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PRESENCIA DE CONTAMINANTES QUÍMICOS EMERGENTES EN MUESTRAS AMBIENTALES Y BIOLÓGICAS

Memoria presentada por

Dña. María del Carmen Gómez Regalado

Para optar al grado de

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Tesis dirigida por

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Resumen

En la presente Memoria de Doctorado, se presentan los resultados obtenidos durante la realización de la Tesis Doctoral titulada "*Presencia de contaminantes químicos emergentes en muestras ambientales y biológicas*". Para lograr los objetivos planteados en este trabajo de investigación, se han estudiado distintas matrices ambientales, como el pepino de mar *(Holothuria tubulosa),* el mejillón *(Mytilus galloprovincialis)* y dos especies de anémona *(Anemonia sulcata* y *Actinia equina)* así como una matriz biológica, la uña humana, con el fin de evaluar la presencia de contaminantes emergentes pertenecientes a distintas familias. La información se ha organizado en dos grandes bloques; uno introductorio y otro experimental, de resultados y discusión.

Por un lado, en la Introducción se incluye una contextualización general de los contaminantes emergentes, su significado, importancia y las distintas familias de compuestos químicos considerados como tal. Presenta las familias de contaminantes consideradas en esta Tesis, como los filtros ultravioleta o los compuestos activos farmacéuticos, así como los efectos adversos sobre la salud de los ecosistemas y humanos con los que han sido relacionados. Un segundo aspecto tratado en la Introducción es la definición de bioindicadores para la determinación de contaminantes emergentes, concretamente el uso del mejillón mediterráneo, el pepino de mar, y de las anémonas; así como el uso de biomarcadores de exposición, como la uña humana, para la evaluación de estos contaminantes y su presencia en el cuerpo humano. Finalmente, se describe brevemente la metodología analítica empleada para la determinación de contaminantes en las matrices seleccionadas, entre ellas la técnica cromatográfica usada (UHPLC-MS/MS) y las técnicas de extracción aplicadas (asistida por ultrasonidos, asistida por microondas y QuEChERs). Por último, se describe el tratamiento estadístico de los datos obtenidos en la fase experimental, los diseños de experimentos y la fase de calibración y validación analítica.

El segundo bloque de la presente Memoria se centra en la **Parte experimental, resultados y discusión,** que a su vez se divide en dos secciones. La **Sección I** expone el desarrollo, validación y aplicación de

métodos analíticos para la determinación de contaminantes emergentes en muestras ambientales y biológicas. Está formada por cinco capítulos:

- El **Capítulo 1** se centra en la revisión bibliográfica de la literatura científica sobre el desarrollo de métodos analíticos para la determinación de compuestos activos farmacéuticos en organismos acuáticos. En esta revisión se describen los bioindicadores usados en los distintos niveles de la cadena trófica, se recogen las metodologías analíticas usadas, desde la recogida de muestra y el pretratamiento de la misma hasta su análisis, y se profundiza en las técnicas de extracción y limpieza contempladas así como en la cromatografía de líquidos acoplada a los distintos detectores.

- El **Capítulo 2** versa sobre el desarrollo y validación de un método analítico para la determinación de filtros ultravioleta en mejillón mediterráneo mediante UHPLC-MS/MS. Se evaluaron las distintas variables que influían en la extracción (tipo de disolvente, cantidad, número de ciclos de extracción, parámetros de la digestión asistida por ultrasonidos). Posteriormente se validó el método y se aplicó a mejillones recogidos de cinco áreas diferentes del litoral granadino.

- El **Capítulo 3** desarrolla un método multi-residuo para la determinación de 17 antibióticos en la pared corporal del pepino de mar *(Holothuria tubulosa).* Se evaluaron las variables influyentes en la extracción y limpieza del extracto por métodos tanto univariantes como multivariantes y finalmente se validó el método y aplicó a muestras de pepino de mar recogidas de nueve zonas de la costa de Granada.

- El **Capítulo 4** detalla el desarrollo de un método UHPLC-MS/MS para la determinación de filtros ultravioleta en uña humana. Igual que en los capítulos anteriores, se evaluaron los distintos parámetros influyentes en la extracción de los compuestos de interés, se validó y se aplicó a muestras de 22 sujetos elegidos al azar.

- En el **Capítulo 5** se presenta una comparación entre dos técnicas de extracción para la determinación de 17 antibióticos usando como matriz de estudio la uña humana. Se evaluaron las variables influyentes en cada una de las dos técnicas de extracción. Posteriormente, se seleccionó la asistida por ultrasonidos teniendo en cuenta la practicabilidad de la

técnica y la eficiencia en la extracción de estos compuestos. Finalmente, se validó el método y se aplicó a la muestra de 10 voluntarios y voluntarias.

La **Sección II** se centra en la exposición de contaminantes emergentes en organismos acuáticos, concretamente compuestos activos farmacéuticos, en organismos marinos (el pepino de mar y dos especies de anémonas). El objetivo principal de esta sección es valorar el peligro potencial que puede tener la presencia de estos contaminantes en el medio ambiente cuando la biota marina está continuamente expuesta a ellos. Son cuatro los capítulos que conforman esta sección.

- El **Capítulo 6** se centra en una revisión bibliográfica sobre la bioacumulación y la bioconcentración de compuestos activos farmacéuticos en organismos acuáticos. Aporta una base de datos sobre la evaluación y factores influyentes así como los distintos ensayos que se realizan para evaluar estos factores.

- El **Capítulo 7** recoge el estudio de bioconcentración de un fármaco antidepresivo, la venlafaxina y su principal metabolito o-desmetilvenlafaxina en los organismos marinos anteriormente descritos. Para ello, se sometieron a los organismos de estudio a una exposición de 28 días y posterior depuración de 52 días. Se recogieron muestras cada cuatro días y se analizaron mediante UHPLC-MS/MS.

- En el **Capítulo 8** se expone un estudio de bioconcentración similar al descrito en el Capítulo 7 siendo los compuestos de estudio tres antibióticos; la ciprofloxacina, el sulfametoxazol y la trimetoprima. Se llevó a cabo la misma etapa experimental de exposición y depuración que en el capítulo anterior y se realizó un posterior análisis con un método analítico previamente validado por el grupo de investigación.

- El **Capítulo 9** está dedicado a un estudio de bioconcentración de características similares a los descritos anteriormente, evaluando como contaminantes un antiepiléptico, la carbamazepina, y un ß-bloqueador, el atenolol. Todas las condiciones experimentales fueron previamente validadas y las muestras fueron analizadas mediante UHPLC-MS/MS.

De forma complementaria al desarrollo de la presente Tesis Doctoral, durante mi estancia en el departamento de Química Analítica, he colaborado en otros trabajos científicos publicados en revistas internacionales de alto impacto que a continuación se enumeran:

- Laura Martín-Pozo, **María del Carmen Gómez-Regalado,** Inmaculada Moscoso-Ruiz, Alberto Zafra-Gómez. Analytical methods for the determination of endocrine disrupting chemicals in cosmetics and personal care products: A review. *Talanta,* 234 (2021) 122642.

- Inmaculada Moscoso-Ruiz, Yolanda Gálvez-Ontiveros, María Giles-Mancilla, **María del Carmen Gómez-Regalado,** Ana Rivas, Alberto Zafra-Gómez. Improved method for the determination of endocrine-disrupting chemicals in urine of school-age children using micro-liquid extraction and UHPLC-MS/MS. *Analytical and Bioanalytical Chemistry,* 414 (2022) 6681-6694.

- Laura Martín-Pozo, **María del Carmen Gómez-Regalado,** María Teresa García-Córcoles, Alberto Zafra-Gómez. Removal of quinolone antibiotics from wastewaters and sewage sludge, in: Emerging Contaminants in the Environment. Edited by Hemen Sarma, Delfina C. Domínguez, Wen-Yee Lee. Elsevier INC. Philadelphia, PA, USA (2022) 335-370.

- Morsina Çipa, **María del Carmen Gómez-Regalado,** Alberto Navalón, Elda Marku, Alberto Zafra-Gómez. Analysis of polycyclic-aromatic hydrocarbons in blood serum samples obtained from oil refinery workers. Determination of occupational exposure. *Atmospheric Pollution Research,* 13 (2022) 101589.

- D. Martin-Way, I. Puche-Sanz, J.M. Cozar, A. Zafra-Gómez, **M.D.C. Gómez-Regalado,** C.M. Morales-Álvarez, A.F. Hernández, L.J. Martínez-González, M.J. Álvarez-Cubero. Genetic variants of antioxidant enzymes and environmental exposures as molecular biomarkers associated with the risk and aggressiveness of bladder cancer. *Science of the Total Environment,* 843 (2022) 156965.

Índice

Índice

Acrónimos y Abreviaturas

ACN	Acetonitrilo
AEMPS	Agencia española de medicamentos y productos sanitarios
API	Ionización a presión atmosférica
ATN	Atenolol
AVB	Avobenzona
BEH	Partícula Híbrida con Puente de Etileno *(Ethylene Bridged Hybrid)*
BP	Benzofenona
BP-1	Benzofenona 1
BP-2	Benzofenona 2
BP-3	Benzofenona 3
BP-6	Benzofenona 6
BP-8	Benzofenona 8
CBZ	Carbamazepina
CEs	Contaminantes emergentes
CIP	Ciprofloxacina
CLA	Claritromicina
DAN	Danofloxacina
DIF	Difloxacina
DoE	Diseño de experimentos *(Design of experiments)*
d-SPE	Dispersión en fase sólida dispersiva *(dispersive solid phase extraction)*
EDAR	Estación depuradora de aguas residuales
EDC	Disruptor endócrino químico, *(Endocrine disruptor chemical)*
ENR	Enrofloxacina
ERY	Eritromicina

ESI	Ionización por electrospray *(electrospray ionization)*
FDA	*Food and Drug Administration*
Filtros UV	Filtros Ultravioleta
FLU	Flumequina
HILIC-LC	Cromatografía de líquidos de interacción hidrofílica
HPLC	Cromatografía de líquidos de alta resolución
HSS	Partículas de sílice de alta resistencia
IUPAC	*International Unionv of Pure and Applied Chemistry*
LEVO	Levofloxacina
LOD	Límite de detección
LOQ	Límite de cuantificación
MAE	Extracción asistida por microondas (*microwave assisted extraction)*
MAR	Marbofloxacina
4-MBC	4-metilbencilideno alcanfor
MeOH	Metanol
MOX	Moxifloxacina
MRM	Modo de reacciones múltiples *(Multiple reaction monitoring)*
MS	Espectrometría de masas
MS/MS	Espectrometría de masas en tándem
NAL	Ácido nalidíxico
NCI	Instituto Nacional del cáncer
NOR	Norfloxacina
NP-LC	Cromatografía de líquidos en fase normal
4-OH-BP	4-hidroxibenzofenona
O-VFX	O-desmetilvenlafaxina

OXA Oxacilina

OXO Ácido oxolínico

PCPs Productos de cuidado personal

PDA Detector de fotodiodos en serie

PEN-V Penicilina V

PhACs Compuestos activos farmacéuticos

QDA Detector de masas de cuadrupolo simple

QqQ Analizador triple cuadrupolo

RA-ß Receptor adrenérgico ß

RDL Rango dinámico lineal

RP-LC Cromatografía de líquidos en fase reversa

RSM Metodología de superficie de respuesta *(response surface methodology)*

SFX Sulfametoxazol

SRM Modo de reacción seleccionada (*Selected reaction monitoring)*

THB Trihidroxibenzofenona

TMP Trimetoprima

UAE Extracción asistida por ultrasonidos *(Ultrasound assisted extraction)*

UHPLC Cromatografía de líquidos de ultra-alta resolución

UNESCO Organización de las Naciones Unidas para la Educación, la Ciencia y la Cultura.

VFX Venlafaxina

VIM Vocabulario internacional de metrología

Objetivos

Actualmente existe una gran preocupación social, política y científica en relación a la presencia ubicua de algunos contaminantes emergentes en el medioambiente y a los efectos adversos que éstos pueden causar tanto en los ecosistemas, como en la salud humana. Es por esto que nace la necesidad de desarrollar nuevos métodos analíticos para el control de estos contaminantes, así como ensayos a pequeña escala de exposición a los mismos, para poder conocer los efectos en la salud cuando la exposición es continuada. En este contexto, el **objetivo principal** de la presente Tesis Doctoral ha sido el estudio de la presencia de numerosos contaminantes en el medio acuático y en el ser humano y su capacidad de bioconcentración en cuatro bioindicadores marinos de elevado interés medioambiental: el mejillón mediterráneo *(Mytilus galloprovincialis),* el pepino de mar *(Holothuria tubulosa),* y dos especies de anémona de mar *(Anemonia sulcata* y *Actinia equina).*

Los contaminantes objeto de estudio seleccionados han sido algunos compuestos de amplia aplicación en la actualidad, concretamente distintos filtros ultravioleta, entre los que cabe destacar la familia de las benzofenonas, considerados también disruptores endocrinos; y algunos principios activos farmacéuticos, diferentes tipos de antibióticos, fármacos neuroactivos y un β-bloqueador. El criterio para seleccionar estos analitos ha sido su presencia en listas de vigilancia del medio marino, la legislación vigente que limita la concentración de algunos de ellos y su elevado uso en las actividades cotidianas del ser humano.

Para lograr el objetivo principal, se plantearon una serie de objetivos específicos, que se describen a continuación:

1. Elaborar una revisión exhaustiva sobre la literatura científica desde los últimos diez años hasta la actualidad, en relación a la determinación de los contaminantes seleccionados en los distintos niveles de la cadena trófica, así como la recopilación de los estudios de bioacumulación/bioconcentración realizados hasta la fecha en distintos bioindicadores marinos. Este primer objetivo es abordado en los **Capítulos 1** y **6**.

2. Desarrollar métodos de buenas características analíticas para la detección y cuantificación de los distintos contaminantes en los bioindicadores marinos mejillón mediterráneo y pepino de mar. Para ello, se evaluarán las distintas técnicas de extracción, las variables influyentes en las mismas, así como los procedimientos de limpieza. Del mismo modo, se desarrollará el método cromatográfico optimizando los distintos parámetros modificables del equipo. Por último, se procederá a la validación (en términos de exactitud, linealidad, sensibilidad y selectividad) y aplicación del método en muestras recogidas en la costa granadina. Este objetivo corresponde a los **Capítulos 2 y 3** de la presente memoria.

3. Desarrollar métodos analíticos para la determinación de los contaminantes emergentes estudiados en una matriz humana, la uña. Para ello, se evaluará la aptitud de la misma como biomarcador de exposición, ya que resulta una matriz biológica usada menos frecuentemente y se optimizará la extracción de los analitos diana mediante estrategias multivariantes principalmente. Finalmente, se procederá a la validación de los métodos atendiendo a las Guías Internacionales de Validación y se aplicará en muestras de voluntarios. Este objetivo se relaciona con los **Capítulos 4 y 5**.

4. Realizar un estudio de bioconcentración en tres bioindicadores marinos; pepino de mar, ortiga de mar y tomate de mar. Para ello, se expondrá a los especímenes a medios contaminados de los fármacos seleccionados en concentración coherente con las guías publicadas en la literatura científica en esta materia. El propósito será la determinación de la relación entre los niveles de estos compuestos en los bioindicadores y del medio acuático en el que habitan, así como la capacidad de depuración que tienen estos animales tras el período de exposición. Este objetivo es abordado en los **Capítulos 7, 8 y 9.**

Introducción

1. Contaminantes emergentes

Los contaminantes emergentes (CEs) son sustancias químicas de síntesis/naturales o microorganismos que entran continuamente en el medioambiente y que por lo general no están controlados y pueden causar efectos adversos, conocidos o no, en la salud humana o en el ecosistema [1]. El término emergente no implica solamente que sean sustancias nuevas o que estén empezando a entrar en el medio recientemente, si no que se ha descubierto hace poco tiempo la amenaza o el impacto nocivo que suponen [2].

En las últimas décadas, el elevado ritmo de crecimiento de la población mundial, las prácticas agrícolas y la industrialización son factores que afectan a la entrada de numerosos contaminantes químicos orgánicos en el medio ambiente. Hoy en día, la mayoría de ellos no están regulados por leyes ambientales, incluso se desconoce el impacto toxicológico que pueden causar en la salud o en el medioambiente [3]. Esto ha despertado el interés de los organismos reguladores de todo el mundo ya que cada vez son más las sustancias detectadas tanto en aguas superficiales como subterráneas [4].

Entre las principales vías de entrada de las distintas familias de CEs destacan el vertido de aguas residuales tanto tratadas como sin tratar, procedentes de estaciones depuradoras de aguas residuales (EDAR), hospitales, fugas de alcantarillado, lixiviados de vertederos y vertidos procedentes de zonas agrícolas y urbanas en las que se usa el estiércol de desecho para actividades de riego [5].

Las investigaciones realizadas en los últimos 20-25 años, han encontrado diferentes contaminantes de origen antropogénico en las aguas tanto superficiales como subterráneas. Entre ellos destacan productos farmacéuticos, hormonas, pesticidas, drogas ilícitas, productos de cuidado personal (PCPs), filtros ultravioleta (UV), productos químicos industriales (tensioactivos, aditivos de la gasolina, retardantes de llama bromados, plastificantes y compuestos perfluorados) y nanomateriales, entre otros [6]. La Figura 1 muestra algunas de estas familias junto con los efectos adversos en la salud con los que se les ha relacionado.

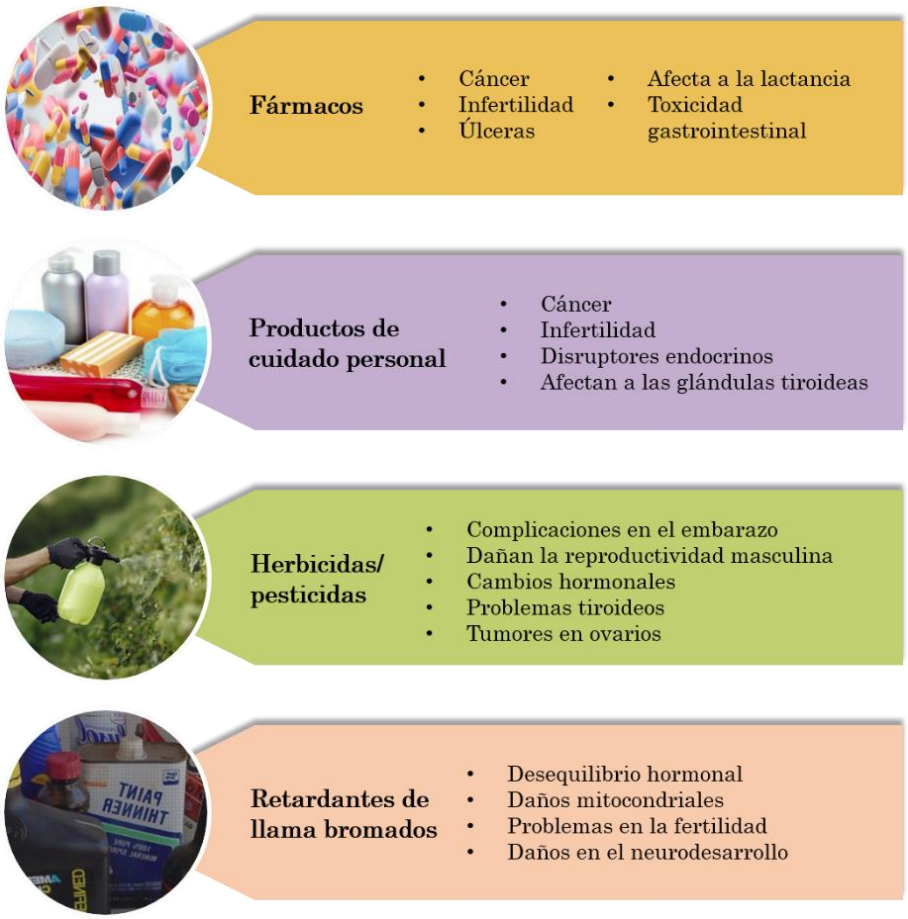


Figura 1. *Familias de contaminantes emergentes.*
Elaboración propia basada en Kumar et al. [1]

La asociación entre la entrada de estos compuestos en el medioambiente y los vertidos de las EDAR se debe al uso ubicuo de muchos de estos compuestos, así como a la falta de eficacia de los tratamientos de eliminación que se emplean actualmente. Además, dado que muchas de estas sustancias no están incluidas en la legislación vigente en materia de tratamiento de aguas residuales, las EDAR no están destinadas explícitamente a eliminarlas [7].

El uso de estos compuestos en nuestro día a día es continuado, y por tanto, también lo es su emisión, lo cual supone una amenaza mundial a la salud pública y de los ecosistemas. Los CEs suelen ser bioactivos y bioacumulativos. Se prevé que el aumento de la población humana

mundial, sobre todo en lugares de alta densidad, provoque un incremento de la concentración de CEs en el medioambiente, así como el número de ecosistemas contaminados [1]. Aunque su presencia suele estar en un rango de concentración bajo, del orden de nanogramos/gramo o incluso cantidades más pequeñas, son motivo de preocupación tanto ambiental como sanitaria debido a su potencial comportamiento tóxico, ya que muchos de ellos tienen la capacidad de interferir con el sistema endocrino de distintos organismos, causando efectos adversos tanto en humanos como en animales aun apareciendo en el medio en concentraciones subterapéuticas [6, 8].

Es por esto que se deben desarrollar métodos analíticos altamente sensibles y selectivos que permitan controlar la presencia de CEs en el medioambiente, y con ellos elaborar una legislación más estricta en cuanto a los límites permitidos para que, junto a la concienciación social y la mejora de los tratamientos de aguas residuales, se pueda llegar a minimizar el impacto que causan.

2. Productos de cuidado personal. Disruptores endocrinos

La Agencia española de medicamentos y productos sanitarios define los PCPs como "*aquellos productos que sin tener la consideración legal de cosméticos, biocidas, productos sanitarios o medicamentos, están destinados a ser aplicados sobre la piel, dientes o mucosas del cuerpo humano con la finalidad de higiene o estética, o para neutralizar o eliminar ectoparásitos*" [9].

Por otro lado, según el Real Decreto (RD 1599/1997), se define producto cosmético como "*toda sustancia o preparado destinado a ser puesto en contacto con las diversas partes superficiales del cuerpo humano (epidermis, sistema piloso y capilar, uñas, labios y órganos genitales externos) o con los dientes y las mucosas bucales, con el fin exclusivo o principal de limpiarlos, perfumarlos, modificar su aspecto, y/o corregir los olores corporales, y/o potegerlos o mantenerlos en buen estado"* [10].

Tanto los PCPs como los cosméticos se han vuelto imprescindibles en nuestra vida diaria. Suelen ser mezclas complejas que en su formulación contienen diversas sustancias químicas, como son los conservantes, las fragancias, los fijadores o los filtros ultravioleta, entre otros [11]. Aunque estos compuestos químicos estén autorizados en los cosméticos y se califican como seguros, algunos de ellos son capaces de alterar el funcionamiento normal del sistema endocrino, considerándose disruptores endocrinos químicos.

La Sociedad Endocrina define los disruptores endocrinos químicos (EDCs) como compuestos químicos exógenos (no naturales), o mezcla de químicos, que interfieren con cualquier aspecto de la acción hormonal [12]. Estos compuestos afectan al equilibrio hormonal corporal por varios mecanismos: pueden alterar la producción hormonal, imitar a las hormonas, influir en el desarrollo de receptores hormonales, funcionar como antagonistas hormonales o modificar la unión hormonal [1]. A pesar de que estos compuestos están presentes en algunos productos destinados a la ingestión directa, se podría decir que la principal vía de entrada en el cuerpo humano es mediante la absorción dérmica. Una vez en el cuerpo,

son mayoritariamente metabolizados y excretados y, en ocasiones, bioacumulados [11]. Entre los posibles efectos adversos que pueden causar, se les ha relacionado con el decrecimiento de la fertilidad, malformaciones en los órganos sexuales así como relación con el cáncer de mama y testicular, disfunciones tiroideas, desajustes en el neurodesarrollo y relación con la obesidad [13, 14]. Los períodos críticos de exposición son el del desarrollo fetal y la pubertad, que se traduce en una mayor vulnerabilidad a la aparición de algunas enfermedades en un período de tiempo medio-largo [15].

Entre estas familias de compuestos presentes en algunos PCPs así como cosméticos, la presente tesis doctoral se ha centrado en la de los filtros ultravioleta como analitos objetivo, que han sido seleccionados atendiendo a criterios como frecuencia de uso, empleo doméstico e industrial, persistencia en el medioambiente y su capacidad de bioacumulación en distintos organismos. La Figura 2 muestra un esquema de las principales vías de entrada de estos compuestos en el medio ambiente.

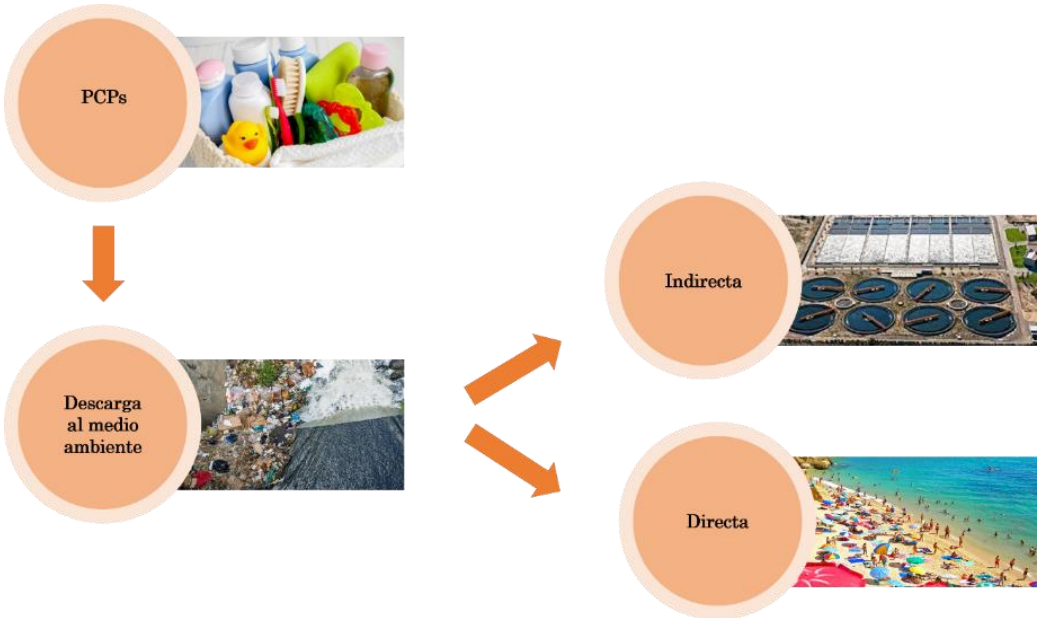


Figura 2. *Vías de entrada de los CEs presentes en PCPs al medio ambiente.* Elaboración propia.

2.1. Filtros ultravioleta

Son filtros UV son una familia de compuestos orgánicos que presentan estructuras aromáticas capaces de absorber la radiación solar, tanto la UV-A (320-400 nm) como la UV-B (280-320 nm). En los últimos años, ha aumentado la preocupación sobre la exposición al sol y los riesgos asociados a esta. Por ello, el consumo de los PCPs dedicados a la protección solar, como las cremas solares, champús o tintes para el pelo, ha incrementado de forma notable [16]. Su elevado uso ha hecho que se reconozcan muchos de ellos como CEs, constituyendo un riesgo para la salud humana y para el medio ambiente. Se ha documentado la presencia de filtros UV en el 95% de los efluentes de aguas residuales y en el 86% de las aguas superficiales del mundo, demostrando su ubicuidad en el medioambiente [16].

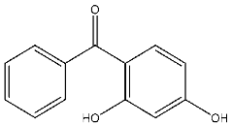
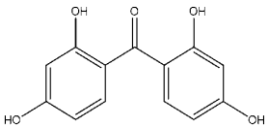
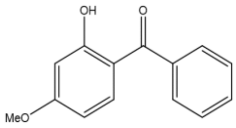
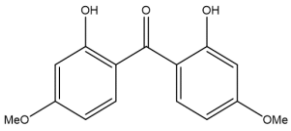
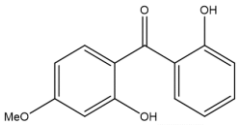
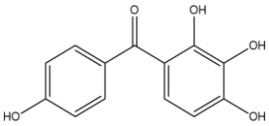
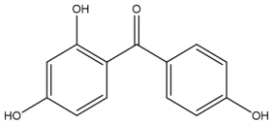
Se estima que actualmente se utilizan hasta 25.000 toneladas de productos que contienen filtros UV a nivel mundial [17]. Muchos de ellos han demostrado tener efectos estrogénicos y efectos adversos en la reproducción, siendo también considerados EDCs [18]. Su modo de entrada en el medioambiente puede ser directo, mediante actividades recreacionales de los bañistas en las zonas costeras, o indirecto, mediante tratamientos ineficaces en las estaciones depuradoras de aguas residuales.

Teniendo en cuenta lo anteriormente expuesto, en la presente Tesis Doctoral, se han seleccionado 9 filtros ultravioleta considerados también disruptores endocrinos como compuestos de estudio. Concretamente siete benzofenonas, la avobenzona y el 4-metilbencilidenalcanfor.

2.2 Benzofenonas

Las benzofenonas (BPs) son cetonas aromáticas. Hoy en día, son los filtros UV químicos más utilizados en PCPs, ya que son capaces de absorber la luz ultravioleta y disiparla en forma de calor. La Tabla 1 muestra las benzofenonas de estudio en el presente trabajo así como algunas de sus propiedades físicoquímicas.

Tabla 1. *Nombre, estructura y propiedades de las BPs de estudio*

Compuesto	Fórmula molecular	Estructura química	Masa molecular (g mol^{-1})	pK_a	Log K_{ow}
BP-1	$C_{13}H_{10}O_3$		214.22	7.72	2.96
BP-2	$C_{13}H_{10}O_5$		246.22	7.10	2.78
BP-3	$C_{14}H_{12}O_3$		228.25	7.10	3.79
BP-6	$C_{15}H_{14}O_5$		274.27	6.81	3.9
BP-8	$C_{14}H_{12}O_4$		244.25	6.78	3.82
4-OH-BP	$C_{13}H_{10}O_5$		246.22	7.38	2.78
THB	$C_{13}H_{10}O_4$		230.22	7.51	2.86

BP-1: benzofenona 1; BP-2: benzofenona 2; BP-3: benzofenona 3; BP-6: benzofenona 6; BP-8: benzofenona 8; 4-OH-BP: 4-hidroxibenzofenona; THB: trihidroxibenzofenona.

Debido a su estructura química y sus propiedades, se trata de compuestos muy estables que además presentan un comportamiento lipofílico, lo que se traduce en que, una vez que ingresan en el organismo (principalmente por absorción dérmica), tienen la facilidad de acumularse en los tejidos [19]. De hecho, han sido detectadas en distintas matrices biológicas como por ejemplo orina [20], leche materna [21, 22], placenta [23], sangre del cordón umbilical [24] o pelo [25] (Li et al., 2022), y numerosas muestras ambientales, como aguas costeras [26], aguas residuales, sedimentos y lodos [27] y organismos marinos como peces [28], cangrejos de río, ostras y mejillones [29].

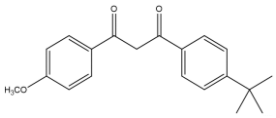
Su ubicuidad en el medio ambiente y su carácter como disruptores endocrinos ha promovido que algunos organismos reguladores, como la Unión Europea limiten el contenido máximo de éstos en cosméticos, que puede contener hasta un 6% en productos faciales, para manos y labiales, hasta un 2.2% en productos corporales y hasta un 0.5% en otros productos en el caso de la benzofenona 3 (BP-3) [30]. Un ejemplo de su elevada presencia y persistencia en el medioambiente es el gran impacto que están teniendo en los blanqueamientos de los arrecifes de coral. Por ello, algunos Estados como Florida o Hawaii han prohibido la presencia de BP-3 en cremas solares [31]. La BP-3 es el filtro UV más usado en PCPs aunque también tiene usos industriales para la prevención del color en productos plásticos, juguetes o materiales de embalaje [32]. Ha demostrado poseer efecto citotóxico además de presentar carácter estrogénico y antiandrogénico. En el caso de peces zebra, se ha demostrado que causa mortalidad, malformaciones en los embriones e incubaciones fallidas [33]. Cabe destacar que uno de sus principales metabolitos, también usado como filtro UV en PCPs, la benzofenona 1 (BP-1), ha demostrado tener mayor actividad estrogénica que la BP-3 [34].

2.3 Avobenzona

La avobenzona (AVB) es un filtro UV también ampliamente usado como aditivo en los PCPs ya que es capaz de absorber la radiación UV en

un rango muy amplio de longitudes de onda (320-400 nm). La Tabla 2 muestra su estructura así como principales propiedades.

Tabla 2. *Estructura y propiedades de la AVB*

Compuesto	Fórmula molecular	Estructura química	Masa molecular ($g\ mol^{-1}$)	pK_a	$logK_{ow}$
AVB	$C_{20}H_{22}O_3$		310.39	9.74	4.51

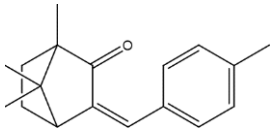
A pesar de que la AVB puede causar dermatitis de contacto, es considerado un compuesto seguro [35]. El límite máximo permitido en Europa y Australia en preparados cosméticos es del 5% y en Estados Unidos, la FDA (*Food and Drug Administration*) permite hasta un máximo de 3% en formulaciones de protección solar. Esto es debido a la inestabilidad del compuesto y los efectos que pueden causar sus derivados [35, 36].

Debido a su estructura química, la AVB disminuye su efecto protector hasta un 60% cuando la exposición al sol es prolongada ya que tiene lugar una isomerización cetoenólica cuando la energía incide sobre el compuesto [37]. Además, la radiación UV es capaz de dividir su enlace homolítico dando lugar a radicales libres capaces de dañar a las proteínas y el ADN, lo cual explica algunos de los efectos adversos encontrados [38]. Se ha demostrado su potencial obesogénico, promoviendo la acumulación de gotas de lípidos y la producción de adiponectina durante la adipogénesis [35]. Posee propiedades similares a las hormonas antiandrogénicas, (anti)glucocorticoides y (anti)tiroideas, por lo que también se considera disruptor endocrino [38]. En muestras biológicas, se ha encontrado que disminuye la calidad de las células reproductoras en mejillones [39], afecta a la actividad de las enzimas antioxidantes e inhibe la función neurológica. En corales, decrece la eficiencia fotosintética [40].

2.4 4-metilbencilidenalcanfor

El 4-metilbencilidenalcanfor (4-MBC) es uno de los filtros UV más usados en cremas solares, lociones corporales y champús. Tiene una buena absorción de la radiación ultravioleta, con un rango de longitudes de onda de 290 a 320 nm. Tras la exposición a la radiación UV, sufre rápidamente una reacción de isomerización fotoquímica y la radiación absorbida se emite en forma de calor [41]. Se trata de un compuesto muy estable que no puede ser completamente eliminado en las plantas depuradoras de aguas residuales [42]. Además, también presenta carácter lipofílico, lo cual implica que tiende a bioacumularse en los tejidos, sedimentos y en organismos acuáticos [41, 43]. La Tabla 3 muestra su estructura y propiedades físicoquímicas.

Tabla 3. *Estructura y propiedades del 4-MBC*

Compuesto	Fórmula molecular	Estructura química	Masa molecular ($g\ mol^{-1}$)	pK_a	$logK_{ow}$
4-MBC	$C_{18}H_{22}O$		254.4	9.74	5.02

Algunos estudios toxicológicos han documentado su comportamiento como disruptor endocrino en mamíferos. Por ejemplo, en 2005, se demostró que tenía actividad estrogénica en ratas [43]. Por otro lado, se han observado efectos estrogénicos en vertebrados acuáticos como el pez cebra, y toxicidad para el desarrollo y la reproducción en invertebrados [41]. En organismos bentónicos, se han encontrado alteraciones en la reproducción y también se les ha relacionado con el blanqueamiento de los corales [44].

3 Compuestos activos farmacéuticos

La FDA los define como "*cualquier sustancia destinada a incorporarse a un activo farmacéutico terminado y que tiene por objeto proporcionar actividad farmacológica u otro efecto directo en el diagnóstico, cura, mitigación, tratamiento o prevención de enfermedades, o afectar la estructura o cualquier función del cuerpo"* [45]. Los productos de uso farmacéutico han sido incluidos en la lista de contaminantes emergentes por la UNESCO (Organización de las Naciones Unidas para la Educación, la Ciencia y la Cultura), y su detección, así como eliminación se han incorporado en la Agenda 2030 como meta de los objetivos de desarrollo sostenible [4, 46].

En el contexto de la política de aguas Europea, la Directiva 2013/39/EU considera los compuestos activos farmacéuticos (PhACs, *pharmaceutical active compounds*) como sustancias prioritarias y revisa las leyes para reducir el impacto medioambiental de estos compuestos [47]. En 2015 se elaboró una primera lista de compuestos bajo vigilancia que ha sido actualizada en 2018, 2020 y 2022 [48-51], priorizando la supervisión de ciertas sustancias, entre las que destacan algunos compuestos farmacéuticos que poseen un riesgo para el medio acuático o de los cuales no se tiene suficiente información como para saber la amenaza que suponen al mismo [52].

A pesar de que muchos de ellos se degradan, su continua emisión al medioambiente hace que se consideren pseudo-persisentes. Además, dado que están diseñados para tener actividad biológica a bajas concentraciones, una exposición crónica puede provocar efectos en la salud de humanos, animales y ecosistemas [52, 53].

Durante las últimas décadas, la producción y el uso de productos farmacéuticos han aumentado rápidamente, sobre todo en los países en desarrollo, debido a la mejora de su nivel de vida. Entre los efectos adversos que pueden causar estos compuestos en el medio ambiente, cabe destacar la alteración endocrina o la aparición de bacterias resistentes a los antibióticos [54]. Los PhACs, se usan ampliamente para el control de enfermedades y como promotores de crecimiento en veterinaria. En la

industria ganadera, la mayoría de los productos suministrados, a través del pienso o mediante inyecciones, no son metabolizados completamente excretándose en las heces y la orina, ya sea en la forma original o conjugados, oxidados o como productos de hidrólisis, vertiéndose niveles considerables a las aguas residuales y entrando, por tanto, en el ciclo del agua y por extensión en el medioambiente [54]. Actualmente son aproximadamente 3000 los compuestos usados como fármacos, con una producción que se estima en miles de toneladas. Como consecuencia, se le ha prestado cada vez más atención a la presencia de estos compuestos en el medioambiente y se les ha considerado uno de los grupos más importantes de CEs [55, 56].

El consumo de fármacos depende de muchos factores, por ejemplo enfermedades estacionales, demografía, nivel económico, sistema de salud, condiciones climáticas o prácticas de prescripción. Además, como el consumo de fármacos varía de unas regiones a otras, el desarrollo de métodos analíticos para la determinación de estos compuestos en distintas matrices debe considerar esas necesidades locales [53].

Teniendo en cuenta la importancia de los fármacos como CEs, debido a su presencia en el medioambiente y sus potenciales efectos adversos en el humano y en otros organismos, la presente Tesis Doctoral se ha centrado en tres grandes familias de compuestos: los antibióticos, los compuestos neuroactivos y los ß-bloqueadores, debido a su extendido uso, sus consideraciones en las listas de vigilancia y su toxicidad. A continuación se describen los compuestos estudiados.

3.1 Antibióticos

Son un tipo de compuestos naturales, de semi-síntesis o de síntesis con actividad antimicrobiana, usados para tratar enfermedades infecciosas en humanos y animales. También se usan como promotores de crecimiento en veterinaria [57]. Clásicamente, se consideraban antibióticos únicamente aquellos compuestos producidos por microorganismos, que eran capaces de inhibir el crecimiento de otros microorganismos [58].

Hay muchos tipos de antibióticos distintos debido a que su mecanismo biológico varía. Algunos de ellos impiden la síntesis de componentes de las paredes celulares de bacterias, como la penicilina, otros son capaces de dañar las membranas citoplasmáticas bacterianas, como la polimixina, o impiden la síntesis de proteínas y ácidos nucleicos, en el caso de las tetraciclinas [59].

La Figura 3 muestra un esquema general de la clasificación de los distintos tipos de antibióticos.

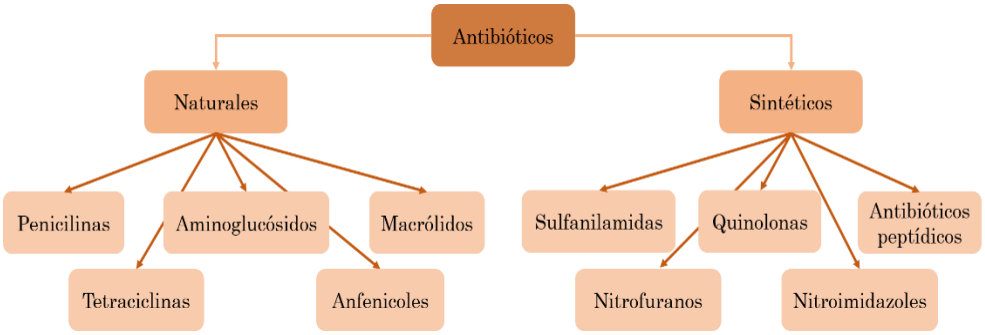


Figura 3. *Esquema de los tipos de antibióticos.*
Elaboración propia basada en Bol'shakov et al. [59]

Los animales suelen excretar entre el 30 y 90% del compuesto de partida que reciben por vía oral o a través de la comida, permitiendo que entren en el medio acuático a través de los excrementos de los animales. La eliminación inadecuada de antibióticos en las granjas de cría también es una causa importante de contaminación de las aguas superficiales [60]. Algunos residuos de antibióticos pueden tener un impacto negativo, tanto en el ecosistema acuático como en la salud humana, aun cuando se encuentran en bajas concentraciones, inhibiendo la reproducción y el crecimiento celular (Kim et al., 2018).

Una de las principales preocupaciones que causa la presencia de antibióticos en el medioambiente es el desarrollo de resistencias bacterianas, considerándose una de las amenazas más serias para la salud pública, ya que cada vez puede llegar a ser más difícil la cura de enfermedades causadas por bacterias [60, 61]. El desarrollo de

resistencias está, por tanto, relacionado directamente con el elevado uso de los antibióticos [6, 60]. En la presente memoria, se han estudiado los grupos de antibióticos que se comentan a continuación.

3.1.1 Quinolonas

La Agencia española de medicamentos y productos sanitarios (AEMPS) define quinolonas y fluoroquinolonas como *"antibióticos sintéticos utilizados para el tratamiento de un amplio espectro de infecciones bacterianas entre las que se incluyen infecciones de las vías urinarias y respiratorias, del aparato genital y gastrointestinal, así como las infecciones cutáneas, óseas y articulares"* [62]. Representan un amplio grupo de antibióticos de síntesis prescritos en todo el mundo, debido a que son muy efectivas frente a todo tipo de enfermedades infecciosas, tienen una buena absorción oral y son antibióticos de amplio espectro [63].

La primera quinolona se sintetizó en los años sesenta y fue el ácido nalidíxico. Su mecanismo de acción consiste en actuar en el ADN cromosómico bacteriano, inhibiendo la acción de las topoisomerasas, enzimas que intervienen en la síntesis del ADN. Las quinolonas pueden clasificarse por su espectro de actividad en generaciones. Las de primera generación son menos usadas actualmente y presentan actividad frente a enterobacterias y algún Gram negativo, pero son inactivas frente a Gram positivos, patógenos atípicos y anaerobios. A partir de las de segunda generación, presentan un átomo de flúor en la posición 6 y se denominan fluoroquinolonas. Éstas presentan mucha mayor actividad frente a Gram negativos, son activas frente a algunos patógenos atípicos y su actividad es moderada frente a Gram positivos. Las de tercera generación presentan características muy similares a las anteriores pero su absorción por vía oral es mejor. Las de cuarta generación presentan mejor actividad frente a Gram positivos y frente a organismos anaerobios [64].

En la Tabla 4 se resumen las quinolonas y fluoroquinolonas de estudio seleccionadas en la presente Tesis Doctoral.

Tabla 4. *Propiedades y estructura de las quinolonas de estudio*

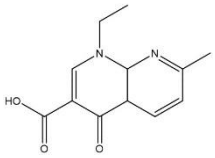
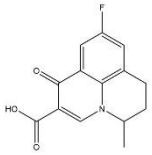
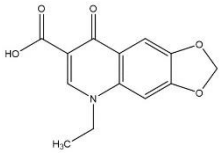
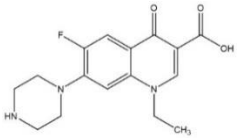
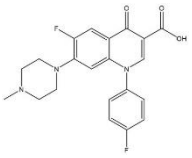
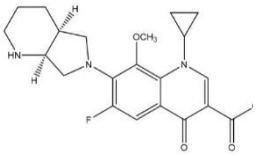
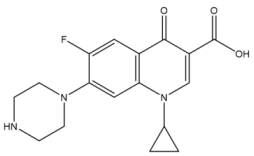
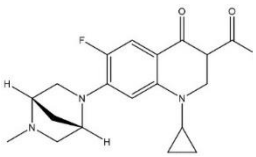
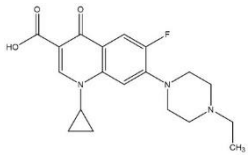
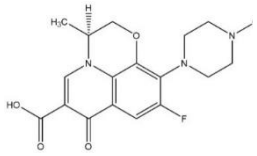
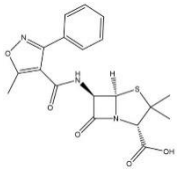
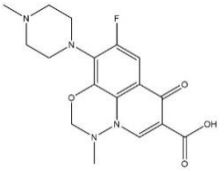
Compuesto	Fórmula molecular	Estructura química	Masa molecular (g mol^{-1})	pK_a	$logK_{ow}$
NAL	$C_{12}H_{12}N_2O_3$		232.23	6.01	1.41
FLU	$C_{14}H_{12}FNO_3$		261.25	6.38	1.6
OXO	$C_{13}H_{11}NO_5$		261.23	5.39	0.95
NOR	$C_{16}H_{18}FN_3O_3$		319.33	6.23	0.46
DIF	$C_{21}H_{19}F_2N_3O_3$		399.4	5.64	1.28
MOX	$C_{21}H_{24}FN_3O_4$		401.4	9.14	0.95
CIP	$C_{17}H_{18}FN_3O_3$		331.34	6.16	0.28
DAN	$C_{19}H_{20}FN_3O_3$		357.4	2.73	0.44

Tabla 4 cont. *Propiedades y estructura de las quinolonas de estudio*

Compuesto	Fórmula molecular	Estructura química	Masa molecular (g mol^{-1})	pK_a	$logK_{ow}$
ENR	$C_{19}H_{22}FN_3O_3$		359.4	3.85	0.7
LEVO	$C_{18}H_{20}FN_3O_4$		361.4	6.1	-0.39
OXA	$C_{19}H_{19}NO_5S$		401.4	2.72	-
MAR	$C_{17}H_{19}FN_4O_4$		362.4	5.96	-2.92

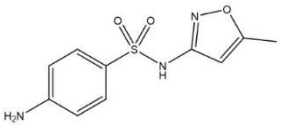
NAL: ácido nalidíxico; FLU: flumequina; OXO: ácido oxolínico; NOR: norfloxacina; DIF: difloxacina; MOX: moxifloxacina; CIP: ciprofloxacina; DAN: danofloxacina; ENR: enrofloxacina; LEVO: levofloxacina; OXA: oxacilina; MAR: marbofloxacina.

3.1.2 Sulfamidas

Se trata de los primeros antibióticos eficaces usados para el tratamiento de infecciones en humano. Son antibióticos de síntesis y de amplio espectro, ya que presentan actividad frente a microorganismos Gram positivos y Gram negativos, si bien es cierto que son propensos a desarrollar resistencias. En cuanto a su mecanismo de acción, cabe destacar que inhiben la síntesis de ácidos nucleicos bacterianos. En este trabajo de investigación, se ha considerado el sulfametoxazol (SFX), cuya estructura y propiedades se encuentran en la Tabla 5, y que actualmente

se combina en una proporción 1/5 con la trimetoprima debido a su efecto sinérgico. A esta combinación se le denomina cotrimoxazol [65].

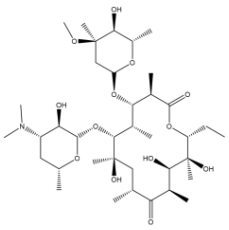
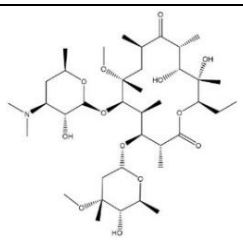
Tabla 5. *Estructura y propiedades del SFX*

Compuesto	Fórmula molecular	Estructura química	Masa molecular ($g\ mol^{-1}$)	pK_a	$logK_{ow}$
SFX	$C_{10}H_{11}N_3O_3S$		253.28	1.6	0.89

3.1.3 Macrólidos

Esta familia de antibióticos se emplea para el tratamiento de infecciones en personas alérgicas a las penicilinas. Su mecanismo de acción se basa en el impedimento de la producción de las proteínas que necesitan las bacterias para crecer y reproducirse [66]. Después de los ß-lactámicos, los macrólidos son los agentes antibacterianos más empleados en humanos. Constan de un anillo de lactona que posee de 14 a 16 carbonos y está sustituido por grupos hidroxilo, alquilo y cetona y por azúcares neutros y/o aminoácidos que se encuentran unidos al núcleo con enlaces de tipo éter [67]. La Tabla 6 muestra la estructura de la eritromicina (ERY) y claritromicina (CLA), macrólidos estudiados en esta Tesis, así como algunas de sus propiedades.

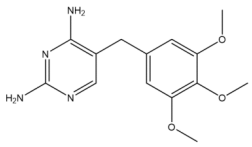
Tabla 6. *Estructura y propiedades de ERY y CLA*

Compuesto	Fórmula molecular	Estructura química	Masa molecular (g mol^{-1})	pK_a	$logK_{ow}$
ERY	$C_{37}H_{67}NO_{13}$		733.9	8.88	3.06
CLA	$C_{38}H_{69}NO_{13}$		748	8.99	3.16

3.1.4 Diaminopirimidinas

Este grupo de antibióticos es frecuentemente usado por su elevada solubilidad en agua y por su muy rápida absorción oral. Se usan como potenciadores de las sulfonamidas frecuentemente. Su mecanismo de acción consiste en inhibir la síntesis de folato en las bacterias a través de una interacción con la enzima dihidrofolato-reductasa [68]. La trimetoprima (TMP), usada normalmente junto con el SFX, se metaboliza en el hígado y se excreta principalmente por vía urinaria [69]. Su estructura y propiedades se resumen en la Tabla 7.

Tabla 7. *Estructura y propiedades de TMP*

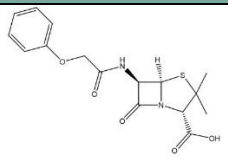
Compuesto	Fórmula molecular	Estructura química	Masa molecular (g mol^{-1})	pK_a	$logK_{ow}$
TMP	$C_{14}H_{18}N_4O_3$		290,32	7.12	0.91

3.1.5 ß-lactámicos

Se trata de un grupo amplio de antibióticos que se caracteriza por contener un anillo ß-lactámico en su estructura. Se incluyen derivados de la penicilina, cefalosporinas, monobactámicos, carbacefem, carbapenems, así como inhibidores de la betalactamasa.

Tradicionalmente, estos antibióticos han sido usados contra las bacterias Gram positivas, si bien es cierto que el desarrollo de antibióticos de amplio espectro ha hecho que también se utilicen contra bacterias Gram negativas. Su mecanismo de acción consiste en la inhibición de la última etapa de la síntesis de la pared celular bacteriana y la inducción de la autolisis bacteriana [70]. En la Tabla 8 se muestra la estructura y propiedades de la Penicilina V (PEN-V), antibiótico ß-lactámico considerado en la presente tesis doctoral.

Tabla 8. *Estructura y propiedades fisicoquímicas de PEN-V*

Compuesto	Fórmula molecular	Estructura química	Masa molecular (g mol^{-1})	pK_a	$logK_{ow}$
PEN-V	$C_{16}H_{18}N_2O_5S$		350.4	2.74	2.09

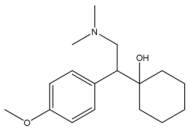
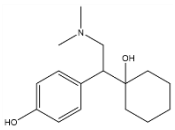
3.2 Antidepresivos

Se trata de una familia de fármacos que actúan aumentando los niveles de alguno de los neurotransmisores cuyos bajos niveles suelen asociarse a la depresión, como la serotonina, noradrenalina o dopamina, y lo hacen mediante distintos mecanismos de acción.

Actualmente, hay dos grandes grupos de fármacos antidepresivos: los heterocíclicos, que fueron los primeros en aparecer y se usan en casos de depresión grave y en casos de ansiedad, y los inhibidores selectivos de la recaptación de serotonina y/o noradrenalina, que son más recientes y su uso está muy extendido. Además, presentan menos efectos secundarios que los primeros. En este segundo grupo, se encuentran la venlafaxina (VFX) y su principal metabolito, la o-desmetilvenlafaxina (O-VFX) [71].

La venlafaxina fue el primer antidepresivo inhibidor de la recaptación de serotonina y noradrenalina autorizado en España, en el año 1995 [72]. Es un antidepresivo con potente efecto inhibidor de la recaptación de noradrenalina cuando se usa a dosis altas, de serotonina a dosis bajas o medias y un efecto inhibidor leve de la recaptación de dopamina. Su metabolismo tiene lugar en el hígado y su vida media es de tres a siete horas y la de su metabolito de nueve a trece horas [73]. Este compuesto se encuentra actualmente en las listas de vigilancia de la Comisión Europea (2022) ya que no se conoce con certeza el efecto que puede tener en los organismos marinos si bien ha sido detectada en *Daphnia magna* u otros organismos como la lubina. Se suele encontrar en el medioambiente en concentraciones más altas que muchos otros fármacos. Algunos estudios centrados en la exposición de organismos marinos a este compuesto han confirmado la influencia de este en la producción de huevos y en la morfología de los túbulos renales del pez cebra [74]. La Tabla 9 muestra la estructura y propiedades de VFX y O-VFX.

Tabla 9. *Estructura y propiedades físicoquímicas de VFX y O-VFX*

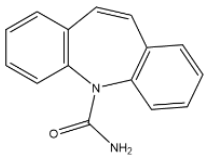
Compuesto	Fórmula molecular	Estructura química	Masa molecular (g mol^{-1})	pK_a	$logK_{ow}$
VFX	$C_{17}H_{27}NO_s$		277.4	9.5	3.2
O-VFX	$C_{16}H_{25}NO_2$		263.37	9.45	2.72

3.3 Antiepilépticos

Según el instituto nacional del cáncer, los antiepilépticos *"son medicamentos usados para prevenir o tratar la convulsiones, crisis convulsivas o crisis epilépticas mediante el control de la actividad eléctrica anómala en el encéfalo"* [75].

Son usados para tratar la epilepsia o tratar afecciones como el trastorno bipolar, migrañas, fibromialgia o el síndrome de las piernas inquietas. El compuesto más destacado de este grupo y objeto de estudio en la presente Tesis Doctoral es la carbamazepina (CBZ). Es un antiepiléptico derivado de carboxamida que se emplea para el tratamiento de trastornos maniacodepresivos y algunos tipos de neuralgias. Disminuye la excitabilidad neuronal por inhibir los canales de sodio dependientes de voltaje. Además, la carbamazepina tiene acción analgésica y antimaniaca. Es un fármaco de primera línea para el tratamiento de las epilepsias parciales o focales [76]. La Tabla 10 muestra su estructura química y propiedades.

Tabla 10. *Estructura y propiedades físicoquímicas de CBZ*

Compuesto	Fórmula molecular	Estructura química	Masa molecular ($g\ mol^{-1}$)	pK_a	$logK_{ow}$
CBZ	$C_{15}H_{12}N_2O$		236.27	13.9	2.45

La CBZ es un fármaco de gran preocupación como CE debido a su toxicidad y persistencia ambiental. Se ha demostrado que tiene efectos adversos sobre los organismos marinos [77] siendo uno de los fármacos más detectados y cuantificados en aguas urbanas. Es usada como marcador de la contaminación antropogénica [78].

3.4 β-bloqueadores

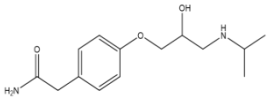
También conocidos como agentes bloqueantes beta adrenérgicos, su función es reducir la presión arterial. Bloquean los efectos de la hormona epinefrina, comúnmente conocida como adrenalina. Pueden afectar únicamente al corazón o también a los vasos sanguíneos. Se usan para prevenir, tratar o mejorar síntomas de arritmia, insuficiencia cardíaca, angina, migraña o temblores, entre otros [79].

Entre ellos, en la presente Tesis Doctoral se estudiado el atenolol (ATN). Este fármaco se usa principalmente para tratar la hipertensión arterial y el dolor torácico asociado al corazón [80]. Muestra afinidad por los receptores adrenérgicos β_1 (RA-β_1), que se localizan en el corazón y los riñones. El estimular estos receptores aumenta la frecuencia cardíaca, la contractilidad miocárdica y la secreción de renina [81].

Los RA-β están relativamente conservados entre los vertebrados, encontrándose en varios órganos de peces como el corazón, branquias o hígado, entre otros, y células como los eritrocitos. Por tanto, la presencia de este compuesto en el agua puede afectar a procesos fisiológicos regulados por estos receptores como puede ser la homeostasis, la presión arterial o el flujo sanguíneo [82].

El atenolol ha sido detectado en aguas residuales y superficiales, en concentraciones que van desde los ng L^{-1} hasta los µg L^{-1}. Vieno et al. y Golovko et al. estimaron que la tasa de eliminación del atenolol es del 10 al 61%, dependiendo de la estación [83, 84]. Se trata además de un compuesto estable a la fotolisis, su biodegradación microbiana es lenta y tiene una baja afinidad de absorción a los sedimentos [85, 86], por lo que se espera que sea frecuentemente detectado. La Tabla 11 muestra la estructura y propiedades físicoquímicas del ATN.

Tabla 11. *Estructura y propiedades fisicoquímicas de ATN*

Compuesto	Fórmula molecular	Estructura química	Masa molecular (g mol^{-1})	pK_a	$logK_{ow}$
ATN	$C_{14}H_{22}N_2O_3$		266.336	9.58	0.16

4 Bioindicadores para la determinación de contaminantes emergentes

Los bioindicadores son organismos vivos, como plantas, plancton, animales y microbios usados para controlar la salud de los ecosistemas naturales en el medio ambiente. No solo se usan como indicadores del cambio, sino que su importancia radica en que muestran el impacto de estos cambios en el medio, ya sean positivos o negativos [87]. Estos cambios en el entorno pueden deberse a la presencia de contaminantes que afecten a la biodiversidad del mismo. Actualmente se utilizan como medio para la gestión de la biovigilancia y evaluar los efectos que tienen sobre la salud humana. Un buen bioindicador debe tener algunas características como estar ampliamente distribuido geográficamente, una esperanza de vida larga, su captura debe de ser fácil y su modo de alimentación debe permitir la acumulación de contaminantes en sus tejidos corporales [88].

Las plantas son herramientas muy sensibles a las tensiones medioambientales. De hecho, las marinas, dado que son inmóviles, proporcionan una información muy útil sobre el estado del medio ambiente oceánico, ya que alcanzan rápidamente el equilibro con su entorno natural [89]. Por otro lado, las variaciones en las poblaciones de animales también pueden indicar cambios que hayan sido causados por la contaminación en el ecosistema. Pueden dar información sobre la bioacumulación de contaminantes en sus tejidos corporales [90]. Por último, los microorganismos son también usados como bioindicadores por su abundancia, su fácil análisis y por la disponibilidad. Además, tienen una tasa de crecimiento muy rápida y su reacción a la presencia de contaminantes se produce incluso a concentraciones bajas [90].

En este trabajo, se han considerado cuatro bioindicadores para la evaluación de la contaminación en el medio marino: el mejillón mediterráneo (*Mytilus galloprovincialis*), el pepino de mar (*Holothuria tubulosa*) y dos tipos de anémonas (*Anemonia sulcata* y *Actinia equina*). A continuación se resume brevemente su utilidad como bioindicadores.

Por otro lado, en la presente Tesis Doctoral, se ha estudiado en profundidad una matriz biológica no invasiva, que es la uña humana,

para evaluar la presencia de CEs de tipo filtro UV así como antibióticos para evaluar su aptitud como biomarcador de la bioacumulación de estos contaminantes en el cuerpo humano.

4.1 Biomarcadores humanos. Uña humana

El término biomarcador se usa para medir la interacción existente entre un sistema biológico y un agente, que puede ser físico, químico o biológico. Esta interacción es evaluada como una respuesta funcional o fisiológica que suele estar asociada con el desarrollo de una enfermedad [91]. El principal uso que se le da a los biomarcadores es la obtención de información sobre el estado ya sea normal o patológico, de un individuo así como la comprensión de enfermedades en distintos aspectos como son el tratamiento, el diagnóstico, la respuesta al tratamiento o la progresión de la enfermedad [92].

En los últimos años, el crecimiento tan rápido de la tecnología y la validación de procesos ha permitido que la química analítica, biología molecular y bioinformática hayan centrado sus investigaciones epidemiológicas y toxicológicas en el uso de biomarcadores, ya que ofrecen una herramienta muy útil en la evaluación de riesgos en salud individual y, sobre todo, pública.

En la presente Tesis Doctoral, se propone la uña humana como biomarcador para la evaluación a la exposición de contaminantes emergentes. Se trata de una matriz keratinizada, que incorpora y acumula sustancias a lo largo del tiempo, y debido al crecimiento, proporciona información retrospectiva del consumo de sustancias en un período de tiempo medio. Por un lado, se propone como alternativa al pelo, que ha sido ampliamente usado para detectar sustancias a largo plazo cuando por ejemplo no se encuentra disponible esta matriz, como pueden ser casos de alopecia o tratamientos de quimioterapia [93].

La incorporación de los compuestos en la uña puede tener lugar por distintas vías, la principal es por difusión desde el riego sanguíneo a medida que va creciendo. La segunda, es la contaminación externa a

través del sudor. El crecimiento de la uña tiene lugar en dos direcciones: longitud y grosor. La longitud se debe a una proliferación de la matriz con un crecimiento distal, que suele ser de 0.1 mm/día en el caso de las uñas de las manos y 0.03-0.04 mm/día para las uñas de los pies. El aumento de grosor se debe a la formación de capas ventrales en el lecho ungueal durante el crecimiento, desde la lúnula hasta el margen libre. Por tanto, la producción continua de uña ventral implica que la vía de acceso y la incorporación de los compuestos sea a lo largo de toda la uña y no en una única banda [94]. La Figura 4 muestra las partes de la uña.

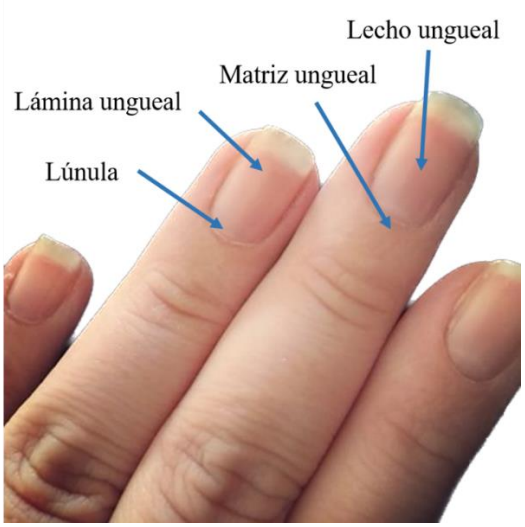


Figura 4. *Partes de la uña.* Elaboración propia.
Fuente: https://cuidateplus.marca.com/belleza-y-piel/cuidados-cuerpo/2021/10/12/han-cambiado-color-grave-179158.html

Como matriz analítica, la uña presenta numerosas ventajas ya que el muestreo es no invasivo y rápido, y el transporte y mantenimiento es sencillo y a temperatura ambiente debido a la estabilidad que presentan. Es cierto que las uñas han sido utilizadas principalmente en análisis forenses y para detectar el consumo de drogas [95], sin embargo, en el campo de la exposición a CEs, como pueden ser algunas familias de disruptores endocrinos, metales o fármacos, no ha sido una matriz muy estudiada.

4.2 Bioindicadores ambientales

4.2.1 Mejillón mediterráneo (*Mytilus galloprovincialis)*

El mejillón mediterráneo es un molusco bivalvo que pertenece al reino *Animalia,* filo *Mollusca,* clase *Bivalbia,* subclase *Pteriomorphia,* orden *Mytiloida,* familia *Mytilidae,* género *Mytilus,* especie *Mytilus galloprovincialis.* Está formado por una concha y una masa visceral. Por un lado, la concha tiene un color azulado, es alargada y posee dos valvas con un extremo triangular y el otro redondeado. Su superficie es lisa y contiene líneas concéntricas que se deben al crecimiento. Por otro lado, la carne es blanda, puede presentar color amarillo o rojizo y su tamaño oscila entre los 5 y los 8 centímetros [96]. Vive adherido a las rocas de las zonas batidas por las olas. En cuanto a la profundidad, no suelen encontrarse a más de 15 metros y su forma se ha ido modificando adaptándose a las necesidades. El extremo triangular se ha estrechado para facilitar el anclaje a las rocas y el circular, se ha ensanchado para aumentar la superficie para la obtención de alimento [97]. Por otra parte, el músculo abductor anterior ha desaparecido prácticamente mientras que el posterior se ha desarrollado para la apertura y el cierre de las valvas. En la Figura 5 se muestra la morfología del cuerpo del mejillón.

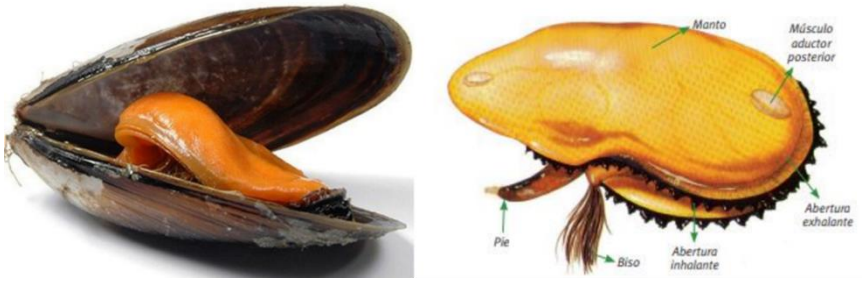


Figura 5. *Morfología del cuerpo del mejillón.*
Fuente: OESA [97]

El mejillón mediterráneo es una especie nativa de la zona costera mediterránea y sus orígenes se asocian con la zona de Galicia, si bien también se encuentra actualmente en el Atlántico Oriental, en el Norte de África y en las zonas de Irlanda y Reino Unido [98].

Se alimentan mediante la filtración del agua. Son capaces de filtrar gran cantidad de agua por unidad de tiempo, de 6 a 8 litros de agua por hora, capturando partículas de hasta 3 μm de diámetro. Filtran el agua a través de sus valvas con la ayuda de una malla denominada biso. Son organismos sésiles y habitan en entornos cercanos a la costa, por lo que su captura es accesible [99]. Algunos investigadores han demostrado que los bivalvos no consumen todas las partículas con las que se encuentran en contacto debido a sus capacidades de selección de partículas [99]. Se alimentan principalmente de materia orgánica en forma de partículas inertes (detritos), fitoplancton (microalgas) y zooplancton (larvas). Su modo de reproducción es mediante fecundación externa y depende de factores ambientales, que dan paso al desove, en el cual se liberan gametos al medio acuático para ser fecundados [97].

Los bivalvos, y más concretamente los mejillones, son los bioindicadores de la contaminación marina por excelencia. Esto se debe a que se encuentran presentes en las costas de todo el mundo, su captura es muy sencilla y su modo de alimentación es mediante filtración, lo cual implica que acumulan en sus tejidos los contaminantes que se encuentran presentes en su hábitat [100].

Son muchos los estudios que han tratado de desarrollar métodos para la determinación de fármacos y otros contaminantes emergentes disruptores endocrinos en distintas especies de mejillón, por ejemplo Malvar et al. [101] desarrollaron un método para la vigilancia del ibuprofeno y sus metabolitos en mejillón; Álvarez Ruiz et al. [102] incluyeron en su método fármacos, pesticidas, sustancias perfluoradas y dos drogas ilegales; Bayen et al. [103] se centraron en la determinación de cafeína, carbamazepina, diltiazem, difenildramina, atrazina y bisfenol A en dos tipos de moluscos, mejillón y almeja; mientras que López-García et al. [104] analizaron 35 sustancias psicoactivas en mejillones. Wolecki et al. [105] evaluaron la presencia de cinco antiinflamatorios no esteroideos (ibuprofeno, paracetamol, diclofenaco, naproxeno y ketoprofeno) junto con tres estrógenos naturales (estrona, 17β-estradiol y estriol) en mejillones y Kim et al. [106], se centraron en pesticidas y fármacos. Por otro lado, García-Fernández et al. [107] y Cañadas et al.

[108] estudiaron la presencia de varios disruptores endocrinos (bisfenoles, parabenos, triclocarbán, bisfenoles y ftalatos) en estos especímenes; Salgueiro-González et al. [109] evaluaron la presencia de bisfenol A y alquilfenoles en mejillón del Golfo de Vizcaya.

En esta Memoria, se ha evaluado el mejillón Mediterráneo como bioindicador de la contaminación marina con el desarrollo de metodología analítica para la determinación de filtros UV.

4.2.2 Pepino de mar *(Holothuria tubulosa)*

El pepino de mar es un equinodermo perteneciente al reino *Animalia,* filo *Echinodermata,* clase *Holothuroidea,* subclase *Actinopoda,* orden *Holothuriida,* familia *Holothuriidae,* género *Holothuria,* especie *Holothuria tubulosa* (Gmelin, 1791). La holoturia posee un cuerpo cilíndrico alargado, parecido al de un gusano. Presenta simetría bilateral. En general, suele tener unos 30 cm de longitud, 6 cm de diámetro y 340 g de peso. Se trata de un animal bentónico que habita en fondos arenosos y no se suele encontrar a mayor profundidad de 100 metros. Es una especie detritívora. Su modo de alimentación tiene lugar mediante la ingestión del sustrato arenoso con los tentáculos bucales, captando la materia orgánica así como organismos presentes en él, y posteriormente expulsa el resto en forma de heces arenosas. Su superficie dorsal presenta prominencias de distintos tamaños con papilas terminales, y su coloración es entre colores pardos o rojizos hasta violeta, mientras que su superficie ventral presenta pies ambulacrales y su coloración es más clara. Entre sus depredadores, destaca la caracola *Charonia lampas* [110]. Presenta una gran capacidad de regeneración. Cuando se sienten amenazados o en peligro, contraen la pared corporal y evisceran, en primer lugar los árboles respiratorios, y posteriormente el tubo digestivo con el resto de los órganos. Rápidamente son capaces de regenerar los órganos internos perdidos [111].

Figura 6. *Pepino de mar (Holothuria tubulosa).*
Fuente: https://biologiamarinamediterranea.webnode.es/fauna-marina/

La *H. tubulosa* presenta sexos separados aunque externamente machos y hembras no presentan diferencias. Su fecundación externa y el período de reproducción tienen lugar en los meses de verano, coincidiendo con un aumento en la temperatura del agua y con la fase de luna llena. Para facilitar la fecundación, se colocan levantando su cuerpo unos dos tercios del sustrato sobre el que se encuentran y expulsan una sustancia de color blanquecina que contiene los espermas y óvulos, que son arrastradas por las corrientes, como se muestra en la Figura 7 [112]. Una vez fecundados los óvulos, forman parte del plancton y tras varias etapas, se depositan en el fondo y se transforman en juveniles.

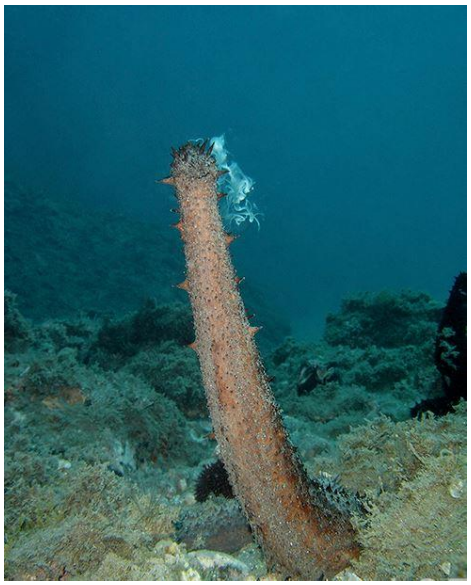


Figura 7. *Reproducción de la H. tubulosa.*
Fuente: https://litoraldegranada.ugr.es/el-litoral/el-litoral-sumergido/fauna/equinodermos/holoturoideos/holothuria-tubulosa/

La *H. tubulosa* se encuentra tanto en el Mar Mediterráneo como el Océano Atlántico, desde la Península Ibérica hasta las Islas Británicas. Se trata de una especie con gran valor comercial e interés económico dado que en los países Orientales es altamente demandada y consumida, lo cual ha implicado el tráfico ilegal del pepino de mar, surgiendo una amenaza a las poblaciones naturales [113].

Dado que estos especímenes se encuentran a lo largo de las costas y su alimentación tiene lugar mediante filtración, son bioindicadores idóneos para la evaluación de la contaminación del mar. Además, debe considerarse la biomagnificación ya que son alimento de otras especies y también son usadas para el consumo humano. Estudios recientes han evaluado la presencia y bioacumulación de algunos contaminantes en el medio marino a través de estos especímenes. Por ejemplo, la presencia de microplásticos en su tracto digestivo [114]; bioacumulación de contaminantes emergentes como conservantes, filtros UV, biocidas, alquilfenoles, tensioactivos aniónicos, plastificantes y sustancias perfluoradas [115, 116]; o metales pesados a nivel traza [117]. Sin embargo, cabe destacar que la literatura científica en evaluación de

fármacos como CEs usando este bioindicador es hoy en día notablemente escasa.

La presente Tesis doctoral ha evaluado las características de este espécimen como bioindicador marino, estudiando la determinación de antibióticos en su pared corporal, así como la capacidad de bioacumulación en sus distintos compartimentos corporales de fármacos de distinta naturaleza.

4.2.3 Anémonas *(Anemonia sulcata* y *Actinia equina)*

El tomate de mar pertenece al reino *Animalia,* filo *Cnidaria*, clase *Anthozoa,* subclase *Hexacorallia*, orden *Actiniaria,* familia *Actiniidae,* género *Actinia* y especie *Actinia equina.* Se trata de un animal que presenta simetría radial y cuerpo cilíndrico de unos 6 cm de diámetro. Es un organismo sésil que presenta un disco basal carnoso con el que se adhiere al sustrato sólido, y en el lado opuesto presenta un orificio con la función de boca y ano. En este orificio contiene seis coronas concéntricas con numerosos tentáculos, entre 190 y 200, que son cortos, de 2 cm de longitud, y gruesos y que contienen células denominadas cnidocitos con orgánulos urticantes, llamados nematocistos, encargados de inyectar veneno a las presas. Tienen la capacidad de retraer los tentáculos hacia dentro, con el fin de protegerse o cuando están emergidos, lo que les hace tener la apariencia de bolo y que, debido a su color rojo, se le llama tomate de mar. La Figura 8 muestra un tomate de mar.

Figura 8. *Tomate de mar (Actinia equina).*
Fotografía propia

Este animal vive fijado a los fondos rocosos, no encontrándose a más de 2 metros de profundidad. Se encuentra distribuido por el Mar Mediterráneo y el Atlántico Norte, y es muy abundante. Se alimenta de pequeñas presas vivas como peces o crustáceos. Tiene los sexos separados, y la fecundación ocurre en la hembra, que va generando óvulos durante todo el año. El macho libera el esperma, que se dispersa en el agua. Su reproducción es ovovivípara, es decir, los embriones se desarrollan en el interior de la hembra, y son expulsados cuando están totalmente formados como pólipos. También es frecuente que se dé la reproducción asexual a partir de la bipartición de la madre, dando lugar a dos ejemplares exactamente iguales.

No es una especie que presente mucho interés económico y según la Unión Internacional para la conservación de la naturaleza (UICN), se considera de preocupación menor [118].

Por otro lado, la anémona de mar, también conocida como ortiga de mar pertenece al reino *Animalia,* filo *Cnidaria*, clase *Anthozoa,* subclase *Hexacorallia*, orden *Actiniaria,* familia *Actiniidae,* género *Anemonia* y especie *Anemonia sulcata.* Este tipo de anémona, igual que el tomate de mar, presenta simetría radial y su cuerpo es cilíndrico. Del mismo modo presenta un disco basal que le permite adherirse al sustrato sólido y un extremo oral con el orificio que hace las veces de boca y ano. También

presenta 6 coronas concéntricas pero sus tentáculos son largos, y presenta en torno a 200. Son finos y poco retráctiles, y también presentan cnidocitos con orgánulos urticantes para inyectar veneno a la presa. Su tamaño es de unos 20 cm cuando el ejemplar es adulto, y unos 10 cm de altura. Los tentáculos pueden llegar a alcanzar 15 cm. Presenta un color verde pardo y en la punta de los tentáculos presenta color violeta [119].

Figura 9. *Ortiga de mar (Anemonia sulcata)*
Fotografía propia

Se trata de una especie muy abundante en el Mediterráneo y el Atlántico Norte. Vive en fondos rocosos de la zona mesolitoral e infralitoral y se adhiere a sustratos duros. Se encuentra a unos 6 metros de profundidad aunque raramente puede encontrarse a más, hasta 20 metros. Es carnívoro y se alimenta de peces y crustáceos. Además, también alberga microalgas endosimbiontes que le aportan nutrientes originados por fotosíntesis.

Del mismo modo que el tomate de mar, es una especie sexuada gonocórica, es decir, con sexos separados y fecundación externa y ovípara. En este caso, la hembra produce óvulos únicamente entre los meses de mayo a julio aunque también presenta reproducción asexual por bipartición. Sus principales depredadores son pulpos y cangrejos. Su capacidad de desplazamiento es muy limitada, tratándose de un

organismo sésil [120]. En cuanto al interés económico, es una especie de consumo, inicialmente típica en Cádiz pero que se ha expandido actualmente. Se trata de una especie de preocupación menor según la IUCN.

En el caso de estos dos tipos de anémonas, son su tolerancia al estrés y su comportamiento sésil lo que las hace excelentes bioindicadores del estado del agua. Sin embargo, apenas se han usado como modelos biológicos para estudios de contaminación [78]. En la literatura disponible, se puede observar que los estudios que han considerado las anémonas como bioindicadores de la contaminación marina, se han centrado principalmente en metales. Por ejemplo, Mitchelmore et al. [121] estudiaron la acumulación de cadmio en la anémona *Anthopleura elegantissima*; Duckworth et al. [122] estudiaron la respuesta de la anémona *Exaiptasia pallida* a la acidificación de los océanos y a la exposición de zinc y níquel; Lozano-Bilbao et al. [123] determinaron metales como cromo, boro, níquel, cobre, plomo, cadmio, hierro, litio y vanadio en *Anemonia sulcata;* y Morgan et al. [124] evaluaron la presencia de hormonas sexuales, oxibenzona y benzilbutilftalato en *Exaiptasia pallida.*

En el presente trabajo, se han considerado ambos tipos de anémonas como bioindicadores de exposición a ciertos fármacos, trabajando con concentraciones conocidas, lo cual ha servido para evaluar su potencial de bioconcentración de estos contaminantes.

5 Metodología analítica para la determinación de contaminantes emergentes en las matrices seleccionadas

5.1 Cromatografía de líquidos

En la bibliografía consultada se encuentra que la cromatografía de líquidos de alta resolución (HPLC, *High Performance Liquid Chromatography*), acoplada a distintos sistemas de detección, ha sido la técnica más usada para la separación y determinación de fármacos y PCPs en matrices biológicas y ambientales. Esto es debido principalmente a que son compuestos con elevado peso molecular, escasamente volátiles y con carácter polar. Además, suelen presentar grupos ionizables, como ácidos carboxílicos o grupos amino.

En los últimos años la técnica de HPLC ha sido reemplazada por la de UHPLC (*ultra high performance liquid chromatography)*, que presenta algunas ventajas sobre la primera. Los análisis son más rápidos y sensibles. Esto se explica por el relleno de la columna, que contiene partículas más pequeñas así como porosas, que permiten mejor resolución de los picos y, por tanto, mejor sensibilidad. Mientras que en HPLC se utilizan tamaños de partícula entre 3 y 5 µm, UHPLC puede usar columnas con tamaños de partícula de 2 µm o inferiores. El efecto asociado es que se trabaja a mayores presiones [125].

La separación de los compuestos objeto de estudio contemplados en esta Tesis Doctoral se ha realizado empleando UHPLC en fase inversa, siendo metanol (MeOH) y acetonitrilo (ACN) los modificadores de la fase móvil y como aditivo se ha usado el ácido fórmico. Los rellenos de columna usados han sido el BEH C18 (n-octadecilo, *Ethylene Bridged Hybrid*), que presentan buena resolución con muchos compuestos y tienen un amplio rango de uso, y el HSS (partículas de sílice de alta resistencia, *high strength silica*), que consiste en una partícula cuyo 100% es sílice y es capaz de soportar altas presiones (hasta 15.000 psi), lo que permite una retención más alta de los compuestos polares y estabilidad mecánica incluso a altas presiones. En la Figura 10 se muestran los equipos empleados en la presente tesis doctoral.

***Figura 10**. UPLC-PDA-QDA (izquierda) y UPLC-QqQ (derecha).*
Fotografía propia

Como sistema de detección, se ha empleado principalmente la espectrometría de masas de cuadrupolo simple (MS) y en tándem de triple cuadrupolo (MS/MS).

5.2 Espectrometría de masas

La espectrometría de masas (MS) es una técnica que se basa en la separación de iones que se encuentran en fase gaseosa de acuerdo a su relación masa/carga (m/z). Dado que los espectrómetros suelen estar acoplados a cromatógrafos, en las fases móviles es típico encontrar aditivos, ya sean de carácter ácido o básico para modificar el pH de dichas fases y mejorar la ionización. Destacan el acetato de amonio o amoníaco cuando la ionización tiene lugar en polaridad negativa, y el ácido fórmico o acético cuando la polaridad es positiva [126].

La espectrometría de masas permite obtener información sobre el peso molecular de las especies presentes en la muestra así como la estructura química, lo cual facilita la identificación y cuantificación de los compuestos. Dado que la cromatografía de líquidos usa disolventes como fases móviles y la espectrometría de masas necesita condiciones de alto vacío, en la cual los iones se encuentran en fase gaseosa, existe cierta incompatibilidad en el acoplamiento entre ambas técnicas. Para ello, se presenta una interfase entre ambos equipos, cuya función es eliminar

cualquier traza de líquido que se encuentre antes del detector y de esta forma no comprometer la sensibilidad del último [127].

Entre los componentes de un espectrómetro de masas, cabe destacar un sistema de introducción de muestra, de ionización, un analizador de masas, un detector y un sistema de adquisición de datos.

La técnica más destacada cuando la ionización tiene lugar a presión atmosférica (API, *atmospheric pressure ionization*) es la ionización por electrospray (ESI, *electrospray ionization*) (Figura 11), que prescinde del vacío para generar iones. En este caso, la muestra se encuentra disuelta en un disolvente, en general polar, y se bombea a través de un capilar. Este líquido, cuando sale del capilar se pulveriza, soltando gotas con iones que se dirigen hacia el espectrómetro de masas [128].

Una vez que los analitos son separados en la columna cromatográfica, estos son ionizados y desolvatados con un gas nebulizador y altas temperaturas, que da lugar a pequeñas gotas las cuales, van aumentando su densidad de carga hasta que las fuerzas repulsivas son mayores que la tensión superficial que las mantiene unidas, fenómeno conocido como el límite de Rayleigh. Llegado a este momento, tiene lugar una explosión, la explosión de Coulomb, que se produce repetidas veces hasta que quedan gotas que contienen un único analito. La evaporación de la última gota de disolvente da lugar a un ion en fase gaseosa. Dado que las disoluciones se cargan con reacciones electroquímicas, donde los electrones se transfieren a una superficie conductora que hace las veces de electrodo, en ocasiones tienen lugar variaciones en el pH. Cuando se trabaja en modo positivo, las gotas salen del aerosol con carga positiva y el electrodo las acepta, dando lugar a una oxidación, mientras que en el modo negativo se produce una reducción [129]. La Figura 11 muestra un ejemplo de ionización con electrospray en modo positivo.

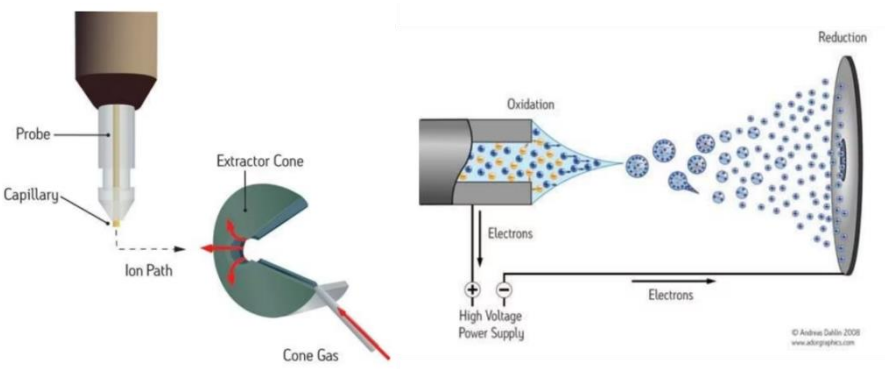


Figura 11*. Esquema de una sonda ESI (izquierda).*
Fuente: Waters
Cromatografía S.A.® Modo de ionización positiva (derecha).
Fuente: Balogh. [129]

Por otro lado, en cuanto a los analizadores de masas, cabe destacar que en los últimos años se ha sustituido en gran medida la espectrometría de masas de cuadrupolo simple por la de espectrometría de masas en tándem de triple cuadrupolo (QqQ). Este analizador de masas goza de gran sensibilidad cuando los análisis son cuantitativos a niveles traza, como es el caso de los CEs en matrices biológicas y ambientales, temática principal de la presente Tesis Doctoral. En él, tras la generación de iones por la interfase, éstos se fragmentan en el primer cuadrupolo (Q_1) en el cual se selecciona un único fragmento de los obtenidos. Se le denomina ion precursor y se obtiene mediante un voltaje concreto en el cono, el cual, debe ser lo suficientemente elevado para que sea capaz de eliminar asociaciones entre nuestro analito objetivo y otros compuestos presentes en la mezcla, y lo suficientemente bajo como para no fragmentar este ion precursor. Una vez pasado el primer cuadrupolo, el ion pasa a una celda de colisión (q_2), que induce una nueva fragmentación causada por la presencia de un gas neutro, dando lugar a varios iones producto. Estos fragmentos generados en el segundo cuadrupolo pasan a ser analizados según su masa/carga (m/z) al tercero (Q_3) aplicando un potencial eléctrico (energía de colisión). La Figura 12 muestra un esquema del proceso que sufre el analito al atravesar los distintos cuadrupolos [130].

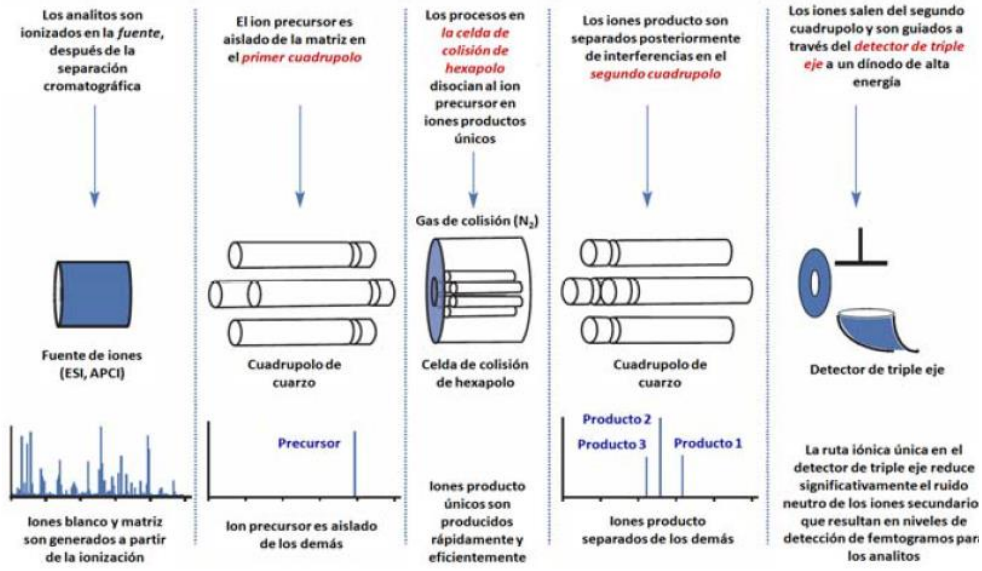


Figura 12. *Esquema del triple cuadrupolo.*
Fuente: Waters Cromatografía S.A.®

El espectrómetro de masas de triple cuadrupolo puede trabajar en diferentes modos. La Figura 13 resume dichos modos de trabajo.

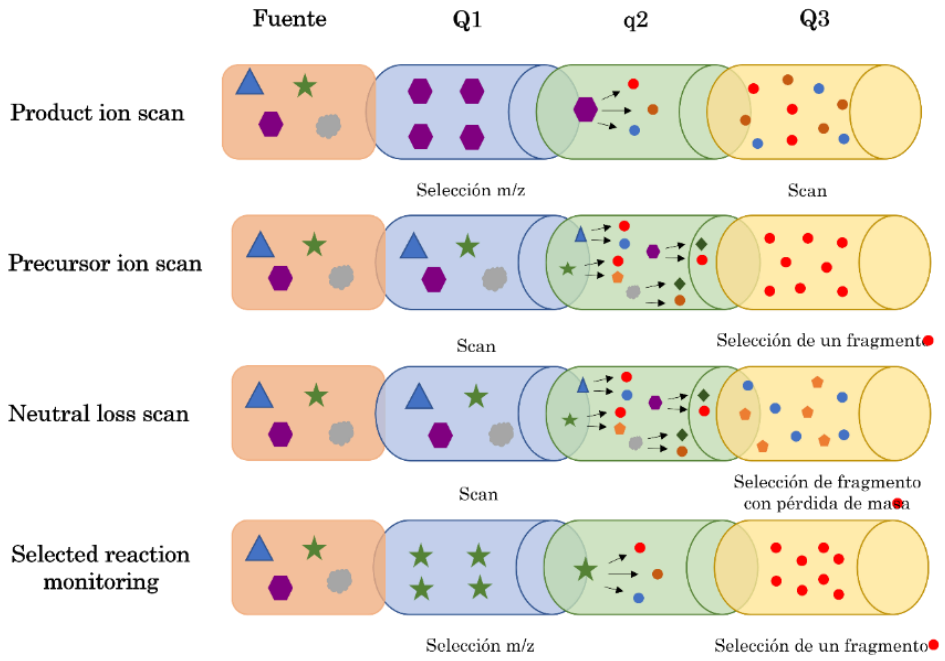


Figura 13*. Modos de trabajo en el triple cuadrupolo.*
Elaboración propia basado en Lin et al., [131]

5.3 Técnicas de extracción

El análisis tanto cualitativo como cuantitativo de los compuestos de interés comienza con un tratamiento que permita separarlos en el mayor grado posible del resto de los componentes de la matriz. La separación selectiva de los compuestos de interés es un paso clave para la determinación analítica. El objetivo principal de cualquier proceso de extracción es maximizar su concentración final sin alterarlo en sus propiedades durante el proceso. Además, es también objetivo que la concentración del resto de componentes de la matriz no deseados sea la mínima posible [132]. En este punto es de gran interés considerar distintas técnicas de extracción y determinar los valores óptimos de todas aquellas variables experimentales que puedan afectar a la calidad del extracto final.

En las últimas décadas, en Química Analítica se ha tratado de sustituir las técnicas clásicas de extracción por nuevas técnicas más eficientes. Uno de los principales motivos era la cantidad de disolvente empleado y, en muchas ocasiones, el tiempo de extracción, que llegaba a ser desde horas a días [133]. Se buscaba principalmente que el gasto en tiempo y disolvente fuese mínimo y respetar en la medida de lo posible los Principios de la Química Verde [134]. En la presente Tesis Doctoral, se han estudiado dos técnicas de extracción instrumentales, la asistida por ultrasonidos y por microondas, así como dos procedimientos de limpieza para eliminar impurezas, la extracción en fase sólida dispersiva y técnica de QuEChERS, basada en la primera. A continuación se comentan brevemente estas 4 técnicas.

5.3.1 Extracción asistida por ultrasonidos

Los ultrasonidos son una forma de energía asociada al sonido en frecuencias superiores a las detectadas por el oído humano. Hoy en día, esta forma de energía se usa en numerosas aplicaciones entre las que se incluye la medicina, la industria o el laboratorio. Un ejemplo es su empleo en procesos de homogeneización, cristalización, desgasificación o de extracción. Es una técnica que cumple varios de los principios de la

química verde y ofrece resultados rápidos, reproducibles y selectivos usando unas condiciones suaves y un gran ahorro de energía [135]. Los ultrasonidos se sitúan por encima de los 16 kHz, no siendo definido el límite superior. La Figura 14 muestra la gama de frecuencias sonoras.

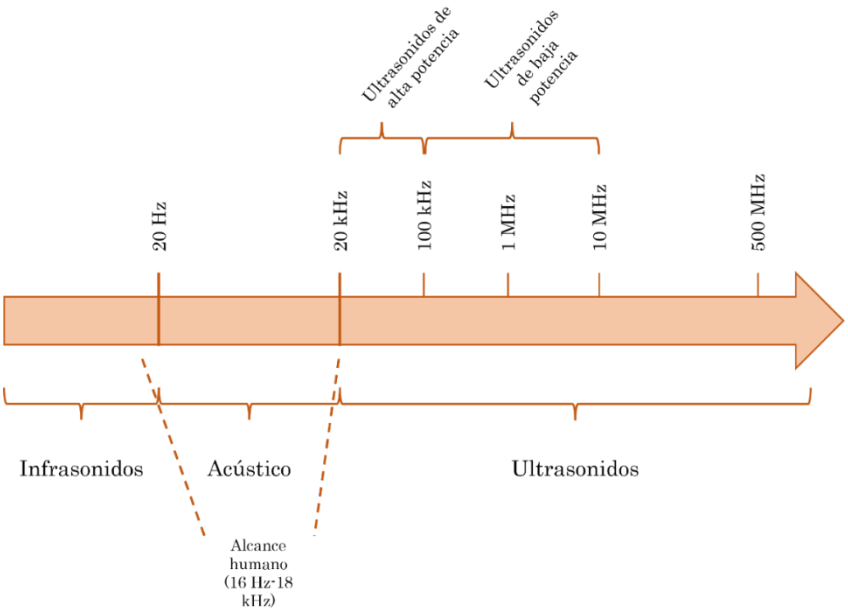


Figura 14. *Gama de frecuencias sonoras.*
Elaboración propia basada en Lavilla et al., [135]

En la aplicación de la extracción asistida por ultrasonidos (UAE, *ultrasound assisted extraction)* se pueden usar disolventes no tóxicos, y en menor cantidad que con las técnicas tradicionales de extracción. Además, se reduce drásticamente el tiempo de extracción [135].

El ultrasonido es una onda de presión que se transmiten a través de un medio como ciclos de compresión y expansión, pudiéndose representar como una onda sinusoidal. No existe interacción directa entre las ondas de ultrasonidos y las moléculas dado que la energía es demasiado baja. El efecto del ultrasonido se debe al fenómeno de cavitación, que consiste en la formación de burbujas de vapor. La fase de compresión ejerce una presión positiva, haciendo que las moléculas del medio se acerquen y la refracción ejerce una presión que las separa [136, 137].

Cuando las ondas tienen suficiente intensidad, la presión generada es mayor que las fuerzas intermoleculares existentes, lo cual produce cavidades en el medio, llamadas burbujas de cavitación. En el medio circundante, tras el colapso, se alcanzan temperaturas de 4000 a 5000 K y presiones superiores a 1000 atm (Figura 15). Las burbujas de cavitación se generan en muy poco tiempo, dando lugar a un proceso cuasiadiabático de alta energía. Las burbujas, por tanto, pueden ser consideradas como microreactores que trabajan a altas temperaturas y presiones, que favorece la aparición de especies muy reactivas y estados excitados.

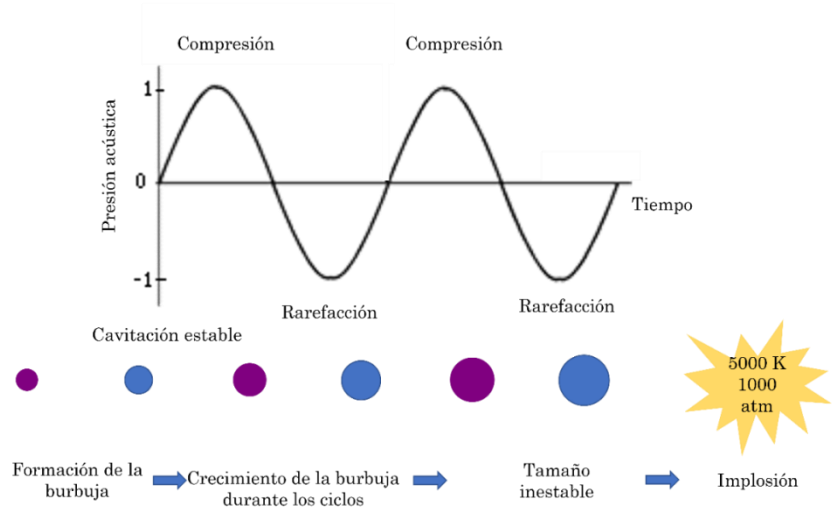


Figura 15. *Cavitación ultrasónica.*
Elaboración propia
Fuente: https://www.hielscher.com/es/ultrasonic-cavitation-in-liquids-2.htm

Una vez que explota la burbuja, la energía liberada se transforma en energía cinética. Las colisiones provocan la ruptura de la matriz dando lugar a partículas más pequeñas, lo cual permite mayor superficie de contacto entre la matriz y el disolvente de extracción. Este hecho, junto con el aumento de la temperatura, que implica mayor solubilidad de las sustancias y con la alta presión, explica que se considere una técnica muy eficaz en la extracción de los compuestos de interés.

Los sistemas de ultrasonidos usados en los laboratorios consisten en baños de ultrasonidos y sondas. En la Figura 16 se muestran dos los sistemas de ultrasonidos usados en la presente Tesis Doctoral.

Figura 16. *Baño de ultrasonidos (izquierda) y sonda de ultrasonidos (derecha).* Fotografía propia

El baño de ultrasonidos está constituido por un tanque, un generador eléctrico y el transductor electrónico. Además, el baño puede estar controlado termostáticamente. Para realizar las extracciones, se introduce la muestra en un recipiente apto para ultrasonidos dentro del baño. Esta técnica se también es conocida como sonicación indirecta. Las ondas de ultrasonidos deben tener la energía suficiente como para que se produzca el fenómeno de cavitación en su interior [138].

La sonda de ultrasonidos está formada por un generador, un transductor y un horno. El sistema permite concentrar la energía del ultrasonido haciendo más efectiva la sonicación incluso cien veces si se compara con el baño de ultrasonidos. En los procedimientos de extracción, se sumerge la sonda en la disolución con la matriz. Se conoce como sonicación directa. Es importante destacar que la mayor parte de la energía se transmite en la punta de la sonda y, por tanto, los efectos sonoquímicos son más fuertes cerca de la misma [135]. Una de las principales ventajas que presenta la sonda de ultrasonidos es que permite controlar los parámetros relacionados con la cavitación, como son la amplitud de onda, frecuencia y potencia [136].

5.3.2 Extracción asistida por microondas

Las microondas son ondas electromagnéticas generalmente entre 300 MHz y 300 GHz. En el espectro electromagnético, se sitúan entre los rayos infrarrojos, cuya frecuencia es mayor, y las ondas de radio (Figura 17) [139]. La extracción asistida por microondas (MAE, *Microwave assisted extraction*) es una técnica que consiste en el calentamiento de los disolventes de extracción empleando las microondas, con el fin de disolver los analitos de interés en estos disolventes. El calentamiento implica una disminución de la viscosidad de los disolventes y un aumento en la solubilidad de los analitos, facilitando la penetración en la matriz, lo cual implica que sea una técnica más rápida que las convencionales.

La irradiación por microondas utiliza un campo magnético con una frecuencia específica similar a la de las reacciones fotoquímicas activadas. Dado que la extracción tiene lugar en recipientes cerrados, se puede llevar a cabo a temperaturas muy elevadas, favoreciendo la rápida transferencia de los analitos de la matriz al disolvente. Además, tras la aplicación de energía, el foco de calor se produce en el centro de la masa, extendiéndose posteriormente hacia el exterior [140].

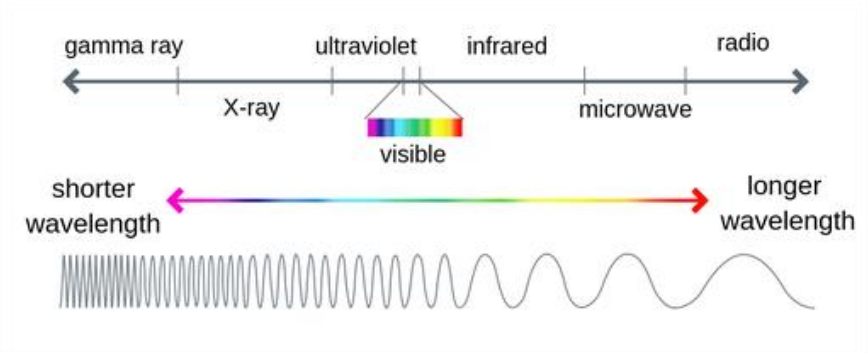


***Figura 17**. Espectro electromagnético.*
Fuente: https://theory.labster.com/electromagnetic-spectrum/

El tiempo de duración del proceso suele estar entre 15 y 30 minutos y los volúmenes de disolvente necesarios entre 10 a 30 mL, siendo una técnica amigable con el medio ambiente. Se ha demostrado que tanto la recuperación de analitos como la reproducibilidad mejora bastante con

respecto a las técnicas convencionales [133]. La Figura 18 muestra el microondas usado en este trabajo.

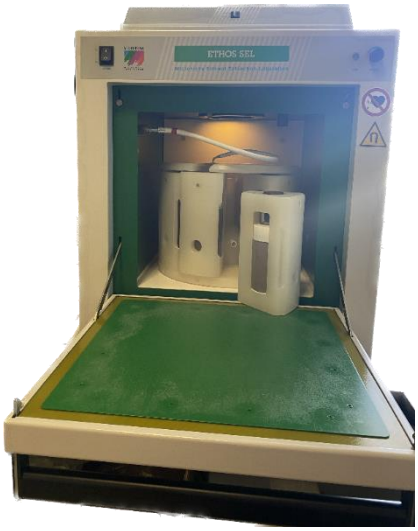


Figura 18*. Horno de microondas de laboratorio. Fotografía propia*

Una de las principales ventajas que ofrece MAE es la reducción del tiempo de extracción. Esto se puede explicar por la diferencia en el rendimiento de calentamiento empleado por la técnica MAE y el calentamiento convencional. En el segundo, se necesita un período de tiempo finito para calentar el vaso previo a que el calor se transfiera a la disolución, mientras que las ondas de microondas calientan directamente la disolución, lo que implica que se dé en muy poco tiempo favoreciendo la no degradación de los analitos (Figura 19). Otra de las ventajas que aporta MAE es que se reduce significativamente el consumo de disolvente orgánico y se pueden procesar simultáneamente varias muestras. MAE es una técnica muy útil cuando los compuestos son inestables térmicamente dado que la transferencia de calor tiene lugar por la colisión y fricción de moléculas dipolares sometidas a dos campos, uno eléctrico y otro magnético [141].

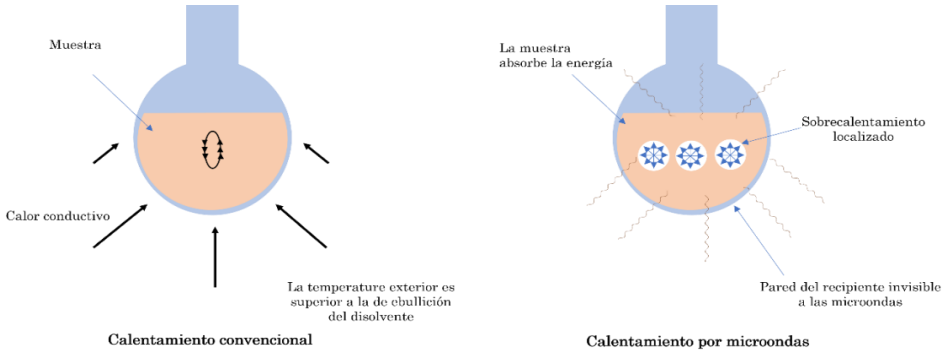


Figura 19. *Extracción convencional y extracción con microondas.* Elaboración propia basada en Anastassiades et al.,[143]

El principio de calentamiento usando la energía de microondas se basa en el efecto directo que tienen las mismas en las moléculas por la conducción iónica y la rotación dipolar, que en muchas ocasiones tienen lugar simultáneamente. La conducción iónica es la migración electroforética de iones cuando se aplica un campo electromagnético. La resistencia de la disolución a este flujo de iones resultará en una fricción, y consecuentemente, en el calentamiento de la disolución. La rotación dipolar, por otro lado, consiste en la alineación de los dipolos con el campo aplicado. Las moléculas polares así como las disoluciones iónicas absorben de manera más fuerte la energía del microondas dado que tienen un momento dipolar permanente, mientras que los disolventes apolares no se calientan cuando se exponen a la energía de microondas. Cuando los vasos se encuentran cerrados, el disolvente puede calentarse muy por encima de su punto de ebullición normal, lo cual permite que aumente la eficacia y la velocidad en la extracción.

El hecho de que diferentes sustancias químicas absorban la energía de microondas en distinta medida implica que el calentamiento transmitido al medio circundante variará en función de la sustancia estudiada. Por tanto, cuando se obtienen muestras con características heterogéneas, dado que son mezclas de sustancias con distintas características estructurales, es posible producir un calentamiento selectivo de algunas zonas de la muestra o algunos componentes de la misma. Este fenómeno se conoce como recalentamiento [133]. La eficiencia en la extracción dependerá de la naturaleza de la matriz y del

analito que es extraído. Por ello, es muy importante la elección correcta del disolvente teniendo en cuenta sus parámetros físicos que incluyen solubilidad, constante dieléctrica y factores de disipación [139]. Además, por un lado, los analitos de interés deben ser solubles en ellos y por otro lado deben ser capaces de absorber la energía transmitida por las microondas, transformándola en calor.

5.3.3 Técnica de QuEChERS

QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*) es una técnica que se desarrolló con el objetivo de que fuera sencilla, obtuviera un alto rendimiento de extracción y cumpliera con los principios de la química verde, es decir, reducir cantidad de muestra y de disolvente usados. El método original fue desarrollado para la recuperación de plaguicidas presentes en frutas y hortalizas, cubriendo un rango amplio de analitos. Posteriormente, debido a la eficacia de la técnica, fue adoptada por la comunidad científica para distintas aplicaciones [143]. La técnica consiste en realizar una dispersión de sales (efecto salting-out), para el aislamiento de los analitos de la matriz a la vez que se realiza una limpieza del extracto. La preparación de la muestra tiene lugar en dos pasos

1. Extracción, vía salting-out con un equilibrio entre fases acuosa y orgánica.
2. Extracción en fase sólida dispersiva (d-SPE, dispersive solid-phase extraction) que implica una limpieza con la combinación de adsorbentes porosos junto con sales para eliminar interferentes.

La efectividad de la técnica dependerá de las propiedades de los analitos, de la composición química de la matriz y del material adsorbente empleado. Es muy importante optimizar aquellos parámetros que pueden afectar al proceso de extracción. Se optimizará el disolvente de extracción, la cantidad de muestra y los tipos de adsorbentes para el proceso final de limpieza [144]. La Figura 20 muestra un resumen de los disolventes y adsorbentes utilizados en la técnica original (naranja) y variaciones de la técnica que se emplean actualmente.

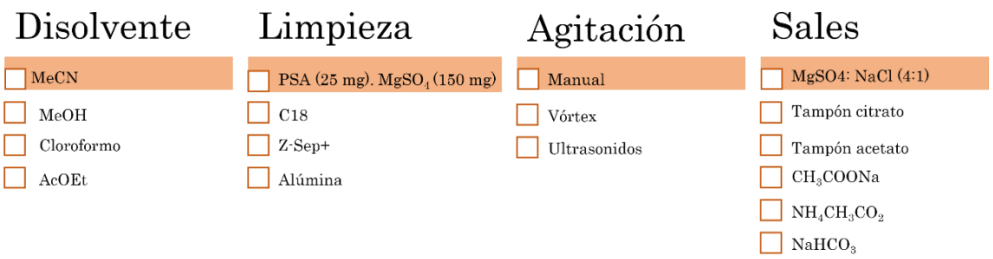


Figura 20. *Resumen de la técnica QuEChERS original y sus modificaciones.*

Elaboración propia basada en Pestrelo et al., [144]

5.4 Tratamiento estadístico de los datos analíticos

5.4.1 Diseño de experimentos

Un diseño de experimentos (DoE, *Design of Experiments)* consiste en una estrategia experimental formada por experiencias previamente planificadas con una serie de variables, denominadas factores. En cada experiencia, la variable toma un valor (nivel) que se encuentra definido por la estructura del diseño [145]. El diseño de experimentos ayuda a la selección de la estrategia óptima obteniendo resultados y conclusiones fiables y reduciendo el tiempo y coste en dicha selección. Se caracteriza por imponer los factores, medir las variaciones inducidas en las respuestas y deducir la relación existente entre causas y efectos (Figura 21). El objetivo del DoE es conocer las variables influyentes en el proceso y el nivel de influencia de cada una de ellas; conocer el grado de interacción entre variables y conocer las condiciones óptimas del proceso para mejorar el resultado final [145].

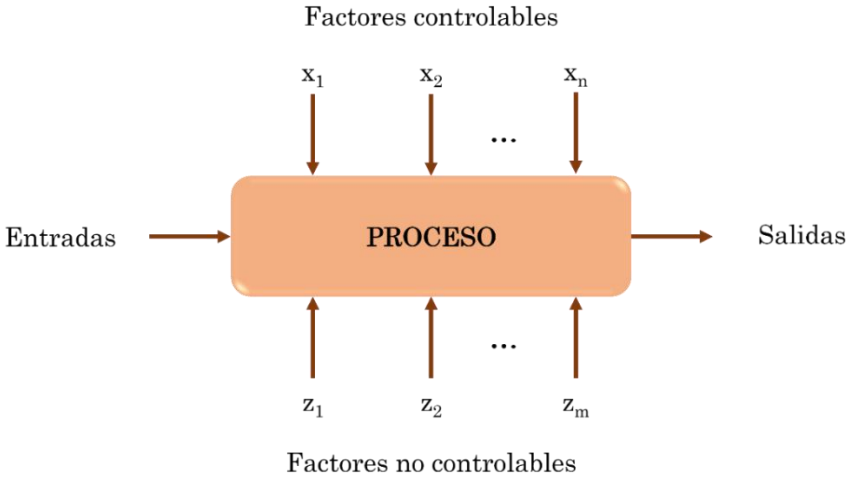


Figura 21. *Esquema general de un proceso*
Elaboración propia

En el procedimiento de optimización de los métodos de extracción de contaminantes en matrices ambientales y biológicas, se deben tener en cuenta numerosos factores y variables que intervienen en la eficiencia de estos. Para la optimización, se pueden ir estudiando las variables implicadas una a una (diseño univariante), que suele ser más largo y tedioso, o estudiar distintas variables simultáneamente así como la interacción existente entre ellas mediante el diseño multivariante. En este tipo de procesos, es frecuente encontrar comportamientos de las variables que únicamente se explican por su interacción, de ahí la importancia de los diseños experimentales. Existen una o varias variables experimentales dependientes, que son las respuestas (y), cuyo valor depende de las variables independientes controlables, que son los factores (x) [146]. La aplicación de herramientas quimiométricas permite la descripción del comportamiento del sistema mediante ecuaciones y modelos matemáticos. Si estas ecuaciones determinan interacciones entre variables, las conclusiones obtenidas mediante el diseño multivariante sean posiblemente muy distintas a las obtenidas si se hubiera hecho un diseño univariante.

Frecuentemente, en los DoE empleados en la optimización de métodos analíticos, se encuentra la metodología de superficie de respuesta (RSM, *Response Surface Methodologies*), que consiste en una

colección de técnicas que permite investigar sobre una respuesta que se puede mostrar como una superficie, cuando los experimentos tratan de comprender el efecto que tiene la variación de los factores cuantitativos en la respuesta obtenida [147].

Una RSM se compone de tres aspectos: diseño, modelo y optimización. En primer lugar, el diseño se relaciona con un modelo matemático que permite conocer el comportamiento de cada variable respuesta. Con estos modelos se puede conocer el grado de influencia considerando efectos lineales, cuadráticos o de interacción de los factores. El segundo aspecto, el modelo, que indica cómo se ajusta la regresión matemática que resume el comportamiento de las variables respuesta. El tercero, la optimización, que se lleva a cabo cuando el ajuste del modelo que se propone es correcto. Con esto se obtienen gráficas de superficie de respuesta en las que se muestra el comportamiento de la variable dependiente a medida que van variando los valores de las variables independientes y se observa visualmente en la gráfica las condiciones óptimas [147]. (Figura 22).

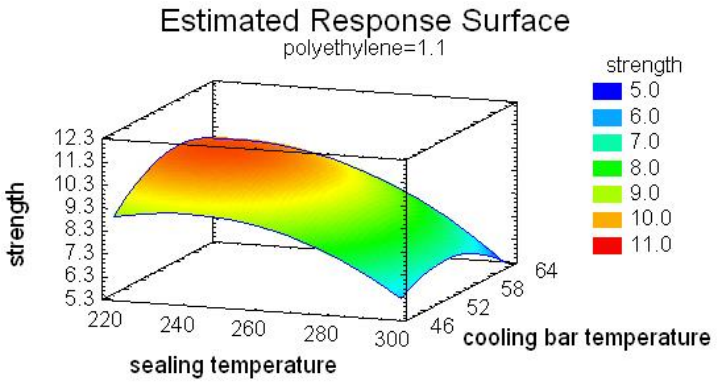


Figura 23. *Ejemplo superficie de respuesta.*
Fuente: https://statgraphics.net/wp-content/gallery/ejemplos-graficos/grafico-de-superficie-de-respuesta-para-diseno-de-experimentos.jpg

En la presente Tesis Doctoral, se han aplicado diseños factoriales completos (3^2) y diseños de Box-Behnken. Se tratan de diseños de segundo orden simétricos y se usan fundamentalmente cuando se conoce o intuye

que los valores óptimos de las variables estudiadas no se encuentran ni en el máximo ni en el mínimo. A continuación se comentan brevemente.

A. ***Diseño factorial completo a tres niveles.*** Se aplica cuando se trata de optimizar dos variables de forma simultánea. Presenta ciertas limitaciones cuando se quieren optimizar más de dos variables. La Figura 23 muestra la representación de este diseño para dos variables. Entre las ventajas que presenta el diseño es que se muestran tanto para los factores de tipo continuo en los que interesa estudiar los efectos cuadráticos, como para los factores discretos que tienen tres niveles de manera natural [148]. La Tabla 12 muestra una réplica completa para un diseño 3^2.

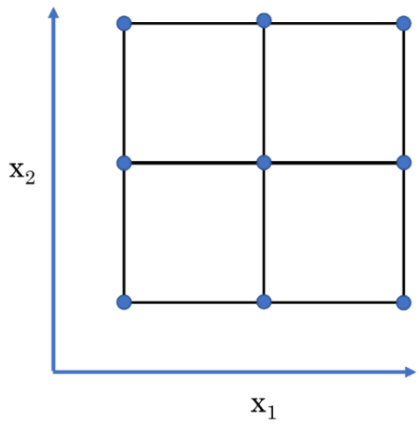


Figura 23*. Diseño factorial completo de tres niveles para la optimización de dos variables (x_1 y x_2).*
Elaboración propia.

Tabla 12*. Matriz de diseño para una réplica completa para un diseño 3^2*

Orden	Factor A	Factor B	Orden	Factor A	Factor B
1	-1	-1	**6**	1	0
2	0	-1	**7**	-1	1
3	1	-1	**8**	0	1
4	-1	0	**9**	1	1
5	0	0			

* En esta matriz no se contemplan las réplicas de los puntos centrales

B. Diseño Box-Behnken. Se trata de un modelo muy usado en Química Analítica. Consiste en un diseño factorial de tres niveles incompleto. La Tabla 13 muestra una matriz de diseño para tres variables. Es un modelo que consiste en tres partes de cuatro experimentos cada una. En cada parte, dos factores se combinan como en un diseño completo de dos niveles mientras que el tercero es fijado a cero. La representación del diseño de Box-Behnken se interpreta de dos maneras, la primera como un cubo con un punto central y el resto de puntos en el centro de cada arista (Figura 24.a) y la segunda como una unión de tres diseños factoriales 2^2 combinados formando un sólido geométrico y un punto central, donde se cruzan los tres diseños factoriales [149] (Figura 25.b).

Tabla 13*. Matriz de experimentos para un diseño de Box-Behnken para tres variables (x_1, x_2, x_3)*

Experimento	x_1	x_2	x_3	Experimento	x_1	x_2	x_3
1	-1	-1	0	8	1	0	1
2	1	-1	0	9	0	-1	-1
3	-1	1	0	10	0	1	-1
4	1	1	0	11	0	-1	1
5	-1	0	-1	12	0	1	1
6	1	0	-1	13	0	0	0
7	-1	0	1				

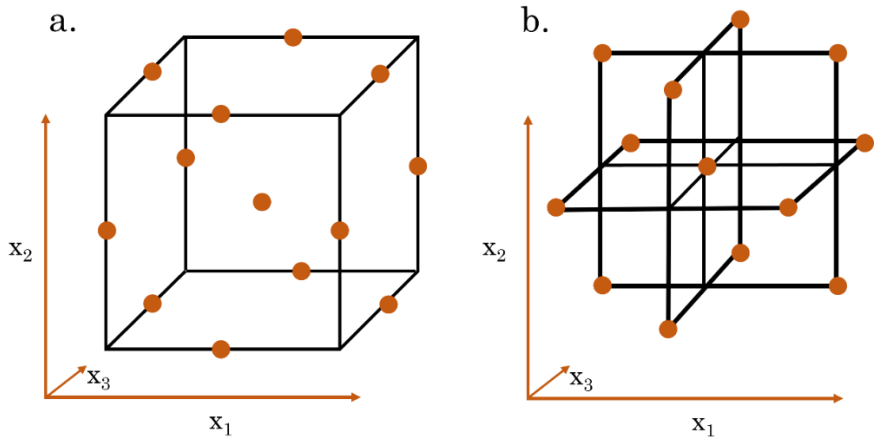


Figura 24. *Diseño de Box-Behnken para la optimización de tres variables*
Elaboración propia

Tras obtener los resultados, se debe comprobar que el modelo ajustado es apropiado según el comportamiento del sistema real y su capacidad de predicción. Para ello, se suele recurrir al análisis de varianza (ANOVA) que comprueba la adecuación del modelo de regresión con la prueba de la falta de ajuste (lack-of-fit test) y la influencia de los coeficientes estimados. Por otro lado, otro parámetro a evaluar es el coeficiente de determinación (R^2), que da un valor que relaciona cuantitativamente los datos experimentales y las respuestas predichas. Por último, se puede comprobar su idoneidad de forma gráfica mediante el diagrama de Pareto. Se trata de un diagrama de barras en el cual podemos comprobar la influencia (positiva o negativa) de cada variable implicada, así como si son estadísticamente significativas o no. En el diagrama se representa una línea vertical que corresponde con el intervalo de confianza especificado, que normalmente suele ser del 95%. La Figura 25 muestra un diagrama de Pareto.

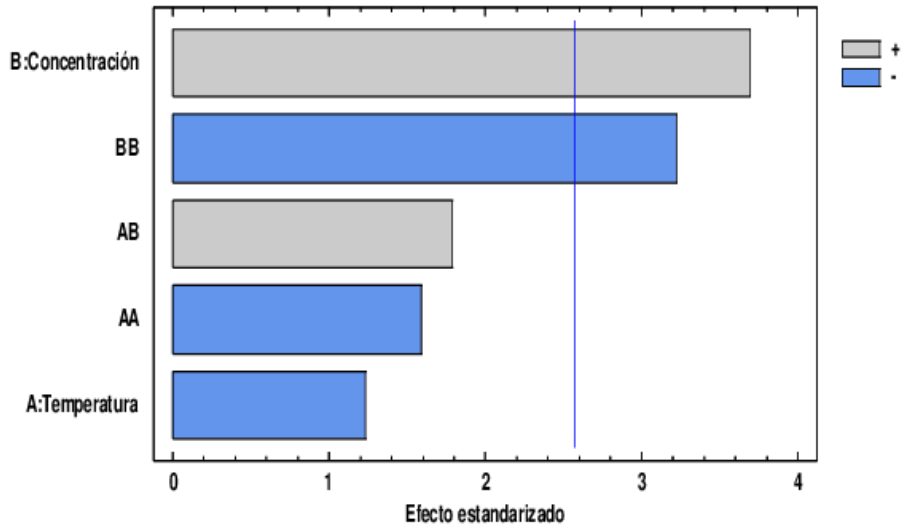


Figura 25. *Ejemplo de Diagrama de Pareto.*
Fuente: Bustamante et al. [150]

5.4.2 Calibración analítica

Cuando se desarrolla o aplica un método analítico, el principal objetivo es obtener información significativa del sistema real. Estos resultados deben ser trazables a referencias o estándares que sean adecuados y por esto una etapa muy importante en el proceso analítico es la de calibración, tanto instrumental como metodológica.

La calibración, según el Vocabulario Internacional de Metrología, VIM (2012) [151], se define como *"operación que bajo condiciones especificadas establece, en una primera etapa, una relación entre los valores y sus incertidumbres de medida asociadas a partir de los patrones de medida, y las correspondientes indicaciones con sus incertidumbres asociadas y, en una segunda etapa, utiliza esta información para establecer una relación que permita obtener un resultado de medida a partir de una indicación".*

En el procedimiento de calibración se diferencian, por tanto, dos fases. La primera es la de calibración metodológica, en el cual se establece un modelo que relaciona la variable independiente (x), en este caso la concentración, con la variable independiente (y) que es la señal analítica:

$$Señal\ analítica = f\ (concentración)$$

La segunda fase consiste en la predicción, es decir, calcular las variables independientes a partir de la ecuación obtenida en la primera etapa y la señal instrumental.

En esta Tesis Doctoral, se ha seleccionado el modelo de regresión lineal univariante por mínimos cuadrados, en el cual la variable independiente (x) se corresponde con la concentración del analito de interés, y se relaciona con la respuesta (y), que es la señal instrumental, mediante una relación del tipo:

$$y = a + b \cdot x$$

Siendo a y b dos parámetros estimados a partir de los datos experimentales.

Por otro lado, en esta etapa del proceso analítico se ha evaluado la homogeneidad de las varianzas (condición de homocedasticidad) a partir del análisis gráfico de los residuos.

5.4.3 Validación del método analítico

La validación del método analítico es la clave para asegurar la calidad del análisis. Validar un método consiste en documentar su adecuación a unos requisitos previamente establecidos, es decir, confirmar que responde adecuadamente al problema analítico que se presenta [152]. El objetivo de la validación es obtener resultados precisos, fiables y reproducibles. Además, existen requerimientos regulatorios y de buenas prácticas y control de calidad, que destacan la importancia de la etapa de validación. El Código de Regulaciones Federales (CFR) 311.165c establece que *"la exactitud, sensibilidad, especificidad y reproducibilidad de los métodos analíticos empleados deberán ser establecidos y documentados".*

En la presente Tesis Doctoral, se ha seguido la Guía ICH para la validación de procedimientos analíticos [153]. Las características típicas que deben evaluarse en la validación de un método, son:

A. Exactitud (veracidad + precisión): La exactitud de un método analítico expresa el grado de concordancia entre el valor verdadero o de referencia y el valor hallado. Se evalúa en términos de veracidad y precisión.

> ***Veracidad.*** Se define como la proximidad entre la media de un número infinito de valores medidos repetidos y un valor de referencia [154]. Dado que es muy complicado establecer un valor como verdadero, normalmente se usan materiales certificados de referencia y la comparación con métodos de referencia. Dentro de la veracidad, se evalúa el efecto matriz, que puede llevar a errores sistemáticos proporcionales, dado que las rectas de calibrado en patrones puros y en presencia de matriz tienen distintas pendientes. Para evitar este tipo de errores, se deben comparar las pendientes de ambas rectas de calibrado y realizar un test de t-Student. Para

eliminar o reducir el efecto matriz, la principal solución que se lleva a cabo es el uso de patrones internos y la preparación de rectas de calibrado con el método de adición de patrón o la calibración en matriz (*matrix matched calibration*).

En los trabajos presentados en la presente Tesis Doctoral, se ha evaluado la veracidad mediante ensayos de recuperación usando las matrices de estudio libres de analitos dopadas con concentraciones conocidas de los mismos. Se trabaja a tres niveles de concentración (bajo, medio y alto) y se calcula la recuperación dividiendo la concentración obtenida mediante la interpolación en la recta de calibrado entre la concentración teórica. La incertidumbre, por tanto, está limitada al error manual cometido en la adición del patrón a la matriz usada como blanco.

Precisión. Es el detalle con el que un instrumento mide una variable. Mide el grado de concordancia que existe entre resultados independientes de un ensayo realizado bajo condiciones conocida [153]. La precisión viene determinada por la desviación estándar relativa de los resultados obtenidos. La desviación estándar relativa (RSD, *relative standard deviation*) se calcula a partir de la expresión:

$$RSD\ (\%) = 100 \cdot {}^{s_c}/_{c}$$

Donde s_c es la desviación estándar obtenida a partir de réplicas y c es la concentración media.

La precisión se puede evaluar en condiciones de repetibilidad, esto es, medidas realizadas en las mismas condiciones experimentales, con el mismo operador y el mismo equipo y en períodos de tiempo cercanos, o de reproducibilidad, en la que se realizan las mismas medidas, pero con distintas condiciones experimentales, operador y equipos.

B. Sensibilidad: La IUPAC la define como *"la relación que existe entre la variación de la señal analítica o cambio en la respuesta instrumental"* [155]. Se determina mediante la pendiente de la recta de calibrado y mediante los límites de detección y cuantificación.

Límite de detección (LOD, limit of detection). Se define como la cantidad más baja de analito en una muestra que puede ser detectada pero no necesariamente cuantificada con un valor exacto (guía ICH).

***Límite de cuantificación* (LOQ, *limit of quantification*).** Se trata de la mínima cantidad de analito en una muestra que puede ser determinada cuantitativamente con exactitud. En los trabajos presentados en esta Tesis, los límites de detección se corresponden a las concentraciones calculadas a partir de una señal igual a tres veces la desviación estándar del blanco y el límite de cuantificación corresponde a diez veces la desviación estándar del blanco [153].

C. Linealidad: Es la capacidad de un procedimiento analítico, dentro de un intervalo determinado, para obtener resultados directamente proporcionales a la concentración de analito en la muestra. Hay dos tipos de linealidad.

a. ***Linealidad in-line:*** se estima con el valor P del test de fallo de ajuste. El valor debe ser mayor o igual al 5%.

b. ***Linealidad on-line:*** se corresponde con el coeficiente de determinación (R^2).

D. Rango dinámico lineal (LDR, linear dynamic range): Se define como el intervalo entre las concentraciones más alta y más baja de analito en la muestra (ambas incluidas) para las cuales se ha demostrado que el procedimiento analítico tiene niveles aceptables de linealidad y exactitud [153].

E. Selectividad: También llamada especificidad. Es la capacidad de evaluar inequívocamente el analito en presencia de componentes que cabe esperar que estén presentes. En los trabajos presentados, se ha evaluado la selectividad mediante la comparación de un cromatograma del blanco y el mismo cromatograma de un blanco dopado con distintas concentraciones de cada analito.

6 Referencias

1. Kumar, R., Qureshi, M., Kumar Vishwakarma, D., Al-Ansari, N., Kuriqi, A., Elbeltagi, A., Saeaswat, A., 2022. A review on emerging water contaminants and the application of sustainable removal technologies. Case Stud. Chem. Environ. Eng., 6, 100219.

2. Rosenfeld, P.E., Feng, L.G.H., 2011. Emerging contaminants, Risk of Hazardous Wastes, Elsevier Editorial, 215-222.

3. Archer, E., Holton, E., Fidal, J., Kasprzyk.Hordern, B., Carstens, A., Brocker, L., Kjeldsen, T.R., Wolfaardt, G.M., 2023. Occurrence of contaminants of emerging concern in the Eerste River, South Africa: Towards the optimization of an urban water profiling approach for public- and ecological health risk characterization. Sci. Total Environ., 859, 160254.

4. Castillo-Zacarías, C., Barocio, M.E., Hidalgo-Vázquez, E., Sosa-Hernández, J.E., Parra-Arroyo, L., López-Pacheco, I.Y., Barceló, D., Iqbal, H.N.M., Parra-Saldívar, R., 2021. Antidepressant drugs as emerging contaminants: Occurrence in urban and non-urban waters and analytical methods for their detection. Sci. Total Environ., 757, 143722.

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

6. Silva, S., Vale Cardoso, V., Duarte, L., Neves Carneiro, R., Martins Almeida, M.M., 2021. Characterization of fice portuguese wastewater treatment plants: Removal efficiency of pharmaceutical active compounds through conventional treatment process and environmental risk. Appl. Sci., 11, 7388.

7. Prasse, C., Stalter, D., Schulte-Oehlmann, U., Oehlmann, J., Ternes, T.A., 2015. Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies. Water Res., 87, 237-270.

8. De Oliveira Santos, A.D., Lima do Nascimento, M.T., Lima Sanson, A., Ferreira dos Santos, R., Cruz Felix L., da Silva de Freitas, A., Hauser-Davis, R.A., Monteiro da Fonseca, E., Baptista Neto, J.A., Maia Bila, D., 2022. Pharmaceuticals, natural and synthetic hormones and phenols in sediments from an eutrophic estuary, Jurujuba Sound, Guanabara Bay, Brazil. Mar. Pollut. Bull., 184, 114176.

9. AEMPS, 2022. Productos de cuidado personal. URL https://www.aemps.gob.es/cosmeticos-cuidado-personal/productos-de-cuidado-personal/ (Último acceso 27.02.2023)

10. BOE., 1997. Real Decreto 1599/1997, de 17 de octubre, sobre productos cosméticos. Páginas 31486-31513. URL https://www.boe.es/buscar/doc.php?id=BOE-A-1997-23067. (Último acceso 27.03.2023).

11. Martín-Pozo, L., Gómez-Regalado, M.C., Moscoso-Ruiz, I., Zafra-Gómez, A., 2021. Analytical methods for the determination of endocrine disrupting chemicals in cosmetics and personal care products: A review. Talanta, 234, 122642.

12. Diamanti-Kandarakis, E., Bourguignon, J-P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, T., Gore, A.C., 2009. Endocrine-disrupting chemicals: An endocrine society scientific statement. Endocr. Rev., 30(4), 293-342.

13. Amorim Amato, A., Brit Wheeler, H., Blumberg, B., 2021. Obesity and endocrine-disrupting chemicals. Endocrinolgy, 10(2), 87-105.

14. Kabir, E.R., Rahman, M.S., Rahman, I., 2015. A review on endocrine disruptors and their possible impacts on human health. Environ. Toxicol. Pharmacol., 40, 241-258.

15. OMS., 2012. State of the Science of Endocrine Disrupting Chemicals. Summary for Decision-Makers. URL https://apps.who.int/iris/bitstream/handle/10665/78102/WHO_HSE_PHE_IHE_2013.1_eng.pdf. (Último acceso 27.02.2023).

16. Sang, Z., Leung, K.S-Y., 2016. Environmental occurrence and ecological risk assessment of organic UV filters in marine organisms from Hong Kong coastal waters. Sci. Total Environ., 566-567, 489-498.

17. Scheele, A., Sutter, K., Karatum, O., Danley-Thomson, A.A., Redfern, L.K., 2023. Environmental impacts of the ultraviolet filter oxybenzone. Sci. Total Environ., 863, 160966.

