Integrated assessment of the presence of emerging compounds and their toxicological effects in estuaries of Biscay

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Tesi hau Eusko Jaurlaritzako doktore berrien prestakuntzarako diru-laguntzari esker egin da. Era berean, Eusko Jaurlaritzak doktore-ikasleei bideratutako mugikortasun bekari esker (Egonlabur) posible izan da Leipzig-eko UfZ ikerketa zentroan (Alemania) 4 hilabeteko egonaldia egitea 2016an, eta bide batez nazioarteko tesiaren aipamena eskuratu ahal izatea. Bestalde, Kultur Paisaien eta Ondarearen UNESCO katedrak emandako mugikortasun-bekari esker beste 2 hilabeteko egonaldia egin ahal izan da Leipzig-en 2017an.

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Esker onak

Azkenean iritsi da tesi honen azken lerroak idazteko unea. Te bero batekin esku artean eta musika “country” amerikarra entzuten (azken boladan nire tesiko soinu-banda bilakatu dena, ezta Eleder?) bizitakoak gogoratzen hasi naiz. 4 urte hauetan denetarik egon da: primerako jendea ezagutu dut, parranda eta bidaiak egon dira, barre asko egin dut, akademikoki asko ikasi dut eta batez ere pertsonalki; baina onartu beharra daukat momentu gogorrak ere egon direla. Argi daukat maratoi hau ez nukeela bukatuko zuen guztion laguntza barik eta horregatik eskerrak eman nahi dizkizuet.

Lehenik eta behin, ama eta aita, Trevi eta Miji, zuei eskerrak eman nahiko nizkizueke. Ez soilik 4 urte hauetan lagundu nauzuen guztiagatik, zuei gehiago zor dizuet. Eskerrik asko edozer gauza lor dezakedala sinestarazteagatik eta beti nire jakin-mina (eta, noski, frustrazioak) bideratzen jakiteagatik. Oraindik ere gogoratzen dut Barandiaranera kremak eta potinjeak egiten joaten ginenean edota etxean, hidrogenozko bateriarekin zebilen urrunetik gidatutako kotxearekin jolasten ibiltzea, adibidez. *Sister!* Zu ere ezin ahaztu, hor egon zara bidelagun: beti prest hegazkin bat hartzeko edo txiste txarren bat kontatzeko, zuk badakizu beti zer esan burua galdu ez dezadan eta ni lasaitzeko ere. Jakiteagatik, lar dakizu niri buruz (eta askotan ez dizkidazu ahazten usten, esate baterako nola ni kimikaria izanik brownie bat egiteko mL eta cL erratu nituenekoa) Gainontzeko senideei ere eskerrak, daudenei eta ez daudenei; nork daki piztutako kandelek eta otoi guztiek nolabaiteko eraginik edukiko izan duten…

Eta nola ez! Eskerrik beroenak Nestorri eta Ailetti, ez dakit zelan eskertu ahalko dizuedan 4 urte hauetan emandako laguntza. Nire “dislexiarekin” guztiz zoratu ez zaituztedan bitartean... Biak ala biak hain desberdinak izanik asko ikasi dut zuengandik. Nestor, beti zaude erronka berrietan sartzeko animatzen (norbere buruan sinetsi behar da), eskerrak behin baino gehiagotan behar genuen bultzada emateko adorea izan duzun edo tesi hau guztiz desberdina izango litzateke. Eskerrik asko ere Santiagon garagardo artean ni lasaitzeagatik. Ailette, gutxitan ezagutu dut torlojuak horrenbeste estutzeko gaitasuna duen norbait, eta zer esan zure energiari buruz, *eres una machine!*

Ta zer esanik ez mintegiko jendeari buruz! Bihotzez esaten dizuet zuei esker plazer bat izan dela lanera etortzea. Mintegian sortutako giroa itzela da benetan! Zenbat barre, juerga, poteo, TXOKOLATE eta palomita-party, talde ekintza (padel surf, indabada, laser-tag... hurrengoa noizko?), bazkari, psikologia taller egin ditugun… Eskerrak eman nahi dizkizuet momenturen batean mintegian batera egon garen orori: Josu, Azibar, Naiara, Arantza, Oscar, Sandra, Oier, Janire, Oihana, Ekhine, eta oraindik ere unibertsitatean zaudetenei: Olivia, Olaia, Josean, Laura, Leire K, Belen, Mire. Animo Denis, Bastian eta Ilaria, testigua zuei pasatzen dizuet, eta Laura zuri betebehar bat: orain zure esku utziko dut egunero Denis apur bat txintxatea. Haizea, adiskide kuttuna! Orain dela hamar urte karrerako lehen egunetik (gurasoen konspirazioarekin barne) nirekin amaiera arte egon zara (erasmusean, masterrean eta orain doktoretzan) ko-zuzendari, psikologo, lagun, eta idazkari lanak egiten. Mintegiaren beste pasilloan, Gasteizen edo Zamudion zaudeten IBeA taldeko kide guztioi ere eskerrik asko momenturen batean eskainitako laguntzagatik edo, besterik gabe, pasilloan irribarre egiteagatik. Maitane eta Olatz, mila eskerrak zuen hurbiltasunagatik. PiE-ko jendeari ere eskar eman anhi dizkiet hainbeste biologoen artean kimikariok eskuzabal hartzeagatik eta Erroman gu hain ondo zaintzeagatik! Laura de Miguel (ez du balio norbaiti testua itzultzeko eskatzea) eta Urtzi Izagirre, ezin zaituztet aipatu barik utzi, plazer bat izan da zuekin erronka berriei aurre egitea.

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Zazpi mendeko gauean gaude.
Gure loreetan sasi,
Irrintzi, oihu, ele ta ulu,
Marmario ta garrasi,
Haize enbata, brisa, galerna,
Gaur garbi, bihar nahasi,
Xake taularen zuri beltzetan
Arrats gozo egun gazi.
Ai itsasorik ez bageneuka
Zeri so negarrez hasi.

Gure zuhaitza landatu dugu
Amildegi muturrean,
Adarrak daude ertzetik haruntz;
Sustraiak, berriz, lurrean,
Bihotz esteak estu helduaz
Esku baten aurrean.
Bertso berriak jartzera noa
Bere indar laburrean
Nola malko bat isuritzen den
Itsasoren aurrean.

Nola haizea gurazalea
Eguzkiaren irteran
Nola kaioak zorabioan
Itsas enbata bezperan
Pentsatzen nago gu ere berdin
Ibiltzen ez ote geran
Susmoa dauka gure patoa
Itsasoa ez ote da
Libre ta zabal dugu aurrean
Baina ezin dugu edan

Heldu herria sustraietatik
Tira eta gora jaso
Jarri Kantauri aurrean eta
Mantendu zutikan gizon
Ispilu hortan ikus gaitezen
Herriz herri auzoz auzo
Sauriak gatzez itxi ditzagun
Malkoak gure zera son
Sano ta libre irla txiki bat
Salbatuko kara kaso

Azken arnasa eman nahi nuke
Eguna hiltzen ari da
Azken arnasa eman nahi nuke
Bertsoak entzuten dira
Azken arnasa eman nahi nuke
Kantari nator herrira
Azken arnasa ematen dugu
Eguzkitik eguzkira
Azken arnasa eman nahi nuke
Itsasoari begira.

(Itsasoari begira, Benito Lertxundi)

Table of contents

Glossary of terms

µ-Extrn	Microextraction

A

A	Chromatographic peak area
AChE	Acetylcholinesterase
ACN	Acetonitrile
ANOVA	Analysis of variance
AP	Aminopropyl
APCI	Atmospheric pressure chemical ionization
ARA-II	Angiotensin II receptor antagonist
ASTM	American Society for Testing and Materials
au	Arbitrary unit

B

BCF	Bioconcentration factor
BHT	Butyl hydroxytoluene
Bi	Bilbao estuary
BPA	Bisphenol-A

C

C_{18}	Octadecylsilyl
CCD	Central Composite Design
CCL	Contaminant Candidate List
CD	Compound Discoverer
CF	Concentration factor
C_{free}	Free-available concentration
CRM	Certified reference material
C_S	Concentration in passive sampler
C_{TWA}	Time-weighted average concentration
C_W	Concentration in water

D

d	Day

D *(continuation)*

ddMS2	Data-dependent MS/MS
DDT	Dichlorodiphenyltrichloroethane
DEET	N,N-diethyl-meta-toluamide
DIA	Desisopropyl-atrazine
diMS2	Data-independent MS/MS
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
DSC	Differential scanning calorimeter
dSPE	Dispersive solid phase extraction
DTG	Derived thermogravimetric

E

E1	Estrone
E2	β-estradiol
EC_i	Median effective concentration
ECs	Emerging contaminants
EDA	Effect-directed analysis
EDCs	Endocrine disruptive compounds
EDTA	Ethylenediaminetetraacetic acid
EE2	17-α-Ethinylestradiol
EEA	European Environment Agency
EG	Ethyleneglycol
EHMC	2-ethylhexyl-4-methoxycinnamate
EQS	Environmental Quality Standards
ERA	Environmental risk assessment
ESI	Electrospray ionization
EtOAc	Ethyl acetate
Extrn	Extraction

F

F	Fraction
FB_{AP}	Aminopropyl column fractionation procedural blank
FB_{C18}	Octadecylsilyl column fractionation procedural blank
FR	Flame retardant
FSW	Filtered sea water

F *(continuation)*

FUSLE	Focused ultrasound solid-liquid extraction
FWHM	Full width at half maximum
Full MS-ddMS2	Full scan – data dependant MS/MS

G

Ga2	Galindo second treatment
Ga3	Galindo third treatment
GC	Gas chromatography
GC-MS	Gas chromatography - mass spectrometry
GES	Good Environmental Status
GPC	Gel permeation chromatography

H

HCD	Higher-energy collisional dissociation
HCH	Hexachlorocyclohexane
HCOOH	Formic acid
HESI	Heated electrospray ionization
HILIC	Hydrophilic interaction liquid chromatography
HOAc	Acetic acid
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry

I

IMHP	2-Isopropyl-6-methyl-4-pyrimidinol
IPE	ion-pair extraction
IT	Index of toxicity

K

K_1	Uptake rate constants
K_2	Dissipation rate constants
KCl	Potassium chloride
K_e	Elimination constant
$K_{e,\ int.\ val}$	Internal validation´s elimination constant
$K_{e,\ cal}$	Calibration experiment´s elimination constant

L

l.o.f	Lack of fit
LC	Liquid chromatography
LC-MS	Liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LC–QqQ–MS/MS	Liquid chromatography - triple quadrupole - tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limits of detection
LOQ	Limits of quantification
LVSPE	Large volume solid phase extraction

M

MAE	Microwave assisted extraction
MAX	Strong anion exchange
Max.	Maximun
MCPA	2-methyl-4-chlorophenoxyacetic acid
MDL	Method detection limit
ME	Matrix effect
MeOH	Methanol
MESCO	Membrane enclosed sorptive coating sampler
Min.	Minimun
MoA	Modes of Action
MS	Mass-spectrometry
MS/MS	Tandem mass-spectrometry
MSFD	Marine Strategy Framework Directive
MSPD	Matrix solid phase dispersion
MTBE	Methyl tert-butyl ether
MQL	Method quantification limit

N

n.a.	not acquired
n.d.	Not detected
n.e.s.	Not enough sensitivity
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCE	Normalized collision energy
N-DBPs	Nitrogen containing disinfection by products

NOEC	No observed effect concentration
NP	Normal phase
NPOC	Non-purgable organic carbon
NTA	Non-targeted analysis

O

OBT	2-hydroxybenzothiazole
ODO	Optical dissolved oxygen
OECD	Organization for Economic Co-operation Development
OPE	Organophosphate esters
ORP	Oxidation-reduction potential
OSPAR	Convention for the Protection of the Marine Environment of the North-east Atlantic

P

PA	Polyamide
PAHs	Polycyclic aromatic hydrocarbons
PC	Principal Component
PCA	Principal Component Analysis
PCP	Personal care product
PDMS	Polydimethylsiloxane
PE	Process efficiency
PEC	Predicted environmental concentration
PES	Polyethersulfone
PFAS	Perfluoroalkyl substance
PFBS	Perfluoro-1-butanesulfonate
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PFOSA	Perfluoroctylsulfonamide
PFP	Pentafluorophenyl
PH	Phenyl hexyl
PiE	Plentzia Marine Station
PISCES	Passive *in situ* concentration extraction sampler
pKa	Acid dissociation constant
Pl	Plentzia estuary
PLE	Pressurized liquid extraction
PNEC	Predicted no-effect concentration

P *(continuation)*

POCIS	Polar Organic Chemical Integrative Samplers
POPs	Persistent organic pollutants
PP	Polypropylene
PPCPs	Pharmaceuticals and personal care products
PRC	Performance Reference Compound
PS	Passive sampling
PTFE	Polytetrafluoroethylene
PVDF	Polydivinylfluoride
PYE	Pyrenyl ethyl

Q

QC	Quality control
QOrbitrap	Quadrupole orbitrap
QqQ	Triple quadrupole
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe

R

r2	Determination coefficient
R_{AP}	Recombined fraction mixture with aminopropyl column
R_{c18}	Recombined fraction mixture with Octadecylsilyl column
REF	Relative enrichment factor
RP	Reverse phase
RQ	Risk Quotient
Rs	Sampling rate
RSD	Relative standard deviation
RT	Room temperature

S

S/N	Signal-to-noise ratio
SBSE	Stir-bar sorptive extraction
sd	Standard deviation
SD	Speedisk
SET	Sea urchin embryo test
SI	Size increase
SLE	Solid-liquid extraction

S *(continuation)*

SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SR	Silicone rubber
SRM	Selected reaction monitoring

T

TCPP	Tris(2-chloroisopropyl)phosphate
TDS	Total dissolved solids
Tg	Glass transition
TGA	Thermogravimetric analysis
TOF	Time-of-flight
TP	Transformation product
t_R	Retention time
TU	Toxic unit
TWA	Time Weight Average

U

UfZ	Helmholtz Centre for Environmental Research
UHPLC	Ultrahigh performance liquid chromatography
Ur	Urdaibai estuary
USB	Ultrasound bath extraction
USEPA	United States Environmental Protection Agency
UV-VIS	Ultraviolet-visible

V

VdW	Van der Waals

W

WFD	Water Framework Directive
WWTP	Wastewater treatment plant

X

X-AW	Weak anion exchange
X-CW	Weak cation exchange

Introduction

The art and science of asking questions

is the source of all knowledge.

Thomas Berger

Pollution has always accompanied human civilizations. However, the concern about the impact of chemicals on human health and the environment has gained an increasing attention in the last decades. In addition to the legacy compounds, the attention of scientific community is focused on the occurrence and effects of compounds that were, until recently, missed, overlooked, ignored or unknown (Daughton, 2004). To many of these compounds we refer as emerging contaminants (ECs). But, *why should be worry about ECs?*

First, the global production of chemicals has risen from 1 million tons per year in the 1930s to over 400 million tons nowadays (Bijlsma and Cohen, 2016). In addition to the increase in the production and use of chemical products, the awareness about the impact of manufactured chemicals on our health and in the environment is, however, rather recent. In fact, the public awareness and the perception about the chemical contaminants have evolved dramatically in the last decades: from the onset of DDT issues to the distribution of microplastics in the ocean (Guillette and Iguchi, 2012).

Certainly, before going on, it is worth clarifying the term *emerging contaminant*. Mostly, it refers to the fact that we know about them thanks to the use of highly sensitive and appropriate instruments and determination methodologies. In this term, however, we also include substances recently introduced into the environment as well as previously known compounds, but whose toxicological effects were previously unknown. Among those contaminants, we can find many different xenobiotics including personal care products (PCPs) (preservatives, sunscreen products, etc.), pharmaceuticals (antibiotics, anti-inflammatory), life style products (food additives, stimulants, detergents, etc.), industrial compounds (perfluoroalkyl substances, PFASs, plasticisers, etc.) and pesticides (Richardson and Ternes, 2018). They are only a few examples from a long list of potential ECs.

As a consequence of the mentioned awareness, different public bodies, such as the European Environment Agency (EEA) and the US Environmental Protection Agency (EPA), have prioritized many contaminants (European Commission, 2013; USEPA, 2015) based on their physico-chemical features (persistence and bioaccumulation) and toxicity. Most of these pollutants are known as *persistent organic pollutants* (POPs) and among them, we can find hydrocarbons, organochlorides,

organic solvents, pesticides, and phthalates, among others. Moreover, in the framework of the Water Framework Directive (WFD) of the European Union (2008/105/EC), environmental quality standard (EQS) values were established for some of the priority contaminants (European Commission, 2008).

Since most of the ECs are labile compounds they cannot be categorized as persistent pollutants, though they are continuously fed into the aquatic environment at low ng-µg/L levels and, thus, they act as if they were persistent and their effects chronic (Daughton and Ternes, 1999). Consequently, the concern about the widespread presence of ECs in the environment and their possible toxic effects is also reflected now in some legislation. For instance, the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) was the first body who formally recognize pharmaceutical contamination, and the compound clotrimazole was included on their priority action list (OSPAR, 2002).

Similarly, the US EPA included 97 contaminants or chemical groups in a Contaminant Candidate List (CCL-4) for future regulatory consideration (USEPA, 2015). This list includes different contaminant classes such as pesticides (e.g. acephate, acrolein, diuron), pesticides by-products (e.g. 3-hydroxycarbofuran, acetochlor ethanesulfonic acid), pharmaceuticals (e.g. erythromycin, nitroglycerin), industrial by-products (e.g. o-toluidine), perfluoroalkyl substances (perfluorooctanoic acid, PFOA and perfluorooctane sulfonic acid, PFOS), plasticisers (e.g. nonylphenols) and hormones (e.g. norethindrone), among many others.

In Europe, the WFD introduced a first dynamic Watch List (WL-1) of ECs (European Commission, 2015), which includes a list of potential water pollutants that Member States should monitor carefully, to determine the risk they pose to the aquatic environment and if the EQS values should be established for them. Five neonicotinoids insecticides, a sunscreen agent (2-ethylhexyl-4-methoxycinnamate, EHMC), three macrolide antibiotics (azithromycin, clarithromycin and erythromycin), one synthetic and two natural hormones (estrone, E1, 17 β-estradiol, E2, and the 17-α-Ethinylestradiol, EE2), a non-steroidal anti-inflammatory drug (diclofenac) and an antioxidant agent (butyl hydroxytoluene, BHT) were identified as candidates under this decision. A second list (WL-2) was also published recently in 2018 with three new substances (metaflumizone, amoxicillin

and ciprofloxacin) (European Commission, 2018).

Nonetheless, the number of listed or regulated compounds is insignificant in comparison with the existing chemicals (currently, more than 100,000 compounds are produced at industrial scale), their metabolites and transformation products. Obviously, one of the consequences of this complex scenario is the difficulties to prioritize the contaminants. The analysis of a tiny fraction of all those compounds and the estimation of the risks associated to them using the procedures included, for instance, in the technical guidance on risk assessment (European Commission, 2003) is unsuitable. In fact, this procedure describes how to prioritize chemicals based on risk ratios, which are estimated comparing the environmental concentrations with the toxicological effects. In addition to this, the estimated risk should include representative test organisms associated to the water type monitored, the different mode of actions (MoA) of the contaminants and the synergies that can occur when mixtures of them are present (Escher and Hermens, 2002; WHO, 2017).

Once the most important contaminants are selected or prioritized, the next task is looking for the sources to implement the best available technologies to minimise their impact. Among the major candidates we can find wastewater treatment plants (WWTPs), domestic wastewaters and hospital discharges. In fact, most of the studies are focused on WWTPs (Tran et al., 2018), and continental and ground water (Gredelj et al., 2018; Manamsa et al., 2016). However, marine environment is often overlooked despite the fact that estuarine and coastal waters are the final destinations (direct spill or inland discharge) of many of these contaminants (Desbiolles et al., 2018). Moreover, the occurrence of ECs in aquatic biota shows even fewer studies, probably due to the greater complexity of the biota matrices and, thus, the lack of suitable analysis protocols (Núñez et al., 2017).

Considering all the viewpoints, there are still many gaps to bridge (Munthe et al., 2017). In fact, the costs of the chemical and biological monitoring required to extend the ecological status to all ECs are unaffordable (Busch et al., 2016). Therefore, there is clearly a need to strengthen the bioanalytical tools to integrate validated bioassays in the regulatory monitoring programs (Di Paolo et al., 2016) and to link the presence of mixtures of chemicals in the environment and the effects observed at different biological levels (molecular, organ, cell,…) (Wernersson et al., 2015).

Finally, the understanding of the fate and effects of emerging contaminants is a complex and challenging issue that requires interdisciplinary approaches and cutting-edge methodologies. In the following sections a more detailed description of some of the hot issues that have been pointed before will be offered from the viewpoint of an analytical chemist.

1.1. Where have all the ECs gone?

A literature search of the term *emerging contaminants* shows a steady increase along the last decade but this probably does not reflect so much the scientific efforts towards ECs but the consequence of a fashionable and trendy use of the term and the topic (Sauvé and Desrosiers, 2014). Most of the literature reports deal with different classes of organic ECs (i.e. pharmaceuticals, UV filters, musks, antimicrobial disinfectants, antioxidants, artificial food additives, PFASs, corrosion inhibitors, flame retardants, plasticiser) that are found in sewage treatment plant effluents, surface and groundwater and biota. There are several works recompiling the ubiquitous occurrence of them in aquatic environments (Desbiolles et al., 2018; Naidu et al., 2016; Richardson and Ternes, 2018; Sousa et al., 2018; Wilkinson et al., 2017).

Nevertheless, it is difficult to establish criteria to compare the occurrence of ECs in water bodies across continents-countries since the mentioned works have analysed or focus on different compounds. In this sense, the work of Loos et al. (Loos et al., 2013a) provides the monitoring of 156 polar organic chemical contaminants in 90 European WWTPs. The highest median concentration levels in the effluents were those of the artificial sweeteners acesulfame (14300 ng/L) and sucralose (2600 ng/L), benzotriazoles (corrosion inhibitors, 2900-6300 ng/L), several organophosphate ester flame retardants (133-2400 ng/L) and plasticisers (e.g. tris(2-chloroisopropyl)phosphate; TCPP, 620 ng/L), pharmaceutical compounds such as carbamazepine (752 ng/L), tramadol (256 ng/L), telmisartan (386 ng/L), venlafaxine (119 ng/L) and irbesartan (480 ng/L), the insect repellent N,N₀-diethyltoluamide (DEET, 678 ng/L), the pesticides 2-methyl-4-chlorophenoxyacetic acid (MCPA, 150 ng/L) and mecoprop (127 ng/L), PFASs (such as PFOS, 62.5 ng/L, and PFOA, 255 ng/L) and caffeine (191 ng/L).

Since direct point sources such as WWTPs are the simplest contributions to evaluate, as recently reviewed by Tran et al. (Tran et al., 2018), a big part of the data available comes from the occurrence in the aquatic phase and there is a lack of information about the distribution of ECs in a variety of solid environmental samples (e.g, biota samples), due to the complexity of these matrices, as mentioned before, (Huerta et al., 2012; Miller et al., 2018; Núñez et al., 2017; Omar et al., 2016).

Finally, it is worth mentioning two important contributions to the monitoring of aquatic systems. One is the growing application of passive sampling (PS) procedures to analyse qualitatively many contaminants and providing the integrative average concentrations of many of them (Aminot et al., 2016; Posada-Ureta et al., 2017; Sultana et al., 2017). The second one is the use of large volume solid phase extraction (LVSPE) devices that allow the on-site catchment of much larger samples (between 25 and 100 L) and the simultaneous extraction on a sorbent phase (Schulze et al., 2017).

1.2. From spot sampling to passive sampling

Sampling is the most crucial step of environmental monitoring programs. Additionally, when highly dynamic media are being monitored (e.g. estuaries, effluents, etc.), the costs and the efforts required to accomplish an efficient spot sampling plan, are very high. In this framework, and especially in the last two decades, PS approaches have been widely applied as feasible alternatives.

PS is usually described as a sampling technique based on the diffusion of an analyte from the sampled medium to a receiving phase with no energy supply other than the difference of the chemical potential (Vrana et al., 2005). The accumulation of analytes in the sampling device is the result of the difference between the chemical potentials of the analytes in both media (i.e., sampled medium and receiving phase). After the accumulation of the target analytes in the receiving phase of a PS device, they are subsequently analysed in order to quantify the compounds found in the sampling medium. From these amounts, the time in which the samplers have been deployed, and the kinetic-thermodynamic features of the sorption (i.e. the sampling rate or Rs), it is possible to estimate the time-weighted average concentration (C_{TWA}) in the sampled medium, as shown in

Figure 1.1.

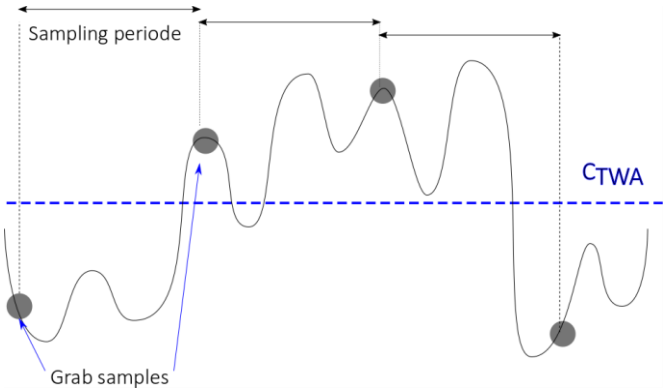

Figure 1.1. *The continuous line shows the dynamic variation of the concentration of a given contaminant, solid spheres represent the active samples and the time-weighted average concentration (C_{TWA}) is the value estimated by PS methods.*

On the contrary, active sampling procedures involve the collection of low volume spot samples (bottle or grab) over a certain period of time (and/or space). This sampling approach is specially challenging when the concentration follows a dynamic pattern in space and in timescale (e.g. groundwaters, tidal effects or the marine coastal currents) or when the contaminants are only present at trace level but still at toxicologically relevant concentrations. In those cases, the C_{TWA} values provided by the passive samplers can offer a more meaningful environmental endpoint than the spot values since the spurious effects of high or low values are limited. In addition to this, it has been emphasized the possibility of obtaining lower detection limits as a consequence of a long accumulation process and the affordability of some passive samplers. Finally, PS offers cost-effective sampling protocols since miniaturize devices with no dependence of power supply are used and long and careless deployments are feasible (Miège et al., 2015).

The backside of PS requires also a deep analysis. First of all, we can mention the real meaning of the fraction that is really being measured (i.e. C_{TWA}) and its relevance, particularly the ranges of fractions going from the free-available concentration (C_{free}) to the total concentration (C_{total}). Moreover, we can also consider the modelling of the fate of microcontaminants and the link of

these values with the accumulation in aquatic organisms, especially when bioavailability and bioaccesibility are discussed (Claessens et al., 2015). Last but not least, we should also recall the low recognition of PS by the regulators (Booij et al., 2016). In the case of the EU WFD it is considered as a complementary tool that needs further research (European Commission, 2013). For instance, there is a lack of compliance to estimate the EQS for the priority contaminants since the standard values refer to total concentrations, and the risk of toxicity for aquatic organisms is based on the bioavailable fraction (Escher and Hermens, 2004).

The overall applicability of passive samplers in environmental monitoring programs can be found in the Norman position paper (Vrana et al., 2009). Broadly speaking, polymer based samplers (i.e. polydimethylsiloxane (PDMS), polyethylene, polyethersulfone (PES), etc.) are most focused on the analysis of hydrophobic compounds. On the contrary, polar organic chemical integrative samplers (POCIS) (Harman et al., 2012), the Chemcatcher (Charriau et al., 2016; Lissalde et al., 2016), the membrane enclosed sorptive coating sampler (MESCO) (Vrana et al., 2001) and the ceramic dosimeter (Martin et al., 2003) are specifically designed to the analysis of more polar compounds.

The passive samplers used for the analysis of non-polar compounds are deeply studied but, passive samplers used to analyse more polar compounds still require a deeper research and the exploration of novel applications. In the case of polar or slightly polar contaminants, POCIS is the most widely used sampler, as shown in **Table 1.1**. It consists on a receiving solid phase (usually HLB) with affinity for a specific group of pollutants and separated from the sample solution by a diffusing-limiting membrane layer (commonly PES membranes). Since their development in the early 2000's (Alvarez et al., 2004), POCIS has been successfully used for the measurement of a wide range of polar to slightly polar compounds (log P 0-4). However, recently, some modifications (see **Table 1.1**) have been proposed to widen the range of micropollutants (e.g, ionic compounds such as herbicides or short chain PFASs) and even non-polar compounds such as PAHs, among others.

Table 1.1. The configuration of POCIS recently proposed and their sampling rates (Rs).

Analyte classes	Matrix	Sorbent	Membrane	Rs range (L/day)	Reference
Pesticides, pharmaceuticals	Water	Oasis HLB	Nylon	0.03 - 3	(Belles et al., 2013)
		Oasis HLB	PES	0.01 - 0.6	
PAHs, PCPs, pesticides	Tap water	Dowex OptiporeL-493	Nylon	0.03 - 2	(Morrison and Belden, 2016)
		Oasis HLB	Nylon	0.06 - 2	
PFASs	Tap water	Strata X-AW	PES	0.2 - 0.4	(Kaserzon et al., 2012)
Pesticides	Tap water	Oasis HLB	PES	0.03 - 0.4	(Fauvelle et al., 2012)
		Oasis MAX		0.02 - 0.3	
		Chromabond HR-Xe		0.004 - 0.1	
	River	Oasis HLB		0.03 - 0.4	
		Oasis MAX		0.05 - 0.3	
		Chromabond HR-Xe		0.004 - 0.1	
		Oasis HLB		0.06 - 0.2	
Alkylphenols, BPA, herbicides, hormones, musks	Tap water	Pest-POCIS and C_{18}	PES	0.04 - 0.2	(Iparraguirre et al., 2017)
	WWTP effluent			0.02 - 0.09	

BPA: bisphenol-A, C_{18}: octadecylsilyl; MAX: strong anion exchange; PAHs: polycyclic aromatic hydrocarbons, PCPs: personal care products, PES: polyethersulfone, POCIS: Polar Organic Chemical Integrative Samplers, Rs: sampling rate, WWTP: wastewater treatment plant, X-AW; weak anion exchange.

1.3. Development of analytical methods

Environmental monitoring of estuaries and/or biota samples has become a challenge owing to the complexity of the matrix (e.g, high physical and chemical dynamism in estuaries and coastal areas, and high lipid and protein contents in marine organisms) as well as to the growing amount of known and unknowns target candidates at trace levels. In this sense, sensitive and robust analytical methods are the key to allow the simultaneous determination of a wide variety of organic micropollutants. To many of these methods we usually called them multiscreening or multiresidue methods (Petrovic, 2014).

In the recent literature there are many works describing deeply the development, optimization and validation workflows of analytical procedures for the analysis of a wide variety of ECs in environmental samples (Huerta et al., 2012; Lorenzo et al., 2018, 2018; Miller et al., 2018;

Núñez et al., 2017; Wilkinson et al., 2017). As a summary, in Figure 1.2 we have included the typical workflow in the development of a multiresidue method considering the protocols recently published in the literature (see Table 1.2).

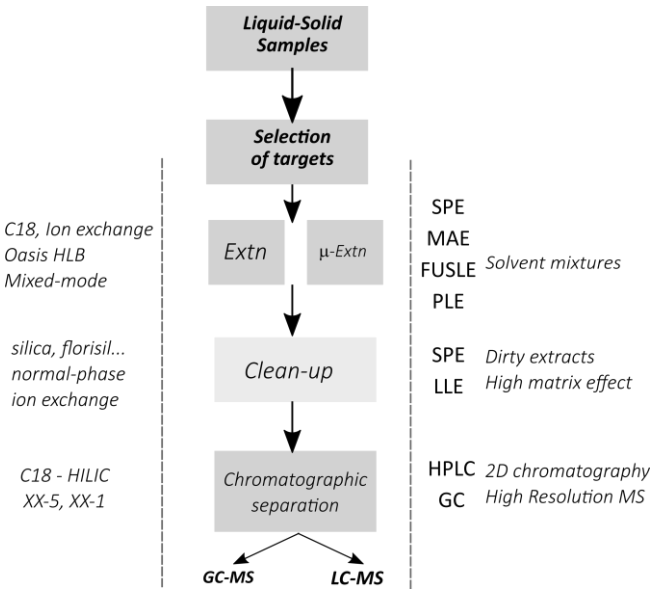

Figure 1.2. *Typical workflow in the development of a multiresidue method. µ-Extn: microextraction, C_{18}: octadecylsilyl, Extn: extraction, FUSLE: focused ultrasound solid-liquid extraction, GC: gas chromatography, GC-MS: gas chromatography – mass spectrometry, HILIC: hydrophilic interaction liquid chromatography, HPLC: high performance liquid chromatography, LC-MS: liquid chromatography – mass spectrometry, LLE: liquid-liquid extraction, MAE: microwave assisted extraction, MS: mass-spectrometry, PLE: pressurised liquid extraction, SPE: solid-phase extraction.*

The first step is the selection of the target analytes. In many cases, though the methods are aimed to covering a wide range of compounds, due to the inherent limitations of the analytical procedures, they are necessarily focused towards a predefined set of contaminant classes. One of the biased consequences of this complexity can be interpreted in terms of the "Matthew effect": we offer a higher prominence to chemicals that have been previously studied instead to those that have been overlooked or omitted due to their low interest in the past (Granjean, 2011; Daughton, 2014).

For instance, as described by Petrovic et al. (Petrovic, 2014), ibuprofen, acetaminophen, diclofenac, sulfamethoxazole, erythromycin, carbamazepine and fluoxetine are the most frequently monitored pharmaceuticals. However, very few multiresidue methods (Bayer et al., 2014; Beckers et al., 2018; Godoy et al., 2015; Grabic et al., 2012) included rarely studied compounds such as telmisartan (an antihypertensive), although it is listed among the top-20 ecotoxicological most relevant compounds, based on their aquatic toxicity (Busch et al., 2016).

Concerning to the extraction step, the purpose is to extract the target compounds in the most efficient way as possible, and minimize the co-extracted matrix components. Since not all the available procedures meet the requirements of the analysis, we have to balance the use of a more efficient solid phase extraction (SPE) protocol, for instance, with a single or a mixture of solid sorbents, or perhaps the use of a microextraction (based on the use of polymeric materials such as PDMS, PES, etc.) procedure. In the case of the SPE solid sorbents, Oasis HLB and Plexa are widely used in the literature (see **Table 1.2**) for low-intermediate polarity compounds (many ECs), and sometimes they are mixed with weak ion exchange sorbents to widen the range of compounds extracted.

The use of a clean-up procedure depends on the matrix effects observed in the chromatographic separation and quantification (typically mass-spectrometer, MS). This is mostly the case of solid samples (e.g. tissues) since the extraction is usually carried out by a solid liquid extraction (SLE) combined with an energy source (focused ultrasound, microwave, pressurised solvents, etc.) and the amount of co-extracted matrix components are too big. Examples of the most commonly used clean-up approaches are also included in **Table 1.2.**

Finally, the last step is the chromatographic separation, mostly GC or LC, coupled to MS (Miller et al., 2018) The use of 2D chromatography has been especially interesting in GC but it is still under development (Dimpe and Nomngongo, 2016). In the case of LCs, the availability of different columns (narrow bore, mixed mode, hydrophilic interaction liquid chromatography, HILIC, etc.) has allowed the development of many specific methods (Pérez-Fernández et al., 2017). Regarding the MS, in most of the cases, the GCs are coupled to a single MS and the LCs to a tandem mass-spectrometry (MS/MS) (Miller et al., 2018; Pérez-Fernández et al., 2017).

Table 1.2. Some examples of multiresidue methods recently published in the literature considering the workflow included in **Figure 1.2.**

Matrix	Classes of ECs	Extraction	Clean-up	Reference
Water (WWTP effluent)	Pharmaceuticals, OPEs, industrial chemicals, pesticides	SPE (Oasis-HLB)	-	(Loos et al., 2013b)
Water (surface water)	Sweeteners, FR, pesticides, industrial chemicals, pharmaceuticals, plasticiser	SPE (Oasis HLB Strata X-AW, Strata X-CW ENV+)	-	(Osorio et al., 2018)
Water (surface water)	Pharmaceutical, PCP, industrial chemical, pesticides, life stimulants products	LVSPE (Chromabond HR-X, X-AW, X-CW)	-	(Schulze et al., 2017)
Water (WWTP effluent)	Pharmaceuticals, illicit drugs	Online SPE (HLB)	-	(López-García et al., 2018)
Water (estuarine, WWTP effluent)	BPA, alkylphenol, hormones	µ-extraction (PES)	-	(Ros et al., 2015)
Water (WWTP influent)	Pharmaceutical and illicit drugs	µ-extraction (Oasis HLB)	-	(Baz-Lomba et al., 2018)
Fish (liver, kidney, brain, muscle, plasma)	Pharmaceuticals	SLE	-	(Grabicova et al., 2018)
Invertebrate	PFASs, estrogens, PCP, UV filter, plasticizers, surfactant, FR and alkylphenols	SLE	dSPE (C18)	(Martín et al., 2017)
Fish (homogenate)	PPCP, drugs of abuse, plastic derivative	USB	SPE (Strata- X)	(Carmona et al., 2017)
Fish (muscle) and invertebrate	PCBs, pesticides, chlorobenzenes, FR, musk, fragrances, antimicrobials	USB	GPC	(Zhang et al., 2015)
Fish (muscle)	Hormones	MAE	SPE (Phree)	(Guedes-Alonso et al., 2017)
Fish (homogenate, liver, muscle)	Pharmaceuticals	PLE	GPC	(Huerta et al., 2013)
Fish (liver, brain, muscle, gills, plasma, bile)	Pharmaceuticals	FUSLE	SPE (Oasis HLB)	(Ziarrusta et al., 2017)
Fish (bile)	Alkylphenols, estrogens, BPA and phthalate	µ-extraction (PES)	-	(Oihana Ros et al., 2015)
Invertebrate	Pesticides, pharmaceuticals, corrosion inhibitor, sweeteners, plasticizer	QuEChERS	LLE (hexane)	(Inostroza et al., 2017)
Invertebrate	FR	MSPD	SPE (NP)	(Villaverde-de-Sáa et al., 2013)

BPA: Bisphenol-A, ECs: emerging compounds, dSPE: dispersive solid phase extraction, FR: flame retardant, FUSLE: focused ultrasound solid-liquid extraction, GPC: gel permeation chromatography, LLE: liquid-liquid extraction, LVSPE: large volume solid phase extraction, MAE: microwave assisted extraction, MSPD: matrix solid phase dispersion, NP: normal phase, OPEs: organophosphate esters, PCP: personal care products, PES: polyethersulfone, PFAS: perfluoroalkyls substance, PLE: pressurized liquid extraction, PPCP: pharmaceutical and personal care products, QuEChERS: quick, easy, cheap, effective, rugged and safe, SLE: solid liquid extraction, SPE: solid phase extraction, USB: ultrasound bath extraction, WWTP: wastewater treatment plant, X-AW: weak anion exchange, X-CW: weak cation exchange.

1.4. From target to non-targeted analysis

The continuous advancement and progress of analytical instrumentation particularly that of mass spectrometry has redesigned the way we think and conceive the chemical analysis. One of the milestones of that progress has been the wide use of high-resolution mass spectrometry (HRMS) coupled to ultra-high performance liquid chromatography (we usually identify this as UHPLC-HRMS). Thanks to these instruments, we can achieve a huge resolution in both the retention time and mass spectra simultaneously, and this offers unsurpassable possibilities to chemical analysis (Hollender et al., 2017). In parallel, the development of algorithms to handle large amount of data and data processing workflows has provided the keys for the paradigm shift in chemical analysis (Blaženović et al., 2018; Gago-Ferrero et al., 2018).

In brief, in the classical approach, i.e. the target analysis, the burden of proof of any analysis is the selection of the target compounds. As mentioned before, this initial selection determines or limits the subsequent developments. On the contrary, when the instrumental setup may eventually provide a full vision of everything that is injected, the burden of proof is shifted to the sample. Under this approach, it is convenient to reduce the analytical procedure to the minimum to avoid any bias. In this way, we can understand that the analysis has become "hypothesis free" and the vision of the analysis can now be the discovery of the compounds that might be in the sample. In fact, we can interrogate the data retrospectively and look for any missed compound (Hollender et al., 2017).

As a consequence of this shift in the analytical paradigm, we now witness the growth of approaches that were hardly imagined before. For instance, it is being very useful to identify the transformation products that take place in the treatment plants or the metabolites that are produced when a drug is taken up by the body. However, the shift from target to non-targeted analysis is not completely ready. In fact, as it is shown in Figure 1.3, the most reliable processing workflow currently applied considers three scenarios: target, suspect and non-targeted screening, according to the prior knowledge we have about the compounds we are looking for (Bletsou et al., 2015). In this sense, when we can foresee the most likely chemical structures that can be produced from a parent compound, and verify any of these structures in our raw data, we would be running

the suspect screening scenario.

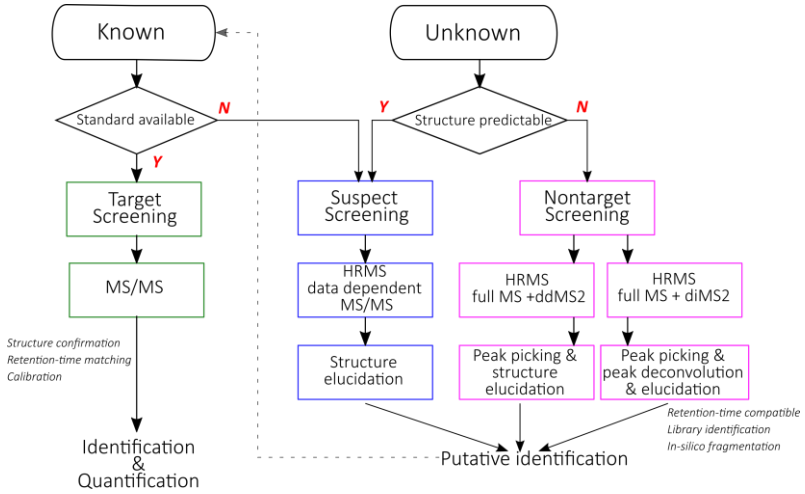

Figure 1.3. *Target, suspect and non-targeted analysis workflow. ddMS2 and diMS2 stand for data-dependent and data-independent MS/MS acquisition modes, respectively (adapted from (Bletsou et al., 2015).*

One important issue is the level of identification experimentally achievable. In **Figure 1.4** we show the combination of the previously described workflow with the confidence levels of the putative identification (Schymanski et al., 2015).

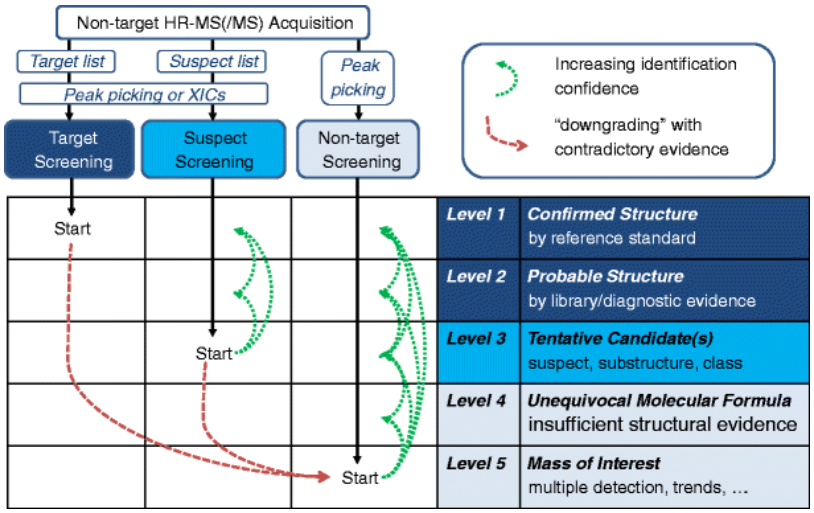

Figure 1.4. *Analytical workflow and confidence levels of compound annotation (taken from Schymanski, 2015).*

Level 1. Full identification. We can confirm the structure of the candidate by MS and MS/MS spectra and retention time matching with a high quality standard. Further evidences would support this identification (i.e. a chromatographic separation with a different column).

Level 2. Probable structure. We can use the unambiguous matching with MS libraries (MS and MS/MS) under comparable experimental conditions (electrospray ionisation, ESI+/- or atmospheric pressure chemical ionisation, APCI) and at different fragmentation energies. We can also annotate an unique structure under this level if we can provide further evidences (experimental data). The lack of a standard hampers the full identification.

Level 3. Tentative identification. We cannot propose an unique structure from this level downwards. At this level, despite we have good MS and MS/MS to annotate a given structure; we cannot differentiate among several equivalent candidates (e.g. isomers).

Level 4. Molecular formula. We can only offer the molecular formula of the compound based on the precise mass spectra and isotopic pattern.

Level 5. Exact mass. We can only provide the mass of the molecule based on the MS and isotopic pattern but more than one feasible formula may fit with that mass.

The instrumental measurement normally starts with the accurate mass from LC-HRMS measurements followed by data-processing to remove noise, blanks, or systematic drifts. Peak picking and peak deconvolution is performed then to extract peaks of all possible compounds. Then the isotopes, adducts, multicharged ions and in-source fragments coming from the same parent compound are grouped. Statistical methods are used then, to evaluate the most relevant features by comparison of different samples and blanks. From the relevant features, the elemental composition is calculated and the most probable molecular formula selected by matching the isotope pattern. For identification, the molecular formulae are searched for in MS/MS databases or libraries (Hollender et al., 2017). The retention time is often used as a further criterion to reduce the number of hits. Some tools predict and evaluate retention-time using log D based models (Aalizadeh et al., 2016).

Finally, identification is achieved when the MS fragmentation and retention time of the unknown compound fit to the library spectrum and the retention time of a reference compound. If no match in an MS/MS database or library is available, searches in large chemical databases are performed. In silico fragmentation has to be used and then the fragments have to be matched against the experimental measured MS fragments (Hug et al., 2014; Krauss et al., 2010; Schymanski et al., 2014).

Concerning to specific tools, a number of open access tools exist for ranking tentative candidates and naming probable structures (e.g., MetFrag [http://c-ruttkies.github.io/MetFrag/] and STOFF-IDENT [https://www.lfu.bayern.de/stoffident/#!home]. These tools, as well as those available from instrument vendors, often rely on large public databases (e.g., ChemSpider [http://www.chemspider.com/] and PubChem [https://pubchem.ncbi.nlm.nih.gov/]) for the initial identification of tentative candidates, and subsequent ranking based on data sources/references. Finally, to enable spectral matching, most tools utilize existing reference spectra, which are available via vendors and open databases (e.g., mzCloud™ [https://www.mzcloud.org/], MassBank [https://massbank.eu/MassBank/], or theoretical spectra, which are generated from fragmentation prediction tools such as MetFrag (Ruttkies et al., 2016).

Nevertheless, among other research needs, further harmonization of data processing is

compulsory. Data exchange among different software platforms, MS instrument suppliers, and open-source MS databases are still the major problems.

1.5. From contaminants to effects (*and vice versa*)

Targeted or non-targeted analysis provides a good insight of the presence of chemical, but they do not include any biological effect data to assess the harm cause by any toxicants.The two main regulations in the EU, i.e. the WFD and the Marine Strategy Framework Directive (MSFD), include the achievement of a Good Environmental Status (GES), in the case of marine environments, and the EQS for fresh, transitional and coastal waters. Both assessments include the implementation of chemical and biological effects monitoring programmes in terms of the concentrations of priority contaminants and the biological effects tools (i.e. ecological indicators, exposition biomarkers and toxicity bioassays) (Lyons et al., 2010).

In this sense, an enduring effort has been made to develop guidelines for ERA. In the case of the EU, and in the framework of the WFD, we can mention Technical Guidance Document on Risk Assessment (European Commission, 2003) that describes important features of the process and discusses theoretical issues, technical matters, and key definitions. In this procedure, the assessment of the ecotoxicological risk is based on the determination of predicted environmental concentrations (PECs) and predicted no-effect concentrations (PNECs). PNEC is the concentration of a substance in any environment below which adverse effects will most likely not occur during long term or short-term exposure. In environmental risk assessment, PNECs are compared to PEC to determine if the risk of a substance is acceptable or not. Consequently, when the PEC/PNEC ratio is lower than 1, the substance is not considered to be of concern but, if the PEC/PNEC ratio is higher than 1, further testing must be carried out to improve the determination of PEC or PNEC with subsequent revision of PEC/PNEC ratio.

PNEC is usually calculated by taking into account the lowest effective concentration obtained among the representative different trophic levels (e.g. algae/bacteria, invertebrate, fish) and divided by an appropriate assessment factor. This approach is based on the concept that ecosystem

sensitivity depends on the most sensitive species and it allows to identify priority substances as recently reported by Busch et al. (Busch et al., 2016) from 1000 chemicals that were determined in different rivers. Nevertheless, this approach has some drawbacks that should be deeply considered, especially the possibility of overlooking toxicology relevant compounds if the focus is just on single chemicals or known mixtures.

In this context, it has been highlighted the lack of direct indicators to assess the biological relevance of chemical monitoring and the need of implementing new effect-directed tools in the current regulations (Di Paolo et al., 2015).

Effect-directed analysis (EDA) (Brack et al., 2016) tries to integrate in the same workflow the toxicity testing and the chemical analysis of a given sample. As it can be seen in **Figure 1.5**, EDA workflow is an iterative procedure that combines orthogonal fractionation schemes to simplify the studied sample with the application of a battery of bioassays, according to the toxicity endpoints, and running non-targeted analysis of the toxic fractions. Once one or more than one fraction is identified as toxic, they can be further fractionated using a separation mode different from the preceding ones.

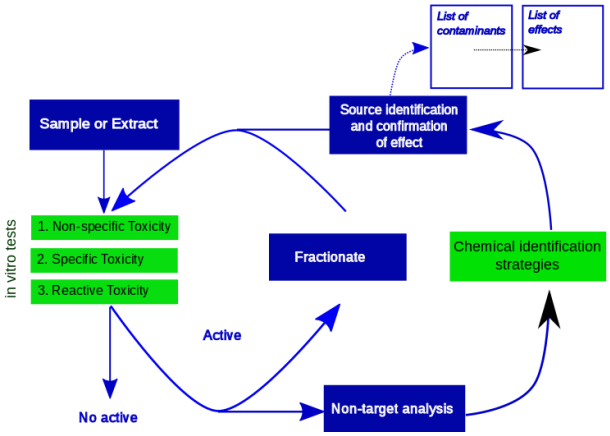

Figure 1.5. *Iterative workflow of the effect-directed analysis procedure (modified from Brack et al., 2016).*

Recently, Brack et al. (Brack et al., 2016) and Simon et al. (Simon et al., 2015) reviewed the most critical aspects of applying the EDA approach in environmental samples. Broadly, the key points of the application of EDA are the selection of the bioassays, the modes of fractionation and the application of non-targeted analysis. The bioassays are basically in-vitro tests to adapt the constraints of the whole workflow to the requirements of each particular assay (high throughput and low volume). There are, however, several in-vivo tests such as *Vibrio fischeri* bacteria (Reineke et al., 2002), fresh water *Pseudokirchneriella subcapitata* algae (Tousova et al., 2018), marine microalgae *Dunaliella tertiolect* (Booij et al., 2014), *Daphnia magna* for aquatic invertebrates (Ouyang et al., 2016) and fish embryo test based on zebrafish, *Danio rerio*, (Di Paolo et al., 2015).

The application of the considered bioassay(s) requires a careful design including the study of the concentration-toxicity response curve, the number of replicates that are required, the volumes that are required to run each test (and therefore the volume of the matrix to extract), the measurement of blanks and positive controls, and the minimization of all losses. In this sense, LVSPE can provide a more logistic alternative than SPE in order to provide the high amount of sample enrichment required in the simultaneous bio-chemical analyses (Busch et al., 2016; Hashmi et al., 2018; König et al., 2017; Neale et al., 2018; Tousova et al., 2018, 2017).

Since the concentration levels are measured in terms of the relative enrichment factor (REF), as a way to estimate the ratio of concentrations of the given sample against the original raw sample, all the sample handling should keep a trace on the concentration/dilution steps. One of the constrains of the pre-concentration and fractionation procedures is the ability to provide a high REF (80-500) value to assure a 100% response (e.g. lethality, inhibition) considering a monotonic response curve. From this curve, it is possible to determine the median effect concentration at a specific level (*i.e.* EC_{10} or EC_{50}) and compare these values with raw samples to show if the concentration in mixtures shows the additive effects.

Table 1.5 shows some meaningful and more recently published EDA studies for water samples. As it can be seen, EDA has been used to identify unexpected causes of biological effect such as mutagenicity in treated drinking water (Vughs et al., 2018) or estrogenic compounds in surface water (Zwart et al., 2018).

Table 1.5. Recently published EDA studies in water samples.

Sample (Volume or exposition time)	Enrichment process	Fractionation (# of fractions)	Endpoint	Identified toxicants	Ref.
Drinking water (15 L)	SPE (OASIS HLB)	C_{18} (250 x 10 mm, 5 µm) (8 F)	Mutagenecity	5 N-DBPs byproducts	Vughs, et al., 2018
River water (850 L)	LVSPE (Chromabond HR-X)	C_{18} (250 x 10 mm, 5 µm) (30 F)	Estrogenic effect Androgenic effect Oxidative stress	E2 Dihydrotestosterone -[a] not observed effects	Hashmi, et al., 2018
Surface water (5 L)	SPE (Chromabond HR-X, X-AW and X-CW and Isolute ENV+)	C_{18} (250 x 10 mm, 5 µm) (24 F) PFP (250 x 10 mm, 5 µm) (24 F) AP (150 x 10 mm, 3 µm) (24 F) PYE (150 x 4.6 mm, 5 µm) (24 F)	Anti-androgenic effect	4-methyl-7-diethylaminocoumarin and one derivate	Muschket et al., 2018
River water and WWTP effluent (4 weeks)	Chemcatcher	C_{18} (250 x 4.6 mm, 5 µm) (40 F)	Estrogenic effect Androgenic Glucocorticoid Dioxin like activity Zebra fish embryo lethal and sublethal effect	E1, EE2 and E2 -[a] Clobetasol propionate and fluticasone propionate -[a] -[a]	(Sonavane et al., 2018)
Surface water (6 weeks)	SD and SR	C_{18} (100 x 2.1 mm, 1.7 µm), (192 F)	Androgen receptor Glucocorticoid Estrogenic effect Antiestrogenic effect	Oxybenzone and piperine	(Zwart et al., 2018)
River water (800 L)	LVSPE (Chromabond HR-X, X-AW and X-CW)	C_{18} (250 x 9.4 mm, 5 µm) (27 F)	Mutagenecity	Comutagenicity of beta carboline alkaloid with aromatic amines	(Muz et al., 2017)
Estuary water (6 weeks)	POCIS (Sepra Zt) SR	C_{18} (100 x 2.1 mm, 1.7 µm) (20 F)	Photosyntesis inhibition	Atrazine, diuron, irgarol, isoproturon, terbutryn and terbutylazine	(Booij et al., 2014)
WWTP effluent (50 L)	LVSPE (Chromabond HR-X, X-AW and X-CW)	LC x LC, C_{18} (150 x 2.1 mm, 1.8 µm) PFP (50 x 4.6 mm, 2.6 µm) (384 F)	AChE inbihition	Tiapride, amisulpride, and lamotrigine	(Ouyang et al., 2016)
WWTP effluent (50 L)	LVSPE (Chromabond HR-X, X-AW and X-CW)	LVSPE (5 F) C_{18}-SPE (9 F) C_{18} (250 x10 mm, 5µm) (30 F)	Photosyntesis inhibition	-[a]	(Tousova et al., 2018)
WWTP effluent (1 L)	SPE (Oasis HLB)	C_{18} (75 x 2.1 mm, 2.5 µm) (25 F)	Estrogenecity effect Androgenecity effect	TCEP -[a]	Itzel et al., 2018)

AChE: acetylcholinesterase, AP: aminopropyl, C_{18}: octadecyl carbon chain, E1: estrone, E2: 17-β-estradiol, EE2: 17-α-ethinylestradiol, F: fractions, LVSPE: large volume solid phase extraction, N-DBPs: nitrogen containing disinfection by products, PFP: pentafluorophenyl; PH: phenyl hexyl, PYE: pyrenyl ethyl, SD: speedisk, SPE, solid phase extraction; SR: silicone rubber, TCEP: phosphorous flame retardant, WWTP: wastewater treatment plant, X-AW: weak anion exchange, X-CW: weak cation exchange.

Reducing the complexity of the extract mixtures by fractionation is one of the key principles in EDA (Brack et al., 2016), as mentioned before. Fractionation of water sample in EDA is predominantly based on reverse phase high performances liquid chromatography (Brack et al., 2016). Other fractionation methods, like lipophilic interaction chromatography, size exclusion, planar chromatography and gas chromatography are also used (Brack et al., 2016). Frequently used fractionation procedures are based on RP C_{18} fractionation (see **Table 1.5**). These types of columns do not only serve the purpose of separating but also they are an important source of information on the compounds eluting in each fraction (i.e C_{18} allows for a separation according to log P or log D in case of ionisable compounds). Sometimes one single fractionation is not sufficient and multistep fractionation procedures are required. They benefit from the combination of chromatographic system with maximum orthogonal selectivity (Brack et al., 2016). For instance, as it can be seen in **Table 1.5**, Muschket et al. (Muschket et al., 2018) applied a parallel fractionation with very different stationary phases covering a wide range of interactions: C_{18}, pentafluorophenyl (PFP), aminopropyl and pyrenyl ethyl columns. Moreover, Ouyang et al. (Ouyang et al., 2016) adopted an unique fractionation step (384 fractions) by two dimensional LC, combining a C_{18} column followed by a PFP.

1.6. References

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Aims and context

Our story may have any number of endings, but its start is a singular choice we make today.

Faisal Khosa

2.1. Aims

In view of the complexity of the occurrence and toxicity of emerging contaminants (ECs) in aquatic environments, we have designed a holistic PhD project to address some of the bioanalytical challenges that were feasible. The main aim was ***to get a close insight about the impact of ECs in estuarine waters and the contribution of wastewater treatment plant (WWTP) effluents in the observed effects***.

Within this preliminary context, the objectives of this PhD work were the following ones:

(i) To develop robust and reliable multiresidue analytical methodologies for the targeted determination of ECs in aqueous samples and in biota samples.

(ii) To extend the use of passive sampling (PS) to determine ECs in estuaries and seawater.

(iii) To monitor the occurrence of ECs in estuaries and WWTP effluents of Biscay using the procedures developed before.

(iv) To implement an effect-directed analysis (EDA) procedure to identify the main toxic compounds in the WWTP effluents using the sea urchin embryo test (SET) as a toxicological *in vivo* bioassay.

2.2. Context

It is worth showing the context of this work to understand the scope of a research group that is aware about the lack of information on the occurrence and the impact ECs in our near aquatic media.

First, the assessment of the ecological quality status of the transitional and coastal waters is a competence of the Basque Government that is currently performed by AZTI-Tecnalia following the criteria enforced by the Water Frame Directive (WFD). The most recent results that are available (AZTI, 2017) include the analysis of polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethane (DDT) and metabolites, hexachlorocyclohexane (HCHs) and some chlorinated pesticides in most of the monitoring stations. As seen, the lack of information regarding the ECs is clear.

When the occurrence of ECs is under discussion the only official reference that is publicly available is a couple of reports performed by Tekniker under the supervision of Ura (Basque Water Agency) dealing with the occurrence of ECs in some WWTP effluents and the impact of hospital effluents in the total sewages treated by the WWTPs (Tekniker, 2011, 2010). In this particular case, it was included the analysis of a wide number of pharmaceutical and personal care products (PCPPs) and industrial compounds (perfluoroalkyl substances, PFASs).

Simultaneously, it is widely reported the environmental stressors that are taking part in most of the estuaries of the Basque Country, from the estuary of Bilbao, one with highest anthropogenic impact of Spain, to the estuary of Urdaibai (a natural biosphere reserve). According to some of them, the prevalence of intersex in fishes is rather widespread and is directly related to the occurrence of endocrine disruptive compounds (EDCs) in the aquatic media (Bizarro et al., 2014; Ortiz-Zarragoitia et al., 2014; Ros et al., 2015). In addition to this, those works pointed to the effluents of WWTP as the most likely source of EDCs but we also lack of a systematic monitoring of these and many other classes of ECs in estuaries and WWTP effluents.

Therefore, the first objective of this PhD work was the development of methods that allowed us the analysis of a set of ECs, first in water and then in fish tissues. We selected a wide range of

emerging compounds, including life style products such as artificial sweeteners (acesulfame, sucralose) and stimulants (caffeine), industrial chemicals such as corrosion inhibitor (2-hydroxybenzothiazole, OBT) and PFASs (perfluoroctylsulfonamide, PFOSA; perfluorooctane sulfonic acid, PFOS; perfluorooctanoic acid, PFOA; perfluoro-1-butanesulfonate, PFBS), pesticides (atrazine, diuron, isoproturon, simazine), phytoestrogens (genistein, genistin, glycitin), hormones (progesterone, testosterone), pharmaceutical (trimethoprim, ciprofloxacin, norfloxacin, sulfadiazine, sulfamethoxazole, amitriptyline, clomipramine, imipramine, nortriptyline, eprosartan, irbesartan, losartan, telmisartan, valsartan, propranolol, acetaminophen, diclofenac, ketoprofen, bezafibrate, clofibric acid, carbamazepine, phenytoin) and personal care products (methylparaben and butylparaben) in order to cope with the analytical requirements. The underlying selection was determined, in a big extent, by the results reported by Tekniker and their ecotoxicological relevance (Petrovic, 2014).

Regarding to the method for biota sample, the needs were not as tight as the ones for waters but the possibility of applying them in a number of circumstances (e.g, fish lab exposure experiments for bioaccumulation and metabolites identification or sentinel biomonitoring) was high enough to be worth the effort. In fact, we were able to apply this method to determine these ECs in mussels in Galicia (results not included in this PhD work).

Since we gained the required expertise to perform PS analysis in estuarine waters, including the calibration of the samplers, we considered that the development of a method based on a POCIS set-up would be a good complement to the analytical method. In this case, we also used polyethersulfone (PES) fibres because they are much cheaper and easier to handle than the POCIS.

Along with the development of these two first objectives, we observed the need to study the stability of the samples and extracts before any monitoring because we could not assure a fast extraction and analysis of a huge volume of samples. That is why we included on the fly this study.

The monitoring of the estuaries and WWTP effluents was a long expected objective, and taking advantage of another longer and more ambitious monitoring of three estuaries of Biscay, we decided to join the efforts and run this for one year.

Finally, we were able to update the instrumental facilities at the Plentzia Marine Station (PiE)

including a high-resolution mass spectrometer. This fact opened the way to run non-targeted analysis and to apply in metabolomic studies. Furthermore, since the marine station is focused on ecotoxicological issues, we were even more ambitious and incorporate certain bioassays. Finally, it was possible to join both, the non-targeted analysis and the bioassays thanks to a stay at Helmholtz Centre for Environmental Research (UfZ, Leipzig) that open the possibility to apply SET in the EDA protocol.

2.3. The works and the main results

The core of this PhD work is arranged in six chapters covering the four objectives mentioned above. The first objective is developed in **chapters 3** and **4**, where the multiresidue methods for water and biota samples are fully described. The development of two PS methods is the main topic included in **chapter 5**, which develops the second objective of this work. In **chapter 6** we have included the study of the stability of target analytes in water samples, different polymeric phases and extracts as a way to apply efficiently the previously developed methods in the monitoring of estuaries and WWTPs. This later work is fully described in **chapter 7**. Finally, the application of the SET to assess the toxicity of several WWTP effluents of Biscay, as well as the application of the EDA protocol using the SET to WWTP of Galindo (Biscay) is described in **chapter 8**.

Following, a brief summary of each of these works is offered.

- *The development of a multiresidue method for the analysis of 41 multi-class emerging contaminants in water.*

In water samplers analysis, a particularly interesting approach is the use of microextraction techniques since they are economically more affordable and cleaner from the environmental point of view. In this sense, a new procedure using PES polymeric material microextraction followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis is shown. Both, the extraction method and the analysis were deeply studied. The optimisation of the analysis included two different chromatographic columns and different variables (polarity, fragmentor voltage, collision energy, and collision cell accelerator) of the mass spectrometer. In the case of PES extraction, ion strength of the water, pH, addition of ethylenediaminetetraacetic acid (EDTA) and

the amount of the polymeric material were thoroughly investigated.

The developed procedure was compared with a previously validated one based on a standard solid-phase extraction (SPE). In contrast to the SPE protocol, PES method allowed a cost-efficient extraction of complex aqueous samples with lower matrix effect from 120 mL of water sample and dual extraction (simultaneously acid and basic pH). Satisfactory and comparable apparent recovery values (80-119% and 70-131%) and method quantification limits (MQL, 0.4-26ng/L and 0.2-23ng/L) were obtained for PES and SPE procedures, respectively, regardless of the matrix. Repeatability values lower than 27% were obtained.

- *The development of a multiresidue method for the analysis of 41 multi-class emerging contaminants in biota*.

Following an approach close to the one used in the previous case, we carried out the full optimisation and validation procedure to analyse of the same set of contaminants in tissues (mussel and fish muscle, liver, gills and brain) and biofluids (fish plasma and bile).

Mussels (*Mytilus galloprovincialis*) and fish were chosen to cover most of the sentinel organism typically analysed in estuarine and coastal environments. In the case of fishes, marine gilt head bream fish were used as they are easily obtained from fish farms, and they were available in lab exposure experiments.

Focused ultrasonic solid-liquid extraction (FUSLE) was chosen due to the high experience of on lab with this technique for the extraction of organic compounds from biota samples (Navarro et al., 2010; Ros et al., 2016; Zabaleta et al., 2014, 2015; Ziarrusta et al., 2016, 2017) and the advantages it provides. As FUSLE is based on the application of ultrasonic radiation using a microtip immersed directly into the sample, it reduce the amount of organic solvent, sample amount and extraction time needed. In this work, the extraction of the solid tissues required a low amount of sample (0.1 or 0.5 g), solvent (7 mL of MeOH: Milli-Q water, 95:5, v:v) and short extraction time (30 s).

Regarding the clean-up step, four alternatives were tested: two protocols validated in the previous work (SPE based on Oasis HLB and microextraction based on PES polymer), a normal SPE

(Florisil phase) and a liquid-liquid extraction (LLE) followed by Oasis-HLB-SPE. The final extracts were analysed by (LC-MS/MS) optimised and validated before.

The methods afforded satisfactory apparent recovery values (71-126%) using isotopically labelled analytes and matrix-matched calibration approach, and repeatability (relative standard deviation, RSD ≤ 22%) regardless of the matrix.

- *The calibration of two new passive samplers for the analysis of ECs in estuarine and coastal waters.*

In order to widen the range of compounds that can be sampled by the commercial polar organic chemical integrative sampler (POCIS), some modifications were included. POCIS containing 100 mg of mixed-mode anion exchanger (Strata X-AW) and 100 mg of polymeric HLB (Plexa) sorbent materials and a highly porous Nylon membrane (30-µm pore size) instead of the conventional PES membranes, were used. Besides, the suitability of PES hollow fibers with more hydrophobic compounds was also studied, for the first time.

In contrast to the previously mentioned two chapters, the studied contaminants were limited to 20 (one compound each class type). Additionally, five deuterated compounds ([$^{2}H_{5}$]-Atrazine, [$^{2}H_{3}$]-Amitriptyline, [$^{2}H_{7}$]-Irbesartan, [$^{2}H_{3}$]-Ketoprofen and [$^{2}H_{9}$]-Progesterone) were studied as candidates for performance reference compounds (PRCs) in both POCIS and PES.

In the case of POCIS, both the sorbents and the Nylon membranes were extracted and analysed independently. The uptake was linear in POCIS sorbent and Nylon membranes but exponential for PES hollow fibres. Furthermore, the highest sampling rates (Rs) values were obtained in POCIS sorbent (between 2.7 for acetaminophen and 491 mL/day for PFOA) followed by Nylon membranes (between 3.6 for OBT and 50 mL/day for telmisartan) and the lowest were those from PES fibres (between 1.7 for bezafibrate and 157 mL/day for butylparaben).

Regarding the PRCs, though [$^{2}H_{5}$]-atrazine, [$^{2}H_{9}$]-Progesterone and [$^{2}H_{3}$]-amitriptyline showed acceptable results in the case of POCIS, only [$^{2}H_{5}$]-atrazine provided a good validation. In the case of PES fibres, the PRC corrections did not provide acceptable results due to a low dissipation of the PRCs.

- *Short term preservation and stability assessment for the analysis of ECs in seawater*.

Because one of our aims was the accurate estimation of the concentrations of emerging compounds in environmental matrices, an important issue is how to store and preserve the environmental samples.

Thus, in this work the stability study was performed in seawater for 23 ECs over one month. Four different alternatives already used previously were tested: (i) seawater at 4°C, (ii) mixed-mode SPE cartridge with Bond Elute Plexa (equivalent of HLB) and Strata X-AW stored at −20°C, (iii) PES hollow fibre stored at −20°C and (iv) methanol extracts once the samples were extracted, and stored at −20°C. Moreover, not only the stability of the analytes was studied, but also the integrity of the supporting polymeric phases by Raman spectroscopy, optical microscopy, differential scanning calorimetric and thermogravimetric analysis.

As could be expected, seawater samples showed the lowest stability (losses between 21-99%). On the contrary, the stability profiles obtained in SPE showed an average loss of 7%, while in PES hollow fibres losses up to 58% were observed. These results suggest that the best way to assure the stability of the water samples is to keep the extracts in the SPE cartridges. A deeper study of the polymeric materials showed the lower efficiency showed in PES fibres might be related with the wettability of this material, based on the thermogravimetric analysis.

- *Occurrence of emerging pollutants in estuaries of the Basque Country. Analysis of sources and distribution, and assessment of the environmental risk*.

Coastal and estuarine areas are ecologically rich and sensitive environments dwelling under the effects of many anthropogenic stressors. Therefore, the protection of those ecosystems from the hazardous effects of chemical contaminants is gaining interest. In this sense, a greater understanding of spatial-temporal patterns in emerging compounds concentration is necessary to characterize the sources, fate and risk and, ultimately, to prevent the anthropogenic impact into the ecosystem. This fact is especially important in the estuaries of the Basque Country because, to the best of our knowledge, it is the first time we achieve this objective. Therefore, we applied the previously developed multiresidue method and the PS methodology to monitor the target ECs selected in three estuaries (Bilbao, Urdaibai and Plentzia) of the Basque Country.

The occurrence of the 41 pre-selected ECs was analysed from winter 2016 to winter 2017 (5 campaigns) by the active sampling protocol developed before. Among the detected compounds, anti-inflammatory drugs (diclofenac and acetaminophen), hypertensive drugs (irbesartan and valsartan), a stimulant (caffeine), an artificial sweetener (acesulfame) and a corrosion inhibitor (OBT) were the ubiquitous compounds. Due to the stratification of the waters in the estuary of Bilbao two independent sources were identified: WWTP and harbour activities. In the case of Gernika and Plentzia, both are estuaries with a high tidal dilution, and the main sources were localized in upper parts of the estuaries.

