calibration curves were noticeably different, as the internal standard (diazepam-d_5) used during quantification with the solvent curve was not correcting completely the ion suppression observed in the real sample. Consequently, the most advantageous approach for an accurate determination of target compounds seemed to be the quantification using a calibration curve prepared with spiked fish extracts and internal standard addition (internal sample calibration), which was able to correct the matrix effects for all the compounds. Internal sample calibration is thus recommended especially in those cases when the analogue internal standard is not available.

Due to the differences observed in lipid content between fish species, it was important to assess if quantification of analytes was affected by the use of the different fish matrices to build the calibration curves. The accuracy of the measurements when preparing the internal sample calibration in three representative species (*C. carpio*, *B. graellsii* and *S. glanis*) was evaluated. An extract of *C. carpio* was spiked (25 ng/g) with a mixture of the analytes and quantified using internal standard calibration and internal sample calibration prepared in extracts of the three fish species. As expected, the most accurate measurement corresponds to the internal sample calibration with *C. carpio* extracts, since the same fish sample was used for building the internal sample calibration curve. Internal sample calibration curve built in *B. graellsii* extracts, with similar amount

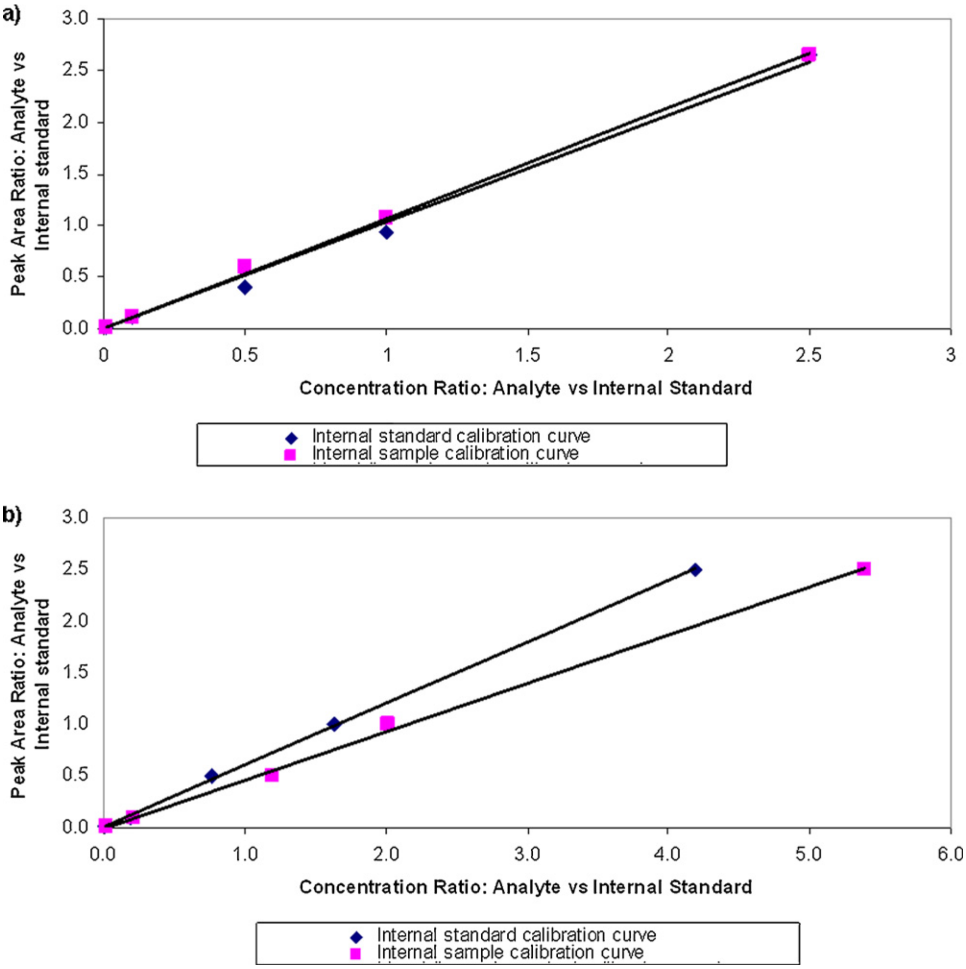


Fig. 5. Comparison between calibration curves in the solvent, and in real sample with internal standard for the pharmaceuticals (a) carbamazepine (carbamazepine-d_{10} as internal standard) and (b) clopidogrel (diazepam-d_5 as internal standard). Calibration curves were prepared in *C. carpio* extracts.

of fat content than *C. carpio*, (Fig S2) was quite optimal at the time of quantifying target compounds in *C. carpio* samples, except for some slight deviation for particular compounds. On the contrary *S. glanis*, with higher fat content that the other two species studied, exhibited great discrepancy in the results between calibration methods, particularly in the case of some β-blockers compounds. These results reiterated the significance of considering the characteristics of the different species, particularly lipid content, when working with internal or external sample calibration. The preparation of internal sample calibration curves in an appropriate matrix, namely extracts of the same specie or at least species with similar characteristics, is suggested as the best approach for an accurate determination of pharmaceutical compounds in this type of biota samples.

3.3. Method validation parameters

Method detection limits (MDL) and method quantification limits (MQL) for fish homogenate, liver and muscle are presented in Table 4. Both, MDL and MQL, were determined in spiked samples ($n = 3$) of the three matrices considered as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. MDLs for the target compounds were in the range of 0.03–0.50 ng/g for fish homogenate, 0.01–0.42 ng/g for fish muscle, and 0.08–0.98 ng/g for fish liver, and were in general similar or lower than those currently reported [5,29,59].

Accuracy and precision were calculated from five repeated injections of a spiked sample at 25 ng/g in the same day. Accuracy was defined as the deviation of the measured mean concentration from the spiked concentration, expressed in percentage, as described by Bogialli et al. [60]. Precision was expressed as the relative standard deviation of the measured concentration. Both values were lower than 20% for the three matrices considered (Table 5). Calibration curves were generated using linear regression analysis ($r^2 > 0.98$) in the concentration range from 0.1 to 50 ng/g.

3.4. Application to environmental samples

The developed method was applied for the determination of pharmaceuticals in fish from four Mediterranean rivers and one reservoir, including fish homogenates, muscle and liver tissues. Nine compounds (carazolol, carbamazepine, citalopram, clopidogrel, diclofenac, propanolol, salbutamol, and venlafaxine) from five therapeutic families were determined at concentrations higher than MDLs (Table 6). Pharmaceutical levels were lower than 10 ng/g in fish homogenates, which is consistent with published scientific literature on the topic [5,26,27]. Highest levels were found in trout liver, where the concentration of carbamazepine was 18 ng/g. Most ubiquitous and recurring compound was diclofenac, detected in 9% of the total number of samples.

Table 4
Method detection and quantification limits (MDL, MQL) in fish homogenate, liver and muscle tissues (ng/g, dry weight).

	MDL (ng/g)			MQL (ng/g)		
	Homogenate	Liver	Muscle	Homogenate	Liver	Muscle
Atenolol	0.05	0.12	0.10	0.17	0.41	0.32
Carazolol	0.04	0.09	0.03	0.15	0.31	0.10
Carbamazepine	0.04	0.08	0.01	0.13	0.25	0.04
Citalopram	0.12	0.09	0.05	0.41	0.29	0.16
Clopidrogel	0.04	0.26	0.08	0.13	0.87	0.26
Codeine	0.06	0.13	0.06	0.18	0.43	0.20
Diazepam	0.08	0.12	0.08	0.25	0.41	0.25
Diclofenac	0.50	0.65	0.19	1.66	2.16	0.62
Epoxycarbamazepine	0.09	0.19	0.11	0.30	0.62	0.38
Hydrochlorothiazide	0.05	0.11	0.17	0.17	0.35	0.57
Hydroxycarbamazepine	0.03	0.25	0.08	0.09	0.83	0.26
Levamisol	0.04	0.29	0.02	0.13	0.97	0.08
Lorazepam	0.49	0.77	0.42	1.62	2.58	1.42
Metropolol	0.20	0.36	0.18	0.67	1.19	0.60
Nadolol	0.03	0.13	0.09	0.10	0.42	0.30
Propanolol	0.09	0.36	0.18	0.29	1.21	0.60
Sertraline	0.32	0.98	0.18	1.08	3.28	0.61
Sotalol	0.26	0.20	0.07	0.88	0.66	0.24
Venlafaxine	0.04	0.40	0.16	0.15	1.33	0.55
Salbutamol	0.07	0.24	0.11	0.25	0.78	0.38

Table 5
Accuracy[a] and precision[b] data of selected pharmaceuticals in fish homogenate, liver and muscle (spiking level 25 ppb).

	Homogenate		Liver		Muscle	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Atenolol	−0.1	2.1	−1.6	14	−1.6	6.6
Carazolol	11	7.3	−12	4.4	−10.9	17
Carbamazepine	−14	4.0	−17	13	−18	5.9
Citalopram	7.1	1.1	−19	14	6.2	1.1
Clopidrogel	−2.5	14.	0.1	12	0.3	9.2
Codeine	−17	15	−0.7	3.9	−17	15
Diazepam	2.3	6.7	−0.4	13	−19	8.6
Diclofenac	0.9	2.7	1.3	18	−20	14
10,11-epoxyCBZ	−5.9	2.0	0.3	4.5	−20	12
Hydrochlorothiazide	−4.2	12	−0.1	20	−6.9	12
2-HydroxyCBZ	−18.9	4.9	−0.1	5.4	−17	9.1
Levamisol	14	3.8	0.2	16	−5.9	6.1
Lorazepam	16	21	8.2	13	4.1	20
Metropolol	0.0	6.3	−0.1	9.7	−9.1	11
Nadolol	1.4	6.4	0.1	7.1	1.0	13
Propanolol	−11	21	0.0	6.9	−0.1	17
Salbutamol	−1.5	3.9	0.0	16	0.1	11
Sertraline	2.9	6.9	3.4	11	−2.6	10
Sotalol	−15	2.9	−19	7.7	−14	7.2
Venlafaxine	13	7.5	−15	10	−11	20

[a] Calculate as [(mean calculated concentration − spiked concentration)/spiked concentration] ×100.

[b] Expressed as relative standard deviation (RSD (%)).

Table 6
Concentration of pharmaceuticals (ng/g, dry weight) in fish homogenates and tissues from fish collected in Mediterranean rivers (Spain).

Therapeutic family	Compound	Species	Matrix	Mean concentration (ng/g) ($n = 3$)	Standard deviation	Detection frequency (%)
Anti-inflamatory	Diclofenac	*Barbus graellsii*	Homogenate	8.8	±0.5	9
		Micropterus salmoides	Homogenate	4.1	±0.9	
Psychiatric drug	Citalopram	*Cyprinus carpio*	Homogenate	0.8	±0.1	3.5
	Carbamazepine	*Salmo trutta*	Liver	17.9	±2.4	–
	Venlafaxine	*Cyprinus carpio*	Homogenate	0.6	±0.02	2
Antiplatelet agent	Clopidogrel	*Cyprinus carpio*	Homogenate	<MLQ		2
β-blockers	Carazolol	*Silurus glanis*	Homogenate	3.8	±0.7	3.5
		Anguilla anguilla	Homogenate	<MLQ		
	Propanolol	*Silurus glanis*	Homogenate	4.2	±1.0	3.5
	Sotalol	*Pseudochondrostoma willkommii*	Homogenate	<MLQ		2
To treat asthma	Salbutamol	*Silurus glanis*	Homogenate	2.6	±0.3	2

4. Conclusions

The multi-residue analytical method described in this paper, based on pressurized liquid extraction, gel permeation chromatography purification and UPLC–MS/MS analysis, provides the necessary sensitivity for the simultaneous trace-level detection of 20 multi-class pharmaceuticals. Recoveries obtained for most of the target compounds were higher than 50%. The application of UPLC–MS/MS operated in the MRM mode, with two transitions monitored for each compound, provided good sensitivity, selectivity and confirmation of positive findings. A thorough evaluation of the matrix effects was performed and lipid content in fish species and tissues was considered to select the best approach to compensate observed matrix effects and to provide an accurate determination of pharmaceutical compounds. Internal sample calibrations in an appropriate matrix were selected as the best strategy in this type of sample. The method presented detection limits in the low ng/g range for the several fish species, thus providing a sensitive, reliable and robust tool that can be used for routine analysis of multi-class pharmaceuticals in different fish tissues. Anti-inflammatories, psychiatric drugs and β-blockers were detected in fish samples from the most polluted sites of Mediterranean rivers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.03.001.

References

[1] European Commision, Proposal for a Directive of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy, in 2011/0429 (COD), Brussels, 2012.
[2] S. Rodriguez-Mozaz, H.S. Weinberg, Environ. Health Perspect. 118 (2010) 1016.
[3] U. Schröder, A. Machetzki, Eur. Food Res. Technol. 225 (2007) 627.
[4] R.B. Bringolf, R.M. Heltsley, T.J. Newton, C.B. Eads, S.J. Fraley, D. Shea, W.G. Cope, Environ. Toxicol. Chem. 29 (2010) 1311.
[5] A.J. Ramirez, M.A. Mottaleb, B.W. Brooks, C.K. Chambliss, Anal. Chem. 79 (2007) 3155.
[6] J. Beyer, G. Jonsson, C. Porte, M.M. Krahn, F. Ariese, Environ. Toxicol. Pharmacol. 30 (2010) 224.
[7] S.D. Ramachandran, M.J. Sweezey, P.V. Hodson, M. Boudreau, S.C. Courtenay, K. Lee, T. King, J.A. Dixon, Mar. Pollut. Bull. 52 (2006) 1182.
[8] C.A.O. Ribeiro, Y. Vollaire, A. Sanchez-Chardi, H. Roche, Aquat. Toxicol. 74 (2005) 53.
[9] M.S. Shailaja, R. Rajamanickam, S. Wahidulla, Environ. Pollut. 143 (2006) 174.
[10] F. Verweij, K. Booij, K. Satumalay, N. van der Molen, R. van der Oost, Chemosphere 54 (2004) 1675.
[11] EU council directive 2008/105/EC on environmental quality standards in the field of water policy, Official Journal of European Union (2008) 84.
[12] U.S. Environmental Protection Agency, Drinking Water Contaminant Candidate List CCL 3 (2010), http://www.epa.gov/safewater/ccl/ccl3.html (accessed 12.11.12).
[13] C.E. Cowan-Ellsberry, S.D. Dyer, S. Erhardt, M.J. Bernhard, A.L. Roe, M.E. Dowty, A.V. Weisbrod, Chemosphere 70 (2008) 1804.
[14] C.G. Daughton, B.W. Brooks, in: W. Nelson Beyer, J.P. Meador (Eds.), Environmental Contaminants in Biota: Interpreting Tissue Concentrations, CRC Press, Boca Raton, 2011, p. 286.
[15] R. van der Oost, J. Beyer, N.P.E. Vermeulen, Environ. Toxicol. Pharmacol. 13 (2003) 57.
[16] R. Pei, S.-C. Kim, K.H. Carlson, A. Pruden, Water Res. 40 (2006) 2427.
[17] M. Farré, L. Kantiani, M. Petrovic, S. Pérez, D. Barceló, J. Chromatogr. A 1259 (2012) 86.
[18] M. Petrovic, M.D. Hernando, M.S. Díaz-Cruz, D. Barceló, J. Chromatogr. A 1067 (2005) 1.
[19] A. Kot-Wasik, J. Dębska, J. Namieśnik, TrAC Trends Anal. Chem. 26 (2007) 557.
[20] D. Fatta, A. Nikolaou, A. Achilleos, S. Meriç, TrAC, Trends Anal. Chem. 26 (2007) 515.
[21] M.D. Celiz, J. Tso, D.S. Aga, Environ. Toxicol. Chem. 28 (2009) 2473.
[22] C. Hao, X. Zhao, P. Yang, TrAC Trends Anal. Chem. 26 (2007) 569.
[23] S.D. Richardson, T.A. Ternes, Anal. Chem. 83 (2011) 4614.
[24] W.W. Buchberger, J. Chromatogr. A 1218 (2011) 603.
[25] B. Huerta, S. Rodríguez-Mozaz, D. Barcelò, Anal. Bioanal. Chem. 404 (2012) 2611.
[26] B.W. Brooks, C.K. Chambliss, J.K. Stanley, A. Ramirez, K.E. Banks, R.D. Johnson, R.J. Lewis, Environ. Toxicol. Chem. 24 (2005) 464.
[27] S. Chu, C.D. Metcalfe, J. Chromatogr. A 1163 (2007) 112.
[28] M.M. Schultz, E.T. Furlong, D.W. Kolpin, S.L. Werner, H.L. Schoenfuss, L.B. Barber, V.S. Blazer, D.O. Norris, A.M. Vajda, Environ. Sci. Technol. 44 (2010) 1918.
[29] J. Wang, P. Gardinali, Anal. Bioanal. Chem. 404 (2012) 2711.
[30] A.M. Al-Ansari, A. Saleem, L.E. Kimpe, V.L. Trudeau, J.M. Blais, J. Chromatogr. B 879 (2011) 3649.
[31] C. Cháfer-Pericás, Á. Maquieira, R. Puchades, B. Company, J. Miralles, A. Moreno, Aquacult. Res. 41 (2010) e217.
[32] R. Fernandez-Torres, M.A. Bello Lopez, M.O. Consentino, M.C. Mochón, Anal. Lett. 44 (2011) 2357.
[33] D.M. Pavlović, S. Babić, A.J.M. Horvat, M. Kaštelan-Macan, TrAC, Trends Anal. Chem. 26 (2007) 1062.
[34] A. Kirbiš, J. Marinšek, V.C. Flajs, Biomed. Chromatogr. 19 (2005) 259.
[35] C. Vannini, G. Domingo, M. Marsoni, F. De Mattia, M. Labra, S. Castiglioni, M. Bracale, Aquat. Toxicol. 101 (2011) 459.
[36] E.B. Dussault, V.K. Balakrishnan, U. Borgmann, K.R. Solomon, P.K. Sibley, Ecotoxicol. Environ. Saf. 72 (2009) 1635.
[37] H. Berrada, F. Borrull, G. Font, R.M. Marcè, J. Chromatogr. A 1208 (2008) 83.
[38] G.C. Nallani, P.M. Paulos, L.A. Constantine, B.J. Venables, D.B. Huggett, Chemosphere 84 (2011) 1371.
[39] R. Cueva-Mestanza, M.E. Torres-Padrón, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, Biomed. Chromatogr. 22 (2008) 1115.
[40] B. Subedi, M.A. Mottaleb, C.K. Chambliss, S. Usenko, J. Chromatogr. A 1218 (2011) 6278.
[41] M. Gros, M. Petrovic, D. Barceló, Anal. Chem. 81 (2009) 898.
[42] V. Osorio, R. Marcé, S. Pérez, A. Ginebreda, J.L. Cortina, D. Barceló, Sci. Total Environ. 440 (2012) 3.
[43] M. Meredith-Williams, L.J. Carter, R. Fussell, D. Raffaelli, R. Ashauer, A.B.A. Boxall, Environ. Pollut. 165 (2012) 250.
[44] A.C. Mehinto, E.M. Hill, C.R. Tyler, Environ. Sci. Technol. 44 (2010) 2176.
[45] A. Lajeunesse, C. Gagnon, F. Gagne, S. Louis, P. Cejka, S. Sauve, Chemosphere 83 (2011) 564.
[46] T.W. Valenti Jr., G.G. Gould, J.P. Berninger, K.A. Connors, N.B. Keele, K.N. Prosser, B.W. Brooks, Environ. Sci. Technol. 46 (2012) 2427.
[47] A. Spiric, D. Trbovic, D. Vranic, J. Djinovic, R. Petronijevic, V. Matekalo-Sverak, Anal. Chim. Acta 672 (2010) 66.
[48] M. Lund, L. Duedahl-Olesen, J.H. Christensen, Talanta 79 (2009) 10.
[49] J. Hong, H.-Y. Kim, D.-G. Kim, J. Seo, K.-J. Kim, J. Chromatogr. A 1038 (2004) 27.
[50] R. Draisci, C. Marchiafava, L. Palleschi, P. Cammarata, S. Cavalli, J. Chromatogr. B: Biomed. Sci. Appl. 753 (2001) 217.
[51] M. Gros, S. Rodríguez-Mozaz, D. Barceló, J. Chromatogr. A 1248 (2012) 104.
[52] A. Wilkowska, M. Biziuk, Food Chem. 125 (2011) 803.
[53] L. Zhao, J. Stevens, Application note, Agilent Technologies, 2010.
[54] V. Matamoros, D. Calderón-Preciado, C. Domínguez, J.M. Bayona, Anal. Chim. Acta 722 (2012) 8.
[55] A.J. Ramirez, R.A. Brain, S. Usenko, M.A. Mottaleb, J.G. O'Donnell, L.L. Stahl, J.B. Wathen, B.D. Snyder, J.L. Pitt, P. Perez-Hurtado, L.L. Dobbins, B.W. Brooks, C.K. Chambliss, Environl Toxicol. Chem. 28 (2009) 2587.
[56] E.B. Dussault, V.K. Balakrishnan, K.R. Solomon, P.K. Sibley, Can. J. Chem. 87 (2009).
[57] R.K. Boyd, Rapid Commun. Mass Spectrosc. 7 (1993) 257.
[58] M. Stüber, T. Reemtsma, Anal. Bioanal. Chem. 378 (2004) 910.
[59] J. Schwaiger, H. Ferling, U. Mallow, H. Wintermayr, R.D. Negele, Aquat. Toxicol. 68 (2004) 141.
[60] S. Bogialli, R. Curini, A. Di Corcia, M. Nazzari, R. Samperi, Anal. Chem. 75 (2003) 1798.

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Development of an extraction and purification method for the determination of multi-class pharmaceuticals and endocrine disruptors in freshwater invertebrates

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ABSTRACT

Aquatic organisms from freshwater ecosystems impacted by waste water treatment plant (WWTP) effluents are constantly exposed to constant concentrations of pharmaceuticals, endocrine disruptors and related compounds, among other anthropogenic contaminants. Macroinvertebrates inhabiting freshwater ecosystems might be useful bioindicators of exposure to contaminants, since their lives are long enough to bioaccumulate, but at the same time may integrate short-term changes in the environment. However, studies about potential bioaccumulation of emerging contaminants in these organisms are very scarce. The objectives of this study were to develop an analytical methodology for the analysis of 41 pharmaceuticals and 21 endocrine disruptors in freshwater invertebrates. In addition, bioaccumulation of these contaminants in three macroinvertebrate taxa inhabiting a waste water treatment plant -impacted river was evaluated. The method for the simultaneous extraction of both families of compounds is based on sonication, purification via removal of phospholipids, and analysis by ultra performance liquid chromatography coupled to a mass spectrometer (UPLC–MS/MS) in tandem. Recoveries for pharmaceuticals were 34–125%, and for endocrine disruptors were 48–117%. Method detection limits (MDLs) for EDCs were in the range of 0.080–2.4 ng g^{-1}, and for pharmaceuticals, 0.060–4.3 ng g^{-1}. These pollutants were detected in water samples taken downstream the waste water treatment plant effluent at concentrations up to 572 ng L^{-1}. Two non-esteroidal anti-inflammatory drugs, diclofenac and ibuprofen, and four endocrine disruptors – estrone, bisphenol A, TBEP, and nonylphenol – were detected in at least one macroinvertebrate taxa in concentrations up to 183 ng g^{-1} (dry weight). An isobaric interference was identified during the analysis of diclofenac in *Hydropsyche* samples, which was successfully discriminated via accurate mass determination by TFC-LTQ Orbitrap.

1. Introduction

Numerous anthropogenic chemicals have been detected in wastewater treatment plants (WWTP) effluents and surface water in recent years, including pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) [1–4]. This situation is of special concern in the Mediterranean region, where WWTP effluents often represent a high proportion of stream flow (particularly under water scarcity scenarios [5,6]), and pollutants such as PhACs and EDCs may be present at relatively high concentrations and cause adverse effects to aquatic organisms [7]. For instance, PhACs have been designed to modify specific biochemical pathways in target organisms [8]. On the other hand, EDCs are compounds that belong to different chemical families, characterized by their capability to alter the endocrine system of exposed organisms by mimicking or counteracting natural hormones, which could have severe consequences even at very low concentrations [9,10]. Several examples support the potential effects of these compounds on the river fauna: the feminisation of male fish in effluent-dominated rivers closely related to the presence of the synthetic hormone 17α-ethinylestradiol (EE2) [11,12], altered fish reproduction by some anti-inflammatory compounds, and altered behaviour and endocrine functions due to exposure to antidepressants [13,14]. Within this context, the recent Directive on the review of priority substances in surface water bodies has selected three of these

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compounds (diclofenac, ethinylestradiol, β-estradiol) as candidates for the "watch list" in the European priority compounds list [15].

A better understanding of how these compounds are transferred from water to biota could help determining the potential of ecosystem damage associated with the discharge of WWTP effluent containing these contaminants [16]. Among freshwater organisms exposed to these bioactive compounds, invertebrates have been less frequently studied than vertebrate species (i.e. fish) [17,18]. However, these organisms are susceptible of being affected by these compounds, and because of their sedentary life, they are representative of the local conditions in freshwater ecosystems [10,19]. Benthic macroinvertebrates are prey of other consumers, constituting a secondary contaminant entry for upper trophic levels. Their relatively long lives facilitate the incorporation of contaminants in their bodies, whereas they also integrate short-term changes in the environment. Therefore, aquatic macroinvertebrates appear to have the potential to be useful biomonitors of pollution [20]. Trichoptera (caddisflies) are already considered a bioindicator group, and these insects are frequently used to assess the ecological status of basins in Mediterranean rivers (e.g. [21]).

Many pollutants have been recurrently detected in macroinvertebrates (invertebrates large enough to be seen without the use of a microscope), and these organisms have been used as bioindicators of a wide variety of compounds, such as pesticides, PCBs, hormones, and metals [18,22–26]. The capability of macroinvertebrates to bioaccumulate PhACs and EDCs has also been established under controlled exposure conditions (see Table S1). However, information about bioaccumulation of PhACs and other EDCs under natural conditions is still non-existent for the great majority of these compounds. A first critical step to understand the bioaccumulation dynamics of these compounds is to develop and validate analytical methods for their extraction and determination in biota matrices [6,27]. Therefore, the objectives of this study were two-fold: (i) to develop an efficient analytical methodology for the simultaneous analysis of PhACs and EDCs in aquatic invertebrates, and (ii) to assess persistence, distribution, and bioaccumulation of these trace contaminants in macroinvertebrate communities affected by WWTP effluents.

2. Materials and methods

2.1. Standards and solutions

A list of the target analytes, their physico-chemical properties and purity of standards and reagents can be found in the supplementary material (Table S2). Individual stock standards and isotopically labeled internal standards were prepared in methanol at a concentration of approximately 1000 mg L^{-1}. Stock solutions and 20 mg L^{-1} mixtures in methanol were stored at −20 °C. Working standard solutions (1 mg L^{-1}) of all compounds, and mixtures of isotopically labelled internal standards were prepared in methanol/water (1:9, v/v for PhACs; 1:1, v/v for EDCs) before each analytical run.

2.2. Sample collection and pre-treatment

The study was conducted in a section of the River Segre, a tributary to the River Ebro in the Pyrenees (NE Iberian Peninsula). Although industrial and agricultural activities are scarce in this area, the river receives the discharge of a WWTP effluent (29 742 population equivalents). Water and invertebrate samples were collected in two sites, respectively: upstream (500 m) and downstream (1500 m) of the local WWTP in October of 2012. Invertebrate samples from three different taxa were collected, namely the gastropod *Ancylus fluviatilis* (*Planorbidae* family), which are air-breathing limpets that graze on biofilms, the filterer trichopteran *Hydropsyche* spp., (*Hydropsychidae* family), in larval and pupal (aquatic) stages, and the predator flatworm *Phagocata* spp. (*Planariidae* family).

River water (100 mL) was collected to determine the presence of PhACs and EDCs in the aqueous phase. River samples were filtered through 1 μm glass fiber filters followed by 0.45 μm nylon membrane filters and kept at −20 °C until analysis. Macroinvertebrates were collected and placed in a 50 mL Falcon tube filled with river water. Individuals were kept for 24 h in filtered stream water to allow for gut clearance. Approximately 70–120 individuals of each taxon were collected per sampling point and composited Composites were freeze-dried, homogenized with a mortar, and kept at −20 °C until analysis. A prior test was performed to determine the water content in the three taxa, which ranged between 77% (*Hydropsyche*) and 90% (*Ancylus*).

2.3. Extraction and purification optimization

Water samples were analyzed for PhACs and EDCs according to Gros et al. [28] for the analysis of PhAC, and also applied for the analysis of EDCs. Ultra-pure water (100 mL) was spiked with a mixture of the target analytes and subsequently subjected to the extraction method described next. Briefly, 3 mL of EDTA 1 M (4%, v/v) were added to water samples. A Baker vacuum system (J.T. Baker, The Netherlands) was used to pre-concentrate the samples in Oasis HLB cartridges (60 mg, 3 mL). They were loaded with 100 mL of water samples and eluted with 6 mL of methanol. The extracts were evaporated under a gentle nitrogen stream and reconstituted with 1 mL of methanol–water mixture (50:50, v/v for EDCs; 10:90, v/v for PhACs), and 10 μL of IS mixture (1 mg L^{-1}) was added to the final extract.

Macroinvertebrates samples were spiked with a mixture of both families of analytes and subsequently subjected to the chosen extraction methodologies described next to obtain the best recovery results for the target compounds. Sonication (Branson Digital Sonifier, model 102C) was the chosen method for extraction following preliminary studies. Samples were placed in a 50-mL Falcon tube in an ice bath, where 3 mL of solvent were added to the samples and vortexed. Ultrasonic probe was plunged in the solution and sonication was applied in 3 cycles of 120 s, with amplitude (intensity) of 30%. Sample size and extraction solvent were tested: 50, 100 and 200 mg of freeze-dried biomass, whereas extraction solvents tested were methanol (100%) and water/acetonitrile (1:3, 1% formic acid, v/v). Samples were centrifuged at 11000 rpm, for 5 min. Supernatant was collected and 1.5 mL of the extract was taken for further purification.

Four procedures were tested during cleanup optimization on the basis of the analytes recovery and the purity of the extract: solid-phase extraction (SPE) performed in a J.T. Baker® vacuum system with (i) Florisil cartridges (1 g, 6 mL), (ii) Oasis HLB (60 mg, 3 mL), and (iii) an Oasis HLB 96-well plate (30 μm, 30 mg). Another technique for the removal of phospholipids and proteins in plasma and serum, common matrix interferences in biological analysis, was also tested: (iv) an Ostro™ 96-well plate, which exhibits selective affinity towards phospholipids while remaining non-selective towards a range of basic, acidic and neutral compounds. Extracts were subjected to different treatments using each of the four clean-up strategies above mentioned:

(i) Extracts were redissolved in 10 mL of acetonitrile and loaded to the cartridge at 1 mL min^{-1} after conditioning with 10 mL of *n*-hexane and 10 mL of acetonitrile.
(ii) Extracts were redissolved in 200 mL of water with EDTA at 1%. After conditioning the cartridges with 6 mL of methanol and 6 mL of water, they were loaded into the cartridges and the eluted in 6 mL of methanol.

(iii) Extracts were redissolved in 1.5 mL of water 1% EDTA. Oasis HLB 96-well plate was placed in a manifold and wells were conditioned with 1.5 mL of methanol and 1.5 mL of water consecutively applying a vacuum extraction. Extracts were loaded and then eluted in 1.5 mL of methanol.

(iv) Extracts were redissolved in 750 μL of water/acetonitrile (1:3, 1% Formic acid, v/v). Ostro^{TM} 96-well plate was placed in a manifold and wells were added 750 μL of fresh solvent and then carefully mixed with the extract in the well. After extraction by vacuum, purified extracts were collected.

All purified extracts were then evaporated and redissolved in 1 mL of methanol-water mixture (50:50, v/v for EDCs; 10:90, v/v for PhACs), and 10 μL of IS mixture (1 mg L^{-1})was added to the final extracts.

2.4. Instrumental analysis

2.4.1. UPLC–QqQ–LIT

For the analysis of PhACs, water and biota extracts were analysed according to Gros et al. [28] using a ultra performance liquid chromatography (UPLC) system (from Waters Milford, MA, USA) coupled to a hybrid quadrupole linear ion trap mass spectrometer Qtrap 5500 (Applied Biosystems, Foster City, CA, USA), equipped with an electrospray ionization source (ESI). Chromatographic conditions for the positive ionization mode were an Acquity HSS T3 column with methanol and 10 mM formic acid/ammonium formate (pH 3.2) as the mobile phase at a flow rate of 0.5 mL min^{-1}. For the negative ionization mode, an Acquity BEH C18 column was applied using acetonitrile and 5 mM ammonium acetate/ammonia (pH=8) at a flow rate of 0.6 mL min^{-1}. For the analysis of EDCs, extracts were analysed using the method developed by Jakimska et al. [29] in the same instrumental set up, using the Acquity BEH C18 column for both positive and negative modes. The chromatographic separation was achieved with methanol and water (pH 9) as mobile phase at a flow rate of 0.4 mL min^{-1}. The acquisition of the compounds into the analyser Qtrap was done in selected reaction monitoring (SRM) mode with the monitoring of two transitions. The most intense transition was used for quantification purposes when it was possible, whereas the second one was used to confirm the identity of the target compounds. Besides the monitoring of the SRM transitions and their retention time, the relative abundance of the two SRM transitions in the sample were compared with those in the standards, and the relative abundances in the samples must be within ± 20% of the two SRM ratios in the analytical standards. Pharmaceutical diclofenac was quantified with the second SRM transition for the insect *Hydropsyche*, as the first SRM transition was overlapped by an isobaric interference.

2.4.2. HPLC–HRMS

For the confirmation of the presence of diclofenac in *Hydropsyche*, a turbulent flow chromatograph coupled to a hybrid linear ion trap—Orbitrap mass spectrometer (TFC-LC-LTQ Orbitrap Velos) equipped with an ESI was used. The chromatographic system operating in off-line was equipped with a Hypersil Gold PFP column (10 mm × 2.1 mm id, 1.9 μm, ThermoFisher Scientific Company; Villebon-France). The LC eluents were solvent A: acetonitrile and solvent B: HPLC-grade water. The mobile phase gradient was programmed as follows: 0 min 5% A, 0.02–1.50 min 60% A, 1.5–2.0 min 100% A, 2.0–3.0 min 100–5% A, 3.0–3.7 min 5% A. The total chromatographic run time was 3.70 min. The analyser was working in negative ionization mode, and all the data was acquired and processed with Xcalibur 2.1 software (Themo Fisher Scientific, River Oaks Parkway, San Jose, USA). The mass spectrometer was operated with the following parameter settings: spray voltage, 2.5 kV; sheath gas, 60 arbitrary units; auxiliary gas, 20 arbitrary units; sweep gas, 0 arbitrary units, capillary temperature, 350 °C; S-lens RF level, 60; heater temperature, 400 °C. Optimal normalized collision energy (NCE) was set at 30 during all the analysis. Two experiments were performed in two corresponding chromatographic runs. First experiment consisted in two scan events. Scan event 1 performed by full scan from 100 to 350 Da with nominal mass resolving power of 60,000 FWHM in order to detect the accurate mass measurements of the [*M*–*H*] ions of diclofenac. Scan event 2 was a “Dependent Scan” at 30,000 of resolution and allowed the fragmentation of peak of diclofenac found in Scan Event 1 into the ion trap and then analysed into the Orbitrap in order to obtain the fragment ions (MS^2). Second experiment consisted in the fragmentation of the product ions from the first experiment obtaining the MS^3 at a resolution of 7500 FWHM. Identification of diclofenac was based on different criteria: the retention time, the isotopic pattern, the fragmentation pattern, the double bound equivalent (DBE) and the accurate mass (with errors below ± 2 ppm at the time of matching the theoretical mass with the measured mass). All the experiments were performed with a diclofenac standard (25 ng mL^{-1}), a sample extract (spiked also at 25 ng mL^{-1}) and the environmental samples taken upstream and downstream the WWTP.

3. Results and discussion

3.1. Extraction and purification optimization

Sonication was selected as extraction method according to preliminary studies. Energy was transmitted from the probe directly into the sample with high intensity and the sample was quickly processed. Two solvent mixtures were considered during the optimization: methanol (100%) and 0.1% formic acid in water/acetonitrile (1:3, v/v). Higher recoveries were obtained using 100% methanol for the majority of the compounds (see Fig. 1). Sample size (50, 100 and 200 mg, always referred as dry weight) was evaluated as an important parameter, due to the small size of the considered organisms, which is usually a limiting factor during sampling. A compromise was reached in between limit of detection and matrix effect, and 100 mg was selected as the final biomass.

For the purification of the extracts, florisil cartridges were discarded as a viable option, despite being commonly used for the elimination of matrix interferences such as lipids [30,31], due to the low recoveries for most of the target compounds. Oasis HLB cartridges have been widely applied for the analysis of pharmaceuticals [32–34]. A comparison between normal 6 mL cartridges and a 96-well microplate with Oasis HLB stationary phase was performed. Recoveries were similar for the majority of the compound, and significant differences were not be detected between both techniques. Finally, OSTRO^{TM} plate was applied, as it was developed to remove phospholipids and precipitate proteins from small volumes of sample [35]. OSTRO^{TM} resulted as the most appropriate technique, with the highest recoveries for the majority of the target compounds and good reproducibility (see Fig. 2).

Presented results led to the selection of an extraction protocol method based on extraction with ultrasonic probe (120 s, 3 cycles, 30% amplitude) of 100 mg (dry weight) of sample homogenized in 3 mL of methanol. Extract (1.5 mL) was the evaporated to dryness and redissolved in 0.75 μL of a mixture of water/acetonitrile containing 0.1% formic acid. Extract was passed through the OSTRO^{TM} plate, followed by 0.75 μL of the same fresh solvent. Purified extract was divided in two aliquots for the analysis of both families of compounds by UPLC–MS/MS. Aliquots were evaporated to dryness and redissolved in: (a) methanol/water (1:1, v/v) for EDCs; (b) methanol/water (1:9, v/v) for PhACs.

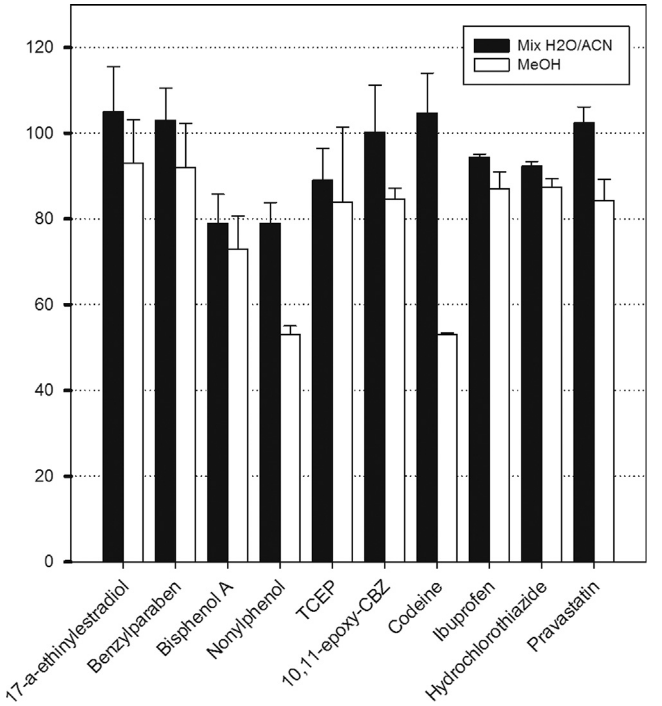

Fig. 1. Comparison of extraction efficiencies (%) of some EDCs and PhACs by sonication using two solvents: a mixture of water/acetonitrile (1:3) and 100% methanol ($n=3$).

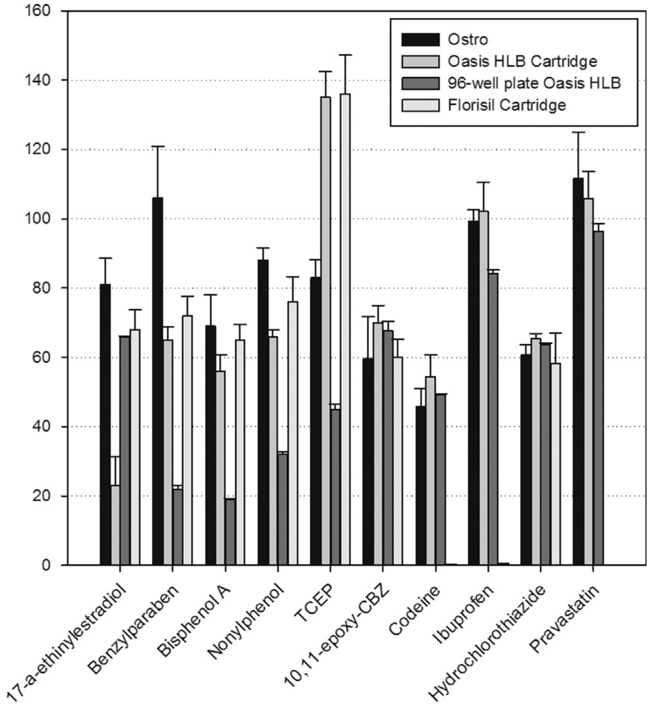

Fig. 2. Comparison of cleanup efficiencies (%) of some EDCs and PhACs by solid phase extraction (SPE) with Oasis HLB and Florisil cartridges, a 96-well-plate Oasis HLB stationary phase, and with Waters OSTRO^{TM} plate ($n=3$).

3.2. Method validation

Method detection limits (MDLs) and method quantification limits (MQLs) in macroinvertebrates for both contaminant families are presented in Tables 1 and 2. Both, MDLs and MQLs, were considered as the minimum amount of analyte with a signal-to-noise ratio of 3 and 10, respectively and were determined in samples spiked at 10 ng g^{-1} ($n=3$). MDLs for EDCs were in the range of 0.08–2.4 ng g^{-1}, and for PhACs, 0.06–4.3 ng g^{-1}.

Calibration curves were generated using linear regression analysis ($r^2>0.98$) in the concentration range from 0.1–100 ng g^{-1}. Ion suppression was measured in 20% of the target compounds (data not shown), so isotopically-labelled compounds were used to compensate possible matrix effects with ESI. Accuracy of the instrumental method was calculated from three repeated injections of a spiked sample at 10 ng g^{-1} in the same day as the deviation of the measured mean concentration from the spiked concentration, expressed in percentage, as described by Bogialli et al. [36]. The instrumental precision (intra-day and inter-day) was calculated from five repeated injections in the same day and in five consecutive days. Both values were lower than 20% for EDCs and PhACs.

3.3. Application to environmental samples

3.3.1. Determination of PhACs and EDCs in water

A total of 81 multiple-class human and veterinary PhACs and 19 EDCs and related compounds, as well as some transformation products, were analysed. PhAC and EDCs water concentrations are presented in Table S3. Recovery percentages and method detection limits for EDCs in water samples are presented in Table S4. Twenty-two PhACs and 6 EDCs were detected downstream the WWTP, in concentrations ranging from non-detected to 573 ng L^{-1} and 261 ng L^{-1}, respectively. In contrast, only a PhAC (2-hydroxy-ibuprofen) and 3 EDCs (caffeine, nonylphenol and 1-*H*-benzotriazole) could be quantified upstream the WWTP. Overall, a clear increase in PhACs & EDCs concentrations was observed in the downstream over upstream sites, indicating that the WWTP effluent is a source of contaminant inputs to the River Segre.

Regarding PhACs, non-steroidal anti-inflammatory drugs (NSAIDs) were the most abundant compounds. The highest value was found for 2-hydroxy-ibuprofen (573 ng L^{-1}) followed by acetaminophen (222 ng L^{-1}) and ibuprofen (193 ng L^{-1}). Lower but still considerable levels were found for the diuretic hydrochlorothiazide (311 ng L^{-1}), and the lipid regulator gemfibrozil (285 ng L^{-1}). Other significant groups were the β-blocking agent atenolol (28 ng L^{-1}) and psychiatric drugs such as carbamazepine and venlafaxine. These concentrations are in agreement with a previous study carried out in the same area, where concentrations of some of these PhACs were between 10 and 500 ng L^{-1} [37]. In similar studies performed in surface waters affected by the effluent of a WWTP, concentrations for the majority of these PhACs were in the range from non-detected to 600 ng L^{-1} [28,38], including ibuprofen metabolites, which have been detected at significant concentrations (up to 1 μg L^{-1}) and higher than the values found for the parent compound [39,40].

In the case of EDCs, the triazoles tolyltriazole (261 ng L^{-1}) and 1-*H*-benzotriazole (194 ng L^{-1}) were the most abundant compounds. Other significant groups were: the stimulant caffeine (207 ng L^{-1}), the flame retardants TBEP and TCPP (65 and 71 ng L^{-1}, respectively), and nonylphenol (116 ng L^{-1}). Similar levels of concentrations for TBEP, caffeine and nonylphenol have been detected by several research groups in river and groundwater from Europe, USA, and Australia [41–44].

3.3.2. Determination of PhACs and EDCs in macroinvertebrates

Several compounds were detected in three species of macroinvertebrates (Table 3). PhACs were not detected in the upstream samples in any species, whereas three EDCs – TBEP, estrone and nonylphenol – were measured in upstream samples in at least one of the three taxa., Two NSAIDs drugs (diclofenac and ibuprofen) were detected downstream of the WWTP in *Hydropsyche*. Four

Table 1
Mean percent recoveries ($n=3$) of EDCs at two spiking levels in macroinvertebrates. Method detection and quantification limits (MDL, MQL) in macroinvertebrate composite (ng g^{-1}, dry weight).

	% Recoveries (± RSD)				**Precision (%RSD)**		
	100 ng g^{-1}	500 ng g^{-1}	**MDL** (ng g^{-1})	**MQL** (ng g^{-1})	**Intra-day**	**Inter-day**	**Accuracy (%)**
1*H*-benzotriazole	48 (± 11)	74 (± 14)	0.41	1.4	16	20	−0.10
17α-Ethinylestradiol	40 (± 10)	77 (± 5.9)	2.4	8.0	4.5	19	22
17β-Estradiol	43 (± 13)	83 (± 1.4)	0.34	1.1	5.1	13	9.1
Benzylparaben	40 (± 3.7)	59 (± 19)	0.50	1.6	3.6	9.4	−0.10
Bisphenol A	79 (± 13)	87 (± 11)	0.32	1.1	2.0	8.4	4.8
Caffeine	61 (± 16)	84 (± 18)	0.75	2.5	12	18	−9.7
Estriol	59 (± 12)	117 (± 4.8)	1.1	3.7	0.70	4.6	−3.8
Estrone	46 (± 17)	82 (± 6.1)	0.43	1.4	2.8	11	2.9
E1-3S	69 (± 15)	64 (± 1.0)	0.57	1.9	5.1	8.2	0.80
E1-3G	56 (± 19)	48 (± 13)	1.4	4.5	13	20	5.4
E2-17G	56 (± 22)	54 (± 7.9)	1.6	5.3	4.7	5.4	2.6
Ethylparaben	42 (± 14)	82 (± 7.9)	0.25	0.85	3.1	6.6	−10
Levonorgestrel	48 (± 15)	59 (± 22)	1.1	3.7	11	14	−3.5
Methylparaben	52 (± 6.5)	78 (± 3.0)	0.42	1.4	2.4	5.8	13
Nonylphenol	69 (± 13)	53 (± 12)	0.40	1.3	2.9	4.0	−11
Progesterone	64 (± 10)	80 (± 16)	0.26	0.88	5.3	15	−25
Propylparaben	50 (± 5.9)	79 (± 16)	0.13	0.42	3.4	5.5	2.3
Tolyltriazole	76 (± 0.1)	105 (± 14)	0.31	1.0	8.0	12	11
TCEP	40 (± 13)	56 (± 17)	0.25	0.84	4.2	8.1	8.9
TBEP	61 (± 18)	98 (± 0.4)	0.10	0.27	3.1	13	7.2
TCPP	55 (± 9.0)	87 (± 18)	0.20	0.67	3.5	15	3.7

Table 2
Mean percent recoveries ($n=3$) of PhACs at two spiking levels in macroinvertebrates.

	% Recoveries (± RSD)				**Precision (% RSD)**		
	100 ng g^{-1}	200 ng g^{-1}	**MDL** (ng g^{-1})	**MQL** (ng g^{-1})	**Intra-day**	**Inter-day**	**Accuracy (%)**
Acetaminophen	68 (± 16)	57 (± 7.4)	1.9	6.4	2.5	14	6.9
Acridone	74 (± 0.2)	82 (± 21)	0.78	2.6	1.7	2.4	−17
Alprazolam	96 (± 19)	121 (± 17)	0.71	2.3	1.2	2.5	−11
Atenolol	45 (± 5.1)	65 (± 12)	0.45	1.0	1.1	2.5	13
Azaperone	73 (± 18)	91 (± 13)	1.6	5.3	1.9	2.3	−4.0
Bezafibrate	47 (± 15)	47 (± 6.3)	0.070	0.24	4.5	14	10
Carbamazepine	61 (± 1)	79 (± 9.9)	0.060	0.21	0.80	1.3	0.10
2-OH-CBZ	51 (± 14)	70 (± 16)	1.4	4.6	2.3	2.7	3.3
10,11-epoxy-CBZ	54 (± 2.1)	97 (± 19)	1.1	3.5	4.3	4.4	0.10
Citalopram	66 (± 19)	94 (± 16)	0.10	0.31	0.5	2.0	−12
Codeine	51 (± 15)	71 (± 20)	2.0	6.8	3.3	8.9	−16
Dexamethasone	43 (± 7.4)	41 (± 18)	0.28	0.94	3.3	10	0.10
Diclofenac	44 (± 15)	51 (± 0.7)	0.51	1.7	1.7	15	4.9
Diltiazem	77 (± 7.2)	93 (± 4.6)	0.83	2.8	0.6	1.1	−8.4
Fluoxetine	37 (± 7.5)	68 (± 7.8)	0.44	1.5	1.7	3.8	−0.10
Gemfibrozil	85 (± 16)	85 (± 18)	0.15	0.50	14	18	0.10
Hydrochlorothiazide	70 (± 15)	58 (± 13)	0.10	0.34	8.5	9.4	6.8
Ibuprofen	88 (± 11)	87 (± 3.4)	0.64	2.1	6.9	12	−14
1-OH-ibuprofen	81 (± 15)	125 (± 18)	1.0	3.4	9.0	12	−4.7
2-OH-ibuprofen	90 (± 14)	93 (± 20)	1.3	4.3	6.8	9.0	0.10
Levamisol	37 (± 14)	55 (± 18)	2.5	8.2	2.4	3.7	1.4
Metronidazole	99 (± 12)	99 (± 21)	2.1	6.9	2.0	2.7	−16
Metoprolol	66 (± 17)	75 (± 5.2)	0.080	0.25	1.4	3.2	−0.1
Nadolol	41 (± 17)	58 (± 3.6)	0.35	1.2	1.6	3.1	−4.8
Naproxen	104 (± 15)	109 (± 17)	0.21	0.69	4.8	20	0.10
Norverapamil	58 (± 8.0)	77 (± 11)	0.79	2.6	1.4	1.6	0.10
Paroxetine	103 (± 17)	133 (± 16)	1.6	5.4	1.8	4.3	−12
Piroxicam	59 (± 3.2)	66 (± 16)	2.8	9.5	2.7	4.8	−0.10
Pravastatin	46 (± 16)	37 (± 14)	4.3	14	3.2	5.1	−24
Propanolol	23 (± 19)	34 (± 20)	2.9	9.5	0.60	1.2	0.10
Propyphenazone	60 (± 17)	83 (± 14)	0.16	0.54	1.3	3.8	16
Ronidazole	46 (± 4.5)	56 (± 18)	0.41	1.3	2.3	2.7	15
Salbutamol	45 (± 16)	59 (± 17)	0.21	0.69	3.7	4.2	11
Sotalol	31 (± 11)	46 (± 9.0)	1.1	3.6	3.4	4.1	−0.10
Sulfamethoxazole	59 (± 10)	49 (± 11)	0.71	2.3	3.6	4.4	−11
Tamsulosin	50 (± 12)	64 (± 6.6)	0.20	0.68	3.0	3.5	−0.10
Torasemide	118 (± 7.6)	113 (± 5.1)	0.10	0.31	3.5	8.2	−3.4
Venlafaxine	63 (± 18)	89 (± 16)	0.10	0.30	0.50	1.9	−3.5
Verapamil	79 (± 20)	118 (± 0.3)	0.71	2.3	1.3	2.0	11
Warfarin	57 (± 4.2)	64 (± 17)	0.15	0.50	1.1	1.9	0.10
Xylazine	43 (± 15)	65 (± 10)	0.54	1.8	1.3	1.6	−14

Table 3
PhACs & EDCs concentration (ng g^{-1}, dry weight) in macroinvertebrates from the Segre River (NE Spain).

	Hydropsyche		Ancylus		Hirudo	
	Upstream	Downstream	Upstream	Downstream	Upstream	Downstream
PhACs						
Ibuprofeno	n.d.	183	n.d.	n.d.	n.d.	n.d.
Diclofenac	n.d.	12.4	n.d.	n.d.	n.d.	n.d.
EDCs						
Estrone	n.d.	n.d.	n.d.	n.d.	17.6	12.4
Bisphenol A	n.d.	8.70	n.d.	n.d.	n.d.	n.d.
TBEP	17.9	31.1	7.70	31.8	22.3	20.6
Nonylphenol	n.d.	n.d.	75.5	96.3	76.7	130

EDCs (estrone, bisphenol A, TBEP, and nonylphenol) were detected overall across the three taxa.

It is generally accepted that substances with a logarithm of octanol–water partition coefficient (log K_{ow}) values equal or higher than 3 have the potential to bioaccumulate in organisms, and particularly in lipid tissues [45]. EDCs detected in macroinvertebrates in this study have a log K_{ow} ranging from 3.9 (TBEP) to 5.7 (nonylphenol) (see Table S2), which could make them susceptible for bioaccumulation in aquatic organisms according to this concept [46]. This seems a plausible hypothesis, as the other three EDCs detected in water (1*H*-benzotriazole, tolyltriazole and caffeine), detected at concentrations around 200 ng L^{-1} but with log $K_{ow} < 2$, were not detected in macroinvertebrates. In fact, TBEP was the most ubiquitous compound, as it was detected in samples upstream and downstream for the three macroinvertebrate taxa in concentrations up to 32 ng g^{-1}. TBEP has previously been detected in other organisms, such as herring gull eggs, flathead gray mullet, common carp and Mediterranean barb in similar concentrations [29,47,48]. Nonylphenol was also detected both upstream and downstream the WWTP effluent in *Ancylus* and *Phagocata*, with maximum concentrations for *Phagocata* of 77 ng g^{-1} in the upstream sample and 130 ng g^{-1} downstream the WWTP. This compound has also been detected in concentrations between 206 and 435 ng g^{-1} in mysid shrimps, a crustacean found in marine and freshwaters in the Netherlands [49]. In Italy, concentrations of nonylphenol up to 240 ng g^{-1} were measured in Mediterranean mussel (*Mytilus galloprovincialis*) in a coastal water ecosystem receiving industrial and municipal WWTP effluents [50]. The natural hormone estrone was only detected in *Phagocata* both upstream and downstream the WWTP at 12–18 ng g^{-1}. Estrone has also been detected in fish muscle elsewhere [29,47,51]. However, the likelihood that this hormone was endogenous of *Phagocata* individuals cannot be dismissed. For example, in the polychaete *Nereis virens*, estradiol has been detected in male and female coelomic fluid [52]. There also evidence that indicates that some mollusks are able to synthesize sex steroids, including estrone [46,53,54]. Estrone, estradiol, testosterone, and progesterone have been found in gastropods [55], cephalopods [56], and various bivalves such as Pacific oysters (*Crassostrea gigas*), Yesso scallops (*Patinopecten yessoensis*) [57], and Blue mussels (*Mytilus edulis*) [54,58,59]. However, some authors affirm that there is no convincing evidence for biosynthesis of vertebrate steroids by invertebrates, for example mollusks [60], which might suggest that the presence of estrone in *Phagocata* could be due to uptake from water. Finally, Bisphenol A was only detected in the downstream sample in *Hydropsyche* at 8.7 ng g^{-1}. Although it was detected in biota samples, this compound was below detectable concentrations in the corresponding water sample, which may suggest that this compound could be more persistent in the macroinvertebrate tissue compared to water. Bisphenol A has been detected in fish tissues of several countries in concentrations up to more than 200 ng g^{-1} [29,47,50,51,61].

In the case of PhACs, four of the analyzed compounds had a log $K_{ow} > 3$. However, only two of them, ibuprofen and diclofenac, were detected in macroinvertebrates, whereas gemfibrozil and bezafibrate, present at relatively high concentrations and with log K_{ow} between 3.9 and 4.3, were not detected in the samples. This suggests that accumulation of PhACs is not only regulated by their log K_{ow}. Pharmaceuticals are, in general, hydrophilic compounds and quite often ionized at the pH of surface waters, with acidic and/or basic chemical functional groups, which affects the lipophilicity of these compounds, and thus, the bioaccumulation in aquatic organisms [62]. For instance, accumulation of fluoxetine in fish and algae is significantly affected by variation in pH [63,64]. Ibuprofen and diclofenac have p*K*a < 5, thus would be primarily ionized at the pH ~7.8 of the river water, and the anions would be more soluble than the neutral molecule, leading to a reduction of the sorption by hydrophobic partition onto organic matter [65]. Actually, their log *D* is 0.85 at pH ~7.8 (calculated by ChemAxon), much lower than for the detected EDCs, which suggests that other mechanisms other that lipophilicity are influencing bioaccumulation in *Hydropsyche*. In this study, concentrations of PhACs ibuprofen and diclofenac in *Hydropsyche* were in the range from non-detected to 183 ng g^{-1}, similar to those detected in fish bile [66], and tissues [67]. Sorption of these compounds to colloids in water may occur, act as a long-term storage and being a sink for PhACs [62]. A recent study estimated calculated that the colloidal phase contributed between 22–33% of diclofenac and 9–28% of ibuprofen in the aquatic system, highlighting the important role of colloids as carrier of contaminants [68]. An increased exposure to these compounds could take place due to the filter-feeding habits of *Hydropsyche* [69], which could enhance its uptake.

3.4. Presence of diclofenac in trichopterans

Extremely high concentrations of the anti-inflammatory diclofenac were measured in the *Hydropsyche* extracts when quantifying with the first transition (SRM_1) (see Section 2), both in the upstream and downstream sample. However, SRM_2 was not detected in the upstream sample, and ratio could not be calculated. According to EU Commission Decision 2002/657/EC [70], a requisite to confirm a compound by low resolution mass spectrometry is that the relative intensities of the detected fragment ions have to correspond to those of the calibration standard at comparable concentrations within a minimum of tolerance: 20%. Since diclofenac is known to have low intensity of the second transition produced during the fragmentation (294→214) [66], as the relative intensity (% of base peak) in a standard is $\leq 10\%$, thus this tolerance can reach 50%. In both the upstream and downstream *Hydropsyche* samples, SRM_1 was detected at similar intensity. SRM_2 was detected in the downstream sample but the ratio was 30 times higher than the ratios obtained with the diclofenac standard. Extracts of

Phagocata and *Ancylus* from the upstream site were spiked with 25 ng mL^{-1} of diclofenac to calculate the SRM$_1$/SRM$_2$ ratio of diclofenac in a complex matrix. Ratios of diclofenac in *Phagocata* and *Ancylus* spiked extracts did comply with the aforementioned rule. However, when the upstream *Hydropsyche* sample was spiked with 25 ng mL^{-1} of diclofenac, the ratio was still much higher than in the standard. Based on these results, the presence of an isobaric interference in *Hydrophsyche* was postulated. In order to confirm this hypothesis and to ensure the correct quantification of diclofenac in the environmental samples, further measures and analytical strategies were taken. A Hypersil Gold PFP column, which provides an alternative selectivity to C$_{18}$ columns to resolve mixtures of halogenated compounds and non-halogenated compounds, was tested to achieve the separation of diclofenac and the suspected interference. The upstream *Hydropsyche* extract spiked with diclofenac at 25 ng mL^{-1} and the non-spiked downstream sample were injected and two peaks were indeed resolved in both cases with the Hypersil Gold PFP column – RT: 1.3 min and 1.6 min (see Fig. 3a) – while in the C$_{18}$ column only one peak appeared. SRM$_1$ was detected in both peaks separated by Hypersil Gold column, whereas SRM$_2$ was only detected in the second peak, and the SRM$_1$/SRM$_2$ ratio in the second peak confirmed that it was diclofenac. An isobaric interference was assumed to co-elute with the target compound using C18 column, and that interference was only separated with the Hypersil Gold PFP column.