18. Johnson, P.I., Favela, K., Jarin, J., Le, A.M., Clark, P.Y., Fu, L., Gillis, A.D., Morga, N., Nguyen, C., Harley, K.G., 2022. Chemicals of concern in personal care products used by womrn of color in three communities of California. J. Expo. Sci. Environ. Epidemiol., 32, 864-876.

19. Hiller, J., Klotz, K., Meyer, S., Uter, W., Hof, K., Greiner, A., Göen, T., Drexler, H., 2019. Systemic availability of lipophilic organic UV filters through dermal sunscreen exposure. Environ. Int., 132, 105068.

20. Hua, L., Liu, W., Liu, Y., Yang, M., Wang, B., Zhu, H., Zhu, L., Yao, Y., Zhang, Y., Zhao, H., 2022. Occurrence and profile characteristics of environmental phenols in human urine from a rural area in Northwestern China. Environ. Pollut., 315, 120405.

21. Rodríguez-Gómez, R., Zafra-Gómez, A., Dorival-García, N., Ballesteros, O., Navalón, A., 2015. Determination of benzophenone-UV filters in human milk samples using ultrasound-assisted extraction and clean-up with dispersive sorbents followed by UHPLC-MS/MS analysis. Talanta, 134, 657-664.

22. Sun, Y., Xie, H., Du, B., Li, J., Liu, L-Y., Gui, Y., Zeng, L., 2022. Widening the Lens on UV filters: Newfound ubiquity of triazine UV filters in breast milk from South China and implications for augmented "cocktail" exposure in infants. Environ. Sci. Technol. Lett., 9, 844-850.

23. Vela-Soria, F., Gallardo-Torres, M.E., Ballesteros, O., Díaz, C., Pérez, J., Navalón, A., Fernández, M.F., Olea, N., 2017. Assessment of

parabens and ultraviolet filters in human placenta tissue by ultrasound assisted extraction and ultra-high performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A, 1487, 153-161.

24. Sunyer-Caldú, A., Peiró, A., Díaz, M., Ibáñez, L., Gago-Ferrero, P., Diaz-Cruz, M.S., 2021. Development of a sensitive analytical method for the simultaneous analysis of Benzophenone-type UV filters and parabens preservatives in umbilical cord blood. MethodsX, 8, 101307.

25. Li, Y., Zhu, Q., Bi, S., Zhou, Q., Liang, Y., Liu, S., Liao, C., 2022. Associations between concentrations of typical ultraviolet filter benzophenones in indoor dust and human hair from China: A human exposure study. Sci. Total Environ., 841, 156789.

26. Celeiro, M., Acerbi, R., Kabir, A., Furton, K.G., Llompart, M., 2020. Development of an analytical methodology based on fabric phase sorptive extraction followed by gas chromatography-tandem mass spectrometry to determine UV filters in environmental and recreational waters. Anal. Chim. Acta, 4, 100038.

27. Xiong, Q., Wu, D., Cheng, Y-X., Hu, L-X., Chen, Q-L., Wu, H-Y., Sun, Y-H., Liu, Y-S., Ying, G-G., 2022. Development and validation of a simultaneous method for the analysis of benzothiazoles and organic ultraviolet filters in various environmental matrices by GC-MS/MS. Anal. Bioanal. Chem., 414, 6541-6555.

28. Huang, Y-F., Chang, J-P., Chen, H-C., Huang, Y-M., 2021. Simultaneous trace analysis of 10 benzophenone-type ultraviolet filters in fish through liquid chromatography-tandem mass spectrometry. Environ. Pollut., 286, 117306.

29. He, K., Timm, A., Blaney, L., 2017. Simultaneous determination of UV-filters and estrogens in aquatic invertebrates by modified quick, easy, cheap, effective, rugged, and safe extraction and liquid chromatography tandem mass spectrometry. J. Chromatogr. A, 1509, 91-101.

30. UE., 2022. Reglamento (UE) 2022/1176 de la Comisión de 7 de julio de 2022 por el que se modifica el Reglamento (CE) nº 1223/2009 del Parlamento Europeo y del Consejo en lo relativo al uso de determinados filtros ultravioleta en productos cosméticos.URL https://www.boe.es/buscar/doc.php?id=DOUE-L-2022-81028. (Último acceso 27.02.2023).

31. Downs, C.A., Bishop, E., Diaz-Cruz, S., Haghshenas, S.A., Stien, D., Rodrigues, A.M.S., Woodley, C.M., Sunyer-Caldú, A., Doust, S.N., Espero, W., Ward, G., Farhangmehr, A., Tabatabaee Samimi, S.M., Risk, M.J., Lebaron, P., DiNardo, J.C., 2022. Oxybenzone contamination from sunscreen pollution and its ecological threat to Hanauma Bay, Oahu, Hawaii, U.S.A. Chemosphere, 291, 132880.

32. Lee, Y-M., Lee, G., Kim, M-K., Zoh, K-D., 2020. Kinetics and degradation mechanism of Benzophenone-3 in chlorination ad UV/chlorination reactions. J. Chem. Eng., 393, 124780.

33. Balázs, A., Krifaton, C., Orosz, I., Szoboszlay, S., Kovács, R., Csenki, Z., Urbányi, B., Kriszt, B., 2016. Hormonal activity, cytotoxicity and developmental toxicity of UV filters. Ecotoxicol. Environ. Saf., 131, 45-53.

34. Watanabe, Y., Kojima, H., Takeuchi, S., Uramaru, N., Sanoh, S., Sugihara, K., Kitamura, S., Ohta, S., 2015. Metabolism of UV-filter benzophenone-3 by rat and human liver microsomes and its effect on endocrine-disrupting activity. Toxicol. Appl. Pharmacol., 282, 119-128.

35. Ahn, S., An, S., Lee, M., Lee, E., Pyo, J.J., Kim, J.H., Ki, M.W., Jin, S.H., Ha, J., Noh, M., 2019. A long-wave UVA filter avobenzone induces obesogenic phenotypes in normal human epidermal keratinocytes and mesenchymal stem cells. Arch. Toxicol., 93, 1903-1915.

36. Comisión Europea (CE), 2023. Annex VI, List of UV filters allowed in cosmetic products. URL https://ec.europa.eu/growth/tools-

databases/cosing/pdf/COSING_Annex%20VI_v2.pdf. (Último acceso 27.02.2023).

37. Guesmi, A., Ohlund, L., Sleno, L., 2020. In vitro metabolism of sunscreen compounds by liquid chromatography/high-resolution tandem mass spectrometry. Rapid Commun. Mass Spectrom., 34, e8679.

38. Klopcic, I., Sollner Dolenc, M., 2017. Endocrine activity of AVB, 2MR, BHA, and their mixtures. Toxicol. Sci., 156(1), 240-251.

39. Bordalo, D., Cuccaro, A., De Marchi, L., Soares, A.M.V.M., Meucci, V., Battaglia, F., Pretti, C., Freitas, R., 2022. In vitro spermiotoxicity and in vivo adults' biochemical pattern after exposure of the Mediterranean mussel to the sunscreen avobenzone. Environ. Pollut., 312, 119987.

40. Carve, M., Nugegoda, D., Allinson, G., Shimeta, J., 2021. A systematic review and ecological risk assessment for organic ultraviolet filters in aquatic environments. Environ. Pollut., 268, 115894.

41. Hong, H., Wang, J., Shi, D., 2021. Effects of salinity on the chronic toxicity of 4-methylbenzylidene camphor (4-MBC) in the marine copepod Tigriopus japonicas. Aquat. Toxicol., 232, 105742.

42. Ramos, S., Homem, V., Alves, A., Santos, L., 2016. A review of organic UV-filters in wastewater treatment plants. Environ. Int., 86, 24-44.

43. Buser, H-R., Müller, M.D., Balmer, M.E., Poiger, T., Buerge, I.J., 2005. Steroisomer composition of the chiral UV filter 4-methylbenzylidene camphor in environmental samples. Environ. Sci. Technol., 39, 3013-3019.

44. Cadena-Aziaga, M.I., Montesdeoca-Esponda, S., Sosa-Ferrera, Z., Santana-Rodríguez, J.J., 2022. Occurrence and environmental hazard of organic UV filters in seawater and wastewater from Gran Canaria Island (Canary Islands, Spain). Environ. Pollut., 300, 118843.

45. FDA (Food and drugs administration), 2022. Code of Federal Regulations Title 21. Drugs: general. URL https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=207. (Último acceso: 27.02.2023).

46. UNESCO, 2020. Emerging pollutants in water and wastewater. URL https://en.unesco.org/emergingpollutantsinwaterandwastewater. (Último acceso 28.02.2023).

47. Unión Europea (UE), 2013. Directiva 2013/39/EU del Parlamento europeo y del Consejo de 12 de agosto de 2013 por la que se modifican las Directivas 2000/60/CE y 2008/105/CE en cuanto a las sustancias prioritarias en el ámbito de la política de aguas. URL https://www.boe.es/doue/2013/226/L00001-00017.pdf. (Último acceso 27.02.2023).

48. Comisión Europea (CE), 2015. Commission Implementing Decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. URL https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32015D0495&qid=1677491761774. (Último acceso: 27.02.2023).

49. Comisión Europea (CE), 2018. Commission Implementing Decision (EU) 2018/840 of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495. URL https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32018D0840&qid=1677491576511. (Último acceso: 27.02.2023).

50. Comisión Europea (CE), 2020. Commission Implementing Decision (EU) 2020/1161 of 4 August 2020 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. URL https://eur-lex.europa.eu/legal-

content/EN/TXT/?uri=uriserv:OJ.L_.2020.257.01.0032.01.ENG&toc=OJ:L:2020:257:TOC. (Último acceso: 27.02.2023).

51. Comisión Europea (CE), 2022. Commission Implementing Decision (EU) 2022/1307 of 22 July 2022 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. URL https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32022D1307&qid=1677491418440. (Último acceso: 27.02.2023).

52. Cravo, A., Silva, S., Rodrigues, J., Cardoso, V.V., Benoliel, M.J., Correia, C., Coelho, M.R., Rosa, M.J., Almeida, C.M.M., 2022. Understanding the bioaccumulation of pharmaceutical active compounds by clams Ruditapes decussatus exposed to a UWWTP discharge. Environ. Res., 208, 112632.

53. Ramírez-Morales, D., Masís-Mora, M., Montiel-Mora, J.R., Cambronero-Heinrichs, J.C., Pérez-Rojas, G., Tormo-Budowski, R., Méndez-Rivera, M., Briceño-Guevara, S., Gutiérrez-Quirós, J.A., Arias-Mora, V., Brenes-Alfaro, L., Beita-Sandí, W., Rodríguez-Rodríguez, C.E., 2021. Multi-residue analysis of pharmaceuticals in water samples by liquid chromatography-mass spectrometry: Quality assessment and application to the risk assessment of urban-influenced surface waters in a metropolitan area of Central America. Process Saf. Environ. Prot., 153, 289-300.

54. Wu, D., Sui, Q., Mei, X., Yu, X., Gu, Y., Zhao, W., 2022. Non-antibiotics matter: Evidence from a one-year investigation of livestock wastewater from six farms in East China. Sci. Total Environ., 846, 157418.

55. Golbaz, S., Yaghmaeian, K., Isazadeh, S., Zamanzadeh, M., 2021. Environmental risk assessments of multiclass pharmaceutical active compounds: selection of high priority concern pharmaceuticals using entropy-utility functions. Environ. Sci. Pollut. Res., 28, 59745-59770.

56. Ortúzar, M., Esterhuizen, M., Olicón-Hernández, D.R., González-López, J., Aranda, E., 2022. Pharmaceutical pollution in aquatic environments: A concise review of environmental impacts and bioremediation systems. Front. Microbiol., 13, 869332.

57. Zhang, C., Deng, Y., Zheng, J., Zhang, Y., Yang, L., Liao, C., Su, L., Zhou, Y., Gong, D., Chen, L., Luo, A., 2019. The application of the QuEChERS methodology in the determination of antibiotics in food: A review. Trends Anal. Chem., 118, 517-537.

58. Moga, A., Vergara-Barberán, M., Lerma-García, M.J., Carrasco-Correa, E.J., Herrero-Martínez, J.M., Simó-Alfonso, E.F., 2021. Determination of antibiotics in meat samples using analytical methodologies: A review. Compr. Rev. Food Sci. Food Saf., 20, 1681-1716.

59. Bol'shakov, D.S., Amelin, V.G., Nikeshina, T.B., 2016. Determination of antibiotics in drugs and biological fluids using capillary electrophoresis. J. Anal. Chem., 71(3), 215-233.

60. Kim, C., Ryu, H-D., Chung, E.G., Kim, Y., Lee, J-K., 2018. A review of analytical procedures for the simultaneous determination of medically important veterinary antibiotics in environmental water: Sample preparation, liquid chromatography and mass spectrometry. J. Environ. Manage., 217, 629-645.

61. PNRA., 2022. ¿Qué es la resistencia a los antibióticos y cómo se propaga? URL https://www.resistenciaantibioticos.es/es/sobre-la-resistencia#que-es-la-resistencia. (Último acceso 27.02.2023).

62. AEMPS, 2018. Quinolonas y fluoroquinolonas de administración sistémica: nuevas restricciones de uso. URL https://www.aemps.gob.es/informa/notasinformativas/medicamentosusohumano-3/seguridad-1/2018/ni_muh_fv-14-2018-quinolonas-fluoroquinolonas/. (Último acceso 27.02.2023).

63. Martín-Pozo, L., Gómez-Regalado, M.C., García-Córcoles, M.T., Zafra-Gómez, A., 2022. Removal of quinolone antibiotics from

wastewaters and sewage sludge. Emerging contaminants in the Environment, Chapter 16. Elsevier Editorial, 381-405.

64. Alós, J.I., 2009. Quinolonas. Enferm. Infecc., 27(5), 290-297.

65. Pérez-Trallero, E., Iglesias, L., 2003. Tetraciclinas, sulfamidas y metronidazol. Enferm. Infecc. Microbiol. Clin., 21(9), 520-529.

66. Kanoh, S., Rubin, B.K., 2010. Mechanisms of action and clinical applications of macrolides as immunomodulatory medications. Clin. Microbiol. Rev., 23, 590-615.

67. Vione, D., Feitosa-Felizzola, J., Minero, C., Chiron, S., 2009. Phototransformation of selected human-used macrolides in surface waters: Kinetics, model predictions and degradation pathways. Water Res., 43, 1959-1967.

68. Hitchings, G.H., 1973. Mechanism of action of trimethoprim-sulfamethoxazole. J. Infect. Dis., 128, 433-436.

69. Gleckman, R., Blagg, N., Joubert, D.W., 1981. Trimethoprim: mechanisms of action, antimicrobial activity, bacterial resistance, pharmacokinetics, adverse reactions, and therapeutic indications. Pharmacotherapy, 1(1), 14-20.

70. Suárez, C., Gudiol, F., 2009. Antibióticos betalactámicos. Enferm. Infecc., 27(2), 116-129.

71. Benedí, J., Romero, C., 2005. Antidepresivos. Farmacia profesional, 19(10), 76-80.

72. Alcántara-Montero, A., 2018. Venlafaxine in the treatment of neuropathic pain. Rev. Soc. Esp. Dolor, 25(2), 94-105.

73. Sierra-Santos, L., Raigal-Martín, Y., Ortega-García, A., Berrichoa Martínez de Pisón, C., Aparicio-Jabalquinto, G., 1999. Venlafaxina y síndrome de discontinuación. Aten. Primaria, 24(10), 617-618.

74. Qu, H., Ma, R., Wang, B., Yang, J., Duan, L., Yu, G., 2019. Enantiospecific toxicity, distribution and bioaccumulation of chiral antidepressant venlafaxine and its metabolite in loach (*Misgurnus*

anguillicaudatus) co-exposed to microplastic and the drugs. J. Hazard. Mater., 370, 203-211.

75. NCI, 2021. Diccionario del cáncer. Antiepiléptico. URL https://www.cancer.gov/espanol/publicaciones/diccionarios/diccionario-cancer/def/antiepileptico. (Último acceso 28.02.2023).

76. Fisterra, 2020. Carbamazepina. URL https://www.fisterra.com/medicamentos/carbamazepina/. (Último acceso 28.02.2023).

77. Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2014. Presence of the pharmaceutical drug carbamazepine in coastal systems: Effects on bivalves. Aquat. Toxicol., 156, 74-87.

78. Vitale, D., Picó, Y., Spanò, N., Torreblanca, A., del Ramo, J., 2020. Carbamazepine exposure in the sea anemones Anemonia sulcata and Actinia equina: Metabolite identification and physiological responses. Sci. Total Environ., 744, 140891.

79. Clínica Mayo, 2021. Betabloqueantes. URL https://www.mayoclinic.org/es-es/diseases-conditions/high-blood-pressure/in-depth/beta-blockers/art-20044522. (Último acceso 28.02.2023).

80. Rojas, S., Navarro, J.A.R., Horcajada, P., 2021. Metal-organic frameworks for the removal of the emerging contaminant atenolol under real conditions. Dalton Trans., 50, 2493.

81. Gibson, J.A., Raphael, B., 2015. Los bloqueadores ß. Nursing, 32(1), 51-55.

82. Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Zlabek, V., Schmidt-Posthaus, H., Grabic, R., Kroupova, H.K., 2014. The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout *(Oncorhynchus mykiss*). Sci. Total Environ., 497-497, 209-218.

83. Vieno, N., Tuhkanen, T., Kronberg, L., 2007. Elimination of pharmaceuticals in sewage treatment plants in Finland. Water Res., 41, 1001-1012.

84. Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014. Removal and seasonal variability of selected analgesics/anti-inflammatory, anti-hypertensive/cardiovascular pharmaceuticals and UV filters in seawater treatment plant. Environ. Sci. Pollut. Res., 21, 7578-7585.

85. 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.

86. Küster, A., Alder, A.C., Escher, B.I., Duis, K., Fenner, K., Garric, J., Hutchinson, T.H., Lapen, D.R., Péry, A., Römbke, J., Snape, J., Ternes, T., Topp, E., Wehrhan, A., Knacker, T., 2009. Environmental risk assessment of human pharmaceuticals in the European Union: A case study with the ß-blocker Atenolol. Integr. Environ. Assess. Manag., 6(1), 514-523.

87. Parmar, T.K., Rawtani, D., Agrawal, Y.K., 2016. Bioindicators: the natural indicator of environmental pollution. Front. Life Sci., 9(2), 110-118.

88. McCarthy, J.F., Shugart, L.R., (Eds.), 2017. Biomarkers of environmental contamination (p. 457). Lewis Publishers.

89. Jain, A., Singh, B.N., Singh, S.P., Singh, H.B., Singh, S., 2010. Exploing biodiversity as bioindicators for water pollution. Uttar Pradesh State Biodiversity Board, 50-56.

90. Khatri, N., Tyagi, S., 2015. Influences of natural and anthropogenic factors on surface and groundwater quality in rural and urban areas. Front. Life Sci., 8(1), 23-39.

91. Arango, S.S., 2011. Biomarcadores para la evaluación de riesgo en la salud humana. Rev. Fac. Nac. Salud Pública, 30(1), 75-82.

92. Lock, E.A., Bonventre, J.V., 2008. Biomarkers in translation; past, present and future. Toxicology, 245, 163-166.

93. Cabo-Golpe, M., de-Castro-Ríos, A., Cruz, A., Páramo, M., López-Rivadulla, M., Lendoiro, E., 2020. Determination of antipsychotic drugs in nails and hair by liquid chromatography tandem mass spectrometry and evaluation of their incorporation into keratinized matrices. J. Pharm. Biomed. Anal., 189, 113443.

94. Palmeri, A., Pichini, S., Pacifici, R., Zuccaro, P., Lopez, A., 2000. Drugs in nails. Physiology, pharmacokinetics and forensic toxicology. Clin. Pharmacokinet., 38(2), 95-110.

95. Cabo-Golpe, M., de-Castro-Ríos, A., Cruz, A., Páramo, M., López-Rivadulla, M., Lendoiro, E., 2021. Determination and distribution of cannabinoids in nail and hair samples. J. Anal. Toxicol., 45, 969-975.

96. Ministerio de agricultura, pesca y alimentación, 2023. Crustáceos y moluscos. Mejillón. URL https://www.mapa.gob.es/es/ministerio/servicios/informacion/mejillon_tcm30-102425.pdf. (Último acceso, 28.02.2023).

97. OESA, 2017. Cultivo del mejillón (*Mytilus galloprovincialis*). Fundación biodiversidad, Madrid. URL https://www.observatorio-acuicultura.es/sites/default/files/images/adjuntos/libros/cuaderno_mejillon.pdf. (Último acceso 28.02.2023).

98. Bonham, V., Shields, J., Riginos, C., 2022. *Mytilus galloprovincialis* (Mediterranean mussel), CABI Compendium. CABI International.

99. Mladinich, K., Holohan, B.A., Shumway, S.E., Brown, K., Ward, J.E., 2022. Determining the properties that Govern selective ingestion and egestion of microplastic by the Blue Mussel (*Mytilus edulis*) and Eastern Oyster (*Crassostrea virginica*). Environ. Sci. Technol., 56, 15770-15779.

100. Viñas, L., Pérez-Fernández, P., Soriano, J.A., López, M., Bargiela, J., Alves, I., 2018. Limpet (*Patella sp*) as a biomonitor for organic pollutants. A proxy for mussel? Mar. Pollut. Bull., 133, 271-280.

101. Malvar, J.L., Santos, J.L., Martín, J., Aparicio, I., Garcia Fonseca, T., Bebianno, M.J., Alonso, E., 2022. Ultrasound-assisted extraction as an easy-to-perform analytical methodology for monitoring ibuprofen and its main metabolites in mussels. Anal. Bioanal. Chem., 414, 5877-5886.

102. Álvarez-Ruiz, R., Picó, Y., Sadutto, D., Campo, J., 2021. Development of multi-residue extraction procedures using QuEChERS and liquid chromatography tandem mass spectrometry for the determination of different types of organic pollutants in mussel. Anal. Bioanal. Chem., 413, 4063-4076.

103. Bayen, S., Segovia Estrada, E., Juhel, G, Kelly, B.C., 2015. Direct injection of tissue extracts in liquid chromatography/tandem mass spectrometry for the determination of pharmaceuticals and other contaminants of emerging concern in mollusks. Anal. Bioanal. Chem., 407, 5553-5558.

104. López-García, E., Postigo, C., López de Alda, M., 2019. Psychoactive substances in mussels: Analysis and occurrence assessment. Mar. Pollut. Bull., 146, 958-992.

105. Wolecki, D., Caban, M., Pazdro, K., Mulkiewicz, E., Stepnowski, P., 2019. Simultaneous determination of non-steroidal anti-inflammatory drugs and natural estrogens in the mussels Mytilus edulis trossulus. Talanta, 200, 316-323.

106. Kim, K., Choi, Y., Mok, S., Moon, H-B., Jeon, J., 2023. Optimization of the QuEChERS method for multi-residue analysis of pharmaceuticals and pesticides in aquaculture products. Food Chem., 399, 133958.

107. García-Fernández, L., García-Córcoles, M.T., Navalón, A., Martín-Pozo, L., Hidalgo, F., Zafra-Gómez, A., 2022. New method for the determination of endocrine disrupting chemicals in Mediterranean

mussel (Mytilus galloprovincialis) using ultra-high performance liquid chromatography-tandem mass spectrometry. Microchem. J., 175, 107102.

108. Cañadas, R., Garrido Gamarro, E., Garcinuño Martínez, R.M., Paniagua González, G., Fernández Hernando, P., 2021. Occurrence of common plastic additives and contaminants in mussel samples: Validation of analytical method based on matrix solid-phase dispersion. Food Chem., 349, 129169.

109. Salgueiro-González, N., Turnes-Carou, I., Viñas, L., Besada, V., Muniategui-Lorenzo, S., López-Mahía, P., Prada-Rodrígez, D., 2016. Occurrence of alkylphenols and bisphenol A in wild mussel samples from the Spanish Atlantic coast and Bay of Biscay. Mar. Pollut. Bull., 106, 360-365.

110. Ocaña, A., Pérez-Ruzafa, A., 2004. Los equinodermos de las costas andaluzas. Acta Granatense, 3, 83-136.

111. Ocaña, A., 2006. Equinodermos. Fauna Andaluza. Publicaciones Comunitarias, SL., Sevilla, 174-190.

112. Ocaña, A., Sánchez-Tocino, S., López-González, J.F., Martín, V., 2000. Guía submarina de invertebrados no artrópodos. Comares (Ed), Granada.

113. Grosso, L., Rampacci, M., Pensa, D., Fianchini, A., BAtir, E., Aydin, I., Ciriminna, L., Felix, P.M., Pombo, A., Lovatelli, A., Vizzini, S., Scardi, M., Rakaj, A., 2023. Evaluating sea cucumbers as extractive species for benthic bioremediation in mussel farms. Sci. Rep., 13, 1457.

114. Lombardo, J., Solomando, A., Cohen-Sánchez, A., Pinya, S., Tejada, S., Ferriol, P., Mateu-Vicens, G., Box, A., Faggio, C., Sureda, A., 2022. Effects of human activity on markers of oxidative stress in the intestine of Holothuria tubulosa, with special reference to the presence of microplastics. Int. J. Mol. Sci., 23, 9018.

115. Martín, J., Hidalgo, F., Alonso, E., García-Córcoles, M.T., Vílchez, J.L., Zafra-Gómez, A., 2020. Assessing bioaccumulation potential of personal care, household and industrial products in a marine echinoderm (Holothuria tubulosa). Sci. Total Environ., 720, 137668.

116. Martín, J., Hidalgo, F., García-Córcoles, M.T., Ibáñez-Yuste, A.J., Alonso, E., Vílchez, J.L., Zafra-Gómez, A., 2019. Bioaccumulation of perfluoroalkyl substances in marine echinoderms: Results of laboratory-scale experiments with Holothuria tubulosa Gmelin, 1791. Chemosphere, 215, 261-271.

117. Chahrour, F., Baaloudj, A., Dermeche, S., Kerfouf, A., 2021. Trace metals (Cu, Cd, Pb, Zn and Ni) concentrations in the Sea cucumber Holothuria tubulosa (Gmelin, 1791) of the Algerian West Coast. Egypt. J. Aquat. Biol. Fish., 25(3), 619-633.

118. UICN, 2014. Actinia equina. URL http://www.iucn.it/scheda.php?id=904591704. (Último acceso 28.02.2023).

119. Göthel, H., 1996. Guide de la faune sous-marine, la Mediterranee, invertebres et poissons. Ulmer (ed), 318p.

120. Navarro, E., Ortega, M.M., Iglesias, J.I.P., 1986. An analysis of variables affecting oxygen consumption in Actinia equina L. (Anthozoa) from two shore positions. Comp. Biochem. Physiol., 86A(2), 233-240.

121. Mitchelmore, C.L., Ringwood, A.H., Weis, V.M., 2003. Differential accumulation of cadmium and changes in glutathione levels as a function of symbiotic state in the sea anemone Anthopleura elegantissima. J. Mar. Biol. Ecol., 284, 71-85.

122. Duckworth, C.G., Picariello, C.R., Thomason, R.K., Patel, K.S., Bielmyer-Fraser, G.K., 2017. Responses of the sea anemone, Exaiptasia pallida, to ocean acidification conditions and zinc or nickel exposure. Aquat. Toxicol., 182, 120-128.

123. Lozano-Bilbao, E., Espinosa, J.M., Lozano, G., Hardisson, A., Rubio, C., González-Weller, D., Gutiérrez, A.J., 2020. Determination of metals in Anemonia sulcata (Pennant, 1777) as a pollution bioindicator. Environ. Sci. Pollut. Res., 27, 21621-21627.

124. Morgan, M.B., Ross, J., Ellwanger, J., Martin-Phrommala, R., Youngblood, H., Qualley, D., Williams, J., 2022. Sea anemones responding to sex hormones, oxybenzone, and benzyl butyl phthalate: transcriptional profiling and in Silico modelling provide clues to decipher endocrine disruption in Cnidarians. Front. Genet., 12, 793306.

125. Walter, T.H., Andrews, R.W., 2014. Recent innovations in UHPLC columns and instrumentation. TrAC, 63, 14-20.

126. Dass, C., 2007. Fundamentals of contemporary mass spectrometry. John Wiley & Sons (editors)

127. Romero-González, R., Fernández-Moreno, J.L., Plaza-Bolaños, P., Garrido-Frenich, A., Martínez-Vidal, J.L., 2007. Empleo de la espectrometría de masas como herramienta para la determinación de tóxicos en alimentos: hacia la seguridad alimentaria. Rev. Esp. Salud Publica, 81(5).

128. Pomilio, A.B., Bernatené, E.A., Vitale, A.A., 2011. Espectrometría de masas en condiciones ambientales con ionización por desorción con electrospray. Acta bioqím. Clín. Latinoam., 45(1).

129. Balogh, M., 2009. The mass spectrometry primer. Lc Gc North America, 22.

130. Haag, A.M., 2016. Mass analyzers and mass spectrometers. In: Mirzaei, H., Carrasco, M. (eds) Modern proteomics-sample preparation, analysis and practical applications. Advances in experimental medicine and biology. 919. Springer.

131. Lin, S-Y., Hsu, W-H., Lin, C-C., Chen, C-J., 2014. Mass spectrometry-based proteomics in Chest Medicine Gerontology, and

Nephrology: subgroups omics for personalized medicine. Biomedicine, 4(4), 25-36.

132. Wang, L., Weller, C.L., 2006. Recent advances in extraction of nutraceuticals from plants. Trends Food Sci. Technol., 17, 300-312.

133. Sparr Eskilsson, C., Björklund, E., 2000. Analytical-scale microwave-assisted extraction. J. Chromatogr. A, 902, 227-250.

134. Viera-Santana, S., Santana-Rodríguez, J.J., 2013. Técnicas analíticas avanzadas para la extracción y preconcentración de contaminantes emergentes en muestras líquidas. Rev. Acad. Canar. Cienc., 25, 77-95.

135. Lavilla, I., Bendicho, C., 2017. Fundamentals of Ultrasound-assisted extraction. Chapter 11. Water extraction of bioactive compounds, Elsevier, 291-316.

136. Flores-Fernández, N., González-Muñoz, M.J., 2017. Ultrasound-assisted extraction of bioactive compounds. Chapter 12. Water extraction of bioactive compounds, Elsevier, 317-329.

137. Tiwari, B.K., 2015. Ultrasound: A clean, green extraction technology. Trends Anal. Chem., 71, 100-109.

138. Mehta, N., Jeyapriya, S., Kumar, P., Kumar Verma, A., Umaraw, P., Kumar Khatkar, S., Khatkar,A. B., Pathak, D., Kaka, U., Qurni Sazili, A., 2022. Ultrasound-assisted extraction and the encasulaption of bioactive components for food applications. Foods, 11, 2973.

139. Veggi,P.C., Martinez, J., Meireles, M.A.A., 2012. Fundamentals of microwave extraction. Chapter 2. Microwave-assisted extraction for bioactive compounds. Springer Ed., 15-52.

140. Llompart, M., Garcia-Jares, C., Celeiro, M., 2019. Microwave-assisted extraction. Encyclopecia of Analitical Science, 3rd edition.

141. Mocan, A., Diuzheva, A., Carradori, S., Andruch, V., Massafra, C., Moldovan, C., Sisea, C., Petzer, J.P., Petzer, A., Zara, S., Diletta

Marconi, G., Zengin, G., Crisan, G., Locatelli, M., 2018. Development of novel techniques to extract phenolic compounds from Romanian cultivars of Prunus domestica L. and their biological properties. Food Chem. Toxicol., 119, 189-198.

142. Budzinski, M., Letelier, H., 1999. Microwave assisted extraction of organic compounds. Analusis, 27(3).

143. Anastassiades, M., Lehotay, S.J., Stajnbaher, D., Schenck, F.J., 2003. Fast and easy multirresidue method employing acetonitrile extraction/partitioning and "Dispersive solid-phase extraction" for the determination of pesticide residues in produce. J. AOAC Int., 86(2), 412-431.

144. Pestrelo, R., Silva, P., Porto-Figueira, P., Pereira, J.A.M., Silva, C., Medina, S., Câmara, J.S., 2019. QuEChERS – Fundamentals, relevant improvements, applications and future trends. Anal. Chim. Acta, 1070, 1-28.

145. Fischer, R.A., 1936. Design of experiments. Br. Med. J., 14(1), 3923.

146. Montgomery, D.C., 2014. Design and analysis of experiments. Wiley (ed).

147. García-Díaz, A., Philips, D.T., 1994. Principles of experimental design and analysis. Chapman & Hall (ed).

148. Myers, R., Montgomery, D., Anderson-Cook, C., 2009. Response surface Methodology: Process and Product optimization using designed experiments. Wiley, 78, 81, 83.

149. Jaramillo, A.C., Echevarría, A.M., Hormaza, A., 2013. Diseño de Box-Behnken para la optimización de la adsorción del colorante azul ácido sobre residuos de flores. Ing. Cienc., 9(18), 75-91.

150. Bustamante, R., Oblitas, J., Rojas, E., 2016. Influcence of temperature and concentration on the rheological behavior of concentrated aguaymanto pulp (Physalis peruviana L.). Agroindustrial Sci., 1(1), 37-43.

151. VIM, 2012. Vocabulario Internacional de Metrología. Conceptos fundamentales y generales, y términos asociados. 3ª Edición en español. URL https://www.cem.es/sites/default/files/vim-cem-2012web.pdf. (Último acceso 28.02.2023).

152. Boqué, R., Maroto, A., Riu, J., Rius, F.J., 2002. Validation of analytical methods. Grasas y aceites, 53(1), 128-143.

153. ICH Quality Guidelines, Topic Q2 (R1), 2005. Validation of analytical procedures: text and methodology. http:// www. ich. org/ products/ guide lines/ quali ty/ artic le/ quali ty- guide lines. html. (Último acceso 28.02.2023).

154. ISO 5725-4. 2020. Accuracy (trueness and precision) of measurement methods and results. Part 4: Basic methods for the determination of the trueness of a standard measurement method. URL https://www.iso.org/standard/69421.html. (Último acceso 28.02.2023).

155. IUPAC, 2014. Sensitivity in metrology and analytical chemistry. URL https://goldbook.iupac.org/terms/view/S05606. (Último acceso 02.03.2023).

Experimental, Resultados y Discusión

Sección I. Desarrollo de metodología analítica para la determinación de CEs

Capítulo 1

An overview of analytical methods to determine Pharmaceutical Active Compounds in Aquatic organisms

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Abstract: There is increasing scientific evidence that some pharmaceuticals are present in the marine ecosystems at concentrations that may cause adverse effects on the organisms that inhabit them. At present, there is still very little scientific literature on the (bio)accumulation of these compounds in different species, let alone on the relationship between the presence of these compounds and the adverse effects they produce. However, attempts have been made to optimize and validate analytical methods for the determination of residues of pharmaceuticals in marine biota by studying the stages of sample treatment, sample clean-up and subsequent analysis. The proposed bibliographic review includes a summary of the most commonly techniques, and its analytical features, proposed to determine pharmaceutical compounds in aquatic organisms at different levels of the trophic chain in the last 10 years.

Keywords: pharmaceuticals; contamination; analytical methods; aquatic organisms; trophic chain.

1. Introduction

Pollution is one of the biggest environmental challenges worldwide. Like climate change or the depletion of water supplies, pollution threatens the stability of the earth's support systems and is a growing concern for human health [1]. Ocean pollution is a very important, but under-recognised, component of global pollution [2]. Seawater covers 97% of surface waters and is considered one of the most abundant resources on our planet [1]. The unsustainable use of marine waters and resources by humans has altered the structure of marine ecosystems, relating to the phenomenon of eutrophication, loss of diversity or the presence of polluting chemicals [3].

Human activities have introduced a large number of contaminants of emerging concern (CECs) into the environment [4]. CECs include a wide variety of compounds such as disinfection by-products, natural toxins, flame retardants, personal care products or pharmaceutical active compounds (PhACs) [5]. Nowadays, an increasing number of people and

animals are in need of health care, which means that the number and amount of PhACs consumed, and consequently excreted, is very high [6–8]. Approximately 3000 compounds are used as pharmaceuticals, with an annual production exceeding hundreds of tonnes [7]. It is well known that the wastewater treatment plants (WWTPs) are often unable to remove them completely, allowing their release into the environment [9,10]. In the case of PhACs, due to their constant release into the seas, even those that can undergo degradation may behave as pseudopersistent contaminants [11]. This continued exposure may present unexpected risks in the organisms that inhabit them such as reproductive disorders, survival of susceptible species, growth rate or development of bacterial resistance and endocrine disruption, among others [8,12,13].

The European Union has developed several laws for the monitoring and protection of the seas and their ecosystem. The Water Framework [14] and the Marine Strategy Framework Directive [15] are based on the maintenance as well as the protection and restoration of the marine environment. In addition, the European Commission has drawn up a first list for the monitoring of CECs in 2015, and then it was updated in 2018, 2020 and 2022. The decision 2022/1307/EC [16], includes some PhACs such as the antibiotics sulfamethoxazole and trimethoprim, or the antidepressant venlafaxine and its main metabolite, O-desmethylvenlafaxine, with a maximum permitted detection limit of 100 ng g^{-1} for the antibiotics and 6 ng g^{-1} for the others. Although quantitative analysis of PhACs in aquatic ecosystems is limited, as dilution makes detection difficult, the use of bioindicator species is valuable in assessing system contamination, since they are able to reflect bioavailability in a variability of concentrations in both water and sediment [11].

Due to the evidence of the presence of these active compounds in the environment and the concern that it raises, as well as the published EU directives, there is a need for the development of analytical methods with the appropriate characteristics to determine these PhACs in biomarkers. Furthermore, taking into account that the PhAC's consumption depends on factors such as seasonal diseases, the health system and prescribing practices or the economic level of the population, the methodology developed must take into account local needs [10]. The present work deals

with a comprehensive overview of the recent methods proposed for the determination of several groups of PhACs in aquatic organisms belonging to different levels of the trophic chain, emphasizing the sample treatment and contrasting the analytical results obtained. For that, we have focused mainly on methodological studies that include analytical quality parameters and relay on liquid chromatography (LC), the most useful separation technique for the multiresidue determination of PhACs [17]. Huerta et al. [18] already reviewed the state of the art of the analysis of PhACs in aquatic biota up to 2012. Thus, the present review provides an update on the current analytical methods since 2012 onwards.

2. Multi-Level Biological Groups as Biomarkers of Exposure

Biomarkers are defined as suborganic changes that occur at the cellular, physiological or molecular level, measurable in cells or tissues of an organism, which may be indicative of exposure [19]. To be a useful bioindicator, an organism must have certain characteristics such as a wide geographical distribution, long life duration, being easy to capture, a feeding mode that allows the accumulation of contaminants present in the environment (e.g., filtration) or the ability to accumulate and tolerate high concentrations of organic and inorganic contaminants in their tissues [20,21]. The use of sentinel species to monitor environmental pollution allows knowledge of the bioavailability of pollutants in the environment over prolonged periods of time [22]. In addition, information on the concentration of pollutants in different organisms is quite useful for considering toxicological and public health aspects of pollution in natural systems [23]. Among the distinct species used as bioindicators, fish and bivalves, particularly mussels, stand out, as the latter are present on coasts all over the world, are easy to capture and are filter-feeders [24,25]. However, it is necessary to study pollution in species other than mussels to assess trophic transfer in aquatic ecosystems. Figure 1 displays the number of studies devoted to the analysis of PhACs for each group of marine organisms according to the literature consulted in scientific databases. It shows that fish have been by far the most investigated in this field. This section summarises the use of some species

belonging to the diverse levels of the trophic chain as bioindicators of pollution.

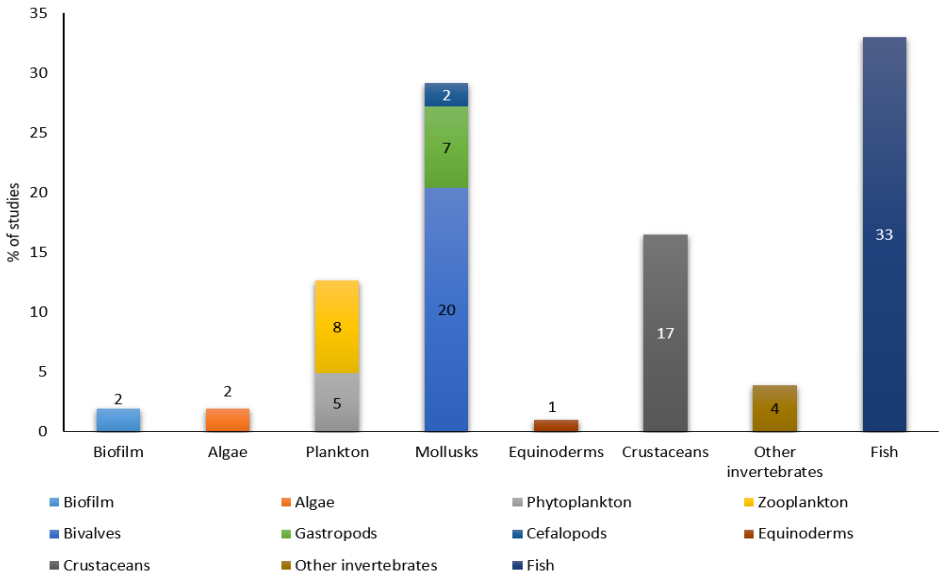

Figure 1. *Number of consulted studies according to the different taxonomic groups in aquatic environment*

2.1. Phytoplankton

Phytoplankton is the group of organisms that form part of the exclusively plant-based plankton. They underlie productivity in aquatic environments and are widely used as biomarkers. Among the different species, pigments and fatty acids are mainly used in the study of pollution [3]. Primary aquatic production is carried out by phytoplanktons, which absorb pollutants from the surrounding water and incorporate large quantities into their cellular compartments. In the case, for example, of arsenic, it has been shown that phytoplankton can excrete it after metabolization into the environment, transferring it to higher trophic levels [26–28]. Yan et al. [29] studied the bioaccumulation of antibiotics and analgesics in cyanobacteria as target organisms.

2.2. Zooplankton

Zooplankton is the fraction of exclusively animal organisms that are part of the plankton. They are very sensitive indicators of the ecological

state of an aquatic system since they are able to respond rapidly to environmental changes with modifications in their composition and structure [30]. Zooplankton has an ecologically important role in marine ecosystems being the primary consumer of the food chain. Furthermore, depending on their life stage and the availability of prey, their feeding behaviour varies, being able to combine the selection with chemoreceptors and mechanoreceptors [31]. The same authors mentioned in the previous section also investigated the bioaccumulation of PhACs in several zooplankton species including Daphnia magna, Cepopeda, Caldocera and Rotifers [27–29].

2.3. Benthos

Benthic macro-invertebrate organisms are those that are found interred in the sand, attached to rocks or those that walk on the bottom, such as clams and cockles, mussels or crabs. Mussels have been recognised as ideal sentinels for the assessment of aquatic pollution because they have a wide geographical distribution, are easy to collect and are filter-feeders that accumulate pollutants in their bodies [32]. In addition, they have a long life-cycle, which allows the study of the effects of pollution over a long period of time [33]. However, although these organisms have often been used as bioindicators of marine pollution, pharmaceutical bioaccumulation is poorly developed, and the presence of these compounds in benthic species differs between sampling sites. Some authors have proposed the used of caged organisms rather than in wild ones, as it varies between species and the distribution and abundance of these specimens' changes spatially and temporally [34]. In the literature, the most studied molluscs were bivalves, specifically mussels, but also oysters, clams, limpets and sea snails [35–37]. Other molluscs also studied have been gastropods (conch, snail) and cephalopods, such as octopus [38–40]. Oher benthos organisms such as crustaceans and echinoderms were studied for the determination on pharmaceuticals in aquatic environments, such as starfish as echinoderm [37] and barnacles, shrimp and crabs as crustaceans [27,38,41]. The most studied drugs include the antibiotic sulfamethoxazole, the analgesic naproxen, the

antiepileptic carbamazepine and the antidepressant venlafaxine [37, 42–44].

2.4. Fish

Fish are considered one of the most important bioindicators in both fresh and salt waters to estimate the level of pollution in the environment [3]. They have the ability to accumulate pollutants present in the surrounding environment in their fatty tissues [45]. Biomonitoring of these species is important due to human consumption, as they are a higher link in the food chain and, besides the inhalation exposure, the presence of contaminants in their bodies may be due to biomagnification (dietary exposure). Human exposure is the main reason to study the bioaccumulation of PhACs in different fish species as well as other biota across trophic levels [34]. Among the different fish species ussually used in bioaccumulation studies are carp [38], flatfish [43], salmon and rainbow trout [46] or mullet [47,48]. Regarding the PhACs studied, they belong to many families of drugs, including antibiotics such as quinolones, sulphonamides, and tetracyclines [39,40], analgesics such as naproxen, diclofenac, and acetaminophen [49–51] and other families such as antidepressants, β-blockers or antiepileptics [52,53].

3. Analytical Methodologies for the Determination of Pharmaceuticals in Biota Samples

The growing concern about the contamination of the environment has led to an increase in the number of publications focused on the detemination of PhACs in aquatic organisms in recent years. Table 1(a–i) summarizes the most relevant methods from the analytical point of view classified by taxonomic groups. Additionally, the graphs shown in Figure 2 represent the extraction techniques (a) and clean-up procedures (b) most commonly used for the sample treatment in the reviewed articles.

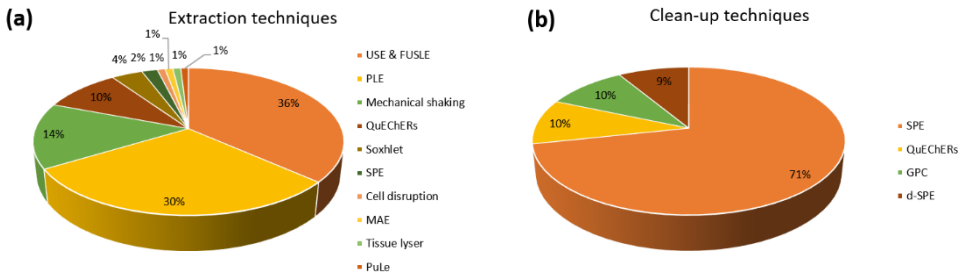

Figure 2. *Extraction techniques (a) and clean-up treatments (b) used in the reviewed publications*

Table 1. *(a) Analytical methods performance for PhACs concentration determination in biofilm. (b) Analytical methods performance for PhACs concentration determination in algae. (c) Analytical methods performance for PhACs concentration determination in plankton. (d) Analytical methods performance for PhACs concentration determination in molluscs. (e) Analytical methods performance for PhACs concentration determination in cephalopods. (f) Analytical methods performance for PhACs concentration determination in echinoderms. (g) Analytical methods performance for PhACs concentration determination in crustaceans. (h) Analytical methods performance for PhACs concentration determination in other invertebrates. (i) Analytical methods performance for PhACs concentration determination in fish*

(a)

Specie	Pharmaceuticals	Type and Amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref.
				Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	
No data	Diclofenac, ibuprofen, 1-OH-ibuprofen, piroxicam, diltiazem, propyphenazone, sulfamethoxazole, verapamil, norverapamil, hydrochlorothiazide, bezafibrate, gemfibrozil, pravastatin, carbamazepine, acridone, 10,11-epoxy-CBZ, 2-OH-CBZ, citalopram, fluoxetine, paroxetine, venlafaxine, azaperone, dexamethasone, metoprolol, propanolol	0.2 (d.w)	Freeze-dried, stored at −20 °C	PLE (citric buffer (pH 4)/ACN)	No data	UHPLC-MS/MS	No data	No data	[72]
Periphyton (No data)	Ethinylestradiol, acetaminophen, diclofenac	0.67 (d.w)	Air dry, powdered	USE (ACN/MeOH 1% acetic acid)	No data	HPLC-MS/MS	62	No data	[88]

(b)

Specie	Pharmaceuticals	Type and amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref
				Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	
Sea lettuce (*Ulva sp.*), Red algae (*Gelidium pristoides*), Hanginng wrack (*Bifurcaria brassicaeformis*), Strap caulerpa (*Caulerpa filiformis*), Slippery orbits (*Aeodes orbotisa*)	Phenytoin, lamivudine, acetaminophen, caffeine, sulfamethoxazole, diclofenac, carbamazepine	10 (d.w)	Rinsed, deshelled and dissected. Freeze-dried	Soxhlet (MeOH/Acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	96.1–100.5	0.62–1.05 ng L^{-1}	[37]
Water starwort (*Callitriche* sp.), Pondweed (*Potamogeton* sp.)	Ethinylestradiol, acetaminophen, diclofenac	0.5 (d.w)	Air dry, powdered	USE (ACN/MeOH 1% acetic acid)	No data	HPLC-MS/MS	81	No data	[88]

(c)

Phytoplankton

Specie	Pharmaceuticals	Type and Amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref
				Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	

No data	Roxithromycin, erythromycin, ofloxacin, norfloxaxin, ciprofloxacin, tetracycline, sulfamethoxazole, sulfadiazine, sulfaquinoxaline, ibuprofen, diclofenac, naproxen, bezafibrate, propranolol, ketoconazole, carbamazepine, caffeine, sertraline, fluoxetine, norfluoxetine, citalopram, paroxetine, venlafaxine, duloxetine, bupropion, amitriptyline, clozapine, fluvoxamine, quetiapine, aripiprazole, chlorpromazine	0.5 (d.w)	Freeze-dried, homogenized, stored at −80 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	66–128	0.07–1.67	[27]
No data	Sulfadiazine, suldapyridine, sulfacetamide, sulfamethazine, sulfamethoxazole, trimethoprim, norfloxacin, ciprofloxacin, ofloxacin, lomefloxacin, oxytetracycline, tetracycline, erythromycin, roxithromycin	0.1–0.5 (d.w)	Freeze-dried, stored at −18 °C	QuEChERs (ACN, acetic acid, 0.1 M EDTA, NaCl, Na_2SO_4)	d-SPE: QuEChERs (ACN, PSA, C18, Na_2SO_4)	LC-MS/MS	80.3–104.9	0.04–0.1	[28]
Cyanobacteria (*Microcystis aeruginosa*), Chlorophyceae (*Pediastrum* spp. *Crucigenia* spp. *Scenedesmus* spp.), Sensu lato (*Coscinodiscus* sspp., *Cyclotella* spp.), Diatoms (*Melosira* spp., *Aulacoseira* spp.), Dinophycaeae (*Peridiniopsis* spp.), Cryptophyceae (*Cryptomonas*), Chrysophyceae (*Dinobryon* spp.), Euglenoidea (*Euglena* spp.)	Sulfadiazine, sulfapyridine, sulfacetamide, sulfamethoxazole, sulfamethazine, trimethoprim, lomefloxacin, ciprofloxacin, norfloxacin, oxytetracycline, tetracycline, roxithromycin, dehydroerythromycin	0.5–1.0 (d.w)	Frozen at −80 °C, stored in a vacuum desiccator	PLE (MeOH/acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	No data	No data	[29]

Cyanobacteria (No data)	Sulfachlorpyridazine, sulfadiazine, sulfadoxine, sulfamerazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfisoxazole, sulfathiazole, trimethoprim, chlortetracycline, doxycycline, oxytetracycline, tetracycline, ciprofloxacin, difloxacin, danofloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, sarafloxacin, azithromycin, clarithromycin, leucomycin, oleandomycin, roxithromycin, tylosin, salinomycin, monensin, florfenicol, chloramphenicol	1 (d.w)	Washed (water), freeze-dried, stored at −20 °C	USE (MeOH, sodium acetate buffer pH 4)	SPE (SAX, HLB cartridges)	RRLC-MS/MS	54.2–117	0.02–9.38	[38]
Green algae (*Chlorophyta*), Diatoms (*Bacillariophyta*), Blue green algae (*Cyanophyta*)	Roxithromycin, erythromycin, ofloxacin, norfloxacin, ciprofloxacin, tetracycline, chloramphenicol, sulfamerazine and sulfadiazine, sulfamethoxazole, ibuprofen, diclofenac, naproxen and indomethacin, clofibric acid, gemfibrozil and bezafibrate, 17ß-estradiol, 17α-ethynylestradiol, propranolol, carbamazepine, ketoconazole, sertraline	0.25 (d.w)	Freeze-dried, ground, stored at −20 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	LC-MS/MS	68–116	0.01–1.12	[66]

Zooplankton

Specie	Pharmaceuticals	Type and Amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref
				Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	
Water flea (*Daphnia magna*)	Roxithromycin, propanolol	Each sample point consisted by 10 daphnia individuals	Homogenized	Sonication (ACN)	SPE (HLB cartridges)	UHPLC-MS/MS	83–106	0.2	[89]
Water flea (*Daphnia magna*)	Tetracycline	30 organisms	Homogenized	MeOH, formic acid, EDTA	No data	LC-MS/MS	84.23	0.31μg L^{-1}	[90]

No data	Sulfachlorpyridazine, sulfadiazine, sulfadoxine, sulfamerazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfisoxazole, sulfathiazole, trimethoprim, chlortetracycline, doxycycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, sarafloxacin, azithromycin, clarithromycin, leucomycin, oleandomycin, roxithromycin, tylosin, salinomycin, monensin, florfenicol, chloramphenicol	1 (d.w)	Washed (water), freeze-dried, stored at −20 °C	USE (MeOH, sodium acetate buffer pH 4)	SPE (SAX, HLB cartridges)	RRLC-MS/MS	54.2–117	0.02–9.38	[38]
No data	Roxithromycin, erythromycin, ofloxacin, norfloxaxin, ciprofloxacin, tetracycline, sulfamethoxazole, sulfadiazine, sulfaquinoxaline, ibuprofen, diclofenac, naproxen, bezafibrate, propranolol, ketoconazole, carbamazepine, caffeine, fluoxetine, norfluoxetine, citalopram, paroxetine, sertraline, venlafaxine, duloxetine, bupropion, amitriptyline, fluvoxamine, clozapine, quetiapine, aripiprazole, chlorpromazine	0.5 (d.w)	Freeze-dried, homogenized, stored at −80 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	66–128	0.07–1.67	[27]
Copepoda, Cladocera, Rotifera (No data)	Sulfadiazine, suldapyridine, sulfacetamide, sulfamethazine, sulfamethoxazole, trimethoprim, norfloxacin, ciprofloxacin, ofloxacin, lomefloxacin, oxytetracycline, tetracycline, erythromycin, roxithromycin	0.1–0.5 (d.w)	Freeze-dried, stored at −18 °C	QuEChERs (ACN, acetic acid, 0.1 M EDTA, NaCl, Na_2SO_4)	d-SPE: QuEChERs (ACN, PSA, C18, Na_2SO_4)	LC-MS/MS	81.1–100.7	0.01–0.12	[28]
Copepoda, Cladocera, Rotifera (No data)	Roxithromycin, erythromycin, ofloxacin, norfloxacin, ciprofloxacin, tetracycline, chloramphenicol, sulfamerazine, sulfadiazine, sulfamethoxazole, ibuprofen, ketoconazole, diclofenac, naproxen, indomethacin, clofibric acid, gemfibrozil, bezafibrate, 17β-estradiol, sertraline, propranolol, 17α-ethynylestradiol, carbamazepine	0.25 g (d.w)	Freeze-dried, ground, stored at −20 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	LC-MS/MS	68–116	0.01–1.12	[39]
Copepoda, Cladocera, Rotifera (No data)	Sulfadiazine, sulfapyridine, sulfacetamide, sulfamethoxazole, sulfamethazine, trimethoprim, lomefloxacin, ciprofloxacin, norfloxacin, oxytetracycline, tetracycline, dehydroerythromycin, roxithromycin	0.5–1.0 (d.w)	Frozen at −80 °C, stored in a vacuum desiccator	PLE (MeOH/acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	No data	No data	[29]
No data	Nicotine, haloperidol, pyremethamine	0.14–0.2 (d.w)	Freeze-dried	USE (ACN, MeOH, H_2O), vortex, USE	SPE (No data)	LC-HRMS/MS	70–130	0.05–5.70 *	[91]
Green algae (*Chlorophyta*), Diatoms (*Bacillariophyta*), Blue green algae (*Cyanophyta*)	Roxithromycin, erythromycin, ofloxacin, norfloxacin, ciprofloxacin, tetracycline, chloramphenicol, ibuprofen, diclofenac, naproxen, indomethacin, clofibric acid, sulfamerazine, sulfadiazine, sulfamethoxazole, gemfibrozil, bezafibrate, propranolol, carbamazepine, sertraline, ketoconazole, 17β-estradiol, 17α-ethynylestradiol	0.25 (d.w)	Freeze-dried, ground, stored at −20 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	LC-MS/MS	68–116	0.01–1.12	[66]
(d)									
Bivalves									
Specie	Pharmaceuticals	Type and	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref

		Amount of Sample (g)		Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	
Oysters (*C. Gigas*), Clams (*C. gallina*), Mussels (*M. galloprovincialis*)	Ronidazole, metronidazole, dimetridazole, sulfamethoxazole, N-acetyl-sulfamethoxazole, azithromycin, erythromycin, venlafaxine, O-desmethylvenlafaxine, carbamazepine, 10,11-Epoxycarbamazepine, citalopram,2-Hydroxycarbamazepine, alprazolam, codeine, phenazone, propyphenazone, piroxicam, azaperone, azaperol, diltiazem, hydrochlorthiazide, tamsulosin	0.5 (d.w)	Shells removed, pooled for homogenizing, freeze-dried, ground and kept at −20 °C	PLE (MeOH/H_2O)	SPE (HLB cartridges)	UHPLC-MS/MS	40–115	0.01–0.80	[35]
Zebra mussels (*Dreissena polymorpha*)	Diclofenac	0.1 (d.w)	Freeze-dried and grinded	QuEChERs (H_2O, ACN, heptane, acetate salt, DMSO)	d-SPE: QuEChERs (acetate salt)	UHPLC-MS/MS	73–117	0.02–1	[64]
Mussels (*Perna viridis*), Oysters (*Cassostrea hongkongensis*)	Acetaminophen, amitrimtyline, aripiprazole, benzoylecgonine, buprenorphine, caffeine, carbamazepine, diclofenac, diltiazem, diphenydramine, fluoxetine, methylphenidate, norfluoxetine, promethazine, sertraline, amlodipine, desmethylsertraline, trimethoprim, erythromycin, sucralose, sulfamethoxazole	1 (w.w)	Separated from their shells, homogenized and frozen at −20 °C	Mechanical shaking (0.1 M acetic acid, MeOH)	No data	LC-MS/MS	80–120	0.01–0.75	[36]
Mussel (*Mytilus galloprovincialis*)	Cocaine, benzoylecgonine, cocathylene, amphetamine, metamphetamine, MDMA, morphine, methadone, 6-monoacetylmorphine, EDDP, ketamine, lysergic acid diethylamide, A tetrahydrocannabinol, 11-hydroxy-A THC, 11-nor-9-carboxy-A THC, AH-7921, mephedrone, MDPV, caffeine, ephedrine, alprazolam, a-hydroxyalprazolam, midazolam, lormetazepam, a-hydroxymidazolam, diazepam, oxazepam, temazepam, citalopram, fluoxetine, sertraline, venlafaxine, zolpidem, chlorpromazine, hydroxyzine	10 (w.w)	Homogenized	Manual shaking (ACN, $MgSO_4$, NaCl, NaCitrate, DCS)	d-SPE: QuEChERs (PSA, C18, MgSO4)	LC-MS/MS	77–118	<2	[78]
Mussel (*Mytilus spp*)	Diclofenac, mefenamic acid, trimethoprim, carbamazepine, gemfibrozil	1 (d.w)	Freeze-dried, ground	PLE (ACN/H_2O), Al_2O_3	SPE (Strata-X SPE cartridges)	LC-MS/MS	83–94	4–29 *	[65]

Mussel (*Mytilus galloprovincialis*)	Carbamazepine, oxcarbazepine + non target compounds (caffeine, metoprolol, cotinine, ketoprofen)	2 (d.w)	Freeze-dried	QuEChERs (ACN, Na_2SO_4, NaCl, $Na_3Cit{\cdot}2H_2O$, $Na_2HCit{\cdot}3H_2O$)	d-SPE: QuEChERs (Na_2SO_4, PSA, C18, formic acid)	LC-HRMS	67–110	0.1–0.3	[92]
Mussel (*Mytilus galloprovincialis*)	Diclofenac, diazepam, sotalol, carbamazepine, citalopram, venlafaxine, azithromycin, sulfamethoxazole	All edible meat (no data)	Pooled, homogenized, freeze-dried, kept at −20 °C	PLE (MeOH/ H_2O)	SPE (HLB cartridges)	UHPLC-MS/MS	No data	0.01–0.65	[55]
Mussel (*Mytilus galloprovincialis*), Razor shell (*Ensis siliqua*), Cockle (*cerastoderma edule*)	Atenolol, metoprolol, nadolol, propanolol, sotalol, salbutamol, diazepam, carbamazepine, azaperol, azaperone, 10,11-epoxycarbamazepine, 2-OH-carbamazepine, citalopram, venlafaxine, alprazolam, chlorothiazide, codeine, phenazone, piroxicam, propyphenazone, ronidazole, dimetridazole, metronidazole, azithromycin, erythromycin	0.5 (d.w)	Freeze-dried	PLE (MeOH/ H_2O)	SPE (HLB cartridges)	UHPLC-MS/MS	No data	0.01–2	[40]
Mussel (*Mytilus galloprovincialis*)	Trimethoprim, ciprofloxacin, norfloxacin, sulfadiazine, sulfamethoxazole, amitriptyline, clomipramine, imipramine, nortriptyline, eprosartan, irbesartan, losartan, diclofenac, telmisartan, valsartan, propanolol, acetaminophen, ketoprofen, bezafibrate, clofibric acid, carbamazepine, phenytoin	0.5 (d.w)	Freeze-dried, ground, homogenized	FUSLE (MeOH/ H_2O)	SPE (HLB cartridges)	LC-MS/MS	71–126	4–48	[70]
Carib pointed-venus (*Anomalocardia brasiliana*), Blue Mussel (*Mytilus edulis*)	Bezafibrate, carbamazepine, chloramphenicol, diclofenac, 4′-Hydroxydiclofenac, furosemide, gemfibrozil, ibuprofen, indapamide, ketoprofen, naproxen, simvastatin	0.5 (d.w)	Dissection to obtain the morphometric measures, freeze-dried	QuEChERs (ACN, formic acid, NH_4Cl)	QuEChERs ($MgSO_4$, Z-Sep)	HPLC-MS/MS	77–126	0.002–1.09	[47]
Limpets (*Cymbula granatina and cymbula oculis*), Sea snail (*Oxystele sinensis and oxytele tigrina*), Mussel (*mytilus galloprovincialis*)	Phenytoin, lamivudine, acetaminophen, caffeine, sulfamethoxazole, diclofenac, carbamazepine	10 (d.w)	Rinsed, deshelled and dissected, freeze-dried	Soxhlet (MeOH/ Acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	96.1–100.5	0.62–1.05 ng L^{-1}	[37]
Oyster (*Ostrea gigas*), Scallop (*Mimachlamys nobilis*), Mussel (*Mytilus edulis*)	Sulfadiazine, sulfamerazine, sulfamethazine, trimethoprim, sulfamethoxazole, sulfathiazole, sulfapyridine, ciprofloxacin, norfloxacin, ofloxacin, tetracycline, flumequine, oxytetracycline, gemfibrozil isochlortetracycline, penicillin G sodium, cefotaxime, spectinomycin, roxithromycin, erythromycin, clarithromycin, thiamphenicol, chloramphenicol, paracetamol, naproxen, ibuprofen, ketoprofen, diclofenac acid, carbamazepine, diltiazem, diphenhydramine	0.2 (d.w)	Freeze-dried, ground into powder, mixed	Sonication (ACN/H_2O)	SPE (HLB cartridges)	UHPLC-MS/MS	43–127	0.01–1.9	[39]

Zebra mussels (*Dreissena polymorpha*)	Nicotine, haloperidol, pyremethamine	0.14–0.2 (d.w)	Gut clearance, frozen, shelled, cryo-storage	USE (ACN, MeOH, H_2O), vortex, USE	SPE (No data)	LC-HRMS/MS	70–130	0.05–5.70 *	[91]
Mussels (*Mytilus galloprovincialis, Mytilus edulis*)	Salicylic acid, clofibric acid, ketoprofen, naproxen, bezafibrate, diclofenac, ibuprofen	1 (d.w)	Lyophilized, homogenized	PLE (Ottawa sand, ultrapure water)	SPE (Oasis MAX cartridges)	LC-MS/MS	61–90	2–50	[93]
Clams (*Ruditapes decussatus, ruditapes philippinarum*)	Acetaminophen, clofibric acid, atenolol, bezafibrate, carbamazepine, cortisone, diclofenac, erythromycin, fluoxetine, ibuprofen, naproxen, propanolol, sulfadiazine, sulfapyridine, caffeine, sulfamethoxazole, testosterone, gestodene, metoprolol, diethylsilbestrol, estradiol, estriol, estrone, 17α-ethinylestradiol	1 (w.w)	Depurated, frozen at −20 °C, homogenized before analysis	Manual shaking (ACN)	QuEChERs (Hexane)	LC-MS/MS	35.2–118	0.35–5.86	[94]
Mussel (*Anodonta*), Snail (*Bellamya sp.*), Bivalve (*Corbiculidae*)	Roxithromycin, erythromycin, ofloxacin, norfloxacin, ciprofloxacin, tetracycline, chloramphenicol, sulfamerazine and sulfadiazine, sulfamethoxazole, ibuprofen, diclofenac, naproxen and indomethacin, clofibric acid, gemfibrozil and bezafibrate, 17β-estradiol and 17α-ethynylestradiol, propranolol, carbamazepine, ketoconazole, sertraline	0.5 (d.w)	Freeze-dried, ground, stored at −20 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	LC-MS/MS	68–116	0.01–1.12	[66]
Asian clam (*Corbicula fluminea*)	Sulfachlorpyridazine, sulfadiazine, sulfadoxine, sulfamerazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfisoxazole, sulfathiazole, trimethoprim, chlortetracycline, doxycycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, sarafloxacin, azithromycin, clarithromycin, leucomycin, oleandomycin, roxithromycin, tylosin, salinomycin, monensin, florfenicol, chloramphenicol	1 (d.w)	Washed (water), homogenized, freeze-dried, stored at −20 °C	USE (AcONa buffer/MeOH)	SPE (SAX/PSA-HLB tandem cartridges)	RRLC-MS/MS	47.9–136.7	0.01–1.99	[38]
Mussel (*Anodonta woodiana*)	Roxithromycin, erythromycin, ofloxacin, norfloxaxin, ciprofloxacin, tetracycline, sulfamethoxazole, sulfadiazine, sulfaquinoxaline, ibuprofen, diclofenac, naproxen, bezafibrate, propranolol, ketoconazole, carbamazepine, caffeine, fluoxetine, norfluoxetine, citalopram, paroxetine, sertraline, venlafaxine, duloxetine, bupropion, amitriptyline, fluvoxamine, trihexylphenidyl, clozapine, quetiapine, aripiprazole, chlorpromazine	0.5 (d.w)	Freeze-dried, homogenized, stored at −80 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	66–128	0.07–1.67	[27]
Clam (*Ruditapes decussatus*), Cockle (*Cerastodema glaucum*), Noble pen shell (*Pinna nobilis*), Sea snail (*Murex trunculus*)	Diclofenac, codeine, carbamazepine, citalopram, diazepam, lorazepam, atenolol, sotalol, propanolol, nadolol, carazolol, hydrochlorothiazide, clopidogrel, salbutamol, levamisole	1 (d.w)	Freeze-dried, milled	PLE (50 °C)	GPC, HPLC-DAD	UHPLC-MS/MS	<20–151.9	0.0004–6	[48]

Pen shell (*Atrina pectinate Linnaeus*), Asian hard clam (*Meretrix lusoria*), Magallana rivularis (*Crassostrea rivvularis Gould*).	Sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfameter, sulfamethazine, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, trimethoprim, chlortetracycline, doxycycline, methacycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, clarythromycin, erythromycin-H_2O, leucomycin, roxithromycin, oleandomycin	2 muscle (w.w)	Frozen, muscle dissected	USE (MeOH/H_2O 0.1 mol L^{-1} acetic acid)	SPE Cartridges (SAX/PSA and HLB cartridges)	LC-MS/MS	50–150	0.05–9.06	[42]
Clam (*Anadara ferruginea*)	Atenolol, metoprolol, venlafaxine, chloramphenicol	2 (d.w)	Washed (water), dissected, homogenized, freeze-dried, stored at −50 °C	USE (MeOH/H_2O)	SPE (MCX cartridges)	LC-MS/MS	68–96	0.05–0.25	[44]
Gastropods									
Specie	**Pharmaceuticals**	**Type and Amount of Sample (g)**	**Pre-Treatment**	**Treatment**		**Analysis**	**Analytical Features**		**Ref**
				Extraction Technique	**Clean-Up**		**Recovery (%)**	**LOD (ng g^{-1})**	
Snail (*Bellamya aeruginosa*)	Sulfachlorpyridazine, sulfadiazine, sulfadoxine, sulfamerazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfapyridine, sulfamonomethoxine, sulfaquinoxaline, sulfisoxazole, sulfathiazole, trimethoprim, chlortetracycline, doxycycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, sarafloxacin, azithromycin, leucomycin, clarithromycin, oleandomycin, roxithromycin, tylosin, salinomycin, monensin,florfenicol, chloramphenicol	1 soft tissues (d.w)	Washed (water), homogenized, freeze-dried, stored at −20 °C	USE (AcONa buffer/MeOH)	SPE (SAX/PSA–HLB tandem cartridges)	RRLC-MS/MS	47.9–136.7	0.01–1.99	[38]
Snail (*Bellamya aeruginosa*)	Roxithromycin, erythromycin, ofloxacin, norfloxaxin, ciprofloxacin, tetracycline, sulfamethoxazole, sulfadiazine, sulfaquinoxaline, ibuprofen, diclofenac, naproxen, bezafibrate, propranolol, ketoconazole, carbamazepine, caffeine, fluoxetine, norfluoxetine, citalopram, paroxetine, sertraline, venlafaxine, duloxetine, bupropion, amitriptyline, clozapine, fluvoxamine, trihexylphenidyl, quetiapine, aripiprazole, chlorpromazine	0.5 (d.w)	Freeze-dried, homogenized, stored at −80 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	66–128	0.07–1.67	[27]

Conch (*Bufonaria perelegans*)	Sulfadiazine, sulfamerazine, sulfamethazine, trimethoprim, sulfamethoxazole, sulfathiazole, sulfapyridine, ciprofloxacin, norfloxacin, ofloxacin, flumequine, tetracycline, oxytetracycline, isochlortetracycline, penicillin G sodium, cefotaxime sodium, spectinomycin, roxithromycin, erythromycin- H_2O, clarithromycin, thiamphenicol, chloramphenicol, paracetamol, naproxen, ibuprofen, ketoprofen, diclofenac acid, carbamazepine, diltiazem, diphenhydramine, gemfibrozil	0.2 (d.w)	Freeze-dried, ground into powder. The whole body was mixed	USE (ACN/H_2O)	SPE (PRiME HLB cartridges)	UHPLC-MS/MS	43–127	0.01–1.9	[39]
Sea snail (*Murex trunculus*)	Diclofenac, codeine, carbamazepine, citalopram, diazepam, lorazepam, atenolol, sotalol, propanolol, nadolol, carazolol, hydrochlorothiazide, clopidogrel, salbutamol, levamisole	1 (d.w)	Freeze-dried and milled	PLE (MeOH)	GPC, HPLC-DAD	UHPLC-MS/MS	<20–151.9	0.0004–6	[48]
Snail (*B. tentaculata*)	Ethinylestradiol, acetaminophen, diclofenac	0.35 (d.w)	Freeze-dried, powered	USE (ACN/MeOH 1% acetic acid)	No data	HPLC-MS/MS	67	No data	[88]
River limpet (*Ancylus fluviatilis*)	Diclofenac, ibuprofen, 1-OH-ibuprofen, piroxicam, acridone, propyphenazone, sulfamethoxazole, diltiazem, verapamil, norverapamil, hydrochlorothiazide, bezafibrate, gemfibrozil, pravastatin, carbamazepine, 10,11-epoxy-CBZ, 2-OH-CBZ, citalopram, fluoxetine, paroxetine, venlafaxine, azaperone, dexamethasone, metoprolol, propanolol	0.1 (d.w)	Homogenized with a mortar, kept at 20 °C	USE (MeOH)	Protein Precipitation and Phospholipid Removal, PlateOSTRO™ plate	UHPLC-MS/MS	No data	No data	[72]
Turritella bacillum Murex trapa, Bufonaria rana (No data)	Atenolol, metoprolol, venlafaxine, chloramphenicol	2 (d.w)	Washed (water), dissected, homogenized, freeze-dried, stored at −50 °C	USE (MeOH/H_2O)	SPE (MCX cartridges)	LC-MS/MS	68–96	0.05–0.25	[44]

(e)

Specie	Pharmaceuticals	Type and Amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref
				Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	

Octopus (*Octopus vulgaris*)	Atenolol, metoprolol, nadolol, propanolol, sotalol, salbutamol, diazepam, carbamazepine, 10,11-epoxycarbamazepine, 2-OH-carbamazepine, citalopram, venlafaxine, alprazolam, azaperol, azaperone, hydrochlorothiazide, codeine, phenazone, propyphenazone, piroxicam, ronidazole, dimetridazole, metronidazole, azithromycin, erythromycin	1 (d.w)	Freeze-dried	PLE (MeOH/H_2O)	GPC	UHPLC-MS/MS	No data	0.02–0.3	[40]
Sepia (*Sepia indica*), Octopus (*Octopus rugosus*), Octopus minor (*Polypus variabilis*), Urotheutis (*Loligo oshimai*)	Sulfamethazine, sulfapyridine, sulfathiazole, sulfanlamide, sulfadiazine, sulfadimethoxine, sulfamonomethoxin, sulfamerazine, sulfamethoxazole, norfloxacin, enoxacin, ofloxacin, ciprofloxacin, enrofloxacin, dehydrated erythromycin, clarithromycin, azithromycin, roxithromycin, florfenicol, chloramphenicol, trimethoprim, lincomycin	5 (d.w)	Washed (water), dissected, homogenized, stored at −20 °C	USE (ACN, citric acid)	SPE (SAX-HLB cartridges)	UHPLC-MS/MS	47.7–172.7	0.04–0.24	[13]

(f)

Specie	Pharmaceuticals	Type and Amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref
				Extraction Technique	Clean-up		Recovery (%)	LOD (ng g^{-1})	
Starfish (*Marthasterias glacialis*), Sea urchins (*parechinus angulosus*)	Phenytoin, lamivudine, acetaminophen, caffeine, sulfamethoxazole, diclofenac, carbamazepine	10 (d.w)	Rinsed, deshelled, dissected, freeze-dried	Soxhlet (MeOH/Acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	96.1–100.5	0.62–1.05 ng L^{-1}	[37]

(g)

Specie	Pharmaceuticals	Type and Amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref
				Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	
Barnacle (*Balanus perforatus*)	Atenolol, ranitidine, acetaminophen, caffeine, trimethoprim, atrazine, amitriptyline, carbamazepine, chloropheniramine malate, ciprofloxacin, diclofenac, fluoxetine, ibuprofen, metronidazole, sulfamethoxazole, warfarin, cephalexin.	1 (d.w)	Dried, ground, pooled, homogenized, freeze-dried	USE (0.1 M acetic acid, MeOH)	SPE (Oasis MCX)	HPLC-MS/MS	30–103	0.1–13 ng mL^{-1}	[52]
Shrimp (*Caridea*), Brown crab (*Cancer pagurus*)	Diclofenac, diazepam, sotalol, carbamazepine, citalopram, venlafaxine, azithromycin, sulfamethoxazole	All edible meat (no data)	Pooled, homogenized by grinding, freeze-dried, −20 °C	PLE (MeOH/H_2O)	SPE (HLB cartridges)	UHPLC-MS/MS	No data	0.01–0.65	[55]

Crabs (*Calappa philargius)*, pen shell *Atrina pectinate Linnaeus*), shrimps (*Fenneropenaeus penicillatus*)	Sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfameter, sulfamethazine, sulfamethoxazole, sulfapyridine, sulfamonomethoxine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, trimethoprim, chlortetracycline, doxycycline, methacycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, clarythromycin, erythromycin-H_2O, leucomycin, roxithromycin, oleandomycin	2 muscle (w.w)	Frozen and muscle dissected	USE (MeOH/H_2O 0.1 mol L^{-1} acetic acid	SPE (SAX/PSA, HLB cartridges)	LC-MS/MS	50–150	0.05–9.06	[42]
No data	Ketoprofen, naproxen, flurbiprofen, diclofenac sodium, ibuprofen	5 muscle tissue (w.w)	Chopped into mince	USE (ACN)	SPE (CF@UiO−66 NH_2)	UHPLC-PDA	95–116.99	0.12–3.50 ng mL^{-1}	[63]
Goose Barnacle (*Pollicipes,*) Carb (*Necora púber*)	Atenolol, metoprolol, nadolol, propanolol, sotalol, salbutamol, diazepam, carbamazepine, 10,11-epoxycarbamazepine, 2-OH-carbamazepine, citalopram, venlafaxine, alprazolam, azaperone, azaperol, hydrochlorothiazide, codeine, phenazone, propyphenazone, piroxicam, ronidazole, dimetridazole, metronidazole, azithromycin, erythromycin	1 (d.w)	Freeze-dried	PLE (MeOH)	GPC	UHPLC-MS/MS	No data	0.03–0.09	[40]
Shrimp (*Palaemon serratus*)	Metronidazole, acetaminophen, amoxicillin, acetazolamide, sulfadiazine, atenolol, caffeine, ampicillin, trimethoprim, norfloxacin, ofloxacin, ciprofloxacin, tetracycline, phenazone, metoprolol, spiramycin, azithromycin, sulfamethoxazole, oxolinic acid, erythromycin A, piperacillin, tylosine, cyclophosphamide, carbamazepine, flumequine, oxazepam, clarithromycin, roxithromycin, lorazepam, losartan, nordiazepam, josamycin, ketoprofen, 19-norethind-rone, amiodarone, hydrochlorothiazide, acetylsalicylic acid, niflumic acid, diclofenac, ibuprofen, gemfibrozil	0.2 (d.w)	Separated abdomen muscle, freeze-dried	Mechanical shaking (MeOH 1% acetic acid)	No data	UHPLC-MS/MS	26–132	0.1–40.2 *	[61]
Freshwater amphipod (*Gammarus pulex*)	Propanolol hydrochloride, ketoprofen, diclofenac salt, bezafibrate, warfarin, flurbiprofen, indomethacin, ibuprofen sodium salt, meclofenamic acid sodium salt, gemfibrozil, atenolol, sulfamethoxazole, sulfamethazine, furosemide, carbamazepone, nimesulide, (+-metoprolol) (+) tartrate, cimetidine, ranitidine, antipyrin, temazepam, diazepam, fluoxetine, nifedipine, mefenamic acid, trimethoprim, caffeine, naproxen	0.1 (d.w)	Freeze-dried, pulverized	PuLE (ACN)	SPE (HLB cartridges)	LC-MS/MS	41–89	1–13	[84]

Green crab (*Carcinus maenas*)	Alprazolan, amoxicillin, atenolol, atorvastatin, azithromycin, bisoprolol, benzylpenicillin, bezafibrate, carbamazepine, carvedilol, cinoxacin, ciprofloxacin, ceftiofur, cephalexin, chlortetracycline, danofloxacin, diclofenac, doxicycline, enoxacin, enrofloxacin, epi-chlortetracycline, epi-tetracycline, erythromycin, epotetracycline, fenofibrate, flumequine, fluoxetine, furosemide, gabapentin, gemfibrozil, ibersartan, ibuprofen, indapamide, lorazepam, losartan, marbofloxacin, nalidixic acid, norfloxacin, nimesulide, ofloxacin, oxolinic acid, oxytetracycline, paracetamol, propanolol, sertraline, simvastatin, spiramycin, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfanilamide, sulfapyridine, sulfisomidine, sulfadoxine, sulfamethoxazole, sulfaquinoxaline, sulfathiazole, sulfisoxazole, tetracycline, tilmicosin, trimethoprim, venlafaxine, topiramate	2 (w.w)	Homogenized	Mechanical shaking (ACN, EDTA)	No data	UHPLC-MS/MS	79.2–109.5	0.59–4.11	[41]
Shrimps: White vannamei prawn, Indian prawn, kiddi shrimp (No data)	Amoxicillin, azithromycin, caffeine, carbamazepine, ciprofloxacin, clarithromycin, diclofenac, erythromycin, furosemide, ketoprofen, ibuprofen, naproxen, sulfamethoxazole, tetracycline	2 (w.w)	Abdomen muscle separated, cut into small parts, frozen at −20 °C	Mechanical shaking (ACN, 0.1 M EDTA, hexane)	No data	UHPLC-MS/MS	81.2–99.4	0.017–1.371	[46]
Shrimp (*Harpiosquilla harpax*), Crab (*Charybdis japonica*), Spear shrimp (*Parapenaeopsis hardwickii*), Giant tiger prawn (*Penaeus monodon*), Green mud crab (*Scylla paramamosain*), Prawn (*Trachypenaeus sedili*)	Sulfamethazine, sulfapyridine, sulfathiazole, sulfanilamide, sulfadiazine, sulfadimethoxine, sulfamonomethoxin, sulfamerazine, sulfamethoxazole, norfloxacin, enoxacin, ofloxacin, ciprofloxacin, enrofloxacin, dehydrated, erythromycin, clarithromycin, azithromycin, roxithromycin, florfenicol, chloramphenicol, trimethoprim, lincomycin	5 (d.w)	Washed (water), dissected, homogenized, stored at −20 °C	USE (ACN, citric acid)	SPE (SAX-HLB cartridges)	UHPLC-MS/MS	47.67–172.67	0.04–0.24	[13]
Mud prawun (*Meapenaeus ensis*), Smoothshell shrimp (*Parapenaeopsis tenella*), Three-spot swimming crab (*Portunus sanguinolentus*), Jinga shrimp (*Metapenaeus affinis*), Robber harpiosquillid mantis shrimp (*Harpiosquilla harpax*)	Atenolol, metoprolol, venlafaxine, chloramphenicol	2 (d.w)	Washed (water), dissected, homogenized, freeze-dried, stored at −50 °C	USE (MeOH/H_2O)	SPE (MCX cartridges)	LC-MS/MS	68–96	0.05–0.25	[44]

White shrimp (*Exopalaemon modestus*) Taihu shrimp (*Macrobranchium nipponense*)	Roxithromycin, erythromycin, ofloxacin, norfloxacin, ciprofloxacin, tetracycline, chloramphenicol, sulfamerazine, sulfadiazine, sulfamethoxazole, ibuprofen, diclofenac, naproxen and indomethacin, clofibric acid, gemfibrozil, bezafibrate, 17β-estradiol,17α-ethynylestradiol, propranolol, carbamazepine, ketoconazole, sertraline	0.5 (d.w)	Separated muscle of shrimp. Freeze-dried, ground and stored at −20 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	LC-MS/MS	68–116	0.01–1.12	[66]
Water flea (*Gammarus pulex*)	Ethinylestradiol, acetaminophen, diclofenac	0.34 (d.w)	Freeze-dry, powered	USE (ACN/MeOH 1% acetic acid)	No data	HPLC-MS/MS	67	No data	[88]
Shrimps (*Paranthura sp.*, *Macrobrachium nipponense*), Crab (*Eriocheir sinensis*)	Sulfachlorpyridazine, sulfadiazine, sulfadoxine, sulfamerazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfisoxazole, sulfathiazole, trimethoprim, chlortetracycline, doxycycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, sarafloxacin, azithromycin, clarithromycin, leucomycin, oleandomycin, roxithromycin, tylosin, salinomycin, monensin, florfenicol, chloramphenicol	1 (d.w)	Washed (water), homogenized, freeze-dried, stored at −20 °C	USE (sodium acetate buffer/MeOH)	SPE (SAX/PSA–HLB tandem cartridges)	RRLC-MS/MS	47.9–136.7	0.01–1.99	[38]
Shrimp (*Macrobranchium nipponense*)	Roxithromycin, erythromycin, ofloxacin, norfloxaxin, ciprofloxacin, tetracycline, sulfadiazine, sulfamethoxazole, sulfaquinoxaline, naproxen, ibuprofen, diclofenac, bezafibrate, propranolol, ketoconazole, carbamazepine, caffeine, fluoxetine, norfluoxetine, citalopram, paroxetine, sertraline, venlafaxine, duloxetine, bupropion, amitriptyline, fluvoxamine, trihexylphenidyl, clozapine, quetiapine, aripiprazole, chlorpromazine	0.5 (d.w)	Freeze-dried, homogenized, stored at −80 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	66–128	0.07–1.67	[27]
Shrimps (No data)	Naproxen, methyltestosterone, 17α-hydroxyprogesterone caproate, progesterone	2 (w.w)	Ground, homogenized	Manual shaking (ACN 0.1% acetic acid)	No data	LC-MS/MS	68–117	1–2	[43]

Crabs: Spectacled box crab (*Calappa philargius*). Shrimps: Redtail shrimpredtail prawn (*Fenneropenaeus penicillatus*)	Sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfameter, sulfamethazine, sulfamethoxazole, sulfapyridine, sulfamonomethoxine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, trimethoprim, chlortetracycline, doxycycline, methacycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, clarythromycin, erythromycin- H_2O, leucomycin, roxithromycin, oleandomycin	2 (w.w)	Frozen and muscle dissected	USE (MeOH/ H_2O, 0.1 M acetic acid)	SPE Cartridges (SAX/PSA, and HLB cartridges)	LC-MS/MS	50–150	0.05–9.06	[42]

(h)

Specie	Pharmaceuticals	Type and Amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref.
				Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	
Ragworm (*Hedryste diversicolor*)	Alprazolan, amoxicillin, atenolol, atorvastatin, azithromycin, bisoprolol, benzylpenicillin, bezafibrate, carbamazepine, carvedilol, cinoxacin, ciprofloxacin, ceftiofur, cephalexin, chlortetracycline, danofloxacin, diclofenac, doxicycline, enoxacin, enrofloxacin, fluoxetine, epi-chlortetracycline, epi-tetracycline, erythromycin, epotetracycline, fenofibrate, flumequine, furosemide, gabapentin, gemfibrozil, ibersartan, ibuprofen, indapamide, lorazepam, losartan, marbofloxacin, nalidixic acid, norfloxacin, nimesulide, ofloxacin, oxolinic acid, oxytetracycline, paracetamol, propanolol, sertraline, simvastatin, spiramycin, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfanilamide, sulfapyridine, sulfisomidine, sulfadoxine, sulfamethoxazole, sulfaquinoxaline, sulfathiazole, sulfisoxazole, tetracline,tilmicosin, trimethoprim, tylosin venlafaxine, topiramate	2.0 (w.w)	Homogenized	Mechanical shaking (ACN, EDTA)	No data	UHPLC-MS/MS	79.2–109.5	0.59–4.11	[41]
Polychaetas (*Perinereis aibuhitensis*, *Notomastus latericeus*, *Sabella pavonina*). Insecta (*Chironomidae sp.*). Worm (*Limnodrilus hoffmeisteri*)	Sulfachlorpyridazine, sulfadiazine, sulfadoxine, sulfamerazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfisoxazole, sulfathiazole, trimethoprim, chlortetracycline, doxycycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, sarafloxacin, azithromycin, clarithromycin, leucomycin, oleandomycin, roxithromycin, tylosin, salinomycin, monensin, florfenicol, chloramphenicol	1.0 (d.w)	Washed (water), homogenized, freeze-dried, stored at −20 °C	USE (sodium acetate buffer/ MeOH)	SPE (SAX/PSA–HLB tandem cartridges)	RRLC-MS/MS	47.9–136.7	0.01–1.99	[38]

Porifera: Sponge (*Cf. Hyrtios*)	Caffeine, fluoxetine, norfluoxetine	0.25 (d.w)	Squeezed, wrapped in aluminium foil, and freeze-dried	USE (acidified methanol, acetonitrile/ methanol, acetonitrile)	SPE (HLB)	UHPLC-MS	80	0.01–10	[85]
Insecta (*Hydropsyche sp., Phagocata vitta*)	Diclofenac, ibuprofen, 1-OH-ibuprofen, piroxicam, propyphenazone, sulfamethoxazole, diltiazem, verapamil, norverapamil, hydrochlorothiazide, bezafibrate, gemfibrozil, pravastatin, carbamazepine, acridone, 10,11-epoxy-CBZ, 2-OH-CBZ, citalopram, fluoxetine, paroxetine, venlafaxine, dexamethasone, azaperone, metoprolol, propanolol	0.1 (d.w)	Homogenized with a mortar, kept at 20 °C	USE (MeOH)	Protein precipitation and phospholipid removal, PlateOSTRO™ plate	UHPLC-MS/ MS	No data	No data	[72]

(i)

Specie	Pharmaceuticals	Type and Amount of Sample (g)	Pre-Treatment	Treatment		Analysis	Analytical Features		Ref.
				Extraction Technique	Clean-Up		Recovery (%)	LOD (ng g^{-1})	
Surgeonfish (*Acanthurus xanthoperus*), Smallmouth catfish (*Ariopsis felis*), Bull fish (*Caranx caninus*), Milkfish (*Chanos chanos*), Yellowfin mojarra (*Gerres cinereus*), Elongated grunt (*Haemulopsis elongatus*), Silk snapper (*Lutjanus peru*), *White mullet* (*mugil curema*), California halibut (*Paralichthys californicus*), Bigscale goatfish (*Pseudupeneus grandisquamis*), Peruvian moonfish (*Selene peruvian*), Common snook (*Centropomus robalito*), Reef Lizardfish (*Synodus lacertinus*), Striped bonito (*Sarda orientalis*)	Diclofenac, ibuprofen, ketorolac, naproxen	25–30 (w.w)	Minced, homogenized	USE (No data)	No data	UHPLC-MS/ MS	92–95	0.97–23.1	[58]

Black Crappie (*Pomoxis nigromaculatus*), Black Redhorse (*Moxostoma duquesni*), Bluegill (*Lepomis macrochirus*), Common Carp (*Cyprinus carpio*), Flathead Catfish (*Pylodictis olivaris*), Freshwater Drum (*Aplodinotus grunniens*), Gizzard Shad (*Dorosoma cepedianum*), Golden Redhorse (*Moxostoma erythrurum*), Hybrid White x Striped Bass (*Morone chrysops x Morone saxatilis*), Largemouth Bass (*Micropterus salmoides*), Mooneye (*Hiodontidae*), Nothern Hogsucker (*Hypentelium nigricans*), Quillback Carpsucker (*Carpiodes cyprinus*), River Carpsucker (*Carpiodes carpio*), Sauger (*Sander canadensis*), Saugeye (*Sander canadensis x Sander vitreus*), Silver Redhorse (*Moxostoma anisurum*), Smallmouth Bass (*Micropetrus dolomieu*), Smallmouth Buffalo (*Ictiobus bubalus*), Smallmouth Redhorse (*Moxostoma breviceps*), Spotted Sucker (*Minytrema melanops*), White Bass (*Morone chrysops*), White Crappie (*Pomoxis annularis*)	Tylosin, lincomycin, furazolidone, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfanilamide, cotinine, carbamazepine, acetaminophen, thiamphenicol, florfenicol, chloramphenicol, caffeine, trimethoprim, azithromycin, triclosan erythrohydrobupropion	0.5 (w.w)	Homogenized	QuEChERs (ACN/H20 1% acetic acid, MgSO4, AcONa)	d-SPE-QuEChERs (MgSO4, PSA, C18)	UHPLC-MS/MS	67–148	0.2–2.6	[60]

Perch (*Perca fluviatilis*), Flounder (*Platichthys flesus*), Turbot (*Scophthalmus maximus*), Plaice (*Pleuronectes platessa*), Cod (*Gadus morhua callarias*), Bream (*Abramis brama*), Crucian (*Carassius carassius*)	Bisoprolol, carbamazepine, clarithromycin, erythromycin, fluoxetine, metronidazole, ofloxacin, promazine, sulfadimethoxine, thiabenzadole, tianeptine, acebutolol, 1-Naphthoxyacetic acid, amitriptyline, amlodipine, atenolol, azithromycin, bosentan, cefotaxime, chlorpromazine, chlortetracycline, clindamycin, clomipramine, codeine, desipramine, dextromethorphan, diclofenac, diltiazem, doxepin, drotaverine, duloxetine, enalapril, escitalopram, fenofibrate, fleroxacin, fluconazole, fluvoxamine, guaifenesin, imipramine, labetalol, losartan, levofloxacin, lincomycin, lomefloxacin, lovastatin, maprotiline, mebendazole, metformin, methoxyverapamil, metoprolol, mianserin, mirtazapine, moclobemide, morantel, mycophenolic acid, nalidixic acid, nifedipine, norfloxacin, nortriptyline, omeprazole, opipramol, oxymetazoline, oxytetracycline, pantoprazole, paroxetine, pefloxacin, piperacillin, propafenone, propanolol, protriptyline, pseudophedrine, quinapril, ramipril, ranitidine, roxithromycin, salbutamol, sotalol, sertraline, sulfadiazine, sulfamethazine, sulfamethoxazole, sulfanilamide, sulfathiazole, telmisartan, tetracycline, tiamulin, tianeptine, tolperisone, trazodone, trimethoprim, tylosin, valsartan, verapamil, xylometazoline	0.05 (w.w)	Homogenized	Mechanical shaking (ACN 0.1% formic acid), frozen, centrifuged. Added ammonium acetate and stirred	d-SPE: C18 sorbent	LC-QTRAP	No data	0.01–0.88	[56]
Rainbow trout (*Oncorhynchus mykis*)	Citalopram	Brain tissue (no data)	Brain separated	TissueLyser II at 30 Hz for 10 min. (ACN:i-propanol 3:1 with 0.1% formic acid)	No data	LDTD-HRPS	97–108	0.39	[54]
Bream (*no data*)	Bezafibrate, carbamazepine, 2-hydroxicarbamazepine, 10,11-dihydroxy−10,11-dihydrocarbamazepine, cetirizine, citalopram, desmehylcitalopram, clopidogrel, diclofenac, diphenhydraine, fexodenadine, fluconazole, norfluoxetine, furosemide, hydrochlorothiazide, metoprolol, oxazepam, primidone, sertraline, sulfamethoxazole, trimethoprim, N-acetylsulfamethoxazole, telmisartan, tramadol, valsartan, venlafaxine, O-desmethylvenlafaxine.	0.05 (fish liver), 0.1 for (fish fillet) (d.w)	Homogenized, lyophilized	Cell disruption (4 m/s for 40 s)	d-SPE: Silica gel	LC-MS/MS	70–130	0.05–5.5 ng mL^{-1} *	[79]

Gilthead sea bream (*Sparus aurata*), Sea bass (*Dicentrarchus labrax*)	Ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, norfloxacin, ofloxacin, oxolinic acid, sarafloxacin, chlortetracycline, doxycycline, minocycline, oxytetracycline, tetracycline, cefaclor, cefadroxil, cefalexin, cefapirin, ceftiofur, cefazolinamoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, penicillin G, penicillin V, azithromycin, clarithromycin, erythromycin- H_2O, tiamulin, tilmicosin, dapsone, sulfachlorpyridazine, sulfaclozine, sulfadiazine, sulfadoxine, sulfadimethoxine, sulfadimidine, sulfaguanidine, sulfameter, sulfamerazine, sulfamethizole, sulfamethoxazole, sulfamethoxypuridazine, sulfaonomethoxine, sulfamoxole, sulfapyridine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, carbadox, olaquindox, florfenicol, thiampenicol, baquiloprin, trimthoprim, lincomycin, novobiocin, rifaximin, albendazole, albendazole oxide, albendazole sulfone, febantel, dimetridazole, fenbendazole, flubendazole, morantel, levamisole, mebendazole, metronidazole, oxfendazole, piperazine, ronidazole, ternidazole, thiabenzadole, triclabendazole, arprinocid, clopidol, decoquinate, diaveridine, ethopabate, halofuginone, imidocarb, lasalocid, monensin, narasin, nigericin, robenidine, salinomycin, 5-hydroxyflunixin, aceclofenac, diclofenac, flunixin, ketoprofen, mefenamic acid, naproxen, meloxicam, niflumic acid, phenylbuntazone, tolfenamic acid, vedaprofen, cimaterol, clenbuterol, clenpenterol, mabuterol, ractopamine, salbutamol, terbutaline, betamethasone, cortisol, cortison, dexamethazone, methyl-thiouracil, methylprednisolone, progesteron, phenyl- thiouracil, propyl-thiouracil, ambroxol, atenolol, atorvastatin, caffeine, carbamazepine, cimetidine, gemfibrozil, haloperidol, indapamide, metformin, metoprolol, paracetamol, propranolol, ranitidine, simvastatin, theophyline, tramadol, triamterene, valsartan, bromhexine, chlorpromazine, colchicine, melamine, coumaphos	1.0 (w.w)	Homogenized, stored at −20 °C	Ultrasonic bath (H_2O containing 0.1% formic acid, 0.1% EDTA (w/v), MeOH, ACN).Precipitation of lipids and proteins	Hexane and further low temperature	UHPLC-MS/MS	No data	20–200	[57]

No data	Chloramphenicol, thiamphenicol, tinidazole, metronidazole, malachite green, crystal violet	2.0 (d.w)	Cleaned, scaled and muscle tissue was taken. Homogenised, blotted dried, freeze at −20 °C	MAE (ACN)	SPE (Activated neutral alumina column), USE (ACN) and DLLME (H_2O, CH_2Cl_2, ACN)	UHPLC-MS/MS	> 87	4.54–101.3 pg kg^{-1}	[59]
Sea bream (*Sparus aurata*)	Erythromycin, N-acetyl sulfamethoxazole, sulfadiazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline, sulfathiazole, trimethoprim, caffeine, paracetamol, phenazone, carbamazepine, carbamazepine-10,11- epoxide, citalopram, fluoxetine, N desmethyl sertraline, norfluoxetine, O desmethyl venlafaxine, sertraline, venlafaxine	1.0 (w.w)	Filleted	QuEChERs (ACN, $MgSO_4$, NaCl)	d-SPE: Z-Sep+	UHPLC-MS/-MS	62–107	0.5–19 *	[62]
Sonek (*Thyrsites atun*), Bonito (*Sarda orientalis*), Panga (*Pachymetopon blochii*), Hottentot (*Pterogymnus laniarius*)	Acetaminophen, caffeine, diclofenac, lamivudine, sulfamethoxazole, carbamazepine	10 (d.w)	Dissection of different parts (fillet, gills, liver and intestine), freeze-dried and ground	Soxhlet (MeOH/Acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	69.2–107.5	0.010–0.036	[49]
Sabalo (*Prochilodus lineatus*), Boga (*Megaleporinus obtusidens*), Dorado (*Salminus brasiliensis*)	Atenolol, carazolol, metoprolol, nadolol, propanolol, sotalol, diazepam, lorazepam, carbamazepine, 10,11-epoxycarbamazepine, 2-hydroxycarbamazepine, venlafaxine, clopidogrel, salbutamol, codeine, diclofenac, hydrochlorothiazide	1.0 (d.w)	Pooled, homogenized	PLE (MeOH)	GPC	UHPLC-MS/MS	26–115	0.028–2.7	[53]
Carps (*Carassius*), Japanese medakas (*Oryzias latipes*), Mosquitofish (*Gambussia affinis*)	Diclofenac, indomethacin, mefenamic acid, ibuprofen, bezafibrate, fenofibric acid, clofibric acid, gemfibrozil, diltiazem, amlodipine, propanolol, carvedilol, losartan, telmisartan, irbesartan, valsartan, rebamipide, cetirizine, diphenhydramine, chlorpheniramine, fexofenadine, epinastine, warfarin, tramadol, O-desmethyl tramadol, N-desmethyl tramadol, sertraline, norsertraline, fluoxetine, norfluoxetine, paroxetine, citalopram, venlafaxine, haloperidol, risperidone, quetiapine, chlorpromazine, aripiprazole, zotepine, phentyon, carbamazepine, clonazepam, diazepam, zolpidem, nitrazepam, oxazepam, flunitrazepam, lorazepam, alprazolam, etizolam, sulfapyridine, sulfamerazine, sulfisozole, sulfamethizole, sulfamethazine, sulfamonomethoxine, sulfamethoxazole, sulfadimethoxine, trimethoprim, lincomycin, fluconazole erythromycin, clarithromycin, rixothromycin, florfenicol,	200 µL plasma (*Carassius carassius*) and 0.1 g whole-body tissue (rest)	Homogenized	USE (MeOH/ACN, and acetic acid-ammonium acetate buffer)	SPE (Hybrid-SPE®-Phospholipid cartridge)	LC-MS/MS	70–120	0.0077–0.93 ng mL^{-1}	[50]

European eel (*Anguilla anguilla*)	Acetaminophen, atenolol, caffeine, diclofenac, etoricoxib, ibuprofen, naproxen, salicylic acid, triclosan, vildagliptin)	1.0 pool (w.w)	Pooled, chopped, and homogenized	d-SPE: QuEChERs (ACN, MgSO4, NaCl, DCS and TCD)	d-SPE: EMR-Lipid ($MgSO_4$ and NaCl)	UHPLC-MS/MS	70–120	1.4–12	[51]
Rainbow trout (*Oncorhynchus mykiss*)	Enrofloxacin, norfloxacin, ciprofloxacin	5.0 muscle (w. w)	Boned	SPE (0.1 M K_2HPO_4 (pH = 6.5))	SPE (Strata XC cartridges)	LC-MS/MS	91.1–108.9	3.3–3.6	[87]
Nile Tilapia (*Oreochromis niloticus*), Milk fish (*chanos chanos*), Common silver biddy (*gerres oyena*), Golden snapper (*lutjanus johni*), Emperor fish (*ethrinus nebulosus*)	Atenolol, ranitidine, acetaminophen, caffeine, trimethoprim, atrazine, amitriptyline, carbamazepine, chlorpheniramine malate, ciprofloxacin, diclofenac, fluoxetine, ibuprofen, metronidazole, sulfamethoxazole, warfarin, cephalexin.	1.0 (d.w)	Filleted and cut into small sections and lyophilized. Pooled and homogenized	USE (0.1 M aqueous acetic acid/MeOH and NH_4OH 0.1 M)	SPE (Oasis MCX cartridges)	HPLC-MS/MS	30–103	0.1–13 ng mL^{-1}	[52]
Mackerel (*Scomber scombrus*), tuna (*Thunnus thynnus*), cod (*Gadus morhua*), perch (*Perca fluviatilis*), Pangas catfish (*Pangasius pangasius*) , sole (*Solea solea*), seabream (*Sparus aurata*), plaice (*Pleuronectes platessa*), salmon (*Salmonidae*)	Diclofenac, diazepam, sotalol, carbamazepine, citalopram, venlafaxine, azithromycin, sulfamethoxazole	Fillet (no data)	Pooled, homogenized by grinding, freeze-dried, kept at −20 °C	PLE (MeOH)	GPC	UHPLC-MS/MS	No data	0.01–0.65	[55]
Rusell's snapper (*Lutjanus russelli*), Saddle tailed sea perch (*Lutjanus erythopterus*), Silverfish (*Trachinotus ovatus*)	Sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfameter, sulfamethazine, sulfamethoxazole, sulfapyridine, sulfamonomethoxine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, trimethoprim, chlortetracycline, doxycycline, methacycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, fleroxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, clarythromycin, erythromycin, leucomycin, roxithromycin, oleandomycin	2 (w.w)	Frozen, muscle dissected	USE: MeOH/H_2O, 0.1 M acetic acid	SPE (SAX/PSA, HLB cartridges)	LC-MS/MS	50–150	0.05–9.06	[42]
No data	Ketoprofen, naproxen, flurbiprofen, diclofenac, ibuprofen	5 (w.w)	Chopped into mince	USE (ACN)	SPE CF@UiO-66 NH_2	UHPLC-PDA	95–116.99	0.12–3.50 ng mL^{-1}	[63]

European pilchardus (*Sardina pilchardus*)	Atenolol, metoprolol, nadolol, propanolol, sotalol, salbutamol, diazepam, carbamazepine, 10,11-epoxycarbamazepine, 2-OH-carbamazepine, citalopram, venlafaxine, alprazolam, azaperone, azaperol, hydrochlorothiazide, codeine, phenazone, propyphenazone, piroxicam, ronidazole, dimetridazole, metronidazole, azithromycin, erythromycin	1 (d.w)	Freeze-dried	PLE (MeOH, 4 extraction cycles)	GPC	UHPLC-MS/MS	No data	0.1–0.6	[40]
Hake (*Merluccius merluccius*), Red mullet (*Mullus surmuletus*), Sole (*Solea solea*)	Metronidazole, acetaminophen, amoxicillin, acetazolamide, sulfadiazine, atenolol, caffeine, ampicillin, trimethoprim, norfloxacin, ofloxacin, ciprofloxacin, tetracycline, phenazone, metoprolol, spiramycin, azithromycin, sulfamethoxazole, oxolinic acid, erythromycin A, piperacillin, tylosine, cyclophosphamide, carbamazepine, flumequine, oxazepam, clarithromycin, roxithromycin, lorazepam, losartan, nordiazepam, josamycin, ketoprofen, 19-norethind-rone, amiodarone, hydrochlorothiazide, acetylsalicylic acid, niflumic acid, diclofenac, ibuprofen, gemfibrozil	0.2 (d.w)	Separated white dorsal muscle, freeze-dried.	Mechanical shaking (MeOH, 1% acetic acid)	No data	UHPLC-MS/MS	28–188	0.1–40.2 *	[61]
Sea bream (*Sparus aurata*)	Trimethoprim, ciprofloxacin, norfloxacin, sulfadiazine, sulfamethoxazole, amitriptyline, clomipramine, imipramine, nortriptyline, eprosartan, irbesartan, losartan, telmisartan, valsartan, propanolol, acetaminophen, diclofenac, ketoprofen, bezafibrate, clofibric acid, carbamazepine, phenytoin	0.5 fish muscle and liver; 0.1 fish gills and brain (d.w)	Freeze-dried, ground, homogenized	FUSLE (MeOH/ H_2O)	SPE (HLB cartridges)	LC-MS/MS	71–126	4–48	[62]
Mullet (*Mugil spp.*, *Mugil curema*), Snook (*Centropomus spp.*)	Bezafibrate, carbamazepine, chloramphenicol, diclofenac, 4′-Hydroxydiclofenac, furosemide, gemfibrozil, ibuprofen, indapamide, ketoprofen, naproxen, simvastatin	0.5 (d.w)	Dissection to obtain the morphometric measures, freeze-dried	QuEChERs (ACN, formic acid, NH_4Cl)	d-SPE: QuEChERs ($MgSO_4$, Z-Sep)	HPLC-MS/MS	70–133	0.004–2.16	[43]
Golden grey mullet (*Liza aurata*), Black goby (*Gobius niger*)	Diclofenac, codeine, carbamazepine, citalopram, diazepam, lorazepam, atenolol, sotalol, propanolol, nadolol, carazolol, hydrochlorothiazide, clopidogrel, salbutamol, levamisole	1.0 golden grey mullet muscle and black goby; 0.5 liver golden grey mullet (d.w)	Freeze-dried, milled	PLE (MeOH)	GPC	UHPLC-MS/MS	<20–200	0.02–6.6	[48]

Yellow grouper (*Epinephelus awoara*), Orbfish (*Ephippus orbis*), Topmouth culter (*Culter alburnus*)	Sulfadiazine, sulfamerazine, sulfamethazine, trimethoprim, sulfamethoxazole, sulfathiazole, sulfapyridine, ciprofloxacin, norfloxacin, ofloxacin, flumequine, tetracycline, penicillin G sodium, oxytetracycline, isochlortetracycline, cefotaxime sodium, spectinomycin, roxithromycin, erythromycin- H_2O, clarithromycin, thiamphenicol, chloramphenicol, paracetamol, naproxen, ibuprofen, ketoprofen, diclofenac acid, diltiazem, carbamazepine, diphenhydramine, gemfibrozil	0.2 (d.w)	Freeze-dried, ground into powder. Separation of back muscles and abdominal muscles	USE (ACN/H_2O)	SPE (PRiME HLB cartridges)	UHPLC-MS/MS	43–127	0.01–1.9	[39]
Senegal seabram (*Diplodus bellottii*), European sea bass (*Dicentrarchus labrax*), Meagre (*Argyrosomus regius*), Lusitanian toadfish (*Halobatrachus didactylus*)	Alprazolan, amoxicillin, atenolol, atorvastatin, azithromycin, bisoprolol, benzylpenicillin, bezafibrate, carbamazepine, carvedilol, cinoxacin, ciprofloxacin, ceftiofur, cephalexin, chlortetracycline, danofloxacin, diclofenac, doxicycline, enoxacin, enrofloxacin, epi-chlortetracycline, epi-tetracycline, erythromycin, epotetracycline, fenofibrate, flumequine, fluoxetine, furosemide, gabapentin, gemfibrozil, ibersartan, ibuprofen, indapamide, lorazepam, losartan, marbofloxacin, nalidixic acid, norfloxacin, nimesulide, ofloxacin, oxolinic acid,oxytetracycline, paracetamol, propanolol, sertraline, simvastatin, spiramycin, sulfachloropyridazine, sulfadoxine, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfanilamide, sulfapyridine, sulfisomidine, sulfamethoxazole, sulfaquinoxaline, sulfathiazole, sulfisoxazole, tetracycline, tilmicosin, trimethoprim, tylosin venlafaxine, topiramate	2 (w.w)	Homogenized	Mechanical shaking (ACN, EDTA)	No data	UHPLC-MS/MS	79.2–109.5	0.59–4.11	[41]
White bream, roach, bleak, perch, asp, pike, pikeperch (No data)	Nicotine, haloperidol, pyremethamine	0.14–0.2 (d.w)	Dissected into fillet and carcass, frozen	USE (ACN, MeOH, H_2O)	SPE (No data)	LC-HRMS/MS	70–130	0.05–5.7*	[91]
Atlantic salmon, Atlantic sea wolf, rainbow trout, Atlantic cod (No data)	Amoxicillin, azithromycin, caffeine, carbamazepine, ciprofloxacin, clarithromycin, diclofenac, erythromycin, furosemide, ketoprofen, ibuprofen, naproxen, sulfamethoxazole, tetracycline	2 (w.w)	Dorsal muscle separated, cut into small parts, frozen at −20 °C	Mechanical shaking (ACN, 0.1 M EDTA, hexane)	No data	UHPLC-MS/MS	81.2–99.4	0.017–1.371	[46]

Flatfish (No data)	Albendazole, 2-amino albendazole sulfone, albendazole sulfone, albendazole sulfoxide, febantel, fenbendazole, flubendazole, 2-amino flubendazole, oxfendazole, oxfendazole sulfone, oxibendazole, cefapirin, desacetylcefapirin, cefazoline, cefoperazone, halofuginone, azithromycin, tildipirosin, dimetridazole, ipronidazole, ipronidazole-OH, metronidazole, metronidazole-OH, tinidazole, ronidazole, dicloxacillin, nafcillin, oxacillin, penicillin V, 2-hydroxymethyl-1-methyl-5-nitromidazole, 4-methylaminoantipyrine, sarafloxacin, orbifloxacin, carbadox, quinoxaline-2-carboxylic acid, olaquindox, 3-methylquinoxaline-2-carboxilic acid, dapsone, N-acetyl dapsone, sulfapyridine, arprinocid, azaperol, azaperon, carazolol, caffeine, clenbuterol, clochicine, diphehydramine, flunixin, imidocarb, isometamidium, ketoprofen, loperamide, metoclopramide, nitroxynil, phenacetin, ractopamine, scopolamine, triamcinolone, valnemuline	2 (w.w)	Homogenized, stored at −20 °C	Mechanical shaking (Water/ACN)	d-SPE: C18	UHPLC-MS/MS	73.2–115	0.5–5 *	[95]

Goldsilk seabream (*Acanthopagrus berda*), Indo-Malaysian barracuda (*Sphyraena jello*), Pale-edged stingray (*Dasyatis zugei*), Japanese scaled sardine (*Sardinella zunasi*), Yellow seabream (*Acanthopagrus latus*), Spotted scat (*Scatophagus argus*), Dotted gizzard shad (*Konosirus punctatus*), Porgies (*Acanthopagrus schleg*), Grey large-eye bream (*Gymnocranius griseus*), Pompano (*Trachinotus ovaus*), Saddleback silver (*Gerres limbatus*), Asian seabasses (*Lateolabrax maculatus*), Silver sea bream (*Rhabdosargus sarba*), Rough flathead (*Grammoplites scaber*), Bloch's gizzard shad (*Nematalosa nasus*), Gangetic anchovy (*Thryssa mysiax*), Japanese goatfosh (*Upeaneus japonicus*), Genus (*Johnius fasciatus*)	Sulfamethazine, sulfapyridine, sulfathiazole, sulfanilamide, sulfadiazine, sulfadimethoxine, sulfamonomethoxin, sulfamerazine, sulfamethoxazole, norfloxacin, enoxacin, ofloxacin, ciprofloxacin, enrofloxacin, dehydrated erythromycin, clarithromycin, azithromycin, roxithromycin, florfenicol, chloramphenicol, trimethoprim, lincomycin	5 (d.w)	Washed (water), dissected, homogenized, stored at −20 °C	USE (ACN, citric acid)	SPE (SAX-HLB cartridges)	UHPLC-MS/MS	47.67–172.67	0.04–0.24	[13]
Eel, flatfish (No data)	Naproxen, methyltestosterone, 17α-hydroxyprogesterone caproate, progesterone	2 (w.w)	Ground, homogenized	Manual shaking (ACN 0.1% acetic acid)	No data	LC-MS/MS	68–117	1–2	[43]

Silver carp (*Hypophthalmichthys molitrix*), Bighead carp (*Aristichthys nobilis*), Common carp (*Cyprinus carpio*), Goldfish (*Carassius auratus*), Common skygazer (*Cultrichthys erythropterus*), Topmouth culter (*Culter alburnus*), Japanese grenadier anchovy (*Coilia ectenes taihuensis*), Asian pencil halfbeak (*Hyporhamphus intermedius*), Clearhead icefish (*Protosalanx hyalocranius*), Common sawbelly (*Hemiculter leucisculus*), Bitterling (*Rhodeus sinensis*), River sand pond snakehead (*Odontobutis potamophila*), Yellow catfish (*Pelteobagrus fulvidraco*), Asian swamp eel (*Monopterus albus*)	Sulfachlorpyridazine, sulfadiazine, sulfadoxine, sulfamerazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfisoxazole, sulfathiazole, trimethoprim, chlortetracycline, doxycycline, oxytetracycline, tetracycline, ciprofloxacin, danofloxacin, enrofloxacin, fleroxacin, difloxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin, pefloxacin, sarafloxacin, azithromycin, leucomycin, clarithromycin, oleandomycin, roxithromycin, tylosin, salinomycin, monensin, florfenicol, chloramphenicol	0.2 liver; 1.0 muscle (d.w)	Washed (water), dissected, homogenized, freeze-dried, stored at −20 °C	No data	No data	RRLC-MS/MS	37.6–135	0.01–1.99	[38]
Grass carp (*Ctenopharyngodon idellus*), Silver carp (*Hypophtha lmichthys molitrix*), Common carp (*Cyprinus carpio*), Crucian carp (*Carassius auratus*), Bighead carp (*Hypophthalmichthys nobilis*), Whitebait (*Reganisalanx brachyrostralis*), Yellow catfish (*Pelteobagrus fulvidraco*), Catfish (*Silurus asotus*), Loach (*Paramisgurnus dabryanus*)	Roxithromycin, erythromycin, ofloxacin, norfloxaxin, ciprofloxacin, tetracycline, sulfamethoxazole, ibuprofen, sulfaquinoxaline, sulfadiazine, diclofenac, naproxen, bezafibrate, propranolol, ketoconazole, carbamazepine, caffeine, fluoxetine, norfluoxetine, citalopram, paroxetine, sertraline, venlafaxine, duloxetine, bupropion, amitriptyline, fluvoxamine, trihexylphenidyl, clozapine, quetiapine, aripiprazole, chlorpromazine	0.5 (d.w)	Freeze-dried, homogenized, stored at −80 °C	PLE (MeOH/acetone)	SPE (HLB cartridges)	UHPLC-MS/MS	66–128	0.07–1.67	[27]

Red bigeye (*Priacanthus macracanthus*), Horn dragonet (*Callionymus curvicornis*), White-spotted spinefoot (*Siganus canaliculatus*), Silver jewfish (*Pennahia argentata*), Burrowing goby (*Trypauchen vagina*), Threadfin porgy (*Evynnis cardinalis*), Palad (*Solea ovata*), Anchovy (*Thryssa kammalensis*), Bony fishes (*Johnius heterolepis*), Japanese flathead (*Inegocia japonica*), Shortnose ponyfish (*Leiognathus brevirostris*), Big head croaker (*Collichthys lucidus*), Goatee croaker (*Dendrophysa russelii*), Yellow croaker (*Larimichthys crocea*), Largehead hairtail (*Trichiurus lepturus*)	Atenolol, metoprolol, venlafaxine, chloramphenicol	2 (d.w)	Washed (water), dissected, homogenized, freeze-dried, stored at −50 °C	USE (MeOH/ H_2O)	SPE (Oasis MCX cartridges)	LC-MS/MS	68–96	0.05–0.25	[44]
Silver carp (*Hypophtha lmichtyts molitrix*), Common carp (*Cyprinus carpio*), Crucian carp (*Carassius auratus*), Lake anchovy (*Coilia extenes*), whitebait (*Reganisalanx brachyrostralis*), Redfin culter (*Cultrichthys erythropterus*), Yellow catfish (*Pelteobagrus fulvidraco*)	Roxithromycin, erythromycin, ofloxacin, norfloxacin, ciprofloxacin, tetracycline, chloramphenicol, sulfamerazine and sulfadiazine, sulfamethoxazole, ibuprofen, diclofenac, naproxen, indomethacin, clofibric acid, gemfibrozil, bezafibrate, 17β-estradiol, 17α-ethynylestradiol, propranolol, carbamazepine, ketoconazole, sertraline	0.5 (d.w)	Separation of liver, brain, gills, and muscle. Freeze-dried, ground, stored at −20 °C	PLE (MeOH/ acetone)	SPE (HLB cartridges)	LC-MS/MS	68–116	0.01–1.12	[66]
Crucian carp (*Carcassius carcassius*)	Florfenicol, thiamphenicol, ofloxacin, pipemidic acid	No data	Liver, muscle, gill and bile separated, washed with 0.15 M KCl, stored at −20 °C	USE (0.1 M AcONa, MeOH)	SPE (SAX/PSA-HLB tandem cartridges)	LC-MS/MS	79.2–91.0	0.5–0.6	[96]

** Limit of quantification; ACN: acetonitrile; DAD: diode-Array detection; DLLME: dispersive liquid-liquid microextraction; DMSO: dimethyl sulfoxide; d-SPE: dispersive solid phase extraction; d.w.: dry weight; FUSLE: focused ultrasonic solid-liquid extraction; GPC: gel permeation chromatography; HPLC: high performance liquid chromatography; HRMS: high resolution mass spectrometry; HRPS: high resolution product scan; LDTD: laser diose thermal desorption; LC: liquid chromatography; MAE: microwave assisted extraction; MeOH: methanol; MS: mass spectrometry; MS/MS: tandem mass spectrometry; PLE: pressurized liquid extraction; PSA: primary secondary amine; PuLE: pulverised liquid extraction; QuEChERs: Quick, easy, cheap, effective, rugged and safe; RRLC: rapid resolution liquid chromatography; UHPLC: ultra-high performance liquid chromatography; USE: ultrasound assisted extraction; w.w.: wet weight.*

3.1. Sample Collection

In most of the literature consulted, specimens were captured by professional divers in different sampling areas, although in some cases, they were purchased in local supermarkets, either to be used as an analyte-free matrix [53], or as study sample [54–56]. Once captured, they were transported on ice, in order to avoid decomposition, at −10 °C and stored frozen at −20 °C [53,57,58], or deep-frozen (around −80 °C) until analysis [55,59,60].

3.2. Sample Pretreatment

Prior to storage, in order to guarantee the homogeneity of the sample as well as to reduce the particle size, and therefore, to achieve better extraction efficiency, most of the articles consulted pulverized the sample. The fish were cleaned before spraying the specimens and then, according to the literature, the vast majority of studies homogenised the sample by analysing a pool of all the body cavities of the different fish. However, in some cases, fish were deboned [87], only the muscle was analysed [39,42,46,59,61] or the different body cavities were analysed separately (fillet, gills, liver, intestine or brain) [49,53,66,96]. In addition, usually the samples were freeze-dried, so that spraying in the absence of humidity would be easier, although several studies worked with wet weight [50,62]. In case of molluscs, cephalopods or crustaceans were generally pooled without differentiating body cavities, removed from the shell if present, freeze-dried and ground into powder [34,63,64]. Ojemaye and Petrick [36], for the study of algae and echinoderms, rinsed, shelled and dissected by freeze-drying, and in the case of plankton, they were washed, homogenized and stored at −20 °C [37,65]. Storage consisted of frozen maintenance until analysis at −20 °C.

3.3. Sample Treatment (Extraction and/or Clean-Up)

3.3.1. Ultrasound. USE and FUSLE

An ultrasound consists of a mechanical wave propagation that is formed by cycles of compression and refraction, that is, waves of high and low pressures combined. The wave frequencies are above 20 kHz.

Ultrasonic solvent extraction (USE) is able to induce these compressions and refractions of solvent molecules resulting in the formation of bubbles due to temperature and pressure variations. Collisions between particles as well as ultrasonic waves are able to induce fragmentation, which reduces the particle size, helping the mass transfer. The implosion of bubbles on the matrix surface results in erosion, which improves solvent accessibility [66]. Ultrasonic irradiation can be indirect or direct, both of which will be explained below. Argüello-Pérez et al. [57] determine four analgesics in fourteen different fish species using USE at 20 °C at 400 W power with a surface area of 3.8 cm2, achieving recoveries close to 100% in all cases. Focused ultrasound solid-liquid extraction (FUSLE) is a relatively new extraction technique, which started gaining popularity because the ultrasonic bath often provides low power. By introducing a probe directly into the extraction mixture, a sonication power up 100 times higher is achieved, as well as greater reproducibility and efficiency. The ultrasound energy is concentrated at the tip of the probe and is hence focused [67], and when ultrasound waves cross the liquid, many gaseous bubbles are formed which, when they implode, produce locally very high temperatures as well as high pressures and velocities of solvent micro-jets [68]. Mijangos et al. used FUSLE to extract antibiotics, analgesics and antiepileptics, among others from mussels and sea bream. For the extraction, authors used 30 s and 10% amplitude with 7 μL of MeOH/H2O (95:5, v/v) as solvent at 0 °C (extraction efficiencies from 71 to 126%) [69]. Some works apply ultrasound in a simpler way, by sonication in a common laboratory ultrasound machine. In this case, ultrasonic irradiation takes place indirectly, i.e., through the sample container. This equipment works at a single frequency, therefore the wave amplitude cannot be controlled. Danesaki et al. [56] used an ultrasonic bath at 60 °C (20 min) followed by a precipitation of lipids and proteins to recover 143 veterinary drugs from fish, while Ali et al. [51], analyzed different PhACs at room temperature (15 min) obtaining recoveries between 30% and 103% and limits of detection (LODs) from 0.1 to 13 ng mL^{-1}.

3.3.2. Pressurized Liquid Extraction

Pressurized liquid extraction (PLE), also called accelerated solvent extraction (ASE), is used for the extraction of analytes from solid or semi-solid matrices, by combining the use of different solvents with high temperatures and pressures. This allows higher recoveries and good extraction efficiencies while decreasing extraction time [70]. MeOH, acetonitrile (ACN) and water, or a mixture of them, have frequently used as extractant solvents. In addition, working temperatures are around 50 °C. Rojo et al. [52] studied different families of PhACs in fish muscle tissue, achieving recoveries between 26 and 115%. Other authors have proposed this technique to investigated different drugs in several types of fish as well as biofilm, plankton, bivalves, crustaceans and cephalopods obtaining LODs between 0.0004 and 6 ng g^{-1} and recoveries ranging from 20 to 151% [26,34,39,47,54,71].

3.3.3. Microwave Assisted Extraction

Microwave-assisted extraction (MAE) was first used to replace Soxhlet extraction with the aim of reducing the amount of extraction solvent, achieving similar or better recoveries than Soxhlet extraction and reducing digestion time. It consists of heating the closed vessel to warm the solvent and decrease its viscosity, while increasing the solubility of the analytes in the extraction solvent and to facilitate the penetration into the matrix [72]. In the literature consulted, only the research by Argüello-Pérez et al. used this assisted extraction technique, for the analysis of several antimicrobials in fish as matrix [58]. ACN was used as solvent and it was carried out for 5 min at 40 °C with a power of 400 W. They obtained recoveries higher than 87% for all analytes and LODs between 4.54 and 101.3 pg kg^{-1}.

3.3.4. Solid-Phase Extraction

Solid phase extraction (SPE) allows the concentration of a target analyte by removing interferents present in the matrix via a solid stationary phase. This is an absorbent, which will be chosen according to the physicochemical properties of target compounds, in order to correctly

separate the analytes from the rest of the interferents [79]. There are different types of sorbents; some of them retain the analytes and others the inteferents.

Boulard et al. [78] used silica gel for cleaning fish liver and fillet extracts in bream together with water and ACN to remove non-polar compounds from the extract. They achieved low LODs for the different PhACs, between 0.05 and 5.5 ng mL^{-1}. Another sorbent used in SPE is the alumina column, which is capable of retaining compounds with an acidic character. It is used for the separation of compounds with medium polarity [80]. Huang et al. [58] used an alumina column in the clean-up phase for the determination of 6 antibiotics in fish muscle. This clean-up took place in two steps, after the alumina column in which ACN was used; a DLLME was carried out. They achieved recoveries higher than 87%.

According to the scientific literature consulted, SPE with cartridges is the most commonly cleun-up technique. Among all the sorbents, the most widely used cartridge is the HLB, as it is a universal for acidic, neutral or alkaline compounds. Other sorbents packed in the cartridges are SAX and PSA, which are multilayer cartridges suitable for polar interactions. Chen et al. used this type combined with the HLB cartridge, facilitating the separation of polar and non-polar compounds for sulfonamides and tetracyclines in crabs, shrimps and different types of fish, reaching recoveries between 50 and 150% [41]. McEneff et al. used a cartridge with Strata-X, which is a reversed-phase polymeric cartridge, at SPE for the determination of different analgesics and antiepileptic drugs in mussels, achieving yields between 83 and 94% [64]. Tanoue et al. used a Hybrid SPE-Phospholipid cartridge, which removed exogenous proteins as well as phospholipid interferences for different drugs and some of their metabolites in fish analysis, with recoveries between 70 and 120% [49].

Gao et al. developed a different type of clean-up based on SPE [62]. These authors used a metal organic framework (MOF) as adsorbent. SPE (CF@UiO-66-NH_2) is a MOF based on Zr and modified with cotton fiber, resulting in CF@UiO-66-NH_2, which has a high adsorption capacity

because it has many active sites. After adsorption, desorption of the analytes takes place by using desorption solvents. Gao et al. [62] used this adsorbent for the extraction of some analgesics such as ketoprofen, naproxen, flurbiprofen, diclofenac sodium and ibuprofen in fish and crustaceans' tissue, achieving recoveries between 95 and 116.99% and LODs between 0.12 and 3.50 ng mL^{-1}.

3.3.5. Dispersive Solid Phase Extraction (dSPE)

This technique consists of the dispersion of a solid sorbent in a liquid or dissolved sample so that impurities or interferents are retained, resulting in a clean extract. After separation, the sorbent is removed, usually by centrifugation [81]. There are different types of sorbents; those used in the consulted literature will be explained below.

C18 sorbent is used for the extraction of non-polar or relatively polar compounds, being able to retain most of the organic compounds present in an aqueous phase.

QuEChERs (quick, easy, cheap, effective, rugged and safe) is one of the most user-friendly techniques. High extraction efficiencies can be achieved and it is also in agreement with green chemistry as it uses a small amount of sample as well as solvent. This makes it one of the most widely used extraction methods nowadays [73]. This technique is applied in two sequential stages. The first one is the extraction phase, which is performed using an organic solvent, normally ACN in the presence of different salts, such as $MgSO_4$ or NaCl, whose function is to regulate pH, control polarity to favor the phase separation and contribute to the recovery of the analyte. Then, a second stage of cleaning is carried out, which consists of purification dSPE. With this step, the residual water and other interfering compounds present in the matrix are removed. For this purpose, some salts are used, such as $MgSO_4$, which removes excess water; PSA (primary/secondary amine), which removes organic acids, fatty acids and sugars from the matrix; C18 (sorbent), which eliminates fats and other non-polar interferences; and graphitized black carbon (GCB), which removes pigments from the sample [73].

3.3.6. Others

Soxhlet. Since it involves much larger quantities of solvent and much longer times than other extraction techniques, and the yields of extraction obtained are not much better, it is a technique rarely used today. It consists of the continuous flow of solvent through the sample, using a distillation flask. When the solvent condenses, it does so with the dissolved analytes. This operation is repeated until extraction process is completed, achieving good extraction efficiencies [74]. Ojemaye and Petrick [36,48] used this technique for the extraction of a group of drugs, such an antiepileptic, antibiotics and an analgesic, in fish, bivalves, algae and echinoderms. They used MeOH and ACN (3:1, *v/v*) as extractant solvents and they achieved recoveries between 69.2 and 107.5% for fish and 96.1 and 100.5% for the rest of the species as well as LODs of 0.01 and 0.036 ng g^{-1} for fish and between 0.62 and 1.05 ng L^{-1} for the other species under study.

TissueLyser II. TissueLyser consists of bead mill equipment which, with adapters, is capable of lysing biological samples by agitation at high speeds. It has many applications, such as the disruption of human, animal, plant and even bacterial tissues. It is a very efficient extraction [75]. Borik et al. [53] used this type of lysis for the extraction of citalopram from rainbow trout fish brain tissue, achieving close to 100% recovery with a LOD of 0.39 ng g^{-1}.

Mechanical shaking. This is one of the simplest extraction techniques as it consists of stirring the sample with the extraction solvent for a certain time to ensure the migration of the analytes from the solid phase to the liquid one. Generally, this agitation is followed by centrifugation so that the decantation can take place and the phases can be separated correctly, leaving the target analytes dissolved in the liquid [76,77]. Not many studies based on the use of this technique have been found, as the time required is usually longer. The most commonly used solvents are can and MeOH, sometimes acidified with formic or acetic acid. López-García et al. [78] used ACN with salts ($MgSO_4$, NaCl, sodium citrate and DCS (sodium citrate sesquihydrate)) for the study of mussel's tissue, with

recoveries between 77% and 118% and and low LODs (<2 ng g^{-1}). Bobrowska-Korczak et al. [55] and Miossec et al. [60], studied the presence of 98 and 41 PhACs, respectively, in fish and shrimps, with LODs between 0.1 and 40.2 ng g^{-1}, reaching recoveries in the range of 28 to 188%.

Cell disruption. This technique is carried out in a high-speed shaking equipment that, in a very short time, is able to extract the maximum amount of DNA, RNA, proteins and other compounds with very good efficiency. This is why after this type of extraction the cleaning and purification protocol plays an essential role in the removal of interferents. Boulard et al. [78] used this extraction technique for the analysis of 26 PhACs in bream and the time required for extraction was 40 s, achieving recoveries from 70% to 130% and LODs from 0.05 to 5.5 ng mL^{-1}.

Pulverised liquid extraction (PuLE). In this extraction technique, the sample is homogenized and the analytes are extracted simoultaneously by shaking. The solid sample is placed in a vessel together with two glass beads and then it is agitated in a homogeniser at a known speed and time. Only one study found in the scientific literature have used this extraction modality. This technique was used to extraxt 29 PhACs in the amphipod *Gammarus pulex*. The recoveries were between 41 and 89% [84].

Gel permeation Chromatography (GPC) is a technique traditionally used for the clean-up of the extracts because it removes biological macromolecules such as fats or proteins, separating them according to size. The column packing is a porous gel, and the beads packaged in it interact with the compounds, so it differs from other separation techniques in that it does not rely on physical or chemical interactions [82]. Rojo et al. used GPC for clean-up of the extracts of fish species when they had determined 15 PhACs and two of their metabolites, achieving recoveries between 26 and 115% [52]. Álvarez-Muñoz et al. studied 8 PhACs from different families in 9 different fish species using GPC as a clean-up technique [54].

Of all the extraction techniques described in this section, those based on the use of ultrasound (USE and FUSLE) have been the most attractive alternatives for the analysis of PhACs in biota (36% of the studies), followed by PLE (30% of the consulted studies). Both techniques are simple, provide automatization, short extraction times and low solvent consumption. For clean-up, SPE using Oasis HLB cartridges has been shown to be an efficient method and the most popular used as a clean-up procedure (71% of the studies), regardless of the aquatic organism under study.