In addition to this, an extra campaign was carried out in spring 2017 combining both active and PS methods. The use of POCIS provided an efficient way to monitor emerging pollutants over a relatively long sampling period. As a result, in addition to the overall good agreement between the passive and active samplings, passive samplers allowed the determination of several compounds that were below the detection limits in the active sampling.

Lastly, we were able to identify the most relevant compounds in terms of their ecotoxicological risk assessment along the selected three estuaries. In the case of acute toxicity the highest risk values (>>1) were obtained for the angiotensin II receptor blockers (telmisartan, eprosartan, etc.), diuron and diclofenac. In the case of the chronic toxicity the highest values (>>1) were estimated for caffeine, diclofenac, bezafibrate and sulfadiazine.

The monitoring was also extended to Mussels from the Basque Country. Along with the last water monitoring campaign, mussels were collected from the harbour of Bilbao, one of the hot spots described before. Among the 41 compounds analysed, 7 were detected in the 1.2 ng/g (PFOA) to 14 ng/g (progesterone) concentrations range. The presence of valsartan (7 ng/g) and telmisartan (6.8 ng/g) in bivalves is reported for the first time here. These results were included in chapter 4 as the application of the method development.

- *Sea urchin embryo test application in the toxicity evaluation of wastewater treatment plant effluents and effect directed analysis.*

SET was used to assess toxicity at four WWTP of Biscay (Gorliz, Mungia, Gernika and Galindo). All the extracts showed embryo growth inhibition and skeleton malformation activity within the

concentration range tested. In relative enrichment factor units (REF), the EC_{50} values ranged from 1.1 REF (Gernika) to 16.8 REF (Gorliz) for skeleton malformation and from 0.3 REF (Gernika) to 8.8 REF (Gorliz) for growth inhibition.

To identify the causative compounds, EDA was successfully applied for the first time using SET assay to the secondary treatment of the Galindo effluent. To this end, two subsequent fractionation steps were performed using a C_{18} and an aminopropyl column. In the first fractionation only one fraction showed a remarkable toxicity, and it was fractionated further into 15 sub-fractions. In this second fractionation, both endpoints were dramatically observed in one fraction

By this fractionation, the number of features detected by high resolution mass spectrometry (HRMS) in the raw sample was drastically reduced from 1500 to 9, among them, two pesticides (mexacarbate and fenpropidin), two antidepressants (amitriptyline and paroxetine) and two anthelmintic agents (mebendazole and albendazole) could be identified in the two toxic fractions.

The comparison of the chemical and biological data using toxic units (TU) showed that mebendazole was the predominant contributor (32%) followed in a less extend by amitriptyline (9%), whereas fenpropidin could only explain the 0.3% of the sea urchin embryogenesis activity in the F13-4 fraction (TU_{bio}=0.03). The high biologically activity shown by mebendazole, which was more toxic than amitriptyline and fenpropidin is in agreement with its specific mode of action (depolymerisation of microtubules) and the contribution of amitriptyline can be interpreted by its high effluent concentration (304 ng/L, $TU_{chem.}$=2.8 e-3), an order of magnitude higher than that of fenpropidin (23 ng/L, $TU_{chem.}$=7.7 e-5) and mebendazole (65 ng/L, TU_{chem}=9.9 e-3).

Finally, SET showed to be an affordable and inexpensive bioassay to screen potential teratogens in ocean and transitional waters.

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3 Multiresidue method in water

Simultaneous determination of 41 multi-class organic pollutants in environmental waters by means of polyethersulfone microextraction followed by liquid chromatography-tandem mass spectrometry

Analytical and Bioanalytical Chemistry,

2018, 410, 615-632

3.1 Introduction

The concern about the impact of chemicals on human health and the environment has gained increasing attention. Among the different existing laws and regulations concerning water management and protection, the Water Framework Directive (WFD) is probably the most important legislation for the EU countries. Directive 2000/60/EU (European Commission, 2000) was the first mark in the European water policy, which set up a strategy to define high-risk substances to be prioritized. A set of 33 priority substances or groups of them and their respective environmental quality standards (EQS) were ratified by Directive 2008/105/EU (European Commission, 2008). Moreover, Directive 2013/39/EU (European Commission, 2013) included 45 pollutants to meet requirements for the protection of the aquatic compartments and human health. More recently, a set of substances for EU monitoring in surface water bodies was defined in the Watch List of Decision 2015/495/EU (European Commission, 2015).

In addition to the traditional pollutants, the attention of scientific community is focussed on the presence of newly found substances with no previous knowledge about their effects in the environment (Dulio and Slobodnik, 2009). These compounds are often referred to as "contaminants of emerging concern". In this sense, it is still observed the lack of monitoring programs including the determination of organic micropollutants, particular regarding these contaminants. Among them, pesticides, pharmaceuticals, personal care products (PPCPs), industrial chemicals, hormones, flame retardants and disinfection by-products are considered (Richardson and Ternes, 2015). Moreover, NORMAN network has worked out a list of the currently most frequently emerging substances and pollutants based on different prioritization criteria (NORMAN, 2013). Similarly, the US EPA defined a Contaminant Candidate List (CCL), where 116 contaminants (104 chemicals and 12 microbiological) were included for consideration in future regulations (United States Environmental Protection Agency, 2009). Consequently, the fate and behaviour of many of these compounds can be very complex since they are widely distributed in aqueous media at concentration levels usually quite low. One typical example is the effluents of wastewater treatment plants (WWTPs) in which a continuous release of many of these compounds has been described in the literature (Lapworth et al., 2012). Since most of the current WWTPs are not specifically designed to eliminate organic

micropollutants, and many of these contaminants are able to pass through WWTPs processes and reach the aquatic environment, they have become in an indirect source of chronic pollution (Smith et al., 2009).

The inclusion of a large variety of chemicals into one multiresidue method will enhance the applicability of sensitive and robust analytical methodologies and thus, providing a broader information about the occurrence and fate of emerging contaminants in the environment (Al Aukidy et al., 2012; Grabic et al., 2012; Petrovic, 2014). A particularly interesting approach is the combination with microextraction techniques since they are economically more affordable and cleaner than traditional approaches from the environmental point of view. Among sorptive microextraction techniques, polydimethylsiloxane (PDMS) based solid-phase microextraction (SPME) (Souza-Silva et al., 2015) and stir-bar sorptive extraction (SBSE) (Prieto et al., 2010) are the most widespread ones. In fact, both share the same extraction features though the amount of phase is 50-250 times larger in SBSE, which increases its preconcentration capacity. However, recently, researchers have focused on the development of new coatings (Camino-Sánchez et al., 2014) to modify the selectivity of the solid phase. In this sense, there are up-to-date three commercially available coatings for SBSE: PDMS, Polyacrylate (PA) and ethyleneglycol/silicone (EG/silicone). The development of new coatings is, in fact, the most relevant improvement to expand the applicability of SBSE, allowing the extraction of more polar compounds. However, some mechanical instability and degradation of these coatings have been reported (Ochiai et al., 2013). Furthermore, new disposable polymeric materials and/or phases such as polypropylene (PP) or polyethersulfone (PES), have also been proposed (Bizkarguenaga et al., 2015; Blanco-Zubiaguirre et al., 2014; Casado et al., 2013; Prieto et al., 2012) as low-cost alternatives to SBSE devices for the aqueous samples extraction or extracts clean-up of different more polar substances.

Concerning to the analysis, and despite gas chromatography (GC) is a well-established analytical technique, during the last decades liquid-chromatography-triple quadrupole-tandem mass spectrometry (LC-QqQ-MS/MS) has become widespread to analyse polar compounds in environmental samples due to its inherent sensitivity and specificity (Carvalho and Santos, 2016; Tomšíková et al., 2012). The main advantage of the LC analysis is that a derivatisation reaction prior to the analysis is not required and, consequently, the whole analytical procedure is simplified

(Hernández et al., 2014). However, it has to be taken into account one of the main drawbacks related with LC based analysis, is the strong matrix effect observed, which leads in many cases to signal suppression or enhancement (Hernández et al., 2012). Therefore, the use of this technique requires a preliminary and exhaustive study of the matrix effects, as well as an adequate sample preparation parameters optimisation (Petrovic, 2014).

Within this context, a new procedure for the simultaneous determination of 41 multiclass organic pollutants in WWTP effluents, estuary and seawater samples using a dual extraction based on PES polymeric material was developed before being applied in further studies dealing with the target analytes environmental distribution. A thorough optimisation of the extraction and desorption processes was assessed. In addition to this, the performance of this new procedure was compared with a validated standard SPE method. To the best of our knowledge, this is the first work using PES polymer combined to LC-MS/MS analysis for the simultaneous determination of a wide variety of organic compounds in aqueous samples.

3.2 Experimental section

3.2.1 Reagents and materials

The target analytes (names and abbreviations) with their corresponding families, the supplier of the standards, CAS number, molecular formula, molecular weight and some of their physic-chemical properties such as the acid dissociation constant (pK_a) and hydrophobicity (included as log P and log $D_{(pH\ 2\ and\ 11)}$) are included in **Table 3.1** and structures in **Figure I** in **Appendix**. The purity of all the target analytes was > 95%.

Table 3.1. Families, names (abbreviation), the supplier of the standards, CAS number, molecular formula, molecular weight, LogP and og D $_{(pH=2 \text{ and } 11)}$ and pKa values of the target compounds.

Family	Analyte	Supplier	CAS	Formula	Mw	Log P[a]	LogD[a] (pH2; 11)	pK_a[a]
Herbicide	Atrazine[b]	Fluka	1912-24-9	$C_8H_{14}ClN_5$	215.68	2.2	1.0; 2.2	4.2
Herbicide	Diuron[b]	Fluka	330-54-1	$C_9H_{10}Cl_2N_2O$	233.09	2.5	2.5; 2.5	13.2
Herbicide	Isoproturon[b]	Fluka	34123-59-6	$C_{12}H_{18}N_20$	206.28	2.6	2.6; 2.6	13.8
Herbicide	Simazine[b]	Fluka	122-34-9	$C_7H_{12}ClN_5$	201.66	1.8	0.6; 1.8	4.2
Hormone	Progesterone	Sigma-Aldrich	57-83-0	$C_{21}H_{30}O_2$	314.46	4.2	4.2; 4.2	-
Hormone	Testosterone	Sigma-Aldrich	58-22-0	$C_{19}H_{28}O_2$	288.42	3.4	3.4; 3.4	-0.9
Industrial chemicals Corrosion inhibitor	2-hydroxybenzothiazole (OBT)	Sigma-Aldrich	934-34-9	C_7H_5NOS	151.19	2.5	2.5; 0.9	6.4
Industrial chemicals PFASs	Perfluoroctylsulfonamide (PFOSA)	Dr. Ehrenstofer	754-91-6	$C_8H_2F_{17}NO_2S$	499.14	4.8	4.8; 3.9	3.4
Industrial chemicals PFASs	Perfluorooctane sulfonic acid (PFOS)[b]	Sigma-Aldrich	1763-23-1	$C_8HF_{17}O_3S$	500.13	5.4	3.0;3.0	-3.3
Industrial chemicals PFASs	Perfluorooctanoic acid (PFOA)	Sigma-Aldrich	335-67-1	$C_8HF_{15}O_2$	414.07	5.1	1.6; 1.6	-4.2
Industrial chemicals PFASs	Perfluoro-1-butanesulfonate (PFBS)	Sigma-Aldrich	29420-49-3	$C_4F_9O_3S$	338.19	2.6	0.2; 0.2	-3.3
Life style products Stimulant	Caffeine	Sigma-Aldrich	58-08-2	$C_8H_{10}N_4O_2$	194.19	-0.6	-0.6; -0.6	-1.2
Life style products Artificial sweetener	Acesulfame	Supelco	55589-62-3	$C_4H_5NO_4S$	163.15	-0.6	-0.6; -1.5	3.0
Life style products Artificial sweetener	Sucralose	Supelco	56038-13-2	$C_{12}H_{19}Cl_3O_8$	397.63	-0.5	-0.5; -0.5	11.9
PCP	Butylparaben	Sigma-Aldrich	94-26-8	$C_{11}H_{14}O_3$	194.23	3.0	3.0; 1.0	8.5
PCP	Methylparaben	Sigma-Aldrich	99-76-3	C_8H_8O3	152.14	1.7	1.7; -0.4	8.5
Pharmaceuticals/ Dihydrofolate reductase	Trimethoprim	Fluka	738-70-5	$C_{14}H_{18}N_4O_3$	290.32	1.3	-0.2; 1.3	7.2
Pharmaceutical Fluoroquinolone	Ciprofloxacin	Fluka	85721-33-1	$C_{17}H_{18}FN_3O_3$	331.34	-0.8	-1.7; -2.0	5.7; 8.7
Pharmaceutical Fluoroquinolone	Norfloxacin	Fluka	70458-96-7	$C_{16}H_{18}FN_3O_3$	319.33	-0.9	-1.8; 2.0	5.7; 8.7
Phamaceutica Sulfonamide	Sulfadiazine	Sigma-Aldrich	68-35-9	$C_{10}H_{10}N_4O_2S$	250.28	0.4	0.1; -0.6	2.0; 7.0
Phamaceutical Sulfonamide	Sulfamethoxazole	Fluka	723-46-4	$C_{10}H_{11}N_3O_3S$	253.28	0.8	0.5; -0.2	2.0; 6.2

Table 3.1. Families, names (abbreviation), the supplier of the standards, CAS number, molecular formula, molecular weight, LogP and og D $_{(pH=2 \text{ and } 11)}$ and pK_a values of the target compounds.

Family	Analyte	Supplier	CAS	Formula	Mw	Log P[a]	LogD[a] (pH2; 11)	pK_a[a]
Pharmaceutical Tricyclyc antidepressant	Amitriptyline	Sigma-Aldrich	50-48-6	$C_{20}H_{23}N$	277.40	4.8	1.3; 4.8	9.8
Pharmaceutical/ Tricyclyc antidepressant	Clomipramine	Sigma-Aldrich	17321-77-6	$C_{19}H_{23}ClN_2$	314.85	4.9	1.4; 4.9	9.2
Pharmaceutical Tricyclyc antidepressant	Imipramine	Sigma-Aldrich	50-49-7	$C_{19}H_{24}N_2$	280.41	4.3	0.8; 4.3	9.2
Pharmaceutical Tricyclyc antidepressant	Nortriptyline	Sigma-Aldrich	72-69-5	$C_{19}H_{21}N$	263.37	4.4	1.2; 4.3	10.5
Pharmaceutical Angiotensin II receptor antagonist	Eprosartan	Solvay Pharma	144143-96-4	$C_{23}H_{24}N_2O_7S$	424.52	3.8	4.1; -1.9	3.6; 6.7
Pharmaceutical Angiotensin II receptor antagonists	Irbesartan	Sanofi	138402-11-6	$C_{25}H_{28}N_6O$	428.53	5.5	3.5; 3.9	4.1; 8.3
Pharmaceutical Angiotensin II receptor antagonist	Losartan	Merck	114798-26-4	$C_{22}H_{23}ClN_6O$	422.91	5.1	3.5; 3.5	3.8; 8.3
Pharmaceutical Angiotensin II receptor antagonist	Telmisartan	Boehringer	144701-48-4	$C_{33}H_30N_4O_2$	514.62	6.1	5.0; 4.3	3.6; 4.7; 5.9
Pharmaceutical Angiotensin II receptor antagonist	Valsartan	Boehringer	137862-53-4	$C_{24}H_{29}N_5O_3$	435.52	5.3	5.3; 0.1	4.4; 8.3
Pharmaceutical β-blocker antihypertensive	Propranolol	Biomedicals	525-66-6	$C_{16}H_{21}N0_2$	256.34	2.6	-0.7; 2.6	9.7;14.1
Pharmaceutical Antiinflammatory	Acetaminophen	Fluka	103-90-2	$C_8H_9NO_2$	151.16	0.9	0.9; -0.6	9.5
Pharmaceutical Antiinflammatory	Diclofenac[c]	Sigma-Aldrich	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	296.15	4.3	4.3; 0.7	4.0
Pharmaceutical Antiinflammatory	Ketoprofen	Biomedicals	22071-15-4	$C_{16}H_{14}O_3$	254.28	3.6	3.6; 0.1	3.9
Pharmaceutical Lipid-regulating	Bezafibrate	Biomedicals	41859-67-0	$C_{19}H_{20}ClNO_4$	361.80	4.0	4.0; 0.5	3.8
Pharmaceutical Lipid-regulating	Clofibric acid	Biomedicals	882-09-7	$C_{10}H_{11}ClO_3$	214.64	2.9	2.9; -0.6	3.4
Pharmaceutical Anticonvulsant	Carbamazepine	Sigma-Aldrich	298-46-4	$C_{15}H_{12}N_2O$	236.26	2.8	2.8; 2.8	16.0
Pharmaceutical Anticonvulsant	Phenytoin	Sigma-Aldrich	57-41-0	$C_{15}H_{12}N_2O_2$	252.20	2.2	2.2; 0.3	8.5
Phytoestrogen	Genistein	Extrasynthese	446-72-0	$C_{15}H_{10}O_5$	270.24	3.1	3.1; -2.9	6.6; 8.0; 9.0
Phytoestrogen	Genistin	Extrasynthese	529-59-9	$C_{21}H_{20}O_{10}$	432.38	0.8	0.8; -3.1	7.3; 9.0;12.5
Phytoestrogen	Glycitin	Extrasynthese	40246-10-4	$C_{22}H_{22}O_{10}$	446.40	1.3	0.3; -1.5	9.0;12.2

[a] Values reported in the Free Data Base www.chemicalize.org;
[b] Priority compounds included in the Directives 2000/60/EU and 2008/105/EU or 2013/39/EU;
[c] Compounds including in the Watch List of Decision 2015/495/EU.

In the case of isotopically mass-labelled analogues (see **Table 3.2**), amitriptyline-d_3 hydrochloride ([$^2H_3$]-amitriptyline, 100 mg/L in methanol), atrazine-d_5 ([$^2H_5$]-atrazine, 99%), carbamazepine-d_{10} ([$^2H_{10}$]-carbamazepine, 100 mg/L in methanol), ketoprofen-d_3 ([$^2H_3$]-ketoprofen, 99.4%), nortriptyline-d_3 hydrochloride ([$^2H_3$]-nortriptyline, 100 mg/L in methanol), progesterone-d_9 ([$^2H_9$]-progesterone, 98%), were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ciprofloxacin-d_8 hydrochloride ([$^2H_8$]-ciprofloxacin, 99%) was obtained from Fluka (Buchs, Switzerland). Sucralose-d_6 ([$^2H_6$]-sucralose, 98%) and irbesartan-d_7 2,2,2-trifluoroacetate salt ([$^2H_7$]-irbesartan, 99.9%) were purchased from Toronto Research Chemicals (Toronto, Canada). The mix (sodium perfluoro-1-hexane [$^{18}O_2$] sulfonate (MPFHxS), sodium perfluoro-1-[1,2,3,4-$^{13}C_4$] octanesulfonate (MPFOS), perfluoro-n-[$^{13}C_4$] butanoic acid (MPFBA), perfluoro-n-[1,2-$^{13}C_2$] hexanoic acid (MPFHxA), perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid (MPFOA), perfluoro-n-[1,2,3,4,5-$^{13}C_5$] octanoic acid (MPFNA), perfluoro-n-[1,2-$^{13}C_2$] decanoic acid (MPFDA), perfluoro-n-[1,2-$^{13}C_2$] undecanoic acid (MPFUdA) and perfluoro-n-[1,2-$^{13}C_2$] dodecanoic acid (MPFDoA)) was obtained at 2 mg/L in methanol from Wellington Laboratories (Ontario, Canada).

Stock standard solutions were dissolved in methanol (UHPLC-MS MeOH, Scharlab, Barcelona, Spain) in order to prepare approximately 1000-2500 mg/L solutions. The addition of 100 µL (in 3.5 mL of total volume) sodium hydroxide 1 mol/L (NaOH, 98%, Panreac, Barcelona, Spain) was necessary for the proper dissolution of fluoroquinolone antibiotics as described by Gros et al. (Gros et al., 2013). 100 mg/L dilutions were prepared in MeOH every month and dilutions at lower concentrations containing all analytes were prepared daily in MeOH: Milli-Q water (30: 70, v: v). All the chemicals standards solutions were stored at −20°C.

Table 3.2. The optimised MS/MS parameters for SRM analysis, retention time (RT) and instrumental limits of quantification (LOQ) for Kinetex biphenyl and Kinetex F5 columns.

Analyte	ESI-MS/MS					Biphenyl		F5	
	Polarity	Precursor ion (m/z)	Product ions (m/z)	Frag. (V)	Collision energy (eV)	RT min	LOQ ng/mL	RT min	LOQ ng/mL
Acesulfame[h]	$[M-H]^-$	162.0	82.0/78.0	72	12/36	1.2	0.81	1.2	0.46
Acetaminophen[d]	$[M+H]^+$	152.1	93.1/110.1	80	33/25	1.4	1.04	1.4	1.96
Amitriptyline[a]	$[M+H]^+$	278.2	91.1/117.1	76	25/29	14.9	0.63	13.9	0.60
Atrazine[b]	$[M+H]^+$	216.1	174.1/68.0	80	17/17	9.3	0.23	9.8	0.21
Bezafibrate[d]	$[M+H]^+$	362.2	139.1/201.1	104	25/37	18.8	0.62	18	0.36
Butylparaben[f]	$[M+H]^+$	195.1	139.0/95.1	72	5/17	12.6	0.92	17.1	2.00
Caffeine[b]	$[M+H]^+$	195.1	138.1/110.0	72	21/25	4.1	1.92	2.3	0.96
Carbamazepine[c]	$[M+H]^+$	237.1	194.4/193.3	104	20/36	11.6	0.25	8.8	0.29
Ciprofloxacin[g]	$[M+H]^+$	332.1	314.2/231.0	136	13/17	4.5	3.01	4.5	3.03
Clofibric acid[d]	$[M-H]^-$	213.0	127.0/85.0	72	8/4	11.3	1.92	13.5	1.29
Clomipramine[a]	$[M+H]^+$	315.2	58.0/227.1	76	41/45	19.4	0.32	21.6	0.33
Diclofenac[d]	$[M+H]^+$	296.0	214.1/250.0	80	33/17	23.2	0.49	23.2	0.37
Diuron[b]	$[M+H]^+$	233.0/235.0	72.1	72/72	17	9.6	0.41	13.2	0.47
Eprosartan[i]	$[M+H]^+$	425.0	207.3/117.0	80	45/45	8.9	1.17	7.6	1.20
Genistein[f]	$[M+H]^+$	271.1	91.1/215.1	140	25/29	10.3	1.60	12.2	2.46
Genistin[f]	$[M+H]^+$	433.1	271.1/153.1	80	13/45	3.6	1.21	3.9	2.03
Glycitin[f]	$[M+H]^+$	447.1	285.1/270.0	80	9/45	3.8	1.01	4.1	1.80
Imipramine[a]	$[M+H]^+$	281.2	86.1/58.1	76	13/41	13.4	0.49	13.1	0.58
Irbesartan[i]	$[M+H]^+$	429.0	207.3/180.1	80	45/45	22.5	0.33	13.5	0.31
Isoproturon[b]	$[M+H]^+$	207.2	72.1/165.1	80	21/13	10.4	0.40	10.4	0.32
Ketoprofen[d]	$[M+H]^+$	255.1	105.0/209.1	104	24/22	18.4	0.60	14.0	0.64
Losartan[i]	$[M+H]^+$	423.0	405.3/207.0	80	45/45	20.3	1.17	12.3	1.20
Methylparaben[f]	$[M+H]^+$	153.1	121.1/651.0	72	13/37	4.8	4.54	6.4	4.68
Norfloxacin[g]	$[M+H]^+$	320.1	302.1/231.0	136	17/41	3.0	1.94	3.9	2.17
Nortriptyline[e]	$[M+H]^+$	264.2	233.1/91.1	102	13/25	14.2	0.38	14.4	0.53
OBT[c]	$[M+H]^+$	152.0	80.0/65.1	110	33/37	6.8	3.03	6.4	2.93
PFBS[b]	$[M-H]^-$	299.9	80.0/99.0	144	37/33	3.0	1.09	8.7	0.56
PFOA[k]	$[M-H]^-$	413.0	369.0/169.0	80	5/13	8.9	1.38	23.1	0.49
PFOS[j]	$[M-H]^-$	498.9	80.0/99.0	170	45/45	11.8	1.96	23.5	0.33
PFOSA[j]	$[M-H]^-$	497.9	78.0/478.1	140	41/45	20.1	2.51	24.5	0.34
Phenytoin[c]	$[M+H]^+$	253.1	182.1/225.1	104	29/9	9.6	0.81	8.3	2.06
Progesterone[b]	$[M+H]^+$	315.2	109.1/97.1	110	25/25	25.9	0.41	23.2	0.87

Table 3.2. The optimised MS/MS parameters for SRM analysis, retention time (RT) and instrumental limits of quantification (LOQ) for Kinetex biphenyl and Kinetex F5 columns.

Analyte	ESI-MS/MS					Biphenyl		F5	
	Polarity	Precursor ion (m/z)	Product ions (m/z)	Frag. (V)	Collision energy (eV)	RT min	LOQ ng/mL	RT min	LOQ ng/mL
Propranolol[a]	$[M+H]^+$	260.2	116.1/72.1	72	16/20	8.3	0.61	9.9	0.55
Simazine[b]	$[M+H]^+$	202.1	68.1/132.0	104	40/20	7.4	0.44	7.6	0.50
Sucralose[h]	$[M-H+HCOOH]^-$	387.1	341.1/179.0	80	9/17	1.0	0.66	1.0	0.33
Sulfadiazine[i]	$[M+H]^+$	251.1	153.0/108.0	104	12/24	2.1	0.26	1.5	0.35
Sulfamethoxazole[i]	$[M+H]^+$	254.1	92.1/156.0	76	28/12	4.6	0.30	3.4	0.69
Telmisartan[i]	$[M+H]^+$	515.0	276.1/497.1	80	45/45	23.8	0.30	21.5	0.34
Testosterone[f]	$[M+H]^+$	289.2	109.1/97.1	80	25/21	23.5	0.83	16.0	0.90
Trimethoprim[b]	$[M+H]^+$	291.2	230.1/261.1	136	20/24	2.2	0.34	1.8	0.26
Valsartan[i]	$[M-H]^-$	434.0	350.2/179.2	80	45/45	23.0	2.60	21.0	2.61
[a]Amitriptyline-d_5	$[M+H]^+$	291.2	91.1/117.1	76	25/29				
[b]Atrazine-d_5	$[M+H]^+$	221.0	179.0/104.0	80	17/17				
[c]Carbamazepine-d_{10}	$[M+H]^+$	237.1	194.4/193.3	104	20/36				
[d]Ketoprofen-d_3	$[M+H]^+$	258.1	105.0/209.1	104	24/22				
[e]Nortriptyline-d_3	$[M+H]^+$	267.2	233.1/91.1	102	13/25				
[f]Progesterone-d_9	$[M+H]^+$	324.3	113.2/106.1	110	25/25				
[g]Ciprofloxacin-d_8	$[M+H]^+$	340.1	322.1/296.1	136	13/17				
[h]Sucralose-d_6	$[M-H+HCOOH]^-$	396.1	347.1/179.0	80	9/17				
[i]Irbesartan-d_7	$[M+H]^+$	436.0	207.3/180.1	80	45/45				
[j]MPFOS	[M-H]-	503.0	99.0/99.0	60	45/45				
[k]MPFOA	[M-H]-	417.0	372.0/372.0	60	5/5				

[a] Corrected with Amitriptyline-d_5.
[b] Corrected with Atrazine-d_5.
[c] Corrected with Carbamazepine-d_{10}
[d] Corrected with Ketoprofen-d_3.
[e] Corrected with Nortriptyline-d_3.
[f] Corrected with Progesterone-d_9.
[g] Corrected with Ciprofloxacin-d_8.
[h] Corrected with Sucralose-d_6.
[i] Corrected with Irbesartan-d_7.
[j] Corrected with MPFOS.
[k] Corrected with MPFOA.

Oasis-HLB (hydrophilic–lipophilic-balanced) 200 mg SPE cartridges were purchased from Waters (Milford, USA). PES hollow fibres (0.7 and 0.5 mm external and internal diameters, 1.43 g/mL density) were obtained from Membrana GmbH (Wuppertal, Germany). Pieces of this polymer (4 cm

length) were cut using a sharp blade and soaked overnight in MeOH (HPLC grade, 99.9%, LabScan, Dublin, Ireland) previous to their use as sorbent material. Afterwards, the polymer was air-dried and stored until their use. Given their reduced cost (c.a. 0.05 €/unit) the pieces were discarded after each use. Ethylenediaminetetraacetic acid (EDTA, 99.0-101.1%, Panreac), formic acid (HCOOH ≥ 98%, Scharlau, Barcelona, Spain), ammonia (25% as NH_3, Panreac) and sodium chloride (NaCl, > 99.8%, Merck) were used for matrix modification.

The extracts were evaporated using a Turbovap LV Evaporator (Zymark, Hopkinton, USA) under a gentle stream of nitrogen (> 99.999% of purity) supplied by Messer (Tarragona, Spain). The reconstituted extracts were (according to the different filters evaluation) filtered through polypropylene (PP, 0.22 µm, 13 mm, Phenomenex, California, USA), polytetrafluoroethylene (PTFE, 0.2 µm, 13 mm, Teknokroma, Barcelona, Spain) or polydivinylfluoride (PVDF, 0.22 µm, 13 mm, Simplepure, Membrane solution, Plano, USA) filters before the LC-MS/MS analysis. Milli-Q (< 0.05 µS/cm, Milli-Q, Millipore) water and UHPLC-MS MeOH (Scharlab) were used as mobile phase eluent and HCOOH (Optima, Fischer Scientific, Gell, Belgium) for mobile phase modification. High purity nitrogen gas (> 99.999%) supplied by Messer was used as collision gas. Nitrogen gas (99.999%) provided by AIR Liquid (Madrid, Spain) was used as both nebuliser and drying gas.

3.2.2 Sample collection and treatment

Effluent samples (Galindo WWTP secondary treatment, 2°57'52.8"W, 43°18'11.0"N), estuary sample (estuary of Bilbao, 2°59'33.77"W, 43°18'50.38"N, downstream of the WWTP) and marine water (3°4'35.58"W, 43°22'50.94"N, harbour of Bilbao estuary) were collected in July 2016. Samples were collected in pre-cleaned amber glass bottles and transported to the laboratory in cooled boxes. Only effluent samples were filtered through 1.2 µm glass microfiber filter (GE Whatman, Maidstone, UK), according to the filter evaluation (see section 3.3.3). All the real samples were kept in the fridge at 4°C before analysis. The analyses were performed within 24 h after sampling.

3.2.3 Polyethersulfone microextraction

Under optimised conditions, two aliquots of 120 mL of water samples (dual extraction) were directly poured into 150 mL glass extraction vessels (ServiQuimia, Tarragona, Spain) containing 30%

NaCl (w/v), in both cases at pH=2 or pH=10 (as they represent two different optimal conditions for the target analytes). Pre-cleaned portions of PES (4 PES tubes of 4 cm each corresponding to a total mass of approx. 48 mg) and a magnetic stirrer were also introduced into the vessels. Additionally, an appropriate volume of a EDTA solution to achieve a final concentration of 0.1% (m/m) was added. Thereafter, vessels were closed and extraction (800 rpm) was performed in a 15 position magnetic stirrer (Gerstel, Mülheim an der Ruhr, Germany) at room temperature and overnight according to the previous experience of the research group (Bizkarguenaga et al., 2015; Blanco-Zubiaguirre et al., 2014; Ros et al., 2015).

Once the sorption step was over, the polymers were removed and rinsed with Milli-Q water in order to eliminate salt residues, and finally, dried with a clean tissue. Subsequently, the sorbents were chemically desorbed. To this aim, the polymers from the two aliquots, i.e. sorbents from pH 2 and 10 modified samples, were introduced together into a 1.5 mL Eppendorf tube (Eppendorf , Berzdorf, Germany) containing 1000 µL of MeOH and soaked for 32 min in an ultrasound bath (Digital Ultrasonic Cleaner, USB Axtor by Lovango, Barcelona, Spain). The extract was evaporated to dryness under a gentle stream of nitrogen at 35 °C and reconstituted in 200 µL of MeOH: Milli-Q water (30:70, v:v). Finally, the reconstituted extracts were filtered through a 0.22 µm PP filter before the LC-MS/MS analysis.

3.2.4 Solid phase extraction procedure

Among the published multiresidue based on SPE methods a suitable SPE protocol (Hernández et al., 2014; Petrovic, 2014) was selected and validated. Prior to the extraction, an appropriate volume of a EDTA solution to achieve a final concentration of 0.1% (g solute/g solution) was added and samples were acidified (pH=2) with formic acid. 200 mg-Oasis HLB cartridges were sequentially conditioned with 5 mL of MeOH, 5 mL of ultrapure water and 5 mL of ultrapure water at pH=2. The sample (100 mL in the case of effluent and 250 mL in the cases of estuary and seawater) was, then, percolated through the cartridge assisted by a vacuum pump at ca. 5 mL/min. Subsequently, the cartridges were washed with 6 mL of ultrapure water, vacuum dried for 40 min and eluted with 6 mL of MeOH. After elution, the extract was evaporated to dryness under a gentle stream of nitrogen at 35°C and reconstituted in 200 µL of MeOH: Milli-Q water (30:70, v:v). Finally, the reconstituted

extracts were filtered through a 0.22 μm PP filter before the LC-MS/MS analysis.

3.2.5 LC-MS/MS analysis

An Agilent 1260 series HPLC chromatograph equipped with a degasser, binary pump, autosampler and column oven coupled to an Agilent 6430 triple quadrupole (QqQ) mass spectrometer equipped with electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA) was employed for the separation and quantification of the 41 target analytes. Two chromatographic columns were tested for the separation: a Kinetex F5 100 Å core-shell (2.1 mm × 100 mm, 2.6 μm) column coupled to a Kinetex F5 pre-column (2.1 mm x 4.6 mm, 2.6 μm), and a Kinetex biphenyl 100Å core-shell (2.1 mm x 100 mm, 2.6 μm) column with a Kinetex biphenyl pre-column (2.1 mm x 5 mm, 2.6 μm), both from Phenomenex (Torrance, 235 CA, USA). The column temperature and the injection volume were set to 35°C and 10 μL, respectively, in the case of both columns. The separation of the target analytes was carried out at a flow rate of 0.3 mL/min. Under optimised conditions, a binary mixture consisting of water: MeOH (95: 5, v: v) (mobile phase A) and mobile phase B of MeOH: water (95: 5, v: v), both containing 0.1% of HCOOH were used for gradient separation of target analytes. The gradient profile started with 30% B which was increased to 50% in 4 min and maintained for 12 min. Then, it was increased to 90% B where it was maintained constant for 10 min. Initial gradient conditions (30% B) were then achieved in 6 min, where it was finally held for another 10 min (post-run step). After the optimisation of ESI parameters, the analysis was carried out in the positive and negative voltages, according to the target analytes, which were simultaneously applied in a single injection using a N_2 flow rate of 12 L/min, a capillary voltage of 3500 V, a nebuliser pressure of 45 psi, and a source temperature of 350°C. Fragmentor voltage (40-200 V) and collision energy (5-45 eV) were optimised (see **Table 3.2**) for ESI source by injection of individual compounds.

Quantification was performed in the selected reaction monitoring (SRM) acquisition mode by recording the two most intense transitions for each analyte (the most sensitive transition was chosen as the quantifier and the second one as qualifier). In addition, the ratio of quantifier/qualifier ion was used to identify according to the limits set by EC guidelines (European Commission, 2002) on performance of analytical methods and the interpretation of the results. Different ion ratio

criteria depending on the relative intensity of the product ion (a relative intensity >50%, ±20%; >20 to 50%, ±25%; >10 to 20% ± 30% and <10% ± 50%) were followed. Instrumental operations, data acquisition and peak integration were performed with the Masshunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

3.2.6 Analyte quantification and method validation

For the quantification of the target analytes in the different aqueous samples an external calibration together with surrogate corrections approach was performed for SPE, while in the case of PES method, a procedural calibration with Milli-Q using isotopically labelled analogues as surrogates was used. In this sense, calibration standards in Milli-Q water were prepared containing concentrations ranged from 0 (procedural blanks) to 1000 ng/L and treated according to the extraction procedure (see sections 3.2.3 and 3.2.4).

Method validation was performed in the case of both PES and SPE protocols for seawater, estuary and wastewater effluents in terms of process efficiency (PE%), apparent recovery and method quantification limits (MQLs). The PE and apparent recovery of the method were evaluated with spiked (100 ng/L) aliquots of effluent, estuary water and seawater real samples and extracted under conditions previously described. The experiments were performed in triplicate (n=3) and blanks (n=3) were processed in parallel for signal subtraction.

Due to the lack of a free analyte matrix, the MQLs could not be calculated by spiking seven replicates of each blank matrix with the analytes at the lowest concentration used in the validation, according to USEPA guidance (United States Environmental Protection Agency, 2016). Thus, MQLs were calculated using the Equation 3.1 (Baker and Kasprzyk-Hordern, 2011a; Huntscha et al., 2012; Kasprzyk-Hordern et al., 2008; Vieno et al., 2006).

$$MQL = \frac{LOQ \times 1000}{PE\ (\%) \times CF} \qquad \text{Equation 3.1}$$

where LOQ (ng/mL) is the instrumental quantification limit (PE (%) is the process efficiency of the analyte in the corresponding matrix and CF is the analyte concentration factor according to the developed procedures.

3.3 Results and discussion

3.3.1 MS/MS parameters optimisation

The parameters related to the mass spectrometry (polarity, fragmentor voltage, collision energy and collision cell accelerator) were fully optimised using a standard containing all the target compounds at a concentration level of 2.5 µg/mL through the specific Agilent optimizer software. Both, target analytes and surrogates were considered. All the precursor ions corresponded to $[M+H]^+$ and $[M-H]^-$ in positive and negative ionization modes, respectively, except in the case of sucralose which increased approximately 10 fold its sensitivity using $[M-H+HCOOH]^-$ adduct as precursor ion. The use of different sucralose adducts as precursor ions has also been reported in the literature (Arbeláez et al., 2015; Ordoñez et al., 2013). Optimum values for the target analytes and surrogates are summarized in Table 3.2.

3.3.2 Calibration ranges, determination coefficients and instrumental limits of quantification

Before LC-MS/MS analysis all the sample extracts and standards solutions were filtered. In order to avoid analyte losses in the filters or any contamination, several 0.22 µm-filters of different providers and materials (PP, PTFE hydrophilic and PVDF filters, see section 3.2.1) were evaluated and compared. In this sense, injection solvent MeOH: Milli-Q water (30:70, v: v) mixture was spiked (200 ng/mL) with the target compounds either after or before filtration and analysed by LC-MS/MS. From the results shown in Table 3.3, PP and PTFE filters gave comparable recovery values (p-value> 0.05 according to ANOVA) and did not show any significant retention. However, some PFASs (PFOA and PFOS) showed recoveries higher than 130% using PTFE filters, and therefore, PP filters were used in further experiments.

Calibration curves were built under optimised chromatographic conditions with MeOH: Milli-Q (30:70, v: v) standard solutions in the instrumental limit of quantification (LOQ)-5000 ng/mL range at 12 concentration levels. The two chromatographic columns (a Kinetex biphenyl, 100 mm x 2.1 mm, 2.5 µm and a Kinetex F5, PFP phase, 100 mm x 2.1 mm, 2.6 µm) were evaluated according to the literature (Borova et al., 2014; Regalado et al., 2014; Ziarrusta et al., 2016).

Table 3.3: Absolute recovery of analytes after 0.22 μm filtration steps.

Analyte	0.22 μm filter		
	PTFE	PP	PVDF
Acesulfame	105±2	101±2	37±8
Acetaminophen	98±5	99±8	90±9
Amitriptyline	104±1	102±2	46±5
Atrazine	103±7	106±10	103±16
Bezafibrate	101±4	108±9	104±8
Butylparaben	106±6	108±11	109±9
Caffeine	92±1	98±5	103±2
Carbamazepine	103±7	109±10	77±11
Ciprofloxacin	101±0.3	98±4	53±6
Clofibric acid	103±3	107±8	78±8
Clomipramine	105±3	99±5	33±6
Diclofenac	102±1	102±2	108±12
Diuron	107±6	108±10	118±20
Eprosartan	96±5	99±3	75±9
Genistein	103±3	108±9	93±7
Genistin	105±6	106±7	92±12
Glycitin	104±2	105±8	107±13
Imipramine	106±6	101±2	46±3
Irbesartan	95±7	105±6	93±11
Isoproturon	103±2	106±7	104±2
Ketoprofen	103±3	107±10	110±4
Losartan	96±7	95±8	97±7
Methylparaben	101±2	102±5	101±6
Norfloxacin	99±1	100±11	46±8
Nortriptyline	100±6	101±8	106±9
OBT	105±1	107±9	106±1
PFBS	120±2	105±3	108±8
PFOA	150±6	105±6	108±11
PFOS	161±10	106±8	89±8
PFOSA	122±3	105±4	109±4
Phenytoin	107±8	111±7	100±14
Progesterone	96±7	101±10	105±7
Propranolol	100±2	105±6	113±15
Simazine	104±4	105±7	115±4
Sucralose	97±4	99±2	38±5
Sulfadiazine	91±9	96±7	99±3
Sulfamethoxazole	102±3	103±3	104±6
Telmisartan	92±5	95±9	98±9
Testosterone	103±5	105±8	112±9
Trimethoprim	93±1	97±5	104±1
Valsartan	92±8	93±9	94±6

PP: polypropylene, PVDF: Polydivinylfluoride, PTFE: Polytetrafluoroethylene

Determination coefficients in the same range of 0.975–0.999 and 0.973–0.999 were obtained for all the target analytes with correction with the corresponding labelled standard for biphenyl and F5 columns, respectively. Limits of detection (LODs) were estimated as the concentration producing a signal-to-noise ratio of 3. LODs below 2.3 and 2.7 ng/mL were obtained for all the analytes in the case of F5 and biphenyl columns, respectively. LOQs were established as the lowest concentration fulfilling all of the following criteria: (i) a linear calibration curve, (ii) an acceptable peak shape, and (iii) a signal-to-noise ratio of at least 10 (S/N=10). As can be observed in **Table 3.2**, in the same order of magnitude LOQ values were obtained by means of both columns for the majority of the analytes, except in the case of artificial sweeteners and PFASs which showed better results in terms of sensitivity and chromatographic peak shape using the PFP column (see **Figure 3.1**). Besides, in general terms, LODs and LOQs obtained were similar to the values reported in the literature (Bizkarguenaga et al., 2015; Grabic et al., 2012; Gros et al., 2013; Huntscha et al., 2012; Tran et al., 2013). Therefore, the F5 (PFP phase) column was selected and used in further experiments.

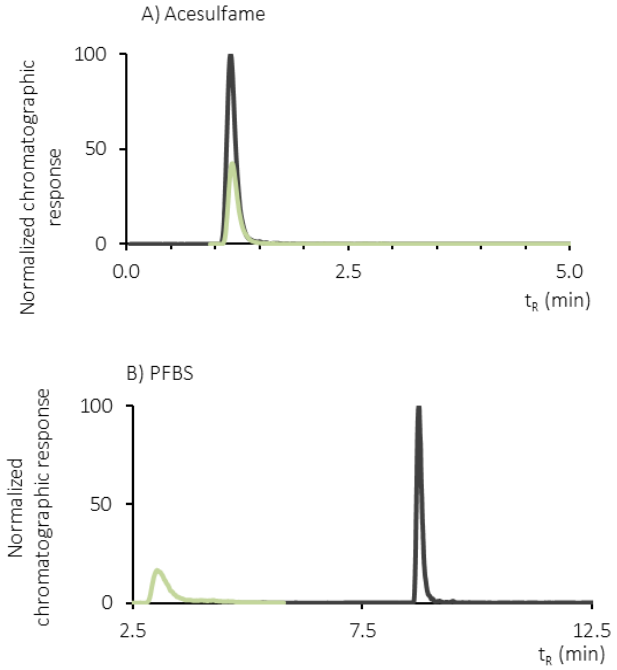

Figure 3.1. *The influence of the column phase on a)acesulfame and b) PFBS analytes for both columns Kinetex F5 (black line) and Kinetex Biphenyl (green line).*

3.3.3 Filtration

An important factor in the analysis of organic compounds in water samples is the filtration step. Water filtration is a highly controversial procedure due to the risk of losing partially some of the analytes in the filters (Baker and Kasprzyk-Hordern, 2011b; Petrovic, 2014). Therefore, possible losses during the water samples filtration step were investigated by means of two widely used filters: 1.2 μm glass microfiber and 0.45 μm cellulose nitrate membrane filters (47 mm diameter, Whatman). The assays were carried out using 100 mL of Milli-Q, WWTP effluent, estuary and seawater samples spiked at 400 ng/L with all the target analytes, before and after filtration. The absolute recovery results included in **Table 3.4** showed two different tendencies based on the particulate matter content and on the filters material nature. Analytes were less retained on both filters in the case of effluent and estuary samples compared with the retention observed in seawater or Milli-Q, probably due to the highest particulate matter content present in the case of effluent and estuary samples, which seems to minimize the retention of analytes independently of the filters nature. Moreover, the retention of the target analytes occurred mostly on 0.45 μm cellulose nitrate membrane filters (0-67%, 0-95% and 0-95% for effluent, estuary samples and seawater, respectively) compared to the 1.2 μm glass microfiber filters (0-46%, 0-68% and 0-7% for effluent, estuary and seawater, respectively). Therefore, only effluent was filtered with 1.2 μm glass microfilters which rendered the lowest analyte losses (the adsorption was negligible, <% 20, for the majority of analytes) as reported in the literature (Baker and Kasprzyk-Hordern, 2011b; Petrovic, 2014). The rest of water matrices were not filtered before analysis by PES or SPE

Table 3.4: Absolute recovery of analytes after 1.2 and 0.45 µm filtration steps.

Analyte	1.2 µm glass microfilter				0.45 µm cellulose nitrate			
	Milli-Q water	Effluent	Estuary	Seawater	Milli-Q water	Effluent	Estuary	Seawater
Acesulfame	78±3	106±5	91±4	65±9	70±3	103±9	73±3	67±7
Acetaminophen	64±2	97±13	85±7	59±2	58±5	95±5	104±11	43±13
Amitriptyline	30±1	79±3	60±2	33±3	4.1±0.3	13.3±0.3	7±2	5±1
Atrazine	99±6	104±3	96±2	93±8	101±10	96±4	105±6	92±10
Bezafibrate	10±7	101±1	84±8	35±5	12.7±0.3	94±4	89±8	11±2
Butylparaben	94±9	95±2	96±4	89±11	95±3	90±2	95±7	88±14
Caffeine	73±6	103±1	93±8	69±11	55±8	102±3	52±8	45±13
Carbamazepine	85±10	98±2	96±3	93±4	87±9	92±3	98±2	79±8
Ciprofloxacin	35±5	106±6	80±7	28±1	12.9±0.1	74±4	33±0.2	15.2±0.8
Clofibric acid	90±8	101±2	92±6	88±9	82±6	102±1	85±5	76±8
Clomipramine	36±1	71±1	45±6	40±2	22.7±0.3	45±1	33±1	25±0.5
Diclofenac	63±5	103±9	85±3	76±8	48±7	105±6	101±7	68±2
Diuron	88±7	103±1	90±6	93±13	92±4	91±3	83±3	89±10
Eprosartan	81±9	104±3	93±5	97±3	50±8	86±2	92±3	65±8
Genistein	76±2	104±4	93±8	85±5	61±6	90±16	111±12	53±4
Genistin	81±7	89±8	91±8	87±12	86±38	91±5	67±9	69±6
Glycitin	75±11	84±2	78±5	62±6	32±6	88±11	31±3	58±9
Imipramine	70±8	85±5	75±3	78±8	28±3	50±1	25±8	33±2
Irbesartan	24±1	105±8	90±8	68±4	1.8±0.1	62±4	53±6	16±3
Isoproturon	53±1	101±1	82±6	73±13	25.5±0.3	94±6	83±8	25±4
Ketoprofen	90±8	98±1	93±7	82±5	93±3	92±6	97±7	97±8
Losartan	75±12	102±4	83±9	69±7	53±2	88±3	42±11	55±6
Methylparaben	77±8	95±2	86±6	91±13	95±1	90±15	88±11	63±5
Norfloxacin	45±3	107±4	82±8	55±3	22±2	74±3	27±3	18±2
Nortriptyline	40±2	76±6	60±6	59±8	14.1±0.1	82±3	36±6	23±5
OBT	99±2	93±10	95±6	97±3	94±6	100±3	94.5±0.6	95±10
PFBS	74±9	106±7	92±7	79±5	89±3	102±11	36±7	78±11
PFOA	15±1	106±19	90±3	34±6	33±1	99±3	48±6	42±2
PFOS	10±1	74±9	42±3	7±1	11.7±0.1	48±9	65±7	12.4±0.6
PFOSA	26±3	105±7	83±5	35±2	13.8±0.8	68±11	23±14	19±2
Phenytoin	39±5	98±6	81±3	66±5	48±5	92±7	58±11	63±9
Progesterone	103±9	100±3	96±5	92±6	60±2	62±1	50±1	36±10
Propranolol	89±8	102±6	99±1	94±6	42.1±0.2	48±4	45±4	38±6
Simazine	47±9	110±9	96±3	99±1	63.4±0.3	98±2	102±10	72±7
Sucralose	67±10	99±1	98±3	59±7	43±9	97±3	85±1	46±10
Sulfadiazine	65±10	95±1	90±3	73±12	50±2	85±11	66±7	66±10
Sulfamethoxazole	75±8	97±4	86±8	71±7	71±4	80±6	85±4	56±12
Telmisartan	69±9	79±15	83±7	78±5	36±2	74±5	52±3	63±10
Testosterone	51±4	104±4	93±11	56±4	43±1	86±1	52±3	38±5
Trimethoprim	56±10	104±3	91±3	74±2	70±10	86±1	63±5	83±7
Valsartan	57±2	105±6	86±8	39±8	12.3±0.1	62±1	44±18	16±6

3.3.4 PES protocol optimisation

3.3.4.1 Desorption conditions

MeOH solvent which is expected to display high affinity for polar analytes and it is compatible with the PES material was chosen as the desorption solvent (Bizkarguenaga et al., 2015; Blanco-Zubiaguirre et al., 2014; Casado et al., 2013; Ros et al., 2015; Villaverde-de-Sáa et al., 2012). The efficiency of desorption step was investigated soaking each piece (24 mg) of sorbent previously exposed to 400 ng/L spiked Milli-Q water samples. Chemical desorption of the analytes from the sorbent was performed 5 consecutive times (8 min each) using 500 µL of MeOH in an ultrasonic bath. 500 µL were chosen as the minimum volume which assures that all PES tubes were completely covered by the MeOH organic solvent. Recoveries in the range of 75-105% were obtained for all the target analytes after the three first desorption cycles except for clomipramine, diclofenac, butylparaben and telmisartan which were detected in the 4th desorption fraction (17-34%). Thus, 4 desorption cycles were selected as optimal desorption time (32 min) and used in further experiments.

3.3.4.2 Microextraction conditions

Factors affecting the analytes extraction (PES amount, sample pH, ionic strength and EDTA addition) were evaluated in order to achieve the optimum extraction conditions. Optimisation of extraction conditions was performed with spiked (400 ng/L) Milli-Q water samples using 120 mL and stirring speed of 800 rpm and considering an extraction period of 12 h (overnight).

In order to improve the extraction efficiency of the target analytes, the amount (2, 3 and 4 pieces of 4 cm each) of PES material (24-48 mg) was evaluated (n=3). The extraction efficiency increased from 15 to 43% with the use of 3 pieces of PES compared with the use of 2 pieces, but no significance differences (F_{Exp}=1.9-7.2 < F_{Crit}=7.7, at 95% of confidence level according to the analysis of variance, ANOVA) were observed between 3 and 4 pieces for most (29 of 41) of the analytes, except in the cases of acesulfame, PFASs, fluoroquinolones, sulfonamides and lipids regulating compounds, which showed slightly higher extraction efficiencies using 4 PES pieces (F_{Exp}=7.9-11.1 > F_{Crit}=7.7, according to ANOVA at 95% of confidence level). Thus, 4 pieces (48 mg) were

selected as optimum PES amount for all the target analytes and used in further experiments.

The addition of a chelating agent such as EDTA prior to the extraction is a very common procedure in SPE protocols (Gros et al., 2013; Hernández et al., 2014) since a considerably improve of the extraction efficiency of antibiotics as well as other pharmaceutical compounds has been observed. This is attributed to the fact that these compounds can potentially bind residual metals present in the sample matrix and glassware, resulting in low extraction recoveries. In this sense, real water samples (effluent, estuary and seawater) were spiked at 400 ng/L with all the target analytes and the samples analytes recoveries calculated (n=3) with and without the addition of EDTA. With this aim, a suitable volume of a EDTA solution was added to achieve a final concentration of 0.1%. As it can be observed in Figure 3.2 (one analyte of each family has been included as example) in the case of effluent, the recoveries of fluoroquinolones, sulfonamides, PFASs and artificial sweeteners were enhanced nearly 21-37% with the addition of EDTA. Not significant differences (p-value>0.05 according to ANOVA) were observed for the rest of the target analytes with and without EDTA addition. Similar results were obtained in the case of estuary and seawater matrices. These results could be due to the fact that antibiotics from the groups of fluoroquinolones and sulfonamides have a high tendency to complex with metal (Ca (II), Mg (II), Al (III), Fe (III), etc.) ions present in the water samples as it has been previously reported in the literature (Seifrtová et al., 2008). Additionally, the presence of EDTA may also minimize the adsorption of these compounds onto the glass wall containing silicate and silanol groups that act as ion-exchange and nucleophilic centres (Mompelat et al., 2009). In this sense, the amount of analyte remaining in the wall phase with and without the addition of EDTA was also estimated. Thus, the extraction vessels were sonicated with 7 mL of MeOH which was transferred to a test tube and evaporated to 200 µL and analysed by means of LC-MS/MS. Since less than 10% of the total amount of target analytes was adsorbed in the wall, not significant differences were observed (p-value>0.05 according to ANOVA) independently of the EDTA addition or not, the affinity between the target analytes and the wall of the extraction vessels was considered negligible. Anyway, since the recoveries of some of the target analytes were enhanced with the addition of EDTA, it was used in further experiments.

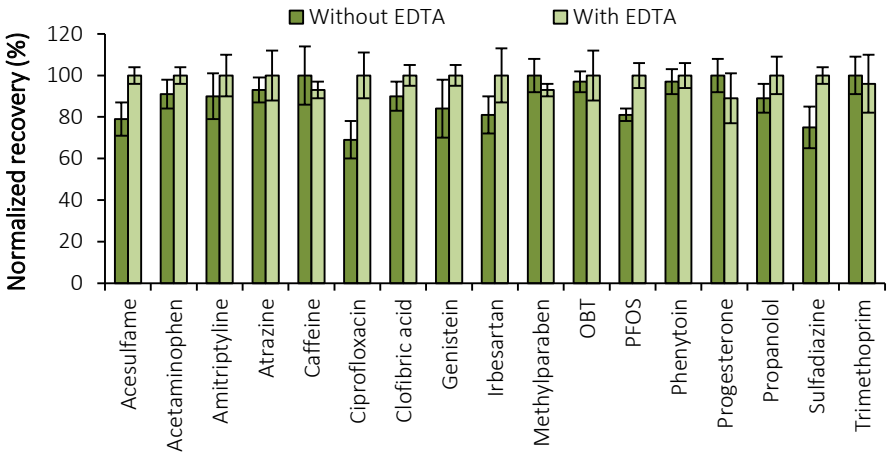

Figure 3.2. *Influence of the addition of EDTA in the case of PES protocol. As an example, only one analyte per family is shown.*

The effect of sample pH (2-12, 2, 7 and 12 pH levels), adjusted with HCOOH or ammonia, and ionic strength (0-30% of NaCl addition, 0, 15 and 30% levels) was studied by means of a Central Composite Design (CCD, 12 experiments, two repetitions of each experiment) with four replicates of the central point. Responses (peak areas) measured for each compound were processed with the Statgraphics Centurion 16.1 software (Startpoint Technologies, Warrenton, VA, USA). Relative standard deviations (RSDs%) of the central experiments (n=4) were in the 1-20% range, except for PFOS (35%) and Caffeine (37%).

The percentage of NaCl had a statistically significant positive effect in the responses of the 76% of the analytes (see **Figure 3.3a** for amitriptyline as example). On the other hand, the addition of NaCl had a negative effect (see **Figure 3.3b** for PFOSA) for the rest of compounds (artificial sweeteners and PFASs) or even it was not statistically significant for OBT, parabens, caffeine, clofibric acid and bezafibrate. This behaviour is explained on the basis of the well-known salting out effect (Prieto et al., 2010). In general terms, it has been observed that for hydrophobic analytes (see the log D values included at **Table 3.1**) the addition of NaCl does not improve, but even reduces, the extraction efficiency, due to the increase in the viscosity of the sample, leading to slower extraction

kinetics for the most lipophilic species. However, polar analytes profit from the higher ionic strength of the sample solution and the response increases with the addition of inert salts. The results observed in this work are in good agreement with the literature (Racamonde et al., 2015; Villaverde-de-Sáa et al., 2012).

The sample pH showed a statistically significant positive linear effect for basic compounds (pK_a=9-12) such as tricyclic antidepresants, propranolol and trimethoprim (see **Figure 3.3a** for amitriptyline) or for acid compounds with basic groups such as atrazine and simazine (see pK_a values included in **Table 3.1**). On the other hand, in the case of acid compounds, 17 analytes showed statistically significant negative effect (see **Figure 3.3b** for PFOSA, as example). Besides, a negative quadratic pH term was obtained in the case of sulfadiazine (see **Figure 3.3c**) which shown its maximum response around pH 6. This could be explained according to multiple pK_a values of some of the target compounds which contain one basic amine group and one acidic sulfonamide group. Thus, while sulfonamides are positively charged at pH 2, they are negatively charged at alkaline conditions above pH 6. Similar behaviour was observed for irbesartan and losartan. For the rest of the analytes, pH did not show any statistically significant response. Finally, the interaction between NaCl and pH terms was only statistically relevant for the anti-inflammatory compounds. Therefore, 30% of NaCl addition was selected as optimum and the extractions were carried out at both pH=2 and pH=10 (dual extraction). Real samples salinity was always measured before extraction, and the salinity adjusted to 30% with the NaCl addition.

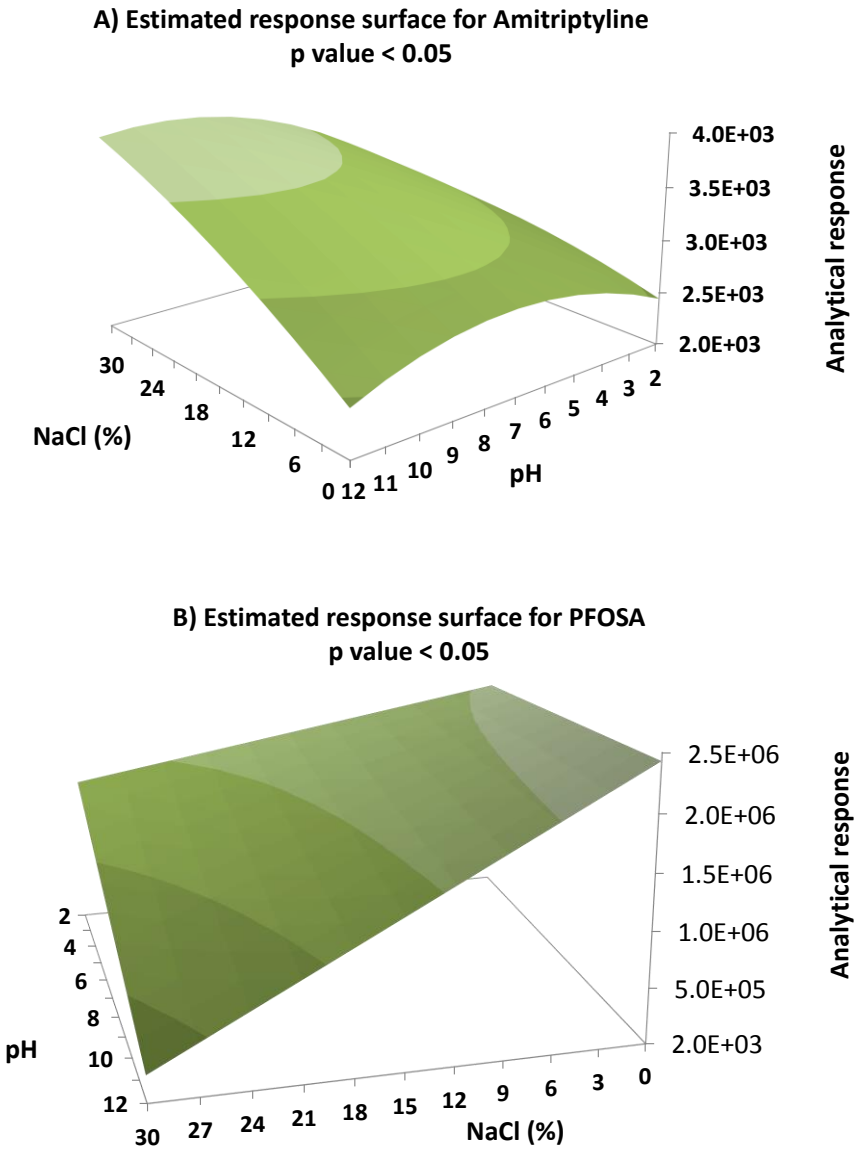

Figure 3.3. *Response surfaces obtained after a CCD for three of the target compounds: (a) Amitriptyline (pK_a=9.8), and (b) PFOSA (pk_a=3.4)*

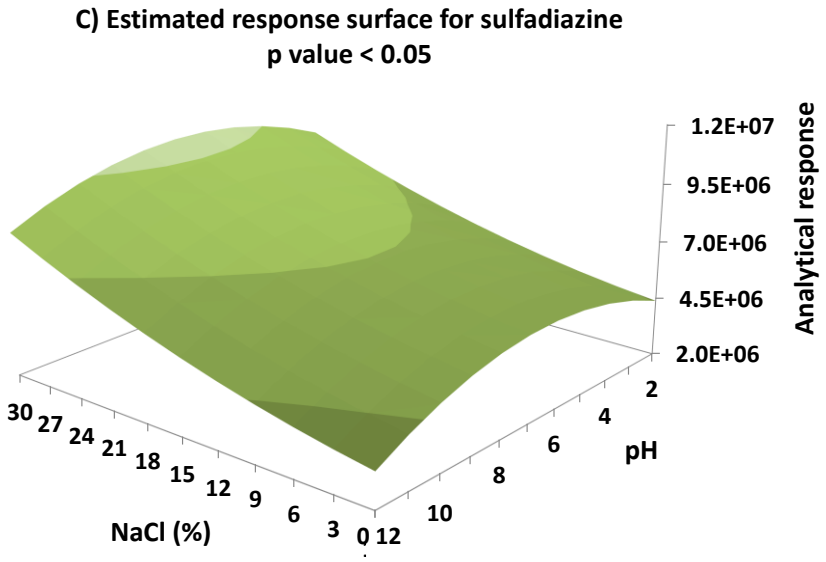

Figure 3.3. *Response surfaces obtained after a CCD for three of the target compounds: (c) Sulfadiazine (pK_a=2.0 and 7.5).*

3.3.5 Matrix effect

The extraction efficiency can be affected by the composition of the sample matrix since high levels of matrix compounds may compete with the sorptive material or can lead to matrix effects during LC-MS/MS determination due to changes of the ESI ionization efficiency.

In this sense, first of all, matrix effects occurring at LC-MS/MS detection were evaluated by comparing the responses obtained for seawater, estuary and effluent samples which were spiked at 100 ng/L of the target analytes after the extraction by means of both PES and SPE procedures (see sections 3.2.3 and 3.2.4) and a standard solution of MeOH: Milli-Q water (30:70, v/v) spiked at the same analyte concentration. Non-spiked blank samples were also analysed and considered in the detection matrix effect calculation. Thus, recoveries were determined according to Equation 3.2, where values close to 100% indicate a lack of matrix effect. Recoveries (71-83% and 75-124% in the cases of PES and SPE protocols, respectively) close to 100% were obtained for the majority of the

analytes in the case of the three evaluated matrices and for both extraction protocols indicating a very low detection matrix effect, except in the case of fluoroquinolones (51-56%) for effluent and SPE procedure which showed a slightly signal suppression.

$$ME\% = 100 \times \frac{Response_{spiked\ sample} - Response_{unspiked\ sample}}{Response_{spiked\ standard\ solution}} \quad \text{Equation 3.2}$$

Subsequently, the matrix effects that take place during the extraction were also estimated by comparing the analytes responses obtained for Milli-Q water and real matrix (seawater, estuary and effluent) spiked at 100 ng/L of each analyte before (n=3) and after (n=3) the extraction using once again both, SPE and PES protocols in order to exclude this time the detection matrix effect. Once again, non-spiked samples were also analysed and considered for calculations (**Equation 3.3**).

$$ME\% = 100 \times \frac{Response_{spiked\ sample} - Response_{unspiked\ sample}}{Response_{spiked\ Milli-Q\ water}} \quad \text{Equation 3.3}$$

As it can be observed in **Figures 3.4a-c** (one analyte of each family has been included as example) for the three evaluated matrices, acceptable recoveries (65-127%) were obtained in the case of PES procedure independently of the matrix considered. Anyway, the lowest or highest recoveries were obtained in the case of effluent (see **Figure 3.4a**). A positive or negative matrix effect in the case of SPE protocol was, however, slightly higher (recoveries in the range of 51-155%, 73-133% and 55-115% for effluent, estuary water and seawater, respectively), showing that PES microextraction provided cleaner extracts and probably less co-extraction of interfering compounds. These matrix effects accounting may be compensated by the use of labelled standards, which will be evaluated in the method validation section (see **section 3.3.6**).

3.3.6 Method validation

The validation results obtained are summarized in **Tables 3.5 and 3.6**. Process efficiencies higher than 50% were achieved by SPE for the majority of target compounds and for the three evaluated matrices, except in the case of ciprofloxacin and losartan for effluent. Process efficiencies in the range of 6–68% were achieved by PES extraction (see **Table 3.5**). Sulfonamides and fluoroquinolones showed a low process efficiency (<20%). In the case of sulfonamides and sartans the low PE could be mainly attributed to the fact that the experimental conditions chosen are not

always the most appropriate for those specific compounds since a compromise has to be found. Nevertheless, the low PE obtained were not considered an obstacle for their determination in environmental waters, as their sensitivity in LC-MS/MS analysis was fairly good (see **Table 3.2** for instrumental LOQs).

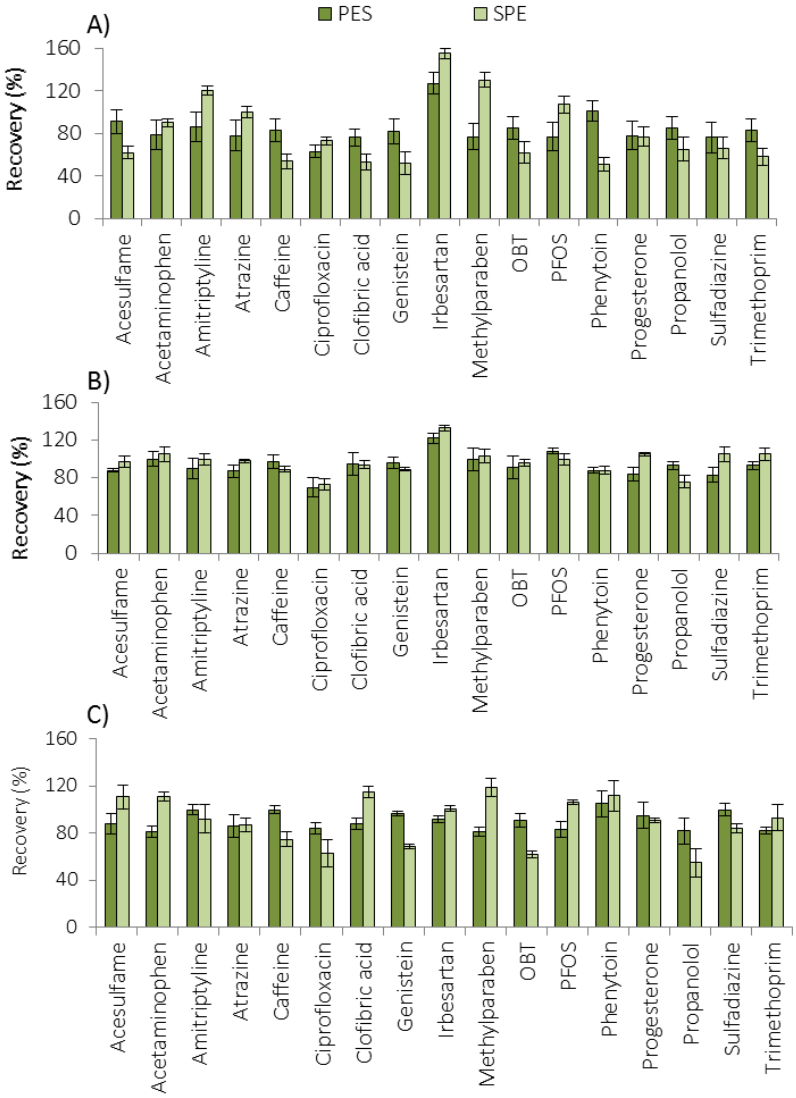

Figure 3.4. *Matrix effect during the PES and SPE protocols in the cases of (a) effluent, (b) estuary and (c) seawater for 17 of the target analytes.*

Concerning to the apparent recoveries, although good apparent recoveries using external calibration and surrogate corrections were obtained in the 70–131% range for all the target analytes and matrices in the case of SPE (see Table 3.6), this approach was not a properly strategy in the case of PES (33–213%). Consequently, a procedural calibration with Milli-Q using isotopically labelled analogues as surrogates approach was considered (see section 3.2.6). Determination coefficients higher than 0.986 were obtained in all the cases after correction with the corresponding isotopically labelled analogues. Acceptable apparent recovery values (see Table 3.6) in the 80–119% range were obtained.

Thus, acceptable apparent recoveries were obtained for all the analytes and matrices by means of both, SPE and PES protocols. In fact, all the values were in compliance with the requirements (i.e. trueness, as mean recovery, must be in the range of 70‑130%) of Commission Decision 2002/657/EC (European Commission, 2002), and similar to the those reported in the literature (Tran et al., 2013; Valls-Cantenys et al., 2016) for some of the target compounds determined in different environmental aqueous samples and using different extraction protocols.

Additionally, the repeatability of PES and SPE methods in terms of RSD% were evaluated for five replicates analysed within a day. RSD values were below 27% for all the target analytes in the case of both, PES and SPE methods. Similar RSD values were also reported in the literature (Valls-Cantenys et al., 2016).