The probability of having an interfering isobaric ion largely decreases with increases in mass resolution and mass accuracy. Therefore, on a second step for confirmation purposes, the accurate mass of the parent compounds in the two resolved chromatographic peaks were determined by injection of the downstream sample in the TFC-LTQ Orbitrap using the same Hypersil Gold PFP column. Measured accurate mass was *m/z*: 294.1714 at RT: 1.3 min and *m/z*: 294.0090 at RT: 1.6 min. Comparing these values with the theoretical mass of diclofenac (*m/z*: 294.0089 and formula $C_{14}H_{11}NCl_2O_2$), an error of 552 ppm and 0.34 ppm was calculated for the isobaric interference (RT: 1.3 min) and suspected diclofenac (RT: 1.6 min), respectively. The low error (< 5 ppm) at RT: 1.6 min added one more confirmation criteria for the identification of diclofenac. In contrast, the most plausible molecular formula suggested by the software for the interfering isobaric mass (ion *m/z*: 294.17069) was $C_{16}H_{25}NO_4$, with a low error (2.8 ppm) and did not include Cl, which is very distinctive for diclofenac.

Accurate mass of the daughter ions was also calculated. The mass spectrometer was operated in a data-dependent-acquisition (DDA) mode in this study, which means that the full-scan analysis with the Orbitrap analyser was followed by a MS–MS scan when the accurate *m/z* of diclofenac was detected. Accurate mass of the product ion (SMR$_1$) of diclofenac was thus determined in the upstream spiked sample as *m/z*: 250.0190. This mass was detected in the downstream non-spiked sample at the RT: 1.6 min with an error of 1.129 ppm for the calculated molecular formula $C_{13}H_{11}NCl_2$. Conversely, the isobaric interference (*m/z*: 294.1714 and RT: 1.3 min) formed ions with different *m/z* values to those of diclofenac. Product ion of that interfering mass was identified as *m/z*: 250.1453 and the

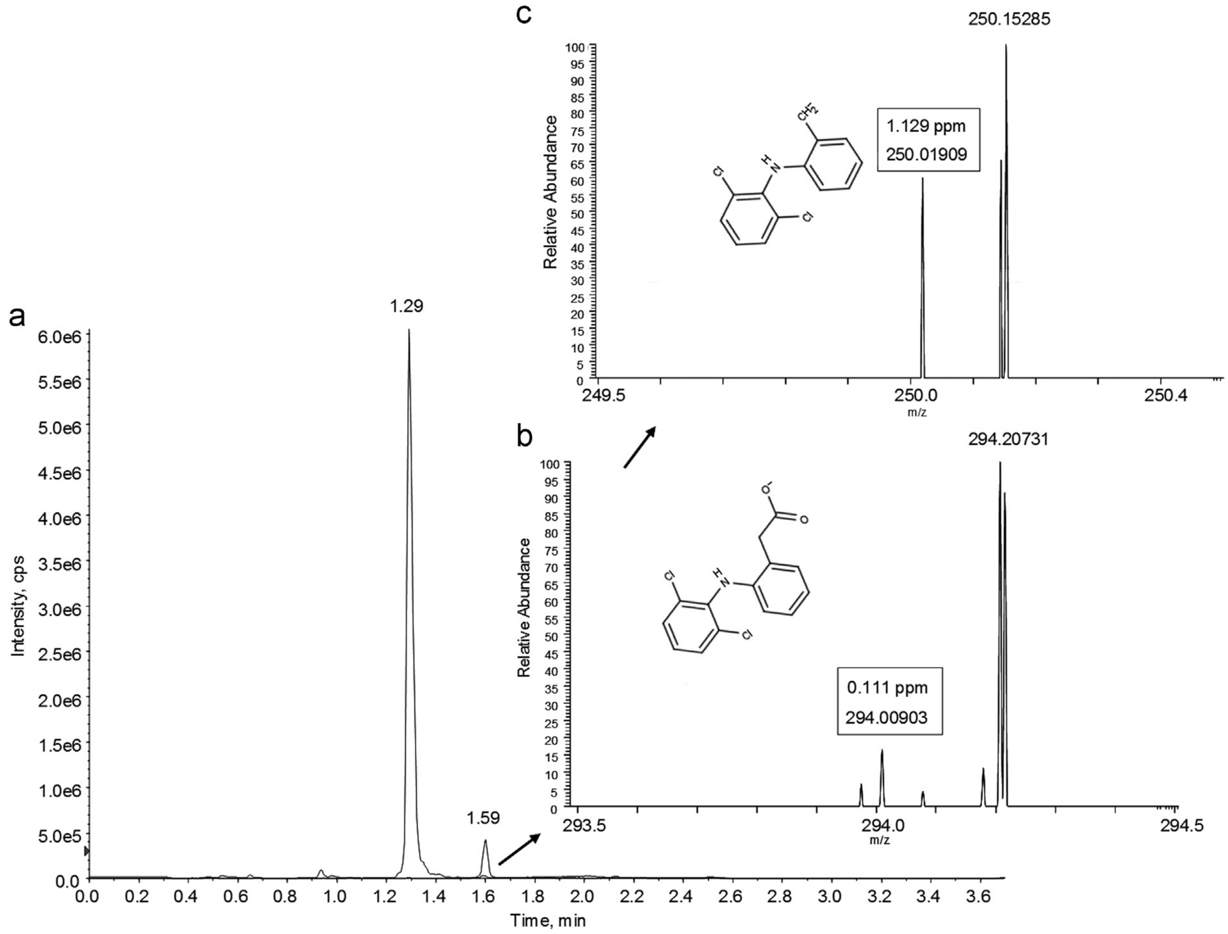

Fig. 3. (a) Chromatographic separation of diclofenac and matrix interference in a *Hydropsyche* sample performed in the Acquity UPLC tandem Qtrap MS; (b) accurate mass of diclofenac, *m/z* 294.009, determined in the Orbitrap MS: (c) Accurate mass of main product ion, *m/z* 250.019, was present in the data dependant scan of this *Hydropsyche* sample which confirmed that diclofenac was present.

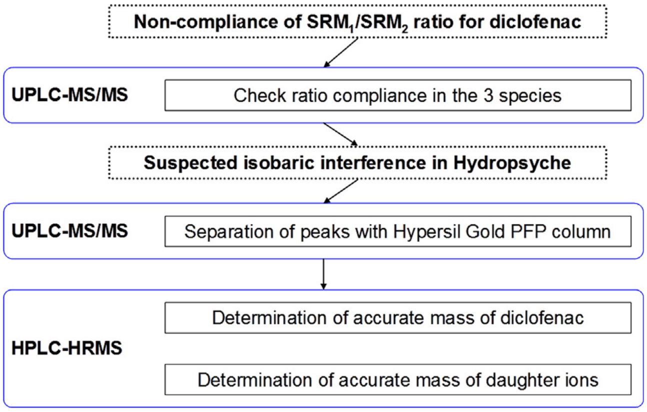

Fig. 4. Workflow applied for the interference discrimination in *Hydropsyche* samples.

molecular formula was $C_{14}H_{20}NO_3$ with an error of 1.092 ppm, being different to the daugher ion of diclofenac.

Other than RTs and accurate mass determination, fragmentation of the ionized molecule can also provide additional information for identification and improve specificity. Product ion of SMR1 of diclofenac (*m/z*: 250.0190 and RT: 1.6 min) was further fragmented in a MS^3 scan event, and gave the ion *m/z*: 214.0430, whose molecular formula was $C_{13}H_{11}NCl$.

According to EU Commission Decision 2002/657/EC, a precursor ion and a product ion at high resolution can achieve the minimum requirement for identification for high resolution mass spectrometry. Both, the accurate mass of *m/z* 250.0190 acquired at 60,000 FWHM, as described in Fig. 3c, and the accurate mass of the precursor ion *m/z*: 294.0090 were determined in the *Hydropsyche* downstream sample. Throughout the experiments, Orbitrap analyser provided excellent accuracy, which means that the matrix components were discriminated drastically. Thus, we were able to determine the presence of diclofenac in *Hydropsyche* samples at the downstream sampling site using SMR2 for quantification and the accurate mass of precursor ion and a product ion at high resolution for confirmation purposes. The workflow applied for the discrimination of the interference is shown in Fig. 4.

4. Conclusions

A simple, rapid, sensitive and efficient analytical method was developed for the determination of 41 PhACs and 21 EDCs in aquatic macroinvertebrates. The final multi-residue procedure consisted of an extraction by sonication, followed by purification of extract lipids, and the analysis was performed by UHPLC–MS/MS, which provided the necessary sensitivity and selectivity for these trace contaminants. The procedure was validated and it is characterized by good accuracy and precision, providing low quantification limits for macroinvertebrates. The method was applied to assess the bioaccumulation of these contaminants in three macroinvertebrate taxa (*Ancylus, Phagocata* and *Hydropsyche*) from a WWTP-impacted river. Two NSAIDs drugs, diclofenac and ibuprofen, and four EDCs – estrone, bisphenol A, TBEP, and nonylphenol – were detected in at least one macroinvertebrate taxa in concentrations up to 183 ng g^{-1}, which seems to indicate that invertebrates have the potential to be useful bioindicators of exposure to PhACs and EDCs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.09.017.

References

[1] M. Gros, M. Petrovic, A. Ginebreda, D. Barceló, Environ. Int. 36 (2010) 15–26.
[2] A. Jelic, M. Gros, A. Ginebreda, R. Cespedes-Sanchez, F. Ventura, M. Petrovic, D. Barcelo, Water Res. 45 (2011) 1165–1176.
[3] A. Joss, E. Keller, A.C. Alder, A. Gobel, C.S. McArdell, T. Ternes, H. Siegrist, Water Res. 39 (2005) 3139–3152.
[4] M. Petrovic, M. Sole, M.J. Lopez de Alda, D. Barcelo, Environ. Toxicol. Chem. 21 (2002) 2146–2156.
[5] V. Osorio, R. Marcé, S. Pérez, A. Ginebreda, J.L. Cortina, D. Barceló, Sci. Total Environ. 440 (2012) 3–13.
[6] M.A. Coogan, R.E. Edziyie, T.W. La Point, B.J. Venables, Chemosphere 67 (2007) 1911–1918.
[7] S.N. Garcia, M. Foster, L.A. Constantine, D.B. Huggett, Ecotoxicol. Environ. Saf. 84 (2012) 207–211.
[8] G. Vernouillet, P. Eullaffroy, A. Lajeunesse, C. Blaise, F. Gagné, P. Juneau, Chemosphere 80 (2010) 1062–1068.
[9] R. Céspedes, S. Lacorte, D. Raldúa, A. Ginebreda, D. Barcelò, B. Piña, Chemosphere 61 (2005) 1710–1719.
[10] D. Pascoe, W. Karntanut, C.T. Müller, Chemosphere 51 (2003) 521–528.
[11] S. Jobling, M. Nolan, C.R. Tyler, G. Brighty, J.P. Sumpter, Environ. Sci. Technol. 32 (1998) 2498–2506.
[12] S. Jobling, R. williams, A. Johnson, A. Taylor, M. Gross-Sorokin, M. Nolan, C. Tyler, Environ. Health Perspect. 114 (Suppl. 1) (2006) 32–39.
[13] T.W. Valenti Jr., G.G. Gould, J.P. Berninger, K.A. Connors, N.B. Keele, K.N. Prosser, B.W. Brooks, Environ. Sci. Technol. 46 (2012) 2427–2435.
[14] J. Corcoran, M.J. Winter, C.R. Tyler, Crit. Rev. Toxicol. 40 (2010) 287–304.
[15] European Commision, in: 2011/0429 (COD), Brussels, 2012.
[16] C.A. Kinney, E.T. Furlong, D.W. Kolpin, M.R. Burkhardt, S.D. Zaugg, S.L. Werner, J.P. Bossio, M.J. Benotti, Environ. Sci. Technol 42 (2008) 1863–1870.
[17] L.B. Barber, S.H. Keefe, R.C. Antweiler, H.E. Taylor, R.D. Wass, Environ. Sci. Technol. 40 (2005) 603–611.
[18] E.B. Dussault, V.K. Balakrishnan, U. Borgmann, K.R. Solomon, P.K. Sibley, Ecotoxicol. Environ. Saf. 72 (2009) 1635–1641.
[19] D.P. Batzer, A. Ruhí, Freshwater Biol. 58 (2013) 1647–1659.
[20] K.L. Goodyear, S. McNeill, Sci. Total Environ. 229 (1999) 1–19.
[21] A. Ruiz-García, J. Márquez-Rodríguez, M. Ferreras-Romero, Ecol. Indic. 14 (2012) 114–123.
[22] E.C. Bizzotto, S. Villa, M. Vighi, Environ. Pollut. 157 (2009) 3192–3198.
[23] D.T. Rhea, D.D. Harper, A.M. Farag, W.G. Brumbaugh, Environ. Monit. Assess. 115 (2006) 381–393.
[24] J. Chetelat, M. Amyot, E. Garcia, Environ. Pollut. 159 (2011) 10–17.
[25] H.A. de Wit, M.J. Kainz, M. Lindholm, Environ. Pollut. 164 (2012) 235–241.
[26] M. Liebig, P. Egeler, J.r. Oehlmann, T. Knacker, Chemosphere 59 (2005) 271–280.
[27] E.B. Dussault, V.K. Balakrishnan, K.R. Solomon, P.K. Sibley, Can. J. Chem. 87 (2009).
[28] M. Gros, S. Rodríguez-Mozaz, D. Barceló, J. Chromatogr. A 1248 (2012) 104–121.
[29] A. Jakimska, B. Huerta, Z. Barganska, A. Kot-Wasik, S. Rodriguez-Mozaz, D. Barcelo, J. Chromatogr. A 1306 (2013) 44–58.
[30] J. Hong, H.-Y. Kim, D.-G. Kim, J. Seo, K.-J. Kim, J. Chromatogr. A 1038 (2004) 27–35.
[31] A. Dirtu, L. Roosens, T. Geens, A. Gheorghe, H. Neels, A. Covaci, Anal. Bioanal. Chem. 391 (2008) 1175–1181.
[32] M.J. Gómez, M. Petrovic, A.R. Fernández-Alba, D. Barcelò, J. Chromatogr. A 1114 (2006) 224–233.
[33] M. Huerta-Fontela, M.T. Galceran, F. Ventura, J. Chromatogr. A 1217 (2006) 4212–4222.
[34] M. Gros, M. Petrovic, D. Barceló, Talanta 70 (2006) 678–690.
[35] Waters Corporation, in, 2010.
[36] S. Bogialli, R. Curini, A. Di Corcia, M. Nazzari, R. Samperi, Anal. Chem. 75 (2003) 1798–1804.

[37] V. Acuña, D. vonSchiller, M.J. García-Galán, S. Rodríguez-Mozaz, L. Corominas, M. Petrovic, M. Poch, D. Barceló, S. Sabater, Sci. Total Environ. 2014, http://dx.doi.org/10.1016/j.scitotenv.2014.05.067, in press.
[38] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Water Res. 42 (2008) 3498–3518.
[39] L. Ferrando-Climent, N. Collado, G. Buttiglieri, M. Gros, I. Rodriguez-Roda, S. Rodriguez-Mozaz, D. Barcelo, Sci. Total Environ. 438 (2012) 404–413.
[40] S. Weigel, U. Berger, E. Jensen, R. Kallenborn, H. Thoresen, H. Huhnerfuss, Chemosphere 56 (2004) 583–592.
[41] M. Gorga, M. Petrovic, D. Barcelo, J. Chromatogr. A 1295 (2013) 57–66.
[42] R.A. Trenholm, B.J. Vanderford, J.C. Holady, D.J. Rexing, S.A. Snyder, Chemosphere 65 (2006) 1990–1998.
[43] K. Quednow, W. Püttmann, Environ. Pollut. 152 (2008) 476–483.
[44] U. Bolz, H. Hagenmaier, W. Körner, Environ. Pollut. 115 (2001) 291–301.
[45] C. Franke, G. Studinger, G. Berger, S. Böhling, U. Bruckmann, D. Cohors-Fresenborg, U. Jöhncke, Chemosphere 29 (1994) 1501–1514.
[46] C. Riva, C. Porte, A. Binelli, A. Provini, Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 152 (2010) 175–181.
[47] W.-L. Chen, G.-S. Wang, J.-C. Gwo, C.-Y. Chen, Talanta 89 (2012) 237–245.
[48] J.-W. Kim, B.R. Ramaswamy, K.-H. Chang, T. Isobe, S. Tanabe, J. Chromatogr. A 1218 (2011) 3511–3520.
[49] T.A. Verslycke, A.D. Vethaak, K. Arijs, C.R. Janssen, Environ. Pollut. 136 (2005) 19–31.
[50] G. Pojana, A. Gomiero, N. Jonkers, A. Marcomini, Environ. Int. 33 (2007) 929–936.
[51] J. Liu, R. Wang, B. Huang, C. Lin, J. Zhou, X. Pan, Environ. Pollut. 162 (2012) 325–331.
[52] J. Keay, J.W. Thornton, Endocrinology 150 (2009) 1731–1738.
[53] J.W. Thornton, E. Need, D. Crews, Science 301 (2003) 1714–1717.
[54] M. Kajiwara, S. Kuraku, T. Kurokawa, K. Kato, S. Toda, H. Hirose, S. Takahashi, Y. Shibata, T. Iguchi, T. Matsumoto, T. Miyata, T. Miura, Y. Takahashi, Gen. Comp. Endocrinol. 148 (2006) 315–326.
[55] D. Le Guellec, M.C. Thiard, J.P. Remy-Martin, A. Deray, L. Gomot, G.L. Adessi, Gen. Comp. Endocrinol. 66 (1987) 425–433.
[56] A. D'Aniello, A. Di Cosmo, C. Di Cristo, L. Assisi, V. Botte, M.M. Di Fiore, Biochem. Biophys. Res. Commun. 227 (1996) 782–788.
[57] T. Matsumoto, M. Osada, Y. Osawa, K. Mori, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 118 (1997) 811–817.
[58] M.A. Reis-Henriques, D. Le Guellec, J.P. Remy-Martin, G.L. Adessi, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 95 (1990) 303–309.
[59] W. Zhu, K. Mantione, D. Jones, E. Salamon, J.J. Cho, P. Cadet, G.B. Stefano, Neuroendocrinol. Lett. 24 (2003) 137–140.
[60] A.P. Scott, Steroids 78 (2013) 268–281.
[61] C. Miège, J.M. Choubert, L. Ribeiro, M. Eusèbe, M. Coquery, Environ. Pollut. 157 (2009) 1721–1726.
[62] A. Zenker, M.R. Cicero, F. Prestinaci, P. Bottoni, M. Carere, J. Environ. Manage. 133 (2014) 378–387.
[63] Y. Nakamura, H. Yamamoto, J. Sekizawa, T. Kondo, N. Hirai, N. Tatarazako, Chemosphere 70 (2008) 865–873.
[64] J. Neuwoehner, B.I. Escher, Aquat. Toxicol. 101 (2011) 266–275.
[65] J.C. Durán-Álvarez, B. Prado, A. Ferroud, N. Juayerk, B. Jiménez-Cisneros, Sci. Total Environ. 473-474 (2014) 189–198.
[66] J.-M. Brozinski, M. Lahti, A. Meierjohann, A. Oikari, L. Kronberg, Environ. Sci. Technol. 47 (2013) 342–348.
[67] B. Huerta, A. Jakimska, M. Gros, S. Rodriguez-Mozaz, D. Barcelo, J. Chromatogr. A 1288 (2013) 63–72.
[68] Y.-P. Duan, X.-Z. Meng, Z.-H. Wen, R.-H. Ke, L. Chen, Sci. Total Environ. 447 (2013) 267–273.
[69] Tachet H, Richoux P, Bournaud M, U.-P. P, Invertebrés d'Eau Douce (2nd corrected impression) ed. CNRS Editions, Paris, 2002.
[70] Council Directive 96/23/EC. Off. J. Union, 2002, L 221/8-36.

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Determination of a broad spectrum of pharmaceuticals and endocrine disruptors in biofilm from a waste water treatment plant-impacted river

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HIGHLIGHTS

- Method for analysis of pharmaceuticals & endocrine disruptors in river biofilm
- Bioaccumulation in biofilm of a WWTP-impacted river evaluated
- Seven PhACs and five EDCs detected in biofilm downstream of the WWTP

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ABSTRACT

Wastewater treatment plants (WWTPs) are one of the main sources of pharmaceuticals and endocrine disrupting compounds in freshwater ecosystems, and several studies have reported bioaccumulation of these compounds in different organisms in those ecosystems. River biofilms are exceptional indicators of pollution, but very few studies have focused on the accumulation of these emerging contaminants. The objectives of this study were first to develop an efficient analytical methodology for the simultaneous analysis of 44 pharmaceuticals and 13 endocrine disrupting compounds in biofilm, and second, to assess persistence, distribution, and bioaccumulation of these contaminants in natural biofilms inhabiting a WWTP-impacted river. The method is based on pressurized liquid extraction, purification by solid-phase extraction, and analysis by ultra performance liquid chromatography coupled to a mass spectrometer (UPLC–MS/MS) in tandem. Recoveries for pharmaceuticals were 31–137%, and for endocrine disruptors 32–93%. Method detection limits for endocrine disruptors were in the range of 0.2–2.4 ng g^{-1}, and for pharmaceuticals, 0.07–6.7 ng g^{-1}. A total of five endocrine disruptors and seven pharmaceuticals were detected in field samples at concentrations up to 100 ng g^{-1}.

1. Introduction

Hundreds of pharmaceuticals (PhACs) are ubiquitously detected in freshwater ecosystems at concentrations ranging between ng L^{-1} to μg L^{-1} (Daughton and Ternes, 1999). Despite these relatively low concentrations, PhACs may pose a risk to aquatic organisms because they are designed to modify biochemical pathways in the human body at low doses. Pharmaceuticals are developed to remain in the human body for an adequate period of time to reach their therapeutic effect, which means that a great majority of them are excreted mostly unchanged and may persist in the environment (Boxall et al., 2004). Another group of emerging contaminants widely detected in freshwater ecosystems are endocrine disrupting compounds (EDCs). These compounds belong to different chemical families, and are able to interfere with the hormonal system of exposed organisms by mimicking or counteracting natural hormones (Céspedes et al., 2005; Pojana et al., 2007). The presence of these compounds in freshwater ecosystems is of special concern considering that organisms are chronically exposed to a mixture of PhACs and EDCs. Well-known examples of harmful effects due to exposure to emerging contaminants are the feminisation of male fish (Kidd et al., 2007; Sumpter, 1998), inhibition of molting in crustaceans (Rodriguez et al., 2007), and altered fish behavior (Margiotta-Casaluci et al., 2014; Valenti et al., 2012). Wastewater treatment plants (WWTP) have been identified as one of the main sources of PhACs and EDCs for freshwater ecosystems (Daughton and Ternes, 1999; Fent et al., 2006; Petrovic et al., 2002). The threat posed by the release of those contaminants through WWTP effluents is particularly worrisome in streams or small rivers, where the dilution capacity of the receiving freshwater ecosystem is small (Brooks et al., 2005).

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Studies reporting toxic effects of PhACs and EDCs have led to some attempts of regulation for some of these compounds in the European Union (Collado et al., 2014), such as the anti-inflammatory diclofenac or the synthetic hormones EE2, which have been included in the so called 'watch list' of priority substances under the Water Framework Directive for the "specific purpose of facilitating the determination of appropriate measures to address the risk posed by these substances" (European Commision, 2013). In the US, the Drinking Water Contaminant Candidate List also contains several PhACs and EDCs, including antibiotics, and hormones (Environmental Protection Agency U.S., 2012). Other PhACs, such as carbamazepine, sulfamethoxazole, diclofenac, ibuprofen, naproxen, bezafibrate, atenolol, erythromycin and gemfibrozil have been classified as high priority pharmaceuticals to the water cycle by the GWRC, Global Water Research Coalition (2008).

A comprehensive knowledge of the fate of these pollutants in all the environmental compartments involved may be crucial to assessing the potential risk associated with the discharge of WWTP effluents. Previous studies have reported bioaccumulation of PhACs and EDCs in different environmental compartments. For instance, some studies have shown that sediments may be a sink of PhACs, due to the links with microbial degradation, in particular for those compounds not affected by hydrolysis or photodegradation (Kunkel and Radke, 2008). Other studies have reported bioaccumulation of PhACs and EDCs in invertebrates (Berlioz-Barbier et al., 2014; Huerta et al., 2015) and fish (Brooks et al., 2005; Chu and Metcalfe, 2007; Du et al., 2012; Huerta et al., 2013; Jakimska et al., 2013; Pojana et al., 2007; Ramirez et al., 2009). The question that remains is whether river biofilms could be a significant compartment for accumulation and transformation of these emerging contaminants.

River biofilms are communities composed mainly of bacteria, algae, and fungi embedded in an organic polymer matrix. This matrix of extracellular polymeric substances (EPS) is particularly relevant in the sorption of compounds from the water phase, acting as a molecular sieve, sequestering cations, anions, apolar compounds and particles (Flemming and Wingender, 2010). Biofilms are fundamental constituents of river ecosystems, as they are involved in vital functions such nutrient retention (Bechtold et al., 2012). Their relatively rapid development, widespread distribution and large biomass, together with their capacity to absorb contaminants, suggest that biofilms are exceptional indicators of pollution (Sabater et al., 2007). Several studies have already shown that the presence of contaminants such as PhACs and EDCs can affect the biofilm negatively, altering its structure and metabolism (Corcoll et al., 2014, 2015; Ricart et al., 2010; Rosi-Marshall et al., 2013). Biofilms have an important role in water purification capacity (Chenier et al., 2003; Tien and Chen, 2013). In fact, transport and fate of contaminants in aquatic environments may be affected significantly by their sorption and remobilization interaction with biofilms (Headley et al., 1998), as they follow a transient development and collapse, and in their detachment may move even kilometers downstream (Sabater et al., 2015), transporting contaminants within them. Thus, biofilms influence the transport and fate of emerging contaminants such as PhACs and EDCs through biotic (bioaccumulation and biotransformation by algae and bacteria) (Chenier et al., 2003; Tien and Chen, 2013) and abiotic (physical sorption to EPS) means (Headley et al., 1998) . In this study, bioaccumulation refers to the concentration of target compounds found within the biofilm, both inside the cells and in the matrix surrounding them, which may be led by active biological uptake or passive physical sorption. Bioaccumulation in biofilms has been reported for a wide variety of contaminants, such as metals (Arini et al., 2012; Morin et al., 2008; Serra and Guasch, 2009; Tien et al., 2013), pesticides (Headley et al., 1998), hormones, surfactants and a psychiatric drug (Correa-Reyes et al., 2007; Writer et al., 2011a, 2011b, 2013). Because of their acknowledged capacity to bioaccumulate different contaminants, they could also play a critical role in transferring PhACs and other EDCs to higher trophic levels of riverine food webs within freshwater ecosystems.

However, information about bioaccumulation of PhACs and other EDCs in river biofilms is still non-existent for the great majority of these compounds. To fill the gaps in knowledge regarding the fate of emerging contaminants in freshwater ecosystems, such as PhACs and EDCs, it is essential to develop and validate appropriate analytical methods. Therefore, the objectives of this study were first to develop an efficient analytical methodology for the simultaneous analysis of PhACs and EDCs in river biofilm, and second, to assess persistence, distribution, and bioaccumulation of these trace contaminants in river biofilms affected by WWTP effluents.

2. Materials and methods

2.1. Standards and solutions

A total of 44 PhACs and 13 EDCs were analyzed. A list of the target analytes, molecular structures, and chemical properties are listed in the supplementary material (Table S1). Individual stock standards and labeled internal standards were prepared in methanol at a concentration of approximately 1000 mg L^{-1}. Stock solutions and 20 mg L^{-1} mixtures in methanol were stored at −20 °C and diluted to 1 mg L^{-1} before each analytical run.

2.2. Sample collection and pre-treatment

The study was conducted in a section of the River Segre (Spain) affected by the discharge of a WWTP effluent. Water and biofilm samples were collected at five sites: one site upstream (500 m from the WWTP) and four downstream of the local WWTP (from 500 to 4500 m). Water samples (100 mL) were filtered through 0.45 μm nylon membrane filters and kept at −20 °C until analysis. Biofilm was collected from surfaces of rocks that were removed from near-shore areas of the stream. Biofilm of at least one river cobble was gently scraped (volume = 40 mL) and used for each replicate. The biofilm was placed directly into Falcon® tubes and transported to the laboratory in a dark cool box. Samples were lyophilised and kept frozen (−20 °C) until analysis.

2.3. Water extraction

Water was extracted according to the method developed by Gros et al. (2012) for the analysis of PhACs, and also applied for the analysis of EDCs. Briefly, 3 mL of EDTA 1 M (4%, v/v) were added to the samples. SPE cartridges (Oasis HLB, 60 mg) were conditioned with 5 mL of methanol followed by 5 mL of ultra-pure water at a flow rate of 2 mL min^{-1}. Samples were loaded onto the cartridge at a flow rate of 1 mL min^{-1}. Cartridges were rinsed with 6 mL of HPLC grade water, and were dried in air for 30 min. Finally, analytes were eluted with 6 mL of methanol and evaporated to dryness under a nitrogen stream and reconstituted in 1 mL of methanol/water (10:90) for the analysis of PhACs and methanol/water (50:50) for the analysis of EDCs. Finally, 5 μL of a 1 mg L^{-1} standard mixture containing labeled compounds were added in the water extracts before analysis.

2.4. Biofilm extraction and clean-up

Initially, sonication was pre-selected as the possible extraction method, together with pressurized liquid extraction (PLE). Four solvents were tested, including methanol, methanol/water (1:1), methanol with 0.1% EDTA, and citric buffer (pH4)/acetonitrile (1:1, v/v), all based on the authors' previous experience with pharmaceutical and EDC extraction. To reduce the number of experiments and solvent consumption, the results of one of these solvents were compared in both extraction methods to find which one had the best extraction recoveries. For sonication extraction, 200 mg of freeze-dried biofilm was placed in a 14-mL Falcon tube with 10 mL of the corresponding solvent. Extraction entailed 3 cycles of 10 min, and the supernatant was collected in a glass vial. The extraction protocol based on pressurized liquid extraction (PLE) was performed with ASE®350 (Thermo Scientific). Biofilm (200 mg) was placed in 10 mL cells together with diatomaceous earth to fill up the rest of the cell. Samples were extracted with the corresponding solvent during 3 cycles of 5 min at 60 °C.

Extracts obtained using either PLE or sonication, were placed under an N2 current until the organic solvent was evaporated till dryness or, in the case of extracts containing an aqueous phase, till 5 mL volume. Evaporated extracts were then diluted to 100 mL of H_2O (organic solvent content < 0.05%). Further purification of the extracts was performed by solid phase extraction (SPE) using the protocol for extraction of water samples described in the previous section (Gros et al., 2012). Purified extracts were then evaporated to dryness under a nitrogen stream and reconstituted in 1 mL of methanol. Two aliquots (250 µl each) were evaporated and reconstituted in the same volume of: a) methanol/water (10:90) for the analysis of PhACs; and b) methanol/water (50:50) for the analysis of EDCs. Finally, 5 µL of a 1 mg L^{-1} standard mixture containing labeled compounds were added to the biofilm extracts as internal standard right before the analysis.

2.5. Instrumental analysis

For the analysis of PhACs, extracts were analyzed using the method developed by Gros et al. (2012) using an ultra-performance liquid chromatography (UPLC) system (Waters Milford, USA) coupled to a hybrid quadrupole linear ion trap mass spectrometer Qtrap 5500 (Applied Biosystems), equipped with an electrospray ionization source (ESI). Briefly, an Acquity HSS T_3 with 10 mM formic acid/ammonium formate (pH 3.2) and methanol as the mobile phase at a flow rate of 0.5 mL $min^{-1}$was applied for the analysis of PhACs in positive mode, whereas an Acquity BEH C_{18} with acetonitrile and 5 mM ammonium acetate/ammonia ($pH = 8$) as mobile phase at a flow rate of 0.6 mL min^{-1} was applied for the PhACs analyzed in negative mode.

For the analysis of EDCs, extracts were analyzed using the LC–MS-MS method developed by Jakimska et al. (2013) in the same instrumental set up, using the Acquity BEH C_{18} column for both positive and negative modes with methanol and water (pH 9) as mobile phase at a flow rate of 0.4 mL min^{-1}.

In both cases, the acquisition of the compounds was done in selected reaction monitoring (SRM) mode with the monitoring of two transitions. The most intense transition was used for quantification purposes when possible, whereas the second one was used to confirm the identity of the target compounds. Quantification was performed with a matrix-matched calibration curve together with the internal standard approach by adding the corresponding labeled compounds at a concentration of 10 ng L^{-1} before analysis.

2.6. Calculation of bioaccumulation factors (BAFs)

Distribution coefficient logarithms (Log Dow) for all the compounds detected in water and/or biofilm were calculated at pH 7.4 and compared with the field-derived bioaccumulation factors (BAFs). BAFs were calculated considering the measured water and biofilm concentrations in this study according to the following equation:

$$BAF\left(\frac{L}{g}\right) = conc.\ biota\left(\frac{ng}{g}\right)/conc.\ water\left(\frac{ng}{L}\right). \qquad (1)$$

3. Results

3.1. Biofilm extraction and purification

Comparison between sonication extraction efficiencies using four solvents (methanol, methanol/water (1:1), methanol with 0.1% EDTA, and citric buffer (pH4)/acetonitrile) resulted in the selection of citric buffer (pH4)/acetonitrile as the final extraction solvent, since the majority of the compounds had higher recoveries (Fig. 1). Mixture methanol/water was discarded, as many of the target analytes were not recovered. The remaining three solvents showed similar recovery efficiencies between them. However, the mixture citric buffer (pH4)/acetonitrile produced slightly higher recoveries for most of the compounds, including most of the EDCs, as well as lower standard deviation between replicates. After selecting citric buffer (pH4)/acetonitrile as an optimal solvent, extraction efficiencies were compared between using sonication and PLE as an extraction methodology (Fig. 1). Although recoveries were similar, better reproducibility with PLE indicated that this extraction method was more robust. Therefore, the final extraction method with PLE involved a sample size of 200 mg, 3 cycles of 5 min, with 100% of fresh solvent each cycle, 90 s purge with N2 at 60 °C. Final recoveries of EDCs and PhACs after extraction and purification with SPE are shown in Tables 1 and 2.

3.2. Method validation

Extraction recoveries, linearity, method detection and quantification limits, accuracy, repeatability and reproducibility as well as matrix effects were calculated for EDCs and PhACs (see Tables 1 and 2). To determine recoveries, biofilm samples were spiked with a mixture of the target analytes at three levels: 10, 100 and 500 ng g^{-1}, selected according to previous studies in other biological matrices. Concentrations obtained after the procedure, calculated by matrix-matched curves (prepared with unexposed biofilm grown in the lab) and internal standard calibration, were compared with the initial spiking levels. Extraction recoveries for all compounds were higher than 50%, except for bisphenol A, triclosan, ibuprofen and sulfamethoxazole, which were between 32 and 42%, possibly due to the fact that the conditions chosen were not the best for these specific compounds, as often occurs in multi-residue methodologies, where a compromise is reached for analytes with very different characteristics.

Method detection limits (MDLs) and method quantification limits (MQLs) were calculated in samples spiked at 10 ng g^{-1} ($n = 3$) as the minimum amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. MDLs for EDCs were in the range of 0.20–2.4 ng g^{-1}, and for PhACs, 0.07–6.7 ng g^{-1}.

Accuracy of the instrumental method was calculated as the deviation of the measured mean concentration from the real concentration, expressed in percentage from two repeated injections of a spiked sample (25 ng g^{-1}). The instrumental precision (intra-day and inter-day) was calculated from five repeated injections on the same day and on five consecutive days of a spiked sample (25 ng g^{-1}). Values were less than 17% and 20% for EDCs and PhACs, respectively.

Signal alterations during analysis by mass spectrometry detectors with electrospray interfaces are particularly noticeable in the case of biological samples. To evaluate the degree of matrix effects during analysis of biofilm, peak areas of biofilm extracts (spiked at 25 ng g^{-1}) were compared to those of the analytes in the solvent mixture at the same concentration. For EDCs, 92% of the compounds suffered ion suppression and only one of them ion enhancement (see Fig S1). In the case of PhACs, 59% suffered ion suppression, 18% ion enhancement and the remaining 23% was within the 20% signal variation that is not considered as a matrix effect (see Fig S2). Due to the obvious effects of the co-eluting interferences in ionization, matrix-matched calibration, together with the addition of labeled compounds, was selected as the most suitable approach to compensate for matrix effects during analysis, as in previous studies (Huerta et al., 2013; Jakimska et al., 2013). Calibration curves prepared in extracts of biofilm from pristine sampling sites (where no analytes were detected) were generated using linear regression analysis ($R^2 > 0.98$) in the concentration range from 0.01 to 100 ng mL^{-1}.

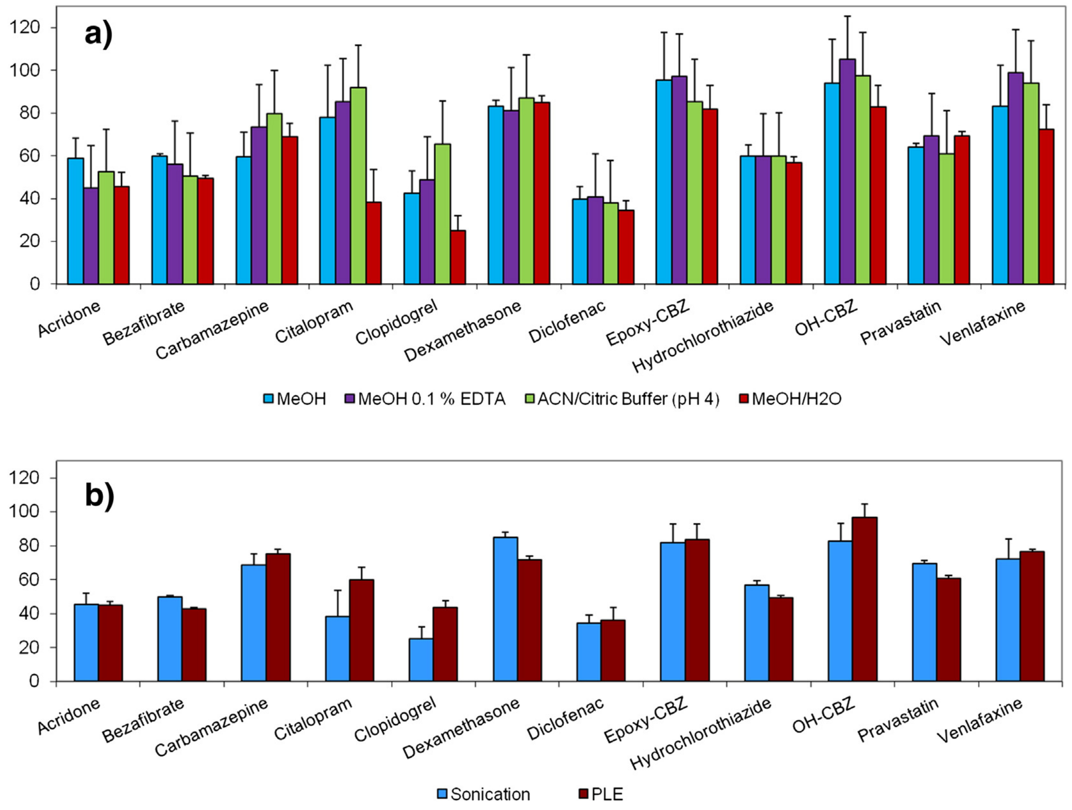

Fig. 1. Comparison of extraction efficiencies (%) for a) four different solvents by sonication and b) two extraction methods (PLE and sonication) using ACN/Citric buffer for selected pharmaceuticals.

3.3. Environmental samples

3.3.1. Water concentrations of PhACs and EDCs

A total of 81 multiple-class human and veterinary PhACs and 19 EDCs and related compounds, as well as some transformation products, were analyzed in water. Recovery percentages and method detection limits in water samples for PhACs and EDCs are published elsewhere (Gros et al., 2012; Huerta et al., 2015). No compounds were detected in the first sampling point, before the WWTP input, as shown in Table 3.

A total of 15 compounds, 14 PhACs from six therapeutic families – analgesics, psychiatric drugs, diuretics, lipid regulators, calcium channel blockers and antibiotics – and one flame retardant were detected in the first sampling site after the WWTP discharge. The highest concentrations were detected for diuretic compounds, lipid regulators and analgesics/anti-inflammatories, with total concentrations of these therapeutic families between 240 and 361 ng L^{-1}, maximum concentration that was measured for hydrochlorothiazide, a diuretic usually present at high concentrations in WWTP-impacted waters (Gros et al., 2012;

Table 1

Mean percent recoveries (n = 3) of 13 EDCs at three spiking levels, and method detection and quantification limits (MDL, MQL) in biofilm (ng/g, dry weight).

	% recoveries (±RSD)			LODs		Precision (% RSD)		
	10 ng/g	100 ng/g	500 ng/g	MDL (ng/g)	MQL (ng/g)	Intra-day	Inter-day	Accuracy (%)
Parabens								
Benzylparaben	49 (±16)	62 (±16)	62 (±11)	0.20	0.67	16.4	16.0	8.8
Ethylparaben	72 (±3.5)	84 (±13)	90 (±5.0)	0.83	2.77	5.1	3.3	12.3
Methylparaben	82 (±9.4)	83 (±9.2)	87 (±6.6)	0.27	0.89	9.3	6.4	9.9
Propylparaben	64 (±13)	69 (±5.9)	85 (±4.7)	0.19	0.64	5.2	4.9	10.7
Plasticizers								
Bisphenol A	32 (±8.6)	32 (±8.5)	53 (±19)	0.71	2.35	14.7	12.4	1.6
Hormones								
Estrone	53 (±11)	68 (±18)	72 (±11)	0.74	2.46	10.7	9.5	9.7
Estrone-3-sulfate	37 (±30)	62 (±2.7)	80 (±1.3)	0.35	1.18	1.4	15.0	2.8
Estriol	78 (±2.2)	87 (±2.7)	93 (±4.9)	2.09	6.97	2.4	2.4	8.8
17α-ethinylestradiol	59 (±14)	64 (±11)	70 (±12)	2.40	7.99	14.1	11.3	10.7
Progesterone	59 (±4.7)	52 (±5.9)	51 (±3.4)	1.69	5.62	8.9	6.2	−1.9
Flame retardants								
TBEP	57 (±11)	56 (±3.8)	65 (±17)	0.70	2.35	3.4	16.8	−4.8
TCEP	70 (±7.8)	79 (±4.9)	75 (±4.1)	2.13	7.11	3.5	1.8	−2.1
Antibacterial								
Triclosan	40 (±6.8)	41 (±9.7)	46 (±6.2)	0.71	2.36	6.6	6.1	8.8

Table 2
Mean percent recoveries (n = 3) of 44 PhACs at three spiking levels, and method detection and quantification limits (MDL, MQL) in biofilm (ng/g, dry weight).

	% recoveries (±RSD)			LODs		Precision (% RSD)		
	10 ng/g	100 ng/g	500 ng/g	MDL (ng/g)	MQL (ng/g)	Intra-day	Inter-day	Accuracy (%)
Analgesics/anti-inflammatories								
1-OH-IBU	83 (±2.8)	97 (±5.3)	105 (±2.0)	1.86	6.19	9.9	5.3	2.8
Ibuprofen	31 (±18)	42 (±6.2)	86 (±3.4)	1.96	6.53	12.8	19.4	13.5
Diclofenac	60 (±0.5)	69 (±19)	74 (±9.5)	1.39	4.63	6.3	2.7	11.0
Diclofenac-glucuronide	75 (±15)	100 (±4.4)	111 (±6.1)	1.63	5.43	5.6	13.3	7.3
Meloxicam	67 (±2.3)	63 (±10)	63 (±7.5)	0.38	1.26	2.3	7.5	3.3
Piroxicam	103 (±14)	109 (±16)	101 (±5.1)	0.39	1.31	5.1	16.7	4.5
Propyphenazone	70 (±5.2)	84 (±3.4)	87 (±2.9)	0.17	0.56	2.9	5.2	6.4
Antibiotics								
Azythromycin	71 (±12)	102 (±14)	101 (±11)	0.15	0.50	2.6	13.2	1.8
Chloramphenicol	94 (±7.7)	94 (±25)	97 (±0.8)	0.78	2.59	6.9	5.3	5.4
Ciprofloxacin	33 (±17)	70 (±12)	56 (±9.1)	0.68	2.26	11.6	16.0	−14.9
Clarithromycin	55 (±9.0)	61 (±16)	64 (±7.8)	0.27	0.92	7.8	9.0	15.8
Erythromycin	63 (±16)	69 (±3.1)	77 (±9.1)	6.71	22.37	4.3	12.1	−3.2
Ofloxacin	65 (±0.3)	70 (±16)	80 (±8.4)	0.25	0.83	16.4	10.5	−2.1
Sulfamethoxazole	24 (±12)	38 (±13)	46 (±19)	0.48	1.59	11.9	14.3	0.1
Antihelmintics								
Thiabendazole	57 (±2.9)	61 (±4.0)	75 (±11)	0.14	0.48	5.4	10.8	12.4
Antiplatelet agent								
Clopidogrel	75 (±16)	74 (±3.2)	109 (±9.5)	0.45	1.50	3.2	9.5	8.2
Calcium channel blockers								
Diltiazem	52 (±10)	105 (±0.7)	113 (±1.5)	0.07	0.24	1.5	10.1	16.8
Norverapamil	77 (±11)	115 (±19)	101 (±5.2)	0.07	0.25	5.6	11.2	12.0
Verapamil	76 (±11)	126 (±22)	108 (±9.0)	0.06	0.21	8.5	11.1	10.6
Diuretic								
Furosemide	57 (±29)	58 (±3.5)	64 (±9.3)	3.54	11.81	3.5	9.3	15.9
Hydrochlorothiazide	39 (±6.2)	79 (±2.7)	83 (±13)	0.14	0.46	2.7	13.2	−2.7
Trazodone	65 (±14)	87 (±14)	87 (±12)	0.10	0.35	14.1	13.9	8.1
Histamine H1 and H2 receptor antagonists								
Desloratadine	80 (±17)	106 (±6.6)	116 (±10)	0.20	0.68	10.1	6.0	4.9
Lipid regulators								
Bezafibrate	78 (±3.7)	90 (±0.8)	97 (±12)	0.12	0.38	0.8	11.9	6.5
Fluvastatin	62 (±9.0)	62 (±9.0)	71 (±10)	0.67	2.23	10.4	14.5	6.2
Gemfibrozil	56 (±1.5)	68 (±2.6)	69 (±13)	0.60	2.00	2.5	12.7	1.2
Loratadine	85 (±13)	90 (±11)	123 (±9.0)	0.18	0.62	2.1	15.0	9.4
Pravastatin	67 (±13)	74 (±7.8)	86 (±9.7)	1.21	4.03	13.1	13.1	−0.1
Psychiatric drugs								
Acridone	69 (±7.6)	92 (±3.3)	88 (±9.8)	0.14	0.46	4.7	4.3	3.1
OH-CBZ	70 (±7.8)	101 (±3.6)	70 (±7.8)	0.17	0.57	3.6	7.8	2.3
Epoxy-CBZ	72 (±6.6)	102 (±6.5)	111 (±16)	0.16	0.55	6.5	6.6	−6.5
Carbamazepine	64 (±4.9)	107 (±4.6)	104 (±8.2)	0.23	0.78	4.1	5.0	7.4
Citalopram	99 (±26)	104 (±4.4)	119 (±23)	0.33	1.11	4.9	9.6	10.6
Diazepam	76 (±8.5)	103 (±7.8)	103 (±0.5)	0.70	2.33	4.9	0.5	1.1
Fluoxetine	63 (±15)	66 (±9.1)	88 (±12)	0.78	2.60	10.1	14.7	4.8
Lorazepam	97 (±7.3)	113 (±6.4)	125 (±5.8)	0.78	2.60	6.4	9.5	1.9
Paroxetine	61 (±11)	68.1 (±15)	71 (±9.5)	0.61	2.04	11.5	9.5	9.1
Sertraline	61 (±11)	69 (±12)	104 (±19)	1.72	5.74	11.1	11.9	−4.0
Venlafaxine	75 (±9.5)	116 (±7.1)	137 (±24)	0.10	0.34	7.1	9.5	11.8
Synthetic glucocorticoid								
Dexamethasone	83 (±15)	78 (±7.3)	89 (±14)	1.18	3.92	7.3	15.7	−0.2
Tranquilizer								
Azaperone	49 (±11)	62 (±9.6)	81 (±15)	0.36	1.19	9.6	16.7	−10.7
β-blocking agents								
Metoprolol	55 (±12)	69 (±15)	77 (±4.1)	0.20	0.68	4.1	11.7	7.3
Metoprolol acid	59 (±10)	59 (±12)	87 (±9.0)	2.88	9.61	9.0	10.2	10.0
Propanolol	62 (±14)	61 (±9.8)	94 (±4.4)	0.27	0.90	9.7	19.4	3.5

Osorio et al., 2012). Total concentration of PhACs and EDCs decreased about 40% in less than 5 km downstream, although the number of compounds detected only decreased 13%. The impact of the WWTP effluent as a source of these contaminants is clearly observed from the consistent die-away in the lower concentrations observed from the WWTP outfall to the last downstream site.

3.3.2. Biofilm concentrations of PhACs and EDCs

Seven PhACs (out of 44) and five EDC (out of 13) were detected in biofilm samples (see Table 3). Fig. 2 shows the levels of major families detected both in water and biofilm in all sampling points. The most concentrated pharmaceutical was the anti-inflammatory diclofenac, which was measured at 100 ng g^{-1} immediately downstream of the WWTP

Table 3
Detected PhACs and EDCs in water and/or biofilm in River Segre.

	Water concentration (ng/L)					Biofilm concentration (ng/g)				
	Control	I1	I2	I3	I4	Control	I1	I2	I3	I4
Endocrine disruptor compounds										
Parabens										
Ethylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	21.3	20.3	6.90	n.d.
Methylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	212	176	9.00	n.d.
Propylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.70	n.d.	n.d.	n.d.
Flame retardants										
TBEP	n.d.	65.3	41.4	21.5	15.9	3.60	50.8	14.1	17.3	4.80
Antibacterial										
Triclosan	n.d.	n.d.	n.d.	n.d.	n.d.	18.0	56.5	76.5	75.0	32.9
Pharmaceutical compounds										
Analgesics/anti-inflammatories										
1-OH-IBU	n.d.	68.7	64.8	46.0	26.0	n.d	n.d	n.d	n.d	n.d
Ibuprofen	n.d.	193	187	115	70.8	n.d	n.d	n.d	n.d	n.d
Diclofenac	n.d.	29.1	25.6	19.9	n.d	n.d.	103	23.5	<LOQ	<LOQ
Antibiotics										
Clarithromycin	n.d.	32.8	24.5	19.1	7.7	n.d	n.d	n.d	n.d	n.d
Sulfamethoxazole	n.d.	18.0	19.6	23.2	9.0	n.d	n.d	n.d	n.d	n.d
Calcium channel blockers										
Diltiazem	n.d.	n.d.	n.d.	n.d.	n.d.	nd	11.8	nd	11.8	4.7
Norverapamil	n.d.	n.d.	n.d.	n.d.	n.d.	nd	20.9	18.7	11.2	4.20
Verapamil	n.d.	n.d.	n.d.	n.d.	n.d.	nd	21.7	20.2	11.1	14.4
Diuretics										
Furosemide	n.d.	45.8	38.6	33.1	<MQL	n.d	n.d	n.d	n.d	n.d
Hydrochlorothiazide	n.d.	311	296	221	199.6	n.d	n.d	n.d	n.d	n.d
Lipid regulator										
Bezafibrate	n.d.	7.20	6.40	4.50	3.4	n.d	n.d	n.d	n.d	n.d
Gemfibrozil	n.d.	286	222	157	76.9	nd	10.3	nd	4.00	nd
Pravastatin	n.d.	44.0	25.7	23.9	13.8	n.d	n.d	n.d	n.d	n.d
Psychiatric drugs										
Carbamazepine	n.d.	38.6	23.6	21.3	15.0	n.d	n.d	n.d	n.d	n.d
OH-CBZ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	1.8	nd
Citalopram	n.d.	4.70	n.d.	n.d.	n.d.	n.d	n.d	n.d	n.d	n.d
Venlafaxine	n.d.	21.1	13.8	9.3	2.4	n.d.	n.d.	43.7	6.50	n.d.

discharge. Diclofenac has been repeatedly detected in wild biota, including invertebrates (Huerta et al., 2015) and fish tissues in Mediterranean rivers (Huerta et al., 2013). Other compounds detected included the antihypertensive drugs diltiazem and verapamil, the lipid regulator gemfibrozil, and the psychiatric drug venlafaxine. Verapamil has previously been detected in invertebrates (*Hydropsyche* sp.) at concentrations around 3 ng g^{-1} downstream of a WWTP (Grabicova et al., 2014). Venlafaxine has also been detected in fish homogenates at 0.6 ng g^{-1} in a heavily polluted river (Huerta et al., 2013). This is the first study to report such compounds from natural river biofilms.

Maximum concentration around 200 ng g^{-1} was detected for paraben preservative methylparaben within 1 km downstream of the WWTP. This compound has previously been detected in fish at concentrations up to 80 ng g^{-1} (Jakimska et al., 2013). Antibacterial triclosan

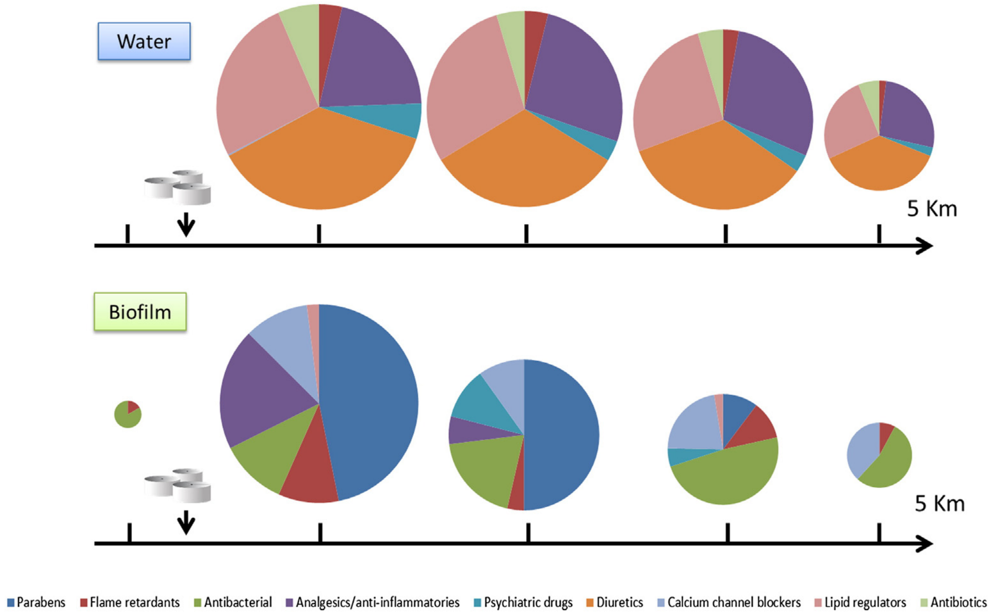

Fig. 2. Relative presence of the groups of contaminants detected in both water and biofilm along 5 km after the discharge of a WWTP.

and the flame retardant TBEP were detected in all sampling points, including the site upstream of the WWTP, in concentrations between 3 and 76 ng g^{-1}. Triclosan has been found to have negative effects both in bacterial and algal communities within the biofilm at environmentally-relevant water concentrations (Ricart et al., 2010).

Several compounds detected in the biofilm were not correspondingly found in water: ethylparaben, methylparaben, propylparaben, triclosan, norverapamil, verapamil and OH-CBZ. This may suggest that biofilm was able to uptake compounds present at very low concentrations in water (<MDLs) and bioaccumulate them. Other field studies indicated that steroidal hormones and 4-nonylphenol were detected in biofilm samples even when they were not in water (Writer et al., 2011a, 2011b).

Still, a number of compounds detected in water were not detected in the biofilm, such as, citalopram, pravastatin, sulfamethoxazole, furosemide, carbamazepine, and bezafibrate, found in water at low concentrations (<50 ng L^{-1}), even in the sampling sites close to the WWTP. Other compounds found at higher concentration in water, such as hydrochlorothiazide (max. conc. 361 ng L^{-1}) and ibuprofen (max. conc. 193 ng L^{-1}), were not detected either in biofilm.

It is important to highlight that collected water samples in this study were discrete, while the biofilm was allowed to grow for a longer period and therefore was exposed to varying concentrations of these contaminants. Therefore correlation between water and biofilm concentration should only be considered as possible tendency of PhAC bioaccumulation. Nevertheless, the levels found for contaminants in river water in the sampling campaign are in line with those previously found in the same site in another period of the day (Acuña et al., 2015) and thus, they can be considered representative of the type of pollution in the area.

3.3.3. Calculation of BAFs

In Fig. 3, three groups were easily identified: i) the compounds detected in water and retained in biofilm (in yellow), with log Dow between 1 and 4; ii) the compounds detected only in water (in blue), generally with log Dow < 1; and iii) the compounds detected only in biofilm (in green), with log Dow > 1.5. The only points out of the norm correspond to clarithromycin (log Dow pH 7.4 = 3), an easily degradable antibiotic, so its absence in biofilm could be due to degradation and not to lack of sorption; and carbamazepine.

4. Discussion

The presence of PhACs and EDCs in surface waters, and therefore in other environmental compartments, may be affected by several processes, such as photolysis, hydrolysis, sorption and biodegradation (Boreen et al., 2003; Radjenović et al., 2009; Yamamoto et al., 2009; Lau et al., 2005; Rosenfeldt and Linden, 2004). Understanding partitioning behaviorof contaminants in the biological compartment, and the role of biota in the removal of these bioactive compounds from surface water is of critical importance to studying their ecological impact.

Octanol-water partition coefficient (Kow) has been successfully applied to predict sorption processes for non-ionisable compounds (Fisk et al., 1998; Meylan et al., 1999). More recently, the use of Dow to predict hydrophobic partition of the neutral species at a specified pH for ionisable compounds has been widely accepted (Fitzsimmons et al., 2001). However, Dow cannot be used alone to completely explain the trends observed in Fig. 3. For instance, among the compounds detected in the biofilm, neutral compounds were present, such as the EDCs, and ionisable compounds, both in positive and negative ionization, as the PhACs (see Table S5). Probably mechanisms other than hydrophobic interactions play important roles in sorption of PhACs, such as electrochemical interactions affected by the presence of aromatic rings and functional groups (Yamamoto et al., 2009). Biofilm bacterial and algal communities together with the EPS matrix are considered to play a substantial role in the sorption of organic and inorganic molecules (Dobor et al., 2012).