4. Instrumental Analysis

4.1. Liquid Chromatography

LC separation technique coupled with an adequate detector allows quantitative determinations of the compounds with high selectivity, sensibility and accuracy. LC is a very suitable technique for the multiresidue PhACs separation. Furthermore, it does not require the previous derivatization step.

Regarding the retention mechanisms, a broad variety may be applicable in LC. Some examples are reverse phase chromatography (RP-LC), normal phase liquid chromatography (NP-LC), hydrophilic interaction liquid chromatography (HILIC), ion-pairing chromatography (IPC), ion exchange chromatography (IEC), or hydrophobic interaction chromatography (HIC), among others. As far as the determination of PhACs in aquatic organisms is concerned, and considering the physicochemical properties of the target compounds (polar compounds), the RP-LC modality has been the best choice for all the authors. This retention mechanism is related to non-polar selectivity consisting of a non-polar stationary phase and, as mobile phases, a solvent mixture of high polarity solvents. Consecuently, the least polar compounds of the mixture appear first in the chromatogram. RP-LC using C18 silica columns is mainly used for separation, although chiral columns based on α1-glycoprotein (AGP) and phenyl or phenyl-hexyl columns have been also used as stationary phases [44,59,60]. Generally, the most commonly

used solvents in the mobile phase are water as the aqueous component (phase A), and in the organic phase, ACN or MeOH (phase B) [59,62,63]. Some authors such as Moreno-González et al. used dichloromethane and methanol (90:20, *v/v*) in isocratic mode as mobile phases for the analysis of 20 PhACs in fishes and molluscs, prior to a study of bioaccumulation [47]. Sometimes, the use of additives in the aqueous phase, or occasionally in both, such as formic acid, ammonium formate, ammonium acetate or acetic acid at low concentrations, assists ionization when mass spectrometry is selected as detection technique. The use of additives provides better analytical signals and thus, make it easier to determine the target analytes [52,60,83,84].

On the other hand, HILIC is considered by far an attractive alternative for the separation of polar compounds, such as pharmaceuticals. This one is associated to polar selectivity, but also using polar mobile phases. Although the reported articles were based on RP-LC, the use of diol and amine columns may be also considered, as they could provide promising results in the separation of PhACs.

In recent years, the HPLC technique has been largely replaced by UHPLC as it has many advantages over the former. The analyses are faster and more sensitive. This is due to the fact that the column packing consists of smaller and more porous particles (sub-2-micron particles) that achieve better chromatographic peaks, and therefore greater sensitivity, although the collateral effect is that the work is carried out at higher pressures. As this review work has focused on the last 10 years of research, most of the studies included the use of UHPLC technique [34,46,58] (56%) while the remaining 44% used classical HPLC (Table 1(a–i)). The chromatographic columns used in the first case are usually 10 cm long [13,27], although some studies achieve separation even with 5 cm columns [35,39,64]. In the case of HPLC, longer chromatographic columns are used, usually 15 cm [28,65,66,88,90], with the exception of some studies using shorter columns of 10 cm [42,52] or 12.5 cm in length [78].

4.2. Detection Systems

After chromatographic separation, spectrophotometric detection has been used on a limited, but interesting, number of cases, depending on the properties of the compounds under study [86]. For example, Gao et al. coupled an ultraviolet detection system for the determination of 5 NSAIDs in fish and shrimp muscle tissues using a new synthetic MOF in the extraction of the compounds, achieving LODs between 0.12 and 3.50 ng mL^{-1} [62]. It is a universal and inexpensive detector that is very useful for routine analysis.

However, MS was the most common detection system used in the literature consulted. For the ionization of the sample, the main interface used is electrospray ionization (ESI). In the literature consulted, 80 studies indicate the use of this interface. ESI involves generating ions by applying a high voltage to a liquid, generating an aerosol. It is often used in the case of macromolecules, as they tend to fragment after ionization. Other interfaces used are atmospheric pressure chemical ionization (APCI) [54] and heated electrospray ionization [92]. In both cases, they use heat and a nebulization gas to form an aerosol and ionize the molecules in the gas phase. In some cases, thermal degradation may occur due to the use of heat, so this interface is often used when the analytes are heat stable and volatile. For that reason, articles consulted in the literature mainly used ESI as an interface, as the PhACs are generally high molecular weight compounds [85].

Based on MS resolution, two main categories are typically distinguished: low resolution (LRMS) and high resolution (HRMS) mass spectrometry. The former gives two decimal m/z digits and is commonly used in targeted analysis, while the latter offers higher resolving power which is advantageous in non-targeted analysis. In the reviewed works, LRMS, in particular, tandem mass spectrometry (MS/MS) using a triple-quadrupole mass analyzer (QqQ), is the most frequently used because of its increased selectivity, low LODs and improved S/N ratio. Multiple reaction monitoring mode (MRM) is particularly useful for the simultaneous determination of different classes of PhACs in one single

run and has been able to detect large amounts of analytes in complex matrices even in trace quantities [46,51,63,69]. López-García et al. [78] used a QqLit analyzer (quadrupole ion trap), consisting of three quadrupoles analyzers in which the last one acts as a linear ion trap, offering better sensitivity. In the determination of psychoactive substances in mussels, they achieved LODs below 2 ng g^{-1}, with high recoveries. Similarly, the use of other systems based on MS/MS, as the HCT (ultra ion trap) [87] and the QTRAP mass spectrometer [48,50,56], have been proposed. In contrast, it should be noted that only one study used a simple quadrupole analyzer. They determined three drugs in sea sponge, achieving detection limits between 0.01 and 10 ng g^{-1} with a recovery of 80% [84].

Likewise, the HRMS counterpart has undergone a noteworthy evolution in the last years. Although it is typically used in non-targeted analyzes when the compounds are unknown a priori, it has been shown to possess sufficient resolving power for quantitative purposes as well. It is especially useful for example to know the transformation products or identify compounds with the same molecular mass, thanks to the structural fragmentation patterns, the accurate mass, and the isotopic distribution. In light of this, analyzers such as Orbitrap or TOF, which also offer very good characteristics, have been employed in some of the revised works [41,48,57,60,62,91,92]. For example, Baesu et al. and Danesaki et al. used the Q-TOF for the determination of drugs from different families in fillet of fish, reaching LODs of 0.2–2.6 ng g^{-1} and 20–200 ng g^{-1}, respectively [57,60]. Kalogeropoulou et al. used a Q-Orbitrap MS achieving limits of quantification (LOQs) between 0.5–19 ng g^{-1} for the analysis of several antibiotics, antiepileptics and antidepressants in fish muscle [62].

5. Conclusions and Future Perspectives

Advances in analytical tools and instrumentation have allowed the development of a high number of sensitive and selective methods to determinate a broad range of PhACs in complex matrices, such as aquatic organisms. The present work provides an overview of the recent available methodologies for the analysis of PhACs in aquatic biota from different levels of the food chain. Among the PhACs, most investigated were antibiotics (ciprofloxacin, trimethoprim, and sulfamethoxazole), non-steroidal anti-inflammatory drugs (NSAIDs), analgesics (diclofenac, ibuprofen, naproxen and acetaminophen), antidepressants (venlafaxine) and antihypertensive drugs (propranolol and metoprolol), in this order, which also corresponds to those most accepted and consumed by the human population. Other groups, such as the cholesterol-lowering, antidiabetic and anticancer drugs, which have greatly increased in the last decade, have occasionally been considered in the studies consulted [98]. In addition, it should be noted that limited research has been conducted to analyze their transformation products (metabolites and degradation products) which emphasizes the need to develop analytical methods to cover this gap.

In relation to the studied taxonomic groups in the determination of PhACs, fish has been the most extensively organism investigated (33%), followed by molluscs (29%) and crustaceans (17%). In contrast, there are few proposed methods to assess the presence of these compounds in echinoderms (1%), and in biota of the first level of the food chain such as algae (2%), phytoplankton (5%), or zooplankton (8%). Therefore, more studies are needed to analyze PhACs at the lowest levels of the food chain, such as producers and benthic primary consumers, since the latter seem to be the main bioaccumulators for filter-feeding [97]. This would help to broaden the knowledge about the trophic transfer of PhACs, a barely explored field.

Given the complexity of biota matrices, special attention has been played to the sample preparation step, both extraction and purification, to obtain clean extracts and not compromise instrument sensitivity due

to matrix effects. The extraction step is key in determining the analytical parameters of the method. As far as extraction techniques, extraction using ultrasound (USE, FUSLE) has been the most attractive alternative, used in 36% of the studies consulted, followed by PLE, used in 30% of the studies. Both techniques provide automatization, short extraction times and low solvent consumption, compared to other techniques, such as traditional Soxhlet extraction. ACN, MeOH and water have been the solvents of choice for UAE while for PLE, in addition to these, the combination of acetone and MeOH has been extensively used. However, other green techniques should be explored for the extraction of these compounds to further reduce solvent and extraction time, such as aqueous two-phase systems (ABS), which remove volatile organic compounds and have very promising prospects. For clean-up, SPE using cartridges has shown to be an efficient method and the most popular used as a clean-up procedure (71% of the studies), regardless of the aquatic organism under study. Polymeric reversed-phase sorbents, and in particular Oasis HLB cartridges, have been the most suitable par excellence. Future trends in PhACs analysis in biota may include the design of on-line extraction techniques to reduce sample handling and avoid tedious sample treatments.

Finally, UHPLC-MS/MS has shown to be the most widely used technology for the analysis of PhACs due to the benefits it can offer. On the one hand, there has been a trend towards the use of UHPLC since, unlike HPLC, it operates at higher pressures and provides better resolution due to shorter column lengths and smaller particle sizes. On the other hand, its coupling with MS/MS detection is advantageous as it provides high sensitivity and selectivity, allowing quantification in the low ng L^{-1} or ng g^{-1}. It should also be noted that some recent works, instead, have used HRMS (Orbitrap or QTOF analyzers) for determining PhACs in the organisms under study being able to distinguish between compounds with comparable masses.

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References

1. Landrigan, P.J.; Stegeman, J.J.; Fleming, L.E.; Allemand, D.; Anderson, D.M.; Backer, L.C.; Brucker-Davis, F.; Chevalier, N.; Corra, L.; Czeruckall, D.; et al. Human health and ocean pollution. *Ann. Glob. Health* **2020**, *86*, 151. https://doi.org/10.5334/aogh.2831.
2. European Environmental Agency. Contaminants in Europe's Seas Moving Towards a Clean, Non-Toxic Marine Environment. EEA Report 2019, 25/2018. Available online: https://www.eea.europa.eu/publications/contaminants-in-europes-seas (accessed on 3 november 2022).
3. Lomartire, S.; Marques, J.C.; Gonçalves, A.M.M. Biomarkers based tools to asses environmental and chemical stressors in aquatic

systems. *Ecol. Indic.* **2021**, *122*, 107207. https://doi.org/10.1016/j.ecolind.2020.107207

4. Zhang, C.; Barron, L.; Sturzenbaum, S. The transportation, transformation and (bio)accumulation of pharmaceuticals in the terrestrial exosystem. *Sci. Total Environ.* **2021**, *781*, 146684. https://doi.org/10.1016/j.scitotenv.2021.146684.
5. Vagi, M.C.; Petsas, A.S.; Kostopoulou, M.N. Potential effect of persistent organic contaminants on Marine Biota: A review on recent research. *Water* **2021**, *13*, 2488. https://doi.org/10.3390/w13182488.
6. Arnold, K.E.; Brown, A.R.; Ankley, G.T.; Sumpter, J.P. Medicating the environment: Assessing risks of pharmaceuticals to wildlife and ecosystems. *Phil. Trans. R. Soc. B* **2014**, *369*, 20130569. http://dx.doi.org/10.1098/rstb.2013.0569.
7. Ortúzar, M.; Esterhuizen, M.; Olicón-Hernández, D.R.; González-López, J.; Aranda, E. Pharmaceutical pollution in aquatic environments: A concise review of environmental impacts and bioremediation systems. *Front. Microbiol.* **2022**, *13*, 869332. https://doi.org/10.3389/fmicb.2022.869332.
8. Golbaz, S.; Yaghmaeian, K.; Isazadeh, S.; Zamanzadeh, M. Environmental risk assessment of multiclass pharmaceutical active compounds: Selection of high priority concern pharmaceuticals using entropy-utility functions. *Environ. Sci. Pollut. Res.* **2021**, *28*, 59745–59770. https://doi.org/10.1007/s11356-021-14693-w.
9. Prionti, C.; Ricciardi, M.; Proto, A.; Bianco, P.M.; Montano, L.; Motta, O. Endocrine-disrupting compounds: An overview on their occurrence in the aquatic environment and human exposure. *Water* **2021**, *13*, 1347. https://doi.org/10.3390/w13101347.
10. Ramírez-Morales, D.; Masís-Mora, M.; Montiel-Mora, J.R.; Cambronero-Heinrichs, J.C.; Pérez-Rojas, G.; Tormo-Budowski, R.; Méndez-Rivera, M.; Briceño-Guevara, S.; Gutiérrez-Quirós, J.A.; Arias-Mora, V.; et al. Multi-residue analysis of pharmaceuticals in water samples by liquid chromatography-mass spectrometry: Quality assessment and application to the risk assessment of urban-influenced surface waters in a metropolitan area of Central America.

Process Saf. Environ. **2021**, *153*, 289–300. https://doi.org/10.1016/j.psep.2021.07.025.

11. Cravo, A.; Silva, S.; Rodrigues, J.; Cardoso, V.V.; Benoliel, M.J.; Correia, C.; Coelho, M.R.; Rosa, M.J.; Almeida, C.M.M. Understanding the bioaccumulation of pharmaceutical active compounds by clams *Ruditapes decussatus* exposed to a UWWTP discharge. *Environ. Res.* **2022**, *208*, 112632. https://doi.org/10.1016/j.envres.2021.112632.
12. Blanco, G.; Junza, A.; Barrón, D. Occurrence of veterinary pharmaceuticals in golden eagle nestlings: Unnoticed scavenging on livestock carcasses and other potential exposure routes. *Sci. Total Environ.* **2017**, *586*, 355–361. http://dx.doi.org/10.1016/j.scitotenv.2017.02.023.
13. Wu, Q.; Pan, C.-G.; Wang, Y.-H.; Xiao, S.-K.; Yu, K-F. Antibiotics in a subtropical food web from the Beibu Gulf, South China: Occurrence, bioaccumulation and trophic transfer. *Sci. Total Environ.* **2021**, *751*, 141718. https://doi.org/10.1016/j.scitotenv.2020.141718.
14. Water Framework Directive (WFD) 2000/60/EC. Available online: https://www.eea.europa.eu/policy-documents/water-framework-directive-wfd-2000 (accessed on 27 September 2022).
15. Marine Strategy Framework Directive 2008/56/EC. Available online: https://www.eea.europa.eu/policy-documents/2008-56-ec (accessed on 27 September 22).
16. European Union. Commission Implementing Decision (EU) 2022/1307 of 22 July 2022 Establishing a Watch List of Substances for Union-Wide Monitoring in the Field of Water Policy Pursuant to Directive 2008/105/EC of the European Parliament and of the Council. *OJEU* 2022; 197/117. Available online: https://euroalert.net/en/oj/105661/commission-implementing-decision-eu-2022-1307-of-22-july-2022-establishing-a-watch-list-of-substances-for-union-wide-monitoring-in-the-field-of-water-policy-pursuant-to-directive-2008-105-ec-of-the-european-parliament-and-of-the-council-notified-under-document-c-2022-5098 (accessed on 3 november 2022).

17. Richardson, S.D.; Ternes, T.A. Water analysis: Emerging contaminants and current issues. *Anal. Chem.* **2018**, *90*, 398–428. https://doi.org/10.1021/acs.analchem.7b04577.
18. Huerta, B.; Rodríguez-Moraz, S.; Barceló, D. Pharmaceuticals in biota in the aquatic environment: Analytical methods and environmental implications. *Anal. Bioanal. Chem.* **2012**, *404*, 2611–2624. https://doi.org/10.1007/s00216-012-6144-y.
19. McCarthy, J.F.; Shugart, L.R. *Biomarkers of Environmental Contamination*, 1st ed.; Publisher: J.F. McCarthy and L.R Shugart. United States, 1990. https://doi.org/10.1201/9781351070263.
20. Vidal-Liñan, L.; Bellas, J.; Campillo, J.A.; Beiras, R. Integrated use of antioxidant enzymes in mussels, *Mytilus galloprovincialis,* for monitoring pollution in highly productive coastal areas of Galicia (NW Spain). *Chemosphere* **2010**, *78*, 265–272. https://doi.org/10.1016/j.chemosphere.2009.10.060.
21. Ríos-Fuster, B.; Alomar, C.; Paniagua González, G.; Garcinuño Martínez, R.M.; Soliz Rojas, D.; Fernández Hernando, P. Assessing microplastic ingestion and occurrence of bisphenols and phthalates in bivalves, fish and holothurians from a Mediterranean marine protected area. *Environ. Res.* **2022**, *214*, 114034. https://doi.org/10.1016/j.envres.2022.114034.
22. Fossi, M.C.; Pedà, C.; Compa, M.; Tsangaris, C.; Alomar, C.; Claro, F.; Ioakeimidis, C.; Galgani, F.; Hema, T.; Deudero, S.; et al. Bioindicators for monitoring marine litter ingestions and its impacts on Mediterranean biodiversity. *Enriron. Pollut.* **2018**, *237*, 1023–1040. https://doi.org/10.1016/j.envpol.2017.11.019.
23. Bartolomé, L.; Etxebarria, N.; Martínez-Arkarazo, I.; Raposo, J.C.; Usobiaga, A.; Zuloaga, O.; Raingeard, D.; Cajaraville, M.P. Distribution of organic microcontaminants, butyltins, and metals in mussels from the Estuary of Bilbao. *Arch. Environ. Contam. Toxicol.* **2010**, *59*, 244–254. https://doi.org/10.1007/s00244-009-9458-9.
24. Viñas, L.; Pérez-Fernández, B.; Soriano, J.A.; López, M.; Bargiela, J.; Alves, I. Limpet (*Patella* sp*)* as a biomonitor for organic pollutants. A

proxy for mussel? *Mar. Pollut. Bull.* **2018**, *133*, 271–270. https://doi.org/10.1016/j.marpolbul.2018.05.046.

25. Qu, Y.; Zhang, T.; Zhang, R.; Wang, X.; Zhang, Q.; Wang, Q.; Dong, Z.; Zhao, J. Integrative assessment of biomarker responses in *Mytilus galloprovincialis* exposed to seawater acidification and copper ions. *Sci. Total Environ.* **2022**, *851*, 158146. http://dx.doi.org/10.1016/j.scitotenv.2022.158146.
26. Ghosh, D.; Ghosh, A.; Bhadury, P. Arsenic through aquatic trophic levels: Effects, tansformations and biomagnification- a concise review. *Geosci. Lett.* **2022**, *9*, 20. https://doi.org/10.1186/s40562-022-00225-y.
27. Yang, H.; Lu, G.; Yan, Z.; Liu, J.; Dong, H.; Bao, X.; Zhang, X.; Sun, Y. Residues, bioaccumulation and trophic transfer of pharmaceuticals and personal care products in highly urbanizad rivers affected by water diversion. *J. Hazard. Mater.* **2020**, *391*, 122245. https://doi.org/10.1016/j.jhazmat.2020.122245.
28. Tang, J.; Wang, S.; Tai, Y.; Tam, N.F.; Su, L.; Shi, Y.; Luo, B.; Tao, R.; Yang, Y.; Zheng, X. Evaluation of factors influencing annual occurrence, bioaccumulation and biomagnification of antibiotics in planktonic food webs of a large subtropical river in South China. *Water Res.* **2020**, *170*, 115302. https://doi.org/10.1016/j.watres.2019.115302.
29. Yan, N.; Long, S.; Xiong, K.; Zhang, T. Antibiotic bioaccumulation in zooplankton from the Yelang Lake Reservoir of Anshun City, Southwest China. *Pol. J. Environ. Stud.* **2022**, *31*, 2367–2380. https://doi.org/10.15244/pjoes/143774.
30. Moreira, F.W.A.; Leite, M.G.P.; Fijaco, M.A.G.; Mendoça, F.P.C.; Campos, L.P.; Eskinazi-Sant'Anna, E.M. Assessing the impacts of mining activities on zooplankton functional diversity. *Acta Limol. Bras.* **2016**, *28*, 107. http://dx.doi.org/10.1590/S2179-975X0816.
31. Cole, M.; Lindeque, P.; Fileman, E.; Halsband, C.; Goodhead, J.M.; Galloway, T.S. Microplastic ingestion by Zooplankton. *Environ. Sci. Technol.* **2013**, *47*, 6646–6655. https://doi.org/10.1021/es400663f.

32. Xu, X.; Pan, B.; Shu, F.; Chen, X.; Xu, N.; Ni, J. Bioaccumulation of 35 metal(loid)s in organis of a freshwater mussel (*Hyriopsis cumingii)* and environmental implications in Poyang Lake, China. *Chemosphere* **2022**, *307*, 136150. https://doi.org/10.1016/j.chemosphere.2022.136150.
33. Sharma, J.; Behera, P.K. Abundance & distribution of aquatic macro-invertebrate families of river Ganga and correlation with environmental parameters. *Environ. Monit. Assess.* **2022**, *194*, 546. https://doi.org/10.1007/s10661-022-10158-w.
34. Grabicová, K.; Stanová, A.V.; Svecová, H.; Nováková, P.; Kodes, V.; Leontovycová, D.; Brooks, B.W.; Grabic, R. Invertebrates differentially bioaccumulate pharmaceuticals: Implications for routine biomonitoring. *Environ. Pollut.* **2022**, *309*, 119715. https://doi.org/10.1016/j.envpol.2022.11971.
35. Álvarez-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* **2015**, *136*, 174–182. http://dx.doi.org/10.1016/j.talanta.2014.12.035.
36. Burket, S.R.; Sapozhnikova, Y.; Zheng, J.S.; Chung, S.S.; Brooks, B.W. At the Intersection of urbanization, water, and food security: Determination of select contaminants of emerging concern in mussels and oysters from Hong Kong. *J. Agric. Food Chem.* **2018**, *66*, 5009–5017. https://doi.org/10.1021/acs.jafc.7b05730.
37. Ojemaye, C.Y.; Petrik, L. Pharmaceuticals and personal care products in the marine environment around False Bay, Cape Town, South Africa: Occurrence and risk assessment study. *Environ. Toxicol. Chem.* **2022**, *41*, 614–634. https://doi.org/10.1002/etc.5053.
38. Zhou, L.-J.; Wang, W.-X.; Lv, Y.-J.; Mao, Z.-G.; Chen, C.; Wu, Q.L. Tissue concentrations, trophic transfer and human risks of antibiotics in freshwater food web in Lake Taihu, China. *Ecotoxicol. Environ. Saf.* **2020**, *197*, 110626. https://doi.org/10.1016/j.ecoenv.2020.110626.
39. Xie, H.; Hao, H.; Xu, N.; Liang, X.; Gao, D.; Xu, Y.; Gao, Y.; Tao, H.; Wong, M. Pharmaceuticals and personal care products in water,

sediments, aquatic organisms, and fish feeds in the Pearl River Delta: Occurrence, distribution, potential sources, and health risk assessment. *Sci. Total Environ.* **2019**, *659*, 230–239. https://doi.org/10.1016/j.scitotenv.2018.12.222.

40. Martínez-Morcillo, S.; Rodríguez-Gil, J.L.; Fernández-Rubio, J.; Rodríguez-Mozaz, S.; Prado Míguez-Santiyán, M.; Valdes, M.E.; Barceló, D.; Valcárcel, Y. Presence of pharmaceutical compounds, levels of biochemical biomarkers in seafood tissues and risk assessment for human health: Results from a case study in North-Western Spain. *Int. J. Hyg. Environ. Health* **2020**, *223*, 10–21. https://doi.org/10.1016/j.ijheh.2019.10.011.
41. Fonseca, V.F.; Duarte, I.A.; Duarte, B.; Freitas, A.; Vila Pouca, A.S.; Barbosa, J.; Gillanders, B.M.; Reis-Santos, P. Environmental risk assessment and bioaccumulation of pharmaceuticals in a large urbanized estuary. *Sci. Total Environ.* **2021**, *783*, 147021. https://doi.org/10.1016/j.scitotenv.2021.147021.
42. Chen, H.; Liu, S.; Xu, X.-R.; Liu, S.-S.; Zhou, G.-J.; Sun, K.-F.; Zhao, J.-L.; Ying, G-G. Antibiotics in typical marine aquaculture farms sourrounding Hailing Island, South China: Occurrence, bioaccumulation and human dietary exposure. *Mar. Pollut. Bull.* **2015**, *90*, 181–187. http://dx.doi.org/10.1016/j.marpolbul.2014.10.053.
43. Zheng, W.; Yoo, K.-H.; Choi, J.-M.; Park, D.-H.; Kim, S.-K.; Kang, Y.-S.; El-Aty, A.M.A.; Hacimüftüoglu, A.; Wang, J.; Shim, J.-H.; Shin, H-C. Residual detection of naproxen, methyltestosterone and 17 α-hydroxyprogesterone caproate in aquatic products by simple liquid-liquid extraction method coupled with liquid Chromatography-tandem mass spectrometry. *Biomed. Chromatogr.* **2019**, *33*, e4396. https://doi.org/10.1002/bmc.4396.
44. Ruan, Y.; Lin, H.; Zhang, X.; Wu, R.; Zhang, K.; Leung, K.M.Y.; Lam, J.C.W.; Lam, P.K.S. Enantiomer-specific bioaccumulation and distribution of chiral pharmaceuticals in a subtropical marine food web. *J. Hazard. Mater.* **2020**, *394*, 122589. https://doi.org/10.1016/j.jhazmat.2020.122589.

45. Mastángelo, M.M.; Valdés, M.E.; Eissa, B.; Ossana, N.A.; Barceló, D.; Sabater, S.; Rodríguez-Mozaz, S.; Giorgi, A.D.N. Occurrence and accumulation of pharmaceutical products in water and biota of urban lowland rivers. *Sci. Total Environ.* **2022**, *828*, 154303. https://doi.org/10.1016/j.scitotenv.2022.154303.
46. Pashaei, R.; Dzingeleviciene, R.; Abbasi, S.; Szultka-Mlynska, M.; Buszewski, B. Determination of 15 pharmaceutical residues in fish and shrimp tissues by high-performance liquid chromatography-tandem mass spectrometry. *Environ. Mon. Assess.* **2022**, *194*, 325. https://doi.org/10.1007/s10661-022-09993-8.
47. Mello, F.V.; Cunha, S.C.; Fogaça, F.H.S.; Alonso, M.B.; Torres, J.P.M.; Fernandes, J.O. Occurrence of pharmaceuticals in seafood from two Brazilian coastal areas: Implication for human risk assessment. *Sci. Total Environ.* **2022**, *803*, 149744. https://doi.org/10.1016/j.scitotenv.2021.149744.
48. Moreno-González, R.; Rodríguez-Mozaz, S.; Huerta, B.; Barceló, D.; León, V.M. Do pharmaceuticals bioaccumulate in marine molluscs and fish from a coastal lagoon? *Environ. Res.* **2016**, *146*, 282–298. http://dx.doi.org/10.1016/j.envres.2016.01.001.
49. Ojemaye, C.Y.; Petrik, L. Occurrences, levels and risk assessment studies of emerging pollutants (pharmaceuticals, perfluoroalkyl and endocrine disrupting compounds) in fish samples from Kalk Bay harbour, South Africa. *Environ. Pollut.* **2019**, *252*, 562–572. https://doi.org/10.1016/j.envpol.2019.05.091.
50. Tanoue, R.; Nozaki, K.; Nomiyama, K.; Kunisue, T.; Tanabe, S. Rapid analysis of 65 pharmaceuticals and 7 personal care products in plasma and whole-body tissue samples of fish using acidic extraction, zirconia-coated silica cleanup, and liquid Chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2020**, *1631*, 461586. https://doi.org/10.1016/j.chroma.2020.461586.
51. Vitale, D.; Picó, Y.; Álvarez-Ruiz, R. Determination of organic pollutants in *Anguilla anguilla* by liquid Chromatography coupled with tandem mass spectrometry (LC-MS/MS). *MEthodsX* **2021**, *8*, 101342. https://doi.org/10.1016/j.mex.2021.101342.

52. Ali, A.M.; Thorsen-Ronning, H.; Sydnes, L.K.; Alarif, W.M.; Kallenborn, R.; Al-Lihaibi, S.S. Detection of PPCPs in marine organisms from contaminated coastal waters of the Saudi Red Sea. *Sci. Total Environ.* **2018**, *621*, 654–662. https://doi.org/10.1016/j.scitotenv.2017.11.298.
53. Rojo, M.; Álvarez-Muñoz, D.; Dománico, A.; Foti, R.; Rodriguez-Mozaz, S.; Barceló, D.; Carriquiriborde, P. Human pharmaceuticals in three major fish species from the Uruguay River (South America) with different feeding habits. *Environ. Pollut.* **2019**, *252*, 146–154. https://doi.org/10.1016/j.envpol.2019.05.099.
54. Borik, A.; Stanová, A.V.; Brooks, B.W.; Grabicová, K.; Randák, T.; Grabic, R. Determination of citalopram in fish brain tissue: Benefits of coupling laser diode thermal desorption with low- and high-resolution mass spectrometry. *Anal. Bioanal. Chem.* **2021**, *412*, 4353–4361. https://doi.org/10.1007/s00216-020-02672-y.
55. Álvarez-Muñoz, D.; Rodríguez-Mozaz, S.; Jacobs, S.; Serra-Compte, A.; Cáceres, N.; Sioen, I.; Verbeke, W.; Barbosa, V.; Ferrari, F.; Fernández-Tejedor, M.; et al. Pharmaceuticals and endocrine disruptors in raw and cooked seafood from European market: Concentrations and human exposure levels. *Environ. Int.* **2018**, *119*, 570–581. https://doi.org/10.1016/j.envint.2018.07.006.
56. Bobrowska-Korczak, B.; Stawarska, A.; Szterk, A.; Ofiara, K.; Czerwonka, M.; Giebultowicz, J.; Determination of pharmaceuticals, heavy metals and oxysterols in fish muscle. *Molecules* **2021**, *26*, 1229. https://doi.org/10.3390/molecules26051229.
57. Danesaki, M.E.; Bletsou, A.A.; Koulis, G.A.; Thomaidis, N.S. Qualitative multiresidue screening method for 143 veterinary drugs and pharmaceuticals in milk and fish tissue using liquid Chromatography quadrupole-time-of-flght mass spectrometry. *J. Agric. Food Chem.* **2015**, *63*, 4493–4508. https://doi.org/10.1021/acs.jafc.5b00962.
58. Argüello-Pérez, M.A.; Ramírez-Ayala, E.; Mendoza-Pérez, J.A.; Monroy-Mendieta, M.M.; Vázquez-Guevara, M.; Lezama-Cervantes, C.; Godínez-Domínguez, E.; Silva-Bátiz, F.D.A.; Tintos-Gómez, A.

Determination of the Bioaccumulative Potential Risk of emerging contaminants in fish muscle as an environmental quality indicator in Coastal Lagoons of he Central Mexican Pacific. *Water* **2020**, *12*, 22721. https://doi.org/10.3390/w12102721.

59. Huang, P.; Zhao, P.; Dai, X.; Hou, X.; Zhao, L. Trace determination of antibacterial pharmaceuticals in fishes by microwave-assisted extraction and solid-phase purification combined with dispersive liquid-liquid microextraction followed by ultra-high performance liquid Chromatography-tandem mass spectrometry. *J. Chromatogr. B* **2016**, *1011*, 136–144. http://dx.doi.org/10.1016/j.jchromb.2015.12.059.
60. Baesu, A.; Ballash, G.; Mollenkopf, D.; Wittum, T.; Sulliván, S.M.P.; Bayen, S. Suspect screening of pharmaceuticals in fish livers based on QuEChERs extraction coupled with high resolution mass spectrometry. *Sci. Total Environ.* **2021**, *783*, 146902. https://doi.org/10.1016/j.scitotenv.2021.146902.
61. Miossec, C.; Mille, T.; Lanceleur, L.; Monperrus, M. Simultaneous determination of 42 pharmaceuticals in seafood samples by solvent extraction coupled to liquid Chromatography-tandem mass spectrometry. *Food Chem.* **2020**, *322*, 126765. https://doi.org/10.1016/j.foodchem.2020.126765.
62. Kalogeropoulou, A.G.; Kosma, C.I.; Albanis, T.A. Simultaneous determinaton of pharmaceuticals and metabolites in fish tissue by QuEChERs extracion and UHPLC Q/Orbitrap MS analysis. *Anal. Bioanal. Chem.* **2021**, *413*, 7129–7140. https://doi.org/10.1007/s00216-021-03684-y.
63. Gao, Y.; Wang, S.; Zhang, N.; Xum, X.; Bao, T. Novel solid-phase extraction filter based on a zirconium meta-organic framework for determination of non-steroidal anti-inflammatory drugs residues. *J. Chromatogr. A* **2021**, *1652*, 462349. https://doi.org/10.1016/j.chroma.2021.462349.
64. Danielle, G.; Fieu, M.; Joachim, S.; James-Casas, A.; Andres, S.; Baudoin, P.; Bonnard, M.; Bonnard, I.; Geffard, A.; Vulliet, E. Development of a multi-residue analysis of diclofenac and some

transformation products in bivalves using QuEChERs extraction and liquid Chromatography-tandem mass spectrometry. Application to samples from mesocosm studies. *Talanta* **2016**, *155*, 1–7. http://dx.doi.org/10.1016/j.talanta.2016.04.016.

65. McEneff, G.; Barron, L.; Kelleher, B.; Paull, B.; Quinn, B. The determination of pharmaceutical residues in cooked and uncooked marine bivalves using pressurized liquid extraction, solid-phase extraction and liquid Chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* **2013**, *405*, 9509–9521. https://doi.org/10.1007/s00216-013-7371-6.
66. Xie, Z.; Lu, G.; Yan, Z.; Liu, J.; Wang, P.; Wang, Y. Bioaccumulation and trophic transfer of pharmaceuticals in food webs from a large freshwater lake. *Environ. Pollut.* **2017**, *222*, 356–366. http://dx.doi.org/10.1016/j.envpol.2016.12.026.
67. Lavilla, I.; Bendicho, C. Chapter 1: Fundamentals of Ultrasound-Assisted Extraction. In *Water Extraction of Bioactive Compounds*, 1st ed.; Domínguez, H., González-Muñoz, M.J., Eds.; Elsevier: Amsterdam, The Netherlands, 2017; pp. 291–316. https://doi.org/10.1016/B978-0-12-809380-1.00011-5.
68. Blanco-Zubiaguirre, L.; Arrieta, N.; Iturregui, A.; Martinez-Arkarazo, M.; Olivares, M.; Castro, K.; Olazabal, M.A.; Madariaga, J.M. Focused ultrasound solid-liquid extraction for the determination of organic biomarkers in beachrocks. *Ultrason. Sonochem.* **2015**, *27*, 430–439. https://doi.org/10.1016/j.ultsonch.2015.06.012.
69. Martínez-Moral, M.P.; Tena, M.T. Focused ultrasound solid-liquid extraction and selective pressurized liquid extraction to determine bisphenol A and alkylphenols in sewage sludge by gas Chromatography-mass spectrometry. *J. Sep. Sci.* **2011**, *34*, 2513–2522. https://doi.org/10.1002/jssc.201100159.
70. Mijangos, L.; Ziarrusta, H.; Zabaleta, I.; Usobiaga, A.; Olivares, M.; Zuloaga, O.; Etxebarria, N.; Prieto, A. Multiresidue analytical method for the determination of 41 multiclass organic pollutants in mussel and fish tissues and biofluids by liquid Chromatography

coupled to tandem mass spectrometry. *Anal. Bioanal. Chem.* **2019**, *411*, 493–506. https://doi.org/10.1007/s00216-018-1474-z.

71. Richter, B.E.; Jones, B.A.; Ezzell, J.L.; Porter, N.L.; Avdalovic, N.; Pohl, C. Accelerated Solvent Extraction: A Technique for Sample Preparation. *Anal. Chem.* **1996**, *68*, 1033–1039. https://doi.org/10.1021/ac9508199.
72. Ruhí, A.; Acuña, V.; Barceló, D.; Huerta, B.; Mor, J.-R.; Rodríguez-Mozaz, S.; Sabater, S. Bioaccumulation and trophic magnification of pharmaceuticals endocrine disruptors in a Mediterranean river food web. *Sci. Total Environ.* **2016**, *540*, 250–259. http://dx.doi.org/10.1016/j.scitotenv.2015.06.009.
73. Destendau, E.; Michel, T.; Elfakir, C. Chapter 4. Microwave-assisted extraction. In *Natural Product Extraction: Principles and Applications*, 2nd ed.; Rostagno, M.A., Prado, J.M., Eds.; Royal Society of Chemistry: Cambridge, UK, 2013; pp. 113–498. https://doi.org/10.1039/9781849737579-00113.
74. Perestrelo, R.; Silva, P.; Porto-Figueira, P.; Pereira, J.A.M.; Silva, C.; Medina, S.; Câmara, J.S. QuEChERS- Fundamentals, relevant improvements, applications and future trends. *Anal. Chim. Acta* **2019**, *1070*, 1–28. https://doi.org/10.1016/j.aca.2019.02.036.
75. Luque de Castro, M.D.; Priego-Capote, F. Soxhlet extraction: Past and present panacea. *J. Chromatogr. A* **2010**, *1217*, 2383–2389. https://doi.org/10.1016/j.chroma.2009.11.027.
76. Gil-Solana, R.; Rodriguez-Mozaz, S.; Diaz-Cruz., M.S.; Sunyer-Caldú, A.; Luarte, T.; Höfer, J., Galbán-Malagón, C.; Gago-Ferrero, P. A protocol for wide-scope non-target analysis of contaminants in small amounts of biota using bead beating tissuelyser extraction and LC-HRMS. *MethodsX* **2021**, *8*, 101193. https://doi.org/10.1016/j.mex.2020.101193.
77. Oluseyi, T.; Olayinka, K.; Alo, B.; Smith, R.M. Comparison of extraction and clean-up techniques for the determination of polycyclic aromatic hydrocarbons in contaminated soil samples. *Afr. J. Environ. Sci. Technol.* **2011**, *5*, 482–493.

78. López-García, E.; Postigo, C.; López de Alda, M. Psychoactive substances in mussels: Analysis and occurrence assessment. *Mar. Pollut. Bull.* **2019**, *146*, 985–992. https://doi.org/10.1016/j.marpolbul.2019.07.042.
79. Boulard, L.; Parrhysius, P.; Jacobs, B.; Dierkes, G.; Wick, A.; Buchmeier, G.; Koschorreck, J.; Ternes, T.A. Development of an analytical method to quantify pharmaceuticals in fish tissues by liquid Chromatography-tandem mass spectrometry detection and application to environmental samples. *J. Chromatogr. A* **2020**, *1633*, 461612. https://doi.org/10.1016/j.chroma.2020.461612.
80. Raisglid, M.E.; Burke, M.F. Fundamentals of solid phase extraction and its application to environmental analyses. *Stud. Surf. Sci. Catal.* **1999**, *120*, 37–75. https://doi.org/10.1016/S0167-2991(99)80356-5.
81. Anand, S.; Srivastava, P. Optimization strategies for purification of Mycophenolic Acid produced by *Penicillium brevicompactum. Appl. Biochem. Biotechnol.* **2020**, *191*, 867–880. https://doi.org/10.1007/s12010-019-03204-w.
82. Islas, G.; Ibarra, I.S.; Hernandez, P.; Miranda, J.M.; Cepeda, A. Dispersive Solid Phase Extraction for the Analysis of Veterinary drugs applied to food samples: A review. *Int. J. Anal. Chem.* **2017**, *2017*, 8215271. https://doi.org/10.1155/2017/8215271.
83. Rimkus, G.G.; Rummler, M.; Nausch, I. Gel permeation Chromatography-high performance liquid Chromatography combination as an automated clean-up technique for the multiresidue analysis of fats. *J. Chromatogr. A* **1996,** *737*, 9–14. https://doi.org/10.1016/0021-9673(96)00020-9.
84. Miller, T.H.; McEneff, G.L.; Brown, R.J.; Owen, S.F.; Bury, N.R.; Barron, L.P. Pharmaceuticals in the freshwater invertebrate, *Gammarus pulex,* determinet using pulverised liquid extraction, solid phase extraction and liquid Chromatograpgy-tandem mass spectrometry. *Sci. Total Environ.* **2015**, *511*, 153–160. http://dx.doi.org/10.1016/j.scitotenv.2014.12.034.
85. Rizzi, C.; Seveso, D.; Galli, P.; Villa, S. First record of emerging contaminants in sponges of an inhabited island in the Maldives. *Mar.*

Pollut. Bull. **2020**, *156*, 111273. https://doi.org/10.1016/j.marpolbul.2020.111273.

86. Clarke, W. Mass spectrometry in the clinical laboratory: Determining the need and avoiding pitfalls. In *Mass Spectrometry in the Clinical Laboratory*, 1st ed.; Nair, H., Clarke, W., Eds.; Academic Press: Cambridge, MA, USA, 2017; pp. 1–15. https://doi.org/10.1016/C2013-0-19099-X.
87. Wagil, M.; Kumirska, J.; Stolte, S.; Puckowskim, A.; Maszkowska,; Stepnowski, P.; Bialk-Bielinska, A. Development of sensitive and reliable LC-MS/MS methods for the determination of three fluoroquinolones in water and fish tissue samples and preliminary environmental risk assessment of their presence in two rivers in nothern Poland. *Sci. Total Environ.* **2014**, *493*, 1006–1013. http://dx.doi.org/10.1016/j.scitotenv.2014.06.082.
88. Wilkinson, J.L.; Hooda, P.S.; Swinden, J.; Barker, J.; Barton, S. Spatial (bio)accumulation of pharmaceuticals, illicit drugs, plasticisers, perfluorinated compounds and metabolites in river sediment, aquatic plants and benthic organisms. *Environ. Pollut.* **2018**, *234*, 864–875. https://doi.org/10.1016/j.envpol.2017.11.090.
89. Ding, D.; Lu, G.; Liu, J.; Yang, H.; Li, Y. Uptake, depuration and bioconcentration of two pharmaceuticals, roxithromycin and propranolol, in *Daphnia magna*. *Ecotoxicol. Environ. Saf.* **2016**, *126*, 85–93. http://dx.doi.org/10.1016/j.ecoenv.2015.12.020.
90. Kim, H.Y.; Jeon, J.; Hollender, J.; Yu, S.; Kim, S.D.; Aqueous and dietary bioaccumulation of antibiotic tetracycline in *D. magna* and its multigenerational transfer. *J. Hazard. Mater.* **2014**, *279*, 428–435. http://dx.doi.org/10.1016/j.jhazmat.2014.07.031.
91. Fu, Q.; Meyer, C.; Patrick, M.; Kosfeld, V.; Rüdel, H.; Koschorreck, J.; Hollender, J. Comprehensive screening of polar emerging organic contaminants including PFASs and evaluation of the trophic transfer behavior in a freshwater food web. *Water Res.* **2022**, *218*, 118514. https://doi.org/10.1016/j.watres.2022.118514.
92. Martínez-Bueno, M.J.; Boillot, C.; Fenet, H.; Chiron, S.; Casellas, C.; Gómez, E. Fast and easy extraction combined with high resolution-

mass spectrometry for residue analysis of two anticonvulsants and their transformation products in marine mussels. *J. Chromatogr. A* **2013**, *1305*, 27–34. http://dx.doi.org/10.1016/j.chroma.2013.06.071.

93. Núñez, M.; Borrull, F.; Pocurull, E.; Fontanals, N. Pressurized liquid extraction followed by liquid Chromatography with tandem mass spectrometry to determine pharmaceuticals in mussels. *J. Sep. Sci.* **2016**, *39*, 741–747. https://doi.org/10.1002/jssc.201501046.
94. Rodrigues, J.; Albino, S.; Silva, S.; Cravo, A.; Cardoso, V.V.; Benoliel, M.J.; Almeida, C.M.M. Development of a multiresidue method for the determination of 24 pharmaceuticals in clams by QuEChERs and liquid Chromatography-triple quadrupole tandem mass spectrometry. *Food. Anal. Methods* **2019**, *12*, 838–851. https://doi.org/10.1007/s12161-018-01418-y.
95. Kim, J.; Park, H.; Kang, H.-S.; Cho, B.-H.; Oh, J-H. Comparison of sample preparation and determination of 60 veterinary drugs residues in flatfish usin liquid Chromatography-tandem mass spectrometry. *Molecules* **2020**, *25*, 1206. https://doi.org/10.3390/molecules25051206.
96. Sun, Y.; Zhang, L.; Zhang, X.; Chen, T.; Dong, D.; Hua, X.; Guo, Z. Enhanced bioaccumulation of fluorinated antibiotics in crucian carp (*Carcassius carcassius):* Influence of fluorine substituent. *Sci. Total Environ.* **2020**, *748*, 141567. https://doi.org/10.1016/j.scitotenv.2020.141567.
97. Świacka, K.; Maculewicz, J.; Kowalska, D.; Caban, M.; Smolarz, K.; Świeżak, J. Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms—Current state of knowledge. *J. Hazard. Mater.* **2022**, *424*, 127350. https://doi.org/10.1016/j.jhazmat.2021.127350.
98. González-Peña, O.I.; López-Zavala, M.A.; Cabral-Ruelas, H. Pharmaceuticasls market, consumption, trends and disease incidence are not driving the pharmaceutical research on water and wastewater. *Int. J. Environ. Res. Public Health* **2021**, *18*, 2532. https://doi.org/10.3390%2Fijerph18052532

Capítulo 2

Ultra-high performance liquid chromatography tandem mass spectrometry analysis of UV filters in marine mussels *(Mytilus galloprovincialis)* from the Southern of Spain

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Abstract: Ultraviolet (UV) filters are a family of organic compounds widely used in sunscreens and personal care products (PCPs) as well as other materials such as plastics, toys and outdoor furniture, for their effectiveness in absorbing UVA and UVB radiation. These compounds directly enter the marine environment because of inefficient wastewater treatments and anthropogenic activities, posing a risk to the marine biota. The present study develops and validates a method to determine some of the most used UV filters (BP-1, BP-2, BP-3, BP-6, BP-8, 4-OH-BP and 4-MBC), in wild *Mytilus galloprovincialis* mussels using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). The sample treatment is based on an ultrasound-assisted extraction followed by a clean-up step using C18 as sorbent. The methodology was satisfactorily validated, obtaining good features, and it was applied for the evaluation of the occurrence of the target analytes in mussels collected in five areas along the tourist coast of Granada (Spain) just after summer holydays period. The results showed a higher bioaccumulation in specimens sampled in recreational areas and with a closed geomorphology, being BP-3 the most predominant in all locations. BP-1 and BP-3 were quantified in all samples and the rest of UV filters were detected in most of them, except for BP-2. The data raises concern about the undesirable effects that UV filter pollution can cause in the area and highlights the need to establish practices that help preserve and sustain the marine ecosystem.

Keywords: Ultraviolet filters; Biomonitoring; Mussels; Acid digestion; Ultrasound assisted extraction; UHPLC-MS/MS.

1. Introduction

UV filters are organic compounds with aromatic structures capable of absorbing solar radiation. Therefore, many personal care products (PCPs) include different families of these substances such as camphor or benzophenone derivatives, among others. UV filters are able to absorb solar radiation UV-A (320 - 400 nm) and UV-B (280 - 320 nm) [1-3]. 4-Methylbenzylidene camphor (4-MBC) is one of the most widely used UV

filter. This compound cannot be completely removed by the traditional wastewater treatment process. Some studies show that in a Chinese treatment plant, the removal efficiency of this compound is 40% [4,5]. Benzophenones (BPs), on the other side, are a family of UV filter compounds which have a high lipophilic character and are able to enter the body and bioaccumulate easily [6-10].

In recent years, there has been increasing concern about sun exposure and the associated risks. Thus, the consumption and use of products which contain UV filters for sun protection, including sunscreens, shampoos or hair dyes as well as plastics, paints or packaging of outdoor materials, has increased considerably [3]. Many UV filters are recognized as emerging pollutants and constitute a risk to human health and the environment. Toxicological studies have demonstrated their estrogenic effects and toxicity for reproduction and development, even when they are found in low concentrations, being considered endocrine disrupting chemicals (EDCs) [5]. EDCs are exogenous compounds capable of causing adverse effects on the health of an organism and its offspring by interfering with the normal functioning of the endocrine system. Within this category, UV filters have received special attention due to their high estrogenic activity, their widespread use and their ubiquitous presence in marine environments. The high occurrence and persistence, in which they are found, represent a potential danger for the organisms that inhabit these environments. An example is the alarming impact they have on coral reefs, inducing genotoxic alterations and rapid bleaching. For this reason, countries such as Hawaii or Florida have taken measures, and have banned the sale of sun creams containing benzophenone-3 (BP-3), one of the most widely used UV filters [2,3,11-13].

The entry of UV filters into the marine environment can be direct, through recreational activities of bathers on beaches and swimming pools, and the consequent release of these compounds through the skin; but also can be indirect, through discharges from wastewater treatment plants, laundry or urine. However, the primary cause is their extensive

use in the manufacture of household and cosmetic products and in the textile industry [14-16]. The efficiency of removal of these compounds depends on several factors such as the operational conditions, the technology used as well as the type of treatment being hardly completely eliminated [17,18]. UV filters pose a great health risk since they are able to enter into trophic chain and biomagnificate [16]. Many of these compounds tend to accumulate in tissues of aquatic organisms because they have several benzene rings and therefore, high lipophilicity. In addition, they pose high Kow values and they are very stable against biotic degradation [2]. The European Union has regulated the maximum concentration of these compounds in sunscreens and other PCPs, which must be between 0.1 and 10% [19]. Thus, according to Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products, the maximum content allowed in commercial products is up to 4% for the case of 4- MBC and 6% for BP-3 [19].

Some studies have demonstrated the accumulation of these compounds in aquatic organisms. Mussels have deserved our attention since they are very abundant in nature and have an important role in food chain due the human consumption. Mussels are filter feeders capable to retaining complex mixtures of contaminants, being bioindicators of high interest. They present some characteristics such as stress resistance, sessile behaviour or tolerance to changes in salinity, and they retain and accumulate pollutants found in the sea with higher concentration levels [2,12,20,21]. In fact, on the coasts of Norway, Portugal and France, concentration levels of contaminants > 800 - 1000 ng g-1 have been determined in cod, shrimp or mussels [3]. However, according to the literature consulted, there are few studies on the quantification of UV filters compounds in marine organisms such as mussels, and on the evaluation of the concentrations found according to the characteristics of the sampling area. Furthermore, none of them cover a large number of UV filters, and there is no data available in relation to the benzophenone family. The objectives of this study have been: (i) develop an UHPLC-MS/MS method for the determination of UV filters in

mussels trough an efficient extraction process capable of achieving low LODs, high recoveries and without interferences from other compounds; (ii) evaluate the presence of these compounds UV filters on the coast of Granada (Spain); and (iii) interpret and compare the results with previously reported available data, and evaluate the evolution.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade of standards: BP-1 (≥ 99%), BP-2 (≥ 97%), BP-3 (≥ 98%), BP-3d_5 (≥ 99%), BP-8 (≥ 98%), 4-OH-BP (≥ 98%) and 4-MBC (≥ 98%) were obtained from Sigma-Aldrich (Madrid, Spain). BP-6 (≥ 98%) was purchased from Alfa Aesar (Massachusetts, MA, USA). Chemical structures of compounds are shown in supplementary material (Table S1). Ultrapure water was purified by a Milli-Q Plus® purification system (Millipore, Madrid, Spain). LC-MS grade methanol (MeOH) was obtained by VWR Prolabo Chemicals (Barcelona, Spain), LC-MS grade acetonitrile (≥ 99.9%) (ACN), n-hexane for HPLC (≥ 99%) and ethyl acetate (AcOEt) were purchased from Honeywell (Madrid, Spain). Acetone LC grade was obtained from Panreac (Barcelona, Spain). Analytical grade formic acid (≥ 98%) used as a modifier of mobile phases and during the optimization of digestion was from Sigma-Aldrich. o-Phosphoric acid (85% w/w), perchloric acid (70%) and hydrochloric acid (6 mol L^{-1}) were obtained from Panreac. Acetic acid was bought from VWR ProLabo Chemicals. Sorbents used for clean-up PSA bonded silica, was obtained from Supelco (Bellfonte, Pennsylvania, USA) and Discovery® DSC-C18 SPE was from Sigma Aldrich. Stock solutions (100 mg L^{-1}) of analytes were prepared in MeOH and stored at -20 ºC until use.

2.2. Instrumentation and software

Ultrasound extraction was performed with a 400 W digital sonifier (Branson Ultrasonic Corporation), with a 0.5-inch (12.7 mm) probe operating at a frequency of 20 kHz. UHPLC-MS/MS analysis was performed using a Waters Acquity UPLC™ H-Class system. Separation of analytes was obtained with an Acquity UPLC® BEH C18 column (50

mm x 2.1 mm i. d., 1.7 µm particle size). A Xevo™ TQS tandem quadrupole mass spectrometer (Waters) equipped with StepWave ion guide and an orthogonal Z-spray™ electrospray ionization (ESI) source was used for UV-filters detection. Other laboratory equipment was a Mettler-Toledo GX400 balance, a Labnet Spectrafuge™ 24D centrifuge, a ScanVac Coolsafe™ freeze dryer for lyophilization of mussels. A vacuum centrifugal evaporator (LaboGene) was used to concentrate and evaporate sample extracts, an IKA vortex-mixer. For reconstitution, an Ultrasounds-HD ultrasonic bath from Selecta was used. MassLynx 4.1 software (Waters) was used for analysis and data acquisition, and Statgraphics plus 5.0 (Statpoint Technologies) combined with excel software were used for statistic treatment of data and experimental designs.

2.3. Specimen collection and acclimatization to experimental conditions

Mussels were collected by professional divers in the period of one month, between August and October 2020, just after summer holidays period, in the Mediterranean coast of the province of Granada (Spain). Five intertidal sites were selected along the coast, combining recreational areas and others non-bathing zones. The exact locations and coordinates are shown in supplementary material (Figure S1).

- Area 1. La Caleta (Salobreña). Area with residential and industrial activities.
- Area 2. Mouth of the river Jate (La Herradura). Area with tourist and vacation activity.
- Area 3. La Calita (La Herradura). Residential area with vacation use.
- Area 4. Las Azucenas beach (Motril). An undeveloped area near to an agricultural zone.
- Area 5. Roadstead of Calahonda (Motril). Area close to agricultural activities.

The specimens were transported refrigerated to the laboratory and then they were stored at -20 ºC until treatment. Acclimatization to experimental conditions consisted of opening the mussels and removing

the shell, collecting the soft tissues and mixing them until obtaining a homogeneous mixture. After being frozen, samples were then freeze-dried at -109 ºC of temperature and <12.8 Pa of pressure for about 72 h. Finally, the lyophilized mussels were crushed until obtaining a fine powder in order to improve the extraction efficiency of the target analytes, since the contact surface in this way will be greater. The crushing was made using a conventional mallet and mortar. Due to the limitation of sampled specimens, the optimization of extraction procedure as well as calibration and validation phases, were carried out with mussels purchased from a local supermarket in the city of Granada. These samples were treated following the same protocol indicated previously and before the optimization tests; different samples were analyzed in order to select those free of our target analytes. Finally, mussels from Delta de l'Ebre in Riumar (Tarragona, Spain) were selected as blank samples.

2.4. Experimental procedure

2.4.1. Preparation of spiked samples

The optimization and validation of the method was carried out using spiked samples. Briefly, the blank (0.5 g of lyophilized mussel) was spiked at 100 ng g^{-1} adding 50 µL of a methanolic solution containing the analytes and the surrogate (BP-$3d_5$). After fortification, the samples were accurately mixed and kept for at least 24 h in the dark at room temperature, in order to guarantee the contact of the analytes with the matrix as well as the evaporation of traces of organic solvent. For calibration and validation purposes samples were spiked in the range of 1-250 ng g^{-1} for all compounds, except for the surrogate standard which was added at 100 ng g^{-1} in all samples. Previously, all blank samples were analyzed to confirm the absence of the target analytes or other compounds that could interfere in the analysis. BP-$3d_5$ was also used as surrogate standard in the analysis of the specimens collected in the coast.

2.4.2. Sample treatment

Aliquots of 0.5 g of lyophilized mussel were weight in glass tubes, and homogenized with 3 mL of a solution of o-phosphoric acid (0.05 mol L^{-1})

in ACN:MeOH (75:25 v/v). The mixture was sonicated using an ultrasound digester at 10 % of amplitude for 15 minutes. After this time, an increase in temperature was observed in the extraction mixture. For this reason, the samples were kept in an ice-water bath during the sonification, and the water was exchanged for fresh water after each extraction stage. Two extraction cycles were done and thirteen samples were treated at the same time. After each extraction cycle, the extracts were centrifuged at 4000 rpm (2600 × *g*) for 5 min and the supernatant was collected and transferred in a new tube. The supernatants of the two extractions were pooled in the same tube, and the solvent was evaporated to dryness in the SpeedVac equipment (at 70 ºC and 2000 rpm (760 x *g*)). Then, in order to eliminate interferences, a clean-up process was carried out. The residue was dissolved in 2 mL of ACN and 0.4 g of C18 were added. The tube was then vortexed for 30 s and subsequently centrifuged at 4000 rpm (2600 × *g*) for 5 minutes. The organic layer was then transferred to another tube and evaporated to dryness. Finally, the residue was reconstituted in 250 µL of the initial mobile phase, transferred into a vial and injected in the UHPLC-MS/MS system.

2.4.3 UHPLC-MS/MS operating conditions

The separation of analytes was achieved using a gradient mobile phase consisting of water (solvent A) and MeOH (solvent B), both acidified with formic acid (0.1% v/v). Chromatographic separation was performed on an Acquity UPLC® BEH C18 column (50 mm x 2.1 mm i. d., 1.7 µm particle size), maintained at 40 ºC. Flow rate was 0.3 mL min^{-1} and injection volume for each vial was 10 µL. The gradient program is shown in Table 1.

Table 1. *Transitions and optimized parameters for UHPLC-MS/MS analysis of UV-filters*

	t_R	Transitions	CV	CE		t_R	Transitions	CV	CE
BP-1	3.9	215.1 → 104.5[b]	34	20	BP-8	4.2	245.2 → 120.5[b]	38	14
		215.1 → 136.6[a]	34	16			245.2 → 150.7[a]	38	18
BP-2	2.4	247.1 → 80.3[b]	8	36	4-OH-BP	3.3	199.2 → 104.5[b]	12	12
		247.1 → 136.6[a]	8	14			199.2 → 120.5[a]	12	18
BP-3	5.0	229.2 → 104.5[b]	20	16	4-MBC	6.1	255.2 → 157.1[b]	28	16
		229.2 → 150.6[a]	20	16			255.2 → 171.0[a]	28	16

BP-6	4.8	275.2 → 94.5[b]	2	36	BP-3d5	4.9	234.2 → 109.4[b]	40	16
		275.2 → 150.6[a]	2	16			234.2 → 150.6[a]	40	18

Chromatographic conditions			
Injection volume	10 µL	Solvent A	Water with 0.1% (v/v) of formic acid
Column	UPLC® BEH C_{18}	Solvent B	Methanol with 0.1% (v/v) of formic acid
Column temperature	40ºC	Flow rate	0.3 mL min^{-1}

Elution gradient	Time (min)	%A	%B
	0.0	60	40
	1.0	60	40
	6.0	10	90
	*8.0	10	90
	8.1	60	40
	10.0	60	40
	*8.0-8.1 min back to initial conditions		

Spectrometric conditions			
Impactor voltage	1 kV	Nebulizer gas pressure	7.0 bar
Source temperature	150ºC	Cone / desolvation gas	N_2 (≥99.995%)
Desolvation temperature	600ºC	Collision gas	Ar (99.999%)
Cone gas flow	150 L h^{-1}	Dwell time	25 ms
Desolvation gas flow	500 L h^{-1}	Inter-scan delay	3 ms

CV: Cone voltage (V); CE: Collision energy (eV). [a] *SRM transition used for quantification.* [b] *SRM transition used for confirmation*

Mass spectrometer (MS) worked with electrospray ionization (ESI) in positive ion mode, and mass spectrometric analysis were performed in multiple-reaction monitoring (MRM) mode in order to increase sensitivity and selectivity. MS/MS parameters were studied and optimized for each compound individually by infusing into the mass spectrometer of 1 µg mL^{-1} of a standard diluted in methanol. The two most sensitive MRM transitions were selected for each analyte for quantification and confirmation of compounds, and a time range for the detection of the target analytes was selected in order to improve the sensitivity of the analysis. The selected transitions as well as the optimum spectrometric parameters are also summarized in Table 1.

2.5. Factorial design 3^2

Factorial design 3^2 is used for the simultaneous optimization of two factors having three different levels under study. This experimental design was used in the optimization of UAE and in the optimization of the clean-up step. A total of 12 run samples with three central points and three levels for each factor were incorporated in the fitted model. The surface desirability responses of each analyte and the combinations of all of them were obtained, and the regression equations, the significance of each parameter and the interactions between them, were assessed through a variance analysis (ANOVA).

2.5. Validity requirements

To guarantee the validity of the results obtained, quality assurance/quality control (QA/QC) procedures were performed. To check for possible background contamination, procedural blanks were injected into the UHPLC equipment, which were processed the same as the samples. The presence of target analytes in the studied matrix was evaluated by analyzing sample blanks to assure that they were detected below the limits of quantification or non-detected. In none of the cases were the analytes detected in quantifiable amounts. A standard addition calibration curve was constructed for each studied compound at seven concentration level, and linearity was evaluated using determination coefficients (R^2). Variability of the instrumental analysis was tested with a standard solution at 100 ng mL^{-1} in the initial mobile phase that was injected every 10 analyses. All samples were prepared in triplicate and each one was injected three times.

3. Results and discussion

3.1. Extraction procedure optimization

A deep study was conducted on the most influential variables that affect the digestion and extraction process of mussel samples. The influence of solvent type, acid concentration or number of extraction cycles were investigated. Aliquots of 0.5 g of fortified blank samples (100 ng g^{-1}) were used.

3.1.1 Selection of extraction and volume solvent

A good solubility of target analytes in the solvent is critical to assure matrix separation and recovery. Therefore, the solvent used for the extraction is a crucial selection. Several solvents and mixtures of them were assayed: MeOH, ACN, AcOEt, acetone:hexane (50:50 v/v) and mixtures of MeOH:ACN (25:75 v/v, 50:50 v/v and 75:25 v/v). The selection was based on the published scientific literature for marine samples [3,11,22]. The results obtained have been included as supplementary material (Figure S2A). The solvent chosen as optimal was MeOH:ACN 25:75 (v/v) since the efficiency of extraction was higher in almost all cases. Individual MeOH and ACN also provided good recoveries. The rest of the solvents were not useful for extraction. Then, volumes from 1.5 to 4 mL were also tested using the optimal solvent. Results are also included in Figure S2 (B). The results show that 3 mL provided the best recovery for all analytes. Volumes below may not be sufficient to efficiently extract the target compounds. Poor recoveries were also obtained with 4 mL, which may be due to a higher matrix effect and the presence of interferences after the extraction procedure. Therefore, 3 mL of the mixture MeOH:ACN 25:75 (v/v) were selected for further experiments.

3.1.2. Optimization of digestion conditions

According to the scientific literature consulted, an acidic digestion could improve the extraction efficiency [15,18]. For this reason, it was compared a neutral extraction and acid extraction testing several acids at the same concentration (0.1 mol L^{-1}) in the extraction solvent. The five acids assayed were: hydrochloric acid, perchloric acid, o-phosphoric acid, formic acid and acetic acid. The results obtained demonstrated that in all cases, an acidic pH favors the extraction. For target analytes, the highest recoveries were provided with o-phosphoric acid. The results of the extraction efficiencies comparison of each acid are also included as supplementary material (Figure S3A). On the other hand, once the acid was chosen, its concentration was optimized. Concentrations between 0.01 mol L^{-1} and 0.20 mol L^{-1} of o-phosphoric acid were evaluated. The results showed that 0.05 mol L^{-1} offered good extraction efficiency in most

of analytes while higher concentrations appeared to have a negative effect. Only in the case of BP-8 and 4-OH-BP, higher acid concentrations achieve better extraction (See Figure S3B). Therefore, as a selection criterion, by compromise of all the target analytes, the intermediate concentration 0.05 mol L^{-1} of acid was chosen because it provided the best results for most of them, and an increase in the recovery of BP-3 up to 50%.

3.1.3. Optimization of the number of extraction cycles

Before studying other variables that affect the UAE, the number of extraction cycles was evaluated. Fortified mussel samples were mixed with 3 mL of the solvent, and subjected to 1, 2 or 3 extractions as appropriate, at 40% of amplitude for 15 min each cycle. For some of the analytes (BP-3, BP-6 and BP-8), the best extraction efficiencies were achieved with the third extraction. For the remaining four UV filters, a second cycle was sufficient. Given the low improvement comparing two and three cycles, and to minimize the use of organic solvent and the time employed, it was decided to use 2 extraction cycles as a compromise. The results obtained are included as supplementary material (Figure S4).

3.1.4. Optimization of the extraction time and amplitude

Once the number of extraction cycles were selected, the time and amplitude of the ultrasound extraction were optimized through a factorial experimental design 3^2. Three levels of each variable were considered: extraction time (1, 10, and 30 min) and power of ultrasound (10, 40, and 70% of amplitude). The matrix design resulted in 12 analytical experiments with three central points. The conditions of experiments are shown in supplementary material (Table S2). ANOVA provided determination coefficients (R^2) higher than 60 % for most of the target analytes. P values for the lack-of-fit test were $P_{lof} > 0.05$ in all cases with a 95 % of confidence level, showing that the model is satisfactory. Pareto charts obtained are included as supplementary material (Figure S6). For most of analytes, amplitude affects negatively while time affects positively. Only in case of BP-6 and 4-OH-BP both parameters affected

negatively for the extraction. In addition, in order to determine the optimal experimental values of each evaluated parameter, it was also studied the global extraction using the desirability function. This function is calculated taking into account the combination of parameters and the contribution of each analyte throughout the extraction process. The response surface was visualized as 2D contour plot (Figure 1), which shows the variation of the two factors simultaneously. In this representation, the responses are normalized between 0 and 1, being the unit the maximum extraction efficiency.

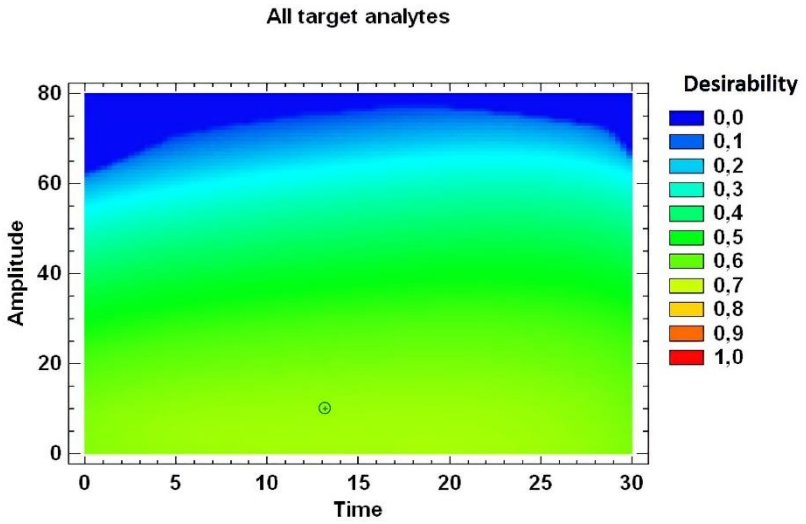

Figure 1. *Global desirability 2D plot for all target UV filters. Samples were spiked with 100 ng g^{-1} of each analyte*

3.1.5. Selection of the clean-up sorbent

A clean-up step is, in many cases, required when analyzing compounds in complex matrices. Therefore, it was used a dispersive solid phase extraction (d-SPE) based on QuEChERs technique for clean-up. Two types of sorbents were tested: a reversed-phase sorbent (C18), a mixed mode sorbent (PSA) and a mix of both (1:1, w/w). They were compared with an extraction procedure without clean-up sorbent. Figure S5 shows the behaviors of target analytes. PSA achieved poor recoveries for some compounds, especially for BP-2, BP-6, and BP-8. Although its combination with C18 improved recoveries, in case of BP-2 they are very

low. In some cases, the extraction solvent without the clean-up step achieved good recoveries, likely because the analytes cannot be retained in any sorbent. However, in order to safeguard the equipment, it was not possible to skip this step since it is a complex matrix and the removal of fat and other interferences was necessary. Thus, as C18 provides good recoveries in all cases, it was the selected sorbent.

The volume of solvent for clean-up (ACN) and the amount of C18 were optimized using a 3^2 experimental design. Three levels of each variable were evaluated: volume of ACN (2, 4 and 6 mL) and amount of C18 (0, 1 and 2 g). The design consisted of 12 experiments which included three central points. The matrix with the operational conditions is shown in supplementary material (Table S3). ANOVA analysis provided coefficients of determination (R^2) around 80 %, and P_{lof} values >5 %, being the model satisfactory with a 95 % confidence level. Additionally, Pareto Charts (Figure S7) show how ACN affects positively while the amount of C18 affects negatively for all the compounds. Individual surface responses for the selected UV filters were similar. Finally, the optimal amount of C18 was 0.4 g and the volume of ACN was 2 mL. Figure 2 shows the 3D graphs of the estimated response surface for each of the target compounds.

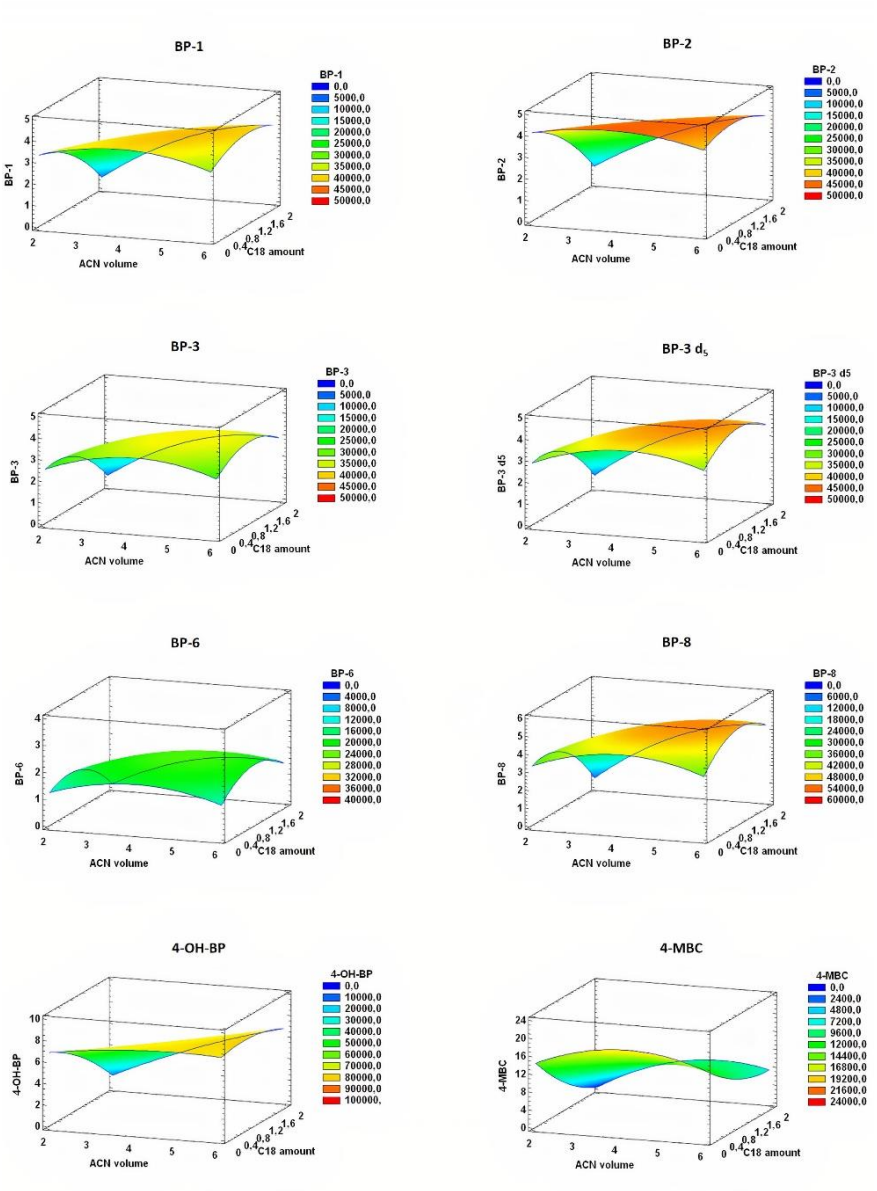

Figure 2. *3^2 design desirability charts for each individual compound. Samples were spiked with 100 ng g^{-1} of each analyte*

3.2. Analytical performance

A seven-point procedural calibration curve (spiking mussel samples before extraction) was constructed for each analyte. Each concentration level was prepared in triplicate and injected three times following the optimized extraction procedure (n = 63). For the case of BP-2 and 4-OH-

BP, the LOQ was 5 ng g^{-1}, and the calibration curve was constructed with six concentration levels (n = 54). BP-$3d_5$ was used as surrogate at 100 ng g^{-1}. Calibration graphs were obtained representing the analyte/surrogate peak area ratio versus the analyte concentration. The analytical parameters obtained are summarized in Table 2.

Table 2. *Calibration parameters*

	BP-1	BP-2	BP-3	BP-6	BP-8	4-OH-BP	4-MBC
n	63	54	63	63	63	54	63
b (g ng^{-1})	$1.49 \cdot 10^{-3}$	$4.07 \cdot 10^{-4}$	$3.34 \cdot 10^{-3}$	$1.25 \cdot 10^{-3}$	$3.16 \cdot 10^{-3}$	$2.22 \cdot 10^{-3}$	$1.54 \cdot 10^{-3}$
$s_{y/x}$	$2.76 \cdot 10^{-2}$	$8.84 \cdot 10^{-2}$	$3.92 \cdot 10^{-2}$	$2.57 \cdot 10^{-2}$	$2.54 \cdot 10^{-2}$	$3.77 \cdot 10^{-2}$	$3.17 \cdot 10^{-2}$
LDR (ng g^{-1})	LOQ-250						
R^2 (%)	98.9	98.6	99.6	98.2	99.8	99.1	98.6
P_{lof} (%)	25.0	98.9	43.7	90.6	72.8	40.6	70.5
LOQ (ng g^{-1})	1.0	5.0	1.0	1.0	1.0	5.0	1.0
LOD (ng g^{-1})	0.3	1.6	0.3	0.3	0.2	1.5	0.3

n: points of calibration; b: slope; $s_{y/x}$: regression standard deviation; LDR: linear dynamic range; R^2: determination coefficient; P_{lof}:p values of thelack-of-fit test; LOD: limit of detection; LOQ: limit of quantification

In order to estimate and evaluate the possible matrix effect (ME), two calibration curves were prepared for each compound, one in the initial mobile phase and the other in the mussel blank. Significant differences between the slopes were estimated using the Student's *t*-test, showing an important matrix effect. Additionally, ME was calculated by comparing the signal of the fortified samples after treatment with the standard solutions at the same concentration (at 100 ng mL^{-1} final concentration) according to the following equation [23]:

$$ME\ (\%) = \frac{Signal\ of\ sample\ extracts\ fortified\ after\ treatment}{Signal\ of\ standard\ solution} \times 100$$

With this estimate, the absolute ME is considered: a ratio equal to 100% indicates the absence of a matrix effect whereas a ratio greater or lower than 100% implies signal enhancement and ion suppression, respectively. Ionic suppression was observed for all analytes to a greater or lesser extent, being BP-6 the least affected, since none presented an

augmented signal. This limitation was compensated by performing the standard addition calibration for quantification purposes, previously described.

3.3. Method validation

The proposed method was validated following the recommendations of the International Conference on Harmonization (ICH) guidelines [24]. Performance characteristics were stablished in terms of linearity, sensitivity (limits of detection and quantification, LOD and LOQ), accuracy (trueness and precision) and specificity.

3.3.1. Linearity

The linearity of the calibration graphs was tested from the lowest quantifiable concentration to 250 ng g^{-1} for all compounds. The correlation coefficient (R^2, %) ranged between 98.2 % and 99.8 % and P_{lof} exceeded 5% in all cases, indicating a good linearity. All values are listed in Table 2.

3.3.2. Sensitivity

The sensitivity of an analytical method is determined by the minimum amount of analyte that it can detect and quantify, by the limits of detection (LOD) and limits of quantification (LOQ). In the present work, these parameters were estimated with an interpolation in the calibration of a signal three times the signal-noise ratio for LOD and ten times the previous ratio for LOQ. The values obtained ranged from 0.2 to 1.6 ng g^{-1} for the LOD, and from 1 to 5 ng g^{-1} for the LOQ (Table 2).

3.3.3. Accuracy

The accuracy and reproducibility of the method were evaluated by spiking mussel blank samples, due to the lack of certified reference materials. Four concentration levels were considered to ensure the accuracy of the method (1, 50, 100, and 250 ng g^{-1}). Precision was assayed on the same day and between days (intra and inter-day precision) as the relative standard deviation, RSD. The procedure was repeated three times a day and for three consecutive days to determine the variability in

both cases. Trueness was evaluated by a recovery assay. The fortified blank samples were analyzed using the proposed method and the concentration of each analyte was determined by interpolation using the calibration regression line (within the linear dynamic range). The concentration quantified was compared with the known amount previously added to the samples, and the percentage was calculated. In all cases, RSD values (%) lower than 15% and recoveries close to 100% (between 90.2% and 111.9%) were obtained (within acceptable limits), which means that the method is highly accurate and reproducible (Table 3).

Table 3. *Recovery assay and accuracy of the proposed method (precision and trueness) in mussel*

Compound	Spiked (ng g^{-1})	RSD (%)	Rec (%)	Compound	Spiked (ng g^{-1})	RSD (%)	Rec (%)
BP-1	1	9.6	111.4	BP-8	1	7.6	92.1
	50	7.0	109.7		50	7.2	97.0
	100	6.9	100.7		100	13.9	99.4
	250	9.0	94.0		250	4.6	100.7
BP-2	5	14.5	92.6	4-OH-BP	5	8.8	108.9
	50	4.2	94.5		50	6.2	109.2
	100	5.3	90.2		100	10.6	107.9
	250	2.2	91.5		250	10.2	100.2
BP-3	1	13.1	103.9	4-MBC	1	3.9	98.4
	50	4.2	91.9		50	7.4	107.9
	100	5.0	93.6		100	3.3	101.5
	250	7.6	96.9		250	11.5	96.3
BP-6	1	6.5	98.2				
	50	3.8	111.9				
	100	10.3	96.4				
	250	1.5	103.3				

RSD: relative standard deviation; Rec: recovery

3.3.4. Specificity

The specificity was evaluated by analyzing the blank. Since no interferences of endogenous substances were observed in the retention times of the analytes of interest, it can be assured that the method is selective. Figure 3 compares a chromatogram of a blank (A) with the

corresponding fortified mussel sample (B), and no peaks of other substances were detected.

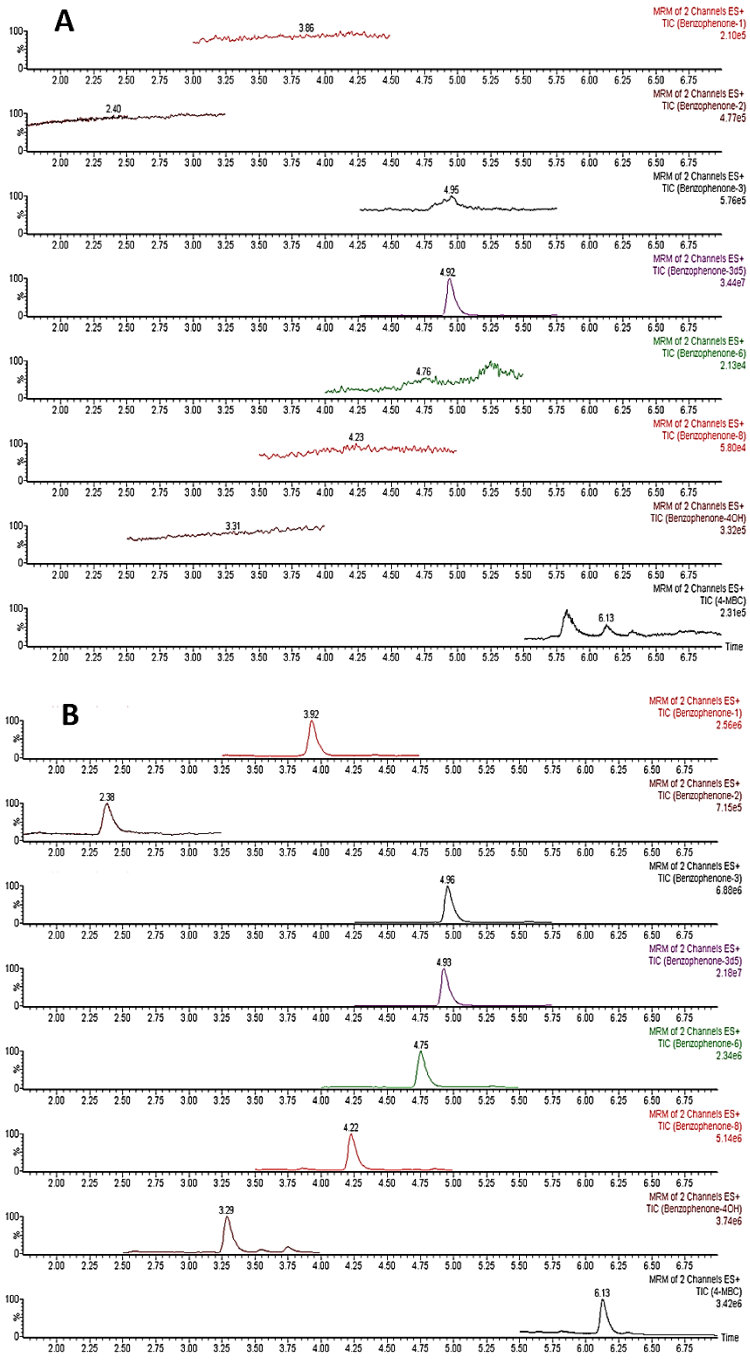

Figure 3. *MRM mode chromatograms of: a blank mussel sample (A); a spiked blank sample containing all the target analytes at 100 ng g^{-1} (B)*

3.4. Biomonitoring of UV-filters in marine mussels. Detection frequency and quantification.

This work proposes a novel UHPLC-MS/MS method for the simultaneously determination of 7 UV-filters in *M. galloprovincialis* mussel, including 6 benzophenones, some of them studied for the first time in this species. The method was satisfactorily applied to determine the levels of the target compounds in mussel specimens collected from 5 different sampling areas from de coast of the province of Granada (Spain). A large amount of mollusks were collected from each site and pool was prepared in each case. The quantities found as mean of 20 determinations are shown in Table 4. Each sample was analyzed in triplicate. Figure 4 shows a chromatogram of a contaminated mussel.

Table 4. *Application of the method to mussel samples*

Concentration (ng g^{-1})								
	BP-1	**BP-2**	**BP-3**	**BP-6**	**BP-8**	**4-OH-BP**	**4-MBC**	**ΣUV-filters**
S1.1	7.7 (4.7)	ND	28.0 (2.4)	ND	ND	5.0 (1.2)	2.7 (6.6)	43.4
S1.2	3.7 (5.0)	ND	23.2 (1.1)	1.9 (7.4)	D	4.6 (5.3)	3.6 (7.7)	37.0
S1.3	3.1 (13.9)	ND	28.6 (2.0)	D	D	D	9.8 (9.1)	41.5
S1.4	2.5 (11.6)	ND	21.1 (3.1)	1.9 (13.3)	ND	D	3.9 (10.5)	29.4
S2.1	8.2 (4.2)	ND	50.2 (4.9)	3.2 (9.2)	ND	D	22.1 (9.1)	83.7
S2.2	9.1 (8.4)	ND	48.7 (2.7)	7.3 (6.3)	ND	D	25.8 (6.7)	90.9
S2.3	13.5 (9.2)	ND	51.8 (5.8)	7.5 (1.8)	ND	D	11.6 (4.8)	84.4
S2.4	7.7 (6.9)	ND	56.4 (0.4)	ND	ND	6.6 (2.1)	5.8 (13.4)	76.5
S3.1	4.4 (9.7)	ND	202.5 (1.7)	ND	D	D	32.3 (13.0)	239.2
S3.2	5.9 (11.4)	ND	219.2 (1.0)	ND	ND	D	23.6 (12.2)	248.7
S3.3	3.9 (8.2)	ND	216.2 (1.3)	ND	1.1(8.9)	D	38.5 (4.5)	259.7
S3.4	3.9 (10.7)	ND	219.5 (0.6)	ND	D	4.5 (3.7)	36.3 (6.9)	264.2
S4.1	8.9 (8.7)	ND	25.9 (2.4)	15.2 (11.7)	D	6.6 (1.4)	4.6 (12.6)	61.2
S4.2	7.0 (8.1)	ND	29.0 (1.2)	8.8 (6.6)	4.5 (9.8)	9.7 (2.7)	4.3 (13.7)	63.3
S4.3	10.3 (2.1)	ND	29.9 (1.9)	34.6 (4.3)	D	41.1 (2.0)	ND	115.9
S4.4	-	-	-	-	-	-	-	-
S5.1	31.8 (1.9)	ND	53.3 (1.2)	30.3 (4.3)	ND	19.4 (1.5)	16.6 (10.8)	151.4
S5.2	20.3 (7.9)	ND	33.3 (12.3)	ND	ND	21.3 (3.2)	4.7 (31.1)	79.6
S5.3	17.5 (10.4)	ND	50.5 (1.3)	33.3 (6.6)	ND	20.0 (7.6)	26.1 (24.5)	147.4
S5.4	21.2 (8.2)	ND	28.0 (1.5)	ND	ND	20.7 (1.6)	ND	69.9

ND: not detected; D: detected (>LOD but <LOQ); the coefficient of variation (%) is indicated in parentheses

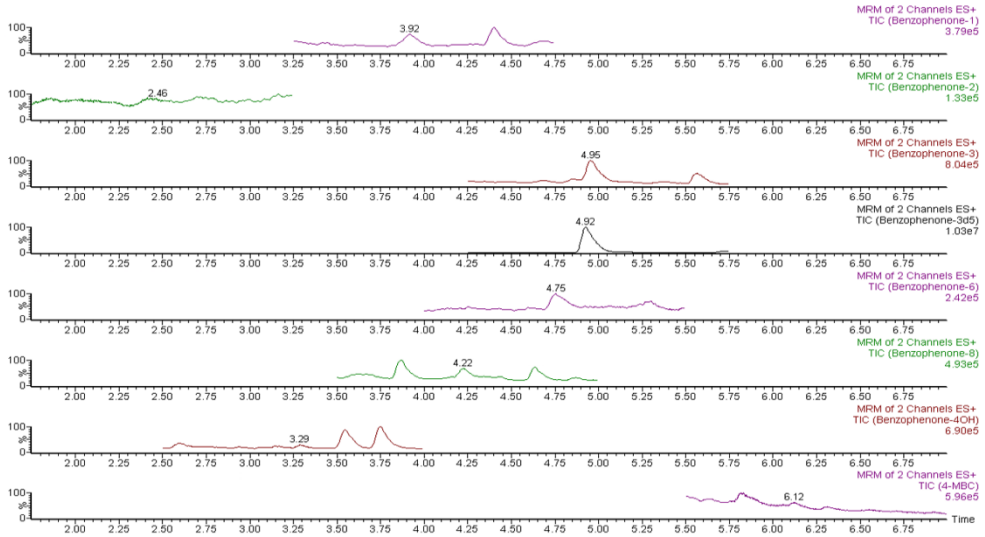

Figure 4. *MRM mode chromatogram of a contaminated sample from a mussel (Area 4)*

Based on the results, all samples were tested positive for at least three of the UV filters. BP-1 and BP-3 were the most frequent benzophenones (about 100% of the samples). The next most frequent UV filter was 4-OH-BP, detected in all samples and quantified in 55% of them (11/20), followed by 4-MBC, detected and quantified in 90% of the samples (18/20). BP-6 and BP-8 were detected and quantified in some of the samples, with BP-8 being the one found at the lowest concentration. Finally, BP-2 was not detected in any of the analyzed samples. In general, the concentrations present in the studied samples did not exceed from 50 ng g^{-1}, with the exception of BP-3 which was found at levels higher than 200 ng g^{-1} (area 3).

The concentrations found according to the different sampling point were compared and interesting differences were observed. Previous works have shown that the waters receiving wastewater effluents or having recreational activities are the most contaminated by UV filters [25]. This trend is also manifested in marine organisms in the area. In addition, a seasonal trend has been observed, showing the highest levels in the warmest seasons after the bathing months [22,26,27]. Here, the highest concentrations of total UV-filters (ΣUV-filters) in mussels were

found in area 3 (La Calita, La Herradura) (239.2-264.2 ng g^{-1}), reporting the highest levels of BP-3 and 4-MBC. This was expected since it is a highly urbanized area with holiday residential use, especially during the collection of samples and the months before. In contrast, in area 2 (mouth of the river Jate, La Herradura), despite being also an urbanized and tourist area, the levels of total UV filters detected were lower (76.5-90.9 ng g^{-1}), probably due to the greater exchange of water for the effluent. In general, moderate concentrations of the target compounds (61.2-151.4 ng g^{-1}) were quantified in non-inhabited areas but close to agricultural areas (area 4 and 5, Motril), while the lowest amounts were detected in area 1 (La Caleta, Salobreña), a rocky coast area, urbanized with residential and industrial uses. The presence of target UV filters was expected in specimens near agricultural areas, since the water used for irrigation usually comes from a water effluent after being treated in a wastewater treatment plant (WWTP), where the removal efficiency of these compounds is not complete. While WWTPs are the main sources for contaminant elimination, including the UV filters, they are also *hot spots* for their release into the environment. Finally, area 1, despite being an urbanized zone, is a rocky area, not intended for bathing and for holiday use, which would explain the low levels of UV filters found in the mussels from this place.

BP-3 was the most predominant UV filter at all sampling locations. These findings are not surprising, as BP-3 is one of the most widely used UV filter for sun protection in sunscreens and cosmetic products [28]. Bioaccumulation of these compounds in specimens depends on the levels to which they are exposed, but also on metabolic biotransformation or biodegradation that can occur in the body and in the marine environment. This phenomenon could explain the high occurrence of BP-1, which may result from the metabolization of BP-3 towards its hydroxylated form. This was also reported for other marine organisms such as zebrafish [29], and is in accordance with the uptake patterns found in wild mussels for the BP-3 [13]. Similarly, the high frequency of 4-OH-BP in the analyzed organisms may be the result of metabolization or transformation in surface water, according to the scientific literature [30]. Interestingly,

these metabolites have been shown to have stronger estrogenic activity than its precursor [31-33]. Nevertheless, only a few preliminary assessments about bioaccumulation and toxicity have been published in mussels, so more detailed investigations are needed to identify the metabolic pathways that are involved.

As previously commented reports on UV filters in mollusks are limited compared to those on other pollutants and, even more in relation to benzophenones. The concentrations of BP-3 found in this study were below those reported by Castro et al. (2018) on mussels from the Portuguese coastline, reaching levels up to 622 ng g^{-1} in the areas with more intensity of recreational activities [2]. On the other hand, the levels found were higher than those reported in mussels from different European fishing areas (BP-3, n.d-85 ng g^{-1}) [34], in clams, mussels and cockles from Galician coast, Spain (BP-3, up to 63 ng g^{-1}) [21], and in mussels and clams from the coast of Hong Kong, China (BP-3 and BP-8 bellow 15 ng g^{-1}) [3]. Liao and Kannan et al. (2019) analyzed four benzophenones (BP-3, 4-OH-BP, BP-1, BP-8 and BP-2) in mollusks collected from 5 coastal areas along the Bohai Sea (China), obtaining similar concentrations (nd-58.8 ng g^{-1}) and not detecting BP-2, in line with our results [11]. In regard to 4-MBC levels, reported in these studies (around 50 ng g^{-1}) were similar to those in our work [2,34,21]. In contrast, Picot Groz et al. (2018) did not detect BP-3 or 4-MBC in mussels from the French coast, collected at the beginning of the tourist season [25]. Similarly, Cunha et al. (2015) also did not find BP-3 or BP-1 in wild mussels from different parts of Europe that were caught during September-December [15]. These differences in concentrations may be due to the period in which they were collected, which would explain why UV-filters have not accumulated in these organisms.

Our results show BP-1 levels lower than BP-3 concentrations in all analyzed specimens. The results are in agreement with the data of Liao and Kannan (2019), who also found higher concentrations of BP-3 compared to BP-1, being the first the most predominant in all locations [11]. This finding is curious since it differs from the results found in relation to the analysis of human biological samples. For instance, in

nails and placenta, higher levels of BP-1 were found, and even BP-3 was not detected [35,36]. In humans a large proportion of BP-3 is metabolized by the cytochrome P450 enzymatic system (Phases I and II), with demethylation being the main pathway, which results in BP-1 [31,33]. In mussels, biotransformation may be slower (intake exceeds metabolization) or involve other metabolic pathways, would account for the differences, but further research is required to investigate the behaviour of UV-filters and their effect in these species, especially during long-term exposition.

4. Conclusions

Although mussels have been previously studied to evaluate the bioaccumulation of pollutants, in relation to UV-filters the information available on these organisms is scarce. An UHPLC-ESI-MS/MS method has been developed and validated for the analysis of UV filters in wild mussels from five areas on the coast of Granada, Spain. UAE followed by a dispersive phase clean up with C18 is proposed, being the sample treatment simple and fast. The method provided acceptable validation data: high linearity (R^2> 98.2%), reproducibility (RSD <15%), recoveries close to 100%, and low detection levels (between 0.2 and 1.6 ng g^{-1}). The paper also reports the bioaccumulation of UV filter compounds in mussels. BP-3 was one of the most frequent UV filters, detected at the highest concentrations (up to 220 ng g^{-1} in area 3). All the UV filters studied, with the exception of BP-2, were detected in most of the samples. Furthermore, spatial variations were observed: the highest concentrations were associated with areas related bathing and more recreational activities, and the lowest concentrations with non-urbanized areas.

This work reveals the persistence and accumulation of UV-filters on the coast, including benzophenones, which raises concern about the negative environmental effects associated with them. In view of the results and based on the high concentrations and frequencies of detection, it would be reasonable to include these compounds in the future monitoring of coastal bivalves. More research should be carried out to

know the behavior of these compounds in this species. The results encourage the need to establish good practices that ensure preservation and sustainability of the marine biodiversity, as well as prevent and mitigate the adverse effects of UV in the environment.

Declaration of competing interest. The authors declare no competing interests.

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References

[1] D. Bordalo, C. Leite, Â. Almeida, A.M.V.M. Soares, C. Pretti, R. Freitas, Impacts of UV filters in *Mytillus galloprovincialis*: Preliminary data on the acute effects induced by environmentally relevant concentrations, Sustainability 12 (2020) 6852.

[2] M. Castro, J.O. Fernandes, A. Pena, S.C. Cunha, Occurrence, profile and spatial distribution of UV-filters and musk fragrances in mussels from Portuguese coastline, Mar. Environ. Res. 138 (2018) 110-118.

[3] Z. Sang, K.S. Leung, Environmental occurrence and ecological risk assessment of organic UV filters in marine organisms from Hong Kong coastal waters, Sci. Total Environ. 566-567 (2016) 489-498.

[4] W. Li, Y. Ma, C. Guo, W. Hu, K. Liu, Y. Wang, T. Zhu, Occurrence and behaviour of four of the most used sunscreen UV filters in a wastewaters reclamation plant, Water Res. 41 (2007) 3506-3512.

[5] H. Hong, J. Wang, D. Shi, Effects of salinity on the chronic of 4-methylbenzylidene camphor (4-MBC) in the marine copepod *Tigriopus japonicas,* Aquat. Toxicol. 232 (2021) 106742.

[6] J. Hiller, K. Klotz, S. Meyer, W. Uter, K. Hof, A. Greiner, T. Göen, H. Drexler, Systemic availability of lipophilic organic UV-filters through dermal sunscreen exposure, Environ. Int. 132 (2019) 105068.

[7] M. Krause, H. Frederiksen, K. Sundberg, F.S. Jørgensen, L.N. Jensen, P. Nørgaard, C. Jørgensen, P. Ertberg, A. Juul, K.T. Drzewiecki, N.E. Skakkebaek, A.M. Andersson, Presence of benzophenones commonly used as UV-filters and absorbers in paired maternal and fetal samples, Environ. Int. 110 (2018) 51-60.

[8] R. Rodríguez-Gómez, A. Zafra-Gómez, F.J. Camino-Sánchez, O. Ballesteros, A. Navalón, Gas chromatography and ultrahigh performance liquid chromatography tandem mass spectrometry methods for the determination of selected endocrine disrupting chemicals in human breast milk after stir-bar sorptive extraction, J. Chromatogr. A. 1349 (2014) 69-79.

[9] F. Vela-Soria, O. Ballesteros, A. Zafra-Gómez, L. Ballesteros, A. Navalón, A new method for the determination of benzophenone-UV-filters in human serum samples by dispersive liquid-liquid microextraction with liquid chromatography-tandem mass spectrometry, Talanta 121 (2014a) 97-104.

[10] F. Vela-Soria, O. Ballesteros, A. Zafra-Gómez, L. Ballesteros, A. Navalón, A multiclass method for the analysis of endocrine disrupting chemicals in human urine samples. Sample treatment by dispersive liquid-liquid microextraction, Talanta 129 (2014b) 209-218.

[11] C. Liao, K. Kannan, Species-specific accumulation and temporal trends of bisphenols and benzophenones in mollusks from the Chinese Bohai Sea during 2006-2015, Sci. Tot. Environ. 653 (2019) 168-175.

[12] K. He, E. Hain, A. Timm, L. Blaney, Bioaccumulation of estrogenic hormones and UV-filters in red swamp crayfish (*Procambarus clarkia),* Sci. Total Environ. 764 (2021) 142871.

[13] L. Vidal-Liñán, E. Villaverde-de-Sáa, R. Rodil, J. Benito Quintana, R. Beiras, Bioaccumulation of UV filters in *Mytilus galloprovincialis* mussel, Chemosphere 190 (2018) 267-271.

[14] S. Bayen, E. Segovia Estrada, G. Juhel, L. Wei Kit, B.C. Kelly, Pharmaceutically active compounds and endocrine disrupting

chemicals in water, sediments and mollusks in mangrove ecosystems from Singapore, Mar. Pollut. Bull. 109 (2016) 716-722.

[15] S.C. Cunha, J.O. Fernandes, L.Vallecillos, G. Cano-Sancho, J.L. Domingo, E. Pocurull, F. Borrull, A.L. Maulvault, F. Ferrari, M. Fernandez-Tejedor, F. Van den Heuvel, Co-occurrence of musk fragances and UV-filters in seafood and macroalgae collected in European hotspots, Environ. Res. 143 (2015) 65-71.

[16] S. Montesdeoca-Esponda, M.E. Torres-Padrón, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, Fate and distribution of benzotriazole UV filters and stabilizers in environmental compartments from Gran Canaria Island (Spain): A comparison study, Sci. Total Environ. 756 (2021) 144086.

[17] X. Bai, K. Acharya, Uptake of endocrine-disrupting chemicals by quagga mussels (Dreissena bugensis) in an urban-impacted aquatic ecosystem, Environ. Sci. Pollut. Res. 26 (2019) 250-258.

[18] K. Styszko, K. Proctor, E. Castrignanò, B. Kasprzyk-Hordern, Occurrence of pharmaceutical residues, personal care products, lifestyle chemicals, illicit drugs and metabolites in wastewater and receiving surface waters of Krakow agglomeration in South Poland, Sci. Total Environ. 768 (2021) 144360.

[19] Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. O. J. EU, L342, 2009, 59/209. Available online at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32009R1223&from=EN (May 2021).

[20] D. Álvarez-Muñoz, S. Rodríguez-Mozaz, S. Jacobs, A. Serra-Compte, N. Cáceres, I. Sioen, W. Verbeke, V. Barbosa, F. Ferrari, M. Fernández-Tejedor, S. Cunha, K. Granby, J. Robbens, M. Kotterman, A. Marques, D. Barceló, Pharmaceuticals and endocrine disruptors in raw and cooked seafood from European market: Concentrations and human exposure levels, Environ. Int. 119 (2018) 570-581.

[21] R. Rodil, E. Villaverde-de-Saá, J. Cobas, J.B. Quintana, R. Cela, N. Carro, Legacy and emerging pollutants in marine bivalves from the Galician coast (NW Spain), Environ. Int. 129 (2019) 364-375.

[22] M. Bachelot, Z. Li, D. Munaron, P. Le Gall, C. Casellas, H. Fenet, E. Gomez, E., Organic UV filter concentrations in marine mussels from French coastal regions, Sci. Total Environ. 420 (2012) 273-279.

[23] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019-3030.

[24] ICH Quality Guidelines, Topic Q2 (R1): Validation of Analytical Procedures: Text and Methodology, 2005. Available online at: http://www.ich.org/products/guidelines/quality/ article/quality-guidelines.html (May 2021).

[25] M. Picot Groz, H. Fenet, M.J. Martínez Bueno, D. Rosain, E. Gomez, Diurnal variations in personal care products in seawater and mussels at three Mediterranean coastal sites, Environ. Sci. Pollut. Res. 25(2018) 9051-9059.

[26] K. Fent, A. Zenker, M. Rapp, Widespread occurrence of estrogenic UV-filters in aquatic ecosystems in Switzerland, Environ. Pollut. 158 (2010) 1817-1824.

[27] M. Picot Groz, M.J. Martinez Bueno, D. Rosain, H. Fenet, C. Casellas, C. Pereira, V. Maria, M.J. Bebianno, E. Gomez, E., Detection of emerging contaminants (UV filters, UV stabilizers and musk) in marine mussels from Portuguese coast by QuEChERs extraction and GC-MS/MS, Sci. Tot. Environ. 493 (2014) 162-169.

[28]Consumer Product Information Database (CPID). Health effects of consumer product. Benzophenone-3 Available online at: https://www.whatsinproducts.com/chemicals/view/1/240/000131-57-7/Benzophenone-3%20(Oxybenzone) (May 2021)

[29] N. Blüthgen, S. Zucchi, K. Fent, Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (Danio rerio), Toxicol. Appl. Pharmacol. 263 (2012) 184-194.

[30] M.H. Wu, D.G. Xie, G. Xu, R. Sun, X.Y. Xia, W.L. Liu, L. Tang, Benzophenone-type UV filters in surface waters: An assessment of

profiles and ecological risks in Shanghai, China, Ecotoxicol. Environ. Saf. 141 (2017) 235-241.

[31] Y. Watanabe, H. Kojima, S. Takeuchi, N. Uramaru, S. Sanoh, K. Sugihara, S. Kitamura, S. Ohta, Metabolism of UV-filter benzophenone-3 by rat and human liver microsomes and its effect on endocrine-disrupting activity, Toxicol. Appl. Pharmacol. 282 (2015) 119-128.

[32] T. Suzuki, S. Kitamura, R. Khota, K. Sugihara, N. Fujimoto, S. Ohta, S., Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens, Toxicol. Appl. Pharmacol. 203 (2005) 9-17.

[33] L. Wang, K. Kannan, Characteristic profiles of benzonphenone-3 and its derivatives in urine of children and adults from the United States and China, Environ. Sci. Technol. 47 (2013) 12532-12538.

[34] S.C. Cunha, L. Trabalón, S. Jacobs, M. Castro, M. Fernandez-Tejedor, K. Granby, W. Verbeke, C. Kwadijk, F. Ferrari, J. Robbens, I. Sioen, E. Pocurull, A. Marques, J.O. Fernandes, J.L. Domingo, UV-filters and musk fragrances in seafood commercialized in Europe Union: Occurrence, risk and exposure assessment, Environ. Res. 161 (2018) 399-408.

[35] L. Martín-Pozo, M.C. Gómez-Regalado, S. Cantarero-Malagón, A. Navalón, A. Zafra-Gómez, Determination of UV filters in human nails using an acid sample digestion followed by ultra-high performance liquid chromatography-mass spectrometry analysis, Chemosphere 273 (2021) 128603.

[36] F. Vela-Soria, I. Jiménez-Díaz, R. Rodríguez-Gómez, A. Zafra-Gómez, O. Ballesteros, A. Navalón, J.L. Vílchez, M.F. Fernández, N. Olea, Determination of benzophenones in human placental tissue samples by liquid chromatography–tandem mass spectrometry, Talanta 85 (2011) 1848-1855.

Table S2. *Matrix design for the optimization of UAE parameters*

Run order	Time (min)	Ampliude (%)
1	15.5	40
2	15.5	40
3	15.5	40
4	1	70
5	30	70
6	30	40
7	1	40
8	15.5	70
9	15.5	40
10	30	10
11	1	10
12	15.5	10

Table S3. *Matrix design for the optimization of clean-up variables*

Run order	ACN volume (mL)	C18 amount (g)
1	4	1
2	4	1
3	4	1
4	4	1
5	4	0
6	2	0
7	6	1
8	6	0
9	2	1
10	2	2
11	4	2
12	6	2

Figure S1. *Sampling area along the coast of Granada, Spain. The 5 selected locations are indicated*

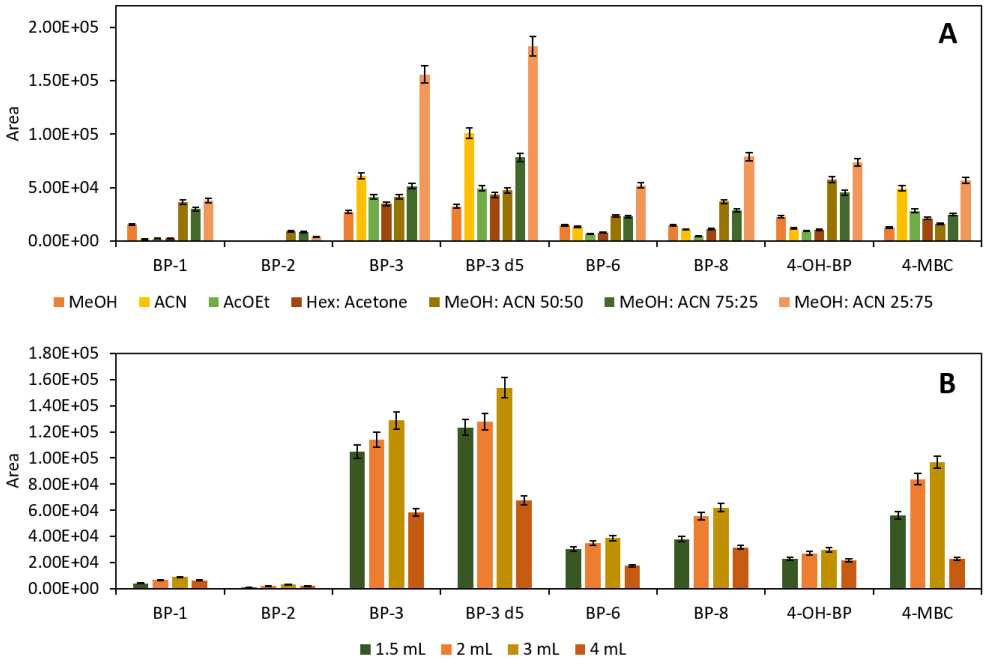

Figure S2*. Selection of the extraction solvent (A) and solvent volume (B). The bar graph depicts the response area obtained for each compound and the average recovery for each extraction solvent. Data are expressed as mean ± error rate from samples in triplicate*

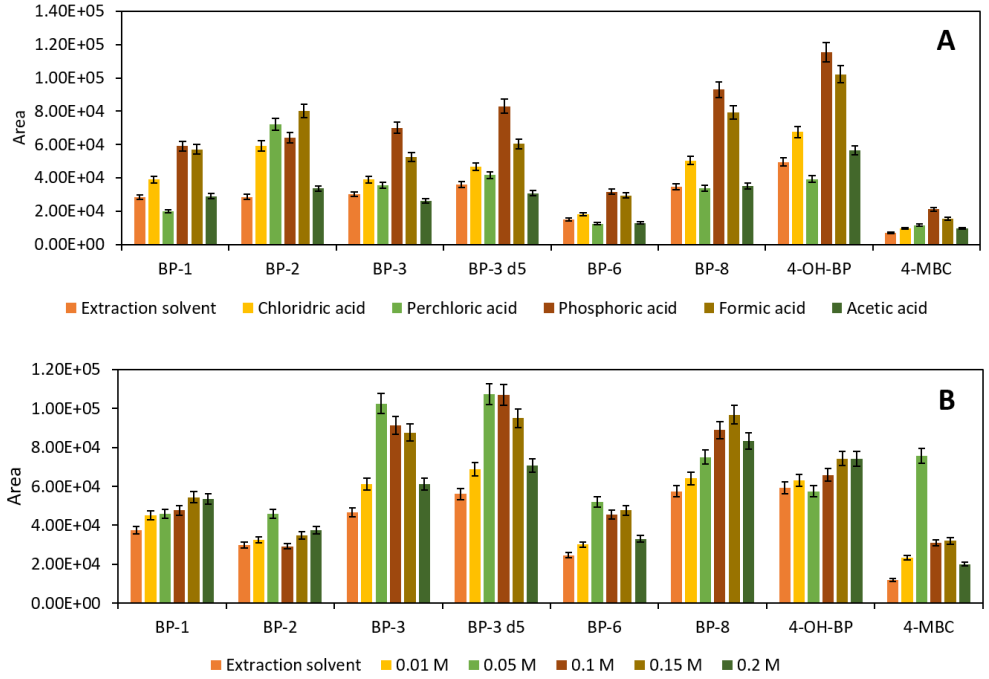

Figure S3. *Selection of optimal acid (A), all solutions were prepared at 0.1 mol L^{-1} in MeOH:ACN 25:75 (v/v); and selection of optimal concentration of o-phosphoric acid (B). Data are expressed as mean ± error rate from samples in triplicate*

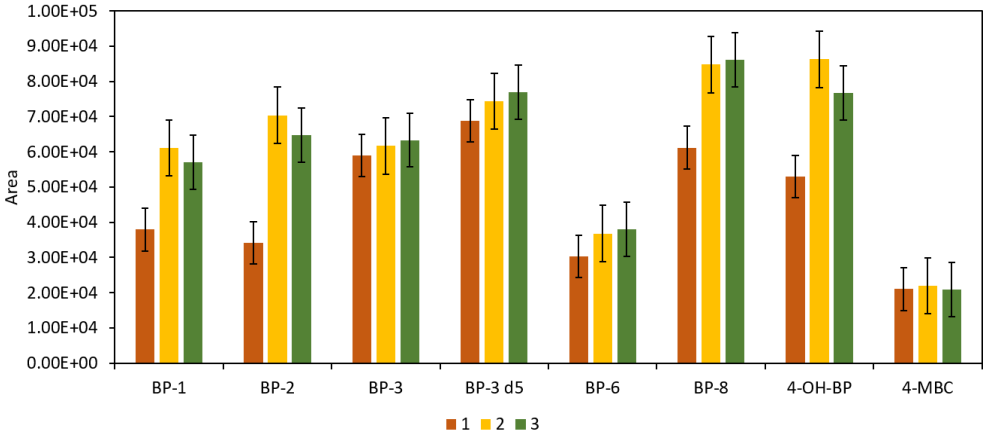

Figure S4. *Study of the number of extraction cycles*

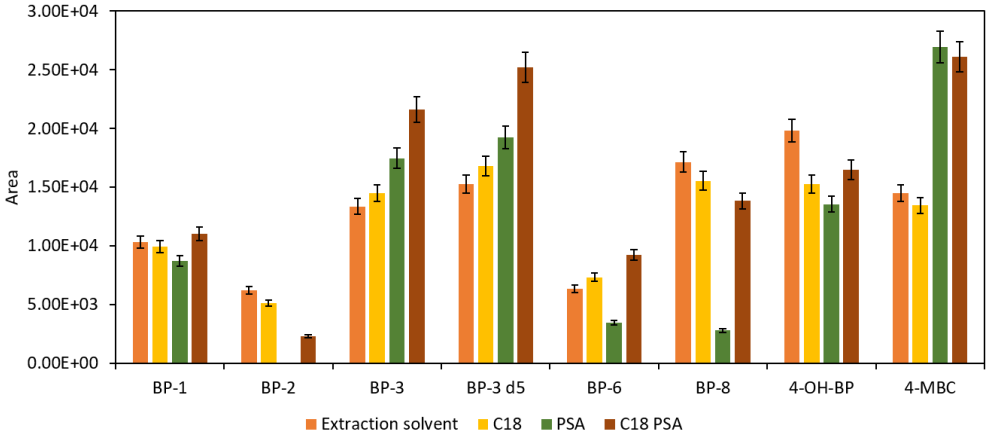

Figure S5. *Selection of clean-up sorbent. The bar graph depicts the response area obtained for each compound and the average recovery for each sorbent. Data are expressed as mean ± error rate for samples in triplicate*

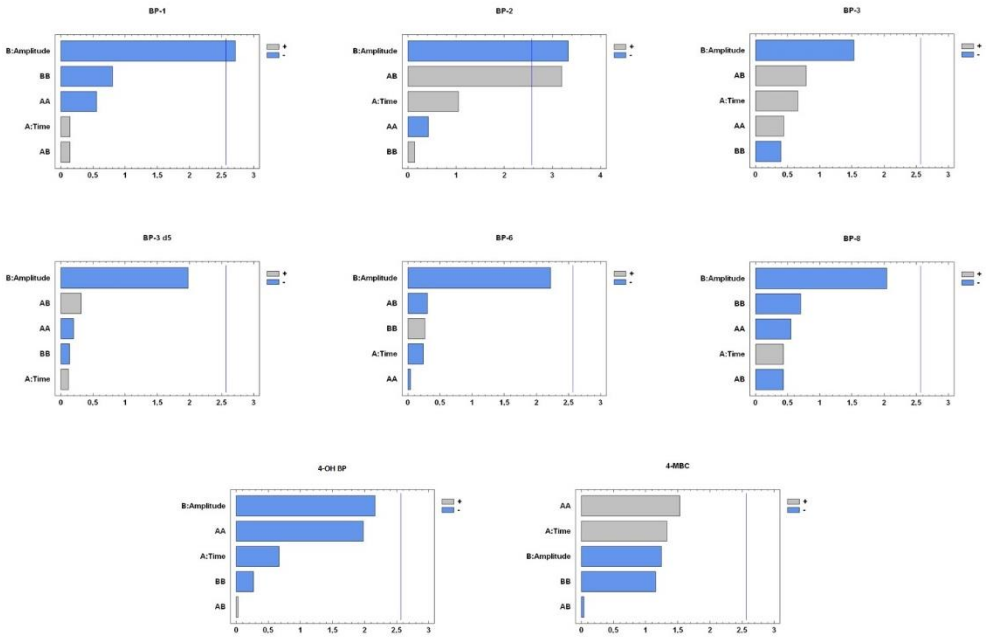

Figure S6. *Pareto-Charts. Standarized effects of ultrasound amplitude and time according to the 3^2 design for the extraction*

Capítulo 3

Multi-residue determination of 17 antibiotics in sea cucumbers (*Holothuria tubulosa)* by ultrahigh performance liquid chromatography-tandem mass spectrometry

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Abstract: Antibiotics are a class of pharmaceuticals routinely prescribed to prevent and treat infections caused by pathogenic bacteria in humans and animals. Their widespread consumption results in a gradual contamination of the aquatic environment, which is of particular concern due to the phenomenon of resistance to antibiotics. Quantitative analysis of antibiotics in marine ecosystems is critical to assess the potential risk of exposure of aquatic species and, consequently, of consumers of seafood. In this context, filter feeders, such as holothurians, are considered ideal bioindicators of pollution in marine waters. In this work, an ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method was developed and validated for the simultaneous determination of 17 multiclass antibiotic residues in *Holothuria tubulosa* specimens. The proposed methodology involved two steps of ultrasound-assisted extraction (UAE) with methanol, followed by a clean-up based on dispersive solid phase extraction (d-SPE) using C18 adsorbent. 3^2 factorial designs were used for the optimization of the most influential variables of the extraction procedure. Linearity of 84.1-99.4 %, precision in terms of RSD lower than 15 %, recoveries between 87.9 and 114.9 %, and detection limits between 0.3 and 3.0 ng g^{-1} were obtained. After validation, the method was used to analyze specimens of *Holothuria tubulosa* collected along the Mediterranean coast of Granada. The obtained results indicate the presence of antibiotics in the area, CIP being the most prevalent.

Keywords: Antibiotics; *Holothuria tubulosa;* exposure; contamination; UHPLC-MS/MS.

1. Introduction

Antibiotics are antimicrobial agents used to treat or prevent infections that are caused by pathogenic bacteria in humans and animals, inhibiting the growth and the survival of other microorganisms [1]. The first antibiotic was penicillin, discovered by Alexander Fleming in 1928. Traditionally, only those synthesized by bacteria or fungi were considered

antibiotics, but today this term also includes those of semi-synthesis and synthesis [2]. In addition to treating infections, antibiotics are used as preventive medicine and as growth promoters in agriculture and livestock [3]. They are very useful drugs, and mortality was greatly reduced thanks to their discovery, yet widespread use has led to them and their metabolites being present in the environment [4].

Antibiotics can enter the environment by excretion, domestic residues or direct discharges and, despite undergoing wastewater treatment processes at conventional waste water treatments plants (WWTPs), complete elimination is unsuccessful. As a consequence, they are transported through effluents and introduced into the environment, contaminating surface and non-surface waters, soils, sediments and even plants fit for consumption, with potential negative effects of both the environment and human health [5-7]. Although the half-life of antibiotics is not very long, their residues can be considered persistent organic pollutants (POPs) due to their wide use and emissions [1]. The public health risks associated with these antibiotics include allergic reactions, hepatic or digestive dysfunction, heart disease as well as the development of bacterial resistance [8,9]. Their overuse has led to the emergence of more and more antibiotic resistant bacteria (ARB) as well as antibiotic resistant genes (ARG), both clinically and in nature [10,11], and this resistance has been identified as one of the main factors in the resurgence of certain contagious diseases [7].

In Europe, antibiotics are allowed for both therapeutic applications and prophylactic use in animal feed. However, the use of them as growth promoters is considered fraudulent, and their use as additives is highly regulated (EC) 1831/2003 [9]. In addition, the European Food Safety Agency stresses the importance of supervising these compounds, for both food safety and the need for adequate analytical methods of determining them [12]. For example, fish is an important route of human exposure to these pollutants, but sampling them in highly polluted coastal areas can lead to an overestimation of the exposure risk they present [13]. In recent years, numerous studies have evaluated marine pollution using

bioindicators such as mussels [14], salmon [13], clams, and fish [15,16]. However, other investigations have assessed the exposure to certain contaminants via marine echinoderms such as sea cucumbers, because of their wide coastal distribution and migratory movements on a local scale [17,18]. Sea cucumbers belong to the class Holothuroidea, are wormlike and have a soft body. There are about 1600 species worldwide of which 66 have commercial uses, such as for consumption in Asian countries or as resources for curing diseases, due to their pharmacological characteristics [19]. Additionally, when these organisms reach their commercial size they are between five and six years old, making them favourable for assessing the accumulation of pollutants over a long period of time. Furthermore, they feed by filtration of sediments, absorbing contaminants present in the area where they live, making them ideal for monitoring contaminants in the environment and good biomarkers of marine quality in general. Although some studies have assessed the presence of certain specific contaminants in *Holothuria tubulosa* (such as perfluoroalkyl compounds, preservatives, UV filters, hormones, surfactants, alkylphenols, brominated flame retardants, and plasticizers [20,21]), to the best of our knowledge none of them have studied the presence of antibiotics in these specimens.

The aim of the present study was to develop and validate a selective and sensitive UHPLC-MS/MS multi-residue method capable of determining 17 antibiotics in *Holothuria tubulosa* and, subsequently, apply this method to wild sea cucumber samples collected along the Granada coastline (Spain). The selection of the target analytes was based on their prevalence in both human and veterinary medicine. European regulations and watch lists for monitoring substances in the marine environment were also consulted. The selected antibiotics are all known to accumulate in the environment, becoming contaminants of concern in marine waters. Their presence in the body walls of the specimens results from uptake from contaminated water and sediments and their subsequent bioaccumulation in body tissues. Furthermore, of the 17 antibiotics selected for this study, only two of them are naturally produced (ERI and PEN V) and, to the best of our knowledge, the

microorganisms that produce them are not present in the holothurian body. Therefore all antibiotics studied in the present work can be considered as exogenous pollutants.

2. Materials and methods

2.1. Chemicals and reagents

Ultrapure water (18.2 MΩ-cm) was purified using an A Milli-Q Plus® system (Millipore, Madrid, Spain). Analytical-grade formic acid (≥ 98 %) used as mobile phase additive, sodium hydroxide reagent grade (NaOH) (≥ 98 %, pellets), and DSC-C18 SPE used as sorbent, were obtained from Sigma-Aldrich (Madrid, Spain). LC-MS grade methanol (MeOH), acetonitrile (ACN, ≥ 99 %), and acetic acid were purchased from VWR Prolabo CHEMICALS (Barcelona, Spain). Ethyl acetate (AcOEt) was obtained from Honeywell (Madrid, Spain) and acetone (ACE, HPLC-GPC-ACS grade), o-phosphoric acid (85 % w/w), hydrochloric acid (6 M), and perchloric acid (70 %) were obtained by Panreac (Barcelona, Spain). PSA bonded silica used as sorbent, was provided by Supelco (Bellfonte, Pennsylvania, USA). Stock solutions were prepared in MeOH and stored at -20ºC until use. Nalidixic acid (NAL), sulfamethoxazole (SUL), flumequine (FLU), oxolinic acid (OXO), trimethoprim (TRI), norfloxacin (NOR), difloxacin (DIF), moxifloxacin (MOX), erythromycin (ERY), marbofloxacin (MAR), and clarithromycin (CLA) were also obtained from Sigma Aldrich (Madrid, Spain). Ciprofloxacin (CIP), penicillin V (PEN), danofloxacin (DAN), enrofloxacin (ENR), levofloxacin (LEVO), oxacillin (OXA) and cinchophen (CIN, surrogate standard) were provided by Alfa Aesar (Massachusetts, MA, USA). The chemical structures of the target analytes, as well as their molecular weight, are shown in Table S1.

2.2. Instrumentation and software (data analysis)

Ultrasound assisted extraction was performed using a 400 W digital sonifier (Branson Ultrasonic Corporation) with a 0.5-inch (12.7 mm) probe operating at a frequency of 20 kHz. The microwave extraction was performed with a Milestone (ETHOS SEL) microwave Labstation (Sheldon, CT, USA) operating at 2455 MHz with a maximum power of

1000 W. Time and temperature control were adjusted using Easy CONTROL-280 software. Lyophilization of samples was performed on a ScanVac CoolSafe™ freeze dryer. An IKA vortex mixer (Staufen, Germay) a Labnet Spectrafuge™ 24D centrifuge (New Jersey, USA), a vacuum centrifugal evaporator (LaboGene, Lynge, Denmark), an Ultrasounds-HD ultrasonic bath from Selecta (Barcelona, Spain), and a Mettler-Toledo GX400 balance (Columbus, OH, USA) were also used.

UHPLC-MS/MS analysis was performed using a Waters ACQUITY UPLC® H-Class (Waters, Manchester, UK), consisting of an ACQUITY™ sample manager, an ACQUITY UPLC™ binary solvent manager (Waters, UK) and an ACQUITY HSS T3 column (100 mm x 2.1 mm i.d., 1.8 µm particle size). A XEVO TQS tandem quadrupole mass spectrometer equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for antibiotic detection. MassLynx 4.1 software (Waters) was used for data acquisition, and Statgraphics plus 5.0 (Statpoint Technologies) together with excel (2019) were employed for both the experimental designs and the statistical calculations. Finally, BioRender.com (2022) online software was use to depict some of the figures.

2.3. Sample collection and storage

The echinoderms were collected by scuba divers from the infra-littoral zones along the coast of Granada (Spain). Sampling areas were: Area 1, Marina del Este: 36° 43' 21" N; 3° 43' 41" W; Area 2, San Cristóbal: 36° 43' 50" N; 3° 41' 58" W; Area 3. Tesorillo: 36° 44' 35" N; 3° 39' 44" W; Area 4, Playa Granada: 36° 42' 58" N; 3° 32' 37" W; Area 5, Carchuna: 36° 41' 40" N; 3° 26' 23" W; Area 6, Calahonda: 36° 41' 53" N; 3° 24' 54" W; Area 7, Mamola: 36° 44' 46" N; 3° 16' 36" W; Area 8. La Herradura: 36° 44' 25" N; 3° 44' 31.6" W; Area 9, Salobreña: 36° 43' 53.9" N; 3° 35' 27.2" W. A map of the locations is included as supplementary information (Figure S1).

The average weight of each *Holothuria tubulosa* specimen was approximately 200 g. The animals were transported to the laboratory in

containers at 4ºC. They were frozen at -20ºC until analysis. Before dissection they were washed to remove traces of sand and dirt. The different body parts were then separated. The body wall was selected for analysis since it was the most abundant body part, taking the volume and weight of sample into account. Samples were placed in sterile polythene containers for their subsequent lyophilisation, in order to ensure minimal degradation of the analytes. They were then pulverised, transferred to opaque containers and stored in a desiccator.

2.4. Experimental conditions

2.4.1. Preparation of fortified samples

Spiked holothurian samples were used for the optimization of the extraction procedure. The blanks (0.2 g of lyophilized samples) were weighted into 10 mL glass tubes and spiked at 100 ng g^{-1} (d.w), adding 0.2 mL of a methanol solution containing all target analytes and the surrogate standard (CIN). For validation and quantitative purposes, blank holothurian samples were spiked in a range from 1 to 250 ng g^{-1} (d.w). Likewise, all analysed holothurian specimens (natural samples) were spiked with the surrogate at 100 ng g^{-1} (d.w) before extraction. After fortification, samples were shaken in a vortex mixer (1 min) and were placed in a freeze dryer (5 h) to guarantee contact of the analytes with the matrix and complete evaporation of the organic solvent. Before extractions, all the fortified samples were left in the dark at room temperature for at least 24 h.

2.4.2. Extraction procedure (Sample treatment)

Aliquots of 0.2 g of dried holothurian samples were weighed into 10 mL glass tubes and spiked with the surrogate (CIN, 100 ng g^{-1} (d.w)) prior to extraction, as above. Then 4 mL of MeOH were added and the samples were vortexed for 30s for homogenization. The mixture was sonicated using an ultrasound digester at 33 % amplitude for 21 min. The samples were subsequently centrifuged at 4000 rpm (2600 $\times g$) for 5 min and the supernatant was then transferred to a different glass tube. Two extraction cycles were carried out, and the two supernatants were unified

in the same tube. The extract was then concentrated to approximately 50 % of the initial volume and a clean-up procedure by dispersive solid phase extraction (d-SPE) was carried out to remove interferences. For this purpose, 0.2 g of the adsorbent C18 was added to the extract resulting from the previous step (about 4 mL). After vortexing for 30 s, the mixture was centrifuged for 5 min at 4000 rpm (2600 × *g*). The supernatant was transferred to another glass tube and evaporated to dryness under a N_2 stream. The final dry residue was reconstituted in 250 µL of a solution of H_2O/MeOH 80/20 (v/v) (initial mobile phase) and sonicated for 10 min. In order to prevent contamination and clogging of the chromatographic column and the system, possible insoluble particles were removed by further centrifugation at 13,500 rpm (16,300 × *g*) for 10 min at room temperature. The obtained solution was introduced into a chromatographic vial and injected into the UPLC-MS/MS system.

2.4.3 UHPLC-MS/MS operating conditions

The chromatographic separation was performed using an ACQUITY UPLC® HSS T3 column (100 mm x 2.1 mm i.d., 1.8 µm particle size). The column was operated at 40ºC and the compounds were separated using a gradient mobile phase consisting of mixtures of an aqueous solution of formic acid (0.1 % v/v) as solvent A, and a solution of formic acid (0.1 % v/v) in methanol as solvent B. The chromatographic gradient was as follows: 0.0 to 1.0 (isocratic), 80 % solvent A; 6.0 min, 75 % A; 8.0 min, 70 % A; 9.0 min, 50 % A and 14.0 min, 0 % A. After that, 6 min were included for column cleaning and conditioning. The procedure is as follows: 14.0-16.0 min, 0 % A; 16.1 min, 80 % A and finally 16.1-20, 80 % solvent A. Constant flow rate was fixed a 0.3 mL min^{-1} and the injection volume was 2 µL. As far as detection is concerned, the mass spectrometer was operated in positive ESI mode and in multiple-reaction monitoring (MRM) mode, providing enhanced selectivity and specificity. Two MRM transitions were selected for each analyte, for quantification (the most abundant) and confirmation. Collision energy and cone voltage were also optimized for each transition. The mass spectrometer parameters are

summarised in Table 1, including the signal ratio between the quantification and confirmation transitions (a/b).

2.4.4 Experimental design. Factorial design 3^2

The 3^2 design is the simplest three-level full factorial design. It is used for simultaneous optimization of two factors, each at three different levels (low, intermediate, and high), resulting in 9 different treatment combinations. In this study, a total of three 3^2 factorial designs were built, in order to optimize the most important variables thorough the extraction procedure: a) solvent amount and number of extraction cycles in UAE, b) time and amplitude in UAE, c) and time and temperature in MAE. Each factorial matrix consisted of 12 runs, including three central point replicates (i.e. 9 + 3 central points). Each run was triplicated, for experimental error.

The analysis of variance (ANOVA) allowed assaying the statistical significance of each variable and the interactions between them by mathematical equations. In addition, Pareto charts were obtained to analyse the statistically significant effects of the variables using a student's t-test for ANOVA. Those variables, with at least 95 % of confidence, were considered to have a significant effect.

2.4.5 Validity requirements

Quality assurance/quality control (QA/QC) procedures were performed in order to guarantee the validity of the results obtained, according to the International Conference on Harmonization (ICH) guidelines for analytical method validation [22]. Procedural blanks were injected to monitor for background contamination, processed along with the samples and injected into the UHPLC-MS/MS system. To guarantee the absence of contamination and to control for equipment variations, standards reconstituted in the initial phase were injected every twenty injections. A procedural standard calibration curve in matrix was constructed for each analyte at eight concentration levels by plotting the analyte/surrogate ratio as a function of analyte concentration. Linearity was studied with correlation coefficients (%R^2) and the lack-of-fit test.

Analytical sensitivity, determined by the slope of the calibration curve, was also calculated. Since both the limit of detection (LOD, the minimum amount of analyte detectable) and the limit of quantification (LOQ, the minimum amount that can be quantified) are fundamental in the validation of the analytical method and to check for sensitivity, these parameters were studied by injecting procedural blanks fortified with decreasing amounts of the target analytes.

Finally, in order to assure the accuracy (trueness and precision) of the method, a recovery assay was carried out. For this purpose, spiked samples at three concentration levels (low, intermediate, and high) were analysed. Precision (% RSD) was determined with inter-day and intra-day assays, and trueness was assessed with recovery data.