The MQL values were determined (see section 3.2.6) and included in Table 3.7. Similar values for both, SPE and PES protocols in the case of effluent matrix were achieved for all the target analytes. However, higher MQLs were obtained for most of the analytes in the case of estuary and seawater using PES compared to SPE. MQLs obtained are in agreement with method detection limits (MDLs) and/or MQLs reported in the literature for some of the target compounds analysed by means of different extraction and microextraction techniques (Bizkarguenaga et al., 2015; Casado et al., 2013; Racamonde et al., 2015; Valls-Cantenys et al., 2016; Villaverde-de-Sáa et al., 2012). It should be underlined the simultaneous determination of 41 organic analytes among a high variety of compounds families considered in this work.

Table 3.5. Process efficiency (%) of the validated procedures by means of PES and SPE for effluent, estuary and seawater.

Analytes	Process efficiency (PE%, n=3)					
	PES			SPE		
	Effluent	Estuary	Seawater	Effluent	Estuary	Seawater
Acesulfame	14±4	17±3	18±3	51±12	56±7	62±9
Acetaminophen	25±3	28±7	32±8	139±28	91±25	97±8
Amitriptyline	47±12	49±11	55±14	68±8	71±16	68±10
Atrazine	25±3	27±6	32±5	93±7	90±16	110±10
Bezafibrate	44±8	48±13	53±7	96±25	107±21	101±12
Butylparaben	41±7	46±10	54±8	71±13	95±19	84±19
Caffeine	30±6	32±5	37±5	78±3	93±20	83±10
Carbamazepine	41±8	43±11	48±12	134±36	108±14	114±27
Ciprofloxacin	9±2	11±2	12±3	26±5	43±3	31±3
Clofibric acid	26±8	32±4	34±4	81±19	86±8	77±20
Clomipramine	51±13	57±14	65±18	53±4	63±5	58±11
Diclofenac	54±11	63±10	68±18	57±10	116±31	95±7
Diuron	37±6	42±11	49±13	76±18	103±9	82±15
Eprosartan	28±7	30±9	34±7	79±13	56±8	88±8
Genistein	35±10	39±11	46±10	42±8	93±14	79±10
Genistin	45±11	48±10	53±11	67±11	99±14	80±22
Glycitin	36±5	45±12	48±9	46±7	87±22	75±11
Imipramine	37±6	41±7	48±10	50±10	64±4	57±13
Irbesartan	26±4	31±8	34±5	58±9	62±11	73±8
Isoproturon	43±11	47±7	51±9	75±5	85±12	90±22
Ketoprofen	42±11	43±8	51±10	108±10	127±19	162±23
Losartan	24±6	26±4	30±6	32±5	51±8	52±9
Methylparaben	48±6	53±12	61±18	96±26	102±22	111±17
Norfloxacin	12±4	13±2	15±3	71±5	53±8	55±14
Nortriptyline	39±10	44±10	51±15	70±10	58±7	56±15
OBT	45±12	49±13	57±10	67±12	90±7	86±13
PFBS	20±5	21±5	25±6	70±13	55±13	79±21
PFOA	18±3	19±4	21±4	78±9	86±6	59±9
PFOS	32±6	35±9	40±11	80±13	96±11	88±7
PFOSA	34±5	36±7	42±11	78±19	55±7	56±4
Phenytoin	44±9	51±10	59±13	141±16	115±24	126±26
Progesterone	51±14	56±12	61±9	62±10	95±26	80±21
Propranolol	27±5	30±6	36±9	70±15	77±7	90±13
Simazine	34±9	36±10	42±12	84±10	96±8	114±24
Sucralose	10±2	12±3	13±3	59±4	59±9	71±9
Sulfadiazine	9±1	10±2	11±2	78±21	48±10	84±23
Sulfamethoxazole	6±1	6±2	7±2	91±6	58±8	136±22
Telmisartan	35±6	39±11	46±7	64±17	57±9	84±11
Testosterone	39±9	40±5	46±12	128±29	80±12	107±22
Trimethoprim	32±8	32±9	38±11	67±7	53±12	66±15
Valsartan	22±3	25±5	26±6	100±14	60±7	91±15

Table 3.6. Apparent recovery (%) of the validated procedures by means of PES and SPE for effluent, estuary and seawater.

Analytes	Apparent recovery (%, n=3)					
	PES-procedural calibration			SPE-external calibration		
	Effluent	Estuary	Seawater	Effluent	Estuary	Seawater
Acesulfame	14±4	17±3	18±3	51±12	56±7	62±9
Acetaminophen	25±3	28±7	32±8	139±28	91±25	97±8
Amitriptyline	47±12	49±11	55±14	68±8	71±16	68±10
Atrazine	25±3	27±6	32±5	93±7	90±16	110±10
Bezafibrate	44±8	48±13	53±7	96±25	107±21	101±12
Butylparaben	41±7	46±10	54±8	71±13	95±19	84±19
Caffeine	30±6	32±5	37±5	78±3	93±20	83±10
Carbamazepine	41±8	43±11	48±12	134±36	108±14	114±27
Ciprofloxacin	9±2	11±2	12±3	26±5	43±3	31±3
Clofibric acid	26±8	32±4	34±4	81±19	86±8	77±20
Clomipramine	51±13	57±14	65±18	53±4	63±5	58±11
Diclofenac	54±11	63±10	68±18	57±10	116±31	95±7
Diuron	37±6	42±11	49±13	76±18	103±9	82±15
Eprosartan	28±7	30±9	34±7	79±13	56±8	88±8
Genistein	35±10	39±11	46±10	42±8	93±14	79±10
Genistin	45±11	48±10	53±11	67±11	99±14	80±22
Glycitin	36±5	45±12	48±9	46±7	87±22	75±11
Imipramine	37±6	41±7	48±10	50±10	64±4	57±13
Irbesartan	26±4	31±8	34±5	58±9	62±11	73±8
Isoproturon	43±11	47±7	51±9	75±5	85±12	90±22
Ketoprofen	42±11	43±8	51±10	108±10	127±19	162±23
Losartan	24±6	26±4	30±6	32±5	51±8	52±9
Methylparaben	48±6	53±12	61±18	96±26	102±22	111±17
Norfloxacin	12±4	13±2	15±3	71±5	53±8	55±14
Nortriptyline	39±10	44±10	51±15	70±10	58±7	56±15
OBT	45±12	49±13	57±10	67±12	90±7	86±13
PFBS	20±5	21±5	25±6	70±13	55±13	79±21
PFOA	18±3	19±4	21±4	78±9	86±6	59±9
PFOS	32±6	35±9	40±11	80±13	96±11	88±7
PFOSA	34±5	36±7	42±11	78±19	55±7	56±4
Phenytoin	44±9	51±10	59±13	141±16	115±24	126±26
Progesterone	51±14	56±12	61±9	62±10	95±26	80±21
Propranolol	27±5	30±6	36±9	70±15	77±7	90±13
Simazine	34±9	36±10	42±12	84±10	96±8	114±24
Sucralose	10±2	12±3	13±3	59±4	59±9	71±9
Sulfadiazine	9±1	10±2	11±2	78±21	48±10	84±23
Sulfamethoxazole	6±1	6±2	7±2	91±6	58±8	136±22
Telmisartan	35±6	39±11	46±7	64±17	57±9	84±11
Testosterone	39±9	40±5	46±12	128±29	80±12	107±22
Trimethoprim	32±8	32±9	38±11	67±7	53±12	66±15
Valsartan	22±3	25±5	26±6	100±14	60±7	91±15

Table 3.7. MQLs (ng/L) obtained by SPE-LC-(ESI)-MS/MS and PES-LC-(ESI)-MS/MS methods obtained with the F5 column in the case of effluent, estuary and seawater sample.

Analyte	MQLs (ng/L)					
	Effluent		Estuary		Seawater	
	PES	SPE	PES	SPE	PES	SPE
Acesulfame	2.6	1.8	2.2	0.7	2.0	0.6
Acetaminophen	6.2	2.8	5.7	1.7	4.9	1.6
Amitriptyline	1.0	1.8	1.0	0.7	0.9	0.7
Atrazine	0.7	0.5	0.6	0.2	0.5	0.2
Bezafibrate	0.7	0.8	0.6	0.3	0.5	0.3
Butylparaben	3.9	5.6	3.5	1.7	3.0	1.9
Caffeine	2.5	2.5	2.4	0.8	2.1	0.9
Carbamazepine	0.6	0.4	0.5	0.2	0.5	0.2
Ciprofloxacin	25.6	23.3	22.2	5.6	19.7	7.8
Clofibric acid	3.9	3.2	3.2	1.2	3.0	1.3
Clomipramine	0.5	1.2	0.5	0.4	0.4	0.5
Diclofenac	0.6	1.3	0.5	0.3	0.4	0.3
Diuron	1.0	1.2	0.9	0.4	0.8	0.5
Eprosartan	3.5	3.0	3.2	1.7	2.8	1.1
Genistein	5.6	11.7	5.0	2.1	4.3	2.5
Genistin	3.6	6.1	3.4	1.6	3.1	2.0
Glycitin	3.9	7.8	3.2	1.7	3.0	1.9
Imipramine	1.3	2.3	1.1	0.7	1.0	0.8
Irbesartan	1.0	1.1	0.8	0.4	0.7	0.3
Isoproturon	0.6	0.9	0.6	0.3	0.5	0.3
Ketoprofen	1.2	1.2	1.2	0.4	1.0	0.3
Losartan	4.0	7.5	3.7	1.9	3.2	1.8
Methylparaben	7.7	9.8	7.0	3.7	6.1	3.4
Norfloxacin	13.9	6.1	13.8	3.3	11.8	3.2
Nortriptyline	1.1	1.5	1.0	0.7	0.8	0.8
OBT	5.2	8.7	4.8	2.6	4.1	2.7
PFBS	2.2	1.6	2.1	0.8	1.8	0.6
PFOA	2.2	1.3	2.1	0.5	1.9	0.7
PFOS	0.8	0.8	0.8	0.3	0.7	0.3
PFOSA	0.8	0.9	0.8	0.5	0.6	0.5
Phenytoin	3.7	2.9	3.2	1.4	2.8	1.3
Progesterone	1.4	2.8	1.3	0.7	1.1	0.9
Propranolol	1.6	1.6	1.5	0.6	1.2	0.5
Simazine	1.2	1.2	1.1	0.4	1.0	0.4
Sucralose	2.7	1.1	2.2	0.4	2.0	0.4
Sulfadiazine	3.2	0.9	2.9	0.6	2.5	0.3
Sulfamethoxazole	9.9	1.5	8.6	1.0	7.9	0.4
Telmisartan	0.8	1.1	0.7	0.5	0.6	0.3
Testosterone	1.9	1.4	1.8	0.9	1.6	0.7
Trimethoprim	0.6	0.8	0.6	0.4	0.5	0.3
Valsartan	9.6	1.8	8.5	3.5	7.9	2.3

3.3.7 Application to real samples

In the absence of a properly certified reference material (CRM), inter-method comparability was carried out. The validated SPE and PES protocols were applied in the analysis (n=3) of seawater, estuarine and WWTP effluent (see **Table 3.8**). In the case of estuary 17 and 10, and in the case of seawater 11 and 6 analytes, were detected above their MQLs with SPE and PES protocols, respectively. Comparable concentrations (p-value>0.05 according to ANOVA) by means of both methodologies were determined for most of the detected analytes in the case of effluent (acetaminophen, bezafibrate, caffeine, carbamazepine, ciprofloxacin, diclofenac, eprosartan, irbesartan, ketoprofen, losartan, norfloxacin, OBT, sulfadiazine, sulfamethozaxole, telmisartan, trimethoprim and valsartan), estuary (acesulfame, caffeine, carbamazepine, diclofenac, irbesartan, OBT, telmisartan and valsartan) and seawater (butylparaben and OBT).

Table 3.8. Real samples concentrations (ng/L) obtained by SPE-LC-(ESI)-MS/MS and PES-LC-(ESI)-MS/MS methods in the case of effluent, estuary and seawater samples.

Analyte	Effluent		Estuary		Seawater	
	PES	SPE	PES	SPE	PES	SPE
Acesulfame	423±33	365±17	213±16	226±57	<MQL	13±1
Acetaminophen	213±19	174±39	n.d.	<MQL	n.d.	<MQL
Amitriptyline	23±4	37±3	<MQL	4.9±0.1	n.d.	2.9±0.1
Atrazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Bezafibrate	53±6	61±2	<MQL	7.5±0.9	n.d.	n.d.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	76±11	62±2	1023±67	914±85	85±6	94±6
Carbamazepine	78±2	87±8	16±3	13.4±0.9	n.d.	3.6±0.2
Ciprofloxacin	74±3	96±21	n.d.	<MQL	n.d.	<MQL
Clofibric acid	n.d.	<MQL	n.d.	<MQL	n.d.	n.d.
Clomipramine	<MQL	<MQL	n.d.	<MQL	n.d.	n.d.
Diclofenac	293±38	350±22	66±7	71.3±0.3	<MQL	25±3
Diuron	96±8	73±9	2.6±0.3	3.44±0.08	n.d.	n.d.
Eprosartan	456±23	515±79	n.d	< MQL	n.d.	n.d.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Imipramine	<MQL	<MQL	n.d.	<MQL	n.d.	n.d.
Irbesartan	1096±98	945±119	496±28	478±50	<MQL	21±1

Table 3.8. Real samples concentrations (ng/L) obtained by SPE-LC-(ESI)-MS/MS and PES-LC-(ESI)-MS/MS methods in the case of effluent, estuary and seawater samples.

Analyte	Effluent		Estuary		Seawater	
	PES	SPE	PES	SPE	PES	SPE
Isoproturon	<MQL	0.9±0.1	n.d.	n.d.	n.d.	n.d.
Ketoprofen	213±19	184±9	<MQL	3.5±0.1	n.d.	n.d.
Losartan	217±24	178±13	n.d.	<MQL	n.d.	<MQL
Methylparaben	n.d.	n.d.	n.d.	n.d.	13±2	<MQL
Norfloxacin	76±4	124±53	n.d.	<MQL	n.d.	n.d.
Nortriptyline	10±2	7.5±0.1	<MQL	<MQL	n.d.	n.d.
OBT	82±12	106±10	81±11	71±5	211±19	179±12
PFBS	56±3	62±2	n.d.	14.4±0.2	n.d.	n.d.
PFOA	<MQL	8.4±0.3	n.d.	n.d.	n.d.	n.d.
PFOS	9.6±0.8	5.5±0.1	<MQL	10±1	n.d.	n.d.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenytoin	n.d.	<MQL	n.d.	n.d.	n.d.	n.d.
Progesterone	<MQL	<MQL	n.d.	n.d.	n.d.	n.d.
Propranolol	26±4	19.5±0.8	n.d.	<MQL	n.d.	n.d.
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sucralose	713±56	621±89	463±53	435±23	<MQL	20±3
Sulfadiazine	56±7	70±8	<MQL	<MQL	n.d.	n.d
Sulfamethoxazole	59±8	62±7	<MQL	2.3±0.3	n.d.	n.d.
Telmisartan	253±19	285±51	412±33	384±23	<MQL	19.4±0.7
Testosterone	<MQL	<MQL	n.d.	n.d.	n.d.	n.d.
Trimethoprim	123±10	98±17	<MQL	10.0±0.1	n.d.	n.d.
Valsartan	489±50	551±52	546±78	623±76	<MQL	30±5

n.d.: not detected, MQL: method quantification limit.

3.4 Conclusions

A new procedure based on a PES microextraction followed by LC-MS/MS was developed and applied for the determination of 41 multiclass organic pollutants (of which 5 are WFD-priority) in seawater, estuary and WWTPs effluents. With this aim, all the steps involving the analytical performance, such as filtration, extraction and analysis were thoroughly optimised. Filtration had a significant impact on the outcome of fate and behaviour of target analytes since it was observed that a high proportion of analytes were bound to filterable particulates. A thorough optimisation of LC–MS/MS analysis was carried out including the chromatographic column, the ionisation conditions

and the mass spectrometric variables. It should be concluded that Kinetex F5 100 mm core-shell column provided better results than Kinetex biphenyl column with the same length and particle diameter in terms of peak resolution, peak symmetry and sensitivity in the case of PFASs and artificial sweeteners. The efficiency of the microextraction step for acid and neutral compounds was mainly conditioned by the sample pH, while for basic compounds both, sample pH and NaCl addition variables were significant. As it is known, a multiresidue or multiclass method comes from the necessity of adjusting the pH to a single value, thus, a compromise has to be found. However, the dual PES microextraction carried out here allowed us the simultaneous determination of acid, neutral and basic compounds. The PES procedure was compared with a previously validated standard SPE procedure. In contrast to SPE protocol, PES method allowed the extraction of complex aqueous samples with lower matrix effect, cost and consumption of organic solvents. Satisfactory and comparable apparent recovery values and MQLs, regardless of the matrix, were obtained.

Finally, the methods were applied to the analysis of the target compounds in several WWTPS effluents and estuaries of Biscay, as will be described in chapter 7.

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Multiresidue method in biota

Multiresidue analytical method for the determination of 41 multiclass organic pollutants in mussel, fish tissues and biofluids by liquid chromatography coupled to tandem mass spectrometry

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4.1 Introduction

The fate and occurrence of non-regulated contaminants in estuarine and coastal waters is still a matter of growing interest (Sousa et al., 2018) In fact, the presence of these chemicals, usually referred as Emerging Contaminants (ECs) (Ternes et al., 2015), in aquatic environments and organisms is often reported but their potential hazards or adverse effects are still under study (Davis et al., 2016). In this sense, the determination of the distribution of the exposed contaminants is required to understand the impact of the exposure, to assess the risks to aquatic life or to implement food safety measures (Miller et al., 2018). Among the reported ECs we can find a wide variety of pharmaceutical and personal care products (PPCPs), currently used pesticides and industrial compounds. Concerning the analytical approaches most of the reported procedures make use of screening or multiresidue methods to quantify known contaminants and, more recently, either suspect or non-targeted methods to identify as many contaminants as possible (Schymanski et al., 2015).

The development of multiresidue methods in complex samples such as fish and mussel tissues follow the typical workflow of sample extraction, clean-up and chromatographic analysis and each method is fine tuned to the specific features of the target contaminants and the sample itself (Núñez et al., 2017). Broadly speaking, the first two steps allow the analysis of a largest amount of contaminants with the minimum payoff in terms of matrix effect or interferences. The final step is usually the liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) since most of the target compounds are slightly or very polar and hardly volatile (Núñez et al., 2017). Regarding the extraction, most of the reported methods combine the use of solvent mixtures with the external energy supply (either microwave, ultrasounds or pressurized solvents) (Núñez et al., 2017). For cleaning the extracts up, solid phase extraction (SPE) has shown good performance in the purification of ECs from biota extracts being the most commonly applied technique (Miller et al., 2018), and allows the preconcentration of the sample. Typical sorbents for SPE include Oasis HLB (hydrophilic–lipophilic balanced) due to their good retentions and highly reproducible recoveries of a wide range of compounds. However, in the case of matrices with high lipid content as fish liver, a further purification procedure is also required. Several techniques have been described in the literature as alternative clean-up protocols to reverse-phase SPE: gel permeation chromatography

(Huerta et al., 2013; Tanoue et al., 2014), solid-phase microextraction (Ros et al., 2015), normal phase SPE (Navarro et al., 2010) and liquid-liquid extraction (LLE) (Al-Ansari et al., 2011; Ziarrusta et al., 2017b).

In the framework of previous analytical developments (Ros et al., 2016), environmental monitoring studies (Mijangos et al., 2018), and the exposure studies of fishes to ECs (Ziarrusta et al., 2017a), we observed the need to develop a target multiresidue method for precise and accurate measurements in biota samples. In this particular work, we developed the simultaneous determination of 41 multi-class organic pollutants in mussel, tissues and fluids of gilt head bream. In the case of the solid tissues, both the optimisation of a focused ultrasound solid-liquid extraction (FUSLE) method and the comparison of different clean-up approaches by means of SPE using Oasis-HLB and Florisil phases, microextraction based on polyethersulfone (PES) polymer and LLE were carried out. In the case of seawater and biofluids, a previously developed SPE method (Mijangos et al., 2018) was applied. The analyses were performed by liquid-chromatography-triple quadrupole tandem mass spectrometry (LC-MS/MS) in all the cases. Furthermore, the uptake and distribution of ten (acesulfame, sulfadiazine, acetaminophen, carbamazepine, PFBS, diuron, amitriptyline, irbesartan, butylparaben and bezafibrate) of the target analytes were investigated in tissues (brain, liver, gill, muscle) and biofluids (plasma, bile) of juvenile gilt-head bream (*Sparus aurata*) exposed to them during 7 days in seawater, under controlled dosing experiments. Finally, mussel (*Mytilus galloprovincialis*) sampled in the Basque coast were also collected and analysed.

4.2 Experimental section

4.2.1 Reagents and materials

The target analytes with their corresponding families, CAS number, molecular formula and weight and some of their physico-chemical properties such as the acid dissociation constant (pK_a) and the log of octanol–water partition coefficient (log P) are included in **Table 3.1, Chapter 3**.

2-hydroxybenzothiazole (OBT), amitriptyline hydrochloride, butylparaben, caffeine, carbamazepine, clomipramine hydrochloride, diclofenac sodium salt, potassium perfluoro-1-

octanesulfonate (PFOS), imipramine hydrochloride, methylparaben, nortriptyline hydrochloride, perfluoro-n-octanoic acid (PFOA), phenytoin, perfluoro-1-butanesulfonate (PFBS), progesterone, sulfadiazine and testosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetaminophen, atrazine, ciprofloxacin hydrochloride, diuron, isoproturon, norfloxacin hydrochloride, simazine, sulfamethoxazole and trimethoprim were acquired from Fluka (Buchs, Switzerland). Acesulfame and sucralose were supplied by Supelco (Bellefonte, PA, USA), whereas clofibric acid, ketoprofen, bezafibrate and propranolol hydrochloride were acquired from MP biomedicals (Illkirch Cedex, France). Genistein, genistin and glycitin were purchased from Extrasynthese (Lyon, France), perfluorooctane sulfonamide (PFOSA) from Dr. Ehrenstorfer (Augsburg, Germany), losartan from Merck (Darmstadt, Germany), valsartan and telmisartan from Boehringer (Ingelheim am Rhein, Germany), irbesartan from Sanofi (Paris, France) and eprosartan mesylate from Solvay pharmaceuticals (Brussels, Belgium). The purity of all the target analytes was > 95%.

In the case of surrogate analogues, amitriptyline-d_3 hydrochloride (100 mg/L in methanol), atrazine-d_5 (99%), carbamazepine-d_{10} (100 mg/L in methanol), ketoprofen-d_3 (99.4%), nortriptyline-d_3 hydrochloride (100 mg/L in methanol), progesterone-d_9 (98%), were purchased from Sigma-Aldrich and ciprofloxacin-d_8 hydrochloride (99%) was obtained from Fluka. Irbesartan-d_7 2,2,2-trifluoroacetate salt (99.9%) were purchased from Toronto Research Chemicals (Toronto, Canada).

Stock standard solutions were dissolved individually on a weight basis in methanol (UHPLC-MS MeOH, Scharlab, Barcelona, Spain) in order to prepare approximately 1000-2500 mg/L solutions. However, the addition of 100 µL sodium hydroxide 1 mol/L (NaOH, 98%, Panreac, Barcelona, Spain) was necessary for the proper dissolution of fluoroquinolone antibiotics as described by Gros et al. (Gros et al., 2013). 100 mg/L dilutions were prepared in MeOH every month and dilutions at lower concentrations containing all analytes were prepared daily in MeOH: Milli-Q (90: 10, v: v). All the chemicals standards solutions were stored at −20 °C.

MeOH (HPLC grade, 99.9%), ethyl acetate (EtOAc; 99.8%) , acetonitrile (ACN, 99.8%) , and n-hexane (HPLC grade, 95%) were supplied by LabScan (Dublin, Ireland), ethylenediaminetetraacetic (EDTA, 99.0-101.1%) and ammonia solution (25% as NH_3) by Panreac (Barcelona, Spain), formic acid (HCOOH ≥ 98%) by Scharlau (Barcelona, Spain) and sodium chloride (NaCl > 99.8%) and acetic acid (HOAc, 100%) by Merck.

A Cryodos-50 laboratory freeze-dryer from Telstar Instrument (Sant Cugat del Valles, Barcelona, Spain) was used to freeze-dry the solid samples. An ultrasonic cell disruptor/homogeniser (100 W, 20 kHz; Bandelin Sonopuls HD 3100 sonifier, Bandelin Electronic, Berlin, Germany) equipped with a 3-mm titanium microtip was used for the extraction of analytes from solid samples. The extractions were performed using 50-mL polypropylene conical tubes (PP, internal diameter 27.2 mm × 117.5 mm length) obtained from Deltalab (Barcelona, Spain) and the extracts were filtered through polypropylene filters (PP, 0.45 μm, Macherey-Nagel, Germany). Fractions were evaporated using a Turbovap LV Evaporator (Zymark, Hopkinton, USA) under a gentle stream of nitrogen (> 99.999% of purity) supplied by Messer (Tarragona, Spain).

Oasis-HLB (hydrophilic–lipophilic-balanced, 200 mg) SPE cartridges were purchased from Waters (Milford, USA) and 2 g-Florisil cartridges from Supelco (Walton-on-Thames, UK). PES tubes were obtained (Membrana, Wuppertal, Germany) in a tubular format (0.7 mm external diameter, 1.43 g/mL density). Pieces of this polymer (4 cm length) were cut using a sharp blade and soaked overnight in MeOH (HPLC grade, 99.9%, LabScan, Dublin, Ireland) before their use as sorbent material. Afterwards, the polymer was dried with air and stored until used. Given their reduced cost (c.a. 0.05 €/unit) the pieces were discarded after each use. Agitation was carried out using 30-mL polystyrene vessels (PS, 60 x 27 mm^2) provided by ServiQuimia (Tarragona, Spain) in a 15 position magnetic stirrer (Gerstel, Mülheim an der Ruhr, Germany). Desorption was made in 1.5 mL Eppendorf tubes purchased from Eppendorf (Berzdorf, Germany) using a Digital Ultrasonic Cleaner (2500 mL, USB Axtor by Lovango, Barcelona, Spain).

The reconstituted extracts were filtered through polypropylene (PP, 0.22 μm, 13 mm, Phenomenex, California, USA) filters before the LC-MS/MS analysis. Milli-Q water (<0.05 S/cm, Milli-Q purification system model 185, Millipore, Bedford, MA, USA) and UHPLC-MS MeOH (Scharlab) were used as mobile phase eluent and HCOOH (Optima, Fischer Scientific, Gell, Belgium) for mobile phase modification. High purity nitrogen gas (> 99.999%) supplied by Messer was used as collision gas and nitrogen gas (99.999%) provided by AIR Liquid (Madrid, Spain) was used as both nebuliser and drying gas.

4.2.2 Application of the method

In order to verify the performance of the method we applied it to determine the concentration of some of the ECs in two different scenarios.

In the first case, live mussels (*Mytilus galloprovincialis*) of 35–45 mm length were manually collected at Arriluze (3° 0'56.68"W, 43°20'15.67"N), in the Bilbao estuary mouth (Bay of Biscay) in April of 2017. Mussels were collected, rinsed with natural water and taken to the laboratory in a cool box before 8 h had elapsed. The soft tissues of the mussels were separated from the shell with a sterile stainless steel scalpel, freeze-dried, ground and homogenised in a ball mill, and stored at 4°C until analysis.

In the second case, juvenile gilt-head bream (*Sparus aurata*) were exposed during 7 days to a mixture (dosing concentrations of 300 ng/L) of ten ECs (acesulfame, sulfadiazine, acetaminophen, carbamazepine, PFBS, diuron, amitriptyline, irbesartan, butylparaben and bezafibrate). Juvenile gilt-head bream weighing around 40 g and measuring 15 cm in length were obtained from Groupe Aqualande (Roquefort, France). For exposure experiments, two polypropylene tanks (control and dosed) each containing 100 L of seawater and 30 gilt-head bream per tank were used. The experiments were performed at controlled temperature (18°C) and light (14:10 h light: dark cycles) during 7 days. The water was continuously aerated using aquarium oxygenators and fish were fed daily with 0.10 g pellets/fish. The analytes dosing was performed using a continuous flow-through system with a peristaltic pump delivering 8.5 L seawater/h and another pump infusing the 10-analytes stock dosing solution (128 ng/mL per analyte in Milli-Q water, refilled every 24 h with newly prepared solution) at 20 mL/h to the exposure tank. Control tank (only seawater) was maintained at identical conditions during the course of the experiment.

Fish processing was carried out according to the Bioethics Committee rules of the University of the Basque Country (procedure approval CEEA/380/2014/ETXEBARRIA LOIZATE). Ten fish were collected from both, exposed and control tanks at the beginning of the experiment (day 0) and on day seven of exposure (day 7). Afterwards, fish were immediately anaesthetized in a tank containing 10 L of seawater with 200 mg/L of tricaine and 200 mg/L of sodium bicarbonate. Blood was sampled from the caudal vein-artery using a syringe previously rinsed with 0.5 mol/L EDTA solution (pH

adjusted to 8.0 using NaOH) and then centrifuged for 5 min at 1000 rpm to get the plasma. Samples of biofluids (bile and plasma) and tissues (liver, gill, muscle and brain) were separated and prepared for analysis. Tissues were stored in liquid nitrogen during dissection, and then transferred to a −80°C freezer. All the tissues were freeze-dried for 48 h, ground and homogenised in a ball mill, and stored at 4°C until analysis.

4.2.3 Sample preparation of tissues samples

4.2.3.1 FUSLE

0.5 g of freeze-dried sample (fish muscle and liver and mussel) or 0.1 g (fish gills and brain) were placed together with 7 mL of MeOH: Milli-Q water (95:5) mixture in a 40-mL PP vessel and isotopically labelled standards mixture (amitriptyline-d_3, atrazine-d_5, carbamazepine-d_{10}, ketoprofen-d_3, progesterone-d_9, ciprofloxacin-d_8, sucralose-d_6 and irbesartan-d_7). The FUSLE extraction required 30 s (with a pulsed time on of 0.8 s and a pulsed time off of 0.2 s) and 10% of amplitude. Extractions were carried out at 0°C in an ice-water bath. After the extraction was over, the supernatant was filtered through 0.45 µm PP filters and the FUSLE extracts were evaporated to ~1 mL under a nitrogen stream and submitted to a clean-up step.

4.2.3.2 Clean-up step

Four different clean-up approaches were tested: (i) reverse phase SPE, (ii) normal phase SPE, (iii) microextraction based on PES and (iv) LLE followed by Oasis-HLB-SPE. For this purpose, FUSLE extracts of mussel, liver and muscle samples were spiked before the clean-up step with a mixture of the target analytes at a concentration level of 500 ng/mL. The experiments were performed in triplicate (n=3) and blanks were processed in parallel for signals subtraction. In all the assays, the eluate recovered after the clean-up step was concentrated to dryness under a gentle stream of nitrogen at 35°C, reconstituted in 200 µL of MeOH: Milli-Q water (90:10, *v:v*) and filtered through a 0.22 µm PP filter, previous to the LC-MS/MS analysis.

4.2.3.2.1 Oasis-HLB-SPE phase

This clean-up approach was performed based on the method published by Mijangos et

al. (Mijangos et al., 2018). Briefly, the extract was evaporated to ~1 mL, diluted in 6 mL of Milli-Q water (previously adjusted at pH 2 with HCOOH) and an appropriate volume of a EDTA solution to achieve a final concentration of 0.1% (g solute/g solution) was added. Oasis HLB-200 mg cartridges were sequentially conditioned with 5 mL of MeOH, 5 mL of ultrapure water and 5 mL of ultrapure water at pH=2. After the extract was loaded, 6 mL of Milli-Q water were added with cleaning purposes before the cartridge was dried for ~40 min under vacuum. Finally, the analytes were eluted using 6 mL of MeOH.

4.2.3.2.2 Florisil-SPE phase

According to the experience of the research group (Navarro et al., 2010) the extract was evaporated to dryness and diluted in 0.5 mL of Hexane. 2 g-Florisil cartridges were conditioned with 10 mL of n-hexane and the extracts loaded on top of the cartridges. Finally, the analytes were eluted with 9 mL of ethyl acetate.

4.2.3.2.3 PES microextraction

Dual PES microextraction was performed according to the method published by Mijangos et al. (Mijangos et al., 2018) with some modifications. Briefly, extracts evaporated to ~1 mL were directly poured into two 30 mL-extraction vessels containing 6 mL of Milli-Q with 30% NaCl (w/v) in both cases, one at pH=2 (adjusted with HCOOH) or pH=10 (adjusted with NH_3). An appropriate volume of a EDTA solution to achieve a final concentration of 0.1% (g solute/g solution), pre-cleaned portions of PES (4 PES tubes of 4 cm each corresponding to a total mass of approx. 50 mg) and a magnetic stirrer were also introduced in each one of the vessels. Thereafter, vessels were closed and extraction (800 rpm) was performed at room temperature overnight. Once the sorption step was over, the polymers were removed and rinsed with Milli-Q water in order to eliminate salt residues, and finally, dried with a clean tissue paper. Subsequently, the sorbents were chemically and simultaneously desorbed in a 1.5 mL Eppendorf tube containing 1 mL of MeOH by soaking for 32 min in an ultrasound bath.

4.2.3.2.4 Liquid-liquid extraction

The extract evaporated to ~1 mL was diluted in 6 mL of Milli-Q water and 2 mL of n-Hexane

were added. The tube was vortexed for 1 min, centrifuged for 5 min at 8000 rpm, and the water layer was collected in a glass test tube. Therefore, the water phase was submitted to the Oasis HLB extraction as described before (see section 4.2.3.2.1).

4.2.3.2.5 Seawater and biofluids sample preparation

Plasma and bile (500 and 100 µL, respectively) and seawater (250 mL) were fortified with deuterated analogues (amitriptyline-d_3, atrazine-d_5, carbamazepine-d_{10}, ketoprofen-d_3, progesterone-d_9, ciprofloxacin-d_8, sucralose-d_6 and irbesartan-d_7) prior to Oasis-HLB SPE extraction (Mijangos et al., 2018) (see chapter 3). The seawater samples or the biofluids (once diluted in 6 mL of Milli-Q water and buffered at pH 2 with HCOOH) were loaded in 200 mg-Oasis HLB cartridges after they were sequentially conditioned with 5 mL of MeOH, 5 mL of ultrapure water and 5 mL of ultrapure water at pH=2. Next, 6 mL of Milli-Q water were added with cleaning purposes before the cartridge was dried for 40 min under vacuum. Then, the analytes were eluted using 6 mL of MeOH. The eluate recovered after the clean-up step was concentrated to dryness under a gentle stream of nitrogen at 35°C, reconstituted in 200 µL of MeOH: Milli-Q water (90:10, *v:v*) and filtered through a 0.22 µm PP filter previous to the LC-MS/MS analysis.

4.2.4 Liquid chromatography coupled to triple quadrupole tandem mass spectrometry

The analysis was performed by LC-QqQ-MS/MS based on a modification of a previously developed method (Mijangos et al., 2018). The separation and quantification of the 41 target analytes were performed using an Agilent 1260 series HPLC coupled to an Agilent 6430 triple quadrupole mass spectrometer with electrospray ionisation (ESI) source (Agilent Technologies, Palo Alto, CA, USA). The extracts (2 µL) were injected into a Kinetex F5 100 Å core-shell (2.1 mm × 100 mm, 2.6 µm) column coupled to a Kinetex F5 pre-column (2.1 mm x 4.6 mm, 2.6 µm), both from Phenomenex (Torrance, 235 CA, USA). The column temperature and the flow rate were set to 35°C and 0.3 mL/min, respectively. A binary mixture consisting of water: MeOH (95: 5, v: v) (mobile phase A) and mobile phase B of MeOH: water (95: 5, v: v), both containing 0.1% of HCOOH were used for gradient separation of target analytes. The gradient profile started with 30% B which was increased to 50% in 4 min and maintained for 12 min. Then, it was increased to 90% B where it was

maintained constant for 10 min. Initial gradient conditions (30% B) were then achieved in 6 min, where it was finally held for another 10 min (post-run step). ESI was carried out using a N_2 flow rate of 12 L/min, a capillary voltage of 3500 V, a nebuliser pressure of 45 psi and a source temperature of 350°C.

Quantification was performed in the selected reaction monitoring (SRM) acquisition mode. Both, negative and positive voltages, according to the target analytes, were simultaneously applied in a single injection. The transitions followed in SRM mode as well as the fragmentor and collision energy (CE, polarity and the ion ratios) values are summarised in **Table 3.2,** in **Chapter 3**. The retention times and instrumental limits of quantification of each analyte are also included. Instrumental operations, data acquisition and peak integration were performed with the Masshunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

4.3 Results and discussion

4.3.1 LC-MS/MS optimisation and instrumental figures of merit

Firstly, it was observed that the presence of water in the reconstitution solvent (30:70-MeOH: Milli-Q) used before (Mijangos et al., 2018) was not suitable for injection into LC-MS/MS due to the presence of a turbidity. This fact was attributed to the presence of water-insoluble matrix components, such as proteins as previously described in the literature (Griebenow and Klibanov, 1996; Ziarrusta et al., 2016a). In this sense, following our previous experience (Ziarrusta et al., 2016a) MeOH: Milli-Q (90:10, v:v) was used as reconstitution solvent since turbidity was observed when water levels were above the 10% .

Furthermore, different injection volumes (2, 5 and 7 µL) were tested. As can be seen in **Figures 4.1a** and **4.1b** for sulfamethoxazole and phenytoin, respectively, the chromatographic resolution of early-eluting analytes (t_R < 3.5 min for 7 µL and 10 µL and t_R < 8 min for 10 µL) was highly affected by the injection volume due to the high eluotropic strength of the injection solvent (MeOH: Milli-Q water (90:10, v:v) in comparison with the initial composition of the mobile phase (MeOH: Milli-Q water, 30:70, v:v). Therefore, 2 µL were used in further assays as injection volume as

a compromise between sensitivity and peak resolution.

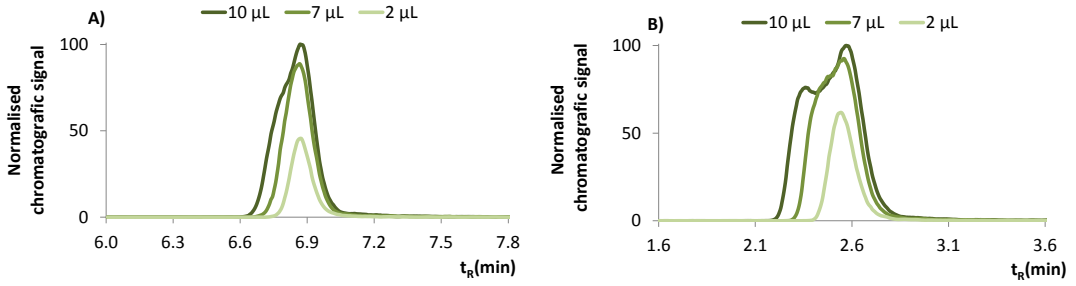

Figure 4.1: *Normalised chromatographic signals of three different injection volumes (2, 5 and 7 µL) for (a) sulfamethoxazole and (b) phenytoin.*

Calibration curves were built with MeOH: Milli-Q (10:90, v: v) standard solutions in the instrumental limit of quantification (LOQ)-5000 ng/mL range at 10 concentration levels. Determination coefficients (r^2) higher than 0.997 were obtained for all the target analytes corrected with the corresponding labelled standard. Instrumental quantification limits (LOQs) were established as the lowest concentration fulfilling all of the following criteria: (i) a linear calibration curve, (ii) an acceptable peak shape, and (iii) a signal-to-noise ratio of at least 10 (S/N = 10). LOQs in the 1-7 ng/mL range were obtained.

4.3.2 Optimisation of FUSLE for tissues

In a first approach for the optimisation of the FUSLE variables, including the solvent type, extraction time and the number of consecutive extractions, we considered the fortification of clean tissues (analyte free). However, in the cases of brain, gills and liver, this approach could not be used since a modification of the matrix was observed. Alternatively, matrix spiking was considered when slurry fortification was not viable, as pointed recently (Ziarrusta et al., 2017b). Both strategies were tested for muscle samples, and comparable results (p-value > 0.3) were obtained for all the target compounds. Hence, the samples were spiked (200 ng/g) using stock solutions of each compound in MeOH.

According to the literature, pure organic solvents such as MeOH and ACN or mixtures of them with water, to extract more polar compounds or with HOAc to promote protein precipitation, are the most commonly used solvents (Núñez et al., 2017). Therefore ACN, MeOH, 95:5-MeOH: Milli-Q and 95:5-MeOH: HOAc mixtures were evaluated here for the extraction of the target analytes. Aliquots of muscle (0.5 g, dry weight) were extracted with 7 mL of the solvents mentioned above during 0.5 min. Other variables such as temperature, solvent volume and amplitude were fixed at 0°C, 7 mL and 10% at 0.8 s/s of duty cycle, respectively, according to our previous experience (Ziarrusta et al., 2016a). In order to optimise the FUSLE conditions, all the extracts were filtered through 0.45 μm PP filters and cleaned-up using the Oasis-HLB-SPE clean-up approach (see section 2.4.2.1). The experiments were performed in triplicate (n=3) and blanks were processed in parallel for signal subtraction.

Figure 4.2 shows the normalised recoveries for FUSLE obtained for some of the target analytes (one analyte per family was included). It was observed that among the tested solvents, mixtures of MeOH: Milli-Q water (95:5, v/v) and MeOH: HOAc (95:5, v/v) rendered the highest recoveries for most of the target compounds. The results showed that the addition of HOAc favoured the extraction of compounds as sulfadiazine and ciprofloxacin, probably due to the disruption of the analyte-protein binding (Martínez Bueno et al., 2013; Tang et al., 2009) promoted by the proteins denaturalisation present in fish muscle (López-Alonso et al., 2010). However, this protein-precipitation caused significant looses (> 30%) during the evaporation step. Therefore, the use of HOAc was discarded and the mixture of MeOH: Milli-Q water (95:5) was selected as the most adequate extractant.

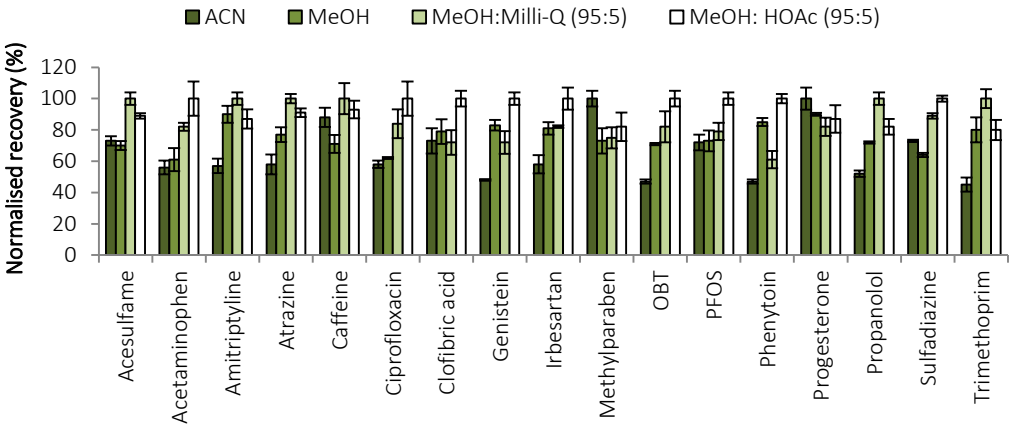

Figure 4.2: *Normalised recoveries of the target compounds (one analyte of each family) obtained with different solvents during FUSLE extraction in fish muscle.*

In order to improve the FUSLE efficiency, three different extraction times (0.5, 2.5 and 5 min) were studied. The optimisation was carried out at the same conditions explained above. Each experiment was carried out in triplicate. As can be seen in **Figure 4.3** the recoveries obtained for most of the target compounds at three extraction times were statistically comparable, except for PFOS, methylparaben, sulfadiazine, propranolol and trimethoprim (extraction time was slightly significant). However, since the differences were not higher than the 20%, extraction time was fixed to the minimum time (i.e. 30 s).

Due to the lack of a certified reference material (CRM), three consecutive extractions (n=3) were performed on the four biological tissues (mussel and fish muscle, gills and liver) in order to determine whether exhaustive extractions were achieved under optimised conditions. Recoveries lower than 10-15% were obtained in the second extraction for all the target analytes except for 6 compounds: ciprofloxacin (23-32%), norfloxacin (25-36%), sulfadiazine (25-29%) and sulfamethoxazole (30-28%) in muscle and brain, respectively, and for progesterone (28%) and PFOS (25%) in liver. Thus, only a single extraction of the same sample was carried out in further assays for all the matrices.

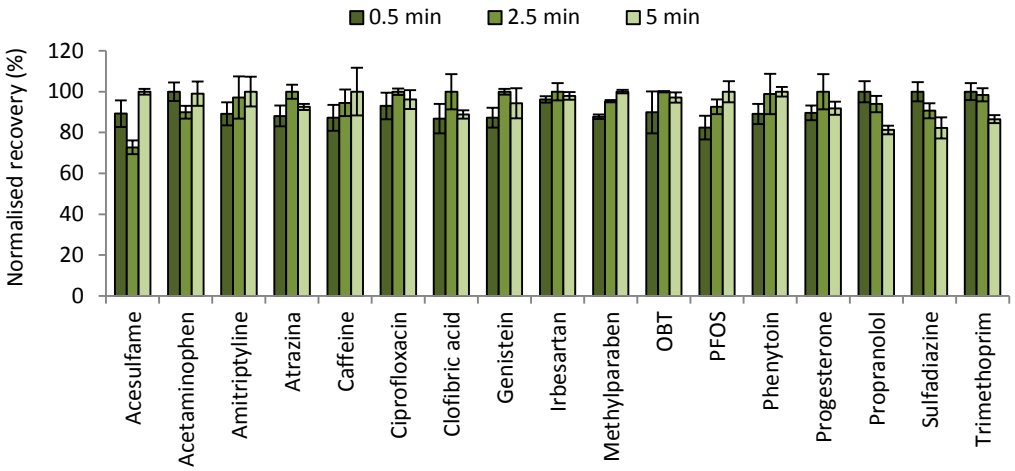

Figure 4.3: *Influence of extraction time during FUSLE in fish muscle for one analyte of each family. Recoveries are expressed as the recovery of the extraction step normalised to the highest values.*

4.3.3 Clean-up optimisation

The efficiency of the clean-up step was evaluated in terms of recoveries and cleanliness of the extracts. Regarding the five tissues studied in this work, fish liver and mussel was chosen due to their high lipid content and muscle due to its high protein content. In this sense, 0.5 g of freeze-dried samples were extracted under the conditions fixed before (see section 4.3.2).

The clean-up recoveries for the procedures tested (i-iv) were calculated by comparing the responses obtained when the extracts were spiked at 500 ng/mL before and after the clean-up. In the case of muscle, a single clean-up using the widely used Oasis-HLB cartridges (Núñez et al., 2017) rendered acceptable results in terms of absolute recoveries (35-85%) and properly cleanliness of the extracts. In consequence, in the case of brain and gills, a single Oasis-HLB purification step was also selected as clean-up protocol. In the case of high lipid content matrices (mussel and liver), acceptable absolute recoveries were obtained when HLB-SPE (41-160%) was used for mussel, while for liver high values were obtained (11-213%). Therefore, different protocols (PES microextraction, Florisil-SPE and the combination of LLE-HLB-SPE) were tested for liver in order to remove most of

the fatty compounds. Florisil cartridges were discarded as a viable option, since negligible recoveries (< 20%) were obtained for 23 out of the 41 target analytes under the elution conditions used. On the contrary, overall, acceptable recoveries with PES microextraction (23-76%) and LLE procedure (40-143%) with n-hexane combined with HLB-SPE, were obtained. It is worth mentioning that, when the LLE step was included before the Oasis-HLB clean-up, most of the analytes were not detected in the *n*-hexane fraction except atrazine, progesterone, telmisartan and irbesartan which showed recoveries between 18 and 35%.

4.3.4 Method validation for tissues and biofluids

The suitability of the methods was evaluated in terms of apparent recoveries (corrected using matrix-matched calibration approach and isotopically labelled standards), precision and method detection limits (MDLs). Apparent recoveries and the repeatability of the method in terms of relative standard deviation (RSD%) for the tissues (mussel and fish muscle, liver, brain and gills) and biofluids (plasma and bile) matrices were determined at two concentration levels: 25 ng/g (n=5) and 100 ng/g (n=3) or 25 ng/L (n=5) and 100 ng/L (n=3), respectively (see **Table 4.1** for the low level results). In the case of liver samples, two different protocols were validated, FUSLE-PES microextraction-LC-MS/MS and FUSLE-LLE-HLB-SPE-LC-MS/MS.

Overall, satisfactory apparent recoveries were achieved for all the target compounds using labelled standards corrections in all the matrices except for liver. Since the use of labelled standards (i.e., isotopically labelled standards of atrazine and irbesartan) could not compensate the recoveries obtained in the case of liver matrix regarding the protocol used (FUSLE-PES and FUSLE-LLE-HLB), matrix-matched calibration approach was applied obtaining acceptable apparent recoveries (64-145%). In all the cases, RSD values varied between 2 and 22%, which can be considered satisfactory values taking into account the complexity of the matrices. Similar results in terms of both apparent recovery and RSD values were obtained in the case of high concentration level tested. The values obtained are comparable to other works focused on the analysis of target analytes in biological samples using other methodologies (Alvarez-Muñoz et al., 2015; Huerta et al., 2013; Turnipseed et al., 2017).

Table 4.1: Apparent recovery ± standard deviation (%, n=3) of the validated procedures for the biotissues and biofluids.

Analyte	Apparent recovery ± sd (%, n=3)							
	Mussel	Plasma	Bile	Brain	Muscle	Gills	Liver (FUSLE-PES)	Liver (FUSLE-LLE-SPE)
Acesulfame	104±7	100±10	102±23	106±21	88±6	88±9	111±15	110±17
Acetaminophen	112±8	105±6	81±17	74±20	81±10	82±14	105±11	125±15
Amitriptyline	99±10	112±9	84±19	118±30	116±15	124±16	95±14	94±22
Atrazine	98±7	113±7	77±19	111±27	110±18	80±12	113±10	90±15
Bezafibrate	99±6	94±12	86±20	81±22	96±14	124±12	111±10	92±17
Butylparaben	93±13	102±11	89±12	100±18	100±12	88±10	95±9	145±20
Caffeine	102±4	95±13	113±17	115±19	118±11	100±17	85±10	110±19
Carbamazepine	105±6	98±7	76±14	108±23	110±17	97±12	108±15	77±17
Ciprofloxacin	94±8	114±12	135±28	91±24	118±19	95±12	89±17	112±14
Clofibric acid	102±10	97±4	135±29	98±23	86±17	71±15	113±17	103±18
Clomipramine	98±12	100±3	118±17	82±16	103±18	87±14	95±14	115±22
Diclofenac	101±8	107±7	124±17	98±22	80±6	73±5	88±12	82±15
Diuron	100±11	104±7	132±36	101±29	104±9	86±9	111±9	110±14
Eprosartan	97±10	109±4	126±20	76±21	107±8	69±10	104±14	118±19

Table 4.1: Apparent recovery ± standard deviation (%, n=3) of the validated procedures for the biotissues and biofluids.

Analyte	Apparent recovery ± sd (%, n=3)							
	Mussel	Plasma	Bile	Brain	Muscle	Gills	Liver (FUSLE-PES)	Liver (FUSLE-LLE-SPE)
Genistein	96±8	97±10	119±17	110±23	110±13	101±13	87±13	101±20
Genistin	101±17	88±10	136±17	98±30	98±15	123±13	82±16	101±15
Glycitin	98±5	87±12	108±12	89±22	86±7	124±8	114±9	95±14
Imipramine	99±10	84±12	134±34	110±29	109±19	85±13	88±15	91±23
Irbesartan	93±11	87±12	125±12	84±23	98±6	122±9	95±15	96±15
Isoproturon	102±6	96±17	95±17	125±23	102±8	74±9	92±17	119±15
Ketoprofen	93±10	116±13	127±13	127±23	100±5	130±9	100±12	121±22
Losartan	96±11	114±21	118±21	100±23	82±11	72±8	83±15	64±11
Methylparaben	105±17	89±21	117±21	124±28	120±18	73±13	107±11	120±21
Norfloxacin	94±8	93±11	87±11	130±23	80±8	99±17	93±14	74±16
Nortriptyline	99±14	93±13	110±13	107±23	92±8	103±12	110±11	116±21
OBT	132±8	83±18	114±18	118±23	84±9	126±17	82±14	112±15
PFBS	95±5	99±23	102±23	99±26	105±9	123±10	94±15	72±11
PFOA	101±5	112±21	102±21	112±21	95±10	127±9	114±14	88±19

Table 4.1: Apparent recovery ± standard deviation (%, n=3) of the validated procedures for the biotissues and biofluids.

Analyte	Apparent recovery ± sd (%, n=3)							
	Mussel	Plasma	Bile	Brain	Muscle	Gills	Liver (FUSLE-PES)	Liver (FUSLE-LLE-SPE)
PFOS	97±9	88±19	106±19	129±29	96±8	119±14	100±14	110±23
PFOSA	94±20	108±15	133±10	91±26	90±9	83±16	103±13	81±16
Phenytoin	109±14	93±12	81±12	101±17	111 ±18	7±15	84±16	111±22
Progesterone	93±14	94±19	88±19	127±33	120±5	69±2	100±16	123±17
Propranolol	94±9	85±17	105±17	94±21	83±11	85±14	100±15	97±15
Simazine	99±7	109±23	128±23	83±18	118±9	126±13	88±10	126±17
Sucralose	138±33	109±20	135±10	99±27	81±12	126±16	103±15	126±22
Sulfadiazine	90±6	96±15	122±15	105±21	90±16	83±18	97±16	92±15
Sulfamethoxazole	98±7	115±16	129±16	70±17	95±15	124±17	102±14	72±19
Telmisartan	97±12	88±18	124±18	98±28	114±13	129±11	106±13	94±20
Testosterone	103±8	93±19	71±19	111±33	85±15	94±13	86±11	92±21
Trimethoprim	96±6	117±19	120±19	109±24	108±16	73±9	113±10	101±17
Valsartan	118±12	101±17	106±17	123±25	88±11	100±12	102±17	116±20

Method detection limits (MDLs) were determined by fortification of five replicates of each blank matrix with each analyte at the lowest concentration levels (10 ng/g and 10 ng/L for tissues and biofluids, respectively) according to the USEPA method (https://www.epa.gov/sites/production/files/2016-12/documents/mdl-procedure_rev2_12-13-2016.pdf). The MDLs were then calculated according to the **Equation 4.1**:

$$MDL = t_{(n-1)}\, x\, sd \quad \textbf{Equation 4.1}$$

where $t_{99,4}$=3.75 and *sd* refers to the standard deviation of the replicate analyses (n=5). MDL values between 0.4-48 ng/g and 0.3-111 ng/L were obtained for biotissues and biofluids, respectively (see **Table 4.2**). Similar MDLs were reported in the literature in a variety of biological matrices (Alvarez-Muñoz et al., 2015; Grabicova et al., 2018; Huerta et al., 2013; Tanoue et al., 2014; Wille et al., 2011).

Table 4.2: Method detection limits (MDLs, n=5) of the validated procedures for the biotissues (ng/g) and biofluids (ng/mL).

Analytes	Tissues (ng/g)						Biofluids (ng/mL)	
	Mussel	Brain	Muscle	Gills	Liver (FUSLE-PES)	Liver (FUSLE-LLE-SPE)	Plasma	Bile
Acesulfame	1	6	2	7	4	1	1	7
Acetaminophen	20	10	3	9	13	2	2	39
Amitriptyline	2	6	1	6	3	2	1	13
Atrazine	0.8	2	0.4	2	5	0.9	0.3	4
Bezafibrate	2	3	0.8	2	4	0.5	0.5	5
Butylparaben	13	12	4	10	8	5	3	46
Caffeine	5	8	2	8	6	1	2	15
Carbamazepine	0.7	3	0.7	3	2	0.6	0.5	5
Ciprofloxacin	0.8	29	16	13	20	17	5	71
Clofibric acid	1	6	2	7	6	5	1	13
Clomipramine	2	3	0.7	4	1	0.9	0.6	7
Diclofenac	1	3	0.5	4	1	0.9	0.5	7
Diuron	0.5	4	0.8	4	2	0.8	0.6	7
Eprosartan	0.9	8	2	9	5	1	1	18
Genistein	5	18	5	14	10	5	3	29
Genistin	1	12	5	15	9	3	3	31
Glycitin	0.9	11	4	7	8	5	3	26
Imipramine	1	6	1	9	3	3	1	16
Irbesartan	0.7	3	0.6	4	2	0.6	0.5	5

Table 4.2: Method detection limits (MDLs, n=5) of the validated procedures for the biotissues (ng/g) and biofluids (ng/mL).

Analytes	Tissues (ng/g)						Biofluids (ng/mL)	
	Mussel	Brain	Muscle	Gills	Liver (FUSLE-PES)	Liver (FUSLE-LLE-SPE)	Plasma	Bile
Isoproturon	0.7	3	0.6	4	2	0.9	0.6	6
Ketoprofen	2	4	0.8	3	3	1	0.7	10
Losartan	0.9	14	3	14	9	4	2	27
Methylparaben	9	16	3	24	8	3	4	34
Norfloxacin	1	32	6	18	16	5	4	60
Nortriptyline	1	6	1	7	3	1	1	19
OBT	2	12	3	13	7	3	3	27
PFBS	2	4	0.7	4	4	0.7	0.7	7
PFOA	7	4	1	4	6	1	0.7	15
PFOS	2	3	0.7	4	2	0.7	0.5	6
PFOSA	0.5	3	0.8	3	2	1	0.7	8
Phenytoin	5	13	4	20	7	2	4	40
Progesterone	10	8	1	7	3	3	1	11
Propranolol	1	5	1	5	4	0.9	0.9	8
Simazine	2	5	0.7	6	3	1	0.7	3
Sucralose	4	3	1	4	2	0.9	0.7	10
Sulfadiazine	0.4	6	0.7	5	3	0.9	0.5	8
Sulfamethoxazole	0.4	11	2	9	4	0.9	1	16
Telmisartan	5	4	0.5	4	2	1	0.6	6
Testosterone	2	4	1	4	3	0.8	0.7	8
Trimethoprim	0.5	2	0.4	2	2	0.5	0.3	4
Valsartan	4	17	3	7	15	4	3	30

4.3.5 Application of the method results

During the exposure experiments, target analytes average concentrations in seawater ranged between 291±13 ng/mL (irbesartan) and 312±15 ng/mL (carbamazepine), which are consistent with nominal dosing concentrations (300 ng/L). In addition to this, the concentration of these analytes in the control tank were below their MQLs (<2-15 ng/L) (see **chapter 3**), except in the case of butylparaben, which was measured at a concentration of 23 ng/mL.

Mortality was not observed in any of the experiments and K and HSI values were not statistically different between control and exposed fish (p-values = 0.06 and 0.18, respectively) at the 95% confidence level, indicating maintenance of fish health over the duration of the 7 days-

exposition experiment.

The uptake concentrations (ng/g or ng/mL) of individual target compounds in liver, muscle, brain, gills, bile and plasma are included in Table 4.3. Acesulfame, acetaminophen and sulfadiazine did not show any tissue distribution. Sulphonamides antibiotics and acetaminophen have also been reported to have low bioaccumulation factors in several species (Armitage et al., 2016).

In general terms, diuron and butylparaben showed the highest concentrations values. Concentrations of PFBS (23 ng/g), diuron (234 ng/g) and butylparaben (214 ng/g) were statistically higher (p-value>0.06-1.2) in liver compared to the rest of fish tissues/fluids. To the best of our knowledge, there is no reported uptake data of PFBS. In the case of carbamazepine (an anticonvulsant) and amitriptyline (an antidepressant), they showed a similar tissue distribution; the highest concentrations (91 and 58 ng/mL, respectively) were detected in brain. These results are consistent with Ziarrusta and co-workers (Ziarrusta et al., 2017a) results, where an extensive diffusion of amitriptyline to fish brain was observed.

Table 4.3. Concentrations (ng/g and ng/mL) of the fish exposed to 10 of the target analytes.

Analytes	Concentration					
	Tissues (ng/g)				Biofluids (ng/mL)	
	Muscle	Gill	Liver	Brain	Plasma	Bile
Acesulfame	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acetaminophen	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Amitriptyline	7.1±0.5	16±2	18±2	58±9	15±1	n.d.
Bezafibrate	6.2±0.4	28±2	41±4	20±3	57±7	10.8±0.8
Butylparaben	73±9	73±7	214±26	<MDL	27±4	55±7
Carbamazepine	8±1	14±1	30±4	91±5	18±1	<MDL
Diuron	34±3	53±4	234±23	22±3	60±8	60±6
Irbesartan	14±2	35±3	53±5	28±2	88±13	24±2
PFBS	3±1	6±1	23±4	<MDL	<MDL	n.d.
Sulfadiazine	n.d.	n.d.	<MDL	<MDL	n.d.	n.d.

n.d.: not detected, MDL: method detection limit

When the method was applied in the analysis of wild mussels, only 7 analytes were detected above their MDLs: amitriptyline (3.2±0.1 ng/g), PFOS (4.2±0.4 ng/g), PFOSA (1.4±0.1 ng/g), progesterone (14±2 ng/g), sulfadiazine (0.6±0.1 ng/g), telmisartan (6.8±0.6 ng/g) and valsartan (7±1 ng/g). See **Figure 4.4** for representative SMR chromatograms of some of the detected analytes.

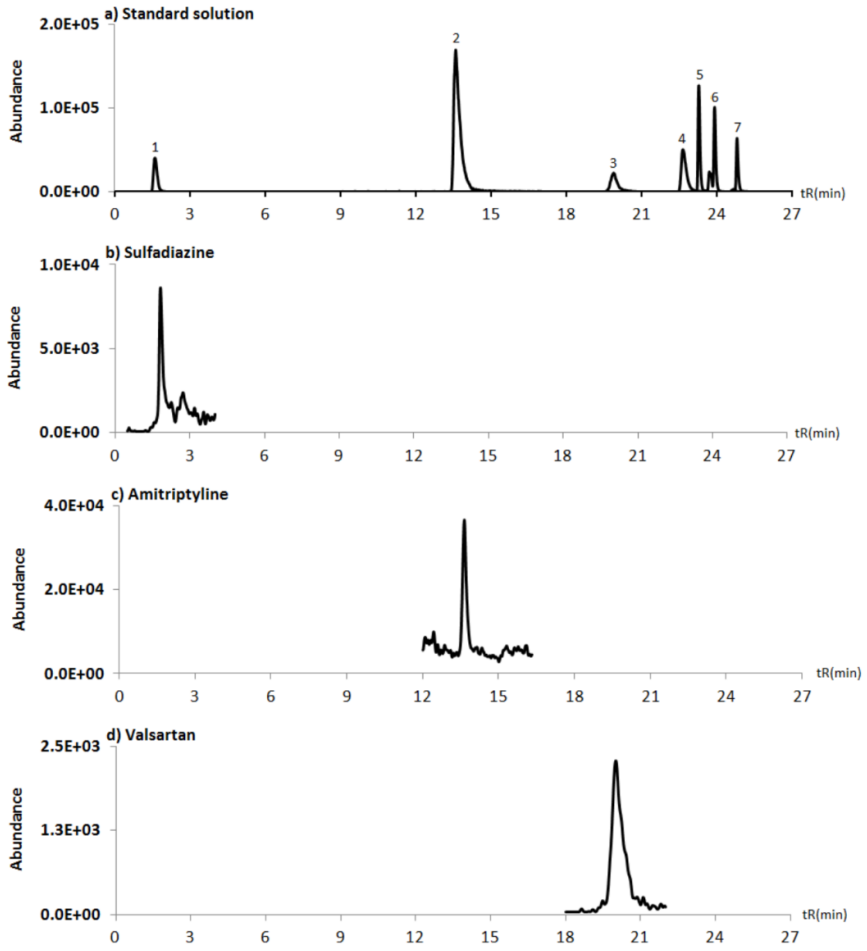

Figure 4.4: *SRM chromatograms of (a) a standard solution of the detected analytes at 200 µg/L each one and (b-d) a mussel real sample extract of (b) sulfadiazine, (c) amitriptyline and (d) valsartan. Other compounds (telmisartan, progesterone, PFOS and PFOSA) were also detected but not included in this figure.*

The presence of pharmaceutical and hormones could be related to the upstream presence of Galindo WWTP, the biggest WWTP in the Basque Country and one of the biggest in Spain. Among the pharmaceuticals, sulfadiazine (0.6±0.1 ng/g, Log P =0.4) was detected at the lowest concentration. Other studies carried out in Singapore (Bayen et al., 2016), California Coast (Dodder et al., 2014), China (Li et al., 2012a) and Spain (Serra-Compte et al., 2017) summarised in **Table 4.4** also reported low detection frequencies and concentrations of sulfadiazine in mussel, suggesting a low accumulation potential or a high metabolic degradation rate in mussels. Concentrations of amitriptyline and progesterone (see **Table 4.4**) were also in accordance with the literature (Álvarez-Muñoz et al., 2015; de Solla et al., 2016; Dévier et al., 2010; Dodder et al., 2014; Klosterhaus et al., 2013). In the case of valsartan and telmisartan, though they are widely used (Godoy et al., 2015), only valsartan has been determined before in invertebrates (Klosterhaus et al., 2013), and it was not detected in any of the studied samples. To the best of our knowledge, this is the first time that the presence of telmisartan (6.8±0.6 ng/g) and valsartan (7±1 ng/g) in mussels is reported.

Concerning the PFASs, the concentration obtained for PFOS (4.2±0.4 ng/g) did not exceed the established Environmental Quality Standard in biota (9.1 μg/kg) in the field of water policy under the directive 2013/39/EU (European Commission, 2013). The concentrations obtained for both, PFOS (4.2±0.4 ng/g), and PFOSA (1.4±0.1 ng/g), are in agreement with previous studies carried out in the Basque coast for PFASs and their potential precursors (Zabaleta et al., 2015) and in Belgium coast (Wille et al., 2011). However, higher concentrations were obtained by Cunha et al. (Cunha et al., 2005), who reported levels of PFOS up to the 126 ng/g in mussels (*Mytilus galloprovincialis)* collected from the Vouga river (Portugal).

Table 4.4. Concentrations (ng/g) of the target analytes detected in mussel samples from various locations (including this study). Results are expressed as concentration ranges or spot concentration values with its standard deviation.

Analyte	Location	Specie	Extraction-clean-up	Concentrations	Reference
Amitriptyline	Basque coast (Basque Country)	M. galloprovicialis	FUSLE-SPE (HLB)	3.2±0.1	In this study
	Basque coast (Basque Country)	M. galloprovicialis	FUSLE-SPE (Evolute-CX)	n.d.	Ziarrusta et al., 2016
	Grand river (USA)	*Lasmigona costata*	USB-SPE (HLB)	5.8-35.1	de Solla et al., 2016
	San Francisco Bay (USA)	*Geukensia demissa*	SLE-SPE (HLB)	n.d.-0.2	Klosterhaus et al., 2013
	California coast (USA)	*M. edulis*	SLE-SPE (HLB)	n.d.-6.2	Dodder et al., 2014
PFOS	Basque coast (Basque Country)	*M. galloprovicialis*	FUSLE-SPE (HLB)	4.2±0.4	In this study
	Basque coast (Basque Country)	*M. galloprovicialis*	FUSLE-SPE (Evolute WAX, Envi-carb)	n.d.-2.4	Zabaleta et al., 2015
	Belgium coast (Belgium)	*M. edulis*	SLE-SPE (HLB)	n.d.-4	Bayen et al., 2016
	California coast (USA)	*M. edulis*	SLE	n.d.-5.5	Dodder et al., 2014
	Vouga (Portugal)	*M. galloprovicialis*	IPE(MTBE)	36.8-125.9	Cunha et al., 2005
PFOSA	Basque coast (Basque Country)	*M. galloprovicialis*	FUSLE-SPE (HLB)	1.4±0.1	In this study
	Basque coast (Basque Country)	*M galloprovicialis*	FUSLE-SPE (Evolute WAX, Envi-carb)	3-8	Zabaleta et al., 2015
	California coast (USA)	*M. edulis*	SLE	n.d.-2.9	Dodder et al., 2014
Progesterone	Basque coast (Basque Country)	*M. galloprovicialis*	FUSLE-SPE (HLB)	14±2	In this study
	Ebro delta (Spain)	*M. galloprovincialis*	PLE-SPE (HLB)	2.6±0.1	Álvarez-Muñoz et al., 2015
	Arcachon Bay (France)	*M edulis*	MAE-SPE (EnviChrom, NH_2)	0-5-8.9	Dévier et al., 2010
Sulfadiazine	Basque coast (Basque Country)	*M. galloprovicialis*	FUSLE-SPE (HLB)	0.6±0.1	In this study
	Singapore coast	*Perna viridis*	SLE	n.d.	Bayen et al., 2016
	California coast	*M. edulis*	SLE-SPE (HLB)	n.d.	Dodder et al., 2014
	Bohai sea, China	*M. edulis*	PLE-SPE (HLB)	n.d.-2.7	Li et al., 2012
	Ebro Delta, Spain	*M. galloprovincialis*	QuEChERS	n.d.	Serra-Compte et al., 2017
Telmisartan	Basque coast	*M. galloprovicialis*	FUSLE-SPE (HLB)	6.8±0.6	In this study
Valsartan	Basque coast	*M. galloprovicialis*	FUSLE-SPE (HLB)	7±1	In this study
	San Francisco Bay	*Geukensia demissa*	SLE-SPE (HLB)	n.d.	Klosterhaus et al., 2013

USB: ultrasonic bath; FUSLE: focused ultrasonic solid liquid extraction; IPE: ion par extraction; MAE: microwave assisted extraction; MTBE: methyl tert-butyl ether; n.d.: not detected; PLE: pressurized liquid extraction; SLE: solid liquid extraction; SPE: solid phase extraction.

4.4 Conclusions

A thorough optimisation and validation of different methodologies was performed for the simultaneous analysis of up to 41 analytes belonging to different families (artificial sweeteners, industrial products, hormones, pharmaceutical and personal care products, pesticides, and phytoestrogens) in biota (mussel and fish tissues and fluids) samples. With this aim, four clean-up protocols were evaluated and HLB for mussel, muscle, plasma, bile, gills and brain, and LLE-HLB or PES microextraction provided satisfactory results not only in terms of apparent recoveries but also in terms of extracts cleanliness and matrix effect. The uptake experiment carried out in this study showed the uptake and distribution of 10 analytes (including PFBS for the first time). Amitriptyline and carbamazepine showed a different tissue distribution, being the brain the compartment with the highest concentration values. When real mussel samples of the Biscay Coast were analysed, the presence of pharmaceutical compounds (amitriptyline, sulfadiazine, telmisartan and valsartan) as well as industrial compounds such as PFOS and PFOSA, was demonstrated. High levels of valsartan and telmisartan were reported for the first time in mussels. The analysis of the environmental samples carried out here showed the necessity of continuing with the monitoring of the area.