This EPS matrix contains apolar regions, groups with hydrogen-bonding potential, anionic groups (in uronic acids and proteins) and cationic groups (for example, in amino sugars), facilitating sites of interaction to the contaminants dissolved in the water (Flemming and Wingender, 2010; Flemming, 1995; Headley et al., 1998). For instance, most biofilm reactors in WWTPs are typically negatively charged and

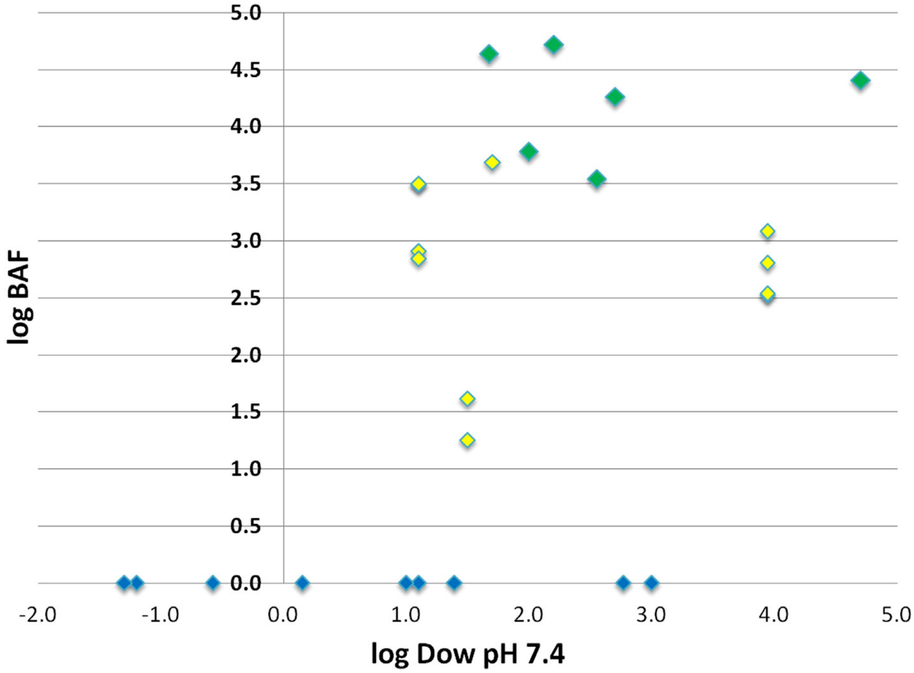

Fig. 3. Correlation between log BAFs and log Dow (at pH 7.4) for the compounds detected in water (in blue), biofilm (in green), or both (in yellow). BAFs of the compounds detected only in biofilm (green), were calculated using MDLs in water as water concentrations to avoid giving BAF values = 0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

thus provide potential sorption sites for neutral and positively-charged compounds, while the sorption of anionic organic molecules is slowed down due to electrostatic repulsion between the negatively-charged molecules and the negatively-charged biofilm (Carlson and Silverstein, 1998; Riml et al., 2013). It is therefore possible that the combination of both ionic binding as well as organic carbon partition may explain the sorption capacity of these contaminants to biofilm, even when they present low log Dow (Kwon and Armbrust, 2008).

In addition to sorption, compounds readily partitioned to the biological compartment may undergo other processes within the biofilm, such as biodegradation. For instance, biofilm biodegradation of diclofenac under controlled conditions was reported after some days of exposure (Paje et al., 2002). Biodegradation may occur, not only due to the presence of the biofilm bacterial community in the EPS matrix, but other components, such as algae and fungi, may also have the capacity to transform some of these contaminants. For example, Della Greca et al. studied the removal and biotransformation of synthetic hormone EE2 by the microalga *Selenastrum capricornutum* and found that EE2 was transformed into 3 products (Della Greca et al., 2008). Elimination of emerging contaminants by the white-rot fungus *Trametes versicolor* has also been applied in different studies (Badia-Fabregat et al., 2014; García-Galán et al., 2011; Llorens-blanch et al., 2015; Yang et al., 2013). In fact, several studies have considered the role of natural biofilm in freshwater ecosystems' water purification capacity (Acuña et al., 2015; Gurr and Reinhard, 2006; Kunkel and Radke, 2011), and Writer et al. showed that attenuation of some EDCs in freshwater ecosystems appears to be a combination of both sorption and biodegradation processes (Writer et al., 2011a, 2011b) by the stream biofilm and sediments.

Bioaccumulation of PhACs and EDCs presented in this study demonstrates that biofilms could be very useful tools in monitoring, as they are naturally available and integrate chronic exposure. However, more research is needed to determine how bioaccumulation and biodegradation are related, i.e. whether bioaccumulation increases bioavailability and therefore increases the chances of biodegradation by bacteria, algae and the enzymes contained in the EPS matrix, and consequently the compounds accumulated in the biofilm are also degraded. Biomagnification potential along the trophic chain should not be forgotten, as biofilm could constitute a secondary exposure pathway for other organisms (invertebrates, fish) coexisting in an ecosystem. Therefore, reliable analytical methods that include metabolites in their analysis are a useful tool for the determination of how biofilm interacts with the contaminants. They would allow determining how the biofilm is actually transforming the parent compounds. The analytical methodology developed here allows the measurement of up to 5 known metabolites and transformation products in biofilm, including 1-OH-ibuprofen, epoxy-carbamazepine, OH-carbamazepine, norverapamil and diclofenac glucuronide.

5. Conclusions

This study developed a simple, rapid, sensitive and efficient analytical method for the determination of 44 PhACs and 13 EDCs in river biofilm. To the best of the authors' knowledge, this is the first study determining such a large number of target compounds in biofilm. The final multi-residue procedure consisted of pressurized liquid extraction, followed by purification of extract with solid-phase extraction, and the analysis was performed by UHPLC–MS/MS, which provided the necessary sensitivity and selectivity for these contaminants. The method was applied to assess the bioaccumulation of these contaminants in natural biofilm from a WWTP-impacted river. Seven PhACs – diclofenac, diltiazem, gemfibrozil, verapamil, norverapamil, OH-CBZ, and venlafaxine – and five EDCs – ethylparaben, methylparaben, propylparaben, TBEP and triclosan – were detected in concentrations up to100 ng g^{-1}.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.05.049.

References

Acuña, V., von Schiller, D., García-Galán, M.J., Rodríguez-Mozaz, S., Corominas, L., Petrovic, M., Poch, M., Barceló, D., Sabater, S., 2015. Occurrence and in-stream attenuation of wastewater-derived pharmaceuticals in Iberian rivers. Sci. Total Environ. 503–504, 133–141.

Arini, A., Feurtet-Mazel, A., Maury-Brachet, R., Pokrovsky, O.S., Coste, M., Delmas, F., 2012. Recovery potential of periphytic biofilms translocated in artificial streams after industrial contamination (Cd and Zn). Ecotoxicology 21, 1403–1414.

Badia-Fabregat, M., Rosell, M., Caminal, G., Vicent, T., Marco-Urrea, E., 2014. Use of stable isotope probing to assess the fate of emerging contaminants degraded by white-rot fungus. Chemosphere 103, 336–342.

Bechtold, H.A., Marcarelli, A.M., Baxter, C.V., Inouye, R.S., 2012. Effects of N, P, and organic carbon on stream biofilm nutrient limitation and uptake in a semi-arid watershed. Limnol. Oceanogr. 57, 1544–1554.

Berlioz-Barbier, A., Buleté, A., Faburé, J., Garric, J., Cren-Olivé, C., Vulliet, E., 2014. Multi-residue analysis of emerging pollutants in benthic invertebrates by modified micro-quick-easy-cheap-efficient-rugged-safe extraction and nanoliquid chromatography–nanospray-tandem mass spectrometry analysis. J. Chromatogr. A 1367, 16–32.

Boreen, A.L., Arnold, W.A., McNeill, K., 2003. Photodegradation of pharmaceuticals in the aquatic environment: a review. Aquat. Sci. 65 (4), 320–341.

Boxall, A.B.A., Sinclair, C.J., Fenner, K., Kolpin, D., Maund, S.J., 2004. Peer reviewed: when synthetic chemicals degrade in the environment. Environ. Sci. Technol. 38, 368A–375A.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., Brooks, B.W., 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24, 464–469.

Carlson, G., Silverstein, J., 1998. Effect of molecular size and charge on biofilm sorption of organic matter. Water Res. 32, 1580–1592.

Céspedes, R., Lacorte, S., Raldúa, D., Ginebreda, A., Barcelò, D., Piña, B., 2005. Distribution of endocrine disruptors in the Llobregat River basin (Catalonia, NE Spain). Chemosphere 61, 1710–1719.

Chenier, M.R., Beaumier, D., Roy, R., Driscoll, B.T., Lawrence, J.R., Greer, C.W., 2003. Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of hexadecane by replicated river biofilms. Appl. Environ. Microbiol. 69, 5170–5177.

Chu, S., Metcalfe, C.D., 2007. Analysis of paroxetine, fluoxetine and norfluoxetine in fish tissues using pressurized liquid extraction, mixed mode solid phase extraction cleanup and liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1163, 112–118.

Collado, N., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J., Rodriguez-Roda, I., Buttiglieri, G., 2014. Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. Environ. Pollut. 185, 202–212.

Corcoll, N., Acuña, V., Barceló, D., Casellas, M., Guasch, H., Huerta, B., Petrovic, M., Ponsatí, L., Rodríguez-Mozaz, S., Sabater, S., 2014. Pollution-induced community tolerance to non-steroidal anti-inflammatory drugs (NSAIDs) in fluvial biofilm communities affected by WWTP effluents. Chemosphere 112, 185–193.

Corcoll, N., Casellas, M., Huerta, B., Guasch, H., Acuña, V., Rodriguez-Mozaz, S., Serra-Compte, A., Barcelo, D., Sabater, S., 2015. Effects of flow intermittency and pharmaceutical exposure on the structure and metabolism of stream biofilms. Sci. Total Environ. 503–504, 159–170.

Correa-Reyes, G., Viana, M.T., Marquez-Rocha, F.J., Licea, A.F., Ponce, E., Vazquez-Duhalt, R., 2007. Nonylphenol algal bioaccumulation and its effect through the trophic chain. Chemosphere 68, 662–670.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect. 107 Suppl, 907–938.

Della Greca, M., Pinto, G., Pistillo, P., Pollio, A., Previtera, L., Temussi, F., 2008. Biotransformation of ethinylestradiol by microalgae. Chemosphere 70, 2047–2053.

Dobor, J., Varga, M., Záray, G., 2012. Biofilm controlled sorption of selected acidic drugs on river sediments characterized by different organic carbon content. Chemosphere 87, 105–110.

Du, B., Perez-Hurtado, P., Brooks, B.W., Chambliss, C.K., 2012. Evaluation of an isotope dilution liquid chromatography tandem mass spectrometry method for pharmaceuticals in fish. J. Chromatogr. A 1253, 177–183.

Environmental Protection Agency U.S., 2012. Water: Contaminant Candidate List 3 — CCL.

European Commision, 2013. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 Amending Directives 2000/60/EC and 2008/105/EC as Regards Priority Substances in the Field of Water Policy (2011/0429 (COD)).

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–159.

Fisk, A.T., Norstrom, R.J., Cymbalisty, C.D., Muir, D.C.G., 1998. Dietary accumulation and depuration of hydrophobic organochlorines: bioaccumulation parameters and their relationship with the octanol/water partition coefficient. Environ. Toxicol. Chem. 17, 951–961.

Fitzsimmons, P.N., Fernandez, J.D., Hoffman, A.D., Butterworth, B.C., Nichols, J.W., 2001. Branchial elimination of superhydrophobic organic compounds by rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 55, 23–34.

Flemming, H.C., 1995. Sorption sites in biofilm. Water Sci. Technol. 32, 27–33.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633.

García-Galán, M.J., Rodríguez-Rodríguez, C.E., Vicent, T., Caminal, G., Díaz-Cruz, M.S., Barceló, D., 2011. Biodegradation of sulfamethazine by *Trametes versicolor*: removal from sewage sludge and identification of intermediate products by UPLC–QqTOF-MS. Sci. Total Environ. 409, 5505–5512.

Grabicova, K., Grabic, R., Blaha, M., Kumar, V., Cerveny, D., Fedorova, G., Randak, T., 2014. Presence of pharmaceuticals in benthic fauna living in a small stream affected by effluent from a municipal sewage treatment plant. Water Res. 1–9.

Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2012. Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem. J. Chromatogr. A 1248, 104–121.

Gurr, C.J., Reinhard, M., 2006. Harnessing natural attenuation. Environ. Sci. Technol. 1, 2872–2876 (May).

GWRC (Global Water Research Coalition), 2008. Development of an International Priority List of Pharmaceuticals Relevant for the Water Cycle.

Headley, J.V., Gandrass, J., Kuballa, J., Peru, K.M., Gong, Y., 1998. Rates of sorption and partitioning of contaminants in river biofilm. Environ. Sci. Technol. 32, 3968–3973.

Huerta, B., Jakimska, A., Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2013. Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1288, 63–72.

Huerta, B., Jakimska, A., Llorca, M., Ruhí, A., Margoutidis, G., Acuña, V., Sabater, S., Rodriguez-Mozaz, S., Barcelò, D., 2015. Development of an extraction and purification method for the determination of multi-class pharmaceuticals and endocrine disruptors in freshwater invertebrates. Talanta 132, 373–381.

Jakimska, A., Huerta, B., Bargańska, Zaneta, Kot-Wasik, A., Rodríguez-Mozaz, S., Barceló, D., 2013. Development of a liquid chromatography–tandem mass spectrometry procedure for determination of endocrine disrupting compounds in fish from Mediterranean rivers. J. Chromatogr. A 1306, 44–58.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104, 8897–8901.

Kunkel, U., Radke, M., 2008. Biodegradation of acidic pharmaceuticals in bed sediments: insight from a laboratory experiment. Environ. Sci. Technol. 42, 7273–7279.

Kunkel, U., Radke, M., 2011. Reactive tracer test to evaluate the fate of pharmaceuticals in rivers. Environ. Sci. Technol. 45, 6296–6302.

Kwon, J.-W., Armbrust, K.L., 2008. Aqueous solubility, n-octanol-water partition coefficient, and sorption of five selective serotonin reuptake inhibitors to sediments and soils. Bull. Environ. Contam. Toxicol. 81, 128–135.

Lau, T.K., Chu, W., Graham, N., 2005. The degradation of endocrine disruptor di-n-butyl phthalate by UV irradiation: A photolysis and product study. Chemosphere 60 (8), 1045–1053.

Llorens-blanch, G., Badia-fabregat, M., Lucas, D., Rodriguez-, S., 2015. Degradation of pharmaceuticals from membrane biological reactor sludge with *Trametes versicolor*. Environ. Sci. Process. Impacts http://dx.doi.org/10.1039/C4EM00579A.

Margiotta-Casaluci, L., Owen, S.F., Cumming, R.I., de Polo, A., Winter, M.J., Panter, G.H., Rand-Weaver, M., Sumpter, J.P., 2014. Quantitative cross-species extrapolation between humans and fish: the case of the anti-depressant fluoxetine. PLoS One 9, e110467.

Meylan, W.M., Howard, P.H., Boethling, R.S., Aronson, D., Printup, H., Gouchie, S., 1999. Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. Environ. Toxicol. Chem. 18, 664–672.

Morin, S., Duong, T.T., Dabrin, A., Coynel, A., Herlory, O., Baudrimont, M., Delmas, F., Durrieu, G., Schafer, J., Winterton, P., Blanc, G., Coste, M., 2008. Long-term survey of heavy-metal pollution, biofilm contamination and diatom community structure in the Riou Mort watershed, South-West France. Environ. Pollut. 151, 532–542.

Osorio, V., Marcé, R., Pérez, S., Ginebreda, A., Cortina, J.L., Barceló, D., 2012. Occurrence and modeling of pharmaceuticals on a sewage-impacted Mediterranean river and their dynamics under different hydrological conditions. Sci. Total Environ. 440, 3–13.

Paje, M.L.F., Kuhlicke, U., Winkler, M., Neu, T.R., 2002. Inhibition of lotic biofilms by diclofenac. Appl. Microbiol. Biotechnol. 59, 488–492.

Petrovic, M., Sole, M., Lopez de Alda, M.J., Barcelo, D., 2002. Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: integration of chemical analysis and biological effects on feral carp. Environ. Toxicol. Chem. 21, 2146–2156.

Pojana, G., Gomiero, A., Jonkers, N., Marcomini, A., 2007. Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. Environ. Int. 33, 929–936.

Radjenović, J., et al., 2009. Solar photocatalytic degradation of persistent pharmaceuticals at pilot-scale: Kinetics and characterization of major intermediate products. Appl. Catal. B Environ. 89 (1–2), 255–264.

Ramirez, A.J., Brain, R.A., Usenko, S., Mottaleb, M.A., O'Donnell, J.G., Stahl, L.L., Wathen, J.B., Snyder, B.D., Pitt, J.L., Perez-Hurtado, P., Dobbins, L.L., Brooks, B.W., Chambliss, C.K., 2009. Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. Environ. Toxicol. Chem. 28, 2587–2597.

Ricart, M., Guasch, H., Alberch, M., Barceló, D., Bonnineau, C., Geiszingerb, A., Farré, M. La, Ferrer, J., Ricciardi, F., Romaní, A.M., Morin, S., Proia, L., Sala, L., Sureda, D., Sabater, S., 2010. Triclosan persistence through wastewater treatment plants and its potential: toxic effects on river biofilms. Aquat. Toxicol. 100, 346–353.

Riml, J., Wörman, A., Kunkel, U., Radke, M., 2013. Evaluating the fate of six common pharmaceuticals using a reactive transport model: insights from a stream tracer test. Sci. Total Environ. 458–460, 344–354.

Rodriguez, E.M., Medesani, D.A., Fingerman, M., 2007. Endocrine disruption in crustaceans due to pollutants: a review. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 146, 661–671.

Rosenfeldt, E.J., Linden, K.G., 2004. Degradation of endocrine disrupting chemicals bisphenol A, ethinyl estradiol, and estradiol during UV photolysis and advanced oxidation processes. Environ. Sci. Technol. 38 (20), 5476–5483.

Rosi-Marshall, E.J., Kincaid, D.W., Bechtold, H.A., Royer, T.V., Rojas, M., Kelly, J.J., 2013. Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilms. Ecol. Appl. 23, 583–593.

Sabater, S., Guasch, H., Ricart, M., Romaní, A., Vidal, G., Klünder, C., Schmitt-Jansen, M., 2007. Monitoring the effect of chemicals on biological communities. The biofilm as an interface. Anal. Bioanal. Chem. 387 (4), 1425–1434.

Sabater, S., Vilalta, E., Gaudes, A., Guasch, H., Muñoz, I., Romani, A., 2015. Ecological implications of mass growth of benthic cyanobacteria in rivers. Aquat. Microb. Ecol. 32, 175–184.

Serra, A., Guasch, H., 2009. Effects of chronic copper exposure on fluvial systems: linking structural and physiological changes of fluvial biofilms with the in-stream copper retention. Sci. Total Environ. 407, 5274–5282.

Sumpter, J.P., 1998. Xenoendocrine disrupters: environmental impacts. Toxicol. Lett. 102–103, 337–342.

Tien, C.J., Chen, C.S., 2013. Patterns of metal accumulation by natural river biofilms during their growth and seasonal succession. Arch. Environ. Contam. Toxicol. 64, 605–616.

Tien, C.J., Lin, M.C., Chiu, W.H., Chen, C.S., 2013. Biodegradation of carbamate pesticides by natural river biofilms in different seasons and their effects on biofilm community structure. Environ. Pollut. 179, 95–104.

Valenti Jr., T.W., Gould, G.G., Berninger, J.P., Connors, K.A., Keele, N.B., Prosser, K.N., Brooks, B.W., 2012. Human therapeutic plasma levels of the selective serotonin reuptake inhibitor (SSRI) sertraline decrease serotonin reuptake transporter binding and shelter-seeking behavior in adult male fathead minnows. Environ. Sci. Technol. 46, 2427–2435.

Writer, J.H., Barber, L.B., Ryan, J.N., Bradley, P.M., 2011a. Biodegradation and attenuation of steroidal hormones and alkylphenols by stream biofilms and sediments. Environ. Sci. Technol. 45, 4370–4376.

Writer, J.H., Ryan, J.N., Barber, L.B., 2011b. Role of biofilms in sorptive removal of steroidal hormones and 4-nonylphenol compounds from streams. Env. Sci. Technol. 45, 7275–7283.

Writer, J.H., Antweiler, R.C., Ferrer, I., Ryan, J.N., Thurman, E.M., 2013. In-stream attenuation of neuro-active pharmaceuticals and their metabolites. Environ. Sci. Technol. 47, 9781–9790.

Yamamoto, H., Nakamura, Y., Moriguchi, S., Nakamura, Y., Honda, Y., Tamura, I., Hirata, Y., Hayashi, A., Sekizawa, J., 2009. Persistence and partitioning of eight selected pharmaceuticals in the aquatic environment: laboratory photolysis, biodegradation, and sorption experiments. Water Res. 43, 351–362.

Yang, S., Hai, F.I., Nghiem, L.D., Price, W.E., Roddick, F., Moreira, M.T., Magram, S.F., 2013. Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. Bioresour. Technol. 141, 97–108.

Chapter 4

Trophic transfer of pharmaceuticals and endocrine disruptors

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Bioaccumulation and trophic magnification of pharmaceuticals and endocrine disruptors in a Mediterranean river food web

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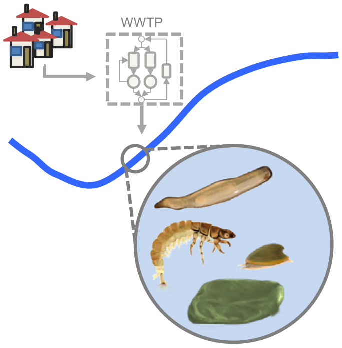
HIGHLIGHTS

- We studied emerging pollutants in an effluent-influenced river food web.
- 12 pharmaceuticals and 5 endocrine-disrupting compounds were detected.
- Observed bioaccumulation factors matched those predicted by an empirical model.
- The flame retardant TBEP experienced trophic magnification.
- Both water and diet may be important sources of exposure for aquatic consumers.

GRAPHICAL ABSTRACT



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ABSTRACT

Increasing evidence exists that emerging pollutants such as pharmaceuticals (PhACs) and endocrine-disrupting compounds (EDCs) can be bioaccumulated by aquatic organisms. However, the relative role of trophic transfers in the acquisition of emerging pollutants by aquatic organisms remains largely unexplored. In freshwater ecosystems, wastewater treatment plants are a major source of PhACs and EDCs. Here we studied the entrance of emerging pollutants and their flow through riverine food webs in an effluent-influenced river. To this end we assembled a data set on the composition and concentrations of a broad spectrum of PhACs (25 compounds) and EDCs (12 compounds) in water, biofilm, and three aquatic macroinvertebrate taxa with different trophic positions and feeding strategies (*Ancylus fluviatilis*, *Hydropsyche* sp., *Phagocata vitta*). We tested for similarities in pollutant levels among these compartments, and we compared observed bioaccumulation factors (BAFs) to those predicted by a previously-developed empirical model based on octanol–water distribution coefficients (D_{ow}). Despite a high variation in composition and levels of emerging pollutants across food web compartments, observed BAFs in *Hydropsyche* and *Phagocata* matched, on average, those already predicted. Three compounds (the anti-inflammatory drug diclofenac, the lipid regulator gemfibrozil, and the flame retardant TBEP) were detected in water, biofilm and (at least) one macroinvertebrate taxa. TBEP was the only compound present in all taxa and showed magnification across trophic levels. This suggests that prey consumption may be, in some

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cases, a significant exposure route. This study advances the notion that both waterborne exposure and trophic interactions need to be taken into account when assessing the potential ecological risks of emerging pollutants in aquatic ecosystems.

1. Introduction

The incidence of pharmaceuticals (PhACs) and endocrine-disrupting compounds (EDCs) in freshwater ecosystems is an issue of increasing concern (e.g., Arnold et al., 2014; Brodin et al., 2014; Lee et al., 2015). Although concentrations of PhACs in the environment are usually very low (i.e., around ng L^{-1} and within the low μg L^{-1} range), these compounds can be reactive to non-target organisms (Hernando et al., 2006) and pseudo-persistent in the environment due to their continuous discharge (Webb, 2001; Daughton, 2002). It is currently widely accepted that EDCs, including environmental estrogens (chemical pollutants that can alter animal endocrine systems by binding to and activating the estrogen receptor) are potentially harmful to exposed organisms, with effects including reduced fertility and fecundity, masculinization of females and feminization of males, and alteration of fish spawning timing (Jobling et al., 2003; Falconer et al., 2006 and references therein). Whereas a number of field studies have addressed the occurrence and effects of emerging pollutants on aquatic vertebrates, particularly fish (e.g. Brooks et al., 2005; Ramirez et al., 2009; Du et al., 2012; Jakimska et al., 2013), bioaccumulation by invertebrates has been largely studied by means of aqueous-exposure experiments (Oetken et al., 2005; Nentwig, 2007; Hoppe et al., 2012) (but see Berlioz-Barbier et al., 2014; Du et al., 2014, 2015). Overall, we need to further study the bioaccumulation of PhACs and EDCs in natural conditions if we are to fully understand the potential impact of these emerging pollutants in freshwater ecosystems.

Two main processes may lead to the bioaccumulation of PhACs and EDCs by aquatic organisms: direct partitioning from the abiotic environment through inhalational exposure (bioconcentration) and dietary sources (trophic transfer). Hydrophobic compounds (especially those poorly metabolized and with high log K_{ow} values) are more likely to present bioaccumulation hazards to aquatic organisms (Mackay and Fraser, 2000). Additionally, higher trophic positions may be differentially affected by trophic transfer (Fisk et al., 2005). Biomagnification of pollutants (and hence toxicity) in top predators has long attracted ecological research (e.g., Cabana and Rasmussen, 1994; Kidd et al., 1995). However, it remains a critical research need with regard to emerging pollutants (Boxall et al., 2012), and still few studies have quantified trophic magnification or dilution of these compounds in an explicit way (but see Vernouillet et al., 2010; Du et al., 2014). Overall, we need to better understand how biotic interactions mediate the bioaccumulation of emerging pollutants (Brodin et al., 2014; Kidd et al., 2014).

Wastewater treatment plants (WWTPs) are a major source of PhACs and EDCs to freshwater ecosystems (Ternes, 1998; Gros et al., 2007). Due to the increased effective exposure faced by organisms in streams and rivers dominated by WWTP effluent discharge, these systems offer an ideal setting to study how these pseudo-persistent PhACs and EDCs accumulate and flow through freshwater ecosystems. With widespread droughts and increased streamflow and groundwater appropriation, these systems may anticipate worst-case scenarios for the accumulation of PhACs and EDCs in rivers with limited dilution capacity in industrialized regions (Brooks et al., 2006; López-Doval et al., 2013; Du et al., 2015).

Here we addressed the general question of the bioaccumulation of PhACs and EDCs by aquatic organisms. More specifically, we examined if PhACs and EDCs were effectively accumulated in an effluent-influenced riverine food web, and whether trophic magnification or trophic dilution took place across the three trophic levels analyzed (primary producers to secondary consumers). To this end, we combined existing (Huerta et al., 2015, in press) and new data on a broad spectrum of emerging pollutants (25 PhACs and 12 EDCs) across 5 compartments that represented a simplification of the effluent-influenced riverine food web: water, biofilm, and three macroinvertebrate taxa differing in trophic position and feeding strategy. These macroinvertebrates were the limpet *Ancylus fluviatilis* O.F. Muller 1774, a primary consumer, mainly microphyte-scraper; the caddisfly *Hydropsyche* sp. Pictet 1834, an omnivore filter-feeder; and the flatworm *Phagocata vitta* (Duges 1830), a macroinvertebrate-predator (Tachet et al., 2002).

We predicted that (i) the composition and levels of PhACs and EDCs in water, biofilm and macroinvertebrates would be related, and that bioaccumulation models developed for invertebrates (based on octanol–water distribution coefficients, D_{ow}) would adequately predict, overall, the range of BAFs observed in our taxa (hereafter, *bioaccumulation hypothesis*). We also predicted that (ii) trophic levels and feeding strategies would account for variation in bioaccumulated PhACs and EDCs within the food web, with compounds being trophically magnified and thus most abundantly found in the macroinvertebrate taxa feeding relatively higher in the chain (hereafter, *trophic magnification hypothesis*).

2. Materials and methods

2.1. Study area

The study was conducted in the Segre River (Ebro River basin, NE Iberian Peninsula) near the town of Puigcerdà, where this river is relatively unimpacted but receives the discharge of a wastewater treatment plant (WWTP) effluent of c. 30,000 population equivalents. At the study site the watershed drains an area of 287 km^2 and the elevation ranges from 1098 to 1130 m a.s.l. The river runs through a gravel bed meandering channel across a broad valley mainly covered with native forests, some pastures and small agricultural fields. The river's mean annual streamflow at the study site is 7.7 $m^3\ s^{-1}$ (ranging from a monthly minimum of 0.7 $m^3\ s^{-1}$ to a monthly maximum of 32.4 $m^3\ s^{-1}$) and presents a hydrograph peaking in late spring because of strong snowmelt influence. The selected river segment holds potentially confounding factors approximately constant (i.e. geomorphology, hydrology, water chemistry; Acuña et al., 2015). The study took place in October 2012, in a control reach (500 m upstream of the WWTP) and three impact reaches (each 1-km long) located downstream of the WWTP.

2.2. Environmental characterization

Environmental variation across reaches was defined according to temperature (°C), pH, electrical conductivity (μS cm^{-1}), nitrite ($N\text{-}NO_3^-$, μg L^{-1}), nitrate ($N\text{-}NO_2^-$, μg L^{-1}), ammonium ($N\text{-}NH_4^+$, μg L^{-1}), phosphate ($P\text{-}PO_4^3$, μg L^{-1}), and dissolved organic carbon (DOC, μg L^{-1}). Temperature, pH, and electrical conductivity were measured in situ using handheld probes (WTW, Weilheim, Germany). Water samples for nutrient analyses were collected at each reach, filtered in 0.7 μm GF/F filters (Whatman Int. Ltd., Maidstone, UK) and kept at −20 °C until analyzed. Phosphate concentration was determined colorimetrically using an Alliance-AMS Smartchem 140 (AMS, Frepillon, France), after Murphy and Riley (1962). Nitrite, nitrate and ammonium concentrations were determined on a Dionex ICS-5000 ion chromatograph (Dionex Co., Sunnyvale, USA; Hach, 2002). DOC concentration was determined on a Shimadzu TOC-V CSH coupled to a TNM-1 module (Shimadzu Co., Kyoto, Japan).

2.3. Food web characterization

Mapping the streambed allowed quantifying variation in resource types and resource availability among reaches. In each reach we visually identified different biofilm types, helophyte and macrophyte patches, moss clumps, benthic organic matter accumulation, and emerged substrata. Then, using a viewer (30 × 30 cm), we estimated the relative cover (%) of each of these substrata classes every 30 cm and across three transverse transects in each reach. The algal biomass in the biofilm was evaluated by using chlorophyll-a as a proxy. To this end, five replicate samples (9 cm^2 each) of biofilm per reach were collected, stored in the dark and frozen (-20 °C). In the laboratory, chlorophyll-a was extracted in 90% acetone for 12 h in the dark at 4 °C, and its concentration was determined using a Lambda UV/VIS spectrophotometer (U-2000; Hitachi, Tokyo, Japan), following Jeffrey and Humphrey (1975).

The faunal community was characterized by means of 6 pooled Surber samples per reach (625 cm^2 each, 330-μm mesh size), covering the variety of microhabitats present. Whole samples were preserved in the field in 70% methylated spirit, and specimens were sorted from debris in the laboratory. Individuals were identified under a binocular microscope to the lowest taxonomic level (to genus level whenever possible; Diptera mostly to subfamily level; Annelida to subclass level), and counted. A subsample of 25 individuals per taxa and reach were measured, and length–mass equations were applied to obtain biomass (as dry mass, after Stockner, 1971; Allan, 1984; Benke and Jacobi, 1986, 1994; Meyer, 1989; Dixon and Wrona, 1992; Burgherr and Meyer, 1997; Benke et al., 1999; Miserendino, 2001; Rosati et al., 2012). We then aggregated taxa biomass in trophic strategies using a fuzzy coding technique (Chevene et al., 1994) that allowed us to consider the range of trophic affinities of each taxa (ranging from 0 = no affinity; to 5 = high affinity; after Tachet et al., 2002), and we compared biomass of macroinvertebrate trophic guilds across reaches.

2.4. Collection of water, biofilm, and macroinvertebrate specimens for analyses

At each reach, water samples (100 mL) were filtered through 1 μm glass fiber filters followed by 0.45 μm nylon membrane filters, and kept at -20 °C until analysis. Samples of epilithic biofilm (volume = 40 mL) were obtained from cobbles near the stream shore by gently scraping the rock surface. These samples were kept in 50 mL Falcon® tubes in a dark cool box and transported to the laboratory, where they were lyophilized and kept frozen (-20 °C) until analysis. Finally, we collected c. 125 individuals of *Ancylus fluviatilis*, 90 individuals of *Hydropsyche* sp., and 70 individuals of *Phagocata vitta* at each reach in order to have sufficient material for analysis. In order to control for variation in size and developmental stages of these macroinvertebrates, in each reach we collected only the largest specimens of *Ancylus* and *Phagocata*; and in the case of *Hydropsyche*, only last larval instars (instar V) and prepupae (i.e. the stage from case-closure to larval-pupae ecdysis). Specimens were placed in 50 mL Falcon® tubes filled with river water and transported in a dark cool box. In the laboratory we confirmed their identification under a binocular microscope, and confirmed taxa were placed in filtered river water during 24 h to allow for gut clearance and thus avoid overestimating tissue concentrations (Van Geest et al., 2010). A composite sample for each taxa and reach was then freeze-dried, homogenized with a mortar, and kept at -20 °C until analysis. The three target macroinvertebrate taxa had been previously determined based on an exploratory field visit (in August 2012) that aimed at identifying consistently-dominant macroinvertebrate taxa across reaches.

2.5. Emerging contaminant extraction

Water samples were extracted according to the method developed by Gros et al. (2012) for the analysis of PhAC, which was also applied for the analysis of EDCs. Briefly, 3 mL of EDTA 1 M (4%, v/v) was added to water samples. A Baker vacuum system (J.T. Baker, Netherlands) was used to pre-concentrate the samples in Oasis HLB cartridges (60 mg, 3 mL). They were loaded with 100 mL of water samples and eluted with 6 mL of methanol. The extracts were evaporated under a gentle nitrogen stream and reconstituted with 1 mL of methanol–water mixture (50:50, v/v for EDCs; 10:90, v/v for PhACs).

Biofilm samples were extracted by pressurized liquid extraction (PLE) with ASE®350, following the method developed by Huerta et al. (in press). Samples (200 mg, dry weight) were placed in 10 mL cells and together with diatomaceous earth to fill up the rest of the cell and extracted with citric buffer (pH 4)/acetonitrile during 3 cycles of 5 min at 60 °C. Extracts were placed under a N_2 current until the volume was approximately 5 mL, and they were then diluted to 100 mL of H_2O (organic solvent content < 0.05%). Further purification of the extracts was performed by solid phase extraction (SPE) using the described protocol for extraction of water samples (Gros et al., 2012). Purified extracts were then evaporated to dryness under a nitrogen stream and reconstituted in 1 mL of methanol. Two aliquots (250 μL each) from this 1 mL of methanol were evaporated in reconstituted in the same volume of: a) methanol/water (10:90) for the analysis of PhACs; b) methanol/water (50:50) for the analysis of EDCs. Finally, 5 μL of a 1 mg/L standard mixture containing labeled compounds was added in the extracts as internal standard.

Macroinvertebrates were processed following Huerta et al. (2015). Samples (100 mg, dry weight) were extracted with an ultrasonic probe (Branson Digital Sonifier, model 102C) (120 s, 3 cycles, 30% amplitude) and homogenized in 3 mL of methanol. Extract (1.5 mL) was evaporated to dryness and dissolved in 0.75 μL of a mixture of water/acetonitrile containing 0.1% formic acid. Extract was passed through the OSTRO™ plate for the removal of phospholipids, followed by 0.75 μL of the same fresh solvent. Purified extract was divided in two aliquots for the analysis of both families of compounds (PhACs and EDCs) by UPLC–MS/MS. Aliquots were evaporated to dryness and dissolved in methanol/water (1:1, v/v) for EDCs, and in methanol/water (1:9, v/v) for PhACs.

Method detection limits (MDL) and method quantification limits (MQL) of all PhACs and EDCs are available in the referenced papers (Gros et al., 2012; Huerta et al., 2015, in press). Here we focused on the 37 compounds that were targeted simultaneously in water, biofilm, and macroinvertebrates (Table S1), and subsequent data analyses were restricted to the subset of compounds that were detected in at least one of these matrices.

2.6. Data analysis

We first explored the across-reach variation in environmental characteristics and presence of pollutants (PhACs + EDCs) in water. To this end we run two Similarity Profile (SIMPROF) tests: one on environmental variables (using Euclidean distances on normalized data), and another one on the presence–absence of PhACs and EDCs present in water (Sorensen coefficient). SIMPROF allows testing if similarities observed in empirical data are smaller or larger than those expected by chance, challenging the null hypothesis of no multivariate structure. We also assessed across-reach variation in resource types (comparing streambed covers across reaches), in resource availability (comparing algal biomass across reaches), and in food web structure (comparing biomass of macroinvertebrate trophic guilds across reaches).

The *bioaccumulation hypothesis* was tested in two ways. First, we compared composition and levels of emerging pollutants among trophic compartments (water, biofilm, *Ancylus*, *Hydropsyche*, and *Phagocata*). To this end we built a similarity matrix using the Bray–Curtis coefficient, to consider not only similarities in pollutants' composition (i.e. presence–absence) but also in their concentrations. We visualized similarities in pollutants among compartments, and among reaches within each compartment, with a non-metric multidimensional scaling (MDS). We subsequently tested for statistical significance in the observed patterns using a Permutational MANOVA (PERMANOVA), considering *food web compartment* as a fixed factor, and *reach* (control vs. impact) as a fixed factor nested within *food web compartment*. This test allowed quantifying overall variation in pollutants across food web compartments, and also differences among pairs of compartments (10 post-hoc comparisons in total, obtained from all possible pairwise groupings of the 5 compartments). If two compartments could not be statistically distinguished based on the composition and levels of PhACs and EDCs present in each, and presented relatively high Bray–Curtis similarities, we would consider them to be positively associated (in order to control for false associations, values below method detection limits were considered as true zeroes in the similarity matrix).

Second, the *bioaccumulation hypothesis* was also tested by comparing observed to predicted bioaccumulation factors (hereafter BAFs, in L kg^{-1}) in the macroinvertebrate taxa. Observed BAFs were obtained as the ratio between concentrations of given pollutants in particular taxa to those detected in water. When a compound was not detected or quantified we used half method detection limits (MDL) or method quantification limits (MQL), and only compounds detected in at least one of the two components were included. In turn, predicted BAFs were obtained from the linear regression proposed by Arnot and Gobas (2006). However, following the approach by Du et al. (2015) here we used the octanol–water distribution coefficient (D_{ow}) instead of the octanol–water partition coefficient (K_{ow}), to account for influences of pH on the bioaccumulation of ionizable compounds (Eq. (1)). D_{ow} was calculated at the approximate field pH = 8 (average pH across sites = 8.05).

$$\log \ \text{BAF} = 0.82 \ \log \ D_{ow} + 0.09 \tag{1}$$

We regressed observed on predicted BAFs of all compounds for each of the three macroinvertebrate taxa, and obtained a slope and corresponding 95% confidence intervals that were used to define whether differences existed or not.

Finally, we examined the *trophic magnification hypothesis* by focusing on compounds that occurred in all living compartments. We tested for differences in pollutant concentration found in biofilm, *Ancylus*, *Hydropsyche*, and *Phagocata* using a mixed model that considered compartment as a fixed effect and reach as a random effect. Post-hoc Bonferroni pairwise tests allowed identifying which compartments differed significantly. We would consider our prediction to be confirmed if global differences existed and if concentrations showed a positive association, overall, with trophic levels (i.e., biofilm < *Ancylus* < *Hydropsyche* < *Phagocata*).

All analyses were developed in Primer v.6 and SPSS v.22 for Windows.

3. Results

3.1. Reach and food web characterization

The SIMPROF tests revealed that reaches were showing, overall, similar environmental conditions (Pi = 0.322, $P = 0.080$; Table 1). However, variation in PhACs and EDCs was significant (Pi = 35.56, $P < 0.001$), indicating that food webs were exposed to different combinations of pollutants across reaches.

Epilithic biofilm cover clearly dominated (>75%) in all reaches, and macrophytes were the second most abundant cover (surpassing 15% in the impact reaches I2 and I3; Fig. 1a). Significantly higher concentrations of chlorophyll-a were found in the impact reaches relative to the control reach (ANOVA on log-transformed data; $F_{3,15} = 18.432$, $P < 0.001$) (Fig. 1b). A total of 34 macroinvertebrate taxa were found (C = 23, I1 = 19, I2 = 14, I3 = 16), with Diptera (10 taxa) and Trichoptera (5 taxa) being the most diverse orders. Macroinvertebrate biomass increased downstream (Fig. 1c). Among the macroinvertebrate trophic guilds, macroinvertebrate feeders (i.e. predator strategies) and detritivores dominated. Predators were more prevalent in the control reach and the impact reach I1, whereas detritivores dominated in the impact reaches I2 and I3 (Fig. 1c). The three selected taxa represented, on average, a pooled 45.9% of the macroinvertebrate biomass (mean % ± SD: *Ancylus* = 6.2 ± 3.3%, *Hydropsyche* = 21.7 ± 6.2%, *Phagocata* = 18.0 ± 11.7%).

Table 1
Water variables at the studied reaches (mean ± SD, n = 3).

Environmental variable	C	I1	I2	I3
T (°C)	13.2 ± 1.7	13.3 ± 1.4	13.4 ± 1.6	13.1 ± 1.3
Conductivity (μS cm^{-1})	183.7 ± 1.1	207.7 ± 1.5	170.7 ± 1.5	202.3 ± 1.5
pH	7.5 ± 0.1	8.6 ± 0.6	8.8 ± 0.1	7.3 ± 0.1
P-PO_4^{3-} (μg L^{-1})	40.1 ± 2.3	172.9 ± 13.1	168.9 ± 1.5	177.8 ± 1.9
N-NO_3^- (μg L^{-1})	583.6 ± 27.1	1230.4 ± 44.5	1329.6 ± 62.9	1409.6 ± 110.6
N-NO_2^- (μg L^{-1})	5.4 ± 0.43	69.8 ± 8.5	76.5 ± 13.6	54.8 ± 5.0
N-NH_4^+ (μg L^{-1})	14.6 ± 2.8	476.9 ± 97.6	393.7 ± 64.0	116.2 ± 31.9
DOC (mg L^{-1})	2.4 ± 0.02	3.0 ± 0.3	2.8 ± 0.03	3.0 ± 0.4

3.2. Bioaccumulation of PhACs and EDCs

A total of 17 compounds were detected (Fig. 2, Table S2). 10 compounds (9 PhACs and 1 EDC) were present in water, 9 in biofilm (6 PhACs and 3 EDCs), and 6 in the macroinvertebrate taxa (1 EDC in *Ancylus*; 2 PhACs and 2 EDCs in *Hydropsyche* and in *Phagocata*) (Fig. 2, Table S2). However, there was a large variation in the identity and levels of detected compounds across compartments (Fig. 2, Table S2). Three compounds (the anti-inflammatory drug diclofenac, the lipid regulator gemfibrozil, and the flame retardant TBEP) were detected simultaneously in biofilm and in (at least) one macroinvertebrate taxa, and these were also found in water. The flame retardant TBEP was the only compound found consistently across all food web compartments (Fig. 2, Table S2). The highest concentrations of PhACs and EDCs occurred in impact reaches (Fig. 2). Hydrochlorothiazide, gemfibrozil and ibuprofen were consistently abundant in water (ranging 115–311 ng L^{-1} across impact reaches), methylparaben was abundant in biofilm in impact reach I1 (176.4 ng g^{-1}), and ibuprofen was also abundant in *Hydropsyche* in the impact reach I2 (182.7 ng g^{-1}) (Fig. 2, Table S2).

3.3. Bioaccumulation hypothesis

The MDS showed the similarities in pollutant composition among food web compartments and among reaches within each compartment (Fig. 3). In this representation of the multivariate space, samples were mainly clustered by food web compartments, indicating that some compounds were specifically accumulated by each group. The vertical axis of the MDS ordered samples by reaches, with those from control reaches (where the lowest number and levels of compounds was detected) lying close, and those from I1/I2 reaches lying relatively closer to those from water (where the highest number and levels of compounds was detected) (Fig. 3). These patterns were confirmed by the PERMANOVA, which delivered significant effects for both food web compartment (Pseudo-$F_{4,10} = 4.59$, $P = 0.001$) and reach (Pseudo-$F_{5,10} = 2.68$, $P = 0.002$). Pairwise post-hoc tests showed that most food web compartments differed significantly in pollutants' composition (Table 2). The three macroinvertebrate taxa presented relatively high Bray–Curtis similarities (40–50%), and two of these pairs (*Ancylus*–*Hydropsyche* and *Hydropsyche*–*Phagocata*) could not be statistically distinguished (Table 2).

When comparing the observed (field) BAFs to predicted BAFs from Eq. (1), statistically significant regressions were obtained for all three taxa (Fig. 4). For *Ancylus*, the slope differed from 1, which implies that the model was not completely capturing the drivers of variation in our

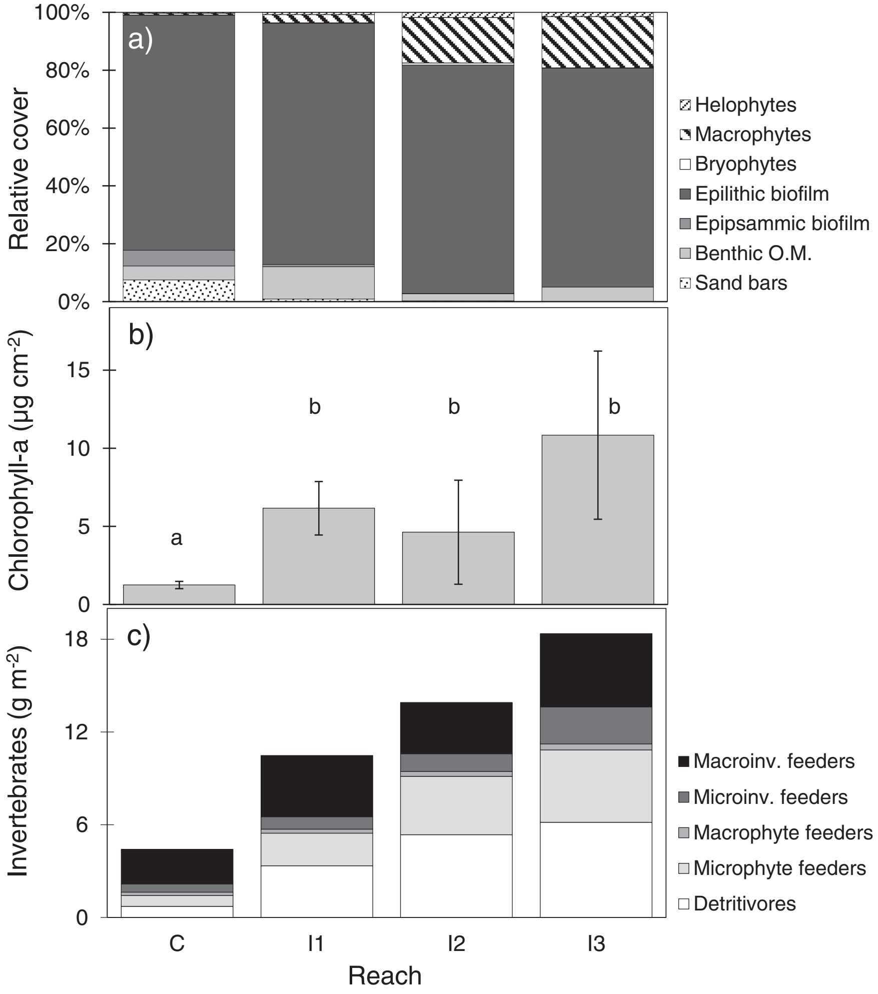

Fig. 1. Cross-reach variation in (a) streambed relative covers, (b) algal biomass (using biofilm chlorophyll-a as a proxy), and (c) food web structure. See text for details. In (b), letters indicate significant grouping according to Bonferroni post-hoc tests. C = control reach; I1 = 1st impact reach after effluent; I2 = 2nd impact reach after effluent; I3 = 3rd impact reach after effluent.

observed BAFs. However, for both *Hydropsyche* and *Phagocata* (especially the latter) the slope confidence intervals included 1, meaning that the dependence of BAF on D_{ow} assumed by the empirical model was applicable to our taxa (Fig. 4). Observed and predicted log BAFs (in L kg^{-1}) are reported in Table S3.

3.4. Trophic magnification hypothesis

Only the flame retardant TBEP occurred in all compartments. TBEP concentration varied among living compartments (Fig. 5) and the mixed model (controlling for reach) showed that these differences were significant ($F_{3,9} = 6.986$, $P = 0.01$). Post-hoc pairwise comparisons further showed that TBEP concentrations increased with trophic levels, being lowest in biofilm, intermediate in *Ancylus*, and highest in *Hydropsyche* and *Phagocata* (Fig. 5). No differences in TBEP concentration were found between the two taxa that putatively held the highest trophic positions (i.e. *Hydropsyche*, omnivore, and *Phagocata*, predator) (Fig. 5).

4. Discussion

A number of field studies have used correlational approaches to infer the effects of emerging pollutants on populations and communities of aquatic organisms (e.g., Muñoz et al., 2009; Ginebreda et al., 2010; Damásio et al., 2011). These studies have reported significant associations between abundance of pollutants and abundance or biomass of certain invertebrate groups (e.g. anti-inflammatories and β-blockers on oligochetes and chironomids; Muñoz et al., 2009), or between integrative hazard indexes and macroinvertebrate diversity indexes (Ginebreda et al., 2010). However, in field conditions emerging contaminants generally co-occur with other chemical contaminants (Kolpin et al., 2002; Focazio et al., 2008; Proia et al., 2013), making the effects of PhACs on aquatic organisms difficult to disentangle from those of co-occurring stressors such as pesticides, salinity, and nutrients (Damásio et al., 2011). For this reason, here rather than inferring impacts of emerging contaminants by comparing community structure between control and impact reaches, we quantified bioaccumulation as a measure of "impact" itself (Van der Oost et al., 2003). Although this approach is still relatively new on invertebrates (but see Du et al., 2014, 2015), it has the advantage of making no assumptions on the effects of emerging pollutants, co-occurring pollutants, or the interaction between both. We detected a wide range of emerging pollutants (17 of the 37 analyzed); an overall low association in the composition of pollutants found among the several food web compartments, but an agreement between observed and predicted BAFs in *Hydropsyche* and *Phagocata* (partly confirming the *bioaccumulation hypothesis*); and an overall trophic magnification of TBEP over the examined trophic levels (confirming the *trophic magnification hypothesis*). Our study was limited in food-web extent (integrating from biofilm to secondary-consumer invertebrates, but no fish) and overlooked temporal variability. Changes

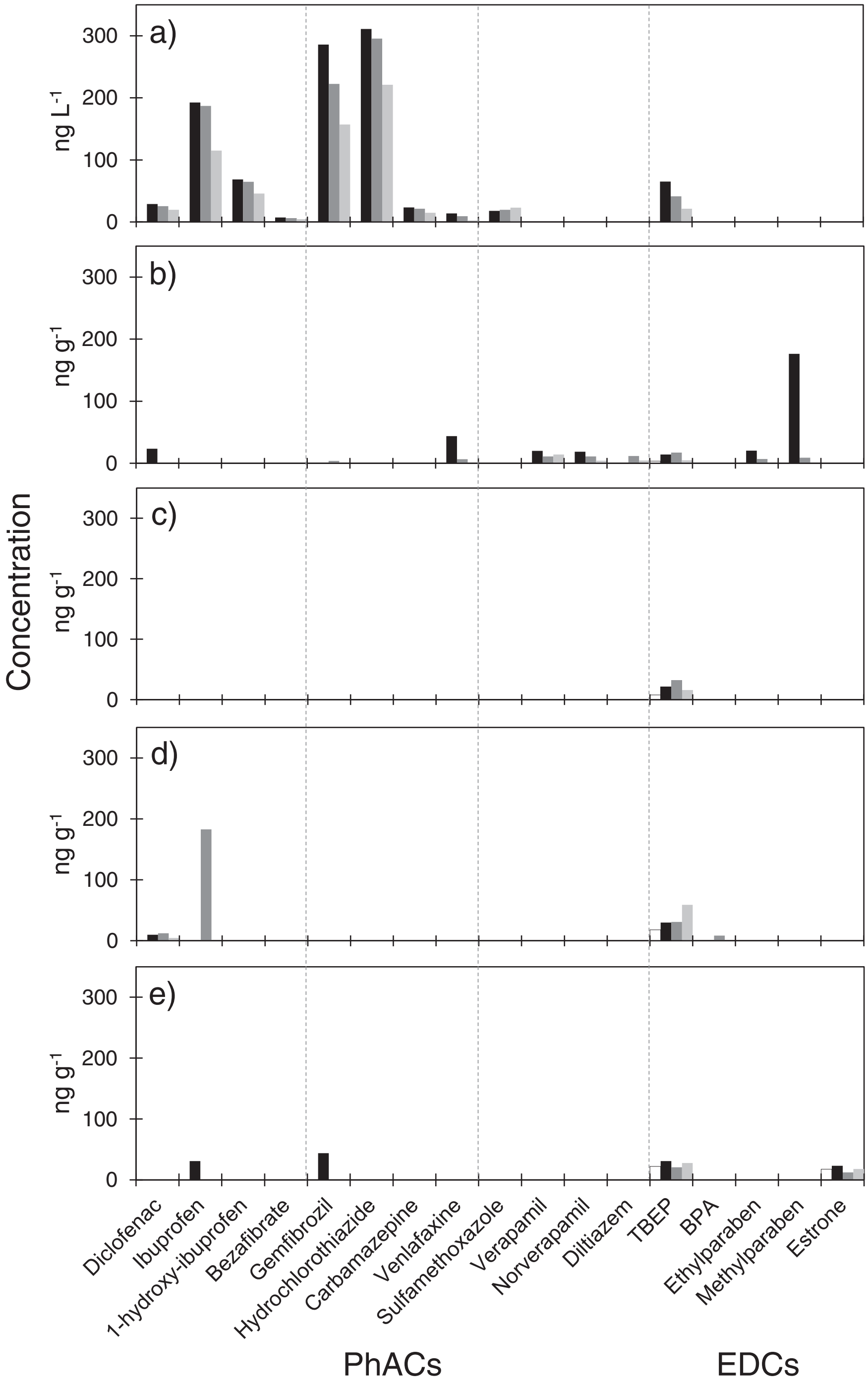

Fig. 2. Levels of the PhACs (12 compounds) and EDCs (5 compounds) that were detected in at least one sample. Each panel represents a food web compartment: a) water; b) biofilm, c) herbivore *Ancylus*; d) omnivore *Hydropsyche*; e) predator *Phagocata*. Shading represents the four sampled reaches: white = control reach (C), black = 1st impact reach after effluent (I1); dark gray = 2nd impact reach after effluent (I2); light gray = 3rd impact reach after effluent (I3). See values in Table S2.

in the load entering the WWTP, in removal efficiencies, and in dilution capacity owing to variation in river discharge (Vieno et al., 2005; Loraine and Pettigrove, 2006; Sui et al., 2011) control effective exposure to biofilm and invertebrates over their lifetimes. Thus, variation (over time) in the number and concentration of compounds found in water would have likely altered BAFs if a longer time window had been considered. However, levels found in river water were close to those found in the same site but in a different period of the year (Acuña et al., 2015), and another study showed that the concentrations of some pharmaceuticals in these study reaches were relatively stable over a 48-h period (Aristi et al., 2015). Overall, our study advances the notion that both waterborne and dietary exposure may contribute to the bioaccumulation of emerging pollutants in aquatic organisms.

4.1. Bioaccumulation of emerging pollutants by aquatic organisms

We found almost half of the analyzed compounds (12 out of 25 PhACs, 5 out of 12 EDCs), but a large variation in their combinations when comparing food web compartments: 5 compounds were present in biofilm but absent in water, 2 EDCs (BPA and estrone) were found in *Hydropsyche* and *Phagocata* but not in water, and 5 out of the 10 compounds detected in water were not found in any other compartment

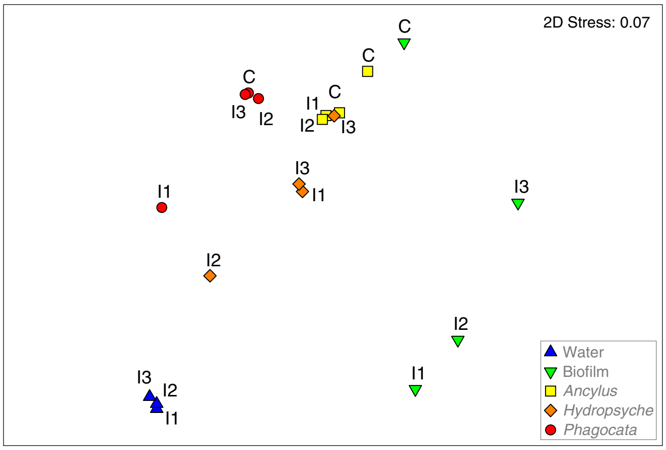

Fig. 3. Non-metric multidimensional scaling (MDS) showing the ordination of the five food web compartments and the four sampled reaches in terms of emerging pollutants' composition (Bray–Curtis similarity index). C = control reach; I1 = 1st impact reach after effluent; I2 = 2nd impact reach after effluent; I3 = 3rd impact reach after effluent. Shapes represent trophic compartments. The control reach for water was not included in this representation because no compounds were detected (see Table S2).