Table 1. *Mass spectrometer parameters*

	t_R (min)	Transitions	CV (V)	CE (eV)	Ratio (a/b)		t_R (min)	Transitions	CV (V)	CE (eV)	Ratio (a/b)
TRI	3.16	291.11→229.98[a] 291.11→92.98[b]	76 76	18 30	27.6	MOX	9.79	402.02→384.09[a] 402.02→358.14[b]	34 34	22 18	1.2
MAR	3.38	363.11→320.00[a] 363.11→72.05[b]	18 18	12 18	1.5	NAL	11.07	233.05→215.02[a] 233.05→187.00[b]	20 20	12 22	1.8
LEVO	4.37	362.05→318.10[a] 362.05→261.07[b]	30 30	16 24	1.3	FLU	11.22	261.92→244.03[a] 261.92→201.96[b]	14 14	18 28	1.9
NOR	4.89	320.17→302.02[a] 320.17→276.08[b]	20 20	16 14	1.3	OXO	11.23	261.97→244.00[a] 261.97→215.91[b]	24 24	14 24	303
CIP	5.49	332.10→314.03[a] 332.10→230.98[b]	26 26	16 32	1.1	ERY	11.27	734.37→158.06[a] 734.37→576.34[b]	18 18	24 14	1.8
SUL	5.60	253.89→155.94[a] 253.89→92.01[b]	20 20	14 26	1.7	PEN	11.48	351.13→229.01[a] 351.13→332.99[b]	54 54	26 14	1.4
ENR	6.03	360.07→316.06[a] 360.07→245.05[b]	18 18	16 22	2.1	OXA	11.74	401.99→144.02[a] 401.99→384.08[b]	60 60	20 14	3.9
DAN	6.24	358.12→340.05[a] 358.12→314.11[b]	40 40	18 36	4.0	CLA	11.81	748.38→158.05[a] 748.38→590.30[b]	28 28	24 14	0.1
DIF	7.10	399.98→356.11[a] 399.98→299.02[b]	42 42	16 26	1.3	CIN	11.91	250.11→128.04[a] 250.11→222.07[b]	40 40	30 26	2.0
Source temperature: 158.9 ºC						Impactor voltage: 2.9 kV					
Desolvation temperature: 600 ºC						Cone gas flow: 150-147 L h^{-1}					
Desolvation gas flow: 1000-989 L h^{-1}						Nebulizer gas pressure: 7.00-6.94 bar					
Cone/desolvation gas: N_2 (≥ 99.995 %)						Collision gas: Ar (≥ 99.999 %)					
Dwell time: 25 ms						Inter-scan delay: 3 ms					

t_R: Retention time; [a] Transition for quantification; [b] Transition for confirmation; CV: Cone voltage; CE: Collision energy

3. Results and discussion

3.1. Optimization of the extraction parameters

The most significant variables affecting the extraction process were studied. Aliquots of 0.2 g of spiked samples (d.w) were used, performing each test in triplicate. The extraction solvent, its amount, the number of cycles as well as the extraction technique and clean-up process were evaluated.

3.1.1 Selection of extraction solvent

Several solvents with different polarities were tested in order to guarantee the correct isolation of the analytes from the matrix with good recoveries. MeOH, ACN, ACE, AcOEt, and mixtures of MEOH/ACN (25/75, v/v), MeOH/ACN (50/50, v/v), MeOH/ACN (75/25, v/v) were tested. The selection was based on the scientific bibliography for marine samples [16,19]. The results obtained are included as supplementary material (Figure S2). For most analytes, MeOH offers the best extraction efficiencies. Only in the case of ENR the mixture of MeOH/ACN (75/25, v/v) provided better results. Therefore, MeOH was selected as the extraction solvent.

3.1.2. Optimization solvent volume and number of extraction cycles

Once the extraction solvent was selected, a factorial experimental design 3^2 was carried out, considering two variables simultaneously: the solvent volume and the number of extraction cycles. The aim was to design a response surface with three central points. For this, three levels of each of the variables were evaluated: 2, 3 and 4 mL of MeOH, and 1, 2, and 3 extraction cycles. In total, 12 experiments were performed, the different conditions of which are shown in Table S2.

The results of ANOVA showed R^2 values between 23.9 and 96.56 %, with most of them located at around 90 %. P-values were higher than 0.05, confirming that the model was satisfactory with a confidence level of 95 %. Pareto charts obtained (supplementary material, Figure S3) showed that the number of extraction cycles was the most significant

variable in most cases, with a positive influence. The desirability function for each compound, as well as the surface responses, showed the results normalized between 0 and 1. The global desirability function was also calculated to consider the best overall extraction efficiency. The results are shown in Figure 1.

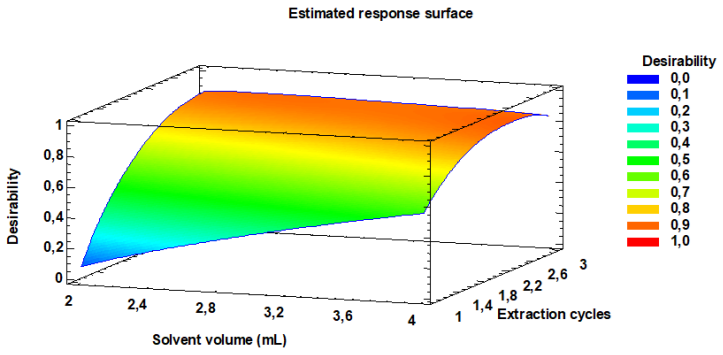

Figure 1. *Global desirability 3D plot. Solvent volume and extraction cycles*

As is shown in the obtained 3D surface response plot, two extraction cycles and 4mL of MeOH achieved better extraction efficiency, with a 90 % desirability rate. Although volume did not account for a significant change in extraction efficiency, greater volume did improve the desirability for most antibiotics. Since the compounds of interest belong to different families of antibiotics, optimal conditions varied according to the group. Nevertheless, the overall result encompassed optimum extraction efficiencies for them all.

3.1.3. Selection of the digestion medium and testing of different acids

In order to improve extraction efficiency, three preliminary tests were carried out to compare extraction in a neutral (MeOH) medium, a basic and acidic one. Two methanolic solutions (0.1 M) were prepared: NaOH for the basic medium and HCl for the acidic one. Then, three different digestion procedures were performed, the difference being the extractant solvent. Each was done in triplicate. The results obtained are included as supplementary material (Figure S4). The neutral medium offered extraction efficiencies similar to the acid medium in most cases.

In light of the results, a second test with different acids was performed, covering a wide range of strengths, to evaluate whether the addition of acids to the solvent favours extraction, compared to the solvent with no additives.

Methanol solutions of different acids (0.1 M) were prepared. The acids tested were HCl, $HClO_4$, H_3PO_4, formic acid and acetic acid. The results obtained are included in the supplementary material (Figure S5). The graph shows that none of the acids caused a significant improvement in extraction yield over the pure solvent. In the case of NAL, CIP, NOR and FLU, better results were obtained with acidic digestions, mainly with HCl (0.1 M). However, for the other analytes the best (or similar) results were obtained with pure MeOH. As a result the decision was taken for most of the target analytes to continue the optimisation without adding acid to the solvent.

3.1.4. Selection of the extraction technique

Two assisted extraction techniques were compared: ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). The main parameters that could affect extraction were optimised for each technique. In the case of UAE the parameters were digestion time and amplitude and, for MAE, digestion time and temperature. Two 3^2 experimental designs were carried out. The experimental matrices are shown in the supplementary material (Tables S3 and S4 for UAE and MAE respectively). Once the optimal conditions for each assisted extraction were deduced, the techniques were compared for extraction efficiency and practicability.

In the case of UAE, the results of ANOVA showed determination coefficients (R^2) between 19.6 % for ERY and 76.5 % for ENR, while P-values were greater than 5 % in all cases, with the model being satisfactory at the 95 % confidence level. This means that all parameters were significant. Figure 2 shows the normalized estimated response for all analytes. The optimal extraction conditions for UAE are 21.6min and 33 % amplitude, achieving a desirability of 64 %.

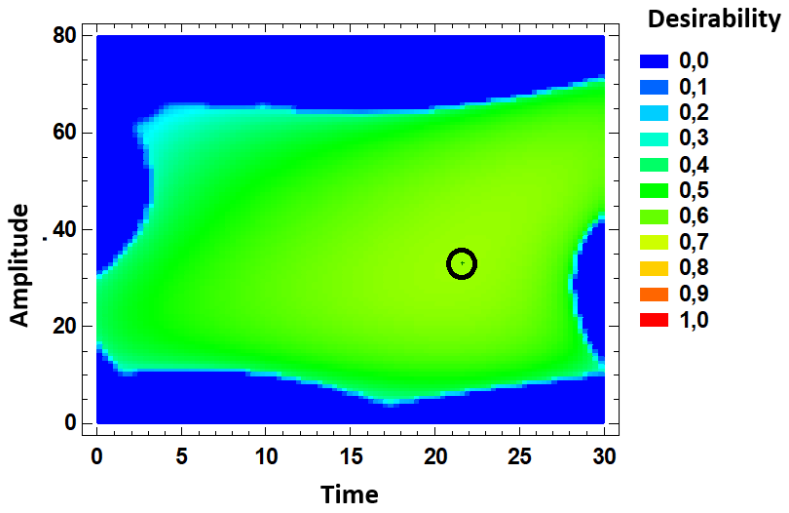

Figure 2. *Global desirability 2D plot for UAE. Amplitude and time*

Regarding MAE, p-values were also higher than 5 %, with a confidence level of 95 %. ANOVA analysis showed determination coefficients between 10.6 % and 93.1 % for MAR and ERY, respectively. Figure 3 depicts the result obtained for the normalised global desirability function in this case. The optimal values were similar for all analytes, 1 min being the optimal time and 142ºC the temperature for maximum extraction efficiency, with 69 % desirability.

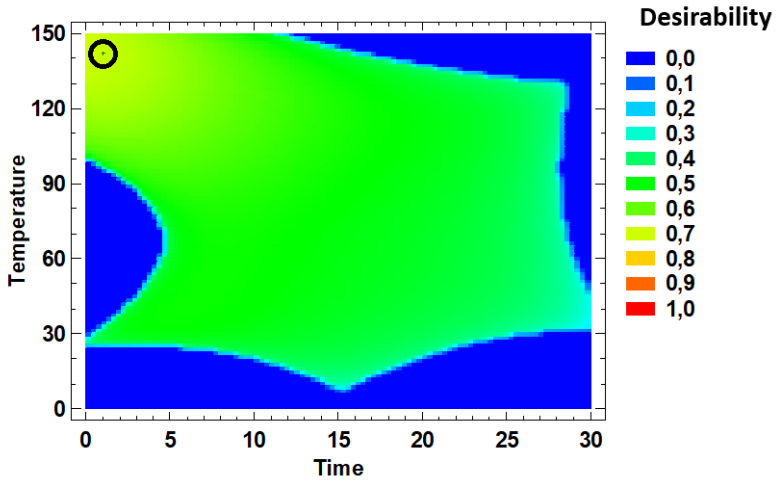

Figure 3. *Global desirability 2D plot for MAE. Temperature and time*

Given that the results obtained were similar for both extraction techniques, UAE was selected. This decision was based on the poorer practicability criteria (time required for one complete cycle) for MAE, including the temperature ramp and time needed to reach 142ºC (12 min), the ventilation time (5 min) and the waiting time for the solvent to cool down (about 30 min).

3.1.5. Clean-up step

The clean-up process of the UAE extracts is important in order to safeguard the chromatographic system, especially when dealing with complex matrices, even if that means a loss of signal and recovery. A preliminary experiment was conducted to select the sorbent that achieves the best extraction efficiency. For this purpose, C18, PSA and a mixture of both adsorbents (50/50, w/w) were compared. The results are shown in the supplementary material section (Figure S6). The addition of C18 does not cause significant signal loss, except in some cases such as ENRO or DIF, and these losses were more noticeable when PSA or a mixture of both sorbents was used. PSA offered better results only in the case of ERY and OXO. C18 was selected for the clean-up step as a compromise solution.

Once the clean-up sorbent was selected, the addition of different amounts of C18 adsorbent was studied. The aim was to determine what quantity was optimal to obtain a clean extract, without excessively compromising either the sensitivity or efficiency of the method. The assays were performed in triplicate using 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0g of C18 sorbent. The results are also shown in the supplementary material (Figure S7). It can be noted that, for most analytes, as the amount of sorbent increases the analytical signal drops significantly. Therefore, the minimal amount (0.2 g) of C18 was selected as the optimal amount of clean-up, since after performing the experiment, the extracts were observed to be clean enough to be injected into the equipment, and the signal did not decrease excessively.

3.2. Analytical performance

The calibration curve was performed using the analyte/surrogate ratio vs analyte concentration. The surrogate was CIN (25 ng g^{-1}). A matrix calibration (spiking the *Holothuria* blank samples prior to extraction) was performed due to the high matrix effect observed during the optimization of the method. To construct the calibration curve, 5, 6 or 7 concentration levels were performed, depending on the LOQ of each compound. Each level was made in triplicate and each replicate was injected three times (n= 45, n=54 or n=63). Table 2 summarises the parameters obtained for each analyte.

Table 2. *Matrix calibration parameters. Accuracy, recovery and precision*

	n	b	R^2	LOD	LOQ	Recovery study, %R (RSD, %, n=15)		
		g ng^{-1}	%	ng g^{-1}	ng g^{-1}	LOQ (ng g^{-1})	50 (ng g^{-1})	100 (ng g^{-1})
TRI	7	$4.68 \cdot 10^{-2}$	98.9	0.3	1.0	87.9 (8.3)	96.4 (7.3)	100.6 (4.8)
MAR	5	$3.14 \cdot 10^{-3}$	97.6	3.0	10.0	107.2 (12.6)	93.6 (11.9)	101.3 (7.7)
LEVO	6	$1.82 \cdot 10^{-3}$	98.9	1.0	4.0	114.9 (6.3)	98.5 (5.6)	100.4 (7.8)
NOR	6	$8.72 \cdot 10^{-3}$	99.2	1.0	4.0	100.9 (9.3)	92.2 (7.9)	101.5 (2.9)
CIP	6	$9.07 \cdot 10^{-3}$	94.1	2.0	5.0	90.2 (6.3)	88.4 (5.2)	106.5 (1.8)
SUL	6	$1.32 \cdot 10^{-3}$	99.0	2.0	5.0	109.0 (4.8)	101.7 (13.8)	99.4 (3.3)
ENR	6	$1.72 \cdot 10^{-2}$	98.6	1.0	4.0	113.4 (5.3)	97.8 (9.4)	100.8 (8.2)
DAN	5	$7.03 \cdot 10^{-3}$	97.5	3.0	10.0	109.9 (10.4)	90.8 (10.0)	102.0 (7.5)
DIF	6	$2.47 \cdot 10^{-3}$	98.6	2.0	5.0	105.2 (6.8)	106 (11.0)	99.3 (3.5)
MOX	6	$7.44 \cdot 10^{-3}$	98.6	2.0	5.0	110.5 (8.1)	92.0 (1.3)	101.5 (8.0)
NAL	7	$2.42 \cdot 10^{-2}$	99.1	0.3	1.0	97.8 (6.9)	98.8 (10.4)	100.4 (5.9)
FLU	7	$1.28 \cdot 10^{-2}$	99.4	0.3	1.0	107.1 (14.4)	99.6 (6.8)	99.8 (5.7)
OXO	7	$1.01 \cdot 10^{-1}$	98.9	0.3	1.0	94.0 (2.2)	98.3 (7.5)	100.1 (8.1)
ERY	6	$4.92 \cdot 10^{-3}$	93.3	2.0	5.0	103.8 (5.8)	87.5 (9.0)	89.7 (6.6)
PEN	5	$3.46 \cdot 10^{-3}$	84.1	3.0	8.0	94.9 (9.8)	91.5 (14.0)	95.9 (10.6)
OXA	6	$1.91 \cdot 10^{-3}$	97.5	1.0	5.0	102.5 (10.5)	91.0 (13.3)	102.2 (5.9)
CLA	6	$4.39 \cdot 10^{-2}$	99.1	1.0	4.0	103.7 (5.4)	100.6 (2.2)	99.9 (6.5)

Linear dynamic range: LOQ-100 ng g^{-1}; n: calibration levels; b: slope of matrix calibration; %R^2 determination coefficient; LOQ: Limit of quantification; LOD: Limit of detection; RSD: Relative Standard Deviation.

3.3. Validation of the method

The proposed method was successfully validated following the recommendations of the ICH [21]. Linearity, sensitivity (limits of detection and quantification), accuracy (in terms of trueness and precision) and selectivity were determined.

3.3.1. Linearity

The concentration range was established from the limit of quantification (LOQ) to 100 ng g^{-1} for all analytes. The linearity of the curve was assessed by the coefficient of determination (% R^2) as well as P-values of the *lack-of-fit* test (P_{lof}). The % R^2 values ranged from 84.1 to 99.4 % and all P_{lof} were greater than 5 %, assuring a good linearity of the curves. These parameters are also included in Table 2.

3.3.2. Sensitivity

The analytical sensitivity was defined by the slope of the calibration line, calculating both limits of detection (LODs) and limits of quantification (LOQs). These parameters were estimated by interpolating the calibration curve, the signal corresponding to three times the signal-to-noise ratio for LODs and ten times the above ratio for LOQs. For LOD, the values were between 0.3 and 3 ng g^{-1} and for LOQ between 1 and 10 ng g^{-1}. The results are also shown in Table 2.

3.3.3. Accuracy (precision and trueness)

Due to the lack of reference materials, accuracy was assessed with spiked Holothuria blank samples. To study the precision of the method, three concentration levels (close to LOQ, 50 and 100 ng g^{-1}) were considered. The intra-day and inter-day precision was studied as the standard deviation, RSD (%). Variability was assessed by repeating the procedure three times on the same day, for four consecutive days. The trueness of the method was evaluated by a recovery assay. The fortified samples with known concentrations were processed and the concentration of each analyte was determined. Subsequently, the calculated concentration was compared with the known one and the

percentage recovery was calculated. Standard deviations below 15 % and recoveries close to 100 % (87.5-114.9 %) in all cases were achieved. Therefore it can be stated that the proposed method is truthful, precise and accurate.

3.3.4. Specificity

Specificity of the method was studied by the blank analysis. The chromatogram confirms the absence of interfering agents at the retention time of the target analytes. A chromatogram of the blank is included as supplementary material (Figure S8). Figure 4 shows a chromatogram of a blank fortified with the analytes at a concentration of 100 ng g^{-1}.

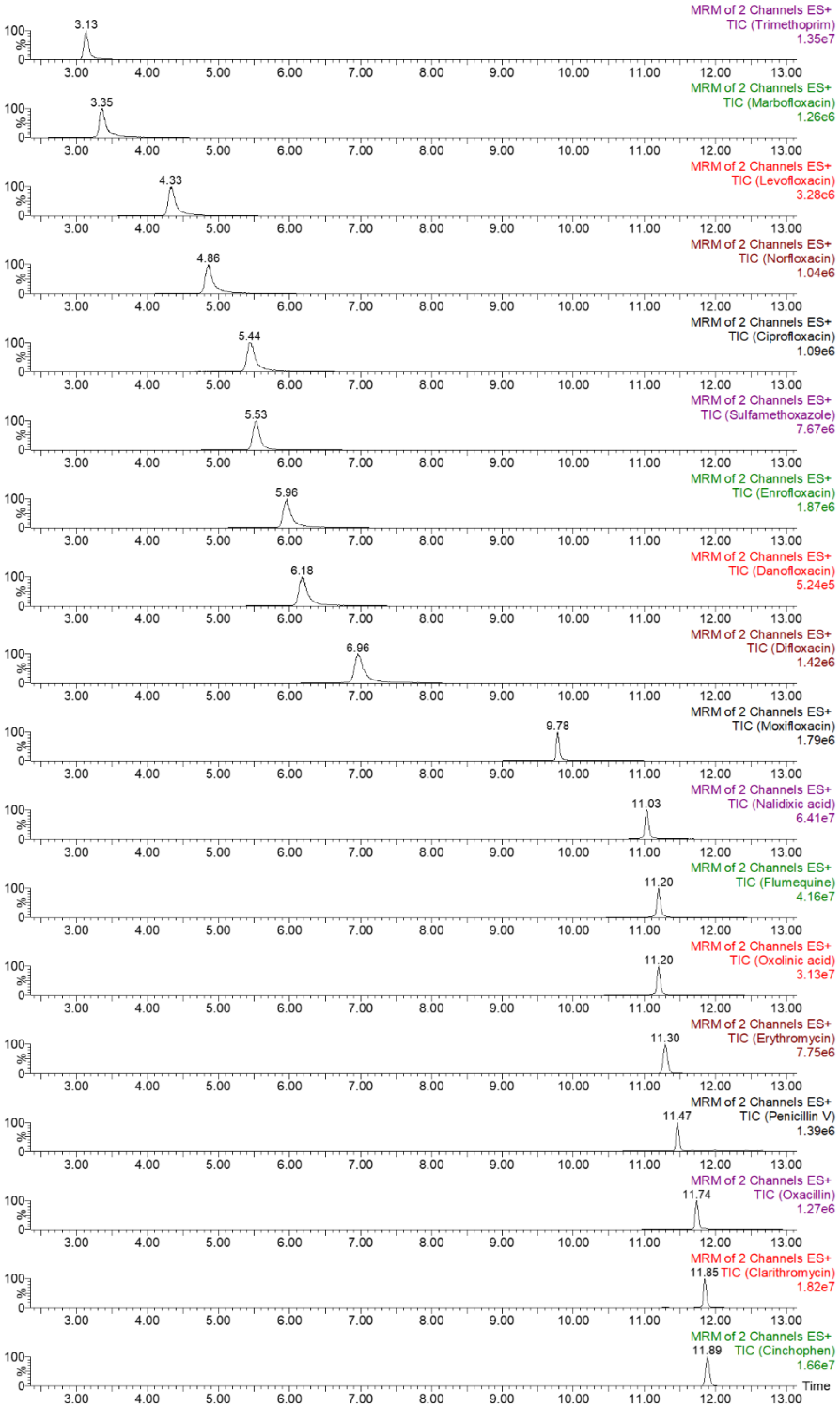

Figure 4. *Spiked blank sample containing all analytes (100 ng g^{-1})*

3.4. Biomonitoring of antibiotics in Holothuria tubulosa. Method application

The method was applied to holothurian specimens from 9 different locations on the coast of Granada (Spain). Six replicates were carried out for each area and each sample was analysed in triplicate. The mean concentration values for each analyte (ng g^{-1}) are shown in Table 3. A chromatogram of an analyte-contaminated sample is depicted in Figure 5. Of the 17 antibiotics studied, 15 of them were detected in at least one site, with OXA and PEN not detected in any case. The detected concentrations ranged from 1.2 (TRI) to 68.0 (DAN) ng g^{-1}.

Table 3. *Application of the method. Concentration in ng g^{-1}. Mean of six determinations*

	M1	M2	M3	M4	M5	M6	M7	M8	M9
CLA	D	D	ND	ND	D	ND	5.0 (6.2)	ND	ND
ERI	D	D	ND	D	ND	ND	22.8 (14.2)	ND	ND
MOX	D	ND	ND	D	D	D	11.2 (1.6)	ND	ND
OXA	ND	ND	ND	ND	ND	ND	ND	ND	ND
DIF	D	ND	ND	D	ND	ND	38.6 (4.7)	9.7 (1.0)	ND
MAR	D	ND	ND	D	ND	ND	11.8 (8.2)	ND	ND
LEV	9.8 (9.8)	D	ND	D	D	D	43.2 (14.9)	ND	ND
ENR	10.2 (14.0)	D	ND	D	ND	ND	28.0 (4.1)	ND	ND
DAN	60.4 (1.0)	D	D	D	ND	D	68.0 (8.7)	D	D
PEN	ND	ND	ND	ND	ND	ND	ND	ND	ND
CIP	39.6 (2.5)	D	D	33.3 (8.4)	19.9 (8.5)	D	19.3 (14.8)	D	11.0 (7.2)
NOR	D	ND	ND	D	D	ND	16.9 (7.4)	ND	ND
TRI	D	1.2 (4.2)	ND	ND	D	D	1.2 (6.7)	D	ND
OXO	D	ND	ND	D	D	ND	10.3 (9.0)	ND	ND
FLU	13.8 (9.0)	D	D	D	1.2 (6.2)	D	5.3 (5.8)	D	D
SUL	8.0 (14.4)	D	D	D	D	D	21.6 (4.7)	D	D
NAL	1.3 (2.2)	1.6 (4.1)	D	1.1 (9.0)	1.7 (8.4)	1.1 (9.7)	3.0 (2.2)	D	2.1 (2.9)

ND: Not detected; D: Detected (LOD < x < LOQ)

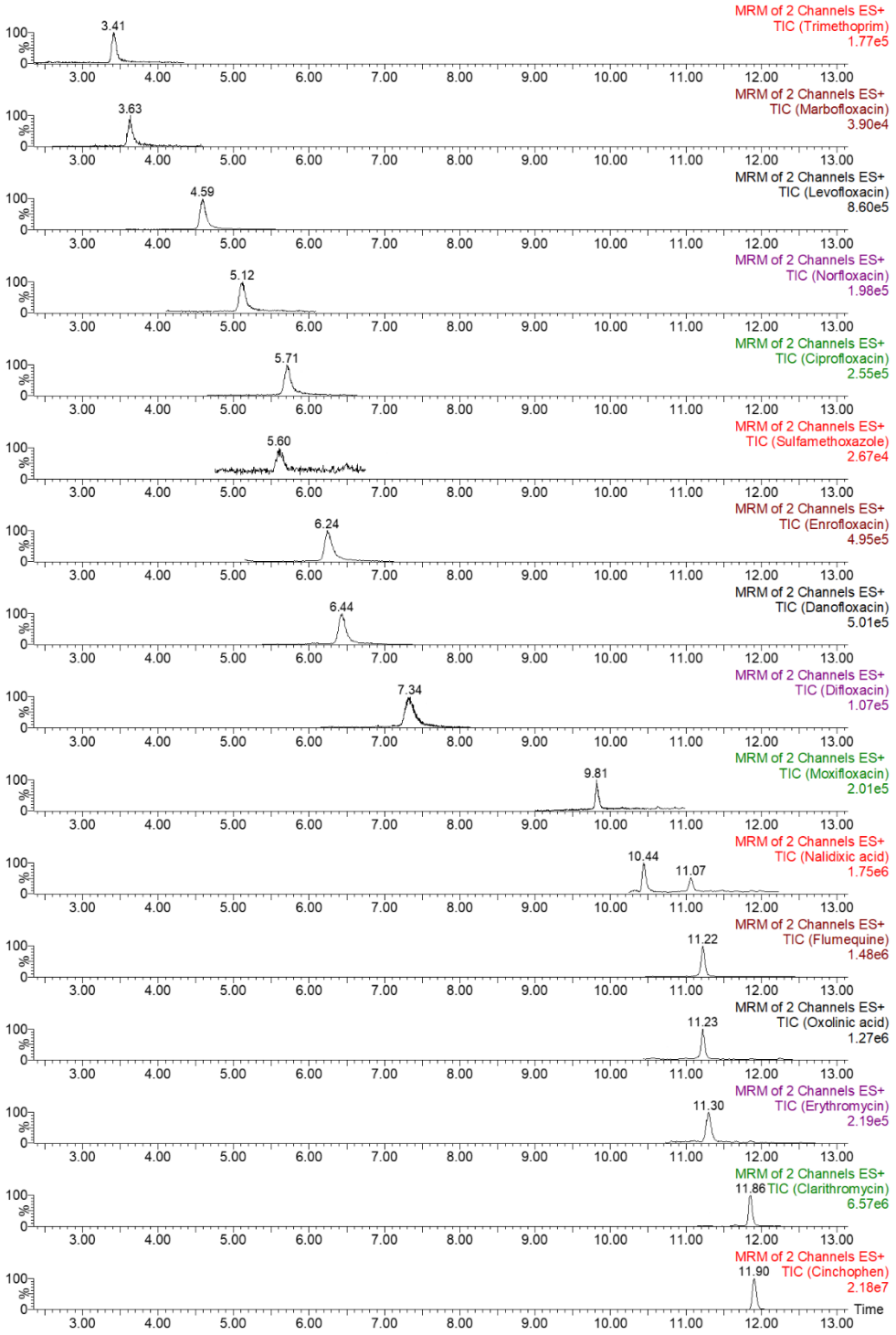

Figure 5. *MRM mode chromatogram of a contaminated sample (Area 7)*

CIP, SUL, FLU and NAL were detected in 100 % of the cases (9/9), being quantified in 55.6 %, 22.2 %, 33.3 % and 77.8 % of the samples respectively. These antibiotics belong to different families, which demonstrate the importance of performing multi-class studies that contemplate the presence of a range of compounds simultaneously. Currently, there is a known problem in the removal of quinolones from wastewater in treatment plants, allowing them to pass into the marine system [23]. In addition, CIP is one of the most frequently used quinolones due to its pharmacokinetic properties and antimicrobial activity, which could explain its presence in all the studied locations [24]. On the other hand, of the sulphonamide family, sulfamethoxazole-trimethoprim, which acts jointly and synergistically, is frequently used in aquaculture, which justifies the presence of this compound in all samples [25]. Baesu *et al.* [26] found a mean SUL concentration of 25.6 ng g^{-1} in fish livers, similar to that found at location 7 which, according to the Andalucía Government and European Union, is a suitable area of the coastline for aquaculture, which could explain why it had the highest concentrations of this antibiotic [27]. Locations 1 and 7 were positive for all analytes (except OXA and PEN), as well as being the most contaminated areas. These two areas also accounted for the highest concentration of any one antibiotic, corresponding to DAN, a fluoroquinolone widely used in veterinary medicine. The detected concentrations of DAN were 60.4 and 68.0 ng g^{-1} in area 1 and 7, respectively [28].

Although the scientific literature on the presence of antibiotics in *Holothuria tubulosa* is limited, other studies in different matrices were consulted to contrast the results. For example, Bortolotte *et al.* [29] studied the presence of 25 antibacterial drugs (8 quinolones and five sulfonamides, among others) in pangasius from Vietnam, and only detected levels of ENR in 5 samples out of 40. This is consistent with the fact that this antibiotic is one of the most widely used in aquaculture [30]. While panga fish are normally raised in fish farms, the specimens of Holothuria in the present study were caught in the wild. Despite this,

ENR was determined in 4 of the 9 locations, which may be caused by the proximity of the captured species to coastal fish farms.

Some concentrations reported in other matrices, such as fish muscle, are lower than those calculated in this study. Miossec *et al.* [31] simultaneously determined 42 drugs in sea food (in three types of fish and one crustacean), detecting only four of the drugs studied and thus suggesting low contamination in these specimens. Miossec and her group carried out their study in open sea, while the echinoderms in our research were collected at points close to the coast. It is to be expected that in the open sea there will be less or more diluted drugs residues. In addition to the geographical and situational factor, other characteristics such as diet, feeding habits, and metabolic capacity, can influence bioaccumulation in organisms. All these variables could explain why the concentration of pollutants is higher in the holothurians of the current study [31]. In a similar work, Danesaki *et al.* studied the presence of 143 veterinary drugs in fish tissue [32]. None of the analytes were detected, suggesting that certain compounds are metabolised by fish and are therefore not detected in their free form, unlike in the case of holothurians, which may be more susceptible to bioaccumulate pharmaceuticals, as has been reported for other types of pollutants [17]. Further studies are needed regarding antibiotics.

Other relevant studies determined antimicrobials in fish or shrimp [33], finding concentrations of 20-75 µg kg^{-1} of CLA, 45-80 µg kg^{-1} of ERY, 15-45 µg kg^{-1} of SUL or 80-115 µg kg^{-1} of CIP, all generally higher concentrations than those found in the current study. They studied 5 types of fish and 4 types of shrimps, purchased in a local Polish market and, according to Serra-Compte *et al.* [16], it can be concluded that samples from aquaculture show higher concentrations than wild specimens.

The concentrations found in different biological matrices of marine origin may depend on many factors, such as the metabolising capacity of the organism, how close they are to coastal areas, whether they are

farmed or wild-caught or the degradation of the compounds in the environment.

4. Conclusions

A multiclass UHPLC-MS/MS method for the analysis of antibiotics in *Holothuria tubulosa* samples was optimized and validated. The proposed method allowed for the simultaneous determination of 17 antibiotics belonging to different chemical classes. To the best of our knowledge no previous study has tested for antibiotics in the *Holothuria tubulosa* species. The extraction procedure was simple and included a two cycle UAE using methanol as the extracting solvent, and a clean-up step based on d-SPE using C18 as the adsorbent. The comparison of MAE and UAE techniques showed UAE as the most effective in terms of applicability and time-saving/recovery. The analytical performance of the method was good in terms of linearity, accuracy (trueness, and precision), sensitivity and selectivity, in compliance with ICH international guideline. The method was applied to wild holothurians collected from nine sites along the Granada coast. NAL, SUL, FLU and CIP were detected in 100 % of the samples, and DAN was quantified at the highest concentrations (68 ng g^{-1}). The high frequency of detection of the studied antibiotics reflects urban pressure along the coastline, causing sea pollution via a variety of avenues, such as veterinary drug residues or wastewater.

The present study demonstrates the bioaccumulation of antibiotic residues in *Holothuria tubulosa* specimens for the first time, revealing the capacity of these organisms to accumulate this type of substances and, in turn, their appropriate use as a biomarker, taking into account their biological characteristics (filter-feeding and sedentary), for future research on the monitoring of these compounds and on ecotoxicological studies in marine waters.

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Declaration of competing interest

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

References

[1] Y. Ben, C. Fu, M. Hu, L. Liu, M.H. Wong, C. Zheng, Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review, Environ. Res. 169 (2019) 483-493.

[2] E.J. Ozumchelouei, A.H. Hamidian, Y. Zhang, M. Yang, Physicochemical properties of antibiotics: A review with an emphasis on detection in the aquatic environment, Water Environ. Res. 92 (2020) 177-188.

[3] V.N. Binh, N. Dang, N.T.K. Anh, L.X. Ky, P.K. Thai, Antibiotics in the aquatic environment of Vietnam: Sources, concentrations, risk and control strategy, Chemosphere 197 (2018) 438-450.

[4] K.I. Ekpeghere, J-W. Lee, H-Y. Kim, S-K. Shin, Determination and characterization of pharmaceuticals in sludge from municipal and livestock wastewater treatment plants, Chemosphere 168 (2017) 1211-1221.

[5] M.A. Bighiu, A.N. Haldén, W. Goedkoop, J. Ottoson, Assessing microbial contamination and antibiotic resistant bacteria using zebra mussels (*Dreissena polymorpha*), Sci. Total Environ. 650 (2019) 2141-2149.

[6] A. Ezzariai, D. Riboul, M.Z. Lacroix, M. Barret, L. El Fels, G. Merlina, A. Bousquet-Melou, D. Patureau, E. Pinelli, M. Hafidi, A pressurized liquid extraction approach followed by standard addition method and UPLC-MS/MS for a fast multiclass determination of antibiotics in a complex matrix, Chemosphere 211 (2018) 893-902.

[7] P. Saxena, I. Hiwrale, S. Das, V. Shukla, L. Tyagi, S. Pal, N. Dafale, R. Dhodapkar, Profiling of emerging contaminants and antibiotic resistance in sewage treatment plants: An Indian perspective, J. Hazard. Mater. 408 (2021) 124877.

[8] S. Decheng, W. Peilong, L. Yang, W. Ruiguo, W. Shulin, X. Zhiming, Z. Su, Simultaneous determination of antibiotics and amantadines in animal-derived feedstuffs by ultraperformance liquid chromatographic-tandem mass spectrometry, J. Chromatogr. B 1095 (2018) 183-190.

[9] D. Magalhães, A. Freitas, A.S. Vila Pouca, J. Barbosa, F. Ramos, The use of ultra-high-pressure-liquid-chromatography tandem time-of-flight mass spectrometry as a confirmatory method in drug residue analysis: Application to the determination of antibiotics in piglet liver, J. Chromatogr. B 1153 (2020) 122264.

[10] P. Chaturvedi, P. Shukla, B.S. Giri, P. Chowdhary, R. Chandra, P. Gupta, A. Pandey, Prevalence and hazardous impact of pharmaceutical and personal care products and antibiotics in environment: A review on emerging contaminants, Environ. Res. 194 (2021) 110664.

[11] J. Li, I.A. Phulpoto, G. Zhang, Z. Yu, Acceleration of emergence of *E. coli* antibiotic resistance in a simulated sublethal concentration of copper and tetracycline co-contaminated environment, AMB Expr. 11 (2021) 14.

[12] EFSA (European Food Safety Authority), Opinion of the scientific panel on contaminants in the food chain on Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts, EFSA J. 653 (2008) 1-131.

[13] L.M. Chiesa, M. Nobile, F. Ceriani, R. Malandra, F. Arioli, S. Panseri, Risk characterisation from the presence of environmental contaminants and antibiotic residues in wild and farmed salmon from different FAO zones, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 36 (2019) 152-162.

[14] Y. Han, W. Zhou, Y. Tang, W. Shi, Y. Shao, P. Ren, J. Zhang, G. Xiao, H. Sun, G. Liu, Microplastic aggravate the bioaccumulation of three veterinary antibiotics thick shell mussel *Mytilus coruscus* and induce synergistic immunotoxic effects, Sci. Total Environ. 770 (2021) 145273.

[15] B. González-Gaya, L. Cherta, L. Nozal, A. Rico, An optimized sample treatment method for the determination of antibiotics in seawater, marine sediments and biological samples using LC-TOF/MS, Sci. Tot. Environ. 643 (2018) 994-1004.

[16] A. Serra-Compte, D. Álvarez-Muñoz, S. Rodríguez-Mozaz, D. Barceló, Multi-residue method for the determination of antibiotics and some of their metabolites in seafood, Food Chem. Toxicol. 104 (2017) 3-13.

[17] J. Martín, F. Hidalgo, M.T. García-Córcoles, A.J. Ibáñez-Yuste, E. Alonso, J.L. Vílchez, A. Zafra-Gómez, Bioaccumulation of perfluoroalkyl substances in marine echinoderms: Results of laboratory-scale experiments with *Holothuria tubulosa* Gmelin, 1791, Chemosphere 215 (2019) 261-271.

[18] J. Martín, F. Hidalgo, E. Alonso, M.T. García-Córcoles, J.L. Vílchez, A. Zafra-Gómez, Assessing bioaccumulation potential of personal care, household and industrial products in marine echinoderm (*Holothuria tubulosa),* Sci. Tot. Environ. 720 (2020) 137668.

[19] I.E.Künili, F.A. Çolakoglu, Chemical and nutritional characteristics of *Holothuria tubulosa* (Gmelin, 1788); A seasonally comparative study, J. Aquat. Food Prod. Technol. 28(7) (2019) 716-728.

[20] J. Martín, A. Zafra-Gómez, F. Hidalgo, A.J. Ibáñez-Yuste, E. Alonso, J.L. Vílchez, Multi-residue analysis of 36 priority and emerging pollutants in marine echinoderms (*Holothuria tubulosa*) and marine sediments by solid-liquid extraction followed by dispersive solid phase extraction and liquid chromatography-tandem mass spectrometry analysis, Talanta 166 (2017) 336-348.

[21] M. Parra-Luna, L. Martín-Pozo, F. Hidalgo, A. Zafra-Gómez, Common sea urchin (*Paracentrotus lividus*) and sea cucumber of the genus *Holothuria* as bioindicators of pollution in the study of chemical contaminants in aquatic media. A revision, Ecol. Indic. 113 (2020) 106185.

[22] ICH Quality Guidelines, Topic Q2 (R1), Validation of analytical procedures: text and methodology (2005). At http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html (accessed on November 2022).

[23] L. Martín-Pozo, M.C. Gómez-Regalado, M.T. García-Córcoles, A. Zafra-Gómez, Removal of quinolone antibiotics from wastewaters and sewage sludge, in: Sarma, H., Domínguez, D.C., Lee, W-Y. (Eds), Emerging Contaminants in the Environment. Challenges and Sustainable Practices. Chapter 16 (2022) 381-406.

[24] T.D.M. Pham, Z.M. Ziora, M. A. T. Blaskovich, Quinolone antibiotics, Med. Chem. Commun. 10 (2019) 1719-1739.

[25] A. Serra-Compte, D. Álvarez-Muñoz, M. Solé, N. Cáceres, D. Barceló, S. Rodríguez-Mozaz, Comprehensive study of sulfamethoxazole effects in merine mussels: Bioconcentration, enzymatic activities and metabolomics, Environ. Res. 173 (2019) 12-22.

[26] A. Baesu, G. Ballash, D. Mollenkopf, T. Wittum, S.M.P. Sullivan, S. Bayen, Suspect screening of pharmaceuticals in fish livers based on QuEChERs extraction coupled with high resolution mass spectrometry, Sci. Tot. Environ. 783 (2021) 146902.

[27] EU, European Union, Regulation (EU) o 1380/2013 of the European Parliament and of the council of 11 December 2013 on the Common Fisheries Policy, amending Council Regulations (EC) No 2371/2002 and (EC) No 639/2004 and Council Decision 2004/585/EC, Off. J. Eur. Union 354 (2013) 22-61.

[28] E.W.F. Cordeiro, R. Medeiros Hilgert, L.A. das Chagas Batista, R. Oliveira Souza, L.F. Souza de Oliveira, M. Mansur Machado, M.D.

Malesuik, F.R. Paula, C.D. Ottonelli Stopiglia, C. Soldateli Paim, Validation of an analytical method by high-performance liquid chromatography and microbiological assay, biological safety and in silico toxicity for danofloxacin, Braz. J. Pharm. Sci. 56 (2020) c:18355.

[29] A.R. Bortolotte, D. Daniel, P. Aparecida de Campos Braga, F.G. Reyes Reyes, A simple and high-throughput method for multiresidue and multiclass quantitation of antimicrobials in pangasius *(Pangasiodon hypophthalamus)* fillet by liquid chromatography coupled with tandem mass spectrometry, J. Chromatogr. B 1124 (2019) 17-25.

[30] M. Andrieu, A. Rico, T.M. Phu, D.T.T. Huong, N.T. Phuong, P.J. Van den Brink, Ecological risk assessment of the antibiotic enrofloxacin applied to *Pangasius* catfish farms on the Mekong Delta, Vietnam, Chemosphere 119 (2015) 407-414.

[31] C. Miossec, T. Mille, L. Lanceleur, M. Monperrus, Simultaneous determination of 42 pharmaceuticals in seafood samples by solvent extraction coupled to liquid chromatography-tandem mass spectrometry, Food Chem. 322 (2020) 126765.

[32] M.E. Danesaki, A.A. Bletsou, G.A. Koulis, N.S. Thomaidis, Qualitative multiresidue screening method for 143 veterinary drugs and pharmaceuticals in milk and fish tissue using liquid chromatography quadrupole-time-of-flight mass spectrometry, J. Agric. Food Chem. 63 (2015) 4493-4508.

[33] R. Pashaei, R. Dzingeleviciene, S. Abbasi, M. Szultka-Mlynska, B. Buszewski, Determination of 15 human pharmaceutical residues in fish and shrimp tissues by high-performance liquid chromatography-tandem mass spectrometry, Environ. Monit. Assess. 194 (2022) 325.

Table S2. *Experimental design 3^2*

Experiment	Solvent volume (mL)	Number of extraction cycles
1	3	2
2	3	2
3	3	2
4	4	3
5	3	3
6	2	1
7	4	1
8	2	3
9	2	2
10	4	2
11	3	2
12	3	1

Table S3. *Experimental design 3^2 for UAE*

Experiment	Time (min)	Amplitude (%)
1	15.5	40
2	15.5	40
3	15.5	40
4	1	40
5	1	10
6	30	70
7	15.5	40
8	30	10
9	30	40
10	15.5	10
11	1	70
12	15.5	70

Table S4. *Experimental design 3^2 for MAE*

Experiment	Time (min)	Temperature (°C)
1	15.5	85
2	15.5	85
3	15.5	85
4	1	20
5	15.5	85
6	15.5	20
7	30	150
8	1	150
9	30	85
10	1	85
11	30	20
12	15.5	150

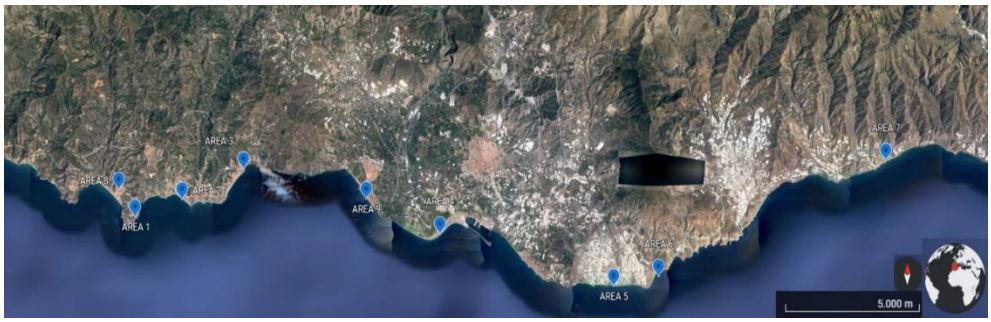

Figure S1. *Map of the locations*

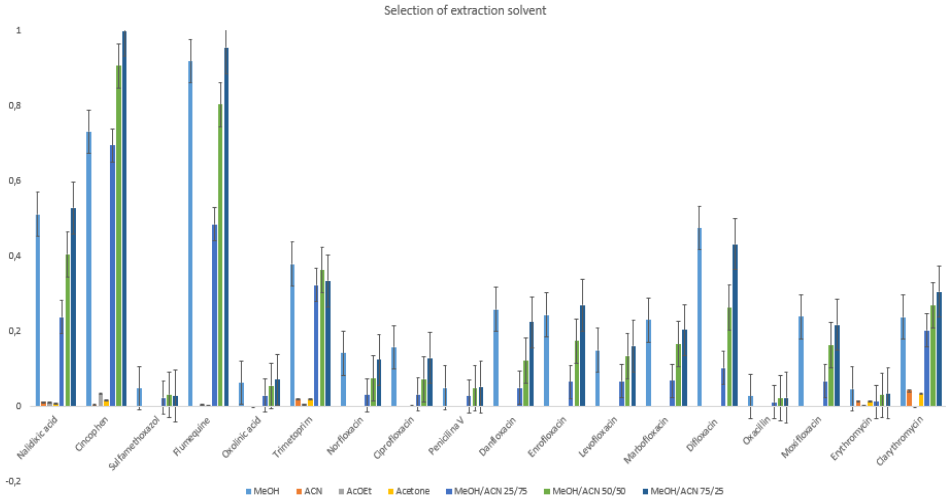

Figure S2. *Selection of extraction solvent. Normalized data for each analyte*

Capítulo 4

Determination of ultraviolet filters in human nails using an acid sample digestion followed by ultra-high performance liquid chromatography-mass spectrometry analysis

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Abstract: Ultraviolet filters (UV-filters) are specific chemicals that absorb and reflect UVA and UVB radiation from the sun. They are regularly used in sunscreens and in other personal care products (PCPs), and in products like plastics, adhesives, toys, or furniture finishes. This work develops and validates a new method to determine concentrations of UV-filters (BP-1, BP-2, BP-3, BP-6, BP-8, 4-OH-BP, THB, AVB) in human nail samples. Nails are easily available and are considered to be suitable indicators of cumulative and continued exposure to harmful chemicals. The treatment of nail samples includes microwave assisted digestion/extraction (MAE) in a methanolic solution of o-phosphoric acid (0.05 mol L^{-1}) followed by analyte determination using ultra-high performance liquid chromatography–mass spectrometry (UHPLC–MS/MS) in multiple reaction monitoring mode. The analytes were separated in less than 10 min. The digestion procedure was optimized using multivariate techniques. Matrix-matched calibration with a pig hoof matrix was used for validating the method. A study of accuracy with spiked blank samples was also conducted. The calculated detection limits varied between 0.2 and 1.5 ng g^{-1}, and quantification limits between 1.0 to 5.0 ng g^{-1}. The trueness of the method was an estimation of the recovery, which was between 90.2% and 112.2%; with an estimated precision (relative standard deviation, % RSD) lower than 12.3% for all UV-filters. Nail samples were obtained from 22 volunteers (male and female). The results showed that BP-1 and BP-3 mainly bioaccumulate in human nails.

Keywords: Ultraviolet filters; Biomonitoring; Human nails; Acid digestion; UHPLC-MS/MS

1. Introduction

Ultraviolet filters (UV-filters) are chemicals that absorb and reflect UVA and UVB radiation from the sun. Due to their excellent capacity to absorb UV light they are used in sunscreens and other personal care products (PCPs) to protect human skin and in plastics and finishes to

prevent photodegradation (Hiller et al., 2019; Kerdivel et al., 2013; Wang et al., 2016; Wu et al., 2017).

One of the most common groups of UV-filters are benzophenones (BPs). These compounds are highly lipophilic and can easily penetrate the dermis and bio-accumulate in the human body being detected in a variety of biological samples a few hours after of application (Hiller et al., 2019; Krause et al., 2018; Rodríguez-Gómez et al., 2014; Vela-Soria et al., 2014a, 2014b). Given the concern about the growing human exposure to these chemicals, the European Commission's Scientific Committee on Consumer Safety restricted the use of BP-3 to 6% in sunscreens and to 0.5% in cosmetic products (Commission Regulation (EU) 2017/238; ECHA, 2020). Commercial BPs include BP-1 to BP-12, but photodegradation products (4-hydroxybenzophenone, 4-OH-BP) and metabolization products (2,3,4-trihydroxybenzophenone, 2,3,4-triOH-BP) of BPs are also used as UV-filters (Hayashi et al., 2006; Vela-Soria et al., 2014a; Watanabe et al., 2015). BP-3 is the most common BP used in PCPs (Wu et al., 2017). Interestingly, BP-3 is mainly transformed into BP-1 after application of sunscreens; this process occurs via demethylation of the methoxy substituent. For this reason, both BP-3 and BP-1 are considered markers of exposure (Wang and Kannan, 2013; Watanabe et al., 2015). Avobenzone (AVB) is a long-wave UV-filter also commonly used in sunscreen and PCP formulations. Its concentration is limited to 5% by the European Union legislation (Ahn et al., 2019; Official website of the European Union, 2020). AVB is easily excited by UV radiation, which produces dissociation of the filter into oxygen-free radicals and derivatives that may damage proteins and DNA. This compound has also been associated to skin irritation in the form of contact dermatitis and photoallergic reactions (Collaris and Frank, 2008; Guesmi et al., 2020). In sunscreen formulations, AVB is used in the enol form, with excellent UVA absorption but easily isomerized to the di-keto form, losing effectiveness (Lebedev et al., 2020).

Large amounts of UV-filters are released into the environment and have been found to accumulate in environmental matrices and in tissues

from marine biota and can also enter the food chain (Cunha et al., 2018; Gago-Ferrero et al., 2015; Martín et al., 2017; Ramos et al., 2016; Vidal-Liñán et al., 2018; Wu et al., 2017). Thus, humans are not exposed only through the skin but also by ingesting fish or shellfish foodstuffs or beverages in contact with the packaging material, contaminated due to the migration of UV-filters into them (Hu et al., 2019; Wu et al., 2019).

The extended use of UV-filters has raised concerns regarding their potential activity as endocrine disruptors (EDs). In this respect, BPs have been found to interact with estrogen, androgen and thyroid hormone receptors (Kerdivel et al., 2013; Schmutzler et al., 2007; Wang et al., 2016; Watanabe et al., 2015). In gene assays conducted in MCF-7 breast cancer cell line and other cells, hydroxylated BPs have displayed estrogenic activity and some metabolites like BP-1 exhibited higher disrupting activity than BP-3 itself (Suzuki et al., 2005; Watanabe et al., 2015). AVB has also been found to act as an ED in humans, with obesogenic, glucocorticoid and anti-androgen-like activities (Ahn et al., 2019; Klopcic and Dolenc, 2017).

Biomonitoring studies are a necessary tool to assess past exposure to these chemicals. Nail samples provide some advantages over more commonly used biological samples like blood and urine. First, sample collection is simple and non-invasive, not requiring specialized staff or special transport or storage conditions (Sukumar, 2005). Second, concentrations of UV-filter in nails reflect human exposure over the previous months given their relatively slow growth rate (Alves et al., 2014; Grashow et al., 2014; He, 2011). Human toenails grow on average 1.6 mm/month therefore toenail samples represent cumulative exposure integrated over the previous 6-12 months (Grashow et al., 2014; Yaemsiri et al., 2010). Toenails can also provide information about *in utero* cumulative exposure since they start forming in the embryo at week 9-10 (Cigan et al., 2018). Lastly, nails are very stable matrices because the compound levels incorporated in the keratin fibers are not affected by changes in metabolism (Liu et al., 2011).

Nail analysis has been extensively used in the forensic and clinical field for the detection of drugs of abuse, trace elements, pharmaceuticals and their metabolites (Cappelle et al., 2015; Daniel et al., 2004). In addition, nails are useful markers to retrospectively assess drug and pharmaceutical use as well as occupational and environmental long-term exposures (Alves et al., 2017; Cappelle et al., 2015; Grashow et al., 2014). Previous studies have shown the presence of different groups of EDCs in human nails including perfluoroalkyl compounds (Li et al., 2012; Liu et al., 2011; Wang et al., 2018), organophosphate flame retardants (Alves et al., 2017; Liu et al., 2016), antimicrobial agents (Shi et al., 2013; Yin et al., 2016), and more recently, bisphenols and parabens (Martín-Pozo et al., 2020). Nevertheless, there is no published literature regarding accumulation of UV-filters.

In this context, the purpose of the present study is to develop and validate an analytical method for the simultaneous measuring of eight UV-filters in human nail samples by UHPLC-MS/MS. BP-1, BP-2, BP-3, BP-6, BP-8, 4-OH-BP, 2,3,4-triOH-BP, and AVB were the selected target analytes. The method was applied to determine these compounds in human nail samples collected from 22 male and female volunteers.

2. Materials and methods

2.1. Chemicals and reagents

Double distilled water (18.2 MΩ-cm) was purified with a Milli-Q Plus® purification system (Millipore, Madrid, Spain). Analytical grade standards of analytes: BP-1 (2,4-dihydroxybenzophenone) (≥99%), BP-2 (2,2', 4,4'- tetrahydroxy-benzophenone) (≥97%), BP-3 (2-hydroxy-4-methoxybenzophenone) (≥98%), BP-8 (2,2'dihydroxy-4-methoxy-benzophenone) (≥98%), 2,3,4-triOH BP (≥99%), 4-OH-BP (≥98%) and deuterated BP-3 [oxybenzone-(*phenyl*-d_5); 2-hydroxy-4-methoxy benzophenone-(*phenyl*-d_5)] were supplied by Sigma-Aldrich (Madrid, Spain). BP-6 (2,2'-dihydroxy-4,4'-dimethoxybenzophenone) (≥98%) were obtained from Alfa Aesar (Massachusetts, MA, USA). AVB

(butylmethoxydibenzoylmethane) was purchased from TCI Chemicals (Oxford, UK). The chemical structures are shown in **Figure 1**.

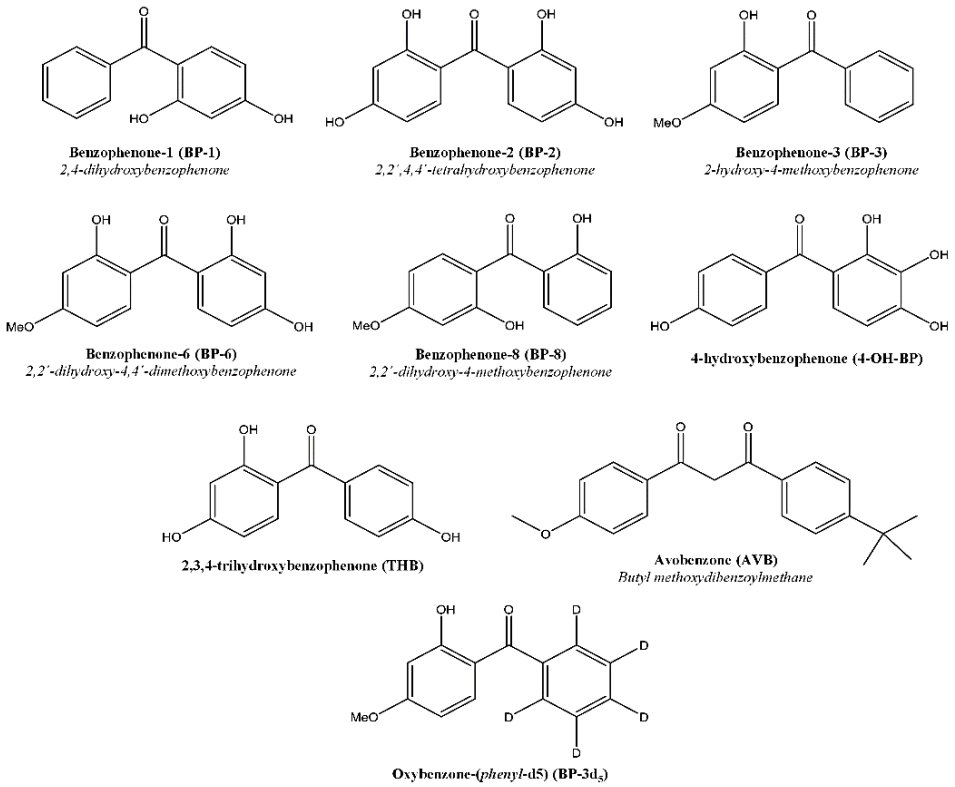

Figure 1. *Chemical structure of the target UV-filters*

Analytical-grade formic acid (≥98%) used as mobile-phase additive and Triton X-100 were purchased from Sigma-Aldrich (Madrid, Spain). LC-MS-grade methanol and acetic acid used for extractions were from VWR ProLabo CHEMICALS (Barcelona, Spain). Perchloric acid (70% w/w), o-phosphoric acid (85% w/w as H_3PO_4), and hydrochloric acid (6 M) were provided by Panreac (Barcelona, Spain). Stock standard solutions (100 mg L^{-1}) of each compound were prepared in methanol and stored in the dark at -20 °C. Working standards were prepared by dilution with methanol immediately before use.

2.2. Instrumentation and software

Chromatographic analyses were carried out on a Waters Acquity UPLC™ I-Class chromatograph (Manchester, UK) with a sample

manager (Acquity™) and a binary solvent manager (Acquity UPLC™). Analyte separation was assayed on a Waters UPLC® BEH C_{18} column (50 x 2.1 mm i.d., 1.7 µm particle size) and a Phenomenex Kinetex EVO C18 LC column (50 x 2.1 mm i.d., 1.7 µm particle size) (Torrance, CA, USA). A triple quadrupole mass spectrometer Xevo TQ-XS (Waters) equipped with an orthogonal Z-Spray™ electrospray ionization (ESI) interface was used for detection. Nail sample lyophilization was performed on a ScanVac CoolSafe™ freeze dryer (Lynge, Denmark). Samples were ground to powder with a Retsch® MM 301 ball mill (Biometa, Asturias, Spain). Additional equipment included a Mettler-Toledo GX400 balance (Columbus, OH, USA), a Labnet Spectrafuge™ 24D centrifuge (New Jersey, USA), an IKA vortex-mixer (Staufen, Germany), a sample concentrator (Stuart, Staffordshire, UK) and a Memmert oven (Schwabach, Germany). An ETHOS SEL microwave Labstation (Sheldon, CT, USA) at 2455MHz was used for MAE. Easy CONTROL-280 software was used for microwave adjustment. An ATC optical fiber temperature sensor was also used. Statgraphics Plus v 5.0 software (Statpoint Technologies Inc., VA, USA) was used for data analysis.

2.3. Sample collection and treatment

Human nail samples were collected during a three-month period from 22 male and female volunteers living in the city of Granada (Spain). All the volunteers were informed of the aims of the study and signed the informed consent form. Fingernails and toenails were clipped with a stainless nail cutter. Specimens were anonymized and stored in polypropylene tubes in the dark until analysis. Since the amount of nail sample is a limiting factor in this study, pig hoof matrix was used for the estimation of digestion efficiency and validation purposes due to its similarity to the human nails (Shi et al., 2013). Pig hoofs were provided by a local slaughterhouse.

As nails may be easily contaminated by external sources, nail samples were washed to remove any substances that may interfere with the analysis. First, nail samples underwent ultrasonic cleaning with Triton X-100 1% solution for 15 min. The samples were then washed

three times with ultrapure distilled water in order to remove any detergent residue and were subsequently rinsed with acetone and with methanol for 15 min each time to remove any nail polish residue. Once nails were washed, they were freeze-dried overnight. The dried samples were ground to small powder using a laboratory ball mill (6 min, at 30 Hz) to improve extraction efficiency. Treatment of the pig hoofs was similar but hoofs took longer to grind because they are harder.

2.4. Preparation of fortified samples

The samples (blank) analyzed for optimization of the extraction parameters were spiked at 100 ng g^{-1} by adding 10 µL of a standard solution of the analytes (100 µg mL^{-1}) to 0.1 g of nail powder. After mixing in vortex for 1 min, the samples were left to stand a minimum of 24 h in the dark to allow the analytes to come into contact with the entire matrix and to achieve sorption equilibrium and solvent evaporation. For method validation, lyophilized nail samples were fortified at a range from 1 to 250 ng g^{-1}. All blank samples were previously analyzed to rule out the presence of target analytes or other compounds that could interfere with the analysis.

2.5. Experimental procedure

2.5.1 Microwave assisted extraction

An aliquot of ground nails (0.1 g) was introduced into a microwave vessel with 2 mL of a solution of o-phosphoric acid (0.05 mol L^{-1}) in methanol. The digestion/extraction was run for 20 min at 44 °C and 1000 W of power. A single extraction cycle was run for each replicate sample. Ten samples were processed simultaneously. The pressure was automatically controlled by the system. After each cycle, ventilation was maintained to cool the vessels (< 40 °C). Once the extraction was finished, each extract was decanted in an Eppendorf tube and centrifuged at 16300 *x g* for 10 min. The extract containing the analytes was transferred to a new tube and dried under a stream of nitrogen. The residue was dissolved in 0.5 mL of the initial mobile phase (water:methanol [60:40 v/v]) and after being vortexed for 30 s it was centrifuged at 16,300 *x g* for 2 min to

eliminate any particles in suspension. The supernatant was passed to a chromatographic glass vial for analysis.

2.5.2 Cromatographic conditions

UHPLC separation was carried out on an Acquity UPLC® BEH C18 column (50 mm x 2.1 mm i.d., 1.7 µm particle size) thermostated at 40 ºC. Water (solvent A) and methanol (solvent B) acidified with 0.1% (v/v) formic acid were used in a gradient mobile phase. For each vial, a volume of 10 µL was injected at a constant flow rate of 0.3 mL min^{-1}. The elution gradient was: 0.0 – 1.0 min, 40% B; 1.0 – 6.0 min, 40 – 90% B; 6.0 – 8.0 min, 90% B; 8.0 – 8.1 min back to initial conditions. The total run time was 10 min.

2.5.3 Mass spectrometric parameters

The mass spectrometer (MS) operated in positive ESI modes. MS/MS acquisition was performed in multiple-reaction monitoring (MRM) mode to enhance specificity and sensitivity. Standard solutions of the compounds (100 ng mL^{-1}) were infused separately to obtain the most sensitive transitions and the optimal cone voltage and collision energy in each case. Two MRM transitions were selected for each compound, the most abundant for quantification and the other for confirmation. At the same time, an acquisition time segment was established based on the retention time of each analyte in the chromatogram which enabled enhanced detection sensitivity. The optimized instrumental settings are summarized in Table 1.

Table 1. *Transitions and optimized parameters for UHPLC-MS/MS analysis of UV-filters*

	t_R	Transitions	CV	CE		t_R	Transitions	CV	CE
BP-1	4.2	215.1 → 104.5[b]	34	20	4-OH-BP	3.6	199.2 → 104.49[b]	12	12
		215.1 → 136.6[a]	34	16			199.2 → 120.52[a]	12	18
BP-2	2.8	247.1 → 80.3[b]	8	36	THB	3.4	231.2 → 104.5[a]	4	18
		247.1 → 136.6[a]	8	14			231.2 → 152.6[b]	4	14
BP-3	5.2	229.2 → 104.5[b]	20	16	AVB	5.5	311.2 → 134.6[b]	8	20
		229.2 → 150.6[a]	20	16			311.2 → 160.7[a]	8	24
BP-6	4.9	275.2 → 94.5[b]	2	36	BP-3 d^5	5.2	234.2 → 109.4[b]	40	16
		275.2 → 150.6[a]	2	16			234.2 → 150.6[a]	40	18
BP-8	4.5	245.2 → 120.5[b]	38	14					
		245.2 → 150.7[a]	38	18					

Chromatographic conditions			
Injection volume	10 µL	Mobile phase	(A) Water with 0.1% of formic acid (B) Methanol with 0.1% of formic acid
Column	UPLC® BEH C_{18}		
Column temperature	40ºC	Flow rate	0.3 mL min^{-1}
Elution gradient	0.0 – 1.0 min, 40% B 1.0 – 6.0 min, 40 – 90% B 6.0 – 8.0 min, 90% B 8.0 – 8.1 min back to initial conditions		

Spectrometric conditions			
Impactor voltage	1 kV	Nebulizer gas pressure	7.0 bar
Source temperature	150ºC	Cone / desolvation gas	N_2 (≥99.995%)
Desolvation temperature	600ºC	Collision gas	Ar (99.999%)
Cone gas flow	150 L h^{-1}	Dwell time	25 ms
Desolvation gas flow	500 L h^{-1}	Inter-scan delay	3 ms

CV: Cone voltage (V); CE: Collision energy (eV). [a] *SRM transition used for quantification.* [b] *SRM transition used for confirmation*

2.6. Box-Behnken design

A Box-Behnken design is an experimental design (2^{nd} order) based on three-level incomplete factorial designs. It is an ideal method for the assessment of critical experimental variables or factors and response surfaces approaches, when minimum and maximum factor-levels are not

desired (Ferreira et al., 2007). In this study, a 15 run Box-Behnken experimental design was used to optimize the extraction/digestion procedure using MAE. Digestion time, temperature and number of extraction cycles were the investigated variables. Three replicates at the center point and three levels for each factor were used to develop predictive models. Relative effects of the linear, quadratic and cross-product interactions between variables were derived from the estimated regression equations and the statistical significance was assessed using the analysis of variance (ANOVA).

2.7. Validity requirements

Instrumental assurance and quality control assessments (QA/QC) were carry out to validate the analytical results. First, to reduce background contamination, procedural blanks were injected into the LC system. All target analytes were detected below the limits of quantification (LOQs). Second, to reduce contamination and variability of the instrument, a standard solution in the initial mobile phase (at 100 ng mL^{-1}) and blank samples fortified with the UV-filters (at 0 and 100 ng g^{-1}) were injected every 10 injections. All the samples (standards, procedural blanks, blank nail samples and spiked nail samples) were prepared in triplicate and analyzed simultaneously.

3. Results and discussion

3.1. Liquid chromatographic-mass spectrometric analysis

The different parameters affecting the chromatographic separation were optimized to obtain the optimal sensitivity and selectivity in the shortest time and with the lowest consumption of resources. To this end, chromatographic and mass spectrometric parameters were evaluated and optimized.

For the optimization of the chromatographic separation different modifiers for the mobile phase as well as elution gradients were evaluated. In addition, two different analytical columns were evaluated to compare the results. The influence of temperature on the chromatographic separation over the peak shape, system pressure and

retention times was also studied. A standard mixture of compounds at 100 ng mL^{-1} was used for optimization of the chromatographic separation. A Kinetex EVO C18 LC column (50 x 2.1 mm i.d., 1.7 µm particle size) from Phenomenex and an Acquity UPLC® BEH C_{18} column (50 x 2.1 mm i.d., 1.7 µm particle size) from Waters were evaluated. Both columns provided similar resolution of the chromatographic peaks but UPLC® BEH C_{18} column provided higher peak heights and it was therefore selected for the determinations. As initial conditions, a mixture of pure methanol (solvent B) and water (solvent A) was used as mobile phase. Subsequently, different compositions of mobile phases were assayed: methanol and acetonitrile, and different mixtures of these solvents as solvent B, and deionized water as solvent A. Several additives in the mobile phase buffered to a pH at which the analytes are completely ionized were tested. First, formic acid 0.1% (v/v) (pH 2.6), ammonia 0.025% (v/v) (pH 10.3) and mixture of both additives (to adjust the pH to 4) were tested in solvent A. The additives were also added to solvent B at similar concentrations. The best separation, peak shapes and ionization of the compounds were obtained using methanol and water acidified with 0.1% (v/v) of formic acid. Different mobile phase compositions were tested for gradient optimization in order to achieve a good separation between peaks in a short analysis time. The final optimized gradient used is described in section *2.5.2.*

Finally, the influence of column temperature on peak shape and retention time was studied. Temperatures between 20 and 50 ºC were tested with increased temperature clearly improving the shape of the peaks and a marked reduction in the system pressure at 40ºC. Temperatures higher than 40ºC did not result in improvement therefore it was selected for the chromatographic separation.

For the optimization of the triple quadrupole tandem mass spectrometer (TQ-XS) parameters, ESI in the positive and the negative modes were assayed, with positive mode providing higher sensitivity for all the compounds. This explains the improvement of the results with an acidified mobile phase that enhances ionization. For each analyte two

transitions (the most sensitive ones) were monitored, the most abundant for quantification, and the other for confirmation (Table 1).

3.2. Optimization of the extraction procedure

Different extraction techniques, including acid and alkaline digestion at room temperature and MAE were examined for the analysis of the UV-filters. The influence of different acids on the extraction was also evaluated.

3.2.1 Selection of alkaline digestion

Due to the nature of samples, nail sample digestion is necessary for an efficient extraction of analytes (Alves et al., 2017; Li et al., 2012; Liu et al., 2016; Martín-Pozo et al., 2020; Shi et al., 2013; Wang et al., 2018; Yin et al., 2016). Acid or alkaline digestions are often used for the determination of drugs and endocrine disrupting chemicals like triclosan and triclocarban (Shi et al., 2013; Yin et al., 2016), perfluorinated compounds (Li et al., 2012; Liu et al., 2011; Wang et al., 2018), bisphenols or parabens (Martín-Pozo et al., 2020) in nails.

In that context, an initial experiment was performed to determine whether to use acid or alkaline digestion for the isolation of UV-filters. NaOH (0.05 mol L^{-1}) and formic acid (0.05 mol L^{-1}) in methanol were assayed (Martín-Pozo et al., 2020) using 2 mL of the digestion/extraction solution mixed with an aliquot of 0.1 g of dried nail containing the analytes and the surrogate (100 ng g^{-1}). The samples were vortexed for 2 min and incubated at 30 ºC for 15 h. After digestion, the solutions were centrifuged for 10 min at 16,300 *x g* and the organic phases containing the target compounds were separated. The solvent was evaporated to dryness under a nitrogen stream. The dried residues were re-dissolved in 0.5 mL of the initial mobile phase (water:methanol, 60:40 v/v). After stirring for 30 s in vortex and centrifugation at 16,300 *x g* for 2 min to remove insoluble impurities, the supernatant was introduced into glass vials for instrumental analysis. Figure 2A compares the extraction efficiencies obtained with the acid and alkaline digestion processes at the optimized chromatographic conditions.

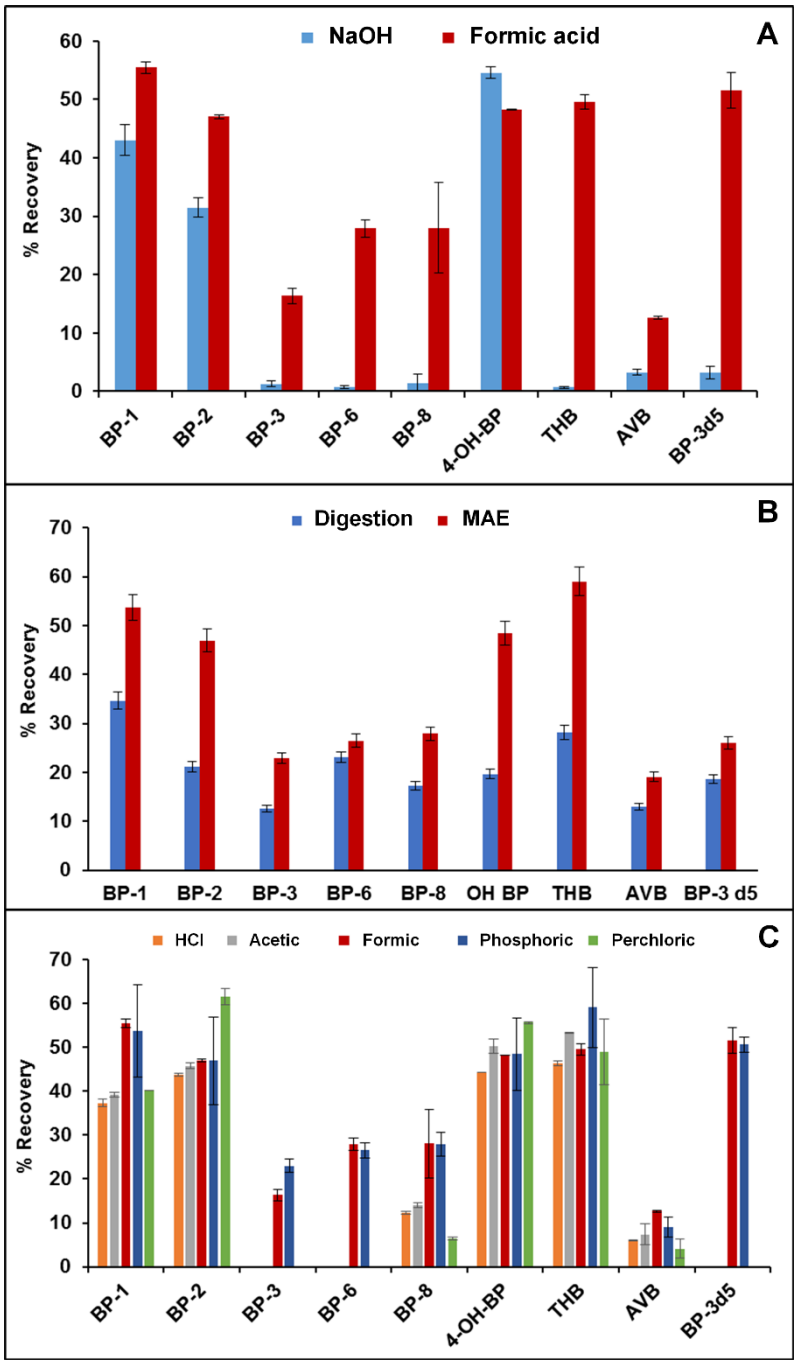

Figure 2. *Selection of the digestion method (A), extraction technique (B), and optimal acid (C) for sample treatment. All solutions were prepared at 0.05 M in methanol. The bar graph depicts the response area obtained for each compound and the average recovery for each extraction solvent. Data are expressed as mean ± SEM from samples in triplicate*

As shown in Fig. 2A, very low recoveries were obtained for some BPs in nail samples (<10%) with alkaline digestion while the acid procedure provided higher normalized areas for all the target analytes, except for 4-OH-BP, where the signal was similar in both types of digestion procedures. Acid digestion was selected for the optimization of the following extraction parameters.

3.2.2 Selection of the extraction technique

To reduce the time of the digestion/extraction process and maximizing the recovery of the analytes, two extraction procedures were compared: digestion at room temperature and microwave-assisted digestion. To this end, two 0.1 g sample aliquots were weighted and spiked with the analytes (100 ng g^{-1}) and 2 mL of the acidic extraction/digestion solvent was added and vortexed for 2 minutes. The first aliquot was incubated at 30ºC for 15 hours and the second one was extracted in the microwave at 70ºC for 30 minutes, at the maximum power of the equipment (1000 W). After the extraction process, the samples were centrifuged at 16300 *x g* for 10 minutes. The supernatant was collected and evaporated to dryness under nitrogen stream. Finally, the dry extract was dissolved in 0.5 mL of a mixture of the initial mobile phase and centrifuged (16300 *x g*, 2 minutes) to remove any impurities in suspension. The supernatant was placed in chromatographic vials and injected into the system. The results are shown in Figure 2B.

Interestingly, MAE provided higher recoveries in a significantly shorter extraction/digestion time (from hours to a few minutes). For some compounds, the % recovery with this technique was double than with the 15-hour digestion. MAE was selected as the extraction technique.

3.2.3 Acid selection for the digestion/extraction process

Different acids for the digestion process were evaluated to select the one that would provide the optimal extraction and greater recovery of the analytes. Aliquots of 0.1 g of nail sample (spiked at 100 ng g^{-1}) were treated with 2 mL of a methanolic solution containing different acids (0.05 mol L^{-1}): hydrochloric acid, acetic acid, formic acid, o-phosphoric

acid, and perchloric acid. All samples underwent MAE (30 min, 70 ºC and 1000 W) as described above. The final extracts were injected into the LC system. Figure 2C shows the average recoveries of the extracted compounds obtained for each acid tested. Our finding showed that formic and o-phosphoric acid provided higher extraction efficiencies, with very similar percentages. However, the recovery of BP-3 and BP-6 was noticeably higher with phosphoric acid. BP-3 and its metabolites are the most widely used UV-filter in PCPs and are of special interest in this study. Therefore, methanol acidified with o-phosphoric acid was selected as the most suitable extraction solvent.

3.2.4 Optimization of microwave-assisted extraction conditions

The three parameters that significantly influence the digestion process using MAE were optimized. Three levels of each variable were investigated: number of extraction cycles (1, 2 and 3), extraction time (5, 17.5 and 30 min) and temperature (30, 50 and 70 ºC). They were we evaluated according to a multivariate optimization using a Box-Behnken design. Microwave power was maintained at 1000 W. The design matrix is included in the Supplementary Material (Table S1).

ANOVA analysis provided determination coefficients (R^2) higher than 0.86. On the other hand, the model was satisfactory with a 95% confidence level, since *P*-values for the *lack-of-fit* test were higher than 0.05 for all target compounds.

The statically significant effects were also screened using a Student's *t*-test distribution and Pareto charts were obtained (Figure S1). A minimum *t*-value -vertical line- was established using a 95% of confidence interval. Those variables with higher *t*-values are considered statistically influential factors in the extraction. The results showed that for most of the analytes extraction time was the most influential variable on the recoveries followed by the temperature. In general, both parameters showed a positive effect whereas the number of cycles had a negative effect. Only for BP-6 the number of cycles positively affected the digestion process.

Additionally, in order to determine the optimal global extraction efficiency, a desirability function approach was used. This function was calculated considering all the experimental values optimized obtained for each analyte for the assayed variables in the Box-Behnken experiment. The graphical representations show the prediction of the overall response when one parameter remains unchanged while the other two change. Plots of the function, normalized between 0 and 1, are shown in Figure 3.

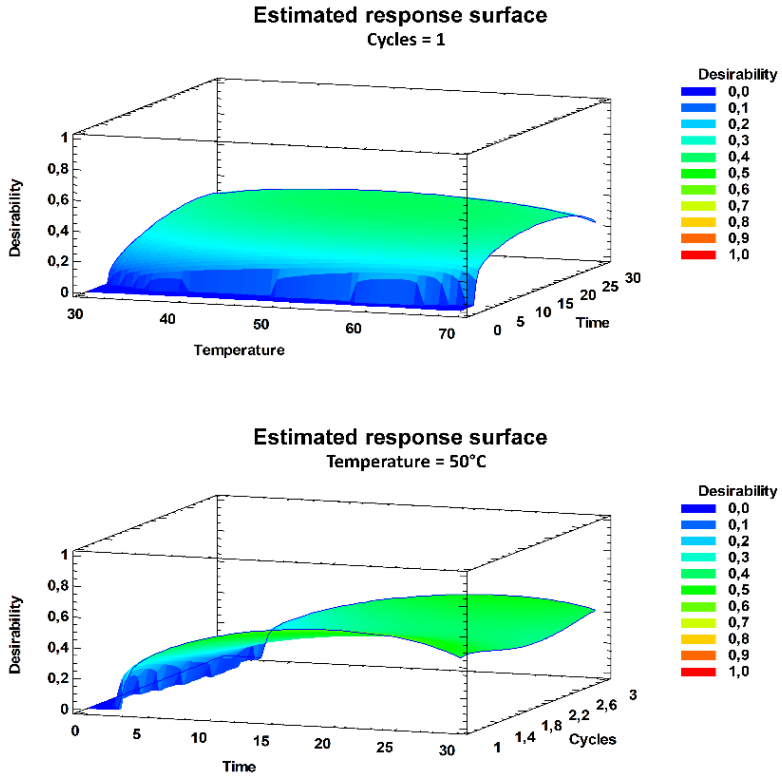

Figure 3. *Box-Behnken design desirability charts. The temperature is set at 50 ˚C (A) and the number of cycles at 1 (B). Samples were spiked with 100 ng g^{-1} of each analyte*

According to the test results, the number of extraction cycles does not improve desirability; therefore one single cycle was selected as optimal. The maximum value of desirability was reached with 20 min of extraction and at a temperature of 44 ºC (approximately 60%), for that reason these conditions were chosen.

3.3. Analytical performance

For calibration purposes, pig hoof free of the target compounds were used (Shi et al., 2013). A previous study of the blank to confirm the absence of analytes in the pig hoof used was carried out. A seven-point matrix-matched calibration curve was prepared using the relative peak area (compound/surrogate) as a function of the analyte concentration. Each point of concentration was repeated four times and analyzed three times. BP-3d5 was added as internal standard at 100 ng g^{-1} in nail matrix. The matrix effect was also estimated by preparing two calibration curves for each UV-filter: one in the pig hoof matrix and the other in the initial mobile phase. The comparison of the slopes of both calibration curves was performed using a Student's *t*-test. This statistical analysis estimated significant differences between the slopes of both curves which confirmed the presence of the matrix effect. Thus, the matrix-match calibration was applied for the quantification of all analytes in the samples. Table 2 summarized the analytical and statistical parameters obtained for each compound.

Table 2. *Calibration parameters*

	BP-1	BP-2	BP-3	BP-6	BP-8	THB	4-OH-BP	AVB
n	42	42	42	42	42	42	42	42
b (g ng^{-1})	0.302	0.249	0.140	0.018	0.2682	0.240	0.592	0.016
$s_{y/x}$	1.220	0.667	0.720	0.088	0.712	0.872	1.477	0.066
LDR (ng g^{-1})	LOQ-250							
R^2 (%)	99.8	99.9	99.7	99.6	99.9	99.8	99.9	99.8
P_{lof} (%)	20.1	10.2	6.2	35.1	27.7	7.45	11.2	28.8
LOQ (ng g^{-1})	4.7	5.0	1.0	1.0	1.0	5.0	1.0	5.0
LOD (ng g^{-1})	1.4	1.5	0.3	0.3	0.2	1.4	0.2	1.5

n: points of calibration; b: slope; $s_{y/x}$: regression standard deviation; LDR: linear dynamic range; R^2: determination coefficient; P_{lof}: p values of the lack-of-fit test; LOD: limit of detection; LOQ: limit of quantification

3.4. Validation of the method

The optimized method was validated in terms of selectivity, linearity, sensitivity and accuracy (trueness and precision). Validation was addressed according to US-FDA guideline for Bioanalytical Method Validation (Guidance for Industry, 2018).

Selectivity. Blank samples were analyzed and no endogenous interferences were detected at the retention times of the analytes of interest. This fact demonstrated the selectivity of the spectrometric conditions. Figure 4 shows the MRM chromatograms obtained from a blank nail sample (A) and a mixture of the target analytes at 100 ng mL^{-1} (B).

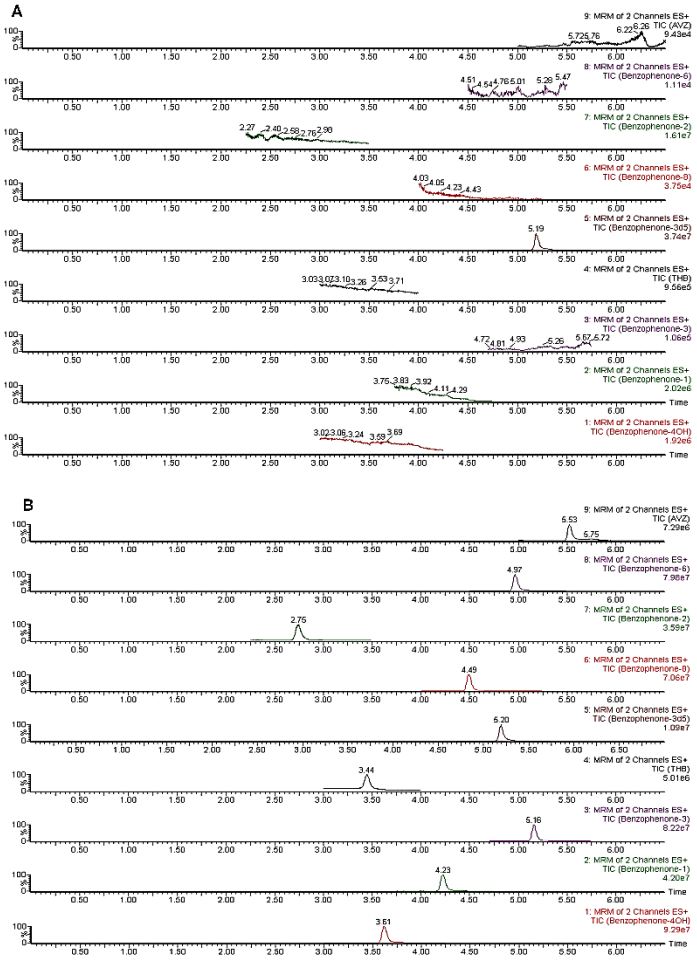

Figure 4. *MRM mode chromatogram of a blank nail sample (A) and a standard mixture of the target compounds (100.0 ng mL^{-1} of each compound)*

Linearity was evaluated by estimating the determination coefficients (R^2) and the *lack-of-fit* P-values (P_{lof} %). The R^2 values obtained were between 99.6 and 99.9% and P-values were > 5% in all cases (Table 2). The statistical data indicated high linearity within the concentration range of the calibration curve.

Sensitivity was assessed in terms of limits of detection (LODs) and limits of quantification (LOQs). In this work, these parameters were calculated from the signal/noise ratio (S/N), as the interpolation on the calibration line of a signal equal to 3·S/N for the LOD and 10·S/N for the LOQ. Predicted LODs were between 0.2 and 1.5 ng g^{-1} while LOQs were between 1.0 and 5.0 ng g^{-1}. Table 2 summarizes the values obtained. These data demonstrate that the proposed method is highly sensitive and can be applied to the selected samples, where analyte concentrations are usually above these limits.

Accuracy (precision and trueness). A recovery assay evaluated the intra- and inter- day variability of the proposed method. For this purpose, blank nail samples were spiked at three different concentration levels (5, 100 and 250 ng g^{-1}) for each compound, extracted and analysed six times during seven consecutive days. For precision, the relative standard deviation (% RSD) was determined. For trueness, recovery rates (% Rec) were calculated and compared with the estimated recoveries given the actual concentration of the spiked samples. The results are shown in Table 3.

RSD values were < 15% for all the compounds. These values were within the acceptable limits established in the validation guide (which allow value < 15% and even 20% in the area close to the LOQ) (Guidance for Industry, 2018). The recoveries were between 90.2% and 112.2%, which are also within the accepted values established in the FDA validation guidelines. High accuracy (trueness and reproducibility) can be therefore attributed to the method.

Table 3. *Recovery assay and accuracy of the proposed method (precision and trueness) in nail samples*

Compound	Spiked (ng g^{-1})	RSD (%)	Rec (%)	Compound	Spiked (ng g^{-1})	RSD (%)	Rec (%)
	5	8.7	101.4		5	6.2	108.0
BP-1	100	4.0	93.5	BP-8	100	3.0	97.6
	250	1.4	100.8		250	1.1	100.4
	5	0.8	110.8		5	3.6	107.2
BP-2	100	2.5	103.2	THB	100	2.7	103.9
	250	1.2	100.2		250	1.7	99.8
	5	2.3	112.2		5	8.0	91.0
BP-3	100	2.9	90.2	4-OH-BP	100	2.6	99.0
	250	2.6	100.9		250	1.0	100.3
	5	5.5	103.0		5	12.3	108.0
BP-6	100	7.7	95.1	AVB	100	5.1	97.8
	250	1.7	100.9		250	2.1	102.5

RSD: relative standard deviation; Rec: recovery

3.5. Biomonitoring of UV-filters in human nails. Detection frequency and quantification

Once the method was satisfactorily validated, it was applied to human nail samples from 22 volunteers. We analyzed three experimental replicas of each subject in triplicate. Table 4 shows the results obtained and Figure 5 shows a chromatogram of a volunteer's sample.

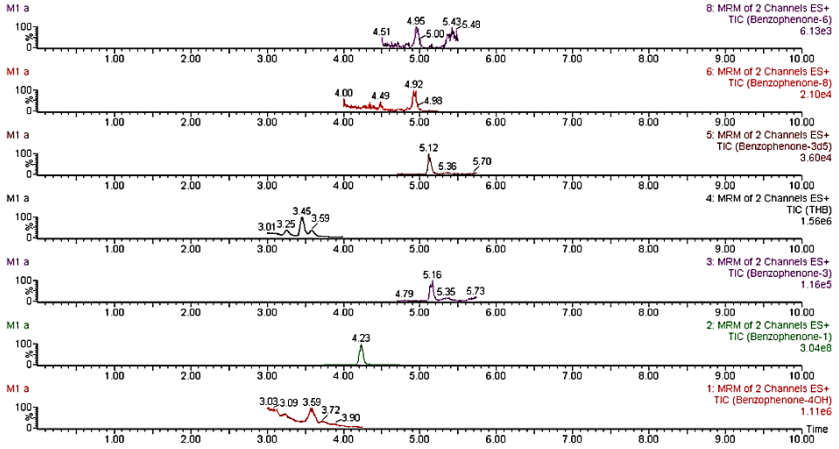

Figure 5. *MRM mode chromatogram of a contaminated sample from a volunteer (sample 1)*

Table 4. *Application of the method to nail samples*

Concentration (ng g-1)								
	BP-1	**BP-2**	**BP-3**	**BP-6**	**BP-8**	**THB**	**4-OH-BP**	**AVB**
S01	D>LDR	ND	225 (7.8)	14 (11.1)	3.9 (8.0)	231 (2.5)	78 (10.3)	D
S02	58 (5.3)	ND	245 (5.4)	14 (11.2)	ND	ND	ND	ND
S03	D>LDR	ND	153 (10.1)	3.9 (10.1)	ND	ND	ND	ND
S04	D>LDR	ND	74 (13.5)	2.6 (8.6)	ND	54 (3.3)	ND	ND
S05	D>LDR	ND	184 (2.6)	1.2 (10.5)	ND	6 (10.5)	ND	D
S06	194 (8.5)	ND	125 (14.4)	8.1 (4.6)	ND	ND	ND	ND
S07	D>LDR	ND	D>LDR	5.3 (14.1)	ND	23 (10.0)	20 (10.6)	ND
S08	D>LDR	ND	D>LDR	5.5 (10.4)	ND	50 (7.3)	31 (9.7)	ND
S09	D>LDR	ND	D>LDR	9.0 (9.5)	ND	42 (3.5)	48 (13.1)	ND
S10	158 (11.0)	ND	228 (10.5)	2.9 (7.6)	ND	ND	ND	ND
S11	D>LDR	ND	D>LDR	2.8 (17.9)	ND	19 (13.9)	2.8 (7.2)	ND
S12	116 (6.1)	ND	72 (2.1)	ND	ND	ND	ND	ND
S13	8.1 (5.2)	ND	25 (8.8)	ND	ND	ND	ND	ND
S14	6.2 (15.9)	ND	83 (3.7)	D	ND	ND	ND	ND
S15	28 (16.4)	ND	59 (11.6)	ND	ND	ND	ND	ND
S16	D>LDR	ND	D>LDR	ND	2 (10.9)	10 (10.7)	1.5 (11.6)	ND
S17	D>LDR	ND	147 (3.0)	ND	ND	ND	ND	ND
S18	29 (8.0)	ND	217 (4.2)	ND	ND	ND	ND	ND
S19	6.3 (8.6)	ND	25 (8.9)	D	ND	ND	ND	D
S20	D>LDR	ND	242 (6.3)	ND	ND	38 (3.8)	ND	D
S21	D>LDR	ND	90 (4.6)	ND	ND	ND	ND	ND
S22	D>LDR	ND	234 (10.5)	ND	ND	ND	4.8 (15.9)	D

ND: not detected; D: detected (>LOD but <LOQ); D>LDR: Detected but higher than upper limit of the studied concentration range; the coefficient of variation (%) is indicated in parentheses

The results showed that all the nail samples tested positive for at least two benzophenone UV-filters. BP-1 was the most frequently found benzophenone (present in all the subjects) and at higher concentrations, exceeding in thirteen individuals the upper limit of the LDR of the present method. BP-3 was also frequently detected but at lower levels. In contrast, BP-2 was not detected in any of the analyzed samples. The rest of the target analytes (BP-6, BP-8, THB, 4-OH-BP, and AVB) were found only in some of the samples and at low concentrations. This finding was expected as these compounds are rarely used in PCPs, according to the scientific literature (Commission Regulation (EU) 2017/238; ECHA,

2020). However, BP-6 was present in 59% of the samples and quantified to very low levels (<LOQ-14 ng g^{-1}), followed by THB, found in 41% of the samples (6-231 ng g^{-1}). AVB was detected in five samples but below the LOQ.

The bioaccumulation of these type of compounds in the body is mainly related to the level of exposure but also to their behavior and metabolism. A possible explanation for the occurrence and the high concentrations of BP-1 found is that a large part of BP-3 (the most widely used compound for sun protection in cosmetics) is metabolized into its hydroxylated forms like BP-1, which accumulate in the nail matrix (Wang and Kannan, 2013; Watanabe et al., 2015).

Volunteer gender has been taken into account in this study. Interestingly, significant differences in benzophenone levels were found between women and men in nail samples. According to our findings, nails from women contained on average up to 18% more BP-3 than men's when considering the quantifiable mean of all participants. In addition, of all the individuals exceeding the linear range (18), for both BP-3 and BP-1, 83.3 % were female while only 16.7 % were male. These differences could be attributed to the continuous use of cosmetics and personal care products by the female population, since many of them contain benzophenones as sunscreen filters.

In nail or similar matrices such as hair, there are no data available on benzophenone analysis. However, our results are consistent with those reported in other tissues, such as placenta, were only BP-3 metabolites were detected but not BP-3 (Valle-Sistac et al., 2016; Vela-Soria et al., 2011). Benzophenones have also been found in other biological samples such as serum, breast milk or urine, although in lower concentration levels than in nails, usually in the order of ng mL^{-1}. Vela-Soria et al. reported concentrations between 0.5-31.0 ng mL^{-1} for the conjugated form of BP-1 and 0.3-99 ng mL^{-1} for the conjugated BP-3 in urine (Vela-Soria et al, 2014). Urine samples from female volunteers showed benzophenone levels significantly higher than those from men, which is consistent with our results in nails. Similar concentrations were quantified in breast milk, where BP-1 concentration was between 0.8-1.5 ng mL^{-1} and BP-3

concentration between 0.8-9.6 ng mL^{-1} (Rodríguez-Gómez et al, 2015). Finally, UV-filters in human serum have been found in concentrations lower than 0.7 ng mL^{-1} for BP-1 and 1.2 ng mL^{-1} for BP-3 (Vela-Soria et al, 2014). The concentration of benzophenone-like compounds detected in the present work confirms that this kind of UV-filters are excreted and bioaccumulated in the keratin matrix, like some pharmaceuticals and drugs (Cappelle et al., 2015). These findings suggest that nails can be used as a marker of exposure to the target analytes.

These high amounts of benzophenones found are indicative of the accumulation of these UV-filters in the human body and support the relevance of this study, given high exposure concentrations and the associated health risks as these compounds act as endocrine disruptors.

4. Conclusions

The detection of UV-filters in human nail samples has been done with an accurate, sensible and specific method using UHPLC-ESI-MS/MS as analytical technique. A non-invasive sampling and a simple sample pre-treatment have been used. The technique allows the detection of nine benzophenone-type compounds simultaneously in less than 10 min with high sensitivity. The validation parameters have been studied, leading to high quality results. Different extraction techniques have been compared. MAE digestion in an acid medium has proven to be optimal for the target compounds. The number of extraction cycles, time and temperature, have been the optimized parameters with highest recoveries in a single cycle, at 44ºC and 20 min. This study demonstrates the long-term bioaccumulation of the investigated compounds in the human body, specifically in stable matrices like nails. The high frequency and concentrations detected in the analyzed samples reveals the high exposure concentrations related to the extended use of these compounds in cosmetics and other personal care products. The results of this work show the high bioaccumulation capacity of UV-filters at levels that could pose a risk to human health as exposure occurs at young ages.

Declaration of competing interest. The authors declare no competing interests.

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References

Ahn, S., An, S., Lee, M., Lee, E., Pyo, J.J., Kim, J.H., Ki, M.W., Jin, S.H., Ha, J., Noh, M., 2019. A long-wave UVA filter avobenzone induces obesogenic phenotypes in normal human epidermal keratinocytes and mesenchymal stem cells. Arch. Toxicol. 93, 1903-1915.

Alves, A., Kucharska, A., Erratico, C., Xu, F., Hond, E.D., Koppen, G., Vanermen, G., Covaci, A., Voorspoels, S., 2014. Human biomonitoring of emerging pollutants through non-invasive matrices: state of the art and future potential. Anal. Bioanal. Chem. 406, 4063-4088.

Alves, A., Covaci, A., Voorspoels, S., 2017. Method development for assessing the human exposure to organophosphate flame retardants in hair and nails. Chemosphere 168, 692-698.

Cappelle, D., Yegles, M., Neels, H., van Nuijs, A.L.N., Doncker, M.D., Maudens, K., Covaci, A., Crunelle, C.L., 2015. Nail analysis for the detection of drugs of abuse and pharmaceuticals: a review. Forensic Toxicol. 33, 12-36.

Cigan, S.S., McGovern, P.M., Choudhary, K., Miller, N.C., Georgieff, M., Rao, R., Stepanov, I., 2018. Toenail manganese as a potential biomarker for in utero and early childhood exposure studies. Sci. Rep. 8, 17034.

Collaris, E.J., Frank, J., 2008. Photoallergic contact dermatitis caused by ultraviolet filters in different sunscreens. Int. J. Dermatol. 1, 35-37.

Commission Regulation (EU) 2017/238 of 10 Feb 2017 amending Annex VI to Regulation (EC) no. 1223/2009 of the European Parliament and of the Council of cosmetic products, 2017.

Cunha, S.C., Trabalón, L., Jacobs, S., Castro, M., Fernandez-Tejedor, M., Granby, K., Verbeke, W., Kwadijk, C., Ferrari, F., Robbens, J., Sioen, I., Pocurull, E., Marques, A., Fernandes, J.O., Domingo, J.L., 2018. UV-filters and musk fragances in seafood commercialized in Europe Union: Occurrence, risk and exposure assessment. Environ. Res. 161, 399-408.

Daniel, C.R., Piraccini, B.M., Tosti, A., 2004. The nail and hair in forensic science. J. Am. Acad. Dermatol. 50, 258-261.

European Chemicals Agency (ECHA), Benzophenone (Online Source), 2020, https://echa.europa.eu/substance-information/-/substanceinfo/100.003.943

Ferreira, S.L., Bruns, R.E., Ferreira, H.S., Matos, G.D., David, J.M., Brandão, G.C., da Silva, E.G., Portugal, L.A., dos Reis, P.S., Souza, A.S., dos Santos, W.N., 2007. Box-Behnken design: an alternative for the optimization of analytical methods. Anal. Chim. Acta 597, 179-186.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2015. UV-filters bioaccumulation in fish from Iberian river basins. Sci. Total. Environ. 518-519, 518-525.

Grashow, R., Zhang, J., Fang, S.C., Weisskopf, M.G., Christiani, D.C., Cavallari, J.M., 2014. Toenail metal concentration as a biomarker of occupational welding fume exposure. J. Occup. Environ. Hyg. 11, 397-405.

Guesmi, A., Ohlund, L., Sleno, L., 2020. In vitro metabolism of sunscreen compounds by liquid chromatography/high resolution tandem mass spectrometry. Rapid Commun. Mass Spectrom. 34, e8679.

Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), 2018.

Hayashi, T., Okamoto, Y., Ueda, K., Kojima, N., 2006. Formation of estrogenic products from benzophenone after exposure to sunlight. Toxicol. Lett. 167, 1-7.

He, K., 2011.Trace elements in nails as biomarkers in clinical research. Eur. J. Clin. Invest. 41, 98-102.

Hiller, J., Klotz, K., Meyer, S., Uter, W., Hof, K., Greiner, A., Göen, T., Drexler, H., 2019. Systemic availability of lipophilic organic UV-filters through dermal sunscreen exposure. Environ. Int. 132, 105068.

Hu, L., Tian, M., Feng, W., He, H., Wang, Y., Yang, L., 2019. Sensitive detection of benzophenone-type ultraviolet filters in plastic food packaging materials by sheathless capillary electrophoresis-electrospray ionization-tandem mass spectrometry. J. Chromatogr. A 1604, 460469.

Karlsson, I., Hillerström, L., Stenfeldt, A.L., Mårtensson, J., Börje, A., 2009. Photodegradation of dibenzoylmethanes: potential cause of photocontact allergy to sunscreens. Chem. Res. Toxicol. 22, 1881–1892.

Kerdivel, G., Le Guevel, R., Habauzit, D., Brion, F., Ait-Aissa, S., Pakdel, F., 2013. Estrogenic potency of benzophenone UV-filters in breast cancer cells: proliferative and transcriptional activity substantiated by docking analysis. PLoS One 8, e60567.

Klopcic, I., Dolenc, M.S., 2017. Endocrine activity of AVB, 2MR, BHA, and their mixtures. Toxicol. Sci. 156, 240-251.

Krause, M., Frederiksen, H., Sundberg, K., Jørgensen, F.S., Jensen, L.N., Nørgaard, P., Jørgensen, C., Ertberg, P., Juul, A., Drzewiecki, K.T., Skakkebaek, N.E., Andersson, A.M., 2018. Presence of benzophenones commonly used as UV-filters and absorbers in paired maternal and fetal samples. Environ. Int. 110, 51-60.

Lebedev, A.T., Bavcon Kralj, M., Polyakova, O.V., Detenchuk, E.A., Pokryshkin, S.A., Trebše, P., 2020. Identification of avobenzone by-products formed by various disinfectants in different types of swimming pool waters. Environ. Int. 137, 105495.

Li, J., Guo, F., Wang, Y., Liu, J., Cai, Z., Zhang, J., Zhao, Y., Wu, Y., 2012. Development of extraction methods for the analysis of perfluorinated compounds in human hair and nail by high performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1219, 54–60.

Liu, W., Xu, L., Li, X., Jin, Y.H., Sasaki, K., Saito, N., Sato, I., Tsuda, S., 2011. Human nails analysis as biomarker of exposure to perfluoroalkyl compounds. Environ. Sci. Technol. 45, 8144-8150.

Liu, L.Y., He, K., Hites, R.A., Salamova, A., 2016. Hair and nails as noninvasive biomarkers of human exposure to brominated and organophosphate flame retardants. Environ. Sci. Technol. 50, 3065-3073.

Martín, J., Zafra-Gómez, A., Hidalgo, F., Ibáñez-Yuste, A.J., Alonso, E., Vilchez, J.L., 2017. Multi-residue analysis of 36 priority and emerging pollutants in marine echinoderms (Holothuria tubulosa) and marine sediments by solid-liquid extraction followed by dispersive solid phase extraction and liquid chromatography–tandem mass spectrometry analysis. Talanta 166, 336-348.

Martín-Pozo, L., Cantarero-Malagón, S., Hidalgo, F., Navalón, A., Zafra-Gómez, A., 2020. Determination of endocrine disrupting chemicals in human nails using alkaline digestion prior to ultra-high performance liquid chromatography-tandem mass spectrometry. Talanta 208, 120429.

Official website of the European Union, List of UV-filters allowed in cosmetic products, Annex VI, Last update: 23/03/2020, Available at: https://ec.europa.eu/growth/tools-databases/cosing/pdf/COSING_Annex%20VI_v2.pdf

Ramos, S., Homem, V., Alves, A., Santos, L., 2016. A review of organic UV-filters in wastewater treatment plants. Environ. Int. 86, 24-44.

Rodríguez-Gómez, R., Zafra-Gómez, A., Camino-Sánchez, F.J., Ballesteros, O., Navalón, A., 2014. Gas chromatography and ultrahigh performance liquid chromatography tandem mass spectrometry methods for the determination of selected endocrine disrupting chemicals in human breast milk after stir-bar sorptive extraction. J. Chromatogr. A 1349, 69-79.

Rodríguez-Gómez, R., Zafra-Gómez, A., Dorival-García, N., Ballesteros, O., Navalón, A., 2015. Determination of benzophenone-UV filters in human milk samples using ultrasound-assisted extraction and clean-

up with dispersive sorbents followed by UHPLC-MS/MS analysis. Talanta. 134, 657-664.

Schmutzler, C., Bacinski, A., Gotthardt, I., Huhne, K., Ambrugger, P., Klammer, H., Schlecht, C., Hoang-Vu, C., Grüters, A., Wuttke, W., Jarry, H., Köhrle, J., 2007. The ultraviolet filter benzophenone 2 interferes with the thyroid hormone axis in rats and is a potent in vitro inhibitor of human recombinant thyroid peroxidase. Endocrinology 148, 2835-2844.

Shi, Y., Liu, X., Zhang, J., Shao, B., 2013. Analysis of triclosan and triclocarban in human nails using isotopic dilution liquid chromatography–tandem mass spectrometry. J. Chromatogr. B 934, 97-101.

Sukumar, A., 2005. Human nails as a biomarker of element exposure. Rev. Environ. Contam. Toxicol. 185, 141-177.

Suzuki, T., Kitamura, S., Khota, R., Sugihara, K., Fugimoto, N., Ohta, S., 2005. Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens. Toxicol. Appl. Pharmacol. 203, 9-17.

Valle-Sistac, J., Molins-Delgado, D., Díaz, M., Ibañez, L., Barceló, D., Díaz-Cruz, M.S., 2016. Determination of parabens and benzophenone-type UV-filters in human placenta. First description of the existence of benzylparaben and benzophenone-4. Environ. Int. 88, 243-249.

Vela-Soria, F., Jiménez-Díaz, I., Rodríguez-Gómez, R., Zafra-Gómez, A., Ballesteros, O., Navalón, A., Vílchez, J.L., Fernández, M.F., Olea, N., 2011. Determination of benzophenones in human placental tissue samples by liquid chromatography-tandem mass spectrometry. Talanta 85, 1848-1855.

Vela-Soria, F., Ballesteros, O., Zafra-Gómez, A., Ballesteros, L., Navalón, A., 2014a. A new method for the determination of benzophenone-UV-filters in human serum samples by dispersive liquid-liquid microextraction with liquid chromatography-tandem mass spectrometry. Talanta 121, 97-104.

Vela-Soria, F., Ballesteros, O., Zafra-Gómez, A., Ballesteros, L., Navalón, A., 2014b. A multiclass method for the analysis of endocrine disrupting

chemicals in human urine samples. Sample treatment by dispersive liquid-liquid microextraction. Talanta 129, 209-218.

Vidal-Liñán, L., Villaverde-de-Sáa, E., Rodil, R., Quintana, J.B., Beiras, R., 2018. Bioaccumulation of UV-filters in Mytilus galloprovincialis mussel. Chemosphere 190, 267-271.

Wang, L., Kannan, K., 2013. Characteristic profiles of benzonphenone-3 and its derivatives in urine of children and adults from the United States and China. Environ. Sci. Technol. 47, 12532-12538.

Wang, J., Pan, L., Wu, S., Lu, L., Xu, Y., Zhu, Y., Guo, M., Zhuang, S., 2016. Recent advances on endocrine disrupting effects of UV-filters. Int. J. Environ. Res. Public Health 13, 782.

Wang, Y., Zhong, Y., Li, J., Zhang, J., Lyu, B., Zhao, Y., Wu, Y., 2018. Occurrence of perfluoroalkyl substances in matched human serum, urine, hair and nail. J. Environ. Sci. 67, 191-197.

Watanabe, Y., Kojima, H., Takeuchi, S., Uramaru, N., Sanoh, S., Sugihara, K., Kitamura, S., Ohta, S., 2015. Metabolism of UV-filter benzophenone-3 by rat and human liver microsomes and its effect on endocrine-disrupting activity. Toxicol. Appl. Pharmacol. 282, 119-128.

Wu, M.H., Xie, D.G., Xu, G., Sun, R., Xia, X.Y., Liu, W.L., Tang, L., 2017. Benzophenone-type UV-filters in surface waters: An assessment of profiles and ecological risks in Shanghai, China. Ecotoxicol. Environ. Saf. 141, 235-241.

Wu, H., Wu, L.H., Wang, F., Gao, C.J., Chen, D., Guo, Y., 2019. Several environmental endocrine disruptors in beverages from South China: occurrence and human exposure. Environ. Sci. Pollut. Res. Int. 26, 5873-5884.

Yaemsiri, S., Hou, N., Slining, M.M., Ke, K., 2010. Growth rate of human fingernails and toenails in healthy American young adults. J. Eur. Acad. Dermatol. Venereol. 24 420-423.

Yin, J., Wei, L., Shi, Y., Zhang, J., Wu, Q., Shao, B., 2016. Chinese population exposure to triclosan and triclocarban as measured via human urine and nails. Environ. Geochem. Health 38, 1125-1135.

Table S1. *Box-Behnken matrix*

Run order	Temperature (°C)	Extraction time (min)	Cycles
1	50	5	3
2	50	17.5	2
3	50	17.5	2
4	50	5	1
5	30	5	2
6	30	17.5	1
7	30	30	2
8	70	17.5	1
9	70	30	2
10	30	17.5	3
11	50	30	3
12	70	5	2
13	50	30	1
14	50	17.5	2
15	70	17.5	3

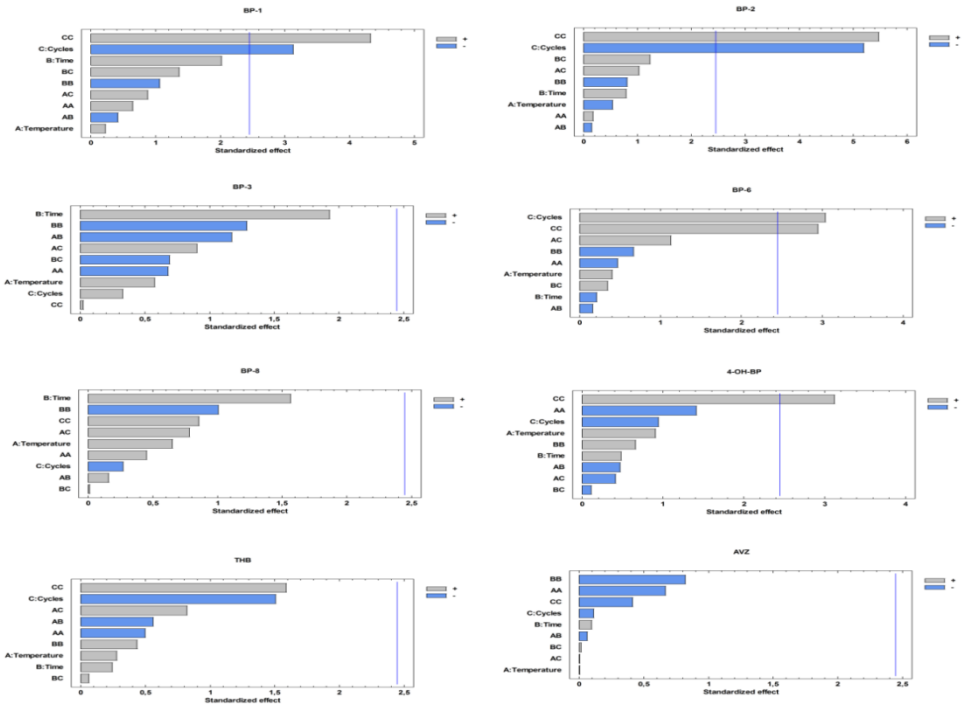

Figure S1. *Pareto Charts show the standardized main effect of compounds most sensitive to the variation of the parameters in the Box-Behnken experimental design*

Capítulo 5

Analytical method for the determination of usually prescribed antibiotics in human nails using UHPLC-MS/MS. Comparison of the efficiency of two extraction techniques

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(Under Review)

Abstract: Antibiotics are a group of drugs used for the treatment of bacterial diseases. They are used in both human and veterinary medicine and, although they are not permitted, they are sometimes used as growth promoters. The present research compares two extraction techniques: ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) in order to evaluate their efficiency in the determination of 17 usually prescribed antibiotics in human nails. The extraction parameters were optimized using multivariate techniques. Once both techniques were compared, MAE was selected as optimal due to its greater experimental practicability together with the better extraction efficiencies it provides. Target analytes were detected and quantified by ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). The run time was 20 minutes. The methodology was then successfully validated, obtaining acceptable analytical parameters according to the guide used. Limits of detection were between 0.3 and 3 ng g^{-1} and limits of quantification were in the range from 1.0 to 4.0 ng g^{-1}. Recovery percentages ranged from 87.5% to 114.2%, and precision (in terms of standard deviation) was less than 15% in all cases. Finally, the optimized method was applied to nails taken from 10 volunteers and the results revealed the presence of one or more antibiotics in all the samples examined. The most commonly found antibiotic was sulfamethoxazole, followed by danofloxacin and levofloxacin. The results demonstrated, on the one hand, the accumulation of these compounds in the human body and, on the other hand, the suitability of nails as a non-invasive biomarker of exposure.

Keywords: Human nails; Antibiotics; Biomarker; Exposure; Emerging contaminants

1. Introduction

Pharmaceuticals, and in particular antibiotics, are produced and consumed daily throughout the world. Once ingested, these compounds are conjugated through the liver and eliminated mainly in the urine. A

non-conjugated active part is also eliminated, reaching wastewater treatment plants, which are often unable to eliminate them completely, entering the environment and being considered emerging pollutants [1,2]. Antibiotics are a group of drugs used for the treatment of bacterial diseases, and are known as agents capable of killing bacteria or inhibiting their growth by different mechanisms [3]. Since Alexander Fleming discovered penicillin in 1928, antibiotics have contributed to the treatment of bacterial infections, reducing their prevalence and the risk of death of the sick [4]. They are used in both human and veterinary medicine and, although not permitted, are sometimes used as growth promoters [5]. This group of drugs is used more and more every year. In 2012, the consumption of antibiotics in non-hospitalized patients reached 3400 t in European countries. France with 719 t, Great Britain with 415 t or Spain with 321 t stands out notably [6]. Due to the massive and continuous use of antibiotics, they are constantly entering the environment and are therefore considered pseudo-persistent contaminants. Although these compounds have a short half-life, their continuous entry means that they are ubiquitous [6]. They cause environmental effects such as decreased microbial diversity, immunity of pathogenic bacteria or destabilization of activated sludge in wastewater treatment plants [5,7].

As for adverse effects in humans, there is the possibility of developing allergic reactions or inducing antibiotic resistance [4,8]. The presence and spread of bacterial resistance to antibiotics in the environment is nowadays considered a serious global health problem [9]. Excessive consumption of these drugs, as well as animal-derived products such as milk or meat, involves the consumption of low doses of antibiotics over a long period of time, which significantly increases the risk of developing global antimicrobial resistance and decreases the efficacy of antibiotics [10]. For that reason, the European Union determined maximum residue limits (MRLs) for veterinary drugs in animal products [3]. In this context, it is necessary to monitor the presence of antibiotics in human and environmental samples to study their effect on health. The development of new low-cost and high-recovery analytical methods, where the sample

treatment procedure plays a fundamental role, is crucial at present, since the concentration of these drugs in real samples is very low and they have a high matrix effect [10].

Human biomonitoring studies (HBM) make it possible to determine the total exposure to a mixture of contaminants in human matrices such as blood, urine, breast milk, saliva, hair or nails, taking into account lifestyle habits [11]. HBM is especially useful because it can help find new chemical exposures, identify the most vulnerable groups, study exposure patterns in the general population or in specific groups, and associate environmental exposures with health risks [12]. Keratinized human matrices, such as hair and nails, allow the study of the accumulation of substances over long periods of time, making possible the retrospective investigation of past drug use [13-15]. On the one hand, hair has been used for decades in forensic and toxicological sciences for routine analysis [16]. On the other hand, nails have recently been used as an alternative to hair in forensic applications, such as in post-mortem cases or when hair is not available (e.g. in case of hyalopecia or after chemotherapy treatment) [17]. The incorporation of substances takes place through diffusion of blood supply during nail growth [18]. This is continuous as well as bidirectional (nails grow 3 mm/month in the hands and 1.5 mm/month in the feet versus 1 cm/month in the hair) [19].

Some studies have used human fingernails as an analytical matrix for the determination of different compounds in the organism. For example, Soleymani et al. studied the presence of heavy metals in the nails of petrochemical workers [20]; Zeng et al. determined PAHs and their hydroxylated metabolites in this matrix [21]; Martín-Pozo et al. investigated the accumulation of endocrine-disrupting UV filters in human nails [22], and Zhe et al. developed a method for the simultaneous determination of diacetylpolyamines in nails of cancer patients [23]. However, to our knowledge, there is no multi-residue method to determine the presence of different antibiotic families in human nails.

The objective of the present study was to develop and validate a selective, sensitive and accurate multiclass UHPLC-MS/MS method

capable of determining the presence of 17 antibiotics, widely used in human and veterinary medicine, in human nail samples. The method involves a sample treatment consisting of microwave-assisted extraction with high recoveries. The method has been applied to samples collected from 10 volunteers.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade standards of analytes: Nalidixic acid (NAL), sulfamethoxazole (SMX), trimethoprim (TMP), flumequine (FLU), oxolinic acid (OXO), norfloxacin (NOR), marbofloxacin (MAR), difloxacin (DIF), moxifloxacin (MOX) erythromycin (ERY) and clarithromycin (CLA) were supplied by Sigma Aldrich (Madrid, Spain). Cinchophen (CIN), penicillin V (PEN-V), danofloxacin (DAN), enrofloxacin (ENR), levofloxacin (LEVO) and oxacillin (OXA) were purchased from Alfa Aesar (Massachusetts, MA, USA) and ciprofloxacin (CIP) was bought at Supelco (Bellfonte, Pennsylvania, USA).

Ultrapure water was purified by a Milli-Q Plus® purification system (Millipore, Madrid, Spain), and solvents used such as LC-MS methanol (MeOH) and acetonitrile (ACN) were obtained from Prolabo Chemicals (Barcelona, Spain), ethyl acetate (EtOAc) from Honeywell (Madrid, Spain), and acetone from Panreac (Barcelona, Spain). Formic acid (98-100 %) used as additive in the aqueous phase and sodium hydroxide (NaOH) (> 98 %, pellets) were obtained from Sigma-Aldrich. Finally, hydrochloric acid (6 N) was obtained from Panreac. The chemical structures of the different compounds under study are shown in supplementary material (Table S1).

2.2. Instrumentation and software

Freeze-drying of the samples was carried out with a ScanVac CoolSafe™ freeze-dyer (Lynge, Denmark). For pulverisation of the samples, a ball mill Retsch® MM 301 (Biometa, Asturias, Spain) was used. An IKA vortex shaker (Staufen, Germany), a balance Mettler-Toledo AND GX400 (Columbus, OH, USA) and a sample concentrator

(Stuart) were also used. Two types of centrifuges were employed, a Spectrafuge 24D Labnet Ultracentrifuge (New Jersey, USA) and a Hettich Universal 320 centrifuge (Tuttingen, Germany) adapted for Eppendorf tubes. For sample digestions, an ultrasound probe 400 W digital sonifier (Branson Ultrasonic Corporation) with a 0.5-inch (12.7 mm) probe operating at a frequency of 20 kHz and a microwave Milestone's ETHOS SEL extraction Labstation (Sheldon, CT, USA), operating at 2455 MHz with a maximum power of 1000 W, were used.

Chromatographic analyses were done with an Acquity™ I-Class chromatography system (Waters, Manchester, UK), equipped with an Acquity sample manager and an Acquity UPLC™ binary solvent manager was used. A Waters UPLC® HSS T3 chromatographic column (2.1 x 100 mm i.d., 1.8 µm particle size) and an Acquity UPLC® BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size) were tested for analyte separation. The chromatographic system was coupled to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters) using Z-spray™ as the ionisation source (ESI) for analyte detection. Data processing was performed with MassLynx V 4.1 SCN 803 (Waters, Manchester, UK) and the statistical package Statgraphics version 16.0 (Virginia, USA).

2.3. Sample collection, storage and pre-treatment

Ten volunteers from the province of Granada (Spain), aged between 14 and 60 years, collected nail samples at home. All volunteers were informed of the study and gave their prior consent. Sample collection was carried out over a period of 3 months during which the subjects cut their fingernails and toenails and stored them together in polypropylene jars. The samples were stored at room temperature and in the dark. Since antibiotics are widely used in both human and veterinary medicine, it is very difficult to find an analyte-free matrix, with the added drawback of limited sample quantity. Therefore, a sample from a volunteer with amounts of antibiotics below the detection limits of the method was used for calibration and validation of the method.

To avoid external contamination (e.g., dust particles, nail polish or other compounds adhering to the nail surface) prior to the analyte extraction, the samples were cleaned with a 1% Triton X-100 solution in an ultrasonic bath twice for 15 minutes, followed by three ultrasonic baths with Milli-Q water to remove detergent residues. Subsequently, to remove possible nail polish, they were washed with acetone in an ultrasonic bath and finally with MeOH for 15 minutes. After washing, the nails were freeze-dried for 24 hours to remove moisture and then pulverized in a ball mill for 10 minutes at 30 Hz. In this way, the particle size is decreased while increasing the contact surface between the analytes under study and the solvents, improving the extraction efficiency. Samples ready for extraction were stored at room temperature in the dark until analysis.

2.4. Preparation of fortified samples

Optimization of the extraction procedure, calibration and method validation were performed with spiked samples. Briefly, 50 µL of a methanolic solution containing the target analytes and the surrogate (cinchophen) were added to 0.05 g of nail blank sample, obtaining a final concentration of 100 ng g^{-1}. After fortification, the samples were kept in the dark at room temperature for 24 hours to ensure contact between the analytes and the matrix and evaporation of the solvent. In addition, for calibration and validation, different concentration levels (1-100 ng g^{-1}) were used for all analytes, excluding the internal standard, whose concentration was kept constant at 100 ng g^{-1}. At this point, the spiked nail samples were ready for extraction procedures.

2.5. Basic procedures

Microwave-assisted extraction (MAE). 0.05 g of lyophilized nail was placed in a microwave vessel. A volume of 1.75 mL of MeOH/ACN (50/50 v/v) was added and vortexed for 30 seconds to ensure contact of the extraction solvent with the entire sample. Ten vessels were processed at the same time in the apparatus, which operates at 88 ºC for 1 min, with a 10 min preheating ramp and 5 min subsequent ventilation to lower the temperature (< 45 ºC). The total digestion time was 16 min. The power of the equipment was 1000 W and the pressure was automatically controlled. Two extraction cycles were performed. The supernatants were centrifuged for 5 min at 3000 rpm and transferred to a glass tube in which they were evaporated to dryness under a stream of N_2. The dried extracts were dissolved in a final volume of 200 µL of the initial mobile phase. They were then centrifuged for 10 min at 13000 rpm and transferred to the vial to be injected directly into the LC-MS system.

Ultrasound-assisted extraction (UAE). 0.05 g of freeze-dried cloves were weighed into a 10 mL glass tube and 2.75 mL of extraction solvent MeOH/ACN (75/25 v/v) was added. It was vortexed for 30 s and placed in a water bath along with the ultrasound probe. Up to 13 samples were processed at the same time. The probe was run at 70 % amplitude for 60 min. Three extraction cycles were performed and, in between, the bath water was changed to avoid overheating. After digestion, the tubes were centrifuged for 5 min at 3000 rpm and the supernatant was removed and mixed after each cycle. Finally, the extract was dried under a stream of N_2 and the residue was dissolved in the initial mobile phase in the same manner as above.

Liquid chromatographic-mass spectrometric analysis. Chromatographic separation was performed using a Waters Acquity HSS T3 column (2.1 x 100 mm i.d., 1.8 µm) maintained at 40 ºC and a mobile phase consisting of water (solvent A) and methanol (solvent B), both acidified with formic acid (0.1 % v/v). The flow rate was set at 0.3 mL min^{-1} and the injection volume was 2 µL. The gradient program was as follows: 0.0-1.0 min, 20 % B, 1.0-6.0 min, 20-25 % B, 6.0-8.0 min, 25-30 % B, 8.0-

9.0 min, 30-50 % B, 9.0-14.0 min, 50-100 % B, 14.0-16.0 min, 100 % B, 16.0-16.1 min, back to 20 % B. The total run time was 20 min.

For MS, positive ionization and multiple reaction monitoring (MRM) mode was used to identify and quantify all target analytes in order to improve specificity and sensitivity. Two different transitions were monitored for each analyte. The most abundant one was used for quantification and the other for identification. In addition, to improve the selectivity of the method, time intervals were selected to measure the different transitions. The instrumental parameters for MS analysis are shown in Table 1.

Table 1. *Transitions and parameters used for MS/MS analysis*

Compound	t_R (min)	MRM Transitions	CV (V)	CE (V)	Ratio (a/b)
TMP	3.16	291.1 → 230.0[a]	76	18	27.6
		291.1 → 93.0[b]	76	30	
MAR	3.38	363.1 → 320.0[a]	18	12	1.5
		363.1 → 72.0[b]	18	18	
LEVO	4.37	362.0 → 318.1[a]	30	16	1.3
		362.0 → 261.1[b]	30	24	
NOR	4.89	320.2 → 302.0[a]	20	16	1.3
		320.2 → 276.1[b]	20	14	
CIP	5.49	332.1 → 314.0[a]	26	16	1.1
		332.1 → 231.0[b]	26	32	
SMX	5.6	253.9 → 155.9[a]	20	14	1.7
		253.9 → 92.0[b]	20	26	
DAN	6.24	358.1 → 340.0[a]	40	18	4.0
		358.1 → 314.1[b]	40	36	
ENR	6.03	360.1 → 316.1[a]	18	16	2.0
		360.1 → 245.1[b]	18	22	
DIF	7.1	399.9 → 356.1[a]	42	16	1.3
		399.9 → 299.0[b]	42	26	
MOX	9.79	402.0 → 384.1[a]	34	22	1.2
		402.0 → 358.1[b]	34	18	
NAL	11.07	233.0 → 215.0[a]	20	12	1.8
		233.0 → 187.0[b]	20	22	
FLU	11.22	261.9 → 244.0[a]	14	18	1.9
		261.9 → 202.0[b]	14	28	
OXO	11.23	261.9 → 244.0[a]	24	14	303
		261.9 → 215.9[b]	24	24	
ERY	11.27	734.4 → 158.1[a]	18	24	1.8
		734.4 → 576.3[b]	18	14	
PEN-V	11.48	351.1 → 229.0[a]	54	26	1.4
		351.1 → 333.0[b]	54	14	
OXA	11.74	402.0 → 144.0[a]	60	20	3.9
		402.0 → 384.1[b]	60	14	
CLA	11.81	748.4 → 158.1[a]	28	24	2.7
		748.4 → 590.3[b]	28	14	
CIN	11.91	250.1 → 128.0[a]	40	30	2.0
		250.1 → 222.1[b]	40	26	
Spectrometric conditions					
Source temperature: 158.9 ºC		Impactor voltage: 2.9 kV			
Desolvation temperature: 600 ºC		Cone gas flow: 150-147 L h^{-1}			
Desolvation gas flow: 1000-989 L h^{-1}		Nebulizer gas pressure: 7.00-6.94 bar			
Cone/desolvation gas: N_2 (≥ 99.995 %)		Collision gas: Ar (≥ 99.999 %)			
Dwell time: 25 ms		Inter-scan delay: 3 ms			

t_R: retention time; CV: cone voltage; CE: collision energy; [a] transition for quantification; [b] transition for confirmation

2.6. Factorial design 3^2

Response surface 3^2 factorial experimental designs were performed for the simultaneous optimization of two significant variables in the three-level extraction procedure, where neither the maximum nor the minimum is desired. Three central points were performed for a total of 12 experiments. The number of extraction cycles was evaluated along with the solvent volume, as well as the modifiable parameters of the extraction equipment, which for the ultrasound probe were time and amplitude, and for the microwaves, time and temperature. Mathematical model equations were used for extraction enhancement and analysis of variance (ANOVA) and correlation coefficients (R^2) detailed the statistical significance of linear, quadratic or cross interactions between variables.

2.7. Validity requirements

Quality Assurance/Quality Control procedures were carried out to study the validity of the method according to the US Food and Drugs Administration (FDA) guidance on validation of bioanalytical methods [24]. For the background contamination study, experimental blanks were performed and processed in the same way as the samples. All target analytes were either not detected or detected but not quantified (< LOQ). In addition, to study contamination and instrumental variability, a standard was prepared in the initial mobile phase at a concentration of 100 ng mL^{-1} and injected every 10 analyses. A calibration curve with five or six concentration levels was performed for each analyte to relate the signal obtained to the amount of the compound. Linearity was evaluated with the R^2 coefficients. All samples were analyzed in triplicate and injected three times in the equipment.

3. Results and discussion

3.1. Liquid chromatographic-mass spectrometric analysis

The optimization of the method started with the comparison of two different chromatographic columns, an Acquity UPLC® HSS T3 column (100 mm x 2.1 mm i.d., 1.8 µm particle size) and an Acquity UPLC® BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size). Although both gave good results, the first showed better peak quality in terms of width and shape for most compounds. The chromatographic separation was optimized using a 100 ng mL^{-1} standard mixture containing all the compounds studied. For the mobile phase, the combination of an aqueous phase (phase A) with the two modifiers usually used in liquid chromatography MeOH and ACN (phase B) was tested. It was observed that the presence of the latter produced a total loss of signal for some of the compounds studied, so its use was discarded. Finally, to improve the ionization of the compounds at the interface, the addition of formic acid in a proportion of 0.1 % (v/v) only in phase A, or in both phases, was evaluated; proving that when it was in both phases it improved the signal corresponding to the analytes in a very notable way.

3.2. Optimization of microwave-assisted extraction

As general variables that can affect the extraction process, the extraction solvent, solvent volume and number of cycles were studied. In addition, the pH of the extraction medium and the different parameters affecting the digestion, such as time of microwave and temperature, were optimized. The power of the equipment was set at 1000 W, which is the maximum power it can reach. MeOH, ACN, mixtures of both (25/75, 50/50 and 75/25, v/v), EtOAc and a mixture of acetone/hexane (50/50 v/v) were tested to cover a wide range of polarities. Figure S1, in the supplementary material, shows how the use of acetonitrile causes the disappearance of the analytical signal for most of the quinolones studied. The 50/50 (v/v) mixture of ACN with MeOH gives better signals for all compounds than both solvents separately or any of the other mixtures studied. In addition, the acetone-hexane mixture also did not give good extraction results and

EtOAc improved extraction efficiencies only in the NAL, FLU, DIF and MOX cases. The effect of extraction volume and number of extraction cycles was also studied using a 3^2 experimental design. One to three extraction cycles and 1 to 3 mL of the solvent selected as optimum (MeOH/ACN 50/50 v/v) were evaluated. The matrix with the experimental conditions is detailed in the supplementary material (Table S2). Three levels of each variable were evaluated in 12 runs. The response surface obtained is shown in Figure 1A. It can be seen how, with a desirability of 64 %, the optimum volume of extraction solvent is 1.75 mL and two cycles should be performed.

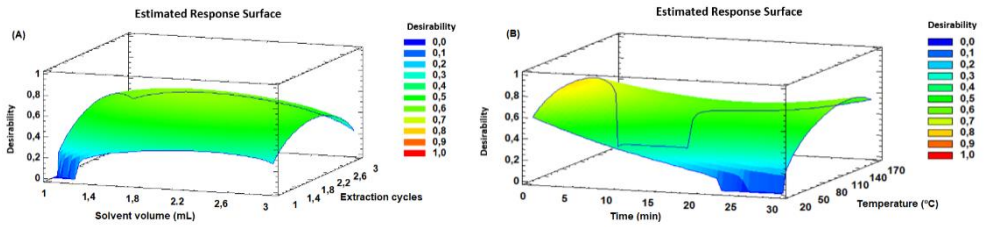


Figure 1. *Response surface 3^2: solvent volume and number of extraction cycles for MAE (a) and response surface 3^2: time and temperature for MAE*

Another experiment consisted of modifying the pH of the extraction solvent by preparing solutions 0.1 mol L^{-1} of HCl and NaOH in the solvent and subjecting the samples to digestion. Data obtained are shown in supplementary material (Figure S2). It was observed that the basic medium clearly impaired the extraction of all analytes, whereas the acidic medium improved recoveries (in particular for quinolones), but eliminated the signal of two compounds, ERY and CLA. Therefore, in compromise with all analytes it was decided not to add acid in the extraction solvent. Finally, the effect of irradiation time and temperature was also tested using a 3^2 experimental design. Three levels of each variable were studied in 12 experiments, as shown in supplementary information (Table S3). The response surface (Figure 1B) indicates that 1 min and 88 ºC give the best extraction efficiency with 74 % desirability. The Pareto plots of the target analytes selected for experiment analysis are shown in supplementary information (Figure S3). These plots indicate that temperature positively affects the extraction efficiencies,

whereas time depends on the analyte, and their combination (parameter AB) negatively influences the extraction efficiency. Probably, high temperatures for prolonged times result in degradation of compounds, which would explain the differences in responses over time among some of them. The ANOVA analysis provides coefficients of determination (R^2) around 60 % and P_{lof} values > 5 %. Therefore, the model used is satisfactory at 95 % confidence level.

Different extraction techniques, including acid and alkaline digestion at room temperature and MAE were examined for the analysis of the UV-filters. The influence of different acids on the extraction was also evaluated.