4.5 References

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5 POCIS and PES passive samplers

Evaluation of polar organic chemical integrative and hollow fibre samplers for the determination of a wide variety of organic polar compounds in seawater

Talanta

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5.1 Introduction

A number of procedures are usually applied for the chemical analysis of micro-organic contaminants in water samples, and the final selection of the most adequate one lies often on the fitness to the analytical purpose, i.e. those that minimise any bias in the analysis and in the interpretation of the results (Zhang et al., 2014). Additionally, when highly dynamic media is being monitored (e.g. transitional waters, effluents, etc.), the costs and the efforts required to accomplish an efficient sampling plan are very high. In this sense, the application of passive sampling (PS) methods has introduced a new paradigm in water analysis, since they can provide reliable estimations of the time-weighted average concentration (C_{TWA}) or the bioavailable fraction of contaminants in complex environmental media. However, proper validated procedures are still required for achieving regulatory compliance (Miège et al., 2015; Söderström et al., 2009; Vrana et al., 2005).

The passive samplers used for the analysis of non-polar compounds are deeply studied and show many applications (Booij et al., 2016). On the contrary, samplers used to analyse more polar compounds still require a deeper research and the exploration of novel applications (Vrana et al., 2005; Zabiegała et al., 2010). Broadly speaking, the most widely used passive samplers are the polar organic chemical integrative samplers (POCIS) (Harman et al., 2012), the Chemcatcher (Charriau et al., 2016; Lissalde et al., 2016), the membrane enclosed sorptive coating sampler (MESCO) (Vrana et al., 2001), the ceramic dosimeter (Martin et al., 2003) and the passive *in situ* concentration extraction sampler (PISCES) (Vrana et al., 2005).

Though POCIS samplers were developed for slightly polar compounds (Alvarez et al., 2004), two configurations are commercially available, the pest-POCIS and the POCIS-pharma. The latter is the most widely used configuration and it consists of 200 mg of Oasis HLB receiving phase enclosed between two polyethersulfone (PES) membranes (Morin et al., 2012). This sampler, however, shows a low affinity for highly polar and ionic compounds (Kaserzon et al., 2012; Mazzella et al., 2007) and low diffusion coefficients through the PES membranes for many hydrophobic compounds (Vermeirssen et al., 2012). To overcome this limitation, the replacement or combination of the Oasis HLB sorbent with a mixed-mode anion exchange sorbent has been recently proposed (Fauvelle et

al., 2012; Kaserzon et al., 2012; Li et al., 2011) to broaden the simultaneous analysis of a large variety of slightly polar and hydrophobic compounds. Similarly, the use of Nylon membranes instead of PES membranes (Belles et al., 2013; Morrison and Belden, 2016a) has been proposed to improve the diffusion across the membranes and the sampling rates. It is also worth mentioning the use of PES material as passive samplers (Posada-Ureta et al., 2016; Rusina et al., 2007).

Another important issue in PS is the use of performance reference compounds (PRCs) to solve the differences in the hydrodynamic regimes between the calibration and the application scenarios (Harman et al., 2008; Huckins et al., 2002). The use of PRC assumes that the exchange between the bulk aqueous phase and the acceptor phase and vice versa are affected in the same way by the variation of the hydrodynamic regimes (i.e. the exchange is isotropic). Therefore, by spiking the sampling devices prior to exposure, the PRC remaining in the passive sampler after a certain deployment time can be used to correct the sampling rates (Rs) estimated during the calibration (Söderström et al., 2009). However, since the uptake in POCIS is basically driven by adsorption processes, the isotropic exchange is not always assured, and this fact explains the lack of reliable PRCs.

Within this context, we considered to extend the use of passive samplers to the analysis of 20-multiclass organic emerging contaminants with a broad range of hydrophobicity (log P ranging between -0.9 and 6.1) in seawater including Nylon membranes instead of the classical PES ones. This way, we could widen the range of compounds that are sampled by the commercial POCIS and we can simultaneously minimise the extraction in the PES membrane. In this sense, the laboratory calibration of two passive samplers was studied: *(i)* POCIS samplers combining 100 mg of a mixed-mode anion exchanger (Strata X-AW) and 100 mg of polymeric HLB (Plexa) and enclosed between two highly porous Nylon membranes (30-µm pore size) and *(ii)* PES hollow fibres to study, for the first time, the suitability of this polymer with more polar compounds following previous works (Posada-Ureta et al., 2017, 2016). In the case of POCIS, the new sorbent mixture and the Nylon membranes were treated independently in order to assess the suitability of the latter. In both cases, the feasibility of five deuterated ([$^2H_3$]-amitriptyline, [$^2H_5$]-atrazine, [$^2H_3$]-ketoprofen, [$^2H_9$]-progesterone and [$^2H_7$]-irbesartan) compounds as PRCs was also considered.

5.2 Experimental section

5.2.1 Reagents and materials

The selected 20 analytes cover a wide variety of physicochemical properties as shown in **Table 3.1** in **Chapter 3**, which includes the molecular weight, the acid dissociation constant (pK_a) and the log P. Other physicochemical properties such as log $D_{(pH=7.4)}$, the water solubility and polar surface area of the target compounds are included in **Table 5.1**.

Table 5.1: Physico-chemical properties log D at pH 7.4, the water solubility (log S), polar surface area and Van der Waals accessible surface area of the target compounds.

Analyte	Log D[a] (pH=7.4)	Log S[a] (pH 7.4) (mol/L)	Polar surface area[a] (A^2)	VdW accessible surface area[a] (A^2)
OBT	0.9	0.0	33	181
Acesulfame	-3.1	0.0	79	184
Acetaminophen	0.9	-1.1	49	222
Amitriptyline	2.5	-2.0	3	282
Atrazine	2.2	-3.8	63	324
Bezafibrate	0.7	-1.3	76	515
Butylparaben	3.0	-2.5	47	315
Caffeine	-0.6	-0.4	58	269
Carbamazepine	2.8	-3.8	46	312
Diuron	2.5	-3.1	32	296
Irbesartan	4.2	-6.0	87	638
Ketoprofen	0.4	-0.3	54	368
Norfloxacin	-0.9	-2.1	73	435
PFOA	1.6	0.0	37	374
PFOS	3.0	0.0	54	454
PFBS	0.2	0.0	57	279
Phenyntoin	2.1	-3.3	58	341
Progesterone	4.2	-5.6	34	524
Sulfadiazine	-0.1	-2.0	98	323
Telmisartan	4.7	-6.5	73	753

[a] Values reported in the Free Data Base www.chemicalize.org
VdW: Van der Walls.

2-Hydroxybenzothiazole (OBT), amitriptyline hydrochloride, butylparaben, caffeine, carbamazepine, perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluoro-1-butanesulfonate (PFBS), phenytoin, progesterone and sulfadiazine were purchased from Sigma-Aldrich (Steinheim, Germany). Acetaminophen, atrazine, diuron and norfloxacin hydrochloride were

acquired from Fluka (Buchs, Switzerland). Acesulfame potassium was supplied by Supelco (Bellefonte, PA, USA), whereas, ketoprofen and bezafibrate were acquired from MP biomedicals (Illkirch Cedex, France). Telmisartan was purchased from Boehringer (Ingelheim am Rhein Germany) and irbesartan from Sanofi (Paris, France). The purity of all the target analytes was >95%.

In the case of the deuterated analogues used as PRCs, [$^2H_3$]-amitriptyline hydrochloride (100 mg/L in methanol), [$^2H_5$]-atrazine, (99%), [$^2H_3$]-ketoprofen (99.4%) and [$^2H_9$]-progesterone (98%) were purchased from Sigma-Aldrich while [$^2H_7$]-irbesartan 2,2,2-(as trifluoroacetate salt, 99.9%) was obtained from Toronto Research Chemicals (Toronto, Canada).

The target analytes were individually dissolved on a weight basis in methanol (MeOH, Romil-UpS, Optima, Fisher Scientific, Loughborough, UK) in order to prepare approximately 250-1000 mg/L of the stock solutions. However, the addition of 100 µL of sodium hydroxide 1 M (NaOH, 98%, Panreac, Barcelona, Spain) was necessary for the proper dissolution of the fluoroquinolones, as described in the recent literature (Gros et al., 2013). Mixed solutions with ~500 mg/L of each target compound were prepared, and lower concentration solutions were afterwards prepared according to the experimentation. The stock solutions were stored in amber vials at -40°C.

MeOH (HPLC grade, 99.9%) and acetone (HPLC grade, 99.8%) were supplied by LabScan (Dublin, Ireland). Acetonitrile (ACN, HPLC grade, 99.9%) was supplied by Sigma–Aldrich (Steinheim, Germany). MeOH and formic acid (Optima formic acid, ≥ 98%) used as mobile phase were provided by Scharlau (Barcelona, Spain) and Fischer Scientific (Geel, Belgium), respectively. Ammonia (25% as NH_3), ethylenediaminetetraacetic sodium salt (EDTA, ≥ 99.9%) were supplied by Panreac. Ultra-pure water was obtained using a Milli-Q water purification system (<0.05 µS/cm, Milli-Q model 185, Millipore, Bedford, MA, USA).

Empty solid phase extraction (SPE) tubes (6 mL) and polypropylene (PP) frits were purchased from Supelco. 50 mL PP conical tubes (internal diameter 27.2 × 117.5 mm length) obtained from Deltalab (Barcelona, Spain) and 1.5 mL Eppendorf tubes supplied by Eppendorf (Berzdorf, Germany) were used for the desorption of Nylon membranes and PES hollow fibres, respectively. Additionally, Oasis-HLB (hydrophilic–lipophilic-balanced, 200 mg) SPE cartridges were purchased from Waters (Milford, USA) for the analysis of exposure media.

High purity nitrogen gas (>99.999%) supplied by Messer was used to evaporate the extracts using a Turbovap LV Evaporator (Zymark, Hopkinton, USA) and as collision gas during the liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Moreover, nitrogen gas (99.999%) provided by Air Liquide (Madrid, Spain) was used as both nebuliser and drying gas during the analysis. Finally, PP filters (0.22 µm, 13 mm) from Phenomenex (California, USA) were used for filtration of all the extracts before the LC-MS/MS analysis.

5.2.2 Passive samplers

Strata X-AW (Phenomenex, Torrance, CA, USA) and Bond-Elute Plexa (Plexa, Agilent, Santa Clara, CA, USA) sorbents were used for the POCIS sampler. Nylon membranes (30 µm of pore size, 65 µm thickness, 45 mm diameter) were acquired from Fisher Scientific (Illkirch, France). The stainless steel rings for the POCIS were homemade by UfZ-Helmholtz Centre for Environmental Research (Leipzig, Germany). PES hollow fibres were purchased from Membrana GmbH (Wuppertal, Germany) (16 cm x 0.7 mm external diameter, 1.43 g/mL density).

POCIS sorbents and PES hollow fibres were thoroughly cleaned before their use. POCIS-sorbents (Strata X-AW and Plexa) were cleaned separately with 100 mL of 2.5% NH_3 in MeOH followed by MeOH and Milli-Q water (30 min for each solvent) in an ultrasonic bath (USB Axtor from Lovango Barcelona, Spain). PES pieces were shaken in pure MeOH for 24 h, renewing the solvent twice during that period of time.

5.2.3 POCIS

Before deployment, each POCIS sorbent was prepared with the corresponding PRC mixture at 4 µg/g of each of the five deuterated analogues. Each PRC was independently spiked to ~3 g of each sorbent and ~25 mL of acetone were finally added. This mixture was stirred for 24 h at 800 rpm, and, then, the acetone was evaporated to dryness at room temperature. Finally, 100 mg of each fortified sorbent was accurately weight and arranged between two Nylon membranes before being fixed with two home made stainless steel rings.

Regarding the extraction of the POCIS after exposure, the sorbent was carefully removed from the membranes using ~10 mL of Milli-Q water and introduced into empty SPE cartridges. The

sorbent was dried under vacuum for ~ 1 h. Afterwards, the cartridges were stored in the freezer at -40°C until analysis. The analytes were firstly eluted with 6 mL of 2.5% NH_3 in MeOH and subsequently with other 6 mL of MeOH. The mixture was evaporated to dryness using a TurboVap LV Evaporator at 35°C and reconstituted in 200 µL of MeOH: Milli-Q water (30:70, *v:v*) mixture. Finally, extracts were filtered through a 0.22 µm PP filter before the LC-MS/MS analysis.

Concerning the two Nylon membranes in each POCIS device, both were immersed together in 10 mL of MeOH and sonicated in an ultrasound bath for 30 min. Then the membranes were removed, and the MeOH extracts were evaporated and reconstituted following the same procedure used for POCIS sorbent.

5.2.4 PES hollow fibres

21 PES hollow fibres of 16 cm (~80 mg) were individually spiked with 10 µg/g of each PRC. Each fibre was immersed in 12 mL of Milli-Q water and 20 µL of a solution containing 60 mg/L of each of the deuterated analogues used as possible PRCs were added. Then, the tubes were shaken for 24 h in an agitator (Reax 2 Overhead Shaker, Heidolph, USA). After the PES fortification with the PRCs, the fibres were removed and dried with a clean lint free tissue paper and every fibre was cut in three smaller pieces. The PES pieces were introduced in 6 different stainless steel tea balls (each ball included three PES pieces).

After the exposure, PES fibres were cut in small pieces (8 pieces per replicate) and introduced in a microcentrifugation tube (Eppendorf, 2 ml) with 1 mL of MeOH. The tubes were sonicated for 30 min in an ultrasound bath to extract the analytes from the fibres, and then the extracts were stored in a freezer at -40°C until analysis. Immediately before the LC-MS/MS analysis, the extracts were evaporated and reconstituted as in the case of POCIS sorbents.

5.2.5 Laboratory calibration

The laboratory calibration of passive samplers was performed at the Plentzia Marine Station (PiE) according to a previous design based on a continuous flow calibration approach (Posada-Ureta et al., 2016; Vallejo et al., 2013). The POCIS and PES samplers were allocated together in a stainless steel carrousel vertically to the water flow direction. Afterwards, the carrousel was immersed in a

metallic tank (~50 L) full of seawater and the carrousel was stirred at a constant rate of 50 rpm (equivalent to a linear water velocity of ~70 cm/s). The exposure tank was continuously fed with seawater at 2 L/h and with a stock solution containing all the analytes at 20 mL/h with a peristaltic pump (323S Watson-Marlow pump, Cornwall, UK) and the exposure was kept for 14 days. The resulting nominal concentration of each analyte in the tank was ~600 ng/L. In order to assure the steady state of that nominal value, the feeding set-up was initiated 72 h before exposing the passive samplers. As the carrousel can only hold 14 POCIS, when some POCIS were removed from the tank, new samplers were placed to assure 3 replicates per day. Moreover, on day 7th of the calibration experiment, additional new replicates (n=2) of POCIS and PES samplers were added and removed 4 and 7 days later in order to validate the calibration model with an independent set of samplers.

Along the calibration and every two or three days, 3 POCIS, 1 tea ball (with the 3 PES fibres) and 250 mL of water were removed from the tank to analyse the target compounds. Simultaneously, blanks (n=3) were air exposed during the deployment and retrieval time to control any potential external contamination. Furthermore, some physicochemical parameters (temperature, dissolved oxygen, pH, redox potential, conductivity and salinity) of the seawater were monitored with a multiparametric probe (EXO 2, YSI, USA).

5.2.6 Water samples

Water sample extraction was carried out according to a previously validated SPE procedure (Mijangos et al., 2018). Briefly, the samples were acidified (pH 2) with 2 mL of formic acid, and EDTA was added at a concentration of 0.1% (m/m). Oasis-HLB cartridges were sequentially conditioned with 5 mL of MeOH, 5 mL of Milli-Q water and finally, with 5 mL of Milli-Q water at pH=2. Once the water samples were percolated through the cartridges, they were washed with 6 mL of Milli-Q water and then vacuum dried for an hour. The cartridges were afterwards stored at -40°C until analysis. The elution was carried out with 6 mL of MeOH, and then the solvent was evaporated to dryness and the sample reconstituted in 200 µL MeOH, as mentioned previously, before analysis by LC-MS/MS. Three replicates of water samples were processed.

5.2.7 LC-MS/MS analysis

All the extracts were analysed in a HPLC-QqQ (Agilent 1260 series LC coupled to an Agilent 6430 triple quadrupole) equipped with an electrospray ionisation (ESI) source (Agilent Technologies, CA, USA). The separation of the 20 target analytes was accomplished according to (Mijangos et al., 2018) at a flow of 0.3 mL/min using a Kinetex F5 100 Å core-shell (2.1 mm × 100 mm, 2.6 μm) column coupled to a Kinetex F5 pre-column (2.1 mm x 4.6 mm, 2.6 μm). The column temperature and the injection volume were set to 35°C and 5 μL, respectively. A binary mixture consisting of a mobile phase A of Milli-Q water: MeOH (95: 5) mixture and a mobile phase B of MeOH: Milli-Q water (95: 5) mixture, both containing 0.1% of formic acid, was used for gradient separation of the target analytes. The gradient profile started with 30% B which was increased to 50% in 4 min and maintained for 12 min. Then it was increased to 90% B where it was maintained constant for 10 min. Initial gradient conditions (30% B) were then achieved in 6 min, where it was finally held for another 10 min (post-run step). ESI was carried out using a N_2 flow rate of 12 L/min, a capillary voltage of 3500 V, a nebuliser pressure of 45 psi, and a source temperature of 350°C. Both, negative and positive voltages, according to the target analytes, were simultaneously applied in a single injection.

Quantification was performed in the selected reaction monitoring (SRM) acquisition mode. The instrumental details regarding the LC-MS/MS measurements, including precursor and product ions (m/z), collision energy (eV) and fragmentor voltage (V) values and polarity, as well as the instrumental limits of quantification (LOQs) are included in **Table 3.2**, **Chapter 3**. Instrumental operations, data acquisition and peak integration were performed with the MassHunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

5.3 Results and discussion

5.3.1 Recovery of the target analytes

Recovery values obtained were in the range of 75-99% for all the analytes and for the three different polymeric materials (POCIS sorbent mix, Nylon membranes and PES hollow fibres). Repeatability in terms of relative standard deviations (n=3; RSDs) between 4 and 19% was obtained

in all the cases. In the case of blank samplers, none of the target analytes were detected in the materials, therefore, they were not considered for the recovery calculations.

5.3.2 Compounds stability

As reported in **Table 5.2**, some physicochemical parameters of seawater at the exposure tank were measured along the experiment. Temperature, conductivity and salinity were constant along the whole experiment with RSDs% lower than 5% and the redox potential values showed an RSD of 21%.

Table 5.2: Physic-chemical parameters of water measured during the experiment.

Exposure Day	Temperature (°C)	pH	Redox potential (ORP, mV)	Conductivity (ms/cm)	Dissolved oxygen (%)	Salinity (psμ)
0	13.8	7.4	143	40.5	98.3	33.8
2	13.7	7.3	154	40.2	97.0	33.6
4	13.6	7.1	141	40.2	99.8	33.8
7	13.4	7.8	97	39.8	93.2	33.6
9	13.3	7.6	156	39.6	92.3	33.4
11	13.5	7.4	172	40.0	93.2	33.8
14	13.6	7.6	98	40.0	86.6	33.6
Average	13.6	7.5	137	40.0	94.3	33.7
RSD%	1.3	3.1	21	0.7	4.7	0.4

The concentrations of the compounds in the exposure tank were also monitored during the calibration period (14 days) (see **Table 5.3**). As a rule, these concentrations were kept under statistical control (average±2 x standard deviation, *sd*), except for PFOA and PFOS. Most of the target analytes showed concentrations around the nominal value (~600 ng/L) except for norfloxacin, OBT and PFBS, which showed values around 800 ng/L, and for caffeine, with values close to 1 μg/L. On the contrary, PFOA, PFOS and progesterone showed concentrations (308-404 ng/L) lower than the nominal ones, which may be explained in terms of the evaporation losses from the stock solution during the fortification step or because the analytes tend to stick on the glass material used but no degradation was suspected since the concentrations remained constant during the calibration period.

Table 5.3: The sampling rate (Rs)±*sd* (mL/day) (n=7 and *sd* from the slope) and the coefficient of determination (r^2) for POCIS sorbent, Nylon membrane and PES fibres, and average concentrations (14 days) in seawater (C_w, ng/L). For PES fibres the release kinetic constant (k_2) is also included.

Analyte	POCIS		Nylon		PES			C_w (ng/L)
	Rs	r^2	Rs	r^2	Rs	k_2	r^2	
Acesulfame	4.2±0.3	0.97	n.e.s		n.e.s			593±53
Acetaminophen	2.7±0.2	0.98	n.e.s.		n.e.s			694±46
Amitriptyline	147±4	0.99	4.9±0.5	0.95	4±2	0.5±0.3	0.95	568±75
Atrazine	294±15	0.98	26±2	0.96	7±2	0.4±0.1	0.98	675±36
Bezafibrate	47±8	0.99	39±1	0.99	1.7±0.6	0.18±0.09	0.97	685±30
Butylparaben	308±12	0.99	47±1	0.95	157±69	0.2±0.1	0.95	506±37
Caffeine	55±4	0.96	n.e.s		n.e.s			1125±68
Carbamazepine	370±18	0.99	15±1	0.97	3±1	0.2±0.1	0.91	634±35
Diuron	409±11	0.99	18±2	0.95	44±9	0.3±0.1	0.99	597±31
Irbesartan	232±8	0.99	46±3	0.99	17±4	0.3±0.1	0.98	595±46
Ketoprofen	411±15	0.99	11.8±0.8	0.98	l.o.f			690±34
Norfloxacin	l.o.f		n.e.s		n.e.s			899±53
OBT	263±15	0.98	3.6±0.3	0.95	43±9	0.3±0.1	0.98	756±62
PFBS	277±14	0.98	n.e.s		10±6	1.1±0.8	0.97	793±51
PFOA	491±24	0.99	23±2	0.97	n.e.s			324±68
PFOS	144±5	0.99	43±2	0.99	l.o.f			212±63
Phenytoin	426±17	0.99	34±2	0.98	8±5	0.2±0.2	0.90	671±29
Progesterone	238±17	0.97	32±2	0.98	76±16	0.4±0.1	0.99	354±12
Sulfadiazine	l.o.f		n.e.s		n.e.s			581±79
Telmisartan	68±1	0.99	50±4	0.98	27±5	0.3±0.1	0.99	469±25

l.o.f The fit was not acceptable (lack of fit).
n.e.s The uptake was too low to be considered (not enough sensitivity).

5.3.3 Uptake kinetics

The first step was the study of the uptake kinetics curves, in order to estimate the Rs of each compound in the POCIS sorbent, in the Nylon membranes and in the PES fibres. The exchange kinetics between a passive sampler and the bulk water phase has been extensively described in the literature (Górecki and Namieśnik, 2002; Vrana et al., 2005) and it is assumed to follow the model shown in **Equation 5.1**.

$$C_s(t) = C_w \cdot {}^{k_1}/_{k_2} \cdot (1 - \exp\,(-k_2 \cdot t)) \quad \textbf{Equation 5.1}$$

where $C_s(t)$ is the concentration in the sampler at exposure time t, C_w is the concentration in water, and k_1 and k_2 the uptake and dissipation rate constants, respectively. Under the kinetic

regime, the uptake is directly proportional to exposure time, i.e. **Equation 5.1** can be written as follows:

$$m_s(t) = C_{TWA} \cdot Rs \cdot t \quad \text{Equation 5.2}$$

where Rs (L/day) is the sampling rate, $m_s(t)$ is the mass of analyte in the sampler after a t exposure time and C_{TWA} is the integrated average water concentration. The values of these integrated concentrations can be calculated from the values shown in **Table I**, **Appendix.**

In the case of PES hollow fibres, Rs and k_2 values were estimated from the non-linear fit to the exponential **Equation 5.3** (Curvefit toolbox for Matlab 2012b):

$$m_s(t) = C_{TWA} \cdot \frac{Rs}{k_2} \cdot (1 - \exp(-k_2 \cdot t)) \text{ Equation 5.3}$$

The estimated fitting parameters (Rs values for POCIS and Rs and k_2 for PES) and their respective *sd* are summarised in **Table 5.3**.

As shown in **Figure 5.1**, as example, the uptake of atrazine is linear without any burst or lag effects in POCIS sorbent and Nylon membranes, but it follows an exponential shape for PES hollow fibres. These patterns are generally extended to all the compounds, except for norfloxacin and sulfadiazine in the case of POCIS sorbent, and acetaminophen, acesulfame, caffeine and PFBS for Nylon membranes.

Most of the fits were statistically acceptable ($r^2 \geq 0.90$) but, since the accumulated mass of some particular compounds was close to the resolution of our analytical method (i.e. $\max(m_s)<10$ ng) the uptake was considered negligible. Consequently, as shown in **Table 5.3**, the three Rs values were reported only for amitriptyline, atrazine, bezafibrate, butylparaben, carbamazepine, diuron, irbesartan, OBT, phenytoin, progesterone and telmisartan. The highest Rs values were obtained in POCIS sorbent (between 2.7 mL/day for acetaminophen and 491 mL/day for PFOA) followed by Nylon membranes (between 3.6 mL/day for OBT and 50 mL/day for telmisartan) and the lowest were obtained in PES fibres (between 1.7 mL/day for bezafibrate and 157 mL/day for butylparaben).

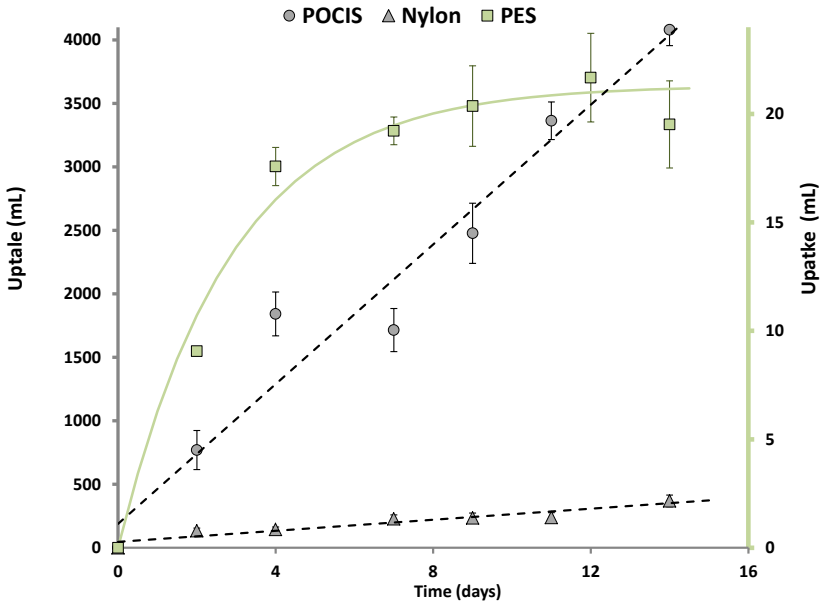

Figure 5.1: *Uptake plots of atrazine for the three evaluate passive samplers (POCIS sorbent, Nylon membrane and PES hollow fiber). The uptakes are plotted according to* ***Equation 5.2*** *for POCIS sorbent and Nylon membranes (left Y axis) and according to* ***Equation 5.3*** *for PES hollow fiber (right Y axis).*

Except for sulfadiazine and norfloxacin, the sorbent mixture used in the POCIS showed linear uptakes for all the compounds over the 14 days regardless of the nature of the compounds (i.e., acesulfame is a highly hydrophilic compound and PFOS is a surfactant negatively charged at the seawater pH value). The fact that sulfadiazine and norfloxacin forms strong metal-ligand complexes with cations typically present in seawater (e.g., Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+}), as reported in the literature (Gros et al., 2013; Gu and Karthikeyan, 2005; Schmitt and Schneider, 2000), may explain the lack of fit observed. Therefore, these two analytes were discarded and not considered in further experiments.

The comparison of the Rs values obtained in this work and those reported in the literature are summarised in **Table 5.4**. Regarding the POCIS sorbent, the Rs were comparable to those reported in the literature following equivalent procedures. Furthermore, to the best of our knowledge, the values of butylparaben, irbesartan, telmisartan and OBT were reported for the first time. The Rs values obtained in this work ranged between 0.0027 and 0.49 L/day with uncertainties between 2-5% (RSD) and they were slightly lower than those reported (0.03-2.78 L/day) by Belles et al. (Belles et al., 2013) using Nylon-POCIS configuration. The highest sampling rates (i.e. > 400 mL/day) were obtained for diuron, ketoprofen, phenytoin, bezafibrate and PFOA, while Rs values of acesulfame (log $D_{(pH=7.4)}$=- 3.1) and acetaminophen (log $D_{(pH=7.4)}$=0.9) were remarkably lower. The use of Strata X-AW sorbent seems to play a remarkable role to improve the uptake of PFBS, PFOA and PFOS (Fedorova et al., 2013; Kaserzon et al., 2013, 2012; Li et al., 2016). Other works in the literature have shown that the modification of the type of sorbent in the POCIS sampler comes accompanied by the increase of the number of compounds that can be analysed (Iparraguirre et al., 2017).

It is also worth mentioning that the Rs values reported in this work for POCIS sorbent are similar to those estimated in different waters (seawater (Martínez Bueno et al., 2009), drinking water (Belles et al., 2014; Fauvelle et al., 2012; Kaserzon et al., 2012; Morin et al., 2012; Morrison and Belden, 2016a) and wastewater (Fedorova et al., 2013; Iparraguirre et al., 2017), which would offer a robust use of these samplers in estuarine or coastal waters. Additionally, the fact that the more acidic analytes could compete with seawater anions, such as phosphate, sulphate and/or chloride, for the anion exchanger sites, seemed to be non-relevant.

In the case of Nylon membranes, it was able to estimate Rs values for also a high number (14 of 20) of compounds (see **Table 5.3**). The net mass found in the Nylon membranes was much lower than that found in the POCIS sorbent (less than 10% for most of the compounds) except for PFOS, irbesartan and butylparaben, where values around 15% were achieved, and particularly, for telmisartan, with comparable accumulations. This fact suggests either a lower affinity for the Nylon membrane or an effective and fast diffusion transfer from the bulk solution to the sorbent through the pores of the membrane.

In the case of PES fibres, it is worth mentioning the faster uptake kinetics compared to the other two samplers. As shown in the uptake profile shown in **Figure 5.1**, the equilibrium was achieved in one week while, for the other two, the kinetic regime is still maintained after two weeks. The use of PES hollow fibres as passive samplers has been described in the literature for non-polar compounds (Posada-Ureta et al., 2016) and the reported Rs values are between 9 mL/day (chlorfenvinphos) to 130 mL/day (4,4'-DDT), which are of the same order of magnitude to those reported in this work.

When the surface of both polymers is taken into account (i.e. 26.4 cm^2 vs. 3.5 cm^2 for Nylon and PES, respectively) the relative suitability or affinity of each sampler can be obtained. In this case, PES (0.48-45 mL/day) showed higher normalised Rs values than Nylon (0.14-1.89 mL/day) for most of the studied compounds (only bezafibrate showed a higher equivalent sampling rate in Nylon; 1.48 vs. 0.48 mL/day). It would be more convenient to use Nylon membranes rather than PES ones in POCIS since the affinity for many contaminants is much lower and the transport across the membrane is faster.

Finally, correlation coefficients of Rs values with some physicochemical features of the studied compounds (physico-chemical parameters included in **Table 5.1**) were calculated. In general terms, low and non-significant correlations (Abs(r) < 0.54, p-level > 0.05) were observed for most of the descriptors and Rs values and, only in the case of Nylon membrane (considered as an independent passive sampler), several significant correlations were observed, particularly the positive high correlation between Rs and polar surface area (r^2=0.70) and Van der Waals accessible surface area (r^2=0.74). These results suggest an uptake mode based on polar interactions between the molecules and the membrane that should be confirmed in future experiments.

Table 5.4. Compound wise comparison between the Rs obtained in this work and in the literature.

Analyte	Rs (mL/day)	Matrix	Sorbent	Membrane (pore size)	Reference
Acesulfame	4.2±0.3	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	80	Tap water	Oasis HLB (220 mg)	PES (0.1 µm)	Sultana et al., 2017
Acetaminophen	2.7±0.2	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	2±14	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Miege et al., 2012
	n.d.	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Morin et al., 2012
Amitriptyline	147±4	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	120±70	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Belles et al., 2014
	180±130	Tap water	Oasis HLB (200 mg)	Nylon (30 µm)	Belles et al., 2014
Atrazine	294±15	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	286±12	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Fauvelle et al., 2014
	263±8	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	186±4	Tap water	Oasis MAX (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	103±4	Tap water	Chromabond HR-X (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	253±10	River	Oasis HLB (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	198±10	River	Oasis MAX (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	102±3	River	Chromabond HR-X (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	300±90	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Belles et al., 2014
	1111±760	Tap water	Oasis HLB (200 mg)	Nylon (30 µm)	Belles et al., 2014
	929	Tap water	Dowex optiporeL-493 (200 mg)	Nylon (35 µm)	Morrison et al., 2016
	994	Tap water	Oasis HLB (200 mg)	Nylon (35 µm)	Morrison et al., 2016
	214	Seawater	Oasis HLB (200 mg)	PES (0.1 µm)	Martinez-Bueno et al., 2009
	189±6	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Morin et al., 2012
Bezafibrate	473±8	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	146±34	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Morin et al., 2012
Butylparaben	308±12	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
Caffeine	55±4	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	167	River water	Oasis HLB (200 mg)	PES (0.1 µm)	Vermeirssen et al., 2012
	44±36	Tap water with 30 g/L NaCl	Oasis HLB (200 mg)	PES (0.1 µm)	Bayen et al., 2014
Carbamazepine	370±18	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	497±15	Tap water with 30 g/L NaCl	Oasis HLB (200 mg)	PES (0.1 µm)	Bayen et al., 2014
	354±42	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Li et al., 2011
	164±3	Tap water	Oasis MAX(200 mg)	PES (0.1 µm)	Li et al., 2011
	248±38	Tap water	Oasis MCX(200 mg)	PES (0.1 µm)	Li et al., 2011
	140±30	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Belles et al., 2014
	190±110	Tap water	Oasis HLB (200 mg)	Nylon (30 µm)	Belles et al., 2014
Diuron	409±11	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	284±9	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Fauvelle et al., 2014
	258	River	Oasis HLB (200 mg)	PES (0.1 µm)	Vermeirssen et al., 2012

Table 5.4. Compound wise comparison between the Rs obtained in this work and in the literature.

Analyte	Rs (mL/day)	Matrix	Sorbent	Membrane (pore size)	Reference
Diuron	208±8	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	162±5	Tap water	Oasis MAX (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	49±3	Tap water	Chromabond HR-X (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	212±6	River	Oasis HLB (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	164±10	River	Oasis MAX (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	56±3	River	Chromabond HR-X (200 mg)	PES (0.1 µm)	Fauvelle et al., 2012
	330±100	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Belles et al., 2014
	1580±1050	Tap water	Oasis HLB (200 mg)	Nylon (30 µm)	Belles et al., 2014
	86	Seawater	Oasis HLB (200 mg)	PES (0.1 µm	Martinez-Bueno et al., 2009
	198±5	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Morin et al., 2012
Irbesartan	232±8	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
Ketoprofen	411±15	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	160±30	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Belles et al., 2014
	520±410	Tap water	Oasis HLB (200 mg)	Nylon (30 µm)	Belles et al., 2014
	118±7	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Morin et al., 2012
OBT	263±15	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
PFBS	277±14	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	n.d.	WWTP effluent	Oasis HLB (200 mg)	PES (0.1 µm)	Fedorova et al., 2013
	370±70	Drinking water	Strata X-AW 600 mg	PES (0.45 µm)	Kaserzon et al., 2012
PFOA	491±24	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	n.d.	WWTP effluent	Oasis HLB (200 mg)	PES (0.1 µm)	Fedorova et al., 2013
	160±10	Tap water	Strata X-AW 600 mg	PES (0.45 µm)	Kaserzon et al., 2012
PFOS	144±5	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	222±35	WWTP effluent	Oasis HLB (200 mg)	PES (0.1 µm)	Fedorova et al., 2013
	360±80	Tap water	Strata X-AW 600 mg	PES (0.45 µm)	Kaserzon et al., 2012
Phenytoin	426±17	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	557±120	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Bayen et al., 2014
Progesterone	238±17	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work
	346±8	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Morin et al., 2012
	47±4	Tap water	Oasis HLB (200 mg)	PES (0.1 µm)	Vallejo et al., 2013
Telmisartan	68±1	Seawater	Strata X-AW and Plexa (200 mg)	Nylon (30 µm)	This work

MAX: strong anion exchange, n.d.: not detected, PES: polyethersulfone, Rs: sampling rate, X-AW: weak anion exchange.

5.3.4 Evaluation of the PRC suitability

It is generally assumed that a PRC can be accepted if the regression lines for dissipation show good fittings ($r^2 > 0.90$) and the dissipation takes place among a significant range of masses (i.e. 20-80% of the initially spiked mass) to assure the analytical resolution of the retained fraction. Besides, PRCs can be applied only when it is demonstrated that the kinetics of a compound uptake and release are isokinetic, i.e. characterised by rate constants that are equal or close to each other.

As shown in **Figure 5.2,** the elimination constants (k_e) from the POCIS sorbent (**Figure 5.2a**) and PES (**Figure 5.2b**) were determined according to **Equation 5.4**, where m_t and m_0 (ng) corresponds to the amount of analyte in the sorbent at *t* days and at 0 day of exposure.

$$\ln\left(\frac{m_t}{m_0}\right) = k_e\, t \qquad \text{Equation 5.4}$$

In the case of POCIS, [$^2H_9$]-progesterone, [$^2H_5$]-atrazine and [$^2H_3$]-amitriptyline fulfilled the above mentioned conditions, with maximum dissipation in the range of 68-90% and linear regression coefficients higher than 0.98. The dissipation of [$^2H_9$]-progesterone showed a short lag of one-two days that were not observed in the case of the other two PRCs. The values of the k_e (L/day) obtained were - 0.19±0.01 for [$^2H_9$]-progesterone, -0.099±0.006 for [$^2H_5$]-atrazine and -0.19±0.01 for [$^2H_3$]-amitriptyline.

On the other hand, in the case of PES fibres, a non-linear dissipation was observed after 7 days of exposure, as it happened for the uptake, and therefore the linear plots could only be built during the first week of exposure. In this case, only [$^2H_9$]-progesterone and [$^2H_5$]-atrazine showed good fittings ($r^2 > 0.95$), but since the maximum dissipation was lower (< 25%) we did not consider them for any further correction. The values of k_e were -0.178±0.005 L/day for [$^2H_5$]-atrazine and -0.103±0.003 L/day for [$^2H_9$]-progesterone.

In the literature, only few studies have reported the use of labelled analogues as PRC candidates in this kind of passive samplers. Belles et al. (Belles et al., 2014) reported the use of [$^2H_5$]-desisopropyl-atrazine ([$^2H_5$]-DIA), [$^{13}C_3$]-caffeine and [$^2H_3$]-salbutamol and they did not observe significant differences in the dissipation rates. On the other hand, Fauvelle et al. (Fauvelle et al., 2012) used two different deuterated analogues ([$^2H_5$]-DIA and [$^2H_3$]-dicamba) in different POCIS

configurations, and only [$^{2}H_5$]-DIA provided good results when Oasis-MAX sorbent was used. Moreover, first order PRC elimination from POCIS-Nylon configuration was demonstrated for [$^{13}C_3$]-caffeine, cotinine-d_3 and [$^{2}H_5$]-DIA (Morrison and Belden, 2016b); however, no discernible elimination was observed for [$^{2}H_5$]-atrazine, [$^{2}H_{10}$]-fluoranthene and lindane. Therefore, it can be concluded that the selection of a PRC and its suitability is intimately related to the features of the membranes and the sorbents used in POCIS.

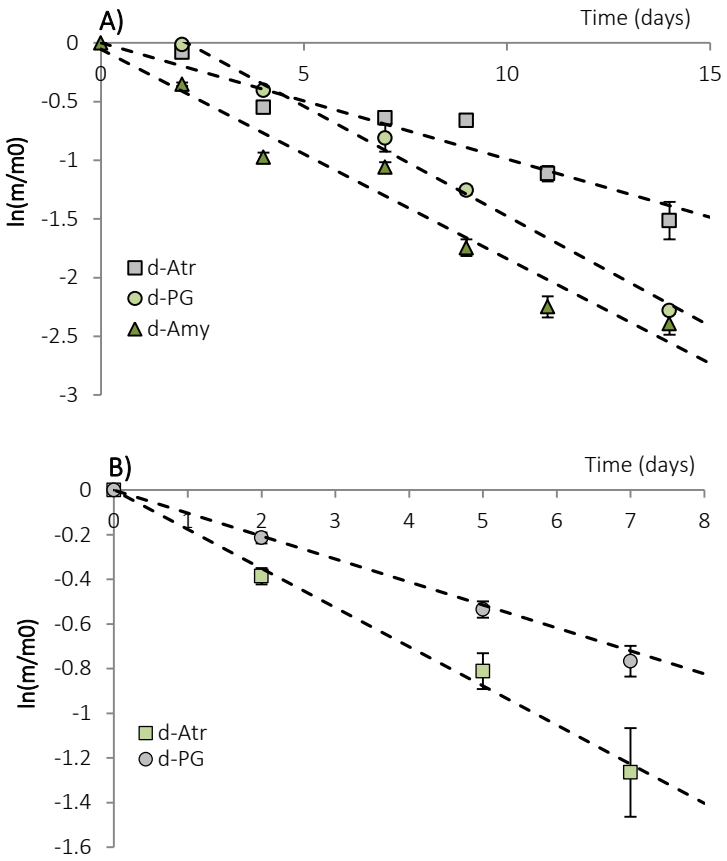

Figure 5.2: *Dissipation plots of the PRCs for a) the POCIS sorbent and b) PES hollow fiber. In the former case the plots of [$^{2}H_5$]-atrazine, [$^{2}H_9$]-progesterone and [$^{2}H_3$]-amitriptyline are shown and in the later those of [$^{2}H_5$]-atrazine and [$^{2}H_9$]-progesterone. In both cases, the dissipation has been plotted according to* ***Equation 5.4****.*

Besides, the suitability of the PRCs was also tested in an internal validation. As mentioned in **section 5.2.3**, some of the POCIS and PES fibres were removed from the tank on day 7th of the calibration experiment and additional new replicates (n=2) of POCIS and PES samplers added and removed 4 and 7 days later, aiming to be used as an "external" validation set and to check the robustness of the calibration process and the estimated Rs values. The samplers that were shifted showed more rust than those introduced at the beginning of the experiment. This particular fact could be considered as a kind of biofouling effect and therefore, it offered the chance to recalculate C_{TWA} for each analyte based on the correction of the laboratory Rs values and the kinetic elimination constants, as shown in **Equation 5.5**:

$$Rs_{corr} = \left(\frac{k_{e\,int.val}}{k_{e\,cal}}\right) \times R_S \quad \text{Equation 5.5}$$

where $k_{e,cal}$ is the elimination constant estimated in the calibration process, and $k_{e\ int.val}$ is the value estimated directly from the validation samples after applying **Equation 5.4**.

Assuming that the average water concentrations, i.e. based on samples at each sampling day (and shown in **Table 5.3**), are good reference values, these average values were plotted against the estimated C_{TWA} values from the non-corrected and corrected Rs values. As shown in **Figure 5.3** for 7-day samples and with the [$^2H_5$]-atrazine correction, the slope of this plot was 1.0, while the non-corrected one was 0.7. Similar results were obtained when [$^2H_3$]-amitriptyline was used as PRC (i.e. slopes of 0.6 and 0.85 for the non-corrected and corrected results, respectively). In the case of PES fibres, as expected from the low dissipation rates, the PRC corrections did not provide good results. It should be highlighted, that no dramatic differences were observed between time-integrated passive sampler and active sampler concentrations, since the C_{TWA} systematically overestimates the water concentrations from spot samples by approximately 50-100 ng/L.

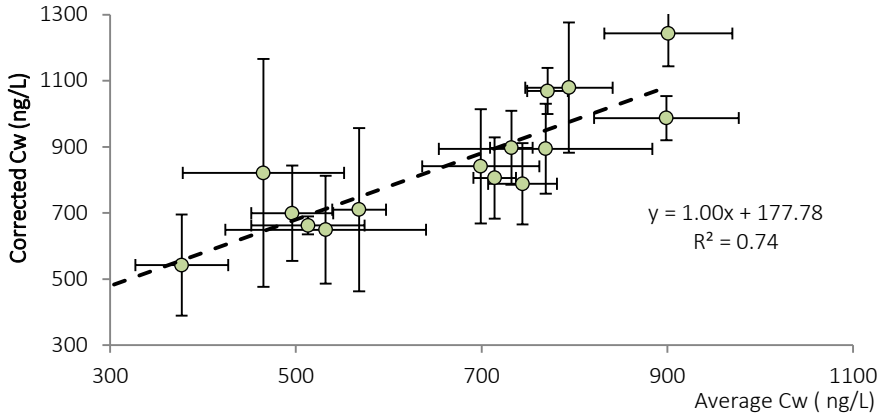

Figure 5.3: *Comparison of spot analysis (SPE) average concentrations and C_w (ng/L) values obtained using POCIS passive sampling approach corrected with [$^2H_5$]-atrazine after 7 days of exposure. The error bars in both axes are the standard deviation of the measured and estimated concentrations.*

5.4 Conclusions

The main achievements of this work can be summarised as follows. First, the calibration of the POCIS uptake of a large number of emerging contaminants was carried out satisfactorily using an innovative sorbent mixture (Strata X-AW and HLB Plexa) and using Nylon as a supporting membrane, which enables the analysis of a wide range of organic compounds. This new POCIS sampling device showed the feasibility of the simultaneous uptake of hydrophilic, acidic and basic compounds, together with the low interference and the good hydrodynamic behaviour of Nylon. Second, the use of POCIS samplers and the [$^2H_5$]-atrazine as PRC allowed the efficient correction of the C_W^{TWA} values of target analytes. It should be highlighted that in spite of not considering a composite active sample, a reasonable agreement was found between grab sampling values and the ones derived from laboratory calibration of POCIS in seawater after PRC corrections.

Finally, POCIS was applied to the analysis of the target compounds in estuaries of Biscay, as will be described in **chapter 7**.

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5 Stability assessment of ECs

Short term preservation and stability assessment for the analysis of emerging contaminants in seawater

Environmental Science and Pollution Research. Submitted

ESPR-D-18-05866

6.1 Introduction

The analysis of the so-called emerging contaminants, i.e. the potentially hazardous compounds that are not under any environmental regulation (Postigo and Barceló, 2015), is facing a number of methodological challenges. Among them, we can highlight the development and implementation of suitable high-throughput methods following the suspect and non-target screening procedures (Schymanski et al., 2015), the design of proper extraction and preconcentration procedures allowing the screening of the widest variety of compounds in a single run, and the strategies to assure the integrity of the samples up to the analysis should be kept into account, since most of the compounds considered are bioactive and their lack of stability can hamper all the analytical efforts (Baker and Kasprzyk-Hordern, 2011a; Fedorova et al., 2014; Petrovic, 2014).

Regarding the last point, however, we tend to assume the integrity of the analytes during the sampling step while the storage of the samples should assure their stability, as pointed out recently (Analytical Methods Committee, 2015). The increasing interest for the analysis of contaminants of emerging concern, the extended use of passive sampling (PS) methods (Miège et al., 2015) and the management requirements when a large number of samples are being processed, have opened the discussion about the stability of the samples as well as the best approaches to assure their preservation. In this sense, we can highlight the review for pharmaceuticals in natural waters by Mompelat et al. (Mompelat et al., 2013), the analysis of illicit drugs in sewers and wastewaters by McCall et al. (McCall et al., 2016) or the analysis of antibiotics by Llorca et al. (Llorca et al., 2014).

Concerning to the PS methods and its increasing use (Miège et al., 2015), new sampling methodologies such as semipermeable membrane devices (Sultana et al., 2017), Chemcatcher (Kaserzon et al., 2014; Vermeirssen et al., 2012), and polar organic chemical integrative samplers (POCIS) (Iparraguirre et al., 2017; Mijangos, 2018b; Vallejo et al., 2013), allow the direct analysis from water matrices avoiding, to some extent, the stability issues. However, issues such as the preservation of compounds in different sorbents during the PS may arise, as pointed by Carlson et al. (Carlson et al., 2013).

As pointed before, labile analytes such as pharmaceuticals and pesticides are bioactive and

hence may undergo different chemical, physical and biological processes from the sampling up to the analysis (Fatta-Kassinos et al., 2011). Thus, depending on stability, quantifying a compound that has been released several hours previously may, in fact, lead to a significant under estimation of the actual amount of residue present. As a consequence, the procedures and strategies to collect and handle the samples are usually guided by the compliance to the existing regulations or to the good laboratory procedures (Baker and Kasprzyk-Hordern, 2011a; Mompelat et al., 2013). Typically, the factors studied are the influence of suspended solids (Baker and Kasprzyk-Hordern, 2011a), the addition of a preserving agent (González-Mariño et al., 2010; Llorca et al., 2014) and the storage conditions (temperature, pH and time) (Baker and Kasprzyk-Hordern, 2011a; Mompelat et al., 2013).

One of the favourite options is the use of solid phase extraction (SPE) cartridges (Ferrer et al., 2011; González-Mariño et al., 2010; Petrović and Barceló, 2000; Turiel et al., 2004) because we gain the extraction of the analytes and we save a lot of space in the labs. On the other hand, the growing interest for passive samplers such as POCIS (Carlson et al., 2013) can provide simplified procedures to sampler store, but we lack the knowledge regarding the sorptive features on the different polymers in short and long-term storage. The effect of wettability on the performance of polymers-stability and properties has been studied in the literature (Sharma and Bijwe, 2012), although it is not explored its effect on the analytes stability.

Therefore, the aim of this work was to evaluate of the stability of 23 organic contaminants (21 emerging compounds and 2 priority contaminants) in seawater during one month and under different preservation procedures. For this purpose, four different preservation modes were tested: (i) seawater samples stored at 4^{o}C, (ii) preconcentration of spiked seawater in a SPE cartridge with a mixture of Bond Elute Plexa and Strata X-AW sorbents (commonly used as passive sampler sorbents (Fauvelle et al., 2012; Kaserzon et al., 2014, 2012; Mijangos, 2018b)) and stored at −20^{o}C, (iii) preconcentration in a polyethersulfone (PES) hollow fibres (disposable polymeric materials used in microextraction techniques (Bizkarguenaga et al., 2015; Blanco-Zubiaguirre et al., 2014a; Mijangos et al., 2018a; Prieto et al., 2010)) and stored at −20°C and finally, (iv) the storage of methanol extracts at −20^{o}C. The target analytes include herbicides, hormones, life style products (stimulants and artificial sweeteners), personal care products, phytoestrogens, industrial chemicals (corrosion inhibitor and perfluoroalkyl substances) and pharmaceuticals (dihydrofolate reductase inhibitor,

fluoroquionolones, sulfonamides, dihydrofolate reductase inhibitor (DHFR inhibitor), tricyclic antidepressants, antihypertensives, anti-inflammatories, β-blocker cardiovascular drugs, lipid-regulatings, angiotensin II receptor antagonists [ARA-IIs] and anticonvulsant psychiatric drug). Additionally, supporting polymeric phases (PES, Plexa and Strata X-AW) integrity was also evaluated by means of Raman spectroscopy, optical microscopy and differential scanning calorimetric and thermogravimetrical analysis.

6.2 Experimental section

6.2.1 Reagents and materials

The selection of the target pollutants was carried out taking into account their presence and relevance on the environment (Brack et al., 2017; Busch et al., 2016; Tousova et al., 2017). According to these criteria, 23 organic pollutants with urban, rural and industrial use were selected, which cover a wide variety of physico-chemical properties as shown in **Table 6.1**, including some physico-chemical parameters.

2-hydroxybenzothiazole (OBT), amitriptyline hydrochloride, butylparaben, caffeine, carbamazepine, perfluorooctane sulfonic acid (PFOS), imipramine hydrochloride, Perfluoro-1-butanesulfonate (PFBS), progesterone and sulfadiazine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Atrazine, diuron, norfloxacin hydrochloride, sulfamethoxazole and trimethoprim were acquired from Fluka (Buchs, Switzerland). Acesulfame potassium was supplied by Supelco (Bellefonte, PA, USA), and ketoprofen, bezafibrate and propranolol were acquired from MP biomedicals (Illkirch Cedex, France). Genistein and genistin were purchased from Extrasynthese (Lyon, France), perfluorooctanesulfonamide (PFOSA) from Dr. Ehrenstofer (Augsburg, Germany), and irbesartan from Sanofi (Paris, France). The purity of all the target analytes was higher than 95%.

Individual stock standard solutions were dissolved on a weight basis in methanol (MeOH, UHPLC-MS MeOH, Scharlab, Barcelona, Spain) in order to prepare approximately 1000-2500 mg/L solutions. However, the addition of 100 µL sodium hydroxide 1 mol/L (NaOH, 98%, Panreac, Barcelona, Spain) was necessary for the proper dissolution of fluoroquinolone antibiotics as

described by Gros et al. (Gros et al., 2013). 100 mg/L dilutions were prepared in MeOH every month and dilutions at lower concentrations containing all analytes were prepared daily in MeOH: Milli-Q (30: 70, v: v). All the chemicals standards solutions were stored at −20 °C.

Table 6.1: Physico-chemical parameters of the target analytes.

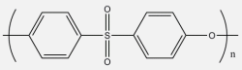
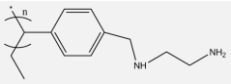
Analyte	pK_a [a]	log P [a]	Log D [a] pH=2; pH=10	Solubility [a] (mg/L)
OBT	6.4	2.5	2.5; 0.9	$2.4 \cdot 10^3$
Acesulfame	3.0	-0.6	-0.6; -1.5	$9.1 \cdot 10^8$
Amitriptyline	9.8	4.8	2.5	0.8
Atrazine	3.2; 14.5	2.2	2.2	214.1
Bezafibrate	-0.8; 3.8	4.0	0.7	$1.2 \cdot 10^3$
Butylparaben	8.5	3.0	3.0	207
Caffeine	-1.1	-0.6	-0.6	$1.6 \cdot 10^3$
Carbamazepine	16.0	2.8	2.8	17.6
Diuron	13.2	2.5	2.5	150
Genistein	6.6; 8.1; 9.0	3.1	2.1	257
Genistin	7.3; 9.0; 12.2	0.8	0.4	$1.4 \cdot 10^3$
Imipramine	9.2	4.3	2.5	$1.0 \cdot 10^3$
Irbesartan	4.1; 5.8	5.4	4.2	0.06
Ketoprofen	3.9	3.6	0.4	120
Norfloxacin	5.8	-0.9	-0.9	$1.2 \cdot 10^8$
PFBS	-3.0	2.6	0.2	$8.4 \cdot 10^4$
PFOS	-3.3	5.4	3.0	520
PFOSA	3.4	4.8	3.9	-
Progesterone	-4.8	4.2	4.2	$5 \cdot 10^3$
Propranolol	9.7; 14.1	2.6	0.4	228
Sulfadiazine	2.0; 7.0	0.4	-0.1	$8.0 \cdot 10^4$
Sulfamethoxazole	2.0; 6.2	0.8	0.0	$3.9 \cdot 10^4$
Trimethoprim	7.2	1.3	1.1	$2.33 \cdot 10^3$

[a] Values reported in the Free Data Base www.chemicalize.org

Individual stock standard solutions were dissolved on a weight basis in methanol (MeOH, UHPLC-MS MeOH, Scharlab, Barcelona, Spain) in order to prepare approximately 1000-2500 mg/L solutions. However, the addition of 100 µL sodium hydroxide 1 mol/L (NaOH, 98%, Panreac, Barcelona, Spain) was necessary for the proper dissolution of fluoroquinolone antibiotics as described by Gros et al. (Gros et al., 2013). 100 mg/L dilutions were prepared in MeOH every month and dilutions at lower concentrations containing all analytes were prepared daily in MeOH: Milli-Q (30: 70, v: v). All the chemicals standards solutions were stored at −20 °C.

The most relevant characteristics and suppliers of the polymers evaluated in the present work are listed in **Table 6.2**. Empty SPE tubes (6 mL) and polypropylene (PP) frites were purchased from Supelco. PES hollow fibres agitation was carried out using 150 mL glass vessels provided by ServiQuimia (Tarragona, Spain) in a 15 position magnetic stirrer (Gerstel, Mülheim an der Ruhr, Germany). Desorption was made in 1.5 mL Eppendorf tubes purchased from Eppendorf (Berzdorf, Germany) using a Digital Ultrasonic Cleaner (2500 mL, USB Axtor by Lovango, Barcelona, Spain). Ethylenediaminetetraacetic acid (EDTA, 99.0-101.1%, Panreac), formic acid (HCOOH ≥ 98%, Scharlau, Barcelona, Spain), ammonia (25% as NH_3, Panreac) and sodium chloride (NaCl, > 99.8%, Merck) were used for matrix modification and elution step. MeOH (HPLC grade, 99.9%) was supplied by LabScan (Dublin, Ireland).

Table 6.2. Main characteristics of the materials evaluated.

Polymer	Chemical structure	Mode of action	Characteristics
Polyethersulfone (Hollow fibre)		Reverse phase	External diameter: 0.7 mm Porosity 87%
Bond Elute Plexa (Bulk)	Hydrophilic styrene divinylbenzene (structure not provide)	Reverse phase	Particle size: 45 µm Pore size: 160 Å
Strata X-AW (Bulk)		Weak anion mixed mode	Particle size: 30 µm Pore size: 85 Å

The extracts were evaporated using a Turbovap LV Evaporator (Zymark, Hopkinton, USA) under a gentle stream of nitrogen (> 99.999% of purity) supplied by Messer (Tarragona, Spain). The extracts were filtered through PP filters (0.22 µm, 13 mm, Phenomenex, California, USA). Milli-Q (< 0.05 µS/cm, Milli-Q, Millipore) water and UHPLC-MS MeOH (Optima, Scharlau, Barcelona, Spain) were used as mobile phase eluents and HCOOH (Optima, Fischer Scientific, Geel, Belgium) for mobile phase modification. High purity nitrogen gas (99.999%) supplied by Messer was used as collision gas and nitrogen gas (99.999%) provided by AIR Liquid (Madrid, Spain) was used as both nebuliser and drying gas.

6.2.2 Stability tests

The removal of suspended particulates from water samples may avoid further degradation or losses of analytes through their adsorption onto the solid particles, but the retention of these analytes in the filters should be also thoroughly considered (Baker and Kasprzyk-Hordern, 2011a; Petrovic, 2014). In this sense, unfiltered seawater samples were collected in 2 L PP bottles as previously described by (Llorca et al., 2014) from the Plentzia Marine Station (PiE-UPV/EHU, Basque Country, Northern Spain) and used for the stability experiments.

The experiments were carried out spiking the seawater (100 mL) with a mixture of the 23 analytes at a final concentration of ~200 ng/L each one. In parallel to the spiked samples, a non-spiked control sample (blank) was also processed in duplicate alongside each of the stability tests and at the same conditions. The experiments were performed in triplicate for each preservation mode at 6 different sampling times (after 0, 3, 10, 17, 24 and 31 days) and all the samples were analysed at the same day by LC-MS/MS (Mijangos et al., 2018a) (see section 6.2.4).

In the case of preservation mode (i), 100 mL of unfiltered seawater were stored at 4°C in the pre-cleaned PP bottles. In the case of mode (ii), 200 mg SPE cartridges (a 1:1 mixture of Strata X-AW and Plexa, as the sorbent composition used for passive sampler previously published (see chapter 5) were prepared from 100 mL spiked seawater samples. SPE cartridges were sequentially conditioned with 5 mL of MeOH and 5 mL of Milli-Q water. Then the water sample (100 mL) was percolated through the cartridge assisted by a vacuum pump at ca. 5 mL/min. In the case of mode (iii), 4 pre-cleaned PES hollow fibres of 4 cm (final weight of aprox. 50 mg) were used, according to the previously published work (Mijangos et al., 2018a). First of all, the fibres were cut using a sharp blade and conditioned by soaking overnight in MeOH and air dried. Two aliquots of 120 mL of spiked seawater (dual extraction) were directly poured into 150 mL extraction vessels and NaCl and EDTA were added to achieve final concentrations of 30% (w/v) and 0.1% (w/w), respectively. The pH of each aliquot was fixed at pH=2 and pH=10 and, finally, hollow fibres and a magnetic stirrer were also added. Thereafter, vessels were closed and extraction was performed at room temperature (RT) and at 800 rpm overnight. Once the sorption step was over, the polymers were removed and rinsed with Milli-Q water in order to eliminate salt residues, and finally, dried with a clean tissue and stored in

an air-tight freezer bag at −20°C. Finally, in the case of mode *(iv)* PES fibres were used following the extraction procedure described before, and once the extraction was accomplished the fibres were introduced into a 1.5 mL Eppendorf tube containing 1 mL of MeOH and soaked for 32 min in an ultrasound bath and then, the methanolic extracts were stored in Eppendorf tubes at −20°C.

To run the analysis (18 aliquots per preservation mode), water samples (mode (i)) were extracted by SPE cartridges according to the previously published work (Mijangos et al., 2018a) . In the case of mode (ii)) the cartridges were washed with 6 mL of Milli-Q water, vacuum dried and eluted with 6 mL of 2.5% (v/v) NH_3 solution in MeOH followed by 6 mL of MeOH (Mijangos, 2018b). All the extraction solutions from modes *(i-iv)* were always evaporated to dryness under a gentle stream of nitrogen at 35°C and reconstituted in 200 µL of MeOH: Milli-Q (30: 70, v: v). Finally, the reconstituted extracts were filtered through a 0.2 µm PP filter before the LC-MS/MS analysis.

The target analytes stability was calculated according to **Equation 6.1**, and a 100% result represents a lack of analytes losses or degradation,

$$\text{Recovery (\%)} = 100 \text{ x } \frac{A^{i}_{x,sp} - A^{i}_{x,nsp}}{A^{0}_{x,sp} - A^{0}_{x,nsp}} \qquad \textbf{Equation 6.1}$$

where $A^{i}_{x,sp}$ and $A^{i}_{x,nsp}$ correspond to the chromatographic peak areas of analyte *x* from the spiked (*sp*) and non-spiked (*nsp*) samples, respectively, at time *i*, and $A^{0}_{x,sp}$ and $A^{0}_{x,nsp}$ are the corresponding peak areas at day 0. A significant (mean) loss of 30% in the recovery of the analytes was chosen to point out the lack of stability during a given storage preservation mode and time, since the precision attributable to an analytical method, expressed as relative standard deviation, RSD (%) (inter-day precision) must be ≤30% according to the European Commission decision 2002/657/EC (European Commission, 2002).

6.2.3 Characterisation of sorptive materials

PES, Plexa and Strata X-AW were individually examined prior and after storage at -20°C and RT for a month. The surface and the cross section of the polymers materials were examined by a Nikon SMZ800 stereomicroscope coupled to a NIKON DS-RI1 at *x* 40 magnifications. Chemical characterisation of the sorptive materials was assured by means of Raman spectroscopy. They were

analysed using a portable Renishaw RA 100 Raman spectrometer (Renishaw, Gloucestershire, UK) using either the 785 nm or the 514 nm excitation laser. Measured scans were accumulated during 50 s at 100% of the maximum power of the used laser. The homogeneity of the PES hollow fibre was tested by acquiring longitudinally ten Raman spectra per fibre (one measurement per 1.5 mm). The software used to collect and process the Raman spectra was BWspec4 and Omnic (Nicolet, Madison, WI, USA).

The wettability and thermal stability of the polymeric materials were studied by differential scanning calorimeter (DSC) by a Mettler Toledo Differential Scanning Calorimeter instrument (model DSC822). 10 mg of each polymeric material were subjected to 5 sequential heating/cooling cycles: the first 4 were done consecutively and the 5^{th} run was performed after having the polymer one hour out of the measuring chamber (nitrogen ambient). Temperatures range was from 0°C to 200°C and the scanning rate was of 20^{o}C/min. Furthermore, a thermogravimetric analysis (TGA) was performed in a Mettler Toledo TGA/SDTA 851 system. 10 mg of solid phase samples were kept during 30 min at 20°C prior to the measurement and then heated from 20 to 800^{o}C. The scanning rate was 10^{o}C/min and all measurements were carried out under nitrogen atmosphere.

6.2.4 Liquid chromatography tandem mass spectrometry analysis

An Agilent 1260 series HPLC chromatograph equipped with a degasser, binary pump, autosampler and a column oven coupled to an Agilent 6430 triple quadrupole (QqQ) mass spectrometer with electrospray ionisation (ESI) source (Palo Alto, CA, USA) was employed. For analyte separation: a Kinetex F5 100 Å core-shell (2.1 mm × 100 mm, 2.6 μm) column coupled to a Kinetex F5 pre-column (2.1 mm x 4.6 mm, 2.6 μm from Phenomenex (Torrance, CA, USA) was used. The column temperature and the injection volume were set to 35°C and 5 μL, respectively. The separation of the target analytes was carried out at a flow rate of 0.3 mL/min. Under optimised conditions (Mijangos et al., 2018a) a binary mixture consisting of water: MeOH (95: 5, v: v) (Phase A) and MeOH: water (95: 5, v: v) (Phase B), both containing 0.1% of HCOOH were used for gradient separation of target analytes. The gradient profile started with 30% B and it was increased to 50% in 4 min and maintained for 12 min. Then it was increased to 90% B, and it was kept constant for 10 min. Initial gradient conditions (30% B) were then recovered in 6 min, held constant for another 10

min (post-run step). ESI was carried out using a nitrogen flow rate of 12 L/min, a capillary voltage of 3500 V, a nebuliser pressure of 45 psi, and a source temperature of 350°C. Quantification was performed in the selected reaction monitoring (SRM) acquisition mode by recording the two most intense transitions for each analyte (the most sensitive was chosen as the quantifier and the second one as qualifier) when it was possible. Both, negative and positive voltages, according to the target analytes, were simultaneously applied in a single injection. Optimum parameter values for each target compound and the limit of quantifications (LOQs) are summarised in Table 3.2 in Chapter 3. Instrumental operations, data acquisition and peak integration were performed with the MassHunter Workstation Software (v B.06.00, Agilent Technologies).

6.3 Results and discussion

6.3.1 Quality control

The analytical figures of merit in real spiked seawater samples (n=3, 100 ng/L) of both, the previously published PES-LC-MS/MS methodology and in the case of SPE-LC-MS/MS method (Mijangos et al., 2018a) are summarised in Chapter 3. The quantification of the target analytes in real seawater was carried out using an external calibration together with surrogate corrections approach for SPE, while in the case of PES method a procedural calibration with Milli-Q using isotopically labelled analogues as surrogates was used. In this sense, extraction efficiency, apparent recovery and method quantification limits (MQLs) were determined. MQLs were calculated using the Equation 6.2 (Baker and Kasprzyk-Hordern, 2011b; Huntscha et al., 2012; Kasprzyk-Hordern et al., 2008; Vieno et al., 2006).

$$MQL = \frac{LOQ \times 1000}{PE\ (\%) \times CF} \qquad \textbf{Equation 6.2}$$

where LOQ (ng/mL) is the instrumental quantification limit (included in Table 3.2, chapter 3) PE (%) is the process efficiency of the analyte in the corresponding matrix (see Table 3.5), and CF is the analyte concentration factor according to the developed procedures.

Additionally, during the samples treatment, control samples (samples spiked at known

concentration level, n=3) and procedural blanks (n=3) were analysed periodically every 12-15 samples. RSDs in the range of 3-30% were obtained for all the analytes and concentrations lower than their MQLs were obtained in the case of blanks for the target compounds.

6.3.2 Characterisation of sorptive materials

The polymers used in the present study (Plexa, Strata X-AW and PES) were characterized chemically before the stability test was assessed. Raman spectrometry was carried out before and after being storing at -20°C and RT for a month (see **Figure 6.1** for the PES hollow fibre).

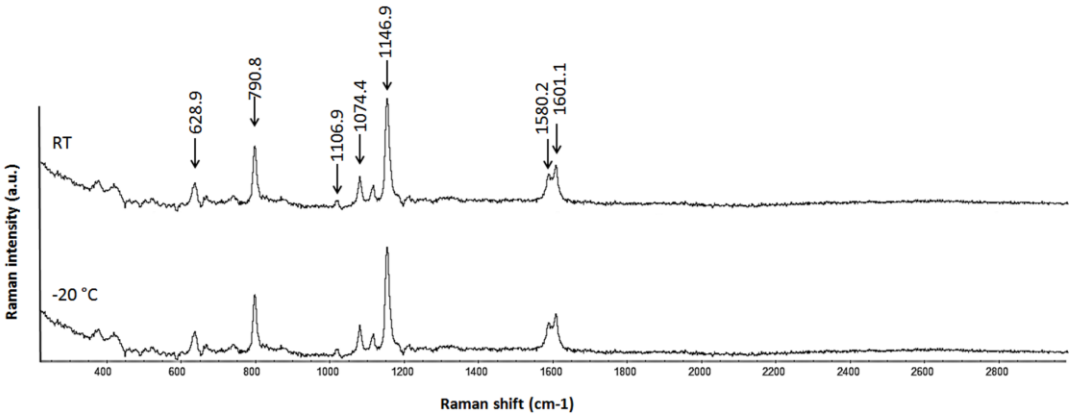


Figure 6.1 *Raman spectra of PES hollow fibres at two different storage temperatures: room temperature (up) and -20°C (down).*

Good quality Raman spectra were obtained for all the sorptive phases before and after storing and no differences were observed at the two temperatures. PES hollow fibres were highly homogeneous since all the measurements taken from a short piece of tube provided the same spectra and the same intensities. The characteristic Raman bands for PES hollow fibre were consistent with the spectra data published in the literature (Blanco-Zubiaguirre et al., 2014a; Cao et al., 2011; Sharma and Bijwe, 2012). With 785 nm laser, the main peak observed were at 1146.9 cm^{-1} corresponding to the symmetric C–O–C stretching (see **Table 6.2** for the chemical structure). Additional peaks at 1074.4 cm^{-1} and 1106.9 cm^{-1} are for symmetric and asymmetric SO_2 stretching,

respectively; peaks at 628.9 cm^{-1} shows C–S stretching; 790.8 cm^{-1} shows the out-of-plane C–H deformation; and 1580.2 cm^{-1} and 1601.1 cm^{-1} are for phenyl ring vibration. According to Strata X-AW-structure (see **Table 6.2**), the main peak was observed at 1440.9 cm^{-1} corresponding to C-H stretching (1400-1470 cm^{-1}) and 1602.4 cm^{-1} correspond to the phenyl ring vibration. Additional peaks at 2868.2-2913.2 cm^{-1} are for the C-H stretching (2800-3000 cm^{-1}). In the case of Plexa as its specific chemical structure is unknown it was not possible to compare the Raman spectra with its structure, however the same spectra feature was observed at the different temperatures (the main peaks observed were at 1001.6, 1589.3 and 1610.0 cm^{-1}).

The alterations of the sorptive surface before and after storage at -20°C were analysed by a Microscopic analysis. The photographs of skin surface of the polymers were taken under identical magnification as shown in **Figure 6.2a-c**. **Figure 6.2c-d** did not show difference related to the ridges along the length of PES hollow fibres, however, the fibre wall thickness (see **Figure 6.2e-f**) was found to be reduced by the addition water (Bolong et al., 2009). As it can be seen in **Figure 6.2h-i** and **6.2k-l** for bulk Plexa and Strata X-AW, respectively, there was no major morphological changes in the surface of the polymers.