(Fig. 2, Table S2). These mismatches could be explained by temporal variation integrated by aquatic organisms but not captured in discrete water samples, as discussed earlier. We also observed significant differences in pollutants' composition and levels between biofilm and the three macroinvertebrate taxa. Although at relatively lower concentrations, biofilm accumulated a higher number of PhACs and EDCs than the three macroinvertebrate taxa (either alone or combined). This high number of pollutants in biofilm might be related not only to biofilm uptake but also to potential physical sorption by the extracellular polymeric substances, EPS (Schorer and Eisele, 1997; Headley et al., 1998; Lawrence et al., 2001; Huerta et al., in press). The lack of detailed data on biofilm structure hinders, in our case, from assessing if variation in the number of pollutants in biofilm could be also partially explained by differences in structure (e.g. related to maturity) across reaches. It is also important to note that *Hydropsyche* and *Phagocata* had a significantly higher number of bioaccumulated pollutants than *Ancylus* (4 vs. 1). For these taxa, but not for *Ancylus*, a previously developed bioaccumulation model for invertebrates (Arnot and Gobas, 2006) correctly predicted the observed BAFs. Du et al. (2015) also found a significant relationship, but with lower coefficients of determination ($R^2 = 0.19$), between predicted and observed BAFs of PhACs in *Planorbarius* snails in an effluent-dependent stream in Texas. Acknowledged factors explaining variation in BAFs have been related (but not restricted) to differences in lipid content, individual size, or life stage (Arnot and Gobas, 2006): higher lipid contents imply a higher capacity to store hydrophobic compounds; larger-bodied animals have slower elimination rates; and age reflects organismal effective exposure under pseudo-persistent conditions. In our study, *Phagocata* was the only taxa with a life-cycle duration > 1 year (Tachet et al., 2002) and likely had the highest lipid content of the three (Makhutova et al., 2009). This taxa also held the highest trophic position (Armitage and Young, 1991; Tachet et al., 2002) and highest mean body size. Since these traits are often correlated in nature (especially body size and trophic position; Woodward and Hildrew, 2002; Woodward et al., 2005; Arim et al., 2010), it may be difficult to parse out their unique influences by means of field studies. Despite these and other sources of uncertainty around BCFs and BAFs (e.g. respiration mode, Meredith-Williams et al., 2012), the relatively simple bioaccumulation model based on D_{ow} (Arnot and Gobas, 2006; as modified by Du et al., 2015) predicted BAFs in the highest trophic levels, which is indeed relevant to ecological risk assessment.

4.2. Trophic magnification of TBEP

In our study, the only compound that could be tested for trophic magnification or dilution (i.e. present in biofilm as well as in the three macroinvertebrate taxa) was the flame retardant TBEP and it showed, overall, trophic magnification. Our finding raises the question of whether TBEP would continue to be magnified over additional (vertebrate) trophic levels, either aquatic (i.e. from *Hydropsyche* larvae or *Phagocata* to invertivore fish) or terrestrial (i.e. from adult, flying *Hydropsyche* to insectivorous birds). Within this context, studying a more extensive food web (including an extra trophic level – e.g., fish – and other primary- and secondary-consumer invertebrates) using C and N stable isotopes could allow for a precise depiction of the magnification pathways. Previous studies had detected TBEP in similar concentrations, in coastal fishes in the Philippines (Kim et al., 2011) and in Mediterranean riverine fishes (also in the Ebro basin, among other rivers; Jakimska et al., 2013). Chen et al. (2012) studied its occurrence in herring gull eggs and pointed out that despite its moderate K_{ow} value (3.9), the ubiquity of TBEP likely indicates bioaccumulation potential. Volatilization of organophosphates from objects protected by flame retardants such as vehicles and buildings causes these compounds to enter aquatic ecosystems via washout from the atmosphere (Regnery and Püttmann, 2009). This could explain why this compound, unlike all PhACs and most of the EDCs detected, was found in all living compartments already in the control reach. TBEP can alter embryonic development and protein expression when present in high concentrations ($150\ \mu g\ L^{-1}$ to $100\ mg\ L^{-1}$; Han et al., 2014), but effects on biota within concentration ranges comparable to ours have not been reported to our knowledge.

Previous studies quantifying trophic magnification of emerging pollutants (either by using trophic magnification factors or by comparing BAFs and ecological effects across trophic levels) show dissimilar results. Du et al. (2014) examined diphenhydramine and carbamazepine concentrations (among other pharmaceuticals) in an effluent-dependent stream food web (considering biofilms, aquatic invertebrates, and vertebrates) and found a slightly positive (non-significant) slope between carbamazepine concentration and trophic position, and a significant and negative one for diphenhydramine. The psychiatric drug carbamazepine was also the focus of a study comparing BAFs and toxic effects of this compound across three trophic levels (algae, a crustacean, and a cnidarian; Vernouillet et al., 2010). In that case

Table 2
Summary of PERMANOVA post-hoc tests comparing pollutant composition and levels among food web compartments (Bray–Curtis similarity index, 999 permutations). Values represent average similarity in pollutants between/within compartments and *P*-value of each pairwise comparison (in parenthesis).
* = $P < 0.05$, reflecting significant differences in pollutant composition for that pair.

		PhAC + EDC matrix B			
		Biofilm	*Ancylus*	*Hydropsyche*	*Phagocata*
PhAC + EDC matrix A	*Water*	2.93 (0.004)*	3.28 (0.004)*	11.15 (0.006)*	7.02 (0.009)*
	Biofilm		23.79 (0.107)	15.97 (0.042)*	15.28 (0.016)*
	Ancylus			50.06 (0.350)	49.01 (0.028)*
	Hydropsyche				42.54 (0.330)

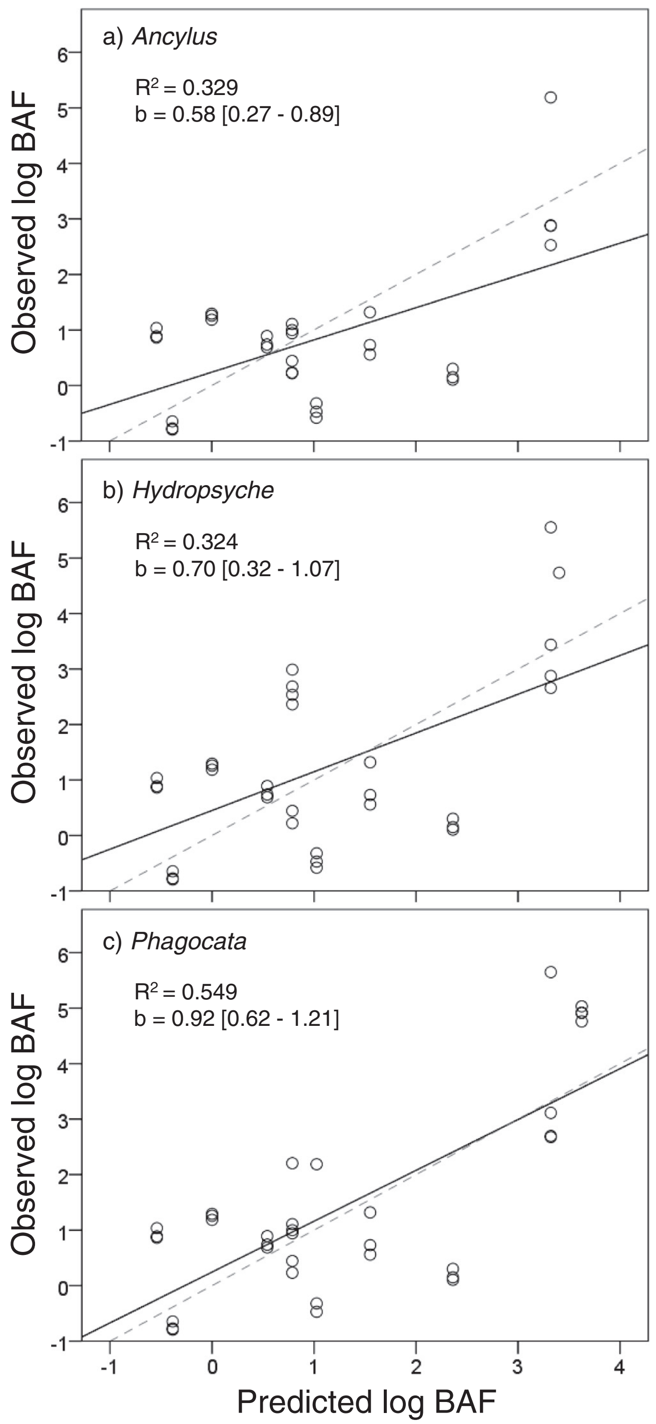

Fig. 4. Predicted vs. observed (field) bioaccumulation factors (BAFs, in L kg^{-1}) for (a) *Ancylus*, (b) *Hydropsyche*, and (c) *Phagocata*. Solid lines show the observed relationship between predicted and observed BAFs, dashed lines indicate a hypothetical 1:1 agreement with the model used to predict BAFs (Arnot and Gobas, 2006; as modified by Du et al. (2015)). The three linear regressions were significant ($P < 0.01$). The coefficients of determination (R^2), and the slopes of the linear regression (b) with associate 95% confidence intervals (in brackets) are shown.

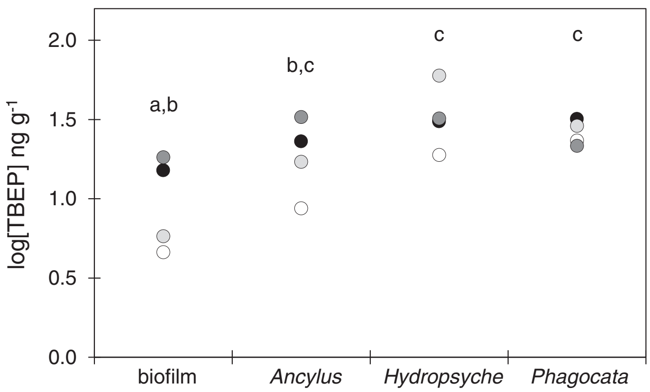

Fig. 5. Concentration (ng g^{-1}) of the flame retardant TBEP bioaccumulated in biofilm, *Ancylus*, *Hydropsyche*, and *Phagocata*. Shading represents the different sampled reaches: white = control reach (C), black = 1st impact reach after effluent (I1); dark gray = 2nd impact reach after effluent (I2); light gray = 3rd impact reach after effluent (I3). Letters indicate significant grouping according to Bonferroni post-hoc tests.

magnification was reported between algae and the crustacean *Thamnocephalus*, but dilution occurred towards the cnidarian *Hydra*, which likely biotransformed this toxic. Another study on a psychiatric drug (the benzodiazepine oxazepam) showed differential behavioral effects between exposed perch and damselflies, and reported that 46% of the pharmaceutical present in ingested prey was accumulated in the predator (Brodin et al., 2014). Since research explicitly targeting bioaccumulation across aquatic trophic levels is still very scarce, the trophic magnification of TBEP we found is a significant finding and should be further investigated.

4.3. Concluding remarks

The bioaccumulation of emerging pollutants by biofilm and aquatic invertebrates under field conditions has attracted little research relative to that devoted to fish. However, sedentary lives may make biofilm and many aquatic macroinvertebrate taxa (including aquatic life stages of insects) particularly realistic indicators of local exposure. In particular, some invertebrate groups have long been known to denote anthropogenic disturbance (Goodnight, 1973; Cairns and Pratt, 1993) and hydroclimatic conditions (Barbour et al., 1992; Batzer and Ruhí, 2013), hence their ability to reflect emerging pollution could even increase their indicator use. Despite the number and variety of emerging pollutants detected in this study (and the presence of compounds like diclofenac, recently included in the watch list of substances for EU-wide monitoring), observed concentrations did not represent a clear risk to exposed organisms. Future research should use stable isotopes to estimate trophic positions and feeding links, and should consider as many food web nodes as possible. However, this study already shows that many compounds can be bioaccumulated, and TBEP can be magnified by macroinvertebrates, in riverine food webs. Our results emphasize the need to consider diet, together with waterborne exposure, as a potential bioaccumulation route in aquatic organisms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.06.009.

References

Acuña, V., von Schiller, D., García-Galán, M.J., Rodríguez-Mozaz, S., Corominas, L., Petrovic, M., Poch, M., Barceló, D., Sabater, S., 2015. Occurrence and in-stream attenuation of wastewater-derived pharmaceuticals in Iberian rivers. Sci. Total Environ. 503, 133–141.

Allan, J.D., 1984. The size composition of invertebrate drift in a Rocky Mountain stream. Oikos 43, 68–76.

Arim, M., Abades, S.R., Laufer, G., Loureiro, M., Marquet, P.A., 2010. Food web structure and body size: trophic position and resource acquisition. Oikos 119, 147–153.

Aristi, I., Schiller, D., Arroita, M., Barceló, D., Ponsatí, L., García-Galán, M.J., Sabater, S., Elosegi, A., Acuña, V., 2015. Mixed effects of effluents from a wastewater treatment plant on river ecosystem metabolism: subsidy or stress? Freshw. Biol. http://dx.doi.org/10.1111/fwb.12576.

Armitage, M.J., Young, J.O., 1991. The feeding biology of the triclad *Phagocata vitta* (Dugès): a laboratory study. Hydrobiologia 215, 63–72.

Arnold, K.E., Brown, A.R., Ankley, G.T., Sumpter, J.P., 2014. Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems. Philos. Trans. R. Soc. B Biol. Sci. 369, 20130569.

Arnot, J.A., Gobas, F.A.P.C., 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environ. Rev. 14, 257–297.

Barbour, M.T., Graves, C.G., Plafkin, J.L., Wisseman, R.W., Bradley, B.P., 1992. Evaluation of EPA's rapid bioassessment benthic metrics: metric redundancy and variability among reference stream sites. Environ. Toxicol. Chem. 11, 437–449.

Batzer, D.P., Ruhí, A., 2013. Is there a core set of organisms that structure macroinvertebrate assemblages in freshwater wetlands? Freshw. Biol. 58, 1647–1659.

Benke, A.C., Jacobi, D.I., 1986. Growth rates of mayflies in a subtropical river and their implications for secondary production. J. North Am. Benthol. Soc. 5, 107–114.

Benke, A.C., Jacobi, D.I., 1994. Production dynamics and resource utilization of snag-dwelling mayflies in a blackwater river. Ecology 75, 1219–1232.

Benke, A.C., Huryn, A.D., Smock, L.A., Wallace, J.B., 1999. Length–mass relationships for freshwater macroinvertebrates in North America with particular reference to the southeastern United States. J. North Am. Benthol. Soc. 18, 308–343.

Berlioz-Barbier, A., Buleté, A., Faburé, J., Garric, J., Cren-Olivé, C., Vulliet, E., 2014. Multi-residue analysis of emerging pollutants in benthic invertebrates by modified micro-quick-easy-cheap-efficient-rugged-safe extraction and nanoliquid chromatography–nanospray–tandem mass spectrometry analysis. J. Chromatogr. A 1367, 16–32.

Boxall, A., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J.P., Verslycke, T., 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? Environ. Health Perspect. 120, 1221–1229.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. Philos. Trans. R. Soc. London B Biol. Sci. 369, 20130580.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24, 464–469.

Brooks, B.W., Riley, T.M., Taylor, R.D., 2006. Water quality of effluent-dominated ecosystems: ecotoxicological, hydrological, and management considerations. Hydrobiologia 556, 365–379.

Burgherr, P., Meyer, E.I., 1997. Regression analysis of linear body dimensions vs. dry mass in stream macroinvertebrates. Arch. Hydrobiol. 139, 101–112.

Cabana, G., Rasmussen, J.B., 1994. Modelling food chain structure and contaminant bioaccumulation using stable nitrogen isotopes. Nature 372, 255–257.

Cairns Jr., J., Pratt, J.R., 1993. A history of biological monitoring using benthic macroinvertebrates. In: Rosenberg, D.M., Resh, V.H. (Eds.), Freshwater Biomonitoring and Benthic Macroinvertebrates. Kluwer Academic Publishers, Boston, Mass, p. 27.

Chen, D., Letcher, R.J., Chu, S., 2012. Determination of non-halogenated, chlorinated and brominated organophosphate flame retardants in herring gull eggs based on liquid chromatography–tandem quadrupole mass spectrometry. J. Chromatogr. A 1220, 169–174.

Chevene, Fran, Doledec, S., Chessel, D., 1994. A fuzzy coding approach for the analysis of long-term ecological data. Freshw. Biol. 31, 295–309.

Damásio, J., Barceló, D., Brix, R., Postigo, C., Gros, M., Petrovic, M., Sabater, S., Guasch, H., de Alda, M.L., Barata, C., 2011. Are pharmaceuticals more harmful than other pollutants to aquatic invertebrate species: a hypothesis tested using multi-biomarker and multi-species responses in field collected and transplanted organisms. Chemosphere 85, 1548–1554.

Daughton, C.G., 2002. Environmental stewardship and drugs as pollutants. Lancet 360, 1035–1036.

Dixon, R.W.J., Wrona, F.J., 1992. Life history and production of the predatory caddisfly *Rhyacophila* vao Milne in a spring-fed stream. Freshw. Biol. 27, 1–11.

Du, B., Perez-Hurtado, P., Brooks, B.W., Chambliss, C.K., 2012. Evaluation of an isotope dilution liquid chromatography tandem mass spectrometry method for pharmaceuticals in fish. J. Chromatogr. A 1253, 177–183.

Du, B., Haddad, S.P., Luek, A., Scott, W.C., Saari, G.N., Kristofco, L.A., Connors, K.A., Rash, C., Rasmussen, J.B., Chambliss, C.K., 2014. Bioaccumulation and trophic dilution of human pharmaceuticals across trophic positions of an effluent-dependent wadeable stream. Philos. Trans. R. Soc. B Biol. Sci. 369, 20140058.

Du, B., Haddad, S.P., Scott, W.C., Chambliss, C.K., Brooks, B.W., 2015. Pharmaceutical bioaccumulation by periphyton and snails in an effluent-dependent stream during an extreme drought. Chemosphere 119, 927–934.

Falconer, I.R., Chapman, H.F., Moore, M.R., Ranmuthugala, G., 2006. Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. Environ. Toxicol. 21, 181–191.

Fisk, A.T., De Wit, C.A., Wayland, M., Kuzyk, Z.Z., Burgess, N., Letcher, R., Braune, B., Norstrom, R., Blum, S.P., Sandau, C., 2005. An assessment of the toxicological significance of anthropogenic contaminants in Canadian arctic wildlife. Sci. Total Environ. 351, 57–93.

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Barber, L.B., Thurman, M.E., 2008. A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States—II) untreated drinking water sources. Sci. Total Environ. 402, 201–216.

Ginebreda, A., Muñoz, I., de Alda, M.L., Brix, R., López-Doval, J., Barceló, D., 2010. Environmental risk assessment of pharmaceuticals in rivers: relationships between hazard indexes and aquatic macroinvertebrate diversity indexes in the Llobregat River (NE Spain). Environ. Int. 36, 153–162.

Goodnight, C.J., 1973. The use of aquatic macroinvertebrates as indicators of stream pollution. Trans. Am. Microsc. Soc. 1, 1–13.

Gros, M., Petrović, M., Barcelo, D., 2007. Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro river basin (northeast Spain). Environ. Toxicol. Chem. 26, 1553–1562.

Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2012. Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem. J. Chromatogr. A 1248, 104–121.

Hach, C., 2002. Water Analysis Handbook. Loveland, Colorado, USA, pp. 61–62.

Han, Z., Wang, Q., Fu, J., Chen, H., Zhao, Y., Zhou, B., Gong, Z., Wei, S., Li, J., Liu, H., 2014. Multiple bio-analytical methods to reveal possible molecular mechanisms of developmental toxicity in zebrafish embryos/larvae exposed to tris (2-butoxyethyl) phosphate. Aquat. Toxicol. 150, 175–181.

Headley, J.V., Gandrass, J., Kuballa, J., Peru, K.M., Gong, Y., 1998. Rates of sorption and partitioning of contaminants in river biofilm. Environ. Sci. Technol. 32, 3968–3973.

Hernando, M.D., Mezcua, M., Fernández-Alba, A.R., Barceló, D., 2006. Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 69, 334–342.

Hoppe, P.D., Rosi-Marshall, E.J., Bechtold, H.A., 2012. The antihistamine cimetidine alters invertebrate growth and population dynamics in artificial streams. Freshwater Res. 31, 379–388.

Huerta, B., Jakimska, A., Llorca, M., Ruhí, A., Margoutidis, G., Acuña, V., Sabater, S., Rodriguez-Mozaz, S., Barceló, D., 2015. Development of an extraction and purification method for the determination of multi-class pharmaceuticals and endocrine disruptors in freshwater invertebrates. Talanta 132, 373–381.

Huerta, B., Rodriguez-Mozaz, S., Nannou, S., Nakis, C., Ruhí, A., Acuña, V., Sabater, S., Barceló, D., 2015w. Determination of a broad spectrum of pharmaceuticals and endocrine disruptors in biofilm from a WWTP-impacted river. Sci. Total Environ http://dx.doi.org/10.1016/j.scitotenv.2015.05.049 (in press).

Jakimska, A., Huerta, B., Bargańska, Ż., Kot-Wasik, A., Rodríguez-Mozaz, S., Barceló, D., 2013. Development of a liquid chromatography–tandem mass spectrometry procedure for determination of endocrine disrupting compounds in fish from Mediterranean rivers. J. Chromatogr. A 1306, 44–58.

Jeffrey, S.W.T., Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanz. BPP 167, 191–194.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Baunbeck, T., Turner, A.P., Tyler, C.R., 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat. Toxicol. 65, 205–220.

Kidd, K.A., Schindler, D.W., Muir, D.C.G., Lockhart, W.L., Hesslein, R.H., 1995. High concentrations of toxaphene in fishes from a subarctic lake. Science 269, 240–242.

Kidd, K.A., Paterson, M.J., Rennie, M.D., Podemski, C.L., Findlay, D.L., Blanchfield, P.J., Liber, K., 2014. Direct and indirect responses of a freshwater food web to a potent synthetic oestrogen. Philos. Trans. R. Soc. B Biol. Sci. 369, 20130578.

Kim, J.-W., Ramaswamy, B.R., Chang, K.-H., Isobe, T., Tanabe, S., 2011. Multiresidue analytical method for the determination of antimicrobials, preservatives, benzotriazole UV stabilizers, flame retardants and plasticizers in fish using ultra high performance liquid chromatography coupled with tandem mass spectrometry. J. Chromatogr. A 1218, 3511–3520.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Lawrence, J.R., Kopf, G., Headley, J.V., Neu, T.R., 2001. Sorption and metabolism of selected herbicides in river biofilm communities. Can. J. Microbiol. 47, 634–641.

Lee, C.-C., Jiang, L.-Y., Kuo, Y.-L., Chen, C.-Y., Hsieh, C.-Y., Hung, C.-F., Tien, C.-J., 2015. Characteristics of nonylphenol and bisphenol A accumulation by fish and implications for ecological and human health. Sci. Total Environ. 502, 417–425.

López-Doval, J.C., Ginebreda, A., Caquet, T., Dahm, C.N., Petrovic, M., Barceló, D., Muñoz, I., 2013. Pollution in mediterranean-climate rivers. Hydrobiologia 719, 427–450.

Loraine, G.A., Pettigrove, M.E., 2006. Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. Environ. Sci. Technol. 40, 687–695.

Mackay, D., Fraser, A., 2000. Bioaccumulation of persistent organic chemicals: mechanisms and models. Environ. Pollut. 110, 375–391.

Makhutova, O.N., Sushchik, N.N., Kalachova, G.S., Ageev, A.V., 2009. Fatty acid content and composition of freshwater planaria Dendrocoelopsis sp. (Planariidae, Turbellaria, Platyhelminthes) from the Yenisei river. J. Sib. Fed. Univ. 2, 135–144.

Meredith-Williams, M., Carter, L.J., Fussell, R., Raffaelli, D., Ashauer, R., Boxall, A.B.A., 2012. Uptake and depuration of pharmaceuticals in aquatic invertebrates. Environ. Pollut. 165, 250–258.

Meyer, E., 1989. The relationship between body length parameters and dry mass in running water invertebrates. Arch. Hydrobiol. 117, 191–203.

Miserendino, M.L., 2001. Length–mass relationships for macroinvertebrates in freshwater environments of Patagonia (Argentina). Ecol. Aust. 11, 3–8.

Muñoz, I., López-Doval, J.C., Ricart, M., Villagrasa, M., Brix, R., Geiszinger, A., Ginebreda, A., Guasch, H., de Alda, M., Romaní, A.M., 2009. Bridging levels of pharmaceuticals in river water with biological community structure in the Llobregat river basin (northeast Spain). Environ. Toxicol. Chem. 28, 2706–2714.

Murphy, J., Riley, J., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta 27, 31–36.

Nentwig, G., 2007. Effects of pharmaceuticals on aquatic invertebrates. Part II: the antidepressant drug fluoxetine. Arch. Environ. Contam. Toxicol. 52, 163–170.

Oetken, M., Nentwig, G., Löffler, D., Ternes, T., Oehlmann, J., 2005. Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. Arch. Environ. Contam. Toxicol. 49, 353–361.

Proia, L., Osorio, V., Soley, S., Köck-Schulmeyer, M., Pérez, S., Barceló, D., Romaní, A.M., Sabater, S., 2013. Effects of pesticides and pharmaceuticals on biofilms in a highly impacted river. Environ. Pollut. 178, 220–228.

Ramirez, A.J., Brain, R.A., Usenko, S., Mottaleb, M.A., O'Donnell, J.G., Stahl, L.L., Wathen, J.B., Snyder, B.D., Pitt, J.L., Perez-Hurtado, P., 2009. Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. Environ. Toxicol. Chem. 28, 2587–2597.

Regnery, J., Püttmann, W., 2009. Organophosphorus flame retardants and plasticizers in rain and snow from Middle Germany. Clean Soil Air Water 37, 334–342.

Rosati, I., Barbone, E., Basset, A., 2012. Length–mass relationships for transitional water benthic macroinvertebrates in Mediterranean and Black Sea ecosystems. Estuar. Coast. Shelf Sci. 113, 231–239.

Schorer, M., Eisele, M., 1997. Accumulation of inorganic and organic pollutants by biofilms in the aquatic environment. Water. Air. Soil Pollut. 99, 651–659.

Stockner, J.G., 1971. Ecological energetics and natural history of *Hedriodiscus truquii* (Diptera) in two thermal spring communities. J. Fish. Board Can. 28, 73–94.

Sui, Q., Huang, J., Deng, S., Chen, W., Yu, G., 2011. Seasonal variation in the occurrence and removal of pharmaceuticals and personal care products in different biological wastewater treatment processes. Environ. Sci. Technol. 45, 3341–3348.

Tachet, H., Richoux, P., Bournaud, M., Usseglio-Polatera, P., 2002. Invertebrés d'eau douce. 2nd correc. ed. CNRS editions, Paris.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Van Geest, J.L., Poirier, D.G., Sibley, P.K., Solomon, K.R., 2010. Measuring bioaccumulation of contaminants from field-collected sediment in freshwater organisms: a critical review of laboratory methods. Environ. Toxicol. Chem. 29, 2391–2401.

Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagné, F., Juneau, P., 2010. Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. Chemosphere 80, 1062–1068.

Vieno, N.M., Tuhkanen, T., Kronberg, L., 2005. Seasonal variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant and in the recipient water. Environ. Sci. Technol. 39, 8220–8226.

Webb, S.F., 2001. A data based perspective on the environmental risk assessment of human pharmaceuticals II—aquatic risk characterisation. Pharmaceuticals in the Environment. Springer, pp. 203–219.

Woodward, G., Hildrew, A.G., 2002. Body-size determinants of niche overlap and intraguild predation within a complex food web. J. Anim. Ecol. 71, 1063–1074.

Woodward, G., Ebenman, B., Emmerson, M., Montoya, J.M., Olesen, J.M., Valido, A., Warren, P.H., 2005. Body size in ecological networks. Trends Ecol. Evol. 20, 402–409.

Chapter 5

Environmental effects of antibiotics and psychiatric drugs: antibiotic resistance and behavioural alterations

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Exploring the links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs

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HIGHLIGHTS

- Antibiotics and prevalence of antibiotic resistance genes in reservoirs
- No clear associations between antibiotic occurrence and resistance genes
- Composition of microbial communities explained by the presence of resistance genes
- Actinobacteria and Firmicutes associated to the presence of resistance genes

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ABSTRACT

Antibiotic resistance represents a growing global health concern due to the overuse and misuse of antibiotics. There is, however, little information about how the selective pressure of clinical antibiotic usage can affect environmental communities in aquatic ecosystems and which bacterial groups might be responsible for dissemination of antibiotic resistance genes (ARGs) into the environment. In this study, chemical and biological characterization of water and sediments from three water supply reservoirs subjected to a wide pollution gradient allowed to draw an accurate picture of the concentration of antibiotics and prevalence of ARGs, in order to evaluate the potential role of ARGs in shaping bacterial communities, and to identify the bacterial groups most probably carrying and disseminating ARGs. Results showed significant correlation between the presence of ARG conferring resistance to macrolides and the composition of bacterial communities, suggesting that antibiotic pollution and the spreading of ARG might play a role in the conformation of bacterial communities in reservoirs. Results also pointed out the bacterial groups *Actinobacteria* and *Firmicutes* as the ones probably carrying and disseminating ARGs. The potential effect of antibiotic pollution and the presence of ARGs on the composition of bacterial communities in lacustrine ecosystems prompt the fundamental question about potential effects on bacterial-related ecosystem services supplied by lakes and reservoirs.

1. Introduction

One of the most significant groups of pollutants within the category of emerging contaminants is antibiotics (Schriks et al., 2010). Antibiotic compounds may be found in different environmental compartments due to their extensive use in human and veterinary medicine and the continuous drainage of surface runoff and release from wastewater treatment plants (WWTP). The presence of these compounds in freshwater systems has attracted increasing attention because of their potential threat to the environment (Gros et al., 2009; Kim and Carlson, 2007). Antibiotics may pose a risk to the ecosystems even at very low concentrations and persistence rates, as they are designed to have a biochemical effect in the microorganisms, and thus have a significant impact in the processes controlled by native biological communities (Maul et al., 2006; Muñoz et al., 2009). To date most research on the impact of antibiotic discharges into the environment are based on the effects on benthic invertebrates and algae (Cleuvers, 2003; Fent et al., 2006). However, little is known about the potential effects on the diversity and functioning of bacterial communities in lacustrine ecosystems, despite its fundamental role as regulators of the processes that define the majority of ecosystem services provided by these freshwater bodies, such as the capacity for self-purification (Bell et al., 2005).

One of the greatest concerns about the presence of antibiotics in the environment is the emergence and dissemination of antibiotic resistance genes (ARGs) due to continuous exposure of bacteria,

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even at very low antibiotic concentrations (Baquero et al., 2008; Kümmerer, 2009a, 2009b; Martinez, 2009; Zuccato et al., 2010). Many of these genes are found on transposons, integrons or plasmids, which can be mobilized and transferred to other bacteria, belonging to the same or different species (Allen et al., 2010). Although, antibiotic resistance development has been extensively investigated in cultivable bacteria, especially those of clinical importance (D'Costa et al., 2007), the vast majority of environmental bacteria cannot be cultured using standard laboratory methods (Amann et al., 1995; Kirk et al., 2004; Oliver, 2010). As a result, there is little information about how the selective pressure of clinical antibiotic usage can affect environmental communities (D'Costa et al., 2011; Martinez, 2009) and which bacterial groups might be responsible for dissemination of ARGs in the environment (Allen et al., 2010; Li et al., 2011; Zhang et al., 2009). This is particularly relevant in the case of reservoirs used for abstraction of drinking water and recreational activities, two principal routes for resistant environmental bacteria and ARGs to reach human populations (Taylor et al., 2011). Water supply in Mediterranean countries is frequently based on resources stored in reservoirs located downstream from populated areas, defining these systems as ideal study sites to investigate the effect of antibiotic pollution on natural microbial communities.

The objective of this study was to assess whether the occurrence of antibiotic compounds and ARGs can have a role in the composition of microbial communities from the water column and sediments of three water supply reservoirs (La Llosa del Cavall, Sau, and Foix) located near Barcelona, Spain. Concentrations of nine antibiotic families in water and sediment samples were measured by ultra-high-performance liquid chromatography coupled to mass spectrometry. Real-time PCR was applied for the quantification of four selected ARGs, whereas pyrosequencing was used to analyze the bacterial community composition. This combined approach allowed to draw an accurate picture of the concentration of antibiotics and prevalence of ARGs in the studied reservoirs, to evaluate the associations between ARGs and bacterial communities, and to identify the bacterial groups most probably carrying and disseminating ARGs.

2. Materials and methods

2.1. Sampling and in situ measurements

Three man-made reservoirs located in Catalonia (Spain) were chosen considering their role in water supply, irrigation, and recreational uses, as well as the intensity of human activity in the upstream watershed. La Llosa Reservoir is located in the headwaters of the Cardener River, and stores 79 hm^3 of water (maximum depth 72 m, mean water residence time (WRT) 333 days) devoted mainly to water supply and irrigation. Sau Reservoir (volume 164 hm^3, maximum depth 50 m, WRT 113 days) is located in a middle stretch of the Ter River, and is a fundamental player in the water supply scheme of the Barcelona Metropolitan Area. Finally, Foix Reservoir is a small reservoir (volume 3.7 hm^3, maximum depth 11 m, WRT 106 days) located in the Foix River devoted to irrigation of local fields and hosts a number of recreational activities. These artificial reservoirs present an increasing pollution gradient from La Llosa Reservoir, which is near pristine conditions, to Foix Reservoir, which is a highly polluted water body, while Sau Reservoir is in an intermediate situation. The main stressor defining the pollution gradient observed in the reservoirs is the impact of effluents from upstream WWTP: in Foix Reservoir the mean percent of water entering the reservoir that comes from upstream WWTP effluents was 60% for the last decade, in Sau Reservoir was 10%, and in La Llosa Reservoir was 0.3%.

Sampling campaigns were performed in May 2010 and May 2011. Samples were taken at three different points in each reservoir: near the main inflow (tail), in middle reach (mid), and near the dam. The rationale behind sampling in these three different points along the reservoirs is that the input of materials from the river promotes the establishment of heterogeneous bacterial communities along the reservoir (Šimek et al., 2011). During the sampling campaigns, reservoirs were stratified, with surface water temperature around 15 °C and the bottom water around 8 °C.

In the three sampling points visited in each reservoir a surface water sample (i.e. 0.5 m depth) was collected with a 5 L hydrographic bottle (except for LC–MS/MS analysis, where an amber glass bottle was used), and a sample from the top 4 cm of the sediments by means of a Ekman–Birge bottom sampler (Hydro-Bios). In the sampling point near the dam an additional water sample one meter above the sediments was collected (i.e., four water samples and three sediment samples for each reservoir in total). ARGs analyses and bacterial community characterization were performed only for samples collected in 2011. At every sampling station profiles of water temperature, conductivity, pH, and dissolved oxygen were measured with a SeaBird 19*plus* SEACAT CTD Profiler. The amount of light down the profiles was assessed by means of a Li-COR spherical Quantum Sensor (LI-193SA), and the light extinction coefficient calculated assuming the Beer–Lambert law. Hydrological data for the reservoirs were supplied by the Catalan Water Agency (ACA).

2.2. General parameters

Water samples were filtered through GF/F filters and analyzed for nitrate and chloride by liquid chromatography in a Konik KNK 500-A, and for soluble phosphate (Murphy and Riley, 1962) and ammonia (Solórzano, 1969) by a colorimetric method. Dissolved organic carbon concentration was quantified by combustion in a Shimadzu TOC-5000 Analyzer after filtration through a GF/F filter and acidification to remove inorganic carbon. Suspended solids were calculated by gravimetry. Total dissolved iron and manganese were measured through the phenantroline method and the persulfate method, respectively (APHA, 1992). Density of live bacteria was assessed after staining water samples with Live/Dead(R) BacLight™ bacterial viability kit. For the top 4 cm of the sediment samples, concentrations of As, Ba, Cd, Ce, Co, Cr, Cu, Ga, Mn, Mo, Nb, Ni, Pb, Rb, Sn, Sr, Th, Ti, V, W, Y, Zn, Zr, and Fe were determined through X-ray fluorescence (model: Axios advanced PANalytical) after adhesion using Elvactite 2044 and posterior pressing.

2.3. Antibiotic analysis

A Baker vacuum system (J.T. Baker, The Netherlands) was used to pre-concentrate the water samples using an adapted method (Gros et al., 2012). Briefly, water sample was pH-adjusted to 3 with 1.0 M HCl and 10 ml of 1 M EDTA (4%, v/v) was added. Oasis HLB cartridges (60 mg, 3 ml) cartridges (Waters, Massachusetts, USA) were loaded with 250 mL of water samples. Cartridges were eluted with 10 ml of methanol. The extracts were evaporated under a gentle nitrogen stream and reconstituted with 1 mL of methanol–water mixture (50:50, v/v). A detailed description of the sediment extraction method can be found elsewhere (Yang et al., 2010). Briefly, 1 g of freeze-dried sediment was sonicated for 15 minutes in 10 mL of 0.2 M citric buffer (pH 4.0) and acetonitrile solution (50:50, v/v). Each extract was centrifuged and the supernatant diluted to 500 mL and purified with SPE following the same procedure as water samples. Extracts were analyzed using the method developed by Gros et al. (Gros et al., 2013) using a UPLC Waters chromatographic system (Milford, MA, USA) coupled to a Qtrap 5500 (Applied Biosystems) mass spectrometer. Chromatographic separation was achieved with an ACQUITY UPLC HSS T3 column (2.1 × 50 mm, particle size 1.8 μm) at 30 °C, supplied by Waters (Milford, MA, USA). Recoveries for the analyzed antibiotic compounds were 50–133% for water samples, and 33–101% for sediment samples.

2.4. Quantification of ARGs by real-time PCR

Water samples were filtered through 0.22 μm nylon filters (Millipore) in order to concentrate bacterial cells. The membranes were then resuspended in lysis buffer (1.2% Triton X-100, 20 mM Tris–Cl, 2 mM sodium EDTA, and 20 mg/ml), and DNA was extracted using a standard phenol-chloroform method. Sediment samples were also collected, weighed, and homogenized in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2). The homogenates were then resuspended in lysis buffer and DNA was extracted as mentioned above. All samples were collected and analyzed in duplicate.

The ARGs considered in this study were *bla*TEM, *ermB*, *qnrS*, and *sulI*, which confer resistance to β-lactam antibiotics, macrolides, quinolones (including fluoroquinolones) and sulfonamides, respectively. ARGs were quantified in all DNA extracted samples using real-time PCR. Copy number of the 16S rRNA gene was also quantified for normalization of the data. All real-time PCR assays were amplified in duplicate using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) on a MX3005P system (Agilent Technologies), with the exception for the *bla*TEM gene, which was amplified using the SYBR® Green Master Mix (Applied Biosystems). The primers sets and the thermal cycling parameters were specific for each gene (Table A.1). After the PCR, a dissociation curve (melting curve) was constructed in the range of 60 °C to 95 °C. *Escherichia coli* strain 488 (*bla*TEM), *Enterococcus* sp. strain CO4097 (*ermB*), *E. coli* strain J53 pMG306 (*qnrS*) and *Aeromonas* sp. strain P2G1 (*sulI*) were used as positive controls to make the standards by transforming gene-bearing plasmids into *E. coli* using the TOPO cloning kit (Invitrogen). Plasmids were then extracted using the PureLink plasmid kit (Invitrogen), and the concentration was determined using a NanoDrop spectrophotometer (NanoDrop). Ten-fold serial dilutions of purified plasmid DNA were used for the generation of standard curves for each ARG. Gene copies were calculated as described previously (Marti and Balcazar, 2012).

Data (copy number of each ARG) were compared using the one-way analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate, because most data were not normally distributed. Data were analyzed using SPSS for Windows version 17.0 (SPSS, Chicago, IL).

2.5. Analysis of bacterial community composition by pyrosequencing

Purified DNA samples from each sampling point were amplified separately with primers 27F (3′-GAG TTT GAT CNT GGC TCAG-5′) and 519R (3′-GTN TTA CNG CGG CKG CTG-5′), and the amplicons were sequenced using Roche 454 GS FLX Titanium chemistry. Raw sequencing reads were quality trimmed using the MOTHUR software package (Schloss et al., 2009). Sequences were restricted to the first 250 bp and aligned using the SILVA reference database (Pruesse et al., 2007). Sequences were then randomly removed from each library to retain a total of 10855 (water samples) and 6058 (sediment samples) sequences for each treatment in order to minimize any bias due to the difference in the total number of sequences. The Ribosomal Database Project (RDP) pipeline and Classifier function (Wang et al., 2007) were used to align and assign identities at a confidence threshold of 80%. MOTHUR was used to assign sequences to operational taxonomic units (OTUs, 97% similarity) and calculate both Shannon's diversity index values (H') and Chao1 richness estimates. The parsimony test, as implemented by MOTHUR, was used to assess whether two or more communities have the same structure. A Bonferroni correction was applied to adjust the significance level for multiple pairwise comparisons. The sequence data from this study have been deposited in the NCBI Short Read Archive under accession number SRA049379.

2.6. Data analyses

To investigate the generality of longitudinal patterns in reservoirs, mean normalized concentrations of antibiotics were calculated to be able to combine data from the different reservoirs in common analyses and figures. Antibiotic concentrations along a given reservoir were normalized by the corresponding average of the whole system, and then data from all reservoirs were used to calculate cross-system means and standard deviations for the tail, mid, and dam regions (water and sediments treated separately). The same procedure was used to calculate mean normalized ARG abundances.

To search for potential links between antibiotic pollution, ARGs, and bacterial communities, a series of Redundancy Analysis (RDA)-based analyses was performed. First, the relative importance of environmental variables (including antibiotic concentrations), space (geographic coordinates) (Van der Gucht et al., 2007), and the presence of ARGs in explaining the bacterial communities in the water column and sediments, separately, was determined. The total variation of the bacterial community matrix was decomposed into unique environmental, spatial, and ARG components with corresponding p values using RDA and partial RDA (Borcard et al., 1992; Cottenie et al., 2003; Legendre and Legendre, 1998). This analysis measures the amount of variation that can be attributed exclusively to one set of explanatory (environment, space, or ARGs) variables and the variation explained by correlations between the different sets (shared or common term). The significance of these components was evaluated with a Monte Carlo permutation test (Legendre and Legendre, 1998), and the variation components were bias-corrected (Peres-Neto et al., 2006). Although the set of samples for the analyses was limited ($n = 12$ for water samples, $n = 9$ for sediments), RDA and partial RDA included in the variation analyses never used a number of explanatory variables in excess of $n/2$ (Borcard et al., 2011) in the case of water samples, and only one RDA failed to fulfill this condition for the sediment set. This was accomplished reducing the number of explanatory variables in each set to the most parsimonious subset of significant variables with a forward selection procedure. This procedure also prevented over-fitting and co-linearity. The bacterial community data consisted in percentages to avoid the deleterious effect of the different density of bacteria on the analyses, while all explanatory variables were log transformed and standardized. No significant eigenvector or polynomial spatial variable was found, hence only the geographic coordinates were considered in the analyses.

The association between antibiotics and ARGs in the water column and sediments was assessed performing two separate RDA using as explanatory variables the corresponding set of environmental variables measured in this study. Due to the large number of potential explanatory variables, the most parsimonious subsets of significant variables were selected with a forward selection procedure. The association between ARGs and bacterial groups in water and sediments was also tested through RDA, using the ARGs data as the explanatory matrix. Forward selection was used as above to avoid over-fitting and co-linearity. All RDA and partial RDA included in this study were performed with R (R Development Core Team, 2007) and the Vegan library (Oksanen et al., 2007).

3. Results

3.1. Pollution gradient

The general physico-chemical characterization (Table A.2) allowed us to confirm a clear eutrophication and pollution gradient from La Llosa to Foix. Water samples for Foix Reservoir showed extremely high values for ammonia, phosphate, and metals. Nonetheless, maximum values for the light extinction coefficient were very high at Foix Reservoir, according to the largest levels of suspended solids. Chloride concentration, a variable closely related to pollution in reservoirs (Marcé et al., 2008), was also largest in Foix Reservoir. On the contrary, in the case of sediment samples, results did not support the existence of an obvious pollution gradient in this compartment (Table A.2). Among elements that can be potentially attributed to human pollution, only Cu, Pb, and Zn showed mild gradients with largest values at Foix Reservoir.

3.2. Antibiotic occurrence

Antibiotic levels at the water column reached their maximum in Foix Reservoir, where the most abundant antibiotic families found were macrolides and sulfonamides, with erythromycin and sulfamethoxazole as the main family representatives, respectively (Fig. 1A–F, Table A.3). In contrast, very low concentrations were found in water from Sau and La Llosa Reservoirs, where maximum concentrations were in the low ng L^{-1} range. The most prevalent antibiotic compound in these two reservoirs was sulfamethozaxole, present in all the water samples from Sau Reservoir and four samples out of twelve from La Llosa. Antibiotic concentrations in sediment samples were slightly higher in Foix Reservoir (Fig. 1G–L, Table A.4), but this tendency was less evident than in water samples. Whereas macrolides followed the same occurrence pattern found in water, sulfonamides and tetracyclines showed a different behavior, with relatively constant concentrations across reservoirs.

Antibiotic concentration along the water body of all reservoirs sampled in this study (Fig. 2A–F) presented two distinct situations: a) the compounds tetracycline, sulfamethoxazole, and erythromycin showed stable concentrations along the water body, a similar result to that found for chloride, a conservative ion often used as a tracer, indicating that these substances did not experienced major transformations during transport along reservoirs; whereas b) ciprofloxacin and ofloxacin seemed to be influenced by attenuation processes, as their concentrations decreased significantly from the tail to the dam. Considering the dynamics of chloride, this reduction in the concentrations cannot be attributed to dilution inside the reservoir but to other biotic or abiotic processes such as sorption in particulate matter and photo and biodegradation. Greater variability within each reservoir was present in sediment samples (Fig. 2G–K), where the fluoroquinolones ciprofloxacin and ofloxacin were absent.

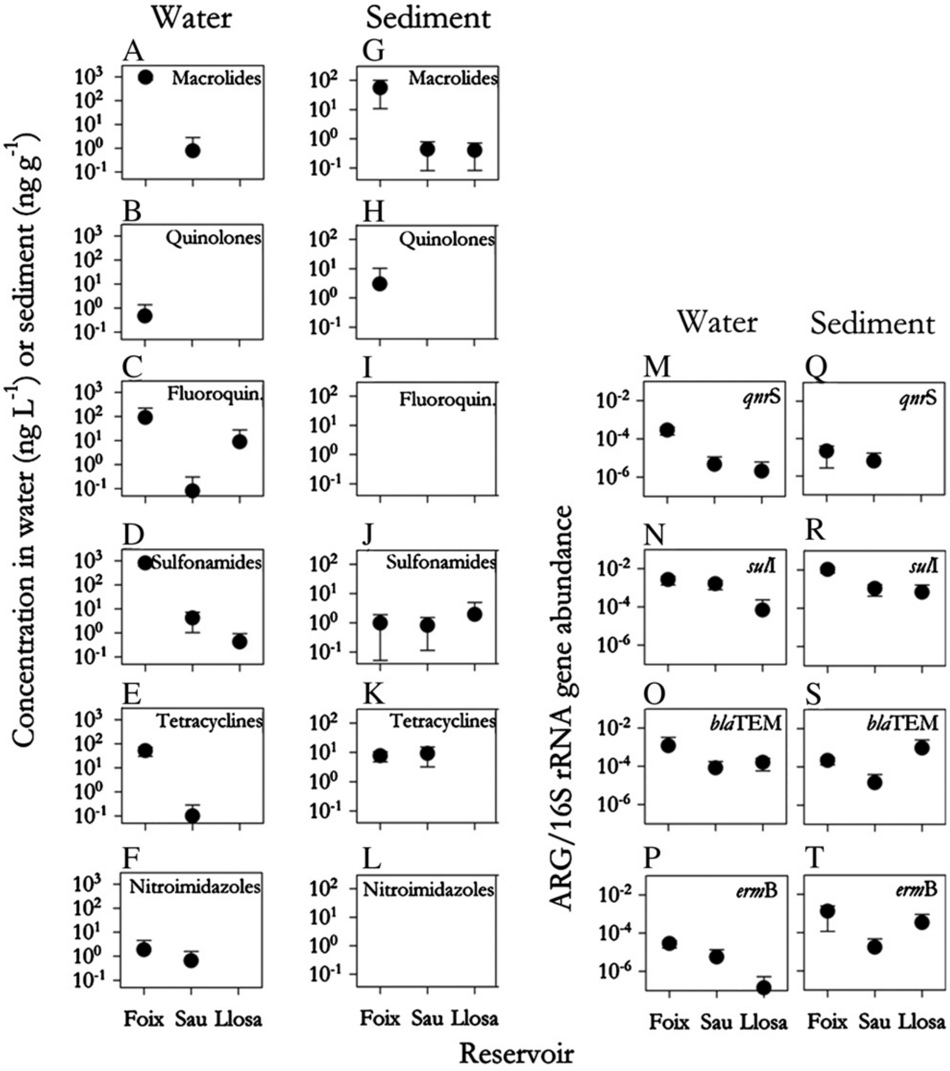

Fig. 1. Average antibiotic concentration and ARG abundance at the water column and sediments found in three water supply reservoirs. For each antibiotic family and reservoir, calculations include all concentrations measured above the limit of quantification from all sampling points. For each ARG and reservoir, calculations included all sampling points. Missing points indicate that the antibiotic family or ARG was not detected. Bars denote standard deviations, and some were not plotted because they extended beyond zero in the logarithmic axis.

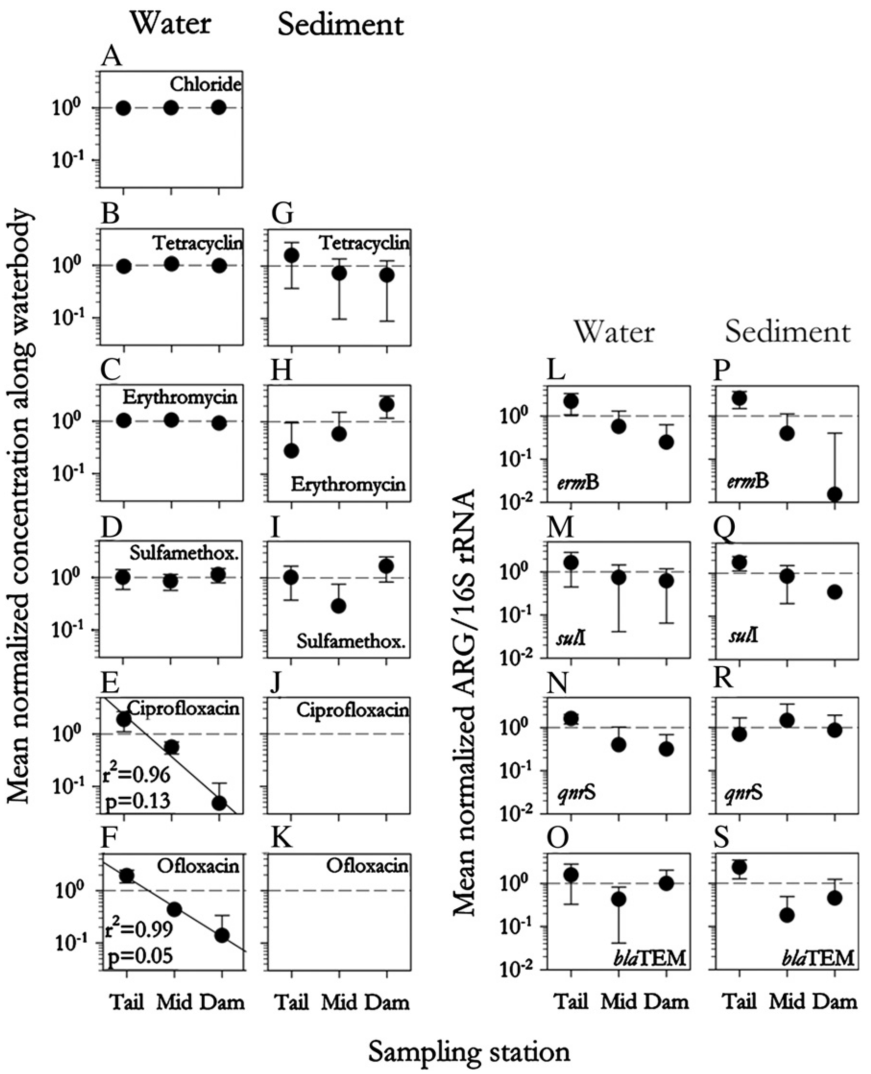

Fig. 2. Occurrence of chloride, selected antibiotics and ARGs in the water column and sediments along the longitudinal axis of water supply reservoirs, expressed as mean normalized concentration (Methods). For reference, the normalized concentration that equals the mean (i.e., one) was indicated as a dashed horizontal line, and we included results of an exponential fit and corresponding curve for fluoroquinolones. Missing points indicate that the antibiotic or ARG was not detected. Bars denote standard deviations, and some were not plotted because they extended beyond zero in the logarithmic axis.

3.3. Prevalence of antibiotic resistance genes (ARG)

Quantitative analysis of ARGs in water samples showed that *qnrS* and *ermB* genes had a higher copy number in Foix Reservoir than in Sau and La Llosa del Cavall reservoirs (Fig. 1M–P). In addition, the abundance of the *sul*I gene was significantly higher ($p < 0.05$) in water samples from Foix and Sau reservoirs compared with the abundance in La Llosa del Cavall Reservoir. However, no significant differences ($p > 0.05$) in abundance of the *bla*TEM gene was found among reservoirs.

For sediment samples, only the abundance of the *sul*I gene was significantly greater ($p < 0.05$) in Foix Reservoir compared to the other two reservoirs (Fig. 1Q-R). The *qnrS* gene was only detected in sediment samples collected from Foix and Sau reservoirs. However, there was no significant difference ($p > 0.05$) in copy number between the two reservoirs. The *bla*TEM and *ermB* genes showed similar copy number in sediment samples from the three reservoirs.

Although the normalized concentration of ARGs in water samples collected at the tail of the reservoirs tended to be larger than in mid and dam regions (Fig. 2L–S), the differences did not reach the level of statistical significance, with the exception of the *qnr*S gene whose concentration was significantly higher ($p < 0.05$) at the tail. A higher concentration of ARGs was indeed observed in sediment samples collected at the tail stations, with the exception of the *qnr*S gene whose concentration did not show any significant difference among sampling points.

3.4. Bacterial community composition

Phylum level affiliations of sequences encountered in each sampling point revealed a diverse bacterial community. The relative abundance of bacterial communities at the phylum level (classes for *Proteobacteria*) showed that the dominant bacterial groups in water samples were *Actinobacteria*, *Betaproteobacteria*, and *Bacteroidetes* (Fig. 3), except for the tail of Foix Reservoir where *Actinobacteria* and *Gammaproteobacteria* were dominant. The bacterial community composition in sediment samples was more variable among the different sampling points (Fig. 3), as measured by the Shannon diversity index and Chao1 richness estimator (Tables A.5-A.8). The dominant bacterial groups in sediment samples were *Actinobacteria, Betaproteobacteria*, and *Gammaproteobacteria* in La Llosa del Cavall Reservoir; *Cyanobacteria, Firmicutes,* and *Planctomycetes* in Sau Reservoir; and *Actinobacteria*, *Firmicutes*, and *Planctomycetes* in Foix Reservoir. A high proportion of unclassified bacteria (mean value for all the sediment samples = 17.41 ± 6.63, $n = 9$) was also observed.

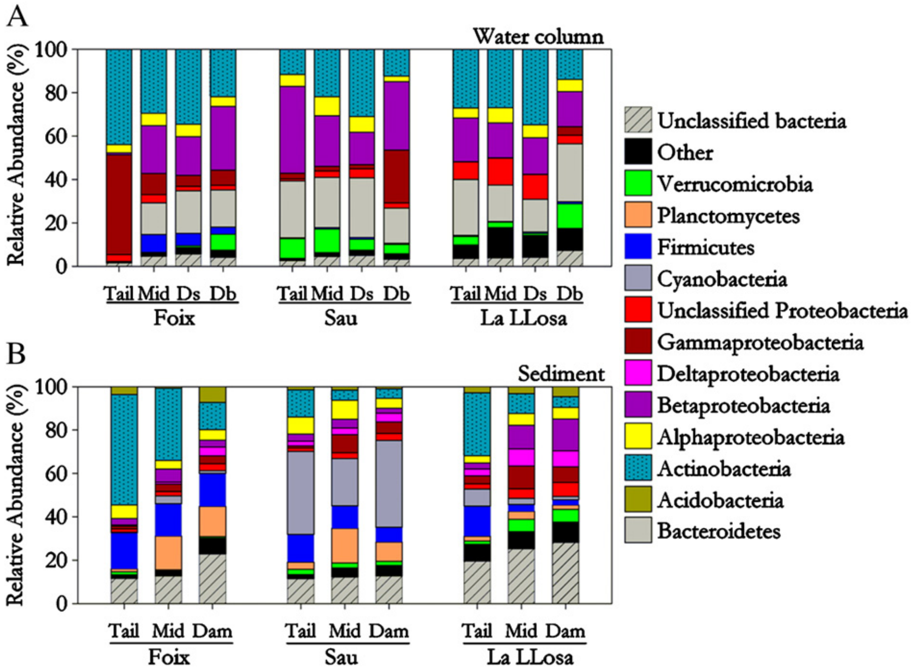

Fig. 3. Bacterial community composition in A) water samples; B) sediment samples from the three reservoirs.##.

Despite the high degree of similarity shared among the bacterial communities from each sampling point at the phylum level, differences with respect to community structure and composition became apparent at the genus level (97% similarity). Pairwise comparison of each bacterial community harbored a phylogenetic structure that was significantly different ($p < 0.01$) from each other, as determined by the phylogeny-based parsimony test (Tables A.5 and A.6). Only water samples collected at the tail, mid, and dam sites from La Llosa del Cavall Reservoir did not show any significant difference in the bacterial community structure.

3.5. Links between antibiotics, ARGs, and bacterial communities

A variation partitioning analysis was performed to identify the environmental and spatial factors that best explain the disparity in bacterial communities found in water and sediment samples (see Section 2.6). The analysis included the geographic location of sampling points, the environmental variables measured in this study (including antibiotic concentration), and the ARG abundance data as a third additional descriptor group. As for water samples, the analysis explained the 80% of the variation in the bacterial community data, and yielded 19% of total variance explained by ARG alone, 17% by environmental variables alone, 11% by geographical location alone, and 33% by a common effect (Fig. 4A and Table A.9). In particular, the variables that significantly contributed to explain the overall bacterial community patterns where: geographic location (x coordinate), water temperature, pH, light extinction coefficient, and normalized *emr*B and *bla*TEM copy numbers. In the case of sediment samples, the analysis explained the 74% of the variation of the bacterial communities, but we could not identify any significant isolated component (Fig. 4B and Table A.9). This was most probably because the explanatory matrices were highly correlated, as showed by the large shared component (69%). Geographical location was not significant in this case, being the variables that significantly contributed to explain the overall bacterial community patterns: W, Rb, and Mn concentration, and normalized *emr*B and *sul*I copy numbers.

Potential links between the environmental conditions and the abundance of ARGs in water and sediment samples were explored performing a RDA, using the environmental variables (including antibiotic concentration) as an explanatory matrix. In the case of water samples, the analysis explained 85% of the variability of the ARGs data (Fig. 4C, $p < 0.0001$), with a disproportional large effect of macrolides concentration (adjusted $r^2 = 0.64$, $p = 0.001$) and minor contributions of density of live bacteria (adjusted $R^2 = 0.15$, $p = 0.001$) and the light extinction coefficient (adjusted $r^2 = 0.06$, $p = 0.001$). It was noteworthy the association between macrolides concentration and normalized *erm*B copy numbers (Pearson's $r^2 = 0.77$, $p = 0.0002$, $n = 12$), and between the density of live bacteria and normalized *sul*I (Pearson's $r^2 = 0.54$, $p = 0.0067$, $n = 12$). For the sediments, the analysis explained 50% of the variability of the ARG data (Fig. 4D, $p = 0.016$), with a significant effect of macrolides concentration (adjusted $r^2 = 0.28$, $p = 0.036$) and Cd concentration (adjusted $r^2 = 0.18$, $p = 0.044$). Macrolides concentration correlated positively with *sul*I copy number (Pearson's $r^2 = 0.52$, $p = 0.0293$, $n = 9$), while Cd concentration was inversely related to *qnr*S (Pearson's $r^2 = 0.51$, $p = 0.0308$, $n = 9$).