3.3. Optimization of ultrasound-assisted extraction

In the same way that the different variables that influence the extraction procedure with MAE were optimized, a study of these parameters was carried out using the UAE technique. In this case, the specific parameters of the extraction equipment are the ultrasound amplitude and the extraction time. First, the same solvents as in the previous section were studied, using 2 mL of each one and subjecting them to a single digestion for 20 min at an amplitude of 30. The results are shown as supplementary material (Figure S4). As shown in the figure, with the MeOH/ACN mixture (75/25 v/v) the best extraction yields are achieved for most analytes than pure MeOH. In contrast, the worst results were obtained with non-polar solvents.

As a second step, a response surface experimental design was performed to simultaneously evaluate the required volume of solvent and the number of extraction cycles, with the same conditions as in the case of MAE, shown in Table S2. Figure 2A presents the response surface obtained for this experiment.

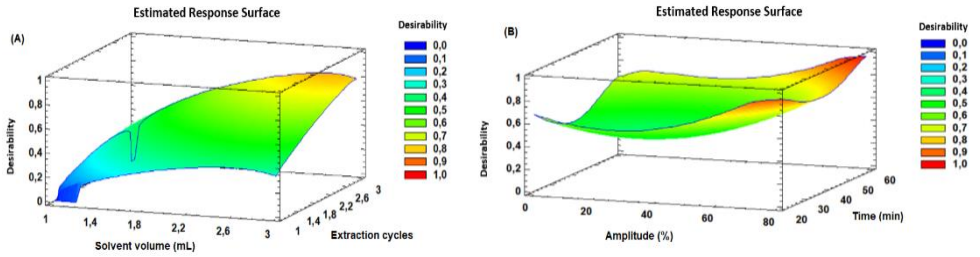


Figure 2. *Response surface 3^2: solvent volume and number of extraction cycles for UAE (a) and response surface 3^2: time amplitude for UAE (b)*

The optimal values were 2.75 mL of extraction solvent with 3 consecutive extraction cycles. The desirability of the experiment was 82% with Plof values > 5 %, with a confidence level of 95%.

As in the case of MAE, a test was carried out in which the pH of the extraction medium was modified by preparing solutions of 0.1 mol L^{-1} of HCl and NaOH in the extraction mixture. Consistent with the results obtained with MAE, it was found that, on the one hand, the basic medium affected negatively in all cases while, on the other hand, the acidic medium mainly benefited the quinolone signal but eliminated ERY and CLA, as can be seen in supplementary information (Figure S5). For this reason, it was also decided in this case not to change the pH value of the extraction solvent.

Finally, the parameters involved in the ultrasound equipment were studied. A response surface design of experiments was performed, whose matrix is shown in supplementary material (Table S4), consisting of 12 experiments where there were three levels of each variable: amplitude 10, 40 and 70% and time, 1, 15.5 and 30 minutes. The results showed that, with a desirability of 80%, the optimum results were 30.8% amplitude and 30 min. In terms of time, the desirability was maximum and, for this reason, it was decided to perform a second experimental design with a longer digestion time, up to 60 min. The matrix with the experimental conditions is also shown as supplementary material (Table S5). In this case, the maximum response surface was reached at 70% amplitude and 60 min, again the maximum time level evaluated, as can be seen in Figure 2B. Longer times were not tested because they would

excessively lengthen the process, which is not desirable. The confidence level was 95% and the Plof values were > 5%. Pareto plots, shown in supplementary information (Figure S6), demonstrate that amplitude is not significant, whereas time positively affects extraction efficiencies. Analysis of variance showed coefficients of determination greater than 18.8-77.1%.

3.4. Comparison of extraction techniques

Extraction efficiencies. Figure 3 shows the comparison of the two extraction techniques under the optimized final conditions. The results show significant differences. Thus, MAE provides much higher recoveries, which means that temperature control in the extraction is very important. Moreover, it should be noted that this is a common behaviour for all the analytes studied, indicating that the extraction efficiency depends on the technique, and not so much on the rest of the variables studied, such as the extraction solvent, the amount of solvent or the number of extraction cycles. The conditions to which the samples are subjected with MAE are more drastic, but when there are not many interferents and matrix effect because it is a simple matrix, the extraction yields improve significantly.

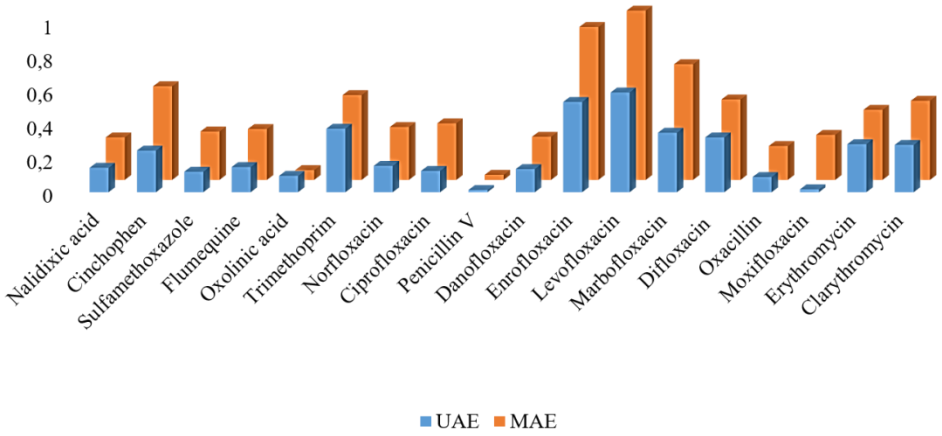


Figure 3. *Comparison of extraction techniques: MAE and UAE. Signal responses are plotted*

Practicability issues and extraction conditions. The in-depth study and optimization of the two extraction techniques leads to the conclusion that the efficiency of MAE is significantly higher for all target analytes. Moreover, the volume used (1.75 mL per extraction cycle) versus 2.75 mL in UAE, implies that a total of 3.75 mL in MAE and 8.25 mL in UAE are used. On the other hand, if the time necessary for the digestion is taken into account, MAE requires 32 min, while UAE takes 3 h. Based on the principles of Green Chemistry and feasibility, it was decided to use MAE conditions for routine analysis of the application of the method on volunteer samples.

3.5. Analytical performance (calibration curves)

Statistical comparison (Student's *t*-test) of the slopes of the solvent-prepared calibration curve and the matrix-prepared calibration curve showed significant differences, indicating the presence of some matrix effect. Consequently, for quantification, a standard procedure matrix calibration was prepared at five or six levels of concentration (from LOQ to 100 ng g^{-1}). Each level was prepared in quadruplicate and injected into the system four times. Cinchophen was used as surrogate at a final sample concentration of 100 ng g^{-1}. Calibration curves were obtained by plotting the antibiotic/surrogate peak area ratio as a function of antibiotic concentration. The calibration parameters are summarized in Table 2.

Table 2. *Matrix calibration parameters. Accuracy, recovery and precision*

	n	b g ng^{-1}	R^2 %	LOD ng g^{-1}	LOQ ng g^{-1}	Recovery study, %R (RSD, %, n=15)		
						LOQ ng g^{-1}	50 ng g^{-1}	100 ng g^{-1}
CIP	5	$3.99\ 10^{-3}$	93.1	1	4.0	95.8 (7.6)	99.2 (12.4)	98.2 (10.2)
CLA	6	$4.03\ 10^{-2}$	99.4	0.3	1.0	103.2 (10.7)	101.0 (10.0)	99.9 (2.4)
DAN	5	$2.98\ 10^{-3}$	96.9	1.5	5.0	108.3 (3.0)	99.3 (5.6)	100.4 (10.6)
DIF	6	$3.32\ 10^{-3}$	98.9	3	9.0	87.5 (12.6)	95.3 (13.0)	98.9 (12.2)
ENR	5	$6.33\ 10^{-3}$	96.3	0.9	4.0	96.2 (14.4)	111.3 (7.3)	97.9 (9.8)
ERY	5	$1.39\ 10^{-3}$	97.9	0.9	4.0	99.6 (7.1)	106.3 (12.0)	98.3 (2.4)
FLU	5	$3.02\ 10^{-2}$	98.1	0.8	3.0	102.6 (7.4)	106.8 (12.0)	98.3 (2.4)
LEVO	5	$8.66\ 10^{-3}$	96.7	0.8	3.0	101.8 (11.3)	114.2 (4.5)	98.1 (7.5)
MAR	5	$6.42\ 10^{-4}$	97.5	3	9.0	108.3 (11.6)	91.4 (8.0)	102.1 (5.2)
MOX	5	$1.97\ 10^{-3}$	97.1	0.9	4.0	91.0 (9.4)	111.1 (3.8)	98.5 (8.6)
NAL	5	$6.14\ 10^{-2}$	99.4	0.7	3.0	99.9 (13.2)	107.9 (9.7)	90.9 (5.3)
NOR	5	$2.65\ 10^{-3}$	90.1	2	8.0	112.1 (8.3)	97.4 (13.9)	96.4 (6.5)
OXA	5	$5.75\ 10^{-4}$	99.4	1.5	5.0	104.1 (5.5)	97.3 (9.1)	92.0 (2.5)
OXO	5	$1.87\ 10^{-2}$	99.6	0.7	3.0	109.4 (13.1)	103.2 (5.8)	92.5 (7.1)
PEN-V	6	$5.54\ 10^{-3}$	99.5	0.4	1.0	106.9 (6.2)	95.3 (5.1)	93.5 (5.1)
SMX	5	$1.15\ 10^{-2}$	99.7	0.9	4.0	111.5 (10.9)	92.1 (5.8)	98.8 (11.1)
TMP	5	$1.93\ 10^{-3}$	99.3	0.9	4.0	113.0 (10.6)	105.8 (11.0)	94.2 (6.5)

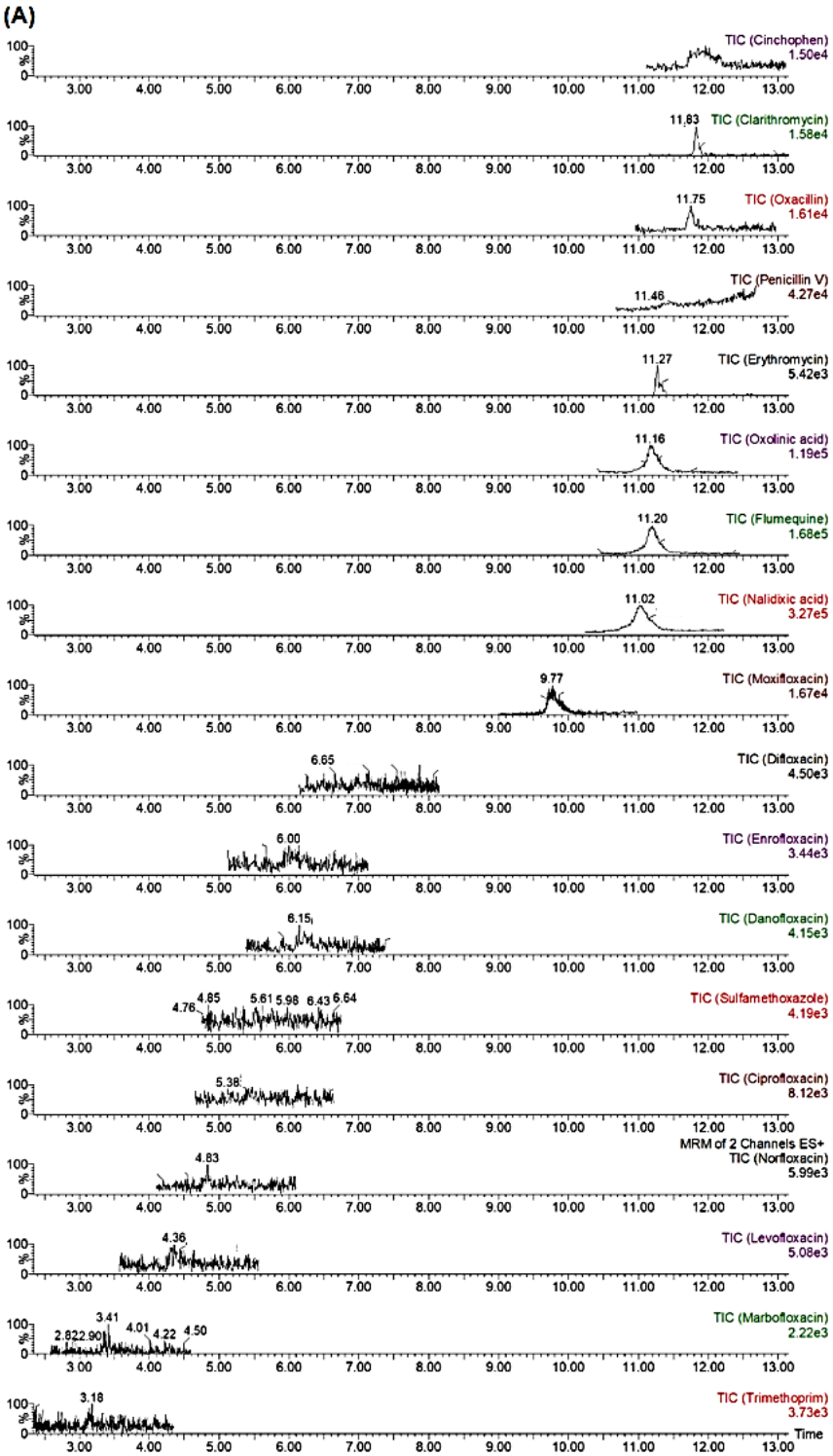
Linear dynamic range: LOQ-100 ng g^{-1}; n: calibration levels; b: slope of matrix calibration; %R^2: determination coefficient; LOQ: limit of quantification; LOD: limit of detection; RSD: Relative Standard Deviation

3.6. Method validation

Antibiotic validation was carried out according to FDA guideline for Bioanalytical Method Validation as indicated in *Section 2.7.* The method was validated in terms of linearity, sensitivity, selectivity and accuracy (trueness and precision).

Linearity. This was checked by means of the coefficients of determination (%R^2) and the p-values of the lack of fit test (% P_{lof}). The R^2 values obtained were between 90.1 and 99.7 and the P_{lof} values were >5% in all cases. This means good linearity within the established ranges.

Selectivity. This was evaluated by analysis of the blank samples. Figure 4 compares the chromatogram of the blank (a) with its corresponding enriched nail matrix containing all target antibiotics (100 ng g^{-1}) (b). No interferences from endogenous compounds are observed in the retention time of the analytes, demonstrating the selectivity of the method.


(A)
TIC (Cinchophen)
1.50e4
11.83 TIC (Clarithromycin)
1.58e4
11.75 TIC (Oxacillin)
1.61e4
TIC (Penicillin V)
11.46 4.27e4
11.27 TIC (Erythromycin)
5.42e3
11.16 TIC (Oxolinic acid)
1.19e5
11.20 TIC (Flumequine)
1.68e5
11.02 TIC (Nalidixic acid)
3.27e5
9.77 TIC (Moxifloxacin)
1.67e4
6.65 TIC (Difloxacin)
4.50e3
6.00 TIC (Enrofloxacin)
3.44e3
6.15 TIC (Danofloxacin)
4.15e3
4.76 4.85 5.61 5.98 6.43 6.64 TIC (Sulfamethoxazole)
4.19e3
5.38 TIC (Ciprofloxacin)
8.12e3
MRM of 2 Channels ES+
4.83 TIC (Norfloxacin)
5.99e3
4.36 TIC (Levofloxacin)
5.08e3
2.82 2.90 3.41 4.01 4.22 4.50 TIC (Marbofloxacin)
2.22e3
3.18 TIC (Trimethoprim)
3.73e3
100 %
0
3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Time

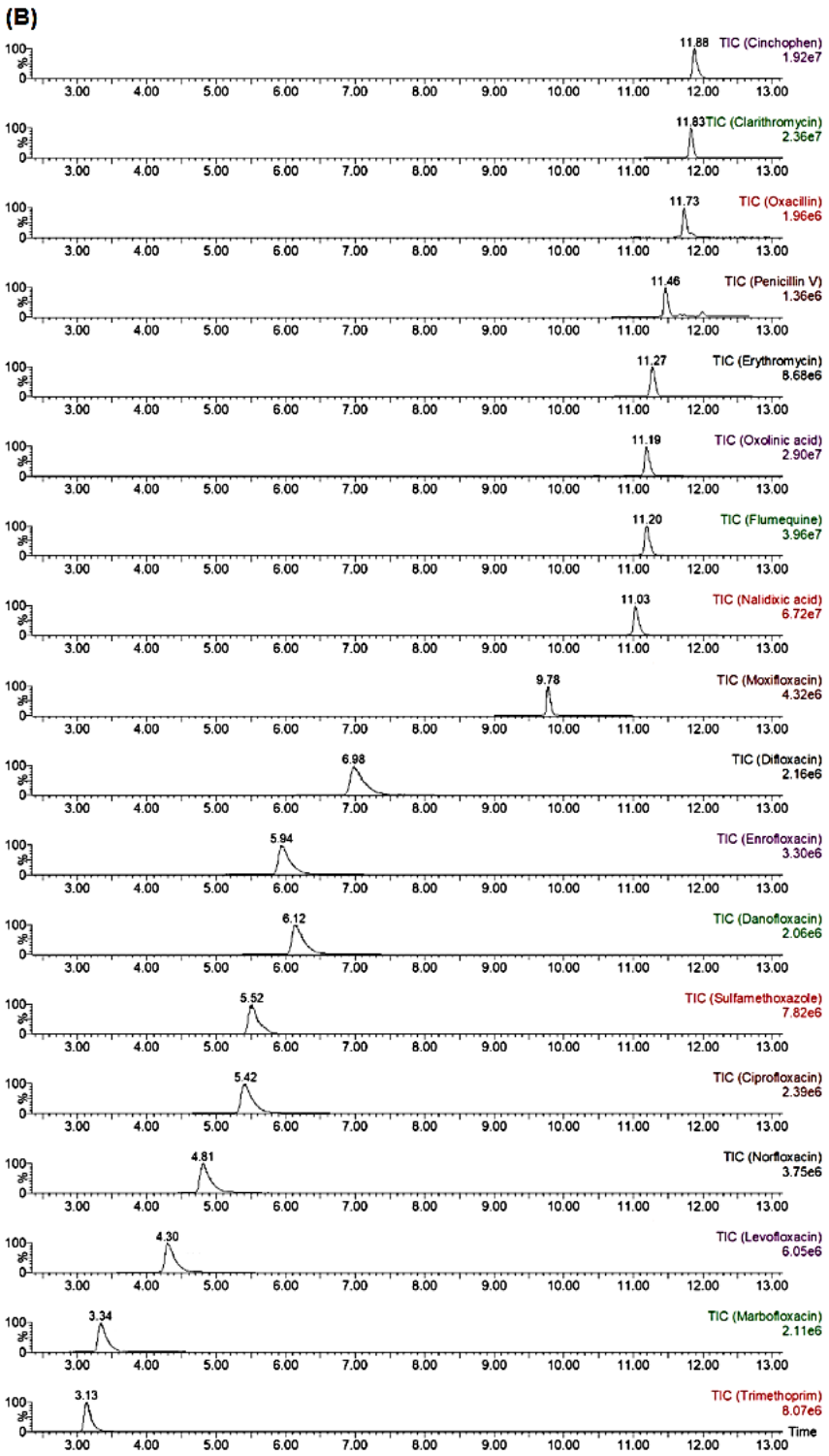


Figure 4. *Comparison of a chromatogram of the blank (a) and a spiked nail matrix (100 ng g^{-1}) (b). MRM of two transitions in ESI+ mode*

Sensitivity. LODs and LOQs were determined experimentally analyzing decreasing concentrations of the antibiotics in the blank samples, and allowed checking the sensitivity of the proposed method. Additionally, for confirmation, they were also calculated according to $3 \cdot s_0$ and $10 \cdot s_0$, for LOD and LOQ respectively, where s_0 is the standard deviation of the blank. The LODs were in the range of 0.3 to 3 ng g^{-1}, while the LOQs were between 1.0 and 9.0 ng g^{-1}. The results are also shown in Table 2.

Accuracy. It was evaluated by performing a recovery assay using nail samples spiked at three concentration levels: LOQ, 50 and 100 ng g^{-1} (low, intermediate and high). Intra- and inter-day precision, expressed as relative standard deviation (%RSD), was calculated by analyzing six replicates of each level per day, and repeated for three consecutive days. Trueness was estimated as recovery rates (%R) by comparing the concentration determined by interpolation from the standard calibration curve with the concentration previously added to the matrix. The results obtained are summarized in Table 2.

3.7. Biomonitoring of antibiotics in human fingernails

Once the proposed method was successfully validated, it was applied to nail samples from 10 subjects who were previously informed of the study to be performed. These volunteers were men (40%), women (60%) and children (two in total). Table 3 shows the results obtained for the samples tested, which were analyzed in triplicate, as well as the standard deviation obtained. In addition, Figure 5 illustrates a chromatogram of a positive sample, belonging to volunteer 5.

Table 3. *Application of the method to nail samples. Concentration in ng g^{-1}. Relative standard deviation in parentheses*

	CIP	CLA	DAN	DIF	ENR	ERY	FLU	LEVO	MAR	MOX	NAL	NOR	OXA	OXO	PEN	SMX
S1	D	2.9 (0.9)	D	ND	D	ND	D	D	6.0 (4.5)	ND	D	85.0 (7.2)	8.9 (8.9)	D	ND	D>LDR
S2	ND	2.1 (0.8)	ND	ND	ND	ND	ND	ND	ND	ND	49.0 (7.8)	ND	ND	ND	ND	25.7 (6.1)
S3	D	ND	ND	ND	ND	ND	D	ND	D	ND	D	23.7 (4.7)	5.0 (4.2)	ND	D	34.2 (4.0)
S4	9.3 (0.4)	ND	5.5 (5.7)	ND	D	ND	4.6 (5.4)	11.3 (7.9)	ND	ND	4.3 (6.8)	22.0 (4.3)	5.0 (7.8)	D	ND	81.4 (8.4)
S5	34.6 (4.9)	2.3 (0.2)	100.0 (11.8)	17.8 (3.2)	90.0 (1.7)	ND	9.1 (2.6)	85.6 (10.6)	ND	ND	D	51.0 (2.2)	ND	D	ND	D>LDR
S6	ND	ND	D	ND	ND	ND	ND	ND	ND	ND	ND	10.7 (1.6)	D	ND	ND	35.4 (2.3)
S7	13.7 (9.6)	ND	ND	ND	ND	ND	3.0 (2.0)	ND	ND	ND	ND	ND	D	4.7 (1.9)	ND	18.2 (6.3)
S8	ND	4.8 (11.9)	D	D	D	D	8.0 (1.4)	22.3 (3.6)	ND	ND	4.9 (1.6)	25.0 (6.7)	9.7 (4.5)	ND	ND	6.3 (12.3)
S9	8.3 (10.8)	ND	D	ND	ND	ND	ND	ND	ND	ND	ND	ND	D	D	ND	12.4 (5.4)
S10	25.1 (1.3)	ND	ND	ND	ND	ND	8.2 (2.8)	ND	12.0 (2.9)	D	30.3 (1.8)	D	9.1 (3.1)	7.5 (9.2)	ND	15.4 (11.3)

D: detected (> LOD but <LOQ); ND: non detected (< LOD)

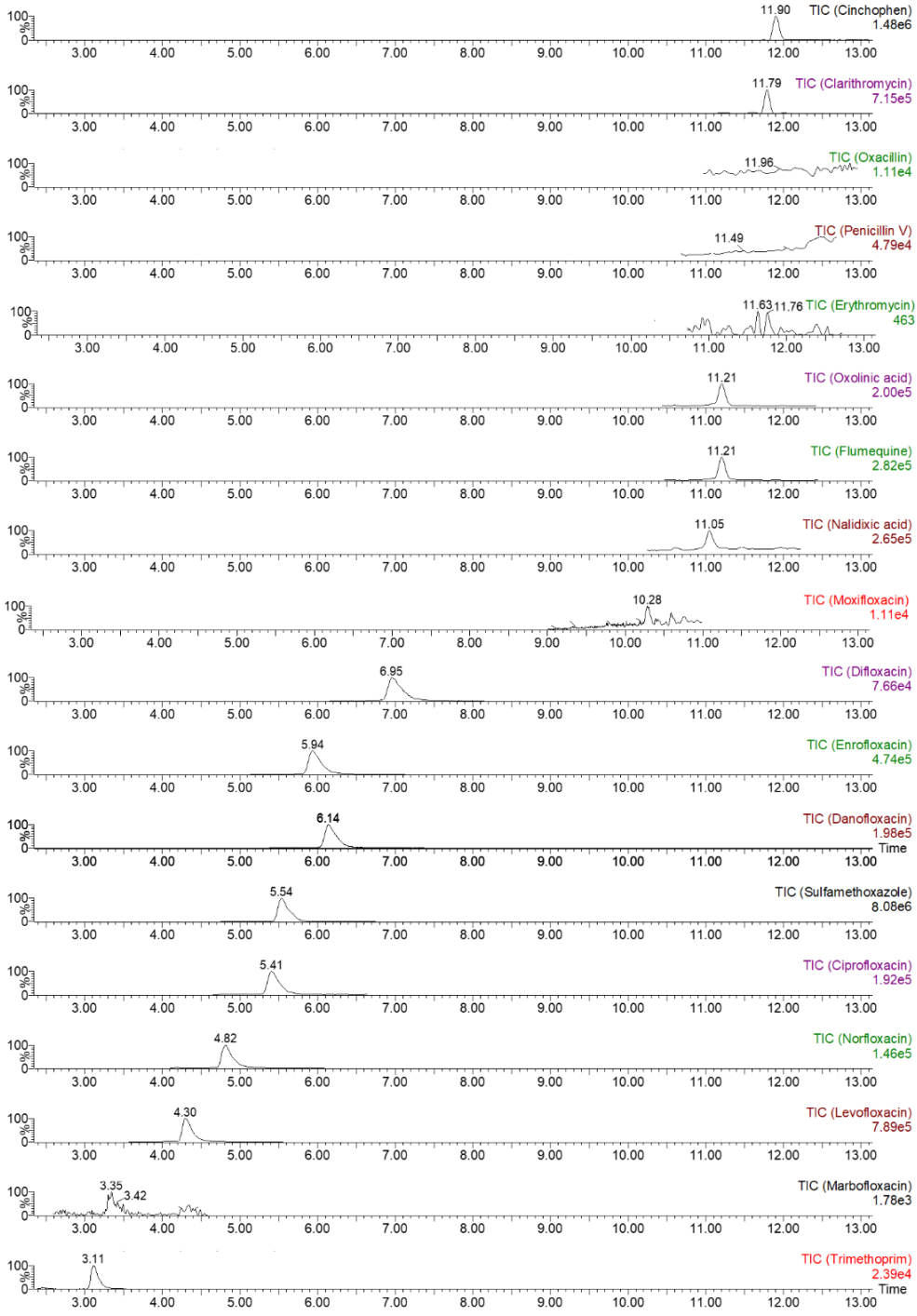


Figure 5. *Chromatogram of a contaminated sample (volunteer 5). MRM of two transitions in ESI+ mode*

Among the results obtained, it should be noted that all the antibiotics studied were detected in at least one of the samples. The most common antibiotic was SMX, which was detected in 100% of the volunteers at concentrations between 6.3 ng g^{-1} and above the upper limit of the linear dynamic range studied in two cases. On the other hand, NOR and OXA were detected in 80% (8/10) of the samples and quantified in 70% and 60% respectively, in a concentration range of 10.7-85.0 ng g^{-1} for NOR and 5.0-9.7 ng g^{-1} for OXA, followed by the quinolones CIP and FLU, detected in 70% of the samples (7/10) and quantified in 50% at concentrations ranging from 8.3-34.6 ng g^{-1} for the former and 3.0-9.1 ng g^{-1} for the latter. DAN, NAL and OXO were detected in 6/10 volunteers, and quantified in 40% of cases or less. Concentrations ranged from 5.5-100 ng g^{-1} for DAN and 4.3-49 ng g^{-1} for NAL, while OXO was found in notably lower concentrations (4.7-5.5 ng g^{-1}). Finally, the rest of the target analytes were detected in 50% or less of the samples and at low concentrations or even below the detection limit, although in the case of LEVO and ENR two samples were found with concentrations of 85.6 and 90.0 ng g^{-1}, which coincidentally corresponded to volunteer number 5, which turned out to be the subject with the highest concentration of practically all the antibiotics studied and who was known from her medical history to be undergoing numerous antibiotic treatments as part of her cancer treatment.

From the results obtained, it has been possible to formulate some observations. SMX was found in all samples, but in contrast, TMP was only detected in 50% of the subjects, despite the fact that they are antibiotics that are used together due to their synergistic effects. This suggests that TMP is metabolized more and/or accumulates less in the body. The fact that SMX is the most common antibiotic in nails is not surprising, as it (along with TMP) is widely used to treat highly prevalent infections and pneumonias. They are also considered first-line antibiotics in veterinary medicine, so the route of exposure for humans is not only through direct consumption, but their use in animal care may also contribute to their presence [25]. This is also the case for some quinolones widely used in the veterinary field, such as DAN, DIF, ENR, FLU, MAR,

NOR and OXO, which have also been detected but at lower concentrations. This is consistent with the intended use, since in the case of quinolones, the European Medicines Agency (EMA) classifies them in category B, meaning that they are relevant in human medicine and should be limited and used only when no other antibiotic is effective, to reduce health risks [25]. It is worth mentioning the kinship relationships of some of the participants: volunteers 1 and 2 are father and daughter, 3 and 6 are sisters, and 8 and 9 are fathers. There seems to be no apparent relationship between the presence of antibiotics or their concentration with the parentage, age, or sex of the volunteers, which is to be expected since the main exposure to these compounds is through prescription consumption. Further research should be conducted with a significant population sample to investigate this aspect.

Information to date on the presence of antibiotics in human nails is limited, and although other studies have considered hair in addition to nails due to their similarity in structural composition, this field also needs to be studied further. Alves et al. used these matrices to evaluate the presence of organophosphorus flame retardants, demonstrating that the concentrations found were higher in nails than in hair [26]. Although the nail is a matrix used in toxicology and for monitoring illegal drug use, its use is not as widespread in biomonitoring exposure. In this regard, there is a gap in the scientific literature. Several compounds have been studied in nails. For example, Kuwayma et al. used the nail for the evaluation of an antihistamine [14], Liu et al. focused on perfluoroalkyl compounds [27] and Lemos et al. investigated on methadone, an illicit drug [28]. Other studies have evaluated the presence in the nail of compounds present in personal care products with endocrine disrupting behaviour, such as triclosan and triclocarban [29], bisphenols and parabens [22] and UV filters [30], demonstrating that these compounds bioaccumulated in the nail during its growth and were found in higher concentrations than in other biological matrices. Li et al. investigated uric acid in nails and found that the concentration in men was higher than in women and propose this matrix as an alternative for non-invasive assessment of systemic diseases [31].

All these studies have shown that human fingernail as a matrix is a good biomarker of exposure, but their results are not comparable with those of the present study because they are different compounds. However, they all share an important idea that nail as a matrix has numerous advantages, such as allowing assessment over a long period of time, providing retrospective information on the consumption of certain compounds and facilitating sampling (as it is non-invasive), transport and storage [27,32].

Antibiotics have been extensively studied and a significant amount of research has been directed towards biomonitoring their exposure by analysing other biological samples. By far the most studied sample has been urine (non-invasive and easy to collect), but being a method of excretion in the body, it allows measuring short-term exposure, unlike fingernails, which could be a reservoir. In addition, some of these studies have tried to link antibiotic exposure to some diseases such as altered gut microbiota [33,34], obesity, mental disorders, allergic diseases [35] and mental health [36], reflecting the concern about exposure to these compounds and the harm they may cause over a medium to long period of time, and the importance of biomonitoring when exposure is prolonged, either through drugs or dietary intake.

As proposals for the future, further research on nails is needed to obtain a more general view of this matrix and to have more experience with the analysis. First, the variety of substances analyzed is still limited, as for pharmaceuticals only sedatives have been studied [37]. Second, in order to assess the accumulation of antibiotics in the human body considering a single route of entry (oral), investigations on drug consumption profiles in volunteers or even in patients with established treatments should be performed to know the behavioural profile and to perform case-control investigations. In general, more studies using nails as a matrix are therefore needed to reach more solid conclusions about their applications.

4. Conclusions

A multiclass UHPLC-MS/MS method for the analysis of antibiotics in human nail samples has been optimized and validated. The proposed method has allowed the simultaneous determination of 17 antibiotics belonging to different families. To our knowledge, no previous study has analyzed antibiotic accumulation in nail samples. Two extraction techniques were optimized and compared, and the results showed that with MAE superior efficacy and viability were obtained. The MAE extraction procedure consisted of two extraction cycles with methanol at 88 ºC for 1 min. The analytical performance parameters were good in terms of linearity, sensitivity, selectivity and accuracy (trueness and precision), in accordance with FDA guidelines for bioanalytical methods. Finally, the method was applied to samples obtained from 10 volunteers. The results showed that SMX appeared in all samples, while the other antibiotics under study were detected in at least one of the samples analyzed. The high frequency of antibiotic detection demonstrates the risk to human health and the threat posed by the development of bacterial resistance. The presence of antibiotics in our volunteers can be considered a serious fact, since to our knowledge; many of them had not consumed antibiotics in the last 2 years. The present study reveals the bioaccumulation of these compounds in the human body and the suitability of nails as biomarkers of exposure as they are deposited through the bloodstream into the matrix which acts as a reservoir. Their use for future research in biomonitoring of emerging contaminants in the human body and exposure is of great interest.

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

References

[1] I. Martínez-Alcalá, J. Soto, A. Lahora, Antibióticos como contaminantes emergentes. Riesgo exotoxicológico y control en aguas residuales, Ecosistemas 29 (2020) 2070.

[2] P. Vaudin, C. Augé, N. Just, S. Mhaouty-Kodja, S. Mortaud, D. Pillon, When pharmaceutical drugs become environmental pollutants: Potential neural effects and underlying mechanisms, Environ. Res. 205 (2022) 112495.

[3] B. Schwaiger, J. König, C. Lesueur, Development and validation of a multiclass UHPLC-MS/MS method for determination of antibiotic residues in dairy products, Food Anal. Methods 11 (2018) 1417-1434.

[4] Z. Lu, F. Deng, R. He, L. Tan, X. Luo, X. Pan, Z. Yang, A pass-through solid-phase extraction clean-up method for the determination of 11 quinolone antibiotics in chicken meat and egg samples using ultra-performance liquid chromatography tandem mass spectrometry, Microchem. J. 151 (2019) 104213.

[5] J. Meléndez-Marmolejo, Y. García-Saavedra, V. Galván-Romero, L. Díaz de León-Martínez, K. Vargas-Berrones, J. Mejía-Saavedra, R. Flores Ramírez, Emerging contaminants. Environmental problems associated with antibiotic use. New detection and remediation techniques and legislative perspectives in Latin America, Rev. Salud ambient. 20 (2020) 53-61.

[6] U. Szymanska, M. Wiergowski, I. Soltyszewski, J. Kuzemko, G. Wiergowska, M.K. Wozniak, Presence of antibiotics in the aquatic environment in Europe and their analytical monitoring: Recent trends and perspectives, Microchem. J. 147 (2019) 729-740.

[7] M. Kang, J. Yang, S. Kim, J. Park, M. Kim, W. Park, Occurrence of antibiotic resistance genes and multidrug-resistant bacteria during wastewater treatment processes. Sci. Tot. Environ. 811 (2022) 152331.

[8] F.V. Mello, S.C. Cunha, F.H.S. Fogaça, M.B. Alonso, J.P.M. Torres, J.O. Fernandes, Occurrence of pharmaceuticals in seafood from two

Brazilian coastal areas: Implication for human risk assessment. Sci. Tot. Environ. 803 (2022) 149744.

[9] K. He, B. Xue, X. Yang, S. Wang, C. Li, X. Zhang, C. Zhao, X. Wang, Z. Qiu, Z. Shen, J. Wang, Low-concentration of trichloromethane and dichloroacetonitrile promote the plasmid-mediated horizontal transfer of antibiotic resistance genes, J. Hazard. Mater. 425 (2022) 128030.

[10] A. Saghafi, R.S. Khoshnood, D.S. Khoshnoud, Z. Eshaghi, Preconcentration and determination of four antibiotics in biological samples using nanofluid-assisted magnetic dispersive micro-solid-phase extraction coupled with high-performance liquid chromatography, Chem. Pap. 76 (2022) 901-911.

[11] J. Angerer, U. Ewers, M. Wilhelm, Human biomonitoring: State of the art, Int. J. Hyg. Environ. Health 210 (2007) 201-228.

[12] A. Pena, S. Duarte, A.M.P.T. Pereira, L.J.G. Silva, C.S.M Laranjeiro, M. Oliveira, C. Lino, S. Morais, Human biomonitoring of selected hazardous compounds in Portugal: Part I- Lessons learned on Polycyclic Aromatic Hydrocarbons, metals, metalloids, and pesticides, Molecules 27 (2022) 242.

[13] M. Cabo-Golpe, A. de-Castro-Ríos, A. Cruz, M. Páramo, M. López-Rivadulla, E. Lendoiro, Determination of antipsychotic drugs in nails and hair by liquid chromatography tandem mass spectrometry and evaluation of their incorporation into keratinized matrices, J. Pharm. Biomed. Anal. 189 (2020) 113443.

[14] K. Kuwayma, H. Miyaguchi, T. Kanamori, K. Tsujikawa, T. Yamamuro, H. Segawa, Y. Okada, Y.T. Iwata, Measurement of three-dimensional distributions of drugs in nails using liquid chromatography/tandem mass spectrometry after micro-segmentation to elucidate drug uptake routes, Anal. Chim. Acta 1108 (2020) 89-97.

[15] M. Shen, H. Chen, P. Xiang, Determination of opiates in human fingernail-Comparison to hair, J. Chromatogr. B 967 (2014) 84-89.

[16] J. Robin, G. Binson, M. Albouy, A. Sauvaget, P. Pierre-Eugène, V. Migeot, A. Dupuis, N. Venisse, Analytical method for the

biomonitoring of bisphenols and parabens by liquid chromatography coupled to tandem mass spectrometry in human hair, Ecotoxicol. Environ. Saf. 243 (2022) 113986.

[17] N. Van Elsué, M. Yegles, Influence of cosmetic hair treatments on cannabinoids in hair: Bleaching, perming and permanent coloring, Forensic Sci. Int. 297 (2019) 270-276.

[18] I. Shu, J. Jones, M. Jones, D. Lewis, A. Negrusz, Detection of drugs in nails: Three-year experience, J. Anal. Tox. 39 (2015) 624-628.

[19] M. Cabo-Golpe, A. de-Castro-Ríos, A. Cruz, M. Páramo, M. López-Rivadulla, E. Lendoiro, Determination of antidepressants and benzodiazepines in paired hair and nail samples, Forensic Sci. Int. 326 (2021) 110935.

[20] D. Soleymani, S. Zargari, A. Faghihi-Zarandi, Separation and determination of mercury from nail and hair in petrochemical workers based on silver carbon nanotubes by microwave-assisted headspace sorbent trap. Anal. Method Environ. Chem. J. 3(2) (2020) 21-33.

[21] Z. Zeng, Y. Gao, J. Cui, M. Lin, J. Tang, F. Wang, Y. Yang, Y. Yu, S. Ma, Liquid-liquid extraction combined with online cleanup for the simultaneous determination of PAHs by GC-MS/MS and their hydroxylated metabolites by LC-MS/MS in human fingernails. J. Chromat. B 1188 (2022) 123057.

[22] L. Martín-Pozo, S. Cantarero-Malagón, F. Hidalgo, A. Navalón, A. Zafra-Gómez, Determination of endocrine disrupting chemicals in human nails using an alkaline digestion prior to ultra-high performance liquid chromatography-tandem mass spectrometry. Talanta 208 (2020) 120429.

[23] J. Zhe Min, Y. Morota, Y-Z. Jiang, G. Li, D. Jin, D. Kang, H-F. Yu, K. Inoue, K. Todoroki, T. Toyo'oka, Rapid and sensitive determination of diacetylpolyamines in human fingernails by ultraperformance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Eur. J. Mass Spectrom. 20 (2014) 477-486.

[24] FDA/CDER (Food and Drug Administration/Center for Drug Evaluation and Research), Guidance for industry, Bioanalytical

method validation, 2013. https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm68107.pdf (accessed on February 2023).

[25] EMA (European Medicines Agency), 2019. Clasificación de los antibióticos para uso en animales para un uso prudente y responsable.https://www.ema.europa.eu/en/documents/report/infographic categorisation-antibiotics-use-animals-prudent-responsible-use es.pdf (Accessed on February 2023).

[26] A. Alves, A. Covaci, S. Voorspoels, Method development for assessing the human exposure to organophosphate flame retardants in hair and nails. Chemosphere 168 (2016) 692-698.

[27] W. Liu, L. Xu, Y.H. Jin, K. Sasaki, N. Saito, I. Sato, S. Tsuda, Human nails analysis as biomarker of exposure to perfluoroalkyl compounds. Environ. Sci. Tech. 45 (2011) 8144-8150.

[28] N.P. Lemos, R.A. Anderson, J.R. Robertson, The analysis of methadone in nail clippings from patients in a methadone-maintenance program. J. Anal. Toxicol. 24 (2000) 656-660.

[29] Y. Shi, X. Liu, J. Zhang, B. Shao, Analysis of triclosan and triclocarban in human nails using isotopic dilution liquid chromatography-tandem mass spectrometry. J. Chromatogr. B 934 (2013) 97-101.

[30] L. Martín-Pozo, M.C. Gómez-Regalado, S. Cantarero-Malagón, A. Navalón, A. Zafra-Gómez, Determination of ultraviolet filters in human nails using an acid sample digestion followed by ultra-high performance liquid chromatography-mass spectrometry analysis. Chemosphere 273 (2021) 128603.

[31] X-L. Li, G. Li, Y-Z. Jiang, D. Kang, C.H. Jin, Q. Shi, T. Jin, K. Inoue, K. Todoroki, T. Toyo'oka, J.Z. Min, Human nails metabolite analysis: A rapid and simple method for quantification of uric acid in human fingernail by high-performance liquid chromatography with UV-detection. J. Chromat. B 1002 (2015) 394-398.

[32] D. Capelle, M. Yegles, H. Neels, A.L.N Van Nuijs, M. De Doncker, K. Maudens, A. Covaci, C.L. Crunelle, Nail analysis for the detection of

drugs of abuse and pharmaceuticals: a review. Forensic Toxicol. 33 (2015) 12-36.

[33] H. Wang, J. Yang, X. Yu, G. Zhao, Q. Zhao, N. Wang, Y. Jiang, F. Jiang, G. He, Y. Chen, Z. Zhou, Q. Jiang, Exposure of adults to antibiotics in a Shanghai suburban area and health risk assessment: A biomonitoring-based study. Environ. Sci. Technol. 52 (2018) 13942-13950.

[34] Y. Zhu, K. Liu, J. Zhang, X. Liu, L. Yang, R. Wei, S. Wang, D. Zhang, S. Xie, F. Tao, Antibiotic body burden of elderly Chinese population and health risk assessment: A human biomonitoring-based study. Environ. Pollut. 256 (2020) 113311.

[35] Y. Hu, Q. Zhu, Y. Wang, C. Liao, G. Jiang, A short review of human exposure to antibiotics based on urinary biomonitoring. Sci. Total Environ. 830 (2022) 154775.

[36] J. Zhang, K. Liu, L. Sun, L. Yang, X. Liu, Y. Zhu, R. Wei, Z. Jin, L. Wang, Y. Ma, S. Wang, A. Liu, F. Tao, Exposure to antibiotics and mental disorders in children: a community-based cross-sectional study. Environ. Geochem. Health 43 (2021) 3237-3253.

[37] R.C. Iriving, S.J. Dickson, The detection of sedatives in hair and nail samples using tandem LC-MS-MS. Forensic Sci. Int. 166 (2007) 58-67.

Table S2. *Experimental design 3^2 for MAE: Solvent volume and number of cycles*

Experiment	Solvent volume (mL)	Number of extraction cycles
1	2	2
2	2	2
3	2	2
4	2	1
5	1	1
6	3	2
7	2	3
8	3	1
9	1	3
10	2	2
11	1	2
12	3	3

Table S3. *Experimental design 3^2 for MAE: Temperature and extraction time*

Experiment	Temperature (°C)	Time (min)
1	85.0	15.5
2	85.0	15.5
3	85.0	15.5
4	150.0	30.0
5	20.0	15.5
6	20.0	1.0
7	85.0	30.0
8	150.0	1.0
9	85.0	1.0
10	150.0	15.5
11	85.0	15.5
12	20.0	30.0

Table S4. *Experimental design 3^2 for UAE: Amplitude and extraction time (I)*

Experiment	Amplitude (%)	Time (min)
1	40.0	15.5
2	40.0	15.5
3	40.0	15.5
4	10.0	1.0
5	70.0	30.0
6	10.0	15.5
7	70.0	1.0
8	40.0	30.0
9	70.0	15.5
10	40.0	15.5
11	40.0	1.0
12	10.0	30.0

Table S5. *Experimental design 3^2 for UAE: Amplitude and extraction time (II)*

Experiment	Amplitude (%)	Time (min)
1	40	40
2	40	40
3	40	40
4	40	20
5	40	60
6	10	60
7	70	40
8	40	40
9	10	40
10	10	20
11	70	20
12	70	60

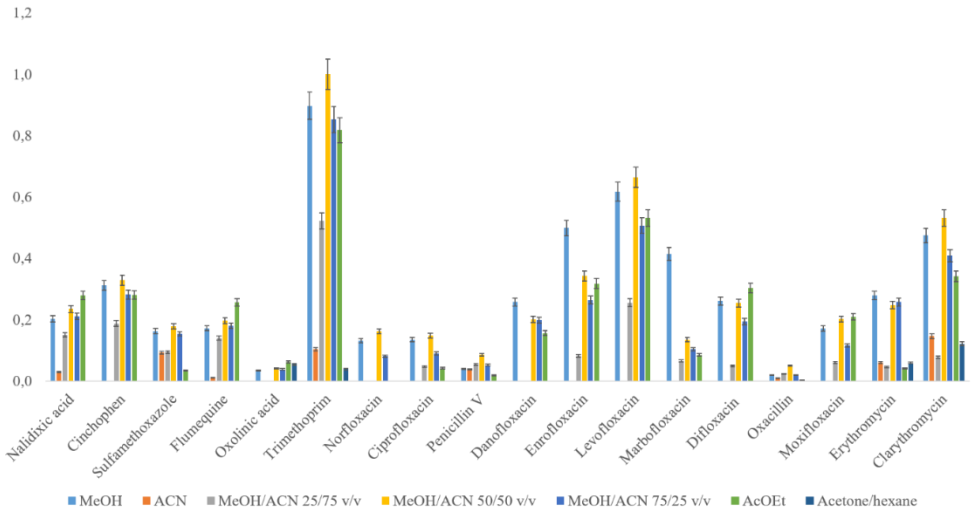

Figure S1. *Solvent test for MAE*

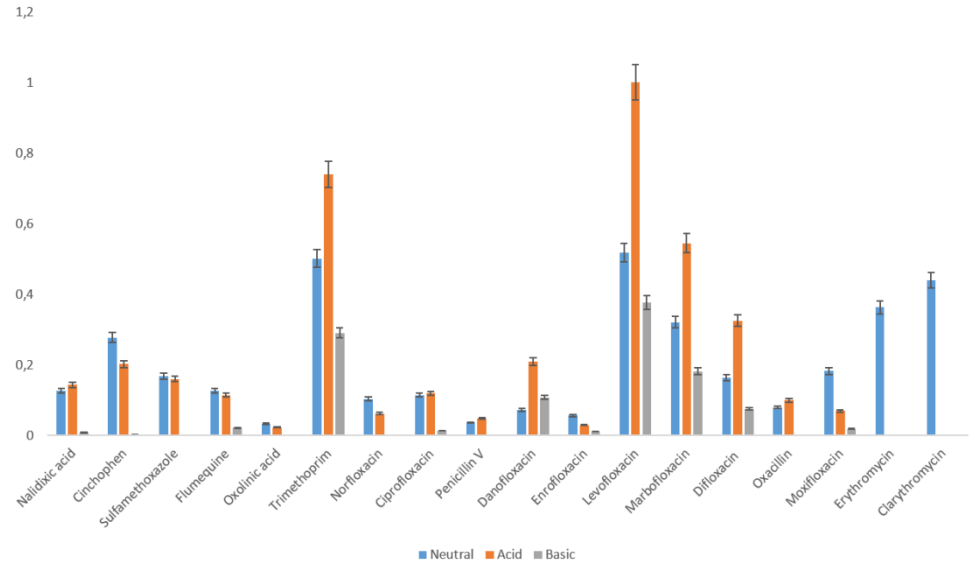

Figure S2. *Extraction medium test with MAE*

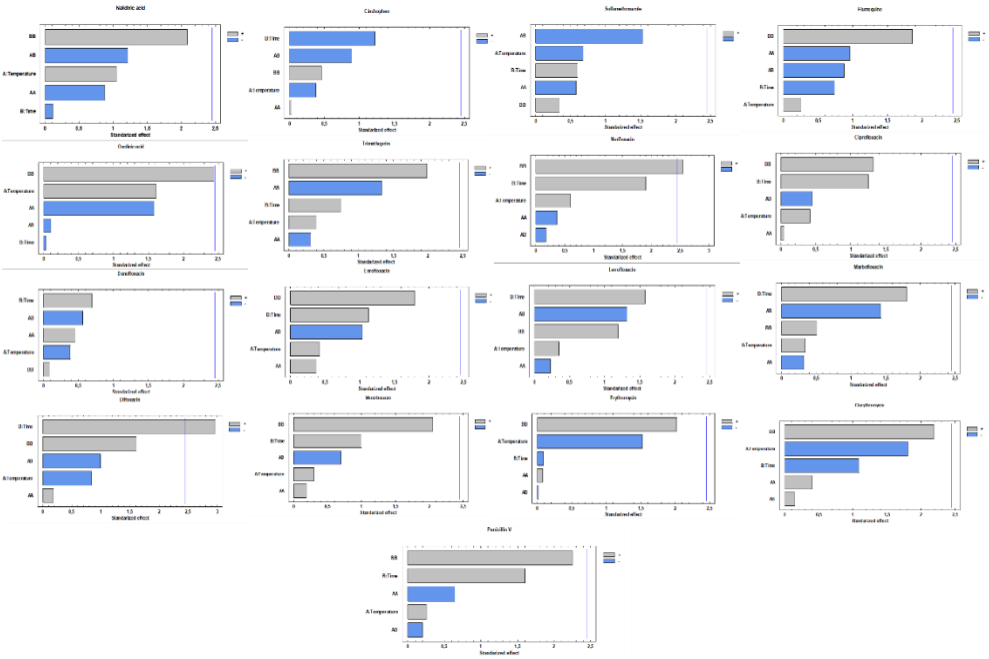

Figure S3. *Pareto Charts for 3^2 experiment (MAE): Time and temperature*

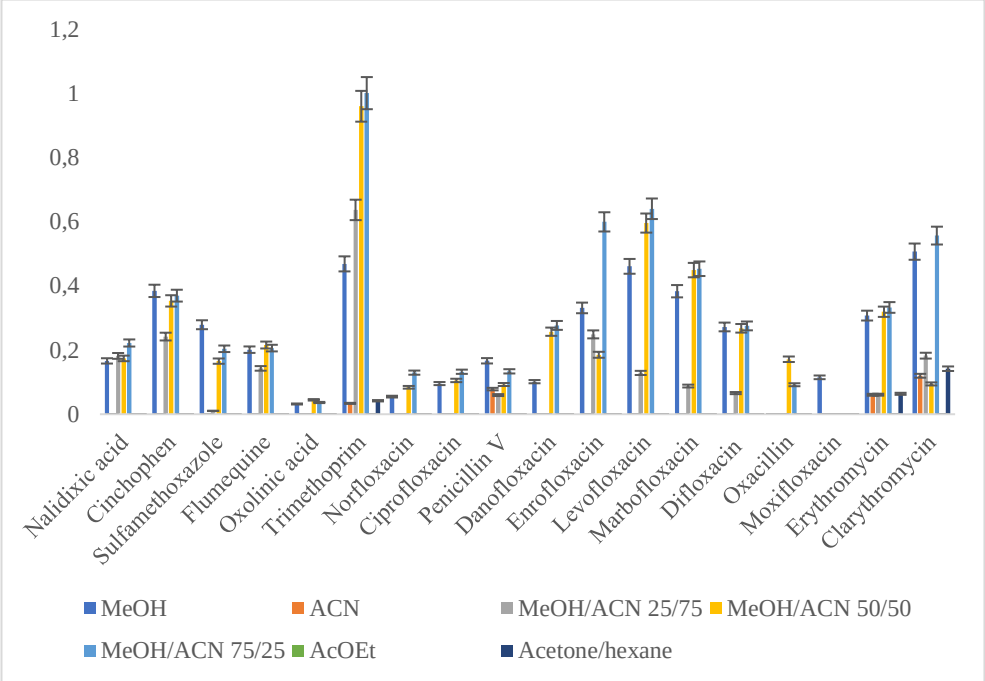

Figure S4. *Solvent test for UAE*

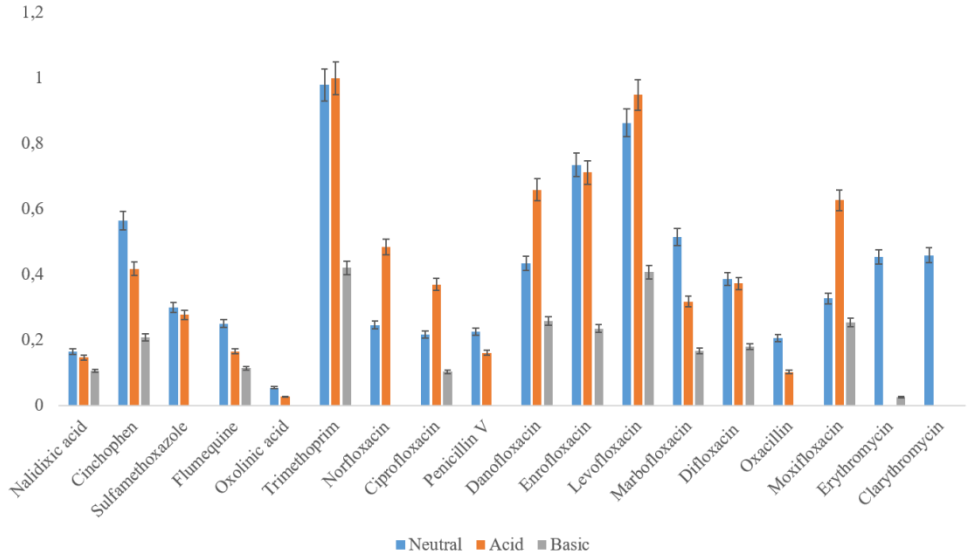

Figure S5. *Extraction medium test with UAE*

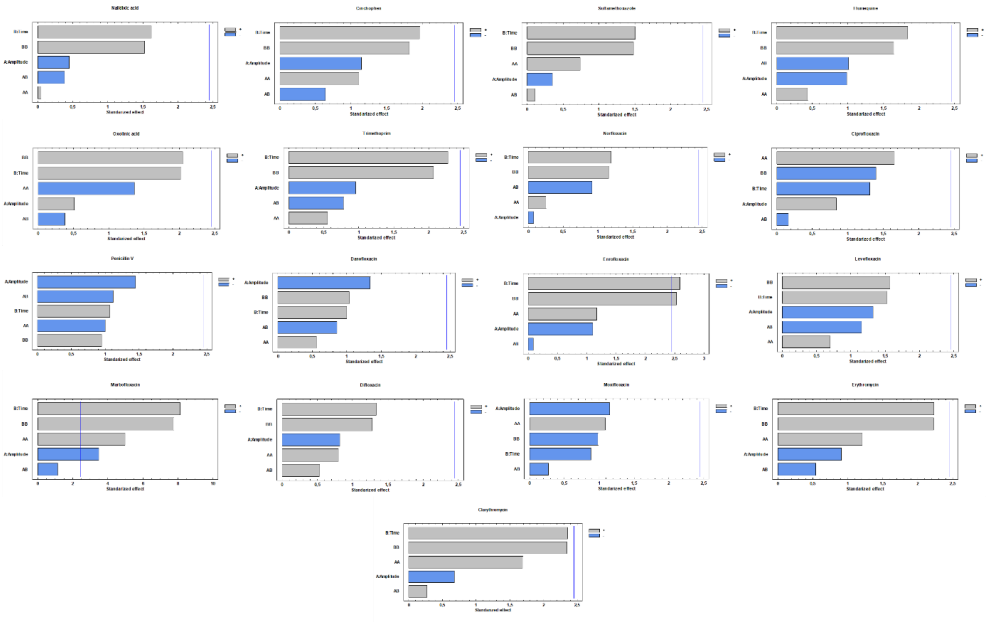

Figure S6. *Pareto Charts for 3^2 experiment (UAE): Time and amplitude*

Sección II. Bioacumulación/ bioconcentración de PhACs en bioindicadores marinos

Capítulo 6

Bioaccumulation/bioconcentration of pharmaceutical active compounds in aquatic organisms: Assessment and factors database

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Abstract: There is increasing evidence that the presence of certain pharmaceuticals in the environment leads to biota exposure and constitute a potential risk for ecosystems. Bioaccumulation is an essential focus of risk assessment to evaluate at what degree emerging contaminants are a hazard both to the environment and the individuals that inhabit it. The main goals of the present review are 1) to summarize and describe the research and factors that should be taken into account in the evaluation of bioaccumulation of pharmaceuticals in aquatic organisms; and 2) to provide a database and a critical review of the bioaccumulation/bioconcentration factors (BAF or BCF) of these compounds in organisms of different trophic levels.

Most studies fall into one of two categories: laboratory-scale absorption and purification tests or field studies and, to a lesser extent, large-scale, semi-natural system tests. Although in the last 5 years there has been considerable progress in this field, especially in species of fish and molluscs, research is still limited on other aquatic species like crustaceans or algae. This revision includes >230 bioconcentration factors (BCF) and >530 bioaccumulation factors (BAF), determined for 113 pharmaceuticals. The most commonly studied is the antidepressant group, followed by diclofenac and carbamazepine. There is currently no reported accumulation data on certain compounds, such as anti-cancer drugs. BCFs are highly influenced by experimental factors (notably the exposure level, time or temperature). Field BAFs are superior to laboratory BCFs, highlighting the importance of field studies for reliable assessments and in true environmental conditions. BAF data appears to be organ, species and compound-specific. The potential impact on food web transfer is also considered. Among different aquatic species, lower trophic levels and benthic organisms exhibit relatively higher uptake of these compounds.

Keywords: Emerging pollutants; biota; exposure; bioconcentration factor; bioaccumulation factor

1. Introduction

Pharmaceutical active compounds (PhACs) are a group of substances of emerging concern in the context of environmental risk assessment (Arguello-Pérez *et al.*, 2020; Mezzelani *et al.*, 2018). Their continuous discharge into aquatic environments has led the European Commission to include them in a watch list of emerging water pollutants, within the Water Framework Directive (EC 2020). This list (last updated in 2020) includes five PhACs (amoxicillin, ciprofloxacin, sulfamethoxazole, trimethoprim, and venlafaxine), as well as o-desmethylvenlafaxine, the main metabolite of venlafaxine. A recent water policy directive establishing a watch list of substances for Union-wide monitoring (dated July 2022) also includes ofloxacin.

The presence of PhACs in the environment can potentially influence both aquatic organisms and the ecosystem function (Chen *et al.*, 2021; Maculewicz *et al.*, 2021; Arguello-Pérez *et al.*, 2020; Mezzelani *et al.*, 2018). Organisms are exposed to PhACs through two main routes: waterborne (bioconcentration) and diet. The combined exposure is defined as bioaccumulation, the resulting discrepancy between the uptake and elimination processes (Arnot and Gobas, 2006). Bioaccumulation measurement is an essential part of risk assessment, to evaluate the scale that these emerging compounds may pose to the environment and their link with human populations (Ruan *et al.*, 2020; Lagesson *et al.*, 2016; Zenker *et al.*, 2014; Nendza *et al.*, 2018).

In a preliminary study conducted in Sweden in 2009, Wennmalm and Gunnarsson described that, of a large group of 300 PhACs, 92% were not biodegradable, 23% of them had bioaccumulation potential, and 61% were classified as toxic for aquatic organisms. Bioaccumulation potential was estimated according to the PhACs' lipophilicity, following the OECD criteria (Organisation for Economic Co-operation and Development, OECD Guideline 315, 2008). Log K_{OW}>3 was interpreted as *"potential to bioaccumulate in aquatic organisms"*. In 2011, Howard and Muir (2011) rated 92 out of 275 PhACs commonly detected in the environment as

potentially bioaccumulative, using quantitative structure property relationships (QSPR).

Nevertheless, discrepancies between predicted and measured concentrations could be significant. In fact, some authors recently stated that for ionic compounds such as PhACs, relying only on the K_{ow} may lead to a degree of underestimation (Kowalska *et al.*, 2021). The potential of bioaccumulation of PhACs is typically determined using two factors: Bioaccumulation factor (BAF) and Bioconcentration factor (BCF), the difference between them being that BCFs are studied in laboratory conditions and exclude dietary intake.

Duarte *et al.* (2022) observed that lipophilicity is not a good predictor of the BCF of a group of neuroactive PhACs in fish, which in turn is highly influenced by experimental parameters (species, life stages and abiotic conditions). Bioaccumulation tests using field studies appear to best reflect environmental conditions, some of the disadvantages being time-consumption and cost.

The availability of quality information is essential to improve accuracy and reduce uncertainty in hazard and risk assessments. Until the present, no criteria have been proposed to evaluate the quality of accumulation assays and, as such, there is a lack of uniformity in the reported studies. In this paper, the accumulation potential of PhACs in aquatic organisms is reviewed and compared. First, the different performed assays to determine bioaccumulation potential, and the experimental conditions they applied, are described. A wide database on BCF/BAF values has been collected and the main variables affecting the data have been considered. Finally, on the basis of the obtained results, the potential impact on food web transfer has been contemplated.

2. Parameters

2.1. Bioconcentration factor (BCF)

Bioconcentration is the process by which a substance is absorbed by an organism from the environment via the respiratory and dermal routes.

Dietary intake is not included. BCF is measured in laboratory conditions and is expressed as:

$$BCF_{SS} = \frac{C_b}{C_w}$$

C_b is the concentration of a chemical in the biota and C_w is the concentration of a chemical in the water at steady-state (SS). Note that guidelines suggest that, for a test to be valid, the concentration of the test substance in the tanks during the uptake phase is maintained within ± 20% of the mean of the measured values. Moreover, the guidelines recommend that the whole body of the organism be used for the process. For special purposes, when the organism is large enough (such as certain species of fish) specific tissues or organs (muscle, kidney, brain or liver) may be used.

Other parameters used to characterize bioaccumulation potential are the absorption rate constant (k_1), the depuration rate constant (k_2) and the bioconcentration kinetic factor (BCF_K):

$$\frac{dC_b}{dt} = k_1 C_w - k_2 C_b$$

$$\frac{dC_b}{dt} = -k_2 C_b$$

C_b is the concentration in biota (ng g^{-1}) ($C_{b,0}$ is the concentration when the depuration phase begins), t the time of exposure (h), k_1 the first-order uptake constant (L kg^{-1} h^{-1}), C_w the concentration in water (µg L^{-1}), and k_2 the first-order elimination rate constant (1 h^{-1}). Assuming a negligible concentration in biota samples at t_0, and considering its constant in the exposure medium, the equations can be expressed as:

$$C_b(\text{uptake}) = \frac{k_1}{k_2} C_w (1 - e^{-k_2 t})$$

$$C_b(\text{depuration}) = C_{b,0} e^{-k_2 t})$$

$$BCF_k = \frac{k_1}{k_2}$$

2.2. Bioaccumulation factor

Bioaccumulation is the process by which a substance is absorbed by the organism, taking into account all exposure pathways, as occurs in the natural environment (dietary and environmental sources). BAF is determined under field conditions and is calculated as:

$$\mathrm{BAF} = \frac{\mathrm{C_b}}{\mathrm{C_w}}$$

C_b is the concentration in biota and C_w is the total concentration in the water phase. BAF measurements presuppose that organisms are at or near SS with the ambient water (acknowledging that natural environments are dynamic and highly variable).

Another bioaccumulation field measurement is the biota-sediment accumulation factor (*BSAF*), calculated as:

$$\mathrm{BSAF} = \frac{\mathrm{C_b}}{\mathrm{C_s}}$$

C_b is the concentration in biota (ng g^{-1}) and $C_{s\,\mathrm{is}}$ the concentration in the surrounding sediments (ng g^{-1}).

2.3. Biomagnification factor (BMF)

Biomagnification is the process by which the thermodynamic activity of the substance in the organism exceeds that of its diet. It can be determined both in the field as well as in laboratory feeding experiments, and is calculated as follows:

$$\mathrm{BMF} = \frac{\mathrm{C_b}}{\mathrm{C_d}}$$

C_b is the chemical concentration in an organism and C_d is the chemical concentration in its diet at SS (when there are no significant differences in PhAC concentration in the organism over three sequential sampling periods).

Trophic Magnification Factor (*TMF*) is the diet-weighted average BMF of a chemical across food webs. TMFs are typically determined from

field measurements and are calculated from the slope of a log-normal regression of chemical residues in organisms, as per their trophic levels:

$$\log [\text{contaminant}] = b\ (\text{Trophic position}) + a$$

$$TFM = 10^{b}$$

The BMF and the TMF describe changes across one or more trophic level, respectively (Burkhard *et al.*, 2013).

3. Experimental designs

3.1. Laboratory test

3.1.1 Acclimation period

Prior to laboratory testing, the organism is acclimatized over a period of 2 to 3 weeks (OECD 305 technical guidance). Uncontaminated water from natural sources is generally used for testing, to ensure specimen survival with no abnormal appearance or behaviour. The baseline laboratory controls include water temperature, pH, dissolved oxygen, lighting type and characteristics, calcium, ammonium, nitrite, alkalinity and salinity (for marine species). The OECD establishes dissolved oxygen values of ≥ 60%. Water temperature depends on the fish or organism species (for most a temperature between 20-25ºC is established, with some species needing lower temperatures (e.g., *Oncorhynchus mykiss* or *Rainbow trout*: 13-17ºC; *Gasterosteus aculeatus* and three-spined stickleback: 18-20ºC). Temperature variation throughout testing should be less than ± 2°C, since larger deviations can affect biological parameters relevant for uptake and depuration, as well as cause organism stress. The pH value should be within the 6.0 to 8.5 range at test initiation. According to some researchers, the use of synthetic water (demineralized water with specific added nutrients) may result more suitable to guarantee uniformity over time. For instance, the composition of fresh river water (ISO 73463) can be prepared using $CaCl_2$ (220.5 mg L^{-1}), $NaHCO_3$ (63 mg L^{-1}), KCl (5.5 mg L^{-1}) and $MgSO_4$ (60.1 mg L^{-1}) in distilled water (Molina Fernández *et al.,* 2021).

There are also recommended guidelines for test organism feeding (1%-2% body weight (bw)/day of lipid and protein content). Food remnants should be removed after feeding to avoid chemical absorption since they could reduce bioavailability and provide a secondary dietary source (OECD 305 technical guidance). Once the acclimation period has ended, the test substance is mixed into the water to begin the assay. To prepare spiked solutions agitation is the preferred method for dissolving the studied substances. The use of organic solvents (acetone, ethanol, methanol, dimethylsulfoxide or acetonitrile) is not generally recommended. However, a maximum level (0.1 mL L^{-1}) is considered acceptable in the preparation of concentrated stock solutions. The solvent concentration must be reproducible in all treatments (OECD 305 technical guidance). Some authors have used a concentration of methanol up to 0.8 mg L^{-1} (Swiacka *et al.*, 2020).

An alternative is the use of a solid-phase desorption dosing system. For example, Maulvault *et al.* (2018) prepared venlafaxine-enriched feed. The spiked solution was previously dissolved in ethanol and diluted with water. The organic solvent was then volatilized by a top-coating process to the pellets with a pressurized spraying container. Regarding the concentration level of the test substance, the OECD establishes concentrations of 1% and 0.1% of its LC_{50} value.

3.1.2 Exposure test

The studied organisms are exposed to PhACs at one or more of the selected concentration levels. A control group (without the test substance) is subjected to identical laboratory conditions. The exposure time of the specimens to contaminants runs until SS is reached. Throughout exposure, samples (of both water and biota) are periodically collected. The concentration of the test substance in the water samples should be determined both prior the addition of the organisms and during the uptake phase. Throughout the test period the water should be sampled (N=5) from the test tanks (from a central point), before feeding, and at the same time the organism is sampled. More frequent sampling after the introduction of the organisms may be useful to ensure stable

concentrations. Organisms should be sampled on at least five occasions during the uptake phase. Exposure time and sampling frequency depend on the mode of action and the chemical-physical properties of the substance. For example, for different species of fish the exposure time can range from 4 days (ibuprofen in Rainbow trout (*O. mykiss*) (Brozinski et al., 2013) to 40 days (venlafaxine and its metabolite in Loach (*M. anguillicaudatus*) (Qu et al., 2019). In the case of larvae, bioconcentration experiments are developed for shorter periods, between 72-120 h post fecundation (Molina-Fernández *et al.*, 2021; Pan *et al.*, 2018). The duration of the test can be shortened or extended if necessary, if it is demonstrated as necessary as per when steady-state is reached. As for sampling, normally three replicates of the organism and at least five water samples are collected at each point in time. The medium is renewed weekly (Lopes *et al.*, 2022), or every 24 h (Gomez *et al.*, 2021; Maulvault *et al.*, 2018; Lu *et al.*, 2018), after which the substance concentration is re-established to maintain nominal values.

As mentioned earlier, aquatic organisms are exposed to chemicals via water uptake (bioconcentration), diet or both. The decision to conduct an aqueous or dietary exposure experiment should be based on water solubility and K_{ow} of the test substance. For compounds with high water solubility and log K_{ow} values between 1.5 and 6.0, as is the case for most PhACs, an aqueous exposure test should be considered first (OECD 305 technical guidance). To the best of our knowledge, only one study in the literature (Maulvault *et al.*, 2018) uses both water and dietary exposure sources (see Table 1).

3.1.3. Up-take and depuration test

This test includes two consecutive phases: exposure (uptake) and post-exposure (depuration). During the uptake phase, the organism is exposed to a chemical compound at one or more concentration levels, under the conditions described above. To initiate the depuration phase, the remaining organisms are transferred to clean water, then the same water renewal and sampling procedures as during the exposure period are followed. The depuration phase is always necessary unless the uptake

is negligible. Organisms should be sampled for the test substance on at least four occasions during this phase. This type of testing is more common in low trophic level organisms, such as molluscs.

3.1.4. Semi-natural large-scale system test

Long-term studies in a controlled aquatic system, evenly contaminated with a chemical mixture, are ideal for identifying general patterns of behaviour. Water, sediments and biota are sampled on a daily or weekly basis for a period of more than three months. This type of test is particularly successful in evaluating biomagnification along the food web, as well as bioaccumulation. However, this type of experiment is infrequent in the scientific literature.

3.2. Field studies

3.2.1. In-situ sampling collection

Currently, there are no criteria regarding field studies. Organisms are exposed throughout their lifetime, so the concentrations within them are near steady-state. Water, sediment and biota are sampled, and a range of sampling points is a key to ensure the representativeness of the data. The analytical rigor applied throughout the sampling and analysis processes is fundamental for the quality of the data. Some practices, such as the use of tags for fish labeling, has been proposed to study the changes in bioaccumulation over time under field conditions (Grabicova *et al.,* 2017). Other authors (de Solla *et al.,* 2016; Gillis *et al.,* 2014) have also field-deployed organisms (i.e., caged, previously sourced locally) in the aquatic environment over the course of several weeks. Furthermore, grab sampling conducted in conjunction with passive sampling may be a more reliable and less time-intense option for assessing relative time-weighted average spatial distribution of organic contaminant concentrations (Wilkinson *et al.*, 2018; Jones-Lepp *et al.*, 2012, Vystavna *et al.*, 2012). The use of passive sampling has been recommended as a method to mimic bioconcentration uptake without the use of live organisms. In the case of polar compounds such as PhACs, the use of Polar Organic Chemical Integrative Sampler (POCIS) has been developed. Grabicova *et al.* (2017)

concluded that integrative passive samplers with fish liver or kidney tissue can be complimentary exploratory tools and can help to distinguish between bioconcentration and bioaccumulation. The main inconvenience of this practice is the inaccessibility of the sampling rates for POCIS over long time periods.

A complex approach that includes not only water and organisms but also sediment interactions is needed to better understand the fate of PhACs in the aquatic environment. Water sediments represent a potential secondary source of PhACs when the hydrological conditions change (Wilkinson *et al.*, 2019; Koba et al., 2018). Moreover, some organisms (e.g. benthic invertebrates) are often exposed to contaminated sediment via ingestion of sediment particles. These organisms are highly important components of the food chain in aquatic environments and contribute significantly to fish diets. Usually, the sediments are collected in the same sampling point as the water samples. A few studies were recently published on this, for example Wilkinson *et al.* (2019) assessed the accumulation and spatial distribution of PhACs and other emerging pollutants in aquatic sediment and five under-studied organisms (periphyton, plants (*Callitriche* sp. and *Potamogeton* sp.), as well as amphipod crustaceans (*G. pulex*) and aquatic snails (B. tentaculate) (n=65 in total) from the Hogsmill, Blackwater and Bourne Rivers in Southern England. Koba *et al.* (2018) analysed 18 PhACs and 7 metabolites in water, sediment and fish of a treated wastewater-affected pond during a one-year experiment in the Czech Republic.

4. Analytical methods

Validated methods and quality assurance/quality control (QA/QC) protocols must be followed to ensure reliable and accurate results. A key aspect that generates uncertainty in the BAF/BCF calculation is the concentration of the compound in the water, since in many cases its value is close to the chosen method's determined detection limit. In these cases, the concentration is generally reported as "non-detected". The statistical treatment used to address "non-detected" samples can have significant effect on the derivation of the BAF.

In order to detect PhACs in the water, matrices between 50 to 1000 mL are often required. After filtration, solid phase extraction (SPE) is the most commonly applied technique. Given the nature of biota samples, a more complex sample preparation is required prior to analysis. In the case of fish, tissues and organs are first separated for individual analyses. In the case of molluscs, cephalopods and crustaceans, they are generally removed from the shells (if present) and then pooled, without differentiating body cavities. Samples are then powdered to homogeneity and, in most cases, freeze-dried. The sample preparation involves the extraction and clean-up steps. Ultrasonic solvent extraction (USE) is still widely applied. Pressurized liquid extraction (PLE), microwave assisted extraction (MAE), matrix solid phase extraction (MSPD) and Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) have also emerged in last few years. For clean-up dispersive-SPE (d-SPE), C18 and PSA sorbents are frequently used. Finally, for adequate identification and quantification of compounds, and in order to define the lower bound detection limits, liquid chromatography coupled with a tandem mass spectrometry detector (LC-MS/MS) is the most suitable technique (Arenas *et al.*, 2021; Álvarez-Ruiz and Picó, 2020; Miossec *et al.*, 2020).

The presence of interferences that co-elute with the test substance is the main drawback of these identification and quantification methods. The evaluation of matrix effects should be included in the methods' validation processes. In practice, the use of matrix-matched calibration curves, and isotopically-labelled internal standards for quantification purposes, are commonly used to reduce matrix effects.

5. Results and discussion

Table 1 summarizes the research found in the scientific literature dedicated to the evaluation of the bioaccumulation potential of PhACs in aquatic organisms.

This data has been obtained from over 100 scientific sources published between 2010 and 2022, with over 95% of the data generated in last 5 years. Information concerning the species, PhACs, concentration levels, acclimation period and experimental designs are included in the table.

Fish are the most commonly used organisms in bioaccumulation studies (n = 32), followed by molluscs (n = 18), crustaceans (n=8) and, finally, minor invertebrates (larvae) (n = 64) (Figure 1). Fish play a key role in the environment, regulating the biological structure of habitats as well as being an important food source for many other aquatic organisms. The most frequently studied species are the crucian carp (*Carassius auratus*), common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) and European perch (*Perca fluviatilis*). The preferred tissues or organs for BCF/BAF measurements were the muscle (35%), brain (18%), liver (15%), whole individual (9%), and other organs (20%) which include gills, gonad, kidney or bile. Molluscs, especially bivalves, are given particular attention in bioaccumulation studies, their sessile lifestyles and either filtering or deposit feeding making them of particular interest (Świacka *et al.*, 2021). In these cases the whole organism is commonly analysed.

Table 1. *Overview of accumulation studies on Pharmaceutically Active compounds (PhACs) in aquatic organisms*

Species name	PhACs	Experiment	Tanks/Aquariums	Acclimataion and conditions	Spiked level (ng mL^{-1})	Organism replicates/tank	Exposure conditions	Reference
Algae								
Macroalgae *Ulva*	Oxytetracyclin	Exposure	25 L glass tanks. Flasks were pre-filled with 244 mL natural filtered seawater	2 weeks of acclimatation in deionized water at 23.2 °C. Photoperiod set to 14:10 light-dark under white fluorescent light	10 and 120	3 replicates per sampling time, each replicate containing 3 algal disks	Sampling times: 0, 0.5, 1, 2, 4, 12, 24, 48, 72, 96 h	Rosa et al., 2019
Biofilm	39 PhACs and two metabolites (Analgesics/anti-inflammatories, antibiotics, antihelmintics, antiplatelet agent, antihypertensive, ß-blocking agents, histamine H1 and H2 receptor antagonists, lipid regulators, psychiatric drugs)	Field	Two lowland urban rivers in Argentine	-	-	5 sampling points/each river	Field study	Mastrángelo et al., 2022
Periphyton	Acetaminophen, diclofenac, 17alpha-ethinylestradiol	Field	Hogsmill River (Greater London), Chertsey Bourne River and the Blackwater River. Rivers received inputs from a total of five sewage treatment works effluent outfalls	-	-	4 samplimg point (N=8): 50 m upstream from effluent outfalls, 50 m downstream of respective STW effluent outfall as well as 250 m and 1000 m downstream from the outfalls. 1 day periphyton was seeded in each stream with samples collected from a regional reference stream. They were colonized on unglazed, ceramic tiles for two weeks prior to the start of this experiment.	Field study	Wilkinson et al., 2018
Periphyton	acetaminophen, caffeine, carbamazepine, diltiazem, diphenhydramine, fluoxetine, norfluoxetine, and sertraline	Mesocosm	BEAR Facility, with 12 outdoor mesocosms with a mixing tank (~378 L), followed by a riffle section, a run or glide section and bottom pool (~378 L), located at the Lake Waco Wetlands, Texas, USA	The flow of water originated from a large holding tank that delivered water to all 12 streams. A controlled volume of water (100 mL/min) was permanently removed from the bottom pool with an overflow drain, while the remaining water flow (~50 L/min) was recirculated to the mixing tank. Periphyton was colonized on unglazed, ceramic tiles for two weeks prior to the start of this experiment.	-		8-day study	Burket et al., 2020
Periphyton	Acetaminophen, amitriptyline, aripiprazole, benzoylecgonine, buprenorphine, caffeine, carbamazepine, diclofenac, diltiazem, diphenhydramine, duloxetine, fluoxetine, methylphenidate, norfluoxetine, promethazine, sertraline	Field	semi-arid urban river influenced by snowmelt sited in East Canyon Creek in Park City, Utah, USA	-	-	3 sampling campaings of 3 days each, including 5 sampling point	Field study	Haddad et al., 2018

Plants								
Macrophyte (*Lemna gibba)*	39 PhACs and two metabolites (analgesics/anti-inflammatories, antibiotics, antihelmintics, antihypertensive, antiplatelet agent, ß-blocking agents, histamine H1 and H2 receptor antagonists, lipid regulators, psychiatric drugs)	Field	Two lowland urban rivers in Argentine.	-	-	5 sampling points in each river	Field study	Mastrángelo et al., 2022
Acer platanoides	Sertrailine and fluoxetine	Exposure	2 constructed aquatic food chains of 3 trophic levels (40 L)	10 d of acclimatation (12:12-h light:dark and 20°C)	10	One and a half grams of freeze-dried leaves. All trophic levels were exposed together within 1 replicate	7 days exposure	Boström et al., 2017
Cattail Typha angustifolia	Caffeine, carbamazepine, ibuprofen, fluoxetine	Field	Lorong Halus Wetland, located in north-eastern Singapore	-	-	3 sampling point	Field study	Wang et al., 2020
Callitriche sp., Potamogeton sp.	Acetaminophen, diclofenac, 17α -ethinylestradiol	Field	Hogsmill River (Greater London), Chertsey Bourne River and the Blackwater River. Rivers received inputs from a total of five sewage treatment works effluent outfalls	-	-	4 samplimg point (N=7,8): 50 m upstream from effluent outfalls, 50 m downstream of respective STW effluent outfall as well as 250 m and 1000 m downstream from the outfalls. 1 day	Field study	Wilkinson et al., 2018
Larvae								
Damselfly larvae, mayfly larvae	Diphenhydramine, oxazepam, trimethoprim, diclofenac, and hydroxyzine	Semi-natural large-scale system	Semi-natural pond 400 m2 (40 × 10 m), with a mean depth of 1.3 m	The pond (pH = 7.2) has no connection to anthropogenically surface waters. The inflow of water comes from rain and ground water	0.4	Individual numbers for each species per sampling were 10 Zygoptera, 20 Planorbidae, 30 Asellus, 30 Ephemeroptera	Sampling was carried out on a daily to weekly basis over a period of 66 days in total. Predators in the system were European perch (Perca fluviatilis) feeding on fish, zooplankton, and benthic macroinvertebrates, and damselfly larvae (Zygoptera: Coenagrion) preying on zooplankton and benthic macroinvertebrates	Lagesson et al., 2016
Damselfly larvae (Zygoptera)	Hydroxyzine and fexofenadine	Exposure	Aquaria (25 × 25 × 8 cm)	1.2 L aged tap water WITH coordinate grid (1 × 1 cm) drawn on the bottom	1.5 and 2.0	n = 10 for Fexofenadine and n = 12 for Hydroxyzine	7 days exposure	Jonsson et al., 2014

Dragonfly larvae (*Sympetrum* sp.)	Temazepan	Exposure	Plastic containers (8×8×8 cm) filled with 200 mL of aged tap water, at different T (10 or 20 °C) and PhAC concentration	48 h of acclimation period being fed with live zooplankton. Larvae were transferred to climate-controlled rooms and the temperature was slowly shifted towards 10 or 20 °C	0.5-5	20 per treatment (two dosis and two temperature + control)	Larvae were exposed for 8 days in an individual static exposure scenario	Cerveny et al., 2021b
Larvae	Diclofenac, ibuprofen, diphenhydramine, gemfibrozil	Exposure	15 microcosms (aquaria of 30 × 20 × 15 cm) with 3 L of water each, and equal amounts of sand (10 tablespoons), stones (3 stones >10 cm and 10 stones 2–5 cm)	20 days of acclimatation in dechlorinated tap water at 9.3 °C. Temperature was increased 0.1 °C every 15 days. Constant oxygen levels	0.5	2 tank/day	4 microcosms controls. 11 exposed to a mixture of PhACs, all randomly placed in 3 incubators, over a 65-day period. The volume of water was kept constant by adding fresh dechlorinated tap water and the concentration of each compound was kept pseudoconstant. Sampled 4 times (day 0, 21, 35, 65). Also with field studies	Previsic et al., 2021
Zebrafish (*Danio rerio*)	Fluoxetine, sertraline, citalopram, paroxetine, norfluoxetine, norsertraline, and desmethylcitalopram	Exposure	-	Simulated river water. Dissolved $O_2 \geq 60$ %, 27°C and pH 7.8. Exposure medium refreshed every 24 h	80 and 300	3 by tank and day	Eleutheroembryos are obtained 72 h post fecundation and then exposed to PhACs and collected at different times (0, 24, 45, 48 h)	Molina-Fernández et al., 2021
Zebrafish (*Danio rerio*)	Fluoxetine	Exposure	6-well plates	Fresh egg water. pH 6.8-7.2; 14:10 h light:dark cycle. 28 °C. Exposure medium refreshed every 24 h	0, 0.1, 1, 10, 100, 1000	3 by tank and day	Zebrafish embryos exposed to fluoxetine from 4 h post-fertilization until 120 hpf	Pan et al., 2018
Worm								
Aquatic worm (*Phagocata vitta*)	Diclofenac, ibuprofen, 1-OH-ibuprofen, piroxicam, propyphenazone, sulfamethoxazole, diltiazem, verapamil, norverapamil, bezafibrate, hydrochlorothiazide, gemfibrozil, parvastatin, acridone, carbamazepine, 10,11-epoxy-carbamazepine, 2-OH-carbamazepine, citalopram, fluoxetine, paroxetine, venlafaxine, azaperone, dexamethasone,	Field	Segre River (Ebro River basin, NE Iberian Peninsula)	-	-	125 individuals of Ancylus fluviatilis, 90 individuals of Hydropsyche sp., and 70 individuals of Phagocata vitta	Field study	Ruhí et al., 2016

	metoprolol, propanolol							
Mollusks								
Mollusks (*Atrina pectinate Linnaeus, Meretrix lusoria, Trisidos kiyoni, and Crassostrea rivularis Gould*)	37 antibiotics: 13 sulfonamides , 5 tetracyclines, 10 fluoroquinolones, 6 macrolides and 3 ionophores	Field	Six marine aquaculture farms at the Hailing Island	-	-	14 fish, 504 shrimp, 4 crabs, 11 shellfish, 5 oyster	Field study	Chen et al., 2015
Cultured shellfish (*Ostrea gigas, Mimachlamys nobilis, Mytilus eduli*, and *Bufonaria perelegans*)	Ciprofloxacin, ofloxacin, norfloxacin, flumequine, Sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, penicillin G, sulfamethazine, sulfamethoxazole, trimethoprim, tetracycline, cefotaxime, oxytetracycline, isochlortetracycline, spectinomycin, roxithromycin, erythromycin, clarithromycin, chloramphenicol, paracetamol, naproxen, ibuprofen, ketoprofen, diclofenac acid, carbamazepine, diltiazem, diphenhydramine, gemfibrozil	Field	Pearl River Delta (Five mariculture sites around)	-	-	N= 459	Field study	Xie et al., 2019
Mussel (*Mytilus trossulus*)	Diclofenac	Exposure-depuration test	Three tanks (glass aquariums) with 15 L (one as control)	3 weeks of acclimatation in artificial sea water at 8-10°C. The water was changed and mussels were fed every 3 days	133	80 individuals in each tank with a sampling of N= 5 by tank and day	Mussels exposed to diclofenac for 5 days, following a 5-day depuration phase	Świacka et al., 2019
Mussel (Mytilus trossulus)	Diclofenac	Exposure-depuration test	Nine tanks (glass aquariums) with 15 L (three as control)	3 weeks of acclimatation in artificial sea water at 5-8°C. The water was changed and mussels were fed every four days	4 and 40	73 individuals by tankwith a sampling of N= 4 by tank and day	25-day experiment: exposure (12d)/depuration (13d). At the beginning of the depuration phase, the glass tanks were refilled with artificial sea water. During the exposure to diclofenac 4 individuals were taken from all tanks every 4d, and at the end of depuration	Świacka et al., 2020

Mussel (*Mytilus trossulus*)	Diclofenac and 4OH-diclofenac	Exposure	Twelve tanks (glass aquariums) with 15 L (three as control)	2 weeks of acclimatation in artificial sea water at 10°C, pH 8.9. Mussels were kept in the dark and fed	20 (diclofenac) and 68 (4OH diclofenac	10 per tank	7 days experiment. During experiment mussels were fed twice (day 0 and 4). On the last 7th day of the experiment, mussel were collected	Świacka et al., 2021
Mussel (*Mytilus galloprovinciali s*	Diclofenac, Etoricoxib and caffeine	Exposure-depuration test	Three tanks (160 L) : control group, the group exposed to the pollutants mixture and the group exposed to pollutants and microplastics	2 weeks of acclimatation in seawater purified using sand filters and UV (salinity 35 ppt, 18 °C, pH 8.0, O_2 > 80% and a 12 h day/night cycle	10 and food (10 ng per specimen and day)	83 per tank	58 days of experiment separated in two stages: 1) exposure stage(days 0–28) when mussels were exposed to contaminants 2) Depuration satge (days 29-58) the tanks were emptied, rinsed and filled with clean water	Álavarez Ruiz et al., 2021
Mussel (*Mytilus galloprovinciali s*	Carbamazepine and 10-hydroxy-10,11- dihydro-carbamazepine	Exposure-depuration test	3 aquaria and 1 control (1.5 L)	1 week of acclimatation in filtered seawater (18°C, 14:10 h light:dark cycle, continuously aerated and renewed daily). Mussels were fed once a day with Tetraselmis suecica	1, 10, 100	18 per tank and 6 in control	7 days of exposure	Boillot et al., 2015
Mussel (*Lasmigona costata*)	Amitriptyline, 10-HO-amitriptyline, amlodipine, amphetamine, anhydrotetracycline, azithromycin, betamethasone, cefotaxime, citalopram, clarithromycin, clotrimazole, cocaine, codeine, desmethyldiltiazem, diltiazem, 1,7-dimethylxanthine, diphenhydramine, enrofloxacin, erythromycin, fluoxetine, gemfibrozil, glyburide, hydrocortisone, iopamidol, metformin, miconazole, minocycline, naproxen, norfluoxetine, norverapamil, oxolinic acid, oxycodone, paroxetine, propranolol, sarafloxacin, sertraline, theophylline, venlafaxine, verapamil, warfarin	Field	Grand River, Ontario, receiving wastewater effluent	-	-	Two sampling point (upstream and downstream) in 1 day. Caged and wild mussels each	Field study	de Solla et al., 2016

Mussel (*Mytilus galloprovinciali s*)	17 α-ethinylestradiol (EE2)	Exposure	Three tanks for each treatmern	2 weeks of acclimatation in artificial sea water: 17°C, pH 8.0, salinity 30. 12 h light / 12 h dark photoperiod with constant aeration. Frequent water renewal. Mussels fed every 2d	0.005, 0.025, 0.125 and 0.625	18 per treatment	28 days of exposure period. Mussels exposed to each concentration were maintained under two temperatures, 17 and 21°C. Weekly, the exposure medium was renewed, after which EE2 concentration was re-established	Lopes et al., 2022
Mussel (*Mytilus galloprovinciali s*)	Venlafaxine	Exposure-depuration test	Four tanks (one as control) of 1.5 L glass aquaria	7 days of acclimatation at 18 °C with a 14:10 h L/D photoperiod	10	18 per tank	7 days exposure phase followed by a 7-day depuration period. The water was almost entirely removed every 24 h and replaced, and venlafaxine was added to reach a 10 µg/L nominal concentration. Organisms were collected at days 0, 1, 3, 7, 8, 10, and 13	Gómez et al., 2021
Mussel (*Mytilus galloprovinciali s*)	Sotalol, sulfamethoxazole, venlafaxine, carbamazepine and citalopram	Exposure and depuration test	Ten tanks	1 week of acclimatation period in sea water at 18 °C, pH 8.00, dissolved oxygen >5 mg/L, salinity = 35‰ and photoperiod of 12 h light and 12 h dark (12L:12D). Mussels were fed three times per day and 25% of water was renewed	Up to 15.7	1 control and 4 treatmenst: two temperature and two pH (50 animals per tank)	20 days of exposure and 20 days of depuration. Sampling at days, 0, 2, 10, 20 (exposure) 22, 30 and 40 (depuration).	Serra-Compte et al., 2018
Clam (*Ruditapes philippinarum)*	17α-ethinylestradiol	Exposure test	Ten tanks (3 L) (at different concentration and two temperature (17 and 21°C)	10 days of acclimatation in artificial seawater (salinity 30, under continuous aeration, 17C and a photoperiod of 12:12 h (light/dark). Clams were fed every 2–3 days. Seawater was renewed every 2–3 days	0, 0.005, 0.025, 0.125 and 0.625	6 individuals per container and three containers per treatment (a total of eighteen individuals/treatment)	28 d of exposure period. The water was renewed once a week after which EE2 concentrations were re-established.	Silva et al., 2022
Clam (*Corbicula fluminea*)	acetaminophen, caffeine, carbamazepine, diltiazem, diphenhydramine, fluoxetine, norfluoxetine, and sertraline	Mesocosm study	BEAR Facility, with 12 outdoor mesocosms with a mixing tank (~378 L), followed by a riffle section, a run or glide section and bottom pool (~378	The flow of water originated from a large holding tank that delivered water to all 12 streams. A controlled volume of water (100 mL/min) was permanently removed from the bottom pool with an overflow drain,	-	3 randomly selected clams were collected from trays in each stream (N = 12)	8-day study. Sampling on days 0, 1, 3 and 8.	Burket et al., 2020

			L), located at the Lake Waco Wetlands, Texas, USA	while the remaining water flow (~50 L/min) was recirculated to the mixing tank. Clam trays were placed at the begin- ning of the run section in each stream				
Clam pen shell	Hydrochlorothiazide	Field	Mar Menor lagoon	-	-	9 sampling points	Field study	Moreno-González et al., 2016
Clam (*Crassostrea gigas, Patinopecten yessoensis and Chlamys farreri*)	Antibiotics (14 sulfonamides, two chloramphenicols and four tetracyclines)	Field	Coastal environment of Dalian (China)	-	-	A total of 20 seawater, 20 sediment and 13 biota samples	Field study	Na et al., 2013
Clam (*Ancylus fluviatilis*)	Diclofenac, ibuprofen, 1-OH-ibuprofen, piroxicam, propyphenazone, sulfamethoxazole, diltiazem, norverapamil, hydrochlorothiazide, verapamil, bezafibrate, gemfibrozil, parvastatin, carbamazepine, acridone, 10,11-epoxy-carbamazepine, 2-OH-carbamazepine, citalopram, fluoxetine, paroxetine, venlafaxine, azaperone, dexamethasone, metoprolol, propanolol	Field	Segre River (Ebro River basin, NE Iberian Peninsula)	-	-	125 individuals of Ancylus fluviatilis, 90 individuals of Hydropsyche sp., and 70 individuals of Phagocata vitta	Field study	Ruhí et al., 2016
Ramshorn snail	Diphenhydramine, oxazepam, trimethoprim, diclofenac, and hydroxyzine	Semi-natural large-scale system	Semi-natural pond 400 m^2 (40 × 10 m), with a mean depth of 1.3 m	The pond (pH = 7.2) has no connection to anthropogenically surface waters. The inflow of water comes from rain and ground water	0.4	Individual numbers for each species per sampling were 10 Zygoptera, 20 Planorbidae, 30 Asellus, and 30 Ephemeroptera	Sampling daily to weekly. Period: 66 days in total. Predators in the system were European perch (Perca fluviatilis) feeding on fish, zooplankton, and benthic macroinvertebrates, and damselfly larvae (Zygoptera: Coenagrion) preying on zooplankton and benthic macroinvertebrates	Lagesson et al., 2016
Snails (*Lymnaeidea & Physidae*)	Acetaminophen, amitriptyline, aripiprazole, benzoylecgonine, buprenorphine, caffeine, carbamazepine, diclofenac,	Field	Semi-arid urban river influenced by snowmelt sited in East Canyon Creek	-	-	3ampling campaings of 3 days each and including five sampling point	Field study	Haddad et al., 2018

	diltiazem, diphenhydramine, duloxetine, fluoxetine, methylphenidate, norfluoxetine, promethazine, sertraline		in Park City, Utah, USA					
Crustaceans								
Crabs (*Calappa philargius*)	37 antibiotics: 13 sulfonamides , 5 tetracyclines, 10 fluoroquinolones, 6 macrolides and 3 ionophores	Field	Marine aquaculture farms	-	-	14 fish, 504 shrimp, 4 crabs, 11 shellfish, 5 oyster	Field study	Chen et al., 2015
Waterlouse	Diphenhydramine, oxazepam, trimethoprim, diclofenac, and hydroxyzine	Semi-natural large-scale system	Semi-natural pond 400 m^2 (40 × 10 m), with a mean depth of 1.3 m	The pond (pH = 7.2) has no connection to anthropogenically surface waters. The inflow of water comes from rain and ground water	0.4	Individual numbers for each species per sampling were 10 Zygoptera, 20 Planorbidae, 30 Asellus, and 30 Ephemeroptera	Sampling: daily to weekly. Period: 66 days in total. Predators in the system were European perch (Perca fluviatilis) feeding on fish, zooplankton, and benthic macroinvertebrates, and damselfly larvae (Zygoptera: Coenagrion) preying on zooplankton and benthic macroinvertebrates	Lagesson et al., 2016
Waterlouse (*Asellus aquaticus*)	sertrailine and fluoxetine	Exposure	2 constructed aquatic food chains of 3 trophic levels (40 L)	10 d of acclimatation (12:12-h light:dark and 20°C)	10	N= 270 *A. aquaticus*. All trophic levels were exposed together within 1 replicate	7 days exposure	Boström et al., 2017
D. Magna	roxithromycin and propranolol	Exposure and depuration	500 mL glass beakers in dark	Artificial freshwater. The culture medium (at 22°C with a light/dark cycle of 16 h/8 h) was renewed three times each week, and the daphnia was fed daily	5 and 100	Approximately 100 adult daphnids (21–28 days old) were placed in each beaker	24 h uptake phase followed by a 24 h depuration phase. Time points of 0, 3, 6, 12 and 24 h. Then, the remaining daphnia were transferred into clean water for the depuration test	Ding et al., 2016
Shrimps (*Gammarus pulex*)	Acetaminophen, diclofenac, 17alpha-ethinylestradiol	Field	Hogsmill River (Greater London), Chertsey Bourne River and the Blackwater River. Rivers received inputs from a total	-	-	4 samplimg point (N=10): 50 m upstream from effluent outfalls, 50 m downstream of respective STW effluent outfall as well as 250 m and 1000 m downstream from the	Field study	Wilkinson et al., 2018

			of five sewage treatment works effluent outfalls			outfalls. 1 day		
Shrimps (*Gammarus pulex*)	Moclobemide, 5-fluoruracil, carbamazepine, diazepam, carvedilol, fluoxetine	Exposure and depuration	5 L tank	water collected from Bishop Wilton Beck. All species were kept at c. 20 °C under a natural light regime and were fed	0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 mmol/L	three replicates of 12 animals	48 h exposure phase followed by a 48 h depuration phase (extended to 72 h fluoxetine and carvedilol. Sampling 0, 3, 6, 12, 24 and 48 h. In depuration stage the organisms were transferred into clean water. The same time points were used in the depuration phase.	Meredith-Williams et al., 2012
Planorbarius corneus	Moclobemide, 5-fluoruracil, carbamazepine, diazepam, carvedilol, fluoxetine	Exposure and depuration	15 L tank	Artificial pond water. All species were kept at c. 20 °C under a natural light regime and were fed	0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 mmol/L	three replicates of 12 animals	Studies consisted of a 48 h exposure phase followed by a 48 h depuration phase (extended to 72 h fluoxetine and carvedilol. Sampling 0, 3, 6, 12, 24 and 48 h. In depuration stage the organisms were transferred into clean water. The same time points were used in the depuration phase.	Meredith-Williams et al., 2012
Shrimps (*Fenneropenaeus penicillatus*)	37 antibiotics: 13 sulfonamides , 5 tetracyclines, 10 fluoroquinolones, 6 macrolides and 3 ionophores	Field	Marine aquaculture farms	-	-	14 fish, 504 shrimp, 4 crabs, 11 shellfish, 5 oyster	Field study	Chen et al., 2015
Insects								
Hydropsyche sp	Diclofenac, ibuprofen, 1-OH-ibuprofen, piroxicam, propyphenazone, sulfamethoxazole, diltiazem, verapamil, norverapamil, bezafibrate, hydrochlorothiazide, gemfibrozil, parvastatin, carbamazepine, acridone, 10,11-epoxy-carbamazepine, 2-OH-carbamazepine, citalopram, fluoxetine, paroxetine, venlafaxine, azaperone, dexamethasone, metoprolol, propanolol	Field	Segre River (Ebro River basin, NE Iberian Peninsula)	-	-	125 individuals Ancylus fluviatilis, 90 Hydropsyche sp., 70 individuals Phagocata vitta	Field study	Ruhí et al., 2016
Aquatic snails (*Bithynia*	Acetaminophen, diclofenac, 17alpha-ethinylestradiol	Field	Hogsmill River (Greater London),	-	-	4 samplimg point (N=9): 50 m upstream from	Field study	Wilkinson et al., 2018

tentaculata)			Chertsey Bourne River and the Blackwater River. Rivers received inputs from a total of five sewage treatment works effluent outfalls			effluent outfalls, 50 m downstream of respective STW effluent outfall as well as 250 m and 1000 m downstream from the outfalls. 1 day		
Water boatman (*Notonecta glauca*)	Moclobemide, 5-fluoruracil, carbamazepine, diazepam, carvedilol, fluoxetine	Exposure and depuration	1.5 L aquarium in water	Artificial pond water. All species were kept at c. 20 °C under a natural light regime and were fed	0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 mmol/L	three replicates of 12 animals	48 h exposure phase followed by a 48 h depuration phase (extended to 72 h fluoxetine and carvedilol. Sampling 0, 3, 6, 12, 24 and 48 h. In depuration stage the organisms were transferred into clean water. The same time points were used in the depuration phase.	Meredith-Williams et al., 2012
Notonecta glauca	Sertrailine and fluoxetine	Exposure	2 constructed aquatic food chains of 3 trophic levels (40 L)	10 d of acclimatation (12:12-h light:dark and 20°C)	10	N= 4 *N. glauca*. All trophic levels were exposed together within 1 replicate	7 days exposure	Boström et al., 2017
Mayflies (*Ephemerella sp.*), crane fly (*Tipula sp.*), snails (Lymnaeidea & Physidae), and caddis fly (*Trichopterans: Helicopsyche sp., Hydropsyche sp.*)	Acetaminophen, amitriptyline, aripiprazole, benzoylecgonine, buprenorphine, caffeine, carbamazepine, diclofenac, diltiazem, diphenhydramine, duloxetine, fluoxetine, methylphenidate, norfluoxetine, promethazine, sertraline	Field	Semi-arid urban river influenced by snowmelt sited in East Canyon Creek in Park City, Utah, USA	-	-	3 sampling campaings of 3 days each and including 5 sampling point	Field study	Haddad et al., 2018
Fish								
14 fish species	NSAIDs (diclofenac, ibuprofen, ketorolac and naproxen)	Field	Coastal Lagoons (Central Mexican Pacific)	-	-	4 specimens per species	Field study	Argüello Pérez et al., 2020

European perch (*Perca fluviatilis*)	Temazepan and oxazepan	Exposure	Aquaria of 50 L were filled with 10 L of aged tap water, with each also being enriched with 1 L of water from the lake of larvae origin. Organic debris from the lakes was also added to provide shelter for individual larvae.	40-day acclimation period (14 °C, oxygen saturation: >100%, pH: 7.8–8.2, light:dark regime of 12:12 h). Fish were fed with frozen chironomid larvae daily, and with live zooplankton collected from a fishless local pond	0.2-2	14 exposure treatments with 15 individuals each under two separate temperature regimes (10 or 20 °C) and different concentration levels	Larvae were exposed for 8 days in an individual static exposure scenario	Cerveny et al., 2021b
Pimephales notatus and *Ictalurus punctatus*	Carbamazepine	Exposure and depuration	Tanks of 20 L	Fish were kept in 16:8 light/dark cycle at 25-21°C, pH 8.0 and O2 7.8 mg/L. Fish were fed	300	control tank and five exposure tanks. minnows (n=60) were randomly distributed	Minnows were exposed for 28 d and then moved into clean tanks containing only dilution water for a 14 d depuration period. In the 14 d CBZ BCF study, juvenile catfish were placed into four 80 L aquaria and exposed to a continuous 83 ug/L carbamazepine solution. Catfish were exposed to CBZ for 7 d and then moved into clean aquaria for a 7 d depuration phase	García et al., 2012
Oreochromis niloticus	Carbamazepine	Field	Pecan Creek Wastewater Reclamation Plant (PCWRP) in Denton, Texas	-	-	1 sampling date	-	García et al., 2012

Common carp (*Cyprinus carpio*) and pikeperch (*Sander lucioperca*), Stone moroko (*Pseudorasbora parva*)	66 PhACs (alfuzosin, amitriptyline, atenolol, atorvastatin, azithromycin, bezafibrate, biperiden, bisoprolol, carbamazepine (þits metabolites 10,11-trans- dihydrocarbamazepine, 10,11-dihydrocarbamazepine), cetirizine, cilazapril, citalopram (þN-desmethylcitalopram), clemastine, clin- damycin (þclindamycin sulfoxide), clonazepam, dicycloverine, diltiazem, donepezil, diphenhydramine, disopyramide, erythro- mycin, fenofibrate, fexofenadine, glibenclamide, halo- peridol, glimepiride, irbesartan, loperamide, maprotiline, meclozine, memantine, methamphetamine, metoprolol, mianserin, miconazole, mirtazapine, orphenadrine, oseltamivir carboxylate, oxazepam, oxcarbazepine, pizotifen, propranolol, ropinirole, roxithromycin, sertraline, sotalol, sulfaclozine, sulfamethazine, sulfamethizole, sulfamethoxazole (þN1-acetylsulfamethoxazole), telmisartan, terbinafine, terbutaline, tramadol, triamterene, trimethoprim, valsartan, venlafaxine (þO-desme- thylvenlafaxine) and verapamil	Field	Wastewater stabilization ponds (Czech Republic)	-	-	12 specimens from each fish species	After six-month exposure to reclaimed wastewater, fish were caught.	Grabicová et al., 2020
Stoneroller minnows (*Campostoma anomalum*)	acetaminophen, caffeine, carbamazepine, diltiazem, diphenhydramine, fluoxetine, norfluoxetine, and sertraline	Mesocosm study	BEAR Facility, with 12 outdoor mesocosms with a mixing tank (~378 L), followed by a riffle section, a run or glide section and bottom pool (~378 L), located at the Lake Waco Wet- lands, Texas, USA	The flow of water originated from a large holding tank that delivered water to all 12 streams. A controlled volume of water (100 mL/min) was permanently removed from the bottom pool with an overflow drain, while the remaining water flow (~50 L/min) was recirculated to the mixing tank. Stoneroller minnows were acclimated for 24 h in out- door storage tanks at the BEAR facility and transplanted to cages in the experimental streams. Fish cages were also placed in the run section of each stream	-	one fish sampled per stream (N = 12) on each sampling day	8-day study. Sampling on days 3 and 8.	Burket et al., 2020

Brown trout (*Salmo trutta* m. fario)	citalopram, clomipramine, haloperidol, hydroxyzine, levomepromazine, mianserin, mirtazapine, paroxetine, sertraline, tramadol and venlafaxine	Field	Zivny Stream (tributary of the Blanice River, the Czech Republic), which is a small stream highly affected by effluent from a sewage treatment plant	-	-	Trouts (N from 11 to 44) were sampled at 1, 3, 6 months in two sampling points. One point was also sampled at 18 months (N=2)	Field study	Grabicova et al., 2017
Rainbow trout (Oncorhynchus mykiss)	carbamazepine, diphenhydramine, diltiazem, and fluoxetine	Field	Effluent-dominated river influenced by snowmelt in East Canyon Creek in Park City, Utah, USA	-	-	In situ upstream and at incremental distances downstream (0.1, 1.4, 13 miles) with two 7-day studies in the Summer and Fall seasons	Fish were acclimated for 24 h before deployment and were not fed before or during the field campaign. Trout were caged in 25.4 cm PVC tubing with a diameter of 10.2 cm and mesh fiberglass. Mesh pore size allowed small aquatic invertebrates to enter cages. On study day 0, cages were deployed at each site with one fish per cage. At each site, triplicate samples were collected days 1, 3, and 7 (n = 3)	Sims et al., 2021
Common carps (*Cyprinus carpio* L.)	Diclofenac, tramadol, atenolol, irbesartan, metoprolol, cetirizine, fexofenadine, meclozine, clarytromycin, clindamycin, erythromicyn, sulfamethoxazole, carbamazepine, oxcarbamazepine, citalopram, methamphetamine, sertraline, and venlafaxine and their metabolites/transformation products metoprolol/atenolol acid, clindamycin sulfoxide, N4 -acetyl sulfamethoxazole, 10,11-epoxycarbamazepine, trans-10,11-dihydro- 10,11-dihydroxy carbamazepine, N-desmethylcitalopram, and O-desmethylvenlafaxine	Field	Cezarka pond (2.6 ha) designed to treat effluent from the Vodnany WWTP, in Czech Republic	-	-	Samplings were performed during 1 year (N=60)	One-thousand tagged carps were stocked in the pond. Twelve fish were caught during each sampling campaign	Koba et al., 2018
(*Pungitius pungitius*)	Sertrailine and fluoxetine	Exposure	2 constructed aquatic food chains of 3 trophic levels (40 L)	10 d of acclimatation (12:12-h light:dark and 20°C)	10	N= 1 *P. pungitius*. All trophic levels were exposed together within 1 replicate	7 days exposure	Boström et al., 2017

Caged goldfish (*Carassius auratus*), Wild carp (*Cyprinus carpio*)	Amitriptyline, benztropine, caffeine, citalopram, diazepam, diphenhydramine, erythromycin, flumequine, ofluoxetine, gemfibrozil, oibuprofen, iopamidol, sertraline, sulfamethazine, valsartan, venlafaxine	Field	Cootes Paradise Marsh, an urban wetland that receives tertiary treated municipal waste waters as well as urban storm runoff	-	-	The goldfish was caged for 21 days in 3 locations and also collected 1 day. Wild carp was collected in two days	fish (13/cage) were fed during the visits (20 g/ cage)	Muir et al., 2017
Fathead minnow and channel catfish	Verapamil and clozapine	Exposure and depuration	20-L and 60-L tanks for fathead minnow and catfish tests, respectively)	Acclimatation for 1 week under a 18:6-h light:dark photoperiod at 20°C in glass aquaria with continuously aerated, carbon-!ltered, dechlorinated tap water under flow- through conditions	500 and 40	For verapamil test, 50 fish (25 each in 2 exposure tanks). For clozapine BCF test, 60 fish (30 each in 2 exposure tanks)	28 days exposure and 14 days of depuration period. Sampling at 1, 3, 7, 14, and 28 days. After up take phase the fish were transferred to clean. Sampling at 35 and 42 days	Nallani et al., 2016
Bluegill sunfish (*Lepomis macrochirus*)	diclofenac, methocarbamol, rosuvastatin, sulfamethoxazole, and temazepam	Exposure and depuration	Flow-through system. Three exposure experiments	1 week of acclimatation. Fish were fed with commercial pellets at a rate of 1−2% bodyweight per day.	1-4		Kinetics of uptake (0−14 d) and elimination (14−28 d). Fish were fed every other day for the duration of the uptake exposure. Sampling days 0, 1, 2, 3, 5, 8, 13, 14 for uptake, and days 14, 15, 16, 17, 20, 22, 28 for elimination	Zhao et al., 2017
Hemiculter leucisculus, Carassius auratus	Oxithromycin, erythromycin and ketoconazole, ibuprofen and diclofenac, propranolol, carbamazepine, 17α-ethinylestradiol	Field	Downstream rivers of sewage treatment plants in Nanjing, China (four Rivers)	-	-	3 sampling point for water and 4 for fishes in 1day and each River	Field study	Liu et al., 2015
Gambusia affinis	Carbamazepine and atenolol	Field	5 L of aquarium water	Acclimatation 1 month in 100 L aerated glass aquaria, fed twice a day. One week before starting the bioassay, fish were acclimatized to 12:12 h light:dark, 21°C. Twenty-four hours before the test fish were randomly separated in 5 L aquaria and stopped feeding	10, 100, 1000	Each aquarium contained five individuals and two replicates were made for each of the five treatments	Exposure for 96 h. Test solutions were half renewed every day	Valdés et al., 2014
Rainbow trout (*Oncorhynchus mykiss*)	NSAID (Ibuprofen)	Exposure	Four tanks (500 L)	Fish were acclimatized changing non-chlorinated artesian well water (pH 7.6, temperature 9.9-11.6) for 1 week. The 0.5-year old fish were fed twice a day and 1-year old fish every other day. Feeding was stopped 3d	0.17, 1.9, 13 and 145	At each level, 9 troutsused	Exposure via intraperitoneal and water for 4 d. A periodic water replacement system was used, where 20% of the water was changed daily.	Brozinski et al., 2013

				before experiments			Analysis of ibuprofen and their metabolites in bile	
Juvenile meagre (*Argyrosomus regius*)	Venlafaxine	Exposure	27 tanks (50 L)	Tanks had independent functioning, being equipped with protein skimmer, UV disinfection, biological filtration and chemical filtration to maintain seawater quality. Furthermore, each tank had independent temperature and pH control	20 (for exposure via water) and 160 µg/kg dw for feed exposure (~4 times the values commonly found in species inhabiting contaminated coastal areas)	3 replicates × 9 treatments = 27 tanks; treatments randomly assigned to each tank/replicate.10 animals analysed for each treatment	Water and dietary exposure sources. Daily spike seawater during the 28 days of exposure. Study of the effcet of temperature (ΔT°C = +5 °C) and high CO2 levels (ΔpCO2 ~1000 µatm; equivalent to ΔpH = −0.4 units). By the end of exposure, behavioural tests were conducted in ten animals randomly selected out of the three replicate tanks composing each treatment.	Maulvault et al., 2018
Crucian carp (*Carassius auratus*)	Diclofenac	Exposure	Three water tanks	2 weeks of acclimatation in dechlorinated municipal water (20°C; pH, 7.2; dissolved O_2, 6.7 mg/L; and total hardness, 119.7 mg/L $CaCO_3$). The photoperiod was a 12:12 h light:dark regime. The fish were fed every day	4, 20 and 100	3 tanks of three fish each were used for every exposure concentration and control group	Fish were exposed to increasing concentrations of diclofenax. The semistatic exposures were renewed every 24 h with 50% of water changes. Sampling at 7, 14, and 21 days	Lu et al., 2018

European perch (*Perca uviatilis*)	Benzodiazepine (temazepam)	Exposure-depuration test	Ten water aquariums (30 L)	2 weeks of acclimatation in aerated flow-through tank of non-chlorinated tap water (pH, 8.2; ammonium < 0.004 mg/L; nitrite < 0.003 mg/L; oxygen saturation, > 100%). The light/dark regime was set to 12/12 h. Fish were fed on a daily basis.	2	Seven individuals in each of 10 exposure tanks	Fish were exposed to 2 µg/L for 10 days. 50% of the water was renewed every 2nd day. 4 to 5 individuals sampled from different tanks at 6, 12, 24, 48, 96, 144, 192, and 240 h after the commencement of exposure. After 10 days of exposure, the remaining fish were transferred to clean water and the depuration period started with exactly the same design as the exposure period. Water samples were taken from 4 randomly selected tanks at 13 sampling points. Fish were fed on a daily basis during both periods	Cerveny et al., 2021a
Largemouth bass, White sucker, Yellow perch, Smallmouth bass	Carbamazepime, Hydrochlorothiazide	Field	164 urban rivers in the U.S.	-	-	Fish samples were collected at 542 randomly selected river locations in 48 states	Field study	Huerta et al., 2018
European perch	Diphenhydramine, oxazepam, trimethoprim, diclofenac, and hydroxyzine	Semi-natural large-scale system	Semi-natural pond 400 m2 (40 × 10 m), with a mean depth of 1.3 m	The pond (pH = 7.2) has no connection to anthropogenically surface waters. The inflow of water comes from rain and ground water	0.4	10 species per sampling. Zygoptera, 20 Planorbidae, 30 Asellus, 30 Ephemeroptera	Sampling was carried out on a daily to weekly basis over a period of 66 days in total. Predators in the system were European perch (Perca fluviatilis) feeding on fish, zooplankton, and benthic macroinvertebrates, and damselfly larvae (Zygoptera: Coenagrion) preying on zooplankton and benthic	Lagesson et al., 2016

							macroinvertebrates	
Sea trout (*Salmo trutta*)	Temazepam and irbesartan	Exposure	Three 400L flow-through tanks	Tanks supplied with river water from the Ume River and equipped with two airstones at 7-11°C, pH 4.5. Fish were fed daily until satiation	temazepam (0.08 and 1.5), irbesartan (0.2 and 20)	16 fish by tank	Exposure over 7 days. Sampling (N=4) after 8 h, 16 h, 1d, 2d, 3d, 4d, 5d, 6d, and 7d of exposure	McCallum et al., 2019
Golden grey mullet	Hydrochlorothiazide	Field	Mar Menor lagoon	-	-	8 sampling points	Field study	Moreno-Gonzalez et al., 2016
Crucian cap (*Carassius auratus*)	Fluoxetine	Exposure or Exposure + depuration	10 L glass tanks	Fresh egg water. pH 6.8-7.2; 14:10-h light:dark cycle at 28 C. Exposure medium was refreshed every 24 h	0.1, 1, 10, 100, 1000	3 tank and day	Long term: 30-day exposure. The test solutions were replaced every 24 h. Short term: 6 days exposure. Fish exposed for 6 days at 0.1 mg/L, followed by a 6-day recovery period (clean water), for a total of 12 days. The test solution and water were replaced every 24 h. Sampling on days 0, 3, 6, 9, and 12	Pan et al., 2018
Loach (*Misgurnus anguillicaudatus*)	Venlafaxine and its metabolite	Exposure	30-L glass tanks	4 weeks of acclimatation in deionized water at 25 °C. The water was renewed and the tanks were cleaned once a day. Each tank was equipped with an aeration stone.	500	-	Exposure experiments: 1) Loaches were exposed to rac-venlafaxine. 2) Loaches were exposed to rac-venlafaxine with microplastic. 3) Loaches exposed to only microplastic. Sampling on days 0, 0.5, 1, 3, 6, 10, 16, 25 and 40	Qu et al., 2019
Epinephelus awoara, Ephippus orbis, Culter alburnus. Shellfish: Ostrea gigas, Mimachlamys nobilis, Mytilus edulis, Bufonaria	Sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole, trimethoprim, sulfathiazole, sulfapyridine, ciprofloxacin, norfloxacin, ofloxacin,flumequine, tetracycline, oxytetracycline, isochlortetracycline, penicillin G sodium, cefotaxime sodium, spectinomycin, roxithromycin, erythromycin-H_2O, clarithromycin, chloramphenicol, paracetamol,	Field	Pearl River Delta (Five mariculture sites around)	-	-	N= 30	Field study	Xie et al., 2019

perelegans	naproxen, ibuprofen, ketoprofen, diclofenac acid, carbamazepine, diltiazem, diphenhydramine, gemfibrozil							
Crucian carp (*Carassius auratus*)	Diclofenac	Exposure	Three tanks per treatment	2 weeks acclimatation in dechlorinated tap water at 20 °C; 12 h:12 h light-dark cycle. pH, 6.9; dissolved O_2 6.8 mg/L; total hardness, 121.6 mg/L$CaCO_3$	1, 10, 100 and 1000	Each treatment was applied in triplicate glass tanks with 6 fish per tank.	Fish were exposed to Cu and diclofenac alone or in combination for 7 days. The fish were not fed throughout the experimental period. The exposure solutions were refreshed every 24 h.	Xie et al., 2020
Lutjanus russelli, *Lutjanus erythopterus* and *Trachinotus ovatus*	37 antibiotics: 13 sulfonamides , 5 tetracyclines, 10 fluoroquinolones, 6 macrolides and 3 ionophores	Field	Marine aquaculture farms	-	-	14 fish, 504 shrimp, 4 crabs, 11 shellfish, 5 oyster	Field study	Chen et al., 2015
Smallmouth bass (Micropterus dolomieu), largemouth bass (Micropterus salmoides), the exotic common rudd (Scardinius erythrophthal mus), rock bass (Ambloplites rupestris), white bass (Morone chrysops), white perch (Morone americana), walleye (Sander vitreus), bowfin (Amia calva), steelhead trout (Oncorhynchus mykiss), and yellow perch	Antidepressants	Field	Niagara River (North American Great lakes)	-	-	Locations of the two WWTPs relative to the Niagara River	Field study	Arnnok et al., 2017

(Perca flavescens).								
Cnesterodom decemmaculatus Jennyns and *Cyphocharax voga Hensel*	39 PhACs and two metabolites (Analgesics/anti-inflammatories, Antibiotics, Antihelmintics, Antihypertensive, Antiplatelet agent, B-blocking agents, Histamine H1 and H2 receptor antagonists, Lipid regulators, Psychiatric drugs)	Field	Two lowland urban rivers in Argentine.	-	-	Five sampling points in each river	Field study	Mastrángelo et al., 2022
Brown trout (*Salmo trutta*) and mottled sculpin (*Cottus bairdii*).	Acetaminophen, amitriptyline, aripiprazole, benzoylecgonine, buprenorphine, caffeine, carbamazepine, diclofenac, diltiazem, diphenhydramine, duloxetine, fluoxetine, methylphenidate, norfluoxetine, promethazine, sertraline	Field	Semi-arid urban river influenced by snowmelt sited in East Canyon Creek in Park City, Utah, USA	-	-	3 sampling campaings of three days each and including five sampling point	Field study	Haddad et al., 2018
Marine medaka (*Oryzias melastigma*)	Sulfamethazine	Exposure	35 L glass vessel	2 weeks of acclimatation in artificial seawater with dissolved O_2 6.0 0.2 mg/L and at 28 °C in a 14 h/10 h light/dark photoperiod cycle	40 and 200	Each treatment was applied in triplicate with four fish per tank.	Fish were exposed to sulfamethazine for 24 h and samples were collected at each time point (0, 1, 2, 7, 12 and 24 h). Fish was not fed during the exposure period	Zhao et al., 2016

Laboratory experiments were conducted by water exposure (33%) or by water uptake and purification phase (25%). Only one of them (Maulvault *et al.*, 2018) used both exposure sources (water and diet). The remaining 37% of the reviewed articles were field studies; the majority of them in freshwater. To the best of our knowledge, only two studies were semi-natural large-scale system tests (Largesson *et al.*, 2016; Burket *et al.*, 2020). Lagesson *et al.* (2016) selected a semi-natural pond of 400 m^2 to assess the extent at which PhACs are taken up by a vertebrate top consumer and four invertebrate species of an aquatic food web. Burket *et al.* (2020) used a municipal wastewater effluent as the source water in an outdoor stream mesocosm to simulate effluent-dependent lotic systems to examine the bioaccumulation of several widely-used PhACs

Table 2 collects a total of >230 BCF and >530 BAF values for 113 PhACs in a range of aquatic species. BAF/BCFs are expressed in wet weight (w.w.) or dry weight (d.w.) bases and the units are L kg^{-1}.