Additionally, thermal degradation of polymers stored at low temperatures was studied by running a TGA curve as shown in **Figure 6.3a-d.** In the case of PES hollow fibre, the thermal characteristics obtained from TGA and first derived thermogravimetric (DTG) curves before storage and after low temperature storage are different as can be seen in **Figure 6.3a** and **6.3b**, respectively. When the fibres were kept at RT a significant weight loss temperature was observed at 550°C and attributed to the decomposition of polymer main chain (Cao et al., 2011; Guan et al., 2005; Sharma and Bijwe, 2012). When PES fibres were kept at -20°C for one month an additional sharp weight (35% of the total mass) can be seen at 100°C, and this loss can be related to the desorption of water. These results are in total agreement with published data (Cao et al., 2011; Guan et al., 2005; Sharma and Bijwe, 2012) where it was observed that water can be bonded through the sulfonic groups of PES polymer. Plexa and Strata X-AW did not show any significant changes (see **Figure 6.3c-d**) in thermal behaviour, the weight loss origin from water content was <5% in both cases.

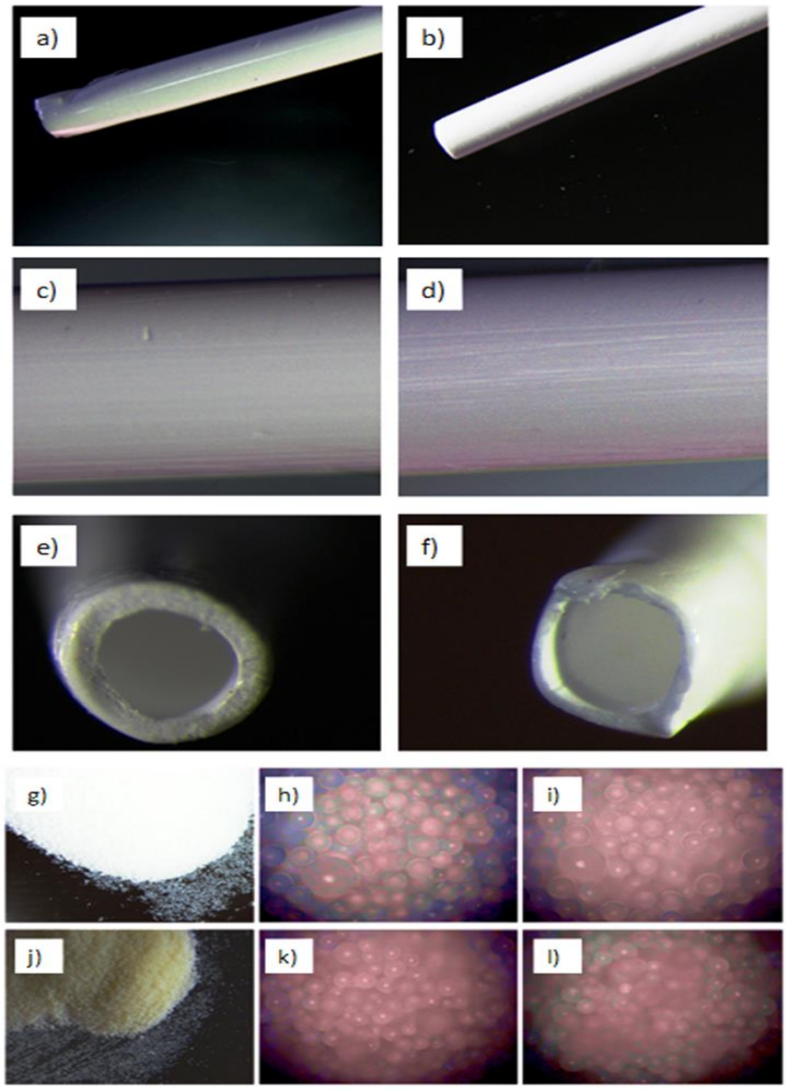


Figure 6.2. *Photographs of polymers surface studied before and after storage at -20°C for one month a) Overall view of PES hollow fibre before storage, b) overall view of PES hollow fibre after storage, c) ridges along the length of PES polymer before storage, d) ridges along the length of PES polymer after storage, e) cross section of PES polymer before storage, f) cross section of PES polymer after storage, g) overall view of Plexa bulk polymer, h) Plexa particles before storage, i) Plexa particles after storage, j) overall view of Strata X-AW bulk polymer, k) Strata X-AW particles before storage and l) Strata X-AW particles after storage.*

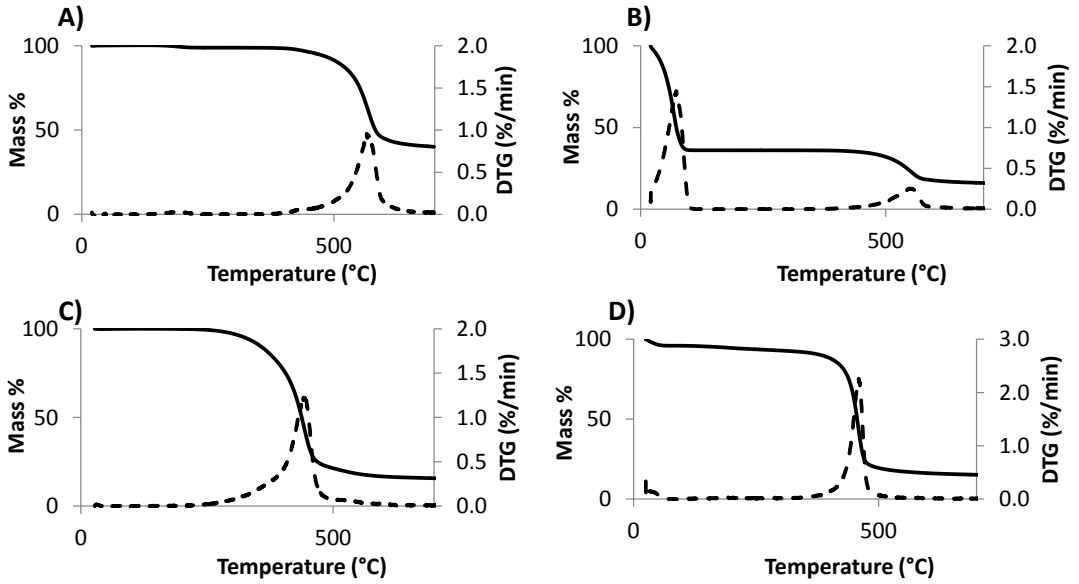


Figure 6.3. Thermogravimetric (TGA) curve (left axis, line) and first derivate thermogravimetric (DTG, right axis, dots) of the studied polymers before and after storage at -20°C for one month: a) PES hollow fibre before storage, b) PES hollow fibre after storage, c) PLEXA after storage, d) Strata X-AW after storage.

Finally, the wettability of these polymers was studied by running differential scanning calorimetric (DSC) analysis in a sequential way. As shown in Figure 6.4a, PES hollow fibres showed two signals in its thermogram: a broad peak around 100°C, due to the desorption of water molecules present in the polymers (Cao et al., 2011; Sharma and Bijwe, 2012) and a glass transition temperature (Tg) at 230°C, which is in agreement with the values reported in the literature for pure PES (Bolong et al., 2009; Cao et al., 2011; Prieto et al., 2012; Sharma and Bijwe, 2012). Regarding the wettability, the removal of water content of the PES fibre was achieved after running the scan several times (runs 1-4 in Figure 6.4a) since the humidity peak was significantly smoothed at every scan. Furthermore, the observed increase of the glass transition temperature is a consequence of the plastification induced by the humidity that lowers Tg. Finally, once the fibre was release from the inert gas chamber of the DSC for an hour (run 5 in Figure 6.4a) the broad peak corresponding to the humidity increased again suggesting that the PES hollow fibre can re-adsorb water. Thus, PES

hollow fibre has the ability to re-uptake water from the air even after being totally dried (Guan et al., 2005). On the contrary, the signals of Plexa and Strata X-AW (see Figure 6.4b and Figure 6.4c, respectively) remain constant after getting dried. These results suggest that the polymers chosen (PLEXA, Strata X-AW and PES hollow fibre) have a good thermal and chemical stability; however, the hydrophobicity of the PES hollow fibre, closely linked to the chemical structure of the polymer, may be an issue.

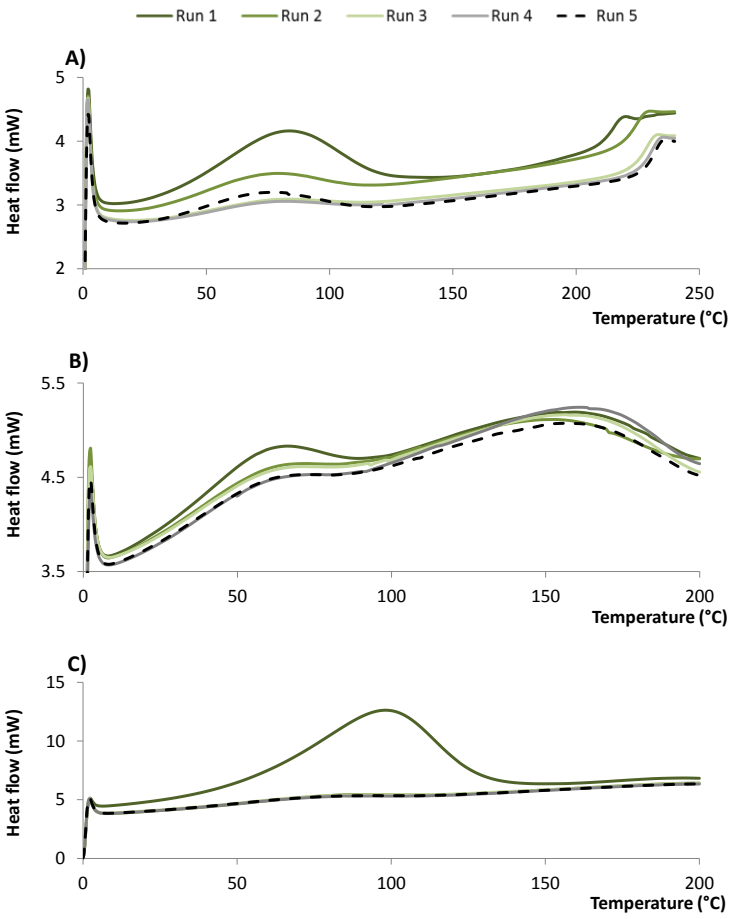


Figure 6.4. *Sequential differential scanning calorimeter (DSC) analysis carried out for the three studied polymers after storing at -20°C for one month: a) PES hollow fibre, b) Plexa and c) Strata X-AW. First four measurements (continuous line) were run sequentially (heating/cooling cycles) but the 5th run (dots) was performed after having each polymer one hour out of the measuring chamber (nitrogen inert gas ambient).*

6.3.3 Stability test

The variation of the concentrations of all the analytes along the storage time in the four modes studied in this work are shown in Figure 6.5a-d. As mentioned before (see section 6.2.2), the storage procedure assures the stability when the losses along the storage time are below 30%.

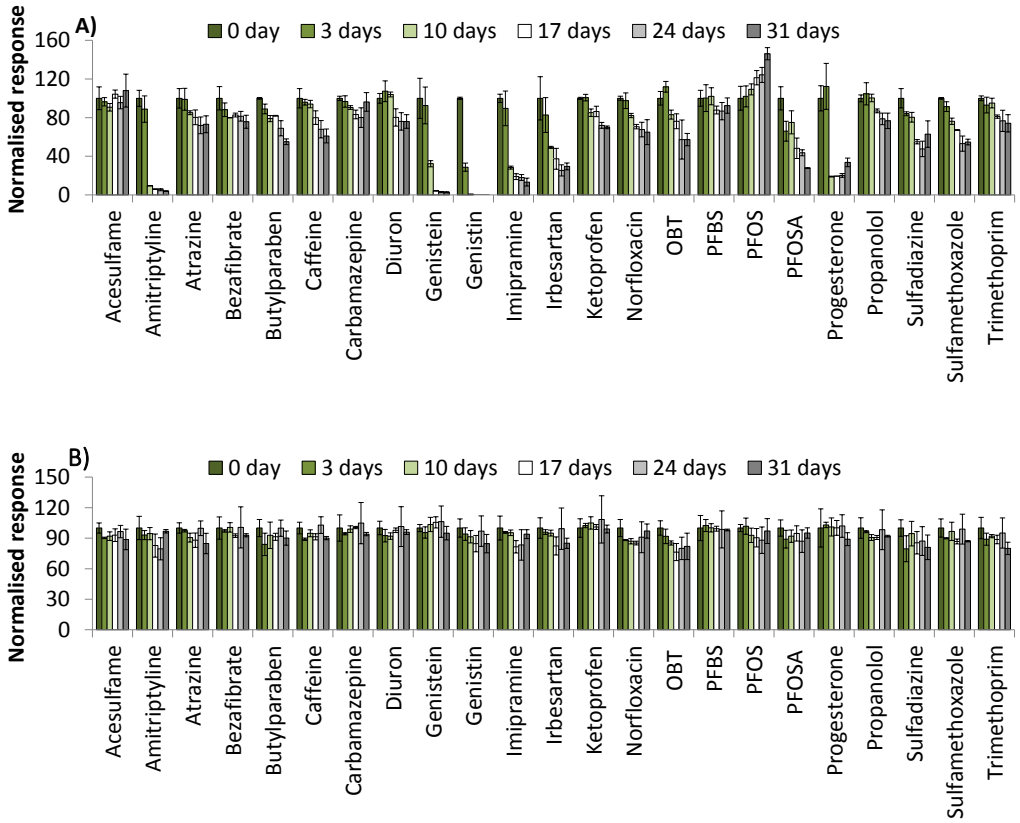


***Figure 6.5**. Relative recovery percentage of each analyte at 6 times (0, 3, 10, 17, 24 and 31 days) preserved at a) raw seawater at 4°C and b) SPE cartridges at -20°C.*

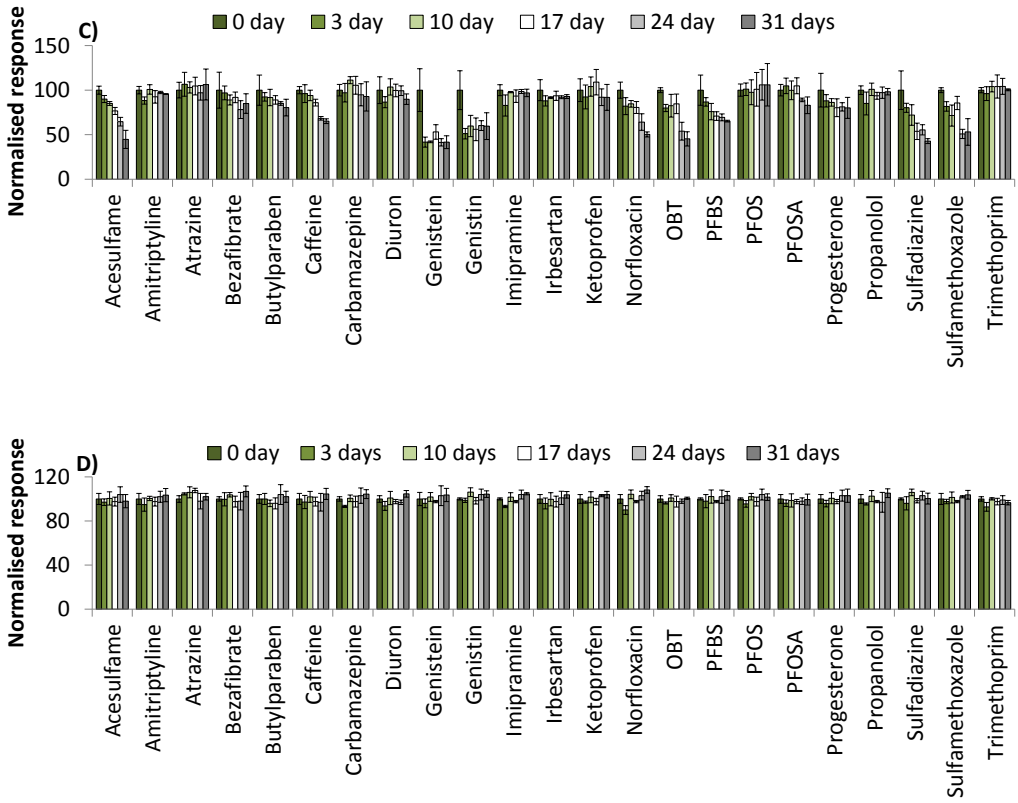


Figure 6.5. *Relative recovery percentage of each analyte at 6 times (0, 3, 10, 17, 24 and 31 days) preserved at c) PES hollow fibres stored at -20°C and d) 100% MeOH extracts stored at -20°C.*

In the case of preservation mode (i) three profiles were observed, as shown in **Figure 6.5a**: a declining profile (78% of the studied analytes), an increasing profile, and constant concentrations throughout the experimental period. After 31 days, statistically significant losses (within 20-45% at a p-level < 0.05 in the analysis of variance) were observed for atrazine, bezafibrate, butylparaben, caffeine, diuron, ketoprofen, norfloxacin, OBT, propranolol, sulfadiazine, sulfamethoxazole and trimethoprim, whereas amitriptyline, imipramine, genistein, genistin, irbesartan and progesterone reduced quantitatively (>99%) their initial concentrations in just three days. These behaviours could be attributed to the chemical structure and reactivity of the studied analytes. With regards to pharmaceutical like compounds, numerous studies have reported the lack of stability in aqueous

samples (Baker and Kasprzyk-Hordern, 2011a; Fedorova et al., 2014; Llorca et al., 2014; Mompelat et al., 2013). Baker et al. (Baker and Kasprzyk-Hordern, 2011a) described a thorough verification of methodologies commonly used for the storage of aqueous samples and for the analysis of pharmaceuticals and illicit drugs, and observed that antidepressant showed a poor stability with a recovery decreased of 61% after 72 hours in unfiltered wastewater samples. Turiel et al. (Turiel et al., 2004) studied the degradation of fluoroquinolones under different storage conditions (time, light and temperature) for 2 weeks and the analytes loses were mainly attributed to photolysis (after two weeks a loss of 50% of the initial concentration was observed).

The increasing profile (up to 146%) was detected in the case of PFOS accompanied by a parallel signal decrease (up to 42%) of its parent compound PFOSA. Similar degradation pathway of PFOSA precursor into the stable PFOS end-product have been reported in the literature (Buck et al., 2011; Zhang et al., 2017). Finally, only acesulfame, carbamazepine and PFBS remain constant during the 31 days evaluation (p-level > 0.05, according to the analysis of variance, ANOVA). These results are in good agreement with those of Van Stempvoord et al. (Van Stempvoort et al., 2011), which compared refrigerated and frozen environmental samples for the stability of artificial sweeteners (acesulfame, cyclamate, saccharin, sucralose) over a storage time of 13 months and found acesulfame was stable during this period. Due to their high stability in aquatic media, acesulfame and carbamazepine compounds have been proposed as tracers of human wastewater contamination in environmental samples (Huntscha et al., 2012; Jekel et al., 2015; Lange et al., 2012; Mawhinney et al., 2011).

In the case of the SPE cartridges the average loss of all compounds after 31 days of storage was 7% with a maximum loss of 24% for OBT, see **Figure 6.5b**. Therefore, the short term preservation of extracted samples in SPE cartridges in the freezer (-20^{o}C) is a good approach. The advantages of using SPE cartridges for these purposes have been previously described in several works (Baker and Kasprzyk-Hordern, 2011a; Fedorova et al., 2014; Mompelat et al., 2013).

On the contrary, though a close stability pattern would have been expected in PES hollow fibres, the stability profiles obtained in PES were quite different from those obtained onto SPE cartridges (see **Figure 6.5b** and **Figure 6.5c** for SPE and PES, respectively). PES hollow fibres showed

remarkable losses on the analytes concentrations after 31 days for acesulfame (45% remaining after 31 days), caffeine (65%), genistein (42%), genistin (60%), norfloxacin (50%), OBT (45%), PFBS (65%), sulfadiazine (43%) and sulfamethoxazole (53%). In contrast to the well-known stability onto SPE cartridges (C_{18} and/or HLB) (Llorca et al., 2014; McCall et al., 2016; Mompelat et al., 2013), there is no published data on stability tests for PES polymer material, even though it is highly used in POCIS as the supporting membrane (Carlson et al., 2013; Posada-Ureta et al., 2017; Vallejo et al., 2013; Vermeirssen et al., 2012) and in sorptive microextraction methods (Bizkarguenaga et al., 2015; Blanco-Zubiaguirre et al., 2014b; Prieto et al., 2012; Ros et al., 2015). Finally, as it can be seen in Figure 6.5d, all the analytes concentrations remain stable up to 31 days in the MeOH extracts.

Regarding the three most relevant matrices (seawater, PES hollow fibres and SPE phases) evaluated and, broadly speaking, only carbamazepine remained constant regardless the preservation mode after 31 days. Remarkable losses onto PES hollow fibres were observed in compounds that showed a high stability in water such as acesulfame (55%) and PFBS (35%). The stability of the phytoestrogens, OBT, fluoroquinolones and sulphonamides was rather low onto PES hollow fibres (42-60% remaining concentrations after 31 days) as well as in seawater. In contrast, amitriptyline, butylparaben, imipramine, irbesartan, progesterone and PFOSA were significantly more stable onto PES hollow fibres (looses <20%) compared to seawater (looses up to 99%).

The patterns observed in the PES hollow fibre might not be related with the degradation of those compounds in the polymer but to the presence of the low amount of water observed in the previous section (see section 6.3.2) that may help to solubilise and to loss some analytes such as genistein, genistin, OBT, PFOSA, sulfadiazine or sulfamethoxazole as it happens in a similar extend in water (see the solubility values collected in Table 6.1).

6.4 Conclusions

According to the results obtained in this work, the best way to assure the stability of the water samples containing polar or slightly polar emerging contaminants is either to keep the MeOH extracts after being extracted the samples by SPE or any other procedure, or to keep the extracted

samples in SPE cartridges. Both procedures assure a high recovery of a wide amount of contaminants typically found in aquatic media for a short term period. This way, the management of the sample analysis can be effectively carried out. Furthermore, PLEXA, Strata X-AW and PES hollow fibre showed a good thermal and chemical stability to be used as potential solid phases but the wettability of the PES fibres has been linked to the lack of stability of a number of compounds. A deeper study of the polymeric materials showed that the losses observed in PES hollow fibres were related to the capability of the polymer to re-absorb water, which might can degraded biotically some analytes or redissolved them due to their high water solubility.

6.5 References

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ECs in effluents and estuaries

Occurrence of emerging pollutants in estuaries of the Basque Country. Analysis of sources and distribution, and assessment of the environmental risk

Water Research 2018, 147, 152-163

7.1 Introduction

The fate and distribution of emerging contaminants is a matter of growing interest since most of the chemicals considered are unregulated and the effects that might be attributed to many of them are missed or overlooked in many monitoring and surveillance programs (Ternes et al., 2015). Among these emerging contaminants we may find many different chemicals including personal care products (PCPs), pharmaceuticals, consumption products (food additives, detergents, etc.), industrial compounds (perfluoroalkyl substances (PFASs), plasticisers, etc.), pesticides and engineered (nano)materials. Although we can find many of these contaminants in the effluents of wastewater treatment plants (WWTPs), in agriculture surface run-offs and in industrial discharges, the impact of the former ones from urban areas (i.e. WWTP effluents) is especially remarkable (Prasse et al., 2015). As a consequence, rivers, estuaries and coastal ecosystems are under the influence of chronic exposures to many emerging contaminants.

Environmental monitoring of special sensitive areas such as estuaries has become a challenge owing to the complexity of the analytical end-points (a growing amount of target candidates at trace level, a high physical and chemical dynamism in estuaries and coastal areas, an unknown number of transformation products, etc.) and the potential effects (antibiotic resistance, endocrine disruption, mutagenicity, etc.) (Maruya et al., 2016). Although the application of the current legal framework, i.e. the Water Framework Directive (WFD) and the Marine Strategy Framework Directive (MSFD), is somewhat limited by the prioritised set of contaminants and the application of compliance methods, we can also widen those limits by a thorough application of screening and non-target methods and new effect-based (bio)analytical approaches (Busch et al., 2016).

In this work, we consider the application of recently developed procedures, for the simultaneous analysis of 41 emerging contaminants and for the calibration of Polar Organic Chemical Integrative Samplers (POCIS) of those emerging contaminants (Mijangos et al., 2018a, 2018b) in several estuaries of the Basque Country because there was a clear lack of analytical data and a high uncertainty about the real impact of many of these contaminants. Though some recent works described a general assessment about the health status (Cajaraville et al., 2016) or the anthropogenic drivers in bacterial communities of this estuary (Aguirre et al., 2017), the occurrence

of emerging contaminants is still missed. In addition to the estuary of Bilbao, we also extended this study to the estuaries of Urdaibai (Gernika) and Plentzia because they fit with completely different geophysical and urban features (Valencia et al., 2004). In this sense, the occurrence of 41 emerging contaminants was analysed from winter 2016 to winter 2017 by grab sampling, with an extra campaign carried out in spring 2017 combining both grab and passive sampling protocols. The main WWTP effluents of each estuary were also monitored to assess the impact of these effluents on the average loading of the estuaries. Finally, we ranked the contaminants in terms of their acute and chronic toxic effects based on the estimated Risk Quotient (RQ) values (European Commission, 2003).

7.2 Experimental section

7.2.1 Study area

As shown in **Figure 7.1a**, three estuaries (Bilbao, Bi; Plentzia, Pl; and Urdaibai, Ur) of the Basque Country were selected for this study and between 4 and 7 sampling sites were chosen at each estuary (**Figure 7.1b-d**). Those sites were selected along a longitudinal gradient from the sea to upstream taking into account the general features of each estuary. In the particular case of the estuary of Bilbao, water samples were collected at two depths (surface and bottom water) due to the high stratification observed in this estuary. In addition to this, the effluent of the main WWTPs of each estuary (i.e. Galindo in Bilbao, Gorliz in Plentzia and Gernika in Urdaibai) were also monitored. An overview of the selected sampling sites is given in **Table 7.1**.

The estuary of Bilbao is 15 km long and an average 100 m wide, and the depth ranges between 2 m (upper estuary) and 30 m (estuary mouth). The estuary is partially mixed in the outer part and highly stratified within the inner half with average tidal ranges between 1.2 m to 4.6 m, and crosses the metropolitan area of Bilbao (>1,000,000 inhabitants). Currently, major pollution inputs have been related with the harbour activities and urban discharges from the WWTP of Galindo, the biggest WWTP in the Basque Country and one of the largest in Spain.

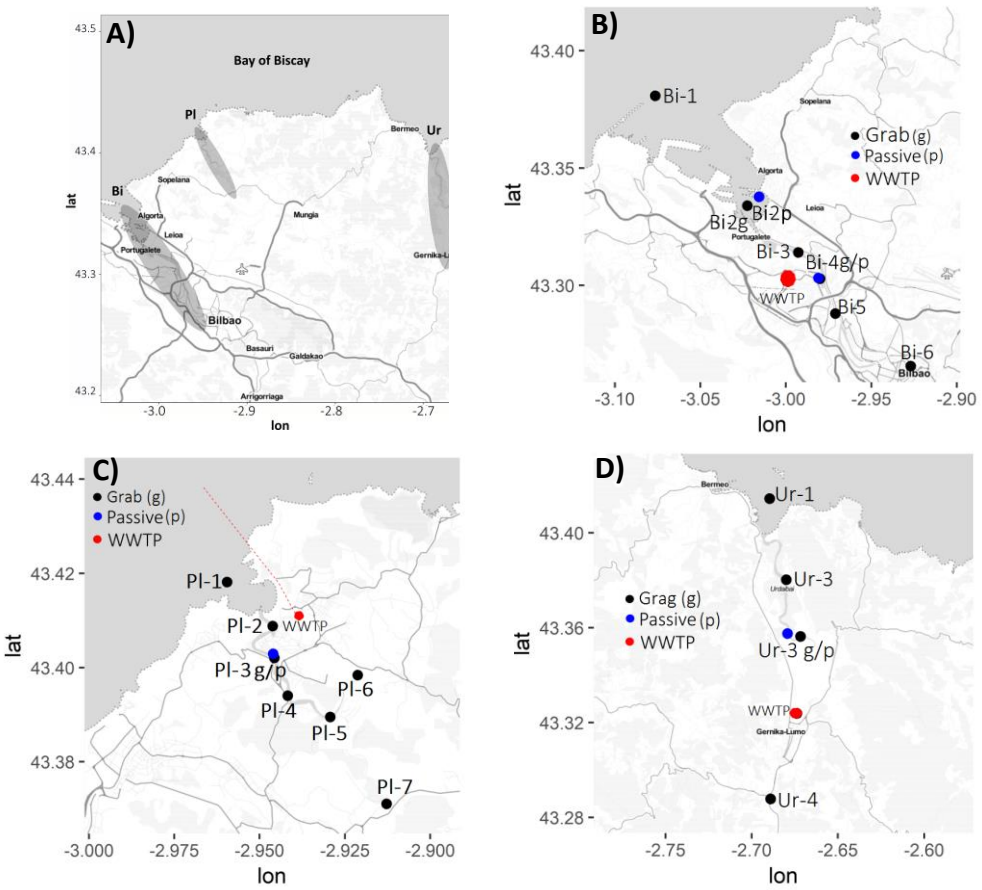

Figure 7.1. *Location of (a) the study areas along the Bay of Biscay and sampling points in (b) Bilbao estuary (Bi), (c) estuary of Plentzia (Pl) and (d) estuary of Urdaibai (Ur). Active sampling points are marked in black, passive samplers' position in blue and the location of the main WWTPs in red. Dashed line shows the position of the effluent discharge of Gorliz WWTP.*

Table 7.1. Sampling codes, depth, distance and a short description of the sampling points for Bilbao estuary (Bi), Plentzia estuary (Pl) and Urdaibai estuary (Ur).

Site code	Depth	Distance [a] (km)	Site characteristics
Bi-1s	Surface	0	Mouth of the estuary. Downstream Galindo WWTP. Close to harbour (the 4th busiest port and largest one in Spain with 3.13 km² of land and 16.94 km² of water along 17 km of waterfront).
Bi-1b	Bottom (22 m)		
Bi-2s[b]	Surface	6.6	Downstream Galindo WWTP. Close to beaches and a marine with high cruise and ship traffic. Site affected by impact of leisure activities and industrial activity.
Bi-2b	Bottom (16 m)		
Bi-3s	Surface	10.5	Downstream Galindo WWTP, where Gobela tributary joins the main chain (0.3% of contribution to the main chain). A remarkable pharmaceutical and food industrial activity remains. To highlight the presence of a firestation during the course of the river.
Bi-3b	Bottom (2 m)		
Bi-4s[b]	Surface	12.3	Downstream Galindo WWTP, where Galindo tributary joins the main chain (4% of contribution). Galindo WWTP releases the effluent discharge (~11,500 L/s) to this tributary which is a low flowing river (500-3000 L/s). A remarkable industrial activity remains in the upper part of Galindo river.
Bi-4b	Bottom (3 m)		
Bi-5s	Surface	14.1	Upstream Galindo WWTP, where Kadagua tributary joins the main chain (27%). A hospital (the largest in the Basque Country) is located in the Kadagua course.
Bi-5b	Bottom (5 m)		
Bi-6s	Surface	19.8	Upstream Galindo WWTP. Site located in the city centre of Bilbao and affected by urban pressure and traffic affluence.
Bi-6b	Bottom (5 m)		
Galindo WWTP	-	13	Biggest WWTP of the Basque Country and one of the biggest of Spain. 2nd Treatment. Source of effluent: industrial 3.7%, hospital 0.5%, domestic 95.8% (>1,000,000 inhabitant). Water flow rate of 1 E9 m^3/day. It discharges directly to Galindo river.
Pl-1	Surface	0	Nearest point to the Gorliz WWTP discharge point. Close to harbour, hospital and beach.
Pl-2	Surface	1	Site affected by leisure activities and shipping navigation.
Pl-3[b]	Surface	2.2	Site affected by urban pressures and recreation shipping navigation.
Pl-4	Surface	3.3	Site affected by urban pressures and navigation.
Pl-5	Surface	4.5	No remarkable activity.
Pl-6	Surface	6.7	No remarkable activity.
Pl-7	Surface	10.1	Close to a dam, which limits the intertidal area.
Gorliz WWTP	-	- 1000	2nd Treatment. Industrial 0%, hospital 1.3%, domestic 98.7% (10,600 inhabitants, featured by an increase in population during the summer). Water flow rate of 1.4e6 m^3/day. It releases the effluent into the estuary mouth (2°57´35.63´´W, 43°25´23.61´´N) through a submarine pipe located to–1000 m from the coast with an 18 m depth.

Table 7.1. Sampling codes, depth, distance and a short description of the sampling points for Bilbao estuary (Bi), Plentzia estuary (Pl) and Urdaibai estuary (Ur).

Site code	Depth	Distance [a] (km)	Site characteristics
Ur-1	Surface	0	Downstream Gernika WWTP. Close to two marines, a little port with middle-size fishing boats and beaches. Site affected by impact of leisure activities.
Ur-2	Surface	5	Downstream Gernika WWTP. Site affected by impact of leisure activities and high traffic pressure (especially during summer-fall time). A shipyard is located between S-2 and S-3 sampling points.
Ur-3 [b]	Surface	8.6	Downstream Gernika WWTP, where a small tributary coming from the agricultural area joins Oka river (main chain).
Ur-4	Surface	18.6	Upstream of Gernika WWTP and upper part of Gernika city (70,000 inhabitants). A remarkable industrial activity (metallurgic and motoring) remains in the surroundings of Oka River.
Gernika WWTP	-	12.9	1st Treatment. Water flow rate of 2200 m^3/day. Source of effluent: industrial and urban 70,000 people). It discharges directly to Oka river.

[a] The distance of each sampling point was calculated to respect sampling point collected in the mouth of the estuary.
[b] POCIS samplers were deployed at these sampling points.

The estuary of Plentzia is classified as a mesotidal system with a tidal variation of 2.5 m, being exposed 80% of the estuary at lowtide. The presence of a dam (~10 km upstream from the sea) in the upper part of the estuary limits the intertidal area. Major pollution inputs can be related to leisure shipping traffic and urban discharges. The Gorliz WWTP, located in the mouth of the estuary, collects urban wastewater from ~ 10,000 inhabitants, featured by an increase in population during the summer period, but it releases the effluent out the estuary mouth through a submarine pipe located at ~ 1000 m from the coast and ~ 18 m depth.

The estuary of Urdaibai (Reserve of The Biosphere declared by Unesco since 1984) is formed by the tidal part of the Oka river with 11.6 km long and 1 km wide alluvial valley (Valencia et al., 2004). The estuary is impacted by urban inputs, especially from the direct discharge of the WWTP of Gernika to the Oka river, fisheries, industrial activities (metallurgic, motoring and shipyards) and leisure activities.

7.2.2 Reagents and materials

The selection of the target pollutants was carried out taking into account their presence and relevance in the environment (Busch et al., 2016). According to these criteria, 41 organic pollutants with urban, rural and industrial use, among several classes (artificial sweeteners, corrosion

inhibitors, hormones, PCPs, PFASs, pesticides, pharmaceuticals and phytoestrogens) were selected. Compound families, names, CAS numbers, suppliers, molecular formulas and other relevant physico-chemical properties for all the target compounds are summarised in **Table 3.1**, **Chapter 3.**

Isotopically mass-labelled analogues amitriptyline-d_3 hydrochloride ([$^2H_3$]-amitriptyline, 100 mg/L in methanol), atrazine-d_5 ([$^2H_5$]-atrazine, 99%), carbamazepine-d_{10} ([$^2H_{10}$]-carbamazepine, 100 mg/L in methanol), ketoprofen-d_3 ([$^2H_3$]-ketoprofen, 99.4%), nortriptyline-d_3 hydrochloride ([$^2H_3$]-nortriptyline, 100 mg/L in methanol), progesterone-d_9 ([$^2H_9$]-progesterone, 98%), were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ciprofloxacin-d_8 hydrochloride ([$^2H_8$]-ciprofloxacin, 99%) was obtained from Fluka (Buchs, Switzerland). Sucralose-d_6 ([$^2H_6$]-sucralose, 98%) and irbesartan-d_7 2,2,2-trifluoroacetate salt ([$^2H_7$]-irbesartan, 99.9%) were purchased from Toronto Research Chemicals (Toronto, Canada). The surrogate mix sodium perfluoro-1-[1,2,3,4-$^{13}C_4$] octanesulfonate (MPFOS) and perfluoro-n-[1,2,3,4-$^{13}C_4$] octanoic acid (MPFOA) was obtained at 2 mg/L in methanol from Wellington Laboratories (Ontario, Canada).

Stock standard solutions were dissolved in methanol (MeOH, UHPLC-MS MeOH, Scharlab, Barcelona, Spain) in order to prepare approximately 1000–2500 mg/L solutions. The addition of 100 µL (in 3.5 mL of total volume) sodium hydroxide 1 mol/L (NaOH, 98%, Panreac, Barcelona, Spain) was necessary for the proper dissolution of fluoroquinolone antibiotics as described by Gros et al. (Gros et al., 2013). Dilutions (100 mg/L) were prepared in MeOH every month and dilutions at lower concentrations containing all analytes were prepared daily in MeOH: Milli-Q water (30:70, v:v). All the chemical standard solutions were stored at − 20°C for no more than one month.

Oasis hydrophilic-lipophilic balanced (HLB) 200 mg SPE cartridges were purchased from Waters (Milford, USA). Formic acid (>98%), and ethylenediaminetetraacetic (EDTA, ≥ 99.9%) used for matrix modification were supplied by Panreac (Barcelona, Spain), MeOH (HPLC grade, 99.9%) by LabScan (Dublin, Ireland) and ultra-pure water was obtained using a Milli-Q water purification system (<0.05 S/cm, Milli-Q model 185, Millipore, Bedford, MA, USA).

Concerning passive sampling, Bond-Elute Plexa (Plexa) bulk sorbent and Strata X-AW bulk sorbent used as POCIS sorbents were purchased to Agilent (Santa Clara, CA, USA) and Phenomenex (Torrance, CA, USA), respectively. Highly porous nylon membranes (30 µm of pore size, 65 µm

thicknesses and 45 mm diameter) were obtained from Fisher Scientific. Empty solid phase extraction (SPE) tubes (6 mL) and polypropylene (PP) frits were purchased from Supelco (Bellefonte, PA, USA) and 50 mL PP conical tubes (internal diameter 27.2×117.5 mm length) were obtained from Deltalab (Barcelona, Spain). Ammonia (25% as NH_3) was supplied by Panreac.

High purity nitrogen gas (>99.999%) supplied by Messer was used to evaporate the extracts using a Turbovap LV Evaporator (Zymark, Hopkinton, USA) and as collision gas during the liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Moreover, nitrogen gas (99.999%) provided by Air Liquide (Madrid, Spain) was used as both, nebuliser and drying gas during the analysis. UHPLC-MS MeOH and formic acid (Optima grade, Fischer Scientific, Geel, Belgium) were used as mobile phase. Finally, PP filters (0.22 µm, 13 mm) from Phenomenex (California, USA) were used for filtration of all the extracts before the LC-MS/MS analysis.

7.2.3 Water sample collection and treatment

Five sampling campaigns were undertaken between February 2016 and February 2017: winter 2016 (February 24-March 18), spring (May 31-Jun 2), summer (September 8-September 12), fall (November 11-November 30) and winter 2017 (February 16-February 23). For each sampling campaign, samples were collected along a salinity gradient from the sea up to the non-tidal zone, always at high tide. Surface water (near 50 cm) and depth water (near the bottom) were collected by means of Van Dorn and Niskin bottles (KC, Denmark A/S, 2L), respectively.

An EXO2 multiparametric probe was deployed during the sampling for in-situ measuring of depth, pH, oxidation-reduction potential (ORP), temperature, conductivity, salinity, total dissolved solids (TDS) and dissolved oxygen concentration (DO) along the water column. The rest of the parameters were determined in the laboratory. Non-purgable organic carbon (NPOC) was determined using a TOC-VCSN (Shimadzu Corporation, Kyoto, Japan), which is based on total oxidation on a platinum catalyst at a temperature of 680°C; the concentrations of ammonia (NH_4^+) and nitrate (NO_3^-) were evaluated potentiometrically using the standard additions method with an ion-selective electrode; and, silicate ($Si(OH)_4$) and phosphate (PO_4^{3-}) concentrations were determined using molybdenum-blue based flow injection analysis methods with UV-VIS

spectrophotometric detection (Kortazar et al., 2016). The physico-chemical properties measured are summarised in Tables II-VII (see Appendix).

Regarding to the analysis of organic pollutants, effluents and estuary water samples were transferred to pre-cleaned PP bottles, transported to the laboratory in cooled boxes and kept in the fridge at -4°C before analysis. The analyses were performed within 24 h after sampling. A previously validated SPE method (Mijangos et al., 2018a) was used for the extraction of the samples, see Chapter 3. Briefly, an appropriate volume of EDTA solution to achieve a final concentration of 0.1% (g solute/g solution) was added and samples were acidified (pH=2) with formic acid prior to the extraction. Oasis HLB 200 mg-cartridges were sequentially conditioned with 5 mL of MeOH, 5 mL of Milli-Q water and 5 mL of Milli-Q water at pH=2. The sample (100 mL in the case of effluent and 250 mL in the case of estuary) was, then, percolated through the cartridge assisted by a vacuum pump at ca. 5 mL/min. Subsequently, the cartridges were washed with 6 mL of ultrapure water, vacuum dried for 40 min and eluted with 6 mL of MeOH. After elution, the extract was concentrated to dryness under a gentle stream of nitrogen at 35°C and reconstituted in 200 µL of MeOH: Milli-Q water (30:70, v:v). Finally, the reconstituted extracts were filtered through a 0.2 µm PP filter before the LC-MS/MS analysis.

7.2.4 Passive sampling

In March-April 2017, POCIS devices were deployed at the estuaries of Bilbao (Bi-2 and Bi-4), Plentzia (Pl-3) and Urdaibai (Ur-3), as shown in Figure 7.1b-d. At each site, a canister containing two POCIS was deployed at ~50-100 cm below the surface and two consecutive deployments of 14 days were carried out. POCIS were prepared according to the procedure described previously (Mijangos et al., 2018b). POCIS were transported at -4°C to the lab. Once in the lab, POCIS sorbent was carefully removed from the membranes using approximately 10 mL of Milli-Q water and introduced into empty SPE cartridges. The sorbent was dried under vacuum for ~ 1 h and storage at -20°C until the analysis. Elution was carried out using 6 mL of MeOH with 2.5% NH_3 followed by 6 mL of MeOH. The mixture was evaporated to dryness using a TurboVap LV Evaporator at 35 °C and reconstituted in 200 µL of MeOH: Milli-Q (30:70, v:v) mixture. Finally, the extracts were filtered through a 0.22 µm PP.

One transport blank was performed per sampling day and, simultaneously, water samples were taken in PP bottles from the sampling sites before each deployment and after the last retrieval (0^{th} , 14^{th} and 28^{th} days). Water samples were carried to the laboratory in cooled boxes and kept at 4°C before analysis, which was performed within 24 h according to a previously validated SPE procedure (Mijangos et al., 2018a), as described previously.

Time-weight average concentrations (C_{TWA}) of acesulfame, acetaminophen, amitriptyline, atrazine, bezafibrate, butylparaben, caffeine, carbamazepine, diuron, irbesartan, ketoprofen, 2-hydroxybenzothiazole (OBT), perfluoro-1-butanesulfonate (PFBS), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), phenytoin, progesterone and telmisartan were calculated based on the sampling rates (Rs) determined previously and the concentration of atrazine-d_5 included as a Performance Reference Compound (PRC), see **Chapter 5**, (Mijangos et al., 2018b).

7.2.5 LC-MS/MS analysis

Analysis were carried out using a HPLC-QqQ (Agilent 1260 series LC coupled to an Agilent 6430 triple quadrupole) equipped with electrospray ionisation (ESI) source (Agilent Technologies) according to a previously optimised method, see **Chapter 3**, (Mijangos et al., 2018a).

The separation of the target analytes was accomplished at a flow of 0.3 mL/min using a Kinetex F5 100 Å core-shell (2.1 mm × 100 mm, 2.6 μm) column coupled to a Kinetex F5 pre-column (2.1 mm x 4.6 mm, 2.6 μm). The column temperature and the injection volume were set to 35°C and 5 μL, respectively. Under optimised conditions, a binary mixture consisting on a mobile phase A of water: MeOH (95: 5) and mobile phase B of MeOH: water (95: 5), both containing 0.1% of formic acid was used for gradient separation of the target analytes. The gradient profile started with 30% B which was increased to 50% in 4 min and maintained for 12 min. Then it was increased to 90% B where it was maintained constant for 10 min. Initial gradient conditions (30% B) were then achieved in 6 min, where it was finally held for another 10 min (post-run step). ESI was carried out using a nitrogen flow rate of 12 L/min, a capillary voltage of 3500 V, a nebulizer pressure of 45 psi, and a source temperature of 350°C. Both, negative and positive voltages, according to the target analytes, were simultaneously applied in a single injection.

Quantification was performed in the selected reaction monitoring (SRM) acquisition mode. Fragmentor voltage and collision energy values for each target analyte are included in **Chapter 3** (see **Table 3.2**). Instrumental operations, data acquisition and peak integration were performed with the MassHunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

7.2.6 Quality Control

Although the analytical method used in this work was previously developed and validated (Mijangos et al., 2018a), procedural blanks and control samples (samples spiked at known concentration) were analysed every 20 samples. Values lower than MQLs were obtained in the case of blanks and apparent recoveries were in agreement with those reported before in **Chapter 3**.

7.2.7 Statistical analysis

Principal Component Analysis (PCA) of the data was performed using The Unscrambler software (v. 9.2.6 Camo, Norway). Prior to any data treatment, the responses were normalised and centred and the models were built using cross-validation (Esbensen et al., 2002). A PCA of the data included in **Tables II-VII** and **IX-XVIII** (**Appendix**) was performed.

7.2.8 Environmental Risk Assessment

Environmental risk assessment (ERA) was carried out evaluating RQ according to the European Union technical Guidance Document (European Commission, 2003). In this study, RQs for acute and chronic effects were calculated for each compound as the ratio of the measured environmental concentration (MEC) and the predicted no-effect concentration (PNEC).

Maximum values obtained along the monitoring period for each compound at each estuary and effluent samples were used as MEC values, which represent the "worst-case scenario" (Alygizakis et al., 2016; Ma et al., 2017). PNEC values were calculated dividing the lowest chronic or acute toxicity data available from the ecotoxicology knowledge-base (ECOTOX database, https://cfpub.epa.gov/ecotox/) for several target species representing different trophic levels (algae/bacteria, invertebrates and fish) by an assessment factor (AF). The reference values were

chosen considering the effects on growth/population, reproduction and behaviour and both fresh water and marine species were considered.

For sub-lethal chronic toxicity levels no-observed effect concentrations (NOEC) were used and the applied AF values were 1000, 100, 50 and 10 depending on the data available: 1000 when only one short-term NOEC value was available, 100 when only one long-term NOEC value was available for a specie in one trophic level, 50 when two long-term NOEC values were available for species in two different trophic levels and, 10 when NOEC values for species in the three evaluated trophic levels were available (European Commission, 2003).

For acute toxicity levels the effect concentration (EC_{50}) or the lethal concentration (LC_{50}) was used and the AF was 1000 (Alygizakis et al., 2016; Ma et al., 2017). L(E)C_{50}s were based on either measured acute concentrations retrieved from the ECOTOX database or, when the data was missing, by QSAR models as described by Busch et al. (Busch et al., 2016).

7.3 Results and discussion

The minimum, maximum and median analyte concentrations (ng/L) of the target compounds determined in each WWTP effluent and estuary are summarised in **Tables 7.2** and **7.3**, respectively. Out of the total 41 compounds, 35 compounds were detected in at least one effluent sample and 36 in at least one estuary sample. Clofibric acid, genistin, glycitin, imipramine, PFOA and perfluoroctylsulfonamide (PFOSA) were below their MQLs in most of the samples.

Table 7.2. Minimum, maximum and median concentrations (ng/L) of the studied target analytes determined in each WWTP effluent.

Analyte	Galindo WWTP (n=15)			Gorliz WWTP (n=15)			Gernika WWTP (n=15)		
	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median
Acesulfame	52	1164	430	51	1261	311	134	11419	4202
Acetaminophen	47	860	111	119	724	222	812	5460	845
Amitriptyline	30	97	50	9	23	16	5	39	22
Atrazine	-aa	-a	-a	-a	-a	-a	18	18	18
Bezafibrate	23	132	82	2	40	14	20	101	78
Butylparaben	-a	-a	-a	9	9	9	97	100	98
Caffeine	25	99	57	71	317	183	1752	65999	26034
Carbamazepin	49	137	94	12	94	52	2	390	46
Ciprofloxacin	155	3803	549	58	3194	120	36	4719	294
Clofibric acid	7	7	7	-a	-a	-a	-a	-a	-a
Clomipramine	3	7	3	1	8	4	-a	-a	-a
Diclofenac	127	1911	1161	8	683	414	10	1932	528
Diuron	55	204	122	31	204	162	7	349	225
Eprosartan	46	339	279	74	570	184	42	879	499
Genistein	-a	-a	-a	-a	-a	-a	5	597	180
Genistin	-a	-a	-a	-a	-a	-a	-a	-a	-a
Glycitin	5	5	5	-a	-a	-a	-a	-a	-a
Imipramine	3	3	3	-a	-a	-a	-a	-a	-a
Irbesartan	410	1275	933	86	750	617	54	940	488
Isoproturon	2	4	3	2	5	4	-a	-a	-a
Ketoprofen	53	281	152	5	13	9	19	374	340
Losartan	43	302	249	32	717	303	21	913	438
Methylparaben	-a	-a	-a	-a	-a	-a	22	189	105
Norfloxacin	32	463	61	15	40	20	2	275	88
Nortriptyline	6	11	9	5	6	6	2	2	2
OBT	53	172	95	61	243	86	8	1082	621
PFBS	33	200	132	-a	-a	-a	28	202	115
PFOA	-a	-a	-a	-a	-a	-a	-a	-a	-a
PFOS	5	9	6	2	2	2	1	168	11
PFOSA	-a	-a	-a	-a	-a	-a	-a	-a	-a
Phenytoin	31	2375	201	31	110	88	111	1020	133
Progesterone	3	11	7	6	25	16	20	20	20

Table 7.2. Minimum, maximum and median concentrations (ng/L) of the studied target analytes determined in each WWTP effluent.

Analyte	Galindo WWTP (n=15)			Gorliz WWTP (n=15)			Gernika WWTP (n=15)		
	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median
Propranolol	13	31	28	5	18	14	2	30	11
Simazine	-[a]	-[a]	-[a]	5	5	5	0	-[a]	-[a]
Sucralose	46	771	638	125	4532	1859	27	1380	52
Sulfadiazine	20	5477	59	7	303	18	6	24	8
Sulfamethoxazole	66	8963	1224	47	244	67	8	190	131
Telmisartan	2	1316	434	146	766	298	127	1208	545
Testosterone	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	2	6	3
Trimethoprim	7	5843	271	6	79	8	2	61	29
Valsartan	89	416	375	154	1811	412	141	9485	8135

[a] Analytes below their method quantification limits (see **chapter 3**)
Max. . maximum; Min. : minimum

7.3.1 Occurrence and seasonal distribution in the WWTPs

Concerning WWTPs, concentration levels were ranged between low-ng/L levels to 8963 ng/L, 4532 ng/L and 11419 ng/L in the case of Galindo, Gorliz and Gernika WWTPs, respectively. Only caffeine (66000 ng/L) found in the Gernika WWTP greatly exceeded these concentrations. In the case of Galindo, sulfamethoxazole (8963 ng/L), trimethoprim (5843 ng/L) and ciprofloxacin (3803 ng/L) were the analytes detected at the highest concentrations. Sucralose (4532 ng/L), ciprofloxacin (3194 ng/L), valsartan (1811 ng/L) and acesulfame (1261 ng/L) were the analytes detected at the highest concentrations in the case of the Gorliz WWTP, whereas in the Gernika WWTP caffeine (65999 ng/L), acesulfame (11419 ng/L), valsartan (9485 ng/L) and acetaminophen (5460 ng/L) showed the highest maximum concentration levels. Genistein (5-597 ng/L), methylparaben (n.d.-189 ng/L) and butylparaben (<MQL-100 ng/L) were only found in the Gernika WWTP.

In general, the levels of commonly detected target pharmaceuticals (i.e. trimethoprim, diclofenac, acetaminophen, sulfamethoxazole, sulfadiazine) were in the same range as those detected in Europe wide surveys of WWTP effluents (Beckers et al., 2018; Loos et al., 2013). Ciprofloxacin, which is highly prescribed for human use, was also found with relatively high

concentrations (32-4719 ng/L) despite its high sorption and degradation coefficients (Boy-Roura et al., 2018). Acesulfame, sucralose, irbesartan, eprosartan, valsartan and telmisartan, which are not so often studied, also exhibited high detection rates (<75%) and high concentrations (see **Table 7.2**), confirming the relevance of their monitoring. In fact, Loos et al. (Loos et al., 2013) considered the angiotensin II receptor antagonists (ARA-IIs) family one of the most relevant emerging contaminants, with median concentrations of 480 ng/L, 368 ng/L and 227 ng/L and maximum concentrations up to 17900 ng/L, 4300 ng/L and 6800 ng/L for irbesartan, telmisartan and eprosartan congeners, respectively.

The levels of caffeine, typically used as an indicator of the presence of untreated domestic wastewater (Nödler et al., 2016), were much higher in the Gernika WWTP than those obtained in Galindo and Gorliz WWTPs (see **Table 7.1** for WWTP details). Moreover, according to the global distribution of caffeine in effluent samples (world-wide analysis of 29132 samples) (Rodríguez-Gil et al., 2018), the reported levels in Gernika WWTP (1752-65999 ng/L) would be ranked above the 95th percentile.

The seasonal patterns at each WWTP were also studied to identify specific features regarding the use of some of the studied contaminants. In the case of the WWTP of Galindo, the highest concentrations were found in summer and winter 2017 (in 9 and 8 compounds, respectively, out of 28) and the lowest in spring (14 out of 28 compounds) and winter 2016 (in 8 out of 28 compounds). In the case of the WWTP of Gorliz, the distribution of the highest concentrations was evenly observed along all the campaigns and the lowest ones in summer (early September) and autumn (late November). It is worth mentioning that in summer seven compounds(acetaminophen, butylparaben, caffeine, carbamazepin, propranolol, simazine and telmisartan) showed the highest levels and another seven (acesulfame, bezafibrate, diclofenac, diuron, losartan, sucralose and sulfadiazine), the lowest ones which could be related with the seasonal mobility patterns of the population in the surrounding urban areas. Finally, in the WWTP of Gernika, it is clearly seen that the highest levels were measured in summer (in 12 out of 28) and the lowest ones in winter (16 out of 28).

7.3.2 Occurrence and distribution of contaminants in the estuaries

Concentrations in the range of 1-3977 ng/L, 1-4138 ng/L and 1-1092 ng/L in the case of the Bilbao, Plentzia and Urdaibai estuaries, respectively, were determined (see **Table 7.3**).

Table 7.3. Minimum, maximum and median concentrations (ng/L) of the studied target analytes determined in each estuary.

Analyte	Estuary of Bilbao (Bi) (surface, n=87)			Estuary of Bilbao (Bi) (bottom, n=78)			Estuary of Plentzia (Pl) (n=99)			Estuary of Urdaibai (Ur) (n=57)		
	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median
Acesulfame	7	191	39	4	20	14	4	70	11	9	126	35
Acetaminophen	9	440	49	5	150	31	14	49	11	14	321	40
Amitriptyline	2	36	8	2	11	5	-a	-a	-a	3	3	3
Atrazine	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Bezafibrate	4	67	9	4	15	11	2	11	3	2	8	4
Butylparaben	-a	-a	-a	-a	-a	-a	-a	-a	-a	2	2	2
Caffeine	25	699	132	8	220	49	20	362	83	27	1092	111
Carbamazepin	1	93	7	2	18	4	1	45	4	1	14	2
Ciprofloxacin	3	540	48	7	298	52	-a	-a	-a	17	17	17
Clofibric acid	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Clomipramine	2	2	2	-a	-a	-a	-a	-a	-a	-a	-a	-a
Diclofenac	1	650	47	3	295	22	1	22	7	2	35	19
Diuron	4	81	14	3	15	4	2	13	4	3	10	7
Eprosartan	8	183	27	10	56	22	3	42	14	3	28	14
Genistein	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Genistin	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Glycitin	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Imipramine	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Irbesartan	2	494	32	2	181	11	2	182	8	2	27	12
Isoproturon	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Ketoprofen	10	57	37	-a	-a	-a	-a	-a	-a	2	4	3
Losartan	8	183	21	6	51	12	2	50	14	6	16	9
Methylparaben	9	66	24	8	34	11	9	65	20	5	6	5
Norfloxacin	4	62	11	25	25	25	-a	-a	-a	5	5	5
Nortriptyline	1	6	6	2	2	2	-a	-a	-a	-a	-a	-a

Table 7.3. Minimum, maximum and median concentrations (ng/L) of the studied target analytes determined in each estuary.

Analyte	Estuary of Bilbao (Bi) (surface, n=87)			Estuary of Bilbao (Bi) (bottom, n=78)			Estuary of Plentzia (Pl) (n=99)			Estuary of Urdaibai (Ur) (n=57)		
	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median
OBT	20	1267	373	38	3977	553	17	4138	301	17	669	160
PFBS	3	158	19	-a	-a	-a	-a	-a	-a	8	13	11
PFOA	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
PFOS	2	28	12	-a	-a	-a	6	8	7	-a	-a	-a
PFOSA	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Phenytoin	6	1401	13	4	84	10	5	13	9	3	20	3
Progesterone	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Propranolol	4	17	9	3	5	4	1	1	1	-a	-a	-a
Simazine	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Sucralose	30	694	137	-a	-a	-a	3	244	58	10	191	101
Sulfadiazine	1	49	21	7	7	7	1	51	5	-a	-a	-a
Sulfamethoxazole	5	226	27	9	227	43	3	19	5	-a	-a	-a
Telmisartan	2	969	34	4	185	15	1	83	9	1	42	8
Testosterone	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a	-a
Trimethoprim	3	2046	21	2	310	16	1	6	2	1	3	2
Valsartan	4	248	65	15	60	46	6	213	41	8	219	75

[a] Analytes below their method quantification limits (see **chapter 3**)
Max. . maximum; Min. : minimum

Taking into account the three estuaries, caffeine and OBT showed the highest detection frequencies (above 97%) followed by irbesartan (70%), telmisartan (68%), acetaminophen (54%), carbamazepine (52%), diclofenac (46%) and acesulfame (42%). Atrazine, clofibric acid, genistein, genistin, glycitin, imipramine, isoproturon, PFOA, PFOSA, progesterone, simazine and testosterone were not detected above their MQLs (see **Table 3.7**, in **Chapter 3**) in any sample. Although the highest concentrations were, in general terms, detected in the Gernika WWTP, the levels observed along this estuary were not the highest ones, as a consequence of the high dilution effect by tidal intrusion in the estuary.

Caffeine was detected in all the evaluated samples at levels ranging between 8 and 699 ng/L, 20-362 ng/L and 27-1092 ng/L for the estuaries of Bilbao, Plentzia and Urdaibai, respectively.

Caffeine levels reported in this study are in the same order of magnitude as those reported by Alygizakis et al. (Alygizakis et al., 2016) in Saronikog gulf (Greece) in the Eastern Mediterranean sea, which also receives inputs from WWTPs.

OBT, a compound widely used as a corrosion inhibitor in many industrial applications as well as in anti-icing fluids and in detergents for household dishwashers, has been frequently reported in effluents and in rivers at concentrations up to 1000 ng/L (Beckers et al., 2018; Loos et al., 2013). However, data on its presence in estuarine environments are scarce. The maximum concentrations at offshore from Venice (113 ng/L), in San Francisco Bay (240 ng/L) and in Baltic sea (Germany) (135 ng/L) (Nödler et al., 2014), are lower than the values obtained in this work (maximum concentrations of 3977 ng/L, 4138 ng/L and 669 ng/L in the estuaries of Bilbao, Plentzia and Urdaibai, respectively).

In general, the pharmaceutical compounds concentrations measured in the three estuaries are similar to those reported in European estuaries and coastal waters (Aminot et al., 2016; Maruya et al., 2016; Munaron et al., 2012; Nödler et al., 2014). For instance, diclofenac and acetaminophen were widely detected at high concentration ranges: 1-650 ng/L, and 54-440 ng/L, 1-22 and 14-49 ng/L, and 2-35 ng/L and 14-321 ng/L in estuaries of Bilbao (taking into account both, surface and bottom water), Plentzia and Urdaibai, respectively. The European Commission recently adopted a watch list (Decision 495/2015/EU) of substances for Union-wide monitoring in the field of water policy, where diclofenac was included.

It is worth mentioning the detection of PFBS, a short chain perfluorinated compound, ranging between its MQL and 158 ng/L and 13 ng/L in the estuaries of Bilbao and Urdaibai, respectively. Besides, the detection of PFOS in estuary waters (not detected (n.d.)-28 ng/L) also indicates that products containing PFOSs are still releasing these substances into the environment, despite the European restrictions on the marketing and use of this compound in 2006 (European Commission, 2006).

Concerning herbicides, a high overall detection of atrazine was observed in the coastal and surface waters of Europe (Beckers et al., 2018; Sousa et al., 2018). However, we only detected diuron in the ranges of 4-81 ng/L, 2-13 ng/L and 3-10 ng/L for the estuaries of Bilbao, Plentzia and

Urdaibai, respectively. Moreover, though diuron is used as active ingredient in antifouling paints as substitutes for tributylin, the concentrations detected in this work are similar to those reported in the literature (Munaron et al., 2012; Nödler et al., 2016) in coastal waters with marinas and/or areas with high ship density, and those concentrations do not exceed the annual average of the environmental quality standards (EQS, 0.2 µg/L) defined by the European Commission (European Commission, 2013).

The three estuaries showed a similar longitudinal pattern, since most concentrations decreased from the upper limit of the estuary to the estuary mouth, as it can be seen in the summation of concentrations included in **Figure 7.2a-d** for the estuaries of Bilbao (surface and bottom water), Plentzia and Urdaibai, respectively.

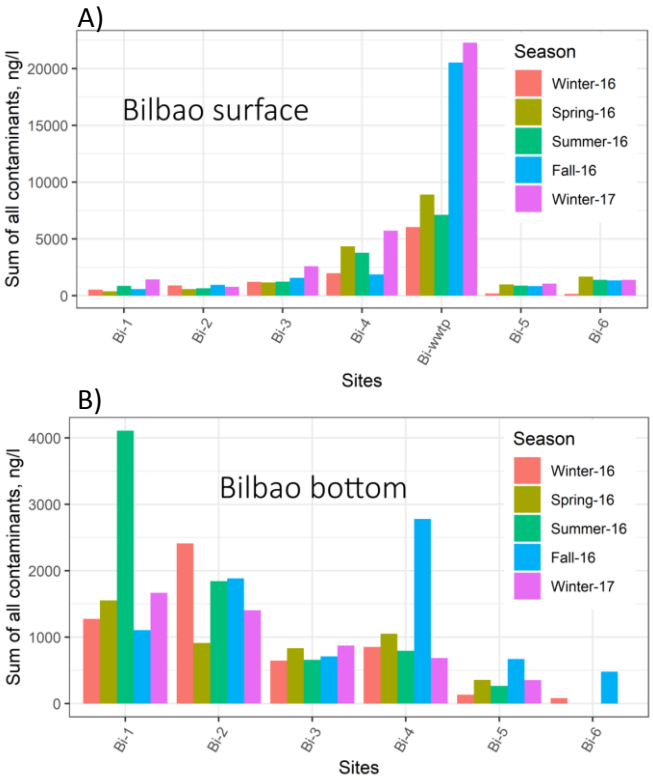

Figure 7.2. *Cumulative (sum of concentrations) spatial concentrations (ng/L) in (a) Bilbao estuary for surface water and (b) Bilbao estuary for bottom water.*

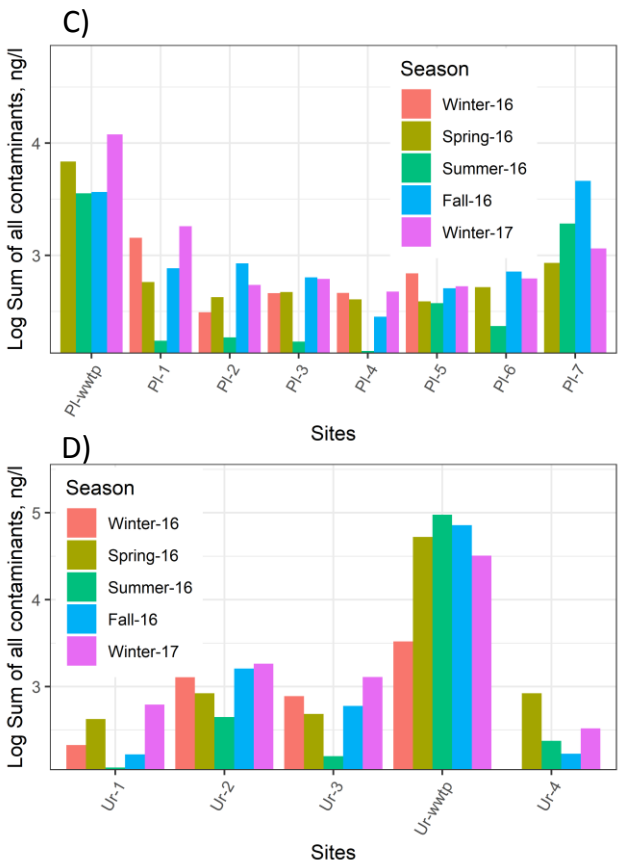

Figure 7.2. *Cumulative (sum of concentrations) spatial concentrations (ng/L) in (c) Plentzia estuary and (d) Urdaibai estuary. In the estuaries of Plentzia and Urdaibai the summation of concentration is plot in log scale.*

In the estuary of Bilbao, the samples collected at two depths were significantly different (analysis of variance, p-value<0.05). Deep and surface water showed two different cumulative (presented as sum of contaminants) longitudinal distributions (see **Figure 7.2a** and **7.2b** for surface and bottom water, respectively), suggesting the presence of two independent sources. On the one hand, surface water showed the highest cumulative concentrations (2742-5558 ng/L) at Bi-4s, which receives the input of the WWTP of Galindo, with a gradual decrease trend from the WWTP discharge point to the sea and upstream. On the contrary, in deep waters, the highest cumulative

concentrations (1044-4050 ng/L) are coming predominantly from OBT corrosion inhibitor (71-93%) and the sources were detected close to the estuary mouth at Bi-1b and Bi-2b, where the industrial harbour, a marina and passenger ship traffic are located. In addition to this, the concentrations of pharmaceuticals and artificial sweeteners were one order of a magnitude higher in the surface water (32% in fall and 75-97% in the rest of seasons for drugs and 57% in fall and 83-97% in the rest of seasons for sweeteners). On the contrary, higher concentrations of OBT (see **Table 7.3**) were observed in deep waters (38-3977 ng/L) compared to the superficial ones (20-1267 ng/L). Therefore, the consequences of a partially mixed estuarine system is observed as well as the impact of the effluent´s flowing mostly along the upper layer and bypassing the bottom saline water.

To the best of our knowledge, there are few works thoroughly evaluating the distribution of emerging contaminants in an estuary environment at different depths of the water columns (Alygizakis et al., 2016; Lara-Martín et al., 2014). For instance, Lara-Martín et al. (Lara-Martín et al., 2014) studied the environmental distribution of non-ionic surfactants and pharmaceuticals in the Long Island Sound Estuary (New York), and they found variations in the profile of concentrations consistent with salinity differences. Alygizakis et al. (Alygizakis et al., 2016) evaluated the occurrence and spatial distribution of 158 pharmaceuticals and drugs of abuse in the Saronikos Gulf (Athenes, Mediterranean Sea) at 3 different depths and amoxicillin, caffeine and salicylic acid showed a significant concentration variation with depth.

In the case of the Plentzia estuary, since the WWTP discharges to the open sea, the effects of this source are hardly seen along the estuary. The concentrations measured in the coastal point (Pl-1) were very low, being caffeine (88-362 ng/L), OBT (23-1361 ng/L) and methylparaben (n.d.-65 ng/L) the only analytes detected above their MQLs. In fact, the highest cumulative concentrations were found at Pl-7 site, 11 km upstream of the estuary mouth (see **Figure 7.2c**) suggesting a non-defined source upstream the estuary. The presence of pharmaceuticals (acetaminophen 25-40 ng/L, bezafibrate 3-11 ng/L, diclofenac n.d.-22 ng/L, eprosartan n.d.-42 ng/L, irbesartan 20-182 ng/L, losartan <MQL-50 ng/L, phenytoin n.d.-3 ng/L, sulfadiazine 2-51 ng/L, telmisartan 3-83 ng/L, trimethoprim n.d.-6 ng/L, valsartan 51-213 ng/L) and, especially, the presence of WWTP effluent marker compounds (Lange et al., 2012; Nödler et al., 2016) such as carbamazepine (3-45 ng/L) and acesulfame (9-70 ng/L) at Pl-7, indicates the possibility of a non-monitored WWTP effluent.

In the Urdaibai estuary, a hot spot was located at site Ur-3 (see **Figure 7.2d**), which receives the discharge from the WWTP effluent. Upstream of that point, at sampling point Ur-4, only caffeine (<MQL-120 ng/L) and OBT (n.d.-669 ng/L) were detected.

7.3.3 Statistical analysis and seasonal patterns

The PCA of the reported data of each estuary was performed independently (see **Tables I-VI** and **IX-XVIII,** in **Appendix**). The mean concentrations at each site, depth and campaign, plus the physico-chemical parameters were included in the PCA.

In the case of the estuary of Bilbao, up to 3 principal components (PCs) were enough to explain up to 70% of the variance of the experiments, where the first PC (PC1) explained up to the 33% of the variance, PC2 up to 25% and the PC3 up to 12%. **Figure 7.3** shows the PC1-PC2 projection of the loadings. As it can be observed, the target chemicals can be clustered in three main groups according to their relationships with the most likely source: compounds with non-identified sources (methylparaben, caffeine and OBT, see **Figure 7.4a** for OBT as example), compounds detected at low frequencies and tentatively linked with a WWTP source (see **Figure 7.4b** for amitriptyline as example), and compounds with a high detection frequency and closely linked with a WWTP source (acesulfame, carbamazepine, diuron, sulfadiazine, sulfamethoxazole, trimethoprim, sucralose; see **Figure 7.4c** for carbamazepine as example). In fact, PO_4^{3-} is also clustered in this latter group, showing high correlation coefficients (r) with the concentrations of carbamazepine (r in the range of 0.82-0.95) and acesulfame (r in the range of 0.79-0.98). Since both organic compounds are also recognized as persistent markers of WWTP effluent discharges (Sun et al., 2016b, 2016a), these results provide a stronger proof of the identification of these sources.