Finally, associations between ARGs and bacterial groups in water samples were assessed performing a RDA using the ARGs data as the explanatory matrix (Fig. 4E). The analysis explained 52% of the variability of the bacterial community data ($p < 0.0027$), with the *bla*TEM (adjusted $r^2 = 0.40$, $p = 0.011$) and *erm*B (adjusted $r^2 = 0.12$, $p = 0.016$) genes as significant explanatory variables. Although the RDA suggested a positive association between normalized *erm*B copy numbers and the percentage of *Firmicutes*, as well as between the *bla*TEM gene and the percentage of *Actinobacteria*, paired Pearson's correlations did not show any significant result. Remarkably, in RDA analysis for the sediment samples, the bacterial groups related to ARGs in water samples were also involved in main associations found in the sediments (Fig. 4F). Explained variability was 69% ($p < 0.0001$), with the *erm*B (adjusted $R^2 = 0.57$, $p = 0.002$) and *sul*I (adjusted $r^2 = 0.12$, $p = 0.019$) genes as significant predictors. The *erm*B gene strongly and significantly correlated with the percentage of *Actinobacteria* (Pearson's $r^2 = 0.88$, $p = 0.0002$, $n = 9$), as well as the *sul*I gene with *Firmicutes* (Pearson's $r^2 = 0.61$, $p = 0.0129$, $n = 9$).

4. Discussion

Significant associations between the ARGs and bacterial community composition in three water supply reservoirs were detected. Although a correlation exercise cannot confirm the hypothesis that

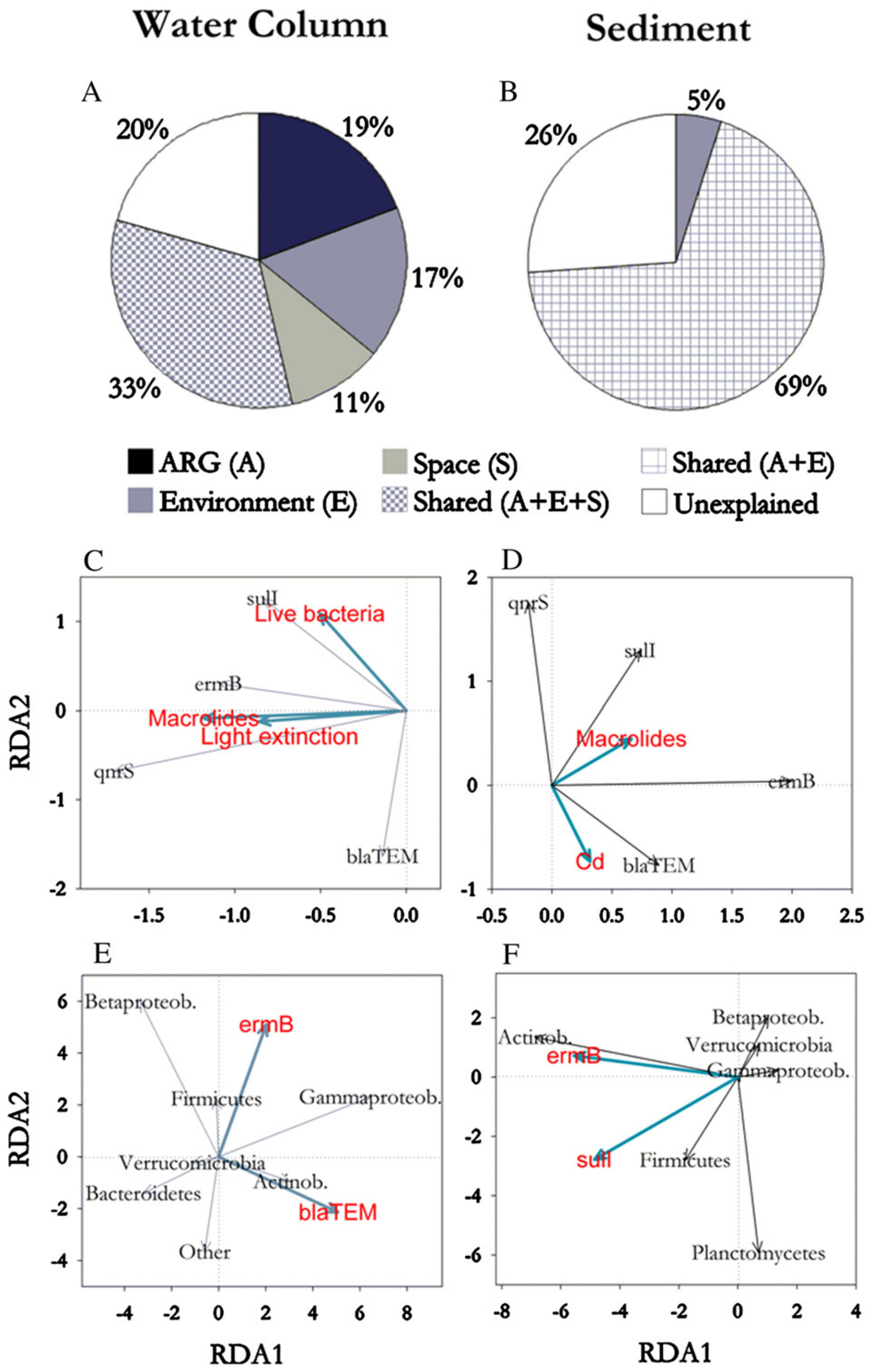

Fig. 4. Analysis of the relationships between environmental variables (including antibiotics), ARG, and bacterial communities in water (left panels) and sediment (right panels) samples: Variation partitioning analyses of the bacterial communities in water (A and B), RDA distance biplots of ARG abundance constrained by environmental variables (C and D) and bacterial community data constrained by ARG abundance (E and F). Explanatory variables in RDA are denoted as bold blue arrows and red marks. We removed unclassified bacterial groups and groups without significant weight in panels e and f to increase readability.##.

antibiotic occurrence and ARGs is shaping the bacterial communities in the reservoirs sampled, results suggested that this is the most plausible explanation for the associations found. A potential alternative explanation is that correlations were spurious, that is, promoted by an unnoticed common cause. This common cause might be the effect of WWTP effluents containing resistant bacteria (Pruden et al., 2006), which means that all associations found between ARGs and bacterial communities were just promoted by the fact that both bacteria and ARGs are released from upstream WWTPs. Although this may be the main explanation for the presence of the detected ARGs in the resistome of the bacterial communities, this cannot explain associations between ARG and bacterial communities found in this study considering the WRT of the water bodies under investigation. Since WRT in the reservoirs is in the order of months, the bacterial communities are the result of the complex ecological interactions (e.g., competence, predation) among bacteria and the other components of the food web after presumably hundreds or thousands of bacterial live cycles. Although it is reasonable to think that the presence of ARGs in the resistome can be facilitated by direct pollution from WWTP effluents, the most plausible explanation for the associations found between ARGs and the bacterial communities is that ARGs and the resistant bacteria spread in the bacterial community facilitated by the presence of the selective agent (antibiotic).

The association between ARGs and the bacterial communities was more evident in the water column, where ARGs was the unique variation component with the strongest explaining power (19%), despite the dominant role of the variation shared by the three explaining sets (33%). The variation shared by the different explaining sets precluded the detection of any significant unique component in the sediments, but considering the highly significant explanatory power of the ARGs data the effect of ARGs on the bacterial communities cannot be discarded (Table A.8). In any case, the pollution gradient in water samples was more evident than in the sediments, where differences in environmental variables and ARGs were less apparent. Normalized ARGs abundance in water and sediments from the same sampling site were closely related (Fig. A.1), suggesting a common process, with the only exception of *qnrS* (related to fluoroquinolones, the antibiotic family with the most variable concentration in water and absent in the sediment).

Maximum antibiotic concentrations found in this study are frequently reported in systems affected by effluents of wastewater treatment plants (Zhou et al., 2011). In this case, the pollution gradient between reservoirs was mainly determined by the wastewater contribution to the total inflow to the reservoir, which peaked at Foix Reservoir (average annual contribution of 31%). Some studies have shown that exposure to highly antibiotic-contaminated effluent promotes resistance genes in environmental bacterial communities (Kristiansson et al., 2011), but other studies suggest that levels of antibiotics below the minimum inhibitory concentration (MIC) can also select resistant bacteria (Gullberg et al., 2011). Thus, even though the levels of antibiotics found in this study are, as expected, far below the MIC for bacteria, it cannot be excluded that they might exert a selection pressure enough to maintain the resistome in environmental communities.

Highest concentrations of antibiotics corresponded to macrolides and sulfamides (water), and tetracyclines (sediment), in similar levels (ng/L to μg/L) to those found in some studies carried out in river water and sediments (Tong et al., 2009; Yang et al., 2010). Although RDA identified macrolides concentration as a principal variable for explaining the variability of ARG in both, water and sediment samples, antibiotic concentration was not a significant variable in variation analyses of the bacterial communities. However, this might be a consequence of the incapability of the analyses to assess the presence of all antibiotic compounds present in the samples, including metabolites and transformation products that can have similar or even higher biological activity (Boxall et al., 2004). Thus, the ARG data may be considered as an integrative proxy of the presence of a complex mixture of antibiotic pollutants, better reflecting the impacts of pollution on the bacterial communities. Metals can also be responsible for the presence of ARG due to co-resistance mechanisms (Baker-Austin et al., 2006; Knapp et al., 2011), although no positive association between ARG and concentration of metals was found in this study. However, the presence of metals could be a factor correlated with the transport and accumulation of antibiotics. For instance, fluoroquinolones are known to form complexes with some iron oxides, which suggest a tendency to adsorb onto clay minerals (Gu and Karthikeyan, 2005). Also, the adsorption of tetracyclines on sediments strongly depends on the pH and presence of metals (Zhang et al., 2011). Values of metals in the target reservoirs showed a maximum of fifty-fold higher concentration of Fe^{2+} in water from Foix than from La Llosa Reservoir, what could explain the higher concentration of antibiotics in the sediment of Foix Reservoir, as a result of not only the greater antibiotic concentration but also other pollutants that might increase antibiotic residence time in the system.

One of the most relevant results from this study is that two bacterial groups associated to the presence of ARGs in both water and sediments were identified as *Actinobacteria* and *Firmicutes*. This is not unexpected, because some members of *Actinobacteria* and *Firmicutes* are producers of antibiotics (Allen et al., 2010; Kieser et al., 2000; Mannanov and Sattarova, 2001). Considering that analyses for the different compartments were independent, this suggests that these two groups may be related to the dissemination of ARGs in natural environments like reservoirs. This is in accordance with previous studies, showing that most resistant bacteria belong to Gram-positive group (Mindlin et al., 2008). These results are remarkable considering that *Actinobacteria* was the bacterial group contributing the most to the bacterial communities sampled in this study (mean abundance = 22 ± 13%), suggesting that indeed ARGs may be significantly linked to bacterial community composition in freshwaters affected by antibiotic pollution. The association between ARG and *Firmicutes* was less conclusive, because in some samples the relatively low number of *Firmicutes* may be explained as a residual population coming directly from the upstream WWTP effluents. If this were the case, the association would be entirely spurious, with no implications related to the dynamics of the natural community.

Although the strong and highly significant relationship found in the sediment samples between *Actinobacteria* and the *erm*B gene as well as between *Firmicutes* and the *sul*I gene seems to support an association between particular taxa and particular ARG, this information should be interpreted with caution. Several of the ARGs abundances were significantly correlated both, in water and sediments, and other ARGs not measured in this study might be present in the samples. Thus, the possibility that significant ARGs detected by RDA simply reflect the effect of non-measured ARGs, or that the uncertainty of the measurements resulted in the observed associations, while masking the causal relationships that would include different measured ARG, cannot be discarded. The fact that *Actinobacteria* is related with the *erm*B gene in sediments but with the *bla*TEM gene in the water column seems to point in this direction (the same reasoning is valid for *Firmicutes*, *sul*I, and *erm*B). The association between *Actinobacteria*, *Firmicutes* and the presence of ARGs can be considered robust, but the bacterial group in charge of carrying each ARG cannot be appointed. It is also difficult to ascertain whether other significant environmental and spatial variables in the analyses have a real effect on the bacterial communities or they simply reflect other environmental conditions, which are either not measured or confounded in spatial differences. The presence of significant variables like metals W or Rb concentration seems to point to the latter.

Although the correlations cannot confirm causal mechanisms, the significant role of macrolides, *erm*B, *Actinobacteria* and *Firmicutes* in independent analyses suggests that antibiotic pollution may play a role in the conformation of bacterial communities in reservoirs via the spreading of ARGs. Macrolides are the most prevalent antibiotic family measured in our samples, and the *erm*B gene is an ARG conferring resistance to, precisely, macrolides. Finally, the *erm*B gene had a significant role in explaining bacterial community variability in both water and sediment samples, and at least in sediments showed a clear relationship with abundance of *Actinobacteria*. In fact, a search using BLASTN (Altschul et al., 1990) for the matching sequences in the GenBank database including updates against the sequence for the *ermB* gene (GenBank accession no. AJ972606) as a query sequence revealed the presence of this gene in several bacterial species, including members of *Actinobacteria*, such as *Eggerthella* sp. (GenBank accession no. AP012211) and *Trueperella pyogenes* (GenBank accession no. AY334073). The spreading of the *ermB* gene may be facilitated due to the fact that it is associated with conjugative transposons located in chromosomes as well as on plasmids (Roberts, 2008). Again, although our results cannot prove the existence of such a causal chain, we can speculate about the existence of a selective pressure of macrolides on the bacterial communities, with spreading of ARGs (already present in the community or coming from ARGs pollution from WWTPs) as a convenient mechanism used by particular bacterial groups to out-compete others. But more research in needed to confirm this mechanism.

Although some studies already described impacts of antibiotics on the functionality of bacterial communities in aquatic environments (Bundschuh et al., 2009; Maul et al., 2006), this is one of the few studies to describe significant associations between antibiotic pollution, the presence of ARGs, and the composition of bacterial communities in lacustrine ecosystems. Moreover, the fact that the bacterial communities were analyzed by pyrosequencing avoided the bias obtained when evaluating antibiotic resistance in environmental microbial communities based only on cultivable bacteria. However, we can only speculate about the effects of the shifts of the bacterial communities promoted by antibiotic pollution and ARGs on the functionality of the ecosystem. While it is well known the principal role of bacteria in the ecosystem processes that define most of the ecosystem services provided by lacustrine ecosystems (e.g., organic matter degradation, denitrification), information to anticipate the impact of the combination of antibiotic pollution and the presence of ARGs on the bacterial communities is lacking. Are ARGs conferring resilience against the impacts of antibiotic pollution thus maintaining the community physiological profiles (i.e. the capacity to degrade a number of carbon sources) and growth indices, or some biogeochemical processes will be severely impacted by the community shift? In the light of these results, these kinds of questions deserve much more attention in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.03.071.

References

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. Call of the wild: antibiotic resistance genes in natural environments. Nat Rev Microbiol 2010;8:251–9.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.

Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Mol Biol Rev 1995;59: 143–69.

APHA. Standard methods for examination of water and wastewater18th ed. ; 1992 [Washington, DC].

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. Co-selection of antibiotic and metal resistance. Trends Microbiol 2006;14:176–82.

Baquero F, Martínez J-L, Cantón R. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol 2008;19:260–5.

Bell T, Newman JA, Silverman BW, Turner SL, Lilley AK. The contribution of species richness and composition to bacterial services. Nature 2005;436:1157–60.

Borcard D, Legendre P, Drapeau P. Partialling out the spatial component of ecological variation. Ecology 1992;73:1045–55.

Borcard D, Gillet F, Legendre P. Numerical ecology with R. New York: Springer Science; 2011.

Boxall ABA, Sinclair CJ, Fenner K, Kolpin D, Maund SJ. Peer reviewed: when synthetic chemicals degrade in the environment. Environ Sci Technol 2004;38:368A–75A.

Bundschuh M, Hahn T, Gessner MO, Schulz R. Antibiotics as a chemical stressor affecting an aquatic decomposer–detritivore system. Environ Toxicol Chem 2009;28:197–203.

Cleuvers M. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol Lett 2003;142:185–94.

Cottenie K, Michels E, Nuytten N, De Meester L. Zooplankton metacommunity structure: regional vs. local processes in highly interconnected ponds. Ecology 2003;84:991-1000.

D'Costa VM, Griffiths E, Wright GD. Expanding the soil antibiotic resistome: exploring environmental diversity. Curr Opin Microbiol 2007;10:481–9.

D'Costa V, King C, Kalan L, Mora M, Sung W, Schwarz C, et al. Antibiotic resistance is ancient. Nature 2011;477:457–61.

Fent K, Weston AA, Caminada D. Ecotoxicology of human pharmaceuticals. Aquat Toxicol 2006;76:122–59.

Gros M, Petrovic M, Barceló D. Tracing pharmaceutical residues of different therapeutic classes in environmental waters by using liquid chromatography/quadrupole-linear ion trap mass spectrometry and automated library searching. Anal Chem 2009;81: 898–912.

Gros M, Rodríguez-Mozaz S, Barceló D. Fast and comprehensive multi- residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J Chromatogr A 2012;1248:104–21.

Gros M, Rodríguez-Mozaz S, Barceló D. Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high- performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J Chromatogr A 2013. http://dx.doi.org/10.1016/j.chroma.2012.12.072.

Gu C, Karthikeyan KG. Sorption of the antimicrobial ciprofloxacin to aluminum and iron hydrous oxides. Environ Sci Technol 2005;39:9166–73.

Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, Hughes D, et al. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 2011;7:9.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. Practical *Streptomyces* genetics. 2nd ed. Norwich: John Innes Foundation; 2000.

Kim S-C, Carlson K. Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. Environ Sci Technol 2007;41:50–7.

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, et al. Methods of studying soil microbial diversity. J Microbiol Methods 2004;58:169–88.

Knapp CW, McCluskey SM, Singh BK, Campbell CD, Hudson G, Graham DW. Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. PLoS One 2011;6:e27300.

Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, Weijdegard B, et al. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. PLoS One 2011;6:7.

Kümmerer K. Antibiotics in the aquatic environment — a review — part I. Chemosphere 2009a;75:417–34.

Kümmerer K. Antibiotics in the aquatic environment — a review — part II. Chemosphere 2009b;75:435–41.

Legendre L, Legendre P. Numerical ecology. New York: Elsevier; 1998.

Li D, Qi R, Yang M, Zhang Y, Yu T. Bacterial community characteristics under long-term antibiotic selection pressures. Water Res 2011;45:6063–73.

Mannanov RN, Sattarova RK. Antibiotics produced by *Bacillus* bacteria. Chem Nat Compd 2001;37:117–23.

Marcé R, Moreno-Ostos E, López P, Armengol J. The role of allochthonous inputs of dissolved organic carbon on the hypolimnetic oxygen content of reservoirs. Ecosystems 2008;11:1035–53.

Marti E, Balcazar JL. Real-time PCR assays for quantification of qnr genes in environmental water samples and chicken feces. Appl Environ Microbiol 2012;79:1743–5.

Martinez JL. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ Pollut 2009;157:2893–902.

Maul JD, Schuler LJ, Belden JB, Whiles MR, Lydy MJ. Effects of the antibiotic ciprofloxacin on stream microbial communities and detritivorous macroinvertebrates. Environ Toxicol Chem 2006;25:1598–606.

Mindlin SZ, Soina VS, Ptrova MA, Gorlenko ZhM. Isolation of antibiotic resistance bacterial strains from East Siberia permafrost sediments. Genetika 2008;44:36–44.

Muñoz I, López-Doval JC, Ricart M, Villagrasa M, Brix R, Geiszinger A, et al. Bridging levels of pharmaceuticals in river water with biological community structure in the Llobregrat River basin (Northeast Spain). Environ Sci Technol 2009:28.

Murphy J, Riley JP. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 1962;27:31–6.

Oksanen J, Kindt R, Legendre P, O'Hara R. Vegan: community ecology package; 2007.

Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 2010;34:415–25.

Peres-Neto PR, Legendre P, Dray S, Borcard D. Variation partitioning of species data matrices: estimation and comparison of fractions. Ecology 2006;87:2614–25.

Pruden A, Pei R, Storteboom H, Carlson KH. Antibiotic resistance genes as emerging contaminants: studies in Northern Colorado. Environ Sci Technol 2006;40:7445–50.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 2007;35:7188–96.

R Development Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2007.

Roberts MC. Update on macrolide–lincosamide–streptogramin, ketolide, and oxazolidinone resistance genes. FEMS Microbiol Lett 2008;282:147–59.

Schloss P, Westcott S, Ryabin T, Hall Jr F, Hartmann M, Hollister E, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75: 7537–41.

Schriks M, Heringa MB, van der Kooi MM, de Voogt P, van Wezel AP. Toxicological relevance of emerging contaminants for drinking water quality. Water Res 2010;44: 461–76.

Šimek K, Comerma M, García J-C, Nedoma J, Marcé R, Armengol J. The effect of river water circulation on the distribution and functioning of reservoir microbial communities as determined by a relative distance approach. Ecosystems 2011;14:1-14.

Solórzano L. Determination of ammonia in natural waters by the phenol hypochlorite method. Limnol Oceanogr 1969;14:799–801.

Taylor NGH, Verner-Jeffreys DW, Baker-Austin C. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol Evol 2011;26:278–84.

Tong L, Li P, Wang Y, Zhu K. Analysis of veterinary antibiotic residues in swine wastewater and environmental water samples using optimized SPE-LC/MS/MS. Chemosphere 2009;74:1090–7.

Van der Gucht K, Cottenie K, Muylaert K, Vloemans N, Cousin S, Declerck S, et al. The power of species sorting: local factors drive bacterial community composition over a wide range of spatial scales. Proc Natl Acad Sci U S A 2007;104:20404–9.

Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7.

Yang J-F, Ying G-G, Zhao J-L, Tao R, Su H-C, Chen F. Simultaneous determination of four classes of antibiotics in sediments of the Pearl Rivers using RRLC–MS/MS. Sci Total Environ 2010;408:3424–32.

Zhang X-X, Zhang T, Fang HP. Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol 2009;82:397–414.

Zhang Z, Sun K, Gao B, Zhang G, Liu X, Zhao Y. Adsorption of tetracycline on soil and sediment: effects of pH and the presence of Cu(II). J Hazard Mater 2011;190: 856–62.

Zhou L-J, Ying G-G, Zhao J-L, Yang J-F, Wang L, Yang B, et al. Trends in the occurrence of human and veterinary antibiotics in the sediments of the Yellow River, Hai River and Liao River in northern China. Environ Pollut 2011;159:1877–85.

Zuccato E, Castiglioni S, Bagnati R, Melis M, Fanelli R. Source, occurrence and fate of antibiotics in the Italian aquatic environment. J Hazard Mater 2010;179:1042–8.

Anti-anxiety drugs and fish behaviour: establishing the link between internal concentrations of oxazepam and behavioural effects

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Abstract

Psychoactive drugs have been persistently detected in surface waters, and biochemical and behavioural effects in a range of aquatic organisms have already been reported. The conservation of these drugs receptors in fish means that a mode-of-action mediated effect could be observed in fish in the same way as in humans, although the key question is at what exposure concentrations this might happen. This study investigated the relationship between the concentrations of oxazepam, a benzodiazepine, in water, fish plasma and other tissues, and the drug-induced behavioural effects after chronic exposure. Higher oxazepam concentrations were found in brain, followed by plasma and liver, while muscle presented the lowest values. Average concentrations measured in plasma after 28 days were 8.7±5.7, 30.3±16.1, and 98.8±72.9 for the 1, 5, and 25 µg L^{-1} exposure nominal water concentrations. Significant positive correlations between plasma and tissue concentration of oxazepam were found in all cases. Exposure of fish to 25 µg L^{-1} produced plasma concentrations within the Human Therapeutic Plasma Concentration (H_TPC) range in many individuals, indicating that an anxiolytic effect in fish, closely related to an increase in the exploratory activity, was expected according to the Read-Across hypothesis. However, no behavioural effects were observed at any exposure concentrations in the exploratory activity, distance travelled or velocity at any exposure level.

Keywords: Read-Across, behaviour, bioaccumulation, fish, psychoactive drug

Introduction

In recent years interest in the potential effects of psychoactive pharmaceuticals in aquatic organisms has grown rapidly, mainly due to the frequent detection of particular compounds in the aquatic environment [173–175]. Several psychoactive drugs have been detected in surface waters, generally in the ng L^{-1} range [175–177], as well as in the biota including fish tissues [41,43,82,100], fish plasma [147], invertebrates [49,86] and biofilm [85], in the low ng g^{-1} range. In addition, an increasing number of studies have attributed biochemical and behavioural effects to some of these compounds in a range of aquatic organisms, and across a wide range of exposure concentrations (pg L^{-1} to mg L^{-1}). Consequently, the environmental implications of exposure to these drugs is, as yet, unclear [175].

Amongst the psychoactive compounds of interest is the benzodiazepine oxazepam has received significant attention after a recent report in which relatively low concentrations (1.8 μg L^{-1}) affected the behaviour of wild fish inhabiting Swedish rivers [103,178]. Oxazepam is a drug widely used primarily for the treatment of anxiety, but is also prescribed for insomnia and acute alcohol withdrawal. This drug acts as an agonist of an allosteric binding site on γ-aminobutyric acid subtype A ($GABA_A$) receptors and increases the efficiency of GABA binding, a major inhibitory neurotransmitter and key regulator of anxiety [179], thus leading to the reduction of the communication between neurons, and hence to a calming effect in the brain [180–182]. Importantly, this neurotransmitter system is well conserved in teleost fish: several studies have identified benzodiazepine-GABA receptors in fish and shown that they have similar binding characteristics as those present in mammals [183,184]; and exposure of zebrafish to GABA-enhancing drugs, such as diazepam (another benzodiazepine drug), results in anxiolytic effects, thus confirming that fish are sensitive to GABA modulation, and specifically the enhanced inhibitory action of benzodiazepines [185]. This apparent conservation of drug target supports the notion that exposure to oxazepam may lead to a similar mode-of-action mediated effect in fish as is the case in humans [181,186].

Considering the importance of characterizing accurate effect concentrations in order to better inform the Environmental Risk Assessment (ERA) of pharmaceuticals, the aim of this study was to investigate the relationship between the external (water) exposure concentration and internal (plasma) exposure concentration of oxazepam in Fathead Minnow (*Pimephales promelas*), and the resultant behavioural effects of the drug after a chronic exposure.

The experimental design used was formulated using the principle of the 'read across hypothesis' [147,187], that is hypothesis that, provided the pharmacological 'machinery' are present and functionally comparable, human pharmaceuticals will elicit the same target-mediated pharmacological response in fish as they do in humans at a similar plasma (or tissue) concentrations (i.e. Human Therapeutic Plasma Concentration, H_TPC) [187]. The main rationale behind the Read-Across Hypothesis is that it should allow the use of data generated in clinical (human) or non-clinical (other mammals) studies to predict the effects and approximate effect concentrations of the same drug in fish, following environmental exposure. In reverse, as is the case here, the approach can be used to estimate the required water concentration that will elicit a pharmacological effect in fish form the human therapeutic plasma concentration using, for example, uptake models like the Fish Plasma Model [145]. This approach has been successfully applied in previous studies on anti-depressant drugs by Margiotta-Casaluci et al., [147] and Valenti et al., [166].

In the current study oxazepam-induced anxiolytic responses in fish were hypothesised as functionally equivalent to the reduction of anxiety in human patients following oxazepam treatment [147]. Fish were exposed to a range of water concentrations of oxazepam predicted to produce plasma concentrations equal to and below (and thus of environmental relevance) those known to induce therapeutic effects in humans. Drug plasma concentrations were measured in individual fish and linked to (anti)anxiety-related endpoints quantified using two types of well-characterised behavioural tests designed to measure anxiety in fish: the Novel Tank Diving Test and the Shelter Seeking test. Oxazepam concentration was also measured in brain, liver, and muscle to characterize drug distribution across major drug reservoirs of the body following chronic exposure.

Materials and Methods

Chemicals

Oxazepam (CAS number 604-75-1) was purchased from Sigma-Aldrich with purity higher than 98%. Stocks solutions were prepared every seven days in N,N-Dimethylformamide (DMF) (Fisher

Scientific) in amber bottles to preserve them from light exposure. HPLC grade methanol, acetonitrile, water and formic acid (98%) were supplied by Merck. The OstroTM 96-well plate used for purification of plasma samples was acquired from Waters Corporation. Dechlorinated tap water (5 and 10 µm carbon filtered) was used as dilution water, and general parameters (pH, temperature and dissolved oxygen) were monitored daily throughout the study.

Test Species and Ethical Statement

Adult Fathead minnows (*Pimephales promelas*) were supplied from breeding stocks maintained at Brunel University, London, UK. Two weeks before the beginning of the study, sexually mature males and females were separated to prevent any spawning activity and acclimated to the test conditions. Fish were fed three times per day: once with adult brine shrimp (Tropical Marine Centre, Gamma irradiated), and twice with flake food (King British Tropical flake food). Fish were not fed on the sampling day. The study was carried out under Project and Personnel Licences granted by the UK Home Office, under the United Kingdom Animals (Scientific Procedures) Act 1986, and the European Animal Directive 2010/63/EU.

Experimental design

The 28-day exposure was carried out using a continuous flow-through system comprising sixteen 20 L glass tanks (dimensions: 455(l) x 260(w) x 250(d) mm. The test was run at a water temperature of 25 ± 1 °C, with a photoperiod of 16 h light:8 h of dark, with 20 min dawn/dusk transition periods. During the experiment, dissolved oxygen concentrations were maintained between 6.4 and 7.8 mg L^{-1}, while pH was 7.5, ±0.5. Water flowed into sixteen glass mixing chambers at a rate of 222 mL min^{-1}, which supplied sixteen tank volumes per day, to each test tank. The same mixing chambers also received concentrated stock solutions of oxazepam delivered via peristaltic pumps at a rate of 0.1 µL min^{-1}. In total, there were four exposure tanks per treatment (two for males and two for females) for each of four treatments (0, 1, 5, and 25 µg L^{-1}). Eight males or females were randomly allocated to the appropriate tanks giving a total of 32 fish per treatment (16 males, and 6 females).

Both the allocation of the treatment groups in the experimental room, and the allocation of each fish into one of the 16 tanks were randomised with the aid of a random number generator.

As previously mentioned, exposure concentrations were selected to cover both, pharmacologically and environmentally relevant concentrations. The selection of external exposure (water) concentrations expected to result in pharmacologically relevant internal (plasma) concentrations was driven by the application of the Fish Plasma Model (FPM) of Huggett et al., [145], and information on the human therapeutic plasma concentration (H_TPC) of oxazepam. The latter was obtained from a study in which patients were administered 15 and 30 mg of oxazepam in two separate occasions with a seven days interval, which produced therapeutic plasma concentrations in the range 100-280 ng mL^{-1} [188].

After the 28-day exposure, fish were sacrificed by an overdose of anaesthetic (Neutral buffered MS222, 100-500 mg L^{-1}), standard length and wet weight measured, and blood taken from the caudal vein using heparinized capillary tubes. Blood samples were centrifuged at 4 °C, 10,000 g for 6 min, plasma collected and stored at -80°C until analysis, and brain, muscle and liver removed, weighed, snap-frozen in liquid nitrogen, and stored at -80°C until subsequent analysis.

Analysis of oxazepam in water, fish plasma and other tissues

Water samples (40 mL) for the chemical analysis were collected in Falcon tubes on days 0, 7, 14, 21, and 28 from all tanks, and kept at -20 °C until analysis. For analysis, 1 mL of each sample was centrifuged at 8000 rpm for 10 min, after which 10 µl of each sample was directly injected in the detector without further extraction. For plasma samples, acetonitrile (400 µl) was added to 50 µl of plasma and vortexed for 30 s for protein precipitation. Samples were then transferred to an Ostro 96-well plate (Waters) connected to a vacuum system for the removal of phospholipids. An aliquot of 300 µl was collected from each extract and was placed under a N_2 current to dry completely. Finally, extracts were dissolved in 100 µl of a 1:1 mixture of methanol/water. Five µl of diazepam-d_5 prepared at 1 µg mL^{-1} in methanol was added as an internal standard to each extract to account for matrix effects during subsequent analysis

Brain, liver and muscle samples were extracted using a method adapted from that of Valdés et al (2015, *in preparation*). Samples (ranging from 20 to 200 mg) were sonicated in 1 ml of methanol for 15 min, and then centrifuged at 10000 rpm for 10 min. After this procedure was repeated 3 times, the supernatant was collected and evaporated to dryness. Extracts were subsequently dissolved in 1 mL of HPLC grade water and then then purified in a SPE Oasis HLB 96 well plate. SPE sorbent in the wells was previously conditioned with 1 ml of methanol and 1 ml of HPLC at a flow of 0.5 mL min^{-1}, with extracts being loaded at the same flow rate. After drying the well for 5 min, 1 mL of methanol was loaded in the plate to elute the samples which were collected and placed under a N_2 current to dry completely. Finally, extracts were dissolved in 750 µl of a mixture methanol/water (1:1) for analysis. Ten µl of diazepam-d$_5$ prepared at 1 µg mL^{-1} in methanol was added to each extract to account for matrix effects during analysis.

All extracts were analysed by ultra-performance liquid chromatography (UPLC) (Waters) coupled to a hybrid quadrupole linear ion trap mass spectrometer Qtrap 5500 (Applied Biosystems), equipped with an electrospray ionization source (ESI) in positive mode. Ten µl was injected in an Acquity HSS T3 column, with 10 mM formic acid/ammonium formate (pH 3.2) and methanol as the mobile phase set at a flow rate of 0.5 mL min^{-1}.

Compound dependent MS parameters (declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP)) as well as compound selected reaction monitoring (SRM) transitions were optimized by direct infusion of individual standard solution of each analyte at 10 ngmL^{-1}. A summary of these parameters is presented in Table S1. All transitions were recorded in Scheduled MRM algorithm with 30s detection window. Source dependent parameters were determined by Flow Injection Analysis (FIA) and were as follows: curtain gas (CUR) – 30 V, nitrogen collision gas (CAD) – medium, source temperature – 500 °C, ion spray voltage – 5500, ion spray gases GS1 – 60 V and GS2 – 50 V. Instrument control data acquisition and data analysis were carried out using Analyst software (Applied Biosystems).

Prediction of oxazepam plasma concentration using the Fish Plasma Model

Measured plasma concentrations were compared to the concentrations predicted by the Fish Plasma Model (FPM) [145]. The model aims to predict the Fish Steady State Plasma Concentration (FssPC) of a drug starting from given water concentration, and is based on the following equations:

(1) $\text{Log } P_{\text{Blood:Water}} = 0.73 \text{ x Log } D_{7.4} - 0.88$

(2) $\text{FssPC} = [\text{Water concentration, µg/L}] \text{ x } P_{\text{Blood:Water}}$

Average water concentrations throughout the 28 days were used to compare measured versus predicted plasma concentrations.

Analysis of fish behaviour

At Day 14, two well characterised fish behavioural tests, the Shelter-seeking Test and the Novel Tank Diving Test, were performed on a sub-sample of all the exposed fish, including fish (n=32) from the control treatment and fish from the treatment at the highest concentration (25 µg L^{-1}). At Day 28, all fish (n=128) were subjected to both tests.

Behavioural tests were performed as described by Valenti et al., [166] and Margiotta-Casaluci et al., [147]. For assessment, fish were transferred from the exposure to the observation tank, which contained only water, with no chemical immediately before performing the tests. For the Shelter-seeking Test, fish were transferred to a 12L observation tank (measuring 390(l) x 200(w) x 200(d) mm). Within each tank, a tile was placed, representing the shelter for the fish, equidistant from the tank walls. Behaviour was recorded for 15 min, which included the time spent under the tile (s). For the Novel Tank Diving Test, fish were transferred to a 9L observation tank (290(l) x 345(w) x 140(d) mm) divided in three areas (bottom, middle, top), and the exploratory behaviour of each fish (e.g. the number of transitions and time spent in each area area) was recorded for 15 min. A Fujifilm Digital Camera (FinePix JV300, 14.0 Mpix) was positioned in front of each observation tank to record fish behaviour, and resultant videos were analysed using VideoTrack version 2.5.0.25 (ViewPoint).

Data analysis

Statistical analyses were conducted using SigmaStat software (version 3.5, Systat, San Jose, USA). Behavioural data were analysed using Kruskal–Wallis tests followed by Dunn's post hoc tests, and also by Wilcoxon-Rank-Sum Test. Bioaccumulation data were analysed using the Holm-Sidaktest One Way ANalysis Of Variance (ANOVA). Statistical significance was set at $p < 0.05$ in all cases.

Results & Discussion

Oxazepam concentrations in water, plasma, brain, and muscle

Recoveries, method detection (MDLs) and quantification limits (MQLs), precision and accuracy for the analysis of oxazepam in water, fish plasma brain, liver and muscle are summarised in Table 1 (see also Supplementary Material). Average water concentrations measured throughout the experiment for the lowest, medium and highest treatment concentrations were 0.8 ±0.2, 4.7 ±0.5 and 30.6 ±2.9 μg L^{-1} (Mean, ± SD, n=20), respectively. During the five sampling events, measured concentrations were within 20% of the nominal concentration in all tanks.

Table 1.Method validation parameters for the analysis of oxazepam in water and fish tissues.

				Precision (% RSD)		
	Recoveries (%) (± RSD)	**MDL (ng mL^{-1})***	**MQL (ng mL^{-1})***	**Intra -day**	**Inter-day**	**Matrix Effect (% ± STDEV)**
Water	-	0.02	0.06	1.2	2.5	103 (±3)
Plasma	78 (13) [a]	0.04	0.14	7.1	8.7	104 (± 9)
Brain	106 (14) [b]	0.54	1.80	0.9	5.3	102 (±8)
Liver	102 (11) [b]	0.12	0.39	0.5	0.3	115 (±1)
Muscle	92 (14) [b]	0.15	0.51	6.1	7.1	99 (±3)

* ng g^{-1} for tissues
[a] Plasma was spiked at 100 ng mL^{-1}
[b] Tissues were spiked at 50 ng g^{-1}

Measured concentrations of oxazepam in plasma of fathead minnows are shown in Table 2 and Table S2. All of the samples in the control group had concentrations below the MDL, while an increasingly high concentration of oxazepam in plasma was observed at higher exposure concentrations. Average concentrations of oxazepam measured in plasma were 8.7 ±5.7, 30.3 ±16.1, and 98.8 ±72.9 (Mean, ± SD, n=32) for the 1, 5, and 25 μg L^{-1} treatment groups, respectively.

Observed inter-individual variability in oxazepam plasma concentrations for fish from the same tank was within the range of 3- and 6-times for 9 out of 12 tanks (Table S3). In three tanks higher variation was likely driven by two samples in which very low concentrations of oxazepam were quantified. If the 5th and 95th percentile values are used, the ratio Max:Min in those three tanks goes back to the expected range of values (2.5-4.2). This degree of intra-tank variability is similar to the one observed for another psychoactive drug, fluoxetine, by Margiotta-Casaluci et al.,[147]. Determining the distribution of the drug in the body and potential bioaccumulation dynamics is critical in order to inform the future development of uptake and pharmacokinetics models able to predict those processes in conditions of chronic exposure [147]. The development of those models is in fact often constrained by the availability of empirical data. Characterization of distribution is particularly important for drugs like oxazepam which act on a specific system (i.e. central nervous system).

The concentrations of oxazepam in the liver, brain, and muscle of exposed fish are summarised in Table 2 and Table S2. Perhaps unsurprisingly the highest concentrations were found in brain, followed by plasma and liver, while muscle presented the lowest values. Oxazepam was detected in all exposed fish except in 12 samples. The majority (>80%) of fish showing no measurable oxazepam uptake were individuals from the lowest treatment concentration group. The tissue-specific bioconcentration factors (BCFs) were calculated according to the measured water concentration and the concentrations in each tissue, and these are summarised in Table 2. BCFs in brain were significantly higher when compared to the other tissues, especially at the lowest exposure concentration, mirroring what has been observed in previous studies [167,189]. This observation suggests that the accumulation of oxazepam in fish tissue at least in part driven by lipophilicity, as brain lipid content is comparatively higher to the other tissue types measured. The tissue/plasma ratios were also calculated and are summarised in Table 2, and Figure 1a. For all tissues, the ratio between the solid tissue and plasma concentration in the lowest concentration, calculated as the logarithmic concentration ($\log_{10}$), resulted significantly different compared to the other two concentrations. A linear regression of log-transformed ($\log_{10}$) solid tissue versus plasma concentrations was undertaken and is represented in Fig. 1b, to provide an indication of oxazepam organ distribution, as in Tanoue et al., [190]. Significant positive correlations between plasma and tissue concentration of oxazepam were found in all cases ($0.578 < R^2 < 0.723$, $p < 0.05$, n= 96, Pearson correlation coefficient). The distribution of oxazepam in fish supports the current knowledge of the pharmacokinetic and distribution of this drug in mammals [191,192].

Benzodiazepines, including oxazepam, are substances able to cross the blood-brain barrier relatively rapidly [179]. Once in the brain, these compounds equilibrate with brain tissue and after equilibrium is attained, a constant brain:plasma ratio is maintained, so that plasma concentrations are proportionately related to concentrations in the brain [193]. This study did not have multi-time sampling, so it is not possible to ascertain if that equilibrium was maintained during the exposure period employed. Nevertheless, the ratio brain/plasma observed here (1.5-2.9:1) is similar to that observed in rats (3-5:1) [191] and cats (1-3:1) [192] supporting the read across approach and suggesting that, at least with oxazepam, the use of preclinical species' data on drug distribution may provide useful insights into pharmacokinetics of the drug in fish.

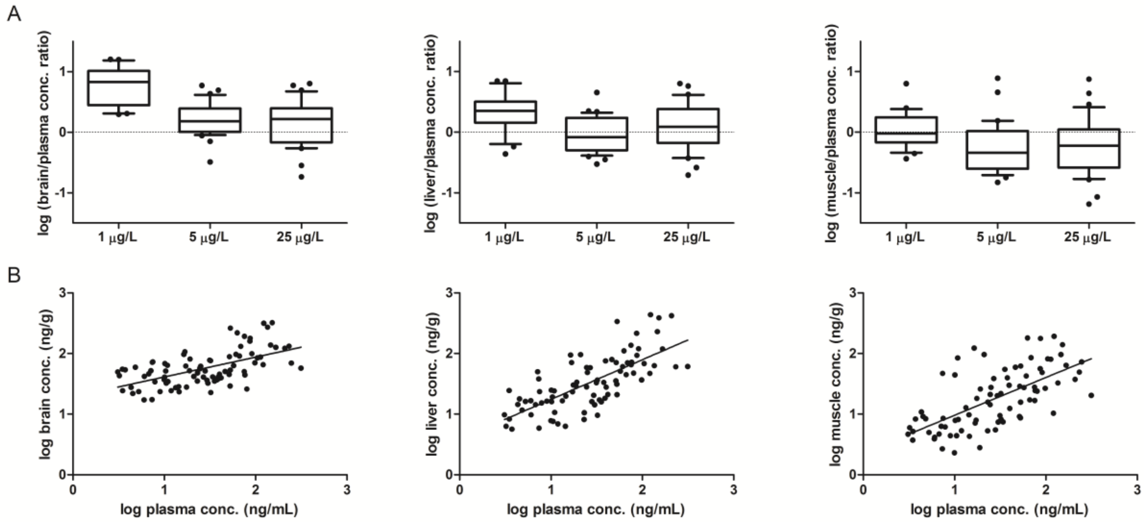

Figure 1 A) Comparative accumulation of oxazepam ($\log_{10}$ tissue/plasma concentration ratio) at the three exposure concentrations; B) Log-linear correlation between plasma and tissue concentration of oxazepam in exposed fish (Pearson correlation coefficient).

Table 2 Measured concentration of oxazepam in plasma, brain, muscle and liver at the three exposure levels. BCF: bioconcentration factors. All values are expressed as median, except for the water concentrations (mean).

Water	**Plasma**			**Brain**			**Muscle**			**Liver**		
Average measured conc. (µg L^{-1})	FPM (ng mL^{-1})	Conc. (ng mL^{-1})*	BCF	Conc. (ng g^{-1})*	BCF	*Brain/Plasma Ratio*	Conc. (ng g^{-1})*	BCF	*Muscle/Plasma Ratio*	Conc. (ng g^{-1})*	BCF	*Liver/Plasma Ratio*
0.8	6	7 (3-18)	9.0	24 (0-73)	30.4	2.9	5 (0-47)	5.8	1.5	9 (0-50)	11.2	0.7
4.7	28	29 (8-80)	6.1	41 (23-78)	8.7	1.5	11 (0-96)	2.3	0.8	22 (8-96)	4.7	0.4
30.6	142	81 (10-313)	2.6	97 (29-322)	3.2	1.6	43 (7-192)	1.4	1.1	69 (0-439)	2.2	0.6

* Median (min-max)

Fish Plasma Concentrations vs Human Therapeutic Plasma Concentrations

Measured plasma concentrations were also compared with the concentrations predicted by the FPM, according to the equations described in Section 2.4 (see Fig. 2). The H_TPC range used as reference [188] in this study is 100-280 ng mL^{-1}. Exposure of fish to 1 and 5 μg L^{-1} resulted in plasma concentrations of oxazepam below the H_TPC, whereas exposure to 25 μg L^{-1} produced plasma concentrations within the H_TPC range in many individual fish, in particular the males (9 out of 16 males and 3 out of 16 females).

The FPM demonstrated good degree of accuracy for the prediction of oxazepam uptake in fish plasma in general, despite the fact that it does not consider inter- or intra-species variability and that it was developed for very hydrophobic compounds [144]. However, when male and female fathead minnows were assessed separately, the model overestimated the measured concentrations in female fish: the measured concentration was on average 2.7-times lower than the predicted concentration for females exposed at the highest concentration. Two hypotheses arise to explain these differences, although no data are available to test their validity: a) gender-specific metabolism of oxazepam lead to different steady-state plasma concentrations; b) smaller gill surface area in females lead to lower uptake rates [194]. Previous studies with a different psychoactive drug, fluoxetine, have also demonstrated the occurrence of dose-dependent modulation (e.g. induction, inhibition, saturation) of metabolic enzymes, which can lead to discrepancies between measured and modelled concentrations [10]. These dynamics are anticipated to occur only at high concentrations and are likely not to be relevant at environmental concentrations. In this study, no metabolites of oxazepam were measured, so the possibility of this effect occurring in the high concentration group remains only theoretical.

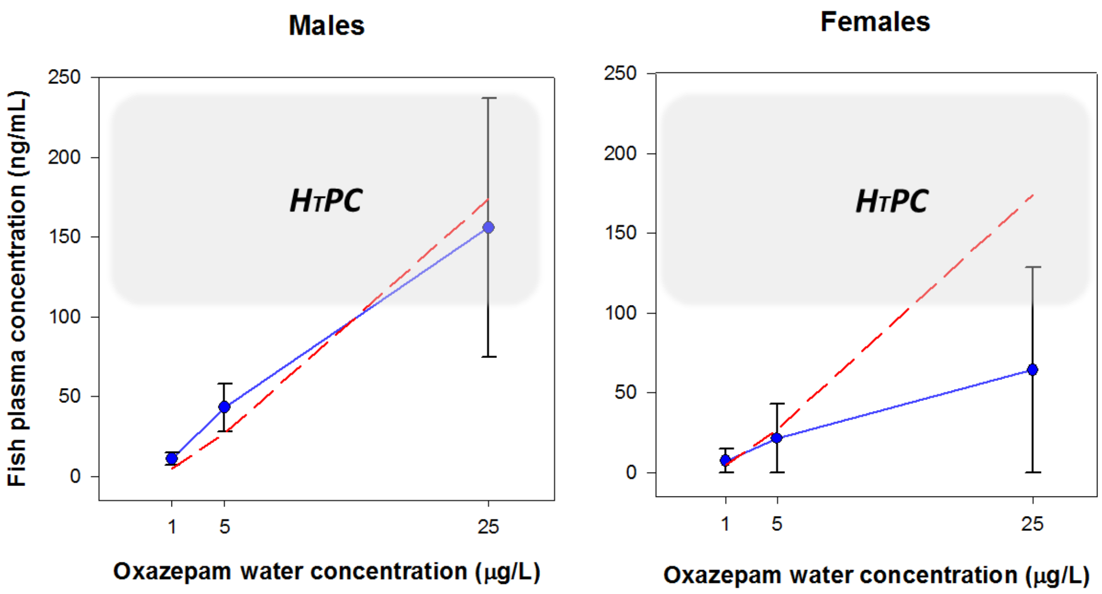

Figure 2 Measured (blue line; mean ± SD; n=20) and predicted (red dashed line) plasma concentrations of oxazepam after 28 days exposure. The grey area indicates the Human Therapeutic Plasma Concentration range (H_TPC).

In humans, sex-specific differences in pharmacokinetics have been identified for many drugs, including benzodiazepines [195]. For instance, a study about the kinetics of oxazepam in men and women determined that elimination half-life was 25% longer in females and clearance of total as well as unbound oxazepam was significantly greater in men than in women [196]. These differences have been attributed to variations in size, composition and hormonal effect, which ultimately can affect drug absorption, distribution, metabolism, and excretion (ADME) profile, although other studies on benzodiazepines, including oxazepam, have not found sex differences in distribution or elimination rates [197,198].

Behavioural Effects

A key hypothesis of this study was that a therapeutically relevant anxiolytic effect of oxazepam would occur in fish only at plasma concentrations similar to the human therapeutic concentrations, but not lower. Oxazepam is a short-intermediate acting benzodiazepine, so after the 28 day exposure, the concentrations found in the plasma are likely to fall in to the steady state. Despite this, no behavioural effects were observed at any exposure concentration, including those that resulted in fish plasma concentrations comparable to H_TPCs in humans.

Shelter Test

No significant differences were observed regarding the time spent under the shelter between the control group and the exposed groups at any concentration, either after 14 (data not shown) or 28 days of exposure (see Fig. 3). Males and females both displayed similar behavioural variability, although male fish spent 20% more time under the refuge than female fish, which was expected as it conforms to normal fathead minnow behaviour [199].

Novel Tank Diving Test

Several endpoints were recorded in this test: number of transitions and time spent in the top and middle area, as well as distance travelled and average speed. Decreased exploratory activity ($p<0.05$) in both the middle and top areas was observed only for the group exposed to 5 µg L^{-1}, although oxazepam plasma concentrations in fish exposed to that concentration were approximately 3-times lower than the H_TPCs, and so no behavioural alterations were expected. In contrast, no significant effects were observed in fish exposed to the highest concentration (25 µg L^{-1}). The analysis of data distribution for individual fish and individual tanks indicated a high degree of both inter-individual and inter-tank variability across treatments, including the control group. The latter included both highly active and poorly active tanks. No behavioural alterations were detected in terms of the distance travelled or velocity at any exposure level.

Behavioural studies provide an important link between the physiology and ecology of the aquatic organisms. Fish in particular provide an excellent test organism for behaviour, as they have high ecological relevance, are constantly exposed to environmental stressors, and possess many of the receptors required for potential responsiveness to many human drugs [186,200]. However, behavioural endpoints are difficult to measure, interpret, and reproduce. In fact, inter-individual behavioural variability may significantly influence the sensitivity of behavioural tests for ecotoxicology and limit their use in risk assessment [166].

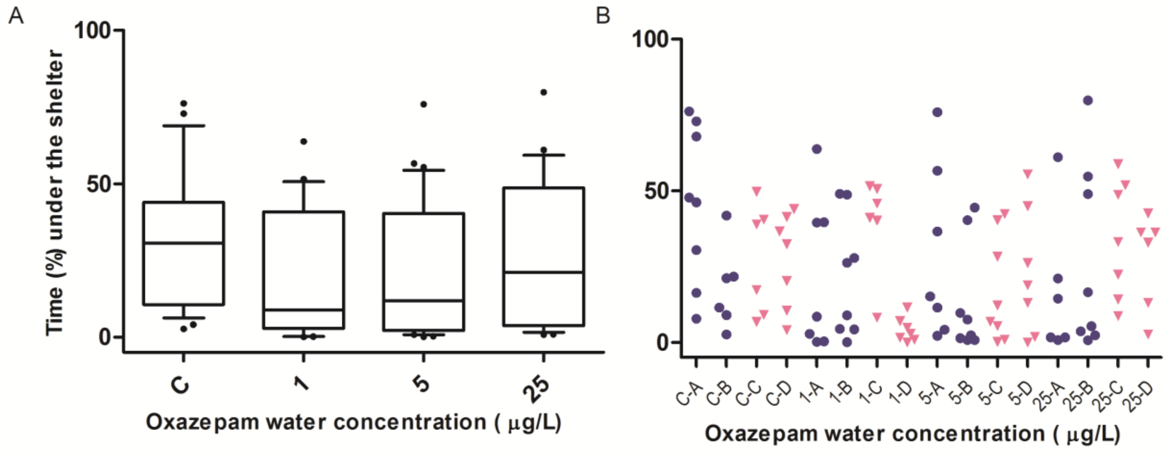

Figure 3 Effect of oxazepam on fish exploratory behaviour quantified during a Shelter-Seeking Test performed after 28 days of exposure. A) Time spent in under the shelter per treatment; Boxes represent medians, with 10th and 90th percentiles (n=32). B) Time spent under the shelter in each individual tank (males represented as blue circles, females as pink inversed triangles). Dashed lines separate control tanks and each one of the three exposure concentrations.

In spite of the potential ecological consequences of behavioural alteration, two main challenges are currently limiting the use of behavioural toxicology data for environmental risk assessment. The first is the availability of validated robust methodologies able to detect the subtle behavioural effects, if any, occurring at low exposure concentrations. The second challenge is represented by the translation of those subtle effects from the laboratory to the field. The first issue can be addressed by employing well-standardised behavioural tests and by performing a careful characterization of baseline values. In the present study we employed two well-established tests, the Novel Tank Diving test and the Shelter Seeking test. Both tests are designed to assess the exploratory behaviour of fish introduced into a novel environment and are recognised as appropriate for the assessment of anxiety in teleosts [201].

The “Novel Tank Diving Test” is based on the instinctive behaviour of fish to search for a refuge when they are transferred to a novel environment (i.e. observation tank) by diving to the bottom or under the shelter until the conditions are perceived as safe enough to initiate exploration. The swimming pattern and several behavioural endpoints are quantified to assess anxiety (number of transitions into the top area, time spent in the top area or inside/outside the shelter) [147,202]. Our results highlight the high inter-individual variability of fish behaviour and suggest that experiments performed with low statistical power (e.g. small sample sizes) may lead to erroneous interpretation of the data at worst, and the absence of meaningful empirical data at best. Nevertheless, method standardisation and validation, if performed, can significantly increase the reliability of behavioural toxicology data.

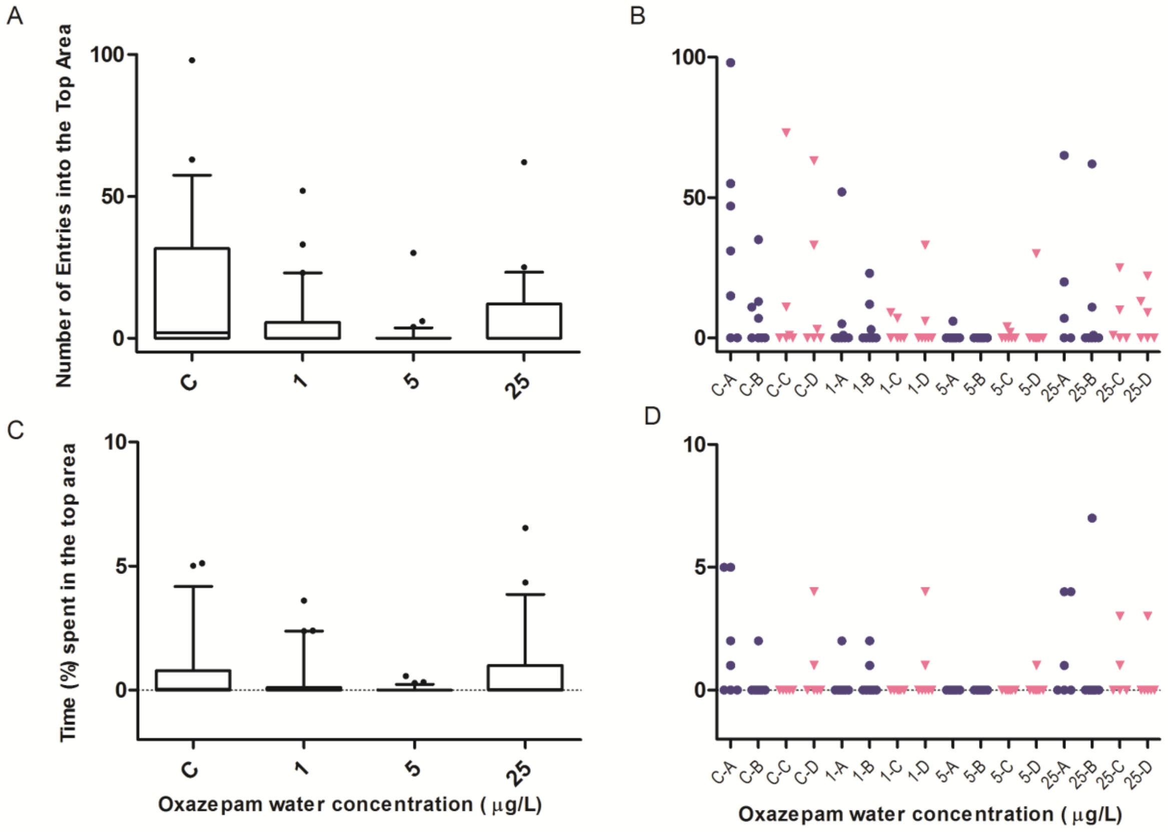

Figure 4 Effect of oxazepam on fish exploratory behaviour quantified during a Novel Tank Diving Test performed after 28 days of exposure. Number of entries into the Top Area: A) per treatment and B) in each individual tank. Time spent in the top area C) per treatment and D) in each individual tank. Males represented as blue circles, females as pink inversed triangles. Boxes represent medians, with 10th and 90th percentiles (n=32). Dashed lines separate control tanks and each one of the three exposure concentrations.

The second challenge is probably the most difficult to tackle. For example, previous studies have shown that fathead minnows exposed to environmentally relevant mixtures of four different psychiatric drugs impacted predator avoidance behaviour (an endpoint of high ecological relevance) [203]. However, results from other studies have been contradictory: while exposure to sertraline induced fathead minnow males to spend less time sheltering due to altered nest guarding behaviour [166], fluoxetine exposure, a drug acting with the same mode of action, resulted in an increase in the same endpoint [203]. The way to translate this contrasting information into ecological predictions is currently uncertain, however, steps forward have been achieved by Hellstrom et al., [204], who successfully applied GPS technology to track the exploratory behaviour of individual fish in a Swedish artificial lake. In the future these technologies may promote the understanding of the ecological relevance of chemical-induced behavioural effects by monitoring such effects with the animals in their natural environment rather than one in which normal behaviour is likely to altered prior even to the introduction to test substances of interest.

Mode of action and prediction of behavioural effect

Knowledge of the mode of action of drugs in humans can be a key component to aid the prediction of potential effects in fish and other wildlife species. In humans benzodiazepines are used as anxiolytics, muscle relaxants, procedural sedation agents, and sedative-hypnotics to treat withdrawal states, among other conditions. They act on the benzodiazepine binding site on the chloride channel of the $GABA_A$ receptor, an inhibitory neurotransmitter receptor, and increase the frequency of chloride channel opening. This hyperpolarizes the cell and prevents nerve firing/stimulation enhancing the action of the endogenous ligand, GABA itself. In the case of fish, this anxiolytic effect has been shown to translate into a reduction of avoidance behaviours and an increase in exploratory behaviour [205] that are indicative of a reduction in anxiety much the same as is the case in human recipients [206]. Consequently, fish exposed to anxiolytic drugs pay less attention to the threats or the uncertainties of a novel environment and show a willingness to make excursions into areas where the threat of predation is potentially higher (e.g. to the surface of the water). These same effects are present in people treated with anxiolytic drugs, and thus, it seems reasonable to conclude that these drugs affect fish in the same way [206].