Table 2. *BAF/BCF observations for PhACs in aquatic organisms belonging to different throphic levels*

Pharmaceutical	Organism	Tissue; dry weight (dw) or wet weight (ww)	BAF	BCF	Reference
1-Hydroxy-ibuprofen	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	7.28-10.8		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	7.28-10.8		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	7.28-10.8		Ruhi et al., 2016
	Predicted	-	0.29		Ruhi et al., 2016
5-Fluorouracil	Shrimps (*Gammarus pulex*)	whole organism (dw)		4.37-9.17	Meredith-Williams et al., 2012
	Water boatman (*Notonecta glauca*)	whole organism (dw)		0.07-0.21	Meredith-Williams et al., 2012
10,11-Epoxycarbamazepine	Macrophyte (Lemna gibba)		308		Mastrángelo et al., 2022
10-Hydroxy-10,11-dihydro-carbamazepine	Mussel (*M. Galloprovincialis*)	whole organism (dw)		4.5	Boillot et al., 2015
10-hydroxy-amitriptyline	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	74-134		De Solla et al., 2016
17α-Ethynylestradiol	Mussel (*Mytilus galloprovincialis*)	whole organism (ww)		30-39	Lopes et al., 2022
	Fish (*Hemiculter leucisculus*)	Liver (ww)	20392		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Brain (ww)	8357		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Muscle (ww)	857		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Gill (ww)	4071		Liu et al., 2015
	Fish (*Carassius auratus*)	Liver (ww)	16642		Liu et al., 2015
	Fish (*Carassius auratus*)	Brain (ww)	7214		Liu et al., 2015
	Fish (*Carassius auratus*)	Gill (ww)	5714		Liu et al., 2015
	Clam (*Ruditapes philippinarum*)	whole organism (ww)		0.024-0.031	Silva et al., 2022
2-Hydroxycarbamazepine	Bifilm		125		Mastrángelo et al., 2022
	Macrophyte (Lemna gibba)		250		Mastrángelo et al., 2022
4-Hydroxy-diclofenac	Mussel (*Mytilus trossulus*)	whole organism (dw)		69.7	Świacka et al., 2021
	Biofilm	(dw)		49.8	Świacka et al., 2021
Amitriptyline	Cottus bairdii	whole organism (ww)	161-2279		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	917		Haddad et al., 2018
	Periphyton	whole organism (ww)	833-17209		Haddad et al., 2018
	Salmo trutta	whole organism (ww)	119-1605		Haddad et al., 2018
	Trichoptera	whole organism (ww)	161-1083		Haddad et al., 2018
	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	4384-8341		De Solla et al., 2016
Amlodipine	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	4077-7578		De Solla et al., 2016
Amphetamine	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	109-284		De Solla et al., 2016
Atenolol	A. Common carp (*Cyprinus carpio*)	liver (ww)	31		Grabicová et al., 2020
	Fish (*Gambusia affinis*)	whole organism (ww)		0.08-0.13	Valdés et al., 2014
Atorvastatin	Fish Predicted (BCFBAF v3.10)	-		56.23	Reis et al., 2021
Azithromycin	A. Common carp (*Cyprinus carpio*)	liver (ww)	1200		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	770		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	1500		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	690		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	brain (ww)	160		Grabicová et al., 2020
	Plankton	(ww)	4800		Grabicová et al., 2020
	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	98-346		De Solla et al., 2016

		contents), and gonadal tissues(ww)			
Benztropine	Caged goldfish (*Carassius auratus*)	plasma	49-165		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	153-1158		Muir et al., 2017
Betamethasone	Fish Predicted (BCFBAF v3.10)	-		8.85	Reis et al., 2021
Bezafibrate	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	4.86-7.78		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	4.86-7.78		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	4.86-7.78		Ruhi et al., 2016
	Predicted	-	3.47		Ruhi et al., 2016
Bisoprolol	Stone moroko (*Pseudorasbora parva*)	(ww)	1.6		Grabicová et al., 2020
	Plankton	(ww)	48		Grabicová et al., 2020
Bupropion	Fish (smallmouth bass)	brain (dw)	3-8		Arnnot et al., 2017
	Fish (smallmouth bass)	gonad (dw)	1-9		Arnnot et al., 2017
	Fish (smallmouth bass)	liver (dw)	1-4		Arnnot et al., 2017
	Fish (largemouth bass)	brain (dw)	6		Arnnot et al., 2017
	Fish (largemouth bass)	gonad (dw)	3		Arnnot et al., 2017
	Fish (largemouth bass)	liver (dw)	2-5		Arnnot et al., 2017
	Fish (white bass)	gonad (dw)	1-26		Arnnot et al., 2017
	Fish (white perch)	gonad (dw)	62		Arnnot et al., 2017
	Fish (walleye)	gonad (dw)	46		Arnnot et al., 2017
	Fish (yellow perch)	gonad (dw)	30		Arnnot et al., 2017
Caffeine	Fish stoneroller minnows (*C. anomalum*)	Fish tissues (ww)	100		Burket et al., 2020
	Cattail (*Typha angustifolia*)	ww		110.68	Wang et al., 2020
	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
	Baetidae	whole organism (ww)	299-451		Haddad et al., 2018
	Cottus bairdii	whole organism (ww)	15-169		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	20		Haddad et al., 2018
	Periphyton	whole organism (ww)	21-642		Haddad et al., 2018
	Salmo trutta	whole organism (ww)	19-169		Haddad et al., 2018
	Trichoptera	whole organism (ww)	20-1056		Haddad et al., 2018
	Caged goldfish (*Carassius auratus*)	plasma	15-16		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	16-51		Muir et al., 2017
	Haemolymph	whole organism (ww)		1.2	Álavarez Ruiz et al., 2021
Carbamazepine	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	1.27-2.00		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	1.27-2.00		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	1.27-2.00		Ruhi et al., 2016
	Predicted	-	229		Ruhi et al., 2016
	A. Common carp (*Cyprinus carpio*)	liver (ww)	1.2		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	2.9		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	2.4		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	brain (ww)	3.9		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	muscle (ww)	1.5		Grabicová et al., 2020
	Stone moroko (*Pseudorasbora parva*)	(ww)	0.47		Grabicová et al., 2020
	Plankton	(ww)	3.6		Grabicová et al., 2020
	Cattail (*Typha angustifolia*)	ww		1289.29	Wang et al., 2020
	Mussel (*M. Galloprovincialis*)	whole organism (dw)		3.9	Boillot et al., 2015
	Shrimps (*Gammarus pulex*)	whole organism (dw)		5.47-8.93	Meredith-Williams et al., 2012
	Water boatman (*Notonecta glauca*)	whole organism (dw)		0.17-0.33	Meredith-Williams et al., 2012
	Fish (*Largemouth bass*)	ventral muscle and skin (dw)		4.3	Huertas et al., 2018
	Fish (*White sucker*)	ventral muscle and skin (dw)		5.1	Huertas et al., 2018
	Fish (*Yellow perch*)	ventral muscle and skin (dw)		11.3	Huertas et al., 2018
	Fish (*Smallmouth bass*)	ventral muscle and skin (dw)		90.9	Huertas et al., 2018
	Fish (predicted)	-		318	Huertas et al., 2018
	Mussel (*M. Galloprovincialis*)	whole organism (dw)		25.8-35.3	Serra-Compte et al., 2018
	Clam (*Corbicula fluminea*)	whole organism (ww)	70		Burket et al., 2020
	Fish stoneroller minnows (*C. anomalum*)	Fish tissues (ww)	4		Burket et al., 2020
	Common carp (*Cyprinus carpio* L.)	liver (ww)	0.8-2.2		Sims et al., 2020

		contents), and gonadal tissues(ww)			
Benztropine	Caged goldfish (*Carassius auratus*)	plasma	49-165		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	153-1158		Muir et al., 2017
Betamethasone	Fish Predicted (BCFBAF v3.10)	-		8.85	Reis et al., 2021
Bezafibrate	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	4.86-7.78		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	4.86-7.78		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	4.86-7.78		Ruhi et al., 2016
	Predicted	-	3.47		Ruhi et al., 2016
Bisoprolol	Stone moroko (*Pseudorasbora parva*)	(ww)	1.6		Grabicová et al., 2020
	Plankton	(ww)	48		Grabicová et al., 2020
Bupropion	Fish (smallmouth bass)	brain (dw)	3-8		Arnnot et al., 2017
	Fish (smallmouth bass)	gonad (dw)	1-9		Arnnot et al., 2017
	Fish (smallmouth bass)	liver (dw)	1-4		Arnnot et al., 2017
	Fish (largemouth bass)	brain (dw)	6		Arnnot et al., 2017
	Fish (largemouth bass)	gonad (dw)	3		Arnnot et al., 2017
	Fish (largemouth bass)	liver (dw)	2-5		Arnnot et al., 2017
	Fish (white bass)	gonad (dw)	1-26		Arnnot et al., 2017
	Fish (white perch)	gonad (dw)	62		Arnnot et al., 2017
	Fish (walleye)	gonad (dw)	46		Arnnot et al., 2017
	Fish (yellow perch)	gonad (dw)	30		Arnnot et al., 2017
Caffeine	Fish stoneroller minnows (*C. anomalum*)	Fish tissues (ww)	100		Burket et al., 2020
	Cattail (*Typha angustifolia*)	ww		110.68	Wang et al., 2020
	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
	Baetidae	whole organism (ww)	299-451		Haddad et al., 2018
	Cottus bairdii	whole organism (ww)	15-169		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	20		Haddad et al., 2018
	Periphyton	whole organism (ww)	21-642		Haddad et al., 2018
	Salmo trutta	whole organism (ww)	19-169		Haddad et al., 2018
	Trichoptera	whole organism (ww)	20-1056		Haddad et al., 2018
	Caged goldfish (*Carassius auratus*)	plasma	15-16		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	16-51		Muir et al., 2017
	Haemolymph	whole organism (ww)		1.2	Álavarez Ruiz et al., 2021
Carbamazepine	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	1.27-2.00		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	1.27-2.00		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	1.27-2.00		Ruhi et al., 2016
	Predicted	-	229		Ruhi et al., 2016
	A. Common carp (*Cyprinus carpio*)	liver (ww)	1.2		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	2.9		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	2.4		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	brain (ww)	3.9		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	muscle (ww)	1.5		Grabicová et al., 2020
	Stone moroko (*Pseudorasbora parva*)	(ww)	0.47		Grabicová et al., 2020
	Plankton	(ww)	3.6		Grabicová et al., 2020
	Cattail (*Typha angustifolia*)	ww		1289.29	Wang et al., 2020
	Mussel (*M. Galloprovincialis*)	whole organism (dw)		3.9	Boillot et al., 2015
	Shrimps (*Gammarus pulex*)	whole organism (dw)		5.47-8.93	Meredith-Williams et al., 2012
	Water boatman (*Notonecta glauca*)	whole organism (dw)		0.17-0.33	Meredith-Williams et al., 2012
	Fish (*Largemouth bass*)	ventral muscle and skin (dw)		4.3	Huertas et al., 2018
	Fish (*White sucker*)	ventral muscle and skin (dw)		5.1	Huertas et al., 2018
	Fish (*Yellow perch*)	ventral muscle and skin (dw)		11.3	Huertas et al., 2018
	Fish (*Smallmouth bass*)	ventral muscle and skin (dw)		90.9	Huertas et al., 2018
	Fish (predicted)	-		318	Huertas et al., 2018
	Mussel (*M. Galloprovincialis*)	whole organism (dw)		25.8-35.3	Serra-Compte et al., 2018
	Clam (*Corbicula fluminea*)	whole organism (ww)	70		Burket et al., 2020
	Fish stoneroller minnows (*C. anomalum*)	Fish tissues (ww)	4		Burket et al., 2020
	Common carp (*Cyprinus carpio* L.)	liver (ww)	0.8-2.2		Sims et al., 2020

	Fish (*Oreochromis niloticus*)	liver (ww)	0.7		García et al., 2012
	Fish (*Oreochromis niloticus*)	muscle (ww)	0.9		García et al., 2012
	Fish (*Oreochromis niloticus*)	blood	2.5		García et al., 2012
	Fish (*Pimephales notatus*)	muscle (ww)		1.9	García et al., 2012
	Fish (*Pimephales notatus*)	liver (ww)		4.6	García et al., 2012
	Fish (*Ictalurus punctatus*)	muscle (ww)		1.8	García et al., 2012
	Fish (*Ictalurus punctatus*)	liver (ww)		1.5	García et al., 2012
	Fish (*Ictalurus punctatus*)	brain (ww)		1.6	García et al., 2012
	Fish (*Ictalurus punctatus*)	plasma (ww)		7.1	García et al., 2012
	Fish (*Hemiculter leucisculus*)	Liver (ww)	615-2750		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Brain (ww)	385-1000		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Muscle (ww)	77-250		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Gill (ww)	269-500		Liu et al., 2015
	Fish (*Carassius auratus*)	Liver (ww)	235-1200		Liu et al., 2015
	Fish (*Carassius auratus*)	Brain (ww)	29-400		Liu et al., 2015
	Fish (*Carassius auratus*)	Muscle (ww)	8.8-200		Liu et al., 2015
	Fish (*Carassius auratus*)	Gill (ww)	200		Liu et al., 2015
	Fish (*Gambusia affinis*)	whole organism (ww)		0.7-0.9	Valdés et al., 2014
	Biofilm		10		Mastrángelo et al., 2022
	Macrophyte (*Lemna gibba*)		141		Mastrángelo et al., 2022
	Cottus bairdii	whole organism (ww)	3-6.5		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	15		Haddad et al., 2018
	Periphyton	whole organism (ww)	3		Haddad et al., 2018
	Trichoptera	whole organism (ww)	10.0-21.0		Haddad et al., 2018
Carbediol	Shrimps (*Gammarus pulex*)	whole organism (dw)		240-303	Meredith-Williams et al., 2012
	Water boatman (*Notonecta glauca*)	whole organism (dw)		1.14-2.18	Meredith-Williams et al., 2012
	Planorbarius corneus	whole organism (dw)		50.4-71.2	Meredith-Williams et al., 2012
Cefotaxime	Fish (*Epinephelus awoara*)	muscle (dw)		3981	Xie et al., 2019
	Fish (*Ephippus orbis*)	muscle (dw)		1585	Xie et al., 2019
	Fish (*Culter alburnus*)	muscle (dw)		1585	Xie et al., 2019
	Shellfish (Ostrea gigas)	whole organism (dw)		1259	Xie et al., 2019
	Shellfish (*Mimachlamys nobilis*)	whole organism (dw)		1585	Xie et al., 2019
	Shellfish (*Mytilus edulis*)	whole organism (dw)		1778	Xie et al., 2019
	Shellfish (*Bufonaria perelegans*)	whole organism (dw)		794	Xie et al., 2019
Cetirizine	Common carp (*Cyprinus carpio* L.)	Liver (ww)	0.58-1.4		Sims et al., 2020
	Plankton	(ww)	47		Grabicová et al., 2020
Chloramphenicol	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	5376		Na et al., 2013
Chlortetracycline	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	0		Na et al., 2013
Ciprofloxacin	Shrimp (Young *Fenneropenseus penicillatus*)	whole organism (ww)		23	Chen et al., 2015
	Biofilm		717		Mastrángelo et al., 2022
	Macrophyte (Lemna gibba)		481		Mastrángelo et al., 2022
Citalopram	Zebrafish (*Danio rerio*)	whole organism (ww)		1.6-3.75	Molina-Fernández et al., 2021
	Zebrafish (*Danio rerio*)	whole organism (ww)		1.45-3.66	Molina-Fernández et al., 2021
	A. Common carp (*Cyprinus carpio*)	liver (ww)	140		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	10		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	140		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	31		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	brain (ww)	81		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	muscle (ww)	43		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	muscle (ww)	1.4		Grabicová et al., 2020
	Stone moroko (*Pseudorasbora*	(ww)	64		Grabicová et al., 2020

	parva)				
	Plankton	(ww)	3000		Grabicová et al., 2020
	Mussel (*M. Galloprovincialis*)	whole organism (dw)		959-2606	Serra-Compte et al., 2018
	Brown trout (*Salmo trutta*)	liver (ww)	260-590		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	kidney (ww)	70-3100		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	brain (ww)			Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	muscle (ww)			Grabicová et al., 2017
	Common carp (*Cyprinus carpio* L.)	Liver (ww)	140-870		Sims et al., 2020
	Caged goldfish (*Carassius auratus*)	plasma	15-58		Muir et al., 2017
	Fish (smallmouth bass)	muscle (dw)	2		Arnnot et al., 2017
	Fish (largemouth bass)	brain (dw)	8		Arnnot et al., 2017
	Fish (largemouth bass)	gonad (dw)	4-5		Arnnot et al., 2017
	Fish (largemouth bass)	liver (dw)	5		Arnnot et al., 2017
	Fish (rudd)	brain (dw)	4		Arnnot et al., 2017
	Fish (rudd)	gonad (dw)	2-4		Arnnot et al., 2017
	Fish (rudd)	liver (dw)	20		Arnnot et al., 2017
	Fish (rudd)	muscle (dw)	1		Arnnot et al., 2017
	Fish (rock bass)	brain (dw)	18		Arnnot et al., 2017
	Fish (rock bass)	gonad (dw)	2-3		Arnnot et al., 2017
	Fish (rock bass)	liver (dw)	9		Arnnot et al., 2017
	Fish (white bass)	brain (dw)	6		Arnnot et al., 2017
	Fish (white bass)	gonad (dw)	9		Arnnot et al., 2017
	Fish (white bass)	liver (dw)	1-19		Arnnot et al., 2017
	Fish (white bass)	muscle (dw)	2		Arnnot et al., 2017
	Fish (white perch)	gonad (dw)	8		Arnnot et al., 2017
	Fish (walleye)	gonad (dw)	5		Arnnot et al., 2017
	Fish (walleye)	liver (dw)	3		Arnnot et al., 2017
	Fish (bowfin)	liver (dw)	1		Arnnot et al., 2017
	Fish (steelhead)	liver (dw)	17		Arnnot et al., 2017
	Fish (yellow perch)	brain (dw)	4		Arnnot et al., 2017
	Fish (yellow perch)	gonad (dw)	1-4		Arnnot et al., 2017
Clarithromycin	Fish Predicted (BCFBAF v3.10)	-		56.49	Reis et al., 2021
	Biofilm		178		Mastrángelo et al., 2022
	Macrophyte (Lemna gibba)		94		Mastrángelo et al., 2022
	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	6.8-61		De Solla et al., 2016
Clindamycin	Common carp (*Cyprinus carpio* L.)	Liver (ww)	2.0-4.9		Sims et al., 2020
	Plankton	(ww)	17		Grabicová et al., 2020
Clindamycin-sulfoxide	Plankton	(ww)	9.1		Grabicová et al., 2020
Clozapine	Fish, channel catfish	plasma		30.5	Nallani et al., 2016
	Fish, channel catfish	brain (ww)		392	Nallani et al., 2016
	Fish, channel catfish	muscle (ww)		81	Nallani et al., 2016
	Fish, channel catfish	gill (ww)		501	Nallani et al., 2016
	Fish, channel catfish	kidney (ww)		958	Nallani et al., 2016
	Fish, channel catfish	liver (ww)		1048	Nallani et al., 2016
	Fish, fathead minnow	brain(ww)		375-538	Nallani et al., 2016
	Fish, fathead minnow	muscle (ww)		71-92.8	Nallani et al., 2016
	Fish, fathead minnow	gill (ww)		475-830	Nallani et al., 2016
	Fish, fathead minnow	kidney (ww)		520-556	Nallani et al., 2016
	Fish, fathead minnow	liver (ww)		605-939	Nallani et al., 2016
Cocaine	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	249-433		De Solla et al., 2016
Codeine	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	31-62		De Solla et al., 2016
Danofloxacin	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
Diazepam	Shrimps (*Gammarus pulex*)	whole organism (dw)		26.5-50.2	Meredith-Williams et al., 2012

	Water boatman (*Notonecta glauca*)	whole organism (dw)		0.70-1.36	Meredith-Williams et al., 2012
	Caged goldfish (*Carassius auratus*)	plasma	38-124		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	109-3120		Muir et al., 2017
Diclofenac	Limpet *(Ancylus fluviatilis)*	whole organism (dw)	8.77-12.8		Ruhi et al., 2016
	Caddisfly *(Hydropsyche sp.)*	whole organism (dw)	231-484		Ruhi et al., 2016
	Flatworm *(Phagocata vitta)*	whole organism (dw)	8.77-12.8		Ruhi et al., 2016
	Predicted	-	6.12		Ruhi et al., 2016
	Fish predicted	-	13		Lagesson et al., 2016
	Invertebrate predicted	-	2		Lagesson et al., 2016
	Mussel (*Mytilus trossulus*)	whole organism (dw)		57	Świacka et al., 2019
	Mussel (*Mytilus trossulus*)	whole organism (ww)		10-180	Ericson et al., 2010
	Mussel (*M. Galloprovincialis*)	visceral mass (ww)		13	Alavarez Ruiz et al., 2021
	Mussel (*M. Galloprovincialis*)	visceral mass (ww)		9.8	Alavarez Ruiz et al., 2021
	Bluegill sunfish (*Lepomis macrochirus*)	Muscle (ww)		0-0.67	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Gill (ww)		2.80-3.66	Zhao et al., 2017
	Plant (*Potamogeton sp.*)	whole organism (dw)	3.92		Wilkinson et al., 2018
	Plant (*Callitriche sp.*)	whole organism (dw)	8.69		Wilkinson et al., 2018
	Periphyton	whole organism (dw)	213		Wilkinson et al., 2018
	Aquatic snails (*Bithynia tentaculata*)	whole organism (dw)	13.2		Wilkinson et al., 2018
	Crustaceans (*Gammarus pulex*)	whole organism (dw)	12.9		Wilkinson et al., 2018
	Fish (*Hemiculter leucisculus*)	Liver (ww)	34-959		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Brain (ww)	14-608		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Muscle (ww)	5-174		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Gill (ww)	6-565		Liu et al., 2015
	Fish (*Carassius auratus*)	Liver (ww)	121-836		Liu et al., 2015
	Fish (*Carassius auratus*)	Brain (ww)	14-291		Liu et al., 2015
	Fish (*Carassius auratus*)	Muscle (ww)	6-36		Liu et al., 2015
	Fish (*Carassius auratus*)	Gill (ww)	18-44		Liu et al., 2015
	Mussel (*M. Galloprovincialis*)	whole organism (dw)		11.3-16.5	Bonnefille et al., 2017
	Mussel (*Dreissena polymorpha*)	-		4.0-13	Daniele et al., 2016
	Trout (*O. Mykiss*)	whole organism (ww)		0.3-2732	Schwaiger et al., 2004
	Trout (*O. Mykiss*)	whole organism (ww)		3.0-5.0	Memmer et al., 2013
	Duckweed (*L. minor*)	whole organism (dw)		3.4-12.1	Kummerova et al., 2016
	Trout (*O. Mykiss*)	whole organism (ww)		320-950	Kallio et al., 2010
	Mussel (*Mytilus trossulus*)	whole organism (dw)		118.5	Świacka et al., 2021
	Biofilm	(dw)		119	Świacka et al., 2021
	Crucian carp (*Carassius auratus*)	liver (ww)		121	Lu et al., 2018
	Crucian carp (*Carassius auratus*)	gill (ww)		52.3	Lu et al., 2018
	Crucian carp (*Carassius auratus*)	muscle (ww)		46.8	Lu et al., 2018
	Crucian carp (*Carassius auratus*)	muscle (dw)		0.56-8.55	Xie et al., 2020
	Crucian carp (*Carassius auratus*)	brain (dw)		2.73-22.1	Xie et al., 2020
	Crucian carp (*Carassius auratus*)	gill (dw)		2.61-28.7	Xie et al., 2020
	Crucian carp (*Carassius auratus*)	kidney (dw)		3.41-61.7	Xie et al., 2020
	Crucian carp (*Carassius auratus*)	liver (dw)		3.09-56	Xie et al., 2020
Diltiazem	Stone moroko (*Pseudorasbora parva*)	(ww)	290		Grabicová et al., 2020
	Baetidae	whole organism (ww)	38-253		Haddad et al., 2018
	Cottus bairdii	whole organism (ww)	25-267		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	53		Haddad et al., 2018
	Periphyton	whole organism (ww)	48-8667		Haddad et al., 2018
	Salmo trutta	whole organism (ww)	11-167		Haddad et al., 2018
	Trichoptera	whole organism (ww)	13-280		Haddad et al., 2018
	Rainbow trout (*Oncorhynchus mykiss*)	Whole body (minus liver) (ww)	10.1-72.5		Sims et al., 2020
	Clam (*Corbicula fluminea*)	whole organism (ww)	350		Burket et al., 2020
	Fish stoneroller minnows (*C. anomalum*)	Fish tissues (ww)	300		Burket et al., 2020
	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland	26-50		De Solla et al., 2016

	Plankton	(excluding stomach and its contents), and gonadal tissues(ww) (ww)	76	Grabicová et al., 2020
Diphenhydramine	Fish predicted	-	285	Lagesson et al., 2016
	Invertebrate predicted	-	36	Lagesson et al., 2016
	Amshorn snail (*Planarbidae*)	whole organism (ww)	1250	Lagesson et al., 2016
	Waterlouse (*Asellus aquaticus*)	whole organism (ww)	600	Lagesson et al., 2016
	Mayfly larvae *(Ephemeropteras)*	whole organism (ww)	125	Lagesson et al., 2016
	Damselfly larvae *(Zygoptera)*	whole organism (ww)	250	Lagesson et al., 2016
	European perch (*Perca fluviatilis*)	muscle (ww)	100	Lagesson et al., 2016
	Plankton	(ww)	1300	Grabicová et al., 2020
	Fish (smallmouth bass)	brain (dw)	4-6	Arnnot et al., 2017
	Fish (smallmouth bass)	gonad (dw)	1-5	Arnnot et al., 2017
	Fish (smallmouth bass)	liver (dw)	2-4	Arnnot et al., 2017
	Fish (smallmouth bass)	muscle (dw)	1	Arnnot et al., 2017
	Fish (largemouth bass)	brain (dw)	5-15	Arnnot et al., 2017
	Fish (largemouth bass)	gonad (dw)	1-2	Arnnot et al., 2017
	Fish (largemouth bass)	liver (dw)	1-4	Arnnot et al., 2017
	Fish (largemouth bass)	muscle (dw)	1	Arnnot et al., 2017
	Fish (rudd)	brain (dw)	4-18	Arnnot et al., 2017
	Fish (rudd)	gonad (dw)	1-2	Arnnot et al., 2017
	Fish (rudd)	liver (dw)	3-21	Arnnot et al., 2017
	Fish (rudd)	muscle (dw)	1	Arnnot et al., 2017
	Fish (rock bass)	brain (dw)	5-29	Arnnot et al., 2017
	Fish (rock bass)	gonad (dw)	1-9	Arnnot et al., 2017
	Fish (rock bass)	liver (dw)	1-7	Arnnot et al., 2017
	Fish (rock bass)	muscle (dw)	1	Arnnot et al., 2017
	Fish (white bass)	brain (dw)	8-9	Arnnot et al., 2017
	Fish (white bass)	gonad (dw)	1-2	Arnnot et al., 2017
	Fish (white bass)	liver (dw)	1-15	Arnnot et al., 2017
	Fish (white bass)	muscle (dw)	1-2	Arnnot et al., 2017
	Fish (white perch)	brain (dw)	4	Arnnot et al., 2017
	Fish (white perch)	gonad (dw)	8	Arnnot et al., 2017
	Fish (white perch)	liver (dw)	1	Arnnot et al., 2017
	Fish (white perch)	muscle (dw)	1	Arnnot et al., 2017
	Fish (walleye)	brain (dw)	5-11	Arnnot et al., 2017
	Fish (walleye)	gonad (dw)	1-2	Arnnot et al., 2017
	Fish (walleye)	liver (dw)	2-4	Arnnot et al., 2017
	Fish (walleye)	muscle (dw)	1	Arnnot et al., 2017
	Fish (steelhead)	gonad (dw)	1	Arnnot et al., 2017
	Fish (yellow perch)	gonad (dw)	1-2	Arnnot et al., 2017
	Fish (yellow perch)	liver (dw)	1	Arnnot et al., 2017
	Fish (yellow perch)	muscle (dw)	0.2	Arnnot et al., 2017
	Periphyton	whole organism (ww)	300	Burket et al., 2020
	Clam (*Corbicula fluminea*)	whole organism (ww)	1600	Burket et al., 2020
	Fish stoneroller minnows (*C. anomalum*)	Fish tissues (ww)	250	Burket et al., 2020
	Rainbow trout (*Oncorhynchus mykiss*)	Whole body (minus liver) (ww)	32-88	Sims et al., 2020
	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	712-1331	De Solla et al., 2016
	Caged goldfish (*Carassius auratus*)	plasma	45-193	Muir et al., 2017
	Baetidae	whole organism (ww)	145-168	Haddad et al., 2018
	Cottus bairdii	whole organism (ww)	48-522	Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	220	Haddad et al., 2018
	Periphyton	whole organism (ww)	239-5581	Haddad et al., 2018
	Salmo trutta	whole organism (ww)	23-105	Haddad et al., 2018
	Trichoptera	whole organism (ww)	81-1279	Haddad et al., 2018
Donepezil	A. Common carp (*Cyprinus carpio*)	liver (ww)	470	Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	320	Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	280	Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	brain (ww)	310	Grabicová et al., 2020
	Stone moroko (*Pseudorasbora*	(ww)	190	Grabicová et al., 2020

	parva)				
	Plankton	(ww)	390		Grabicová et al., 2020
Doxycycline	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	4456		Na et al., 2013
Enoxacin	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
Enrofloxacin	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
	Shrimp (Young *Fenneropenaeus penicillatus*)	whole organism (ww)		650	Chen et al., 2015
	Fish (Adult *Trachinotus ovatus*)	muscle (ww)		861	Chen et al., 2015
Erythromycin	Fish (*Hemiculter leucisculus*)	Liver (ww)	7559-7900		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Brain (ww)	1088-2900		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Muscle (ww)	700-971		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Gill (ww)	1200-1412		Liu et al., 2015
	Fish (*Carassius auratus*)	Liver (ww)	31-120		Liu et al., 2015
	Fish (*Carassius auratus*)	Brain (ww)	160-226		Liu et al., 2015
	Fish (*Carassius auratus*)	Muscle (ww)	528-760		Liu et al., 2015
	Fish (*Carassius auratus*)	Gill (ww)	1480-1616		Liu et al., 2015
	Caged goldfish (*Carassius auratus*)	plasma	34-101		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	53-121		Muir et al., 2017
Estrone	Flatworm (*Phagocata vitta*)	whole organism (dw)	4207		Ruhi et al., 2016
	Predicted	-	81846		Ruhi et al., 2016
Etoricoxib	Mussel (*M. Galloprovincialis*)	visceral mass (ww)		6.4	Alavarez Ruiz et al., 2021
	Mussel (*M. Galloprovincialis*)	visceral mass (ww)		6.7	Álavarez Ruiz et al., 2021
	Haemolymph	whole organism (ww)		0.90	Álavarez Ruiz et al., 2021
Fenofibrate	Fish Predicted (BCFBAF v3.10)	-		322.2	Reis et al., 2021
Fexofenadine	Plankton	(ww)	68		Grabicová et al., 2020
	Damselfly larvae	whole organism (dw)		120	Jonsson et al., 2014
Florophenicol	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	266		Na et al., 2013
Fluconazole	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
Flumequine	Caged goldfish (*Carassius auratus*)	plasma	68-626		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	72-562		Muir et al., 2017
Fluoxetine	Zebrafish (*Danio rerio*)	whole organism (ww)		8-8.1	Molina-Fernández et al., 2021
	Zebrafish (*Danio rerio*)	whole organism (ww)		7.27-7.3	Molina-Fernández et al., 2021
	Cattail (*Typha angustifolia*)	ww		658.56	Wang et al., 2020
	Shrimps (*Gammarus pulex*)	whole organism (dw)		161800-209500	Meredith-Williams et al., 2012
	Water boatman (*Notonecta glauca*)	whole organism (dw)		1.08-1.75	Meredith-Williams et al., 2012
	Zebrafish (*Danio rerio*)	whole organism (ww)		0.019-0.22	Pan et al., 2018
	Zebrafish (*Danio rerio*)	whole organism (ww)		1.73-6.88	Pan et al., 2018
	Red crucian cap (*Carassius auratus*)	brain (ww)		12.4-110	Pan et al., 2018
	Red crucian cap (*Carassius auratus*)	liver (ww)		11.1-1.37	Pan et al., 2018
	Red crucian cap (*Carassius auratus*)	muscle (ww)		12.1-166	Pan et al., 2018
	Caged goldfish (*Carassius auratus*)	plasma	310-1480		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	176-334		Muir et al., 2017
	Rainbow trout (*Oncorhynchus mykiss*)	Whole body (minus liver) (ww)	251-426		Sims et al., 2020
	Plant (*A. platanoides*)	pooled samples (dw)	1300		Boström et al., 2017
	Crustacean (*A. aquaticus*)	whole organism (ww)	110		Boström et al., 2017

	Insect (*N. glauca*)	whole organism (ww)	11		Boström et al., 2017
	Fish (*P. pungitus*)	-	41		Boström et al., 2017
	Clam (*Corbicula fluminea*)	whole organism (ww)	200		Burket et al., 2020
	Baetidae	whole organism (ww)	356		Haddad et al., 2018
	Cottus bairdii	whole organism (ww)	38-2929		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	3431		Haddad et al., 2018
	Periphyton	whole organism (ww)	118-6192		Haddad et al., 2018
	Salmo trutta	whole organism (ww)	19-2134		Haddad et al., 2018
	Trichoptera	whole organism (ww)	47-4352		Haddad et al., 2018
Gemfibrozil	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	0.26-0.48		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	0.26-0.49		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	0.33-153		Ruhi et al., 2016
	Predicted	-	10.59		Ruhi et al., 2016
	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
Haloperidol	B. Pikeperch (*Sander lucioperca*)	liver (ww)	170		Grabicová et al., 2020
	Caged goldfish (*Carassius auratus*)	plasma	190-1110		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	208-1088		Muir et al., 2017
	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	0-62		De Solla et al., 2016
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	280		Grabicová et al., 2020
Hydrochlorothiazide	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	0.16-0.22		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	0.16-0.23		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	0.16-0.24		Ruhi et al., 2016
	Predicted	-	2.43		Ruhi et al., 2016
	Fish (*Largemouth bass*)	ventral muscle and skin (dw)		0.4	Huertas et al., 2018
	Fish (*White sucker*)	ventral muscle and skin (dw)		4.5	Huertas et al., 2018
	Fish (*Yellow perch*)	ventral muscle and skin (dw)		9.7	Huertas et al., 2018
	Fish (*Smallmouth bass*)	ventral muscle and skin (dw)		12.5	Huertas et al., 2018
	Fish (predicted)	-		16.7	Huertas et al., 2018
	Cockle (*Cerastoderma glaucum*)	whole organism (dw)	321-590		Moreno-González et al., 2016
	Noble pen shell (*Pinna nobilis*)	whole organism (dw)	109.7		Moreno-González et al., 2016
	Golden grey mullet (*Liza aurata*)	whole organism (dw)	182.5		Moreno-González et al., 2016
	Biofilm		34		Mastrángelo et al., 2022
	Macrophyte (Lemna gibba)		70		Mastrángelo et al., 2022
Hydroxyzine	Fish predicted	-	40		Lagesson et al., 2016
	Invertebrate predicted	-	6		Lagesson et al., 2016
	Damselfly larvae	whole organism (dw)		2000	Jonsson et al., 2014
	Amshorn snail (*Planorbidae*)	whole organism (ww)	97000		Lagesson et al., 2016
	Waterlouse (*Asellus aquaticus*)	whole organism (ww)	7000		Lagesson et al., 2016
	Mayfly larvae (*Ephemeropteras*)	whole organism (ww)	5000		Lagesson et al., 2016
	Damselfly larvae (*Zygoptera*)	whole organism (ww)	18000		Lagesson et al., 2016
	European perch (*Perca fluviatilis*)	muscle (ww)	1000		Lagesson et al., 2016
Ibuprofen	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	1.66-2.75		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	1.66-954		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	1.70-160		Ruhi et al., 2016
	Predicted	-	6.12		Ruhi et al., 2016
	Cattail (*Typha angustifolia*)	ww		157.57	Wang et al., 2020
	Wild carp (*Cyprinus carpio*)	plasma	115		Muir et al., 2017
	Rainbow trout (*Oncorhynchus mykiss*)	bile		14000-49000	Brozinski et al., 2013
	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
	Biofilm	-	13		Mastrángelo et al., 2022
Iopamidol	Caged goldfish (*Carassius auratus*)	plasma	37-40		Muir et al., 2017
Irbesartan	Plankton	(ww)	2.5		Grabicová et al., 2020
Ketoprofen	Fish (*Epinephelus awoara*)	muscle (dw)		39811	Xie et al., 2019
	Fish (*Culter alburnus*)	muscle (dw)		1995	Xie et al., 2019

	Shellfish (*Ostrea gigas*)	whole organism (dw)		70795	Xie et al., 2019
	Shellfish (*Mimachlamys nobilis*)	whole organism (dw)		70795	Xie et al., 2019
	Shellfish (*Mytilus edulis*)	whole organism (dw)		39811	Xie et al., 2019
	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
Loratadine	Fish Predicted (BCFBAF v3.10)	-		1253	Reis et al., 2021
	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
Metformin	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	0.15-1.29		De Solla et al., 2016
Methocarbamol	Bluegill sunfish (*Lepomis macrochirus*)	Muscle (ww)		0.13-0.35	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Liver (ww)		0.19-0.58	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Brain (ww)		0.23-0.53	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Bile (ww)		5.90-48.6	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	plasma		0.13-0.24	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Gut (ww)		0.34-0.75	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Gill (ww)		0.14-0.21	Zhao et al., 2017
Methylphenidate	Cottus bairdii	whole organism (ww)	15-21		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	29		Haddad et al., 2018
	Periphyton	whole organism (ww)	50-155		Haddad et al., 2018
	Salmo trutta	whole organism (ww)	3190-21429		Haddad et al., 2018
	Trichoptera	whole organism (ww)	29		Haddad et al., 2018
Metoprolol	Common carp (*Cyprinus carpio* L.)	Liver (ww)	1.4-11		Sims et al., 2020
	Stone moroko (*Pseudorasbora parva*)	(ww)	6.3		Grabicová et al., 2020
	Plankton	(ww)	81		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	liver (ww)	11		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	21		Grabicová et al., 2020
	Plankton	(ww)	3.8		Grabicová et al., 2020
Metoprolol acid	Common carp (*Cyprinus carpio* L.)	Liver (ww)	7.3-16		Sims et al., 2020
Mianserin	Plankton	(ww)	520		Grabicová et al., 2020
	Brown trout (*Salmo trutta*)	kidney (ww)	630-670		Grabicová et al., 2017
Miconazole	Plankton	(ww)	1000		Grabicová et al., 2020
Mirtazapine	Stone moroko (*Pseudorasbora parva*)	(ww)	140		Grabicová et al., 2020
	Plankton	(ww)	440		Grabicová et al., 2020
	Brown trout (*Salmo trutta*)	liver (ww)	110-300		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	kidney (ww)	2100-6000		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	brain (ww)	4.0-6.0		Grabicová et al., 2017
Moclobemide	Shrimps (*Gammarus pulex*)	whole organism (dw)		3.18-6.32	Meredith-Williams et al., 2012
	Water boatman (*Notonecta glauca*)	whole organism (dw)		0.19-0.52	Meredith-Williams et al., 2012
N-Desmethylcitalopram	Common carp (*Cyprinus carpio* L.)	Liver (ww)	250-1700		Sims et al., 2020
	A. Common carp (*Cyprinus carpio*)	liver (ww)	370		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	1400		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	71		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	muscle (ww)	22		Grabicová et al., 2020
	Stone moroko (*Pseudorasbora parva*)	(ww)	100		Grabicová et al., 2020
	Plankton	(ww)	2600		Grabicová et al., 2020
	Zebrafish (*Danio rerio*)	whole organism (ww)		0.35-0.7	Molina-Fernández et al., 2021
	Zebrafish (*Danio rerio*)	whole organism (ww)		0.21-0.69	Molina-Fernández et

					al., 2021
N-Desmethyldiltiazem	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	33-66		De Solla et al., 2016
Naproxen	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	0-9.2		De Solla et al., 2016
Norfloxacin	Fish (*Epinephelus awoara*)	muscle (dw)		3981	Xie et al., 2019
	Fish (*Culter alburnus*)	muscle (dw)		1000	Xie et al., 2019
	Shellfish (Ostrea gigas)	whole organism (dw)		200	Xie et al., 2019
	Shellfish (*Mimachlamys nobilis*)	whole organism (dw)		8	Xie et al., 2019
	Shellfish (*Mytilus edulis*)	whole organism (dw)		16	Xie et al., 2019
	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
Norfluoxetine	Zebrafish (*Danio rerio*)	whole organism (ww)		12.0-20.0	Molina-Fernández et al., 2021
	Zebrafish (*Danio rerio*)	whole organism (ww)		7.6-8.4	Molina-Fernández et al., 2021
	Fish (smallmouth bass)	brain (dw)	15-49		Arnnot et al., 2017
	Fish (smallmouth bass)	gonad (dw)	5-39		Arnnot et al., 2017
	Fish (smallmouth bass)	muscle (dw)	1-7		Arnnot et al., 2017
	Fish (largemouth bass)	brain (dw)	15-97		Arnnot et al., 2017
	Fish (largemouth bass)	gonad (dw)	8		Arnnot et al., 2017
	Fish (largemouth bass)	muscle (dw)	1-3		Arnnot et al., 2017
	Fish (rudd)	brain (dw)	21-24		Arnnot et al., 2017
	Fish (rudd)	gonad (dw)	5-9		Arnnot et al., 2017
	Fish (rock bass)	brain (dw)	15-130		Arnnot et al., 2017
	Fish (rock bass)	gonad (dw)	49		Arnnot et al., 2017
	Fish (white bass)	brain (dw)	18-66		Arnnot et al., 2017
	Fish (white bass)	muscle (dw)	3-6		Arnnot et al., 2017
	Fish (white perch)	gonad (dw)	1		Arnnot et al., 2017
	Fish (walleye)	muscle (dw)	1		Arnnot et al., 2017
	Clam (*Corbicula fluminea*)	whole organism (ww)	700		Burket et al., 2020
	Cottus bairdii	whole organism (ww)	68-6328		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	2034		Haddad et al., 2018
	Periphyton	whole organism (ww)	46-8249		Haddad et al., 2018
	Salmo trutta	whole organism (ww)	31-2712		Haddad et al., 2018
	Trichoptera	whole organism (ww)	1808-4351		Haddad et al., 2018
Norsertraline	Zebrafish (*Danio rerio*)	whole organism (ww)		38	Molina-Fernández et al., 2021
	Zebrafish (*Danio rerio*)	whole organism (ww)		26.5	Molina-Fernández et al., 2021
	Fish (smallmouth bass)	brain (dw)	470-910		Arnnot et al., 2017
	Fish (smallmouth bass)	gonad (dw)	150		Arnnot et al., 2017
	Fish (smallmouth bass)	liver (dw)	240-320		Arnnot et al., 2017
	Fish (smallmouth bass)	muscle (dw)	84-170		Arnnot et al., 2017
	Fish (largemouth bass)	brain (dw)	590-1200		Arnnot et al., 2017
	Fish (largemouth bass)	gonad (dw)	130-160		Arnnot et al., 2017
	Fish (largemouth bass)	liver (dw)	310-470		Arnnot et al., 2017
	Fish (largemouth bass)	muscle (dw)	140-160		Arnnot et al., 2017
	Fish (rudd)	brain (dw)	420-680		Arnnot et al., 2017
	Fish (rudd)	gonad (dw)	150		Arnnot et al., 2017
	Fish (rudd)	liver (dw)	300-5000		Arnnot et al., 2017
	Fish (rudd)	muscle (dw)	110-310		Arnnot et al., 2017
	Fish (rock bass)	brain (dw)	500-1800		Arnnot et al., 2017
	Fish (rock bass)	gonad (dw)	130-200		Arnnot et al., 2017
	Fish (rock bass)	liver (dw)	770-1600		Arnnot et al., 2017
	Fish (rock bass)	muscle (dw)	94-150		Arnnot et al., 2017
	Fish (white bass)	brain (dw)	510-920		Arnnot et al., 2017
	Fish (white bass)	liver (dw)	150-520		Arnnot et al., 2017
	Fish (white bass)	muscle (dw)	140-190		Arnnot et al., 2017
	Fish (white perch)	brain (dw)	840		Arnnot et al., 2017
	Fish (white perch)	liver (dw)	1100		Arnnot et al., 2017
	Fish (white perch)	muscle (dw)	150		Arnnot et al., 2017
	Fish (walleye)	brain (dw)	350-670		Arnnot et al., 2017
	Fish (walleye)	gonad (dw)	200		Arnnot et al., 2017

	Fish (walleye)	liver (dw)	290		Arnnot et al., 2017
	Fish (walleye)	muscle (dw)	96-330		Arnnot et al., 2017
	Fish (bowfin)	brain (dw)	1500		Arnnot et al., 2017
	Fish (bowfin)	liver (dw)	260		Arnnot et al., 2017
	Fish (bowfin)	muscle (dw)	150		Arnnot et al., 2017
	Fish (steelhead)	brain (dw)	160-600		Arnnot et al., 2017
	Fish (steelhead)	liver (dw)	270-630		Arnnot et al., 2017
	Fish (steelhead)	muscle (dw)	140-160		Arnnot et al., 2017
	Fish (yellow perch)	brain (dw)	250		Arnnot et al., 2017
O-Desmethylvenlafaxine	Common carp (*Cyprinus carpio* L.)	Liver (ww)	1.3-5.6		Sims et al., 2020
	A. Common carp (*Cyprinus carpio*)	liver (ww)	6		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	4.9		Grabicová et al., 2020
	Plankton	(ww)	100		Grabicová et al., 2020
	Loach (*Misgurnus anguillicaudatus*)	liver (ww)		0.15-0.97	Qu et al., 2019
	Loach (*Misgurnus anguillicaudatus*)	liver (ww)		4.51-22.81	Qu et al., 2019
Ofloxacin	Fish (Adult *Trachinotus ovatus*)	muscle (ww)		1164	Chen et al., 2015
	Macrophyte (Lemna gibba)		429		Mastrángelo et al., 2022
Oxazepam	European perch (*Perca fluviatilis*)	muscle (ww)		13-19	Cerveny et al., 2021b
	European perch (*Perca fluviatilis*)	brain (ww)		56-70	Cerveny et al., 2021b
	Fish predicted	-	25		Lagesson et al., 2016
	Invertebrate predicted	-	4		Lagesson et al., 2016
	Amshorn snail (*Planorbidae*)	whole organism (ww)	37.5		Lagesson et al., 2016
	Waterlouse (*Asellus aquaticus*)	whole organism (ww)	45		Lagesson et al., 2016
	Mayfly larvae (*Ephemeropteras*)	whole organism (ww)	20		Lagesson et al., 2016
	Damselfly larvae (*Zygoptera*)	whole organism (ww)	10		Lagesson et al., 2016
	European perch (*Perca fluviatilis*)	muscle (ww)	18		Lagesson et al., 2016
Oxycodone	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	0-23.6		De Solla et al., 2016
Oxytetracycline	Shrimp (Young *Fenneropenseus penicillatus*)	whole organism (ww)		2	Chen et al., 2015
	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	509		Na et al., 2013
Paracetamol	Fish (*Epinephelus awoara*)	muscle (dw)		3162	Xie et al., 2019
	Plant (*Potamogeton sp.*)	whole organism (dw)	45.9		Wilkinson et al., 2018
	Plant (*Callitriche sp.*)	whole organism (dw)	22.5		Wilkinson et al., 2018
	Periphyton	whole organism (dw)	22.1		Wilkinson et al., 2018
	Aquatic snails (*Bithynia tentaculata*)	whole organism (dw)	37.0		Wilkinson et al., 2018
	Crustaceans (*Gammarus pulex*)	whole organism (dw)	26.4		Wilkinson et al., 2018
	Clam (*Corbicula fluminea*)	whole organism (ww)	1000		Burket et al., 2020
Paroxetine	Zebrafish (*Danio rerio*)	whole organism (ww)		10.0-25	Molina-Fernández et al., 2021
	Zebrafish (*Danio rerio*)	whole organism (ww)		7.6-9.53	Molina-Fernández et al., 2021
Phenazone	Fish Predicted (BCFBAF v3.10)	-		3.16	Reis et al., 2021
Phenylbutazone	Fish Predicted (BCFBAF v3.10)	-		56.49	Reis et al., 2021
Prednisone	Fish Predicted (BCFBAF v3.10)	-		4.27	Reis et al., 2021
Propranolol	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	1059-1939		De Solla et al., 2016
	Crustacean (*D. Magna*)	whole organism (ww)		18-85	Ding et al., 2016
	Fish (*Hemiculter leucisculus*)	Liver (ww)	1000-4000		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Brain (ww)	500-1000		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Gill (ww)	500		Liu et al., 2015
	Fish (*Carassius auratus*)	Brain (ww)	200		Liu et al., 2015
	Fish (*Carassius auratus*)	Gill (ww)	133		Liu et al., 2015
Rosuvastatin	Bluegill sunfish (*Lepomis macrochirus*)	liver (ww)		0-0.20	Zhao et al., 2017
	Bluegill sunfish (*Lepomis*	bile (ww)		0-6.70	Zhao et al., 2017

	macrochirus)				
	Bluegill sunfish (*Lepomis macrochirus*)	plasma		0.40-0.56	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	gut (ww)		0.79-1.30	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	gill (ww)		0-0.14	Zhao et al., 2017
Roxithromycin	Crustacean (*D. Magna*)	whole organism (ww)		13.4-93.5	Ding et al., 2016
	Fish (*Hemiculter leucisculus*)	Liver (ww)	729-7091		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Brain (ww)	497-2091		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Muscle (ww)	21-1273		Liu et al., 2015
	Fish (*Hemiculter leucisculus*)	Gill (ww)	41-1636		Liu et al., 2015
	Fish (*Carassius auratus*)	Liver (ww)	725-920		Liu et al., 2015
	Fish (*Carassius auratus*)	Brain (ww)	566-630		Liu et al., 2015
	Fish (*Carassius auratus*)	Muscle (ww)	142-282		Liu et al., 2015
	Fish (*Carassius auratus*)	Gill (ww)	225-540		Liu et al., 2015
Salicylic acid	Shrimp (Young *Fenneropenaeus penicillatus*)	whole organism (ww)		661	Chen et al., 2015
	Crab (Adult Calappa philargius)	whole organism (ww)		536	Chen et al., 2015
	Mollusks (Adult *Meretrix lusoria*)	whole organism (ww)		1854	Chen et al., 2015
	Fish (Adult *Trachinotus ovatus*)	muscle (ww)		1238	Chen et al., 2015
	Fish (Adult *Lutjanus russelli*)	muscle (ww)		1103	Chen et al., 2015
Sertraline	Zebrafish (*Danio rerio*)	whole organism (ww)		37.5-50	Molina-Fernández et al., 2021
	Zebrafish (*Danio rerio*)	whole organism (ww)		36.7-48.9	Molina-Fernández et al., 2021
	A. Common carp (*Cyprinus carpio*)	liver (ww)	870		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	490		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	400		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	490		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	brain (ww)	2400		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	brain (ww)	710		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	muscle (ww)	65		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	muscle (ww)	38		Grabicová et al., 2020
	Stone moroko (*Pseudorasbora parva*)	(ww)	100		Grabicová et al., 2020
	Plankton	(ww)	6000		Grabicová et al., 2020
	Caged goldfish (*Carassius auratus*)	plasma	109-659		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	120-349		Muir et al., 2017
	Brown trout (*Salmo trutta*)	liver (ww)	880-2400		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	kidney (ww)	2800-4400		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	brain (ww)	240		Grabicová et al., 2017
	Common carp (*Cyprinus carpio* L.)	Liver (ww)	430-140000		Sims et al., 2020
	Plant (*A. platanoides*)	pooled samples (dw)	2200		Boström et al., 2017
	Crustacean (*A. aquaticus*)	whole organism (ww)	360		Boström et al., 2017
	Insect (*N. glauca*)	whole organism (ww)	26		Boström et al., 2017
	Fish (*P. pungitius*)	-	49		Boström et al., 2017
	Clam (*Corbicula fluminea*)	whole organism (ww)	650		Burket et al., 2020
	Mussel (*Lasmigona costata*)	Tisseus (gill, digestive gland (excluding stomach and its contents), and gonadal tissues(ww)	19565-51231		De Solla et al., 2016
	Fish (smallmouth bass)	brain (dw)	24-27		Arnnot et al., 2017
	Fish (smallmouth bass)	gonad (dw)	27		Arnnot et al., 2017
	Fish (largemouth bass)	brain (dw)	68		Arnnot et al., 2017
	Fish (rudd)	brain (dw)	18		Arnnot et al., 2017
	Fish (rock bass)	brain (dw)	29		Arnnot et al., 2017
	Fish (rock bass)	gonad (dw)	14-15		Arnnot et al., 2017
	Fish (white bass)	brain (dw)	23		Arnnot et al., 2017
	Fish (white bass)	gonad (dw)	2		Arnnot et al., 2017
	Fish (walleye)	brain (dw)	15-29		Arnnot et al., 2017
	Fish (walleye)	gonad (dw)	2		Arnnot et al., 2017
	Baetidae	whole organism (ww)	3026-		Haddad et al., 2018

			9211		
	Cottus bairdii	whole organism (ww)	66-6947		Haddad et al., 2018
	Lymnaeidea & Physidae	whole organism (ww)	15789		Haddad et al., 2018
	Periphyton	whole organism (ww)	600-19737		Haddad et al., 2018
	Salmo trutta	whole organism (ww)	160-5751		Haddad et al., 2018
	Trichoptera	whole organism (ww)	224-11053		Haddad et al., 2018
Sotalol	Mussel (*M. Galloprovincialis*)	whole organism (dw)		18.8-59.2	Serra-Compte et al., 2018
Spectinomycin	Shellfish (*Mytilus edulis*)	whole organism (dw)		2512	Xie et al., 2019
Sulfacetamide	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	1401		Na et al., 2013
Sulfadiazine	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	10757		Na et al., 2013
	Shrimp (Young *Fenneropenseus penicillatus*)	whole organism (ww)		1392	Chen et al., 2015
	Fish (Adult *Trachinotus ovatus*)	muscle (ww)		781	Chen et al., 2015
Sulfadimethoxine	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	0		Na et al., 2013
Sulfadoxine	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	0		Na et al., 2013
Sulfameter	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	92034		Na et al., 2013
Sulfamerhazine	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	3501		Na et al., 2013
	Caged goldfish (*Carassius auratus*)	plasma	197-546		Muir et al., 2017
	Fish marine medaka (*Oryzias melastigma*)	Female gills (ww)		0.74-5.54	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Female liver (ww)		1.08-26.30	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Female bile (ww)		10.69-42.95	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Female gonad (ww)		0.70-8.05	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Female muscle (ww)		0.15-4.10	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Male gills (ww)		0.57-7.95	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Male liver (ww)		1.03-60.64	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Male bile (ww)		2.78-145.36	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Male gonad (ww)		3.4-27.45	Zhao et al., 2016
	Fish marine medaka (*Oryzias melastigma*)	Male muscle (ww)		0.07-0.73	Zhao et al., 2016
Sulfamethiazole	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	2332		Na et al., 2013
Sulfamethoxazole	Limpet (*Ancylus fluviatilis*)	whole organism (dw)	15.3-19.7		Ruhi et al., 2016
	Caddisfly (*Hydropsyche sp.*)	whole organism (dw)	15.3-19.8		Ruhi et al., 2016
	Flatworm (*Phagocata vitta*)	whole organism (dw)	15.3-19.9		Ruhi et al., 2016
	Predicted	-	1		Ruhi et al., 2016
	Fish (Young *Lutjanus russelli*)	muscle (dw)		185	Chen et al., 2015
	Fish (*Epinephelus awoara*)	muscle (dw)		1000	Xie et al., 2019
	Fish (*Ephippus orbis*)	muscle (dw)		178	Xie et al., 2019
	Fish (*Culter alburnus*)	muscle (dw)		398	Xie et al., 2019
	Shellfish (Ostrea gigas)	whole organism (dw)		1000	Xie et al., 2019
	Shellfish (Mimachlamys nobilis)	whole organism (dw)		126	Xie et al., 2019
	Shellfish (Mytilus edulis)	whole organism (dw)		13	Xie et al., 2019
	Mussel (*M. Galloprovincialis*)	whole organism (dw)		6.2-9.0	Serra-Compte et al.,

					2018
	Bluegill sunfish (*Lepomis macrochirus*)	Muscle (ww)		0-0.99	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Liver (ww)		0-4.48	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Brain (ww)		0-2.45	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Bile (ww)		0-0.49	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	plasma		0	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Gut (ww)		0.90-3.41	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Gill (ww)		0-0.36	Zhao et al., 2017
	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	350		Na et al., 2013
Sulfamethoxypyridazine	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	7025		Na et al., 2013
Sulfamonomethoxine	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	3076		Na et al., 2013
Sulfathiazole	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	488		Na et al., 2013
Sulfisoxazole	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	0		Na et al., 2013
Telmisartan	A. Common carp (*Cyprinus carpio*)	liver (ww)	7.8		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	6.6		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	0.65		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	11		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	brain (ww)	0.44		Grabicová et al., 2020
	Stone moroko (*Pseudorasbora parva*)	(ww)	12		Grabicová et al., 2020
	Plankton	(ww)	91		Grabicová et al., 2020
Temazepam	European perch (*Perca fluviatilis*)	muscle (ww)		24-25	Cerveny et al., 2021b
	European perch (*Perca fluviatilis*)	brain (ww)		83-100	Cerveny et al., 2021b
	Dragonfly larvae (*Sympetrum* sp.)	whole organism (ww)		0.39-0.44	Cerveny et al., 2021b
	Bluegill sunfish (*Lepomis macrochirus*)	Muscle (ww)		1.08-5.69	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Liver (ww)		9.17-25.5	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Brain (ww)		4.42-15.1	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Bile (ww)		2350-4940	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	plasma		3.85-7.92	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Gut (ww)		23.9-139.5	Zhao et al., 2017
	Bluegill sunfish (*Lepomis macrochirus*)	Gill (ww)		2.56-5.81	Zhao et al., 2017
	Sea trout (*Salmo trutta*)	muscle (ww)		7.68	McCallum et al., 2019
Terbinafine	Plankton	(ww)	340		Grabicová et al., 2020
Tetracycline	Clams (*Crassostrea gigas, Patinopecten yessoensis, Chlamys farreri*)	whole organism (ww)	1677		Na et al., 2013
Tramadol	A. Common carp (*Cyprinus carpio*)	liver (ww)	2.8		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	2.4		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	5.9		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	(ww)	6.2		Grabicová et al., 2020
	Stone moroko (*Pseudorasbora*	(ww)	3.8		Grabicová et al., 2020

	parra)				
	Plankton	(ww)	29		Grabicová et al., 2020
	Brown trout (*Salmo trutta*)	liver (ww)	1.2-5.0		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	kidney (ww)	10-110		Grabicová et al., 2017
	Common carp (*Cyprinus carpio* L.)	Liver (ww)	2.6-16		Sims et al., 2020
Trimethoprim	Fish predicted	-	1		Lagesson et al., 2016
	Invertebrate predicted	-	0.2		Lagesson et al., 2016
	Shrimp (Young *Fenneropenseus penicillatus*)	whole organism (ww)		63	Chen et al., 2015
	Fish (Young *Lutjanus russelli*)	muscle (ww)		6488	Chen et al., 2015
	Fish (*Epinephelus awoara*)	muscle (dw)		1413	Xie et al., 2019
	Fish (*Ephippus orbis*)	muscle (dw)		1259	Xie et al., 2019
	Shellfish (*Ostrea gigas*)	whole organism (dw)		794	Xie et al., 2019
	Shellfish (*Mimachlamys nobilis*)	whole organism (dw)		794	Xie et al., 2019
	Shellfish (*Mytilus edulis*)	whole organism (dw)		1585	Xie et al., 2019
	Shellfish (*Bufonaria perelegans*)	whole organism (dw)		398	Xie et al., 2019
	Plankton	(ww)	42		Grabicová et al., 2020
	Wild carp (*Cyprinus carpio*)	plasma	129-577		Muir et al., 2017
Venlafaxine	Limpet *(Ancylus fluviatilis)*	whole organism (dw)	0.559-1.319		Ruhi et al., 2016
	Caddisfly *(Hydropsyche sp.)*	whole organism (dw)	0.559-1.319		Ruhi et al., 2016
	Flatworm *(Phagocata vitta)*	whole organism (dw)	0.559-1.319		Ruhi et al., 2016
	Predicted	-	1.55		Ruhi et al., 2016
	Mussel (*Mytilus galloprovincialis*)	whole organism (dw)		265	Gomes et al., 2021
	Loach (*Misgurnus anguillicaudatus*)	liver (ww)		0.04-0.14	Qu et al., 2019
	Loach (*Misgurnus anguillicaudatus*)	liver (ww)		0.06-0.92	Qu et al., 2019
	A. Common carp (*Cyprinus carpio*)	liver (ww)	16		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	5		Grabicová et al., 2020
	A. Common carp (*Cyprinus carpio*)	kidney (ww)	26		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	9.2		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	brain (ww)	12		Grabicová et al., 2020
	B. Pikeperch (*Sander lucioperca*)	muscle (ww)	0.4		Grabicová et al., 2020
	Stone moroko (*Pseudorasbora parva*)	(ww)	3.8		Grabicová et al., 2020
	Plankton	(ww)	180		Grabicová et al., 2020
	Mussel (*M. Galloprovincialis*)	whole organism (dw)		213-528	Serra-Compte et al., 2018
	Brown trout (*Salmo trutta*)	liver (ww)	13-15		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	kidney (ww)	22-44		Grabicová et al., 2017
	Brown trout (*Salmo trutta*)	muscle (ww)	3.3		Grabicová et al., 2017
	Common carp (*Cyprinus carpio* L.)	Liver (ww)	15-51		Sims et al., 2020
	Caged goldfish (*Carassius auratus*)	plasma	5-31		Muir et al., 2017
	Wild carp (*Cyprinus carpio*)	plasma	5-32		Muir et al., 2017
	Juvenile meagre (*Argyrosomus regius*)			64.6	Maulvault et al., 2018
	Fish (smallmouth bass)	gonad (dw)	1		Arnnot et al., 2017
	Fish (smallmouth bass)	liver (dw)	7-20		Arnnot et al., 2017
	Fish (smallmouth bass)	muscle (dw)	1		Arnnot et al., 2017
	Fish (largemouth bass)	gonad (dw)	3-4		Arnnot et al., 2017
	Fish (largemouth bass)	liver (dw)	3		Arnnot et al., 2017
	Fish (largemouth bass)	muscle (dw)	1-2		Arnnot et al., 2017
	Fish (rudd)	muscle (dw)	1		Arnnot et al., 2017
	Fish (rock bass)	liver (dw)	4		Arnnot et al., 2017
	Fish (rock bass)	muscle (dw)	1		Arnnot et al., 2017
	Fish (white bass)	gonad (dw)	6		Arnnot et al., 2017
	Fish (white bass)	liver (dw)	1		Arnnot et al., 2017
	Fish (white perch)	liver (dw)	11		Arnnot et al., 2017
	Fish (white perch)	muscle (dw)	1		Arnnot et al., 2017
	Fish (walleye)	gonad (dw)	7-14		Arnnot et al., 2017
	Fish (walleye)	muscle (dw)	1		Arnnot et al., 2017

	Fish (steelhead)	muscle (dw)	1	Arnnot et al., 2017
	Fish (yellow perch)	gonad (dw)	9	Arnnot et al., 2017
	Fish (yellow perch)	liver (dw)	84-150	Arnnot et al., 2017
	Biofilm		**2316**	Mastrángelo et al., 2022
	B. Pikeperch (*Sander lucioperca*)	liver (ww)	700	Grabicová et al., 2020
Verapamil	B. Pikeperch (*Sander lucioperca*)	kidney (ww)	870	Grabicová et al., 2020
	Plankton	(ww)	**9100**	Grabicová et al., 2020
	Fish, channel catfish	plasma	0.7	Nallani et al., 2016
	Fish, channel catfish	heart (ww)	5.2	Nallani et al., 2016
	Fish, channel catfish	muscle (ww)	1.3	Nallani et al., 2016
	Fish, channel catfish	gill (ww)	6.7	Nallani et al., 2016
	Fish, channel catfish	kidney (ww)	46.5	Nallani et al., 2016
	Fish, channel catfish	liver (ww)	13.4	Nallani et al., 2016
	Fish, fathead minnow	heart (ww)	14.6	Nallani et al., 2016
	Fish, fathead minnow	muscle (ww)	17.3-23.1	Nallani et al., 2016
	Fish, fathead minnow	gill (ww)	29.3-40.3	Nallani et al., 2016
	Fish, fathead minnow	kidney (ww)	34.4-74	Nallani et al., 2016
	Fish, fathead minnow	liver (ww)	40-75	Nallani et al., 2016

In bold values classified as accumulative

In bold values classified as accumulative

Some PhACs have a considerable number of reported BCF/BFA values. Specifically, there are more than 35 observations for the antihistamine diphenhydramine (n = 52), followed by carbamazepine (n = 48), diclofenac (n = 41), and the antidepressant therapeutic group (venlafaxine (n = 42), citalopram (n = 40) and sertraline (n = 40) and their metabolite norsertraline (n=22). There are only one or two BCF/BAF values for 52% of the PhACs and there are five or fewer reported BCF/BAF values for 62% of the PhACs. To the best of our knowledge, there are no observations for the anticancer drug group, with the exception of 5-fluorouracile.

Figures 1 and 2 show the general trend of the bibliographic data in this field of research. It can be observed that the distribution for individual PhACs is not uniform.

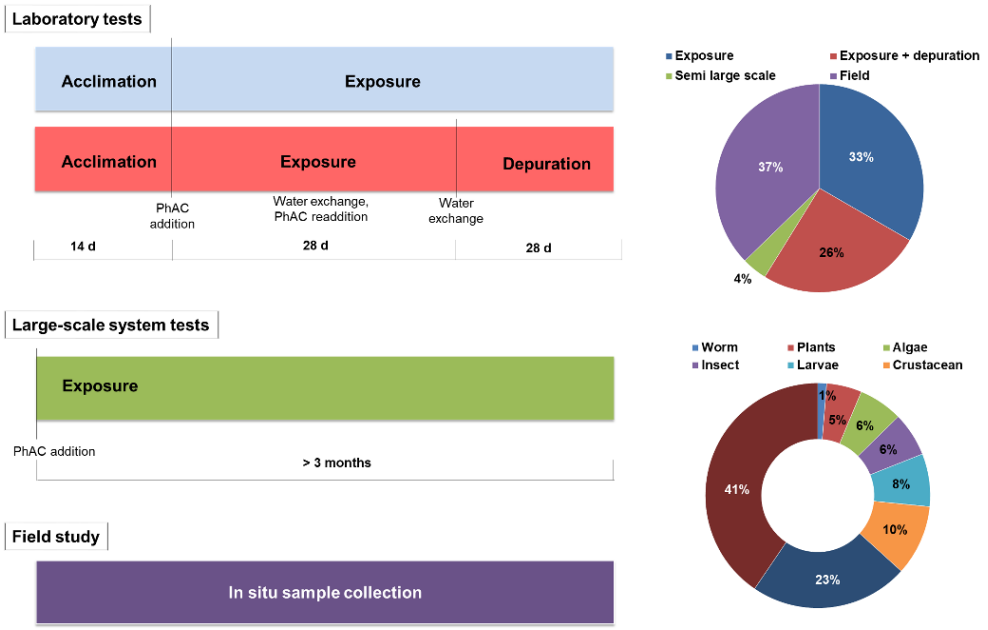

Figure 1. *Schematic representation of the possible scenarios to assess bioconcentration/bioaccumulation potential*

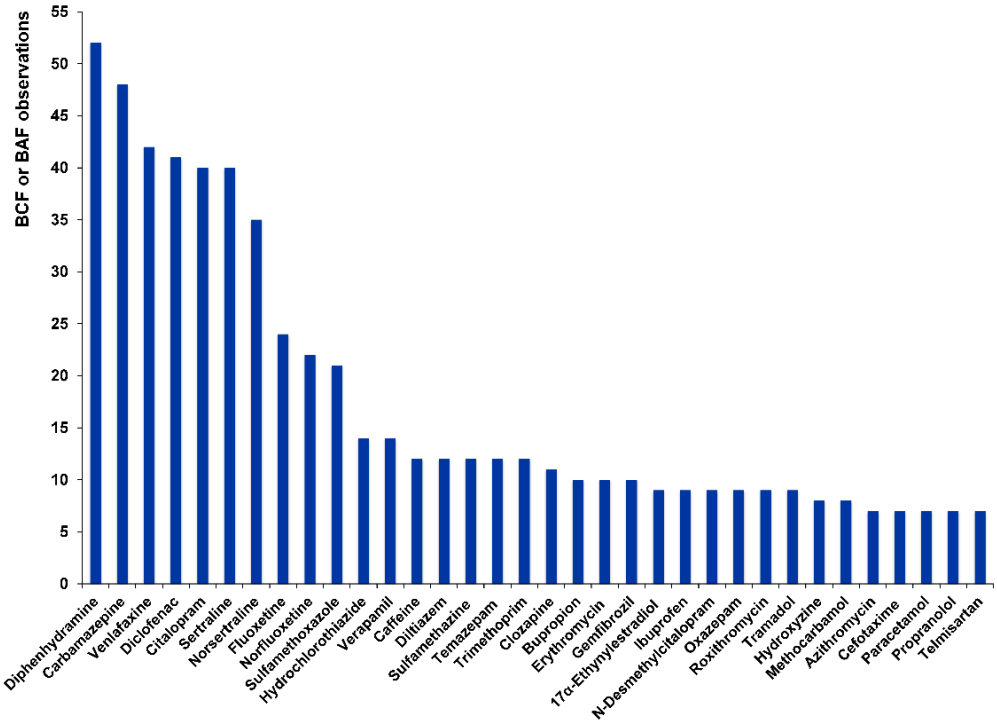

Figure 2. *PhACs most frequently studied in bioaccumulation assays*

One of the main explanations for the lack of data uniformity is the disparate factors used in the design of the surveys (water temperature, exposure time, exposure route, dissolved organic matter, feeding and growth rates, or selected tissue or organ). BCF values are highly influenced by experimental and biotic factors (Duarte *et al.*, 2022). Some authors have pointed to temperature and exposure time as the variables higher weight in BCF data. Maulvault *et al.* (2018) assessed the effect of increased temperature (ΔT°C = 5 ºC) and high CO_2 levels (equivalent to ΔpH = - 0.4 units). Their data suggest an increase of BCF with the combination of warming and acidification. Recently, Cerveny *et al.* (2021) assessed how temperature affects temazepam biotransformation and the subsequent accumulation of its metabolite (oxazepam) in two organisms of the food chain ecosystem (the European perch (*Perca fluviatilis*) and the dragonfly larvae (*Sympetrum* sp.). Their results showed that exposure to PhACs may change across temperature gradients in the environment. While the bioconcentration of temazepam in perch was reduced at higher temperatures as a consequence of the biotransformation and accumulation of its main metabolite in the fish (two-fold higher at 20ºC compared to 10ºC), no temperature influence was found for larvae. Temperature may affect metabolic activity and, as a consequence, the bioconcentration pattern (Buckman *et al.*, 2004). In a previous study, Opperhuizen *et al.* (1988) observed an increase in BCF values for chlorinated benzenes with increasing water temperature. In other research, Lu *et al.* (2018) reported that in the presence of higher dissolved organic matter content (DOM), the potential bioconcentration of diclofenac in fish decreased significantly. Feeding and the hydrodynamic experiment also led to lower bioaccumulation of diclofenac in fish tissues.

Taking into account the route of exposure, in the case pf PhACs exposure through water may be more important for uptake and bioaccumulation rates than dietary exposure (Du et al., 2015). For example, Maulvault *et al.* (2018) observed plasma concentration levels of venlafaxine 50 times higher in fish exposed via water (46 µg/L day) compared to fish exposed via diet (0.5 µg/L day). Nune *et al.* (2020)

suggested that direct (waterborne) and trophic (via contaminated feed) exposures to diclofenac caused significantly different physiological modifications in aquatic organisms. Variability of results is reduced if standard protocols are met and with better knowledge of the key experimental parameters. Heynen *et al.* (2016) compare uptake of oxazepam from water (bioconcentration) and via a contaminated diet (trophic transfer) in Eurasian perch *(Perca fluviatilis*) and dragonfly larvae (*Aeshna grandis*). Bioconcentration and trophic transfer of oxazepam were found in both predator species. However, higher bioconcentrations were observed for perch (BCF 3.7) than for dragonfly larvae (BCF 0.5). The relative contribution via prey consumption was 14% and 42% for perch and dragonflies, respectively.

Though theoretically BCF should not be affected by the concentration to which the organism is exposed since it is a result of the difference between the absorption and elimination processes, different levels were regularly tested in the reviewed literature. In some cases, a different bioconcentration pattern has been observed, suggesting that this factor must be taken into consideration (Świacka *et al.,* 2020; Lopes *et al.,* 2020; Lu *et al.,* 2018; McCallum *et al.,* 2019; Pan *et al.,* 2018; Molina-Fernández *et al.,* 2021; Xie *et al.,* 2020; Brozinski *et al.,* 2013). Pan *et al.* (2018) reported that the BCF of fluoxetine in red crucian carp was inversely proportional to the exposure concentrations. Similarly, Zhao et al. (2016) observed that the BCF of sulfamethazine at a concentration of 40 ng mL^{-1} was higher than at 200 ng mL^{-1}. Rosa *et al.* (2019) observed that the oxytetracycline levels in the macroalgae *Ulva* decreased significantly slower at 40 ng mL^{-1} (48 h) than at 120 ng mL^{-1} (24 h).

On the other hand, for organic substances there is a clear correlation between the lipid content of biota and BCF values (Miller *et al.,* 2019; Gobas *et al.*, 1999). Hydrophobic substances (log K_{ow}>3) reach equilibrium in the lipid fraction and the bioaccumulation potential is theoretically indicated by log Kow. Arnot and Gobas (2006) reported that doubling the lipid content approximately doubles the BCF. Thus it is important to normalize lipids to reduce variability and allow better

comparison of results. However, bioaccumulation is also empirically demonstrated by organism concentrations exceeding the surrounding water concentration (Meredith-Williams *et al.*, 2012; Grabicoba *et al.*, 2015). Variations of the pH in the exposure system may significantly change the ionization states of PhACs and consequently influence their bioaccumulation. A number of studies on aquatic organisms and on a range of PhACs (sertraline, fluoxetine, sulfathiazole, ciprofloxacin, lincomycin, enrofloxacin and chlortetracycline, ibuprofen or acetaminophen) have demonstrated that uptake and toxicity of ionizable PhACs can also be very sensitive to changes in pH of the environment (Meredith-Williams *et al.*, 2012; Rendal *et al.*, 2009; Valenti *et al.*, 2009). For example, Ding et *al.* (2016) examined the bioconcentration profiles of roxithromycin and propranolol in *D. magna* after 24 h of exposure under different pH levels (7-9). Their results showed that daphnia body burdens were strongly dependent on pH and increased with increasing pH values. The fractions of neutral species, which are more lipophilic than the corresponding ionic species, increase with increasing pH levels. Because almost 80% of all pharmaceuticals are ionizable (Manallack, 2008), thus having a pH-dependent neutral species distribution, the K_{ow} may be a less reliable predictor of bioaccumulation than for neutral organic chemicals. There is no apparent relationship between BCF values and log K_{ow} for PhACs (Duarte *et al.,* 2022). The statistical correlation between accumulation data reported in Table 2 (BCF and BAF) and log Kow was $R^2<0.1$. Therefore, understanding and establishing a framework for the bioaccumulative behavior of PhACs is crucial for assessing risks for aquatic ecosystems.

Bioconcentration is also controlled by organism tissue components other than lipids. For example, Arnnok *et al.* (2017) and Lu *et al.* (2018) found higher bioaccumulation patterns in brain, liver or gill tissue compared with muscle tissue, indicating the possibility of distribution variance across tissues. The data are not normalized for lipids because, as the authors explain, it is not appropriate to do this for ionizable compounds (Haddad *et al.,* 2018; Ramirez *et al.,* 2009; Grabicová *et al.,* 2020). In fact, a new "non-classical" bioaccumulation behaviour is

observed where some authors have suggested that proteins may have a significant effect on the PhAC bioaccumulation process (Duarte *et al.,* 2022; Maculewicz *et al.,* 2021). Kowalska *et al.* (2021) demonstrated higher affinity of drug metabolites for blood proteins than for lipids. Haddad *et al.* (2018) showed that normalization of ionizable PhACs to neutral lipid fractions is inappropriate.

Special attention is also being paid to metabolites and transformation products. These can be just as dangerous, if not more so, than their parent compounds (Swiacka *et al.,* 2022; Maculewicz *et al.,* 2021). Metabolites usually have high hydrolytic stability and this increases the likelihood that they will accumulate in the tissues of organisms (Kowalska *et al.,* 2021). An example of this is antidepressants. A study conducted by Arnnok *et al.* (2017) reported a high bioaccumulation (BCF up to 3000) of norsertraline (sertraline metabolite). This metabolite accumulates mainly in the liver and brain of fish. The BCF for sertraline (more lipophilic) was lower than 100. The authors attribute this to the metabolization of sertraline by fish, although they also state that further studies are needed to confirm the process. The same mechanism was not observed for norfluoxetine (the main metabolite of fluoxetine) whose BCF was <130. A study developed by Zhao *et al.* (2016), in medaka (*O. melastigma*) after exposure to sulfamethazine showed that its metabolite (acetylsulfamethazine) accumulated more readily in the organism (mainly in the gonad), presenting a different distribution pattern from that of the parent compound.

Reported investigations also suggest the probability of misestimating the risks to aquatic organisms when not considering certain environmental scenarios. The presence of other substances, for instance, seems to affect PhAC bioaccumulation in organisms. Co-exposure of Cu and diclofenac (100 and 1000 µg L^{-1}) significantly decreased drug accumulation in crucian carp compared to single exposure to diclofenac (Xie et al., 2020). The mixture at higher levels also led to severer hepatic oxidative stress. However, co-exposure studies are relatively recent and in some cases contradictory. Zhang *et al.* (2019) found that the presence

of microplastics increases the bioaccumulation of roxithromycin in red tilapia fish tissue, while the study by Wang *et al.* (2020) indicated that co-exposure with nanoplastics leads to reduced bioaccumulation and accelerated biodegradation of ibuprofen in freshwater algae. These results suggest the likelihood of erroneously estimating risks to aquatic organisms without taking environmental factors into account. Thus, when BAF data are available they should be considered the better source of information of the bioaccumulative potential of a substance. According with reported data, BAF values are superior to BCF values, highlighting the importance of field studies for reliable assessment under real conditions (Figure 3). For example, for citalopram and its main metabolite, the mean BAF values are almost 100 and 1000 times higher respectively than the corresponding BCF values. This was also observed by Arnot and Gobas (2006) when both factors were compared for a total of 350 organic substances.

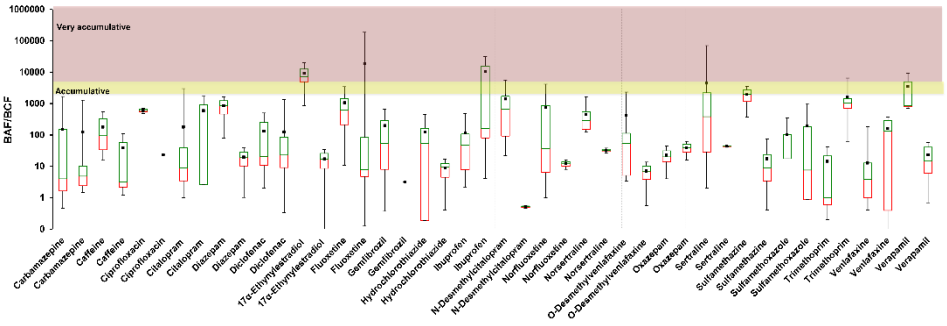

Figure 3. *Box-and-whisker plots of BAF (left box) and BCF (right box) observations of PhACs*

While the majority of PhACs are relatively water soluble and are generally non-bioaccumulative, resident biota can be chronically exposed to them due to the continuous release of these active compounds into ecosystems. Overall, a substance is usually considered very bioaccumulative if BAF values are >5000 L/kg in aquatic organisms, and as bioaccumulative if the BAF values are between 2000 to 5000 L/kg (Government of Canada, 2000). Some authors have used this general classification given that, in practice, there is no unified classification criterion for the bioaccumulation potential of PhACs (Burket *et al.*, 2020;

de Solla *et al.*, 2016; Chen *et al.*, 2015; Na *et al.*, 2013). According to reported data, although most PhACs are considered non-bioaccumulative (BAF <2000 L/kg), we found that 38 out of 113 PhACs identified in this review ever exceed the BAF of 2000 criterion (although it was <12% and 6% of the BAF and BCF data). In a study with 20 antibiotics in the coastal environment of Dalian (China) using three clam species as target aquatic organisms, Nu *et al.* (2013) categorized sulfamethazine, sulfamethiazole, sulfamonomethoxine, and doxycycline as potentially bioaccumulative, while sulfadiazine, sulfameter, sulfamethoxypyridazine, and chloramphenicol were bioaccumulative.

Like BCF data, reported BAF data vary between studies and organisms. One of the main reasons for this variability is ecosystem type. The spatial and temporal variability associated with sampling is a major difficulty in obtaining reliable bioaccumulation information from field data. The steady-state assumption may not always be correct. For the evaluation to be representative, long-term conditioning of the study area is recommended (Burkhard *et al.,* 2013; Arnot and Gobas, 2006; USEPA, 2000). Reported studies describe between five (Xie *et al.*, 2019) and twenty (Na *et al.*, 2013) sampling areas and a sampling frequency between one (Chen *et al.,* 2015) and two (Na *et al.,* 2013). However, it is very difficult to carry out true random sampling as a consequence of the high economic and ecological costs (Swiacka *et al.,* 2022). BAF data are complex and seem organ, species and compound-specific. A large study conducted by Huerta *et al.* (2018) investigated the prevalence of PhACs in fish representing different trophic niches from 25 U.S. rivers and streams. The results suggested that the uptake of PhACs may be selective. Freshwater omnivorous fish accumulated a greater variety of PhACs of different therapeutic categories than the co-habitant carnivores and invertivores. Rojo *et al.* (2019) obtained similar conclusions.

Grabicová *et al.* (2020) reproduced a common aquaculture practice to evaluate the accumulation of PhACs in common prey of one omnivorous and one piscivorous fish for a period of 6 months. The authors found different bioaccumulation rates of PhACs between fish species. It is also

interesting to note that in this study the highest levels of PhACs were found in plankton with BAF >4000. In addition, organ-specific bioaccumulation was very clear for sertraline among other PhACs (brain > liver > kidney) for both species, while low concentrations were found in muscle tissue. Bao *et al.* (2020) reported differences in PhAC bioaccumulation among 5 wild fish species from Taihu Lake (China). Medroxy-progresterone was the PhAC with the highest BAF (1474 L kg^{-1}) in *C. carpio,* hexesestrol (1400 L kg^{-1}) in *C. auratus,* dienoestrol (893 L kg^{-1}) in *H. molitrix* and *A. nobilis,* and D-norgestrel (2460 L kg^{-1}) in *Anabarilius sp.* Similar results were also obtained by Du *et al.* (2016), Haddad *et al.* (2017) and Rojo *et al.* (2019). It has also been reported that kinetic differences and particular metabolic biotransformation can lead to differences in the bioaccumulation potential of certain life-stages (Swiacka *et al.,* 2022).

Finally, an important limitation is that most of the studies only determined PhACs in biota and water and did not examine suspended solids or sediments on which the more hydrophobic PhACs will be adsorbed. In a screening study considering 66 PhACs in the Tejo Estuary, Fonseca *et al.* (2021) reported that only 2 compounds were found simultaneously in water and biota, demonstrating the complex dynamics and behaviour of PhACs. Nevertheless, higher detection frequencies were observed in benthic and demersal species living directly on or just above the substrate, supporting the combined roles of sediment and dietary routes of PhAC uptake. Many other studies (Oetken *et al.,* 2005; Lagesson *et al.,* 2016; Xie *et al.,* 2017; Wilkinson *et al.,* 2018) have emphasized that filter-feeding organisms concentrate higher amounts of PhACs, due to their higher polluted environment and ingestion of organic matter from sediments.

5.1. Food web transfer

An important aspect to be addressed in the present review is the transfer of PhACs to the food web. The data on trophic transfer of PhACs are still very limited (Ruan *et al.,* 2020; Du *et al.,* 2014; Ruhi *et al.,* 2016; Lagesson *et al.,* 2016) although a general trend indicates that lower

trophic position organisms bioaccumulate PhACs to a greater extent than higher trophic position organisms (Ding *et al.,* 2015; Vernouillet *et al.,* 2010; Xie *et al.,* 2017; Du *et al.,* 2014; Ruhi *et al.,* 2016). Detritivores and herbivores, benthic primary consumers at lower trophic levels, were confirmed as the primary bioaccumulators of PhAC contamination in a semi-natural pond ecosystem (Lagesson *et al.,* 2016). PhACs were quantified at concentration levels ranging from <0.03 to 5.88 ng g^{-1} w.w. in 24 species of molluscs, crustaceans and fish in a subtropical marine food web (Ruan *et al.,* 2020). Trophic dilution was observed. Generally, invertebrate organisms had higher concentration levels than fish (TMFs 0.164 and 0.517 for atenolol and chloramphenicol, respectively). This can be explained by the fact that higher-level organisms have a greater capacity to metabolize substances. Similarly, trophic dilution was reported for 6 antidepressants (TMFs 0.01-0.71) in a semi-arid urban river, influenced by snowmelt and downstream from a municipal effluent discharge. The results were comparable at all locations and in all seasons, regardless of the different exposure conditions and concentrations (Haddad *et al.,* 2018). Du et al. (2014) reported that PhACs accumulated in higher concentrations in invertebrates compared to fish in samples from an effluent-dependent stream. The authors reported a TMF of 0.38 and 1.17 for diphenhydramine and carbamazepine, respectively. The compounds detected in all the analysed species showed that none of them experienced trophic biomagnification. The study carried out by Xie *et al.* (2017) in the second largest lake in China (Taihu Lake) revealed the presence of antibiotics, NSAIDs and hormones in plankton, zoobenthos, shrimp and fish, the second of these recording the highest concentrations. No biomagnification was observed.

These results support the data that waterborne and not dietary exposures represent the primary route of fish uptake. Nevertheless more research is needed on the use of TMFs in bioaccumulation assessments and regulatory considerations (Swiacka *et al.,* 2022; Haddad *et al.,* 2018).

6. Conclusions

This review summarises all the recent advances examining bioaccumulation of PhACs in aquatic organisms. A total of 231 BCFs and 531 BAF determined for 113 PhACs have been collected. Without a doubt, there is much more data on fish and molluscs (63% of the collected data) compared with crustaceans (10%), insects (8%) and algae or larvae (6%). Large differences in reported data (organ, species and compound-specific) have been found. Some PhACs such as the antidepressant group, diclofenac or carbamazepine have been extensively studied in comparison with other groups of pharmaceuticals.

The results of the literature survey showed that, despite the number of works published on bioaccumulation in aquatic organisms which corroborate the importance of this topic, some aspects still require additional consideration.

1) There is an urgent need for more data on certain therapeutic groups of PhACs, such as anticancer drugs. In addition, certain PhACs can accumulate significantly in the body of aquatic organisms through biotransformation of the parent compound, without being present in the water at all. Metabolization and biotransformation have been shown to be an important exposure pathway, contributing significantly to direct uptake from the water. Therefore, PhAC metabolites should be given more attention in future research, as many can exert pharmacological effects comparable to parent drugs.

2) Water characteristics such as temperature and pH or DOM have been shown to significantly affect bioconcentration of certain PhACs in aquatic organisms. While physico-chemical properties of water vary greatly, knowledge about their role in the uptake, metabolic transformation, and excretion of PhACs is still limited. Temperature, for instance, is cause for concern given that this factor is a fundamentally important environmental variable influencing standard metabolic rates, for example in fish (Clarke and Johnston, 1999; Killen *et al.*, 2010; Ohlberger *et al.*, 2012). On the other hand, improving scientific

knowledge requires stricter adherence to standard protocols and better documentation of the key experimental parameters. The complexity and variability of the results will be reduced with compliance to specific criteria.

3) BCF data from PhACs have been poorly correlated with lipophilicity. It seems that ionizable chemicals follow a new "non-classical" bioaccumulation behaviour where proteins may have a significant effect on the process. Therefore, it is extremely important to conduct further studies using a broader group of compounds to elucidate these relationships and assess to what extent their affinity for blood proteins translates into their potential for bioaccumulation.

4) Recent co-exposure studies have also flagged the likelihood of underestimating the risks to aquatic organisms by not taking into account an environmental scenario since, in the natural environment, PhACs occur as complex mixtures and in the company of other contaminants that could cause a dissimilar effect on the organism. Reported BAF values are superior to laboratory BCF values, highlighting the importance of field studies for reliable assessment and the best reflection of natural conditions. Some practices, such as taking into account long-term average conditions of the studied area, or the use of well-calibrated passive samplers, are crucial for reliable results and accurately calculated field-derived BCF values, respectively.

5) Finally, and regarding trophic transfer in aquatic ecosystems, benthic primary consumers at lower trophic levels concentrate higher amounts of PhACs due to the higher polluted environment and the ingestion of organic matter from sediments. Waterborne rather than dietary exposure represent the primary route of uptake of fish although, to date, the studies are too limited and the data insufficient to draw clear conclusions. Further research should be also conducted to study the bioaccumulation of PhACs in non-target species and other trophic positions.

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

References

Álvarez-Ruiz, R., Picó, Y., 2020. Analysis of emerging and related pollutants in aquatic biota. Tr. Environ. Anal. Chem. 25, e00082. https://doi.org/10.1016/j.teac.2020.e00082

Álvarez-Ruiz, R., Picó, Y., Campo, J., 2021. Bioaccumulation of emerging contaminants in mussel (Mytilus galloprovincialis): Influence of microplastics. Sci. Total Environ. 796, 149006. https://doi.org/10.1016/j.scitotenv.2021.149006.

Arenas, M., Martín, J., Santos, J.L., Aparicio, I., Alonso, E., 2021. An overview of analytical methods for enantiomeric determination of chiral pollutants in environmental samples and biota. Tr. Anal. Chem. 43, 116370. https://doi.org/10.1016/j.trac.2021.116370

Arguello-Pérez, M.Á., Ramírez-Ayala, E., Mendoza-Pérez, J.A., Monroy-Mendieta, M.M., Vázquez-Guevara, M., Lezama-Cervantes, C., Godínez-Domínguez, E., de Asís Silva-Bátiz, F., Tintos-Gómez, A., 2020. Determination of the bioaccumulative potential risk of emerging contaminants in fish muscle as an environmental quality indicator in

coastal lagoons of the central mexican pacific. Water 12(10), 2721. https://doi.org/10.3390/w12102721

Arnnok, P., Singh, R.R., Burakham, R., Pérez-Fuentetaja, A., Aga, D.S., 2017. Selective uptake and bioaccumulation of antidepressants in sh from ef uent-impacted Niagara River. Environ. Sci. Technol. 51(18), 10652–10662. https://doi.org/10.1021/acs.est.7b02912

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(4), 257-297. https://doi.org/10.1139/A06-005

Boillot, C. Martinez Bueno, M.J. Munaron, D. Le Dreau, M. Mathieu, O. David, A. Fenet, H. Casellas, C. Gomez, E., 2015. In vivo exposure of marine mussels to carbamazepine and 10-hydroxy-10,11-dihydro-carbamazepine: Bioconcentration ad metabolization. Sci. Total Environ. 532, 564–570. http://dx.doi.org/10.1016/j.scitotenv.2015.05.067

Bonnefille, B., Arpin-Pont, L., Gomez, E., Fenet, H., Courant, F., 2017. Metabolic profiling identification of metabolites formed in Mediterranean mussels (Mytilus galloprovincialis) after diclofenac exposure. Sci. Total Environ. 583, 257-268. http://dx.doi.org/10.1016/j.scitotenv.2017.01.063

Boström, M.J., Ugge, G., Jönsson, J.A., Berglund, O., 2017. Bioaccumulation and trophodynamics of the antidepressants sertraline and fluoxetine in laboratory-constructed, 3-level aquatic food chains. Environ. Toxicol. Chem. 36(4), 1029-1037. doi: 10.1002/etc.3637.

Brozinski, J.M., Lahti, M., Oikari, A., Kronberg, L., 2013. Identification and dose dependency of ibuprofen biliary metabolites in rainbow trout. Chemosphere 93(9), 1789-95. http://dx.doi.org/10.1016/j.chemosphere.2013.06.018

Buckman, A.H., Brown, S.B., Hoekstra, P.F., Solomon, K.R., and Fisk, A.T., 2004. Toxicokinetics of three polychlorinated biphenyl technical mixtures in Rainbow trout (Oncorhynchus mykiss). Environ. Toxicol. Chem., 23:1725-1736.

Burket, S.R., Wright, M.V., Baker, L.F., Chambliss, C.K., King, R.S., Matson, C.W., Brooks, B.W., 2020. Periphyton, bivalves and fish differentially accumulate select pharmaceuticals in effluent-dependent stream mesocosms. Sci. Total Environ. 745, 140882. https://doi.org/10.1016/j.scitotenv.2020.140882.

Burkhard, L.P., Borga, K., Powell, D.E., Leonards, P., Muir, D.C.G., Parkerton, T.F., Woodburn, K.B., 2013. Improving the quality and scientific understanding of trophic magnification factors (TMFs). Environ. Sci. Technol. 47, 1186-1187. http://dx.doi.org/10.1021/es305253r

Cerveny, D., Fick, J., Klaminder, J., Bertram, M.G., Brodin, T., 2021a. Exposure via biotransformation: Oxazepam reaches predicted pharmacological effect levels in European perch after exposure to temazepam. Ecotoxicol. Environ. Saf. 217, 112246. https://doi.org/10.1016/j.ecoenv.2021.112246

Cerveny, D., Fick, J., Klaminder, J., McCallum, E.S., Bertram, M.G., Castillo, N.A., Brodin, T., 2021b. Water temperature affects the biotransformation and accumulation of a psychoactive pharmaceutical and its metabolite in aquatic organisms. Environ. Int. 155, 106705. https://doi.org/10.1016/j.envint.2021.106705

Chen, H., Liu, S., Xu, X.R., Liu, S.S., Zhou, G.J., Sun, K.F., Zhao, J-L., Ying, G-G., 2015. Antibiotics in typical marine aquaculture farms surrounding Hailing Island, South China: Occurrence, bioaccumulation and human dietary exposure. Mar. Pollut. Bull. 90(1–2), 181-187. http://dx.doi.org/10.1016/j.marpolbul.2014.10.053

Chen, L., Guo, C., Sun, Z., Xu, J., 2021. Occurrence, bioaccumulation and toxicological effect of drugs of abuse in aquatic ecosystem: A review. Environ. Res. 200, 111362. https://doi.org/10.1016/j.envres.2021.111362

Daniele, G., Fieu, M., Joachim, S., James-Casas, A., Andres, S., Baudoin, P., Bonnard, M., Bonnard, I., Geffard, A., Vulliet, E., 2016. Development of a multi-residue analysis of diclofenac and some transformation products in bivalves using QuEChERS extraction and liquid chromatography-tandem mass spectrometry. Application to samples from mesocosm studies. Talanta 155, 1-7. https://doi.org/10.1016/j.talanta.2016.04.016

de Solla, S.R., Gilroy, È.A.M., Klinck, J.S., King, L.E., McInnis, R., Struger, J., Backus, S.M., Gillis, P.L., 2016. Bioaccumulation of pharmaceuticals and personal care products in the unionid mussel Lasmigona costata in a river receiving wastewater effluent, Chemosphere, 146, 486-496. https://doi.org/10.1016/j.chemosphere.2015.12.022.

Ding, J., Lu, G., Li, S., Nie, Y., Liu, J., 2015. Biological fate and effects of propranolol in an experimental aquatic food chain. Sci. Total Environ. 532, 31-39. https://doi.org/10.1016/j.scitotenv.2015.06.002

Ding, J., Lu, G., Liu, J., Yang, H., Li, Y., 2016. Uptake, depuration, and bioconcentration of two pharmaceuticals, roxithromycin and propranolol, in Daphnia magna, Ecotoxicol. Environ. Saf. 126, 85-93. https://doi.org/10.1016/j.ecoenv.2015.12.020.

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., Brooks, B.W., 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. http://dx.doi.org/10.1098/rstb.2014.0058

Duarte, I.A., Fick, J., Cabral, H.N., Fonseca, V.F., 2022. Bioconcentration of neuroactive pharmaceuticals in fish: Relation to lipophilicity, experimental design and toxicity in the aquatic environment. Sci. Total Environ. 812, 152543. http://dx.doi.org/10.1016/j.scitotenv.2021.152543

Ericson, H., Thorsén, G., Kumblad, L., 2010. Physiological effects of diclofenac, ibuprofen and propranolol on Baltic Sea blue mussels. Aquat. Toxicol. 99(2), 223-231. https://doi.org/10.1016/j.aquatox.2010.04.017

European Commission, 2018. EUR-Lex – Commission implementing decision (EU) 2018/ 840 of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495. Off. J. Eur. Union L141, 9-12.

European Commission, 2020. EUR-Lex – Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 may 2020 on minimum requirements for water reuse. Off. J. Eur. Union L177, 32-54.

European Commission, 2022. EUR-Lex – Regulation (EU) 2022/1307 of the European Parliament and of the Council of 22 july 2022 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. Off. J. Eur. Union L197, 117-121.

Fonseca, V.F., Duarte, I.A., Duarte, B., Freitas, A., Pouca, A.S.V., Barbosa, J, Gillanders, B.M., Reis-Santos, P., 2021. Environmental risk assessment and bioaccumulation of pharmaceuticals in a large urbanized estuary. Sci. Total Environ. 783, 147021. https://doi.org/10.1016/j.scitotenv.2021.147021

Garcia, S.N., Foster, M., Constantine, L.A., Huggett, D.B., 2012. Field

and laboratory fish tissue accumulation of the anti-convulsant drug carbamazepine. Ecotoxicol. Environ. Saf. 84, 207-211. https://doi.org/10.1016/j.ecoenv.2012.07.013.

Gillis, P.L., Gagne, F., McInnis, R., Hooey, T.M., Choy, E.S., Andre, C., Hoque, M.E., Metcalfe, C.D., 2014. The impact of municipal wastewater effluent on field- deployed freshwater mussels in the Grand River (Ontario, Canada). Environ. Toxicol. Chem. 33, 134-143. DOI: 10.1002/etc.2401

Gobas F.A.P.C., de Wolf W., Burkhard L.P., Verbruggen E. and Plotzke K., 2009. Revisiting bioaccumulation criteria for POPs and PBT assessments. Integr. Environ. Assess. Manag. 5, 624-637. https://doi.org/10.1021/acs.est.7b02912

Gobas, F.A.P.C., Wilcockson, J.B., Russell, R.W., and Haffner, G.D., 1999. Mechanism of biomagnification in fish under laboratory and field conditions. Environ. Sci. Technol. 33, 133-141. https://doi.org/10.1021/es980681m

Gomez, E., Boillot, C., Martinez Bueno, M.J., Munaron, D., Mathieu, O., Courant, F., Fenet, H., 2021. In vivo exposure of marine mussels to venlafaxine: bioconcentration and metabolization. Environ. Sci. Pollut. Res. 28(48), 68862-68870. https://doi.org/10.1007/s11356-021-14893-4

Government of Canada, 1999. Canadian Environmental Protection Act, 1999. Canada Gazette Part III. 22. Public Works and Government Services, Canada, Ottawa, Ont., Canada. pp. 249.

Government of Canada, 2000. Persistence and bioaccumulation regulations. Canada Gazette Part II. 134. Public Works and Government Services, Canada, Ottawa, Ont., Canada. pp. 607-612.

Grabicova, K., Grabic, R., Blaha, M., Kumar, V., Cerveny, D., Fedorova, G., Randak, T., 2015. Presence of pharmaceuticals in benthic fauna living in a small stream affected by ef!uent from a municipal sewage treatment plant. Water Res. 72,145–153. doi: 10.1016/j.watres.2014.09.018.

Grabicova, K., Grabic, R., Fedorova, G., Fick, J., Cerveny, D., Kolarova, J., Turek, J., Zlabek, V., Randak, T., 2017. Bioaccumulation of psychoactive pharmaceuticals in fish in an effluent dominated stream. Water Res. 124, 654-662. http://dx.doi.org/10.1016/j.watres.2017.08.018

Grabicová, K., Grabic, R., Fedorova, G., Vojs Staňová, A., Bláha, M., Randák, T., Brooks, B.W., Zlabek, V., 2020. Water reuse and

aquaculture: Pharmaceutical bioaccumulation by fish during tertiary treatment in a wastewater stabilization pond. Environ. Pollut. 267, 115593. https://doi.org/10.1016/j.envpol.2020.115593

Haddad, S.P., Luek, A., Scott, W.C., Saari, G.N., Burket, S.R., Kristofco, L.A., Corrales, J., Rasmussen, J.B., Chambliss, C.K., Luers, M., Rogers, C., Brooks, B.W., 2018. Spatio- temporal bioaccumulation and trophic transfer of ionizable pharmaceuticals in a semi-arid urban river influenced by snowmelt. J. Hazard Mater. 359, 231-240. https://doi.org/10.1016/j.jhazmat.2018.07.063

Howard, P.H., Muir, D.C.G., 2011. Identifying new persistent and bioaccumulative organics among chemicals in commerce II: pharmaceuticals. Environ. Sci. Technol. 45, 6938-6946. https://doi.org/10.1021/es201196x

Heynen, M., Fick, J., Jonsson, M., Klaminder, J., Brodin, T., 2016. Effect of bioconcentration and trophic transfer on realized exposure to oxazepam in 2 predators, the dragonfly larvae (aeshna grandis) and the eurasian perch (perca fluviatilis). Environ. Toxicol. Chem. 35(4), 930–937. DOI: 10.1002/etc.3368

Huerta, B., Rodriguez-Mozaz, S., Lazorchak, J., Barcelo, D., Batt, A., Wathen, J., Stahl, L., 2018. Presence of pharmaceuticals in fish collected from urban rivers in the U.S. EPA 2008–2009 National Rivers and Streams Assessment. Sci. Total Environ. 634, 542-549. https://doi.org/10.1016/j.scitotenv.2018.03.387

Jonsson, M., Fick, J., Klaminder, J., Brodin, T., 2014. Antihistamines and aquatic insects: Bioconcentration and impacts on behavior in damselfly larvae (Zygoptera). Sci. Total Environ. 472, 108-111. https://doi.org/10.1016/j.scitotenv.2013.10.104.

Kallio, J.M., Lahti, M., Oikari, A., Kronberg, L., 2010. Metabolites of the aquatic pollutant diclofenac in fish bile. Environ. Sci. Technol. 44(19), 7213-7219. https://doi.org/10.1021/es903402c

Koba, O., Grabicova, K., Cerveny, D., Turek, J., Kolarova, J., Randak, T., Zlabek, V., Grabic, R., 2018. Transport of pharmaceuticals and their metabolites between water and sediments as a further potential exposure for aquatic organisms. J. Haz. Mater. 342, 401-407. http://dx.doi.org/10.1016/j.jhazmat.2017.08.039

Kowalska, D., Maculewicz, J., Stepnowski, P., Dolzonek, J., 2021. Interaction of pharmaceutical metabolites with blood proteins and membrane lipids in the view of bioconcentration: a preliminary study based on in vitro assessment. Sci. Total Environ. 783, 146987. https://doi.org/10.1016/j.scitotenv.2021.146987

Kummerová, M., Zezulka, Š., Babula, P., Tříska, J. Possible ecological risk of two pharmaceuticals diclofenac and paracetamol demonstrated on a model plant Lemna minor. 2016. J. Hazard. Mater. 302, 351-361. http://dx.doi.org/10.1016/j.jhazmat.2015.09.057

Lagesson, A., Fahlman, J., Brodin, T., Fick, J., Jonsson, M., Byström, P., Klaminder, J., 2016. Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web - Insights from a field experiment. Sci. Total Environ. 568, 208-215. http://dx.doi.org/10.1016/j.scitotenv.2016.05.206

Liu, J., Lu, G., Xie, Z., Zhang, Z., Li, S., Yan, Z., 2015. Occurrence, bioaccumulation and risk assessment of lipophilic pharmaceutically active compounds in the downstream rivers of sewage treatment plants. Sci. Total Environ. 511, 54-62. https://doi.org/10.1016/j.scitotenv.2014.12.033.

Lopes, J., Coppola, F., Soares, A.M.V.M., Meucci, V., Pretti, C., Polese, G., Freitas, R., 2022. How temperature rise will influence the toxic impacts of 17 α-ethinylestradiol in Mytilus galloprovincialis? Environ. Res. 204, 112279. https://doi.org/10.1016/j.envres.2021.112279

Lu, G., Xie, Z., Zhang, Z., 2018. Effects of dissolved organic matter, feeding, and water flow on the bioconcentration of diclofenac in crucian carp (*Carassius auratus*). Environ. Sci. Pollut. Res. 25(8), 7776-7784. https://doi.org/10.1007/s11356-017-1081-0

Maculewicz, J., Kowalska, D., Świacka, K., Toński, M., Stepnowski, P., Białk-Bielińska, A., Dołżonek, J., 2022. Transformation products of pharmaceuticals in the environment: Their fate, (eco)toxicity and bioaccumulation potential. Sci. Total Environ. 802, 149916. https://doi.org/10.1016/j.scitotenv.2021.149916

Manallack, D.T., 2008. The pK(a) distribution of drugs: Application to drug discovery. Perspect. Med. Chem. 1, 25–38.

Mastrángelo, M.M., Valdés, M.E., Eissa, B., Ossana, N.A., Barceló, D., Sabater, S., Rodríguez-Mozaz, S., Giorgi, A.D.N., 2022. Occurrence and accumulation of pharmaceutical products in water and biota of urban lowland rivers. Sci. Total Environ. 828, 154303. http://dx.doi.org/10.1016/j.scitotenv.2022.154303

Maulvault, A.L., Santos, L.H.M.L.M., Paula, J.R., Camacho, C., Pissarra, V., Fogaça, F., Barbosa, V., Alves, R., Ferreira, P.P., Barceló, D., Rodriguez-Mozaz, S., Marques, A., Diniz, M., Rosa, R., 2018. Differential behavioural responses to venlafaxine exposure route, warming and acidification in juvenile fish (Argyrosomus regius). Sci. Total Environ. 634, 1136-1147.

https://doi.org/10.1016/j.scitotenv.2018.04.015

McCallum, E.S., Sundelin, A., Fick, J., Alanärä, A., Klaminder, J., Hellström, G., Brodin, T., 2019. Investigating tissue bioconcentration and the behavioural effects of two pharmaceutical pollutants on sea trout (*Salmo trutta*) in the laboratory and field. Aquat. Toxicol. 207, 170-178. https://doi.org/10.1016/j.aquatox.2018.11.028

Memmert, U., Peither, A., Burri, R., Weber, K., Schmidt, T., Sumpter, J.P., Hartmann, A., 2013. Diclofenac: New data on chronic toxicity and bioconcentration in fish. Environ. Toxicol. Chem. 32(2), 442-452. https://doi.org/10.1002/etc.2085

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. https://doi.org/10.1016/j.envpol.2011.11.029.

Mezzelani, M., Gorbi, S., Regoli, F., 2018. Pharmaceuticals in the aquatic environments: Evidence of emerged threat and future challenges for marine organisms. Mar. Environ. Res. 140, 41-60. https://doi.org/10.1016/j.marenvres.2018.05.001

Miller, T.H., Gallidabino, M.D., MacRae, J.R., Owen, S.F., Bury, N.R., Barron, L.P., 2019. Prediction of bioconcentration factors in fish and invertebrates using machine learning. Sci. Total Environ. 648, 80-89. https://doi.org/10.1016/j.scitotenv.2018.08.122

Miossec, C., Mille, T., Lanceleur, L., Monperrus, M., 2020. Simultaneous determination of 42 pharmaceuticals in seafood samples by solvent extraction coupled to liquid chromatography–tandem mass spectrometry. Food Chem. 322, 126765. https://doi.org/10.1016/j.foodchem.2020.126765

Molina-Fernández, N., Rainieri, S., Muñoz-Olivas, R., de Oro-Carretero, P., Sanz-Landaluze, J., 2021. Development of a method for assessing the accumulation and metabolization of antidepressant drugs in zebrafish (*Danio rerio*) eleutheroembryos. Anal. Bioanal. Chem. 413(20), 5169-5179. https://doi.org/10.1007/s00216-021-03486-2

Moreno-González, R., Rodríguez-Mozaz, S., Huerta, B., Barceló, D., León, V.M., 2016. Do pharmaceuticals bioaccumulate in marine molluscs and fish from a coastal lagoon? Environ. Res. 146, 282-298. http://dx.doi.org/10.1016/j.envres.2016.01.001

Muir, D., Simmons, D., Wang, X., Peart, T., Villella, M., Miller, J., Sherry, J. Bioaccumulation of pharmaceuticals and personal care product chemicals in fish exposed to wastewater effluent in an urban

wetland. Sci Rep 7, 16999 (2017). https://doi.org/10.1038/s41598-017-15462-x

Na, G., Fang, X., Cai, Y., Ge, L., Zong, H., Yuan, X., Yao, Z., Zhang, Z., 2013. Occurrence, distribution, and bioaccumulation of antibiotics in coastal environment of Dalian, China. Mar. Pollut. Bull. 69(1-2), 233-237. http://dx.doi.org/10.1016/j.marpolbul.2012.12.028

Nallani, G.C., Edziyie, R.E., Paulos, P.M., Venables, B.J., Constantine, L.A., Huggett, D.B., 2016. Bioconcentration of two basic pharmaceuticals, verapamil and clozapine, in fish. Environ. Toxicol. Chem. 35(3), 593–603. DOI: 10.1002/etc.3244.

Nendza, M., Kühne, R., Lombardo, A., Strempel, S., Schüürmann, G., 2018. PBT assessment under REACH: screening for low aquatic bioaccumulation with QSAR classifications based on physicochemical properties to replace BCF in vivo testing on fish. Sci. Total Environ. 616, 97–106. https://doi.org/10.1016/j.scitotenv.2017.10.317

Nunes, B., Daniel, D., Canelas, G.G., Barros, J., Correia, A.T., 2020. Toxic effects of environmentally realistic concentrations of diclofenac in organisms from two distinct trophic levels, *Hediste diversicolor* and *Solea senegalensis*. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 231, 108722. https://doi.org/10.1016/j.cbpc.2020.108722

OECD., 2012. Test No 305: Bioaccumulation in fish: Aqueous and dietary exposure. Section 3, 1-72. http://www.oecd-ilibrary.org/environment/test-no-305-bioaccumulation-in-fish-aqueous-and-dietary-exposure_9789264185296-en

Oetken, M., Nentwig, G., Löffer, D., Ternes, T., Oehlmann, J., 2005. Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. Arch. Environ. Contam. Toxicol. 49(3), 353-361. https://doi.org/10.1007/s00244-004-0211-0

Opperhuizen, A., Serne, P., and van der Steen, J.M.D., 1998. Thermodynamics of fish/water and octanol/water partitioning of some chlorinated benzenes. Environ. Sci. Technol. 22, 286-292. https://doi.org/10.1021/es00168a008

Pan, C., Yang, M., Xu, H., Xu, B., Jiang, L., Wu, M., 2018. Tissue bioconcentration and effects of fluoxetine in zebrafish (*Danio rerio*) and red crucian cap (*Carassius auratus*) after short-term and long-term exposure. Chemosphere 8-14. https://doi.org/10.1016/j.chemosphere.2018.04.082

Previšić, A., Vilenica, M., Vučković, N., Petrović, M., Rožman, M., 2021. Aquatic insects transfer pharmaceuticals and endocrine disruptors

from aquatic to terrestrial ecosystems. Environ. Sci. Technol. 55(6), 3736-3746. https://dx.doi.org/10.1021/acs.est.0c07609

Qu, H., Ma, R., Wang, B., Yang, J., Duan, L., Yu, G., 2019. Enantiospecific toxicity, distribution and bioaccumulation of chiral antidepressant venlafaxine and its metabolite in loach (Misgurnus anguillicaudatus) co-exposed to microplastic and the drugs. J. Hazard. Mater. 370, 203-211. https://doi.org/10.1016/j.jhazmat.2018.04.041

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 prod- ucts in fish: results of a national pilot study in the United States. Environ. Toxicol. Chem. 28, 2587-2597. https://doi.org/10.1897/08-561.1

Reis, E.O., Santos, L.V.S., Lange, L.C., 2021. Prioritization and environmental risk assessment of pharmaceuticals mixtures from Brazilian surface waters. Environ. Pollut. 288, 117803. https://doi.org/10.1016/j.envpol.2021.117803

Rendal, C., Kusk, K.O., Trapp, S., 2011. The effect of pH on the uptake and toxicity of the bivalent weak base chloroquine tested on Salix viminalis and Daphnia magna. Environ. Toxicol. Chem. 30 (2), 354-359. doi: 10.1002/etc.391.

Rojo, M., Álvarez-Muñoz, D., Domanico, A., Foti, R., Rodriguez-Mozaz, S., Barceló, D., Carriquiriborde, P., 2019. Human pharmaceuticals in three major fish species from the Uruguay River (South America) with different feeding habits. Environ. Pollut. 252, 146–154. https://doi.org/10.1016/j.envpol.2019.05.099

Rosa, J., Leston, S., Freitas, A., Vila Pouca, A.S., Barbosa, J., Lemos, M.F.L., Pardal, M.A., Ramos, F., 2019. Oxytetracycline accumulation in the macroalgae Ulva: Potential risks for IMTA systems. Chemosphere 226, 60-66. https://doi.org/10.1016/j.chemosphere.2019.03.112

Ruan, Y., Lin, H., Zhang, X., Wu, R., Zhang, K., Leung, K.M.Y., Lam, J.C.W., Lam, P.K.S., 2020. Enantiomer-specific bioaccumulation and distribution of chiral pharmaceuticals in a subtropical marine food web. J. Hazard. Mater. 394, 122589. https://doi.org/10.1016/j.jhazmat.2020.122589

Ruhí, A., Acuña, V., Barceló, D., Huerta, B., Mor, J.R., Rodríguez-Mozaz, S., Sabater, S., 2016. Bioaccumulation and trophic magnification of pharmaceuticals and endocrine disruptors in a Mediterranean river food web. Sci. Total Environ. 540, 250-259.

http://dx.doi.org/10.1016/j.scitotenv.2015.06.009

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Part I: Histopathological alterations and bioaccumulation in rainbow trout. Aquat. Toxicol. 68(2), 141-150. https://doi.org/10.1016/j.aquatox.2004.03.014

Serra-Compte, A., Maulvault, A.L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz, S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (Mytilus galloprovincialis), Environ. Pollut. 236, 824-834. https://doi.org/10.1016/j.envpol.2018.02.018.

Silva, M.G., Esteves, V.I., Meucci, V., Battaglia, F., Soares, A.M.V.M., Pretti, C., Freitas, R., 2022. Metabolic and oxidative status alterations induced in Ruditapes philippinarum exposed chronically to estrogen 17α-ethinylestradiol under a warming scenario. Aquat. Toxicol. 244, 106078. https://doi.org/10.1016/j.aquatox.2022.106078

Sims, J.L., Burket, S.R., Franco, M.E., Lovin, L.M., Scarlett, K.R., Steenbeek, R., Chambliss, C.K., Ashcroft, C., Luers, M., Lavado, R., Brooks, B.W., 2020. Pharmaceutical uptake kinetics in rainbow trout: In situ bioaccumulation in an effluent -dominated river influenced by snowmelt. Sci. Total Environ. 736, 10. https://doi.org/10.1016/j.scitotenv.2020.139603

Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., Świeżak, J., 2022. Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms – Current state of knowledge. J. Hazard. Mater. 424, 127350. https://doi.org/10.1016/j.jhazmat.2021.127350

Świacka, K., Michnowska, A., Maculewicz, J., Caban, M., Smolarz, K., 2021. Toxic effects of NSAIDs in non-target species: A review from the perspective of the aquatic environment. Environ. Pollut. 273, 115891. https://doi.org/10.1016/j.envpol.2020.115891

Świacka, K., Smolarz, K., Maculewicz, J., Caban, M., 2020. Effects of environmentally relevant concentrations of diclofenac in *Mytilus trossulus*. Sci. Total Environ. 737, 139797. https://doi.org/10.1016/j.scitotenv.2020.139797

Świacka, K., Smolarz, K., Maculewicz, J., Michnowska, A., Caban, M., 2021. Exposure of *Mytilus trossulus* to diclofenac and 4′-hydroxydiclofenac: Uptake, bioconcentration and mass balance for the evaluation of their environmental fate. Sci. Total Environ. 791,

148172. https://doi.org/10.1016/j.scitotenv.2021.148172

Świacka, K., Szaniawska, A., Caban, M., 2019. Evaluation of bioconcentration and metabolism of diclofenac in mussels *Mytilus trossulus* - laboratory study. Mar. Pollut. Bull. 141, 249-255. https://doi.org/10.1016/j.marpolbul.2019.02.050

US-EPA, 2000. Methodology for deriving ambient water quality criteria for the protection of human health. US Environmental Protection Agency, Washington, D.C., USA. pp. 180.

Valdés, M.E., Amé, M.V., Bistoni, M.A., Wunderlin, D.A., 2014. Occurrence and bioaccumulation of pharmaceuticals in a fish species inhabiting the Suquía River basin (Córdoba, Argentina). Sci. Total Environ. 472, 389-396. https://doi.org/10.1016/j.scitotenv.2013.10.124.

Valenti, T.W., Perez-Hurtado, P., Chambliss, K.C., Brooks, B.W., 2009. Aquatic toxicity of sertraline to Pimephales promelas at environmentally relevant surface water pH. Environ. Toxicol. Chem. 28 (12), 2685-2694. doi: 10.1897/08-546.1.

Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagne, F., Juneau, P., 2010. Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. Chemosphere 80, 1062-1068. https://doi.org/10.1016/j.chemosphere.2010.05.010

Wang, F., Wang, B., Qu, H., Zhao, W., Duan, L., Zhang, Y., Zhou, Y., Yu, G., 2020. The influence of nanoplastics on the toxic effects, bioaccumulation, biodegradation and enantioselectivity of ibuprofen in freshwater algae *Chlorella pyrenoidosa*. Environ. Pollut. 263, 114593. https://doi.org/10.1016/j.envpol.2020.114593

Wang, Y., Yin, T., Kelly, B.C., Gin, T.Y-H., 2019. Bioaccumulation behaviour of pharmaceuticals and personal care products in a constructed wetland. Chemosphere 222, 275-285. https://doi.org/10.1016/j.chemosphere.2019.01.116.

Wennmalm, A., Gunnarsson, B., 2009. Pharmaceutical management through environmental product labeling in Sweden. Environ. Int. 35, 775-777. https://doi.org/10.1016/j.envint.2008.12.008

Wilkinson, J.L., Hooda, P.S., Swinden, J., Barker, J., Barton, S., 2018. Spatial (bio)accumulation of pharmaceuticals, illicit drugs, plasticisers, perfluorinated compounds and metabolites in river sediment, aquatic plants and benthic organisms. Environ. Pollut. 234, 864-875. https://doi.org/10.1016/j.envpol.2017.11.090.

Wilkinson, J.L., Hooda, P.S., Swinden, J., Barker, J., Barton, S., 2018. Spatial (bio) accumulation of pharmaceuticals, illicit drugs, plasticisers, per uorinated compounds and metabolites in river sediment, aquatic plants and benthic organisms. Environ. Pollut. 234, 864-875. https://doi.org/10.1016/j.envpol.2017.11.090

Xie, H., Hao, H., Xu, N., Liang, X., Gao, D., Xu, Y., Gao, Y., Tao, H., Wong, M., 2019. Pharmaceuticals and personal care products in water, sediments, aquatic organisms, and fish feeds in the Pearl River Delta: Occurrence, distribution, potential sources, and health risk assessment. Sci. Total Environ. 659, 230-239. https://doi.org/10.1016/j.scitotenv.2018.12.222

Xie, Z., Luan, H., Zhang, Y., Wang, M., Cao, D., Yang, J, Tang, J., Fan, S., Wu, X., Hua, R., 2020. Interactive effects of diclofenac and copper on bioconcentration and multiple biomarkers in crucian carp (*Carassius auratus*). Chemosphere 242, 125141. https://doi.org/10.1016/j.chemosphere.2019.125141

Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J. Environ. Manage. 133, 378-387. http://dx.doi.org/10.1016/j.jenvman.2013.12.017

Zhang, S., Ding, J., Razanajatovo, R.M., Jiang, H., Zou, H., Zhu, W., 2019. Interactive effects of polystyrene microplastics and roxithromycin on bioaccumulation and biochemical status in the freshwater fish red tilapia (*Oreochromis niloticus*). Sci. Total Environ. 648, 1431-1439. https://doi.org/10.1016/j.scitotenv.2018.08.266

Zhao, J-L., Furlong, E.T., Schoenfuss, H.L., Kolpin, D.W., Bird, K.L., Feifarek, D.J., Schwab, E.A., Ying, G-G., 2017. Uptake and Disposition of Select Pharmaceuticals by Bluegill Exposed at Constant Concentrations in a Flow-Through Aquatic Exposure System. Environ. Sci. Technol. 51 (8) 4434–4444. https://doi.org/10.1021/acs.est.7b00604

Zhao, S., Wang, X., Li, Y., Lin, J., 2016. Bioconcentration, metabolism, and biomarker responses in marine medaka (*Oryzias melastigma*) exposed to sulfamethazine. Aquat. Toxicol. 181, 29-36. https://doi.org/10.1016/j.aquatox.2016.10.026.

Capítulo 7

Exposure of non-target marine organisms *Holothuria tubulosa, Anemonia sulcata* and *Actinia equina* to the antidepressant venlafaxine and its main metabolite o-desmethylvenlafaxine

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Abstract: The exposure to the antidepressant venlafaxine (VFX) and its major metabolite o-desmethylvenlafaxine (O-VFX) in common marine organisms including *Holothuria tubulosa*, *Anemonia sulcata* and *Actinia equina* is evaluated. The methodology involves exposure for 28 days (10 µg/L day) followed by a 52-day depuration period. The accumulation shows a first-order kinetic process and reached a mean tissue concentration of 49125/54342 ng/g dw in *H. tubulosa* and 64810/93007 ng/g dw in *A. sulcata*. Studied compounds are considered cumulative (BCF_{VFX} = 2947, 4728 and 2080 L/kg dw in *H. tubulosa*, *A. sulcata* and *A. equina*, and $BCF_{O\text{-}VFX}$ = 2392 L/kg dw in *A. sulcata*). The study revealed differences between tissues in metabolizing abilities in *H. tubulosa*, this effect increases significantly with time in the digestive tract while it was negligible in the body wall. The results provide a detailed description of VFX and O-VFX accumulation in common and non-target organisms in the marine environment.

Keywords: Antidepressant drugs; Metabolite; Bioconcentration; Organism-distribution; Marine environment.

1. Introduction

Pharmaceuticals active compounds (PhACs) include a diverse and wide group of chemical substances used for the treatment and prevention of diseases in human and animals (Silva et al., 2015). There are more than 4000 substances with different chemical and therapeutic properties that have greatly improved global public health in recent decades (Duarte et al., 2023; Fonseca et al., 2021). However, their high consumption means that the excretion of these drugs from the human body contributes to their release into wastewater, allowing them to enter the environment and more specifically the marine ecosystem, which has led to them being considered a group of emerging pollutants (EPs) of high concern (Archer et al., 2023; Antonopoulou et al., 2022).

Among the PhACs, antidepressants are a family of drugs widely prescribed to treat depression, anxiety and even chronic pain (Zheng et

al., 2023). The antidepressants consumption was doubled between 2000 and 2017 in OECD countries and this trend will probably continue to increase (OECD 2019; Gomez et al., 2021). Thus, gaining further insight into their bioaccumulation capacity is critical to prioritize chemicals and evaluate their chronic risks to non-target organisms. Specifically, venlafaxine (VFX) and O-desmethylvenlafaxine (O-VFX) belong to the family serotonin-norepinephrine reuptake inhibitors (SNRIs) and they are two of the most widely used treatment for early-onset depression (Ahmadimanesh et al., 2020). According to the Decision 2022/1307/EC, the European Commission has included both compounds in a watch list of substances as emerging pollutants (European Commission, 2022). These drugs are long-term treatments and are classified as genotoxic and carcinogenic, and it is therefore appropriate to assess their environmental toxicity (Castillo-Zacarías et al., 2021). VFX has become an environmental pollutant because of its wide consumption and incomplete degradation in wastewater treatments (Golovko et al., 2020; Boulard et al., 2020; Kosma et al. 2019; Osorio et al., 2016).

In recent years, biomonitoring strategies have focused on the use of bioindicators to assess the impact of these compounds in marine ecosystem (Chahrour et al., 2021). Bioconcentration is the accumulation of chemicals in an organism that is constantly exposed to the contaminated environment. The bioconcentration factor (BCF) is a term used to assess the amount of accumulated contaminants in an organism in relation to the environment (Nikokherad et al., 2022). Absorption of chemical compounds by marine organisms can take place through various pathways, such as the dermal, gut, gill or pulmonary surfaces (Chidya et al., 2022). There are several studies of bioconcentration of neuroactive pharmaceuticals in aquatic organisms, e.g. Qu et al. (2019) evaluated the BCF of VFX and its metabolite in loach with values between 0.04-0.14 L/kg, or Maulvaut et al. (2018), Gravicoba et al. (2014) and Lajeunesse et al. (2011) studied the bioconcentration of different antidepressants in fish species such as rainbow trout, loach or brook trout. Other studied have focused in the bioconcentration in bivalves (Serra-Compte et al., 2018; Gomez et al., 2021) obtaining BCFs between 213.3-528.1 and 265 L/kg,

respectively. The importance of this type of study lies in understanding the behavior of animals in controlled laboratory experiments in order to be able to know the potential effects of the presence of these pollutants in nature and the harm or risk they may cause on a large scale (McCallum et al., 2018). However, to the best of our knowledge, an important gap within marine environment is that the accumulation pattern of PhACs have been studied only in a few of the many species that exist.

Cotton spinner sea cucumber *(Holothuria tubulosa)* belong to the class Holothuroidea, echinoderms found in almost all marine environments (Künili et al., 2019). They probe to be ideal bioindicators for biomonitoring EPs since its feeding mode consists of ingesting sediments from which it extracts organic matter (Bulleri et al., 2021). Some studies have found the presence of pollutants in different body compartments such as the digestive tract or gonads (Martín et al., 2020), suggesting that higher trophic levels can be reached. On the other hand, snakelocks anemone *(Anemonia sulcata)* and beadlet anemone (*Actinia equina*) are sessile organisms that can be affected by the presence of pollutants in the environment they inhabit, which also makes them interesting for the study of bioaccumulation of PhACs and as informants of the state of the water (Morais et al., 2022).

In this context, the present study was conducted to investigate the bioconcentration of the antidepressant VFX and its metabolite in non-target but common organisms found in the marine environment, exposed under controlled laboratory conditions. The study aimed to determine the bioaccumulation kinetics and its BCF in sea cucumber, snakelocks anemone and beadlet anemone. Selected species are excellent sentinels for monitoring bioavailable contaminants as can reflect changes in the concentration of the contaminant from the surrounding environment, accumulate contaminants without being seriously affected by the concentrations as well as are considered representative and abundant in the marine environment (OSPAR commission, 2012).

2. Materials and methods

2.1. Chemical and reagents

Ultrapure water (18.2 MΩ-cm) was obtained by using a Milli-Q Plus® system (Millipore, Madrid, Spain). Analytical-grade formic acid (≥ 98%) used as mobile phase additive (Honeywell, Charlotte, USA). LC-MS grade methanol (MeOH) was purchased from VWR Prolabo CHEMICALS (Barcelona, Spain). VFX (≥ 98.0%) and O-VFX (≥ 98.5%) were purchased by Sigma Aldrich (Madrid, Spain). Cinchophen (CIN, surrogate standard) was provided from Alfa Aesar (Massachusetts, MA, USA). Chemical structures and the physicochemical properties of the analytes are shown in Table S1. Nitrate, nitrite and ammonium tests were conducted with Hanna instruments® test kits (HI 3874, 3873 and 3826, respectively). Stock solutions were prepared in MeOH and stored at -20 ºC until use.

2.2. Laboratory test

2.2.1. Specimens' collection and acclimation period

The specimens under study were captured in February 2022 by divers at a depth of 10-15 m at random locations along the coast of Granada (Spain). Then, they were transported in refrigerated containers, at 4 ºC, to the laboratory facilities where the experiment was carried out. They were then placed in the experimental tanks and left to acclimatize and depurate for a period of 15 days.

2.2.2. Up-take and depuration test

The study was developed at the Zoology Department of the University of Granada (Spain), where a 300 L glass tank containing recirculating natural seawater was installed with artificial illumination by a 6000 ºK metal halide light bulb. The system was filtered with a biological filter with foam rubber and bioballs and aeration was performed with compressed air. Photoperiods of 10 hours of light and 14 hours of darkness were carried out. During the experiment, the physicochemical characteristics of the water were checked two times a week, with mean values of temperature 19.7 ± 0.2 ºC, pH 8.2 ± 0.0,

salinity 37.5 ± 0.2 PSU, dissolved O_2 6.3 ± 0.1 mg/L, ammonium 0.20 ± 0.03 mg/L, nitrites 0.23 ± 0.01 mg/L, nitrates 51 ± 3 mg/L.

Feeding. A spoonful containing a mixture of fishmeal and dried spirulina (1:1) was added to the aquarium bi-weekly to feed the animals.

Experimental design. A low exposure concentration was used, 1% of LC_{50}, according with the OECD 305 method (LC_{50} 10-16 mg/L in algae, fish and *Daphnia* (Golovko et al., 2020). During the first 28 days of the experiment (uptake), after acclimation of the specimens over two weeks, 3 mg of each compound was added daily. As the volume of the tank was 300 L, the final concentration added was 10 µg/L per day. Every four days, sample specimens were collected from the tank. In the case of sea cucumber, body compartments (body wall and digestive tract) were separated. After day 28, all remaining specimens were moved to a new tank with the same characteristics as the previous one, starting the depuration period, which took place for 52 days, with a total duration of 80 days for the experiment. In the same way, samples were collected every 4 days and the same pre-treatment of the specimens was carried out, which consisted of freeze-drying and pulverization. All samples were stored at -20 ºC until analysis.

Sample collection. In each sampling, 3 sea cucumbers, 3 snakelocks anemones and 2 beadlet anemones were collected as well as 100 mL vessel containing sediments and water. Digestive tract and body wall were separated from the sea cucumbers and the anemones were stored in plastic bags. Once in the laboratory, all samples were freeze-dried except for the water, which was frozen at -20 ºC. Freeze-drying took place for 48 hours at -109 ºC. After drying, the samples were first pulverized with a mortar and pestle to reduce the particle size as much as possible, and then with a ball mill for 15 minutes with a frequency of 25.0 s^{-1}. Then, they were stored at -20 ºC until analysis.

2.3. Analytical methods

Animal and sediment samples were aliquoted (0.2 g dw) and weighed into 10 mL glass tubes together with a methanolic solution of surrogate (CIN), obtaining a final concentration of 200 ng/g in the sample. The samples were left in the dark for 24 hours to ensure contact between the matrix and the internal standard. Subsequently, 2.2 mL of MeOH were added, vortexed for 30 seconds and an ultrasound-assisted extraction (UAE) was performed for 12 minutes at 40 % amplitude. The samples were then centrifuged for 5 minutes at 4000 rpm and the supernatant was removed to another tube. This procedure was performed in duplicate, mixing the collected supernatants. Afterwards, the extracts were dried under a N_2 stream. The dried residue was reconstituted in an 80/20 (v/v) H_2O/MeOH solution to a final volume of 0.2 mL and 2 µL were injected into the LC apparatus.

Water samples were collected in sterilized 100 mL water bottles. Aliquots of 10 mL of sample were separated and 50 µL of a methanolic solution of CIN was added, being 200 ng/mL the final concentration of the internal standard. The sample was then frozen and freeze-dried for 48 hours at -109 ºC. After drying, 1 mL of MeOH was added and vortexed for 1 min. Subsequently, it was centrifuged for 5 min at 4000 rpm and the supernatant was collected. The procedure was repeated and the supernatants mixed and then evaporated under a N_2 stream. After drying, the same reconstitution procedure was performed as for the solid matrices.

Liquid chromatography-tandem mass spectrometry analysis was performed using an ACQUITY HSS T3 column (100 mm x 2.1 mm internal diameter, 1.8 µm particle size). The compounds were separated using a mobile phase gradient consisting of an aqueous buffer with 0.05% formic acid (v/v) as phase A and MeOH as phase B. The method was carried out in 8 minutes with the following gradient program: 0-1.5 min, isocratic gradient 80% of A. 1.5-3 min, linear gradient from 80% to 60% A, 3-4 min, linear gradient from 60 to 15% A, and 4-6 min, isocratic 15% A. 6-6.1 min, return to initial conditions for 2 min in order to conditioning

for the next injection. Two multiple reaction monitoring (MRM) transitions were selected for each analyte, the first one for quantification, and the second one for confirmation. All these data are shown as supplementary material together with the spectrometer parameters (Table S2). The mass spectrometer was operated in positive ESI mode. The method was successfully validated according to the ICH guideline (ICH Guidelines, 2005) in terms of linearity, range, sensitivity and accuracy (trueness and precision). The validation process is explained in Supplementary Information (Validity requirements). Table S3 summarizes the limits of detection and quantification, as well as linearity and linear dynamic range. Accuracy assays showed recoveries close to 100% for all compounds and matrices, with standard deviations below 15% in all cases, which proves that the method is accurate.

2.4. Bioconcentration factor calculation

The BCF was calculated in two different ways: the first one is from the concentration levels measured in water and biota at steady-state (SS) through the equation 1:

$$BCF_{SS} = \frac{C_b}{C_w} \quad (1)$$

C_b is the concentration in the biota at ng/g dry weight (dw) and C_w is the concentration in the water at ng/mL, both measured at the same time. The steady stated was estimated at 28 days in all cases. Moreover, the C_w was kept within 20% of the measured mean (n=3). Two different parts of the organisms (body wall and digestive tract) were considered in sea cucumber, while for anemones the whole body of the organism was considered.

The second way to calculate the BCF is through the kinetic parameters, absorption rate constant (k_1) and the depuration rate constant (k_2):

$$\frac{dC_b}{dt} = k_1 C_w - k_2 C_b \quad (2) \qquad \frac{dC_b}{dt} = -k_2 C_b \quad (3)$$

Assuming a negligible concentration in biota samples at t_0, and considering its constant in the exposure medium, the equations can be expressed as:

$$C_b(\text{uptake}) = \frac{k_1}{k_2} C_w(1 - e^{-k_2 t}) \quad (4) \qquad C_b(\text{depuration}) = C_{b,0} e^{-k_2 t} \quad (5)$$

C_b is the concentration in biota (ng/g dw), $C_{b,0}$ is the concentration when the depuration phase begins (ng/g dw), t the time of exposure (days), k_1 the first-order uptake constant (mL/g·d), C_w the concentration in water (ng/mL), and k_2 the first-order elimination rate constant (1/d).

$$BCF_k = \frac{k_1}{k_2} \quad (6)$$

The biological half-lives of the chemicals ($t_{1/2}$) were determined as:

$$t_{1/2} = \frac{\ln 2}{k_2} \quad (7)$$

3. Results

3.1. Bioconcentration in cotton spinner sea cucumber

The mean concentrations measured, with their relative standard deviation, in water and sediment samples during the experiment are shown in Table 1.

The concentration levels measured for VFX and O-VFX before the first addition of compounds to the experimental system (t = 0) were 2.40 and 4.59 ng/mL in the water phase, and 0.38 and 2.97 ng/g dw in the sediments, respectively. These concentrations are negligible compared to those added during the exposure period. The concentration levels measured during the exposure period were maintained relatively stable and meet the 20% maximum variation commitment of OECD 305. Similar variations have been observed in other comparable studies in which the concentration was monitored during the experiment (Molina-Fernández et al., 2021; Molina-Fernández et al., 2017).

Table 1. *Mean concentration and relative standard deviation of VFX and O-VFX in the aqueous phase and in the sediments throughout the experiment*

Sampling (days)	Water phase				Sediments			
	VFX		O- VFX				VFX	
	Mean (ng/mL)	RSD (%)	Mean (ng/mL)	RSD (%)	Mean (ng/g)	RSD (%)	Mean (ng/g)	RSD (%)
0	2.40	11	4.59	5	0.38	16	2.97	12
4	11.0	10	2.42	1	62.1	3	63.4	11
8	19.4	13	29.5	8	86.0	2	65.2	9
12	14.3	5	4.20	3	88.2	12	83.2	10
16	11.7	4	35.9	5	189	3	158	2
20	26.8	6	39.9	6	171	6	188	2
24	17.7	3	30.8	6	236	2	212	9
28	15.7	8	29.4	4	227	10	214	9
32	8.32	8	7.45	13	56.5	4	29.4	5
36	4.25	1	<LOD	-	41.0	12	9.15	2
40	3.94	16	6.34	9	36.4	9	13.2	8
44	2.46	11	<LOD	-	35.3	5	14.9	13
48	3.37	14	7.57	8	29.1	1	15.7	5
52	2.24	2	6.95	1	23.9	4	14.6	13
56	3.50	1	7.32	13	64.7	5	17.0	6
60	1.10	12	5.07	3	19.1	9	9.26	7
70	0.55	6	<LOD	-	26.7	14	12.6	8
80	<LOD	-	<LOD	-	8.90	8	5.67	4

< LOD: below the limit of detection

Overall, VFX and O-VFX were detected at higher concentrations in the sediment compartment than in water samples, which suggest that sediments may be an important source of selected compounds for benthonic biota like is the case of sea cucumber. The experimental partition factor between the marine sediment and the water, calculated as the ratio between the concentration measured in both compartments was 1.16 and 0.86 L/kg (log K_d) for VFX and O-VFX, respectively (in concordance with their octanol-water partition coefficient (log Kow 3.2 and 2.7 for VFX and O-VFX, respectively). To our knowledge, this is the first K_d data reported in marine sediments. The calculated values are similar or slightly lower (1.21-2.19) to those reported by Osorio et al. (2016) and Boulard et al. (2020), who studied VFX sorption in Iberian and German Rivers, respectively. Some higher values up to 3.2 has been

published in water and sediment from Lake Malaren, Sweden (Golovko et al., 2020). VFX was only found at one of three locations at concentration ranges between 2.8-7.8 ng/g dw while O-VFX was quantified at all sampling locations (up to 3.7 ng/g dw). Authors concluded that O-VFX has stronger persistency than its parent compound. The accumulation and depuration profile of VFX and O-VFX in sea cucumber are drawn in Figure 1.

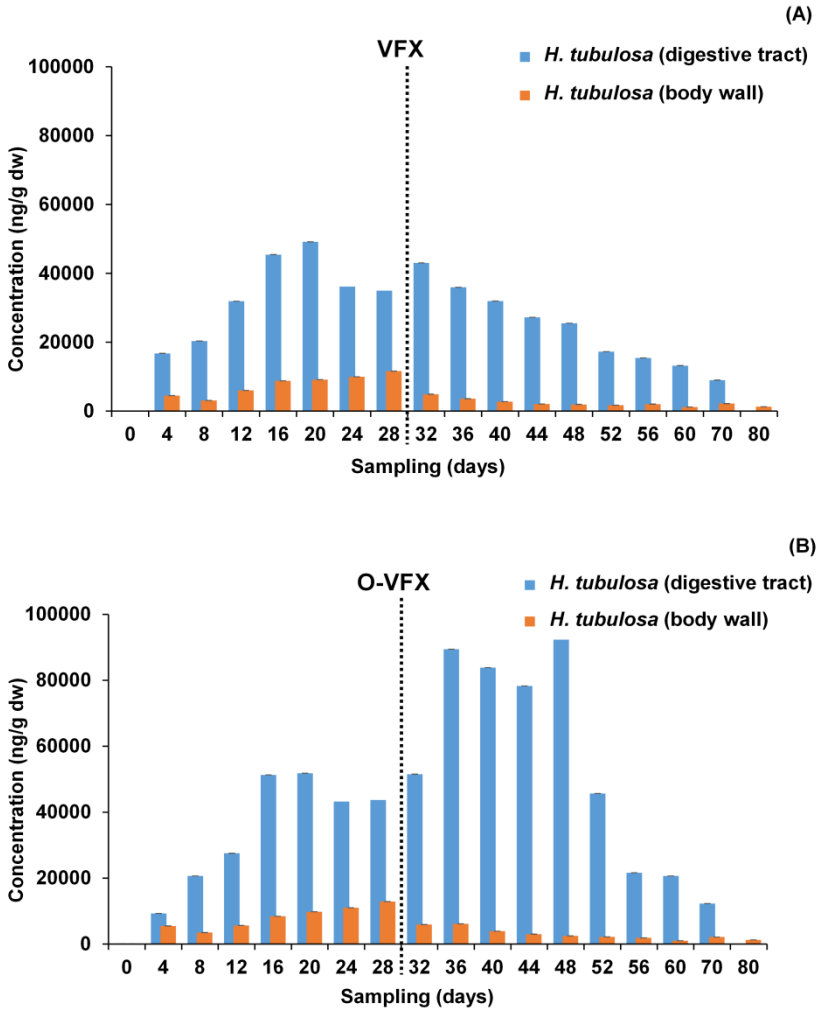


Figure 1. *Kinetics of accumulation (days 0–28) and depuration (days 32–80) of VFX (A) and O-VFX (B) in sea cucumber (digestive tract and body wall)*

In the uptake phase, the concentrations kept increasing, and a rapid uptake was observed. After 28 days, the concentration of VFX and O-VFX reached as high as 40823 and 54342 ng/g dw, respectively, in the digestive tract and 11577 and 12870 ng/g dw, respectively, in the body wall. These concentrations were almost 100-fold higher than those found in the water and sediment phase. Both compounds showed similar accumulation patterns, however, at the initial stage of the experiment the concentration measured for VFX was higher than O-VFX, while along days the order was reverted in the digestive tract which can be explained by a possible metabolization of VFX.