For the target analytes, 76%, 86% and 81% of the compounds showed a significantly positive correlation with PO_4^{3-} in the case of the estuary of Bilbao (r>0.863), Plentzia (r>0.714) and Urdaibai (r>0.854), respectively. Five analytes (acetaminophen, caffeine, methylparaben, OBT and PFOS) showed low r values suggesting the presence of an additional or different input source. In the case of acetaminophen and PFOS, two different sources are identified in the estuary of Bilbao, a WWTP discharge in spring and summer (positive r in the range of 0.71 and 0.86-0.87 for acetaminophen

and PFOS, respectively) and a non-identified source in Bi-3 in winter 2016 and 2017. Methylparaben (r from -0.60 to -0.24) showed the highest concentrations close to the estuary mouth and harbour sites at the three estuaries. Lastly, in the case of caffeine (r from -0.71 to 1.00) and OBT (r from -0.83 to 0.99) steady emissions were observed at Bi-1s/b and Ur-1 (both sampling points close to a harbour/marine) and at the hot-spots of the three estuaries (Bi-4s, Pl-7 and Ur-3). Additionally, non-specific inputs of caffeine and OBT over the whole estuary were observed, mainly related to leisure or urban activities in the case of caffeine and shipping activity over the navigable estuary channel in the case of OBT. The high detection frequencies and wide distribution of both, underlines the relevance of their monitoring in estuaries.

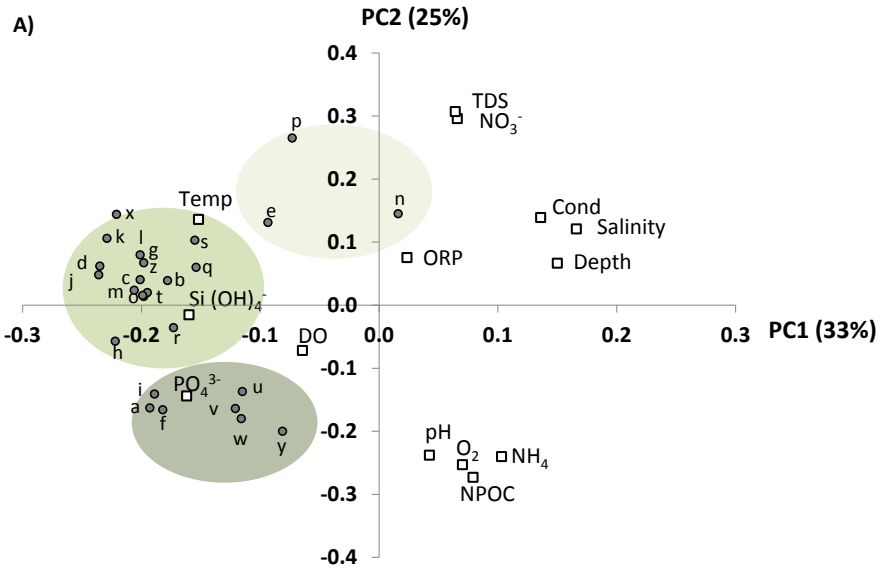

Figure 7.3. *PC1-PC2 projection of loadings of the target analyte concentrations and water physico-chemical distributions in the estuary of Bilbao. Abbreviations: a, acesulfame; b, acetaminophen; c, amitriptyline; d, bezafibrate; e, caffeine; f, carbamazepine; g, ciprofloxacin; h, diclofenac; i, diuron; j, eprosartan; k, irbesartan; l, ketoprofen; m, losartan; n, methylparaben; o, norfloxacin; p, OBT; q, PFBS, r, PFOS; s, phenytoin; t, propranolol; u, sucralose; v, sulfadiazine; w, sulfamethoxazole; x, telmisartan; y, trimethoprim; z, valsartan. Cond., conductivity; DO, dissolved oxygen; NH_4, ammonia; NO_3^-, nitrate; NPOC, non-purgable organic carbon; PO_4^{3-}, phosphate; TDS, total dissolved solids; $Si(OH)_4^-$, silicates.*

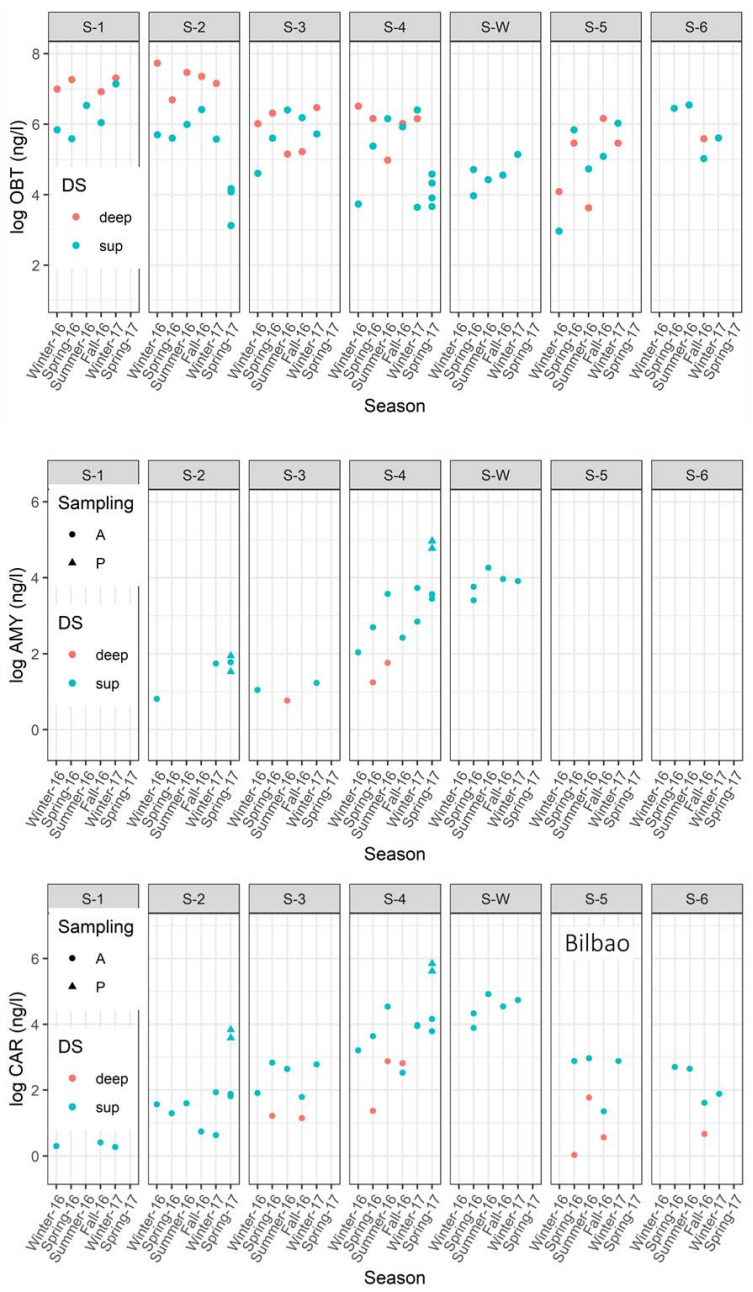

Figure 7.4. *Mean concentrations (ng/L) obtained for each sampling site (deep and surface) and each season at Bilbao estuary with active (circle) and passive samplers (triangle) for (a) OBT, (b) amitriptyline (AMY) and (c) carbamazepine (CAR).*

In the estuary of Bilbao, unlike to the other two estuaries, surface and bottom water samples were clustered separately in fall (as seen in Figure 7.2). Bottom water showed higher cumulative concentrations in comparison to the other campaigns, see Figure7.2b, probably due to a higher mixing rate of the water column (see Table III in appendix for water physico-chemical parameters).

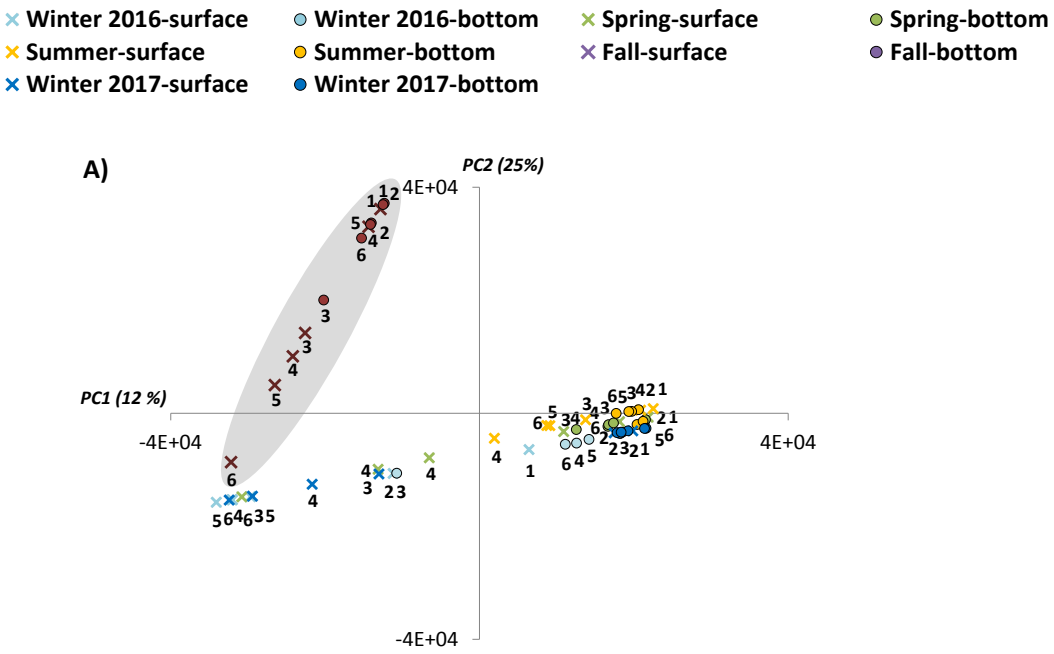

Figure 7.5. Principal component analysis of Bilbao estuary. (a) PC1-PC2 projection of the scores. Numbers refer to the sampling point in Bilbao estuary, being 1 the estuary mouth and 6 the upper part of the estuary.

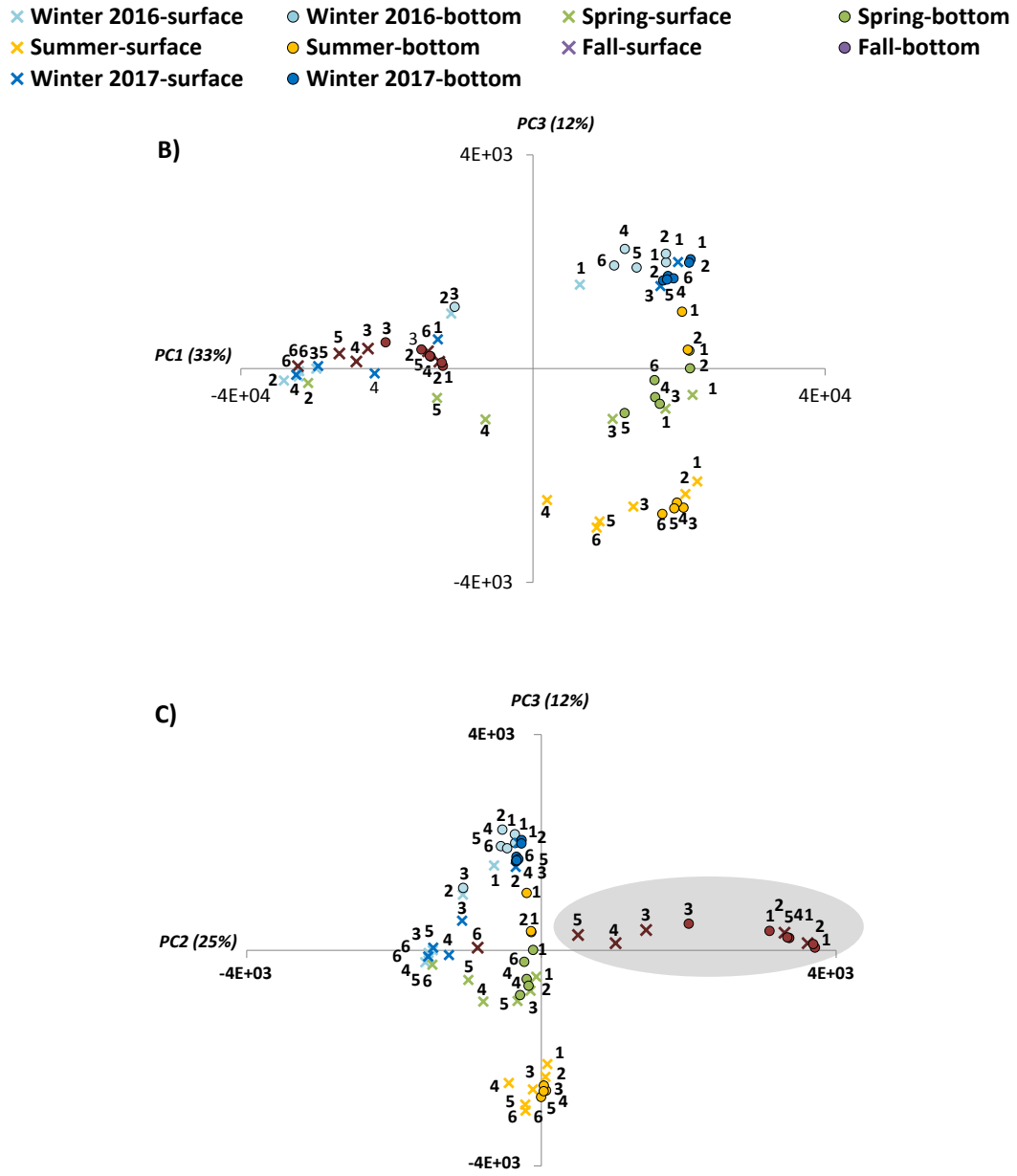

Figure 7.5. Principal component analysis of Bilbao estuary. (b) PC1-PC3 projection of the scores and (c) PC2-PC3 projection of scores. Numbers refer to the sampling point in Bilbao estuary, being 1 the estuary mouth and 6 the upper part of the estuary.

Among the target analytes, the highest levels of acetaminophen and diclofenac were observed during the winter (see **Figure 7.6a** for diclofenac) and fall seasons in Plentzia and Urdaibai, probably related to an increment in the consumption of them and a decreased biodegradation (Aminot et al., 2016; Beckers et al., 2018; Sun et al., 2016a).

Moreover, in summer, significantly high concentrations (analysis of variance, ANOVA, p_{value} <0.05) of amitriptyline, bezafibrate, ARA-IIs and ciprofloxacin pharmaceuticals were detected in the estuary of Bilbao (see **Figure 7.6b,** where telmisartan was included as example). This fact might be related to the lower flow in this season and the lowest impact of the tidal dilution. A similar effect was observed by Aminot et al. (Aminot et al., 2016) where the majority of pharmaceuticals exhibited lower in-stream attenuation during summer in the estuary of the Garonne river.

In the case of carbamazepine and phenytoin (see **Figure 7.6c** in the case of phenytoin), psychiatric drugs which are mainly used to treat epilepsy, the highest concentrations were observed during summer and spring at the three estuaries, which might be related to a higher prescription and usage in those seasons (Aminot et al., 2016; Beckers et al., 2018).

The same pattern is also observed with trimethoprim and sulfamethoxazole (see **Figure 7.6d**, where sulfamethoxazole is included as example), which are often co-administered to enhance the treatment against a variety of bacterial infection. They showed significantly lower concentrations (p_{valu} <0.05) in summer, suggesting a joint prescription of these two antibiotics in Biscay throughout the year. Beckers et al. (Beckers et al., 2018) also observed a join temporal exposure patterns for trimethoprim and sulfamethoxazole after analysing the presence of 146 organic micropollutants from two separate WWTP effluents in Germany.

Lastly, OBT (see **Figure 7.6e**) and caffeine showed a constant emission with large general variation but we were unable to see a clear pattern, probably because of their wide variety of applications and their potential different sources.

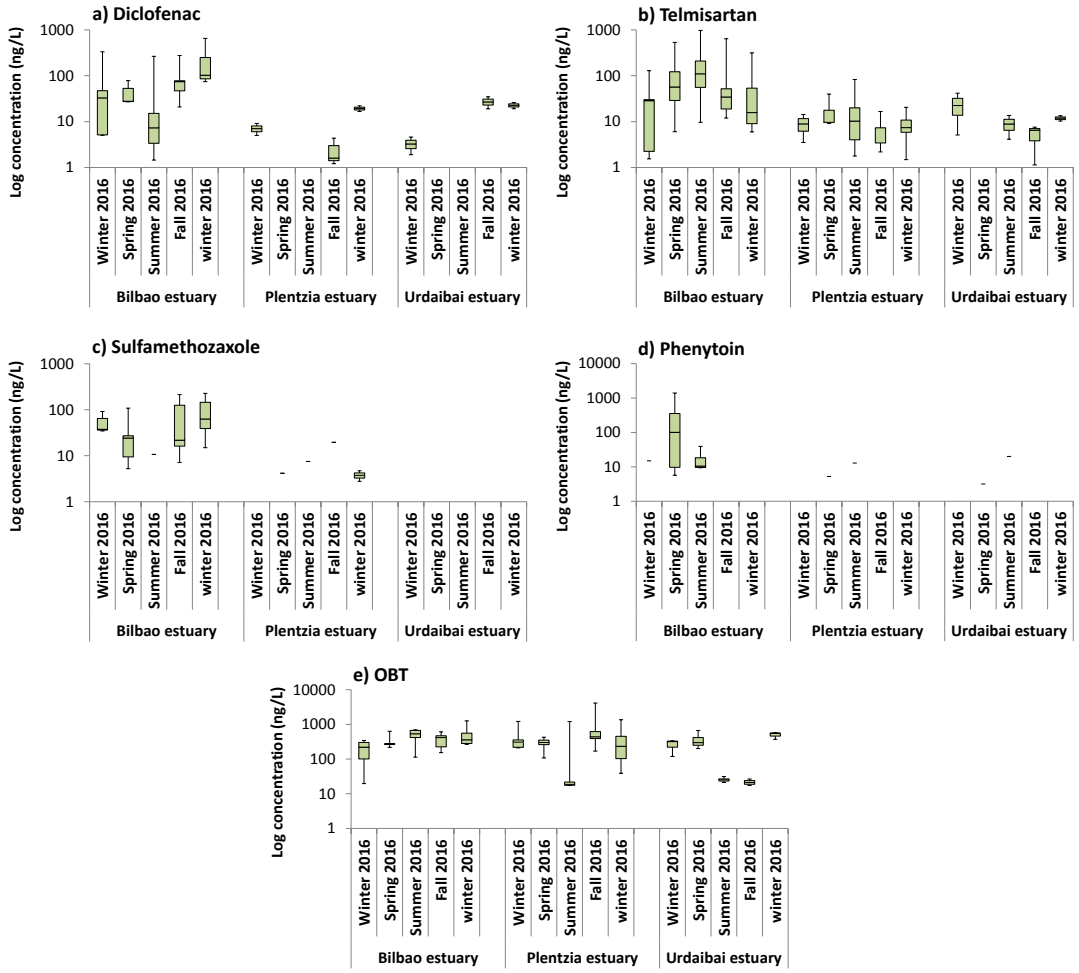

Figure 7.6. *Logarithmic concentrations ranges (ng/L) within sampling campaigns at each sampling estuary (Bilbao, Plentzia and Urdaibai) for (a) diclofenac, (b) telmisartan, (c) phenytoin, (d) sulfamethoxazole and (e) OBT.*

7.3.4 Passive sampling results

The concentrations obtained from the POCIS were included together with the grab sampling concentrations (days 0th, 14th and 28th) in Tables XIX-XX, Appendix. Grab sampling and C_{TWA} s in water (ng/L) are only shown for compounds for whose Rs values were previously determined, see

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The highest concentrations ranges obtained with passive samplers were observed in the upstream site in Bilbao (Bi-4) (progesterone 2 ng/L-telmisartan 3118 ng/L) and in Urdaibai (Ur-3) (amitriptyline 1 - telmisartan 1088 ng/L), followed by Plentzia (Pl-3) (1.0 ng/L amitriptyline - OBT 530 ng/L) and the harbour of Bilbao (Bi-2) (bezafibrate 0.9 ng/L- caffeine 129 ng/L). From the 21 monitored compounds by passive sampling, ketoprofen, PFBS, PFOS and progesterone were exclusively quantified by passive sampling. On the contrary, only atrazine, butylparaben, PFOA and phenytoin were not quantifiable at any sampling site with the passive sampling approach. Finally, though we were able to identify genistein, glycitin and clofibric acid in the POCIS, we could not estimate their C_{TWA} since we lacked their Rs. Therefore, we can highlight the need for further POCIS calibration to include these compounds.

The comparison between C_{TWA} and direct ones (days 0^{th}, 14^{th} and 28^{th}) showed a good agreement in all the estuaries. Only two compounds, caffeine (416 ng/L in POCIS vs 22-174 ng/L with grab sample) in Plentzia and progesterone (26 ng/L in POCIS vs n.d.-8 ng/L with grab sample) in Urdaibai showed higher concentrations with POCIS than with active sampling (see Figures 7.7a-d).

POCIS has been used primarily for continental surface water monitoring or sewage discharges (Harman et al., 2012), and few works (Munaron et al., 2012; Shi et al., 2014) have used POCIS as a tool to monitor emerging contaminants in estuarine environments since they can be highly dynamic. The concentrations measured by spot sampling over 3 different days fluctuated less than 35% in the case of the estuary of Bilbao (taking into account both sampling points) and more than 56% and 91% for Plentzia and Urdaibai estuaries, respectively. However, it is clear from this study that POCIS provides an efficient way to monitor emerging pollutants over a relatively long time period.

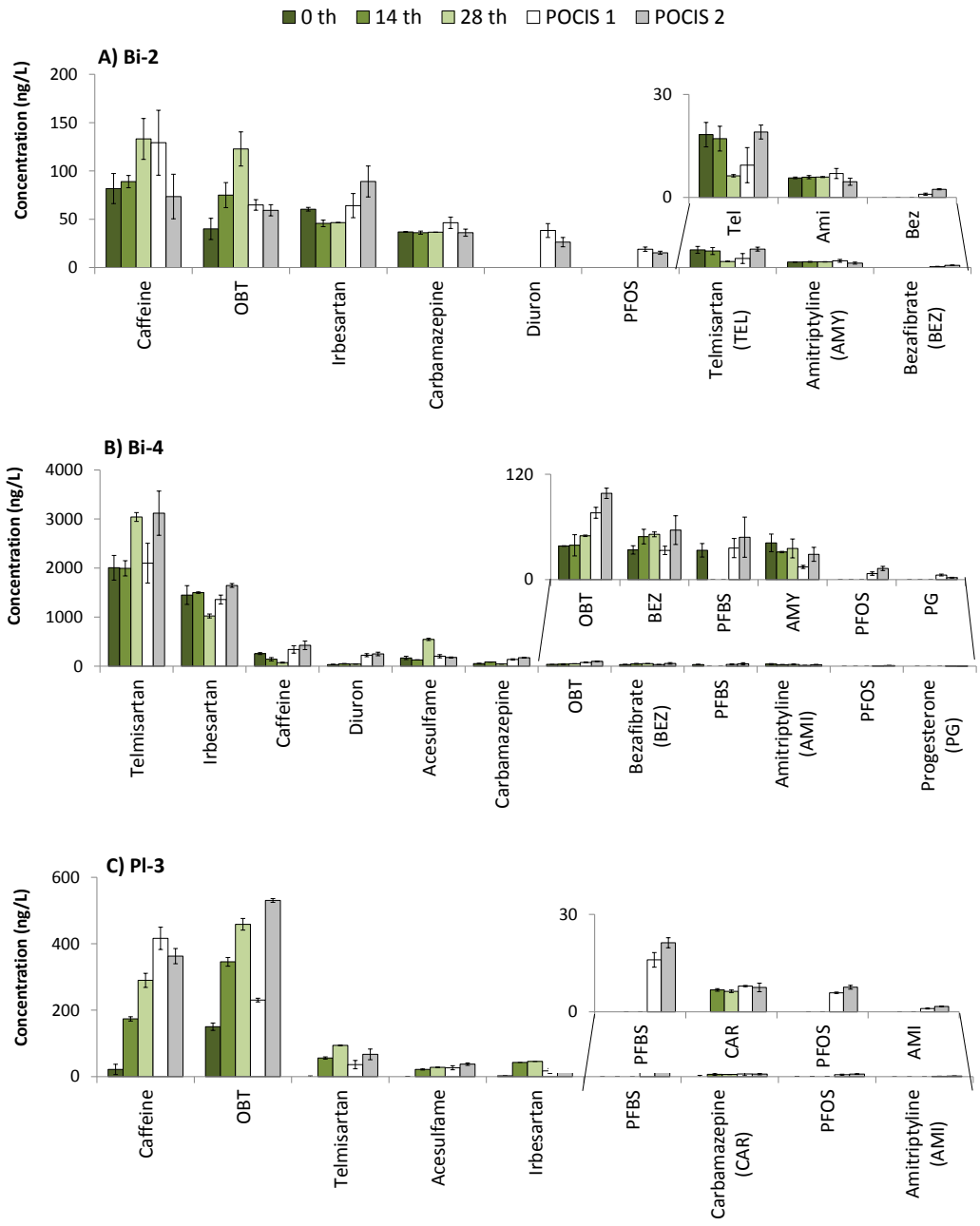

***Figure 7.7**. Concentration (ng/L) obtained by active sampling (days 0th, 14th and 28th) and POCIS (1st and 2nd deployment) at sampling points: (a) Bi-2, (b) Bi-4 and (c) Pl-3.*

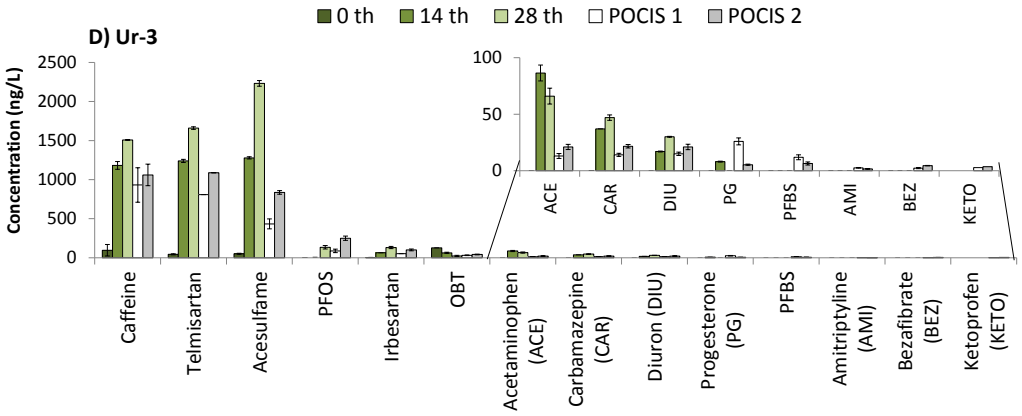

***Figure 7.7**. Concentration (ng/L) obtained by active sampling (days 0th, 14th and 28th) and POCIS (1st and 2nd deployment) at sampling points: (d) Ur-3.*

7.3.5 Environmental risk assessment

Concerning the toxicity database selection, most of the literature related to emerging compounds toxicity focused on fresh water organisms (Alygizakis et al., 2016; Beckers et al., 2018; Busch et al., 2016; Ma et al., 2017). However, in this work, bioassays carried out with marine representative organisms were also taken into consideration to include the effects of the measured contaminants in estuarine ecosystems. From the evaluated initial dataset (833 for LC_{50}s - EC_{50}s and 904 for NOECs values), only 27% of the bioassays reported were performed in seawater. Furthermore, measured data were favoured over QSAR based ones due to the limitations of the QSAR models to account for a large variety of chemical structures (Busch et al., 2016). **Tables 7.4 and 7.5** show the most sensitive NOEC and acute L(E)C_{50} values, respectively, reported for the studied contaminants and for target species (zebra danio, water flea, copepod, bivalve, sea urchin, water flea, green algae, haptophyte and cyanobacteria) and test media (fresh water and seawater). Chronic data were available only for 21 of the 41 target compounds, whereas acute toxicological data were missed only for clomipramine, imipramine and nortriptyline. European guidelines (European Commission, 2013) recommend the use of chronic toxicity to calculate PNEC values, as they are most likely to induce chronic effects rather than acute ones. However, due to the current

limited availability of chronic toxicity data, short-term (EC_{50} and LC_{50}) values are widely used to estimate PNEC values and, therefore, the potential adverse effects to aquatic organisms (Beckers et al., 2018; Busch et al., 2016).

Table 7.4. The lowest available chronic effect concentrations of target analytes for each representative specie and water media collected from the ECOTOX database

Analyte	Taxonomic Level	Species Common Name (*scientific name*)	Media Type	Duration (Days)	Concentration (µg/L)	References
Acetaminophen	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	5720	(Kim et al., 2012)
	Fish	Zebra Danio (*Danio rerio*)	FW	21	5000	(Zhang and Gong, 2013)
Amitriptyline	Fish	Zebra Danio (*Danio rerio)*	FW	14	1000	(Yang et al., 2014)
Atrazine	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	49	10	(Pannard et al., 2009)
	Invertebrate (crustaceans)	Copepod (*copepoda*)	SW	28	25	(Bejarano et al., 2005)
	Invertebrate (crustaceans)	Copepod (*cyclopoida*)	FW	21	25	(Choung et al., 2013)
	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	99	(Olmstead and LeBlanc, 2003)
	Invertebrate (molluscs)	Bilvalve (*Mytilus galloprovincialis*)	SW	56	3583	(Ei-Shenawy et al., 2007)
	Fish	Zebra Danio (*Danio rerio*)	FW	28	30	(Plhalova et al., 2011)
Bezafibrate	Invertebrate (molluscs)	Bilvalve (*Mytilus galloprovincialis*)	SW	2	1	(Fabbri et al., 2014)
Caffeine	Algae	Green Algae (*Cyanophycota*)	FW	56	5	(Lawrence et al., 2012)
	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	120	(Lu et al., 2013)
Carbamazepine	Algae,	Green Algae (*Chlorella pyrenoidosa*)	FW	30	1000	(Zhang et al., 2012)
	Invertebrate (crustacean)	Copepod (*Calanoida*)	FW	31	2	(Jarvis et al., 2014)
	Fish	Zebra Danio (*Danio rerio*)	FW	21	1780	(Madureira et al., 2012)
Ciprofloxacin	Algae,	Green Algae (*Pseudokirchneriella subcapitata*)	FW	3	5000	(Yang et al., 2008)
Clofibric acid	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	40000	(Han et al., 2006)
Diclofenac	Algae	Green Algae (*Cyanophycota*)	FW	56	5	(Lawrence et al., 2012)
	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	10000	(Han et al., 2006)
	Invertebrate (molluscs)	Bilvalve (*Mytilus galloprovincialis*)	SW	21	0.25	(Gonzalez-Rey and Bebianno, 2014)
Diuron	Algae	Green Algae (*Chlorella pyrenoidosa*)	FW	21	2.33	(Davis et al., 1976)
Genistein	Fish	Zebra Danio (*Danio rerio*)	FW	75	1.3	(Schiller et al., 2014)
Isoproturon	Algae	Green Algae (*Chlorella fusca var. Vacuolata*)	FW	1	4	(Junghans et al., 2006)

Table 7.4. The lowest available chronic effect concentrations of target analytes for each representative specie and water media collected from the ECOTOX database

Analyte	Taxonomic Level	Species Common Name (*scientific name*)	Media Type	Duration (Days)	Concentration (µg/L)	References
Methylparaben	Algae,	Green Algae (*Pseudokirchneriella subcapitata*)	FW	3	5000	(Madsen et al., 2001)
Norfloxacin	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	120	(Lu et al., 2013)
PFOA	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	6250	(Ji et al., 2008)
PFOS	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	1250	(Ji et al., 2008)
	Invertebrate (molluscs)	Bilvalve (*Lampsilis siliquoidea)*	FW	36	69.5	(Hazelton et al., 2012)
	Fish	Zebra Danio (*Danio rerio*)	FW	152	5	(Wang et al., 2011)
Progesterone	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	22	100	(Kashian and Dodson, 2004)
Propranolol	Invertebrate (molluscs)	Bilvalve (*Mytilus edulis*)	SW	21	100	(Ericson et al., 2010)
	Fish	Zebra Danio (*Danio rerio*)	FW	21	31.8	(Madureira et al., 2012)
Simazine	Algae,	Green Algae (*Stigeoclonium sp.)*	FW	42	100	(Goldsborough and Robinson, 1986)
	Fish	Zebra Danio (Danio rerio)	FW	28	60	(Plhalova et al., 2011)
Sulfadiazine	Algae,	Haptophyte (*Isochrysis galbana*)	SW	4	100	(Orte et al., 2013)
Sulfamethoxazole	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	120	(Lu et al., 2013)
	Fish	Zebra Danio (*Danio rerio*)	FW	21	533	(Madureira et al., 2012)
Trimethoprim	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	21	3120	(De Liguoro et al., 2012)
	Fish	Zebra Danio (*Danio rerio*)	FW	21	157	(Madureira et al., 2012)

FW=fresh water; SW=seawater.

Table 7.5 The lowest available acute effect concentrations (EC_{50}) of target analytes for each representative specie and water media collected from the ECOTOX database

Analyte	Taxonomic level	Species Common Name (Scientific name)	Media Type	EC_{50} (µg/L)	Reference
Acesulfame	Algae	Green algae (*Isochrysis galbana*)	-a	2068174	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	19450783	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	8622577	(Busch et al., 2016)
Acetaminophen	Algae	Green algae (*Isochrysis galbana*)	-a	478042	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	11850	(Kim et al., 2012)
	Fish	Zebra Danio (*Danio rerio*)	FW	1529769	(Selderslaghs et al., 2012) (
Amitriptyline	Algae	Green algae (*Isochrysis galbana*)	-a	1860	(Beckers et al., 2018)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	942	(Beckers et al., 2018)
	Fish	Zebra Danio (*Danrio rerio*)	FW	1400	(Yang et al., 2014)
Atrazine	Algae	Green Algae (*Chlorella vulgaris*)	FW	4	(Bérard et al., 2003)
	Algae	Green Algae (*Tetraselmis chuii*)	SW	20	(Debelius et al., 2008)
	Algae	Haptophyte (*Isochrysis galbana*)	FW	91.1	(Weiner et al., 2004)
	Algae	Haptophyte (*Isochrysis galbana*)	SW	30	(Debelius et al., 2008)
	Bacteria	Cyanobecteria (*Microcystis aeruginosa*)	FW	20	(Chalifour et al., 2016)
	Bacteria	Cyanobecteria (*Synechococcus elongates*)	SW	49	(González-Barreiro et al., 2004)
	Invertebrate (crustacean)	Copepod (*Eurytemora affinis*)	FW	125	(Forget-Leray et al., 2005)
	Invertebrate (crustacean)	Copepod (*Acartia tonsa*)	SW	94	(Ward and Ballantine, 1985)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	420	(Palma et al., 2009)
	Invertebrate (mollusc)	Bilvalve (*Mytilus galloprovincialis*)	SW	3100	(Losso et al., 2004)
	Fish	Zebra Danio (*Danio rerio*)	FW	6090	(Wang et al., 2017)
Bezafibrate	Algae	Green algae (*Isochrysis galbana*)	-a	247	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	30300	(Han et al., 2006)
	Fish	Zebra Danio (*Danio rerio*)	-a	1684	(Busch et al., 2016)
Butylparaben	Algae	Green algae (*Isochrysis galbana*)	-a	21424	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	24297	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	11337	(Busch et al., 2016)
Caffeine	Algae	Green algae (*Isochrysis galbana*)	-a	1124	(Busch et al., 2016)

Table 7.5 The lowest available acute effect concentrations (EC_{50}) of target analytes for each representative specie and water media collected from the ECOTOX database

Analyte	Taxonomic level	Species Common Name (Scientific name)	Media Type	EC_{50} (µg/L)	Reference
Caffeine	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	440	(Lu et al., 2013)
	Fish	Zebra Danio (*Danio rerio*)	FW	155352	(Teixidó et al., 2013)
Carbamazepine	Algae	Green Algae (*Chlorella pyrenoidosa*)	FW	33110	(Zhang et al., 2012)
	Algae	Green Algae (*Dunaliella tertiolecta*)	SW	53200	(Tsiaka et al., 2013)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	111000	(Han et al., 2006)
	Fish	Zebra Danio (*Danio rerio*)	FW	50089	(Pruvot et al., 2012)
Ciprofloxacin	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	6700	(Yang et al., 2014)
Clofibric acid	Algae	Green Algae (*Desmodesmus subspicatus*)	FW	115000	(Cleuvers, 2003)
	Algae	Green Algae (*Tetraselmis chuii*)	SW	318200	(Nunes et al., 2005)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	141200	(Han et al., 2006)
	Fish	Zebra Danio (*Danio rerio*)	-a	322	(Busch et al., 2016)
Diclofenac	Algae	Green Algae (Pseudo*kirchneriella subcapitata*)	FW	64800	(Quinn et al., 2011)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	80100	(Han et al., 2006)
	Fish	Zebra Danio (*Danio rerio*)	-a	44	(Busch et al., 2016)
Diuron	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	0.7	(Ma et al., 2006)
	Algae	Green Algae (*Dunaliella tertiolecta*)	SW	2.9	(Booij et al., 2013)
	Algae	Haptophyte (*Isochrysis galbana)*	SW	10	(Walsh, 1972)
	Bacteria	Cyanobecteria (*Synechocystis sp.)*	FW	8	(Podola and Melkonian, 2005)
	Bacteria	Cyanobecteria (*Chroococcus minor*)	SW	5	(Bao et al., 2011)
	Invertebrate (crustacean)	Copepod (*Nitocra spinipes*)	SW	4000	(Karlsson et al., 2006)
	Invertebrate (echinoderm)	Sea Urchin (*Paracentrotus lividus*)	SW	1940	(Manzo et al., 2008)
	Fish	Zebra Danio (*Danio rerio*)	-a	48758	(Busch et al., 2016)
Eprosartan	Algae	Green algae (*Isochrysis galbana*)	-a	11	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	0.2	(Busch et al., 2016)
	Fish	Zebra Danio (Danio rerio)	-a	1	(Busch et al., 2016)
Genistein	Algae	Green algae (*Isochrysis galbana*)	-a	12087	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	350	(Busch et al., 2016)

Table 7.5 The lowest available acute effect concentrations (EC_{50}) of target analytes for each representative specie and water media collected from the ECOTOX database

Analyte	Taxonomic level	Species Common Name (Scientific name)	Media Type	EC_{50} (µg/L)	Reference
Genistein	Fish	Zebra Danio (*Danio rerio*)	FW	2800	(Schiller et al., 2014)
Irbesartan	Algae	Green algae (*Isochrysis galbana*)	-a	124	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	2	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	5	(Busch et al., 2016)
Isoproturon	Algae	Green Algae (*Chlorella pyrenoidosa*)	FW	5	(Ma et al., 2001)
	Algae	Green Algae (*Dunaliella tertiolecta*)	SW	8.7	(Sjollema et al., 2014)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna)*	-a	26698	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio)*	-a	30937	(Busch et al., 2016)
Ketoprofen	Algae	Green algae (*Isochrysis galbana*)	-a	14947	(Busch et al., 2016)
	Invertebrate (crustacean)	Copepod (*Tisbe battagliai*)	SW	15800	(Schmidt et al., 2011)
	Fish	Zebra Danio (*Danio rerio*)	-a	279	(Busch et al., 2016)
Losartan	Algae	Green algae (*Isochrysis galbana*)	-a	2434	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	42	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio)*	-a	64	(Busch et al., 2016)
Methylparaben	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	91000	(Madsen et al., 2001)
Norfloxacin	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	10400	(Eguchi et al., 2004)
	Bacteria	Cyanobacteria (*Chlorella vulgaris*)	FW	38	(Ando et al., 2007)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	880	(Lu et al., 2013)
OBT	Algae	Green algae (*Isochrysis galbana*)	-a	11000	(Beckers et al., 2018)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	56700	(Beckers et al., 2018)
	Fish	Zebra Danio (*Danio rerio)*	-a	24100	(Beckers et al., 2018)
PFBS	Algae	Green algae (*Isochrysis galbana*)	-a	269318	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	364012	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	333285	(Busch et al., 2016)
PFOA	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	96200	(Rosal et al., 2010)
	Algae	Green Algae (*Chlorella vulgaris*)	SW	877205	(Latała et al., 2009)
	Algae	Haptophyte (*Isochrysis galbana*)	SW	163600	(Mhadhbi et al., 2012)
	Bacteria	Cyanobecteria (*Geitlerinema amphibium*)	SW	248442	(Latała et al., 2009)

Table 7.5 The lowest available acute effect concentrations (EC_{50}) of target analytes for each representative specie and water media collected from the ECOTOX database

Analyte	Taxonomic level	Species Common Name (Scientific name)	Media Type	EC_{50} (µg/L)	Reference
PFOA	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	268686000	(Sanderson et al., 2003)
	Invertebrate (echinoderm)	Sea Urchin (*Paracentrotus lividus*)	SW	20000	(Mhadhbi et al., 2012)
	Invertebrate (mollusc)	Bilvalve (*Ligumia recta*)	FW	161000	(Hazelton et al., 2012)
	Invertebrate (mollusc)	Bilvalve (*Lampsilis siliquoidea*)	FW	162600	(Hazelton et al., 2012)
	Fish	Zebra Danio (*Danio rerio*)	FW	157320	(Kalasekar et al., 2015)
PFOS	Algae	Haptophyte (*Isochrysis galbana*)	SW	37500	(Mhadhbi et al., 2012)
	Invertebrate (echinoderm)	Sea Urchin (*Paracentrotus lividus*)	SW	20000	(Mhadhbi et al., 2012)
	Invertebrate (mollusc)	Bilvalve (*Ligumia recta*)	FW	13500	(Hazelton et al., 2012)
	Fish	Zebra Danio (*Danio rerio*)	FW	1120	(Huang et al., 2010)
Phenytoin	Invertebrate (echinoderm)	Sea Urchin (*Arbacia punctulata*)	SW	9081	(Estus and Blumer, 1989)
Progesterone	Algae	Green algae (*Chlorella vulgaris*)	-a	6415000	(Busch et al., 2016)
	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	-a	10524000	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	14197000	(Busch et al., 2016)
Propranolol	Algae	Green Algae (*Desmodesmus subspicatus*)	FW	5800	(Cleuvers, 2003)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	1600	(Huggett et al., 2002)
	Invertebrate (echinoderm)	Sea Urchin (*Paracentrotus lividus*)	SW	232	(Karaaslan and Parlak, 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	14197000	(Busch et al., 2016)
Simazine	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	0.614	(Turbak et al., 1986)
	Algae	Green Algae (*Dunaliella tertiolecta*)	SW	760	(McFeters et al., 1983)
	Algae	Haptophyte (*Isochrysis galbana*)	SW	500	(Walsh, 1972)
	Bacteria	Cyanobacteria (*Synechocystis sp.)*	FW	131	(Podola and Melkonian, 2005)
	Invertebrate (crustacean)	Copepod (*Heliodiaptomus viduus*)	FW	1000	(George et al., 1982)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	FW	94000	(Karaaslan and Parlak, 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	716	(Busch et al., 2016)
Sucralose	Algae	Green algae (*Isochrysis galbana*)	-a	23414190000	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	22881218000	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	10951640000	(Busch et al., 2016)
Sulfadiazine	Algae	Green algae (*Pseudokirchneriella subcapitata*)	FW	2190	(Eguchi et al., 2004)

Table 7.5 The lowest available acute effect concentrations (EC_{50}) of target analytes for each representative specie and water media collected from the ECOTOX database

Analyte	Taxonomic level	Species Common Name (Scientific name)	Media Type	EC_{50} (µg/L)	Reference
Sulfadiazine	Algae	Haptophyte (*Isochrysis galbana*)	SW	1440	(Orte et al., 2013)
	Bacteria	Cyanobacteria (*Microcystis aeruginosa*)	FW	135	(Lützhøft et al., 1999)
	Invertebrate (echinoderm)	Sea Urchin (*Arbacia lixula*)	SW	12700	(Carballeira et al., 2012)
	Fish	Zebra Danio (*Danio rerio*)	-a	189420	(Busch et al., 2016)
Sulfamethoxazole	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	520	(Isidori et al., 2005)
	Invertebrate (crustaceans)	Water Flea (*Daphnia magna*)	FW	1290	(Lu et al., 2013)
	Fish	Zebra Danio (*Danio rerio*)	-a	385124	(Busch et al., 2016)
Testosterone	Algae	Green algae (*Isochrysis galbana*)	-a	9121	(Busch et al., 2016)
	Invertebrate (crustacean)	Copepod (*Acartia tonsa*)	SW	1500	(Andersen et al., 2001)
	Fish	Zebra Danio (*Danio rerio)*	-a	186	(Busch et al., 2016)
Trimethoprim	Algae	Green Algae (*Pseudokirchneriella subcapitata*)	FW	40000	(Yang et al., 2008)
	Bacteria	Cyanobacteria (*Anabaena variabilis*)	FW	11000	(Ando et al., 2007)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna*)	-a	37966	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	27064	(Busch et al., 2016)
Valsartan	Algae	Green algae (*Isochrysis galbana*)	-a	574	(Busch et al., 2016)
	Invertebrate (crustacean)	Water Flea (*Daphnia magna)*	-a	96	(Busch et al., 2016)
	Fish	Zebra Danio (*Danio rerio*)	-a	134	(Busch et al., 2016)

-a=predicted value with QSAR according to Bunsh et al (Busch et al., 2016)
FW=fresh water; SW=seawater.

Criteria for interpreting the RQ values in order to establish different levels of environmental risk were: low risk (RQ values below to 0.1), medium risk (RQ values between 0.1 and 1) and high risk (RQ values higher than 1) (Alygizakis et al., 2016; European Commission, 2013; Ma et al., 2017). RQ values of the detected compounds are summarised in **Tables XXI-XXII** (**Appendix**) and **Figure 7.8a-d.**

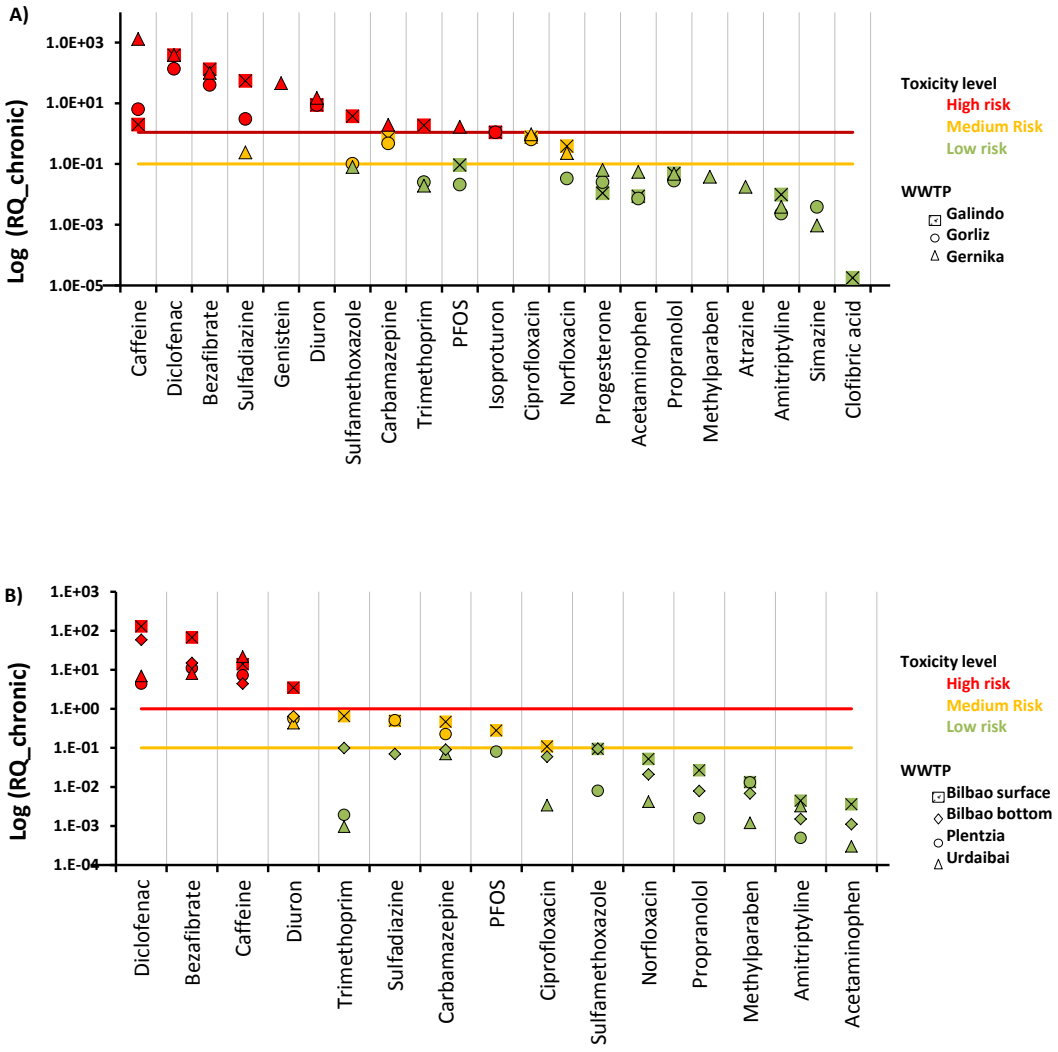

Figure 7.8. Risk Quotients (RQ) of the target compounds calculated for (a) chronic toxicity in effluents samples and (b) chronic toxicity in estuary samples. Colours refer to the risk level: green RQ<0.1; orange 0.1<RQ<1 and red RQ>1.

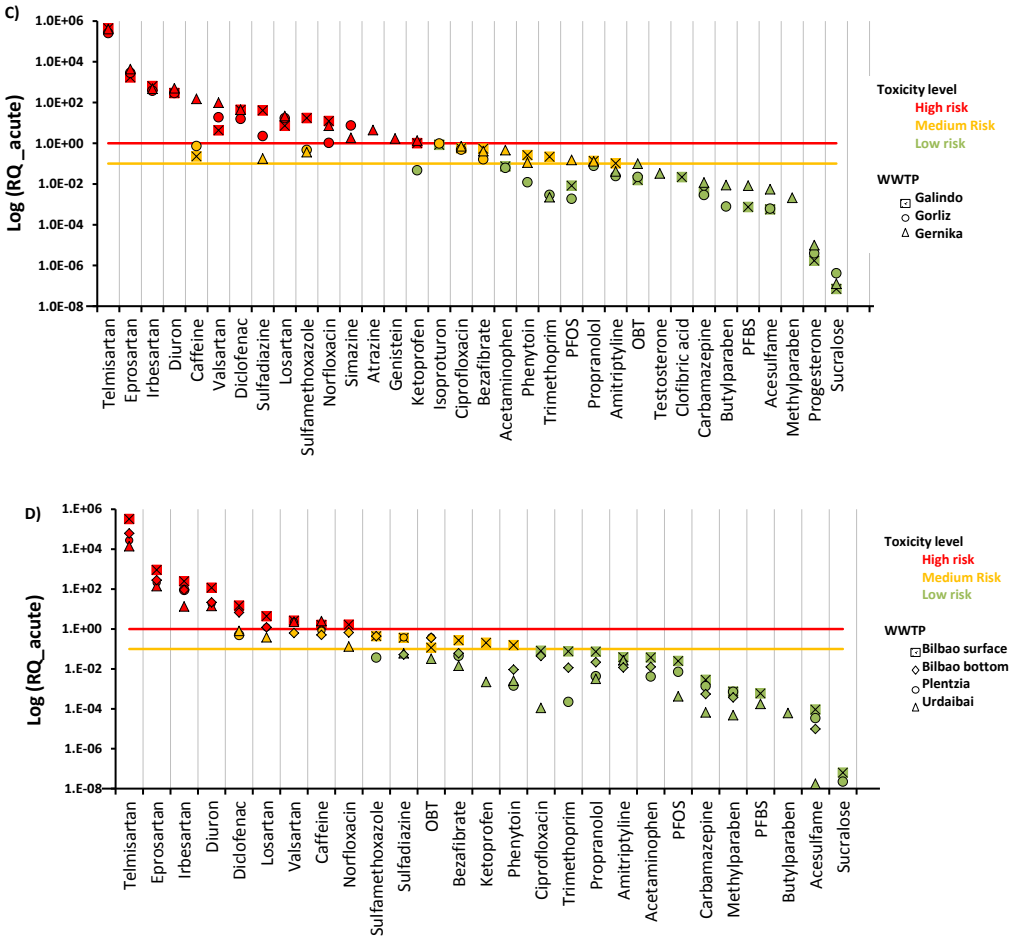

Figure 7.8. Risk Quotients (RQ) of the target compounds calculated for (c) acute toxicity in effluent samples and (d) acute toxicity for estuary samples. Colours refer to the risk level: green RQ<0.1; orange 0.1<RQ<1 and red RQ>1.

Regarding the chronic toxicity, as can be seen in **Figures 7.8a** and **7.8b** for WWTP effluent and estuary samples, respectively, caffeine, diclofenac, bezafibrate, sulfadiazine and genistein are the compounds that showed the most negative impact. It is worth mentioning the impact of caffeine (detection frequency of 99%) and the fact that its main source is not only related to WWTP effluents.

RQ values obtained for pharmaceutical compounds are consistent with other RQs reported in the literature (Beckers et al., 2018; Busch et al., 2016). Among them, the anti-inflammatory agent diclofenac was previously identified as one of the main risk drivers in environmental mixtures (Beckers et al., 2018; Busch et al., 2016) and has been associated with growth inhibition in daphnia and cell multiplication in algae (see **Tables 7.4 and 7.5**).

Compounds detected at low concentrations and frequency as genistein (only detected in effluent samples at maximum concentrations of 5-597 ng/L) could imply a higher acute risk due to their higher toxicity (see **Figure 7.8a**). Moreover, PFOS also showed a RQ_{acute}>0.1 although the maximum detected concentrations during the monitoring campaign (28 ng/L and 168 ng/L for estuary and effluent samples, respectively) did not exceed the its established Environmental Quality Standard (Maximum Allowable Concentration 65000 ng/L) (European Commission, 2013).

Regarding the acute toxicity, in at least one of the evaluated sample points, telmisartan, eprosartan, irbesartan, diuron, caffeine, valsartan, diclofenac, sulfadiazine, sulfamethoxazole, losartan, norfloxacin, simazine, atrazine, genistein and ketoprofen showed a RQ_{acute}>1, while isoproturon, ciprofloxacin, bezafibrate, acetaminophen, phenytoin, trimethoprim, PFOS, propranolol and amitriptyline showed a RQ_{acute}>0.1 (**Figures 7.8c** and **7.8d** for effluent and estuary samples, respectively).

In this work, three sartans-compounds (telmisartan, eprosartan and irbesartan) were ranked as the most toxic compounds on the bases of RQ_{acute} estimation. Similar results were also found by Busch et al. (Busch et al., 2016) from a list of 214 top toxic compounds. Although the occurrence of sartans has been reported before (Loos et al., 2013), to the best of our knowledge there are no measured L(E)50 values and in this work their RQ ranking relies only on toxicity estimations that are retrieved from QSAR prediction (Busch et al., 2016). Therefore, they should be included in further monitoring campaigns as well as in bioassays.

Norfloxacin, sulfamethoxazole and sulfadiazine, also showed high RQ_{acute} values. These results are in agreement with Ma et al. (Ma et al., 2017), where sulfadiazine and sulfamethoxazole (RQs ranging from 1.0 to 9.2) were identified as the antibiotics with the higher ecological risks.

Besides, it is noteworthy that the aquatic risk assessment pointed out a highly possible risk in all the sites where diuron, simazine and atrazine were detected (see **Table 7.2** and **7.3** for the concentrations), even if the Environmental Quality Standard for pesticides (EQS, 0.2 μg/L) established by Directive 2013/39/EC (European Commission, 2013) were not exceeded.

Lastly, in the case of OBT, RQ_{acute} values>0.1 were only obtained in the estuary samples, confirming the importance of monitoring not only WWTPs but also other potential sources such as harbours.

In any case, it should be underlined that RQs act as a normalised measurement of risk allowing a comparison between different compounds with different toxicities and exposure levels and thereby it might be good a starting point for further prioritisation.

7.4 Conclusions

The analysis of a wide number of emerging contaminants in three estuaries and three WWTP effluents allowed us to describe the complexity of the studied scenarios. The time and space distribution of the studied contaminants allowed us to identify that one of the most likely sources are the urban wastes released by the WWTPs, though other remarkable contributions such as the harbour activities were also identified. It is important to emphasize that WWTPs are important secondary sources of anthropogenic compounds and the composition of their effluents depend on the primary urban inputs and the efficiency of the treatments. In this sense, the confirmation of valuable markers of these effluents has also been highlighted to identify non-monitored effluents. In addition to this, we can also point out the utility of passive samplers because they allowed us to estimate C_{TWA} of a number of contaminants and to detect contaminants that would have not been measured by active sampling methods. Besides, based on the estimated RQ values the contaminants were ranked in terms of their acute and chronic toxic effects to complement their occurrence data along the estuaries and their most likely sources. On the one hand, well known pharmaceuticals, such as diclofenac show high acute and chronic effects, but ubiquitous caffeine seems to be a contaminant of increasing concern. On the other hand, the estimated toxicity of the

antihypertensive drugs (sartans) together with their detection frequencies in effluents and estuaries, are warning evidences of their environmental impact.

7.5 References

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Evaluation of WWTP effluent toxicity

Application of the sea urchin embryo test in toxicity evaluation and effect-directed analysis of wastewater treatment plant effluents

Environmental Science and Technology (to be submitted)

8.1. Introduction

The presence of emerging contaminants (ECs) in the aquatic environment is an issue of growing concern due to the chronic exposure of many aquatic ecosystems and the subsequent risks for environmental and human health (Ternes et al., 2015). Considering that around 500 known organic micropollutants can be typically reported in aquatic systems, and more than 10,000 are likely to be found, a targeted analysis of all these compounds seems difficult to achieve in regular monitoring and using methods that comply with quality standards (Hernández et al., 2012). In addition to the difficulties of the screening analysis of complex samples, especially the most demanding ones such as effluents of wastewater treatment plants (WWTPs), the picture of the toxicological risk that is obtained is still very limited. In this context, the application of bioassays allows us not only to determine the modes of action of complex samples but to reduce the domain of ECs to be analysed and to focus the analytical efforts towards the most toxic ones (Busch et al., 2016). In fact, the development and application of high throughput bioanalytical techniques and screening tools, i.e., the use of high resolution mass spectrometry (HRMS) combined with bioassays application, is one of the ways to tie the demanding needs of information for ECs (i.e., the links between the occurrence and the toxicity) (Escher and Leusch, 2011).

To estimate the risk associated with the exposure to ECs, we have to integrate the fate and behaviour of the contaminants (both primary contaminants and by-products) and the effects that may occur at different taxonomic levels and the ecological relevance of the tested bioassays (Arnold et al., 2014; Pusceddu et al., 2018). While freshwater organisms have been widely studied as biological models in ecotoxicity, there is still a gap with marine organisms (Beiras and Tato, 2018; Gaw et al., 2014). In this sense, the use of sea urchin (*Paracentrotrus lividus*) embryo test (SET) (Carballeira et al., 2012; Saco-Álvarez et al., 2010) has been chosen in a number of works since they are key benthic species in the coastal environment and they are sensitive to many emerging contaminants (Cunha et al., 2017; Gambardella et al., 2016; Vethaak et al., 2017).

Sea urchins live in wave-exposed rock pools, coral reefs and in sea grass bed, where they function as grazer and prey play a key role (Agnello, 2017). On the one hand, they graze and prune the algae, thus remodelling the bottom. On the other hand, sea urchin embryo and larvae swim in

the water column up to their metamorphosis, being a good part of the plankton and constituting food for other organisms (Agnello, 2017).

The use of sea urchin models has been included in the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and it has been standardised by several national environmental agencies (ASTM, 1995; Environment Canada, 2011; USEPA, 2002). However, it still requires the standardisation and validation to achieve the rank of zebrafish based tests (Di Paolo et al., 2015).

Paracentrotus Lividus is widely found in Europe (Mediterranean and Atlantic coast) and there are equivalent species in eastern (*Lytechinus variegates*) and western American coasts (*Strongylocentrotus purpuratus*), and even in the Antarctic (*Sterechinus neumayeri*). Therefore, the sea urchin bioassay would support worldwide applications. In spite of easiness to capture these invertebrates from the field (they inhabit hard bottom from few centimetres deep to 20 or more meters (Gambardella et al., 2016)), the availability of embryos with reliable good quality outside the natural spawning season is rather limited (Anselmo et al., 2011; Garmendia et al., 2010). Nevertheless, it is affordable to maintain them in captivity in aquaculture facilities. Besides, the growing sea urchin and hatching the eggs and embryos aer quite simple to carry out (e.g., high amount of eggs, and high and external fecundity) and therefore they may become a promising model. Gametes can be obtained easily from mature adults either by direct stripping of the gonad (Bellas, 2008) or osmotic-shock induced spawning (Carballeira et al., 2012) (see **Figure 8.1a-d**). Moreover, eggs and larvae are transparent and the early embryo development is highly synchronous and predictable (see **Figure 8.2a-l** for the predictably development of the *Paracentrotus Lividus* over 48 h), which makes the observation and evaluation of the larvae easy to follow. Finally, it is worth mentioning the sophistication of the urchin genome and the number of complex immune responses that integrates, which may be equivalent to that of vertebrates (Pennisi, 2006).

Most standard methods (ASTM, 1995; Environment Canada, 2011; USEPA, 2002) mentioned before are based on the evaluation of the sea urchin morphological toxicity. This end-point involves distinguishing between normal and malformed larvae. The normal larvae should exhibit four fully formed arms (two longer post-oral arms and two shorter oral arms that are half the length of the

long axis of the larvae) and a regular outer contour of the body (see **Figure 8.2l**). Recently, several other end-points have been proposed (e.g., fertilisation rate (Mohd Zanuri et al., 2017), skeletogenesis (Carballeira et al., 2012), neurotoxicity response (Falugi and Aluigi, 2012), swimming behaviour (Faimali et al., 2017)) in order to enhance the SET applicability. For instance, since the identification of more detailed abnormalities can complicate the observation, and considering that this even depends on the position of the larvae under microscope, Saco-Alvarez and co-workers (Saco-Álvarez et al., 2010) proposed an alternative growth inhibition endpoint based on the size increase. The maximum dimension of all the individuals (not only normal larvae but also any other earlier or abnormal development stage) is measured, and the size increased respect to the egg diameter is measured. This allows a more independent observation response, and makes automatic reading feasible, which could improve the high-throughput required in monitoring programs.

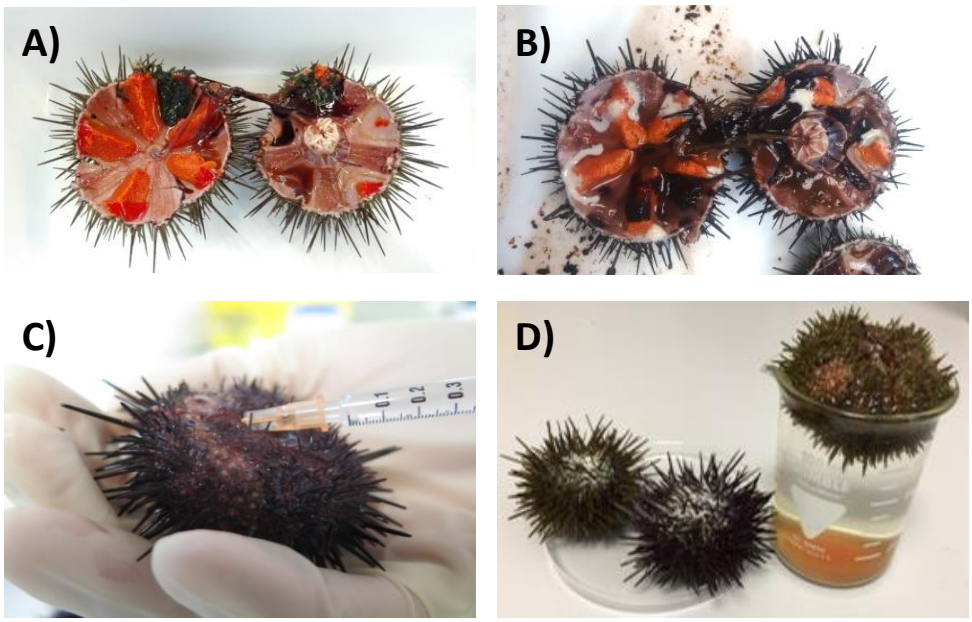

***Figure 8.1**. Obtaining gametes by direct stripping (a-b) and Osmotic-shock-induced (c-d). a) Mature gonads of a female, b) mature gonads of a male, c) injection of 1 mL KCl 0.5 M through the peri-oral membrane into coelom and d) collection of the gametes from inverted female over a beaker containing seawater and sperm directly from the gonopores of the males.*

Figure 8.2. *Paracentrotus lividus embryo development, from fertilised egg until it reachs the four arm pluteus stage in 48 h: a) fertilised egg (within the first 30 min), b) 2 cell division (within 1.30 min), c) 4 cell division (2.30 h), d) eight cell division (3 h), e) sixteen cells division (3.30 h), f) 32 cell division (4.30 h), g) morula (4.45 h), h) blastula (5 h), i) glastula (5-22 h), j-k) primsa larvae, pre pluteos stage (22-48 h), and l) normal four arm pluteous stage (48 h).*

The integration of these kind of bioassays in chemical monitoring can be achieved through the application of the effect-directed analysis (EDA) since it is a streamlined procedure that integrates a chromatographic fractionation with bioassays driven non-targeted analysis (Brack et al., 2016). Furthermore, the application of EDA may go from the discovery of unknown chemicals to the prioritisation of contaminants, according to the scope and criteria (i.e., the selected end-point) established beforehand. Recently published EDA applications focused on the toxicity evaluation of

wastewater effluents and surface waters (Hashmi et al., 2018; Osorio et al., 2018), the elucidation of the causes of mutagenicity in the Rhine river (Muz et al., 2017) and the development of specific protocols based on in vitro tests (Muschket et al., 2018; Ouyang et al., 2016; Zwarg et al., 2018; Zwart et al., 2018). However, the application of EDA on estuaries and coastal waters is still rather limited (Booij et al., 2014).

Within this context, this chapter includes the application of SET to study the toxicity of the WWTP effluents affecting some important estuaries of Biscay. in addition to this, the implementation of SET into an EDA protocol was carried out for the first time in order to integrate a relevant organism in coastal environments and to widen the scope of this procedure.

8.2. Experimental section

8.2.1 Reagents and materials

Name, classes, main use, molecular formulas and CAS numbers for the set of organic compounds used are summarised in **Table 8.1**. Brands of the compounds are also included.

Oasis hydrophilic-lipophilic balanced (HLB) 200 mg solid phase extraction (SPE) cartridges were purchased from Waters (Milford, USA). Bond-Elut Plexa and Strata X-AW bulk sorbents used in the EDA approach were purchased from Agilent (Santa Clara, CA, USA) and Phenomenex (Torrance, CA, USA), respectively. Empty SPE tubes (6 mL and 20 mL) and polypropylene (PP) frits were purchased from Supelco (Bellefonte, PA, USA).

Formic acid (>98%) and sodium thiosulfate (≥98%) were supplied by Panreac (Barcelona, Spain). Methanol (MeOH, HPLC grade, 99.9%), ethyl acetate (EtOAc, HPLC grade, 99.9%), acetone (HPLC grade, 99.9%) and ammonium solution (25% as NH_3) used in the SPE extraction were obtained from Sigma Aldrich (St. Louis, MO, USA). Ultra-pure water was obtained using a Milli-Q water purification system (<0.05 S/cm, Milli-Q model 185, Millipore, Bedford, MA, USA). Dimethyl sulfoxide (DMSO, cell culture grade) used in the bioassays was supplied by Panreac.

Table 8.1. Name, class, use, molecular formula, CAS number and brand of each of the synthetic compounds studied. They all have a purity of at least 97%.

Name	Class	Use[a]	Molecular formula	CAS number
2-hydroxybenzothiazole[B]	Industrial chemical	Corrosion inhibitor	C_7H_5NOS	934-34-9
4-Chlorophenol[B]	Pharmaceutical	Anti-infective	C_6H_5ClO	106-48-9
4-hydroxytamoxifen[C]	Pharmaceutical	Antineoplastic	$C_{26}H_{29}NO_2$	97151-02-5
Acesulfame[D]	Life style product	Artificial sweetener	$C_4H_5NO_4S$	33665-90-6
Acetaminophen[E]	Pharmaceutical	Anti-inflammatory	$C_8H_9NO_2$	103-90-2
Acetamiprid[B]	Pesticide	Insecticide	$C_{10}H_{11}ClN_4$	135410-20-7
Acetochlor[B]	Pesticide	Herbicide	$C_{14}H_{20}ClNO_2$	34256-82-1
Acyclovir[B]	Pharmaceutical	Antiviral	$C_8H_{11}N_5O_3$	59277-89-3
Alachlor[B]	Pesticide	Herbicide	$C_{14}H_{20}ClNO_2$	15972-60-8
Albendazole[B]	Pharmaceutical	Anthelminthic	$C_{12}H_{15}N_3O_2S$	54965-21-8
Amantadine[B]	Pharmaceutical	Antiviral	$C_{10}H_{18}ClN$	665-66-7
Ambroxol[B]	Pharmaceutical	Expectorants	$C_{13}H_{18}Br_2N_2O$	18683-91-5
Ametryn[B]	Pesticide	Herbicide	$C_9H_{17}N_5S$	834-12-8
Amiodarone[B]	Pharmaceutical	Antiarrhythmic	$C_{25}H_{30}Cl_2NO_3$	19774-82-4
Amitriptyline[B]	Pharmaceutical	Antidepressant	$C_{20}H_{23}N$	50-48-6
Amoxicillin[B]	Pharmaceutical	Antibiotic	$C_{16}H_{25}N_3O_8S$	61336-70-7
Ampicillin[B]	Pharmaceutical	Antibiotic	$C_{16}H_{19}N_3O_4S$	69-53-4
Anastrozole[B]	Life style product	Stimulant	$C_{17}H_{19}N_5$	120511-73-1
Atenolol[B]	Pharmaceutical	Antihypertensive	$C_{14}H_{22}N_2O_3$	29122-68-7
Atorvastatin[B]	Pharmaceutical	Anti-cholesteric	$C_{33}H_{35}FN_2O_5$	134523-00-5
Atrazine[E]	Pesticide	Herbicide	$C_8H_{14}ClN_5$	1912-24-9
Azelastine[B]	Pharmaceutical	Antihistaminic	$C_{22}H_{25}Cl_2N_3O$	79307-93-0
Azithromycin[B]	Pharmaceutical	Antibiotic	$C_{38}H_{72}N_2O_{12}$	83905-01-5
Azoxystrobin[B]	Pesticide	Fungicide	$C_{22}H_{17}N_3O_5$	131860-33-8
Bendiocarb[B]	Pesticide	Insecticide	$C_{11}H_{13}NO_4$	22781-23-3
Bentazone[B]	Pesticide	Herbicide	$C_{10}H_{12}N_2O_3S$	25057-89-0
Benzethonium[B]	Pharmaceutical	Bactericide	$C_{27}H_{42}ClNO_2$	121-54-0
Benzothiazole[B]	Industrial chemical	Corrosion inhibitor	C_7H_5NS	95-16-9
Bezafibrate[F]	Pharmaceutical	Anti-cholesteric	$C_{19}H_{20}ClNO_4$	41859-67-0
Bicalutamide[B]	Pharmaceutical	Antineoplastic	$C_{18}H_{14}F_4N_2O_4S$	90357-06-5
Bisoprolol[B]	Pharmaceutical	Antihypertensive	$C_{18}H_{31}NO_4$	66722-44-9
Boscalid[B]	Pesticide	Fungicide	$C_{18}H_{12}Cl_2N_2O$	188425-85-6
Bosentan[B]	Pharmaceutical	Antihypertensive	$C_{27}H_{29}N_5O_6S$	147536-97-8
Bupropion[C]	Pharmaceutical	Antidepressant	$C_{13}H_{19}Cl_2NO$	31677-93-7

Table 8.1. Name, class, use, molecular formula, CAS number and brand of each of the synthetic compounds studied. They all have a purity of at least 97%.