Fathead minnows react in different ways when exposed to stress: sometimes they become motionless (“freezing”), for periods ranging from approximately 0.5 min to greater than 8 min. In other cases, they swim more slowly, or they spend less time outside the shelter or the opposite: sudden unpredictable dashing may happen as initial response to danger. In other cases, there is no apparent response [207]. This serves to highlight the inherent variability of behavioural data when measured in fish, but nonetheless, such measures of anxiety were the most appropriate in order to determine the mode of action related effects of oxazepam following chronic exposure in fathead minnow.

Interpretation is further complicated by the diverse array of behavioural effects that oxazepam can induce. In humans, the decrease of anxiety is the intended effect of the drug, but in some cases excitement or side-effects such as drowsiness, ataxia, fatigue, memory loss, and slurred speech may occur [208]. Considering the potential paradoxical response, it is difficult to determine the phenotypic manifestation of these effects in fish. These complex issues make it very difficult to know what behaviours might be affected by different groups of psychoactive drugs, and hence to decide which behavioural test to use when investigating the possible effects of these drugs on fish.

Final remarks

In this study, we successfully characterized the uptake and distribution of oxazepam in several tissues in fathead minnow, following chronic exposure. These data are key for understanding the uptake and body distribution of benzodiazepines, an important group of pharmaceutical environmental contaminants, in potentially exposed fish. In particular, the detection of bioconcentration in brain tissue shows that oxazepam is able to reach target tissues even in fish exposed to environmentally relevant concentrations. Despite this, no drug-related behavioural effects were observed in the Shelter-Seeking Test at any concentration, and significant decreased exploratory behaviour in the Novel Tank Diving Test only in fish exposed to the middle concentration. Therefore, although (expected) anxiolytic behavioural effects cannot be completely excluded and additional experiments would be necessary to elucidate the reproducibility of the results, the data suggesting a comparable therapeutic effect in fish as is seen in humans, is not strong.

Our results did not replicate the behavioural alterations reported by Brodin et al., [103] in fish exposed to 1.8 μgL^{-1} of oxazepam. This may be due to either methodological differences or to interspecies differences in sensitivity or ADME. The determination of plasma concentrations of oxazepam in future studies with different fish species will help to ascertain whether a difference in species sensitivity truly exists or other factors resulted in the differences between the two studies.

Overall, these results highlight the difficulties in interpreting behavioural changes even when medium-high power standardised behavioural tests are conducted at sub-therapeutic concentrations.

The use of the Read-Across approach [187] and the measurement or prediction of internal drug concentrations were demonstrated to be valuable tools to allow some interpretation of the results obtained and remain extremely valuable data with which to help better understand the impact of human pharmaceuticals in non-target, environmentally-relevant species groups.

Acknowledgments

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Chapter 6

General discussion

DISCUSSION

I. DEVELOPMENT OF ANALYTICAL METHODS FOR THE DETERMINATION OF PHARMACEUTICALS AND ENDOCRINE DISRUPTORS IN FISH, INVERTEBRATES AND BIOFILM

The constant release of PhACs and EDCs into surface waters and subsequent detection in many environmental compartments have derived in some regulatory efforts concerning water bodies by several governmental bodies [34,36,214]. An additional step would be to consider whether the environmental exposure to these chemicals leads to their bioaccumulation and persistence in biological tissues [2]. For that reason, the need for analytical methods for extraction and determination of PhACs and EDCs in aquatic organisms is very relevant. At the beginning of this thesis, very few multi-residue methods for the analysis of PhACs and EDCs were published, probably due to the lack of appropriate instrumentation with enough selectivity, specificity, and sensitivity [2,98,215] available at the time, in part because the interest in these groups of emerging contaminants is relatively recent when compared to priority and legacy contaminants. In fact, only a handful of papers dedicated to the bioaccumulation of PhACs [41,43,100] and EDCs [45,67,70,77,138] in wild organisms were published before 2010. In this context, the first objective of this thesis was to develop and validate analytical methods to measure a broad set of pharmaceuticals and endocrine disruptors in relevant biological matrices: fish tissues, invertebrates, biofilm.

Aquatic biota comprises many organisms with different physical characteristics, habitats, lifespans, dietary and living habits, etc. These differences are very attractive when studying bioaccumulation, as they introduce a number of biological factors that are going to influence the sensitivity of a species to a chemical. For instance, fish have long lifespan and are able to swim along the river, so they are capable of integrating exposure in a relatively large area during extended periods. On the other hand, they are able to metabolise the chemicals they may uptake, and therefore the bioaccumulation may be less significant than other organisms with a less evolved metabolism. Invertebrates possess the capacity to integrate rapid environmental variations as their growth is quicker, but their size may be a limiting factor when detecting trace level contaminants. Biofilm also have a rapid development and usually large biomass, and are also pointed out as early sentinels of contamination and other physicochemical impact in the river. Besides, they represent the first step in the trophic chain so they could play a critical role in transferring PhACs and other EDCs to higher trophic levels. However determining if the bioaccumulation is happening by absorption mechanisms in the intercellular polymeric matrix or by uptake in the cells embedded in such may be difficult to discern. These are just a few examples of how the study of bioaccumulation in different organisms may answer different questions about the processes taking place in the environment.

This inherent complexity of organisms is at the same time a decisive factor when developing an analytical technique. It is obviously different to analyse fish than algae, or insect larvae, as the composition of these organisms is diametrically opposed. The extraction and purification procedure to isolate the target analytes from complex biological matrix is going to heavily depend on the non-targeted molecules present in the matrix that could interfere with the detection system. As an example, when analysing a plasma sample, the presence of blood proteins could impede the accurate determination of a target compound, so the main strategy in this case would be a quick protein denaturalisation by adding a small volume of organic solvent which would lead to the protein precipitation. The resulting extract would be purified and, therefore, suitable for analysis (see Chapter 5). However, when the sample is a fish tissue, such as liver, a more thorough extraction and purification is needed. First, the extraction must be efficient enough to remove the target compounds from a solid matrix and transfer them into a liquid phase. Then this extract must be purified to remove a high proportion of lipids and other molecules that could interfere with the detector, by clogging the instrument or by distorting the analytical response (suppression or enhancement) (see Chapter 3). These interferences are usually called *matrix effects*. In fact, when dealing with biota samples, the assessment of the matrix effects is a fundamental part, as it was effectively demonstrated during Chapter 3, in particular in the case of fish, but was also considered for invertebrates and biofilm. Signal suppression up to 83% was calculated for pharmaceuticals analysed in fish liver tissues, which may be attributed to the higher lipid content of liver (48%) compared to the fish whole body (15%), while it was lower in the case of biofilm (~ 20%) [216], less than 20% for invertebrates (and therefore is not considered significant), and negligible (less than 5%) in the case of fish plasma. These results highlight the relevance of using a suitable approach to compensate for matrix effects during analysis of biota samples, considering the variability in composition that different species might have [114]. The most advantageous approach for an accurate determination of target compounds seemed to be the quantification using a calibration curve prepared with spiked matrix extracts and internal standard addition (internal sample calibration), which was able to correct the matrix effects for all the compounds [82]. Internal sample calibration is thus strongly recommended especially in those cases when the analogue internal standard is not available.

Quality parameters and method performance, including recoveries, method detection limits, accuracy, etc., are also critical variables during analytical method development. The main challenge when analysing biota is to reach the balance between achieving good pre-concentration factors and recoveries and performing an exhaustive extraction and purification that results in method limits of detection low enough to detect the target compounds. In fact, many authors consider that recoveries are acceptable in a wider range than in other environmental compartments, due to the analytical challenge that poses the development of a multi-analyte methods for compounds with very diverse physicochemical characteristics and to the intensive sample pre-treatment demanded in biota matrices [48,217]. In this project, PLE was chosen as the preferred extraction method for fish and biofilm, due to the better reproducibility as a consequence of the automatized extraction, as well as overall better recoveries (30-140%), and the significantly lesser matrix interferences observed during analysis. This was impossible in the case of invertebrates, as the sample size was a limiting factor, so it was necessary to downsize the extraction and use a sonication probe.

The purification method was also adapted for each matrix. GPC was selected as clean-up method for fish tissues, as it removed a high proportion of the lipids, but it was deemed not necessary in the case of less complex matrices, where a simpler and less resource-consuming step would be enough. That was the case for biofilm, where SPE with Oasis HLB cartridges was applied, or invertebrates and fish plasma, where and OSTRO^{TM} plate developed to remove phospholipids and precipitate proteins resulted as the most appropriate technique. These techniques allowed reaching very low MDLs, in the range of 0.01–6.7 ng g^{-1} for PhACs, and between 0.08-2.4 ng g^{-1} in the case of EDCs.

After applying these methods to environmental samples, a total of 12 EDCs and 14 PhACs were detected in fish, invertebrates and biofilm. Table 1 shows the compounds detected in the three matrices, including some data about the accumulation of EDCs in fish, which was not directly included in this thesis but they are relevant for this discussion. Concentrations of these compounds were lower for PhACs than EDCs in all matrices, but they followed the same general pattern: fish < invertebrates < biofilm. Among all the compounds detected, only two were in common in the three matrices: the anti-inflammatory diclofenac and the flame retardant TBEP.

As explained in the Chapter 1, the main factor used to predict or to explain bioaccumulation of a compound is the octanol-water partitioning coefficient (K_{ow}), a measure of hydrophobicity that drives sorption and accumulation. For those compounds that are ionisable, as in the case of many PhACs, the $log\ D_{ow}$ (which is the $logK_{\mathrm{ow}}$ at a defined pH) is a better parameter to apply to predict or explain bioaccumulation at environmental pH. Accumulation of EDCs in several aquatic organisms seems to support that hypothesis, as those compounds with a $log\ K_{ow} \geq 3$ have a higher prevalence in biological tissues. That is the case of compounds like TBEP ($log\ K_{ow} = log\ D_{ow\ pH\ 7.4} = 4$), detected in the great majority of the samples analysed in fish, invertebrates and biofilm. It is also the case of other compounds detected at least in two of the biota matrices, such as estrone, bisphenol A, and triclosan. This seems a reasonable assumption, particularly when other compounds where present in the water at higher concentrations but were almost never detected in macroinvertebrates and biofilm, especially if their $log\ K_{ow} < 2$, as the case of 1H-benzotriazole, tolyltriazole and caffeine, for example.Therefore, log K_{ow} of EDCs and tissue lipid content of organisms provide a strong basis for estimating tissue/water partitioning [126]. To certain point, this may be true also for PhACs. According to the results presented in this thesis, 90% of the PhACs compounds detected have a $log\ K_{ow} \geq 3$, but when $log\ D_{ow}$ at environmental pH (7.4) is considered, this value is only greater than 3 for 20% of the compounds. This suggests that although lipophilicity and hydrophobic interactions are influencing bioaccumulation even in the case of these compounds, possibly other mechanisms are also contributing, and further research is needed to determine if processes such as active transport are inducing the uptake of these compounds.

Table 5. List of compounds detected in the organisms analysed. Cross in red for data published in Jakimska et al., (2013) [141], paper not included in this thesis. Water data refer only to invertebrates and biofilm. Blanks mean the compound was not analysed. When the sign (-) is present means that the compounds was not detected.

	Water	***Fish***	***Invertebrates***	***Biofilm***	***log K_{ow}***	***log D_{ow}***
	Endocrine Disruptor Compounds					
Hormones						
Estrone		x	x	-	4	4
Parabens						
Ethylparaben		x	-	x	2	2
Methylparaben		x	-	x	2	2
Propylparaben		x	-	x	3	2
Benzylparaben		x	-		3	3
Flame Retardants						
TBEP	x	x	x	x	4	4
TCEP		x	-	-	2	2
Surfactants						
Nonylphenol	x		x		6	6
Plastiziers						
Bisphenol A		x	x	-	4	4
Antibacterial						
Triclosan		x		x	5	5
Others						
Caffeine	x	x	-		-1	0
Tolyltriazole		x	-		2	2
	Pharmaceutical Compounds					
Analgesics/anti-inflammatories						
Ibuprofen	x		x	-	4	1
Diclofenac	x	x	x	x	4	1
Calcium channel blockers						
Diltiazem	x		-	x	3	2
Norverapamil			-	x	5	2
Verapamil			-	x	5	3
Lipid regulator						
Gemfibrozil	x		-	x	4	1
Psychiatric drugs						
Carbamazepine	x	x	-	-	3	3
OH-CBZ	x	-	-	x	2	2
Citalopram	x	x	-	-	4	2
Venlafaxine	x	x	x	-	3	2
β-blockers						
Propanolol		x	-	-	3	1
Salbutamol		x	-		1	-1
Carazolol		x			3	0.2
Other						
Clopidogrel		-		-	4	4

II. DETERMINATION OF PHARMACEUTICALS AND ENDOCRINE DISRUPTORS IN BIOFILM AND INVERTEBRATES: TROPHIC TRANSFER

The second objective of this thesis was to assess the bioaccumulation and potential biomagnification of these compounds in different trophic levels in the natural environment. Studies previous to 2010 showed that very little, if any, biomagnification occurred in the trophic chain in the case of the psychiatric drug carbamazepine [110] or the surfactant nonylphenol [77,149]. Parallel studies to the one presented in this thesis also confirm that bioaccumulation of PhACs through from the lower to the upper trophic levels did not occur in the field [135,150].

Results addressed in Chapter 3 (except fish data) regarding the uptake of PhACs and EDCs were recovered in Chapter 4 to analyse differential accumulation between different trophic levels (primary producers to secondary consumers). Occurrence of 25 PhACs and 12 EDCs in water, biofilm, and three macroinvertebrate taxa differing in trophic position and feeding strategy was studied in a small river heavily impacted by the discharge of a nearby WWTP to fully understand the potential impact of these emerging pollutants in freshwater ecosystems in the “worst-case scenario” [218].

From all the compounds in common for the 3 different matrices, only 10 compounds were detected in water, 9 in biofilm and 6 in macroinvertebrates, including the anti-inflammatory drug diclofenac, and the flame retardant TBEP, which were detected simultaneously in water, biofilm and macroinvertebrates. Biofilm accumulated a higher number of PhACs and EDCs than the three macroinvertebrate taxa, although, as pointed out in Chapter 3, this could be related to the influence of the EPS matrix and its capacity to interact with the contaminants in the water, which could lead to physical sorption instead of their uptake by the cells.

Contaminants concentrations in both water and biota were significantly affected by the WWTP effluents, as the increased concentration in the first reach after the discharge point clearly indicated. Subsequently, a decrease in the number a concentration of compounds detected along all studied impacted river segments was observed. This decrease cannot be only attributed to dilution of contaminants with river water, as this parameter was determined as 13% in a previous study in the same section of the river [219] and could not account for the attenuation observed in this study for both PhACs and EDCs. In fact, in-stream attenuation was different for water, biofilm and macroinvertebrates. Water concentration was reduced between 33-64% (EDCs and PhACs respectively) from the first impacted reach to the last (in less than 3 km), whereas the concentration in biofilm decreased between 2-22%. Macroinvertebrates concentrations was barely affected, when the three taxa where considered as a whole, in the case of EDCs, driven mostly by TBEP slight biomagnification. In the case of PhACs in macroinvertebrates, the concentration in the last reach (I_3) was 5% of the one detected right after the WWTP.

In agreement with the results from Chapter 3, hydrophobic compounds ($log\ K_{ow} \geq 3$) were present more often in biological tissues. It is well-known that factors such as lipid content (more attractive for hydrophobic compounds), individual size (and therefore, different metabolism) or life stage (exposure time) can affect uptake levels [220], and this may difficult the accurate prediction by simple models as the one applied in this case. However, the model [135] used to predict the BAFs, which based on the $log\ D_{ow}$ to account for the possible ionisation of the PhACs, was able to predict BAFs in all taxa, which could be very useful for future research and risk assessment.

Among the 34 macroinvertebrate taxa present in the sampling area, only 3 were selected according to their feeding strategy and habitat, contemplating both feeders (*Phagocata*) and detritivores (*Ancylus, Hydropsyche),* as this may affect the dietary intake of the target compounds.

No biomagnification of PhACs was observed, but one EDC, flame retardant TBEP, was found consistently across all food web compartments, although at relatively low concentrations. TBEP concentrations increased slightly with the trophic levels, being the lowest in biofilm, intermediate in *Ancylus*, and the highest in *Hydropsyche* and *Phagocata.*

There are, however, some limitations, such as lacking data from higher trophic levels like vertebrate organisms or the lack of integrated data about water contamination (only "snapshot" water concentrations were measured). However, the trophic magnification of TBEP we found is a significant finding and should be further investigated. The results highlight the need to consider diet, together with waterborne exposure, as a potential bioaccumulation route in aquatic organisms for some emerging contaminants.

Another consideration and advisable future step is the study of the role that the organisms play in the attenuation observed of most of contaminants in river water, i.e., the level of biodegradation taken place in the environment, and that could be also related to the attenuation previously seen in the case of biota. Evidence of biodegradation of emerging contaminants by river biofilm has been already reported [221]. This could be interesting when trying to understand not only the effects in the organisms, but also the contaminants fate in the aquatic environment. Uptake is irrevocably associated to degradation/removal, including biodegradation, which highlight the role of the determination of metabolites and degradation products in the study of the uptake/elimination kinetics. As stated in Chapter 3, more research is needed to determine how bioaccumulation and biodegradation are related, i.e. whether bioaccumulation increases bioavailability and therefore increases the chances of biodegradation.

Although the correlation between bioaccumulation and measurable effects is still very much unknown, a few studies have tried to correlate both in recent years, particularly in the case of PhACs. Some of the effects observed in bioaccumulation experiments after exposure to a compound or a mixture of drugs ranged from behavioural modifications [167,222] to histopathological changes [96], reproductive alterations [93,223], mortality [122], estrogenicity[102], neurotoxicity [223] and modifications of gene expression and proteomic changes [54,66,71,169,222]. However, these studies used generally greater exposure concentrations than those normally detected in the environment (µg L^{-1} to mg L^{-1}), and consequently the tissues concentrations were also usually higher. Thus, the detected concentrations of PhACs and EDCs in this thesis may not represent an immediate risk for the exposed organisms. Nevertheless, future research should include more studies on this issue to build a robust correlation between the fate and effects of pharmaceuticals in aquatic ecosystems.

III. ENVIRONMENTAL EFFECTS OF THE OCCURRENCE OF ANTIBIOTICS AND PSYCHIATRIC DRUGS: ANTIBIOTIC RESISTANCE AND BEHAVIOURAL ALTERATIONS

The last specific aim of this thesis was to assess the impacts of exposure. Two different approaches were used to achieve this during this thesis: a field-exposure study and a lab-exposure experiment under controlled conditions, which emphasizes the variety of fronts and effects, both direct and indirect, that PhACs may entail to the environment. For instance, when an impact is studied in the field, under natural conditions, the setting is more realistic and the results of the study are more likely to generalize and account for many variables that are not controlled or even known, which means that the observations and results obtained can be used to assess the risk to the environment more accurately. However the field experiment has less control and so it may be difficult to determine what factors are influencing the outcome. In a lab experiment, the researcher is able to control most of the conditions, and so can better isolate factors, although the results might not reflect what is actually happening in nature.

The impact of the presence of antibiotics, a group of PhACs, in the freshwater environment was studied with samples taken from a field exposure situation. Despite the relevance of the issue, which will be expanded in the next section, not many studies have tried to investigate this issue outside of the clinical context. Culturing natural bacterial communities in the lab still remains a challenge [224], although culture-independent analysis based on molecular methods have allowed a deeper understandings into the issue. On top of that, the assessment of a real situation with current levels of pollution in 3 water reservoirs, one of them provider of drinking water for a city such as Barcelona (population: 1.6 millions), was deemed very pertinent and relevant for the public health.

The second study related to the effect of a psychoactive drug in the fish behaviour after 28-day exposure was performed under controlled conditions in a lab exposure. The nature of this endpoint made it impossible to perform the experiment in the field:

- Exposure time and concentration had to be stable for 28 days, so the drug would have enough time to build up in the fish before the behaviour could be tested.
- Parameters such as temperature, pH, ammonia may influence not only the fitness (and survival) of the fish, but also affect the drug uptake. Therefore, they had to be monitored daily and kept within certain realistic ranges.
- Behavioural testing in this case entailed filming the fish, which logistically was quite complicated (camera placement, lighting, etc.). Any minimum alteration of the fish environment may affect the results, which could confound even more the inherently difficult interpretation of behavioural data. For instance, fish get used being alone in a room, with only a person coming

in/out at regular periods, so the filming had to be done when nobody was present in the room to avoid any interference, as fish may react in a different way otherwise. In the same way, filming must be performed quickly, to keep the fish stress to a minimum, which could alter the results obtained.

In this case, the lab study contributed in a different way to the question whether PhACs are having a harmful effect in the aquatic environment. The concentrations used during the exposure were usually above the considered environmentally-relevant level, except in the case of the lower one (1 μg L^{-1}), which could be a real life scenario. The experimental design, with three different concentrations, two different behavioural tests performed in a great number of fish, gave this study a significant statistic power and reliable data despite the intrinsic variability of the biological responses, which would not possible in a field study.

Antibiotic Resistance in Water Reservoirs

Although antibiotic resistance is a natural and old phenomenon [173], the common use (and misuse) of antibiotics in modern times, not only in the clinical context, but also in agriculture and veterinary practices is affecting the natural environment to a degree which could have severe consequences for human health. In fact, antibiotic resistance has become one of the most important public health issues, causing high mortality due to the impossibility of treating antibiotic-resistant infections [224].

Many studies have demonstrated that human activity cause an impact in the dissemination of antibiotic resistance determinants, as heavily-impacted areas usually present more antibiotic resistance than pristine environments [225,226]. In this context, WWTPs might act as reservoirs of antibiotic resistance bacteria and ARGs, as they receive sewage discharges from households and hospitals, as well as inputs from diffuse contamination from surrounding crops, and they may have a role in the spread of antibiotic resistance in the environment [227]. However, little is known about the diversity, distribution and origins of resistance genes for the majority of environmental bacteria [228]. In recent years, the application of highly specific and sensitive molecular techniques, such as the quantitative PCR (qPCR), has allowed the precise quantification of ARGs abundance in both aquatic and terrestrial samples that resulted in a wealth of studies about the impact of WWTPs discharges on aquatic environments and, particularly, on the prevalence and distribution of antibiotic resistance determinants in the receiving waters [229–232].

This thesis examined the “impact” of the discharge of antibiotics and ARGs by WWTP effluents on the aquatic resistome and the composition of microbial communities in three water reservoirs. The comparison and multivariate analysis of water ecosystems with increasing levels of pollution helped to explain the relationship between antibiotic resistance, antibiotic residues and bacterial populations in planktonic and sediment compartments. The main conclusions of this study were that concentration of both, antibiotics and ARGs, were higher in the reservoir most heavily impacted by WWTP discharges. It has been also possible to confirm that two bacterial groups, *Actinobacteria* and *Firmicutes*, were closely associated to the presence of ARGs in water and sediment [233]. As in the case of other studies [229–231], statistical analyses showed significant correlations between the presence of antibiotics and ARGs; although no direct correlation was found between antibiotic concentration and the bacterial groups present in the samples. Nevertheless, the absence of direct associations between antibiotic concentrations and resistant bacteria may be due to the high persistence of ARGs within the bacterial community, even in the absence of selective factors [229,234].

Despite the limitations of the study, as only a few compounds and ARGs were measured, and that causal mechanisms could not be identified, the fact that the applied statistical analysis identified macrolides, *ermB* resistance gene, and *Actinobacteria* and *Firmicutes* as relevant variables, suggest that antibiotic pollution may play a role in shaping the bacterial communities inhabiting the studied reservoirs, as recently demonstrated in hospital and urban wastewater [229,230]. However, several ecological and environmental factors other than the direct (or indirect) effect of antibiotics may also contribute in modulating the phylogenetic composition and structure of bacterial communities, most of which could also stimulate gene transfer, promoting the spread of antibiotic resistance among environmental bacteria [230,233,235]. Further research is needed before assigning causality between the presence of antibiotics in the environment and the prevalence of antibiotic resistance. It is fundamental to determine if the presence of antibiotic resistance in a given environment is mostly due to the dissemination of ARGs released by WWTP discharges or, on the contrary, environmental resistance is stimulated by the presence of antibiotic residues in the WWTP effluent. The application of new and more powerful molecular techniques, such as massive parallel sequencing and metagenomics, could be very useful to resolve the source of ARGs and to precisely identify those members of the community that harbours resistance determinants. Moreover, the applications of these state-of-the-art molecular techniques would allow the identification of novel genes and genetic variants linked to antibiotic resistance [227,228]. These data will be fundamental to have a better understanding of the diversity, dynamics and fate of ARGs in the natural environment, and the actual role of sub-inhibitory concentrations of antibiotics on aquatic microbial communities [233].

Behavioural Alterations by a Psychoactive Drug

Fish behaviour has been used to study normal or pathological behaviours and modelling brain disorders in neuroscience research due to the similarities with human physiology [192,236–238]. In fact, the sensitivity of fish behaviour to pharmacological influence, including anxiolytic drugs, has undoubtedly been established [239–241]. However, despite the high ecological relevance of the application of this type of behavioural studies in the environment, the difficulty of measuring, interpreting and reproducing behavioural endpoints is something to take into consideration. In fact, inter-individual behavioural variability significantly influence the sensitivity of behavioural tests for ecotoxicology and may limit their use in risk assessment [148,167].This variability of fathead minnow behaviour when exposed to an stimulus includes a wide range of behaviours, from “freezing” to an increase in the exploratory activity, but also covers the total lack of response, slower swimming activity, or even “dashing” across the tank, which may be easily overlooked [212]. For that same reason, an appropriate selection of the behavioural endpoints is of vital importance, in order to ensure that the results are not due to erroneous interpretation on innate behaviour [242].

In this study, regardless of the statistical power derived from the experimental design, no behavioural effects could be determined at any exposure concentrations, including the one that produced H_TPCs in fish plasma. Lack of dose-response, high inter-individual and inter-tank behavioural variability, and drug plasma concentrations $<H_TPC$ in the responsive group, all collectively suggest that the decreased exploratory activity at 5 $\mu g\ L^{-1}$ may be independent by the drug exposure.

Nevertheless, the exposure experiment under controlled conditions was an efficient tool for investigating the bioaccumulation of a psychoactive drug, oxazepam, in different fish tissues, including muscle, brain, liver, and plasma. Although it is increasingly more obvious that internal concentrations of a contaminant in an exposed organism are the key to understand the link between external exposure and toxicological effects [243], this is currently not very well understood [244]. Firstly, information about the relationship between individual blood and tissue concentrations of psychiatric drugs in fish is very limited. In our study, highest concentrations of oxazepam were found in brain, followed by plasma and liver, while muscle presented the lowest values, which supports the current knowledge of the pharmacokinetic and distribution of this drug in mammals [199,200]. The mean tissue/plasma concentration ratios calculated ranged from 0to 16 for brain, 0 to 6.4 for liver and 0 to 7.8 for muscle, suggesting relatively low transportability of these compounds into muscle and liver, but higher transportability to the brain, as observed in previous studies [197]. Despite the drug not being overly lipophilic ($log\ D_{7.4}$= 2.24), the fact that that accumulation in the brain was significantly higher than in the other tissues, supports the idea that accumulation is mainly driven by lipophilicity in this case, as brain tissue contains a high proportion of lipids.

Significant positive correlations were found between plasma and tissue concentrations, although remarkably, brain had the lowest positive correlation with plasma ($r = 0.578$) compared to muscle and liver tissues, in agreement with a recent study in wild fish were concentrations of psychoactive drugs, sertraline, haloperidol and carbamazepine in brain, did not have a significant correlation with plasma concentrations [197]. It was suggested that this lack of strong correlation was due to the wide variation in brain/plasma ratios between individuals (up to 32-fold at the highest exposure concentration).

Regardless, these distribution and bioaccumulation data could be very useful to feed the future development of uptake and pharmacokinetics models able to predict distribution and bioaccumulation dynamics in conditions of chronic exposure, which would reduce the amount of experimental work needed for the ERA, and therefore speed up the process for current and future emerging contaminants.

Chapter 7

Conclusions

CONCLUSIONS

1. Three different analytical multi-residue methods were developed for the analysis of 61 pharmaceuticals (from 12 therapeutic families) and 22 endocrine disruptors (including 5 groups, such as hormones, flame retardants, parabens, antibacterials and plasticizers; and other related compounds such as caffeine) in fish tissues, invertebrates, and biofilm.
2. These methods achieved very low MDLs, in the range of 0.01–6.7 ng g^{-1} for PhACs, and between 0.08-2.4 ng g^{-1} in the case of EDCs. Each method was successfully applied to the trace determination of PhACs and EDCs in environmental samples from different freshwater sites.
3. A fourth analytical method was also developed for the analysis of the psychiatric drug oxazepam in fish plasma as well as in other fish tissues.
4. The methods developed are highly comprehensive, measuring up to 61 compounds simultaneously, in the case of biofilm.
5. Concentrations of endocrine disruptors in all matrices were in general higher (up to one order of magnitude) than those of pharmaceuticals. Most prevalent families were anti-inflammatories (diclofenac) in the case of pharmaceuticals, whereas the most predominant endocrine disruptors were flame retardants. The anti-inflammatory diclofenac and the flame retardant TBEP were the only compounds detected in all biological tissues analysed in wild biota.
6. Concentrations were significantly higher for all compounds in samples collected after WWTP effluent discharges to the surface water than those collected upstream the WWTP (where in the majority of the cases the compounds were not detected). For instance, in the case of biofilm samples only two EDCs (TBEP and triclosan) were detected at concentrations < 20 ng g^{-1}, while a total of 12 compounds were detected after the WWTP discharge at concentrations up to 200 ng g^{-1}. This data on occurrence highlights the influence of WWTP in the bioaccumulation of these types of emerging contaminants in freshwater organisms.

7. Calculated BAFs for both pharmaceuticals and endocrine disruptors were generally lower than 1000 (bioaccumulation is not considered significant when BAFs < 1000), apart from very few exceptions: TBEP, Bisphenol A, estrone.
8. In general, values of these emerging contaminants measured in wild biota, usually in the low ng per gram range (< 200 ng g^{-1}), were lower than those measured for other organic pollutants, such as pesticides, PCBs, PBDEs, etc., which are usually 1-2 orders of magnitude above these values.
9. No biomagnification of pharmaceuticals could be established. However, the flame retardant TBEP was consistently found across all food web compartments studied in slightly increasing concentrations.
10. Compounds with a $log\ K_{ow} \geq 3$ (estrone, TBEP, bisphenol A, triclosan, diclofenac) were present more often in biological tissues, however, when $log\ D_{o\ w} \geq 3$was considered, only EDCs followed this pattern.
11. Mechanisms other than lipophilicity and hydrophobic interactions are also influencing bioaccumulation of pharmaceuticals, as many pharmaceuticals with a $log\ D_{ow} < 3$ were also present in biological tissues.
12. Further research is needed to determine if processes such as active transport are inducing the uptake of these compounds, in particular in the case of ionisable compounds in a given environmental pH for instance such as pharmaceuticals.
13. Concentrations (< 200 ng g^{-1}) detected for the great majority of the compounds do not represent an immediate risk for the exposed organisms.
14. Not only antibiotics concentrations, but also ARGs *bla*TEM, *erm*B, *qnr*S, and *sul*I, were higher in the reservoir most heavily impacted by WWTP discharges (out of the three reservoirs studied). Dominant bacterial groups in water and sediments were identified. It was also possible to confirm that two bacterial groups, *Actinobacteria* and *Firmicutes*, were closely associated to the presence of ARGs in water and sediment.

15. Multivariate statistical analysis allowed the assessment of the role of macrolides, *ermB* resistance gene, *Actinobacteria* and *Firmicutes* in the conformation of bacterial communities in reservoirs via the spreading of ARGs. However, further research is needed before assigning causality, and the application new technologies, such as metagenomics, could be very useful to determine the spread of antibiotic resistance and to quantify if this is due to the dissemination of ARGs in WWTPs or to the presence of antibiotic residues in the environment.
16. In the oxazepam exposure experiments the highest concentration was found in brain, followed by plasma and liver, while muscle presented the lowest values. This distribution of oxazepam in fish supports the current knowledge of the pharmacokinetic and distribution of this drug in mammals.
17. Significant positive correlations between plasma and tissue concentration of oxazepam were found between plasma and tissue concentrations, and these data could be used to feed the future development of uptake and pharmacokinetics models able to predict distribution and bioaccumulation dynamics in conditions of chronic exposure.
18. The FPM demonstrated good degree of accuracy for the prediction of oxazepam uptake in fish plasma, despite the fact that it does not consider inter- or intra-species variability and that it was developed for very hydrophobic compounds.
19. No behavioural effects were observed at any exposure concentrations, including the one that produced HTPCs in fish plasma. Lack of dose-response, high inter-individual and inter-tank behavioural variability, and drug plasma concentrations <H_TPC in the responsive group, all collectively suggest that the decreased exploratory activity at 5 μg L^{-1} may be independent by the drug exposure.
20. Despite the high ecological relevance of behavioural studies, the difficulty of measuring, interpreting and reproducing behavioural endpoints is something to consider. In fact, inter-individual behavioural variability may significantly influence the sensitivity of behavioural tests for ecotoxicology and limit their use in risk assessment.

FUTURE RECOMENDATIONS

Based on the results obtained in this thesis, it is evident that bioaccumulation of PhACs and EDCs is occurring in aquatic environments, particularly in those streams heavily impacted by WWTPs. The long-term consequences of this issue for aquatic organisms are still widely unknown. The link between bioaccumulation and harmful biological effects is a knowledge gap that should be considered in the future in order to determine if the presence of these compounds in the environment should be regulated. Particularly in the case of PhACs, the internal concentration of a drug (e.g. in the blood or in a target tissue) is what ultimately induces pharmacological or toxicological responses in the organisms, so the analysis of fish plasma in the wild could be very useful to determine the bioavailability of these compounds, and therefore, characterise the effect concentrations in order to feed Environmental Risk Assessment (ERA) studies. This is crucial for those compounds recurrently detected in wild biota, such as diclofenac, TBEP or triclosan, especially considering that very few studies exist on their harmful effects to different aquatic organisms following chronic exposure or even in the successive generations.

Chapter 8

Appendix

APPENDIX A: Supplementary Material for the article *Huerta et al., 2013. Journal of Chromatography A 1288, pp. 62 - 72*

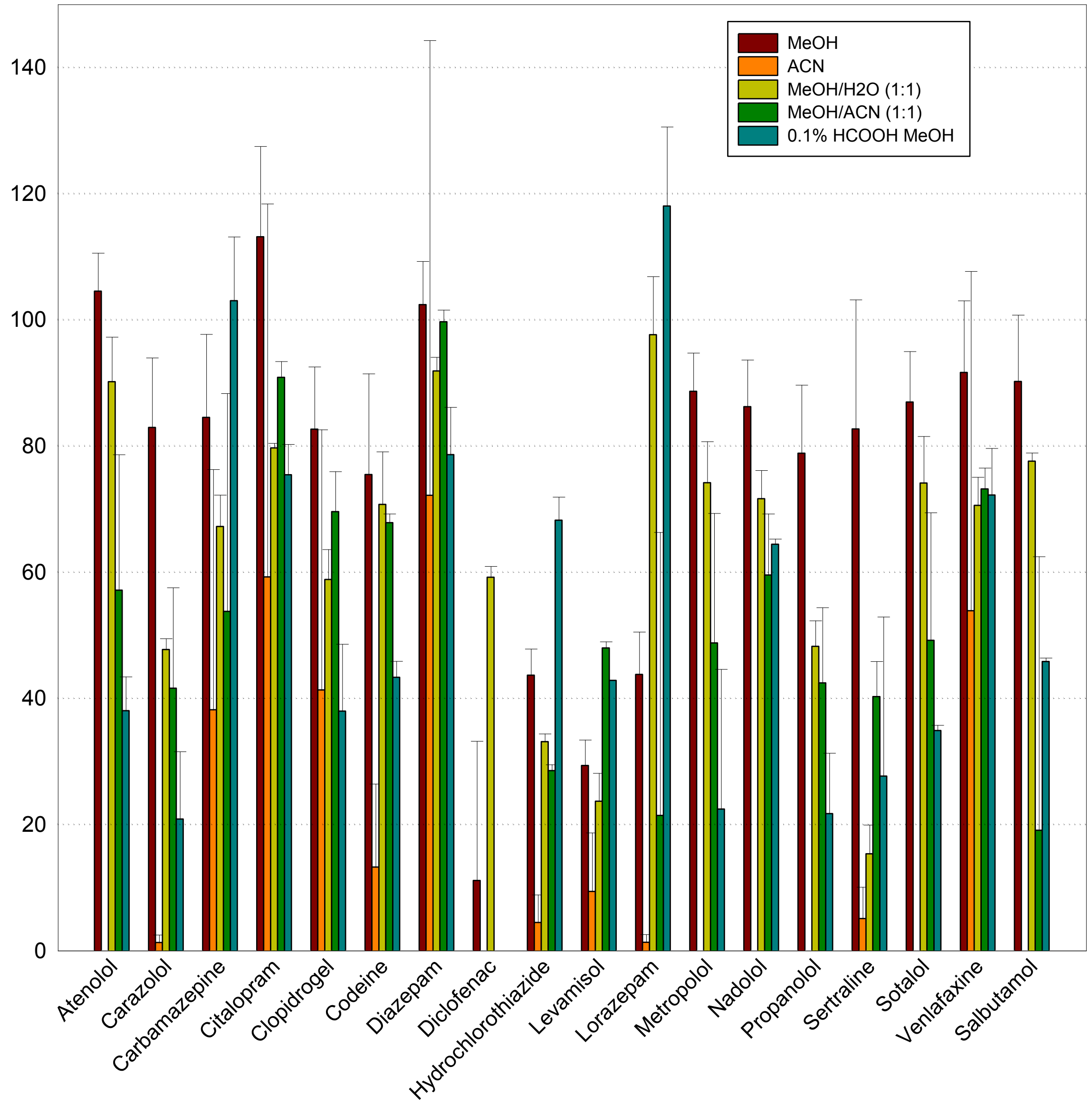


Fig. S1 Comparison of extraction efficiencies (%) between five extraction solvents at 50 °C for selected pharmaceutical compounds (n=3)

Table S1 Matrix effects (%) of the analytes in fish homogenate, liver and muscle tissues (n=3; spiking level= 5 ng/g), calculated as [100 – [(area analyte in matrix - area analyte in blank) x 100]/ area analyte in blank]. Negative values indicate signal suppression, and positive values indicate signal enhancement.

	Matrix Effects (%) (RSD %)					
	TISSUES			**SPECIES**		
	Liver	Muscle	Homogenate	*Cyprinus carpio*	*Barbus graellsii*	*Silurus glanis*
Atenolol	-37.1 (2.6)	-27.1 (2.8)	-8 (14.4)	-0.1 (17.1)	4.2 (1.5)	-5.6 (4.5)
Carazolol	10.9 (2.7)	-0.7 (1.4)	-13.1 (10.9)	-3.5 (15.3)	-8.0 (13.2)	-27.3 (9.3)
Carbamazepine	-54.5 (3.1)	-26.4 (0.8)	-8 (5.5)	1.4 (16.5)	-4.3 (8.9)	-14.9 (5.8)
Citalopram	-50.2 (0.7)	-45.1 (1.4)	-10.4 (4.3)	-8.8 (4.3)	-7.6 (17.2)	-32.9 (8.9)
Clopidrogel	-83.7 (1.0)	-86.8 (0.8)	-3.9 (0.1)	4.9 (14.5)	1.6 (7.8)	-9.1 (4.0)
Codeine	-31.8 (1.1)	-23.4 (2.7)	-18.4 (1.4)	-17.6 (9.3)	-13.7 (7.1)	-24.0 (4.5)
Diazepam	-64.1 (3.3)	-69.5 (3.1)	-11.4 (0.3)	- 8.4 (5.7)	-11.2 (13.5)	-28.0 (9.9)
Diclofenac	19.3 (5.5)	4.2 (3.4)	1 (6.8)	5.0 (6.0)	2.5 (11.9)	-5.2 (15.8)
10,11-epoxyCBZ	-59.8 (1.1)	-29.8 (1.1)	-20 (1.8)	-10.1 (18.5)	-5.3 (17.1)	-27.0 (6.1)
Hydrochlorothiazide	-50.4 (4.6)	-16.5 (2.3)	6.5 (8.9)	42.3 (6.9)	27.7 (19.9)	86.5 (5.4)
2-HydroxyCBZ	-49 (0.2)	-23.7 (3.9)	-9.9 (5.1)	-4.5 (10.1)	-1.9 (13.1)	-14.2 (6.3)
Levamisol	-1.9 (3.2)	4.5 (0.5)	-1.1 (7.5)	6.8 (13.7)	3.2 (11.3)	-14.7 (9.2)
Lorazepam	-69.5 (0.7)	-60.6 (1.3)	-1.8 (3.4)	22.0 (9.5)	12.9 (14.8)	-78.6 (7.2)
Metropolol	-28.1 (1.6)	-19.6 (1.9)	-16.4 (2.2)	-4.2 (7.2)	-11.6 (10.3)	-25.1 (13.1)
Nadolol	-39.1 (2.9)	-24.5 (2.1)	-24.1 (1.3)	-22.9 (2.4)	-20.4 (2.6)	-18.7 (5.4)
Propanolol	-40.2 (0.2)	-61.0 (4.5)	-24.8 (2.9)	-1.5 (12.1)	-14.0 (14.5)	-50.8 (5.7)
Salbutamol	-38.3 (10.1)	-21.4 (12.1)	-18.6 (0.6)	-10.3 (3.3)	-15.8 (7.2)	-37.1 (13.3)
Sertraline	-55.2 (4.7)	-15.6 (3.7)	54.3 (24.2)	60.2 (8.0)	41.3 (9.6)	58.1 (12.2)
Sotalol	-29.7 (2.2)	-49.6 (8.0)	-17.1 (4.2)	-1.9 (4.5)	-8.9 (2.2)	-20.4 (10.0)
Venlafaxine	-25.8 (0.4)	-15.3 (3.0)	-4.9 (2.7)	-3.4 (3.3)	-4.4 (11.1)	-25.7 (18.7)

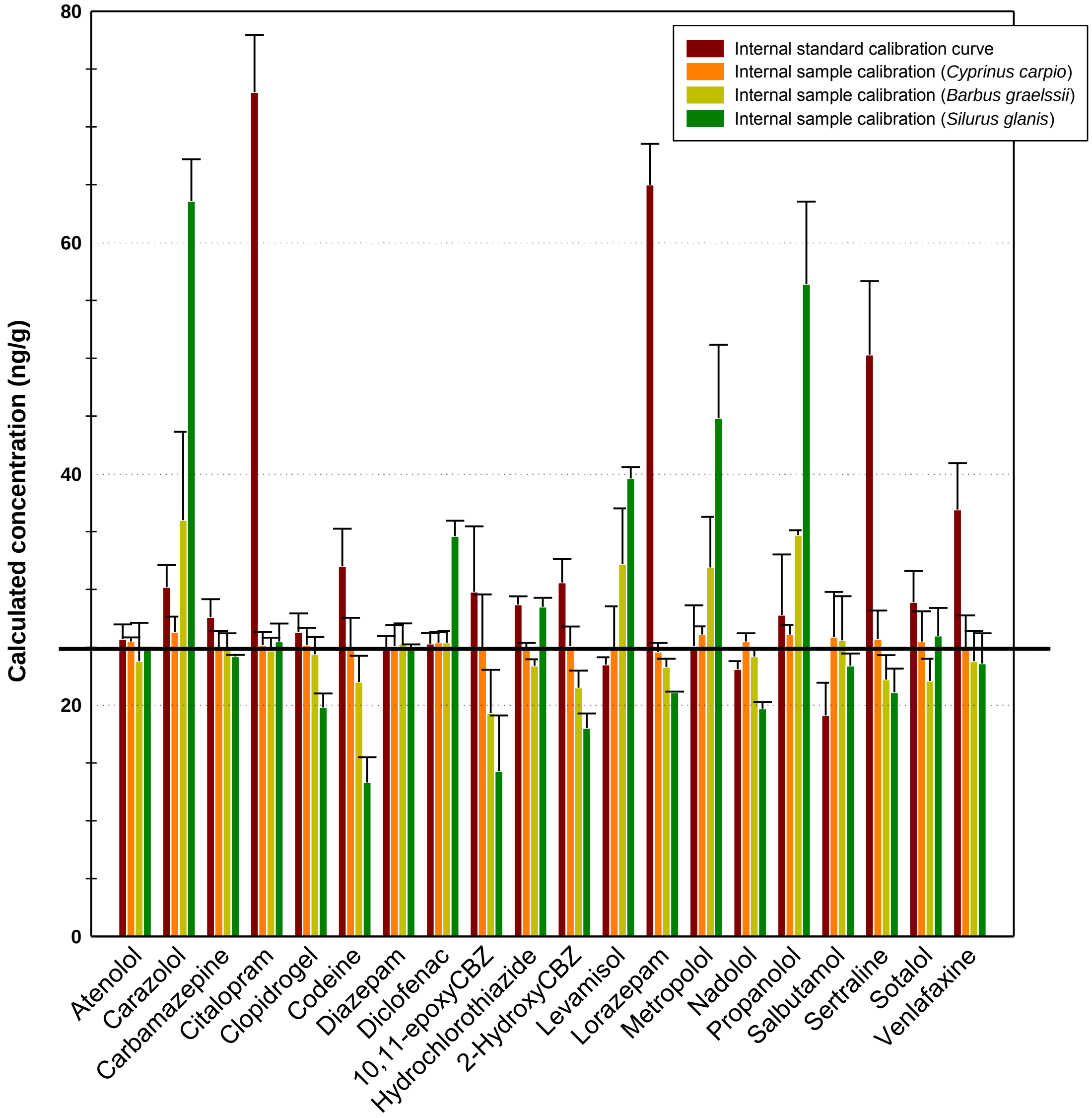


Fig. S2 Quantification values of GPC purified extracts from *Cyprinus carpio* spiked with a pharmaceutical mixture at 25 ppb (black line) obtained by different calibration techniques (internal standard calibration, and internal sample calibration prepared in extracts of the following species: *Cyprinus carpio*, *Barbus graellsii* and *Silurus glanis*.

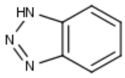
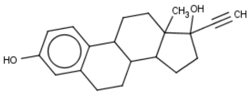
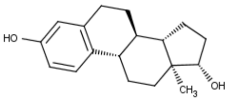
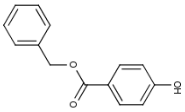
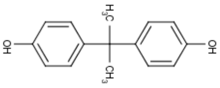
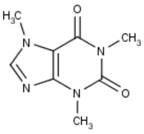
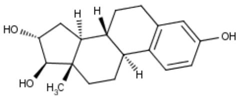
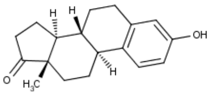
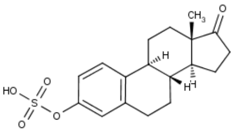
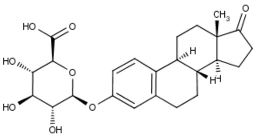
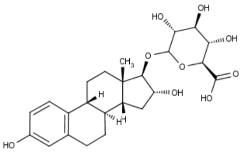
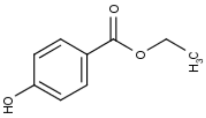
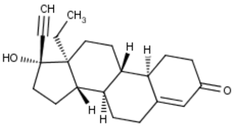
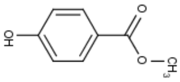
APPENDIX B: Supplementary Material for the article *Huerta et al., 2015. Talanta 132, pp. 373 - 381*

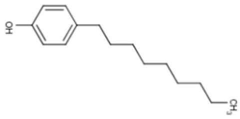
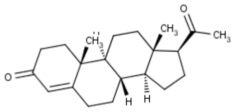
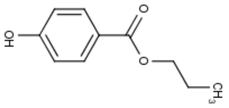
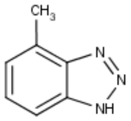
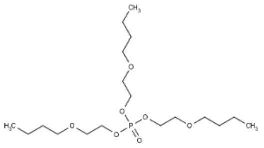
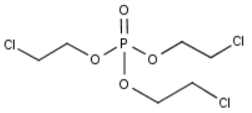
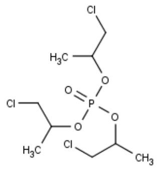
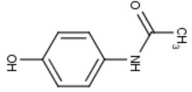
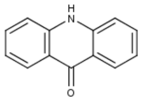
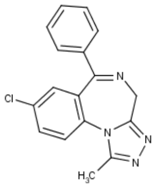
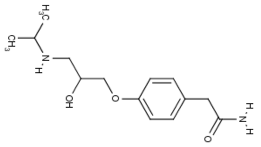
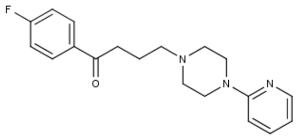
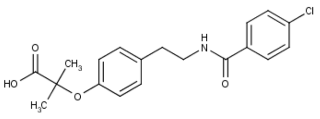
Table S1 Bioaccumulation of PhACs and EDCs in macroinvertebrates in previous studies

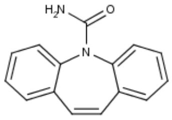
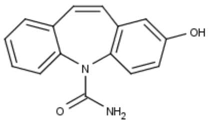
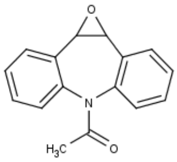
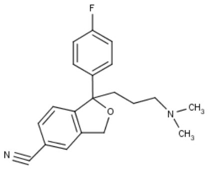
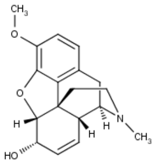
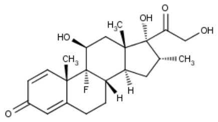
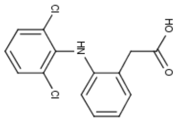
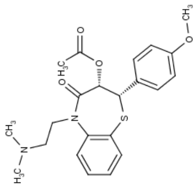
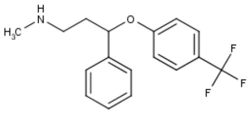
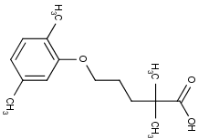
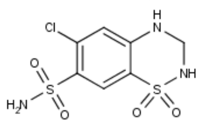
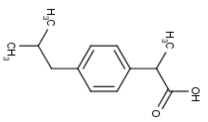
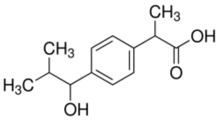
Exposure Studies			
Compound	**Organism**	**Bioconcentration Factor**	**Reference**
17-α-ethinylestradiol	*Chironomus tentans, Hyalella azteca*	18-215	[1]
Estrone	*Daphnia magna*	228	[2]
Levonorgestrel	Zebra mussel	25-208	[3]
Carbamazepine	*Thamnocephalus platyurus*	12.6	[4]
Fluoxetine	*G. pulex*	185,900	[5]
Hydroxyzine, Fexofenadine	Damselfly larvae	2000	[6]
Field Studies			
Compound	**Organism**	**Concentration**	**Reference**
Fluoxetine	*Elliption complanata*	79 ng g^{-1}	[7]
Antibiotics	Marine mollusks	1.5 µg g^{-1}	[8]

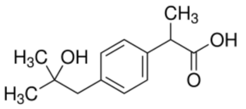
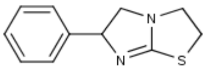
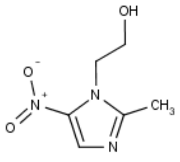
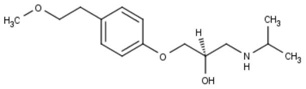
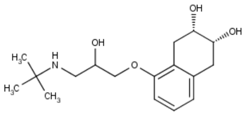
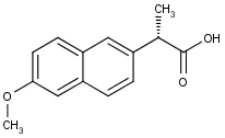
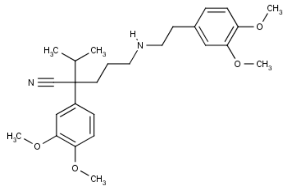
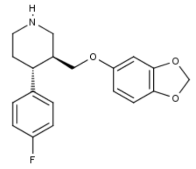
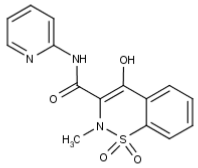
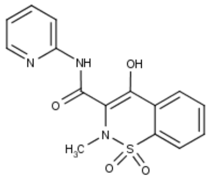
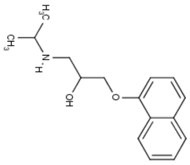
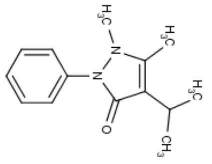
1. Dussault EB, Balakrishnan VK, Borgmann U, Solomon KR & Sibley PK (2009) Bioaccumulation of the synthetic hormone 17alfa-ethinylestradiol in the benthic invertebrates Chironomus tentans and Hyalella azteca. *Ecotoxicol Environ Saf* **72**, 1635-1641.
2. Gomes RL, Deacon HE, Lai KM, Birkett JW, Scrimshaw MD & Lester JN (2004) An assessment of the bioaccumulation of estrone in Daphnia magna. *Environ Toxicol Chem* **23**, 105-108.
3. Contardo-Jara V, Lorenz C, Pflugmacher S, Nützmann G, Kloas W & Wiegand C (2011) Molecular effects and bioaccumulation of levonorgestrel in the non-target organism Dreissena polymorpha. *Environmental Pollution* **159**, 38-44.
4. Vernouillet G, Eullaffroy P, Lajeunesse A, Blaise C, Gagné F & Juneau P (2010) Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. *Chemosphere* **80**, 1062-1068.
5. Meredith-Williams M, Carter LJ, Fussell R, Raffaelli D, Ashauer R & Boxall ABA (2012) Uptake and depuration of pharmaceuticals in aquatic invertebrates. *Environ Pollut* **165**, 250-258.
6. Jonsson M, Fick J, Klaminder J & Brodin T (2013) Antihistamines and aquatic insects: Bioconcentration and impacts on behavior in damselfly larvae (Zygoptera). *Science of The Total Environment* **472**, 108-111.
7. Bringolf RB, Heltsley RM, Newton TJ, Eads CB, Fraley SJ, Shea D & Cope WG (2010) Environmental occurrence and reproductive effects of the pharmaceutical fluoxetine in native freshwater mussels. *Environ Toxicol Chem* **29**, 1311-1318.
8. Li W, Shi Y, Gao L, Liu J & Cai Y (2012) Investigation of antibiotics in mollusks from coastal waters in the Bohai Sea of China. *Environ Pollut* **162**, 56-62.

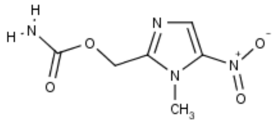
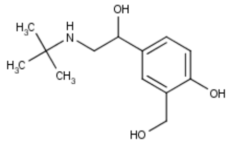
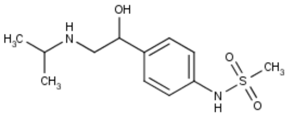
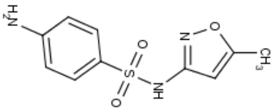
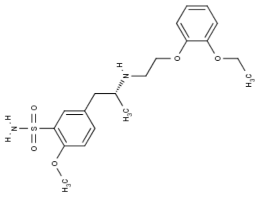
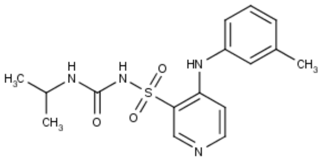
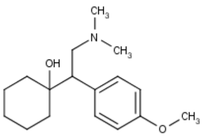
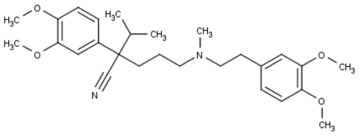
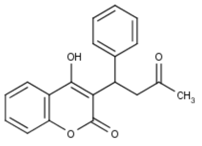
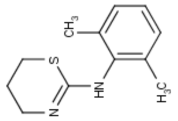
Table S2 List of compounds (PhACs & EDCs) and physic-chemical characteristics

	Family	**Chemical Structure**	**Precursor ion**	**Internal standard**	**log P**
		Endocrine Disruptors & related compounds			
1-H-benzotriazole	Triazoles		120.1 [M+H]$^+$	1-H-benzothiazole-d$_4$	1.3
17α-ethinylestradiol	Hormones		295.1 [M-H]$^-$	17α-ethinylestradiol-d$_4$	3.9
17β- Estradiol	Hormones		271.0 [M-H]$^-$	17β- Estradiol-d$_2$	3.7
Benzylparaben	Preservatives		227.0 [M-H]$^-$	Methylparaben-d$_4$	3.4
Bisphenol A	Plasticizers		227.0 [M-H]$^-$	Bisphenol A-d$_4$	4.0
Caffeine	Stimulants		195.0 [M+H]$^+$	Caffeine-d$_3$	-0.5
Estriol	Hormones		287.0 [M-H]$^-$	Estrone-d$_4$	2.6
Estrone	Hormones		269.1 [M-H]$^-$	Estrone-d$_4$	4.3
E1-3S	Hormones		349.0 [M-H]$^-$	Estrone-d$_4$	3.8
E1-3G	Hormones		445.0 [M-H]$^-$	Estrone-d$_4$	2.3
E2-17G	Hormones		447.0 [M-H]$^-$	17α-ethinylestradiol-d$_4$	1.2
Ethylparaben	Preservatives		165.0 [M-H]$^-$	Methylparaben-d$_4$	2.0
Levonorgestrel	Hormones		313.0 [M+H]$^+$	Progesterone-d$_8$	3.6
Methylparaben	Preservatives		151.0 [M-H]$^-$	Methylparaben-d$_4$	1.6

Nonylphenol	Alkylphenols		219.0 [M-H]$^-$	Octylphenol-d$_{17}$	5.7
Progesterone	Hormones		315.0 [M+H]$^+$	Progesterone-d$_8$	4.1
Propylparaben	Preservatives		179.0 [M-H]$^-$	Methylparaben-d$_4$	2.5
Tolyltriazole	Triazoles		134.1 [M+H]$^+$	1-H-benzothiazole-d$_4$	1.7
TBEP	Flame retardants		399.0 [M+H]$^+$	Trisphenyl phosphate-d$_{15}$	3.9
TCEP	Flame retardants		284.8 [M+H]$^+$	Trisphenyl phosphate-d$_{15}$	2.1
TCPP	Flame retardants		326.9 [M+H]$^+$	Trisphenyl phosphate-d$_{15}$	3.3
		Pharmaceuticals			
Acetaminophen	Analgesics/anti-inflammatories		154 [M-H]$^-$	Acetaminophen-d$_4$	1.6
Acridone*	Psychiatric drugs		196 [M+H]$^+$	Carbamazepine-d$_{10}$	4.2
Alprazolam	Psychiatric drugs		309 [M+H]$^+$	Diazepam-d$_5$	2.3
Atenolol	β-Blocking agents		267 [M+H]$^+$	Atenolol-d$_7$	0.4
Azaperone	Tranquilizer		328 [M+H]$^+$	Azaperone-d$_4$	3.2
Bezafibrate	Lipid regulators		360 [M-H]$^-$	Bezafibrate-d$_6$	3.9

Carbamazepine	Psychiatric drugs		237[M+H]$^+$	Carbamazepine-d_{10}	2.7
2-OH-CBZ*	Psychiatric drugs		253 [M+H]$^+$	Carbamazepine-d_{10}	2.4
10,11-epoxy-CBZ*	Psychiatric drugs		253 [M+H]$^+$	Carbamazepine-d_{10}	2.3
Citalopram	Psychiatric drugs		325 [M+H]$^+$	Citalopram-d_4	3.7
Codeine	Analgesics/anti-inflammatories		300 [M+H]$^+$	Carbamazepine-d_{10}	1.3
Dexamethasone	Synthetic glucocorticoid		451 [M-H]$^-$	Dexamethasone-d_4	1.6
Diclofenac	Analgesics/anti-inflammatories		294 [M-H]$^-$	Ibuprofen-d_3	4.2
Diltiazem	Calcium channel blockers		415 [M+H]$^+$	Carbamazepine-d_{10}	2.7
Fluoxetine	Psychiatric drugs		310 [M+H]$^+$	Fluoxetine-d_5	4.1
Gemfibrozil	Lipid regulators		249 [M-H]$^-$	Gemfibrozil-d_6	4.3
Hydrochlorothiazide	Diuretic		296 [M-H]$^-$	Hydrochlorothiazide-d_2	-0.5
Ibuprofen	Analgesics/anti-inflammatories		205 [M-H]$^-$	Ibuprofen-d_3	3.8
1-OH-Ibuprofen*	Analgesics/anti-inflammatories		221 [M-H]$^-$	Ibuprofen-d_3	2.6

2-OH-Ibuprofen*	Analgesics/anti-inflammatories		221 [M-H]$^-$	Ibuprofen-d_3	2.3
Levamisol	Antihelmintics		205 [M+H]$^+$	Ronidazole-d_3	2.3
Metronidazole	Antibiotics		172 [M+H]$^+$	Ronidazole-d_3	-0.4
Metoprolol	β-Blocking agents		268 [M+H]$^+$	Atenolol-d_7	1.7
Nadolol	β-Blocking agents		310 [M+H]$^+$	Atenolol-d_7	0.8
Naproxen	Analgesics/anti-inflammatories		229 [M-H]$^-$	Ibuprofen-d_3	2.9
Norverapamil*	Calcium channel blockers		441 [M+H]$^+$	Verapamil-d_6	4.6
Paroxetine	Psychiatric drugs		330 [M+H]$^+$	Fluoxetine-d_5	3.1
Piroxicam	Analgesics/anti-inflammatories		330 [M-H]$^-$	Meloxicam-d_3	0.6
Pravastatin	Lipid regulators		423 [M-H]$^-$	Gemfibrozil-d_6	1.6
Propanolol	β-Blocking agents		260 [M+H]$^+$	Atenolol-d_7	2.5
Propyphenazone	Analgesics/anti-inflammatories		231 [M+H]$^+$	Phenazone-d_3	2.3

Ronidazole	Antibiotics		201 [M+H]$^+$	Ronidazole-d$_3$	-0.4
Salbutamol	To treat asthma		240 [M+H]$^+$	Atenolol-d$_7$	0.3
Sotalol	β-Blocking agents		273 [M+H]$^+$	Atenolol-d$_7$	-0.4
Sulfamethoxazole	Antibiotics		254 [M+H]$^+$	Sulfamethoxazole- d$_4$	0.7
Tamsulosin	Prostatic hyperplasia		409 [M+H]$^+$	Sulfamethoxazole-d$_4$	2
Torasemide	Diuretic		347 [M-H]$^-$	Furosemide-d$_5$	1.8
Venlafaxine	Psychiatric drugs		278 [M+H]$^+$	Venlafaxine-d$_6$	2.7
Verapamil	Calcium channel blockers		455 [M+H]$^+$	Verapamil-d$_6$	5.0
Warfarin	Anticoagulant		309 [M+H]$^+$	Warfarin-d$_5$	2.7
Xylazine	Sedation and muscle relaxation		221 [M+H]$^+$	Xylazine-d$_6$	3.6

*metabolite

Standards and reagents

- EDCs

1-H-benzotriazole, 17α-ethinylestradiol, 17β-Estradiol, Benzylparaben, Bisphenol A, Caffeine, Estriol, Estrone, Estrone sulfate, Estrone glucuronide, Estradiol glucuronide, Ethylparaben, Levonorgestrel, Methylparaben, Nonylphenol, Progesterone, Propylparaben, Tolyltriazole, TBEP, TCEP and TCPP were purchased from Sigma–Aldrich and presented a purity > 95 %. Isotopically labelled compounds, used as internal standards, estrone-d_4, 17 β-estradiol-d_2, 17 α-ethinylestradiol-d_4, BPA-d_4, methylparaben-d_4, 1H-benzotriazole-d_4 and caffeine-d_3 were purchased from CDN isotopes. Progesterone-d8 was from Cambridge Isotope Laboratories. Trisphenylphosphate-d15 was obtained from Sigma–Aldrich.