Meanwhile, during the depuration phase, the concentrations of VFX and O-VFX showed a slow drop. Up to 17 days were needed to eliminate half of the amount ($t_{1/2}$) in the digestive tract and even a longer time (30 and 20 days for VFX and O-VFX, respectively) was estimated in the body wall, probably explained by the low metabolization and degradation in this tissue. At the end of the experiment, after 52 days of depuration, concentrations in *H. tubulosa* were 8982 and 12257 ng/g dw for VFX and O-VFX, respectively, in the digestive tract and 1231 and 1258 ng/g dw for VFX and O-VFX, respectively, in the body wall.

Table 2 summarizes the kinetic parameters after adjusting to the first-order kinetic model.

***Table 2**. Kinetic data of VFX and O-VFX in studied animals*

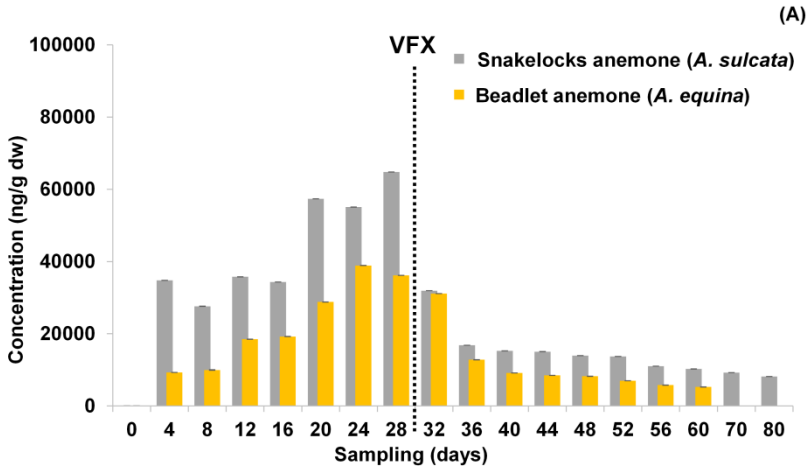
Compound	k_2 (1/d)	R^2 (k_2)	$t_{1/2}$ (d)	k_1 (L/kg d)	R^2 (k_2)	$BCF_{kinetic}$ (L/kg)	BCF_{ss} (L/kg)	BCF_{ss} sed
	H. tubulosa (digestive tract)							
VFX	0.041	0.941	17	121.7	0.686	2947	1831	287
O-VFX	0.053	0.905	13	103.8	0.823	1515	1298	275
	H. tubulosa (body wall)							
VFX	0.023	0.608	30	21.74	0.688	949	738	51
O-VFX	0.033	0.716	20	14.32	0.735	431	437	60
	A. sulcata							
VFX	0.027	0.808	26	125.4	0.784	4728	4133	286
O-VFX	0.042	0.727	16	100.1	0.688	2392	3160	434
	A. equina							
VFX	0.051	0.799	13	106.9	0.834	2080	2307	160
O-VFX	0.055	0.558	13	83.92	0.816	1522	1837	252

k_1: first-order uptake constant; k_2: first-order elimination rate constant; R^2: Determination coefficient of linear representations; BCF_k: bioconcentration factor based in kinetic parameter; BCF_{ss}: bioconcentration factor based in concentration measured at the steady state; BCF_{ss} sed: bioconcentration factor in the sediment compartment

The accumulation rate constants (k_1) were relatively high, especially in the digestive tract 122 and 103 L/kg·d for VFX and O-VFX, respectively, while the contrary effect was observed for depuration constants (k_2) with a rate of were 0.04 and 0.06 1/d for VFX and O-VFX, respectively. The estimated BCF_k varied slightly between studied tissues, been notably higher in the digestive tract in comparison with the body wall pointing out the absorption (by diet) as the main up-take route of studied compounds. The BCF was also calculated at the steady stated, estimated after 28 days, getting BCF_{SS} and BCF_k for comparison purposes. Some slightly differences could be observed between the two ways of calculation, probably explained because the steady state was not ready reached. The 305 guideline in 2002 suggests to use toxicokinetic models as the first-order two-compartment model to estimate the BCF if the steady state is not clearly reached. However, kinetic model is not commonly applied in the previous reported studies (Molina-Fernández et al., 2021).

3.2. Bioconcentration in snakelocks anemone and beadlet anemone

The accumulation and depuration profile of VFX and O-VFX in snakelocks anemone and beadlet anemone are drawn in Figure 2.



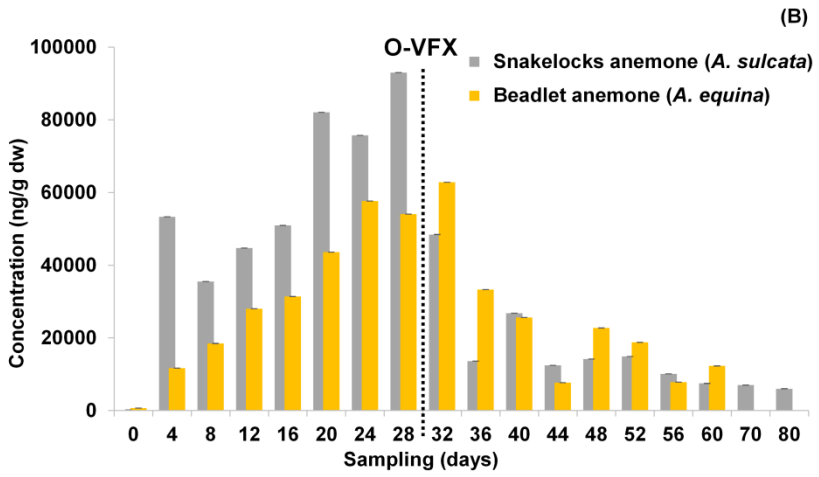


Figure 2. *Kinetics of accumulation (days 0–28) and depuration (days 32– 80) of VFX (A) and O-VFX (B) in snakelocks anemone and beadlet anemone*

The bioaccumulation pattern in anemones showed a similar trend that in the digestive tract of sea cucumber. VFX and O-VFX were detected in control anemones samples (not exposed) at concentration levels between 17-24 and 116-661 ng/g dw, respectively. Both compounds bioaccumulated, increasing their concentration with the increasing of the exposure time, resulting in 64810 and 93007 ng/g dw uptake for VFX and O-VFX, respectively, in snakelocks anemone and in 36176 and 54054 ng/g dw uptake for VFX and O-VFX, respectively, in beadlet anemone after 28 days of exposure. This phenomenon suggests the latent high accumulation of these compounds in anemones without being seriously affected.

On the other hand, depuration was very slow for both compounds. Within 26 and 16 days, the concentration in whole organisms decreased 50% of the initial at the depuration phase in snakelocks anemone for VFX and O-VFX, respectively, and a few days less (13) in beadlet anemone. As is shown in Table 2, the calculated BCF of VFX and O-VFX varied slightly between organisms. This might be due to the different capacities to accumulate and/or metabolize compounds of the different species. In fact, at difference of A. equina, A. sulcata is an endosymbiotic species that

interchanges nutriments and metabolites with its endosymbiotic microalgae (Stambler and Dubinsky, 1987).

4. Discussion

Considering REACH classification that estimates BCFs > 2000 L/kg for cumulative compounds, VFX would be considered as such (2947, 4728 and 2080 L/kg dw in *H. tubulosa*, *A. sulcata* and *A. equina*, respectively) or O-VFX (2392 L/kg dw) in *A. sulcata* and consequently they may represent a potential risk in the marine environment (Molina Fernandez et al., 2021). Then, sea cucumber and anemones represent interesting models for studying bioconcentration.

On the one hand, the anemones studied are sessile organisms (like mussels) and can be associated with a specific place, unlike fish. Indeed, as they are secondary or tertiary consumers (they feed on carrion or animals that may be herbivores -primary consumers- or carnivores -secondary consumers-), they may have a greater capacity for bioconcentration of each trophic level. Sea cucumber although is not sessile, it is sedentary with low mobility and it can also be associated with a specific place because its locomotion capacity is much reduced. In this case, we are dealing with a detritivorous deposit feeder animal that obtain food particles by ingesting the surface layer of the marine sediment on which it crawls and lives and where it accumulates (Palmer et al., 2022; Jiang et al., 2015; Martín et al., 2017; Sugni et al., 2007).

Although extensive bioconcentration studies have been undertaken in organism like fish or mussels, studies occurring in other nontarget organisms have been limited in number of species and/or type of contaminant, also including metabolites. The log K_{ow} has been the most widely used descriptor to predict the bioaccumulation potential, especially for lipophilic compounds (higher log K_{ow} corresponds to a higher BCF) (Arnot and Gobas, 2006). Meylan et al. (1999) developed an improved screening-level BCF estimation method and proposed that BCF values were 3.16 for the ionizable compounds with log K_{ow} < 5. However, in the present study, VFX (log K_{ow}: 2.47) presented higher

bioaccumulation (up to 1831 and 4133 L/kg dw in sea cucumber and snakelocks anemone) than predicted with Meylan et al. (1999) or the QSAR models of Mackay and Veith (i.e., 26.3–42.6), as reported in Serra-Compte et al. (2018). Relying only on the K_{ow} may lead to a degree of underestimation (Gómez-Regalado et al., 2023; Kowalska *et al.*, 2021). For example, in ionized chemicals such as pharmaceuticals, the BCF is pH dependent (Molina-Fernandez et al., 2021). Nakamura et al. (2008) reported that BCF values of fluoxetine (secondary amine) measured in Japanese medaka *(Oryzias latipes)* increases as the pH increases from 9 to 260 L/kg at pH 7 and 9, respectively.

The availability of quality information is essential to improve accuracy and reduce uncertainty in hazard and risk assessments. Studies with fish yield BCF values lower than the ones obtained in this study, but quite different between them can be found in the literature. Table 3 summarizes the research found in the scientific literature dedicated to the evaluation of the bioaccumulation potential of VFX and O-VFX in different aquatic organisms.

Table 3. *BCF observations for VLF and O-VLF in aquatic organisms belonging to different trophic levels*

Compound	Organism	Type of study	Nominal exposure concentration (ng/mL)	Exposure/depuration period	Tissue; dry weight (dw) or wet weight (ww)	BCF	Reference
VFX	Loach (*Misgurnus anguillicaudatus*)	steady state	500	16	liver (ww)	0.04-0.14	Qu *et al.,* 2019
			500 (co-exposure with microplastics at 50 mg/L)	16		0.06-0.92	
	Juvenile meagre (*Argyrosomus regius*)	steady state	20 (for exposure via water) and 160 µg/kg dw for feed exposure	28	-	65	Maulvault *et al.,* 2018
	Juvenile rainbow trout (*Oncorhynchus mykiss*)	steady state	trout were exposed to treated effluent wastewater	13	plasma	8	Grabicoba et al., 2014
			trout were exposed to treated effluent wastewater		liver (ww)	63	
			trout were exposed to treated effluent wastewater		brain (ww)	9	
	Brook trout (*Salvelinus fontinalis*)	steady state	trout were exposed to 10% and 20% v/v of effluent treated with 15 mg/L of ozone.	3 months	liver (ww)	18	Lajeunesse et al., 2011
			trout were exposed to 10% and 20% v/v of effluent treated with 15 mg/L of ozone.		brain (ww)	11	
			trout were exposed to 10% and 20% v/v of effluent treated with 15 mg/L of ozone.		muscle (ww)	2	
	Mussel (*Mytilus galloprovincialis*)	steady state	up to 15.7	20/20	whole organism (dw)	213-258	Serra-Compte et al., 2018

	Mussel (*Mytilus galloprovincialis*)	kinetic	10	7/7	whole organism (dw)	275	Gomez *et al.,* 2021
	Mussel (*Mytilus galloprovincialis*)	steady state	1, 10, 100 (unique intial exposure concentration)	7	whole organism (dw)	178-189	Gomez *et al.,* 2021
	Sea cucumber (*Holothuria tubulosa*)	kinetic	10	28/52	digestive tract (dw)	87	The present study
	Sea cucumber (*Holothuria tubulosa*)	steady state		28	digestive tract (dw)	1831	
	Sea cucumber (*Holothuria tubulosa*)	kinetic		28/52	body wall (dw)	91	
	Sea cucumber (*Holothuria tubulosa*)	steady state		28	body all (dw)	738	
	Sea anemone (*Anemonia sulcata*)	kinetic		28/52	whole organism (dw)	91	
	Beadlet anemone (*Actinia equina*)	steady state		28	whole organism (dw)	2307-4133	
O-VFX	Loach (*Misgurnus anguillicaudatus*)	steady state	500	16	liver (ww)	0.15-0.97	Qu *et al.,* 2019
			500 (co-exposure with microplastics at 50 mg/L)			4.31-22.81	
	Sea cucumber (*Holothuria tubulosa*)	kinetic	10	28/52	digestive tract (dw)	65	The present study
	Sea cucumber (*Holothuria tubulosa*)	steady state		28	digestive tract (dw)	1298	
	Sea cucumber (*Holothuria tubulosa*)	kinetic		28/52	body wall (dw)	91	
	Sea cucumber (*Holothuria tubulosa*)	steady state		28	body all (dw)	437	
	Sea anemone (*Anemonia sulcata*)	kinetic		28/52	whole organism (dw)	91	
	Beadlet anemone (*Actinia equina*)	steady state		28	whole organism (dw)	1837-3160	

Qu et al. (2019), for example, estimated a BCFss between 0.04-0.14 for VFX and between 0.15-0.97 for O-VFX in liver of loach (*Misgurnus anguillicaudatus*) exposed to a nominal VFX concentration of 16 µg/L day, similar to those used in the present study. BCF variability between tissues was also noted between liver, plasma and brain of trout in two different exposure studies with animals exposed to treated effluent wastewater (Grabicova et al., 2014 and Lajeunesse et al., 2011). In both cases, authors found higher bioaccumulation patterns in liver (BCF 53 and 17 L/kg) followed by brain (9 and 11 L/kg) compared with muscle tissue (2 L/kg). Maulvault et al. (2018) uses both water and dietary exposure sources to study VFX accumulation in juvenile meagre (*Argyrosomus regius*) after 20 days besides the influence of environmental climate conditions. Authors concluded that VFX bioaccumulation (BCF = 64.6, exposure via water) in fish plasma was enhanced under the combination of warming and acidification. Their results also showed that VFX bioaccumulation in fish plasma was positively correlated with the fish growth, regardless of the exposure pathway. BCF levels similar to those estimated in the present study in sea cucumber have been reported for VFX in laboratory studies conducted on mussels (*Mytilus galloprovincialis*) (178-189 L/kg (Serra-Compte et al., 2018) and 178-189 L/kg (Gomez et al., 2021), although the exposure and depuration times were seven and twenty days, respectively).

In comparison and according with reported data summarized in Table 3, our results demonstrate that anemones and sea cucumber tend to have the highest BCF. Similar behaviour has been reported for other emerging pollutants in the literature (Xie et al., 2017; Martín et al., 2019). However, it should be noted that there are inherent difficulties in interpreting and comparing BCF. This variability, besides the natural dispersion due to the different species, can be due to the lack of uniformity in the reported studies such as the exposure concentrations, in the case of ionizable compounds bioaccumulation is found to be concentration-dependent (Burden et al., 2014), the exposure and depuration times (Gómez-Regalado et al., 2023), the temperature (Maulvault et al., 2018) or pH (Maulvault et al., 2018).

Another important limitation found is that most of the accumulation studies did not examine suspended solids or sediments on which contaminants are often adsorbed. Many authors (Oetken et al., 2005; Lagesson et al., 2016; Xie et al., 2017; Wilkinson et al., 2018) have emphasized that deposit-feeding organisms concentrate higher amounts of PhACs, due to their ingestion of organic matter from sediments. Recently, Fonseca et al. (2021) observed higher detection frequencies in benthic and demersal species living directly on or just above the substrate, supporting the combined roles of sediment and dietary routes of pharmaceutical uptake. In the present work, the sediments were also collected in the same sampling point as the water samples. A BCF from sediment up to 287 in the digestive tract of the sea cucumber for VFX and up to 434 in snakelocks anemone for O-VFX were noted.

Finally, besides the accumulation of the VFX parent compound, the presence of its related metabolites in biota is a focus of interest, as some metabolites can be biologically active. The O-VFX metabolite shares the primary biochemical actions of its parent compound and appears to contribute to the therapeutic efficacy of those medications in humans (Rudorfer and Porter 1997). Thus, the presence of this metabolite in marine organisms could have adverse effects. We have determined the ratio between the metabolite concentration and the concentration of the parent compound in the studied animals at the different exposure times. This ratio increases with time, especially in the bioaccumulation experiment carried out in the digestive tract of *H. tubulosa* while a constant value was observed in the body wall, reflecting a low metabolic activity in this tissue and highlighting between-tissues differences in metabolization capacities. Therefore, the BCF must be supplemented with knowledge of the metabolism to have a clear view of their environmental risk.

5. Conclusions

The bioconcentration of the antidepressant VFX and its main metabolite O-VFX in lower non-target marine organisms was studied under laboratory-controlled conditions. Both compounds were rapidly accumulated from the first days up to 40823 and 54342 ng/g dw uptake in the digestive tract of the sea cucumber within 28 days and 64810 and 93007 ng/g dw, respectively, in the snakelocks anemone showing a first-order kinetics process in all cases.

The kinetic BCF reached 2947 (VFX) and 1515 L/kg dw (O-VFX) in the digestive tract of the sea cucumber, and between 2080-4728 (VFX) and 1522-2392 L/kg dw (O-VFX) in anemones which classified the VFX as cumulative. The animal-specific BCF generally followed the order of snakelocks anemone > beadlet anemone > sea cucumber. The results also highlighted between-tissues differences in metabolization capacities, being noteworthy this effect in the digestive tract while irrelevant in the body wall of sea cucumber.

The potential adverse effects of VFX and O-VFX on non-target organisms due to exposure in the marine environment should therefore not be overlooked. Our results demonstrate that anemones and sea cucumber tend to have the highest BCF in comparison with other target organisms like fish or mussels, although the reported data are quite different between them. Up to now, no criteria have been proposed to evaluate the quality of accumulation assays and, as such, there is a lack of uniformity in the reported studies. The need for increased standardization of experimental settings is key towards improving accuracy of environmental risk assessments. In the light of the increasing need to propose animal models, the use of echinoderm and cnidaria represent promising alternative for future bioaccumulation and ecotoxicological studies of emerging pollutants.

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CRediT authorship contribution statement. MCGR: Methodology, resources, conceptualization, writing, review & editing; JM: Methodology, resources, conceptualization, writing, review & editing; FH: Methodology, resources, conceptualization, review & editing; JLS: Conceptualization, supervision, review & editing; IA: Conceptualization, supervision, writing, review & editing; EA: Conceptualization, supervision, funding acquisition and project administration; AZG: Conceptualization, writing, review & editing, supervision, funding acquisition and project administration.

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

References

Ahmadimanesh, M., Balarastaghi, S., Rashedinia, M., Yazdian-Robati, R., 2020. A systematic review on the genotoxic effect of serotonin and norepinephrine reuptake inhibitors (SNRIs) antidepressants. Psychopharmacology. 237, 1909-1915. https://doi.org/10.1007/s00213-020-05550-8

Antonopoulou, M., Dormousoglou, M., Spyrou, A., Dimitroulia, A.A., Vlastos, D., 2022. Na overall assessment of the effects of antidepressant paroxetine on aquatic organisms and human cells. Sci. Total Environ. 852, 158393. http://dx.doi.org/10.1016/j.scitotenv.2022.158393

Archer, E., Holton, E., Fidal, J., Kasprzyk-Hordern, B., Carstens, A., Brocker, L., Kjeldsen, T.R., Wolfaardt, G.M., 2023. Occurrence of contaminants of emerging concern in the Eerste River, South Africa: Towards the optimisation of an urban water profiling approach for public- and ecological health risk characterisation. Sci. Total Environ. 859, 160254. http://dx.doi.org/10.1016/j.scitotenv.2022.160254

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 (4), 257-297. https://doi.org/10.1139/a06-005

Boulard, L., Dierke, G., Schlüsener, M.P., Wick, A., Koschorreck, J., Ternes, T.A., 2020. Spatial distribution and temporal trends of pharmaceuticals sorbed to suspended particulate matter of German rivers. Water Res. 171, 115366. http://dx.doi.org/10.1016/j.watres.2019.115366

Bulleri, F., Ravaglioli, C., Anselmi, S., Renzi, M., 2021. The sea cucumber *Holothuria tubulosa* does not reduce the size of microplastics but enhances their resuspension in the water column. Sci. Total Environ. 781, 146650. https://doi.org/10.1016/j.scitotenv.2021.146650

Burden, N., Creton, S., Weltje, L., Maynard, S.K., Wheeler, J.R., 2014. Reducing the number of fish in bioconcentration studies with general chemicals by reducing the number of test concentrations. Regul Toxicol Pharmacol. 70(2), 442-445. https://doi.org/10.1016/j.yrtph.2014.08.008

Castillo-Zacarías, C., Barocio, M.E., Hidalgo-Vázquez, E., Sosa-Hernández, J.E., Parra-Arroyo, L., López-Pacheco, I.Y., Barceló, D., Iqbal, H.N.M., Parra-Saldívar, R., 2021. Antidepressant drugs as emerging contaminants: Occurrence in urban and non-urbam waters and analytical methods for their detection. Sci. Total Environ. 757, 143722. https://doi.org/10.1016/j.scitotenv.2020.143722

Chahrour, f., Baaloudj, A., Dermeche, S., Kerfouf, A., 2021. Trace metals (Cu, Cd, Pb, Zn, and Ni) concentrations in the Sea Cucumber *Holothuria tubulosa* (Gmelin, 1791) of the Algerian West Coast. Egypt. J. Aquat. Biol. Fish. 25(3), 619-633. https://doi.org/10.21608/EJABF.2021.179882

Chidya, R., Derbalah, A., Abdel-Dayem, S., Kaonga, C., Tsuji, H., Takeda, K., Sakugawa, H., 2022. Contamination, dynamics, and health risk assessment of pesticides in seawater and marine samples from the

Seto Inland Sea, Japan. Environ. Sci. Pollut. Res. 29, 67894-67907. https://doi.org/10.1007/s11356-022-20617-z

Duarte, I.A., Reis-Santos, P., Fick, J., Cabral, H.N., Duarte, B., Fonseca, V.F., 2023. Neuroactive pharmaceuticals in estuaries: Occurrence and tissue-specific bioaccumulation in multiple fish species. Environ. Pollut. 316, 120531. https://doi.org/10.1016/j.envpol.2022.120531

European Union, 2022. Commission implementing decision (EU) 2022/1307 of 22 July 2022 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. Off. J. Eur. Union L197/117-121.

Fonseca, V.F., Duarte, I.A., Duarte, B., Freitas, A., Pouca, A.S.V., Barbosa, J., Gillanders, B.M., Reis-Santos, P., 2021. Environmental risk assessment and bioaccumulation of pharmaceuticals in a large urbanized estuary. Sci. Total Environ. 783, 147021. https://doi.org/10.1016/j.scitotenv.2021.147021

Golovko, O., Rehrl, A. L., Köhler, S., & Ahrens, L., 2020. Organic micropollutants in water and sediment from Lake Mälaren, Sweden. Chemosphere 258, 127293. https://doi.org/10.1016/j.chemosphere.2020.127293

Gómez-Regalado, M. del C., Martín, J., Santos, J. L., Aparicio, I., Alonso, E., Zafra-Gómez, A., 2022. Bioaccumulation/bioconcentration of pharmaceutical active compounds in aquatic organisms: Assessment and factors database. Sci. Total Environ. 861:160638. https://doi.org/10.1016/j.scitotenv.2022.160638

Gomez, E., Boillot, C., Martinez Bueno, J., M., Munaron, D., Mathieu, O., Courant, F., & Fenet, H., 2021. In vivo exposure of marine mussels to venlafaxine: bioconcentration and metabolization. Environ. Sci. Pollut. Res. 28, 68862-68870. https://doi.org/10.1007/s11356-021-14893-4

Grabicova, K., Lindberg, R.H., Ostman, M., Grabic, R., Randak, T.,

Larsson, D.G., Fick, J., 2014. Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant. Sci. Total Environ. 488-489, 46-50. https://doi.org/10.1016/j.scitotenv.2014.04.052

ICH Quality Guidelines, Topic Q2 (R1), Validation of analytical procedures: text and methodology (2005). At http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html (accessed on February 2023).

Jiang, H., Tang, S., Qin, D., Chen, Z., Wang, J., Bai, S., Mou, Z., 2015. Heavy metals in sea cucumber juveniles from coastal areas of Bohai and Yellow Seas, North China. Bull. Environ. Contam. Toxicol. 94, 577-582. https://doi.org/10.1007/s00128-014-1432-1.

Kosma, C.I., Nannou, C.I., Boti, V.I., Albanis, T.A., 2019. Psychiatrics and selected metabolites in hospital and urban wastewaters: occurrence, removal, mass loading, seasonal influence and risk assessment. Sci. Total Environ. 659, 1473-1483. https://doi.org/10.1016/j.scitotenv.2018.12.421

Kowalska, D., Maculewicz, J., Stepnowski, P., Dolzonek, J., 2021. Interaction of pharmaceutical metabolites with blood proteins and membrane lipids in the view of bioconcentration: a preliminary study based on in vitro assessment. Sci. Total Environ. 783, 146987. https://doi.org/10.1016/j.scitotenv.2021.146987

Künili, I.E. and Çolakoglu, F.A., 2019. Chemical and nutritional characteristics of *Holothuria tubulosa* (Gmelin, 1788); a seasonally comparative study. J. Aquat. Food Prod. Technol. 28, 716-728. https://doi.org/10.1080/10498850.2019.163738

Lagesson, A., Fahlman, J., Brodin, T., Fick, J., Jonsson, M., Byström, P., Klaminder, J., 2016. Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web - Insights from a field experiment. Sci. Total Environ. 568, 208-215. http://dx.doi.org/10.1016/j.scitotenv.2016.05.206

Lajeunesse, A., Gagnon, C., Gagne, F., Louis, S., Cejka, P., Sauve, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment - evidence of biological effects. Chemosphere 83 (4), 564-571. https://doi.org/10.1016/j.chemosphere.2010.12.026

Mackay, D., 1982. Correlation of bioconcentration factors. Environ. Sci. Technol. 16, 274e278. https://doi.org/10.1021/es00099a008

Martín, J., Hidalgo, F., Alonso, E., García-Córcoles, M.T., Vílchez, J.L., Zafra-Gómez, A., 2020. Assessing bioaccumulation potential of personal care, household and industrial products in a marine echinoderm (*Holothuria tubulosa*). Sci. Total Environ. 720, 137668. https://doi.org/10.1016/j.scitotenv.2020.137668

Martín, J., Hidalgo, F., García-Córcoles, M.T., Ibáñez-Yuste, A.J., Alonso, E., Vílchez, J.L., Zafra-Gómez, A., 2019. Bioaccumulation of perfluoroalkyl substances in marine echinoderms: Results of laboratory-scale experiments with *Holothuria tubulosa* Gmelin, 1791. Chemosphere. 215, 261-271. https://doi.org/10.1016/j.chemosphere.2018.10.037.

Martín, J., Zafra-Gómez, A., Hidalgo, F., Ibáñez-Yuste, A.J., Alonso, E., Vílchez, J.L., 2017. Multi-residue análisis of 36 priority and emerging pollutants in marine echinoderms *(Holothuria tubulosa)* and marine sediments by solid-liquid extraction followed by dispersive solid phase extraction and liquid chromatography-tandem mass spectrometry analysis. Talanta, 166, 336-348. https://doi.org/10.1016/j.talanta.2017.01.062

Maulvault, A.L., Santos, L.H.M.L.M., Paula, J.R., Camacho, C., Pissarra, V., Fogaça, F., Barbosa, V., Alves, R., Ferreira, P.P., Barceló, D., Rodriguez-Mozaz, S., Marques, A., Diniz, M., Rosa, R., 2018. Differential behavioural responses to venlafaxine exposure route, warming and acidification in juvenile fish (*Argyrosomus regius*). Sci. Total Environ. 634, 1136-1147. https://doi.org/10.1016/j.scitotenv.2018.04.015

McCallum, E.S., Sundelin, A., Fick, J., Alanärä, A., Klaminder, J., Hellström, G., Brodin, T., 2019. Investigating tissue bioconcentration and the behavioural effects of two pharmaceutical pollutants on sea trout (*Salmo trutta)* in the laboratory and field. Aquat. Toxicol. 207, 170-178. https://doi.org/10.1016/j.aquatox.2018.11.028

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. https://doi.org/10.1002/etc.5620180412

Miller, T.H., Bury, N.R., Owen, S.F.; Barron, L.P., 2017. Uptake, biotransformation and elimination of selected pharmaceuticals in a freshwater invertebrate measured using liquid chromatography tandem mass spectrometry. Chemosphere 183, 389-400. https://doi.org/10.1016/j.chemosphere.2017.05.083

Molina-Fernandez, M., Perez-Conde, C., Rainieri, S., Sanz-Landaluze, J., 2017. Method for quantifying NSAIDs and clofibric acid in aqueous samples, lumpfish (*Cyclopterus lumpus*) roe, and zebrafish (*Danio rerio*) eleutheroembryos and evaluation of their bioconcentration in zebrafish eleutheroembryos. Environ. Sci. Pollut. Res. 12, 10907-10918. https://doi.org/10.1007/s11356-016-6671-8

Molina-Fernández, N., Rainieri, S., Muñoz-Olivas, R., de Oro-Carretero, P., & Sanz-Landaluze, J., 2021. Development of a method for assessing the accumulation and metabolization of antidepressant drugs in zebrafish (*Danio rerio*) eleutheroembryos. https://doi.org/10.1007/s00216-021-03486-2

Morais, L.M.S., Sarti, F., Chelazzi, D., Cncinelli, A., Giarrizzo, T., Martinelli Filho, J.E., 2020. The sea anemone *Bunodosoma cangicum* as a potential biomonitor for microplastics contamination on the Brazilian Amazon coast. Environ. Pollut. 265, 114817. https://doi.org/10.1016/j.envpol.2020.114817.

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., NHirai, N.,

Tatarazako, N., 2008. The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): acute toxicity in fish larvae and bioaccumulation in juvenile fish. Chemosphere 70, 865-873. https://doi.org/10.1016/j.chemosphere.2007.06.089

Nikokherad, H., Esmaili-Sari, A., Moradi, A.M., Bahramifar, N., Mostafavi, P.G., 2022. Bioaccumulation capacity of *Chlorella vulgaris* and *Spirulina platensis* exposed to silver nanoparticles and silver nitrate: Bio- and health risk assessment approach. Algal Res. 64, 102671. https://doi.org/10.1016/j.algal.2022.102671.

OECD Health Statistics 2019.

OECD. Test No. 305: Bioaccumulation in fish: aqueous and dietary exposure. OECD Guidelines for the Testing of Chemicals. 2012; Section 3, O.P.

Oetken, M., Nentwig, G., Löffer, D., Ternes, T., Oehlmann, J., 2005. Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. Arch. Environ. Contam. Toxicol. 49(3), 353-361. https://doi.org/10.1007/s00244-004-0211-0

Osorio, V., Larrañaga, A., Aceña, J., Pérez, S., & Barceló, D., 2016. Concentration and risk of pharmaceuticals in freshwater systems are related to the population density and the livestock units in Iberian Rivers. Sci. Total Environ. 540, 267-277. https://doi.org/10.1016/j.scitotenv.2015.06.143

OSPARCOM, 2012. JAMP Guidelines for Monitoring Contaminants in Biota. OSPAR Commission, London, p. 122.

Palmer, T. A., Klein, A. G., Sweet, S. T., Frazier, A. J., Montagna, P. A., Wade, T. L., & Beseres Pollack, J., 2022. Using epibenthic fauna as biomonitors of local marine contamination adjacent to McMurdo Station, Antarctica. Mar. Pollut. Bull. 178, 113621. https://doi.org/10.1016/j.marpolbul.2022.113621

Qu, H., Ma, R., Wang, B., Yang, J., Duan, L., Yu, G., 2019. Enantiospecific

toxicity, distribution and bioaccumulation of chiral antidepressant venlafaxine and its metabolite in loach (*Misgurnus anguillicaudatus*) co-exposed to microplastic and the drugs. J. Hazard. Mater. 370, 203-211. https://doi.org/10.1016/j.jhazmat.2018.04.041

Rudorfer, M.V., Porter, W.Z., 1997. The role of metabolites of antidepressants in the treatment of depression. CNS Drugs 7(4), 273-312. https://doi.org/10.2165/00023210-199707040-00003

Serra-Compte, A., Maulvault, A. L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz, S., & Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*). Environ. Pollut. 236, 824-834. https://doi.org/10.1016/j.envpol.2018.02.018.

Silva, B., Costa, F., Neves, I.C., Tavares, T., 2015. Psychiatric pharmaceuticals as emerging contaminants in wastewater. Springer Briefs in Green Chemistry for Sustainability. https://doi.org/10.1007/978-3-319-20493-2_1

Stambler, N., Dubinsky, Z., 1987. Energy relationships between *Anemonia sulcata* and its endosymbiotic zooxanthellae. Symbiosis 3, 233-248.

Sugni, M., Mozzi, D., Barbaglio, A., Bonasoro, F., Carnevali, M.D.C., 2007. Endocrine disrupting compounds and echinoderms: new ecotoxicological sentinels for the marine ecosystem. Ecotoxicology 16, 95-108. https://doi.org/10.1007/s10646-006-0119-8

Veith, G.D., DeFoe, D.L., Bergstedt, B.V., 1979. Measuring and estimating the bio- concentration factor of chemicals in fish. J. Fish. Res. Board Can. 36, 123e155. https://doi.org/10.1139/f79-146

Wilkinson, J.L., Hooda, P.S., Swinden, J., Barker, J., Barton, S., 2018. Spatial (bio)accumulation of pharmaceuticals, illicit drugs, plasticisers, perfluorinated compounds and metabolites in river sediment, aquatic plants and benthic organisms. Environ. Pollut. 234,

864-875. https://doi.org/10.1016/j.envpol.2017.11.090

Xie, Z., Lu, G., Yan, Z., Liu, J., Wang, P., Wang, Y., 2017. Bioaccumulation and trophic transfer of pharmaceuticals in food webs from a large freshwater lake. Environ. Pollut. 222, 356-366. https://doi.org/10.1016/j.envpol.2016.12.026

Zhao, J.-L., Furlong, E. T., Schoenfuss, H. L., Kolpin, D. W., Bird, K. L., Feifarek, D. J., Schwab, E. A., Ying, G.-G., 2017. Uptake and disposition of select pharmaceuticals by bluegill exposed at constant concentrations in a flow-through aquatic exposure system. Environ. Sci. Technol. 51, 4434−4444. http://dx.doi.org/10.1021/acs.est.7b00604

Zheng, Y., Chang, X., Huang, Y., He, D., 2023. The application of antidepressant drugs in cancer treatment. Biomed. Pharmacother. 157, 113985. https://doi.org/10.1016/j.biopha.2022.113985.

Instrumentation and software

The samples were lyophilised with a ScanVac CoolSafe™ freeze dryer. The pulverisation of the samples was performed with a Retsch MM200 ball mixer (Haan, Germany). An IKA vortex (Staufen, Germany), a Labnet Spectrafuge™ 24D centrifuge (New Jersey, USA) were also used. Ultrasound extraction was performed with a 400 W digital sonicator, with a 0.5-inch (12.7 mm) probe operating at a frequency of 20 kHz. In addition, an Ultrasounds-HD ultrasonic bath from Selecta (Barcelona, Spain) and a Mettler-Toledo precision balance (Columbus, OH, USA) were set up. Finally, a Stuart sample concentrator-nitrogen evaporator (Staffordshire, UK) was used for the evaporation of the samples.

UHPLC-MS/MS analysis was conducted with a Waters ACQUITY UPLC® H-Class (Waters, Manchester, UK), consisting of an AQUITY™ sample manager, an ACQUITY UPLC™ binary solvent manager (Waters, UK) and an ACQUITY HSS T3 column (100 mm x 2.1 mm internal diameter, 1.8 µm particle size). A XEVO TQ-XS quadrupole tandem mass spectrometer was used for antidepressant detection equipped with an orthogonal Z-spray™ electrospray ionisation (ESI) source. MassLynx 4.1 software (Waters) was employed for data acquisition.

Hanna Instruments® digital thermometer (model HI Checktemp 1), HACH® sensION™ þ EC5 portable conductivity/TDS meter with conductivity probe (model 50 60), HACH® HQ40d portable multimeter with pH probe (model pHC101) and luminescent dissolved oxygen probe (model LDO101) were used for the measurements.

Validity requirements

The first step was to check that all the matrices studied had a very low matrix effect. For this, a calibration with pure standard and a calibration with matrix were compared, and the slope of the line obtained was similar for all compounds. Calibration points were prepared at eight concentration levels. To evaluate the sensitivity of the method from the

signal-to-noise ratio obtained from the injection of standards with decreasing amounts of the selected target analytes, the limit of detection (LOD) and the limit of quantification (LOQ) were determined. The selectivity of the method was studied by comparing the chromatograms of the blank with a standard sample. Furthermore, the accuracy in terms of trueness and precision was evaluated by recovery tests at three concentration levels (low, medium and high) for trueness, and the relative standard deviation (% RSD) of the measurements was evaluated three times on the same day and over three days for precision.

The quality assurance/quality control (QA/QC) of the data obtained in the validation was checked by injecting spiked blank samples, which were treated the same as the samples to control background contamination. It was found that there were no detectable amounts in these blanks. Also, instrumental stability was assessed by injecting each sample in triplicate and every 20 analyses a mixture of the pure standards of the target analytes was injected.

Table S1*. Structure and physicochemical properties of the studied compounds*

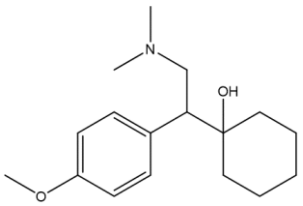
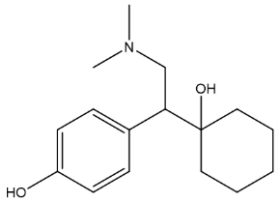
Compound	Mw (g/mol)	pK_a	Log K_{ow}	Structure
VFX	277.4	9.50	3.20	
O-VFX	263.4	9.45	2.72	

***Table S2**. Retention time, transitions and MS parameters*

Compound	t_R (min)	Transitions	CV (V)	CE (eV)	Ratio
VFX	4.45	278.2 > 58.1[a]	24	16	1.1
		278.2 > 260.3[b]	24	12	
O-VFX	3.88	264.2 > 58.1[a]	20	16	1.6
		264.2 > 246.2[b]	20	12	
Spectrometric conditions					
Impactor voltage	1 kV	Nebulizer gas pressure	7.0 bar		
Source temperature	150 ºC	Cone/desolvation gas	N_2 (≥ 99.995 %)		
Desolvation temperature	600 ºC	Collision gas	Ar (99.999 %)		
Cone gas flow	150 L h^{-1}	Dwell time	25 ms		
Desolvation gas flow	500 L h^{-1}	Inter-scan delay	3 ms		

Transitions: [a]for quantification; [b]for confirmation; CV: Cone voltage; CE: collision energy

***Table S3**. Limit of detection and limit of quantification, precision and linearity*

Compound	LOD (ng/g dw)	LOQ (ng/g dw)	RSD (%)	Linearity (%)
VFX	0.03	0.10	10.71	99.62
O-VFX	1.67	5.00	7.46	99.10

LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative Standard Deviation.

Capítulo 8

Uptake and depuration of three common antibiotics in benthic organisms: sea cucumber *(Holothuria tubulosa),* snakelocks anemone *(Anemonia sulcata)* and beadlet anemone *(Actinia equina)*

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Abstract: Antibiotics are widely used in humans and animals, which has attracted a great deal of attention due to the development of bacterial resistance which is currently a problem of great concern to governments and states, as it is linked to the resurgence of infectious diseases. Understanding bioaccumulation of antibiotics in aquatic organisms is a key for understanding their risk assessment. The present study was designed to study the bioaccumulation of target antibiotics in relevant organisms that inhabit benthic marine environments. The uptake and depuration of ciprofloxacin (CIP), sulfamethoxazole (SMX) and trimethoprim (TMP) in cotton spinner sea cucumber (*Holothuria tubulosa*), snakelocks anemone (*Anemonia sulcata)* and beadlet anemone (*Actinia equina*) under controlled laboratory conditions, was investigated. The results showed that antibiotics had a particular tendency over time during the whole uptake and depuration periods. The tissue distribution of antibiotics in sea cucumber was strongly influenced by the structure of the compounds, while CIP was highly concentrated in the body wall; TMP was highly concentrated in the digestive tract. Two different approaches were used to estimate the biocencentration factors (BCF) in the different animal models based on toxicokinetic data and the the concentrations measured at the steady-state. The ranges of BCF were 456-2731 L/kg, 6-511 L/kg and 9-100 L/kg for TMP, CIP and SMX, respectively. The estimated BCF values obtained classify TMP as cumulative in *A. equina* and *H. tubulosa* emphasizing the potential bioconcentration in these marine organisms. A lack of correlation was observed between BCFs of target antibiotics and K_{ow}. The animal-specific BCF followed the order of beadlet anemone > sea cucumber > snakelocks anemone.

Keywords: Ciprofloxacin; Sulfamethoxazole; Trimethoprim; Bioconcentration; Lower trophic organisms; Animal-distribution.

1. Introduction

Antibiotics are antimicrobials widely used for the treatment and prevention of bacterial diseases (WHO, 2020). They are used as preventive medicine and as growth promoters (Bihn et al., 2018). The extensive use of these drugs poses a potential risk to public health due to its possible allergic reactions, as well as the development of bacterial resistance (Decheng et al., 2018) which is currently a problem of great concern to governments and states, as it is linked to the resurgence of infectious diseases (Saxena et al., 2021). After use, whether by animals or humans, these compounds are excreted and/or discharged, ending up in wastewater treatment plants (WWTP), either in their original form or in their metabolites, and from there to aquatic environments, including marine environments. Their entry into the environment and more specifically into the marine ecosystem has raised great scientific concern since detectable concentrations are found to be potentially harmful (Ziarrusta et al., 2018, Cravo et al., 2022).

This concern has motivated the European Union to develop regulations on the protection of marine ecosystems and the scientific community to develop research on the biomonitoring of these compounds in water, sediments and different species of biota. The Water Framework Directive (WFD; 2000/60/EC) and the Marine Strategy Framework Directive (European Union, 2008/56/EC) were elaborated in order to promote environmentally sustainable use of water and maintain good status of the marine environment. In addition, in 2015, the European Commission (EC) drew up a first list of emerging substances to be controlled by the marine environment. This list was updated in 2018 and 2020, and Decision 2020/1161/EC includes some antibiotics to be monitored such as ciprofloxacin (CIP), sulfamethoxazole (SMX) and trimethoprim (TMP), whose maximum acceptable method detection limits are 78 and 89 ng/L for the first and 100 ng/L for sulfamethoxazole and trimethoprim (European Union, 2020).

Biomonitoring of these substances in the marine environment is usually carried out using bioindicators. A bioindicator is a living

organism, such as a plant, animal or microorganism, capable of providing information on the quality of the environment (Bonanno et al., 2018). The use of bioindicators as sentinels of environmental health provides qualitative and quantitative information (Bonanno & Pavone, 2015). Anemones are sessile benthic organisms that possess high stress tolerance and low mobility and are potential organisms for monitoring environmental pollution. The snakelocks anemone *Anemonia sulcata* (Pennant, 1777) as well as the beadlet anemone *Actinia equina* (Linnaeus, 1758) are widely distributed in the Mediterranean Sea and the Atlantic Ocean, thus making them very interesting species for bioaccumulation studies (Vitale et al., 2020). Sea cucumbers of the gender *Holothuria*, are a key component of benthic communities as they play a very important role in the dynamics of the seabed removing its organic matter. The cotton spinner sea cucumber (*Holothuria tubulosa*) (Gmelin, 1791) is a detritivore deposit feeder that ingests substrate sediments to profit its food particles and organic matter. It is one of the most common sea cucumbers in the Mediterranean Sea and their sensitivity to pollutants has also made it model specie for testing the effects of marine pollution (Lombardo et al., 2022; Morroni et al., 2020; Rakaj et al., 2021).

In this context, and taking into account the importance of controlling the presence and accumulation of antibiotics in the environment and the convenience of certain species being sentinels of this contamination, it is necessary to highlight the relevance of developing laboratory tests to evaluate the bioconcentration of the compounds both in the marine environment and in the organisms that inhabit it (Duarte et al., 2023). Bioconcentration is the accumulation of chemicals in the body of organisms that are constantly exposed to the contaminated environment (Nikokherad et al., 2022). Several studies have focused on the bioconcentration of antibiotics in marine organisms. For example, Serra-Compre et al. (2018) studied the bioconcentration of sulfamethoxazole in mussels, obtaining BCFs between 6.2-9.0; Zhao and colleagues (2017) investigated the bioconcentration of the same antibiotic in laboratory tests using different tissues of bluegill sunfish *(Lepomis macrochirus)* observing significant differences in BCFs between the different body

compartments, with values in the range of 0 to 4.48 obtained in the liver. Zhu et al. (2020) and Miller et al. (2017) focused their study on the bioaccumulation of different antibiotics the former and different pharmaceutical families the latter. These studies concentrated on the different body compartments of Juvenile sea cucumber and freshwater invertebrate, respectively, finding BCFs values for trimethoprim ranged from 38.3 to 724.8 in the case of sea cucumber and 16-21 in the invertebrate.

The aim of the present study was to carry out a laboratory bioconcentration test of three antibiotics widely used in medicine (CIP, SMX and TMP) in three bioindicators belonging to the benthic marine environment, *H. tubulosa, A. sulcata* and *A. equina*. The experiment lasted a total of 80 days and the antibiotics in the organisms were determined. Bioconcentration factor was calculated in two ways, using the mass balance model and the steady state and results were compared with the available scientific literature. To the best of our knowledge, this is the first study to determine the bioconcentration of these antibiotics in non-target but common marine organisms.

2. Material and methods

2.1. Chemical and reagents

Ultrapure water (18.2 MΩ cm) was produced by using a Milli-Q Plus® system (Millipore, Madrid, Spain). Ciprofloxacin (CIP, ≥ 98.0%), sulfamethoxazole (SMX, ≥ 98.0%) and trimethoprim (TRI, ≥ 99.8%) were purchased from Sigma-Aldrich (Madrid, Spain). Cinchophen (CIN) used as surrogate, was bought from Alfa Aesar (Massachusetts, MA, USA). LC-MS methanol (MeOH) was obtained from VWR ProLabo CHEMICALS (Barcelona, Spain). LC-MS formic acid (98%) used as mobile phase modifier was from Honeywell (Charlotte, USA). Stock solutions (100 mg L^{-1}) of target analytes were prepared in methanol and stored at -20 ºC. The physicochemical properties and the chemical structure of antibiotics are included as supplementary material (Table S1). Nitrate, nitrite and ammonium analyses were performed using Hanna instruments® Test Kits (HI 3874, 3873 and 3826).

2.2. Laboratory test

2.2.1. Sample collection and acclimation period

The animals used in this study were collected off the coast of Granada (Spain) by divers. They were captured at random locations at depths of 10 to 15 meters in February 2022. Then, they were transported in refrigerated containers to the Zoology Department of the University of Granada at a temperature of 4 ºC. Once there, they were transferred to the experimental aquaria, where the acclimatization period, which lasted 15 days, began. During sample collection, 50 holothurians, of which 45 were used as samples (mean 193.7 ± 10.7 g), of the species *Holothuria tubulosa* as well as 50 snakelocks anemones (*Anemonia sulcata*) and beadlet anemones (*Actinia equina*) were captured.

2.2.2. Up-take and depuration test

The experiment was carried out in the Zoology Department of the University of Granada (Spain). A 300 L glass aquarium containing natural seawater in recirculation with artificial lighting by means of a 6000 ºK metal halide lamp was used. The system was filtered with a biological filter with foam rubber and bioballs, and the aeration was carried out with compressed air. Photoperiods of 10 hours of light and 14 hours of darkness were carried out. Throughout the experiment, the physicochemical characteristics of the water were monitored twice a week, with mean values of temperature 19.7 ± 0.2 ºC, pH 8.2 ± 0.0, salinity 37.5 ± 0.2 PSU, dissolved O_2 6.3 ± 0.1 mg L^{-1}, ammonium 0.20 ± 0.03 mg L^{-1}, nitrites 0.23 ± 0.01 mg L^{-1}, nitrates 51 ± 3 mg L^{-1}. Twice a week a scoop containing a mixture of fishmeal and dried spirulina (1:1) was added to the aquarium to feed the animals.

The experiment took place over 80 days. Due to the LD_{50} values of the study compounds CIP 10.30 mg/L for *Drosophila melanogaster* (Liu et al., 2019) and 6.43 and 6.38 mg/L for SMX and TMP, respectively for Daphnias (Martínez-Alcalá et al., 2020), a low exposure concentration was used, 1% of LC_{50}, according with the OECD 305 method. For the first 28 days (the uptake period), 3 mg of each of the target compounds was

added daily to the aquarium. For this, approximately 10 L of water was withdrawn and the compounds were diluted with vigorous manual dilution. Once dissolved, the water was returned by spreading it throughout the aquarium. Since the capacity of the aquarium was 300 L, the final concentration of the different compounds added each day was 10 μg L^{-1}. Holothurians (3 specimens), snakelocks anemones (3 specimens) and beadlet anemone (2 specimens) as well as water (approximately 80-100 mL) and sediments were sampled every 4 days.

Once the uptake period finished a depuration stage was carried out. For this purpose, all remaining specimens were moved to a new aquarium with the same characteristics as the previous one. During this time, the antibiotics studied were not added, only specimens, water and sediments were sampled every 4 days in the same way as in the previous absorption period. For each sampling, 3 sea cucumbers, 3 snakelocks anemones and 2 beadlet anemones as well as water and sediments were collected. In the case of sea cucumbers, their body compartments (body wall and digestive tract) were separated. Once in the laboratory, the animals and the sediments were freeze-dried for 48 hours at -109 ºC. After lyophilizing, the samples were sprayed, first manually with a pestle and mortar and then with a ball mill and then, stored at -20 ºC. The water was also conserved under these conditions.

2.3. Analytical methods

For the extraction of the target compounds, in the case of solid samples, 0.2 g (dry weight, dw) were weighed into 10 mL glass tubes to which 50 μL of a CIN standard solution at 4 mg L^{-1} were added, giving a final concentration of 200 ng g^{-1} in the sample. The samples were kept for 24 hours in the dark, in order to ensure maximum contact between the matrix and the surrogate. The next day, 2.2 mL of MeOH were added and vortexed for 30 seconds. Ultrasound-assisted extraction (UAE) was performed for 12 minutes at 40% amplitude. After digestion, the tubes were centrifuged and the supernatant was removed. Two extraction cycles were performed by pooling the supernatants, which were subsequently evaporated to dryness under a stream of N_2. The dry

extracts were dissolved in 0.2 mL of the initial mobile phase (80/20 H_2O/MeOH, v/v) and 2 µL were injected into the chromatographic system.

For the analysis of water, 10 mL of the sample were placed in a glass tube and 50 µL of CIN (surrogate) was added to have a final concentration of 200 ng mL^{-1}. The samples were frozen and then freeze-dried for 48 hours until dry. Once dry, 1 mL of MeOH was added and vortexed vigorously for 1 minute and then centrifuged for 5 minutes to separate the high amount of salt present in the sample. The process was performed twice and the supernatants were mixed. The samples were evaporated under a N_2 stream and dissolved in the initial mobile phase as for the solid samples.

Analysis by ultra-high performance liquid chromatography coupled to tandem mass spectrometry was performed using an ACQUITY UPLC HSS T3 column (100 mm x 2.1 mm i.d x 1.8 µm particle size). The mobile phase used for the separation of the compounds was water modified with 0.05% formic acid (v/v) as phase A and methanol as phase B. The separation of the compounds took place over 8 minutes and the gradient program carried out is specified below: 0.0-1.5 min, 20% B, 1.5-3.0 min, 20-40% B, 3.0-4.0 min, 40-85% B, 4.0-6.0 min, 85% B, 6.0-6.1 min, 85-20% B, back to initial conditions (total run 8 min). The electrospray (ESI) positive mode was used as an interface and the multiple-reaction monitoring mode was used to identify and quantify all compounds, in order to increase sensitivity and specificity. Two transitions were selected for each compound; the most abundant was used for quantification and the other one for confirmation. The parameters used as well as the transitions are included as supplementary information (Table S2).

The method was also validated according the ICH guidelines (ICH, 2005) in terms of linearity, linear dynamic range, sensitivity and accuracy (trueness and precision). Table 1 summarizes the limits of detection and quantification, as well as linearity and linear dynamic range. Recovery assays were carried out at three concentration levels (low, medium and high) for the assurance of the accuracy of the method.

Recoveries close to 100% in the different matrices were obtained, being the relative standard deviation below 15% in all cases.

Table 1. *Validation parameters of the analytical method*

Compound	LOD (ng/g)	LOQ (ng/g)	RSD (%)	Linearity (%)
CIP	0.03	0.1	9.9	94.4
SMX	0.03	0.1	10.4	99.6
TMP	0.03	0.1	7.7	99.7

LOD: limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation

2.4. Toxicokinetic modelling and bioconcentration factor calculation

The bioconcentration kinetic parameters for the studied antibiotics in each animal model were calculated based on a mass balance model (Mackay and Fraser, 2000). In this model, the uptake and depuration process can be described as follow:

$$\frac{dC_b}{dt} = k_1 C_w - k_2 C_b \quad (1)$$

$$\frac{dC_b}{dt} = -k_2 C_b \quad (2)$$

Where C_b is the concentration in biota (ng/g dw), t the time of exposure (days), k_1 the first-order uptake constant (L/kg·d), C_w the concentration in water (ng/mL), and k_2 the first-order elimination rate constant (1/d).

Assuming a negligible concentration in biota samples at t_0, and considering its constant in the exposure medium, the equations can be expressed as:

$$C_b(\text{uptake}) = \frac{k_1}{k_2} C_w(1 - e^{-k_2 t}) \quad (3)$$

$$C_b(\text{depuration}) = C_{b,0} e^{-k_2 t}) \quad (4)$$

$C_{b,0}$ is the concentration when the depuration phase begins.

The BCF can then be estimated as:

$$BCF_k = \frac{k_1}{k_2} \quad (5)$$

Two different parts of the organisms (body wall and digestive tract) were considered in the case of *H. tubulosa*, while for anemones the whole body of the organism was considered.

The half-lives ($t_{1/2}$) of antibiotics in biological samples were determined as:

$$t_{1/2} = \frac{ln2}{k2} \quad (6)$$

A second approach was also used to calculate the BCF using the concentration levels measured in water and biota at steady-state through the equation 7:

$$BCF_{SS} = \frac{C_b}{C_w} \quad (7)$$

C_b is the concentration in the biota (ng/g dw) and C_w is the concentration in the water (ng/mL), at the same time and at the steady-state. The steady stated was estimated at 28 days in all cases. Moreover, the C_w was kept within 20% of the measured mean (N = 3).

2.5. Quality and exposure control

Prior to the experiment, selected antibiotics were measured in the water and sediment of the aquaria as well as in the control organisms (N = 5). No adverse behaviour was observed in both the control and treatment groups during the period of the experiment. Mortality was less

than 1% in all cases. The condition index (control and contaminated) remained stable, with low variability, under these conditions being thus optimal for animals during the experiment. To guarantee the quality assurance of the results, a protocol involving the use of control samples including fortified organisms samples with the target compounds (at 100 ng/g dw), a mixture of the standards in pure solvent (at 100 ng/mL), solvent (methanol:water 50:50 v/v) and procedural blanks injections were included in each analytical batch. For quantification purposes, eight-point calibration curves in matrix were prepared containing CFX, SMX and TMP in the range from method quantitation limit (LOQ) to 500 ng/g dw.

3. Results and discussion

The mean concentrations of the antibiotics studied, in the water phase of the experimental aquarium and the surrounding sediments throughout the experiment, are shown in Table 2.

Table 2. *Mean concentration and relative standard deviation (RSD, %) of CIP, SMX and TMP in the water and sediments phases throughout the experiment*

Sampling (day)	Water phase (ng/mL)						Sediments (ng/g dw)					
	CIP		SMX		TMP		CIP		SMX		TMP	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
0	ND	-	ND	-	ND	-	3.6	2.3	ND	-	4.08	6.3
4	22.4	5.3	<LD	-	13.3	5.6	31.3	30.0	5.95	2.3	89.0	26.7
8	39.6	6.9	4.49	13.5	18.1	9.2	34.9	11.6	4.99	3.0	94.0	8.8
12	28.6	5.0	9.80	3.2	16.5	9.6	44.4	4.9	10.3	5.2	60.3	2.2
16	38.1	3.7	12.4	5.4	19.8	3.2	90.6	9.4	12.3	15.2	90.8	6.7
20	34.5	6.7	16.9	1.7	21.3	11.0	41.9	4.9	11.6	12.3	162	5.5
24	32.7	2.5	15.0	28.3	18.3	5.8	83.7	3.8	31.3	21.0	172	5.5
28	33.0	8.2	17.0	6.1	19.0	6.6	93.7	5.7	29.0	26.6	106	12.5
32	ND	-	ND	-	5.69	5.1	14.5	3.4	ND	-	14.3	8.5
36	ND	-	ND	-	0.28	0.2	13.8	0.8	ND	-	4.07	2.1
40	ND	-	ND	-	0.17	1.0	13.7	1.9	ND	-	5.38	26.4
44	ND	-	ND	-	-	-	15.1	10.2	ND	-	2.50	7.1
48	ND	-	ND	-	-	-	13.5	1.0	ND	-	1.49	10.0
52	ND	-	ND	-	ND	-	13.4	1.6	ND	-	1.39	1.5
56	ND	-	ND	-	ND	-	14.9	13.4	ND	-	2.68	3.0
60	ND	-	ND	-	ND	-	13.5	0.9	ND	-	7.06	7.0
70	ND	-	ND	-	ND	-	13.5	1.2	ND	-	1.57	10.2
80	ND	-	ND	-	ND	-	13.6	2.7	ND	-	1.32	1.0

ND: no detected; <LD: Below limit of detection

3.1. Bioconcentration of ciprofloxacin

CIP was not detected in the water control samples, thus indicating the absence of contamination prior to the controlled addition of the pollutants. In the case of sediments, the concentrations initially found of CIP was negligible in relation to those measured after controlled addition. During the exposure stage (spiking with a nominal concentration of 10 ng/mL) the values measured showed a minimal variation between 22.4 to 39.6 ng/mL. CIP was detected at higher concentrations in the sediment compartment than in water samples from 3.6 ng/g in control sediments to 93.7 ng/g on the last day of the exposure experiment (28 days), which suggest that sediments may be an important source of selected compounds for deposit feeders like is the case of sea cucumber. The results of the up-take exposure in two different tissues

(digestive tract and body wall) of sea cucumber and in the whole body of both types of anemones are plotted in Figure 1.

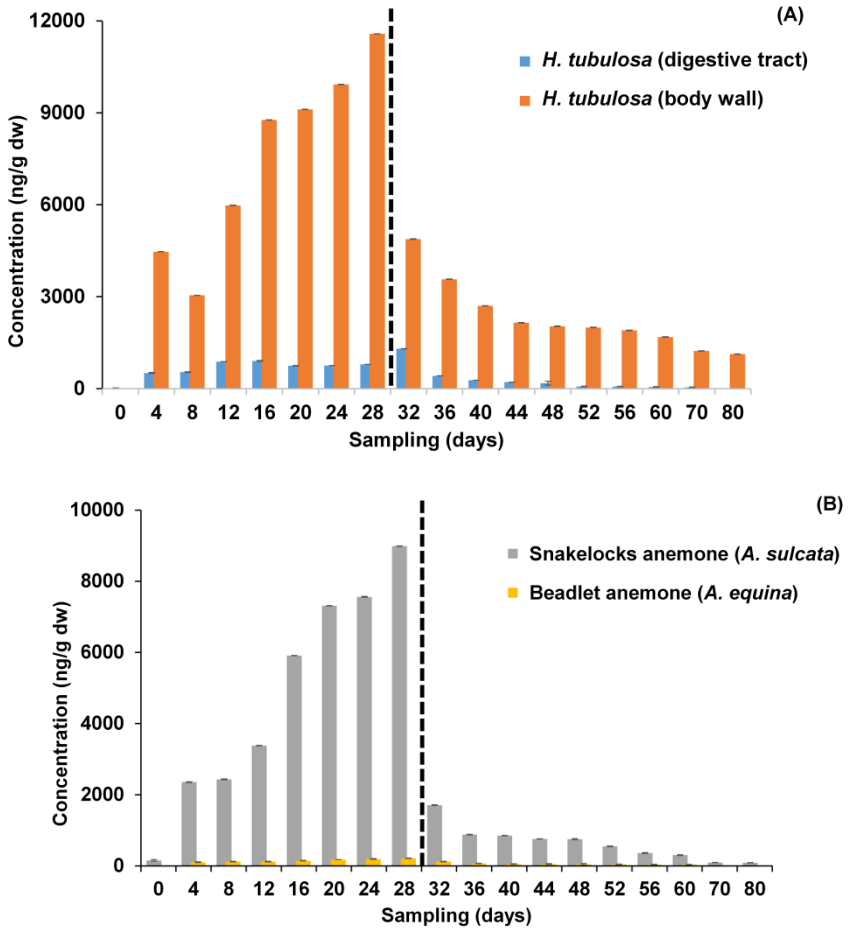


Figure 1. *Kinetics of accumulation (days 0-28) and depuration (days 32-80) of CIP in sea cucumber (Holothuria tubulosa) (A) and snakelocks anemone (Anemonia sulcata) and beadlet anemone (Actinia equina) (B)*

CIP was not detected in the control samples of *A. equina* (whole body) and *H. tubulosa* (body wall), while a mean concentration of 19 ng/g dw and 155 ng/g dw was found in the digestive tract of *H. tubulosa* and in the whole body of *A. sulcata*, respectively. In the uptake phase, a rapid accumulation was observed from the first 4 days. The concentration of CIP reached up to 11577 and 8987 ng/g dw in the body wall of sea

cucumber and in snakelocks anemone, respectively, after 28 days with an accumulation rate constants (k_1) of 14.2 and 19.4 L/kg·d. In the depuration phase, CIP seems to follow a biphasic elimination pattern with an initial rapid decrease the first four days (concentration dropped down nearly 80% and 70% between days 28 and 32 in *A. sulcata* and body wall of *H. tubulosa*) followed by a slower decrease. Table 3 summarizes the toxicokinetic parameters after adjusting to the first-order kinetic model.

***Table 3**. Toxicokinetic data of CPX, SMX and TMP in studied animals*

Compound	k_2 (1/d)	R^2	$t_{1/2}$ (d)	k_1 (L/kg d)	R^2	$BCF_{kinetic}$ (L/kg)	BCF_{ss} (L/kg)	BCF_{ss} sed
	H. tubulosa (digestive tract)							
CIP	0.084	0.886	8	1.146	0.440	14	24	8
SMX	0.071	0.891	10	1.901	0.566	27	36	21
TMP	0.154	0.895	4	300.6	0.880	1948	2489	448
	H. tubulosa (body wall)							
CIP	0.028	0.896	25	14.20	0.823	511	351	124
SMX	0.049	0.696	14	0.497	0.772	9	14	8
TMP	0.084	0.972	8	38.39	0.696	456	597	107
	Snakelocks anemone (*A. sulcata*)							
CIP	0.066	0.946	11	19.49	0.806	297	272	96
SMX	0.040	0.974	17	3.962	0.895	100	78	46
TMP	0.112	0.785	6	91.15	0.581	812	982	177
	Beadlet anemone (*A. equina*)							
CIP	0.036	0.838	19	0.216	0.858	6	6	2
SMX	0.049	0.937	14	1.279	0.841	26	28	17
TMP	0.221	0.943	3	602.6	0.502	2731	2635	474

k_1: first-order uptake constant; k_2: first-order elimination rate constant; R^2: Determination coefficient of linear representations; BCF_k: bioconcentration factor based in kinetic parameter; BCF_{ss}: bioconcentration factor based in concentration measured at the steady state; BCF_{ss} sed: bioconcentration factor in the sediment compartment

In contrast, the pattern was different in the digestive tract of sea cucumber and in the whole body of beadlet anemone. Slow absorption was observed with an accumulation of less than 1.14 and 0.22 L/kg·d, respectively, while a shorter half-life time (8 days) was required in the digestive tract with respect to the body wall of *H. tubulosa*, probably explained by higher metabolization and degradation in the former. The bioaccumulation pattern of CIP showed a striking difference between both anemones, which may be related with different capacities to accumulate and/or metabolize compounds since *A. sulcata* exchanges nutriments and metabolites with its endosymbiotic microalgae while *A. equina* does not.

The BCF was calculated using the two methods described in section 2.4. Some slight differences were observed between the two calculation methods, probably due to the fact that the steady state had not been reached. According to testing guidelines published by OECD (OECD, 1996), the times to 95% of steady state (i.e., $t_{95} = 3.0/k_2$) in the uptake periods were in the ranges between 100 d (for body wall) and 36 d (for digestive tract). The 305 guideline in 2002 suggested using toxicokinetic models o estimate the BCF if the steady state is not clearly reached. Different regulatory authorities have established a threshold of BCF > 2000 (EU, 2011; EPA 2012) to consider a compound to be bioaccumulative in organisms. Based on these thresholds, PIC could be classified as non-bioaccumulative in lower trophic organisms. A summary of research dedicated to the evaluation of the potential bioaccumulation of CIP, SMX and TMP in aquatic organisms is shown in Table 4.

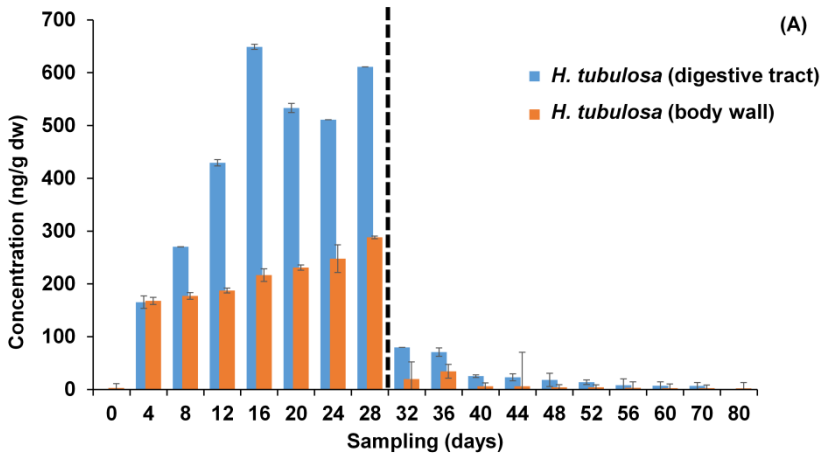
Table 4. *BCF observations for antibiotic studied in aquatic organisms belonging to different trophic levels*

Compound	Organism	Type of study	Nominal exposure concentration (ng/mL)	Exposure/depuration period	Tissue; dry weight (dw) or wet weight (ww)	BCF	Reference
SMX	Mussel (*M. galloprovincialis*)	steady-state	<15.7	20/20	whole organism (dw)	6.2-9.0	Serra-Compte et al., 2018
	Bluegill sunfish (*Lepomis macrochirus*)	steady-state and kinetic	1000	30	muscle (ww)	0-0.99	Zhao et al., 2017
					liver (ww)	0-4.48	
					brain (ww)	0-2.45	
					bile (ww)	0-0.49	
					plasma	0	
					gut (ww)	0.90-3.41	
					gill (ww)	0-0.36	
TMP	Juvenile sea cucumber (*A. japonicus*)	Kinetic	1	28/28	body wall (dw)	724.8	Zhu et al., 2020
			10	28/28	body wall (dw)	98.7	
			1	28/28	mouth (dw)	237	
			10	28/28	mouth (dw)	38.3	
			1	28/28	digestive tract (dw)	108	
			10	28/28	digestive tract (dw)	118	
			1	28/28	respiratory tree (dw)	193	
			10	28/28	respiratory tree (dw)	246	
	Freshwater invertebrate (*Gammarus pulex*)	steady-state	1	48 h/48 h	whole organism (ww)	16-21	Miller et al., 2017

Although some bioaccumulation studies in fish or benthic mussels have been conducted for TMP or SMX, studies on CIP are lacking. To our knowledge, this is the first study to evaluate the bioconcentration potential of CIP in controlled laboratory experiments. Bioconcentration potential is an important criterion for assessing food safety and ecosystem risk. Although the octanol-water partition coefficient (K_{ow}) has been proven to be a good predictor of bioconcentration for very lipophilic organic chemicals (Arnot and Gobas, 2006), some authors (Gómez-Regalado et al., 2023; Kowalska *et al.*, 2021; Molina Fernandez et al., 2021; Nakamura et al., 2008) have reported that it is not a good indicator in case of ionizable compounds like pharmaceuticals. CIP is a type of ionizable pharmaceutical, which can present various ionization states at ambient pH values. In the present work, it was observed that the CBF values of selected antibiotics increase with log K_{ow}, although there was no significant correlation ($r^2 = 0.3$, $p > 0.05$).

3.2. Bioconcentration of sulfamethoxazole

SMX is widely detected in the marine environment. Its presence and fate in the environment have been intensively studied because of its potential, even at low concentrations, to promote antibiotic resistance (Zhu et al., 2022). Figure 2 shows the bioconcentration profiles during 28 days of exposure and 52 days of depuration of the three species under study to a constant concentration of SMX (10 µg/L).



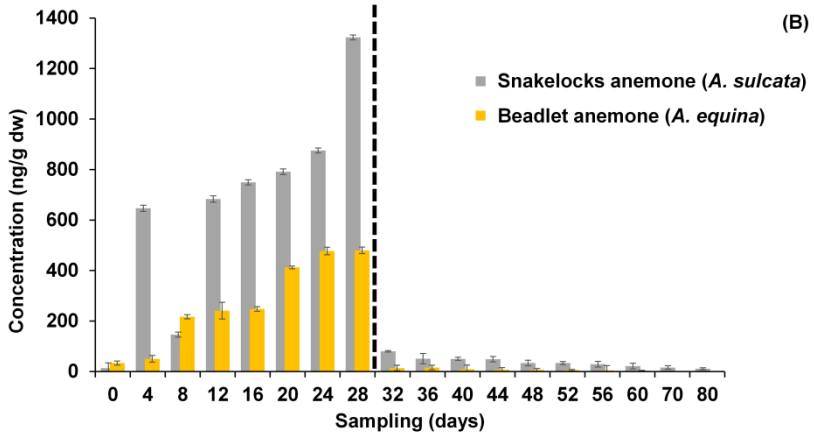


Figure 2. *Kinetics of accumulation (days 0-28) and depuration (days 32-80) of SMX in sea cucumber (Holothuria tubulosa) (A) and snakelocks anemone (Anemonia sulcata) and beadlet anemone (Actinia equina) (B)*

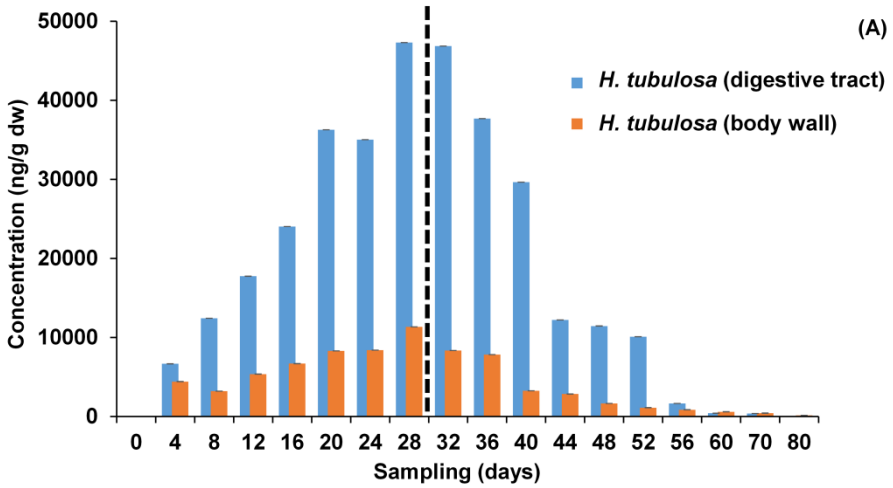
Among the target antibiotics, SMX showed the slowest increase in concentration over time. Rapid absorption was observed at baseline, but concentrations almost stabilized by day 8 in all animals. The uptake/depuration rate constant (k_1/k_2) and half-lives ($t_{1/2}$) of the target chemicals are provided in Table 3. The k_1 values ranged from 0.49 L/kg·d in the body wall of sea cucumber to 3.96 L/kg·d in beadlet anemone. During the depuration period, SMX showed a sharp drop in concentration, falling by almost 90% between days 28 and 32, and then continuing slowly to below detection limits. The half-lives of SMX were completed between 10 d (in digestive tract of sea cucumber) to 17 d (in snakelocks anemone). The k_2 values of SMX were in the range of 0.040-0.071 1/d. The estimated kinetic parameters were similar to that reported by Zhu et al. (2020) for the antibiotic sulfadiazine in the juvenile sea cucumber.

The BCF values in studied animals are also shown in Table 3. The BCFs follow the order 100 (snakelocks anemone) > 27 (digestive tract of sea cucumber) > 26 (beadlet anemone) > 9 (body wall of sea cucumber). The body wall was observed to have the lowest BCF values compared to the digestive tract, which was relative to its low absorption rates and high depuration rates. Although the BCFs are higher for SMX than for CIP, both are well below the thresholds established by the EU. Experimental-

based BCFs are limited, but preliminary studies carried out by Zhu et al. (2020) and Zhao et al. (2017) also observed restricted uptake of SMX on juvenile sea cucumber (*Apostichopus japonicus*) and on bluegill sunfish (*Lepomis macrochirus*) exposed to environmentally relevant concentrations. BCF slightly lower (6.2-9 L/kg) have been reported for SMX in laboratory studies conducted on benthic mussels (*Mytilus galloprovincialis*) (Serra-Compte et al., 2018). This phenomenon may be due to the predominant presence of this drug as an anionic species (>99%, pKa = 5.7) in the seawater (pH = 8.1 in the current study) because ionic species were reported to have a slower rate to cross membranes than the corresponding neutral molecule (Zhu et al., 2020).

3.3. Bioconcentration of trimethoprim

The measured concentration levels for TMP in the aquatic compartment increased to 13.3 ng/mL after the first 4 days and to 19 ng/mL after the end of the exposure period (Table 2), very close to the nominal concentration added. Figure 3 shows the bioconcentration kinetic curves of TMP in the three animal models.



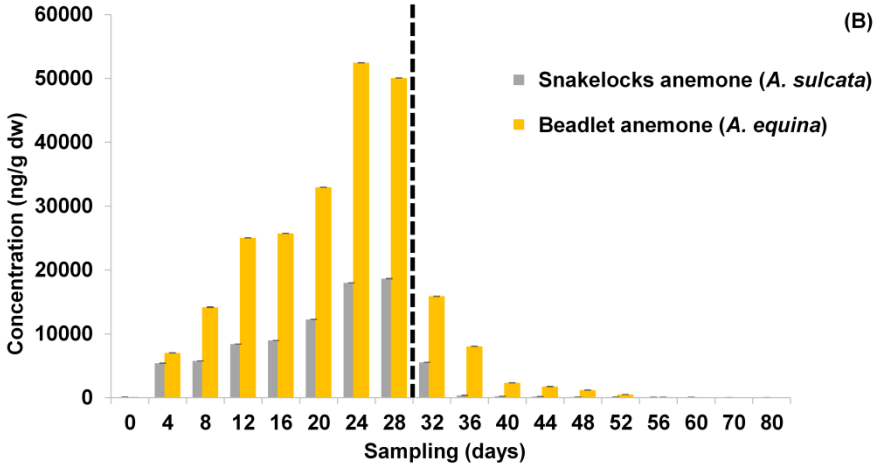


Figure 3. *Kinetics of accumulation (days 0-28) and depuration (days 32-80) of TMP in sea cucumber (Holothuria tubulosa) (A) and snakelocks anemone (Anemonia sulcata) and beadlet anemone (Actinia equina) (B)*

An increasing trend was observed from the beginning, which was strong during the first day, going from 0 to a mean value of almost 7000 ng/g after only 4 days. Thereafter, the concentration in the organisms continued to increase. At the end of the experiment an average concentration up to 47291 ng/g (dw) and 52452 ng/g (dw) was detected in the digestive tract of the sea cucumber and in beadlet anemone, respectively. We thought that the TMP had reached a pseudo-stationary state. his phenomenon, which has also been observed in the uptake of other antibiotics such as roxithromycin in *D. magna* (Ding et al., 2016) or enrofloxation in carp (Chen et al., 2019), could be attributed to increased uptake due to growth dilution or compensation by depuration (Maes et al., 2014). To understand the tissue distribution in sea cucumber, the concentration percentages of the antibiotics studied in the digestive tract and body wall after 28 days of exposure were calculated, as shown in Figure 4.

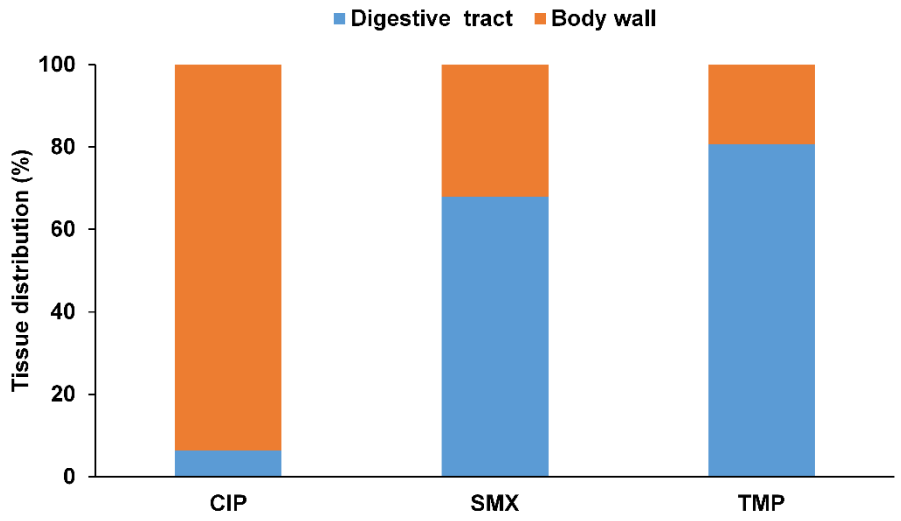


Figure 4. *Tissue distribution of the three antibiotics in H. tubulosa after 28 days exposure in term of concentration*

While CIP was highly concentrated in the body wall of *H. tubulose*, TMP was in its digestive tract. This fact can be explained due to ionic form can hardly diffuse across biomembrane, bioconcentration of ionizable compounds like CIP inside organisms was relative with the fraction of neutral molecules (Chen et al., 2019; Trapp, 2009).

As shown in Figure 3, depuration of TMP happened quickly in anemones (almost 70 % of clearance after 4 days of elimination period), while a more gradual decrease was observed in the digestive tract of *H. tubulosa*, up to 93% of TMP could still be detected after 4 days of elimination.

The uptake rate constants (k_1), depuration rate constants (k_2), and depuration half-lives ($t_{1/2}$) are also listed in Table 3. TMP exhibited the highest k_1 (38-603 L/kg·d) and k_2 (0.08–0.22 1/d), which are approximately 100 and 2 times, respectively, higher than those estimated for CIP and SMX. Based on the regulatory limits, TMP could be classified as either bioaccumulative or non-bioaccumulative. TMP was found to be bioaccumulative in the beadlet anemone *A. equina* and in the sea cucumber *H. tubulosa* with a BCF of 2731 L/kg and 1948 L/kg, respectively, thereby indicating that it is an ideal substance for exploring

bioaccumulation mechanisms. Field-based BCFs are limited. Similar results were also reported by Zhu et al. (2020) in sea cucumber (*Apostichopus japonicus*). Miller et al. (2017) reported lower BCFs than those determined in the current study of TMP (16–21 L/kg) in *Gammarus pulex*.

Finally, it is important to note that most of the accumulation studies did not examine suspended solids or sediments on which contaminants are often adsorbed. Deposit-feeding detritivore organisms may concentrate higher amounts of pharmaceuticals, due to their ingestion of organic matter from sediments. Sediments were also collected in the same sampling point as the water samples. A BCF from sediment up to 474 in beadlet anemone and up to 448 in the digestive tract of the sea cucumber were noted.

4. Conclusions

The present study evaluates the toxicokinetics of three common antibiotics in common marine organisms under controlled experimental conditions. The results indicated that the tissue distribution was strongly influenced by the type of antibiotic, while TMP was highly concentrated in the digestive tract of sea cucumber; CIP was in its body wall. BCF values of three antibiotics decreased in the order: TMP > SMX > CIP.

The maximum k_1 was 603 L/(kg·d) found in beadlet anemone for TMP while the minimum k_1 was 0.21 L/(kg·d) discovered in the same organism for CIP. The maximum and minimum k_2 were 0.221 1/d and 0.036 1/d, estimated in beadlet anemone for TMP and in common snakelocks anemone for SMX, respectively. This variability may be explained by interspecies variations in the biological absorption and clearance and physicochemical properties of the antibiotics. In addition, our results also indicated that tissue distribution in sea cucumber was strongly influenced by the structure of the compounds, whereas CIP was highly concentrated in the body wall, TMP was in its digestive tract. This fact can be explained by the fact that the ionic form of the former can hardly diffuse through the biomembrane. A lack of correlation between the CBFs

of target antibiotics and K_{ow} has also been observed. The present study demonstrates that some common non-target marine organisms tend to have highest BCF than classical animal models such as fish or mussels. However, there are inherent difficulties in interpreting and comparing BCF. This variability, besides the natural dispersion due to the different species, can be due to the lack of uniformity in the reported studies. Overall, the results of this study provide basic data for antibiotic risk assessment. However, the metabolic or biotransformation behaviour of antibiotics needs to be further investigated.

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CRediT authorship contribution statement. María del Carmen Gómez Regalado: Methodology, resources, conceptualization and writing; Julia Martín: Methodology, resources, conceptualization, writing, review & editing; Felix Hidalgo: Methodology, resources, conceptualization, review & editing; Juan Luis Santos: Conceptualization, supervision, review & editing; Irene Aparicio: Conceptualization, supervision, writing, review & editing; Esteban Alonso: Conceptualization, supervision, funding acquisition and project administration; Alberto Zafra-Gómez: Conceptualization, writing, review & editing, supervision, funding acquisition and project administration.

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

References

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(4), 257-297.

https://doi.org/10.1139/A06-005

Bihn, V.N., Dang, N., Anh, N.T.K., Ky, L.X., Thai, P.K., 2018. Antibiotics in the aquatic environment of Vietnam: Sources, concentrations, risk and control strategy. Chemosphere 197, 438-450. https://doi.org/10.1016/j.chemosphere.2018.01.061.

Bonanno, G. and Orlando-Bonaca, M., 2018. Perspectives on using marine species as bioindicators of plastic pollution. Mar. Pollut. Bull. 137, 209-221. https://doi.org/10.1016/j.marpolbul.2018.10.018.

Bonanno, G. and Pavone, P., 2015. Leaves of *Phragmites australis* as potential atmospheric biomonitors of platinum group elements. Ecotoxicol. Environ. Saf. 114, 37-37. https://doi.org/10.1016/j.ecoenv.2015.01.005.

Chen, H., Liu, S., Xu, X.R., Liu, S.S., Zhou, G.J., Sun, K.F., Zhao, J-L., Ying, G-G., 2015. Antibiotics in typical marine aquaculture farms surrounding Hailing Island, South China: Occurrence, bioaccumulation and human dietary exposure. Mar. Pollut. Bull. 90(1–2), 181-187. http://dx.doi.org/10.1016/j.marpolbul.2014.10.053

Chen, M., Zhao, H., Wang, Y., Bekele, T. G., Liu, W., Chen, J., 2019. Uptake and depuration of eight fluoroquinolones (FQs)in common carp (*Cyprinus carpio*). Ecotoxicol. Environ Saf. 180, 202-207. https://doi.org/10.1016/j.ecoenv.2019.04.075

Cravo, A., Silva, S., Rodrigues, J., Vale Cardoso, V., Benoliel, M.J., Correia, C., Coelho, M.R., Rosa, M.J., Almeida, C.M.M., 2022. Understanding the bioaccumulation of pharmaceutical active compounds by clams *Ruditapes decussatus* exposed to a UWWTP discharge. Environ. Res. 208, 112632. https://doi.org/10.1016/j.envres.2021.112632.

Decheng, S., Peilong, W., Yang, L., Ruiguo, W., Shulin, W., Zhiming, X., Su, Z., 2018. Simultaneous determination of antibiotics and amantadines in animal-derived feedstuffs by ultraperformance liquid chromatographic-tandem mass spectrometry. J. Chromat. B. 1095,

183-190. https://doi.org/10.1016/j.jchromb.2018.07.025.

Ding, J., Lu, G., Li, Y., 2016. Interactive effects of selected pharmaceutical mixtures on bio- accumulation and biochemical status in crucian carp (*Carassius auratus*). Chemosphere 148, 21-31. https://doi.org/10.1016/j.chemosphere.2016.01.017

Ding, J.N., Lu, G.H., Liu, J.C., Yang, H., Li, Y., 2016. Uptake, depuration, and bioconcentration of two pharmaceuticals, roxithromycin and propranolol, in *Daphnia magna*. Ecotoxicol. Environ. Saf. 126, 85-93. https://doi.org/10.1016/j.ecoenv.2015.12.020

Duarte, I.A., Reis-Santos, P., Fick, J., Cabral, H.N., Duarte, B., Fonseca, V.F., 2023. Neuroactive pharmaceuticals in estuaries: Occurrence and tissue-specific bioaccumulation in multiple fish species. Environ. Pollut. 316, 120531. https://doi.org/10.1016/j.envpol.2022.120531

European Union, 2011. Commission regulation (EU) No 253/2011 of 15 March 2011 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards Annex XIII. OJEU, L69/7-12. Available at https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R0253&from=EN (accessed on February 2023)

European Union, 2008. Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive). OJEU, L164/19-39. https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32008L0056 (accessed on February 2023).

European Union, 2020. Commission implementing decision (EU) 2020/1161 of 4 August 2020 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. OJEU, 257-32/35. Available at https://eur-lex.europa.eu/legal-

content/EN/TXT/PDF/?uri=CELEX:32020D1161&from=EN (accessed on February 2023)

Gómez-Regalado, M.C., Martín, J., Santos, J. L., Aparicio, I., Alonso, E., Zafra-Gómez, A., 2023. Bioaccumulation/bioconcentration of pharmaceutical active compounds in aquatic organisms: Assessment and factors database. Sci. Total Environ. 861, 160638. https://doi.org/10.1016/j.scitotenv.2022.160638

ICH Quality Guidelines, 2005. Topic Q2 (R1): Validation of analytical procedures: text and methodology. Available at http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html (accessed on February 2023).

Kowalska, D., Maculewicz, J., Stepnowski, P., Dolzonek, J., 2021. Interaction of pharmaceutical metabolites with blood proteins and membrane lipids in the view of bioconcentration: a preliminary study based on in vitro assessment. Sci. Total Environ. 783, 146987. https://doi.org/10.1016/j.scitotenv.2021.146987

Liu, J., Li, X., Wang, X., 2019. Toxicological effects of ciprofloxacin exposure to *Drosophila melanogaster.* Chemosphere 237, 124542. https://doi.org/10.1016/j.chemosphere.2019.124542

Lombardo, J., Solomando, A., Cohen-Sánchez, A., Pinya, S., Tejada, S., Ferriol, P., Mateu-Vicens, G., Box, A., Faggio, C., Sureda, A., 2022. Effects of human activity on markers of oxidative stress in the intestine of *Holothuria tubulosa*, with special reference to the presence of microplastics. Int. J. Mol. Sci. 23, 9018. https://doi.org/10.3390/ijms23169018.

Mackay, D., Fraser, A., 2000. Bioaccumulation of persistent organic chemicals: mechanisms and models. Environ. Pollut. 110 (3), 375-391. https://doi.org/10.1016/S0269-7491(00)00162-7

Maes, H.M., Maletz, S.X., Ratte, H.T., Hollender J., Schaeffer A., 2014. Uptake, elimination, and bio- transformation of 17α-ethinylestradiol by the freshwater alga *Desmodesmus sub- spicatus*. Environ. Sci.

Technol. 48 (20), 12354-12361. https://doi.org/10.1021/es503574z

Martínez-Alcalá, I., Soto, J., Lahora, A., 2020. Antibióticos como contaminantes emergentes. Riesgo exotoxicológico y control en aguas residuales y depuradas. Ecosistemas 29(3), 2070. https://doi.org/10.7818/ECOS.2070

Miller, T.H., Bury, N.R., Owen, S.F., Barron, L.P., 2017. Uptake, biotransformation and elimination of selected pharmaceuticals in a freshwater invertebrate measured using liquid chromatography tandem mass spectrometry. Chemosphere 183, 389-400. https://doi.org/10.1016/j.chemosphere.2017.05.083

Molina-Fernández, N., Rainieri, S., Muñoz-Olivas, R., de Oro-Carretero, P., Sanz-Landaluze, J., 2021. Development of a method for assessing the accumulation and metabolization of antidepressant drugs in zebrafish (*Danio rerio*) eleutheroembryos. Anal. Bioanal. Chem. 413, 5169-5179. https://doi.org/10.1007/s00216-021-03486-2

Morroni, L., Rakaj, A., Grosso, L., Fianchini, A., Pellegrini, D., Regoli, F., 2020. Sea cucumber *Holothuria polii* (Delle Chiaje, 1823) as new model for embryo bioassays in ecotoxicological studies. Chemosphere 240, 124819. https://doi.org/10.1016/j.chemosphere.2019.124819

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., NHirai, N., Tatarazako, N., 2008. The effects of pH on fluoxetine in Japanese medaka
(*Oryzias latipes*): acute toxicity in fish larvae and bioaccumulation in juvenile fish. Chemosphere 70, 865-873. https://doi.org/10.1016/j.chemosphere.2007.06.089

Nikokherad, H., Esmaili-Sari, A., Moradi, A.M., Bahramifar, N., Mostafavi, P.G., 2022. Bioaccumulation capacity of *Chlorella vulgaris* and *Spirulina platensis* exposed to silver nanoparticles and silver nitrate: Bio- and health risk assessment approach. Algal Res. 64, 102671. https://doi.org/10.1016/j.algal.2022.102671

OECD, 2012. Guidelines for the Testing of Chemicals, Section 3:

Environmental fate and behavior. Test No. 305: Bioaccumulation in fish: Aqueous and dietary exposure. Available at https://doi.org/10.1787/9789264185296-en (accessed on February 2023)

Rakaj, A., Morroni, L., Grosso, L., Fianchini, A., Pensa, D., Pellegrini, D., Regoli, F., 2021. Towards sea cucumbers as a new model in embryo-larval bioassays: *Holothuria tubulosa* as test species for the assessment of marine pollution. Sci. Total Environ. 787, 147593. https://doi.org/10.1016/j.scitotenv.2021.147593.

Saxena, P., Hiwrale, I., Das, S., Shukla, V., Tyagi, L., Pal, S., Dafale, N., Dhodapkar, R., 2021. Profiling of emerging contaminants and antibiotic resistance in sewage treatment plants: An Indian perspective. J. Hazard. Mater. 408, 124877. https://doi.org/10.1016/j.jhazmat.2020.124877.

Serra-Compte, A., Maulvault, A. L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz, S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*). Environ. Pollut. 236, 824-834. https://doi.org/10.1016/j.envpol.2018.02.018

Trapp, S., 2009. Bioaccumulation of polar and ionizable compounds in plants. In: Devillers, J. (Ed.), Ecotoxicology Modeling, vol. 2. Springer, New York, pp. 299–353.

US-EPA, 2012. TSCA Work plan chemicals: methods document. U.S.Environmental Protection Agency. Office of Pollution Prevention and Toxics Report No, Washington, p 39.

Vitale, D., Picó, Y., Spanò, N., Torreblanca, A., del Ramo, J., 2020. Carbamazepine exposure in sea anemones *Anemonia sulcata* and *Actinia equina*: Metabolite identification and physiological responses. Sci. Total Environ. 744, 140891. https://doi.org/10.1016/j.scitotenv.2020.140891

WFD (Water Framework Directive), 2000. Directive 2000/60/EC of the European parliament and of the council of 23 October 2000 establishing a framework for Community action in the field of water policy. OJEC L327/1-70. Available online at https://eur-lex.europa.eu/resource.html?uri=cellar:5c835afb-2ec6-4577-bdf8-756d3d694eeb.0004.02/DOC_1&format=PDF (accessed on February 2023).

WHO (World Health Organization), 2020. Antibiotic resistance. Available online at https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance (accessed on February 2023).

Xie, H., Hao, H., Xu, N., Liang, X., Gao, D., Xu, Y., Gao, Y., Tao, H., Wong, M., 2019. Pharmaceuticals and personal care products in water, sediments, aquatic organisms, and fish feeds in the Pearl River Delta: Occurrence, distribution, potential sources, and health risk assessment. Sci. Total Environ. 659, 230-239. https://doi.org/10.1016/j.scitotenv.2018.12.222

Zhao, J-L., Furlong, E.T., Schoenfuss, H.L., Kolpin, D.W., Bird, K.L., Feifarek, D.J., Schwab, E.A., Ying, G-G., 2017. Uptake and disposition of select pharmaceuticals by bluegill exposed at constant concentrations in a flow-through aquatic exposure System. Environ. Sci. Technol. 51 (8), 4434-4444. https://doi.org/10.1021/acs.est.7b00604

Zhao, S., Wang, X., Li, Y., Lin, J., 2016. Bioconcentration, metabolism, and biomarker responses in marine medaka (*Oryzias melastigma*) exposed to sulfamethazine. Aquat. Toxicol. 181, 29-36. https://doi.org/10.1016/j.aquatox.2016.10.026

Zhu, M., Chen, J., Peijnenburg, W. J. G. M., Xie, H., Wang, Z., Zhang, S., 2022. Controlling factors and toxicokinetic modeling of antibiotics bioaccumulation in aquatic organisms: A review. Crit. Rev. Environ. Sci. Technol. 1-21. https://doi.org/10.1080/10643389.2022.2142033

Zhu, M., Wang, Z., Chen, J., Xie, H., Zhao, H., Yuan, X., 2020. Bioaccumulation, biotransformation, and multicompartmental toxicokinetic model of antibiotics in sea cucumber (*Apostichopus*

japonicus). Environ. Sci. Technol. 54(20), 13175-13185. https://doi.org/10.1021/acs.est.0c04421

Ziarrusta, H., Mijangos, L., Montes, R., Rodil, R., Anakabe, E., Izagirre, U., Prieto, A., Etxebarria, N., Olivares, M., Zuloaga, O., 2018. Study of bioconcentration of oxybenzone in gilt-head bream and characterization of its by-products. Chemosphere 208, 399-407. https://doi.org/10.1016/j.chemosphere.2018.05.154

Instrumentation and software

An Acquity UPLC™ I-Class chromatography system (Waters, Manchester, UK) equipped with an Acquity™ sample manager and an Acquity UPLC™ binary solvent manager was used. A Waters Acquity UPLC HSS T3 column (100 mm x 2.1 mm i.d, 1.8 µm particle size) was used. A Xevo TQ-XS triple quadrupole mass spectrometer equipped with an orthogonal Z-Spray™ electrospray ionization source (ESI).

Freeze-drying of the samples took place with a ScanVac CoolSafe™ freeze-dryer. For the extraction procedure of the target analytes, an IKA Vortex (Staufen, Germany), an ultrasound probe with a 400 W digital sonicator with 0.5 inch (12.7 mm) tip operating at a frequency of 20 kHz were used. A Labnet Spectrafuge™ 24D centrifuge (New Jersey, USA), a Selecta ultrasonic bath (Barcelona, Spain) and a Mettler-Toledo precision balance (Columbus, OH, USA) were also employed. A Retsch ball mill (Germany) was used to pulverize the samples. Finally, evaporation of the samples was carried out with a Stuart sample concentrator nitrogen evaporator (Staffordshire, UK).

For the measurements, the following instruments were used: Hanna Instruments® digital thermometer (model HI Checktemp 1), HACH® sensION™ þ EC5 portable conductivity/TDS meter with a conductivity probe (model 50 60), HACH® HQ40d portable multimeter with a pH probe (model pHC101) and a luminescent dissolved oxygen probe (model LDO101).

Validation requirements

All matrices studied were first checked for a low matrix effect. In order to do so, a calibration with pure standard and a calibration with matrix were compared, and the slope of the line obtained was similar for all compounds. Eight calibration levels were prepared.

Limit of detection (LOD) and limit of quantification (LOQ) were used to evaluate the sensitivity of the method from the signal-to-noise ratio obtained from the injection of standards with decreasing amounts of the selected target analytes. The method selectivity was studied by

comparison of the chromatograms of the blank with a standard sample. Moreover, the accuracy in terms of trueness and precision was evaluated by recovery assays at three concentration levels (LOQ, medium and high) for trueness, and the relative standard deviation (% RSD) of the measurements was evaluated three times on the same day and for three days for precision.

The quality assurance/quality control of the data obtained in the validation was verified (QA/QC) by injecting spiked blank samples, which were treated the same as the samples to control background contamination. It was observed that there were no detectable amounts in these blanks. Additionally, instrumental stability was assessed by injecting each sample in triplicate and every 20th analysis a mixture of the pure standards of the target analytes was injected.

Table S1*. Physicochemical properties and structure of the target compounds*

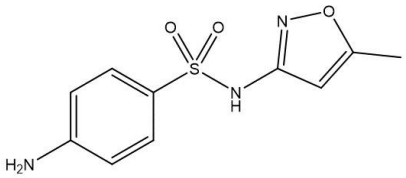
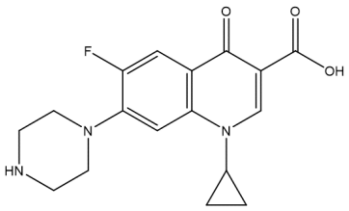
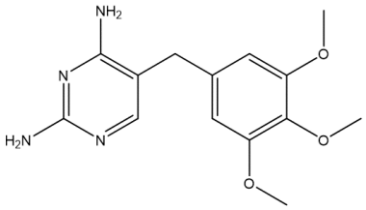
Compound	MW (g/mol)	pK_a	Log K_{ow}	Structure
SMX	253.28	1.6	0.89	
CIP	331.34	6.16	0.28	
TMP	290.32	7.12	0.91	

***Table S2**. MRM conditions and ESI mode used for LC-MS/MS of antibiotics*

Compound	t_R (min)	Transitions	CV (V)	CE (V)	Ratio (a/b)
SMX	3.92	253.9 → 92.0[a]	20	26	1.02
		253.9 → 155.9[b]	20	14	
CIP	3.68	332.1 → 314.0[a]	26	16	1.34
		332.1 →230.9[b]	26	32	
TMP	2.99	291.1 → 229.9[a]	24	22	1.18
		291.1 → 123.0[b]	24	22	

Spectrometric conditions			
Impactor voltage	1 kV	Nebulizer gas pressure	7.0 bar
Source temperature	150 ºC	Cone/desolvation gas	N_2 (≥ 99.995 %)
Desolvation temperature	600 ºC	Collision gas	Ar (99.999 %)
Cone gas flow	150 L/h	Dwell time	25 ms
Desolvation gas flow	500 L/h	Inter-scan delay	3 ms

[a] transition used for quantification; [b] transition used for confirmation; CV: Cone voltage; CE: Collision energy

Capítulo 9

Bioconcentration of pharmaceuticals in benthic marine organisms *(Holothuria tubulosa, Anemonia sulcata Actinia equina)* exposed to environmental contamination by atenolol and carbamazepine

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Environmental Toxicology and Pharmacology (Under review)

Abstract: The extensive use of pharmaceuticals leads to their ubiquitous occurrence in coastal aquatic environments. Their accumulation in aquatic organisms is an issue that has received increasing attention in recent years since previous attempts to estimate this phenomenon have been ineffective. Invertebrates such as benthic organisms are key in the functioning of marine ecosystems and can be used as sentinel species of water quality status. The aim of the present work is to assess the bioconcentration kinetics of atenolol (ATN) and carbamazepine (CBZ) in three common marine organisms including cotton spinner sea cucumber (*Holothuria tubulosa*), snakelocks anemone (*Anemonia sulcata)* and beadlet anemone (*Actinia equina*) under controlled laboratory conditions. The type of organism, the tissue and structure of the compound have a significant impact upon their toxicokinetic processes. CBZ exhibited higher uptake and excretion rates resulting BCF (41-537 L/kg for CBZ vs 7-50 L/kg for ATN) although both are far below these threshold limits established by the UE. The measured BCF using kinetic data showed some slightly differences with those predicted using the concentrations measured at the steady-state, probably explained because the steady state was not ready reached. The animal-specific BCF generally followed the order of cotton spinner sea cucumber > beadlet anemone > snakelocks anemone for ATN while was the opposite for CBZ. The study also highlighted between-tissues differences in the digestive tract and the body wall of the sea cucumber. The work presented here was the first to model bioconcentration of ATN and CBZ in holothurian and anemone animal models.

Keywords: Atenolol; Carbamazepine; Up-take/Depuration; Animal model; Benthic organisms.

1. Introduction

In recent years, the consumption of a number of pharmaceutically active compounds (PhACs) has increased considerably due to their high medical and veterinary prescription and uses (Duarte et al. 2023; Xu et al., 2019). Some of these PhACs have been recognized as an important

group of emerging pollutants (EPs) (Cerveny et al., 2021). The release of these pollutants into the environment can occur through a variety of pathways, including municipal wastewater treatment plants (WWTPs), as these compounds are not completely removed by traditional treatments, meaning that many of them are found in surface waters around the world (McCallum et al., 2019). The emissions into the environment is continuous (Xu et al., 2019), which has led them to be considered pseudo-persistent pollutants, despite the fact that many of them degrade (Cravo et al., 2022). Controlled laboratory studies on exposure to these compounds have shown that they affect the development, physiology and behaviour of aquatic species. Even so, it is very complex to demonstrate that these observed effects will be the same in nature (Duarte et al., 2023).

Atenolol (ATN) is a ß-blocker used for heart rhythm-related diseases, but also used in combination with other drugs to treat hypertension (Mastrángelo et al., 2022). Picó et al. (2019) detected it in treated wastewater at concentrations ranging 0.1 to 0.9 μg L^{-1} and Biel-Maeso et al. (2018) demonstrated its persistence even in treated water, which makes it an emerging pollutant of global concern. On the other hand, carbamazepine (CBZ) is a widely used anti-epileptic drug in clinical practice, in fact, worldwide consumption of CBZ was estimated at 1014t (Yang et al., 2023). It is considered one of the most important and relevant compounds due to its high toxicity as well as its persistence in the environment, and its toxic effects on aquatic organisms have been demonstrated. Almeida et al. (2014) studied its effects in clams and Dumas and colleagues (2022) focused on molecular alterations in mussels. In recent years, much research has been devoted to determining the effects and risks produced by pharmaceuticals once they reach the environment, and it is one of the most quantifiable pharmaceuticals in urban waters, it has been widely used as a marker of anthropogenic pollution (Vitale et al., 2020).

According to the Marine Strategy Framework Directive (European Union, MSFD 2008), research on the accumulation of emerging

contaminants in water, sediments and aquatic organisms is essential. For this purpose, the quantification of pollutants takes place by means of some factors such as the bioconcentration factor (BCF) (Martín et al., 2020). Valdés et al. (2014), Meylan et al. (1999) and Fu et al. (2009) studied the bioconcentration of ATN in the whole organism of different fish species, obtaining BCFs between 0.008 and 3.16, while Steinbach et al. (2014) calculated bioconcentration by differentiating fish parts and found that ATN accumulation varied according to the body compartment of the fish. Moreover, some studies have focused on the bioaccumulation of CBZ in different aquatic organisms due to its toxicity. Boillot et al. (2015), Serra-Compte et al. (2018), Daniele et al. (2017) and Contardo-Jara et al. (2011) focused their studies in mussels, in which they obtained BCFs for CBZ in the range of 3.9 to 90, while García et al. (2012) and Valdés et al. (2014, 2016) studied the CBZ bioconcentration in fish, with BCFs between 0.7-9, differentiating in most cases the body compartments.

Bioindicators are important tools for detecting changes in the environment. They allow the health of ecosystems to be assessed through the biological responses they provide (Bonanno et al., 2018). The essential characteristics that a good bioindicator should have, are abundance, ease of sampling, moderate tolerance to disturbance and a wide distribution throughout the ecosystem, which allows for a true assessment of exposure (Urban et al., 2012). Holothurians have proven to be useful bioindicators of marine pollution as, on the one hand, they are widely distributed in coastal areas and on the other hand, their feeding mode consists of ingesting sediments from which they capture organic matter (Bulleri et al., 2021; Chahrour et al., 2021). Sea anemones also exhibit characteristics that make them potential biomonitors of pollution in the sea due to their wide distribution, generalist feeding behaviour, ease of sampling and sessile habit (Morais et al., 2022).

In this context, the main objective of the present study was to assess the bioaccumulation of ATN and CBZ, in a laboratory model, using cotton spinner sea cucumber (*Holothuria tubulosa*), snakelocks anemone

(*Anemonia sulcata)* and beadlet anemone (*Actinia equina*) as bioindicators. The duration of the experiment was 80 days to determine the relationship between compound concentrations in specimens, water and sediment. BCFs were calculated by steady-state and a mass balance models, using the different body compartments of the sea cucumber as well as the two types of anemones.

2. Material and methods

2.1. Chemical and reagents

Ultrapure water (18.2 MΩ cm) was obtained using a Milli-Q Plus® system (Millipore, Madrid, Spain). Analytical grade formic acid (≥ 98%) was used as mobile phase additive. LC-MS grade methanol (MeOH) was acquired from VWR Prolabo CHEMICALS (Barcelona, Spain). ATN (≥ 98.0%) and CBZ (≥ 98.0%) were purchased from Sigma Aldrich (Madrid, Spain). Cinchophen (CIN, internal standard) was supplied by Alfa Aesar (Massachusetts, MA, USA). The chemical structure and physicochemical properties of the analytes are shown in Table S1. Nitrate, nitrite and ammonium analyses were essayed with Hanna instruments® test kits (HI 3874, 3873 and 3826, respectively). Stock solutions were prepared in MeOH and stored at -20 °C until use.

2.2. Laboratory test

2.2.1. Acclimation period

The specimens under study were captured in February 2022 by divers at a depth of 10-15 m at random points along the coast of Granada (Spain). The animals were transported, in refrigerated containers at 4 ºC, to the facilities where the laboratory test was carried out. Once in the laboratory, they were introduced into the experimental tanks and allowed to acclimatize and purify for a period of 15 days. In this process, 50 cotton spinner sea cucumbers were captured, 45 of them were used as samples (mean 193.7 ± 10.7 g). Fifty snakelocks anemones (*Anemonia sulcata*) and beadlet anemones (*Actinia equina*) were also captured.

2.2.2. Up-take and depuration test

The research was carried out at the Zoology Department of the University of Granada (Spain), where a 300 L glass aquarium containing recirculating natural seawater was installed with artificial illumination by a 6000ºK metal halide lamp. The system was filtered with a biological filter with foam rubber and bioballs, and the aeration was carried out with compressed air. Photoperiods of 10 hours of light and 14 hours of darkness were carried out. Throughout the experiment, the physicochemical characteristics of the water were checked twice a week, with mean values of temperature 19.7 ± 0.2 ºC, pH 8.2 ± 0.0, salinity 37.5 ± 0.2 PSU, dissolved O_2 6.3 ± 0.1 mg/L, ammonium 0.20 ± 0.03 mg/L, nitrites 0.23 ± 0.01 mg/L, nitrates 51 ± 3 mg/L. Twice a week a scoop containing a mixture of dehydrated fish meal and spirulina (1:1) was added to the aquarium to feed the holothurians.

The experimental design was as follow. During the 28 days of the assay (uptake), after acclimatization time, 3 mg of each compound were added daily to the tank. As the tank capacity was 300 L, the final concentration of each compound was 10 µg/L per day. The exposure concentration used is in agreement with the OECD 305 method taking into account the LC_{50} reported values in aquatic organisms (Kim et al., 2009; Heye et al., 2016). Specimens were sampled every four days (3 sea cucumbers, 3 snakelocks anemones, and 2 beadlet anemones). For sea cucumbers, body wall and digestive tract were separated. After day 28, all the remaining specimens were transferred to a new tank with the same characteristics as the previous one, initiating the depuration period, which took place for another 52 days, being the total duration of the experiment 80 days. Similarly, samples were collected every 4 days. Immediately after sample collection, a simple pre-treatment of each specimen, consisting of freeze-drying and pulverization, was carried out. All samples were stored at -20 ºC until analysis.

2.3. Analytical methods

Aliquots of pulverized material from cotton spinner sea cucumber, snakelocks anemone and beadlet anemone, as well as sediment (0.2 g dry weight) were taken and weighed into 10 mL glass tubes together with a methanolic surrogate solution (CIN), giving a final sample concentration of internal standard of 200 ng/g. The samples were left in the dark for 24 hours to ensure contact between the matrix and the internal standard. Then, 2.2 mL of MeOH was added, vortexed for 30 seconds and an ultrasound-assisted extraction (UAE) was done for 12 minutes at 40% amplitude. The samples were then centrifuged for 5 minutes at 4000 rpm and the supernatant was collected in another tube. This procedure was repeated, mixing the collected supernatants. The samples were then completely dried under a stream of N_2. Finally, the residue was dissolved in an 80/20 (v/v) H_2O/MeOH mixture (composition of the initial mobile phase) to a final volume of 0.2 mL. Finally, 2 µL were injected into the UHPLC-MS/MS equipment.

Water samples were treated as follows. They were collected in 100 mL plastic bottles. Aliquots of 10 mL of sample were separated and 50 µL of a methanolic solution of CIN were added, the final sample concentration of the internal standard was 200 ng/mL. The solution was frozen and then freeze-dried for 48 hours at -109 ºC. After drying, 1 mL of MeOH was added and vortexed for 1 min. Afterwards the sample was centrifuged for 5 min at 4000 rpm and the supernatant was separated. The procedure was repeated once more and the two supernatants collected were pooled and evaporated under a stream of N_2. The dry extract was dissolved in the initial chromatographic mobile phase as for the solid samples.

The analysis by ultrahigh performance liquid chromatography-tandem mass spectrometry was performed using an ACQUITY HSS T3 column (100 mm x 2.1 mm internal diameter, 1.8 µm particle size). Compounds were separated by using a mobile phase gradient which consisted of an aqueous buffer with 0.05% formic acid (v/v) as phase A and MeOH as phase B. Thus, the method was performed in 8 min with the subsequent

gradient program: 0-1.5 min, isocratic gradient at 80% A. 1.5-3 min, linear gradient from 80% to 60% A, 3-4 min, linear gradient from 60 to 15% A, and 4-6 min, isocratic gradient at 15% A. 6-6.1 min, return to initial conditions for 2 min in order to prepare for the next injection. For each analyte, two multiple reaction monitoring (MRM) transitions were selected, the first for quantification, and the second for confirmation. Transitions and MS parameters are shown in Table 1.

Table 1. *UHPLC-MS/MS conditions for the determination of ATN and CBZ*

Compound	t_R (min)	Transitions	CV (V)	CE (eV)	Ratio (a/b)
ATN	0.96	267.1 > 145.1[a]	20	24	2.1
		267.1 > 190.0[b]	20	14	
CBZ	4.91	237.1 > 194.0[a]	38	18	8.6
		237.1 > 178.9[b]	38	34	
Spectrometric conditions					
Impactor voltage		1 kV	Nebulizer gas pressure	7.0 bar	
Source temperature		150 ºC	Cone/desolvation gas	N_2 (≥ 99.995 %)	
Desolvation temperature		600 ºC	Collision gas	Ar (99.999 %)	
Cone gas flow		150 L h^{-1}	Dwell time	25 ms	
Desolvation gas flow		500 L h^{-1}	Inter-scan delay	3 ms	

[a] *Transition used for quantification;* [b] *transition used for confirmation; CV: cone voltage; CE: collision energy*

Mass spectrometer operated in positive ESI mode. The method was successfully validated according to the ICH guideline (ICH, 2005) in terms of linearity, range, sensitivity and accuracy (trueness and precision). The validation procedure is described in supplementary material section. Table S2 summarizes the limits of detection and quantification as well as the linearity and linear dynamic range. Recovery assays showed recoveries close to 100%, with standard deviations below 15%, for all compounds and matrices.

2.4. Toxicokinetic and bioconcentration calculation

The bioconcentration kinetic parameters in each animal model were estimated using a mass balance model (Mackay and Fraser, 2000). Two different parts of sea cucumber (body wall and digestive tract) were studied, while for anemones the whole body of the organism was considered. The competing uptake and depuration processes resulting in bioconcentration could be described mathematically as:

$$\frac{dC_b}{dt} = k_1 C_w - k_2 C_b \quad (1)$$

In order to perform the absorption and elimination toxicokinetic analysis, the mean chemical concentrations of each sample at each sampling time were used. The classical pseudo-first order toxicokinetic model was applied. A nonlinear regression was fitted to Equation 2 to obtain the constant elimination rate (k_2):

$$C_b(\text{depuration}) = C_{b,0} e^{-k_2 t} \quad (2)$$

$C_{b,0}$ is the concentration of pharmaceutical in the organism at the beginning of the experiment, C_b is the concentration in time, and t is time (days).

In the case of the calculation of the uptake rate constant (k_1), a nonlinear regression was fitted to equation 3 taking into account the previously calculated k_2 values.

$$C_b(\text{uptake}) = \frac{k_1}{k_2} C_w (1 - e^{-k_2 t}) \quad (3)$$

The half-lives ($t_{1/2}$) of pharmaceuticals in biological samples were estimated as:

$$t_{1/2} = \frac{\ln 2}{k2} \quad (4)$$

The BCF through the kinetic parameters was calculated as:

$$BCF_k = \frac{k_1}{k_2} \quad (5)$$

In addition, the BCFs of each compound were calculated from the average concentration in each animal and the corresponding concentration in the aqueous phase at steady state by the equation 6:

$$BCF_{SS} = \frac{C_b}{C_w} \quad (6)$$

C_b is the concentration in the organism (ng/g dw) and C_w is the concentration in the water (ng/mL), both measured at the same time and at the steady-state. The steady stated was estimated at 28 days. Moreover, the C_w was kept within 20% of the measured mean (n = 3).

2.5. Quality and exposure control

Before the experiment, pharmaceuticals were measured in the water and sediment of the aquaria as well as in control organisms (N = 5) of each class. No adverse behaviour was observed in either the control or treated groups during the entire assay period. Mortalities of each group were lower than 1%. The condition index (control and contaminated) remained stable, with low variability, under these conditions being thus optimal for the cotton spinner sea cucumber and the anemones during the experiment. Unfortunately, there are currently no standard exposure tests for this type of animals. Different publications put forward a cut-off level of 10% mortality in the blank controls, as quality measure (Vellinger et al., 2012; Vellinger et al., 2013).

In order to guarantee the quality assurance of the results, a protocol involving the use of control samples including fortified organisms samples with the target compounds (100 ng/g dw), a mixture of the standards in pure solvent (100 ng/mL), solvent (MeOH:water, 50:50 v/v) and procedural blanks injections were included in each analytical batch. For quantification purposes, eight-point calibration in matrix curves were prepared containing ATN and CBZ in the range from method quantitation limit to 500 ng/g dw.

3. Results and discussion

3.1. Pharmaceutical concentrations in exposure water and sediment compartments

Data for concentrations of the target analytes in the aqueous and the surrounding sediments throughout the experiment are displayed in Table 2.

Table 2. *Mean concentration and relative standard deviation of ATN and CBZ in the aqueous phase and in the sediments throughout the experiment*

Sampling (days)	Water phase				Sediments			
	ATN		CBZ		ATN		CBZ	
	Mean (ng/mL)	RSD (%)	Mean (ng/mL)	RSD (%)	Mean (ng/mL)	RSD (%)	Mean (ng/mL)	RSD (%)
0	ND	-	ND	-	ND	-	0.22	23.0
4	ND	-	27.1	5.3	12.0	10.8	101	9.7
8	ND	-	58.6	4.8	5.30	2.6	149	10.6
12	ND	-	56.9	6.3	4.57	22.0	207	5.6
16	ND	-	81.8	3.6	5.00	15.2	407	14.0
20	3.60	4.1	92.2	2.6	5.14	0.7	624	3.1
24	5.98	2.9	85.7	5.1	11.3	5.0	578	14.3
28	8.90	0.7	74.6	2.2	6.00	6.3	751	7.4
32	ND	-	5.77	5.6	3.60	1.2	23.3	10.0
36	ND	-	0.18	5.3	3.64	2.2	3.00	19.5
40	ND	-	0.18	8.7	3.77	2.4	2.00	10.2
44	ND	-	0.20	-	3.59	2.3	2.26	15.8
48	ND	-	0.21	10.7	3.58	1.2	1.72	12.2
52	ND	-	0.49	0.7	3.72	4.3	1.60	14.5
56	ND	-	0.20	76.8	3.65	2.0	2.00	4.8
60	ND	-	ND	-	3.62	3.3	1.02	12.4
70	ND	-	ND	-	3.51	1.8	2.11	3.8
80	ND	-	ND	-	4.00	15.6	1.26	10.2

ND: not detected

PhACs were not detected in control aquaria, thus indicating the absence of contamination. During the exposure stage (spiking with a nominal concentration of 10 ng/mL of each of them) the values measured showed a minimal variation between 3.60 and 8.90 ng/mL for ATN and

between 27.1 to 92.2 ng/mL for CBZ. ATN was less stable in water samples and only was detected in few samples after 20 days of the exposure experiment. The concentration levels measured during the exposure period were maintained relatively stable and meet the 20% maximum variation commitment of OECD 305.

ATN was not detected in water from the depuration tank. CBZ present a decrease in the concentrations up to 5.8 ng/mL after 4 days of depuration and not detected after 28 days. CBZ was however detected at higher concentration in the sediment compartment than in water samples from 0.22 ng/g dw in control sediment to 751 ng/g on the last day (28) of the exposure experiment, which suggest that sediments may be an important source of selected compounds for benthonic biota like is the case of cotton spinner sea cucumber.

3.2. Uptake and depuration kinetics of carbamazepine and atenolol

Concentrations of ATN and CBZ in control animals were below limits of detection. The uptake and depuration pattern in the sea cucumber and the anemones were determined and the results for both compounds are presented in Figures 1 and 2, respectively.

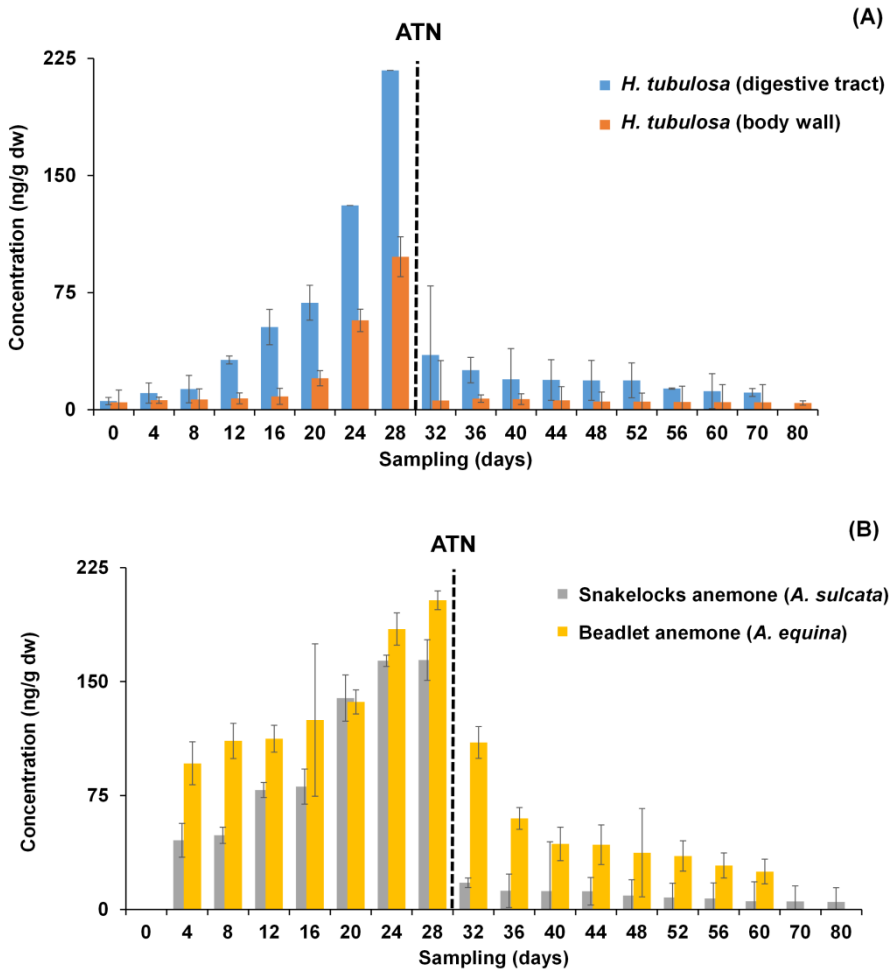

Figure 1. *Kinetics of accumulation (days 0-28) and depuration (days 32-80) of ATN in sea cucumber (Holothuria tubulosa) (A) and snakelocks anemone (Anemonia sulcata) and beadlet anemone (Actinia equina) (B)*

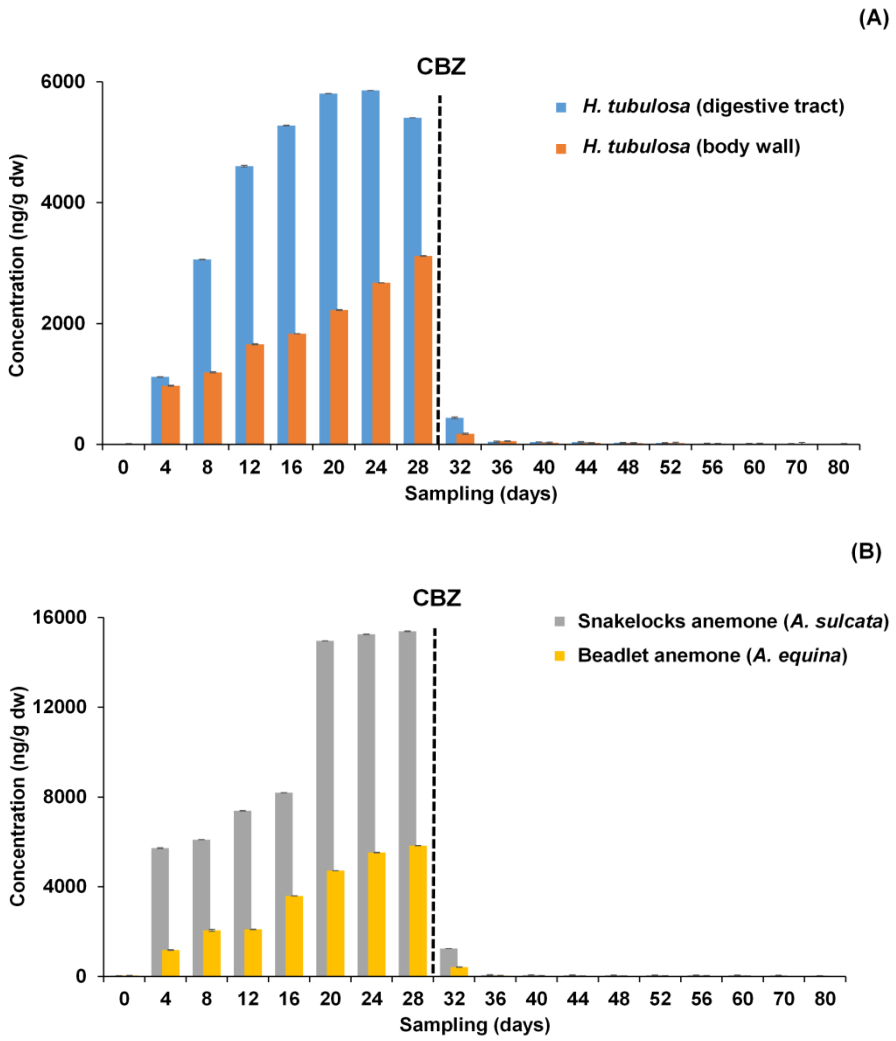

Figure 2. *Kinetics of accumulation (days 0-28) and depuration (days 32-80) of CBZ in sea cucumber (Holothuria tubulosa) (A) and snakelocks anemone (Anemonia sulcata) and beadlet anemone (Actinia equina) (B)*

Throughout the exposure phase, it was observed that the concentrations of the compounds under study increased significantly, with rapid absorption being observed in all groups of animals and tissues. As for the levels of contaminants found after the exposure treatment, all compounds showed bioconcentration, although to different degrees. CBZ was detected at the highest concentration after 20 days of exposure (5803 ng/g dw) in the digestive tract of cotton spinner sea cucumber and 28 days (15387 ng/g dw) in snakelocks anemone. These concentrations were 100-

fold higher than those found in the water phase. A concentration up to 215 and 204 ng/g dw was reached for ATN in the digestive tract of cotton spinner sea cucumber and in beadlet anemone, respectively. CBZ visually appeared to reach a maximum level after approximately 20 days of exposure for the digestive tract of the sea cucumber and snakelocks anemone, however ATN did not level off even within 28 days uptake phase. According to OECD 305E testing guidelines (OECD, 2012), the estimated times to 95% of steady state (i.e., $t_{95} = 3.0/k2$) in the uptake phase were not less than 45 d for CBZ and 93 d for ATN. Thus, we speculated that ATN visually appearing to level off should be a pseudo-steady state which might be attributed to lack of longer exposure time. The capacity of stress tolerance of selected organisms together with their low mobility, allows this group of animals to be suggested as potential sentinel organisms for the monitoring of environmental pollution.

During the depuration phase, a significant decrease in the concentrations of both drugs was observed, with a high initial elimination rate of over 83 % for ATN and 91 % for CBZ (except for beadlet anemone, 46 %) on day 32 (i.e. 4 days of depuration). From this day on, the fall curves slowed down markedly. On the other hand, it was observed that the concentrations of the two PhACs in cotton spinner sea cucumber were significantly dependent on the selected tissue. Higher concentrations were measured in the digestive tract, almost twice than in body wall. For example, the concentration of ATN in digestive tract on last exposure day was of 214 ng/g dw to 96 ng/g dw in the body wall. In the anemone group, concentration levels of CBZ tend to be higher in snakelocks anemone than those to beadlet anemone (5 fold) after 28 days of exposure, which might be related with the different capacities to accumulate and/or metabolize compounds of the different type of anemones. Sea anemones have the ability to absorb dissolved organic matter through ectodermal cells (Schlichter, 1978) and *A. sulcata* has a larger relative body surface area than *A. equina* due to its larger and thinner tentacles. In fact, unlike *A. equina*, *A. sulcata* is an endosymbiotic species that exchanges nutrients and metabolites with its endosymbiotic microalgae (Stambler and Dubinsky, 1987; Vitale et al., 2020).

The uptake and depuration kinetics constants, bioconcentration factor, and biological half-lives ($t_{1/2}$) are summarized in Table 3.

***Table 3**. Kinetic data of ATN and CBZ in sea cucumber and anemones*

Compound	k_2 (1/d)	R^2	$t_{1/2}$ (d)	k_1 (L/kg·d)	R^2	$BCF_{kinetic}$ (L/kg)	BCF_{ss} (L/kg)	BCFs Sed
H. tubulosa **(digestive tract)**								
ATN	0.024	0.890	29	1.202	1.000	50	50	53
CBZ	0.066	0.928	11	4.851	0.885	74	69	7
H. tubulosa **(body wall)**								
ATN	0.011	0.846	64	0.504	0.994	47	11	24
CBZ	0.037	0.877	19	1.729	0.748	41	42	4
A. sulcata								
ATN	0.030	0.884	23	0.206	0.685	7	18	41
CBZ	0.012	0.938	60	6.204	0.884	537	206	20
A. equina								
ATN	0.032	0.949	21	0.585	0.892	18	23	50
CBZ	0.024	0.760	29	5.195	0.888	127	78	8

k_1: first-order uptake constant; k_2: first-order elimination rate constant; R^2: Determination coefficient of linear representations; BCF_k: bioconcentration factor based in kinetic parameter; BCF_{ss}: bioconcentration factor based in concentration measured at the steady state; BCF_{ss} sed: bioconcentration factor in the sediment compartment

The bioconcentration data fit the toxicokinetic model well with high correlation coefficients ($r^2 \geq 0.748$) for all animals. The uptake rate constants of ATN were between 0.206 L/kg·d in snakelocks anemone and 1.202 L/kg·d in the digestive tract of sea cucumber. The uptake rate constants of CBZ were between 1.729 L/kg·d in the body wall of sea cucumber and 6.204 L/kg·d in snakelocks anemone. This could be because the difference in absorption rates may be associated with the lower permeability of ATN across biological membranes.

The depuration rate constants were between 0.011-0.032 and 0.012-0.066 1/d for ATN and CBZ, respectively. CBZ ($\log K_{ow} = 2.45$) were less hydrophilic than ATN ($\log K_{ow} = 0.16$) and thus excreted faster which was

agreement with several previous publications in which hydrophilic compounds had been proved to have low excretion rates (Hendriks et al., 2001). Moreover, it was noted that the uptake and depuration rates of two PhACs in the digestive tract were greater than those estimated in body wall, which could indicate that digestive metabolism might be a main transformation pathway for them. Biological half-lives were 21–64 d for ATN and 11-60 d for CBZ. The longest half-lives were in body wall.

3.3. Bioconcentration factors

It is important to note that, in the literature, BCF values are usually calculated as the ratio of the instantaneous concentrations measured in biota to those in water. In a regulatory context, BCF is a kinetic measure established using a standardized test protocol (e.g., OECD 305) in which the ratio of the rate of absorption to the rate of elimination is determined. Thus, it is in vivo laboratory exposure studies that can elucidate toxicokinetic properties to identify hazards associated with exposure and help prioritize compounds of concern for specific analytical approaches in environmental monitoring campaigns. In the present work, the two different approaches were used to estimate the BCF in the different animal models and similar values were get in case of CBZ while significant differences were observed in some cases probably explained as consequence that the steady state was not reached (Table 3). Animal-specific BCF were in the ranges of 41-537 L/kg for CBZ and 7-50 L/kg for ATN. Following the indications from REACH legislation, those compounds with a BCF > 2000 are considered bioaccumulative, and very bioaccumulative if the corresponding BCF > 5000. Although the BCFs are higher for CBZ than for ATN, both are well below these EU thresholds.

Table 4 summarizes the research found in the literature dedicated to the evaluation of the bioaccumulation potential of these two PhACs in aquatic organisms under laboratory studies.