Name	Class	Use[a]	Molecular formula	CAS number
Butylparaben[B]	Personal care product	Preservative	$C_{11}H_{14}O_3$	94-26-8
Caffeine[B]	Life style product	Stimulant	$C_8H_{10}N_4O_2$	58-08-2
Captopril[B]	Pharmaceutical	Antihypertensive	$C_9H_{15}NO_3S$	62571-86-2
Carbamazepine[B]	Pharmaceutical	Anticonvulsant	$C_{15}H_{12}N_2O$	298-46-4
Carbaryl[B]	Pesticide	Insecticide	$C_{12}H_{11}NO_2$	63-25-2
Carbendazim[B]	Pesticide	Fungicide	$C_9H_9N_3O_2$	10605-21-7
Celecoxib[B]	Pharmaceutical	Cyclooxygenase 2 inhibitors	$C_{17}H_{14}F_3N_3O_2S$	169590-42-5
Cetirizine[B]	Pharmaceutical	Antihistaminic	$C_{21}H_{27}Cl_3N_2O_3$	83881-52-1
Chloridazon[B]	Pesticide	Herbicide	$C_{10}H_8ClN_3O$	1698-60-8
Chloroxuron[B]	Pesticide	Herbicide	$C_{15}H_{15}ClN_2O_2$	1982-47-4
Chlortoluron[B]	Pesticide	Herbicide	$C_{10}H_{13}ClN_2O$	15545-48-9
Ciprofloxacin[E]	Pharmaceutical	Antibiotic	$C_{17}H_{18}FN_3O_3$	85721-33-1
Clarithromycin[B]	Pharmaceutical	Antibiotic	$C_{38}H_{69}NO_{13}$	81103-11-9
Clofibric acid[F]	Pharmaceutical	Anti-cholesteric	$C_{10}H_{11}ClO_3$	882-09-7
Clomazone[B]	Pesticide	Herbicide	$C_{12}H_{14}ClNO_2$	81777-89-1
Clomipramine[B]	Pharmaceutical	Antidepressant	$C_{19}H_{23}ClN_2$	303-49-1
Clonidine[B]	Pharmaceutical	Antihypertensive	$C_9H_9Cl_2N_3$	4205-90-7
Clopidogrel[C]	Pharmaceutical	Antithrombotic	$C_{16}H_{16}ClNO_2S$	113665-84-2
Clozapine[B]	Pharmaceutical	Antipsychotic	$C_{18}H_{19}ClN_4$	5786-21-0
Cortisone[B]	Pharmaceutical	Anti-inflammatory	$C_{21}H_{28}O_5$	53-06-5
Cotinine[B]	Life style product	Stimulant	$C_{10}H_{12}N_2O_3S$	486-56-6
Crotamiton[B]	Pharmaceutical	Anti-parasitic	$C_{13}H_{17}NO$	483-63-6
Cyclophosphamide[B]	Pharmaceutical	Antineoplastic	$C_7H_{15}Cl_2N_2O_2P$	50-18-0
Cyproterone[G]	Pharmaceutical	Hormone	$C_{22}H_{27}ClO_3$	2098-66-0
Desloratadine[C]	Pharmaceutical	Antihistaminic	$C_{19}H_{19}ClN_2$	100643-71-8
Dexamethasone[B]	Pharmaceutical	Anti-inflammatory	$C_{22}H_{29}FO_5$	50-02-2
Diazepam[C]	Pharmaceutical	Antianxiety	$C_{16}H_{13}ClN_2O$	439-14-5
Dichlorvos[B]	Pesticide	Insecticide	$C_4H_7Cl_2O_4P$	62-73-7
Diclofenac[B]	Pharmaceutical	Anti-inflammatory	$C_{14}H_{11}Cl_2NO_2$	15307-86-5
Didecyldimethylammonium[B]	Industrial chemical	Biocide	$C_{22}H_{48}BrN$	2390-68-3
Diethyl toluamide [B] (DEET)	Pesticide	Insecticide	$C_{12}H_{17}NO$	134-62-3
Diflufenican[B]	Pesticide	Herbicide	$C_{19}H_{11}F_5N_2O_2$	83164-33-4
Dimethachlor[B]	Pesticide	Herbicide	$C_{13}H_{18}ClNO_2$	50563-36-5
Dimethoate[B]	Pesticide	Insecticide	$C_5H_{12}NO_3PS_2$	60-51-5

Table 8.1. Name, class, use, molecular formula, CAS number and brand of each of the synthetic compounds studied. They all have a purity of at least 97%.

Name	Class	Use[a]	Molecular formula	CAS number
Diphenhydramine[B]	Pharmaceutical	Antihistaminic	$C_{17}H_{22}ClNO$	147-24-0
Diuron[F]	Pesticide	Herbicide	$C_9H_{10}Cl_2N_2O$	330-54-1
Dodemorph[B]	Pesticide	Fungicide	$C_{18}H_{35}NO$	1593-77-7
Domperidone[B]	Pharmaceutical	Anti-inflammatory	$C_{22}H_{24}ClN_5O_2$	57808-66-9
Drospirenone[B]	Pharmaceutical	Hormone	$C_{24}H_{30}O_3$	67392-87-4
Duloxetine[B]	Pharmaceutical	Antidepressant	$C_{18}H_{19}BOS$	116539-59-4
EDDP[B]	Pesticide	Fungicide	$C_{14}H_{15}O_2PS_2$	17109-49-8
Efavirenz[B]	Pharmaceutical	Antiviral	$C_{14}H_9ClF_3NO_2$	15498-52-4
Eprosartan[B]	Pharmaceutical	Antihypertensive	$C_{23}H_{24}N_2O_7S$	133040-01-4
Erythromycin[B]	Pharmaceutical	Antibiotic	$C_{37}H_{67}NO_{13}$	114-07-8
Ethion[B]	Pesticide	Insecticide	$C_9H_{22}O_4P_2S_4$	563-12-2
Exemestane[B]	Pharmaceutical	Antineoplastic	$C_{20}H_{24}O_2$	107868-30-4
Fenoxycarb[B]	Pesticide	Insecticide	$C_{17}H_{19}NO_4$	72490-01-8
Fenpropidin[B]	Pesticide	Fungicide	$C_{19}H_{31}N$	67306-00-7
Fenpropimorph[B]	Pesticide	Fungicide	$C_{20}H_{33}NO$	67564-91-4
Fenthion[B]	Pesticide	Insecticide	$C_{10}H_{15}O_3PS_2$	55-38-9
Finasteride[B]	Pharmaceutical	Antiviral	$C_{23}H_{36}N_2O_2$	98319-26-7
Fluconazole[B]	Pharmaceutical	Antifungal	$C_{13}H_{12}F_2N_6O$	86386-73-4
Flufenoxuron[B]	Pesticide	Insecticide	$C_{21}H_{11}ClF_6N_2O_3$	101463-69-8
Flumequine[B]	Pharmaceutical	Antibiotic	$C_{14}H_{12}FNO_3$	42835-25-6
Flusilazole[B]	Pesticide	Fungicide	$C_{16}H_{15}F_2N_3Si$	85509-19-9
Flutamide[B]	Pharmaceutical	Antineoplastic	$C_{11}H_{11}F_3N_2O_3$	13311-84-7
Fluvoxamine[C]	Pharmaceutical	Antidepressant	$C_{15}H_{21}F_3N_2O_2$	54739-18-3
Furosemide[B]	Pharmaceutical	Antibiotic	$C_{12}H_{10}ClN_2O_5S$	54-31-9
Gabapentin[B]	Pharmaceutical	Anticonvulsant	$C_9H_{17}NO_2$	60142-96-3
Gemfibrozil[B]	Pharmaceutical	Hypolipidemic	$C_{15}H_{22}O_3$	25812-30-0
Genistein[H]	Phytoestrogens	Phytoestrogen	$C_{15}H_{10}O_5$	446-72-0
Genistin[H]	Phytoestrogens	Phytoestrogen	$C_{21}H_{20}O_{10}$	529-59-9
Glibenclamide[B]	Pharmaceutical	Antidiabetic	$C_{23}H_{28}ClN_3O_5S$	10238-21-8
Glimepiride[B]	Pharmaceutical	Antidiabetic	$C_{23}H_{34}N_4O_5S$	93479-97-1
Glycitin[H]	Phytoestrogens	Phytoestrogen	$C_{22}H_{22}O_{10}$	40246-10-4
Hexazinone[B]	Pesticide	Herbicide	$C_{12}H_{20}N_4O_2$	51235-04-2
Hydroxychloroquine[B]	Pharmaceutical	Antilipemic	$C_{18}H_{28}ClN_3O_5S$	747-36-4
Ifosfamide[B]	Pharmaceutical	Antineoplastic	$C_7H_{15}Cl_2N_2O_2P$	3778-73-2

Table 8.1. Name, class, use, molecular formula, CAS number and brand of each of the synthetic compounds studied. They all have a purity of at least 97%.

Name	Class	Use[a]	Molecular formula	CAS number
Imatinib[B]	Pharmaceutical	Antineoplastic	$C_{29}H_{31}N_7O$	152459-95-5
Imazalil[B]	Pesticide	Fungicide	$C_{14}H_{14}Cl_2N_2O$	35554-44-0
Imidacloprid[B]	Pesticide	Insecticide	$C_9H_{10}ClN_5O_2$	138261-41-3
Iminostilbene[B]	Life style product	Stimulant	$C_{14}H_{11}N$	256-96-2
Imipramine[B]	Pharmaceutical	Antidepressant	$C_{19}H_{24}N_2$	50-49-7
Indometacin[B]	Pharmaceutical	Anti-inflammatory	$C_{19}H_{16}ClNO_4$	53-86-1
Iprodione[B]	Pesticide	Fungicide	$C_{13}H_{13}Cl_2N_3O_3$	36734-19-7
Irbesartan[I]	Pharmaceutical	Antihypertensive	$C_{25}H_{28}N_6O$	138402-11-6
Isoproturon[E]	Pesticide	Herbicide	$C_{12}H_{18}N_2O$	34123-59-6
Ketoconazole[B]	Pharmaceutical	Antifungal	$C_{26}H_{28}Cl_2N_4O_4$	65277-42-1
Ketoprofen[F]	Pharmaceutical	Anti-inflammatory	$C_{16}H_{14}O_3$	22071-15-4
Lenacil[B]	Pesticide	Herbicide	$C_{13}H_{18}N_2O_2$	2146-08-1
Lidocaine[B]	Pharmaceutical	Anesthetise	$C_{14}H_{22}N_2O$	137-58-6
Linuron[B]	Pesticide	Herbicide	$C_9H_{10}Cl_2N_2O_2$	330-55-2
Lorazepam[B]	Pharmaceutical	Antianxiety	$C_{15}H_{10}Cl_2N_2O_2$	846-49-1
Losartan[J]	Pharmaceutical	Antihypertensive	$C_{22}H_{23}ClN_6O$	114798-26-4
Mebendazole[C]	Pharmaceutical	Anthelminthic	$C_{16}H_{13}N_3O_3$	31431-39-7
Mebeverine[B]	Pharmaceutical	Anticonvulsant	$C_{25}H_{36}ClNO_5$	2753-45-9
Meclocycline[B]	Pharmaceutical	Antibiotic	$C_{29}H_{27}ClN_2O_{14}S$	73816-42-9
Mecoprop[B]	Pesticide	Herbicide	$C_{10}H_{11}ClO_3$	93-65-2
Medroxyprogesterone[B]	Pharmaceutical	Hormone	$C_{22}H_{32}O_3$	520-85-4
Mefenamic acid[B]	Pharmaceutical	Anti-inflammatory	$C_{15}H_{15}NO_2$	61-68-7
Memantine[B]	Pharmaceutical	Antiparkinsonian	$C_{12}H_{22}ClN$	41100-52-1
Metalaxyl[B]	Pesticide	Fungicide	$C_{15}H_{21}NO_4$	57837-19-1
Metamitron[B]	Pesticide	Herbicide	$C_{10}H_{10}N_4O$	41394-05-2
Metazachlor[B]	Pesticide	Herbicide	$C_{14}H_{16}ClN_3O$	67129-08-2
Metconazole[B]	Pesticide	Fungicide	$C_{17}H_{22}ClN_3O$	125116-23-6
Metformin[B]	Pharmaceutical	Antidiabetic	$C_4H_{12}ClN_5$	1115-70-4
Methiocarb[B]	Pesticide	Insecticide	$C_{11}H_{15}NO_2S$	2032-65-7
Methotrexate[B]	Pharmaceutical	Antineoplastic	$C_{20}H_{22}N_8O_5$	59-05-2
Methylparathion[B]	Pesticide	Insecticide	$C_8H_{10}NO_5PS$	298-00-0
Methylpirimiphos[B]	Pesticide	Insecticide	$C_{11}H_{20}N_3O_3PS$	29232-93-7
Metolachlor[B]	Pesticide	Herbicide	$C_{15}H_{22}ClNO_2$	51218-45-2
Metoprolol[B]	Pharmaceutical	Antihypertensive	$C_{15}H_{25}NO_3$	51384-51-1

Table 8.1. Name, class, use, molecular formula, CAS number and brand of each of the synthetic compounds studied. They all have a purity of at least 97%.

Name	Class	Use[a]	Molecular formula	CAS number
Metribuzin[B]	Pesticide	Herbicide	$C_8H_4N_4OS$	21087-64-9
Miconazole[B]	Pharmaceutical	Antibiotic	$C_{18}H_{14}Cl_4N_2O$	22916-47-8
Mirtazapine[B]	Pharmaceutical	Antidepressant	$C_{17}H_{19}N_3$	85650-52-8
Montelukast[B]	Pharmaceutical	Anti-inflammatory	$C_{35}H_{35}ClNO_3$	151767-02-1
Myclobutanil[B]	Pesticide	Fungicide	$C_{15}H_{17}ClN_4$	88671-89-0
Mycophenolic acid[B]	Pharmaceutical	Antibiotic	$C_{17}H_{20}O_6$	24280-93-1
Naproxen[B]	Pharmaceutical	Anti-inflammatory	$C_{14}H_{14}O_3$	22204-53-1
Nitrofurantoin[B]	Pharmaceutical	Anti-infective	$C_8H_6N_4O_5$	67-20-9
Perfluoro-1-butanesulfonate [B] (PFBS)	Industrial chemical	PFAS	$C_4H_9O_3S$	375-73-5
Norfloxacin[E]	Pharmaceutical	Antibiotic	$C_{16}H_{18}FN_3O_3$	70458-96-7
Norgestimate[B]	Pharmaceutical	Hormone	$C_{23}H_{31}NO_3$	35189-28-7
Nortriptyline[B]	Pharmaceutical	Antidepressant	$C_{18}H_{21}N$	894-71-3
Omeprazol[B]	Pharmaceutical	Diuretic	$C_{17}H_{19}N_3O_3S$	73590-58-6
Ondansetron[B]	Pharmaceutical	Anthelminthic	$C_8H_{20}ClN_3O$	99614-01-4
Oryzalin[B]	Pesticide	Herbicide	$C_{12}H_{18}N_4O_6S$	19044-88-3
Oxazolam[B]	Pharmaceutical	Sedative	$C_{18}H_{17}ClN_2O_2$	24143-17-7
Oxybutynin[B]	Pharmaceutical	Anticholinergic	$C_{22}H_{32}ClNO_3$	1508-65-2
Parathion[B]	Pesticide	Insecticide	$C_{10}H_{14}NO_5PS$	56-38-2
Paroxetine[B]	Pharmaceutical	Antidepressant	$C_{19}H_{21}ClFNO_3$	78246-49-8
Pendimethalin[B]	Pesticide	Herbicide	$C_{13}H_{19}N_3O_4$	40487-42-1
Pentoxifylline[B]	Pharmaceutical	Vasodilator	$C_{13}H_{18}N_4O_3$	6493-05-6
Perfluoroctylsulfonamide[C] (PFOSA)	Industrial chemical	PFAS	$C_8H_2F_{17}NO_2S$	754-91-6
Perfluorooctane sulfonic acid (PFOS) [B]	Industrial chemical	PFAS	$C_8HF_{17}O_3S$	1763-23-1
Perfluorooctanoic acid (PFOA) [B]	Industrial chemical	PFAS	$C_8HF_{15}O_2$	335-67-1
Phenytoin[B]	Pharmaceutical	Anticonvulsant	$C_{15}H_{12}N_2O_2$	57-41-0
Pindolol[B]	Pharmaceutical	Antihypertensive	$C_{14}H_{20}N_2O_2$	13523-86-9
Pipamperone[B]	Pharmaceutical	Antidepressant	$C_{21}H_{32}Cl_2FN_3O_2$	2448-68-2
Pirimicarb[B]	Pesticide	Insecticide	$C_{11}H_{18}N_4O_2$	23103-98-2
Pravastatin[B]	Pharmaceutical	Anti-cholesteric	$C_{23}H_{35}NaO_7$	81131-70-6
Prednisone[B]	Pharmaceutical	Anti-inflammatory	$C_{21}H_{26}O_5$	53-03-2
Primidone[B]	Pharmaceutical	Anticonvulsant	$C_{12}H_{14}N_2O_2$	125-33-7
Prochloraz[B]	Pesticide	Fungicide	$C_{15}H_{16}Cl_3N_3O_2$	67747-09-5
Progesterone[B]	Pharmaceutical	Hormone	$C_{21}H_{30}O_2$	57-83-0
Propachlor[B]	Pesticide	Herbicide	$C_{11}H_{14}ClNO$	1918-16-7

Table 8.1. Name, class, use, molecular formula, CAS number and brand of each of the synthetic compounds studied. They all have a purity of at least 97%.

Name	Class	Use[a]	Molecular formula	CAS number
Propamocarb[B]	Pesticide	Fungicide	$C_9H_{20}N_2O_2$	245979-73-5
Propanil[B]	Pesticide	Herbicide	$C_9H_9Cl_2NO$	709-98-8
Propiconazole[B]	Pesticide	Fungicide	$C_{15}H_{17}Cl_2N_3O_2$	60207-90-1
Propofol[B]	Pharmaceutical	Anesthetise	$C_{12}H_{18}O$	2078-54-8
Propranolol[F]	Pharmaceutical	Antihypertensive	$C_{16}H_{21}NO_2$	525-66-6
Propyphenazone[B]	Pharmaceutical	Anti-inflammatory	$C_{14}H_{18}N_2O$	479-92-5
Propyzamide[B]	Pesticide	Herbicide	$C_{12}H_{11}Cl_2NO$	23950-58-5
Prosulfocarb[B]	Pesticide	Herbicide	$C_{14}H_{21}NOS$	52888-80-9
Pyrantel[B]	Pharmaceutical	Anthelminthic	$C_{34}H_{30}N_2O_6S$	22204-24-6
Pyrazophos[B]	Pesticide	Fungicide	$C_{14}H_{20}N_3O_5PS$	13457-18-6
Quinmerac[B]	Pesticide	Herbicide	$C_{11}H_8ClNO_2$	90717-03-6
Quinoxyfen[B]	Pesticide	Fungicide	$C_{15}H_8Cl_2FNO$	124495-18-7
Raloxifene[B]	Pharmaceutical	Selective Estrogenic Receptor Modulators	$C_{28}H_{28}ClNO_4S$	84449-90-1
Ranitidine[B]	Pharmaceutical	Antiulcer	$C_{13}H_{23}ClN_4O_3S$	66357-59-3
Remifentanyl[B]	Pharmaceutical	Analgesic	$C_{20}H_{28}N_2O_5$	132875-61-7
Risperidone[B]	Pharmaceutical	Antipsychotic	$C_{23}H_{27}FN_4O_2$	106266-06-2
Ropinirole[B]	Pharmaceutical	Antiparkinsonian	$C_{16}H_{25}ClN_2O$	91374-20-8
Roxithromycin[B]	Pharmaceutical	Antibacterial	$C_{41}H_{76}N_2O_{15}$	80214-83-1
Sertraline[B]	Pharmaceutical	Antidepressant	$C_{17}H_{17}Cl_2N$	79617-96-2
Simazine[E]	Pesticide	Herbicide	$C_7H_{12}ClN_5$	122-34-9
Sotalol[B]	Pharmaceutical	Antihypertensive	$C_{12}H_{21}ClN_2O_3S$	959-24-0
Spiroxamine[B]	Pesticide	Fungicide	$C1_8H_{35}NO_2$	118134-30-8
Sulfadiazine[B]	Pharmaceutical	Antibiotic	$C_{10}H_{10}N_4O_2S$	68-35-9
Sulfamethazine[B]	Pharmaceutical	Antibiotic	$C_{12}H_{14}N_4O_2S$	57-68-1
Sulfamethoxazole[B]	Pharmaceutical	Antibiotic	$C_{10}H_{11}N_3O_3S$	723-46-6
Sulfapyridine[B]	Pharmaceutical	Antibiotic	$C_{11}H_{11}N_3O_2S$	144-83-2
Sulfathiazole[B]	Pharmaceutical	Antibiotic	$C_9H_9N_3O_2S_2$	72-14-0
Tamoxifen[B]	Pharmaceutical	Selective Estrogenic Receptor Modulators	$C_{26}H_{29}NO$	10540-29-1
Tebuconazole[B]	Pesticide	Fungicide	$C_{16}H_{22}ClN_3O$	107534-96-3
Telmisartan[K]	Pharmaceutical	Antihypertensive	$C_{33}H_{30}N_4O_2$	144701-48-4
Terbinafine[B]	Pharmaceutical	Antifungal	$C_{21}H_{26}ClN$	78628-80-5
Terbuthylazine[B]	Pesticide	Herbicide	$C_9H_{16}ClN_5$	5915-41-3
Terbutryn[B]	Pesticide	Herbicide	$C_{10}H_{19}N_5S$	886-50-0
Testosterone[B]	Pharmaceutical	Hormone	$C_{19}H_{28}O_2$	58-22-0

Table 8.1. Name, class, use, molecular formula, CAS number and brand of each of the synthetic compounds studied. They all have a purity of at least 97%.

Name	Class	Use[a]	Molecular formula	CAS number
Thiabendazole[B]	Pharmaceutical	Anthelminthic	$C_{10}H_7N_3S$	148-79-8
Thiacloprid[B]	Pesticide	Insecticide	$C_{10}H_9ClN_4S$	111988-49-9
Thiamethoxam[B]	Pesticide	Fungicide	$C_8H_{10}ClN_5O_3S$	153719-23-4
Thymol[B]	Pesticide	Fungicide	$C_{10}H_{14}O$	89-83-8
Tramadol[B]	Pharmaceutical	Analgesic	$C_{16}H_{26}ClNO_2$	36282-47-0
Triadimenol[B]	Pesticide	Fungicide	$C_{14}H_{18}ClN_3O_2$	55219-65-3
Triethylphosphate[B]	Industrial chemical	Plasticiser	$C_6H_{15}O_4P$	78-40-0
Trimethoprim[E]	Pharmaceutical	Antibiotic	$C_{14}H_{18}N_4O_3$	738-70-5
Triphenylphosphate[B]	Industrial chemical	Fire retardant	$C_{18}H_{15}O_4P$	115-86-6
Valsartan[K]	Pharmaceutical	Antihypertensive	$C_{24}H_{29}N_5O_3$	137862-53-4
Verapamil[B]	Pharmaceutical	Antihypertensive	$C_{27}H_{38}N_2O_4$	52-53-9

[a] The classification of the compounds was done according to the information obtained in Pubchem (https://pubchem.ncbi.nlm.nih.gov/)
[B] Sigma-Aldrich
[C] Dr. Ehrenstofer
[D] Supelco
[E] Fluka
[F] MP biomedicals
[G] Toronto Research Chemicals
[H] Extrasynthese
[I] Sanofi
[J] Merck
[K] Boehringer

LC-MS grade MeOH, water and formic acid (Optima grade) purchased from Sigma Aldrich were used as mobile phase in the fractionation, whereas Optima grade water, acetonitrile (ACN), isopropanol and formic acid provided by Fischer Scientific (Geel, Belgium) were used as mobile phase in the LC-HRMS.

8.2.2. Sampling and sample preparation

8.2.2.1 WWTP effluent toxicity

For the toxicity analysis of the WWTP effluents four treatment plants of Biscay were selected (i.e. Gorliz, Mungia, Gernika and Galindo, see the location on **Chapter 7, Figure 7.1**) and, in the case of Galindo, the samples were taken from the secondary treatment (Ga2) and from an experimental third treatment effluent (Ga3) that uses a chlorination process. Further details about the treatments, water flow and sources of all those effluents are summarised in **Table 8.2.**

Table 8.2. Name, location, treatment, effluents discharge estuaries, water flow and influents sources of the WWTPs studied in this work.

WWTP	Treatment	Effluents discharge estuaries	Water flow (m^3/day)	Influent sources (%)
Galindo Ga2	2nd	Bilbao estuary	1.0 E9	Industrial 3.2%, Hospital 0.5%, Domestic 96.3% (>1000000 inhabitant)
Galido Ga3	3rd chlorination	-[a]	-[a]	Industrial 3.2%, Hospital 0.5%, Domestic 96.3% (>1000000 inhabitant)
Gorliz	2nd	Plentzia estuary It releases the effluent into the estuary mouth through a submarine pipe located to 1000 m from the coast with an 18 m depth.	1.4 E6	Industrial 0%, Hospital 1.3%, Domestic 98.7% (10600 inhabitants)
Mungia	2nd	Plentzia estuary It releases the effluent into the upper part (22 km with to respect the mouth) of Plentzia estuary	5.4 E3	Industrial 3.1%, Hospital 0%, Domestic 96.9% (17000 inhabitants)
Gernika	1st	Urdaibai estuary. It discharges directly to the estuary of Urdaibai, which is declared Reserve of The Biosphere by Unesco since 1984.	-[b]	Industrial 25.33%, Hospital 0.2%, Domestic 74.46% (70000 inhabitants)

a) Experimental state treatment, private use in the WWTP
b) Unknown (but <10% of the total flow)

From each effluent 5 L samples were taken in pre-cleaned plastic bottles and transported to the laboratory in cooled boxes and filtered within 48 h with a 1.2 µm glass microfiber filter (GE Whatman, Maidstone, UK) before extraction.

The filtered samples were extracted with 200 mg HLB-SPE according to a previously validated method with slight modifications (Mijangos et al., 2018a). Each cartridge was sequentially conditioned with 5 mL of acetone, 5 mL of EtOAc, 5 mL of MeOH and 5 mL of Milli-Q water. In the case of Ga3, sodium thiosulfate (30 mg/L) was added to the sample, prior to perform the SPE, to neutralise the presence of chlorine (Fernández et al., 2008). A maximum of 500 mL of each effluent sample were passed through each cartridge (several cartridges were used in parallel) assisted by a vacuum pump at ca. 5 mL/min. Subsequently, the cartridges were washed with 6 mL of ultrapure water, vacuum dried for 40 min and eluted with 6 mL of MeOH. All the eluted extracts were pooled together and the final extract was then concentrated to dryness under a gentle stream of nitrogen at 35°C, re-dissolved in pure MeOH, and submitted to the sea urchin bioassay (see **section 8.2.4**).

8.2.2.2. Application of EDA

For EDA, 225 L of the effluent of the secondary treatment of Galindo (Ga2) was sampled and filtered in the lab. For the SPE extraction, the cartridges were prepared in-house by filling an empty PP column (20 mL) with 1.5 g Strata X-AW (bottom) and 3.5 g of Bond-Elut Plexa (top). Previous to the extraction, both bulk materials were individually cleaned with 400 mL of acetone followed by EtOAc, MeOH, MeOH with 2% NH_3 (v/v) solution and Milli-Q water (30 min for each solvent, 3 cycles) in an ultrasonic bath. The 225 L of the effluent sample were percolated through the cleaned cartridges assisted by a vacuum pump at ca. 5 mL/min (the ratio mass of sorbents/volume of effluent was scaled up from an amount of 0.2 g of total sorbent amount per 0.5 L of water). After the extraction, all cartridges were kept at -40°C for 24 h and freeze-dried (Cryodos-50 laboratory freeze-dryer from Telstar Instrument, Sant Cugat del Vallés, Barcelona, Spain). Elution was carried out with 90 mL of MeOH: EtOAc (50:50, v:v) solvent mixture followed by 60 mL of MeOH with 2% NH_3 (v/v). All extracts were neutralised by adding formic acid and the pooled extracts were evaporated using a rotary evaporation (Büchi, Switzerland) and adjusted to a final volume of 225 mL (i.e. the raw sample with a concentration factor of 1000).

8.2.3 EDA workflow

As illustrated in **Figure 8.3**, the previously obtained raw sample (effluent sample submitted to a SPE extraction) was subjected to a two-fold fractionation step (see **section 8.2.3.1**). The SET bioassay was applied to all the fractions obtained at both fractionations (see **section 8.2.4**), while non-targeted chemical analysis was restricted to biologically active and non-active neighbouring fractions and the parent extract (see **section 8.2.5**). In the same way, a procedural blank was also submitted to fractionation and analysis.

At each fractionation step, a recombined mixture of all the fractions was prepared and tested in the bioassay to assure that no major losses of bioactivity occurred during fractionation. Finally, SET dose-effect curves of putatively identified candidate drivers were recorded in those cases where standards were available in order to confirm the toxicity of these compounds and to assess their contribution to the entire bioactivity of the active fractions (see **section 8.2.6**) in terms of toxic units (TU). In order to account for the concentration of contaminants along the whole procedure, dose-

range values are given in terms of the relative enrichment factor (REF), which is the product of the enrichment factor of the SPE process and the dilution of the extract in the bioassay test media (see section 8.2.4).

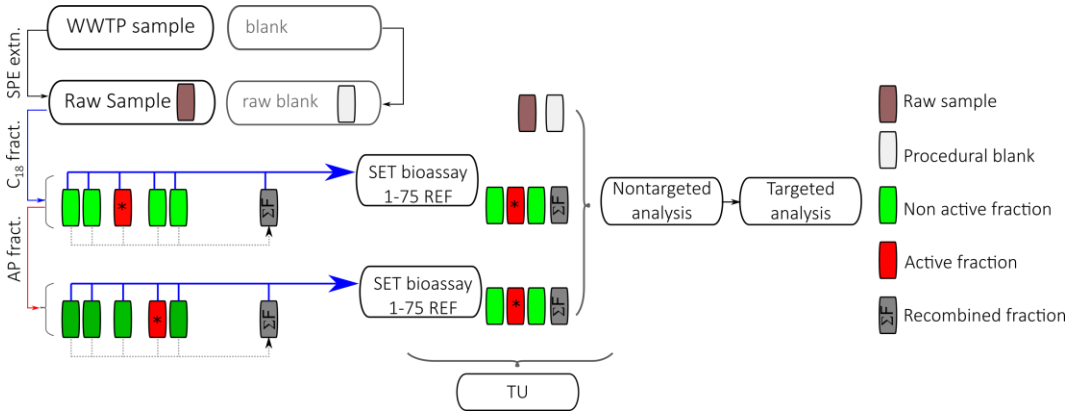

Figure 8.3. *Schematic representation of the experimental design of the EDA approach. ΣF, recombined fractions; AP fract., fractionation with aminopropyl column; C_{18} fract., fractionation with C_{18} column; REF, relative enrichment factor; SET, sea urchin embryo toxicity; SPE, solid phase extraction; TU toxic units.*

8.2.3.1 Fractionation

The extracts were fractionated by semi-preparative reverse phase liquid chromatography. The HPLC was operated under the control of Chromeleon 6.7 (Dionex) software and was comprised of a Rheodyne manual valve, a Varian Prostar 210 Pump and a Foxy 2000 fraction collector (Teledyne Isco Inc.Lincon USA). A Dionex UVD 340U UV/VIS detector was used for the recording of chromatograms at 210 nm and 254 nm.

The sequential fractionation was performed combining two different columns with an orthogonal selectivity (Muschket et al., 2018): a reverse phase C_{18} column (Macherey-Nagel Nucleodur C_{18} column, 250 x 10 mm, 5 µm particle size) and an aminopropyl column (AP, Imtakt, 150 x 10 mm, 3 µm particle size) using a gradient elution with water and MeOH, both containing 0.1% of formic acid, at a flow rate of 2.36 mL/min. In the first fractionation, the gradient started at

30% of MeOH, held for 5 min, linearly increasing to 95% of MeOH within 30 min and maintained for the next 15 min before returning back to the initial conditions for 15 min re-equilibration. In total, 18 fractions (F1-F18) of two minute intervals were collected followed by two fractions of three minutes (F19-F20) and a last fraction (F21) of 8 minutes (see **Table 8.3**). In the second fractionation, the gradient started at 5% of MeOH, held for 2 min, linearly increasing to 95% MeOH within 32 min and maintained for the next 10 min before returning to the initial conditions for 20 min re-equilibration. 15 fractions (F13-1-F13-15) of three minute intervals were collected.

Fractionation blanks (FB_{C18} and FB_{AP}) were obtained and processed prior to the sample fractionation. The recombined fraction mixtures (R_{C18} and R_{AP}) were constituted from equal volumes of all 21 and 15 fractions collected, respectively, and processed in the same way as the individual fractions.

Aliquots of 500 µL of the 10000 fold enriched extract were injected at each run and the resulting fractions from each of the 12 injections were combined. In order to remove the water that hampers the evaporation of the extracts (see **Table 8.3**), the fractions, blanks (FB_{C18} and FB_{AP}) and recombined mixtures (R_{C18} and R_{AP}) were first diluted with LC-MS grade water to less than 5% of MeOH (Hashmi et al., 2018) and then re-extracted with SPE on Plexa:Strata-X-AW (70:30, m:m, conditioned with 12.5 mL of LC-MS grade acetone, ethyl acetate, MeOH and 25 mL of LC-MS grade water) . The loaded cartridges were dried and eluted with 9 mL of MeOH:EtOAc (1:1, v:v) and 6 mL of MeOH containing 2% (v/v) 7N ammonia in MeOH (Supelco, Bellefonte, PA, USA). The extracts were neutralised with formic acid and evaporated to dryness under a gentle stream of nitrogen at 35°C. The final extract was split in two fractions: one part was reconstituted in 200 µL of MeOH: Milli-Q water (15:85, v/v) 1000 fold enriched for the chemical analysis (see **section 8.2.5**) and the other was re-dissolved in pure MeOH, stored at -40°C and then submitted to the sea urchin embryo test (see **section 8.2.4**).

Additionally, the recovery of the whole procedure (extraction with SPE and fractionation) was assesed with a synthetic mixture containing 216 micropollutants (see **Table 8.1**) including several classes of environmentally relevant compounds. The set of compounds (each at 500 ng/mL) was submitted to each fractionation procedure using the same elution program explained above and the

resulting fractions were analysed by LC-HRMS (see section 8.2.5).

Table 8.3. Fraction names, elution time windows and water content of the resulting fractions after the consecutive fractionation performed with two columns (Nucleodur C_{18} gravity and Imtakt aminopropyl).

Fraction	Fractionation approaches					
	1st fractionation step Nucleodur C_{18} Gravity column			2nd fractionation step Imtakt aminopropyl column		
	Name	Fraction t_R [a] (min)	Water content (%)	Name	Fraction t_R [a] (min)	Water content (%)
1	F1-1	0-2	70	F13-1	0-3	92
2	F1-2	2-4	70	F13-2	3-6	84
3	F1-3	4-6	68	F13-3	6-9	77
4	F1-4	6-8	64	F13-4	9-12	69
5	F1-5	8-10	60	F13-5	12-15	61
6	F1-6	10-12	56	F13-6	15-18	53
7	F1-7	12-14	52	F13-7	18-21	45
8	F1-8	14-16	48	F13-8	21-24	37
9	F1-9	16-18	44	F13-9	24-27	29
10	F1-10	18-20	40	F13-10	27-30	21
11	F1-11	20-22	36	F13-11	30-33	13
12	F1-12	22-24	32	F13-12	33-36	6
13	F1-13	24-26	28	F13-13	36-39	5
14	F1-14	26-28	24	F13-14	39-42	5
15	F1-15	28-30	20	F13-15	42-45	5
16	F1-16	30-32	16			
17	F1-17	32-34	12			
18	F1-18	34-36	10			
19	F1-19	36-39	5			
20	F1-20	39-42	5			
21	F1-21	42-50	5			

[a] The fractions collector was started with a delay of 4 min.

8.2.4 Sea-urchin embryo test (SET)

Adults of sea urchins (*P lividus*) were provided by the ECIMAT (Galicia, Spain) or collected from an intertidal area of Armintza (43.43347N, 2.89889W, Basque Country) and maintained in aquaria at the Plentzia Marine Station (PiE). Seawater tanks were maintained at 15±1°C and natural photoperiod. Every two days sea urchins were fed with macroalgae and dregs were siphoned.

Gametes were obtained by osmotic-shock-induced spawning injecting 1 mL of potassium chloride (KCl, 0.5 mol/L) through the peri-oral membrane into coelom (Carballeira et al., 2012). Afterwards, gametes were observed under a microscope to check their viability (eggs roundness and sperm mobility) and the viable ones were pooled.

The fertilisation procedure was carried out as described by Fernández and Beiras (Fernández and Beiras, 2001). A dense suspension of oocytes in control FSW was fertilised with a few µL of non-diluted sperm. 20 µL-aliquots (n=4) were taken to record fertilisation success (assessed by the percentage of eggs showing a fertilisation membrane, see **Figure 8.2a**) and egg density through an inverted microscope (Nikon eclipse Ti-S). Eggs were counted using a Sedgewick-rafter counting cell (Pyser Optics, Edenbridge, United Kingdom). Within 30 minutes, the fertilised egg suspension was distributed in glass vials (20 mL) containing a known volume of test sample (3 mL), assuring a final concentration of 40 eggs/mL.

In parallel, the methanolic solutions obtained from the extraction and fractionation (see **sections 8.2.2** and **8.2.3.1**) were concentrated to dryness under a gentle stream of nitrogen at 35°C and redissolved with 3 mL of filtered seawater (0.2 µm, FSW) containing 0.1% of DMSO, (v/v). In order to perform the dose-response curve, two different concentration ranges were used: REF 0.05-75 (3 mL, n=3) for the analysis of toxicity in the effluents and REF 1-75 (3 mL, n=3) for the EDA approach. Afterwards, the samples were placed in an incubator at 20°C for 48 h in darkness until larvae reach the four arm-pluteus stage (see **Figure 8.2l**). After the incubation, larvae were preserved by adding a one drop per sample of 40% formalin.

The quantitative assessment of the toxic effects was evaluated by measuring two different sublethal points: the index of toxicity (IT) account for the skeleton malformations and by measuring the growth inhibition or size increase (SI) of the larvae.

For the calculation of the IT, 100 individual embryos were categorised for their level of malformation according to Carballeira et al. (Carballeira et al., 2012) (see **Figure 8.4**). Normal larvae or Level 0 correspond to larvae at four arm-pluteus stage with fully developed arms, complete skeletal rods and of similar size to control larvae. Level 1 toxicity (slightly toxic) was characterised by larvae presenting an incorrect arrangement of skeletal rods (crossed tip, separated tip, fused arms

and incomplete skeletal rods). Level 2 (moderate toxicity) was featured by larvae with no skeleton or in which skeletal rods were absent or incomplete, or anomalous shape. Level 3 toxicity (highly toxic) was characterised by the blockage of development at early stages and larvae that did not reach the pluteus stage. Then, the general index of toxicity (IT) was calculated according to **Equation 8.1**.

$$IT = \frac{(0 \text{ x } \%\text{Level } 0)+(1 \text{ xLevel } 1)+(2 \text{ x } \%\text{Level } 2)+(3 \text{ x } \%\text{Level } 3)}{100} \quad \text{(Equation 8.1)}$$

where IT ranges from no toxicity (IT=0) to highly toxic (IT=3).

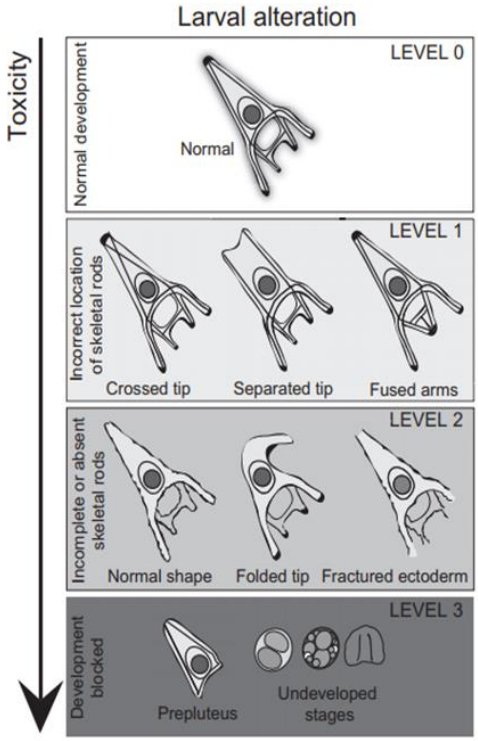

***Figure 8.4**. Classification of larval malformations according to their degree of alteration in order to establish the severity of toxicity proposed by Carballeira et al.* (Carballeira et al., 2012).

The growth inhibition was recorded according to Saco-Álvarez (Saco-Álvarez et al., 2010). The maximum dimension of 35 early embryos (either normal or abnormal, see **Figure 8.5a-e**) was measured and the size increase was calculated by subtracting the fertilised egg diameter at t=0 (fertilised egg were fixed once the initial size was measured).

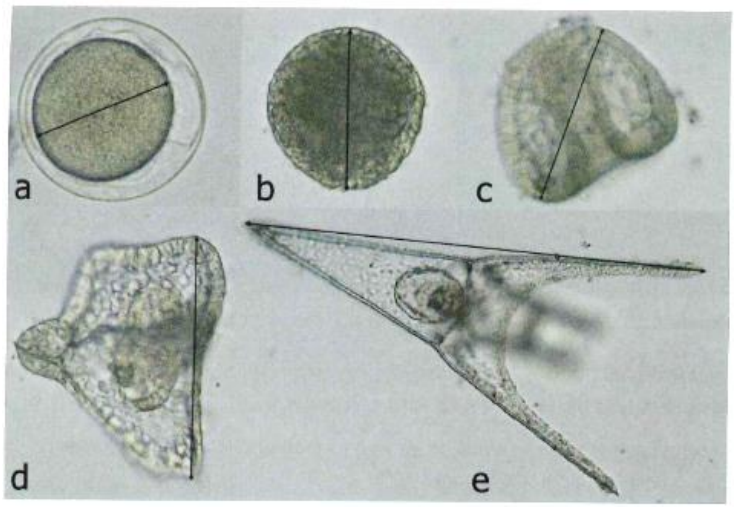

Figure 8.5*. Examples of how to measure the maximum dimension according to Saco-Álvarez (Saco-Álvarez et al., 2010) in P. Lividus at different stages: (a) fertilised eggs, (b) morulae, (c) gastrule (d) prism larve, and (e) 4 arms pluteus larvae. In case of 4 arms pluteus larvae, either in the case of normal or abnormal larvae the distance is always measured as the distance between the apex and the end of the post oral arm.*

As quality control tests, four different control samples (n=3) were included: i) eggs (fertilised eggs development was blocked just after fertilisation), ii) FSW, iii) solvent control (FSW with DMSO at 0.1% v/v) and iv) procedural blank control. Procedural blanks were processed in parallel to the effluent samples and fractions. A test was acceptable when the mean size of all the controls respect to the egg exceeds in 218 µm (in the case of larvae growth rate) or the length of control larvae was >340 µm (in the case of IT criterion) (Saco-Álvarez et al., 2010). Water quality was also measured at the beginning and at the end of the bioassay to ensure acceptability of incubation (temperature

20°C, salinity >32%, dissolved oxygen >5 mg/L, pH >7 and ammonia <40 µg/L (NOEC 40 µg/L) (Saco-Álvarez et al., 2010)). Additionally, to assure the accuracy of the test, copper (Cu) solutions (0-1000 µg/L) were used as quality positive control samples (Beiras et al., 2003).

All statistical analyses were performed with the SPSS Statistics 23 package (v17, IBM SPSS), using data corrected by the control response. To test the normal distribution of the data, a normality analysis was conducted using the Shapiro–Wilk test and non-normal data were modified with an angular transformation ($SI' = \arcsin SI^{0.5}$). The EC_i values (EC_{50} and EC_{10}) with 95% confidence limits were calculated by the probit model. Sizes measuring and images were taken with NIS-Elements Imaging Software v4.30 (Nikon Instruments BV, Europe).

8.2.5. LC-HRMS analysis

Raw effluent sample extract (without fractionation) from Ga2, the fractions showing a significant toxicity in the SET and the non-toxic neighbour fractions were analysed by LC-HRMS. Non-targeted analysis was performed in a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Thermo Scientific Q Exactive quadrupole-Orbitrap mass spectrometer equipped with a heated ESI source (HESI, Thermo, CA, USA).

The separation was carried out at 0.3 mL/min and 35°C of flow rate and temperature, respectively, on an ACE UltraCore 2.5 SuperPhenylhexyl (2.1 mmx 100 mm, 2.5 µm) column coupled to a pre filter (Vivi Jour, Schenkon, Switzerland) from Waters (Milford, Massachusetts, United States). Milli-Q water was used as mobile phase A and ACN as mobile phase B, both containing 0.1% formic acid. The injection volume was set to 5 µL. The eluent gradient profile was as follows: linear change of 85% A to 70% up to 4 min, another linear change to 50% A up to 4 min (hold 12 min), another linear change to 10% A up to 10 min (hold 15 min) and a final linear change to 85% A up to 3 min. Lastly, 5 min to regain initial conditions.

The Orbitrap was operated in the corresponding ionisation mode in full scan – data dependant MS/MS (Full MS-$ddMS^2$) discovery acquisition mode. One full scan at a resolution of 70,000 full width at half maximum (FWHM) at m/z 200 over a scan range of m/z 70-1000 was followed by three $ddMS^2$ scans at a resolution of 17,500 FWHM at m/z 200, with an isolation window of 0.8 Da. The stepped normalised collision energy (NCE) in the higher-energy collisional

dissociation (HCD) cell was set to 10, 35 and 75 eV. Negative and positive voltages were measured in different injections runs. The HESI source parameters in positive mode were set to 3.2 kV spray voltage, 300°C capillary temperature, 35 arbitrary units (au) sheath gas (nitrogen), 10 au auxiliary gas, 1 au sweep gas, 280°C auxiliary gas heater and S-lens RF level 55.0. The HESI source parameters in negative mode were set to 3.2 kV spray voltage, 330°C capillary temperature, 48 au sheath gas, 11 au auxiliary gas, 2 au sweep gas, 310°C auxiliary gas heater and S-lens RF level 55.0. External calibration of the instrument was conducted immediately prior to analysis using Pierce LTQ ESI Calibration Solutions (Thermo Scientific, Waltham, Massachusetts, United States). The instrument was controlled by Xcalibur 4.0 software (Thermo).

Data analysis was done using Compound Discoverer 2.1 (CD; Thermo-Fisher Scientific). The workflow (see **Figure II**) and settings (see **Table XXIII**) used for the data analysis with the CD are summarised in **Appendix**. Briefly, peak picking and peak alignment were performed with a retention time deviation of 0.5 min, a mass tolerance of 5 ppm and a signal higher than $5 \cdot 10^5$. The m/z values of the predicted compounds were searched in the peak list considering the criteria of 5 ppm for mass tolerance and 30% for the intensity tolerance for the isotope search. The peaks that fulfilled both criteria were manually checked and only those with available MS^2 spectra, a maximum of 10 background contamination to sample ratio and resembling Lorentzian or Gaussian peak shape, were further considered. Structural assignments were carried out based on $ddMS^2$ fragments annotated by Compound Discoverer. Afterwards, we compared the exact mass, isotope pattern, MS^2 fragmentation and abundances of the selected features with those available in the mzCloud (best match >70%) library. When the substance was not available in the mzCloud library, experimental fragmentation pattern was compared against *in silico* fragmentation obtained in MetFrag (https://msbi.ipb-halle.de/MetFragBeta/). Besides, plausible candidates were selected based on the number of references in *ChemSpider* as an indicator of human use and commercial importance.

From this step, only the peaks with an intensity ratio 4 times higher in the active (toxic) fractions compared to neighbouring inactive ones were considered. Since the C_{18} column is expected to separate complex mixtures according to hydrophobicity (Brack et al., 2016), retention time and Log $D_{(pH=3)}$ values were used as criteria for candidate selection based on log D calibration of the C_{18} chromatographic system, with the synthetic mixture (500 ng/mL) of 216 micropollutants (see

Table 8.1). Besides, the analysis of the compounds presence in each fractions allowed estimating the interval of retention times. Lastly, tentatively identified mixture components were confirmed with neat standards using retention times and MS/MS spectra.

8.2.6. Chemical and effect confirmation

TUs were calculated according to the following equations:

$$TU_{chem} = \frac{C_i}{EC_{50(i)}} \qquad \text{Equation 8.2}$$

$$TU_{bio} = \frac{1}{EC_{50(sample)}} \qquad \text{Equation 8.3}$$

Where C_i is the concentration of a compound *i* in the sample extract or fraction, the $EC_{50\ (sample)}$ is the 50% effect concentration of the sample expressed as REF and $EC_{50\ (i)}$ is the 50% effect concentration of the target compound i.

The determination of the concentrations was carried out in the TraceFinder 4.1 software (Thermo). EC_{50} values were calculated by recording the modelling dose-response curves. Stock solutions were made up by dissolving standards in non-toxic DMSO approximately 2 hours before the beginning of the experiment. The experimental concentrations were obtained by diluting the stock solutions in FSW maintaining a final DMSO concentration in the exposure vessels lower than 0.1% (v/v). Dose range concentrations were chosen on the basis of their measured concentrations in the extracts and their water solubility.

8.3. Results and discussion

8.3.1. Effluent toxicity evaluation

The procedural blanks did not induce any effect with the tested endpoints below the maximum concentration level (REF 75) and all the extracts showed embryo growth or size increase inhibition and skeleton malformation activity within the concentration range tested, which allowed us to calculate EC_{50} values.

The modelled dose-response curves are shown in Figure 8.6a-b and the EC_{10} and EC_{50} values

determinated are summarised in **Table 8.4. Figure 8.7a-i** shows representative malformations observed for the tested effluents in this work.

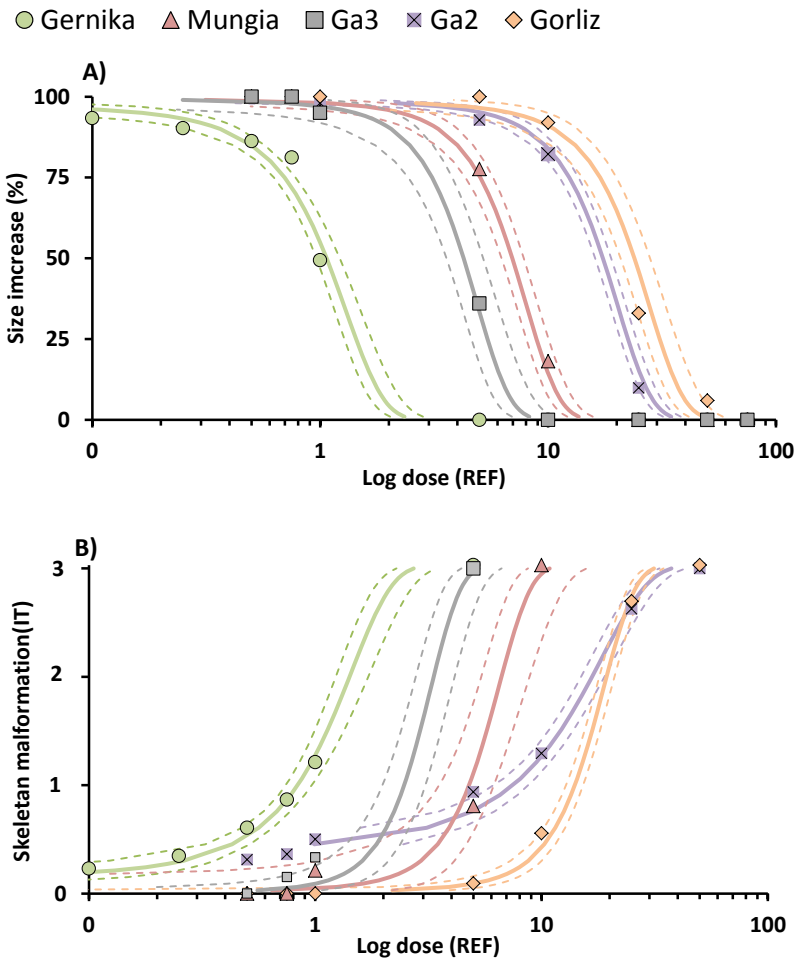

***Figure 8.6**. The log dose-response curves of the tested effluents samples (Gernika, Mungia, Ga2, Ga3 and Gorliz) obtained with a) size increase endpoint and b) skeleton malformation endpoint. Continuous lines show the EC fit values obtained with probit model and dashed lines indicate the confidence level (95%).*

Table 8.4. EC_{10} and EC_{50} obtained with both endpoints (larvae malformations, IT, and size increase, SI) along with their confidence limits (95%) and the dose concentration range (expressed as relative enrichment factor, REF) used for each sample.

	Sample	Dose range (REF)	Effective concentrations (EC_i) (Confidence limits)			
			Skeleton malformation		Size increase	
			$EC_{10\text{-}IT}$	$EC_{50\text{-}IT}$	$EC_{10\text{-}SI}$	$EC_{50\text{-}SI}$
Effluent screening	Gernika	0.05-75	0.3 (0.1-0.4)	1.1 (1.0-1.4)	0.36 (0.26-0.44)	1.1 (1.0-1.2)
	Mungia	0.05-75	2.9 (1.0-4.1)	5.7 (4.6-7.5)	3.3 (2.5-4.0)	7.0 (6.2-7.8)
	Gorliz	0.05-75	8.8 (7.3-10.0)	16.8 (15.5-18.2)	10.6 (7.5-13-5)	23.9 (20.8-28.0)
	Ga2	0.05-75	<0.05	12.2 (10.8-13.9)	7.9 (6.6-9.1)	17.4 (16.1-18.9)
	Ga3	0.05-75	1.6 (1.1-2.1)	2.9 (2.4-3.6)	2.1 (1.2-2.7)	4.3 (4.3-5.2)

The effluent of Gernika WWTP was identified as the most toxic one followed by Ga3 ($EC_{50\text{-}SI}$=1.1 REF and 4.3, respectively) and the effluents with the secondary treatment ($EC_{50\text{-}SI}$=7.0, 17.4 and 23.9 for Mungia, Galindo and Gorliz, respectively). These investigations revealed a 6-23 times higher bioactivity of the effluent of the Gernika WWTP effluent compared to the other two effluents after secondary treatments. An EC_{10} values 0.4 and 0.3 REF indicate significant effects even in diluted samples and thus this effect might be of highly concern, even considering the tidal dilution of the discharge in the estuary (as discussed in **Chapter 7**) (Mijangos et al., 2018b).

Effluents from Galindo (Ga2 and Ga3) exhibited two different patterns regarding the selected endpoints. Ga2 showed a lower $EC_{10\text{-}IT}$ value for larvae development compared to Ga3 (<0.05 vs 1.6, see **Table 8.4**), while growth or size increase was inhibited at lower concentrations by Ga3 ($EC_{50\text{-}SI}$ 2.1 vs 7.9). Even though larvae treated with Ga2 reached the 4 arm pluteous stage at any dose concentration lower than REF 50, a high number of crossed tip malformations (level 1) were observed even at low REF values (see **Figure 8.7b**). This fact would suggest a slightly different susceptibility of both endpoints to the concentration of complex mixtures with malformations being more sensitive than the growth inhibition or size increase at low concentrations. Enhanced growth inhibition at Ga3 might be driven by chlorination by-products formed during the treatment (Fernández et al., 2008).

Overall, the $EC_{10\text{-}IT}$ values obtained in this study are higher than those reported by (Carballeira et al., 2012) for different effluents of marine fish farms without any preconcentration/clean-up treatment. However, they reported similar malformations.

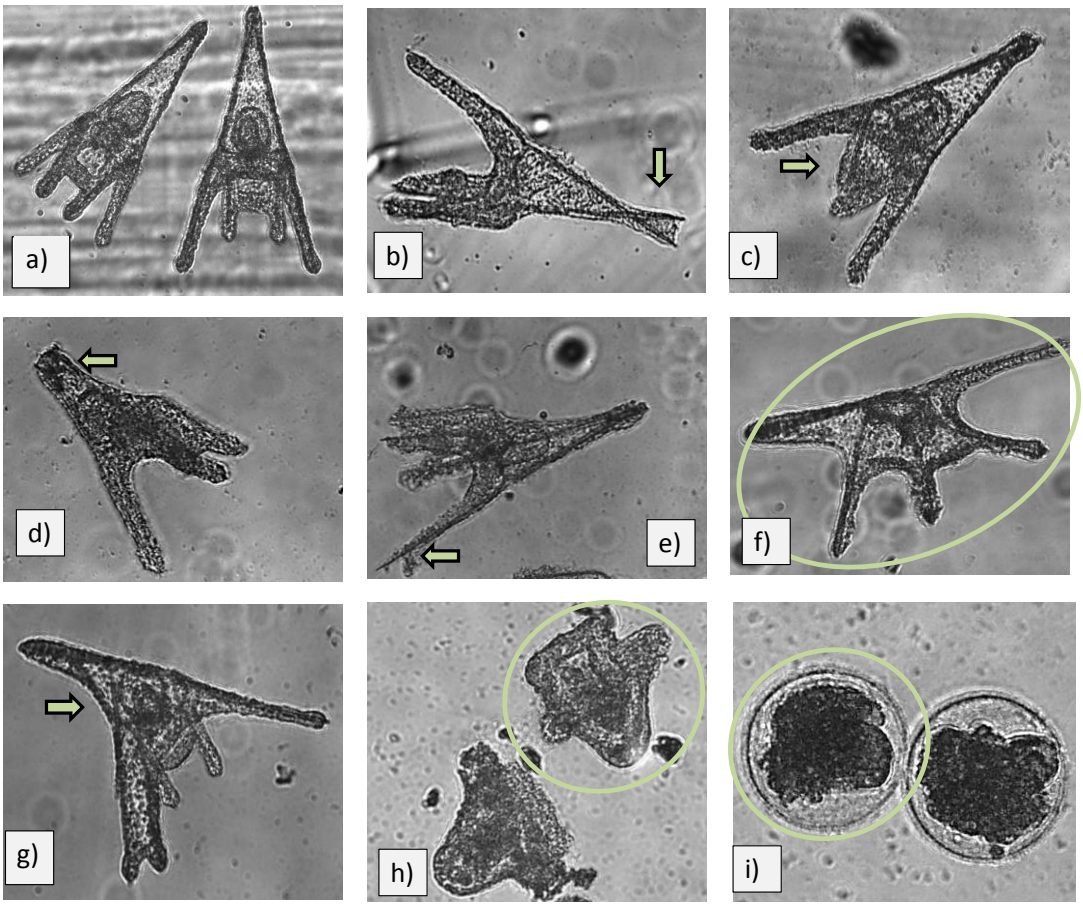

Figure 8.7. *Types of embryonic stages and abnormalities of Paracentrotus lividus. a) normal 4 arm pluteus stage (level 0); b) crossed tip (level 1); c) fused arms (level 1); d) separated tip (level 1); e) incomplete skeletal rods (level 2); f) absence of skeletal rods (level 2); g) folded tip (level 2); h) pre-pluteus stage; i) undeveloped stage.*

8.3.2. Identification of active fractions

The effluent extract from the secondary treatment of Galindo (Ga2) was selected to demonstrate the power of SET-based EDA to identify drivers of sea urchin toxicity. SET of the tested extract exhibited monotonic dose–response curves with REF 75 causing full inhibition (100%) and 17.2 REF and 19.2 for $EC_{50\text{-}IT}$ and $EC_{50\text{-}SI}$, respectively, indicating no significant difference in sensitivity between skeleton malformation and growth. Procedural blanks (FB_{C18} and FB_{AP}) did not induce any effect on the tested endpoints up to REF 75.

As can be seen in **Figure 8.8**, in the first fractionation step only fraction 13 (F13) showed a remarkable toxicity. In order to further reduce complexity the active primary fraction F13 was separated into 15 sub-fractions using the AP column. In this second fractionation, embryo growth or size increase inhibition was only observed in two fractions, namely F13-4 and F13-5. However, this last fraction (F13-5) was not further considered due to its relatively low bioactivity.

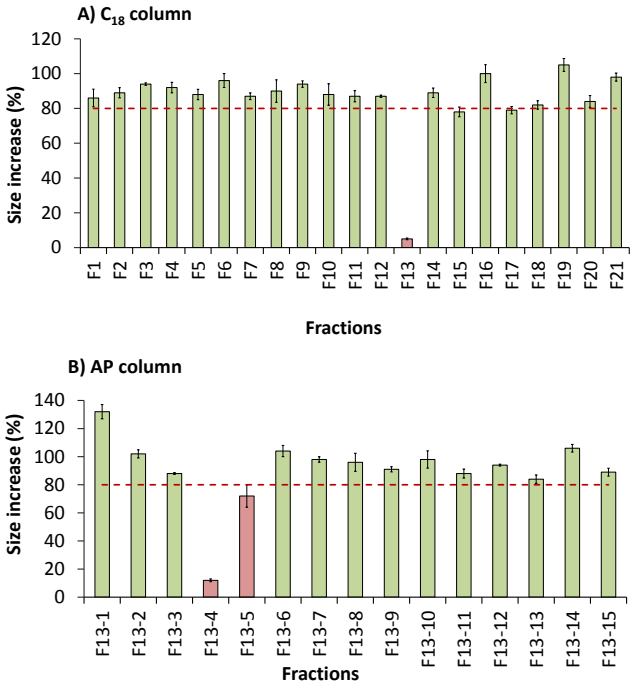

Figure 8.8. *Size increase (%) response of the fractions obtained with a) C_{18} column b) aminopropyl (AP) column. Red bars represent the identified active fractions. All the fractions are at REF 75.*

The modelled dose-response curve are shown in **Figure 8.9a-b** and the determined EC_{10} and EC_{50} values of the identified active samples are summarised in **Table 8.5.**

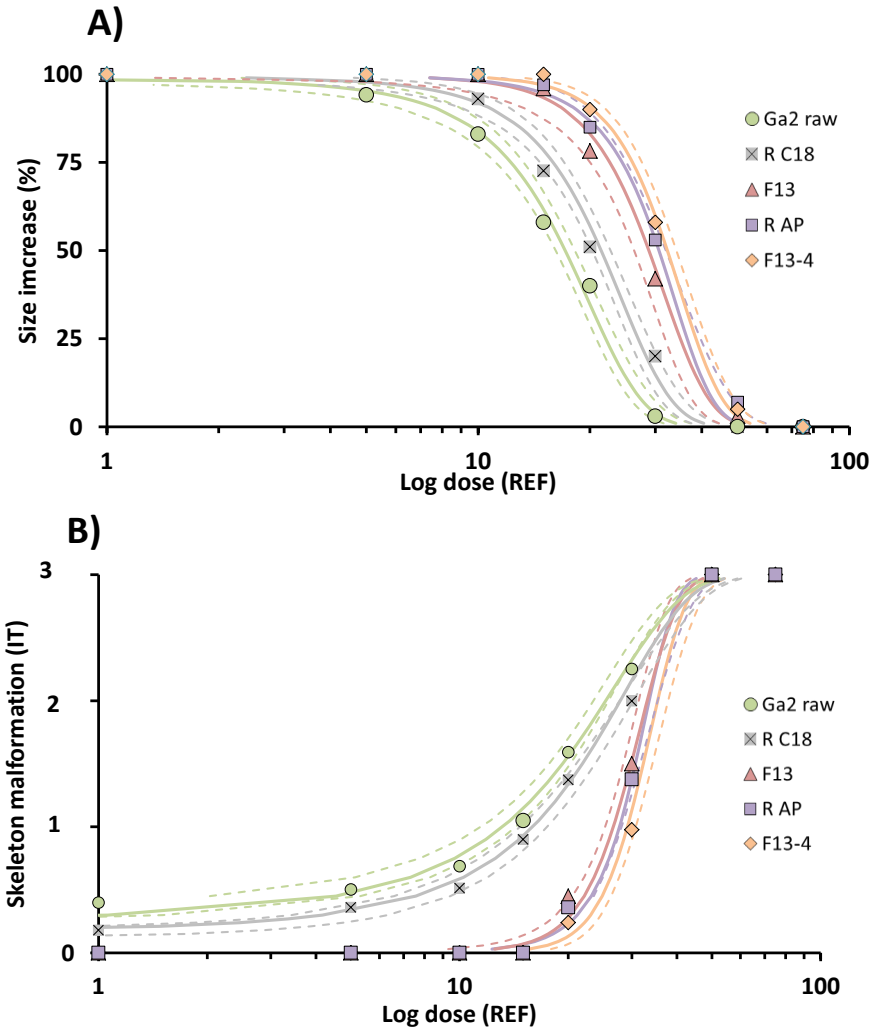

Figure 8.9. *The log dose-response curve of the active samples (Ga2 raw, R_{C18}, F13, R_{AP} and F13-4) obtained with a) size increase endpoint and b) Skeleton malformation endpoint. Straight lines show the EC fit values obtained with probit and dashed line the confidence level (95%).*

Table 8.5. EC_{10} and EC_{50} obtained with both endpoints (larvae malformations, IT, and size increase, SI) with their 95% confidence limits and the dose concentration range (expressed as relative enrichment factor, REF) used for each sample.

	Sample	Dose range (REF)	Effective concentrations (EC_i) (Confidence limits)			
			Skeleton malformation		Size increase	
			$EC_{10\text{-}IT}$	$EC_{50\text{-}IT}$	$EC_{10\text{-}SI}$	$EC_{50\text{-}SI}$
EDA approach	RAW	1-75	<1	19.3 (17.7-21.0)	7.8 (6.3-9.1)	17.2 (16.1-18.2)
	R_{C18}	1-75	4.2 (1.5-6.3)	21.9 (20.3-23.7)	10.9 (9.0-12.3)	21.4 (20.3-22.7)
	F13	1-75	20.3 (18.5-21.8)	29.8 (28.4-31.6)	17.0 (13.5-19.6)	28.8 (26.3-32.0)
	R_{AP}	1-75	24.2 (22.3-25.9)	31.1 (28.9-32.6)	21.6 (20.3-24.9)	26.2 (23.1-29.3)
	F13-4	1-75	23.1 (21.1-24.6)	32.7 (30.7-34.2)	20.4 (18.4-22.1)	32.3 (30.7-34.2)

The biological activities of the recombined primary fractions (R_{C18}) and of the raw sample were identical in a window of ±20% confirming the excellent recovery of the fractionation procedure. The latter has been confirmed chemically with the mixture of 216 standard compounds (acceptable recoveries from 53% to 89%) were obtained for most of the tested compounds, see Table XXIV in Appendix). Since about 75% of the EC_{50} of the raw extract were recovered in F13, the rest is probably distributed over the other fractions without getting significant in any of them. Besides, more than 90% of EC_{50} value of F13 for skeleton malformation and 86% for growth inhibition could be recovered in F13.4. This indicates only minor contributions of the other secondary fractions to the activity of F13.

Interestingly, on the basis of $EC_{10\text{-}IT}$ much lower values are observed for raw extract and R_{C18} than in the F13 active fractions (<1-4.2 vs 20.3 REF), indicating that slightly increased skeleton malformations might be already induced at very low concentrations of the complex mixture even if this effect cannot be recovered in the fractions. This is also evident from the reduced slope of the dose-response curves for raw extract and R_{C18} and skeleton malformation (see Figure 8.9b). With no other complementary information, it would be feasible to include the possibility of synergic effects of the different mixtures, i.e. part of the effects are seen in raw and recombined fractions were

missed in the toxic fraction and in the second fractionation. This toxicity distribution has already been reported in other EDA works (Brack et al., 2016). For instance, Hashmi et al. (Hashmi et al., 2018) observed a similar effect when they evaluated the oxidative stress response in Danube river.

8.3.3. Non-targeted analysis

The toxic fractions (F13 and F13-4), the neighbouring non-toxic fractions, the recombined fractions and the raw and blank samples were analysed in order to identify the most likely toxic candidates.

More than 15,000 features (in both positive and negative ionisation modes) were detected in the raw sample. Among them, 49 could be identified (Level 1), 67 tentatively identified as probable structures (Level 2a) and 59 as tentative candidates (Level 3), according to Schymanski classification (Schymanski et al., 2015) (**see Table 8.6**).

The list of feasible features present in the raw sample was drastically reduced when the restriction to be found in the two toxic fractions was introduced, i.e. maintaining only the features with peak intensity at least 4 times higher in the active fractions (F13 and F13-4) than in the neighbouring non-active fractions and focusing on retention time's windows of 4.5-7.5 min (see **Table XXIV** in **Appendix**). Lastly, the pre-calibrated C_{18} fractionation step (C_{18} vs. log $D_{(pH=3)}$, r^2=0.89) showed that the F13 fraction may content chemicals with Log $D_{(pH=3)}$ in the range of 1.27-2.49 (see **Table XXIV** in **Appendix**).

Table 8.6. Retention time (RT), ionisation, molecular weight, formula, name and uses of the compounds classified as level 1 or level 2a according to Schymanski criteria (Schymanski et al., 2015) with a mzCloud best match >70% and a maximum error of 5 ppms. Compounds classified as level 3 due to isomerisation (cis-trans) or enantiomers (R, S) were also included.