- PhACs

High purity grade (>95%) pharmaceutical standards acetaminophen, acridone, alprazolam, atenolol, bezafibrate, carbamazepine, citalopram, codeine, dexamethasone, diclofenac, diazepam, diltiazem, fluoxetine, gemfibrozil, hydrochlorothiazide, ibuprofen, levamisole, metronidazole, nadolol, naproxen, norverapamil, piroxicam, pravastatin, propanolol, propyphenazone, ronidazole, salbutamol, sotalol, sulfamethoxazole, tamsulosin, warfarin and xylazine were acquire from Sigma–Aldrich. Paroxetine, sertraline, torasemide and velafaxine were purchased from the European Pharmacopeia (EP). Metoprolol and verapamil were purchased from the US Pharmacopeia (USP). Metabolites 2-hydroxycarbamazepine (2-HydroxyCBZ) and 10, 11-epoxycarbamazepine (10, 11-EpoxyCBZ), 1-OH-ibuprofen, 2-OH-ibuprofen were purchased from Toronto Research Chemicals (TRC). Isotopically labeled compounds, used as internal standards, were xylazine-d_6, azaperone-d_4, ibuprofen-d_3, diazepam-d_5, meloxicam-d_3, ronidazole-d_3, ofloxacin-d_3, and fluoxetine-d_5 (as hydrochloride salts) from Sigma–Aldrich. Dexamethasone-d_4, bezafibrate-d_6, gemfibrozil-d_6, carbamazepine-d_{10}, citalopram-d_4 (as hydrobromide), atenolol-d_7, warfarin-d_5, and hydrochlorothiazide-d_2 were purchased from CDN isotopes (Quebec, Canada) and sulfamethoxazole-d_4, acetaminophen-d_4, venlafaxine-d_6, verapamil-d_6 (as hydrochloride salt) and furosemide-d_5 were from Toronto Research Chemicals (Ontario, Canada).

Table S3 PhACs and EDCs concentration (ng L^{-1}) measured before and after a WWTP effluent discharged in the river.

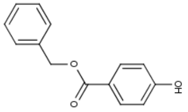
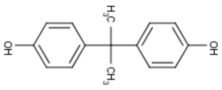
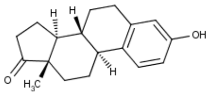
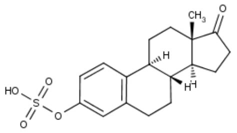
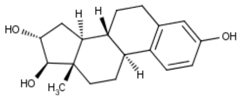
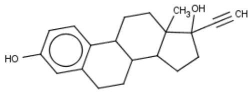
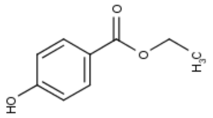
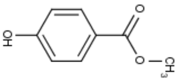
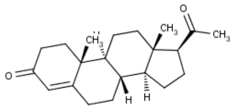
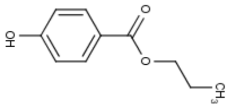
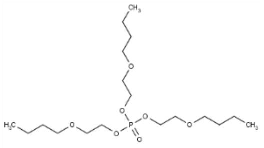
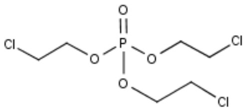
Family	**Compound**	**Upstream WWTP**	**Downstream WWTP**
Pharmaceuticals			
Analgesics/anti-inflammatories	Acetaminophen	<MQL	222
	Codeine	n.d.	33.7
	Diclofenac	n.d.	29.1
	Ibuprofen	n.d.	193
	2-hdroxy-ibuprofen	17	573
	1-hydroxy-ibuprofen	n.d.	68.7
Lipid regulators	Bezafibrate	n.d.	7.20
	Gemfibrozil	n.d.	286
	Pravastatin	n.d.	25.7
Psychiatric drugs	Carbamazepine	n.d.	23.6
	Venlafaxine	n.d.	13.8
ß-Blocking agents	Atenolol	n.d.	27.5
Diuretic	Hydrochlorothiazide	n.d.	311
Antibiotics	Sulfamethoxazole	n.d.	18.0
Endocrine Disruptors & related compounds			
Triazoles	1H benzotriazole	3.8	195
	Tolyltriazole	n.d.	261
Stimulants	Caffeine	51.5	207
Flame retardants	TBEP	n.d.	65.3
	TCPP	n.d.	71.5
Alkylphenols	Nonylphenol	90.56	117

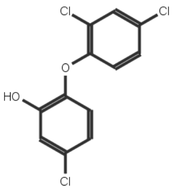
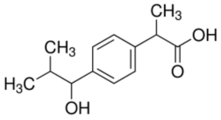
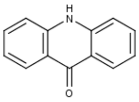
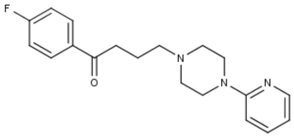
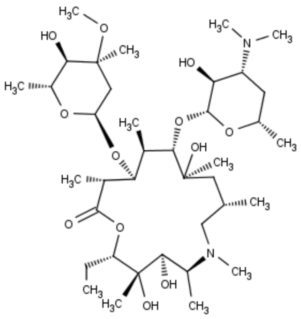
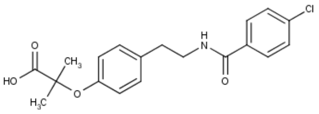
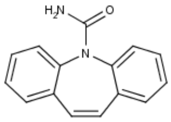
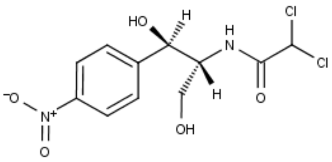
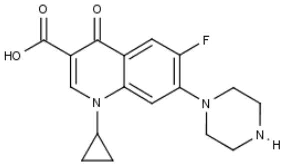
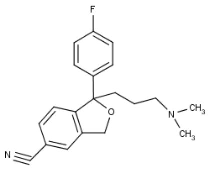
Table S4 Method detection and extraction efficiencies of EDCs in water samples

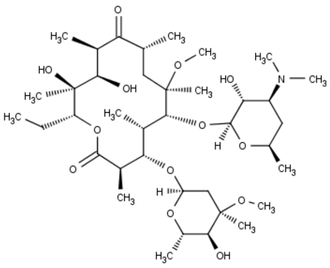
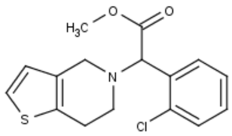
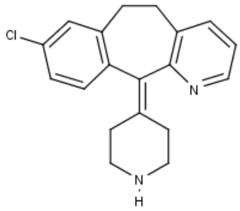
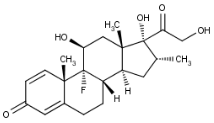
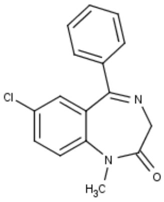
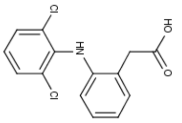
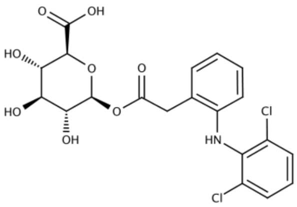
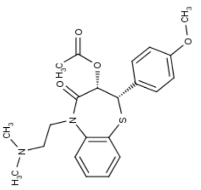
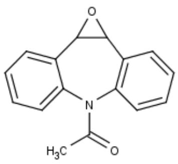
Compound	**MDL (ng L $^{-1}$)**	**MQL (ng L $^{-1}$)**	**Rec (%) ± SD**
1H-benzotriazole	2.4	8.0	97 ± 6.1
17α-ethinylestradiol	4.4	14	82 ± 2.4
17β- Estradiol	5.6	18	88 ± 2.1
Benzylparaben	1.8	6.0	132 ± 4.3
Bisphenol A	7.2	24	65 ± 6.6
Caffeine	4.8	16	105 ± 5.9
Estriol	12	43	96 ± 2.0
Estrone	1.5	5.1	97 ± 1.8
E1-3S	4.0	13	63 ± 1.9
E1-3G	4.6	15	37 ± 7.3
E2-17G	4.9	16	23 ± 8.2
Ethylparaben	3.5	112	122 ± 2.2
Levonorgestrel	9.2	31	76 ± 1.4
Methylparaben	4.9	13	83 ± 5.2
Nonylphenol	7.6	25	73 ± 7.9
Progesterone	0.8	2.6	110 ± 1.6
Propylparaben	2.5	8.4	139 ± 1.2
Tolyltriazole	5.6	19	47 ± 19
TCEP	3.3	11	72 ± 3.8
TBEP	0.80	2.8	65 ± 2.8
TCPP	1.9	6.4	66 ± 10

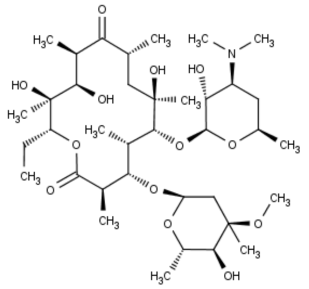
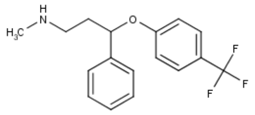
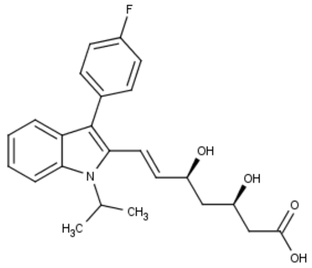
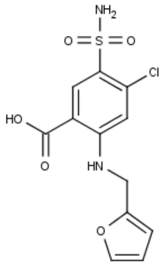
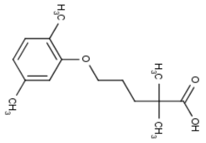
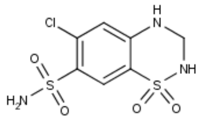
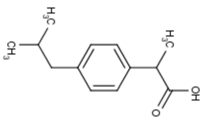
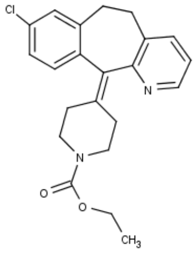
APPENDIX C: Supplementary Material for the article *Huerta et al., 2015. Science of Total Environment, doi: 10.1016/j.scitotenv.2015.05.049*

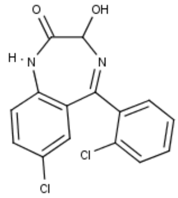
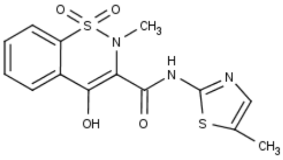
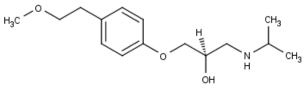
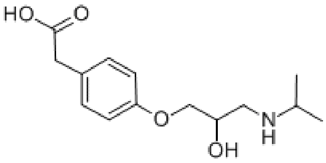
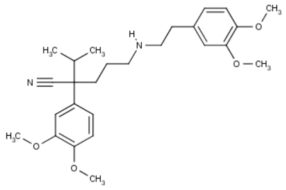
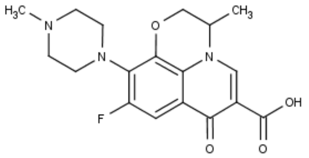
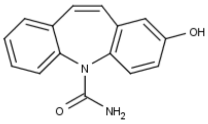
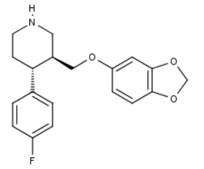
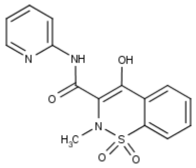
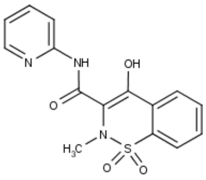
Table S1 List of compounds (PhACs & EDCs) and physic-chemical characteristics

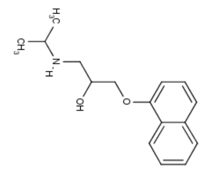
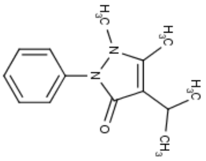
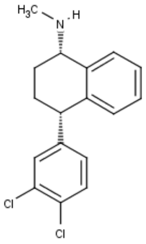
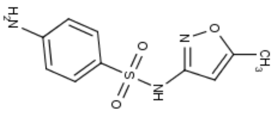
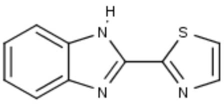
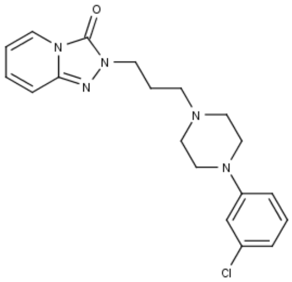
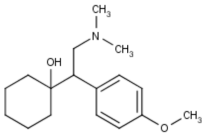
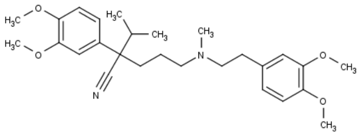
	Family	**Chemical Structure**	**Precursor ion**	**Internal standard**	**log P**
		Endocrine Disruptors & related compounds			
Benzylparaben	Preservatives		227.0 $[M-H]^-$	Methylparaben-d4	3.4
Bisphenol A	Plasticizers		227.0 $[M-H]^-$	Bisphenol A-d4	4.0
Estrone	Hormones		269.1 $[M-H]^-$	Estrone-d4	4.3
Estrone-3-sulfate	Hormones		349.0 $[M-H]^-$	Estrone-d4	3.8
Estriol	Hormones		287.0 $[M-H]^-$	Estrone-d4	2.6
17α-ethinylestradiol	Hormones		295.1 $[M-H]^-$	17α-ethinylestradiol-d4	3.9
Ethylparaben	Preservatives		165.0 $[M-H]^-$	Methylparaben-d4	2.0
Methylparaben	Preservatives		151.0 $[M-H]^-$	Methylparaben-d4	1.6
Progesterone	Hormones		315.0 $[M+H]^+$	Progesterone-d8	4.1
Propylparaben	Preservatives		179.0 $[M-H]^-$	Methylparaben-d4	2.5
TBEP	Flame retardants		399.0 $[M+H]^+$	Trisphenyl phosphate-d15	3.9
TCEP	Flame retardants		284.8 $[M+H]^+$	Trisphenyl phosphate-d15	2.1

Triclosan	Antibacterial		288.5 [M-H]$^-$	Triclosan Methyl-d3-ether	4.9
		Pharmaceuticals			
1-OH-Ibuprofen*	Analgesics/anti-inflammatories		221 [M-H]$^-$	Ibuprofen-d3	2.6
Acridone*	Psychiatric drugs		196 [M+H]$^+$	Carbamazepine-d10	4.2
Azaperone	Tranquilizer		328 [M+H]$^+$	Azaperone-d4	3.2
Azythromycin	Antibiotics		749 [M+H]$^+$	Azithromycin-d3	2.44
Bezafibrate	Lipid regulators		360 [M-H]$^-$	Bezafibrate-d6	3.9
Carbamazepine	Psychiatric drugs		237[M+H]$^+$	Carbamazepine-d10	2.7
Chloramphenicol	Antibiotics		321 [M-H]$^-$	-	0.88
Ciprofloxacin	Antibiotics		332 [M+H]$^+$	Ofloxacin-d3	-0.8
Citalopram	Psychiatric drugs		325 [M+H]$^+$	Citalopram-d4	3.7

Clarithromycin	Antibiotics		748 [M+H]$^+$	Azithromycin-d3	3.2
Clopidogrel	Antiplatelet agent		322 [M+H]$^+$	Diazepam-d5	4.0
Desloratadine*	Histamine H1 and H2 receptor antagonists		311 [M+H]$^+$	Cimetidine-d3	4.0
Dexamethasone	Synthetic glucocorticoid		451 [M-H]$^-$	Dexamethasone-d4	1.6
Diazepam	Psychiatric drugs		285 [M+H]$^+$	Diazepam-d5	3.1
Diclofenac	Analgesics/anti-inflammatories		294 [M-H]$^-$	Ibuprofen-d3	4.2
Diclofenac glucuronide*	Analgesics/anti-inflammatories		470 [M-H]$^-$	Ibuprofen-d3	2.3
Diltiazem	Calcium channel blockers		415 [M+H]$^+$	Carbamazepine-d10	2.7
10,11-epoxy-CBZ*	Psychiatric drugs		253 [M+H]$^+$	Carbamazepine-d10	2.3

Erythromycin	Antibiotics		734 [M+H]$^+$	Erythromycin-N,N$^{13}C_2$	2.6
Fluoxetine	Psychiatric drugs		310 [M+H]$^+$	Fluoxetine-d5	4.1
Fluvastatin	Lipid regulators and cholesterol lowering statin drugs		410 [M-H]$^-$	Gemfibrozil-d6	3.8
Furosemide	Diuretic		329 [M-H]$^-$	Furosemide-d5	1.7
Gemfibrozil	Lipid regulators		249 [M-H]$^-$	Gemfibrozil-d6	4.3
Hydrochlorothiazide	Diuretic		296 [M-H]$^-$	Hydrochlorothiazide-d2	-0.5
Ibuprofen	Analgesics/anti-inflammatories		205 [M-H]$^-$	Ibuprofen-d3	3.8
Loratadine	Histamine H1 and H2 receptor antagonists		483 [M+H]$^+$	Cimetidine-d3	4.5

Lorazepam	Psychiatric drugs		321 [M+H]$^+$	Diazepam-d5	3.5
Meloxicam	Analgesics/anti-inflammatories		350 [M-H]$^-$	Meloxicam-d3	1.6
Metoprolol	β-Blocking agents		268 [M+H]$^+$	Atenolol-d7	1.7
Metoprolol acid*	β-Blocking agents		268 [M+H]$^+$	Atenolol-d7	-1.2
Norverapamil*	Calcium channel blockers		441 [M+H]$^+$	Verapamil-d6	4.6
Ofloxacin	Antibiotics		362 [M+H]$^+$	Ofloxacin-d3	0.6
2-OH-CBZ*	Psychiatric drugs		253 [M+H]$^+$	Carbamazepine-d10	2.4
Paroxetine	Psychiatric drugs		330 [M+H]$^+$	Fluoxetine-d5	3.1
Piroxicam	Analgesics/anti-inflammatories		330 [M-H]$^-$	Meloxicam-d3	0.6
Pravastatin	Lipid regulators		423 [M-H]$^-$	Gemfibrozil-d6	1.6

Propanolol	β-Blocking agents		260 $[M+H]^+$	Atenolol-d7	2.5
Propyphenazone	Analgesics/anti-inflammatories		231 $[M+H]^+$	Phenazone-d3	2.3
Sertraline	Psychiatric drugs		307 $[M+H]^+$	Fluoxetine-d5	5.1
Sulfamethoxazole	Antibiotics		254 $[M+H]^+$	Sulfamethoxazole-d4	0.7
Thiabendazole	Antihelmintics		202 $[M+H]^+$	Ronidazole-d3	2.8
Trazodone	Psychiatric drugs		372 $[M+H]^+$	Fluoxetine-d5	3.1
Venlafaxine	Psychiatric drugs		278 $[M+H]^+$	Venlafaxine-d6	2.7
Verapamil	Calcium channel blockers		455 $[M+H]^+$	Verapamil-d6	5.0

Standards and reagents

- EDCs

17α-ethinylestradiol, Benzylparaben, Bisphenol A, Estriol, Estrone, Estrone sulfate, Ethylparaben, Methylparaben, Progesterone, Propylparaben, TBEP, TCEP and Triclosan were purchased from Sigma–Aldrich and presented a purity > 95 %. Isotopically labeled compounds, used as internal standards, estrone-d_4, 17 α-ethinylestradiol-d_4, BPA-d_4, Triclosan Methyl-d_3-ether and methylparaben-d_4 were purchased from CDN isotopes. Progesterone-d_8 was from Cambridge Isotope Laboratories. Trisphenylphosphate-d_{15} was obtained from Sigma–Aldrich.

- PhACs

High purity grade (>95%) pharmaceutical standards acridone, bezafibrate, carbamazepine, chloramphenicol, ciprofloxacin, citalopram, chlarithromycin, clopidogrel, desloratadine, dexamethasone, diclofenac, diazepam, diltiazem, erythromycin, fluoxetine, fluvastatin, furosemide, gemfibrozil, hydrochlorothiazide, ibuprofen, loratadine, lorazepam, meloxicam, norverapamil, ofloxacin, piroxicam, pravastatin, propanolol, propyphenazone, sulfamethoxazole, thiabendazole, trazodone and verapamil were acquire from Sigma–Aldrich. Paroxetine, sertraline and velafaxine were purchased from the European Pharmacopeia (EP). Metoprolol and verapamil were purchased from the US Pharmacopeia (USP). Metabolites 2-hydroxycarbamazepine (2-HydroxyCBZ) and 10, 11-epoxycarbamazepine (10, 11-EpoxyCBZ), 1-OH-ibuprofen, 2-OH-ibuprofen, diclofenac glucuronide, and metoprolol acid were purchased from Toronto Research Chemicals (TRC). Isotopically labeled compounds, used as internal standards, were azaperone-d_4, ibuprofen-d_3, diazepam-d_5, meloxicam-d_3, ronidazole-d_3, ofloxacin-d_3, and fluoxetine-d_5 (as hydrochloride salts) from Sigma–Aldrich. Dexamethasone-d_4, bezafibrate-d_6, gemfibrozil-d_6, carbamazepine-d_{10}, citalopram-d_4 (as hydrobromide), atenolol-d_7, cimetidine-d_3 and hydrochlorothiazide-d_2 were purchased from CDN isotopes (Quebec, Canada) and sulfamethoxazole-d_4, venlafaxine-d_6, verapamil-d_6 (as hydrochloride salt), azythromycin-d_3 and furosemide-d_5 were from Toronto Research Chemicals (Ontario, Canada).

Table S4 Method detection and extraction efficiencies of EDCs in water samples.

Compound	**MDL (ng L $^{-1}$)**	**MQL (ng L $^{-1}$)**	**Rec (%) ± SD**
Benzylparaben	1.8	6.0	132 ± 4.3
Bisphenol A	7.2	24	65 ± 6.6
Estrone	1.5	5.1	97 ± 1.8
Estrone-3-sulfate	4.0	13	63 ± 1.9
Estriol	12	43	96 ± 2.0
17α-ethinylestradiol	4.4	14	82 ± 2.4
Ethylparaben	3.5	112	122 ± 2.2
Methylparaben	4.9	13	83 ± 5.2
Progesterone	0.8	2.6	110 ± 1.6
Propylparaben	2.5	8.4	139 ± 1.2
TBEP	0.80	2.8	65 ± 2.8
TCEP	3.3	11	72 ± 3.8

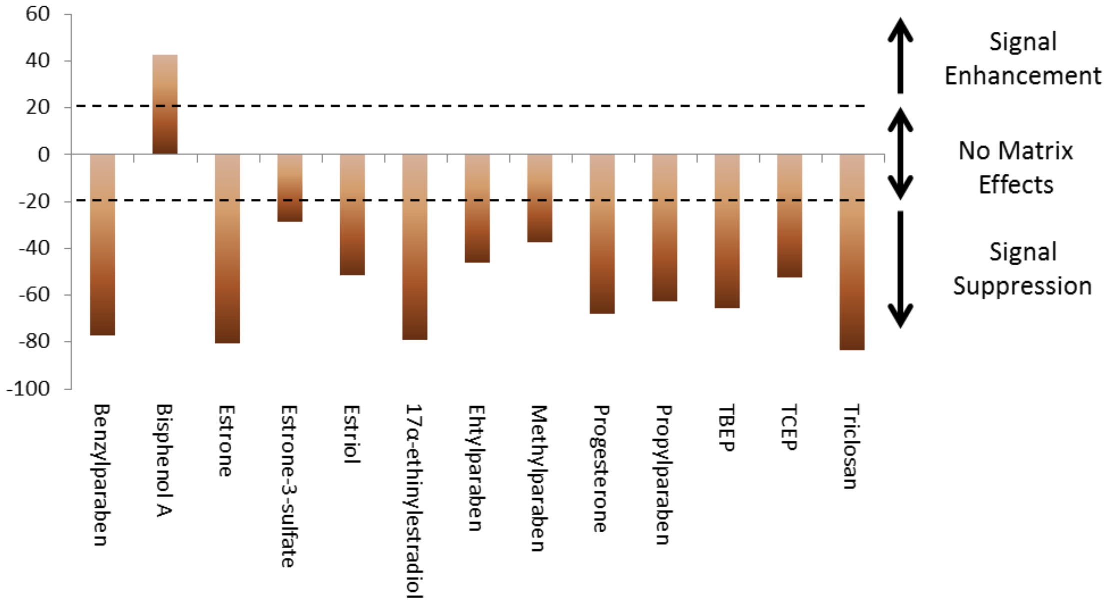


Fig S1. Signal enhancement or suppression for EDCs calculated as 1-[100-((Area std biofilm/Area std solvent)*100)].

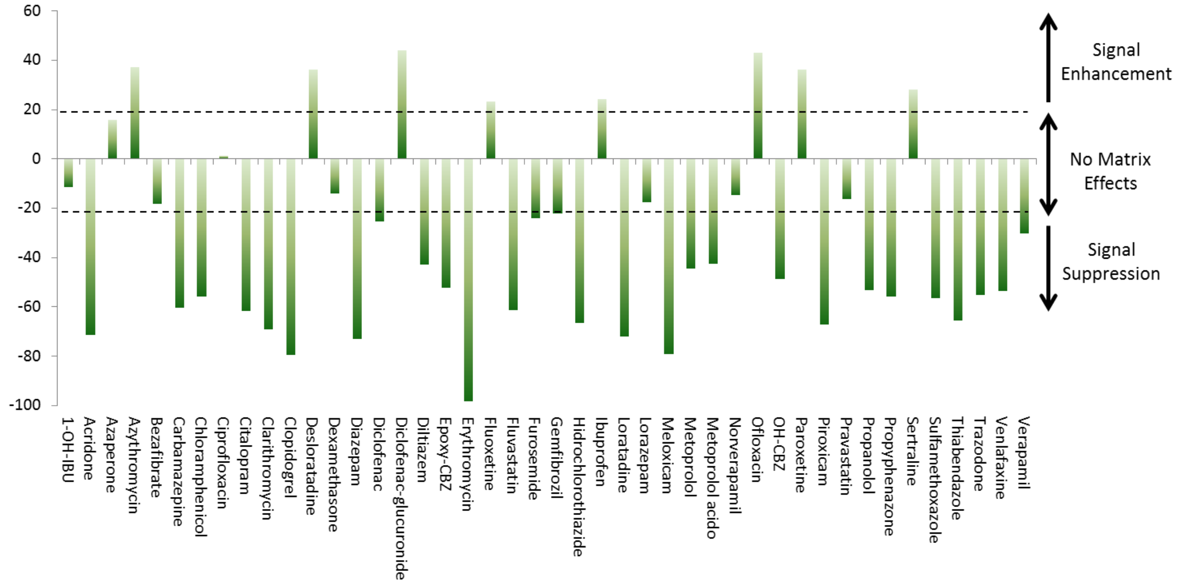


Fig S2. Signal enhancement or suppression for PhACs calculated as 1-[100-((Area std biofilm/Area std solvent)*100)].

Table S5 Physico-chemical properties (ionisation properties and polarity) of the compounds detected in biofilm from the River Segre.

	pka	log Kow	log D 7.4	Ionisable at pH 7.4	"+/-"
Endocrine Disruptors					
Ethylparaben	8.5	2.0	2.0		
Methylparaben	8.5	1.7	1.7		
Propylparaben	8.5	2.6	2.6		
TBEP	<0	3.9	3.9		
Triclosan	7.7	5.0	4.7		
Pharmaceuticals					
Diclofenac	4.0	4.3	1.1	x	-
Diltiazem	8.2	2.7	1.7	x	+
Gemfibrozil	4.4	4.4	1.5	x	-
Norverapamil	10.3	4.7	2.2	x	+
Venlafaxine	8.9	2.7	1.1	x	+
Verapamil	9.7	5.0	2.7	x	+

APPENDIX D: Supplementary Material for the article *Ruhi et al., Science of Total Environment; doi:10.1016/j.scitotenv.2015.06.009*

Table S1. List of pharmaceutical compounds (PhACs) and endocrine-disrupting compounds (EDCs) considered in this study, ordered by families within compound types. Associate physicochemical characteristics are shown (log D_{ow} were calculated at field pH = 8). * denotes metabolites.

Compound type	**Compound**	**Family**	**Precursor ion**	**Internal standard**	**log K_{ow}**	**log D_{ow}**
PhAC	Diclofenac	Analgesics/anti-inflammatories	294 $[M-H]^-$	Ibuprofen-d3	4.2	0.85
PhAC	Ibuprofen	Analgesics/anti-inflammatories	205 $[M-H]^-$	Ibuprofen-d3	3.8	0.85
PhAC	1-OH-Ibuprofen*	Analgesics/anti-inflammatories	221 $[M-H]^-$	Ibuprofen-d3	2.6	-0.5
PhAC	Piroxicam	Analgesics/anti-inflammatories	330 $[M-H]^-$	Meloxicam-d3	0.6	-1.74
PhAC	Propyphenazone	Analgesics/anti-inflammatories	231 $[M+H]^+$	Phenazone-d3	2.3	2.35
PhAC	Sulfamethoxazole	Antibiotics	254 $[M+H]^+$	Sulfamethoxazole- d4	0.7	-0.11
PhAC	Diltiazem	Calcium channel blockers	415 $[M+H]^+$	Carbamazepine-d10	2.7	2.33
PhAC	Verapamil	Calcium channel blockers	455 $[M+H]^+$	Verapamil-d6	5.0	3.36
PhAC	Norverapamil*	Calcium channel blockers	441 $[M+H]^+$	Verapamil-d6	4.6	2.41
PhAC	Hydrochlorothiazide	Diuretic	296 $[M-H]^-$	Hydrochlorothiazide-d2	-0.5	-0.58
PhAC	Bezafibrate	Lipid regulators	360 $[M-H]^-$	Bezafibrate-d6	3.9	0.55
PhAC	Gemfibrozil	Lipid regulators	249 $[M-H]^-$	Gemfibrozil-d6	4.3	1.14
PhAC	Pravastatin	Lipid regulators	423 $[M-H]^-$	Gemfibrozil-d6	1.6	-1.69
PhAC	Carbamazepine	Psychiatric drugs	237$[M+H]^+$	Carbamazepine-d10	2.7	2.77
PhAC	Acridone*	Psychiatric drugs	196 $[M+H]^+$	Carbamazepine-d10	4.2	4.2
PhAC	10,11-epoxy-CBZ*	Psychiatric drugs	253 $[M+H]^+$	Carbamazepine-d10	2.3	2.31
PhAC	2-OH-CBZ*	Psychiatric drugs	253 $[M+H]^+$	Carbamazepine-d10	2.4	2.43
PhAC	Citalopram	Psychiatric drugs	325 $[M+H]^+$	Citalopram-d4	3.7	1.98
PhAC	Fluoxetine	Psychiatric drugs	310 $[M+H]^+$	Fluoxetine-d5	4.1	2.38

PhAC	Paroxetine	Psychiatric drugs	330 [M+H]$^+$	Fluoxetine-d5	3.1	1.38
PhAC	Venlafaxine	Psychiatric drugs	278 [M+H]$^+$	Venlafaxine-d6	2.7	1.78
PhAC	Dexamethasone	Synthetic glucocorticoid	451 [M-H]$^-$	Dexamethasone-d4	1.6	1.68
PhAC	Azaperone	Tranquilizer	328 [M+H]$^+$	Azaperone-d4	3.2	3.15
PhAC	Metoprolol	β-Blocking agents	268 [M+H]$^+$	Atenolol-d7	1.7	0.09
PhAC	Propanolol	β-Blocking agents	260 [M+H]$^+$	Atenolol-d7	2.5	0.92
EDC	TBEP	Flame retardants	399.0 [M+H]$^+$	Trisphenyl phosphate-d15	3.9	3.94
EDC	TCEP	Flame retardants	284.8 [M+H]$^+$	Trisphenyl phosphate-d15	2.1	2.11
EDC	Estrone	Hormones	269.1 [M-H]$^-$	Estrone-d4	4.3	4.31
EDC	Estrone-3-sulfate	Hormones	349.0 [M-H]-	Estrone-d4	3.8	1.46
EDC	Estriol	Hormones	287.0 [M-H]$^-$	Estrone-d4	2.6	2.67
EDC	17α-ethinylestradiol	Hormones	295.1 [M-H]$^-$	17α-ethinylestradiol-d4	3.9	3.9
EDC	Progesterone	Hormones	315.0 [M+H]$^+$	Progesterone-d8	4.1	4.15
EDC	Bisphenol A	Plasticizers	227.0 [M-H]$^-$	Bisphenol A-d4	4.0	4.04
EDC	Benzylparaben	Preservatives	227.0 [M-H]$^-$	Methylparaben-d4	3.4	3.28
EDC	Ethylparaben	Preservatives	165.0 [M-H]$^-$	Methylparaben-d4	2.0	1.91
EDC	Methylparaben	Preservatives	151.0 [M-H]$^-$	Methylparaben-d4	1.6	1.56
EDC	Propylparaben	Preservatives	179.0 [M-H]$^-$	Methylparaben-d4	2.5	2.43

Table S2. Detected compounds (ng L^{-1} for water, ng g^{-1} for biofilm and invertebrates). PhACs = pharmaceutical compounds, EDCs = endocrine-disrupting compounds. C= control reach, I1 = 1st impact reach after effluent, I2 = 2nd impact reach after effluent, I3 = 3rd impact reach after effluent (I3). < *MDL* = below method detection limit; < *MQL* = below method quantification limit.

		PhACs											
		Diclofenac	*Ibuprofen*	*1-hydroxy-ibuprofen*	*Bezafibrate*	*Gemfibrozil*	*Hydrochlorothiazide*	*Carbamazepine*	*Venlafaxine*	*Sulfamethoxazole*	*Verapamil*	*Norverapamil*	*Diltiazem*
Water	**C**	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	I1	29.1	192.6	68.7	7.2	285.7	311.1	23.6	13.8	18	<MDL	<MDL	<MDL
	12	25.6	187.2	64.8	6.4	222.4	295.6	21.3	9.3	19.6	<MDL	<MDL	<MDL
	I3	19.9	115.1	46	4.5	157.1	221.1	15	2.4	23.2	<MDL	<MDL	<MDL
Biofilm	**C**	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	I1	23.5	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	43.7	<MDL	20.2	18.7	<MDL
	12	<MQL	<MDL	<MDL	<MDL	4	<MDL	<MDL	6.5	<MDL	11.1	11.2	11.8
	I3	<MQL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	14.4	4.2	4.7
Ancylus	**C**	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	I1	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	12	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	I3	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Hydropsyche	**C**	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	I1	10	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	12	12.4	182.7	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	I3	4.6	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Phagocata	**C**	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	I1	<MDL	30.9	<MDL	<MDL	43.9	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	12	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
	I3	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL

		EDCs				
		TBEP	*BPA*	*Ethylparaben*	*Methylparaben*	*Estrone*
Water	**C**	<MDL	<MDL	<MDL	<MDL	<MDL
	I1	65.3	<MDL	<MDL	<MDL	<MDL
	12	41.4	<MDL	<MDL	<MDL	<MDL
	I3	21.5	<MDL	<MDL	<MDL	<MDL
Biofilm	**C**	3.6	<MDL	<MDL	<MDL	<MDL
	I1	14.1	<MDL	20.3	176.4	<MDL
	12	17.3	<MDL	6.9	9	<MDL
	I3	4.8	<MDL	<MDL	<MDL	<MDL
Ancylus	**C**	7.7	<MDL	<MDL	<MDL	<MDL
	I1	22	<MDL	<MDL	<MDL	<MDL
	12	31.8	<MDL	<MDL	<MDL	<MDL
	I3	16.1	<MDL	<MDL	<MDL	<MDL
Hydropsyche	**C**	17.9	<MDL	<MDL	<MDL	<MDL
	I1	29.8	<MDL	<MDL	<MDL	<MDL
	12	31.1	8.7	<MDL	<MDL	<MDL
	I3	58.9	<MDL	<MDL	<MDL	<MDL
Phagocata	**C**	22.3	<MDL	<MDL	<MDL	17.6
	I1	30.9	<MDL	<MDL	<MDL	23.2
	12	20.6	<MDL	<MDL	<MDL	12.4
	I3	27.9	<MDL	<MDL	<MDL	17.8

Table S3. Observed (Obs) and predicted (Pred) log bioaccumulation factors (log BAFs, in L kg^{-1}) of detected compounds across reaches and invertebrate taxa (see text for details). C = control reach, I1 = 1st impact reach after effluent, I2 = 2nd impact reach after effluent, I3 = 3rd impact reach after effluent (I3).

Reach	**Compound**	**Ancylus**		**Hydropsyche**		**Phagocata**	
		obs	pred	obs	pred	obs	pred
C	Estrone					4.913	3.624
	TBEP	5.188	3.321	5.554	3.321	5.649	3.321
I1	1-hydroxy-ibuprofen	0.862	-0.541	0.862	-0.541	0.862	-0.541
	Bezafibrate	0.687	0.541	0.687	0.541	0.687	0.541
	Carbamazepine	0.104	2.361	0.104	2.361	0.104	2.361
	Diclofenac	0.943	0.787	2.536	0.787	0.943	0.787
	Estrone					5.033	3.624
	Gemfibrozil	-0.581	1.025	-0.581	1.025	2.187	1.025
	Hydrochlorothiazide	-0.794	-0.386	-0.794	-0.386	-0.794	-0.386
	Ibuprofen	0.220	0.787	0.220	0.787	2.205	0.787
	Sulfamethoxazole	1.295	0.000	1.295	0.000	1.295	0.000
	TBEP	2.528	3.321	2.659	3.321	2.675	3.321
	Venlafaxine	0.559	1.550	0.559	1.550	0.559	1.550
I2	1-hydroxy-ibuprofen	0.887	-0.541	0.887	-0.541	0.887	-0.541
	Bezafibrate	0.738	0.541	0.738	0.541	0.738	0.541
	BPA			4.735	3.403		
	Carbamazepine	0.149	2.361	0.149	2.361	0.149	2.361
	Diclofenac	0.998	0.787	2.685	0.787	0.998	0.787
	Estrone					4.761	3.624
	Gemfibrozil	-0.472	1.025	-0.472	1.025	-0.472	1.025
	Hydrochlorothiazide	-0.772	-0.386	-0.772	-0.386	-0.772	-0.386
	Ibuprofen	0.233	0.787	2.989	0.787	0.233	0.787
	Sulfamethoxazole	1.258	0.000	1.258	0.000	1.258	0.000
	TBEP	2.885	3.321	2.876	3.321	2.697	3.321
	Venlafaxine	0.730	1.550	0.730	1.550	0.730	1.550
I3	1-hydroxy-ibuprofen	1.036	-0.541	1.036	-0.541	1.036	-0.541

Bezafibrate	0.891	0.541	0.891	0.541	0.891	0.541
Carbamazepine	0.301	2.361	0.301	2.361	0.301	2.361
Diclofenac	1.108	0.787	2.364	0.787	1.108	0.787
Estrone					4.918	3.624
Gemfibrozil	-0.321	1.025	-0.321	1.025	-0.321	1.025
Hydrochlorothiazide	-0.646	-0.386	-0.646	-0.386	-0.646	-0.386
Ibuprofen	0.444	0.787	0.444	0.787	0.444	0.787
Sulfamethoxazole	1.185	0.000	1.185	0.000	1.185	0.000
TBEP	2.874	3.321	3.438	3.321	3.113	3.321
Venlafaxine	1.319	1.550	1.319	1.550	1.319	1.550

APPENDIX E: Supplementary Material for the article *Huerta et al. 2013 Science of the Total Environment 456-457 (2013) 161–170*

Table S.1. Real-time PCR primer sequences and reaction conditions

Target gene	Primers	Sequence	Conditions	Efficiency	Reference
16S rRNA	F1048 R1194	GTGSTGCAYGGYTGTCGTCA ACGTCRTCCMCACCTTCCTC	95ºC 3 min (1 cycle); 95ºC 15 sec and 60ºC 1 min (35 cycles)	98%	1
blaTEM	bla-TEM, F bla-TEM, R	GCKGCCAACTTACTTCTGACAACG CTTTATCCGCCTCCATCCAGTCTA	95ºC 3 min (1 cycle); 95ºC 15 sec and 60ºC 20 sec (40 cycles)	98%	2
qnrS	qnrSrtF11 qnrSrtR11	GACGTGCTAACTTGCGTGAT TGGCATTGTTGGAAACTTG	95ºC 3 min (1 cycle); 95ºC 15 sec and 62ºC 20 sec (40 cycles)	96%	3
sul(I)	Sul(I) FW Sul(I) RV	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	95ºC 3 min (1 cycle); 95ºC 15 sec and 65ºC 20 sec (40 cycles)	97%	4
ermB	erm(B)-91f erm(B)-454r	GATACCGTTTACGAAATTGG GAATCGAGACTTGAGTGTGC	95ºC 3 min (1 cycle); 95ºC 15 sec and 58ºC 20 sec (40 cycles)	98%	5

1. Maeda, H., C. Fujimoto, Y. Haruki, T. Maeda *et al*. 2003. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tet*Q gene and total bacteria. *FEMS Immunology and Medical Microbiology* 39: 81-86.
2. Xi, C. and others 2009. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Applied and Environmental Microbiology* 75: 5714-5718.
3. Marti, E., and J.L. Balcazar. 2012. Real-time PCR assays for quantification of qnr genes in samples from different sources. Appl. Environ. Microbiol. 2013; 79: 1743-1745.
4. Pei, R., S.C. Kim, K. H. Carlson, and A. Pruden. 2006. Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Research* 40: 2427-2435.
5. Chen, J., Z. Yu, F. C. Michel, Jr., T. Wittum, and M. Morrison. 2007. Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. *Applied and Environmental Microbiology* 73: 4407-4416.

Table S.2. Physico-chemical characterization of water samples and sediments from the three reservoirs (La Llosa del Cavall, Sau, and Foix). Data are for the period 2010-2011 and includes samples from all sampling points in each reservoir. For some analyses of the sediments at La Llosa, only samples from the dam station were available (marked with an asterisk), and samples for percent organic carbon and nitrogen were lost (na).

		La Llosa		**Sau**		**Foix**	
		Min	Max	Min	Max	Min	Max
				Water column			
Water temperature	ºC	6	18.3	7	22.2	11.2	22.4
Dissolved oxygen	mg L^{-1}	7.9	9.6	0.1	11	5.7	15.7
pH		8.3	8.8	8.1	9.4	8.1	9.4
Nitrate	mmol L^{-1}	19	28	104	209	11	354
Ammonia	mmol L^{-1}	0.2	4.5	1.2	9.9	1.7	177.3
Phosphate	mmol L^{-1}	0.4	26	13	3086	99	22632
Dissolved organic carbon	mg L^{-1}	1.1	2.2	2.6	8.2	3.6	8.4
Chloride	mg L^{-1}	24	30	17	36	112	208
Suspended solids	mg L^{-1}	0.7	1.9	1.4	27.1	4.4	65.2
Fe	mg L^{-1}	8	30	13	157	72	428
Mn	mg L^{-1}	4	11	30	242	105	630
Ligth extintion coefficient	m^{-1}	0.15	0.23	0.96	2.8	1.2	9.23
Density of live bacteria	10^{6} ind mL^{-1}	0.19	0.46	0.76	5.93	0.08	5.05
				Sediments			
Al	mg g^{-1}	43.8*	-	21.7	34.3	27.7	41.9
K	mg g^{-1}	14.9*	-	7.9	11.1	9.1	14.0
Ca	mg g^{-1}	132.0*	-	145.9	170.6	59.7	121.0
Mg	mg g^{-1}	12.1*	-	18.0	22.8	10.9	15.5
Si	mg g^{-1}	214.0*	-	151.2	167.7	201.6	209.9
Fe	mg g^{-1}	21.6*	-	11.0	17.2	14.0	19.8
Mn	mg g^{-1}	0.3*	-	0.4	0.5	0.5	1.0
Ti	mg g^{-1}	2.3*	-	3.1	3.7	4.0	4.8
P	mg g^{-1}	0.6*	-	0.2	0.3	0.3	0.4
Co	mg kg^{-1}	9.2	11.4	7.1	12.4	10.0	13.2
Cr	mg kg^{-1}	37.2	48.8	46.8	47.9	39.0	74.9
Cu	mg kg^{-1}	21.3	22.4	74.6	97.7	35.8	46.3
Nb	mg kg^{-1}	7.9	10.7	9.9	11.9	11.9	15.0
Ni	mg kg^{-1}	18.7	30.1	19.3	27.8	23.9	31.4
Pb	mg kg^{-1}	17.6	21.6	29.1	32.9	27.4	39.2
Sr	mg kg^{-1}	430.2	584.3	258.5	305.5	232.2	315.8
Th	mg kg^{-1}	3.9	5.6	4.7	6.5	7.0	13.5
V	mg kg^{-1}	81.7	116.4	65.4	95.6	90.0	114.2
Zn	mg kg^{-1}	48.3	70.2	96.0	109.2	111.6	117.9
Organic carbon	%	na	na	4.4	5.3	4.1	4.9
Organic nitrogen	%	na	na	0.2	0.2	0.3	0.6

Table S.3. Average (N= 4) antibiotic concentration (ng L^{-1}) in water samples from both sampling campaigns (May 2010 & 2011).

	Foix Reservoir		Sau Reservoir		Llosa Reservoir	
	2010	2011	2010	2011	2010	2011
Macrolides	1112.2	779.1	nd	nd	nd	nd
Quinolones & Fluoroquinolones	167.9	nd	nd	nd	nd	17.5
Sulfonamides	743.7	869.9	1.9	6.7	bql	nd
Tetracyclines	59.8	55.4	nd	nd	nd	nd
Nitroimidazoles	2.8	bql	nd	1.3	nd	nd

*blq: below quantitation level

[#] nd: non-detected

Table S.4. Average (N= 3) antibiotic concentration (μg kg^{-1}) in sediment samples from both sampling campaings (May 2010 & 2011).

	Foix Reservoir		Sau Reservoir		Llosa Reservoir	
	2010	2011	2010	2011	2010	2011
Macrolides	64.9	45.3	0.7	bql	bql	bql
Quinolones & Fluoroquinolones	5.8	nd	nd	nd	nd	nd
Sulfonamides	0.8	0.9	1.4	bql	0.6	0.9
Tetracyclines	8.8	6.5	11	7.4	nd	nd

*blq: below quantitation level

[#] nd: non-detected

Table S.5. Parsimony test for bacterial assemblages found in water samples

Tree	SAMPLES	Score	*P*-value
1	SAU (MID) - SAU (DAM)	90	0.001
1	SAU (MID) - SAU (DAM, bottom)	94	0.001
1	SAU (DAM) - SAU (DAM, bottom)	94	0.001
1	SAU (MID) - LA LLOSA (TAIL)	65	0.001
1	SAU (DAM) - LA LLOSA (TAIL)	72	0.001
1	SAU (DAM, bottom) - LA LLOSA (TAIL)	70	0.001
1	SAU (MID) - LA LLOSA (MID)	78	0.001
1	SAU (DAM) - LA LLOSA (MID)	88	0.001
1	SAU (DAM, bottom) - LA LLOSA (MID)	97	0.001

1	LA LLOSA (TAIL) - LA LLOSA (MID)	101	0.038*
1	SAU (MID) - LA LLOSA (DAM)	63	0.001
1	SAU (DAM) - LA LLOSA (DAM)	75	0.001
1	SAU (DAM, bottom) - LA LLOSA (DAM)	72	0.001
1	LA LLOSA (TAIL) - LA LLOSA (DAM)	97	0.802*
1	LA LLOSA (MID) - LA LLOSA (DAM)	112	0.197*
1	SAU (MID) - LA LLOSA (DAM, bottom)	101	0.001
1	SAU (DAM) - LA LLOSA (DAM, bottom)	109	0.001
1	SAU (DAM, bottom) - LA LLOSA (DAM, bottom)	203	0.001
1	LA LLOSA (TAIL) - LA LLOSA (DAM, bottom)	86	0.001
1	LA LLOSA (MID) - LA LLOSA (DAM, bottom)	116	0.001
1	LA LLOSA (DAM) - LA LLOSA (DAM, bottom)	85	0.001
1	SAU (MID) - FOIX (TAIL)	22	0.001
1	SAU (DAM) - FOIX (TAIL)	20	0.001
1	SAU (DAM, bottom) - FOIX (TAIL)	28	0.001
1	LA LLOSA (TAIL) - FOIX (TAIL)	19	0.001
1	LA LLOSA (MID) - FOIX (TAIL)	21	0.001
1	LA LLOSA (DAM) - FOIX (TAIL)	22	0.001
1	LA LLOSA (DAM, bottom) - FOIX (TAIL)	27	0.001
1	SAU (MID) - FOIX (MID)	96	0.001
1	SAU (DAM) - FOIX (MID)	94	0.001
1	SAU (DAM, bottom) - FOIX (MID)	170	0.001
1	LA LLOSA (TAIL) - FOIX (MID)	87	0.001
1	LA LLOSA (MID) - FOIX (MID)	117	0.001
1	LA LLOSA (DAM) - FOIX (MID)	98	0.001
1	LA LLOSA (DAM, bottom) - FOIX (MID)	171	0.001
1	FOIX (TAIL) - FOIX (MID)	59	0.001
1	SAU (MID) - FOIX (DAM)	90	0.001
1	SAU (DAM) - FOIX (DAM)	96	0.001
1	SAU (DAM, bottom) - FOIX (DAM)	144	0.001
1	LA LLOSA (TAIL) - FOIX (DAM)	77	0.001
1	LA LLOSA (MID) - FOIX (DAM)	107	0.001

1	LA LLOSA (DAM) - FOIX (DAM)	82	0.001
1	LA LLOSA (DAM, bottom) - FOIX (DAM)	136	0.001
1	FOIX (TAIL) - FOIX (DAM)	40	0.001
1	FOIX (MID) - FOIX (DAM)	240	0.001
1	SAU (MID) - FOIX (DAM, bottom)	100	0.001
1	SAU (DAM) - FOIX (DAM, bottom)	107	0.001
1	SAU (DAM, bottom) - FOIX (DAM, bottom)	234	0.001
1	LA LLOSA (TAIL) - FOIX (DAM, bottom)	81	0.001
1	LA LLOSA (MID) - FOIX (DAM, bottom)	112	0.001
1	LA LLOSA (DAM) - FOIX (DAM, bottom)	92	0.001
1	LA LLOSA (DAM, bottom) - FOIX (DAM, bottom)	189	0.001
1	FOIX (TAIL) - FOIX (DAM, bottom)	27	0.001
1	FOIX (MID) - FOIX (DAM, bottom)	259	0.001
1	FOIX (DAM) - FOIX (DAM, bottom)	213	0.001
1	SAU (MID) - SAU (TAIL)	98	0.001
1	SAU (DAM) - SAU (TAIL)	117	0.001
1	SAU (DAM, bottom) - SAU (TAIL)	183	0.001
1	LA LLOSA (TAIL) - SAU (TAIL)	96	0.001
1	LA LLOSA (MID) - SAU (TAIL)	129	0.001
1	LA LLOSA (DAM) - SAU (TAIL)	91	0.001
1	LA LLOSA (DAM, bottom) - SAU (TAIL)	195	0.001
1	FOIX (TAIL) - SAU (TAIL)	26	0.001
1	FOIX (MID) - SAU (TAIL)	208	0.001
1	FOIX (DAM) - SAU (TAIL)	172	0.001
1	FOIX (DAM, bottom) - SAU (TAIL)	224	0.001

*Bonferroni correction for multiple comparisons was used among the pairwise comparisons.

Table S.6. Parsimony test for bacterial assemblages found in sediment samples

Tree	SAMPLES	Score	*P*-value
1	SAU (TAIL) - SAU (MID)	367	0.001
1	SAU (TAIL) - SAU (DAM)	338	0.001
1	SAU (MID) - SAU (DAM)	568	0.001
1	SAU (TAIL) - LA LLOSA (TAIL)	217	0.001
1	SAU (MID) - LA LLOSA (TAIL)	189	0.001
1	SAU (DAM) - LA LLOSA (TAIL)	169	0.001
1	SAU (TAIL) - LA LLOSA (MID)	335	0.001
1	SAU (MID) - LA LLOSA (MID)	572	0.001
1	SAU (DAM) - LA LLOSA (MID)	604	0.001
1	LA LLOSA (TAIL) - LA LLOSA (MID)	183	0.001
1	SAU (TAIL) - LA LLOSA (DAM)	291	0.001
1	SAU (MID) - LA LLOSA (DAM)	569	0.001
1	SAU (DAM) - LA LLOSA (DAM)	630	0.001
1	LA LLOSA (TAIL) - LA LLOSA (DAM)	148	0.001
1	LA LLOSA (MID) - LA LLOSA (DAM)	1072	0.001
1	SAU (TAIL) - FOIX (TAIL)	254	0.001
1	SAU (MID) - FOIX (TAIL)	232	0.001
1	SAU (DAM) - FOIX (TAIL)	202	0.001
1	LA LLOSA (TAIL) - FOIX (TAIL)	170	0.001
1	LA LLOSA (MID) - FOIX (TAIL)	206	0.001
1	LA LLOSA (DAM) - FOIX (TAIL)	187	0.001
1	SAU (TAIL) - FOIX (MID)	362	0.001
1	SAU (MID) - FOIX (MID)	425	0.001
1	SAU (DAM) - FOIX (MID)	351	0.001
1	LA LLOSA (TAIL) - FOIX (MID)	219	0.001
1	LA LLOSA (MID) - FOIX (MID)	338	0.001
1	LA LLOSA (DAM) - FOIX (MID)	311	0.001
1	FOIX (TAIL) - FOIX (MID)	274	0.001
1	SAU (TAIL) - FOIX (DAM)	326	0.001

1	SAU (MID) - FOIX (DAM)	401	0.001
1	SAU (DAM) - FOIX (DAM)	382	0.001
1	LA LLOSA (TAIL) - FOIX (DAM)	187	0.001
1	LA LLOSA (MID) - FOIX (DAM)	415	0.001
1	LA LLOSA (DAM) - FOIX (DAM)	375	0.001
1	FOIX (TAIL) - FOIX (DAM)	236	0.001
1	FOIX (MID) - FOIX (DAM)	371	0.001

Table S.7. Number of OTUs* and estimators of sequence diversity and richness in water samples

Water samples	Reads	OTU	H’	Chao
Foix (tail)	10855	126	2.79	150
Foix (mid)	10855	499	3.88	900
Foix (dam, surface)	10855	382	3.68	596
Foix (dam, bottom)	10855	606	4.41	893
Sau (tail)	10855	536	3.79	833
Sau (mid)	10855	440	4.11	707
Sau (dam, surface)	10855	350	3.96	624
Sau (dam, bottom)	10855	476	4.16	826
La Llosa (tail)	10855	290	3.69	493
La Llosa (mid)	10855	309	3.47	488
La Llosa (dam, surface)	10855	301	3.41	543
La Llosa (dam,bottom)	10855	503	4.76	756

*OTU signifies the number of OTUs observed at 97% similarity, H' signifies the nonparametric Shannon diversity index, and Chao signifies the Chao1 estimated minimum number of OTUs.