Table 4. *BCF observations for ATN and CBZ in aquatic organisms.*

	Organism	Type of study	Nominal exposure concentration (ng/mL)	Exposure/ depuration period	Tissue; dry weight (dw) or wet weight (ww)	BCF	Reference
ATN	Fish (*Gambusia affinis*)	steady-state	10-1000	96 h/0	whole organism (ww)	0.08-0.13	Valdés et al., 2014
	Juvenile rainbow trout *(O. mykiss)*	steady-state	1-1000	42/0	liver	0.21	Steinbach et al., 2014
					kidney	0.10	
					muscle	0.002	
	Sea cucumber (*H. tubulosa*)	Kinetic	10	28/52	digestive tract (dw)	50	The present work
	Sea cucumber (*H. tubulosa*)				body wall (dw)	47	
	Snakelocks anemone (*A. sulcata*)				whole organism (dw)	7	
	Beadlet anemone (*A. equina*)				whole organism (dw)	18	
	Estimated					3.16	Meylan et al., 1999
	Estimated					0.34	Fu et al., 2009
CBZ	Mussel (*M. Galloprovincialis*)	kinetic and steady-state	10	7 / 7	whole organism (dw)	3.9	Boillot et al., 2015
	Shrimps (*Gammarus pulex*)	kinetic	100	48 h / 48 h	whole organism (dw)	5.47-8.93	Meredith-Williams et al., 2012
	Water boatman (*Notonecta glauca*)					0.17-0.33	
	Mussel (*M. Galloprovincialis*)	steady-state	<15.7	20 / 20	whole organism (dw)	25.8-35.3	Serra-Compte et al., 2018
	Fish (*Pimephales notatus*)	steady-state	1	14 / 0	muscle (ww)	1.9	García et al., 2012
					liver (ww)	4.6	
	Fish (*Ictalurus punctatus*)	steady-state	1	14/0	muscle (ww)	1.8	
					liver (ww)	1.5	
					brain (ww)	1.6	

					plasma (ww)	7.1	
	Fish (*Gambusia affinis*)	steady-state	10-1000	96 h/0	whole organism (ww)	0.7-0.9	Valdés et al., 2014
	Zebra mussel *(Dreissena polymorpha)*		0.05-5	6 months	whole organism (dw)	3.4-14.8	Daniele et al., 2017
	Zebra mussel *(Dreissena polymorpha)*	steady-state	0.236-236	7/0	whole organism (dw)	90	Contardo-Jara et al., 2011
	Fish *(Jenynsia multidentata)*	steady-state	0.5-100	48h/0	gill (ww)	5	Valdés et al., 2016
					intestine (ww)	5	
					liver (ww)	9	
					brain (ww)	9	
					muscle (ww)	6	
	Snakelocks anemone *(A. sulcata)*	steady-state	0-100	8/0	whole body	0.65-29	Vitale et al., 2020
	Beadlet anemone *(A. equina)*	steady-state	0-100	8/0	whole body	0.62-24	Picó et al., 2020
	Ruditapes sp.	steady-state	0.3-9	96 h/0	soft tissue	0.1-1.2	Almeida et al. (2014)
	Ruditapes decussatus	steady-state	30-50	14	gills	20-32	Abdelhafidh et al. (2018)
	Daphnia magna	steady-state	5-100	48 h/0	whole body	202-20	Nkoom et al. (2019)
	Mytilus edulis	kinetic	1-100	7/7	whole body	2.15-2.17	Boillot et al., 2018
	Ruditapes philippinarum	steady-state	1	28/0	gills	1.7	Almeida et al. (2018)
	Sea cucumber (*H. tubulosa*)	kinetic	10	28/52	digestive tract (dw)	74	The present work
	Sea cucumber (*H. tubulosa*)	kinetic	10	28/52	body wall (dw)	41	
	Snakelocks anemone (*A. sulcata*)	kinetic	10	28/52	whole organism (dw)	537	
	Beadlet anemone (*A. equina*)	kinetic	10	28/52	whole organism (dw)	127	
	Estimated					3.16	Meylan et al., 1999
	Estimated					1.57	Fu et al., 2009

Comparatively, there are many efforts made for CBZ regarding ATN. Although the list of antiepileptic drugs is broad, CBZ is mainly used in the context of environmental studies due to its high consumption and low degradation rate which in turn results in its presence in the environment. Therefore, there are many studies confirming its adverse effects on aquatic organisms (Ferrari et al., 2003). However, bibliographic research demonstrates that uptake data available for aquatic organisms is mostly devoted to fish and mussels. In addition, most of the published works have estimated the BCF taking into account the concentration measured at steady-state, but have not fully addressed their bioconcentration kinetics.

The highest laboratory BCF reported was for CBZ that reached 25.8-35.3 L/kg in the mussel (*Mytilus galloprovincialis*) (Serra-Compte et al., 2018). These values are similar to those of cotton spinner sea cucumbers, but lower than those of anemones. However, other estimates of accumulation were found to be 5 times lower (Table 4), although for different animals and under different experimental conditions. Recently, Vitale et al. (2020) evaluated the ability of anemones to accumulate CBZ at two level exposure concentrations in a short period experiment (1 and 100 ng/mL). It seems that at low concentrations anemones do not exhibit CBZ bioconcentration but at high concentrations, some mechanisms such as biotransformation processes or multi-resistance systems get saturated or are not efficient enough. Therefore, CBZ is not eliminated at the same rate as it is taken up, so bioconcentration occurs (BCF = 29 and 24 in *A. equina* and *A. sulcate*, respectively). In the present work, slightly higher values were observed when anemones were exposed for 28 days at intermediate concentration levels (10 ng/mL). These differences could be observed between the two ways of calculation, probably explained because the steady state was not ready reached. The 305 guideline in 2002 suggests to use toxicokinetic models as the first-order two-compartment model to estimate the BCF if the steady state is not clearly reached. However, kinetic model is not commonly applied in the previous reported studies (Molina-Fernández et al., 2021).

As for ATN, the BCF reported in fish are significantly lower than those found in the lower invertebrate organisms studied in the present work. However, it is largely unknown whether these compounds can bioaccumulate in echinoderms. Previously published studies have shown that the log BCF values of hydrophobic compounds are usually closely related to their octanol-water partition coefficient (log K_{ow}) (Barron, 1990). However, estimating the BCF for ionized compounds is highly complicated based on the difference in lipophilicity of neutral and ionized species. Meylan et al. (1999) developed an improved method of estimating BCF at the screening-level and proposed BCF values of 3.16 for ionizable compounds with log $K_{ow} < 5$.

Fu et al. (2009) designed a model for calculating the BCF of ionizing organic compounds and separately established BCF regression models for acids and bases based on pK_a and log K_{ow}. This model performed better than some previously established ones. In the present work, the BCF values of ATN and CBZ were predicted according to the methods of Meylan and Fu under similar experimental conditions (0.34 for ATN and 1.57 for CBZ), and then the data obtained have been compared with the BCF determined by the predictions of the two models. The results showed that our measured BCF values were not consistent with the predicted values. However, the predicted models have been developed considering data reported in fish species, thus the importance of measured data in other animal models. Sea cucumber and anemones have resulted interesting models for studying bioconcentration since they can reflect changes in the concentration of the contaminant from the surrounding environment, accumulate contaminants without being seriously affected by the concentrations as well as are considered representative and abundant in the marine environment (OSPAR commission, 2012). In addition, anemones are sessile organisms (like the mussel) and we can associate them with a specific place as opposed to a fish. They could be considered secondary or tertiary consumers (they feed on animals that can be herbivores -primary consumers- or carnivores -secondary consumers), thus can have a higher bioconcentration capacity from each trophic level. Sea cucumber although is not sessile, it is sedentary with

very low mobility and can also be associated with a specific place because its locomotion capacity is much reduced. In this case, we are dealing with detritivore animals that feed by ingesting organic matter and food particles from the surface layer of the marine sediment on which it crawls and lives and where it accumulates.

4. Conclusions

The present study is centered in the determination of the bioconcentration (uptake/depuration) kinetic of ATN and CBZ in cotton spinner sea cucumber and anemones as animal model. The research demonstrates the highest capacity of non-target but common marine organisms to accumulate CBZ than other pharmaceuticals such as ATN. The results also show that the type of organism, the tissue and structure of the compound have a significant impact upon their toxicokinetic processes and could significantly affect the uptake and depuration rates and BCF. CBZ exhibited higher uptake and excretion rates resulting BCF of 41-537 L/kg for CBZ vs 7-50 L/kg for ATN, although both are far below these threshold limits established by the UE. The animal-specific BCF generally followed the order of snakelocks anemone > beadlet anemone > sea cucumber for CBZ while was the opposite for ATN. The uptake and depuration rates of two compounds in the digestive tract were both much greater than those in body wall, which indicated digestive metabolism might be a main transformation pathway for them. Therefore, a full understanding of this phenomenon also requires an understanding of metabolic processes. Therefore, in the next step, the data must be complemented by experiments to determine whether and to what extent certain compounds undergo biotransformation.

An important gap within aquatic ecosystems is that, despite the above, the effects of PhACs have been studied only in a few of the many species that exist. Moreover, in our opinion, a credible estimation of the problem of bioconcentration remains a major challenge. Although numerous models exist, their accuracy is still a questionable issue, especially for ionic compounds. In this work, significant differences were found with those predicted BCF values of ATN and CBZ according to

Meylan's and Fu's methods under similar experiment conditions, highlighting that experimental practices are crucial for reliable results and accurately calculated field-derived BCF values.

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

References

Almeida, A., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2014. Presence of the pharmaceutical drug carbamazepine in coastal systems: Effects on bivalves. Aquat. Toxicol. 156, 74-87. https://doi.org/10.1016/j.aquatox.2014.08.002

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(4), 257-297. https://doi.org/10.1139/A06-005

Barron, M.G., 1990. Bioconcentration. Will water-borne organic chemicals accumulate in aquatic animals? Environ. Sci. Technol. 24, 1612-1618. https://doi.org/10.1021/es00081a001

Biel-Maeso, M., Corada-Fernández, C., Lara-Martín, P.A., 2018. Monitoring the occurrence of pharmaceuticals in soils irrigated with reclaimed wastewater. Environ. Pollut. 235, 312-321. https://doi.org/10.1016/j.envpol.2017.12.085

Boillot, C., Martinez Bueno, M.J., Munaron, D., Le Dreau, M., Mathieu, O., David, A., Fenet, H., Casellas, C., Gomez, E., 2015. In vivo exposure of marine mussels to carbamazepine and 10-hydroxy-10,11-dihydro-carbamazepine: Bioconcentration and metabolization. Sci. Total Environ. 532, 564-570. http://dx.doi.org/10.1016/j.scitotenv.2015.05.067

Bonanno, G., Orlando-Bonaca, M., 2018. Perspectives on using marine species as bioindicators of plastic pollution. Mar. Pollut. Bull. 137, 209-221. https://doi.org/10.1016/j.marpolbul.2018.10.018

Bulleri, F., Ravaglioli, C., Anselmi, S., Renzi, M., 2021. The sea cucumber *Holothuria tubulosa* does not reduce the size of microplastics but enhances their resuspension in the water column. Sci. Total Environ. 781, 146650. https://doi.org/10.1016/j.scitotenv.2021.146650

Cerveny, D., Fick, J., Klaminder, J., McCallum, E.S., Bertram, M.G., Castillo, N.A., Brodin, T., 2021. Water temperature affects the biotransformation and accumulation of a psychoactive pharmaceutical and its metabolite in aquatic organisms. Environ. Int. 155, 106705. https://doi.org/10.1016/j.envint.2021.106705

Chahrour, F., Baaloudj, A., Dermeche, S., Kerfouf, A., 2021. Trace metals (Cu, Cd, Pb, Zn, and Ni) concentrations in the sea cucumber *Holothuria tubulosa* (Gmelin, 1791) of the Algerian west coast. Egypt. J. Aquat. Biol. Fish. 25(3), 619-633. https://doi.org/10.21608/EJABF.2021.179882

Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nützmann, G., Kloas, W.,

Wiegand, C., 2011. Exposure to human pharmaceuticals carbamazepine, ibuprofen and bezafibrate causes molecular effects in *Dreissena polymorpha.* Aquat. Toxicol. 105, 428-437. https://doi.org/10.1016/j.aquatox.2011.07.017

Cravo, A., Silva, S., Rodrigues, J., Cardoso, V.V., Benoliel, M.J., Correia, C., Coelho, M.R., Rosa, M.J., Almeida, C.M.M., 2022. Understanding the bioaccumulation of pharmaceutical active compounds by clams *Ruditapes decussatus* exposed to a UWWTP discharge. Environ. Pollut. 208, 112632. https://doi.org/10.1016/j.envres.2021.112632

Daniele, G., Fieu, M., Joachim, S., Bado-Nilles, A., Beaudouin, R., Baudoin, P., James-Casas, A., Andres, S., Bonnard, M., Bonnard, I., Geffard, A., Vulliet, E., 2017. Determination of carbamazepine and 12 degradation products in various compartments of an outdoor aquatic mesocosm by reliable analytical methods based on liquid chromatography-tandem mass spectrometry. Environ. Sci. Pollut. Res. 24, 16893-16904. https://doi.org/10.1007/s11356-017-9297-6

Duarte, I.A., Reis-Santos, P., Fick, J., Cabral, H.N., Duarte, B., Fonseca, V.F., 2023. Neuroactive pharmaceuticals in estuaries: Occurrence and tissue-specific bioaccumulation in multiple fish species. Environ. Pollut. 216, 120531. https://doi.org/10.1016/j.envpol.2022.120531

Dumas, T., Courant, F., Almunia, C., Boccard, J., Rosain, D., Duporté, G., Armengaud, J., Fenet, H., Gomez, E., 2022. An integrated metabolomics and proteogenomics approach reveals molecular alterations following carbamazepine exposure in the male mussel *Mytilus galloprovincialis.* Chemosphere. 286, 131793. https://doi.org/10.1016/j.chemosphere.2021.131793

European Union, 2008. Directive 2008/56/EC of the European parliament and of the council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy (MSFD). OJEU L164/19-*40.* Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32008L0056&from=en (accessed on February 2023).

Ferrari, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicol. Environ. Saf. 55, 359-370. https://doi.org/10.1016/S0147-6513(02)00082-9

Fu, W., Franco, A., Trapp, S., 2009. Methods for estimating the bioconcentration factor of ionizable organic chemicals. Environ. Toxicol. Chem. 28(7), 1372-1379. https://doi.org/dx.doi.org/10.1897/08-233

García, S.N., Foster, M., Constantine, L.A., Huggett, D.B., 2012. Field and laboratory fish tissue accumulation of the anti-convulsant drug carbamazepine. Ecotoxicol. Environ. Saf. 84, 207-211. https://doi.org/10.1016/j.ecoenv.2012.07.013.

Hendriks, A.J., van der Linde, A., Cornelissen, G., Sijm, D.T.H.M., 2001. The power of size. 1. Rate constants and equilibrium ratios for accumulation of organic substances related to octanol–water partition ratio and species weight. Environ. Toxicol. Chem. 20, 1399–1420. https://doi.org/dx.doi.org/10.1897/1551-5028

Heye, K., Becker, D., Everlosh, C.L., Durmaz, V., Ternes, T.A., Oetken, M., Oehlmann, J., 2016. Effects of carbamazepine and two of its metabolites on the non-biting midge *Chironomus riparius* in a sediment full life cycle toxicity test. Water Res. 98, 19-27. http://dx.doi.org/10.1016/j.watres.2016.03.071

Huerta, B., Rodriguez-Mozaz, S., Lazorchak, J., Barcelo, D., Batt, A., Wathen, J., Stahl, L., 2018. Presence of pharmaceuticals in fish collected from urban rivers in the U.S. EPA 2008–2009 National Rivers and Streams Assessment. Sci. Total Environ. 634, 542-549. https://doi.org/10.1016/j.scitotenv.2018.03.387

ICH Quality Guidelines, 2005. Topic Q2 (R1). Validation of analytical procedures: text and methodology. Available at http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html (accessed on February 2023).

Kim, J-W., Ishibashi, H., Yamauchi, R., Ichikawa, N., Takao, Y., Hirano, M., Koga, M., Arizono, K., 2009. Acute toxicity of pharmaceutical and personal care products on freshwater crustacean *(Thamnocephalus platyurus)* and fish *(Oryzias latipes).* J. Toxicol. Sci. 34(2), 227-232. https://doi.org/10.2131/jts.34.227

Mackay, D., and Fraser, A., 2000. Bioaccumulation of Persistent Organic Chemicals: Mechanisms and Models. Environ. Pollut. 110, 375-391. http://dx.doi.org/10.1016/S0269-7491(00)00162-7

Martín, J., Hidalgo, F., Alonso, E., García-Córcoles, M.T., Vílchez, J.L., Zafra-Gómez, A., 2020. Assessing bioaccumulation potential of personal care, household and industrial products in a marine echinoderm (*Holothuria tubulosa*). Sci. Total Environ. 720, 137668. https://doi.org/10.1016/j.scitotenv.2020.137668

Mastrángelo, M.M., Valdés, M.E., Eissa, B., Ossana, N.A., Barceló, D., Sabater, S., Rodríguez-Mozaz, S., Giorgi, A.D.N., 2022. Occurrence and accumulation of pharmaceutical products in water and biota of urbn lowland rivers. Sci. Total Environ. 828, 154303. https://doi.org/10.1016/j.scitotenv.2022.154303

McCallum, E.S., Sundelin, A., Fick, J., Alanärä, A., Klaminder, J., Hellström, G., Brodin, T., 2019. Investigating tissue bioconcentration and the behavioural effects of two pharmaceutical pollutants on sea trout (*Salmo trutta*) in the laboratory and field. Aquat. Toxicol. 207, 170-178. https://doi.org/10.1016/j.aquatox.2018.11.028

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. https://doi.org/10.1016/j.envpol.2011.11.029

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(4), 664-672. https://doi.org/10.1002/etc.5620180412.

Molina-Fernández. N., Rainieri, S., Muñoz-Olivas, R., de Oro-Carretero, P., Sanz-Landaluze, J., 2021. Development of a method for assessing the accumulation and metabolization of antidepressant drugs in zebrafish *(Danio rerio)* eleutheroembryos. Anal. Bioanal. Chem. 413, 5169-5179. https://doi.org/10.1007/s00216-021-03486-2

Morais, L.M.S., Sarti, F., Chelazzi, D., Cincinelli, A., Giarrizzo, T., Martinelli Filho, J.E., 2020. The sea anemone *Bunodosoma cangicum* as a potential biomonitor for microplastics contamination on the Brazilian Amazon coast. Environ. Pollut. 265, 114817. https://doi.org/10.1016/j.envpol.2020.114817

Nkoom, M., Lu, G., Liu, J., Yang, H., Dong, H., 2019. Bioconcentration of the antiepileptic drug carbamazepine and its physiological and biochemical effects on *Daphnia magna.* Ecotoxicol. Environ. Saf. 172, 11-18. https://doi.org/10.1016/j.ecoenv.2019.01.061

OECD, 2012. Guidelines for the Testing of Chemicals, Section 3: Environmental fate and behavior. Test No. 305: Bioaccumulation in fish: Aqueous and dietary exposure. Available at https://doi.org/10.1787/9789264185296-en (accessed on February 2023)

OSPARCOM, 2012. JAMP Guidelines for Monitoring Contaminants in Biota. OSPAR Commission, London, p. 122.

Picó, Y., Alvarez-Ruiz, R., Alfarhan, A.H., El-Sheikg, M.A., Alobaid, S.M., Barceló, D., 2019. Uptake and accumulation of emerging contaminants in soil and plant treated with wastewater under real-world environmental conditions in the Al Hayer area (Saudi Arabia). Sci. Total Environ. 652, 562-572. https://doi.org/10.1016/j.scitotenv.2018.10.224

Serra-Compte, A., Maulvault, A.L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz, S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*), Environ. Pollut. 236,

824-834. https://doi.org/10.1016/j.envpol.2018.02.018

Schlichter, D., 1978. On the ability of *Anemonia sulcata* (Coelenterata: Anthozoa) to absorb charged and neutral amino acids simultaneously. Mar. Biol. 45, 97-104.

Stambler, N., Dubinsky, Z., 1987. Energy relationships between *Anemonia sulcata* and its endosymbiotic zooxanthellae. Symbiosis 3, 233-248.

Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Zlabek, V., Schmidt-Posthaus, H., Grabic, R., Kroupova, H.K., 2014. The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout *(Oncorhynchus mykiss).* Sci. Total Environ. 497-498, 209-218. http://dx.doi.org/10.1016/j.scitotenv.2014.07.111

Urban, N.A., Swihart, R.K., Malloy, M.C., Dunning Jr. J.B., 2012. Improving selection of indicator species when detection is imperfect. Ecol. Indic. 15, 188-197. https://doi.org/10.1016/j.ecolind.2011.09.031

Valdés, M.E., Amé, M.V., Bistoni, M.A., Wunderlin, D.A., 2014. Occurrence and bioaccumulation of pharmaceuticals in a fish species inhabiting the Suquía River basin (Córdoba, Argentina). Sci. Total Environ. 472, 389-396. https://doi.org/10.1016/j.scitotenv.2013.10.124

Valdés, M.E., Huerta, B., Wunderlin, D.A., Bistoni, M.A., Barceló, D., Rodriguez-Mozaz, S., 2016. Bioaccumulation and bioconcentration of carbamazepine and other pharmaceuticals in fish under field and controlled laboratory experiments. Evidences of carbamazepine metabolization by fish. Sci. Total Environ. 557-558, 58-67. http://dx.doi.org/10.1016/j.scitotenv.2016.03.045

Vellinger, C., Parant, M., Rousselle, P., Immel, F., Wagner, P., Usseglio-Polatera, P., 2012. Comparison of arsenate and cadmium toxicity in a freshwater amphipod (*Gammarus pulex*). Environ. Pollut. 160, 66-73. https://doi.org/10.1016/j.envpol.2011.09.002

Vellinger, C., Gismondi E., Felten, V., Rouselle, P., Mehennaoui, K.,

Parant, M., Usseglio-Polatera, P., 2013. Single and combined effects of cadmium and arsenate in *Gammarus pulex* (Crustacea, Amphipoda): understanding the links between physiological and behavioural responses. Aquat. Toxicol. 140–141, 106–116. https://doi.org/10.1016/j.aquatox.2013.05.010

Vitale, D., Picó, Y., Spanò, N., Torreblanca, A., del Ramo, J., 2020. Carbamazepine exposure in the sea anemones *Anemonia sulcata* and *Actinia equina:* Metabolite identification and physiological responses. Sci. Total Environ. 744, 140891. https://doi.org/10.1016/j.scitotenv.2020.140891

Wang, Y., Yin, T., Kelly, B.C., Gin, T.Y-H., 2019. Bioaccumulation behaviour of pharmaceuticals and personal care products in a constructed wetland. Chemosphere 222, 275-285. https://doi.org/10.1016/j.chemosphere.2019.01.116

Xu, C., Niu, L., Guo, H., Sun, X., Chen, L., Tu, W., Dai, Q., Ye, J., Liu, W., Liu, J., 2019. Long-term exposure to the non-steroidal anti-inflammatory drug (NSAID) naproxen causes thyroid disruption in zebrafish at environmentally relevant concentrations. Sci. Total Environ. 676, 387-395. https://doi.org/10.1016/j.scitotenv.2019.04.323

Yang, J., Wang, J., Wang, F., Meng, F., Yang, X., Pan, Q., Liu, X., Duan, A., Wang, D., 2023. Insights into the effect of carbamazepine on the anaerobic fermentation of waste activated sludge: Performance, mechanisms and regulation. J. Chem. Eng. 452, 139549. https://doi.org/10.1016/j.cej.2022.139549

Instrumentation and software

Samples were freeze-dried using a ScanVac CoolSafe™ freeze-dryer. Pulverisation of the samples was performed with a Retsch MM200 ball mixer (Haan, Germany). An IKA vortex (Staufen, Germany) and a Labnet Spectrafuge™ 24D centrifuge (New Jersey, USA) were also used. Ultrasonic extraction was performed with a 400 W digital sonicator, with a 0.5 inch (12.7 mm) probe operating at a frequency of 20 kHz. In addition, an Ultrasounds-HD ultrasonic bath from Selecta (Barcelona, Spain) and a Mettler-Toledo precision balance (Columbus, OH, USA) were used. Evaporation of the samples was carried out with a Stuart sample concentrator-nitrogen evaporator (Staffordshire, UK).

UHPLC-MS/MS analysis was performed with a Waters ACQUITY UPLC® H-Class (Waters, Manchester, UK), consisting of an AQUITY™ sample manager, an ACQUITY UPLC™ binary solvent manager (Waters, UK) and an ACQUITY HSS T3 column (100 mm x 2.1 mm internal diameter, 1.8 µm particle size). An XEVO TQ-XS quadrupole tandem mass spectrometer equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for antiepileptic detection. MassLynx 4.1 software (Waters) was used for data acquisition.

The following instruments were used for the measurements Hanna Instruments® digital thermometer (model HI Checktemp 1), HACH® sensION™ þ EC5 portable conductivity/TDS meter with conductivity probe (model 50 60), HACH® HQ40d portable multimeter with pH probe (model pHC101) and luminescent dissolved oxygen probe (model LDO101).

Validation requirements

First of all, it was checked with all the matrices under study that the matrix effect was low. For this purpose, a pure standard calibration and a matrix calibration were compared and the slope of the line obtained was similar for all compounds. Eight levels of the calibration were prepared.

The selectivity of the method was studied by comparing the chromatograms of the blank with a standard sample. The sensitivity of

the method was evaluated by the limit of detection (LOD) and limit of quantification (LOQ) from the signal-to-noise ratio obtained from the injection of standards with decreasing amounts of the target analytes. On the other hand, the accuracy in terms of trueness and precision was evaluated by recovery tests at three concentration levels (LOQ, medium and high) for trueness, and the relative standard deviation (% RSD) of the measurements was evaluated three times on the same day and for three days for precision.

The assurance of the data obtained in the validation was verified (QA/QC) by injecting spiked sample blanks, which were treated the same as the samples to control background contamination. It was observed that there were no detectable amounts in these blanks. In addition, instrumental stability was assessed by injecting each sample in triplicate and every twentieth analysis a mixture of the pure standards of the target analytes was injected.

Table S1*. Physicochemical properties and structure of target analytes*

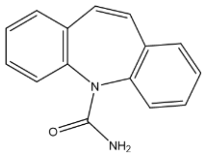
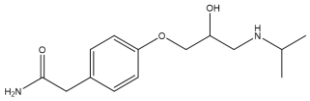
Compound	MW (g/mol)	pK_a	Log K_{ow}	Structure
CBZ	236.27	13.9	2.45	
ATN	266.34	9.58	0.16	

***Table S2**. Validation parameters of the analytical method. Limit of detection and quantification, precision and linearity*

Compound	LOD ($ng\ g^{-1}$)	LOQ ($ng\ g^{-1}$)	RSD (%)	Linearity (%)
ATN	0.03	0.1	14.3	99.1
CBZ	0.03	0.1	8.3	99.7

LOD: limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation

Conclusiones

La presente Tesis Doctoral se ha centrado en dos aspectos principales. Por un lado se han optimizado metodologías de buensas características analíticas para el estudio de diferentes contaminantes emergentes en bioindicadores ambientales y biomarcadores biológicos. Se ha incidido principalemente en la etapa de tratamiento de muestra y se han estudiado diferentes técnicas de extracción y *clean-up* para garantizar la obtención de extractos limpios y una separación eficiente de los analitos objeto de estudio. Por otro lado, se han realizado ensayos de bioconcentración de compuestos activos farmacéuticos en bioindicadores marinos bajo condiciones controladas de laboratorio.

A continuación se resumen las conclusiones más relevantes obtenidas en este trabajo de investigación.

1. Se han realizado dos revisiones bibliográficas para conocer el estado del arte en las dos temáticas principales abordadas en este trabajo: desarrollo de metodologías para el análisis de contaminantes emergentes en distintas matrices y estudios de acumulación de compuestos activos farmacéuticos en organismos marinos.

   a. Con respecto al desarrollo de metodología, tema abordado en el **Capítulo 1,** se ha demostrado la necesidad de investigar de manera más profunda la presencia de estos contaminantes en los niveles más bajos de la cadena trófica como son los organismos bentónicos. Se ha corroborado la importancia de la etapa de tratamiento de muestra, (extracción y purificación de los extractos), siendo la extracción por ultrasonidos y de líquidos presurizados las técnicas más usadas y los adsorbentes poliméricos de fase inversa para la purificación. Para la separación, se ha recurrido exclusivamente a la cromatografía de líquidos debido a las características fisicoquímicas de los compuestos de estudio.

   b. En cuanto a los ensayos de bioconcentración, considerados en el **Capítulo 6,** se ha comprobado que los organismos más estudiados han sido peces y moluscos. Se han corroborado con este trabajo algunas consideraciones como que se le ha prestado poca atención a los

metabolitos y productos de degradación y que las características del agua como son temperatura y pH han resultado ser muy influyentes en la bioconcentración de determinados compuestos. Los organismos bentónicos han mostrado concentrar mayores cantidades de contaminantes debido a la ingestión de la materia orgánica procedente de los sedimentos, demostrando su aptitud como bioindicadores de la contaminación marina.

2. Se han desarrollado, optimizado y validado métodos de buenas características analíticas para la determinación de dos grandes familias de contaminantes emergentes, los filtros UV, considerados también disruptores endocrinos y PhACs, concretamente antibióticos, en las matrices seleccionadas.

   a. En cuanto a los filtros UV, se han puesto a punto metodologías LC-MS/MS para su determinación en mejillón mediterráneo *(M. galloprovincialis)* y en uña humana. Estas investigaciones han sido recogidas en los **Capítulos 2 y 4**, respectivamente.

   En el primero, la técnica de extracción seleccionada fue la asistida por ultrasonidos, seguida de una extracción en fase sólida dispersiva usando como adsorbente C-18. Se aplicó el método en muestras de mejillones salvajes de la costa de Granada, siendo detectados todos los compuestos diana, excepto la BP-2, y mostrando variaciones dependiendo de la zona de captura. Se obtuvieron concentraciones mayores en las zonas cercanas a las áreas recreacionales y de bañistas.

   En el segundo, se ha demostrado que la digestión asistida por microondas en medio ácido ofrece las mejores recuperaciones de los analitos. La uña ha resultado ser una matriz apta para estudios de bioacumulación de estos contaminantes en el cuerpo humano. Las concentraciones obtenidas son elevadas, lo cual implica que el ser humano se encuentra muy expuesto a estos compuestos.

   b. Con respecto a los antibióticos, se ha desarrollado un método UHPLC-MS/MS para la determinación de 17 residuos de antibióticos en muestras de carcasa de pepino de mar *(H. tubulosa)*. Estos trabajos

de investigación se desarrollan en los Capítulos 3 y 5, respectivamente. En cada uno de ellos se ha estudiado la etapa de extracción de los compuestos y la validación.

En el **Capítulo 3**, la extracción fue asistida por ultrasonidos y la limpieza del extracto se realizó mediante una extracción en fase sólida dispersiva con C-18. Se aplicó el método a muestras recogidas en el litoral granadino, siendo todas positivas en más de un antibiótico estudiado. Este trabajo demuestra la capacidad de bioacumulación de antibióticos por esta especie y su capacidad como bioindicador de la contaminación marina.

Capítulo 5, se optimizaron dos técnicas de extracción (UAE y MAE) y se compararon en términos de eficacia y viabilidad. La digestión en microondas demostró poseer mayor eficacia y necesitar menos tiempo de digestión así como de cantidad de disolvente, siendo seleccionada para la aplicación. Se aplicó el método en muestras de 10 voluntarios y de los 17 antibióticos estudiados, todos fueron detectados en al menos una de las muestras analizadas. Esta elevada frecuencia en la detección demuestra el riesgo global a la salud humana debido al posible desarrollo de resistencias bacterianas.

3. Se han realizado estudios de bioconcentración de distintos PhACs bajo condiciones de laboratorio controladas en tres bioindicadores marinos: pepino de mar *(H. tubulosa)*, anémona de mar *(A. sulcata)* y tomate de mar *(A. equina)*. Se expusieron a los animales durante 28 días a los fármacos de estudio y posteriormente tuvo lugar una depuración de 52 días.

   a. En el **Capítulo 7**, se ha estudiado la bioconcentración de la VFX y su principal metabolito (O-VFX). Los compuestos han mostrado un proceso cinético de primer orden en todos los casos. El BCF siguió el orden anémona de mar > tomate de mar > pepino de mar. Se demostró que la bioconcentración dependía del tejido estudiado, siendo mayor en el tracto digestivo que en la carcasa en el caso del pepino de mar, que puede deberse a la capacidad de metabolización de la especie.

b. El **Capítulo 8** se ha centrado en el estudio de bioconcentración de tres antibióticos de muy amplio uso en medicina actualmente, CIP, TMP y SFX. Los resultados indicaron que la distribución tisular estaba muy influenciada por el tipo de antibiótico. TMP se concentró en el tracto digestivo del pepino de mar mientras que CIP en la carcasa. Los valores de BCF obtenidos son muy distintos en las especies estudiadas, que se explica por la variación en la absorción biológica. Estos organismos bentónicos han demostrado poseer BCF más elevados que otros animales más estudiados como son los peces o mejillones, aunque la falta de uniformidad entre los estudios publicados y la dispersión natural hacen que la interpretación de los resultados sea compleja y que en ocasiones no se puedan correlacionar.

c. En el **Capítulo 9,** se ha estudiado la cinética de bioconcentración de CBZ y ATN. Se ha demostrado que estos organismos tienen mayor capacidad para acumular el primer fármaco. Los procesos toxicocinéticos de estas especies varían entre ellas e incluso se diferencian en sus compartimentos corporales. El BCF de cada animal siguió el orden ortiga de mar > tomate de mar > pepino de mar para CBZ y para ATN sucedió lo contrario. Este estudio sugiere que los sedimentos pueden ser una fuente importante de contaminantes para la biota bentónica y subraya la necesidad de prestar atención al fenómeno de biomagnificación en estas especies.

Anexo I. Publicaciones de la Tesis

molecules

MDPI

Review

An Overview of Analytical Methods to Determine Pharmaceutical Active Compounds in Aquatic Organisms

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Abstract: There is increasing scientific evidence that some pharmaceuticals are present in the marine ecosystems at concentrations that may cause adverse effects on the organisms that inhabit them. At present, there is still very little scientific literature on the (bio)accumulation of these compounds in different species, let alone on the relationship between the presence of these compounds and the adverse effects they produce. However, attempts have been made to optimize and validate analytical methods for the determination of residues of pharmaceuticals in marine biota by studying the stages of sample treatment, sample clean-up and subsequent analysis. The proposed bibliographic review includes a summary of the most commonly techniques, and its analytical features, proposed to determine pharmaceutical compounds in aquatic organisms at different levels of the trophic chain in the last 10 years.

Keywords: pharmaceuticals; contamination; analytical methods; aquatic organisms; trophic chain

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1. Introduction

Pollution is one of the biggest environmental challenges worldwide. Like climate change or the depletion of water supplies, pollution threatens the stability of the earth's support systems and is a growing concern for human health [1]. Ocean pollution is a very important, but under-recognised, component of global pollution [2]. Seawater covers 97% of surface waters and is considered one of the most abundant resources on our planet [1]. The unsustainable use of marine waters and resources by humans has altered the structure of marine ecosystems, relating to the phenomenon of eutrophication, loss of diversity or the presence of polluting chemicals [3].

Human activities have introduced a large number of contaminants of emerging concern (CECs) into the environment [4]. CECs include a wide variety of compounds such as disinfection by-products, natural toxins, flame retardants, personal care products or pharmaceutical active compounds (PhACs) [5]. Nowadays, an increasing number of people and animals are in need of health care, which means that the number and amount of PhACs consumed, and consequently excreted, is very high [6–8]. Approximately 3000 compounds are used as pharmaceuticals, with an annual production exceeding hundreds of tonnes [7]. It is well known that the wastewater treatment plants (WWTPs) are often unable to remove them completely, allowing their release into the environment [9,10]. In the case of PhACs, due to their constant release into the seas, even those that can undergo degradation may behave as pseudopersistent contaminants [11]. This continued exposure may present unexpected risks in the organisms that inhabit them such as reproductive disorders, survival of susceptible species, growth rate or development of bacterial resistance and endocrine disruption, among others [8,12,13].

96. Clarke, W. Mass spectrometry in the clinical laboratory: Determining the need and avoiding pitfalls. In *Mass Spectrometry in the Clinical Laboratory*, 1st ed.; Nair, H., Clarke, W., Eds.; Academic Press: Cambridge, MA, USA, 2017; pp. 1–15. [CrossRef]
97. González-Peña, O.I.; López-Zavala, M.A.; Cabral-Ruelas, H. Pharmaceuticasls market, consumption, trends and disease incidence are not driving the pharmaceutical research on water and wastewater. *Int. J. Environ. Res. Public Health* **2021**, *18*, 2532. [CrossRef]
98. Świacka, K.; Maculewicz, J.; Kowalska, D.; Caban, M.; Smolarz, K.; Świeżak, J. Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms—Current state of knowledge. *J. Hazard. Mater.* **2022**, *424*, 127350. [CrossRef]

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Ultra-high performance liquid chromatography tandem mass spectrometry analysis of UV filters in marine mussels (*Mytilus galloprovinciallis*) from the southern coast of Spain

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ABSTRACT

Ultraviolet (UV) filters are a family of organic compounds widely used in sunscreens and personal care products (PCPs) as well as other materials such as plastics, toys and outdoor furniture, for their effectiveness in absorbing UVA and UVB radiation. These compounds directly enter the marine environment because of inefficient wastewater treatments and anthropogenic activities, posing a risk to the marine biota. The present study develops and validates a method to determine some of the most used UV filters (BP-1, BP-2, BP-3, BP-6, BP-8, 4-OH-BP and 4-MBC), in wild *Mytilus galloprovincialis* mussels using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). The sample treatment is based on an ultrasound-assisted extraction followed by a clean-up step using C18 as sorbent. The methodology was satisfactorily validated, obtaining good features, and it was applied for the evaluation of the occurrence of the target analytes in mussels collected in five areas along the tourist coast of Granada (Spain) just after summer holydays period. The results showed a higher bioaccumulation in specimens sampled in recreational areas and with a closed geomorphology, being BP-3 the most predominant in all locations. BP-1 and BP-3 were quantified in all samples and the rest of UV filters were detected in most of them, except for BP-2. The data raises concern about the undesirable effects that UV filter pollution can cause in the area and highlights the need to establish practices that help preserve and sustain the marine ecosystem.

1. Introduction

UV filters are organic compounds with aromatic structures capable of absorbing solar radiation. Therefore, many personal care products (PCPs) include different families of these substances such as camphor or benzophenone derivatives, among others. UV filters are able to absorb solar radiation UV-A (320–400 nm) and UV-B (280–320 nm) [1–3]. 4-Methylbenzylidene camphor (4-MBC) is one of the most widely used UV filter. This compound cannot be completely removed by the traditional wastewater treatment process. Some studies show that in a Chinese treatment plant, the removal efficiency of this compound is 40% [4,5]. Benzophenones (BPs), on the other side, are a family of UV filter compounds which have a high lipophilic character and are able to enter the body and bioaccumulate easily [6–10].

In recent years, there has been increasing concern about sun exposure and the associated risks. Thus, the consumption and use of products which contain UV filters for sun protection, including sunscreens, shampoos or hair dyes as well as plastics, paints or packaging of outdoor materials, has increased considerably [3]. Many UV filters are recognized as emerging pollutants and constitute a risk to human health and the environment. Toxicological studies have demonstrated their estrogenic effects and toxicity for reproduction and development, even when they are found in low concentrations, being considered endocrine disrupting chemicals (EDCs) [5]. EDCs are exogenous compounds capable of causing adverse effects on the health of an organism and its offspring by interfering with the normal functioning of the endocrine system.

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https://doi.org/10.1016/j.microc.2021.106800
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Available online 6 September 2021

[4] W. Li, Y. Ma, C. Guo, W. Hu, K. Liu, Y. Wang, T. Zhu, Occurrence and behaviour of four of the most used sunscreen UV filters in a wastewaters reclamation plant, Water Res. 41 (2007) 3506–3512.

[5] H. Hong, J. Wang, D. Shi, Effects of salinity on the chronic of 4-methylbenzylidene camphor (4-MBC) in the marine copepod *Tigriopus japonicas*, Aquat. Toxicol. 232 (2021), 106742.

[6] J. Hiller, K. Klotz, S. Meyer, W. Uter, K. Hof, A. Greiner, T. Göen, H. Drexler, Systemic availability of lipophilic organic UV-filters through dermal sunscreen exposure, Environ. Int. 132 (2019), 105068.

[7] M. Krause, H. Frederiksen, K. Sundberg, F.S. Jørgensen, L.N. Jensen, P. Nørgaard, C. Jørgensen, P. Ertberg, A. Juul, K.T. Drzewiecki, N.E. Skakkebaek, A. M. Andersson, Presence of benzophenones commonly used as UV-filters and absorbers in paired maternal and fetal samples, Environ. Int. 110 (2018) 51–60.

[8] R. Rodríguez-Gómez, A. Zafra-Gómez, F.J. Camino-Sánchez, O. Ballesteros, A. Navalón, Gas chromatography and ultrahigh performance liquid chromatography tandem mass spectrometry methods for the determination of selected endocrine disrupting chemicals in human breast milk after stir-bar sorptive extraction, J. Chromatogr. A. 1349 (2014) 69–79.

[9] F. Vela-Soria, O. Ballesteros, A. Zafra-Gómez, L. Ballesteros, A. Navalón, A new method for the determination of benzophenone-UV-filters in human serum samples by dispersive liquid-liquid microextraction with liquid chromatography-tandem mass spectrometry, Talanta 121 (2014) 97–104.

[10] F. Vela-Soria, O. Ballesteros, A. Zafra-Gómez, L. Ballesteros, A. Navalón, A multiclass method for the analysis of endocrine disrupting chemicals in human urine samples. Sample treatment by dispersive liquid-liquid microextraction, Talanta 129 (2014) 209–218.

[11] C. Liao, K. Kannan, Species-specific accumulation and temporal trends of bisphenols and benzophenones in mollusks from the Chinese Bohai Sea during 2006–2015, Sci. Tot. Environ. 653 (2019) 168–175.

[12] K. He, E. Hain, A. Timm, L. Blaney, Bioaccumulation of estrogenic hormones and UV-filters in red swamp crayfish (*Procambarus clarkia)*, Sci. Total Environ. 764 (2021), 142871.

[13] L. Vidal-Liñán, E. Villaverde-de-Sáa, R. Rodil, J.B. Quintana, R. Beiras, Bioaccumulation of UV filters in *Mytilus galloprovincialis* mussel, Chemosphere 190 (2018) 267–271.

[14] S. Bayen, E.S. Estrada, G. Juhel, L.W. Kit, B.C. Kelly, Pharmaceutically active compounds and endocrine disrupting chemicals in water, sediments and mollusks in mangrove ecosystems from Singapore, Mar. Pollut. Bull. 109 (2) (2016) 716–722.

[15] S.C. Cunha, J.O. Fernandes, L. Vallecillos, G. Cano-Sancho, J.L. Domingo, E. Pocurull, F. Borrull, A.L. Maulvault, F. Ferrari, M. Fernandez-Tejedor, F. Van den Heuvel, Co-occurrence of musk fragances and UV-filters in seafood and macroalgae collected in European hotspots, Environ. Res. 143 (2015) 65–71.

[16] S. Montesdeoca-Esponda, M.E. Torres-Padrón, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, Fate and distribution of benzotriazole UV filters and stabilizers in environmental compartments from Gran Canaria Island (Spain): a comparison study, Sci. Total Environ. 756 (2021) 144086, https://doi.org/10.1016/j.scitotenv.2020.144086.

[17] X. Bai, K. Acharya, Uptake of endocrine-disrupting chemicals by quagga mussels (Dreissena bugensis) in an urban-impacted aquatic ecosystem, Environ. Sci. Pollut. Res. 26 (1) (2019) 250–258.

[18] K. Styszko, K. Proctor, E. Castrignanò, B. Kasprzyk-Hordern, Occurrence of pharmaceutical residues, personal care products, lifestyle chemicals, illicit drugs and metabolites in wastewater and receiving surface waters of Krakow agglomeration in South Poland, Sci. Total Environ. 768 (2021), 144360.

[19] Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. O. J. EU, L342, 2009, 59/209. Available online at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32009R1223&from=EN (May 2021).

[20] D. Álvarez-Muñoz, S. Rodríguez-Mozaz, S. Jacobs, A. Serra-Compte, N. Cáceres, I. Sioen, W. Verbeke, V. Barbosa, F. Ferrari, M. Fernández-Tejedor, S. Cunha, K. Granby, J. Robbens, M. Kotterman, A. Marques, D. Barceló, Pharmaceuticals and endocrine disruptors in raw and cooked seafood from European market: Concentrations and human exposure levels, Environ. Int. 119 (2018) 570–581.

[21] R. Rodil, E. Villaverde-de-Sáa, J. Cobas, J.B. Quintana, R. Cela, N. Carro, Legacy and emerging pollutants in marine bivalves from the Galician coast (NW Spain), Environ. Int. 129 (2019) 364–375.

[22] M. Bachelot, Z. Li, D. Munaron, P. Le Gall, C. Casellas, H. Fenet, E. Gomez, E.,, Organic UV filter concentrations in marine mussels from French coastal regions, Sci. Total Environ. 420 (2012) 273–279.

[23] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (13) (2003) 3019–3030.

[24] ICH Quality Guidelines, Topic Q2 (R1): Validation of Analytical Procedures: Text and Methodology, 2005. Available online at: http://www.ich.org/products/guidelines/quality/ article/quality-guidelines.html (May 2021).

[25] M. Picot Groz, H. Fenet, M.J. Martínez Bueno, D. Rosain, E. Gomez, Diurnal variations in personal care products in seawater and mussels at three Mediterranean coastal sites, Environ. Sci. Pollut. Res. 25(2018) 9051-9059.

[26] K. Fent, A. Zenker, M. Rapp, Widespread occurrence of estrogenic UV-filters in aquatic ecosystems in Switzerland, Environ. Pollut. 158 (5) (2010) 1817–1824.

[27] M. Picot Groz, M.J. Martinez Bueno, D. Rosain, H. Fenet, C. Casellas, C. Pereira, V. Maria, M.J. Bebianno, E. Gomez, E.,, Detection of emerging contaminants (UV filters, UV stabilizers and musk) in marine mussels from Portuguese coast by QuEChERs extraction and GC-MS/MS, Sci. Tot. Environ. 493 (2014) 162–169.

[28] Consumer Product Information Database (CPID). Health effects of consumer product. Benzophenone-3 Available online at: https://www.whatsinproducts.com/chemicals/view/1/240/000131-57-7/Benzophenone-3%20(Oxybenzone) (May 2021).

[29] N. Blüthgen, S. Zucchi, K. Fent, Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (Danio rerio), Toxicol. Appl. Pharmacol. 263 (2) (2012) 184–194.

[30] M.H. Wu, D.G. Xie, G. Xu, R. Sun, X.Y. Xia, W.L. Liu, L. Tang, Benzophenone-type UV filters in surface waters: An assessment of profiles and ecological risks in Shanghai, China, Ecotoxicol. Environ. Saf. 141 (2017) 235–241.

[31] Y. Watanabe, H. Kojima, S. Takeuchi, N. Uramaru, S. Sanoh, K. Sugihara, S. Kitamura, S. Ohta, Metabolism of UV-filter benzophenone-3 by rat and human liver microsomes and its effect on endocrine-disrupting activity, Toxicol. Appl. Pharmacol. 282 (2) (2015) 119–128.

[32] T. Suzuki, S. Kitamura, R. Khota, K. Sugihara, N. Fujimoto, S. Ohta, Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens, Toxicol. Appl. Pharmacol. 203 (2005) 9–17.

[33] L. Wang, K. Kannan, Characteristic profiles of benzonphenone-3 and its derivatives in urine of children and adults from the United States and China, Environ. Sci. Technol. 47 (21) (2013) 12532–12538.

[34] S.C. Cunha, L. Trabalón, S. Jacobs, M. Castro, M. Fernandez-Tejedor, K. Granby, W. Verbeke, C. Kwadijk, F. Ferrari, J. Robbens, I. Sioen, E. Pocurull, A. Marques, J. O. Fernandes, J.L. Domingo, UV-filters and musk fragrances in seafood commercialized in Europe Union: Occurrence, risk and exposure assessment, Environ. Res. 161 (2018) 399–408.

[35] L. Martín-Pozo, M.C. Gómez-Regalado, S. Cantarero-Malagón, A. Navalón, A. Zafra-Gómez, Determination of UV filters in human nails using an acid sample digestion followed by ultra-high performance liquid chromatography-mass spectrometry analysis, Chemosphere 273 (2021), 128603.

[36] F. Vela-Soria, I. Jiménez-Díaz, R. Rodríguez-Gómez, A. Zafra-Gómez, O. Ballesteros, A. Navalón, J.L. Vílchez, M.F. Fernández, N. Olea, Determination of benzophenones in human placental tissue samples by liquid chromatography–tandem mass spectrometry, Talanta 85 (4) (2011) 1848–1855.

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Multi-residue determination of 17 antibiotics in sea cucumbers (*Holothuria tubulosa)* by ultrahigh performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

Antibiotics are a class of pharmaceuticals routinely prescribed to prevent and treat infections caused by pathogenic bacteria in humans and animals. Their widespread consumption results in a gradual contamination of the aquatic environment, which is of particular concern due to the phenomenon of resistance to antibiotics. Quantitative analysis of antibiotics in marine ecosystems is critical to assess the potential risk of exposure of aquatic species and, consequently, of consumers of seafood. In this context, filter feeders, such as holothurians, are considered ideal bioindicators of pollution in marine waters. In this work, an ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method was developed and validated for the simultaneous determination of 17 multiclass antibiotic residues in *Holothuria tubulosa* specimens. The proposed methodology involved two steps of ultrasound-assisted extraction (UAE) with methanol, followed by a clean-up based on dispersive solid phase extraction (d-SPE) using C18 adsorbent. 3^2 factorial designs were used for the optimization of the most influential variables of the extraction procedure. Linearity of 84.1–99.4 %, precision in terms of RSD lower than 15 %, recoveries between 87.9 and 114.9 %, and detection limits between 0.3 and 3.0 ng g^{-1} were obtained. After validation, the method was used to analyze specimens of *Holothuria tubulosa* collected along the Mediterranean coast of Granada. The obtained results indicate the presence of antibiotics in the area, CIP being the most prevalent.

1. Introduction

Antibiotics are antimicrobial agents used to treat or prevent infections that are caused by pathogenic bacteria in humans and animals, inhibiting the growth and the survival of other microorganisms [1]. The first antibiotic was penicillin, discovered by Alexander Fleming in 1928. Traditionally, only those synthesized by bacteria or fungi were considered antibiotics, but today this term also includes those of semi-synthesis and synthesis [2]. In addition to treating infections, antibiotics are used as preventive medicine and as growth promoters in agriculture and livestock [3]. They are very useful drugs, and mortality was greatly reduced thanks to their discovery, yet widespread use has led to them and their metabolites being present in the environment [4].

Antibiotics can enter the environment by excretion, domestic residues or direct discharges and, despite undergoing wastewater treatment processes at conventional waste water treatments plants (WWTPs), complete elimination is unsuccessful. As a consequence, they are transported through effluents and introduced into the environment, contaminating surface and non-surface waters, soils, sediments and even plants fit for consumption, with potential negative effects of both the environment and human health [5–7]. Although the half-life of antibiotics is not very long, their residues can be considered persistent organic pollutants (POPs) due to their wide use and emissions [1]. The public health risks associated with these antibiotics include allergic reactions,

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[4] K.I. Ekpeghere, J.-W. Lee, H.-Y. Kim, S.-K. Shin, J.-E. Oh, Determination and characterization of pharmaceuticals in sludge from municipal and livestock wastewater treatment plants, Chemosphere 168 (2017) 1211–1221.
[5] M.A. Bighiu, A.N. Haldén, W. Goedkoop, J. Ottoson, Assessing microbial contamination and antibiotic resistant bacteria using zebra mussels (*Dreissena polymorpha*), Sci. Total Environ. 650 (2019) 2141–2149.
[6] A. Ezzariai, D. Riboul, M.Z. Lacroix, M. Barret, L. El Fels, G. Merlina, A. Bousquet-Melou, D. Patureau, E. Pinelli, M. Hafidi, A pressurized liquid extraction approach followed by standard addition method and UPLC-MS/MS for a fast multiclass determination of antibiotics in a complex matrix, Chemosphere 211 (2018) 893–902.
[7] P. Saxena, I. Hiwrale, S. Das, V. Shukla, L. Tyagi, S. Pal, N. Dafale, R. Dhodapkar, Profiling of emerging contaminants and antibiotic resistance in sewage treatment plants: An Indian perspective, J. Hazard. Mater. 408 (2021) 124877.
[8] S. Decheng, W. Peilong, L. Yang, W. Ruiguo, W. Shulin, X. Zhiming, Z. Su, Simultaneous determination of antibiotics and amantadines in animal-derived feedstuffs by ultraperformance liquid chromatographic-tandem mass spectrometry, J. Chromatogr. B 1095 (2018) 183–190.
[9] D. Magalhães, A. Freitas, A.S. Vila Pouca, J. Barbosa, F. Ramos, The use of ultra-high-pressure-liquid-chromatography tandem time-of-flight mass spectrometry as a confirmatory method in drug residue analysis: Application to the determination of antibiotics in piglet liver, J. Chromatogr. B 1153 (2020) 122264.
[10] P. Chaturvedi, P. Shukla, B.S. Giri, P. Chowdhary, R. Chandra, P. Gupta, A. Pandey, Prevalence and hazardous impact of pharmaceutical and personal care products and antibiotics in environment: A review on emerging contaminants, Environ. Res. 194 (2021), 110664.
[11] J. Li, I.A. Phulpoto, G. Zhang, Z. Yu, Acceleration of emergence of *E. coli* antibiotic resistance in a simulated sublethal concentration of copper and tetracycline co-contaminated environment, AMB Expr. 11 (2021) 14.
[12] EFSA (European Food Safety Authority), Opinion of the scientific panel on contaminants in the food chain on Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts, EFSA J. 653 (2008) 1-131.
[13] L.M. Chiesa, M. Nobile, F. Ceriani, R. Malandra, F. Arioli, S. Panseri, Risk characterisation from the presence of environmental contaminants and antibiotic residues in wild and farmed salmon from different FAO zones, Food Addit, Contam. Part A Chem. Anal. Control Expo. Risk Assess. 36 (1) (2019) 152–162.
[14] Y. Han, W. Zhou, Y. Tang, W. Shi, Y. Shao, P. Ren, J. Zhang, G. Xiao, H. Sun, G. Liu, Microplastic aggravate the bioaccumulation of three veterinary antibiotics thick shell mussel *Mytilus coruscus* and induce synergistic immunotoxic effects, Sci. Total Environ. 770 (2021), 145273.
[15] B. González-Gaya, L. Cherta, L. Nozal, A. Rico, An optimized sample treatment method for the determination of antibiotics in seawater, marine sediments and biological samples using LC-TOF/MS, Sci. Tot. Environ. 643 (2018) 994–1004.
[16] A. Serra-Compte, D. Álvarez-Muñoz, S. Rodríguez-Mozaz, D. Barceló, Multi-residue method for the determination of antibiotics and some of their metabolites in seafood, Food Chem. Toxicol. 104 (2017) 3–13.
[17] J. Martín, F. Hidalgo, M.T. García-Córcoles, A.J. Ibáñez-Yuste, E. Alonso, J. L. Vílchez, A. Zafra-Gómez, Bioaccumulation of perfluoroalkyl substances in marine echinoderms: Results of laboratory-scale experiments with *Holothuria tubulosa* Gmelin, 1791, Chemosphere 215 (2019) 261–271.
[18] J. Martín, F. Hidalgo, E. Alonso, M.T. García-Córcoles, J.L. Vílchez, A. Zafra-Gómez, Assessing bioaccumulation potential of personal care, household and industrial products in marine echinoderm (*Holothuria tubulosa)*, Sci. Tot. Environ. 720 (2020), 137668.
[19] I.E. Künili, F.A. Çolakoglu, Chemical and nutritional characteristics of *Holothuria tubulosa* (Gmelin, 1788); A seasonally comparative study, J. Aquat. Food Prod. Technol. 28 (7) (2019) 716–728.
[20] J. Martín, A. Zafra-Gómez, F. Hidalgo, A.J. Ibáñez-Yuste, E. Alonso, J.L. Vílchez, Multi-residue analysis of 36 priority and emerging pollutants in marine echinoderms (*Holothuria tubulosa*) and marine sediments by solid-liquid extraction followed by dispersive solid phase extraction and liquid chromatography-tandem mass spectrometry analysis, Talanta 166 (2017) 336–348.
[21] M. Parra-Luna, L. Martín-Pozo, F. Hidalgo, A. Zafra-Gómez, Common sea urchin (*Paracentrotus lividus*) and sea cucumber of the genus *Holothuria* as bioindicators of pollution in the study of chemical contaminants in aquatic media. A revision, Ecol. Indic. 113 (2020), 106185.
[22] ICH Quality Guidelines, Topic Q2 (R1), Validation of analytical procedures: text and methodology (2005). At http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html (accessed on November 2022).
[23] L. Martín-Pozo, M.C. Gómez-Regalado, M.T. García-Córcoles, A. Zafra-Gómez, Removal of quinolone antibiotics from wastewaters and sewage sludge, in: Sarma, H., Domínguez, D.C., Lee, W-Y. (Eds), Emerging Contaminants in the Environment. Challenges and Sustainable Practices. Chapter 16 (2022) 381-406.
[24] T.D.M. Pham, Z.M. Ziora, M.A.T. Blaskovich, Quinolone antibiotics, Med. Chem. Commun. 10 (10) (2019) 1719–1739.
[25] A. Serra-Compte, D. Álvarez-Muñoz, M. Solé, N. Cáceres, D. Barceló, S. Rodríguez-Mozaz, Comprehensive study of sulfamethoxazole effects in merine mussels: Bioconcentration, enzymatic activities and metabolomics, Environ. Res. 173 (2019) 12–22.
[26] A. Baesu, G. Ballash, D. Mollenkopf, T. Wittum, S.M.P. Sullivan, S. Bayen, Suspect screening of pharmaceuticals in fish livers based on QuEChERs extraction coupled with high resolution mass spectrometry, Sci. Tot. Environ. 783 (2021), 146902.
[27] EU, European Union, Regulation (EU) o 1380/2013 of the European Parliament and of the council of 11, on the Common Fisheries Policy, amending Council Regulations (EC) No 2371/2002 and (EC) No 639/2004 and Council Decision 2004/585/EC, Off. J. Eur. Union 354 (2013) (December 2013) 22–61.
[28] E.W.F. Cordeiro, R.M. Hilgert, L.A.D.C. Batista, R.O. Souza, L.F.S.d. Oliveira, M. M. Machado, M.D. Malesuik, F.R. Paula, C.D.O. Stopiglia, C.S. Paim, Validation of an analytical method by high-performance liquid chromatography and microbiological assay, biological safety and in silico toxicity for danofloxacin, Braz. J. Pharm. Sci. 56 (2020).
[29] A.R. Bortolotte, D. Daniel, P. Aparecida de Campos Braga, F.G. Reyes Reyes, A simple and high-throughput method for multiresidue and multiclass quantitation of antimicrobials in pangasius *(Pangasiodon hypophthalamus)* fillet by liquid chromatography coupled with tandem mass spectrometry, J. Chromatogr. B 1124 (2019) 17–25.
[30] M. Andrieu, A. Rico, T.M. Phu, D.T.T. Huong, N.T. Phuong, P.J. Van den Brink, Ecological risk assessment of the antibiotic enrofloxacin applied to *Pangasius* catfish farms on the Mekong Delta, Vietnam, Chemosphere 119 (2015) 407–414.
[31] C. Miossec, T. Mille, L. Lanceleur, M. Monperrus, Simultaneous determination of 42 pharmaceuticals in seafood samples by solvent extraction coupled to liquid chromatography-tandem mass spectrometry, Food Chem. 322 (2020), 126765.
[32] M.E. Dasenaki, A.A. Bletsou, G.A. Koulis, N.S. Thomaidis, Qualitative multiresidue screening method for 143 veterinary drugs and pharmaceuticals in milk and fish tissue using liquid chromatography quadrupole-time-of-flight mass spectrometry, J. Agric. Food Chem. 63 (18) (2015) 4493–4508.
[33] R. Pashaei, R. Dzingeleviciene, S. Abbasi, M. Szultka-Mlynska, B. Buszewski, Determination of 15 human pharmaceutical residues in fish and shrimp tissues by high-performance liquid chromatography-tandem mass spectrometry, Environ. Monit. Assess. 194 (2022) 325.

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Determination of ultraviolet filters in human nails using an acid sample digestion followed by ultra-high performance liquid chromatography–mass spectrometry analysis

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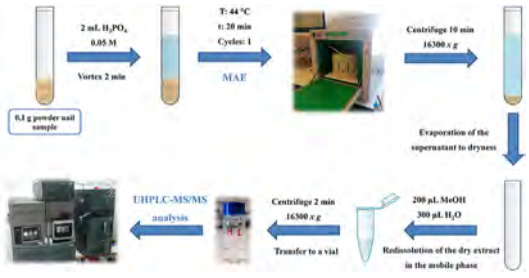
HIGHLIGHTS

- A method for the analysis of UV-filters in nails from human origin is validated.
- An acid digestion of nails followed by UHPLC-MS/MS analysis is proposed.
- Multivariate optimization strategies are used for sample treatment.
- With application to samples, the bioaccumulation of these compounds is demonstrated.

GRAPHICAL ABSTRACT



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ABSTRACT

Ultraviolet filters (UV-filters) are specific chemicals that absorb and reflect UVA and UVB radiation from the sun. They are regularly used in sunscreens and in other personal care products (PCPs), and in products like plastics, adhesives, toys, or furniture finishes. This work develops and validates a new method to determine concentrations of UV-filters (BP-1, BP-2, BP-3, BP-6, BP-8, 4-OH-BP, THB, AVB) in human nail samples. Nails are easily available and are considered to be suitable indicators of cumulative and continued exposure to harmful chemicals. The treatment of nail samples includes microwave assisted digestion/extraction (MAE) in a methanolic solution of o-phosphoric acid (0.05 mol L^{-1}) followed by analyte determination using ultra-high performance liquid chromatography–mass spectrometry (UHPLC–MS/MS) in multiple reaction monitoring mode. The analytes were separated in less than 10 min. The digestion procedure was optimized using multivariate techniques. Matrix-matched calibration with a pig hoof matrix was used for validating the method. A study of accuracy with spiked blank samples was also conducted. The calculated detection limits varied between 0.2 and 1.5 ng g^{-1}, and quantification limits between 1.0 and 5.0 ng g^{-1}. The trueness of the method was an estimation of the recovery, which was between 90.2% and 112.2%; with an estimated precision (relative standard deviation, % RSD) lower than 12.3% for all UV-filters. Nail samples were obtained from 22 volunteers (male and female). The results showed that BP-1 and BP-3 mainly bioaccumulate in human nails.

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Stepanov, I., 2018. Toenail manganese as a potential biomarker for in utero and early childhood exposure studies. Sci. Rep. 8, 17034.
Collaris, E.J., Frank, J., 2008. Photoallergic contact dermatitis caused by ultraviolet filters in different sunscreens. Int. J. Dermatol. 1, 35–37.
Commission Regulation (EU) 2017/238 of 10 Feb 2017 Amending Annex VI to Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of Cosmetic Products, 2017.
Cunha, S.C., Trabalón, L., Jacobs, S., Castro, M., Fernandez-Tejedor, M., Granby, K., Verbeke, W., Kwadijk, C., Ferrari, F., Robbens, J., Sioen, I., Pocurull, E., Marques, A., Fernandes, J.O., Domingo, J.L., 2018. UV-filters and musk fragances in seafood commercialized in Europe Union: occurrence, risk and exposure assessment. Environ. Res. 161, 399–408.
Daniel, C.R., Piraccini, B.M., Tosti, A., 2004. The nail and hair in forensic science. J. Am. Acad. Dermatol. 50, 258–261.
European Chemicals Agency (Echa), 2020. Benzophenone (online source). https://echa.europa.eu/substance-information/-/substanceinfo/100.003.943.
Ferreira, S.L., Bruns, R.E., Ferreira, H.S., Matos, G.D., David, J.M., Brandão, G.C., da Silva, E.G., Portugal, L.A., dos Reis, P.S., Souza, A.S., dos Santos, W.N., 2007. Box-Behnken design: an alternative for the optimization of analytical methods. Anal. Chim. Acta 597, 179–186.
Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2015. UV-filters bioaccumulation in fish from Iberian river basins. Sci. Total Environ. 518–519, 518–525.
Grashow, R., Zhang, J., Fang, S.C., Weisskopf, M.G., Christiani, D.C., Cavallari, J.M., 2014. Toenail metal concentration as a biomarker of occupational welding fume exposure. J. Occup. Environ. Hyg. 11, 397–405.
Guesmi, A., Ohlund, L., Sleno, L., 2020. In vitro metabolism of sunscreen compounds by liquid chromatography/high resolution tandem mass spectrometry. Rapid Commun. Mass Spectrom. 34, e8679.
Guidance for Industry, 2018. Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Center for Veterinary Medicine (CVM).
Hayashi, T., Okamoto, Y., Ueda, K., Kojima, N., 2006. Formation of estrogenic products from benzophenone after exposure to sunlight. Toxicol. Lett. 167, 1–7.
He, K., 2011. Trace elements in nails as biomarkers in clinical research. Eur. J. Clin. Invest. 41, 98–102.
Hiller, J., Klotz, K., Meyer, S., Uter, W., Hof, K., Greiner, A., Göen, T., Drexler, H., 2019. Systemic availability of lipophilic organic UV-filters through dermal sunscreen exposure. Environ. Int. 132, 105068.
Hu, L., Tian, M., Feng, W., He, H., Wang, Y., Yang, L., 2019. Sensitive detection of benzophenone-type ultraviolet filters in plastic food packaging materials by sheathless capillary electrophoresis-electrospray ionization-tandem mass spectrometry. J. Chromatogr. A 1604, 460469.
Kerdivel, G., Le Guevel, R., Habauzit, D., Brion, F., Ait-Aissa, S., Pakdel, F., 2013. Estrogenic potency of benzophenone UV-filters in breast cancer cells: proliferative and transcriptional activity substantiated by docking analysis. PloS One 8, e60567.
Klopcic, I., Dolenc, M.S., 2017. Endocrine activity of AVB, 2MR, BHA, and their mixtures. Toxicol. Sci. 156, 240–251.
Krause, M., Frederiksen, H., Sundberg, K., Jørgensen, F.S., Jensen, L.N., Nørgaard, P., Jørgensen, C., Ertberg, P., Juul, A., Drzewiecki, K.T., Skakkebaek, N.E., Andersson, A.M., 2018. Presence of benzophenones commonly used as UV-filters and absorbers in paired maternal and fetal samples. Environ. Int. 110, 51–60.
Lebedev, A.T., Bavcon Kralj, M., Polyakova, O.V., Detenchuk, E.A., Pokryshkin, S.A., Trebše, P., 2020. Identification of avobenzone by-products formed by various disinfectants in different types of swimming pool waters. Environ. Int. 137, 105495.
Li, J., Guo, F., Wang, Y., Liu, J., Cai, Z., Zhang, J., Zhao, Y., Wu, Y., 2012. Development of extraction methods for the analysis of perfluorinated compounds in human hair and nail by high performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1219, 54–60.
Liu, W., Xu, L., Li, X., Jin, Y.H., Sasaki, K., Saito, N., Sato, I., Tsuda, S., 2011. Human nails analysis as biomarker of exposure to perfluoroalkyl compounds. Environ. Sci. Technol. 45, 8144–8150.
Liu, L.Y., He, K., Hites, R.A., Salamova, A., 2016. Hair and nails as noninvasive biomarkers of human exposure to brominated and organophosphate flame retardants. Environ. Sci. Technol. 50, 3065–3073.
Martín, J., Zafra-Gómez, A., Hidalgo, F., Ibáñez-Yuste, A.J., Alonso, E., Vilchez, J.L., 2017. Multi-residue analysis of 36 priority and emerging pollutants in marine echinoderms (*Holothuria tubulosa*) and marine sediments by solid-liquid extraction followed by dispersive solid phase extraction and liquid chromatography–tandem mass spectrometry analysis. Talanta 166, 336–348.
Martín-Pozo, L., Cantarero-Malagón, S., Hidalgo, F., Navalón, A., Zafra-Gómez, A., 2020. Determination of endocrine disrupting chemicals in human nails using alkaline digestion prior to ultra-high performance liquid chromatography-tandem mass spectrometry. Talanta 208, 120429.
Official website of the European Union, List of UV-filters allowed in cosmetic products, Annex VI, Last update: 23/03/2020, Available at: https://ec.europa.eu/growth/tools-databases/cosing/pdf/COSING_Annex%20VI_v2.pdf.
Ramos, S., Homem, V., Alves, A., Santos, L., 2016. A review of organic UV-filters in wastewater treatment plants. Environ. Int. 86, 24–44.
Rodríguez-Gómez, R., Zafra-Gómez, A., Camino-Sánchez, F.J., Ballesteros, O., Navalón, A., 2014. Gas chromatography and ultrahigh performance liquid chromatography tandem mass spectrometry methods for the determination of selected endocrine disrupting chemicals in human breast milk after stir-bar sorptive extraction. J. Chromatogr. A 1349, 69–79.
Rodríguez-Gómez, R., Zafra-Gómez, A., Dorival-García, N., Ballesteros, O., Navalón, A., 2015. Determination of benzophenone-UV filters in human milk samples using ultrasound-assisted extraction and clean-up with dispersive sorbents followed by UHPLC-MS/MS analysis. Talanta 134, 657–664.
Schmutzler, C., Bacinski, A., Gotthardt, I., Huhne, K., Ambrugger, P., Klammer, H., Schlecht, C., Hoang-Vu, C., Grüters, A., Wuttke, W., Jarry, H., Köhrle, J., 2007. The ultraviolet filter benzophenone 2 interferes with the thyroid hormone axis in rats and is a potent in vitro inhibitor of human recombinant thyroid peroxidase. Endocrinology 148, 2835–2844.
Shi, Y., Liu, X., Zhang, J., Shao, B., 2013. Analysis of triclosan and triclocarban in human nails using isotopic dilution liquid chromatography–tandem mass spectrometry. J. Chromatogr. B 934, 97–101.
Sukumar, A., 2005. Human nails as a biomarker of element exposure. Rev. Environ. Contam. Toxicol. 185, 141–177.
Suzuki, T., Kitamura, S., Khota, R., Sugihara, K., Fugimoto, N., Ohta, S., 2005. Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens. Toxicol. Appl. Pharmacol. 203, 9–17.
Valle-Sistac, J., Molins-Delgado, D., Díaz, M., Ibañez, L., Barceló, D., Díaz-Cruz, M.S., 2016. Determination of parabens and benzophenone-type UV-filters in human placenta. First description of the existence of benzylparaben and benzophenone-4. Environ. Int. 88, 243–249.
Vela-Soria, F., Jiménez-Díaz, I., Rodríguez-Gómez, R., Zafra-Gómez, A., Ballesteros, O., Navalón, A., Vílchez, J.L., Fernández, M.F., Olea, N., 2011. Determination of benzophenones in human placental tissue samples by liquid chromatography-tandem mass spectrometry. Talanta 85, 1848–1855.
Vela-Soria, F., Ballesteros, O., Zafra-Gómez, A., Ballesteros, L., Navalón, A., 2014a. A new method for the determination of benzophenone-UV-filters in human serum samples by dispersive liquid-liquid microextraction with liquid chromatography-tandem mass spectrometry. Talanta 121, 97–104.
Vela-Soria, F., Ballesteros, O., Zafra-Gómez, A., Ballesteros, L., Navalón, A., 2014b. A multiclass method for the analysis of endocrine disrupting chemicals in human urine samples. Sample treatment by dispersive liquid-liquid microextraction. Talanta 129, 209–218.
Vidal-Liñán, L., Villaverde-de-Sáa, E., Rodil, R., Quintana, J.B., Beiras, R., 2018. Bioaccumulation of UV-filters in *Mytilus galloprovincialis* mussel. Chemosphere 190, 267–271.
Wang, L., Kannan, K., 2013. Characteristic profiles of benzonphenone-3 and its derivatives in urine of children and adults from the United States and China. Environ. Sci. Technol. 47, 12532–12538.
Wang, J., Pan, L., Wu, S., Lu, L., Xu, Y., Zhu, Y., Guo, M., Zhuang, S., 2016. Recent advances on endocrine disrupting effects of UV-filters. Int. J. Environ. Res. Publ. Health 13, 782.
Wang, Y., Zhong, Y., Li, J., Zhang, J., Lyu, B., Zhao, Y., Wu, Y., 2018. Occurrence of perfluoroalkyl substances in matched human serum, urine, hair and nail. J. Environ. Sci. 67, 191–197.
Watanabe, Y., Kojima, H., Takeuchi, S., Uramaru, N., Sanoh, S., Sugihara, K., Kitamura, S., Ohta, S., 2015. Metabolism of UV-filter benzophenone-3 by rat and human liver microsomes and its effect on endocrine-disrupting activity. Toxicol. Appl. Pharmacol. 282, 119–128.
Wu, M.H., Xie, D.G., Xu, G., Sun, R., Xia, X.Y., Liu, W.L., Tang, L., 2017. Benzophenone-type UV-filters in surface waters: an assessment of profiles and ecological risks in Shanghai, China. Ecotoxicol. Environ. Saf. 141, 235–241.
Wu, H., Wu, L.H., Wang, F., Gao, C.J., Chen, D., Guo, Y., 2019. Several environmental endocrine disruptors in beverages from South China: occurrence and human exposure. Environ. Sci. Pollut. Res. Int. 26, 5873–5884.
Yaemsiri, S., Hou, N., Slining, M.M., Ke, K., 2010. Growth rate of human fingernails and toenails in healthy American young adults. J. Eur. Acad. Dermatol. Venereol. 24, 420–423.
Yin, J., Wei, L., Shi, Y., Zhang, J., Wu, Q., Shao, B., 2016. Chinese population exposure to triclosan and triclocarban as measured via human urine and nails. Environ. Geochem. Health 38, 1125–1135.

Talanta

Analytical method for the determination of usually prescribed antibiotics in human nails using UHPLC-MS/MS. Comparison of the efficiency of two extraction techniques

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Manuscript Number:	
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	Lydia Espín-Moreno, PhD Student
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	Alberto Zafra-Gómez, PhD
Abstract:	Antibiotics are a group of drugs used for the treatment of bacterial diseases. They are used in both human and veterinary medicine and, although they are not permitted, they are sometimes used as growth promoters. The present research compares two extraction techniques: ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) in order to evaluate their efficiency in the determination of 17 usually prescribed antibiotics in human nails. The extraction parameters were optimized using multivariate techniques. Once both techniques were compared, MAE was selected as optimal due to its greater experimental practicability together with the better extraction efficiencies it provides. Target analytes were detected and quantified by ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). The run time was 20 minutes. The methodology was then successfully validated, obtaining acceptable analytical parameters according to the guide used. Limits of detection were between 0.3 and 3 ng g-1 and limits of quantification were in the range from 1.0 to 4.0 ng g-1. Recovery percentages ranged from 87.5% to 114.2%, and precision (in terms of standard deviation) was less than 15% in all cases. Finally, the optimized method was applied to nails taken from 10 volunteers and the results revealed the presence of one or more antibiotics in all the samples examined. The most commonly found antibiotic was sulfamethoxazole, followed by danofloxacin and levofloxacin. The results demonstrated, on the one hand, the accumulation of these compounds in the human body and, on the other hand, the suitability of nails as a non-invasive biomarker of exposure.
Suggested Reviewers:	Mateusz Kacper Woźniak, PhD Professor, Gdańsk University of Technology mateusz.wozniak@pg.edu.pl Dr. Woźniak has published in subjects related with the present research. He has worked in the presence of antibiotics in the aquatic environment and their analytical monitoring. He is referenced in the bibliographic list.
	Delphine Cappelle, PhD Professor, University of Antwerp delphine.cappelle@uantwerpen.be Dr. Capelle has published in the subject of this research. The research group has work in the nail analysis for the detection of drugs of abuse and pharmaceuticals. Her research is referenced in the bibliographic list of the manuscript.
	Julia Martín Bueno, PhD Professor, University of Seville jbueno@us.es Prof. Martín has work in the subject of this review. The have publish a lot of research

	about the determination of pharmaceuticals in biological and environmental matrices. She has been referenced in the bibliographic list of the present manuscript.
Opposed Reviewers:	

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Review

Bioaccumulation/bioconcentration of pharmaceutical active compounds in aquatic organisms: Assessment and factors database

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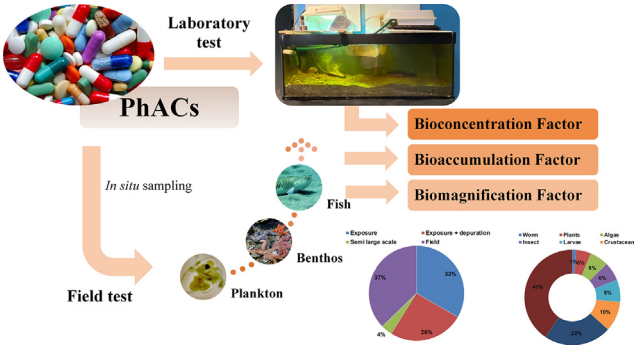
HIGHLIGHTS

- Pharmaceutical active compounds accumulation in aquatic organisms is discussed.
- Need to further standardize BCF testing for key influencing parameters.
- BAFs > BFCs denote the importance of the field study for a reliable assessment.
- BAF data seem organ-, specie- and compound-specific.
- Lower trophic positions bioaccumulate PhACs to a greater extent than higher positions.

GRAPHICAL ABSTRACT



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ABSTRACT

There is increasing evidence that the presence of certain pharmaceuticals in the environment leads to biota exposure and constitute a potential risk for ecosystems. Bioaccumulation is an essential focus of risk assessment to evaluate at what degree emerging contaminants are a hazard both to the environment and the individuals that inhabit it. The main goals of the present review are 1) to summarize and describe the research and factors that should be taken into account in the evaluation of bioaccumulation of pharmaceuticals in aquatic organisms; and 2) to provide a database and a critical review of the bioaccumulation/bioconcentration factors (BAF or BCF) of these compounds in organisms of different trophic levels.

Most studies fall into one of two categories: laboratory-scale absorption and purification tests or field studies and, to a lesser extent, large-scale, semi-natural system tests. Although in the last 5 years there has been considerable progress in this field, especially in species of fish and molluscs, research is still limited on other aquatic species like crustaceans or algae. This revision includes >230 bioconcentration factors (BCF) and >530 bioaccumulation factors (BAF), determined for 113 pharmaceuticals. The most commonly studied is the antidepressant group, followed by diclofenac and carbamazepine. There is currently no reported accumulation data on certain compounds, such as anti-cancer drugs. BCFs are highly influenced by experimental factors (notably the exposure level, time or temperature). Field BAFs are superior to laboratory BCFs, highlighting the importance of field studies for reliable assessments and in true environmental conditions. BAF data appears to be organ, species and compound-specific. The potential impact on food web transfer is also considered. Among different aquatic species, lower trophic levels and benthic organisms exhibit relatively higher uptake of these compounds.

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Reis, E.O., Santos, L.V.S., Lange, L.C., 2021. Prioritization and environmental risk assessment of pharmaceuticals mixtures from Brazilian surface waters. Environ. Pollut. 288, 117803. https://doi.org/10.1016/j.envpol.2021.117803.

Rendal, C., Kusk, K.O., Trapp, S., 2011. The effect of pH on the uptake and toxicity of the bivalent weak base chloroquine tested on Salix viminalis and Daphnia magna. Environ. Toxicol. Chem. 30 (2), 354–359. https://doi.org/10.1002/etc.391.

Rojo, M., Álvarez-Muñoz, D., Domanico, A., Foti, R., Rodriguez-Mozaz, S., Barceló, D., Carriquiriborde, P., 2019. Human pharmaceuticals in three major fish species from the Uruguay River (South America) with different feeding habits. Environ. Pollut. 252, 146–154. https://doi.org/10.1016/j.envpol.2019.05.099.

Rosa, J., Leston, S., Freitas, A., Vila Pouca, A.S., Barbosa, J., Lemos, M.F.L., Pardal, M.A., Ramos, F., 2019. Oxytetracycline accumulation in the macroalgae ulva: potential risks for IMTA systems. Chemosphere 226, 60–66. https://doi.org/10.1016/j.chemosphere.2019.03.112.

Ruan, Y., Lin, H., Zhang, X., Wu, R., Zhang, K., Leung, K.M.Y., Lam, J.C.W., Lam, P.K.S., 2020. Enantiomer-specific bioaccumulation and distribution of chiral pharmaceuticals in a subtropical marine food web. J. Hazard. Mater. 394, 122589. https://doi.org/10.1016/j.jhazmat.2020.122589.

Ruhí, A., Acuña, V., Barceló, D., Huerta, B., Mor, J.R., Rodríguez-Mozaz, S., Sabater, S., 2016. Bioaccumulation and trophic magnification of pharmaceuticals and endocrine disruptors in a Mediterranean river food web. Sci. Total Environ. 540, 250–259. https://doi.org/10.1016/j.scitotenv.2015.06.009.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat. Toxicol. 68 (2), 141–150. https://doi.org/10.1016/j.aquatox.2004.03.014.

Serra-Compte, A., Maulvault, A.L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz, S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (Mytilus galloprovincialis). Environ. Pollut. 236, 824–834. https://doi.org/10.1016/j.envpol.2018.02.018.

Silva, M.G., Esteves, V.I., Meucci, V., Battaglia, F., Soares, A.M.V.M., Pretti, C., Freitas, R., 2022. Metabolic and oxidative status alterations induced in Ruditapes philippinarum exposed chronically to estrogen 17α-ethinylestradiol under a warming scenario. Aquat. Toxicol. 244, 106078. https://doi.org/10.1016/j.aquatox.2022.106078.

Sims, J.L., Burket, S.R., Franco, M.E., Lovin, L.M., Scarlett, K.R., Steenbeek, R., Chambliss, C.K., Ashcroft, C., Luers, M., Lavado, R., Brooks, B.W., 2020. Pharmaceutical uptake kinetics in rainbow trout: in situ bioaccumulation in an effluent -dominated river influenced by snowmelt. Sci. Total Environ. 736, 10. https://doi.org/10.1016/j.scitotenv.2020.139603.

Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., Świeżak, J., 2022. Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms – current state of knowledge. J. Hazard. Mater. 424, 127350. https://doi.org/10.1016/j.jhazmat.2021.127350.

Świacka, K., Michnowska, A., Maculewicz, J., Caban, M., Smolarz, K., 2021a. Toxic effects of NSAIDs in non-target species: a review from the perspective of the aquatic environment. Environ. Pollut. 273, 115891. https://doi.org/10.1016/j.envpol.2020.115891.

Świacka, K., Smolarz, K., Maculewicz, J., Caban, M., 2020. Effects of environmentally relevant concentrations of diclofenac in Mytilus trossulus. Sci. Total Environ. 737, 139797. https://doi.org/10.1016/j.scitotenv.2020.139797.

Świacka, K., Smolarz, K., Maculewicz, J., Michnowska, A., Caban, M., 2021b. Exposure of Mytilus trossulus to diclofenac and 4′-hydroxydiclofenac: uptake, bioconcentration and mass balance for the evaluation of their environmental fate. Sci. Total Environ. 791, 148172. https://doi.org/10.1016/j.scitotenv.2021.148172.

Świacka, K., Szaniawska, A., Caban, M., 2019. Evaluation of bioconcentration and metabolism of diclofenac in mussels Mytilus trossulus - laboratory study. Mar. Pollut. Bull. 141, 249–255. https://doi.org/10.1016/j.marpolbul.2019.02.050.

US-EPA, 2000. Methodology for Deriving Ambient Water Quality Criteria for the Protection of Human Health. US Environmental Protection Agency, Washington, D.C., USA, p. 180.

Valdés, M.E., Amé, M.V., Bistoni, M.A., Wunderlin, D.A., 2014. Occurrence and bioaccumulation of pharmaceuticals in a fish species inhabiting the Suquía River basin (Córdoba, Argentina). Sci. Total Environ. 472, 389–396. https://doi.org/10.1016/j.scitotenv.2013.10.124.

Valenti, T.W., Perez-Hurtado, P., Chambliss, K.C., Brooks, B.W., 2009. Aquatic toxicity of sertraline to Pimephales promelas at environmentally relevant surface water pH. Environ. Toxicol. Chem. 28 (12), 2685–2694. https://doi.org/10.1897/08-546.1.

Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagne, F., Juneau, P., 2010. Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. Chemosphere 80, 1062–1068. https://doi.org/10.1016/j.chemosphere.2010.05.010.

Vystavna, Y., Huneau, F., Grynenko, V., Vergeles, Y., Celle-Jeanton, H., Tapie, N., Budzinski, H., Le Coustumer, P., 2012. Pharmaceuticals in Rivers of two regions with contrasted socio-economic conditions: occurrence, accumulation, and comparison for Ukraine and France. Water Air Soil Pollut. 223, 2111–2124. https://doi.org/10.1007/s11270-011-1008-1.

Wang, F., Wang, B., Qu, H., Zhao, W., Duan, L., Zhang, Y., Zhou, Y., Yu, G., 2020. The influence of nanoplastics on the toxic effects, bioaccumulation, biodegradation and enantioselectivity of ibuprofen in freshwater algae Chlorella pyrenoidosa. Environ. Pollut. 263, 114593. https://doi.org/10.1016/j.envpol.2020.114593.

Wang, Y., Yin, T., Kelly, B.C., Gin, T.Y.-H., 2019. Bioaccumulation behaviour of pharmaceuticals and personal care products in a constructed wetland. Chemosphere 222, 275–285. https://doi.org/10.1016/j.chemosphere.2019.01.116.

Wilkinson, J.L., Hooda, P.S., Swinden, J., Barker, J., Barton, S., 2018. Spatial (bio)accumulation of pharmaceuticals, illicit drugs, plasticisers, perfluorinated compounds and metabolites in river sediment, aquatic plants and benthic organisms. Environ. Pollut. 234, 864–875. https://doi.org/10.1016/j.envpol.2017.11.090.

Xie, H., Hao, H., Xu, N., Liang, X., Gao, D., Xu, Y., Gao, Y., Tao, H., Wong, M., 2019. Pharmaceuticals and personal care products in water, sediments, aquatic organisms, and fish feeds in the Pearl River Delta: occurrence, distribution, potential sources, and health risk assessment. Sci. Total Environ. 659, 230–239. https://doi.org/10.1016/j.scitotenv.2018.12.222.

Xie, Z., Luan, H., Zhang, Y., Wang, M., Cao, D., Yang, J., Tang, J., Fan, S., Wu, X., Hua, R., 2020. Interactive effects of diclofenac and copper on bioconcentration and multiple biomarkers in crucian carp (Carassius auratus). Chemosphere 242, 125141. https://doi.org/10.1016/j.chemosphere.2019.125141.

Xie, Z., Lu, G., Yan, Z., Liu, J., Wang, P., Wang, Y., 2017. Bioaccumulation and trophic transfer of pharmaceuticals in food webs from a large freshwater lake. Environ. Pollut. 222, 356–366. https://doi.org/10.1016/j.envpol.2016.12.026.

Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J. Environ. Manag. 133, 378–387. https://doi.org/10.1016/j.jenvman.2013.12.017.

Zhang, S., Ding, J., Razanajatovo, R.M., Jiang, H., Zou, H., Zhu, W., 2019. Interactive effects of polystyrene microplastics and roxithromycin on bioaccumulation and biochemical status in the freshwater fish red tilapia (Oreochromis niloticus). Sci. Total Environ. 648, 1431–1439. https://doi.org/10.1016/j.scitotenv.2018.08.266.

Zhao, J.-L., Furlong, E.T., Schoenfuss, H.L., Kolpin, D.W., Bird, K.L., Feifarek, D.J., Schwab, E.A., Ying, G.-G., 2017. Uptake and disposition of select pharmaceuticals by bluegill exposed at constant concentrations in a flow-through aquatic exposure system. Environ. Sci. Technol. 51 (8), 4434–4444. https://doi.org/10.1021/acs.est.7b00604.

Zhao, S., Wang, X., Li, Y., Lin, J., 2016. Bioconcentration, metabolism, and biomarker responses in marine medaka (Oryzias melastigma) exposed to sulfamethazine. Aquat. Toxicol. 181, 29–36. https://doi.org/10.1016/j.aquatox.2016.10.026.

Marine Pollution Bulletin

Exposure of non-target marine organisms Holothuria tubulosa, Anemonia sulcata and Actinia equina to the antidepressant venlafaxine and its main metabolite o-desmethylvenlafaxine

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	Antidepressant drugs; Metabolite; Bioconcentration; Organism-distribution; Marine environment
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Abstract:	The exposure to the antidepressant venlafaxine (VFX) and its major metabolite o-desmethylvenlafaxine (O-VFX) in common marine organisms including Holothuria tubulosa, Anemonia sulcata and Actinia equina is evaluated. The methodology involves exposure for 28 days (10 µg/L day) followed by a 52-day depuration period. The accumulation shows a first-order kinetic process and reached a mean tissue concentration of 49125/54342 ng/g dw in H. tubulosa and 64810/93007 ng/g dw in A. sulcata. Studied compounds are considered cumulative (BCFVFX = 2947, 4728 and 2080 L/kg dw in H. tubulosa, A. sulcata and A. equina, and BCFO-VFX = 2392 L/kg dw in A. sulcata). The study revealed differences between tissues in metabolizing abilities in H. tubulosa, this effect increases significantly with time in the digestive tract while it was negligible in the body wall. The results provide a detailed description of VFX and O-VFX accumulation in common and non-target organisms in the marine environment.
Suggested Reviewers:	Vanessa F. Fonseca, PhD Professor, University of Lisbon vffonseca@fc.ul.pt Dr. Fonseca is an important worldwide expert in Ecological Chemistry and water protection. She has work in the study of bioconcentration of pharmaceuticals. She is referenced in the present manuscript.
	David Chelazzi, PhD Professor, University of Florence chelazzi@csgi.unifi.it The research group of Dr. Chelazzi has work with sea anemone as a potential biomonitor for microplastics contamination on the Brazilian Amazon coast. He has been referenced in the present manuscript.

Highlights

- Bioconcentration of VFX and O-VFX in non-target organisms was investigated.
- Tissue-specific concentration of pollutants in *H. tubolosa*: digestive tract > body wall.
- Between-tissues differences in metabolization capacities were found.
- Organism-specific BCF: snakelocks anemone > beadlet anemone > sea cucumber.
- VFX could be classified as accumulative in sea cucumber and anemones.

Science of the Total Environment

Uptake and depuration of three common antibiotics in benthic organisms: sea cucumber (Holothuria tubulosa), snakelocks anemone (Anemonia sulcata) and beadlet anemone (Actinia equina)

--Manuscript Draft--

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Abstract:	Antibiotics are widely used in humans and animals, which has attracted a great deal of attention due to the development of bacterial resistance which is currently a problem of great concern to governments and states, as it is linked to the resurgence of infectious diseases. Understanding bioaccumulation of antibiotics in aquatic organisms is a key for understanding their risk assessment. The present study was designed to study the bioaccumulation of target antibiotics in relevant organisms that inhabit benthic marine environments. The uptake and depuration of ciprofloxacin (CIP), sulfamethoxazole (SMX) and trimethoprim (TMP) in sea cucumber (Holothuria tubulosa), snakelocks anemone (Anemonia sulcata) and beadlet anemone (Actinia equina) under controlled laboratory conditions, was investigated. The results showed that antibiotics had a particular tendency over time during the whole uptake and depuration periods. The tissue distribution of antibiotics in sea cucumber was strongly influenced by the structure of the compounds, while CIP was highly concentrated in the body wall; TMP was highly concentrated in the digestive tract. Two different approaches were used to estimate the biocencentration factors (BCF) in the different animal models based on toxicokinetic data and the the concentrations measured at the steady-state. The ranges of BCF were 456-2731 L/kg, 6-511 L/kg and 9-100 L/kg for TMP, CIP and SMX, respectively. The estimated BCF values obtained classify TMP as cumulative in A. equina and H. tubulosa emphasizing the potential bioconcentration in these marine organisms. A lack of correlation was observed between BCFs of target antibiotics and Kow. The animal-specific BCF followed the order of beadlet anemone > sea cucumber > snakelocks anemone.
Suggested Reviewers:	Phong K. Thai, PhD Professor, Queensland University of Technology phong.thai@qut.edu.au The group of Dr. Thai works in the field of antibiotics in the aquatic environment, sources, concentrations, risk and control strategy. They have been cited in the reference list.
	Giuseppe Bonanno, PhD Professor, University of Catania bonanno.giuseppe@unict.it The group of Dr. Bonano has investigated the use of marine species as bioindicators of pollution as potential biomonitors. They have been cited in the reference list.

	Alexandra Cravo, PhD Professor, University of Algarve acravo@ualg.pt The group of Dr. Cravo has work in the bioaccumulation of pharmaceutical active compounds exposed to wastewaters discharges. They have been cited in the reference list.
	Abbas Esmaili-Sari, PhD Professor, Tarbiat Modares University esmaili@modares.ac.ir The group of Dr. Esmaili has work in the bioaccumulation capacity of different species. The have also worked in the bio- and health risk assessment approach. They have been cited in the reference list.
	Sukdeb Pal, PhD Researcher, CSIR-National Environmental Engineering Research Institute s_pal@neeri.res.in The group of Dr. Pal has work in the bioaccumulation field and in the study of the presence of emerging contaminants including antibiotics in environmental compartments. They have been cited in the reference list.
	Jakub Maculewicz, PhD Professor, Medical University of Gdansk jakub.maculewicz@phdstud.ug.edu.pl The group of Dr. Maculewicz has work in the bioaccumulation field and in the study of the presence of emerging contaminants including antibiotics in environmental compartments. They have been cited in the reference list.
Opposed Reviewers:	

Environmental Toxicology and Pharmacology

Bioconcentration of pharmaceuticals in benthic marine organisms (Holothuria tubulosa, Anemonia sulcata and Actinia equina) exposed to environmental contamination by atenolol and carbamazepine.

--Manuscript Draft--

Manuscript Number:	
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Keywords:	Atenolol; Carbamazepine; Up-take/Depuration; Animal model; Benthic organisms
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	Esteban Alonso
	Alberto Zafra-Gómez, PhD
Abstract:	ABSTRACT The extensive use of pharmaceuticals leads to their ubiquitous occurrence in coastal aquatic environments. Their accumulation in aquatic organisms is an issue that has received increasing attention in recent years since previous attempts to estimate this phenomenon have been ineffective. Invertebrates such as benthic organisms are key in the functioning of marine ecosystems and can be used as sentinel species of water quality status. The aim of the present work is to assess the bioconcentration kinetics of atenolol (ATN) and carbamazepine (CBZ) in three common marine organisms including cotton spinner sea cucumber (Holothuria tubulosa), snakelocks anemone (Anemonia sulcata) and beadlet anemone (Actinia equina) under controlled laboratory conditions. The type of organism, the tissue and structure of the compound have a significant impact upon their toxicokinetic processes. CBZ exhibited higher uptake and excretion rates resulting BCF (41-537 L/kg for CBZ vs 7-50 L/kg for ATN) although both are far below these threshold limits established by the UE. The measured BCF using kinetic data showed some slightly differences with those predicted using the concentrations measured at the steady-state, probably explained because the steady state was not ready reached. The animal-specific BCF generally followed the order of sea cucumber > beadlet anemone > snakelocks anemone for ATN while was the opposite for CBZ. The study also highlighted between-tissues differences in the digestive tract and the body wall of the sea cucumber. The work presented here was the first to model bioconcentration of ATN and CBZ in holothurian and anemone animal models.
Suggested Reviewers:	Christoph Steinbach, PhD Professor, University of South Bohemia in Ceske Budejovice Research Institute of Fish Culture and Hydrobiology steinbach@frov.jcu.cz The research group of Dr. Steinbach has conducted research in the field of bioaccumulation and behaviour of drugs of interest such as atenolol in aquatic environments. They have been cited in the reference list.
	Daniel Albert Wunderlin, PhD Professor, National University of Cordoba dwunder@fcq.unc.edu.ar The research group of Dr. Wunderlin has conducted research in the field the occurrence and bioaccumulation of pharmaceuticals in a fish species. They have been cited in the reference list.
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Opposed Reviewers:	

Anexo II. Otras publicaciones

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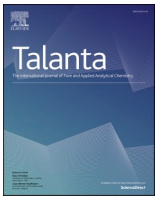

Analytical methods for the determination of endocrine disrupting chemicals in cosmetics and personal care products: A review

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ABSTRACT

Personal care products (PCPs) and cosmetics are indispensable product in our daily routine. Their widespread use makes them a potential route of exposure for certain contaminants to which human would not be normally exposed. One of these contaminants includes endocrine disrupting chemicals, molecules capable of mimicking the body's natural hormones and interfering with the endocrine system. Some of them are ingredients included in the product's formulation, such as UV-filters (sunscreens), phthalates (plasticizers and preservatives), synthetic musks (fragrances), parabens and other antimicrobial agents (antimicrobial preservatives). Others are non-intended added substances that may result from the manufacturing process or migration from the plastic packaging, as with bisphenols and perfluorinated compounds. Some of these endocrine disruptors have been restricted or even banned in cosmetics and PCPs given the high risk they pose to health. Thus, the development of fast, sensitive and precise methods for the identification and quantification of these compounds in cosmetics is a substantial need in order to ensure consumer safety and provide insight into the real risk of human exposure. The present work aims at reviewing the more recently developed analytical methods published in the literature for the determination of endocrine disrupting chemicals in cosmetics and PCPs using chromatographic techniques, with a focus on sample treatment and the quality of analytical parameters.

1. Introduction

Personal care products (PCPs) and cosmetics have become indispensable in our daily routine. Manufactured by the cosmetic industry, they are designed to care for our body health and wellness and are used to cleanse, exfoliate and protect our skin and hair. Soaps, gels, shampoos, makeup, nail polish are merely a few examples of these products. Typically, they are complex mixtures that contain a variety of chemical substances depending on their intended use, such as preservatives, colorants, fixatives, fragrances, UV-filters, solvents, and more [1].

In general, the chemical compounds authorized in cosmetics are considered safe as they undergo a regulated risk assessment for both hazard identification and exposure [2]. Despite these regulatory actions, some of the approved chemicals are able to interfere with the normal functioning of the endocrine system by mimicking the action of the body's natural hormones. They are commonly known as endocrine disrupting chemicals (EDCs). Exposure to these compounds has been associated with adverse health effects in humans particularly related to reproductive toxicity, such as decreased fertility, malfunction of the sexual organs, breast, endometrial and testicular cancer. Other effects include corticoid and thyroid dysfunction, neurodevelopmental disorders, and more recently increased risk of obesity (obesogens) [3,4].

Most EDCs are intentionally included in the formulations of PCPs like benzophenones in sunscreens or parabens used as preservatives and antimicrobials. However, other EDCs are not allowed but can be detected in cosmetics as a result of degradation of some ingredients, presence of minor impurities, contamination during packaging, or migration from the plastic packaging to the final product, like bisphenols derived from polycarbonates and epoxy resins [1,5]. Additionally, when some of these compounds are not chemically bound to the polymer matrix, they can passively migrate into the PCP over time. In this case they are referred as leachable and extractable when the compound can be extracted under extreme conditions, usually via laboratory

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[154] M. Nyeborg, M. Pissavini, Y. Lemasson, O. Doucet, Validation of HPLC method for simultaneous and quantitative determination of 12 UV-filters in cosmetics, Int. J. Cosmet. Sci. 32 (2010) 47–53.
[155] M.T. García-Córcoles, R. Rodríguez-Gómez, B. de Alarcón-Gómez, M. Çipa, L. Martín-Pozo, J.M. Kauffmann, A. Zafra-Gómez, Chromatographic methods for the determination of emerging contaminants in natural water and wastewater samples: a review, Crit. Rev. Anal. Chem. 49 (2) (2019) 160–186.
[156] G. Álvarez Rivera, M. Bueno, D. Ballesteros-Vivas, J.A. Mendiola, E. Ibañez, Pressurized Liquid Extraction. Liquid-phase Extraction, 2020, pp. 375–398 (Chapter 13).
[157] B. Subedi, L. Aguilar, E.M. Robinson, D. J Hagemann, E. Bjorklund, R.J. Sheesley, S. Usenko, Selective pressurized liquid extraction as a simple-preparation technique for persistent organic pollutants and contaminants of emerging concern, Trends Anal. Chem. 68 (2015) 119–132.
[158] L. Martín-Pozo, B. de Alarcón-Gómez, R. Rodríguez-Gómez, M. T García-Córcoles, M. Çipa, A. Zafra- Gómez, Analytical methods for the determination of emerging contaminants on sewage sludge samples. A review, Talanta. 192 (2019) 508–533.
[159] J. I Cacho, M. Hernández-Córdoba, Técnicas de microextracción: una metodología sencilla para la mejora de prestaciones en el laboratorio químico-analítico de alimentos, CTC Alimentación. 58 (2014) 21–26.
[160] U. Vincent, JCR Guidelines for Selecting And/or Validating Analytical Methods for Cosmetics. Recommending Standardization Steps for Analytical Methods for Cosmetics (EUR 27284), Publications Office of the European Union, 2015, 978-92-79-48534-3. Available on-line at: https://publications.jrc.ec.europa.eu/repository/handle/JRC96082. (Accessed April 2021).
[161] N. Salgueiro-González, S. Castiglioni, E. Zuccato, I. Turnes-Carou, P. López-Mahía, S. Muniategui-Lorenzo, Recent advances in analytical methods for the determination of 4-alkylphenols and bisphenol A in solid environmental matrices: a critical review, Anal. Chim. Acta 1024 (2018) 39–51.
[162] J.C. Hubinger, A survey of phthalate esters in consumer cosmetic products, J. Cosmet. Sci. 61 (2010) 457–465.
[163] Regulation (EC), 2002/657 Commission Decision of 14 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Available online at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02002D0657-20040110&from=EN. (Accessed April 2021).

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RESEARCH PAPER

Improved method for the determination of endocrine-disrupting chemicals in urine of school-age children using microliquid–liquid extraction and UHPLC-MS/MS

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Abstract

The presence of endocrine-disrupting chemicals in our daily life is increasing every day and, by extension, human exposure and the consequences thereof. Among these substances are bisphenols and parabens. Urine is used to analyze the exposure. The determination of 12 bisphenol homologues and 6 parabens is proposed. A procedure based on a method previously developed by our research group in 2014 is improved. The extraction yield is higher, because the new protocol is 5 times more efficient. Also, a comparison between calibration with pure standards and matrix calibration, to calculate the matrix effect, was also made. A high grade of matrix effect for all analytes was observed. In terms of validation, the limits of detection (LOD) were between 0.03 and 0.3 ng mL^{-1} and limits of quantification (LOQ) 0.1 to 1.0 ng mL^{-1}, respectively, and the recovery is higher than 86.4% and lower than 113.6%, with a RSD lower than 13.5% in all cases. A methodology for accurate and sensitive quantification of bisphenol homologues together with parabens in human urine using UHPLC-MS/MS was developed. The method was successfully applied to 30 urine samples from children.

Keywords UHPLC-MS/MS · Dispersive liquid–liquid microextraction · Parabens · Bisphenols · Endocrine-disrupting chemicals · Urine samples

Introduction

Endocrine-disrupting chemicals (EDCs) are compounds that exert actions mainly through nuclear hormone receptors and cause adverse effects on the health of an organism or its progeny [1]. People are constantly exposed to these chemicals in daily life from packaging to fungicides, and they are found abundantly in our environment. Scientific studies have demonstrated that a high exposition to EDCs can alter the hormonal balance and the endocrine system function [2–4].

Bisphenols (BPs) are a family of synthetic compounds with two hydroxyphenyl functional groups. Bisphenol A (BPA) is the most important compound of this family. The use of BPA is well-extended for food packaging, infant bottles, reusable water bottles, or microwaveable products [5]. Several studies have demonstrated that BPA can migrate from plastics to food, being the diet one of the main routes of exposure. Because of the lipophilic behaviour of BPA, it is accumulated in fat tissues, and it causes effects related to alterations in the endocrine system function, alterations in neurodevelopment, and cognitive disorders such as Alzheimer or the development of obesity [6–8]. Although, once in the body, a high percentage of BPA is metabolized by conjugation through glucuronidation and sulfatation, resulting in hydrophilic metabolites that are water-soluble and lack of estrogenic activity, an important amount of the compound is accumulated by the organism as a free form [9, 10]. For those reasons, its use in children's articles was banned by the European Commission in 2011. The European Food Safety Authority (EFSA) recommended 0.04 ng kg^{-1} day^{-1} as tolerable daily intake (TDI) [11], and the European Commission

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23. Moscoso-Ruiz I, Gálvez-Ontiveros Y, Cantarero-Malagón S, Rivas A, Zafra-Gómez A. Optimization of an ultrasound-assisted extraction method for the determination of parabens and bisphenol homologues in human saliva by liquid chromatography-tandem mass spectrometry. Microchem J. 2022;175: 107122. https://doi.org/10.1016/j.microc.2021.107122.
24. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003;75(13):3019–30. https://doi.org/10.1021/ac020361s.
25. ICH Quality Guidelines, Topic Q2 (R1): validation of analytical procedures: text and methodology, 2005. http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html. Accessed on June 2022.
26. Gálvez-Ontiveros Y, Moscoso-Ruiz I, Rodrigo L, Aguilera M, Rivas A, Zafra-Gómez A. Presence of parabens and bisphenols in food commonly consumed in Spain. Foods. 2021;10:92. https://doi.org/10.3390/foods10010092.
27. Yang Y, Guan J, Yin J, Shao B, Li H. Urinary levels of bisphenol analogues in residents living near a manufacturing plant in south China. Chemosphere. 2014;112:481–6. https://doi.org/10.1016/j.chemosphere.2014.05.004.
28. Mustieles V, D'Cruz SC, Couderq S, Rodríguez-Carrillo A, Fini JB, Hofer T, Steffensen IL, Dirven H, Barouki R, Olea N, Fernández MF, David A. Bisphenol A and its analogues: a comprehensive review to identify and prioritize effect biomarkers for human biomonitoring. Environ Int. 2020;144: 105811. https://doi.org/10.1016/j.envint.2020.105811.
29. Li A, Wang F, Tao L, Ma C, Bi L, Song M, Jiang G. Rapid and simultaneous determination of multiple endocrine-disrupting chemicals and their metabolites in human serum and urine samples. Talanta. 2022;248: 123639. https://doi.org/10.1016/j.talanta.2022.123639.
30. Gálvez-Ontiveros Y, Páez S, Monteagudo C, Rivas A. Endocrine disruptors in food: impact on gut microbiota and metabolic diseases. Nutrients. 2020;12(4):1158. https://doi.org/10.3390/nu12041158.
31. Catenza CJ, Farooq A, Shubear NS, Donkor KK. A targeted review on fate, occurrence, risk and health implications of bisphenol analogues. Chemosphere. 2021;268: 129273. https://doi.org/10.1016/j.chemosphere.2020.129273.
32. Santoro A, Chianese R, Troisi J, Richards S, Nori SL, Fasano S, Guida M, Plunk E, Viggiano A, Pierantoni R, Meccariello R. Neuro-toxic and reproductive effects of BPA. Curr Neuropharmacol. 2019;17(12):1109–32. https://doi.org/10.2174/1570159X17666190726112101.
33. Rochester JR, Bolden AL. Bisphenol S and F: a systematic review and comparison of the hormonal activity of bisphenol a substitutes. Environ Health Perspect. 2015;123(7):643–50. https://doi.org/10.1289/ehp.1408989.
34. Wei F, Mortimer M, Cheng H, Sang N, Guo LH. Parabens as chemicals of emerging concern in the environment and humans: a review. Sci Total Environ. 2021;778: 146150. https://doi.org/10.1016/j.scitotenv.2021.146150.
35. Pellicer-Castell E, Belenguer-Sapiña C, Amorós P, Haskouri JE, Herrero-Martínez JM, Mauri-Aucejo AR. A β-cyclodextrin sorbent based on hierarchical mesoporous silica for the determination of endocrine-disrupting chemicals in urine samples. J Chromatogr A. 2022;1671: 463007. https://doi.org/10.1016/j.chroma.2022.463007.
36. Chen HC, Chang JW, Sun YC, Chang WT, Huang PC. Determination of parabens, bisphenol a and its analogues, triclosan, and benzophenone-3 levels in human urine by isotope-dilution-UPLC-MS/MS method followed by supported liquid extraction. Toxics. 2022;10(1):21. https://doi.org/10.3390/toxics10010021.
37. Jorvekar SB, Jala A, Borkar RM. Determination of bisphenols and parabens in cow urine distillate from India: implication of human exposure and risk assessment. Environ Sci Pollut Res Int. 2022. https://doi.org/10.1007/s11356-022-19441-2.
38. Bocato MZ, Cesila CA, Lataro BF, de Oliveira A, Campíglia AD, Barbosa Jr F. A fast-multiclass method for the determination of 21 endocrine disruptors in human urine by using vortex-assisted dispersive liquid-liquid microextraction (VADLLME) and LC-MS/MS. Environ Res. 2020;189: 109883. https://doi.org/10.1016/j.envres.2020.109883.
39. Silveira RS, Rocha BA, Rodrigues JL, Barbosa Jr F. Rapid, sensitive and simultaneous determination of 16 endocrine-disrupting chemicals (parabens, benzophenones, bisphenols, and triclocarban) in human urine based on microextraction by packed sorbent combined with liquid chromatography tandem mass spectrometry (MEPS-LC-MS/MS). Chemosphere. 2020;240: 124951. https://doi.org/10.1016/j.chemosphere.2019.124951.
40. Sanchis Y, Coscollà C, Yusà V. Analysis of four parabens and bisphenols A, F, S in urine, using dilute and shoot and liquid chromatography coupled to mass spectrometry. Talanta. 2019;202:42–50. https://doi.org/10.1016/j.talanta.2019.04.048.
41. Rocha BA, de Oliveira A, Barbosa Jr F. A fast and simple air-assisted liquid-liquid microextraction procedure for the simultaneous determination of bisphenols, parabens, benzophenones, triclosan, and triclocarban in human urine by liquid chromatography-tandem mass spectrometry. Talanta. 2018;183:94–101. https://doi.org/10.1016/j.talanta.2018.02.052.
42. Azzouz A, Rascón AJ, Ballesteros E. Simultaneous determination of parabens, alkylphenols, phenylphenols, bisphenol A and triclosan in human urine, blood and breast milk by continuous solid-phase extraction and gas chromatography-mass spectrometry. J Pharm Biomed Anal. 2016;119:16–26. https://doi.org/10.1016/j.jpba.2015.11.024.
43. Azzouz A, Rascón AJ, Ballesteros E. Determination of free and conjugated forms of endocrine-disrupting chemicals in human biological fluids by GC-MS. Bioanalysis. 2016;8(11):1145–58. https://doi.org/10.4155/bio-2015-0008.
44. Ren L, Fang J, Liu G, Zhang J, Zhu Z, Liu H, Lin K, Zhang H, Lu S. Simultaneous determination of urinary parabens, bisphenol A, triclosan, and 8-hydroxy-2'-deoxyguanosine by liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Anal Bioanal Chem. 2016;408(10):2621–9. https://doi.org/10.1007/s00216-016-9372-8.
45. Moos RK, Angerer J, Wittsiepe J, Wilhelm M, Brüning T, Koch HM. Rapid determination of nine parabens and seven other environmental phenols in urine samples of German children and adults. Int J Hyg Environ Health. 2014;217(8):845–53. https://doi.org/10.1016/j.ijheh.2014.06.003.
46. Vela-Soria F, Ballesteros O, Zafra-Gómez A, Ballesteros L, Navalón A. A multiclass method for the analysis of endocrine disrupting chemicals in human urine samples. Sample treatment by dispersive liquid-liquid microextraction. Talanta. 2014;129:209–18. https://doi.org/10.1016/j.talanta.2014.05.016.
47. Quigley A, Cummins W, Connolly D. Dispersive liquid-liquid microextraction in the analysis of milk and dairy products: a review. J Chem. 2016;4040165.https://doi.org/10.1155/2016/4040165
48. Farajzadeh A, Nemati M, Reza M, Mogaddam A. Air-assisted liquid-liquid microextraction; principles and applications with analytical instruments. Trends Anal Chem. 2020;122: 115734. https://doi.org/10.1016/j.trac.2019.115734.

CHAPTER

Removal of quinolone antibiotics from wastewaters and sewage sludge

16

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16.1 Introduction

Quinolones represent an important group of synthetic antibacterial agents widely prescribed worldwide. They are characterized by having a basic bicyclic ring structure. Most of the quinolones administered clinically are fluoroquinolones yielded by the addition of a fluoride group in the central ring (Kocsis, Domokos, & Szabo, 2016). These compounds are highly effective for the treatment of all types of infectious diseases because of their broad spectrum of activity (Gram-negative and Gram-positive) and their good oral adsorption. Inside bacteria, they act by inhibiting the synthesis of nucleic acids, breaking the DNA, and thereby, disrupting the microbial replication (Pham, Ziora, & Blaskovich, 2019). Therefore they are very popular for pulmonary, urinary, and digestive infections in both humans and animals (Hooper & Wolfson, 1993).

In Europe the average consumption of quinolone antibiotics has hardly changed in the last decade. According to the European Surveillance of Antimicrobial Consumption (ESAC) database, in 2018 the consumption of quinolones was estimated at 1.7 DDD (expressed as a daily dose per 1000 inhabitants), the same as 2008. Half of these countries have reported a marked tendency to decrease that intake, but the rest have maintained or even increased their consumption. Even so they are still clinically valuable and extensively used in clinical medicine (ESAC-Net, 2018).

Faced with this situation, the emergence of bacterial resistance is a very common phenomenon (Koch et al., 2021). The number of bacterial strains resistant to quinolones has progressively grown, and this has serious implications in the clinical setting. This resistance has developed in all species of bacteria that are treated by this type of antibiotics (Correia, Poeta, Hébraud, Capelo, & Igrejas, 2017). In response, efforts have been placed on the introduction of chemical modifications in the quinolone structure in order to generate more powerful compounds, with a broader spectrum of activity, an improved pharmacokinetics, and a lower frequency of inducing resistance. Some modifications investigated have been substitutions in certain parts of the ring, such as a cypropyl group in C1 position, a fluorine atom in C6 position, or a halogen, methoxide, or fused third ring in position 8, among others (Kocsis et al., 2016). This has led to the distinction of quinolones in four generations, based on their spectrum of activity (Pham et al., 2019).

Wastewater and residual solids generated by human population (residential and industrial areas, hospitals, farms, etc.) are the main sources of quinolone contamination. Once administered, the

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Zhang, X., Guo, W., Ngo, H. H., Wen, H., Li, N., & Wu, W. (2016). Performance evaluation of powdered activated carbon for removing 28 types of antibiotics from water. *Journal of Environmental Management*, *172*, 193–200.

Zhao, W., Yu, G., Blaney, L., & Wang, B. (2021). Development of emission factors to estimate discharge of typical pharmaceuticals and personal care products from wastewater treatment plants. *Science of the Total Environment*, *769*, 144556.

Zheng, X., Xu, S., Wang, Y., Sun, X., Gao, Y., & Gao, B. (2018). Enhanced degradation of ciprofloxacin by graphitized mesoporous carbon (GMC)-TiO_2 nanocomposite: Strong synergy of adsorption-photocatalysis and antibiotics degradation mechanism. *Journal of Colloid and Interface Science*, *527*, 202–213.

Zhou, G. J., Ying, G. G., Liu, S., Zhou, L. J., Chen, Z. F., & Peng, F. Q. (2014). Simultaneous removal of inorganic and organic compounds in wastewater by freshwater green micro-algae. *Environmental Science: Processes & Impacts*, *16*(8), 2018–2027.

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Analysis of polycyclic-aromatic hydrocarbons in blood serum samples obtained from oil refinery workers. Determination of occupational exposure

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ARTICLE INFO

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ABSTRACT

The present study was undertaken to evaluate the occupational exposure to PAHs of long-term workers in a specific petroleum refinery in Albania. The main route of exposure to these pollutants is via inhalation within a highly contaminated work environment. Serum samples from workers were collected and analyzed. A cloud-point extraction method was applied to isolate and concentrate PAHs from samples using Triton X-100 as the extraction agent. The phase separation of micellar serum solutions was induced with the addition of sodium chloride. The analyses were performed by gas chromatography/mass spectrometry using a capillary column HP-5MS. The PAHs analyzed were acenaphthene, acenaphthylene, anthracene, benzo[α]anthracene, benzo[α]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]perylen, chrysene, dibenzo[α,h]anthracene, fluorene, indeno [1,2,3-cd]pyrene, naphthalene, phenanthrene and pyrene. Mean concentration of analytes ranged from 4 ng mL^{-1} for fluorene and anthracene to 110 ng mL^{-1} for dibenzo[α,h]anthracene. The compositional profile of the PAHs was primarily 4–6 rings PAHs. Diagnostic isomeric ratios of benzo[α]anthracene/(benzo[α]anthracene + chrysene) and phenanthrene/anthracene suggest that the PAHs in the serum were primarily pyrogenic PAHs. The bioaccumulation trend of the PAHs in the workers proved conditional to their physical location within the workplace.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread contaminants emitted from a variety of sources, prevalent in petroleum oil industries (Chen et al., 2014). PAHs are organic molecules composed of fused aromatic rings containing only carbon and hydrogen. They are lipophilic and therefore have a great capacity to cross cell membranes. Consequently, these compounds easily bioaccumulate in the adipose tissue of humans and animals (Neal et al., 2008). Anthropogenic activity has been considered one of the primary sources of PAHs emitted into the atmosphere, specifically in relation to the incomplete combustion of organic matter like fossil fuels, biofuels and petroleum. As a result, these compounds are commonplace in both workplaces and the environment (Abdel-Shafy and Mansour 2016; Wu et al., 2019).

The general composition of petroleum depends on its geological age and the conditions in which it was formed, greatly affecting the distribution of PAHs in crude oil (Wang and Fingas 2003). The presence of PAHs in petroleum varies from 0.2% to more than 7%, consisting primarily of two/three-ringed PAHs (Stogiannidis and Laane 2015; Zakaria et al., 2002).

The physical-chemical behavior of PAHs is conditioned by their molecular weight. Those with higher molecular mass (HMW-PAHs) tend to be associated with particulate matter of a variety of sizes that enters the body through the respiratory system. The smaller the particles, the deeper they penetrate the organism. Those with lower molecular mass (LMW-PAHs) remain dissolved in the air and are absorbed via the respiratory system (Kamal et al., 2011; Låg et al., 2020). Taking into account that the main routes of exposure to these compounds are diet, dermal contact or inhalation, in the present study the first two could be ruled out due to the protective suits worn by the refinery workers. All workers used protective and adequate gloves and appropriate clothing during the working day, yet masks were generally not employed to

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apr.2022.101589.

References

Abdel-Shafy, H.I., Mansour, M.S.M., 2016. A review on polycyclic aromatic hydrocarbons: source, environmental impact, effect on human health and remediation. Egypt. J. Pet. 25, 107–123.

Adeniji, A., Okoh, O., Okoh, A., 2019. Levels of polycyclic aromatic hydrocarbons in the water and sediment of Buffalo River Estuary, South Africa and their health risk assessment. Arch. Environ. Contam. Toxicol. 76, 657–669.

Aquilina, N.J., Delgado-Saborit, J., Meddings, M.C., Baker, S., Harrison, R.M., Jacob, P., Wilson, M., Yu, L., Duan, M., Benowitz, N.L., 2010. Environmental and biological monitoring of exposures to PAHs and ETS in the general population. Environ. Int. 36, 763–771.

Bauer, A.K., Siegrist, K.J., Wolff, M., Nield, L., Brüning, T., Upham, B.L., Käfferlein, H.U., Plöttner, S., 2022. The carcinogenic properties of overlooked yet prevalent polycyclic aromatic hydrocarbons in human lung epithelial cells. Toxics 10 (16), 28.

Castaño-Vinyals, G., D'Errico, A., Malats, N., Kogevinas, M., 2004. Biomarkers of exposure to polycyclic aromatic hydrocarbons from environmental air pollution. Occup. Environ. Med. 61, e12.

Chen, Y.M., Lin, W.Y., Chan, Ch, 2014. The impact of petrochemical industrialization on life expectancy and per capita income in Taiwan: an 11-year longitudinal study. BMC Publ. Health 14, 8, 247.

Geer Wallace, M.A., Kormos, T.A., Pleil, J.P., 2016. Blood-borne biomarkers and bioindicators for linking exposure to health effects in environmental health science. J. Toxicol. Environ. Health B 19, 380–409.

Kamal, A., Qayyum, M., Cheema, I.U., Rashid, A., 2011. Biological monitoring of blood naphthalene levels as a marker of occupational exposure to PAHs among auto-mechanics and spray painters in Rawalpindi. BMC Publ. Health 11, 10, 467.

Låg, M., Øvrevik, J., Refsnes, M., Holme, J.A., 2020. Potential role of polycyclic aromatic hydrocarbons in air pollution-induced non-malignant respiratory diseases. Respir. Res. 21, 299.

Lorenzi, D., Entwistle, J.A., Cave, M., Dean, J.R., 2011. Determination of polycyclic aromatic hydrocarbons in urban street dust: implications for human health. Chemosphere 83, 970–977.

Nadal, M., Schuhmacher, M., Domingo, J.L., 2004. Levels of PAHs in soil and vegetation samples from Tarragona County, Spain. Environ. Pollut. 132, 1–11.

Neal, M.S., Zhu, J., Foster, W.G., 2008. Quantification of benzo[α]pyrene and other PAHs in the serum and follicular fluid of smokers versus non-smokers. Reprod. Toxicol. 25, 100–106.

Nguyen, T.Ch, Loganathan, P., Nguyen, T.V., Vigneswaran, S., Kandasamy, J., Slee, D., Stevenson, G., Naidu, R., 2014. Polycyclic aromatic hydrocarbons in road-deposited sediments, water sediments, and soils in Sydney, Australia: comparisons of concentration distribution, sources and potential toxicity. Ecotoxicol. Environ. Saf. 104, 339–348.

Pocurull, E., Fontanals, N., Calull, M., Aguilar, C., 2020. Liquid-phase extraction. Chapter 20 - environmental applications. Handbooks in separation science, ed. Elsevier Inc. Amsterdam, The Netherland 591–641, 2020.

Pulkrabova, J., Stupak, M., Svarcova, A., Rossner, P., Rossnerova, A., Ambroz, A., Sram, R., Hajslov, J., 2016. Relationship between atmospheric pollution in the residential area and concentrations of polycyclic aromatic hydrocarbons (PAHs) in human breast milk. Sci. Total Environ. 562, 640–647.

Ramesh, A., Archibong, A.E., Hood, D.B., Guo, Z., Loganathan, B.G., 2011. Global environmental distribution and human health effects of polycyclic aromatic hydrocarbons. In: Loganathan, B.G., Lam, P.K.S. (Eds.), Global Contamination Trends of Persistent Organic Chemicals. CRC Press, Boca Raton, Florida, pp. 95–124 (Chapter 5).

Ramesh, A., Kumar, A., Aramandla, M.P., Nyanda, A.M., 2015. Polycyclic aromatic hydrocarbon residues in serum samples of autopsied individuals from Tennessee. Int. J. Environ. Res. Publ. Health 12, 322–334.

Rengarajan, T., Rajendran, P., Nandakumar, N., Lokeshkumar, B., Rajendran, P., Nishigaki, I., 2015. Exposure to polycyclic aromatic hydrocarbons with special focus on cancer. Asian Pac. J. Trop. Biomed. 5, 182–189.

Singh, V.K., Patel, D.K., Jyoti, Ram, S., Mathur, N., Siddiqui, M.K.J., 2008. Blood levels of polycyclic aromatic hydrocarbons in children and their association with oxidative stress indices: an Indian perspective. Clin. Biochem. 41, 152–161.

Srogi, K., 2007. Monitoring of environmental exposure to polycyclic aromatic hydrocarbons: a review. Environ. Chem. Lett. 5, 169–195.

Stogiannidis, E., Laane, R., 2015. Characterization of polycyclic aromatic hydrocarbons by using their molecular indices: an overview of possibilities. Rev. Environ. Contam. Toxicol. 234, 49–133.

Tiwari, J.N., Chaturvedi, P., Ansari, N.G., Patel, D.K., Jain, S.K., Murthy, R.C., 2011. Assessment of polycyclic aromatic hydrocarbons (PAH) and heavy metals in the vicinity of an oil refinery in India. Soil Sediment Contam. 20, 315–328.

Vane, ChH., Kim, A.W., Beriro, D.J., Cave, M.R., Knights, K., Moss-Hayes, V., Nathanail, P.C., 2014. Polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB) in urban soils of Greater London, UK. Appl. Geochem. 51, 303–314.

Wang, Z., Fingas, M.F., 2003. Development of oil hydrocarbon fingerprinting and identification techniques. Mar. Pollut. Bull. 47, 423–452.

Wu, M.T., Lin, P.C., Pan, C.H., Peng, C.Y., 2019. Risk assessment of personal exposure to polycyclic aromatic hydrocarbons and aldehydes in three commercial cooking workplaces. Sci. Rep. 9, 1661.

Xia, X., Carroll-Haddad, A., Brown, N., Utell, M.J., Mallon, C.T.M., Hopke, P.K., 2016. Polycyclic aromatic hydrocarbons and polychlorinated dibenzo-p-dioxins/ dibenzofurans in microliter samples of human serum as exposure indicators. JOEM (J. Occup. Environ. Med.) 58, 8S.

Yang, B.C., Fang, S.F., Wan, X.J., Luo, Y., Zhou, J.Y., Li, Y., Li, Y.J., Wang, F., Huang, O. P., 2017. Quantification of monohydroxylated polycyclic aromatic hydrocarbons in human urine samples using solid-phase microextraction coupled with glass-capillary nanoelectrospray ionization mass spectrometry. Anal. Chim. Acta 973, 68–74.

Yunker, M.B., Macdonald, R.W., Vingarzan, R., Mitchell, R.H., Goyette, D., Sylvestre, S., 2002. PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Org. Geochem. 33, 489–515.

Zakaria, M.P., Takada, H., Tsutsumi, S., Ohno, K., Yamada, J., Kouno, E., Kumata, H., 2002. Distribution of polycyclic aromatic hydrocarbons (PAHs) in rivers and estuaries in Malaysia: a widespread input of petrogenic PAHs. Environ. Sci. Technol. 1, 1907–1918.

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Genetic variants of antioxidant enzymes and environmental exposures as molecular biomarkers associated with the risk and aggressiveness of bladder cancer

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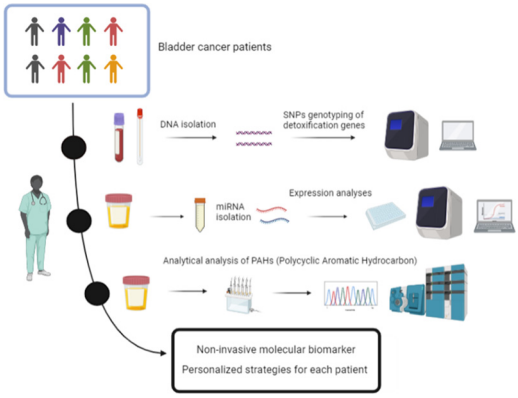
HIGHLIGHTS

- Genetic variants of CYP17A1, CAT and SOD1 were associated with bladder cancer (BC).
- A different set of genetic variants studied were associated with BC aggressiveness.
- Exposure to smoke, dust and alcohol intake were risk factors associated with BC.
- Urine levels of BaP and BPA were associated with bladder cancer.
- Higher expression of miRNA-93-5p, miRNA-193b and miRNA-27a increased BC risk.

GRAPHICAL ABSTRACT



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ABSTRACT

Bladder cancer (BC) is one of the top 10 most common tumours worldwide; however, no molecular markers are currently available for tumour management and follow-up. BC could benefit from molecular biomarkers in environmental disease, which provide mechanistic understanding of individual susceptibility to exposure-related cancers and allow characterizing genetic alterations in the molecular pathway for malignancy. This case-control study performed a molecular analysis in 99 BC and 125 controls. Buccal swabs were collected to assess SNPs in eleven genes coding for xenobiotic detoxification enzymes, cellular antioxidant defences, and hormone synthesis and signalling (*NAT2* (rs1801280), *GPX1* (rs1050450 and rs17650792), *TXNRD1* (rs7310505), *PRDX3* (rs3740562), *PON1* (rs662), *SOD1* (rs10432782), *SOD2* (rs4880), *CAT* (rs1001179), *CYP17A1* (rs743572) and *ESR1* (rs746432)). A structured questionnaire was administered to study participants to assess environmental and dietary chemical exposures.

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Sabo, A.A., Birolo, G., Naccarati, A., Dragomir, M.P., Aneli, S., Allione, A., et al., 2020. Small non-coding RNA profiling in plasma extracellular vesicles of bladder cancer patients by next-generation sequencing: expression levels of miR-126-3p and piR-5936 increase with higher histologic grades. Cancers (Basel) 12, 1507. https://doi.org/10.3390/cancers12061507.

Saginala, K., Barsouk, A., Aluru, J.S., Rawla, P., Padala, S.A., Barsouk, A., 2020. Epidemiology of bladder cancer. Med. Sci. (Basel) 8. https://doi.org/10.3390/medsci8010015.

Saito, Y., Friedman, J.M., Chihara, Y., Egger, G., Chuang, J.C., Liang, G., 2009. Epigenetic therapy upregulates the tumor suppressor microRNA-126 and its host gene EGFL7 in human cancer cells. Biochem. Biophys. Res. Commun. 379, 726–731. https://doi.org/10.1016/j.bbrc.2008.12.098.

Salvi, S., Casadio, V., Conteduca, V., Burgio, S.L., Menna, C., Bianchi, E., et al., 2015. Circulating cell-free AR and CYP17A1 copy number variations may associate with outcome of metastatic castration-resistant prostate cancer patients treated with abiraterone. Br. J. Cancer 112, 1717–1724. https://doi.org/10.1038/bjc.2015.128.

Sarabandi, S., Effatpanah, H., Sereshki, N., Samavarchi Tehrani, S., Moradi-Sardareh, H., 2022. 50-bp insertion/deletion polymorphism of the superoxide dismutase-1 is associated with bladder cancer risk in an Iranian population. Nucleosides Nucleotides Nucleic Acids 41, 154–165. https://doi.org/10.1080/15257770.2021.2014521.

Song, Y., Qi, X., Liu, X., 2020. N-acetyltransferase 2 polymorphism is associated with bladder cancer risk: an updated meta-analysis based on 54 case-control studies. Gene 757, 144924. https://doi.org/10.1016/j.gene.2020.144924.

Strehlow, K., Rotter, S., Wassmann, S., Adam, O., Grohé, C., Laufs, K., Böhm, M., Nickenig, G., 2003. Modulation of antioxidant enzyme expression and function by estrogen. Circ. Res. 93, 170–177. https://doi.org/10.1161/01.RES.0000082334.17947.11.

Svendsen, C., James, A., Matulewicz, R.S., Moreton, E., Sosnowski, R., Sherman, S., et al., 2022. Carcinogenic biomarkers of exposure in the urine of heated tobacco product users associated with bladder cancer: a systematic review. Urol. Oncol. 40, 149–160. https://doi.org/10.1016/j.urolonc.2021.11.018.

Taheri, M., Shirvani-Farsani, Z., Ghafouri-Fard, S., Omrani, M.D., 2020. Expression profile of microRNAs in bladder cancer and their application as biomarkers. Biomed. Pharmacother. 131, 110703. https://doi.org/10.1016/j.biopha.2020.110703.

Tan, W., Li, Z., Xia, W., Zhu, J., Fan, R., 2022. miR-221-3p regulates hepatocellular carcinoma cell proliferation, migration and invasion via targeting LIFR. Ann. Hepatol. 27 (Suppl. 1), 100567. https://doi.org/10.1016/j.aohep.2021.100567.

Teleka, S., Hindy, G., Drake, I., Poveda, A., Melander, O., Liedberg, F., et al., 2020. Blood pressure and bladder cancer risk in men by use of survival analysis and in interaction with NAT2 genotype, and by Mendelian randomization analysis. PLoS One 15, e0241711. https://doi.org/10.1371/journal.pone.0241711.

Tyagi, H., Daulton, E., Bannaga, A.S., Arasaradnam, R.P., Covington, J.A., 2021. Urinary volatiles and chemical characterisation for the non-invasive detection of prostate and bladder cancers. Biosensors (Basel) 11, 437. https://doi.org/10.3390/bios11110437.

Vela-Soria, F., Ballesteros, O., Zafra-Gómez, A., Ballesteros, L., Navalón, A., 2014. UHPLC-MS/MS method for the determination of bisphenol A and its chlorinated derivatives, bisphenol S, parabens, and benzophenones in human urine samples. Anal. Bioanal. Chem. 406, 3773–3785. https://doi.org/10.1007/s00216-014-7785-9.

Verma, N., Pink, M., Boland, S., Rettenmeier, A.W., Schmitz-Spanke, S., 2017. Benzo[a]pyrene-induced metabolic shift from glycolysis to pentose phosphate pathway in the human bladder cancer cell line RT4. Sci. Rep. 7, 1–14. https://doi.org/10.1038/s41598-017-09936-1.

Vicuña, L., Fernandez, M.I., Vial, C., Valdebenito, P., Chaparro, E., Espinoza, K., et al., 2019. Adaptation to extreme environments in an admixed human population from the Atacama Desert. Genome Biol.Evol. 11, 2468–2479. https://doi.org/10.1093/gbe/evz172.

Wang, Z., Zhao, Y., Shen, X., Zhao, Y., Zhang, Z., Yin, H., et al., 2022. Single-cell genomics-based molecular algorithm for early cancer detection. Anal. Chem. 94, 2607–2614. https://doi.org/10.1021/acs.analchem.1c04968 2607-261410.1021/acs.analchem.1c04968.

Wieczorek, E., Jablonowski, Z., Tomasik, B., Gromadzinska, J., Jablonska, E., Konecki, T., et al., 2017. Different gene expression and activity pattern of antioxidant enzymes in bladder cancer. Anticancer Res. 37, 841–848 Doi:21873/anticanres.11387.

Zhai, X., Xu, W., 2018. Long noncoding RNA ATB promotes proliferation, migration, and invasion in bladder cancer by suppressing microRNA-126. Oncol. Res. 26, 1063–1072 Doi:3727/096504018X15152072098476.