Table S.8. Number of OTUs* and estimators of sequence diversity and richness in sediment samples

Sediment samples	Reads	OTU	H’	Chao
Foix (tail)	6058	507	4.86	740
Foix (mid)	6058	817	5.40	1363
Foix (dam)	6058	810	5.85	1188
Sau (tail)	6058	910	4.39	1420
Sau (mid)	6058	1556	5.70	3136
Sau (dam)	6058	1363	4.86	3200
La Llosa (tail)	6058	422	5.06	603
La Llosa (mid)	6058	1967	7.03	3460
La Llosa (dam)	6058	2355	7.22	5397

*OTU signifies the number of OTUs observed at 97% similarity, H' signifies the nonparametric Shannon diversity index, and Chao signifies the Chao1 estimated minimum number of OTUs.

Table S.9. Percentage of variation in bacterial community composition explained by the sets of ARG, environmental, and spatial variables at the two different compartments

Variation	Water samples (N = 12)			Sediment samples (N = 9)		
	Explanatory variables	*P*-value	Variance explained	Explanatory variables	*P*-value	Variance explained
ARG+Environmental+Space	6	0.00005	0.80	na	na	na
ARG+Environmental	na	na	na	5	0.00696	0.74
ARG	2	0.00282	0.52	2	0.00001	0.69
Environmental	3	0.00040	0.62	3	0.00001	0.77
Space	1	0.05247	0.12	na	na	na
Pure ARG	2	0.01262	0.19	2	0.64450	0.00
Pure Environmental	3	0.01154	0.17	3	0.23160	0.05
Pure Spatial	1	0.01896	0.11	na	na	na
Shared ARG+Environmental+Space	nt	nt	0.33	na	na	na
Shared ARG+Environmental	na	na	na	nt	nt	0.69
Unexplained	nt	nt	0.20	nt	nt	0.26

na - not applicable

nt - not testable

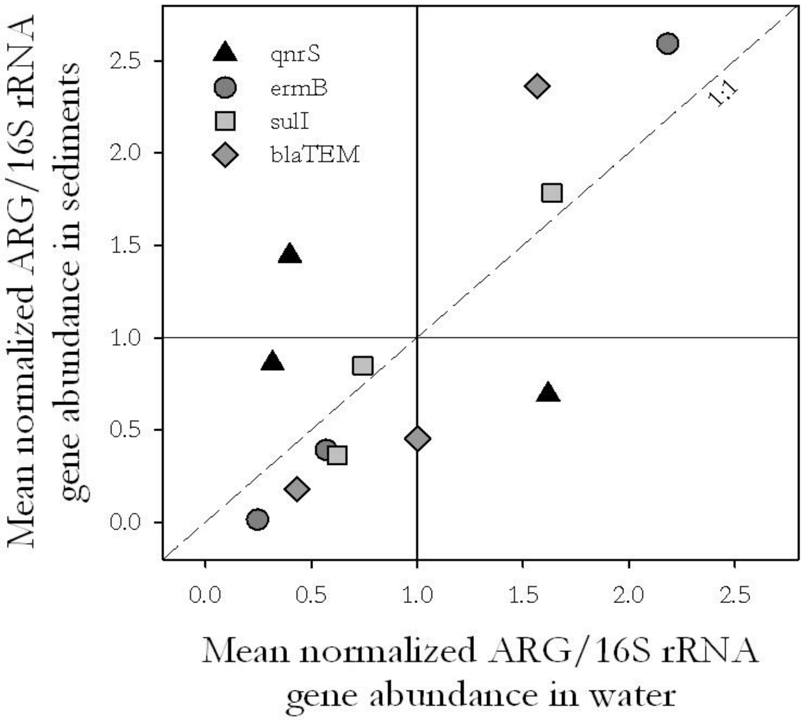


Figure S.1. Mean normalized abundance of ARGs in the water column vs. sediments. The 1:1 line was added for reference as a dashed line.

APPENDIX F: Supplementary Material for the article Huerta et al 2015. *Submitted*

Method validation parameters

Both MDL and MQL were determined in spiked samples (n = 3) of all matrices considered as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. Precision was calculated from five repeated injections of a spiked sample at 10 ng mL^{-1} or ng g^{-1}, and was expressed as the relative standard deviation of the measured concentration. Calibration curves in both solvent and clean matrix extract were generated using linear regression analysis ($R^2 > 0.98$) in the concentration range from 0.1 to 100 ng mL^{-1} for water and plasma, or 0.1 to 50 ng g^{-1} for tissues. When matrix-matched calibration curves were applied to the quantification, matrix effects (percentage of signal reduction or enhancement when comparing the compound in the matrix *vs* the solvent) were considered negligible (see Table 1).

Table S1 SRM transitions and compound dependent MS parameters for target analytes

Compounds			Quantification		Confirmation		SRM1/2 ratio (±SD)n=3
	Rt (min)	Precursor ion (m/z)	Q3	DP/CE/CXP	Q3	DP/CE/CXP	
Oxazepam	1.33	287 [M+H]+	241	96/33/12	104	111/43/14	2.86
Diazepam-d5 (IS)	1.44	290 [M+H]+	198	101/47/26			

Table S2 Concentration of oxazepam in plasma and tissues (wet weight) of each of the fish individuals after 28-days exposure

Water Conc. (µg L^{-1})	Gender	Plasma (ng mL^{-1})	Brain (ng g^{-1})	Liver (ng g^{-1})	Muscle (ng g^{-1})
30.6	M	63.0	75	111	48
30.6	M	122.6	109	215	184
30.6	M	135.1	166	184	154
30.6	M	86.6	136	266	113
30.6	M	151.6	82	74	81
30.6	M	53.0	97	101	60
30.6	M	112.9	238	370	63
30.6	M	313.1	177	272	38
30.6	M	91.1	144	256	79
30.6	M	10.6	117	224	98
30.6	M	208.3	219	420	181
30.6	M	232.4	314	439	193
30.6	M	60.8	271	63	33
30.6	M	87.3	160	216	56
30.6	M	166.0	323	388	140
30.6	M	245.7	262	337	62
30.6	F	51.8	88	0	80
30.6	F	16.8	57	61	20
30.6	F	104.6	99	120	49
30.6	F	75.5	97	94	123
30.6	F	68.7	122	199	37
30.6	F	146.1	132	61	50
30.6	F	32.2	97	61	42
30.6	F	10.0	178	93	177
30.6	F	121.0	127	119	64
30.6	F	75.3	70	137	73
30.6	F	76.4	89	71	29
30.6	F	19.1	107	71	7
30.6	F	52.6	85	68	81
30.6	F	24.6	192	65	17
30.6	F	98.2	131	70	85
30.6	F	66.5	137	230	96
4.7	M	40.2	76	36	22
4.7	M	26.9	41	25	44
4.7	M	25.1	65	47	10
4.7	M	37.8	50	52	28
4.7	F	21.1	44	72	24
4.7	M	57.5	81	34	26
4.7	F	39.6	29	31	9
4.7	M	29.3	56	59	21
4.7	M	46.3	70	37	27
4.7	M	52.8	91	45	17

4.7	M	39.0	46	28	12
4.7	M	51.4	41	58	39
4.7	M	80.1	61	49	28
4.7	F	24.5	37	33	40
4.7	M	32.3	42	96	96
4.7	M	41.8	63	51	27
4.7	F	17.6	39	75	30
4.7	F	17.2	36	20	20
4.7	F	23.1	51	80	60
4.7	F	34.0	53	21	16
4.7	F	35.5	41	18	55
4.7	F	31.4	45	35	9
4.7	F	23.1	26	47	24
4.7	F	30.1	57	28	14
4.7	F	7.9	23	10	8
4.7	F	10.8	40	21	84
4.7	F	28.0	28	30	18
4.7	F	18.9	62	23	8
4.7	F	10.5	48	11	16
4.7	F	10.0	41	17	7
4.7	F	10.4	35	16	11
4.7	F	14.5	78	33	9
0.8	M	7.1	35	8	5
0.8	M	9.0	32	14	7
0.8	M	7.5	34	15	6
0.8	M	5.3	64	24	4
0.8	F	7.3	51	16	5
0.8	M	4.3	52	29	3
0.8	M	14.4	32	8	4
0.8	M	11.9	37	8	2
0.8	F	7.4	51	19	47
0.8	F	6.1	23	20	4
0.8	F	3.5	52	50	6
0.8	F	3.7	24	16	4
0.8	F	11.0	73	23	9
0.8	F	3.2	24	16	5
0.8	F	6.0	17	38	3
0.8	F	13.3	28	14	11
0.8	F	4.5	28	6	10
0.8	F	10.2	24	7	5
0.8	F	3.1	72	6	5
0.8	F	3.5	42	10	4
0.8	F	6.8	24	8	5
0.8	F	4.8	54	6	8
0.8	F	4.3	34	11	8
0.8	F	9.4	43	6	6

0.8	M	13.5	17	17	4
0.8	M	17.9	27	17	13
0.8	M	10.4	22	18	9
0.8	M	13.3	68	16	8
0.8	F	12.5	50	10	5
0.8	F	3.7	55	24	4
0.8	F	7.0	46	14	5
0.8	M	13.4	60	12	8

Table S3 Inter-individual and intra-treatment variability of oxazepam plasma concentrations.

Treatment	Mean	Median	Min	Max	25%	75%	SD
1 µg/L - Rep. A Males	8.4	7.4	4.3	14.4	6.7	9.7	
1 µg/L - Rep. B Males	11.5	12.9	3.7	17.9	9.5	13.5	
1 µg/L - Rep. C Females	5.8	4.7	3.1	10.2	4.1	7.4	
1 µg/L - Rep. D Females	9.3	6.0	3.2	31.0	3.7	8.9	
1 µg/L - Males	10.9	11.1	5.3	17.9	7.5	13.5	3.8
1 µg/L - Females	7.4	5.4	3.1	31.0	3.7	7.9	6.3
1 µg/L - All Fish	8.7	7.2	3.1	31.0	4.5	12.1	5.7
5 µg/L - Rep. A Males	34.7	33.6	21.1	57.5	26.4	39.8	
5 µg/L - Rep. B Males	46.0	44.1	24.5	80.1	37.3	51.8	
5 µg/L - Rep. C Females	13.9	10.7	7.9	28.0	10.3	15.6	
5 µg/L - Rep. D Females	26.5	26.6	17.2	35.5	21.7	32.1	
5 µg/L - Males	43.1	40.2	25.1	80.1	32.3	51.4	15.0
5 µg/L - Females	21.5	21.1	7.9	39.6	12.7	29.0	9.6
5 µg/L - All Fish	30.3	28.7	7.9	80.1	18.6	39.2	16.1
25 µg/L - Rep. A Males	136.6	128.6	1.6	245.7	80.7	214.3	
25 µg/L - Rep. B Males	129.7	117.8	53.0	313.1	80.7	139.2	
25 µg/L - Rep. C Females	62.1	60.2	1.0	146.1	28.4	82.8	
25 µg/L - Rep. D Females	66.7	70.9	19.1	121.0	45.6	81.8	
25 µg/L - Males	133.2	117.8	1.6	313.1	80.7	176.6	82.6
25 µg/L - Females	64.4	67.6	1.0	146.1	30.3	81.8	40.2
25 µg/L - All Fish	98.8	81.5	1.0	313.1	52.9	125.7	72.9

Chapter 9

References

REFERENCES

1. Pal, A., Gin, K. Y.-H., Lin, A. Y.-C. & Reinhard, M. Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects. *Sci. Total Environ.* **408,** 6062–6069 (2010).

2. Richardson, S. D. & Ternes, T. A. Water Analysis: Emerging Contaminants and Current Issues. *Anal. Chem.* **83,** 4614–4648 (2011).

3. Petrie, B., Barden, R. & Kasprzyk-Hordern, B. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Res.* **72,** 3–27 (2014).

4. Petrovic, M., Hernando, M. D., Díaz-Cruz, M. S. & Barceló, D. Liquid chromatography-tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. *J. Chromatogr. A* **1067,** 1–14 (2005).

5. Sauvé, S. & Desrosiers, M. A review of what is an emerging contaminant. *Chem. Cent. J.* **8,** 15 (2014).

6. Fent, K., Weston, A. A. & Caminada, D. Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.* **76,** 122–159 (2006).

7. Halling-Sørensen, B. *et al.* Occurrence, fate and effects of pharmaceutical substances in the environment: A review. *Chemosphere* **36,** 357–393 (1998).

8. Lapworth, D. J., Baran, N., Stuart, M. E. & Ward, R. S. Emerging organic contaminants in groundwater: A review of sources, fate and occurrence. *Environ. Pollut.* **163,** 287–303 (2012).

9. Arpin-Pont, L., Bueno, M. J. M., Gomez, E. & Fenet, H. Occurrence of PPCPs in the marine environment: a review. *Environ. Sci. Pollut. Res.* (2014). doi:10.1007/s11356-014-3617-x

10. Gros, M., Rodríguez-Mozaz, S. & Barceló, D. Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem. *J. Chromatogr. A* **1248,** 104–121 (2012).

11. Carmona, E., Andreu, V. & Picó, Y. Occurrence of acidic pharmaceuticals and personal care products in Turia River Basin: From waste to drinking water. *Sci. Total Environ.* **484,** 53–63 (2014).

12. Kostich, M. S., Batt, A. L. & Lazorchak, J. M. Concentrations of prioritized pharmaceuticals in effluents from 50 large wastewater treatment plants in the US and implications for risk estimation. *Environ. Pollut.* **184,** 354–359 (2014).

13. López-Serna, R., Petrović, M. & Barceló, D. Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain). *Sci. Total Environ.* **440,** 280–289 (2012).

14. López-Serna, R., Pérez, S., Ginebreda, A., Petrović, M. & Barceló, D. Fully automated determination of 74 pharmaceuticals in environmental and waste waters by online solid phase extraction-liquid chromatography- electrospray-tandem mass spectrometry. *Talanta* **83,** 410–424 (2010).

15. López-Serna, R. *et al.* Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain. *Environ. Pollut.* **174,** 305–315 (2013).

16. Buchberger, W. W. Current approaches to trace analysis of pharmaceuticals and personal care products in the environment. *J. Chromatogr. A* **1218,** 603–618 (2011).

17. Daughton, C. G. in *Anal. Fate Remov. Pharm. Water Cycle* (Petrovic, M. & Barceló, D.) **50,** 1–49 (Elsevier, 2007).

18. Daughton, C. G. Environmental Stewardship and Drugs as Pollutants. *Lancet* **360,** 1035–1036 (2002).

19. Corcoran, J., Winter, M. J. & Tyler, C. R. Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. *Crit. Rev. Toxicol.* **40,** 287–304 (2010).

20. Daughton, C. G. & Ternes, T. A. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Env. Heal. Perspect.* **107 Suppl ,** 907–38. (1999).

21. IPCS. *Global assessment of the state-of-the-science of endocrine disruptors*. (2002).

22. Blumberg, B., Iguchi, T. & Odermatt, a. *Endocrine disrupting chemicals*. *J. steroid Biochem. ...* (2011).

23. Campbell, C. G. *et al.* Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: A review. *Chemosphere* **65,** 1265–1280 (2006).

24. Gorga, M., Petrovic, M. & Barcelo, D. Multi-residue analytical method for the determination of endocrine disruptors and related compounds in river and waste water using dual column liquid chromatography switching system coupled to mass spectrometry. *J. Chromatogr. A* **1295,** 57–66 (2013).

25. Esteban, S. *et al.* Analysis and occurrence of endocrine-disrupting compounds and estrogenic activity in the surface waters of Central Spain. *Sci. Total Environ.* **466-467,** 939–951 (2014).

26. Huerta-Fontela, M., Galceran, M. T. & Ventura, F. Occurrence and removal of pharmaceuticals and hormones through drinking water treatment. *Water Res.* **45,** 1432–1442 (2011).

27. Vulliet, E. & Cren-Olivé, C. Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. *Environ. Pollut.* **159,** 2929–2934 (2011).

28. Loos, R. *et al.* Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. *Water Res.* **44,** 4115–4126 (2010).

29. Félix-Cañedo, T. E., Durán-Álvarez, J. C. & Jiménez-Cisneros, B. The occurrence and distribution of a group of organic micropollutants in Mexico City's water sources. *Sci. Total Environ.* **454-455,** 109–118 (2013).

30. Reh, R., Licha, T., Geyer, T., Nödler, K. & Sauter, M. Occurrence and spatial distribution of organic micro-pollutants in a complex hydrogeological karst system during low flow and high flow periods, results of a two-year study. *Sci. Total Environ.* **443,** 438–445 (2013).

31. Rodriguez-Mozaz, S., Marco, M. P., Lopez De Alda, M. J. & Barceló, D. Biosensors for environmental monitoring of endocrine disruptors: A review article. *Anal. Bioanal. Chem.* **378,** 588–598 (2004).

32. Lee, H. R. *et al.* Molecular mechanism(s) of endocrine-disrupting chemicals and their potent oestrogenicity in diverse cells and tissues that express oestrogen receptors. *J. Cell. Mol. Med.* **17,** 1–11 (2013).

33. Collado, N. *et al.* Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. *Environ. Pollut.* **185,** 202–212 (2014).

34. European Commision. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. *2011/0429 (COD)* (2013).

35. Environmental Protection Agency U.S. Water: Contaminant Candidate List 3 - CCL. (2012).

36. GWRC (Global Water Research Coalition). Development of an International Priority List of Pharmaceuticals Relevant for the Water Cycle . (2008).

37. European Medicines Agency. *Guideline on the environmental risk assessment of medicinal products for human use*. (2006).

38. Runnqvist, H. *et al.* Determination of pharmaceuticals in environmental and biological matrices using pressurised liquid extraction: Are we developing sound extraction methods? *J. Chromatogr. A* **1217,** 2447–2470 (2010).

39. Moreno-Bondi, M., Marazuela, M., Herranz, S. & Rodriguez, E. An overview of sample preparation procedures for LC-MS multiclass antibiotic determination in environmental and food samples. *Anal. Bioanal.Chem.* **395,** 921–946 (2009).

40. Pavlović, D. M., Babić, S., Horvat, A. J. M. & Kaštelan-Macan, M. Sample preparation in analysis of pharmaceuticals. *TrAC, Trends Anal. Chem.* **26,** 1062–1075 (2007).

41. Brooks, B. W. *et al.* Determination of select antidepressants in fish from an effluent-dominated stream. *Environ. Toxicol. Chem.* **24,** 464–469 (2005).

42. Schultz, M. M. *et al.* Antidepressant Pharmaceuticals in Two U.S. Effluent-Impacted Streams: Occurrence and Fate in Water and Sediment, and Selective Uptake in Fish Neural Tissue. *Environ. Sci. Technol.* **44,** 1918–1925 (2010).

43. Chu, S. & Metcalfe, C. D. Analysis of paroxetine, fluoxetine and norfluoxetine in fish tissues using pressurized liquid extraction, mixed mode solid phase extraction cleanup and liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **1163,** 112–118 (2007).

44. Blewett, T. a., Chow, T. L., Maclatchy, D. L. & Wood, C. M. A species comparison of 17-??-ethynylestradiol uptake and tissue-specific distribution in six teleost fish. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* **161,** 33–40 (2014).

45. Ahel, M., Mc Envoy, J. & Giger, W. Bioaccumulation of the lipophylic metabolites of non ionic surfactants in freshwater organisms. *Environ. Pollut.* **79,** 243–248 (1993).

46. Santín, G., Barón, E., Eljarrat, E. & Barceló, D. Emerging and historical halogenated flame retardants in fish samples from Iberian rivers. *J. Hazard. Mater.* **263,** 116–121 (2013).

47. Lin, A. Y.-C., Plumlee, M. H. & Reinhard, M. Natural attenuation of pharmaceuticals and alkylphenol polyethoxylate metabolites during river transport: photochemical and biological transformation. *Environ. Toxicol. Chem.* **25,** 1458–1464 (2006).

48. Ramirez, A. J., Mottaleb, M. A., Brooks, B. W. & Chambliss, C. K. Analysis of Pharmaceuticals in Fish Using Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Chem.* **79,** 3155–3163 (2007).

49. Alvarez-Muñoz, D., Huerta, B., Fernandez-Tejedor, M., Rodríguez-Mozaz, S. & Barceló, D. Multi-residue method for the analysis of pharmaceuticals and some of their metabolites in bivalves. *Talanta* **136,** 174–182 (2015).

50. Berlioz-Barbier, A. *et al.* MicroQuEChERS–nanoliquid chromatography–nanospray–tandem mass spectrometry for the detection and quantification of trace pharmaceuticals in benthic invertebrates. *Talanta* **132,** 796–802 (2015).

51. Du, B., Haddad, S. P., Scott, W. C., Chambliss, C. K. & Brooks, B. W. Pharmaceutical bioaccumulation by periphyton and snails in an effluent-dependent stream during an extreme drought. *Chemosphere* **119,** 927–934 (2015).

52. Liu, J. *et al.* Occurrence, bioaccumulation and risk assessment of lipophilic pharmaceutically active compounds in the downstream rivers of sewage treatment plants. *Sci. Total Environ.* **511,** 54–62 (2015).

53. Tanoue, R. *et al.* Simultaneous determination of polar pharmaceuticals and personal care products in biological organs and tissues. *J. Chromatogr. A* **1355,** 193–205 (2014).

54. Mehinto, A. C., Hill, E. M. & Tyler, C. R. Uptake and Biological Effects of Environmentally Relevant Concentrations of the Nonsteroidal Anti-inflammatory Pharmaceutical Diclofenac in Rainbow Trout (Oncorhynchus mykiss). *Environ. Sci. Technol.* **44,** 2176–2182 (2010).

55. Kallio, J.-M., Lahti, M., Oikari, A. & Kronberg, L. Metabolites of the Aquatic Pollutant Diclofenac in Fish Bile. *Environ. Sci. Technol.* **44,** 7213–7219 (2010).

56. Lahti, M., Brozinski, J.-M., Jylha, A., Kronberg, L. & Oikari, A. Uptake from water, biotransformation, and billiary excretion of pharmaceuticals by rainbow trout. *Environ. Toxicol. Chem.* **30,** (2011).

57. Mimeault, C. *et al.* The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, Carassius auratus. *Aquat. Toxicol.* **73,** 44–54 (2005).

58. Brozinski, J.-M., Lahti, M., Meierjohann, A., Oikari, A. & Kronberg, L. The Anti-Inflammatory Drugs Diclofenac, Naproxen and Ibuprofen are found in the Bile of Wild Fish Caught Downstream of a Wastewater Treatment Plant. *Environ. Sci. Technol.* **47,** 342–348 (2013).

59. Zhao, J.-L. *et al.* Tissue-specific bioaccumulation of human and veterinary antibiotics in bile, plasma, liver and muscle tissues of wild fish from a highly urbanized region. *Environ. Pollut.* **198,** 15–24 (2015).

60. Owen, S. F. *et al.* Uptake of propranolol, a cardiovascular pharmaceutical, from water into fish plasma and its effects on growth and organ biometry. *Aquat. Toxicol.* **93,** 217–224 (2009).

61. Nallani, G. C., Paulos, P. M., Constantine, L. A., Venables, B. J. & Huggett, D. B. Bioconcentration of ibuprofen in fathead minnow (Pimephales promelas) and channel catfish (Ictalurus punctatus). *Chemosphere* **84,** 1371–1377 (2011).

62. Gelsleichter, J. & Szabo, N. J. Uptake of human pharmaceuticals in bull sharks (Carcharhinus leucas) inhabiting a wastewater-impacted river. *Sci. Total Environ.* **456-457,** 196–201 (2013).

63. Togunde, O. P., Oakes, K. D., Servos, M. R. & Pawliszyn, J. Optimization of solid phase microextraction for non-lethal in vivo determination of selected pharmaceuticals in fish muscle using liquid chromatography-mass spectrometry. *J. Chromatogr. A* **1261,** 99–106 (2012).

64. Kirbiš, A., Marinšek, J. & Flajs, V. C. Introduction of the HPLC method for the determination of quinolone residues in various muscle tissues. *Biomed. Chromatogr.* **19,** 259–265 (2005).

65. Valdés, M. E., Amé, M. V., Bistoni, M. D. L. A. & Wunderlin, D. A. Occurrence and bioaccumulation of pharmaceuticals in a fish species inhabiting the Suquía River basin (Córdoba, Argentina). *Sci. Total Environ.* **472,** 389–396 (2014).

66. Vannini, C. *et al.* Effects of a complex mixture of therapeutic drugs on unicellular algae Pseudokirchneriella subcapitata. *Aquat. Toxicol.* **101,** 459–465 (2011).

67. Pojana, G., Gomiero, A., Jonkers, N. & Marcomini, A. Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. *Environ. Int.* **33,** 929–936 (2007).

68. Fernandez-Torres, R., Lopez, M. A. B., Consentino, M. O., Mochon, M. C. & Payan, M. R. Enzymatic-microwave assisted extraction and high-performance liquid chromatography-mass spectrometry for the determination of selected veterinary antibiotics in fish and mussel samples. *J. Pharm. Biomed. Anal.* **54,** 1146–1156 (2011).

69. Liu, J. *et al.* Biological effects and bioaccumulation of steroidal and phenolic endocrine disrupting chemicals in high-back crucian carp exposed to wastewater treatment plant effluents. *Environ. Pollut.* **162,** 325–331 (2012).

70. Cathum, S. & Sabik, H. Determination of steroids and coprostanol in surface water, effluent and mussel using gas chromatography-mass spectrometry. *Chromatographia* **53,** S394–S399 (2001).

71. Contardo-Jara, V. *et al.* Exposure to human pharmaceuticals Carbamazepine, Ibuprofen and Bezafibrate causes molecular effects in Dreissena polymorpha. *Aquat. Toxicol.* **105,** 428–437 (2011).

72. Cueva-Mestanza, R., Torres-Padrón, M. E., Sosa-Ferrera, Z. & Santana-Rodríguez, J. J. Microwave-assisted micellar extraction coupled with solid-phase extraction for preconcentration of pharmaceuticals in molluscs prior to determination by HPLC. *Biomed. Chromatogr.* **22,** 1115–1122 (2008).

73. Huet, A. C. *et al.* Development of an optical surface plasmon resonance biosensor assay for (fluoro)quinolones in egg, fish, and poultry meat. *Anal. Chim. Acta* **623,** 195–203 (2008).

74. Azzouz, A., Souhail, B. & Ballesteros, E. Determination of residual pharmaceuticals in edible animal tissues by continuous solid-phase extraction and gas chromatography-mass spectrometry. *Talanta* **84,** 820–828 (2011).

75. Garcia, S. N., Foster, M., Constantine, L. A. & Huggett, D. B. Field and laboratory fish tissue accumulation of the anti-convulsant drug carbamazepine. *Ecotox. Environ. Safe.* **84,** 207–211 (2012).

76. Du, B., Perez-Hurtado, P., Brooks, B. W. & Chambliss, C. K. Evaluation of an isotope dilution liquid chromatography tandem mass spectrometry method for pharmaceuticals in fish. *J. Chromatogr. A* **1253,** 177–183 (2012).

77. Shao, B., Hu, J., Yang, M., An, W. & Tao, S. Nonylphenol and nonylphenol ethoxylates in river water, drinking water, and fish tissues in the area of Chongqing, China. *Arch. Environ. Contam. Toxicol.* **48,** 467–473 (2005).

78. Poma, G., Binelli, A., Volta, P., Roscioli, C. & Guzzella, L. Evaluation of spatial distribution and accumulation of novel brominated flame retardants, HBCD and PBDEs in an Italian subalpine lake using zebra mussel (Dreissena polymorpha). *Environ. Sci. Pollut. Res.* **21,** 9655–9664 (2014).

79. Wang, S. *et al.* Validation and use of in vivo solid phase micro-extraction (SPME) for the detection of emerging contaminants in fish. *Chemosphere* **85,** 1472–1480 (2011).

80. Zhou, S. N., Oakes, K. D., Servos, M. R. & Pawliszyn, J. Application of Solid-Phase Microextraction for In Vivo Laboratory and Field Sampling of Pharmaceuticals in Fish. *Environ. Sci. Technol.* **42,** 6073–6079 (2008).

81. Subedi, B., Mottaleb, M. A., Chambliss, C. K. & Usenko, S. Simultaneous analysis of select pharmaceuticals and personal care products in fish tissue using pressurized liquid extraction combined with silica gel cleanup. *J. Chromatogr. A* **1218,** 6278–6284 (2011).

82. Huerta, B., Jakimska, A., Gros, M., Rodríguez-Mozaz, S. & Barceló, D. Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **1288,** 63–72 (2013).

83. Matamoros, V., Calderón-Preciado, D., Domínguez, C. & Bayona, J. M. Analytical procedures for the determination of emerging organic contaminants in plant material: A review. *Anal. Chim. Acta* **722,** 8–20 (2012).

84. Carabias-Martinez, R., Rodriguez-Gonzalo, E., Revilla-Ruiz, P. & Hernandez-Mendez, J. Pressurized liquid extraction in the analysis of food and biological samples. *J. Chromatogr. A* **1089,** 1–17 (2005).

85. Huerta, B. *et al.* Determination of a broad spectrum of pharmaceuticals and endocrine disruptors in biofilm from a waste water treatment plant-impacted river. *Sci. Total Environ.* (2015). doi:10.1016/j.scitotenv.2015.05.049

86. Huerta, B. *et al.* Development of an extraction and purification method for the determination of multi-class pharmaceuticals and endocrine disruptors in freshwater invertebrates. *Talanta* **132,** 373–381 (2015).

87. Delepee, R., Pouliquen, H. & Le Bris, H. The bryophyte Fontinalis antipyretica Hedw. bioaccumulates oxytetracycline, flumequine and oxolinic acid in the freshwater environment. *Sci. Total Environ.* **322,** 243–253 (2004).

88. Faroongsarng, D., Chandumpai, A., Chiayvareesajja, S. & Theapparat, Y. Bioavailability and absorption analysis of oxytetracycline orally administered to the standardized moulting farmed Pacific white shrimps (Penaeus vannamei). *Aquaculture* **269,** 89–97 (2007).

89. Wang, M. *et al.* Improved compatibility of liquid chromatography with electrospray tandem mass spectrometry for tracing occurrence of barbital homologous residues in animal tissues. *J. Chromatogr. A* **1217,** 2821–2831 (2010).

90. Miller, T. H. *et al.* Pharmaceuticals in the freshwater invertebrate, Gammarus pulex, determined using pulverised liquid extraction, solid phase extraction and liquid chromatography–tandem mass spectrometry. *Sci. Total Environ.* **511,** 153–160 (2015).

91. Al-Ansari, A. M., Saleem, A., Kimpe, L. E., Trudeau, V. L. & Blais, J. M. The development of an optimized sample preparation for trace level detection of 17alfa-ethinylestradiol and estrone in whole fish tissue. *J. Chromatogr. B* **879,** 3649–3652 (2011).

92. Huerta, B., Jakimska, A., Gros, M., Rodriguez-Mozaz, S. & Barcelo, D. Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. *J Chromatogr A* **1288,** 63–72 (2013).

93. Bringolf, R. B. *et al.* Environmental occurrence and reproductive effects of the pharmaceutical fluoxetine in native freshwater mussels. *Environ. Toxicol. Chem.* **29,** 1311–1318 (2010).

94. Togunde, O. P., Oakes, K. D., Servos, M. R. & Pawliszyn, J. Determination of Pharmaceutical Residues in Fish Bile by Solid-Phase Microextraction Couple with Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS). *Environ. Sci. Technol.* **46,** 5302–5309 (2012).

95. Grabicova, K. *et al.* Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant. *Sci. Total Environ.* **488-489,** 46–50 (2014).

96. Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H. & Negele, R. D. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. *Aquat. Toxicol.* **68,** 141–150 (2004).

97. Van Dorst, B. *et al.* Recent advances in recognition elements of food and environmental biosensors: A review. *Biosens. Bioelectron.* **26,** 1178–1194 (2010).

98. Díaz-Cruz, M. S. & Barceló, D. in *Anal. Fate Remov. Pharm. Water Cycle* (Petrovic, M. & Barceló, D.) **50,** (Elsevier, 2007).

99. Metcalfe, C. D. *et al.* Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. *Environ. Toxicol. Chem.* **29,** 79–89 (2010).

100. Ramirez, A. J. *et al.* Occurrence of Pharmaceuticals and Personal Care Products in Fish: Results of a National Pilot Study in the United States. *Environ. Toxicol. Chem.* **28,** 2587–2597 (2009).

101. David, A., Abdul-Sada, A., Lange, A., Tyler, C. R. & Hill, E. M. A new approach for plasma (xeno)metabolomics based on solid-phase extraction and nanoflow liquid chromatography-nanoelectrospray ionisation mass spectrometry. *J. Chromatogr. A* **1365,** 72–85 (2014).

102. Dussault, E. B., Balakrishnan, V. K., Borgmann, U., Solomon, K. R. & Sibley, P. K. Bioaccumulation of the synthetic hormone 17alfa-ethinylestradiol in the benthic invertebrates Chironomus tentans and Hyalella azteca. *Ecotoxicol. Environ. Saf.* **72,** 1635–1641 (2009).

103. Brodin, T., Fick, J., Jonsson, M. & Klaminder, J. Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations. *Science (80-.).* **339,** 814–815 (2013).

104. Schröder, U. & Machetzki, A. Determination of flumequine, nalidixic acid and oxolinic acid in shrimps by high-performance liquid chromatography with fluorescence detection. *Eur. Food Res. Technol.* **225,** 627–635 (2007).

105. Rambla-Alegre, M., Peris-Vicente, J., Esteve-Romero, J. & Carda-Broch, S. Analysis of selected veterinary antibiotics in fish by micellar liquid chromatography with fluorescence detection and validation in accordance with regulation 2002/657/EC. *Food Chem.* **123,** 1294–1302 (2010).

106. Kümmerer, K. The presence of pharmaceuticals in the environment due to human use: present knowledge and future challenges. *J. Environ. Manag.* **90,** 2354–2366 (2009).

107. Berrada, H., Borrull, F., Font, G. & Marcè, R. M. Determination of macrolide antibiotics in meat and fish using pressurized liquid extraction and liquid chromatography-mass spectrometry. *J. Chromatogr. A* **1208,** 83–89 (2008).

108. Peters, R. J. B., Bolck, Y. J. C., Rutgers, P., Stolker, A. A. M. & Nielen, M. W. F. Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry. *J. Chromatogr. A* **1216,** 8206–8216 (2009).

109. Lajeunesse, A. *et al.* Determination of carbamazepine in aquatic organisms by liquid-liquid extraction and liquid chromatography-tandem mass spectrometry. *J. Environ. Monit.* **11,** (2009).

110. Vernouillet, G. *et al.* Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. *Chemosphere* **80,** 1062–1068 (2010).

111. Subedi, B. *et al.* Occurrence of Pharmaceuticals and Personal Care Products in German Fish Tissue: A National Study. *Environ. Sci. Technol.* **46,** 9047–9054 (2012).

112. Jeon, J., Kurth, D. & Hollender, J. Biotransformation Pathways of Biocides and Pharmaceuticals in Freshwater Crustaceans Based on Structure Elucidation of Metabolites Using High Resolution Mass Spectrometry. *Chem. Res. Toxicol.* **26,** 313–324 (2013).

113. Dussault, E. B., Balakrishnan, V. K., Solomon, K. R. & Sibley, P. K. Matrix effects on mass spectrometric determinations of four pharmaceuticals and personal care products in water, sediments and biota. *Can. J. Chem.* **87,** (2009).

114. Celiz, M. D., Tso, J. & Aga, D. S. Pharmaceutical metabolites in the environment: Analytical challenges and ecological risks. *Environ. Toxicol. Chem.* **28,** 2473–2484 (2009).

115. Kwon, J. W., Armbrust, K. L., Vidal-Dorsch, D. & Bay, S. M. Determination of 17alpha-ethynylestradiol, carbamazepine, diazepam, simvastatin, and oxybenzone in fish livers. *J AOAC Int* **92,** 359–369 (2009).

116. Miao, X. S. & Metcalfe, C. D. in *Anal. Fate Remov. Pharm. Water Cycle* (Petrovic, M. & Barceló, D.) **50,** (Elsevier, 2007).

117. Hao, C., Zhao, X. & Yang, P. GC-MS and HPLC-MS analysis of bioactive pharmaceuticals and personal-care products in environmental matrices. *TrAC, Trends Anal. Chem.* **26,** 569–580 (2007).

118. Boyd, R. K. Quantitative trace analysis by combined chromatography and mass spectrometry using external and internal standards. *Rapid Commun.Mass Sp.* **7,** 257–271 (1993).

119. Stüber, M. & Reemtsma, T. Evaluation of three calibration methods to compensate matrix effects in environmental analysis with LC-ESI-MS. *Anal. Bioanal. Chem.* **378,** 910–916 (2004).

120. Baird, D. J. & Van den Brink, P. J. Using biological traits to predict species sensitivity to toxic substances. *Ecotoxicol Env. Saf* **67,** 296–301 (2007).

121. Meredith-Williams, M. *et al.* Uptake and depuration of pharmaceuticals in aquatic invertebrates. *Environ. Pollut.* **165,** 250–258 (2012).

122. Rubach, M. N. *et al.* Framework for traits-based assessment in ecotoxicology. *Integr. Env. Assess Manag.* **7,** 172–186 (2011).

123. Berlioz-Barbier, A. *et al.* Multi-residue analysis of emerging pollutants in benthic invertebrates by modified micro-quick-easy-cheap-efficient-rugged-safe extraction and nanoliquid chromatography–nanospray–tandem mass spectrometry analysis. *J. Chromatogr. A* **1367,** 16–32 (2014).

124. Grabicova, K. *et al.* Presence of pharmaceuticals in benthic fauna living in a small stream affected by effluent from a municipal sewage treatment plant. *Water Res.* **72,** 145–53 (2015).

125. Bertelsen, S. L., Hoffman, A. D., Gallinat, C. A., Elonen, C. M. & Nichols, J. W. Evaluation of Log K Ow and Tissue Lipid Content As Predictors of Chemical Partitioning To Fish Tissues. *Environ. Toxicol.* **17,** 1447–1455 (1998).

126. Franke, C. *et al.* The assessment of bioaccumulation. *Chemosphere* **29,** 1501–1514 (1994).

127. Hermens, J. L. M., de Bruijn, J. H. M. & Brooke, D. N. The octanol-water partition coefficient: Strengths and limitations. *Environ. Toxicol. Chem.* **32,** 732–733 (2013).

128. Van der Oost, R., Beyer, J. & Vermeulen, N. P. E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **13,** 57–149 (2003).

129. Sugano, K. *et al.* Coexistence of passive and carrier-mediated processes in drug transport. *Nat. Rev. Drug Discov.* **9,** 597–614 (2010).

130. Fernandez-Torres, R., Bello Lopez, M. A., Consentino, M. O. & Mochón, M. C. Simultaneous Determination of Selected Veterinary Antibiotics and their Main Metabolites in Fish and Mussel Samples by High-Performance Liquid Chromatography with Diode Array-Fluorescence (HPLC-DAD-FLD) Detection. *Anal. Lett.* **44,** 2357–2372 (2011).

131. Cháfer-Pericás, C. *et al.* Multiresidue determination of antibiotics in aquaculture fish samples by HPLC–MS/MS. *Aquacult Res* **41,** e217–e225 (2010).

132. Franzellitti, S. *et al.* A multibiomarker approach to explore interactive effects of propranolol and fluoxetine in marine mussels. *Environ. Pollut.* **205,** 60–69 (2015).

133. Al-Ansari, A. M. *et al.* Bioaccumulation of the pharmaceutical 17alfa-ethinylestradiol in shorthead redhorse suckers (Moxostoma macrolepidotum) from the St. Clair River, Canada. *Environ. Pollut.* **158,** 2566–2571 (2010).

134. Du, B. *et al.* Bioaccumulation and trophic dilution of human pharmaceuticals across trophic positions of an effluent-dependent wadeable stream. *Phil. Trans. R. Soc. B* **369,** (2014).

135. Fair, P. a *et al.* Occurrence of triclosan in plasma of wild Atlantic bottlenose dolphins (Tursiops truncatus) and in their environment. *Environ. Pollut.* **157,** 2248–2254 (2009).

136. Salgueiro-González, N. *et al.* Occurrence, distribution and bioaccumulation of endocrine disrupting compounds in water, sediment and biota samples from a European river basin. *Sci. Total Environ.* **529,** 121–130 (2015).

137. Coogan, M. A., Edziyie, R. E., La Point, T. W. & Venables, B. J. Algal bioaccumulation of triclocarban, triclosan, and methyl-triclosan in a North Texas wastewater treatment plant receiving stream. *Chemosphere* **67,** 1911–1918 (2007).

138. Kookana, R. S. *et al.* Bioconcentration of triclosan and methyl-triclosan in marine mussels (Mytilus galloprovincialis) under laboratory conditions and in metropolitan waters of Gulf St Vincent, South Australia. *Mar. Pollut. Bull.* **74,** 66–72 (2013).

139. Ramaswamy, B. R. *et al.* Determination of preservative and antimicrobial compounds in fish from Manila Bay, Philippines using ultra high performance liquid chromatography tandem mass spectrometry, and assessment of human dietary exposure. *J. Hazard. Mater.* **192,** 1739–1745

140. Jakimska, A. *et al.* Development of a liquid chromatography-tandem mass spectrometry procedure for determination of endocrine disrupting compounds in fish from Mediterranean rivers. *J. Chromatogr. A* **1306,** 44–58 (2013).

141. Keith, T. L. *et al.* Identification and Quantitation of Nonylphenol Ethoxylates and Nonylphenol in Fish Tissues from Michigan. *Environ. Sci. Technol.* **35,** 10–13 (2001).

142. Maruya, K. A. *et al.* Organic contaminants of emerging concern in sediments and flatfish collected near outfalls discharging treated wastewater effluent to the Southern California Bight. *Environ. Toxicol. Chem.* **31,** 2683–2688 (2012).

143. Lajeunesse, A. *et al.* Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment: Evidence of biological effects. *Chemosphere* **83,** 564–571 (2011).

144. Fitzsimmons, P. N., Fernandez, J. D., Hoffman, A. D., Butterworth, B. C. & Nichols, J. W. Branchial elimination of superhydrophobic organic compounds by rainbow trout (Oncorhynchus mykiss). *Aquat Toxicol* **55,** 23–34 (2001).

145. Huggett, D. B., Cook, J. C., Ericson, J. F. & Williams, R. T. A Theoretical Model for Utilizing Mammalian Pharmacology and Safety Data to Prioritize Potential Impacts of Human Pharmaceuticals to Fish. *Hum. Ecol. Risk Assess. An Int. J.* **9,** 1789–1799 (2003).

146. Christen, V., Hickmann, S., Rechenberg, B. & Fent, K. Highly active human pharmaceuticals in aquatic systems: A concept for their identification based on their mode of action. *Aquat. Toxicol.* **96,** 167–181 (2010).

147. Margiotta-Casaluci, L. *et al.* Quantitative Cross-Species Extrapolation between Humans and Fish: The Case of the Anti-Depressant Fluoxetine. *PLoS One* **9,** e110467 (2014).

148. Correa-Reyes, G. *et al.* Nonylphenol algal bioaccumulation and its effect through the trophic chain. *Chemosphere* **68,** 662–670 (2007).

149. Ding, J., Lu, G., Li, S., Nie, Y. & Liu, J. Biological fate and effects of propranolol in an experimental aquatic food chain. *Sci. Total Environ.* **532,** 31–39 (2015).

150. Crane, M., Watts, C. & Boucard, T. Chronic aquatic environmental risks from exposure to human pharmaceuticals. *Sci. Total Environ.* **367,** 23–41 (2006).

151. Santos, L. H. M. L. M. *et al.* Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *J. Hazard. Mater.* **175,** 45–95 (2010).

152. Jones, O. A. H., Voulvoulis, N. & Lester, J. N. Potential Ecological and Human Health Risks Associated With the Presence of Pharmaceutically Active Compounds in the Aquatic Environment. *Crit. Rev. Toxicol.* **34,** 335–350 (2004).

153. Roos, V., Gunnarsson, L., Fick, J., Larsson, D. G. J. & Rudén, C. Prioritising pharmaceuticals for environmental risk assessment: Towards adequate and feasible first-tier selection. *Sci. Total Environ.* **421-422,** 102–10 (2012).

154. Carlsson, C., Johansson, A.-K., Alvan, G., Bergman, K. & Kühler, T. Are pharmaceuticals potent environmental pollutants?: Part I: Environmental risk assessments of selected active pharmaceutical ingredients. *Sci. Total Environ.* **364,** 67–87 (2006).

155. Owen, S. F. *et al.* Comparative physiology, pharmacology and toxicology of beta-blockers: Mammals versus fish. *Aquat. Toxicol.* **82,** 145–162 (2007).

156. Hutchinson, T. H. *et al.* Ecological risk assessment of endocrine disruptors. *Environ. Health Perspect.* **108,** 1007–1014 (2000).

157. Legler, J. & Brouwer, A. Are brominated flame retardants endocrine disruptors? *Environ. Int.* **29,** 879–85 (2003).

158. Colborn, T., vom Saal, F. S. & Soto, A. M. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **101,** 378–384 (1993).

159. Milla, S., Depiereux, S. & Kestemont, P. The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. *Ecotoxicology* **20,** 305–319 (2011).

160. Janer, G. & Porte, C. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. *Ecotoxicology* **16,** 145–160 (2007).

161. Depledge, M. . & Billinghurst, Z. Ecological Significance of Endocrine Disruption in Marine Invertebrates. *Mar. Pollut. Bull.* **39,** 32–38 (1999).

162. LeBlanc, G. Crustacean endocrine toxicology: a review. *Ecotoxicology* **16,** 61–81 (2007).

163. Sumpter, J. P. Feminized responses in fish to environmental estrogens. *Toxicol. Lett.* **82-83,** 737–742 (1995).

164. Jobling, S. *et al.* Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Env. Heal. Perspect.* **114 (Suple,** 32–39 (2006).

165. Oaks, J. L. *et al.* Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **427,** 630–633 (2004).

166. Valenti Jr., T. W. *et al.* Human Therapeutic Plasma Levels of the Selective Serotonin Reuptake Inhibitor (SSRI) Sertraline Decrease Serotonin Reuptake Transporter Binding and Shelter-Seeking Behavior in Adult Male Fathead Minnows. *Environ. Sci. Technol.* **46,** 2427–2435 (2012).

167. Contardo-Jara, V. *et al.* Molecular effects and bioaccumulation of levonorgestrel in the non-target organism Dreissena polymorpha. *Environ. Pollut.* **159,** 38–44 (2011).

168. Cuklev, F. *et al.* Diclofenac in fish: Blood plasma levels similar to human therapeutic levels affect global hepatic gene expression. *Environ. Toxicol. Chem.* **30,** 2126–2134 (2011).

169. Baquero, F., Martínez, J.-L. & Cantón, R. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* **19,** 260–265 (2008).

170. Pruden, A., Pei, R., Storteboom, H. & Carlson, K. H. Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado. *Environ. Sci. Technol.* **40,** 7445–7450 (2006).

171. Kristiansson, E. *et al.* Pyrosequencing of Antibiotic-Contaminated River Sediments Reveals High Levels of Resistance and Gene Transfer Elements. *PLoS One* **6,** 7 (2011).

172. D’Costa, V. *et al.* Antibiotic resistance is ancient. *Nature* (2011).

173. Homberg, A. *et al.* Does waterborne citalopram affect the aggressive and sexual behaviour of rainbow trout and guppy? *J. Hazard. Mater.* **187,** 596–599 (2011).

174. Brandao, F. P. *et al.* Short-term effects of neuroactive pharmaceutical drugs on a fish species: Biochemical and behavioural effects. *Aquat. Toxicol.* **144-145,** 218–229 (2013).

175. Sumpter, J. P., Donnachie, R. L. & Johnson, A. C. The apparently very variable potency of the anti-depressant fluoxetine. *Aquat. Toxicol.* **151,** 57–60 (2014).

176. Calisto, V. & Esteves, V. I. Psychiatric pharmaceuticals in the environment. *Chemosphere* **77,** 1257–1274 (2009).

177. Gardner, M. *et al.* The significance of hazardous chemicals in wastewater treatment works effluents. *Sci. Total Environ.* **437,** 363–372 (2012).

178. Jonsson, M., Fick, J., Klaminder, J. & Brodin, T. Antihistamines and aquatic insects: Bioconcentration and impacts on behavior in damselfly larvae (Zygoptera). *Sci. Total Environ.* **472,** 108–111 (2013).

179. Argyropoulos, S. V. & Nutt, D. J. The use of benzodiazepines in anxiety and other disorders. *Eur. Neuropsychopharmacol.* **9,** S407–S412 (1999).

180. Facciolo, R. M., Crudo, M., Zizza, M., Giusi, G. & Canonaco, M. alpha GABA(A) subunit-orexin receptor interactions activate learning/motivational pathways in the goldfish. *Behav Brain Res* **234,** 349–356 (2012).

181. Oggier, D. M., Weisbrod, C. J., Stoller, A. M., Zenker, A. K. & Fent, K. Effects of Diazepam on Gene Expression and Link to Physiological Effects in Different Life Stages in Zebrafish Danio rerio. *Environ. Sci. Technol.* **44,** 7685–7691 (2010).

182. Thomson, J. S., Watts, P. C., Pottinger, T. G. & Sneddon, L. U. Plasticity of boldness in rainbow trout, Oncorhynchus mykiss: do hunger and predation influence risk-taking behaviour? *Horm. Behav.* **61,** 750–757 (2012).

183. Anzelius, M., Ekström, P., Möhler, H. & Richards, J. G. Immunocytochemichal Localization of the GABAA/Benzodiazepine Receptor β2/β3 Subunits in the Optic Tectum of the Salmon. *J. Recept. Signal Transduct.* **15,** 413–425 (1995).

184. Friedl, W., Hebebrand, J., Rabe, S. & Propping, P. Phylogenetic conservation of the benzodiazepine binding sites: Pharmacological evidence. *Neuropharmacology* **27,** 163–170 (1988).

185. Stewart, A. *et al.* Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **35,** 1421–31 (2011).

186. Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O. & Larsson, D. G. J. Evolutionary Conservation of Human Drug Targets in Organisms used for Environmental Risk Assessments. *Environ. Sci. Technol.* **42,** 5807–5813 (2008).

187. Rand-weaver, M. *et al.* The Read-Across Hypothesis and Environmental Risk Assessment of Pharmaceuticals. *Environ. Sci. Technol.*, **47** (20), pp 11384–11395 (2013).

188. Smink, B. E. *et al.* The concentration of oxazepam and oxazepam glucuronide in oral fluid, blood and serum after controlled administration of 15 and 30 mg oxazepam. *Br. J. Clin. Pharmacol.* **66,** 556–560 (2008).

189. Freitas, R. *et al.* The effects of carbamazepine on macroinvertebrate species: Comparing bivalves and polychaetes biochemical responses. *Water Res.* **85,** 137–147 (2015).

190. Tanoue, R. *et al.* Uptake and Tissue Distribution of Pharmaceuticals and Personal Care Products in Wild Fish from Treated-wastewater Impacted Streams. *Environ. Sci. Technol.* (2015). doi:10.1021/acs.est.5b02478

191. Sisenwine, S. F. & Tio, C. O. The metabolic disposition of oxazepam in rats. *Drug Metab. Dispos.* **14 ,** 41–45 (1986).

192. Ramsay, R., EJ, H., RJ, P. & Wilder, B. BRain uptake of phenytoin, phenobarbital, and diazepam. *Arch. Neurol.* **36,** 535–539 (1979).

193. Greenblatt, D. J., Miller, L. G. & Shader, R. I. Neurochemical and pharmacokinetic correlates of the clinical action of benzodiazepine hypnotic drugs. *Am J Med.* **88,** S18–S24 (1990).

194. Dick, T., Sijm, M. & Van der Linde, A. Size-Dependent Bioconcentration Kinetics of Hydrophobic Organic Chemicals in-Fish Based on Diffusive Mass Transfer and Allometric Relationships. *Env. Sci Technol* **29,** 2769–2777 (1995).

195. Divoll, M., Greenblatt, D. J., Harmatz, J. S. & Shader, R. I. Effect of age and gender on disposition of temazepam. *J. Pharm. Sci.* **70,** 1104–1107 (1981).

196. Greenblatt, D. J., Divoll, M., Harmatz, J. S. & Shader, R. I. Oxazepam kinetics: effects of age and sex. *J. Pharmacol. Exp. Ther.* **215 ,** 86–91 (1980).

197. Ochs, H. R., Greenblatt, D. J. & Otten, H. Disposition of oxazepam in relation to age, sex, and cigarette smoking. *Klin. Wochenschr.* **59,** 899–903 (1981).

198. Rubinow, D. R. & Moore, M. Sex-dependent modulation of treatment response. *Dialogues Clin. Neurosci.* **6,** 39–51 (2004).

199. Carreau-Green, N. D., Mirza, R. S., MartÍnez, M. L. & Pyle, G. G. The ontogeny of chemically mediated antipredator responses of fathead minnows Pimephales promelas. *J. Fish Biol.* **73,** 2390–2401 (2008).

200. Sweet, L. Antidepressant Effects in Hybrid Striped Bass: Moving from External Exposures to Internal Doses. (2015).

201. Maximino, C. *et al.* Measuring anxiety in zebrafish: A critical review. *Behav. Brain Res.* **214,** 157–171 (2010).

202. Levin, E. D., Bencan, Z. & Cerutti, D. T. Anxiolytic effects of nicotine in zebrafish. *Physiol. Behav.* **90,** 54–58 (2007).

203. Weinberger, J. & Klaper, R. Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish Pimephales promelas (fathead minnow). *Aquat. Toxicol.* **151,** 77–83 (2014).

204. Hellstrom, G. Behaviour modifications: bridging the gap between laboratory assays and natural behaviour in Ecotoxicological risk assessment studies. (2015).

205. Bencan, Z., Sledge, D. & Levin, E. D. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacol. Biochem. Behav.* **94,** 75–80 (2009).

206. Gray, J. A. & McNaughton, N. *The Neuropsychology of Anxiety: An Enquiry into the Functions of the Septo-Hippocampal System, Second Edition*. (Oxford University Press Inc., 2000).

207. Lawrence, B. J. & Smith, R. J. F. Behavioral response of solitary fathead minnows,Pimephales promelas, to alarm substance. *J. Chem. Ecol.* **15,** 209–219 (1989).

208. Hall, R. C. & Zisook, S. Paradoxical reactions to benzodiazepines. *Br. J. Clin. Pharmacol.* **11,** 99S–104S (1981).

209. U.S. Environmental Protection Agency. Drinking Water Contaminant Candidate List CCL 3. (2010).

210. Kümmerer, K. Antibiotics in the aquatic environment - A review - Part I. *Chemosphere* **75,** 417–434

211. Conrad, A. *et al.* Fatty Acids of Lipid Fractions in Extracellular Polymeric Substances of Activated Sludge Flocs. **38,** (2003).

212. Wang, J. & Gardinali, P. Analysis of selected pharmaceuticals in fish and the fresh water bodies directly affected by reclaimed water using liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* **404,** 2711–2720 (2012).

213. López-Doval, J. C. *et al.* Pollution in mediterranean-climate rivers. *Hydrobiologia* **719,** 427–450 (2013).

214. Acuña, V. *et al.* Occurrence and in-stream attenuation of wastewater-derived pharmaceuticals in Iberian rivers. *Sci Total Env.* **503-504,** 133–41 (2015).

215. Arnot, J. A. & Gobas, F. A. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environ. Rev.* **14,** 257–297 (2006).

216. Writer, J. H., Ryan, J. N. & Barber, L. B. Role of biofilms in sorptive removal of steroidal hormones and 4-nonylphenol compounds from streams. *Env. Sci Technol* **45,** 7275–7283 (2011).

217. Xie, Z. *et al.* Behavioral and biochemical responses in freshwater fish Carassius auratus exposed to sertraline. *Chemosphere* **135,** 146–155 (2015).

218. Wang, Q. *et al.* Bioconcentration, metabolism and neurotoxicity of the organophorous flame retardant 1,3-dichloro 2-propyl phosphate (TDCPP) to zebrafish. *Aquat. Toxicol.* **158,** 108–115 (2015).

219. De Castro, A. P., Fernandes, G. D. R. & Franco, O. L. Insights into novel antimicrobial compounds and antibiotic resistance genes from soil metagenomes. *Front. Microbiol.* **5,** 1–9 (2014).

220. Port, J. a., Wallace, J. C., Griffith, W. C. & Faustman, E. M. Metagenomic Profiling of Microbial Composition and Antibiotic Resistance Determinants in Puget Sound. *PLoS One* **7,** (2012).

221. Chen, B., Liang, X., Huang, X., Zhang, T. & Li, X. Differentiating anthropogenic impacts on ARGs in the Pearl River Estuary by using suitable gene indicators. *Water Res.* **47**, (8):2811-20 (2013).

222. Szczepanowski, R. *et al.* Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* **155,** 2306–2319 (2009).

223. Schmieder, R. & Edwards, R. Insights into antibiotic resistance through metagenomic approaches. *Future Microbiol.* **7,** 73–89 (2012).

224. Novo, A., André, S., Viana, P., Nunes, O. C. & Manaia, C. M. Antibiotic resistance, Antimicrobial residues and bacterial community composition in urban wastewater. *Water Res.* **47,** 1875–1887 (2013).

225. Varela, A. R., André, S., Nunes, O. C. & Manaia, C. M. Insights into the relationship between antimicrobial residues and bacterial populations ina hospital-urban wastewater treatment plant system. *Water Res.* **54,** 327–336 (2014).

226. Rodriguez-Mozaz, S. *et al.* Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* **69,** 234–242 (2015).

227. Graham, D. W. *et al.* Antibiotic resistance gene abundances associated with waste discharges to the Almendares river near Havana, Cuba. *Environ. Sci. Technol.* **45,** 418–424 (2011).

228. Berendonk, T. U. *et al.* Tackling antibiotic resistance: the environmental framework. *Nat. Rev. Microbiol.* (2015). doi:10.1038/nrmicro3439

229. Andersson, D. I. & Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* **8,** 260–271 (2010).

230. Manaia, C. M. Antibiotic resistance in wastewater : origins , fate , and risks. 1–5 (2014). doi:10.1007/s11553-014-0452-3

231. Gerlai, R. A small fish with a big future: zebrafish in behavioral neuroscience. *Rev. Neurosci.* **22,** 3 (2011).

232. Webb, K. J. *et al.* Zebrafish reward mutants reveal novel transcripts mediating the behavioral effects of amphetamine. *Genome Biol.* **10,** R81 (2009).

233. Michael Stewart, A. & V. Kalueff, A. The Developing Utility of Zebrafish Models for Cognitive Enhancers Research. *Curr. Neuropharmacol.* **10,** 263–271 (2012).

234. Sackerman, J. *et al.* Zebrafish Behavior in Novel Environments: Effects of Acute Exposure to Anxiolytic Compounds and Choice of Danio rerio Line. *Int. J. Comp. Psychol.* **23,** 43–61 (2010).

235. Gerlai, R., Lee, V. & Blaser, R. Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (Danio rerio). *Pharmacol. Biochem. Behav.* **85,** 752–761 (2006).

236. Cachat, J. *et al.* Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nat. Protoc.* **5,** 1786–1799 (2010).

237. Stadnicka, J., Schirmer, K. & Ashauer, R. Predicting Concentrations of Organic Chemicals in Fish by Using Toxicokinetic Models. *Environ. Sci. Technol.* **46,** 3273–3280 (2012).

238. McCarty, L. S. & Mackay, D. Enhancing ecotoxicological modeling and assessment. Body Residues and Modes Of Toxic Action. *Environ. Sci. Technol.* **27,** 1718–1728 (1993).