#	RT (min)	Ionisation	Molecular Weight	Formula	Name	Level	Use
1	0.7	Positive	129.10149	$C_4H_{11}N_5$	Metformin	2a	Hypoglycemic
2	0.8	Positive	140.10617	$C_6H_{12}N_4$	Hexamethylenetetramine	2a	Preservative
3	0.8	Positive	266.16265	$C_{14}H_{22}N_2O_3$	Atenolol	1	Antihypertensive
4	0.8	Positive	314.14077	$C_{13}H_{22}N_4O_3$ S	Ranitidine	1	Histamine H2 Antagonists
5	0.8	Positive	292.23589	$C_{14}H_{32}N_2O_4$	Edetol	2a	Intermediate
6	0.9	Negative	163.8960	$C_4H_4NO_4S$	Acesulfame	2a	Artificial sweetener
7	1.1	Positive	303.19448	$C_{17}H_{25}N_3O_2$	Vildagliptin	2a	Antidiabetic
8	1.3	Positive	328.14525	$C_{15}H_{24}N_2O_4S$	Tiapride	2a	Antipsychotic
9	1.3	Positive	274.04082	$C_{13}H_{10}N_2O_3S$	Ensulizole	2a	Sunscreen
10	1.4	Positive	270.17284	$C_{17}H_{22}N_2O$	Doxylamine	2a	Antihistamine
11	1.5	Positive	201.03588	$C_{10}H_7N_3S$	Thiabendazole	1	Bendazole
12	1.5	Positive	151.13602	$C_{10}H_{17}N$	Amantadine	1	Antiviral
13	1.6	Positive	290.13732	$C_{14}H_{18}N_4O_3$	Trimethoprim	1	Antibiotic
14	1.7	Positive	152.09493	$C_8H_{12}N_2O$	IMHP	2a	Insecticide
15	1.7	Positive	245.11623	$C_{13}H_{15}N_3O_2$	4-Acetamidoantipyrine	2a	Analgesic
16	1.7	Positive	203.10581	$C_{11}H_{13}N_3O$	4-Aminoantipyrine	2a	Anti-inflammatory
17	1.7	Positive	194.08031	$C_8H_{10}N_4O_2$	Caffeine	1	Stimulant
18	1.7	Positive	231.10066	$C_{12}H_{13}N_3O_2$	Isocarboxazide	2a	Antidepressant
19	1.7	Positive	113.08433	$C_6H_{11}NO$	Caprolactam	2a	Flavouring agent
20	1.8	Positive	219.12587	$C_{13}H_{17}NO_2$	Alminoprofen	2a	Anti-inflammatory
21	1.8	Positive	331.13283	$C_{17}H_{18}FN_3O_3$	Ciprofloxacin	1	Antibiotic
22	1.8	Positive	234.17302	$C_{14}H_{22}N_2O$	Lidocaine	1	Anesthetics
23	1.9	Positive	361.14336	$C_{18}H_{20}FN_3O_4$	Levo/Ofloxacin	3	Antibiotic
24	1.9	Positive	237.09189	$C_{13}H_{16}ClNO$	Ketamine	2a	Stimulant
25	2.0	Positive	369.17180	$C_{17}H_{27}N_3O_4S$	Amisulpride	2a	Antipsychotic
26	2.1	Positive	249.05697	$C_{11}H_{11}N_3O_2S$	Sulfapyridine	1	Antibiotic
27	2.1	Positive	119.04854	$C_6H_5N_3$	Benzotriazole	2a	Anticorrosive
28	2.3	Positive	255.00765	$C_9H_7Cl_2N_5$	Lamotrigine	2a	Anticonvulsant
29	2.3	Positive	267.18312	$C_{15}H_{25}NO_3$	Metoprolol	1	Antihypertensive
30	2.4	Positive	263.18830	$C_{16}H_{25}NO_2$	Desvenlafaxine	2a	Antidepressant
31	2.5	Positive	188.09482	$C_{11}H_{12}N_2O$	Antipyrine	2a	Anti-inflammatory
32	2.6	Positive	221.17781	$C_{14}H_{23}NO$	Tapentadol	2a	Sedative
33	2.7	Positive	250.13171	$C_{13}H_{18}N_2O_3$	Lacosamide	2a	Anticonvulsant

Table 8.6. Retention time (RT), ionisation, molecular weight, formula, name and uses of the compounds classified as level 1 or level 2a according to Schymanski criteria (Schymanski et al., 2015) with a mzCloud best match >70% and a maximum error of 5 ppms. Compounds classified as level 3 due to isomerisation (cis-trans) or enantiomers (R, S) were also included.

#	RT (min)	Ionisation	Molecular Weight	Formula	Name	Level	Use
34	2.8	Positive	748.50754	$C_{38}H_{72}N_2O_{12}$	Azithromycin	2a	Antibacterial
35	2.8	Positive	270.10012	$C_{15}H_{14}N_2O_3$	10,11-Dihydroxycarbamazepine	3	Anticonvulsant
36	2.8	Positive	306.10369	$C_{13}H_{12}F_2N_6O$	Fluconazole	1	Antifungal
37	3.1	Negative	133.06244	$C_7H_7N_3$	4-Methylbenzotriazole	2a	Anticorrosive
38	3.2	Positive	307.04302	$C_{15}H_{14}ClNO_2S$	Clopidogrel carboxylic acid	2a	Antithrombotic
39	3.2	Positive	239.10745	$C_{13}H_{18}ClNO$	Bupropion	1	Antidepressant
40	3.3	Positive	424.17967	$C_{18}H_{33}ClN_2O_5S$	Clindamycin	2a	Antibiotic
41	3.3	Positive	325.22484	$C_{18}H_{31}NO_4$	Bisoprolol	1	Antihypertensive
42	3.4	Positive	277.20391	$C_{17}H_{27}NO_2$	Venlafaxine	2a	Antidepressant
43	3.4	Positive	254.10527	$C_{15}H_{14}N_2O_2$	10-Hydroxycarbazepine	2a	Anticonvulsant
44	3.6	Positive	182.07080	$C_6H_{15}O_4P$	Triethyl phosphate	1	Organophosphorus
45	3.6	Positive	326.12935	$C_{18}H_{19}ClN_4$	Clozapine	1	Antipsychotic
46	4.0	Positive	253.05187	$C_{10}H_{11}N_3O_3S$	Sulfamethoxazole	1	Antibiotic
47	4.0	Positive	446.20632	$C_{24}H_{26}N_6O_3$	Olmesartan	2a	Antihypertensive
48	4.1	Positive	372.15029	$C_{20}H_{24}N_2O_3S$	Deacetyl Diltiazem	2a	Antihypertensive
49	4.2	Positive	151.00910	C_7H_5NOS	OBT	1	Anticorrosive
50	4.2	Positive	222.1367	$C_{12}H_{18}N_2O_2$	Mexacarbate	2a	Insecticide
51	4.2	Positive	259.15691	$C_{16}H_{21}NO_2$	Propanolol	1	Antihypertensive
52	4.3	Positive	371.15090	$C_{19}H_{22}ClN_5O$	Trazodone	2a	Antidepressant
53	4.3	Positive	252.08963	$C_{15}H_{12}N_2O_2$	Carbamazepine epoxide	2a	Anticonvulsant
54	4.5	Positive	348.12529	$C_{16}H_{20}N_4O_3S$	Torsemide	2a	Antihypertensive
55	4.6	Negative	266.0804	$C_{14}H_{10}N_4O_2$	Valsartan acid	2a	Antihypertensive
56	4.7	Positive	252.08954	$C_{15}H_{12}N_2O_2$	Oxcarbazepine	2a	Anticonvulsant
57	4.9	Positive	733.46031	$C_{37}H_{67}NO_{13}$	Erythromycine	1	Antibacterial
58	5.0	Positive	310.14775	$C_{19}H1_9FN_2O$	Demethylcitalopram	2a	Antidepressant
59	5.0	Negative	265.08845	$C_{12}H_{15}N_3O_2S$	Albendazole	1	Antihelminthic
60	5.0	Positive	199.11084	$C_{12}H_{13}N_3$	Pyrimethanil	2a	Fungicide
61	5.2	Positive	324.16341	$C_{20}H_{21}FN_2O$	Citalopram	3	Antidepressant
62	5.2	Positive	414.13738	$C_{17}H_{20}F_6N_2O_3$	Flecainide	2a	Antiarrhythmic
63	5.3	Positive	232.15727	$C_{14}H_{20}N_2O$	Norfentanyl	2a	Sedative
64	5.3	Positive	143.13100	$C_8H_{17}NO$	Valpromide	2a	Anticonvulsant
65	5.4	Positive	414.16106	$C_{22}H_{26}N_2O_4S$	Diltiazem	2a	Antihypertensive
66	5.4	Positive	295.09547	$C_{16}H_{13}N_3O_3$	Mebendazole	1	Antihelminthic

Table 8.6. Retention time (RT), ionisation, molecular weight, formula, name and uses of the compounds classified as level 1 or level 2a according to Schymanski criteria (Schymanski et al., 2015) with a mzCloud best match >70% and a maximum error of 5 ppms. Compounds classified as level 3 due to isomerisation (cis-trans) or enantiomers (R, S) were also included.

#	RT (min)	Ionisation	Molecular Weight	Formula	Name	Level	Use
67	5.5	Positive	277.18261	$C_{20}H_{23}N$	Maprotiline	2a	Antidepressant
68	5.6	Positive	309.18376	$C_{19}H_{23}N_3O$	Benzydamine	2a	Anti-inflammatory
69	5.8	Positive	236.09469	$C_{15}H_{12}N_2O$	Carbamazepine	1	Anticonvulsant
70	5.8	Positive	329.1423	$C_{19}H_{20}FNO_3$	Paroxetine	2a	Antidepressant
71	5.8	Positive	292.09743	$C_{15}H_{17}ClN_2O_2$	Climbazole	2a	Fungicide
72	5.9	Positive	256.12087	$C_{15}H1_6N_2O_2$	Ancymidol	2a	Herbicide
73	5.9	Positive	241.13585	$C_{10}H_{19}N_5S$	Terbutryn	1	Herbicide
74	6.1	Positive	747.47635	$C_{38}H_{69}NO_{13}$	Clarithromycin	1	Antibiotic
75	6.2	Negative	330.0078	$C_{12}H_{11}ClN_2O_5S$	Furosemide	1	Diuretic
76	6.2	Positive	309.20885	$C_{21}H_{27}NO$	Methadone	2a	Stimulant
77	6.3	Positive	230.14171	$C_{14}H_{18}N_2O$	Propyphenazone	1	Anti-inflammatory
78	6.3	Positive	277.18271	$C_{20}H_{23}N$	Amitriptyline	1	Antidepressant
79	6.3	Positive	286.05054	$C_{15}H_{11}ClN_2O_2$	Oxazepam	2a	Benzodiazepine
80	6.4	Positive	388.15474	$C_{21}H_{25}ClN_2O_3$	Cetirizine/ Levocetirizine	3	Antihistaminic
81	6.4	Positive	514.23640	$C_{33}H_{30}N_4O_2$	Telmisartan	1	Antihypertensive
82	6.5	Positive	428.23194	$C_{25}H_{28}N_6O$	Irbesartan	1	Antihypertensive
83	6.6	Positive	320.01146	$C_{15}H_{10}Cl_2N_2O_2$	Lorazepam	1	Sedative
84	6.7	Positive	191.13078	$C_{12}H_{17}NO$	DEET	1	Insecticide
85	6.8	Positive	197.17781	$C_{12}H_{23}NO$	Laurolactam	2a	Intermediate
86	6.9	Positive	206.14178	$C_{12}H_{18}N_2O$	Isoproturon	1	Herbicide
87	6.9	Positive	422.16173	$C_{22}H_{23}ClN_6O$	Losartan	1	Antihypertensive
88	7.1	Positive	232.01692	$C_9H_{10}Cl_2N_2O$	Diuron	1	Herbicide
89	7.6	Positive	203.13095	$C_{13}H_{17}NO$	Crotamiton	1	Antipruritic
90	7.7	Positive	334.02693	$C_{16}H_{12}Cl_2N_2O_2$	Lormetazepam	2a	Sedative
91	7.8	Positive	389.10053	$C_{21}H_{15}N_3O_5$	Azoxystrobin acid	2a	Fungicide
92	7.8	Positive	284.07139	$C_{16}H_{13}ClN_2O$	Diazepam	1	Sedative
93	7.9	Negative	142.01703	C_7H_7ClO	Chlorocresol	2a	Fungicide
94	7.9	Negative	214.03885	$C_{10}H_{11}ClO_3$	Mecoprop	2a	Herbicide
95	8.0	Positive	361.10772	$C_{19}H_{20}ClNO_4$	Bezafibrate	1	Hypolipidemic
96	8.1	Positive	321.05870	$C_{16}H_{16}ClNO_2S$	Clopidogrel	1	Antithrombotic
97	8.2	Positive	547.23468	$C_{27}H_{37}N_3O_7S$	Darunavir	2a	Antiviral
98	8.3	Positive	323.12978	$C_{15}H_{21}N_3O_3S$	Gliclazide	2a	Hypoglycemic
99	8.3	Positive	386.17221	$C_{22}H_{26}O_6$	GENISER MD	2a	Clarifier

Table 8.6. Retention time (RT), ionisation, molecular weight, formula, name and uses of the compounds classified as level 1 or level 2a according to Schymanski criteria (Schymanski et al., 2015) with a mzCloud best match >70% and a maximum error of 5 ppms. Compounds classified as level 3 due to isomerisation (cis-trans) or enantiomers (R, S) were also included.

#	RT (min)	Ionisation	Molecular Weight	Formula	Name	Level	Use
100	8.4	Positive	148.08873	$C_{10}H_{12}O$	Anethole	3	Flavouring agent
101	8.4	Negative	435.22713	$C_{24}H_{29}N_5O_3$	Valsartan	1	Antihypertensive
102	8.8	Negative	430.06107	$C_{18}H_{14}F_4N_2O_4S$	Bicalutamide	1	Antineoplastic
103	9.1	Positive	307.14485	$C_{16}H_{22}ClN_3O$	Tebuconazole	1	Fungicide
104	9.2	Negative	276.07246	$C_{11}H_{11}F_3N_2O_3$	Flutamide	1	Antineoplastic
105	9.3	Negative	250.15658	$C_{15}H_{22}O_3$	Gemfibrozil	1	Antilipemic
106	9.5	Positive	342.03212	$C_{18}H_{12}Cl_2N_2O$	Boscalid	1	Fungicide
107	9.6	Positive	295.01621	$C_{14}H_{11}Cl_2NO_2$	Diclofenac	1	Anti-inflammatory
108	10.0	Positive	341.06957	$C_{15}H_{17}Cl_2N_3O_2$	Propiconazole	1	Fungicide
109	10.0	Positive	268.15718	$C_{17}H_{20}N_2O$	Centralite	2a	Gunshot residue
110	10.2	Positive	210.10190	$C_8H_{19}O_4P$	Dibutyl phosphate	2a	Intermediate
111	10.2	Positive	266.16425	$C_{12}H_{27}O_4P$	Tributyl Phosphate	2a	Organophosphorus
112	10.2	Positive	283.32352	$C_{19}H_{41}N$	Cetrimonium	2a	Surfactant
113	10.5	Negative	241.10985	$C_{15}H_{15}NO_2$	Mefenamic acid	1	Anti-inflammatory
114	11.3	Positive	304.10024	$C_{12}H_{21}N_2O_3PS$	Dimpylate	2a	Insecticide
115	11.8	Negative	499.93779	$C_8HF_{17}O_3S$	PFOS	1	Surfactant
116	12.2	Positive	326.07044	$C_{18}H_{15}O_4P$	Triphenyl phosphate	1	Organophosphorus
117	13.4	Positive	325.37027	$C_{22}H_{47}N$	Didecyldimethylammonium	2a	Antiseptic
118	14.3	Negative	326.19145	$C_{18}H_{30}O_3S$	4-Dodecylbenzenesulfonic acid	2a	Surfactant
119	14.7	Negative	220.18197	$C_{15}H_{24}O$	Caryophyllene oxide	3	Flavouring agent
120	14.9	Positive	312.13566	$C_{19}H_{20}O_4$	Butylbenzylphthalate	2a	Plasticiser
121	23.4	Positive	283.28688	$C_{18}H_{37}NO$	Stearamide	2a	Surfactant
122	25.1	Positive	297.30254	$C_{19}H_{39}NO$	Tridemorph	2a	Fungicide

DEET: N,N-Diethyl-meta-toluamide, GENISER MD: Bis(methylbenzylidene)sorbitol, IMHP: 2-Isopropyl-6-methyl-4-pyrimidinol, OBT: 2-hydroxyobenzothiazole.

In this sense, the initial amount of peaks detected at the raw sample was limited to nine features (see **Table 8.7**). Two of these features could be associated to tentative structures and four more were confirmed with standards, as can be seen in the MS^2 spectra shown in **Figure 8.10a-f**. The determination of the unequivocal molecular formula was not possible in the case of the remaining 3 features due to poor MS^2 spectra. The nine identified compounds include two pesticides, mexacarbate and fenpropidin and 4 pharmaceuticals, amitriptyline and paroxetine antidepressants

drugs, and mebendazole and albendazole antihelminthic agents. All these compounds were detected in all the active fractions (RAW, F13, F13-4), except fenpropidin, which could not be detected in the raw sample. We attribute this to the complex matrix of the raw sample compared to that of the individual fractions (F13, F13-4).

As an example, the identification of mexacarbate (m/z 233.1440, RT 4.2 min) is explained in detail. Only one plausible molecular formula ($C_{12}H_{18}N_2O_2$) remained after the mass accuracy (<5 ppm) and isotopic fit criteria and only two structures showed an mzCloud score above 70%: mexacarbate (a pesticide, 4-(dimethylamino)-3,5-dimethylphenyl methylcarbamate) and neostigmine (a parasympathomimetic pharmaceutical, N,N,N-trimethybenzenamino 3-(dimethylcarbomoyloxy)). The main differences between their structures arise in the position of two methyl groups. Metfrag explained the fragments found in the MS^2 spectra of both candidates: Mexacarbate explained nine out of the ten most intense fragments and neostigmine explained eight. Neostigmine could not explain the peak m/z 178.12175 (see **Figure 8.10a)** present in the spectra, and in the case of mexacarbate it was feasible by the loss of N-methylamine [$C_9H_{11}NO + H^+$], m/z 178.1227. Lastly, F13 fraction may content chemicals with Log $D_{(pH=3)}$ in the range of 1.27-2.49, and thus, neostigmine (Log $D_{(pH=3)}$=-1.6) was discarded as a possible candidate.

Table 8.7. Overview of the 9 non-targeted peaks detected in the active samples (raw, F13 and F13-4).

#	Polarity	Molecular weight	RT [min]	Formula	Compound name	Log D (pH=3)	Level	MOA	Raw concentration (µg/L)
1	$[M+H]^+$	2.221.367	4.2	$C_{12}H_{18}N_2O_2$	Mexacarbate	1.8	2a	Acetylcholinesterase inhibition	NA
2	$[M+H]^+$	2.041.513	4.4	$C_{14}H_{20}O$	Unknown		4		
3	$[M-H]^-$	2.650.885	5	$C1_2H_{15}N_3O_2S$	Albendazole	2.2	1	Mitosis, cell cycle	0.018
4	$[M-H]^+$	2.950.958	5.3	$C_{16}H_{13}N_3O_3$	Mebendazole	2.4	1	Mitosis, cell cycle	0.065
5	$[M+H]^+$	3.291.423	5.8	$C_{19}H_{20}FNO_3$	Paroxetine	1.9	2a	Serotonin reuptake inhibitors	0.009
6	$[M+H]^+$	2.771.827	6.2	$C_{20}H_{23}N$	Amitriptyline	1.3	1	Serotonin reuptake inhibitors	0.304
7	$[M+H]^+$	2.732.452	6.9	$C_{19}H_{31}N$	Fenpropidin	1.9	1	Sterol biosynthesis inhibition	0.023
8	$[M-H]^-$	3.170.343	7.5	$C_6H_{11}N_3O_{12}$	Unknown		4		
9	$[M-H]^-$	3.070.056	7.5	$C_{10}H_6ClN_7OS$	Unknown		4		

NA: not analysis due to a luck of standard.

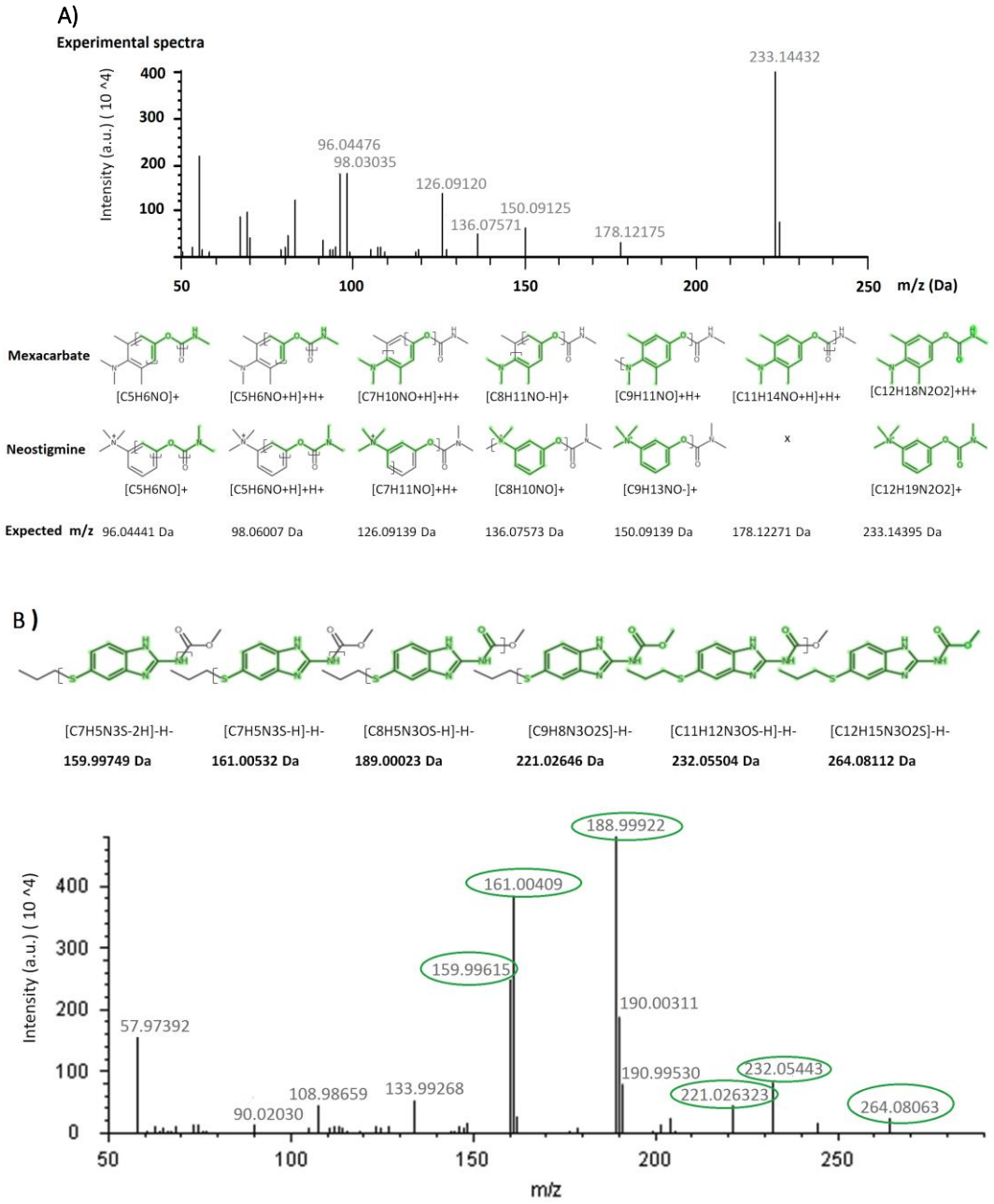

Figure 8.10. MS^2 spectra (HCD 10, 35 and 75) of a) Fragments explanation of two potential candidates (mexacarbate and neostigmine) which match with the precursor ion #1 included in **Table 8.6**, and b) albendazole. Only the major fragments have been rounded.

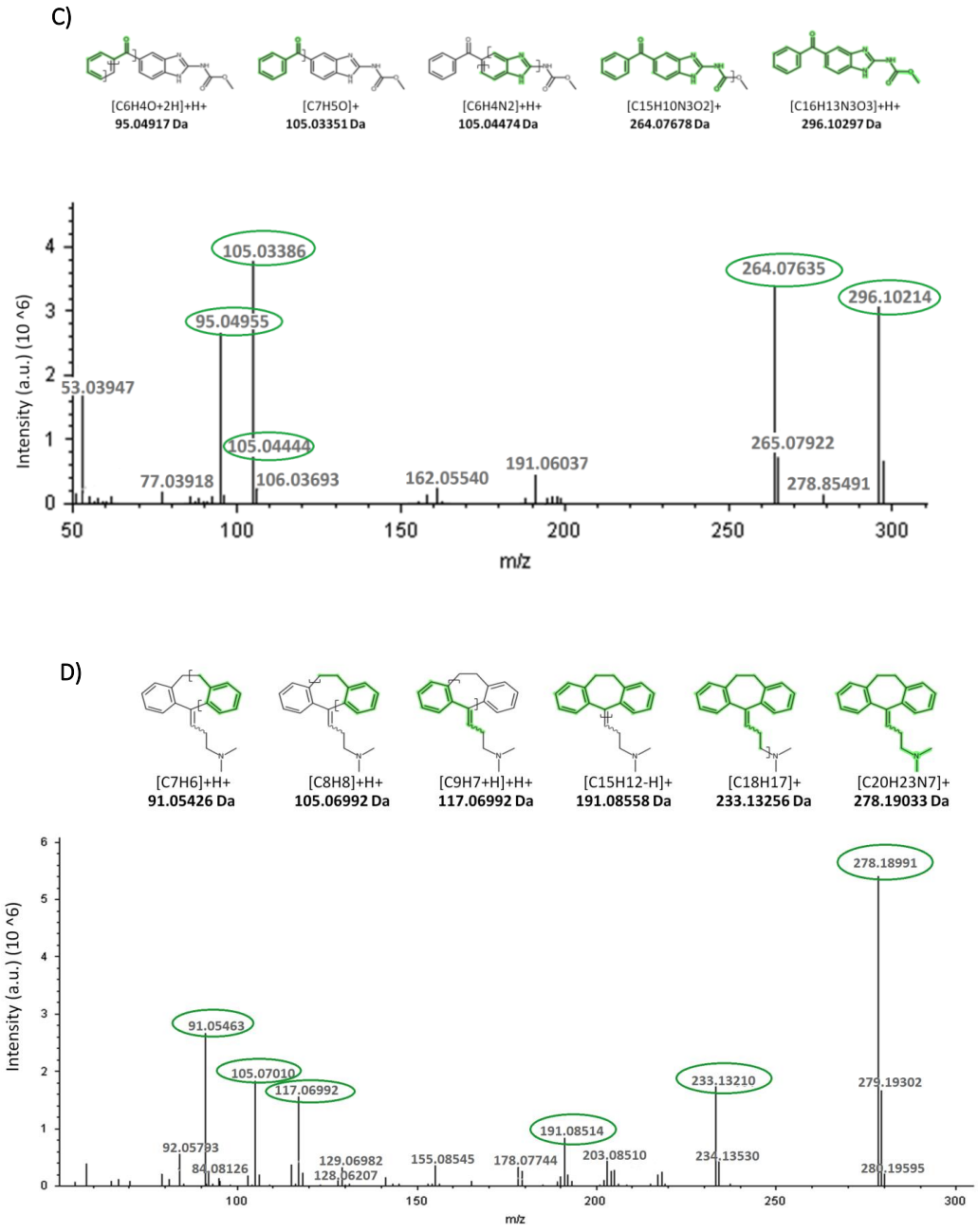

Figure 8.10. MS^2 spectra (HCD 10, 35 and 75) of c) mebendazole and d) amitriptyline. Only the major fragments have been rounded.

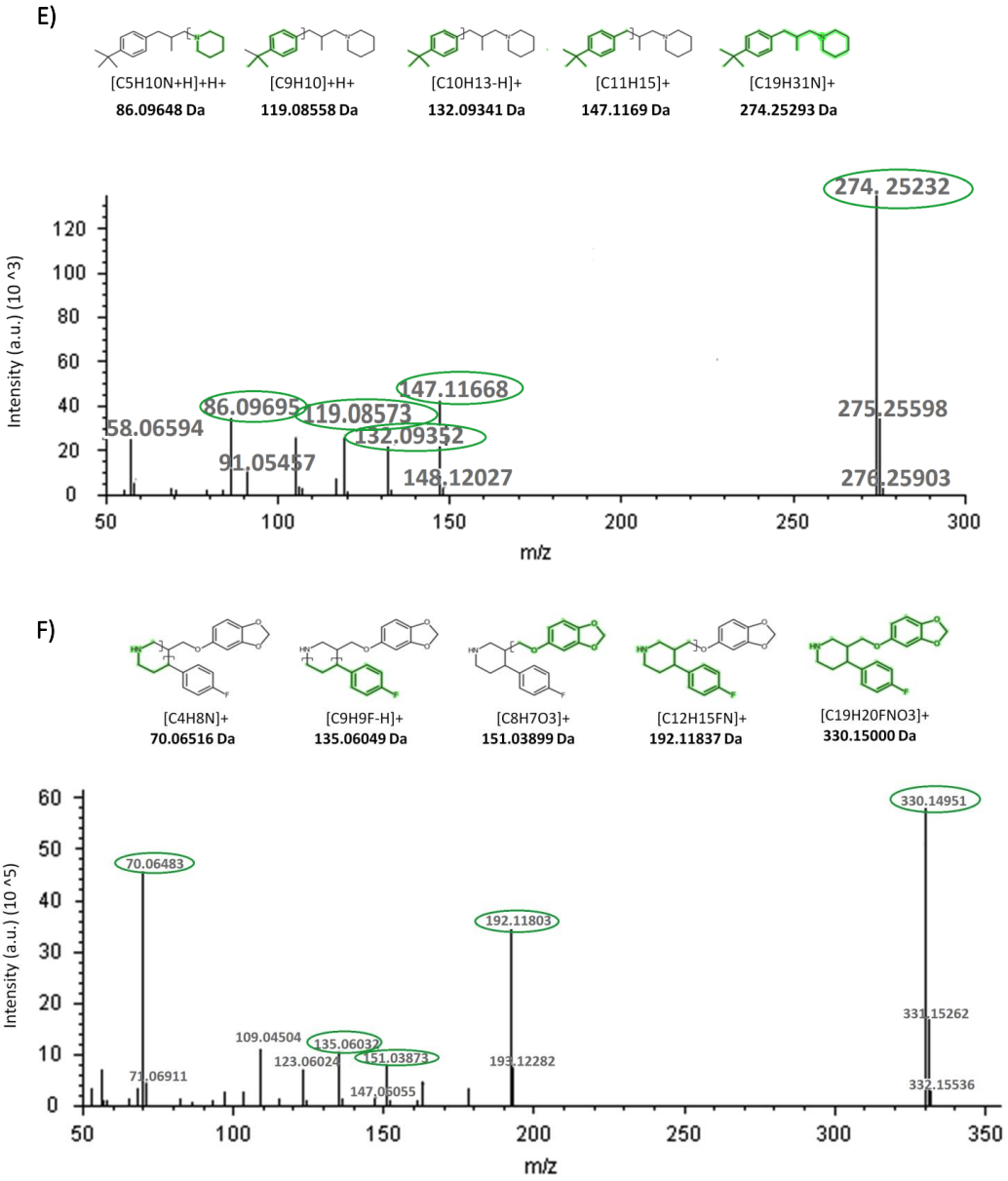

Figure 8.10. MS^2 spectra (HCD 10, 35 and 75) of e) fenpropidin and f) paroxetine. Only the major fragments have been rounded.

8.3.4. Assessment of toxicities and conclusions

The current knowledge of SET in response to individual organic chemicals is still very limited. In fact, we could not narrow down the identified list with the information available in EPA Dashboard web application (https://comptox.epa.gov/dashboard) or ecotoxicology knowledgebase (ECOTOX database, https://cfpub.epa.gov/ecotox/). Therefore, one compound for each of the MoA (see **Table 8.7**) in the toxic fraction was tested: the antihelminthic mebendazole (Ga2 effluent concentration 65 ng/L at REF=1), the anti-depressant amitriptyline (304 ng/L) and the fungicide fenpropidin (23 ng/L). They were tested for toxicity in SET with $EC_{50\text{-}SI}$ of 213, 3523 and 9653 µg/L. The comparison of the chemical and biological data using TUs showed that mebendazole was the predominant contributor (32%) followed in a less extend by amitriptyline (9%), whereas fenpropidin could only explain the 0.3% of the sea urchin embryogenesis activity in the F13-4 fraction (TU_{bio}=0.03).

The high biologically activity shown by mebendazole, which was more toxic than amitriptyline and fenpropidin is in agreement with its specific MoA. Mebendazole is a benzimidazole extensively used as an anthelmintinc agent in veterinary and human practices in order to treat parasitic infections (Akhtar et al., 2017). Adults and worm eggs are affected by depolymerisation of microtubules (Tydén et al., 2016), a process that plays an essential role in sea urchin embryos since this process is involved in many cellular processes such as cell division during early embryogenesis, intracellular transport and four arm-pluteus stage shape maintenance (Kiselyov et al., 2010; Semenova et al., 2006; Sheremetev et al., 2010). For instance, Stepanov et al. (Stepanov et al., 2015) evaluated the microtubule-destabilizing properties of a series of benzimidazole drugs and reported alterations in swimming pattern of blastulae treated after hatching. The rapid spinning of embryos around the axis suggests a microtubule destabilizing activity as it can be seen in the video available at http://www.chemblock.com/urchin.php.

The contribution (9%) of amitriptyline, with a TU_{chem}.=2.8 E-3, can be interpreted by its high effluent concentration (304 ng/L), an order of magnitude higher than that of fenpropidin (23 ng/L, TU_{chem}.=7.7 E-5) and mebendazole (65 ng/L, TU_{chem}=9.9 E-3). This neuroactive antidepressant has been reported to be toxic for crustaceans (Busch et al., 2016; Minguez et al., 2014) and, specially,

for zebrafish (Beckers et al., 2018). Among other alterations, it was demonstrated to alter the swimming behaviour and body length of Danio rerio embryo (Yang et al., 2014). However, this is the first time that the potential toxicity of amitriptyline on sea urchin embryos has been evaluated.

The share of non-explained toxicity can be attributed to paroxetine and albendazole (both of them present in the raw sample at a much lower concentration than those of amitriptyline and mebendazole), plus mexacarbate and the non-identified compounds.

Finally, the use of SET in the EDA streamline procedure was successfully implemented to study the toxicity of WWTP effluents and to identify the most toxic contaminants. The performance of the SET allowed us to measure effectively the toxicity of all the fractions reducing significantly the chemical domain of potential contaminants. In fact, the non-targeted analysis of the toxic fractions allowed us to fully identify six contaminants from nine potential candidates. In addition to this, we also determined the toxic units to estimate the contribution of the identified contaminants in the total toxicity. In this sense, mebendazole was identified as the predominant contributor followed in a less extend by amitriptyline, though it is true that we are lacking evidence due to non-tested compounds.

8.4. References

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Conclusions

It is good to have an end to journey towards;

but it is the journey that matters, in the end.

Ursula K. Le Guin

If we recall the aims and objectives of this PhD Thesis we would see that our main aim was to **get a close insight about the impact of emerging contaminants in estuarine waters and the contribution of WWTP effluents in the observed effects** and, once the work is concluded, we honestly think that the aims were satisfactorily accomplished.

In the case of the large monitoring which we performed in three estuaries and WWTP effluents we were able to determine the concentration of a wide number of emerging contaminants. These achievements are not trivial because we now have a much closer knowledge about the occurrence of emerging contaminants in our estuaries and the effluents of WWTPs and we are now able to focus the analytical efforts towards a set of contaminants that were never considered before.

Deeply, we obtained a better understanding of the temporal and spatial distribution of the 41 contaminants along the studied estuaries of the Bay of Biscay (Bilbao, Plentzia and Urdaibai). Furthermore, since we monitored the effluents with higher impact on those estuaries we were able to identify the most likely sources of contamination. In the particular case of the estuary of Bilbao, at least two independent sources were categorised, one in the effluents and the other in the harbour activities. The scenario of the other two estuaries was comparable, though the tidal dilution was remarkably higher and the impact of other sources was much lower. One of the outcomes of this work was the maturation of the WWTP fingerprint that might be useful in future works. Another outcome was the identification of pharmaceuticals, such as diclofenac and sartants, and caffeine as contaminants of increasing concern that should be carefully monitored in the future.

On the other hand, in the case of the application of the effect-directed analysis, we were able to implement a bioassay designed for coastal waters as the way to drive the non-targeted analysis and to identify the main toxic emerging contaminants in a WWTP effluent. Most of the experimental work was carried out from scratch and without hardly any previous expertise in this field. However, thanks to the network of partners and coworkers, we were able to implement for the first time the sea urchin embryo test at the Plentzia Marine Station as the way to measure the toxicity in the EDA approach. The combination of a bioassay that is ecologically relevant for the coastal waters and the non-targeted analysis workflow in the toxic fractions allowed us to identify

six of the nine most toxic compounds present in the effluent of a WWTP. In fact, two of those compounds are anthelmintic drugs (albendazole and mebendazole), other two are antidepressant (paroxetine and amitriptyline) and last two are pesticides (mexacarbate and fenpropidin).

These two achievements would have not been possible if we had not succeeded in the analytical developments tackled in the previous four chapters. The development of robust analytical methods is the core work of our research and, in this particular case, we were able to develop most of the methods that were thoroughly used in the monitoring of the estuaries and in other spinoff applications.

Briefly, we developed two methods to run a targeted multiresidue analysis of more than 40 emerging contaminants in a variety of water and biota environmental samples. In the case of water, one of the developed methods, dual PES microextraction, gains a lower matrix effect, cost and consumption of organic solvents. In the case of biota samples, both tissues and biofluids, the combination of an exhaustive FUSLE protocol with different clean-up alternatives rendered accurate results and demonstrated to be useful for the treatment of complex biota samples. The full development of a new POCIS configuration to perform passive sampling analysis was also carried out, allowing the monitoring of hydrophilic, acid and basic compounds in complex systems such as the estuaries. Finally, an established and validates SPE methodology allowed us to safe the integrity of a large amount of real water samples.

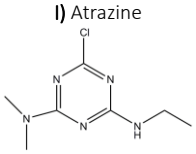
Appendix

A good decision is based on knowledge

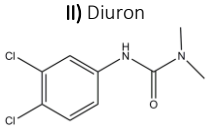
and not on numbers.

Plato

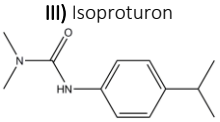
I) Atrazine



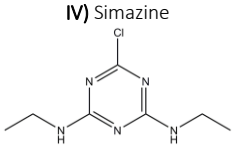
II) Diuron



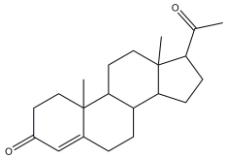
III) Isoproturon



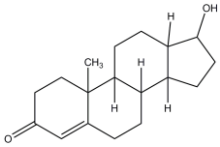
IV) Simazine



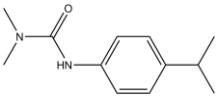
V) Progesterone



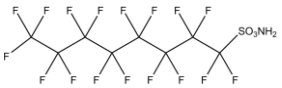
VI) Testosterone



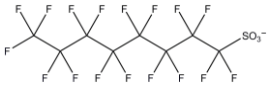
VII) 2-Hydroxybenzothiazole (OBT)



VIII)Perfluoroctylsulfonamide (PFOSA)

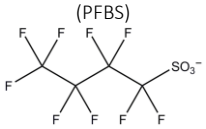


IX) Perfluorooctane sulfonic acid (PFOS)

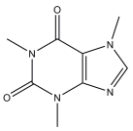


X) Perfluorooctanoic acid (PFOA)

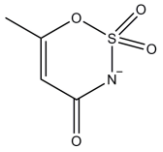
XI)Perfluorobutanesulfonate (PFBS)



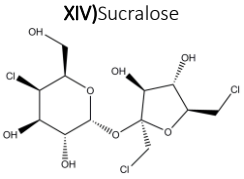
XII) Caffeine



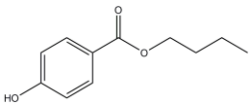
XIII) Acesulfame



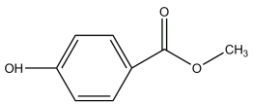
XIV)Sucralose



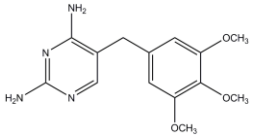
XV) Butylparaben



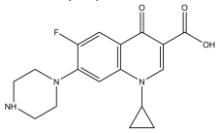
XVI) Methylparaben



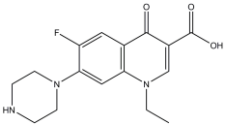
XVII) Trimethoprim



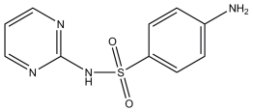
XVIII) Ciprofloxacin



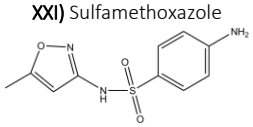
XIX) Norfloxacin



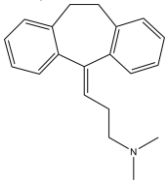
XX) Sulfadiazine



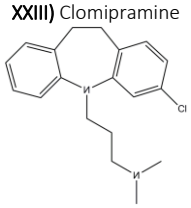
XXI) Sulfamethoxazole



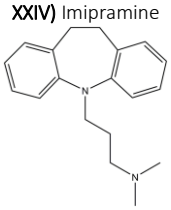
XXII) Amitriptyline



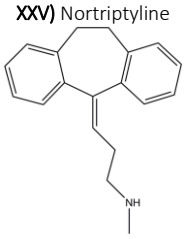
XXIII) Clomipramine



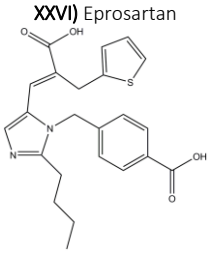
XXIV) Imipramine



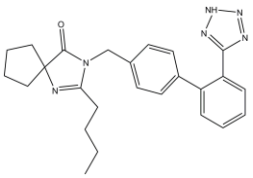
XXV) Nortriptyline



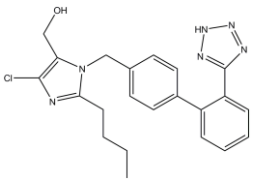
XXVI) Eprosartan



XXVII) Irbesartan



XXVIII) Losartan



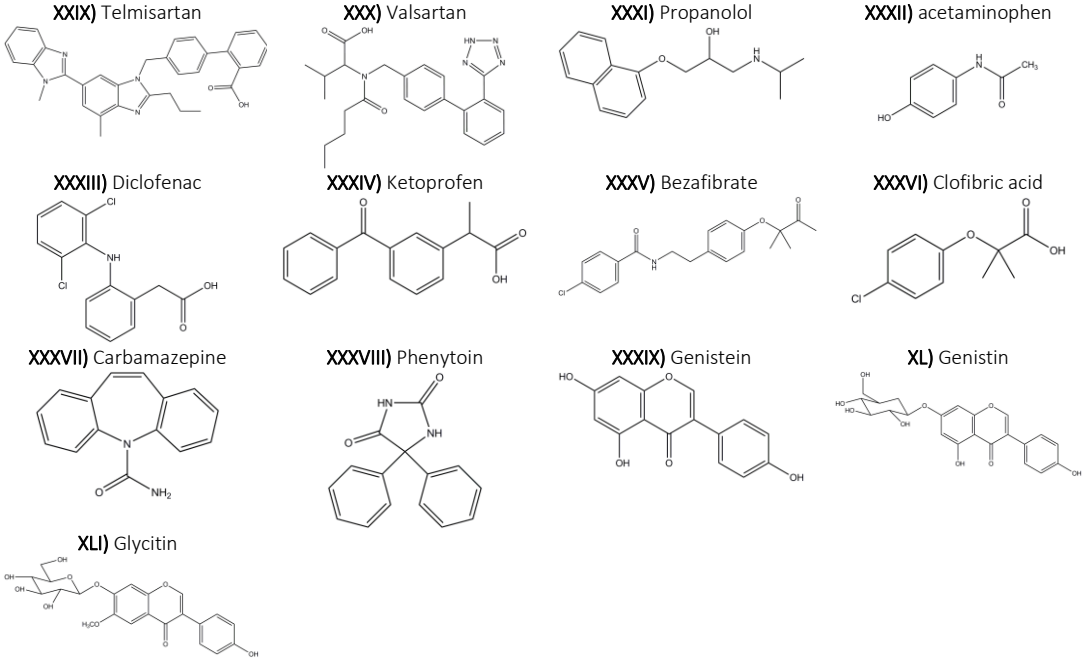


Figure I *Structures of the 41 target compounds.*

Table I: The concentrations (ng/L) of the target analytes in the tank at each sampling day (±*sd.*, n=3).

Analyte	d=0	d=2	d=4	d=7	d=9	d=11	d=14
Acetaminophen	490	679±49	550±91	668±31	805±8	616±11	575±57
Acesulfame	761	632±108	445±31	840±25	814±22	824±57	597±248
Amitriptyline	728	459±1	431±7	560±57	530±11	424±33	539±168
Atrazine	748	541±2	643±113	694±54	737±20	732±21	694±173
Bezafibrate	709	576±15	664±62	745±65	779±5	765±148	796±37
Butylparaben	588	432±4	469±30	434±71	518±2	498±9	533±42
Caffeine	1060	1022±27	1123±69	1344±62	1430±23	1212±48	994±158
Carbamazepine	650	517±17	612±55	719±57	766±11	719±93	726±61
Diuron	618	501±6	558±34	636±59	709±4	670±62	783±59
Irbesartan	696	511±11	518±24	571±78	604±11	564±124	533±22
Ketoprofen	696	586±11	691±41	728±88	802±2	810±127	837±38
Norfloxacin	833	848±2	917±9	1031±65	1135±16	979±51	755±267
OBT	705	641±24	774±79	928±61	979±26	879±55	817±61
PFBS	740	717±20	812±106	921±54	991±18	880±114	805±62
PFOA	242	337±19	252±61	339±74	563±50	558±15	667±8
PFOS	159	263±42	111±26	52±23	414±58	898±63	457±70
Phenytoin	714	572±16	610±16	739±60	735±21	708±56	796±127
Progesterone	374	345±2	317±17	312±67	379±15	382±28	434±55
Sulfadiazine	711	342±64	365±34	765±57	718±26	576±75	541±161
Telmisartan	502	504±72	409±97	361±29	467±12	458±4	573±146

Table II. Sample location, sampling depth and water physico-chemical parameters for each sampling campaign in the Bilbao estuary (Bi).

Season (yyyy/mm/dd)	Site	Depth (m)	pH	ORP (mV)	Temperature (°C)	Conductivity (µS/cm)	Salinity (psµ)	TDS (mg/L)	ODO (mg/L)
Winter 2016/03/18	Bi-1s	0.3	7.9	266	12.3	32363	27	27787	9
	Bi-1b	20.0	8.0	253	12.6	41744	36	35516	8
	Bi-2s	0.3	7.8	268	11.3	18419	15	16214	10
	Bi-2b	16.2	8.0	278	12.6	41688	36	35492	8
	Bi-3s	0.9	8.1	273	11.6	4208	3	3680	11
	Bi-3b	1.8	7.8	198	11.0	18709	16	16616	10
	Bi-4s	1.0	7.4	194	11.7	2345	2	2047	9
	Bi-4b	3.3	7.5	214	11.5	36833	32	32228	8
	Bi-5s	1.0	8.5	243	10.6	771	1	691	11
	Bi-5b	4.7	7.7	291	12.2	38414	33	33010	8
	Bi-6s	1.0	8.1	252	9.2	2326	2	2166	12
	Bi-6b	4.5	7.6	285	11.9	35855	31	31115	8
Spring 2016/05/31	Bi-1s	0.8	8.2	131	17.4	46933	36	35693	8
	Bi-1b	22.2	8.1	132	16.0	45938	37	36026	8
	Bi-2s	0.9	8.2	145	18.0	44138	34	33130	9
	Bi-2b	16.2	8.1	146	16.5	46245	37	35855	8
	Bi-3s	0.9	8.1	117	18.6	38371	28	28409	9
	Bi-3b	2.6	8.1	118	17.9	43438	33	32691	9
	Bi-4s	0.1	8.0	141	19.5	24047	17	17476	10
	Bi-4b	3.5	7.9	110	17.6	42794	33	32418	6
	Bi-5s	0.9	8.1	151	18.5	18312	13	13591	10
	Bi-5b	3.1	8.0	156	18.3	39629	29	29542	9
	Bi-6s	0.9	8.2	156	18.3	3599	2	2680	10
	Bi-6b	6.1	7.5	191	16.8	42373	33	32687	5
Summer 2016/09/07	Bi-1s	0.0	7.6	236	20.8	49044	35	34629	7
	Bi-1b	23.1	7.7	224	15.2	44689	36	35718	7
	Bi-2s	0.1	7.8	236	21.4	47878	34	33416	7
	Bi-2b	15.6	7.9	231	16.1	45775	37	35861	7
	Bi-3s	0.9	7.8	230	22.8	42267	29	28696	7
	Bi-3b	1.8	7.8	228	21.8	47066	33	32594	6
	Bi-4s	0.1	7.6	219	24.1	32417	21	21439	6
	Bi-4b	2.1	7.6	169	21.9	47873	33	33077	6
	Bi-5s	0.2	8.0	105	24.1	38695	25	25613	8
	Bi-5b	3.9	7.9	112	22.0	46836	33	32268	6
	Bi-6s	0.1	7.8	211	24.7	38538	25	25177	5
	Bi-6b	4.1	7.7	217	22.5	45593	31	31141	4
Fall 2016/11/30	Bi-1s	0.2	7.1	165	14.5	43988	36	35725	8
	Bi-1b	21.0	8.1	194	14.7	44927	37	36327	8
	Bi-2s	0.1	8.7	239	14.1	41095	34	33739	8
	Bi-2b	15.0	8.6	236	14.6	44659	37	36209	8
	Bi-3s	0.2	9.0	152	13.1	25011	20	21021	8
	Bi-3b	1.8	9.1	153	13.2	29834	25	25057	8

Table II. Sample location, sampling depth and water physico-chemical parameters for each sampling campaign in the Bilbao estuary (Bi).

Season (yyyy/mm/dd)	Site	Depth (m)	pH	ORP (mV)	Temperature (°C)	Conductivity (µS/cm)	Salinity (psµ)	TDS (mg/L)	ODO (mg/L)
Fall 2016/11/30	Bi-4s	0.2	9.0	184	13.7	21742	17	18015	8
	Bi-4b	3.7	8.7	171	14.3	41758	35	34108	7
	Bi-5s	0.1	9.1	183	12.6	17245	14	14685	9
	Bi-5b	6.0	8.6	137	14.3	41524	34	33941	7
	Bi-6s	0.1	9.6	85	11.1	5902	4	5221	11
	Bi-6b	5.7	8.6	108	14.0	39343	33	32403	4
Winter 2017/02/23	Bi-1s	0.9	7.9	268	12.8	43105	37	36531	9
	Bi-1b	22.1	7.8	228	12.9	44488	39	37634	8
	Bi-2s	0.2	8.0	260	13.2	41422	35	34722	9
	Bi-2b	14.5	7.9	33	12.9	44365	38	37482	8
	Bi-3s	0.2	7.9	71	13.0	17372	14	14641	9
	Bi-3b	2.1	7.9	95	13.2	41683	36	35020	8
	Bi-4s	0.1	7.1	120	14.9	10866	8	8616	8
	Bi-4b	2.8	7.8	117	13.1	42799	37	35977	7
	Bi-5s	0.1	8.2	96	11.7	4405	3	3843	11
	Bi-5b	4.8	7.8	135	13.1	42058	36	35419	7
	Bi-6s	0.9	8.6	197	11.5	2073	1	1816	12
	Bi-6b	4.9	7.8	223	12.9	42088	36	35590	6

Bi-b: bilbao estuary bottom, Bi-s: Bilbao estuary surface, ODO: optical dissolved oxygen, TDS: total dissolved solid. Numbers refer to the sampling point in Bilbao estuary, being 1 the estuary mouth and 6 the upper part of the estuary (see **Figure7.1**. in **chapter 7**).

Table III. Sample location, sampling depth and non-purgable organic carbon (NPOC) ammonia (NH_4^+), nitrate (NO_3^-) silicate ($Si(OH)_4$) and phosphate (PO_4^{3-}) concentrations for each sampling campaign in the Bilbao estuary (Bi).

Season (yyyy/mm/dd)	Site	Depth (m)	NPOC (mg/L)	NH_4^+ (mg/L)	NO_3^- (mg/L)	$Si(OH)_4$ (mg/L)	PO_4^{3-} (mg/L)
Winter 2016/03/18	Bi-1s	0.3	2.20	24	185	2.12	0.04
	Bi-1b	20.0	1.04	46	238	0.25	0.02
	Bi-2s	0.3	2.73	5	60	7.18	0.31
	Bi-2b	16.2	1.27	34	235	0.31	0.03
	Bi-3s	0.9	2.78	2	28	10.94	0.24
	Bi-3b	1.8	2.74	4	52	7.36	0.23
	Bi-4s	1.0	4.65	2	47	10.87	3.30
	Bi-4b	3.3	1.49	25	208	1.88	0.17
	Bi-5s	1.0	2.39	0.4	9	9.21	0.06
	Bi-5b	4.7	1.42	29	221	1.25	0.08
	Bi-6s	1.0	3.05	1	14	8.64	0.09
	Bi-6b	4.5	1.63	24	192	2.08	0.08
Spring 2016/05/31	Bi-1s	0.8	1.51	24	274	0.11	0.03
	Bi-1b	22.2	1.47	46	288	0.01	0.03
	Bi-2s	0.9	1.97	5	235	0.14	0.21
	Bi-2b	16.2	1.47	34	272	0.15	0.06
	Bi-3s	0.9	3.10	2	180	1.25	0.88
	Bi-3b	2.6	2.12	4	228	0.01	0.29
	Bi-4s	0.1	5.31	2	123	1.78	7.90
	Bi-4b	3.5	1.95	25	226	0.73	0.43
	Bi-5s	0.9	3.13	0.4	227	0.20	0.20
	Bi-5b	3.1	2.24	29	345	2.57	0.68
	Bi-6s	0.9	4.11	1	29	4.76	0.15
	Bi-6b	6.1	1.98	24	212	1.32	0.31
Summer 2016/09/07	Bi-1s	0.0	2.35	48	221	0.21	0.07
	Bi-1b	23.1	1.83	49	228	0.23	0.04
	Bi-2s	0.1	2.88	43	216	0.67	0.39
	Bi-2b	15.6	2.00	44	224	0.42	0.04
	Bi-3s	0.9	4.20	31	171	2.89	1.08
	Bi-3b	1.8	2.70	46	221	0.81	0.38
	Bi-4s	0.1	8.64	24	123	6.18	7.59
	Bi-4b	2.1	3.31	43	186	1.45	0.93
	Bi-5s	0.2	5.70	32	154	2.11	1.42
	Bi-5b	3.9	3.42	44	199	1.20	0.38
	Bi-6s	0.1	7.03	27	141	2.89	0.51
	Bi-6b	4.1	4.39	45	204	1.84	0.56
Fall 2016/11/30	Bi-1s	0.2	1.39	36	242	0.23	0.03
	Bi-1b	21.0	1.13	36	243	0.17	0.02
	Bi-2s	0.1	1.58	30	223	1.78	0.19
	Bi-2b	15.0	1.04	36	252	0.20	0.02
	Bi-3s	0.2	2.94	20	147	5.04	1.21
	Bi-3b	1.8	2.23	20	159	4.43	0.58
	Bi-4s	0.2	3.18	15	119	6.46	1.55

Table III. Sample location, sampling depth and non-purgable organic carbon (NPOC) ammonia (NH_4^+), nitrate (NO_3^-) silicate ($Si(OH)_4$) and phosphate (PO_4^{3-}) concentrations for each sampling campaign in the Bilbao estuary (Bi).

Season (yyyy/mm/dd)	Site	Depth (m)	NPOC (mg/L)	NH_4^+ (mg/L)	NO_3^- (mg/L)	$Si(OH)_4$ (mg/L)	PO_4^{3-} (mg/L)
Fall 2016/11/30	Bi-4b	3.7	1.41	34	249	0.99	0.15
	Bi-5s	0.1	2.95	13	106	6.57	0.43
	Bi-5b	6.0	1.51	32	239	1.00	0.10
	Bi-6s	0.1	3.17	3	46	10.99	0.15
	Bi-6b	5.7	2.25	32	210	2.20	0.20
Winter 2017/02/23	Bi-1s	0.9	2.46	35	236	0.12	0.04
	Bi-1b	22.1	2.12	34	238	0.21	0.03
	Bi-2s	0.2	2.72	32	223	0.48	0.09
	Bi-2b	14.5	2.17	36	255	0.21	0.04
	Bi-3s	0.2	6.46	12	92	5.30	0.80
	Bi-3b	2.1	2.64	32	221	1.69	0.12
	Bi-4s	0.1	9.99	7	74	7.55	4.96
	Bi-4b	2.8	2.89	32	224	0.75	0.15
	Bi-5s	0.1	7.22	2	24	1.93	0.03
	Bi-5b	4.8	3.07	34	237	0.74	0.08
	Bi-6s	0.9	8.68	1	17	4.72	0.05
	Bi-6b	4.9	3.89	31	213	1.10	0.08

Bi-b: bilbao estuary bottom, Bi-s: Bilbao estuary surface. Numbers refer to the sampling point in Bilbao estuary, being 1 the estuary mouth and 6 the upper part of the estuary (see **Figure7.1**. in **chapter 7**).

Table IV. Sample location, sampling depth and water physico-chemical parameters for each sampling campaign in the Plentzia estuary (Pl).

Season (mm/dd)	Site	Depth (m)	pH	ORP (mV)	Temperature (°C)	Condutivity (µS/cm)	Salinity (psµ)	TDS (mg/L)	ODO (mg/L)
Winter 2016/03/15	Pl-1	1.1	8.0	262	12.1	39944	35	34421	9
	Pl-2	1.0	8.0	242	12.0	39390	34	34044	9
	Pl-3	1.0	7.9	252	11.8	38687	34	33638	9
	Pl-4	1.1	7.8	223	11.7	35901	31	31311	9
	Pl-5	1.0	7.4	194	9.6	11661	20	10735	11
	Pl-6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Pl-7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Spring 2016/06/01	Pl-1	0.06	8.2	185	17.8	47272	36	35597	8
	Pl-2	0.1	7.8	245	17.8	45040	34	33965	8
	Pl-3	0.07	8.1	214	17.9	44860	34	33749	8
	Pl-4	0.04	8.1	268	18.2	44243	33	33038	8
	Pl-5	0.06	8.1	290	18.7	37573	28	27784	8
	Pl-6	0.04	8.1	277	18.9	32398	23	23811	8
	Pl-7	0.3	6.0	128	17.5	459	0.3	348	8
Summer 2016/09/12	Pl-1	0.2	8.4	61	17.9	47066	36	35413	7
	Pl-2	0.1	7.8	235	17.8	45040	36	35965	7
	Pl-3	0.9	8.0	242	20.1	48993	36	35158	7
	Pl-4	0.8	8.0	249	21.3	48821	35	34125	7
	Pl-5	0.8	8.1	266	22.3	45173	31	30970	7
	Pl-6	0.7	8.1	279	23.5	42693	28	28554	7
	Pl-7	1.0	7.4	51	19.2	621	0.3	454	6
Fall 2016/11/28	Pl-1	0.4	8.5	258	14.5	43737	36	35599	8
	Pl-2	0.8	7.8	311	14.3	43152	36	35217	8
	Pl-3	0.9	8.8	289	14.4	43734	36	35623	8
	Pl-4	0.8	8.6	303	14.4	43599	36	35523	8
	Pl-5	0.9	8.8	330	12.6	31478	26	26819	9
	Pl-6	0.9	8.6	329	12.4	26930	22	23034	9
	Pl-7	1.0	6.3	177	10.1	331	0.2	301	10
Winter 2017/02/21	Pl-1	0.3	8.0	266	12.9	43921	38	37145	9
	Pl-2	0.5	8.1	294	12.9	43666	38	36893	9
	Pl-3	0.4	8.1	320	12.5	43047	37	36752	9
	Pl-4	0.5	8.1	311	12.5	35614	30	30388	9
	Pl-5	0.5	8.1	304	12.1	25494	21	22001	10
	Pl-6	0.06	8.1	278	11.6	21050	17	18413	10
	Pl-7	0.2	7.9	225	10.3	392	0.3	355	10

n.a.: not adquired, ODO: optical dissolved oxygen, TDS: total dissolved solid. Pl-i: plentzia estuary sampling point,;numbers refer to the sampling point, being 1 the estuary mouth and 7 the upper part of the estuary (see **Figure7.1**. in **chapter 7**).

Table V. Sample location, sampling depth and non-purgable organic carbon (NPOC) ammonia (NH_4^+), nitrate (NO_3^-) silicate ($Si(OH)_4$) and phosphate (PO_4^{3-}) concentrations for each sampling campaign in the Plentzia estuary (Pl).

Season (yyyy/mm/dd)	Site	Depth (m)	NPOC (mg/L)	NH_4^+ (mg/L)	NO_3^- (mg/L)	$Si(OH)_4$ (mg/L)	PO_4^{3-} (mg/L)
Winter 2016/03/15	Pl-1	1.1	1.3	41	223	0.4	0.02
	Pl-2	1.0	1.2	40	216	0.7	0.02
	Pl-3	1.0	1.1	40	220	0.7	0.02
	Pl-4	1.1	1.7	22	139	6	0.1
	Pl-5	1.0	2.4	8	53	10	0.2
	Pl-6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Pl-7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Spring 2016/06/01	Pl-1	0.06	1.5	46	362	0.1	0.02
	Pl-2	0.1	1.9	44	347	0.4	0.02
	Pl-3	0.07	1.9	41	359	0.7	0.03
	Pl-4	0.04	1.8	40	376	1	0.04
	Pl-5	0.06	2.2	33	404	3	0.04
	Pl-6	0.04	2.6	29	368	4	0.06
	Pl-7	0.3	3.4	0.5	25	16	0.5
Summer 2016/09/12	Pl-1	0.2	1.5	51	249	0.1	0.01
	Pl-2	0.1	1.4	53	239	0.2	0.02
	Pl-3	0.9	1.5	50	233	0.1	0.02
	Pl-4	0.8	1.7	50	249	0.4	0.02
	Pl-5	0.8	2.0	46	207	1	0.05
	Pl-6	0.7	2.4	41	192	2	0.07
	Pl-7	1.0	4.4	0.9	8	18	0.5
Fall 2016/11/28	Pl-1	0.4	1.5	34	238	0.4	0.03
	Pl-2	0.8	1.3	35	240	0.4	0.03
	Pl-3	0.9	1.3	34	238	0.3	0.03
	Pl-4	0.8	1.4	35	239	0.4	0.03
	Pl-5	0.9	3.1	20	151	6	0.1
	Pl-6	0.9	3.1	20	146	6	0.1
	Pl-7	1.0	5.9	0.4	10	2	0.3
Winter 2017/02/21	Pl-1	0.3	0.9	33	224	0.2	0.02
	Pl-2	0.5	0.8	34	239	0.2	0.03
	Pl-3	0.4	0.9	34	231	0.4	0.03
	Pl-4	0.5	1.2	24	177	3	0.05
	Pl-5	0.5	2.0	16	119	5	0.07
	Pl-6	0.06	2.0	10	70	7	0.08
	Pl-7	0.2	2.8	0.2	7	10	0.1

n.a.: not adquired. Pl-i: plentzia estuary sampling point,;numbers refer to the sampling point, being 1 the estuary mouth and 7 the upper part of the estuary (see **Figure7.1**. in **chapter 7**).

Table VI. Sample location, sampling depth and water physico-chemical parameters as non-purgable organic carbon (NPOC) ammonia (NH_4^+), nitrate (NO_3^-) silicate ($Si(OH)_4$) and phosphate (PO_4^{3-}) concentrations for each sampling campaign in the Urdaibai estuary (Ur).

Season yyyy/mm/dd	Sampling point	Depth (m)	pH	ORP (mV)	Temperature (°C)	Conductivity (µS/cm)	Salinity (psµ)	TDS (mg/L)	ODO (mg/L)
Winter 2016/02/24	Ur-1	0.9	8.3	276	13	40586	35	34338	9
	Ur-2	0.9	8.3	299	13	25869	21	21778	9
	Ur-3	1.1	8.1	279	13	10845	8	9229	8
	Ur-4	0.2	8.7	105	10	302	0.2	275	12
Spring 2016/06/02	Ur-1	1.2	8.3	290	19	46902	35	36891	6
	Ur-2	0.1	8.0	270	19	43340	32	32046	7
	Ur-3	0.1	7.8	265	19	31251	22	22716	6
	Ur-4	0.1	7.2	98	15	367	0.2	296	10
Summer 2016/09/08	Ur-1	0.2	7.6	307	21	49901	36	34933	7
	Ur-2	0.1	7.5	310	21	49710	36	34923	7
	Ur-3	0.2	7.2	262	24	49276	33	32635	5
	Ur-4	0.2	8.8	170	19	537	0.3	391	9
Fall 2016/11/17	Ur-1	1.1	7.9	209	15	47202	39	38222	8
	Ur-2	0.4	9.1	234	14	34151	28	28363	8
	Ur-3	0.2	9.4	157	13	19477	16	16590	8
	Ur-4	0.2	9.6	113	10	352	0.2	322	12
Winter 2017/02/16	Ur-1	0.9	7.9	226	13	41526	36	35333	9
	Ur-2	0.2	7.9	229	13	28009	23	23478	8
	Ur-3	0.1	7.9	217	13	14103	11	11912	8
	Ur-4	0.2	8.5	148	10	322	0.2	290	13

ODO: optical dissolved oxygen, TDS: total dissolved solid. Ur-i: urdaibai estuary sampling point; numbers refer to the sampling point, being 1 the estuary mouth and 4 the upper part of the estuary (see **Figure7.1**. in **chapter 7**).

Table VII. Sample location, sampling depth and water physico-chemical parameters as non-purgable organic carbon (NPOC) ammonia (NH_4^+), nitrate (NO_3^-) silicate ($Si(OH)_4$) and phosphate (PO_4^{3-}) concentrations for each sampling campaign in the Urdaibai estuary (Ur).

Season (yyyy/mm/dd)	Site	Depth (m)	NPOC (mg/L)	NH_4^+ (mg/L)	NO_3^- (mg/L)	$Si(OH)_4$ (mg/L)	PO_4^{3-} (mg/L)
Winter 2016/02/24	Ur-1	0.9	1.5	44	221	0.5	0.01
	Ur-2	0.9	2.0	25	133	4.3	0.08
	Ur-3	1.1	2.3	10	57	12.1	0.16
	Ur-4	0.2	1.5	0.2	6	11.8	0.05
Spring 2016/06/02	Ur-1	1.2	1.7	47	323	0.3	0.02
	Ur-2	0.1	1.9	37	317	1.5	0.08
	Ur-3	0.1	2.9	26	241	6.0	0.17
	Ur-4	0.1	1.7	0.5	20	18.7	0.06
Summer 2016/09/08	Ur-1	0.2	1.6	46	232	0.1	0.01
	Ur-2	0.1	1.9	54	240	0.1	0.02
	Ur-3	0.2	3.6	50	207	1.9	0.16
	Ur-4	0.2	7.3	0.6	9	23.0	0.12
Fall 2016/11/17	Ur-1	1.1	1.7	34	233	0.3	0.02
	Ur-2	0.4	3.7	22	174	3.8	0.12
	Ur-3	0.2	5.9	15	98	8.3	0.31
	Ur-4	0.2	9.0	0.2	7	1.5	0.09
Winter 2017/02/16	Ur-1	0.9	0.9	32	217	1.0	0.04
	Ur-2	0.2	1.6	21	139	4.5	0.10
	Ur-3	0.1	2.5	10	75	7.6	0.15
	Ur-4	0.2	1.5	0.1	6	4.5	0.06

Ur-i: urdaibai estuary sampling point; numbers refer to the sampling point, being 1 the estuary mouth and 4 the upper part of the estuary (see **Figure7.1**. in **chapter 7**).

Table VIII. Mean concentrations (ng/L) of the target analytes determined in the three studied WWTPs effluents (Galindo, Gorliz and Gernika).

Analyte	Galindo WWTP (ng/L)					Gorliz WWTP (ng/L)					Gernika WWTP (ng/L)				
	Winter 2016	Spring 2016	Summ 2016	Fall 2016	Winter 2017	Winter 2016	Spring 2016	Summ 2016	Fall 2016	Winter 2017	Winter 2016	Spring 2016	Summ 2016	Fall 2016	Winter 2017
Acesulfame	430	80	573	52	1164	849	311	51	283	1261	134	7747	11419	3386	4202
Acetaminophen	860	215	111	53	47	222	247	724	119	134	812	845	1126	5460	832
Amitriptyline	43	30	71	53	50	9	20	16	13	23	5	22	39	24	19
Atrazine	<mql	<mql	<mql	n.d.	n.d.	n.d.	<mql	<mql	n.d.	n.d.	<mql	<mql	18	<mql	<mql
Bezafibrate	42	23	132	88	82	20	14	2	7	40	20	68	101	<mql	89
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9	n.d.	n.d.	<mql	97	100	<mql	<mql
Caffeine	25	36	57	82	99	210	175	317	71	183	1752	26034	65999	46065	21974
Carbamazepin	76	49	137	94	114	71	48	94	12	52	2	80	390	46	39
Ciprofloxacin	155	3803	508	789	549	58	3194	88	224	120	36	4719	488	n.d.	100
Clofibric acid	7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<mql	<mql	<mql	n.d.	n.d.
Clomipramine	3	4	7	3	3	<mql	8	<mql	1	4	<mql	<mql	<mql	<mql	<mql
Diclofenac	1161	127	811	1911	1479	660	n.d.	8	168	683	10	<mql	252	1932	803
Diuron	121	55	133	122	204	131	204	31	n.d.	193	7	250	199	349	225
Eprosartan	218	46	331	339	279	570	184	128	74	485	42	879	499	722	386
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<mql	n.d.	5	479	180	597	8
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<mql	<mql	<mql
Glycitin	n.d.	n.d.	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<mql	n.d.	n.d.
Imipramine	3	n.d.	<mql	n.d.	n.d.	<mql	n.d.	n.d.	n.d.	<mql	<mql	<mql	n.d.	n.d.	n.d.
Irbesartan	584	410	933	1275	1060	750	355	617	86	666	54	711	465	940	488
Irbesartan	584	410	933	1275	1060	750	355	617	86	666	54	711	465	940	488
Isoproturon	n.d.	2	3	4	4	n.d.	4	2	5	<mql	<mql	<mql	n.d.	n.d.	n.d.

n.a.=not adquired; n.d.= non detected, < mql= below method quantification limit; summ: summer

Table VIII. Mean concentrations (ng/L) of the target analytes determined in the three studied WWTPs effluents (Galindo, Gorliz and Gernika).

Analyte	Galindo WWTP (ng/L)					Gorliz WWTP (ng/L)					Gernika WWTP (ng/L)				
	Winter 2016	Spring 2016	Summ 2016	Fall 2016	Winter 2017	winter 2016	Spring 2016	Summ 2016	Fall 2016	Winter 2017	Wwinter2016	Spring 2016	Summ 2016	Fall 2016	Winter 2017
Ketoprofen	152	53	238	109	281	13	5	n.d.	n.d.	n.d.	19	340	340	374	243
Losartan	100	43	302	301	249	303	717	109	32	415	21	913	438	610	344
Methylparaben	n.d.	<mql	<mql	<mql	n.d.	n.d.	<mql	n.d.	n.d.	n.d.	<mql	<mql	22	189	n.d.
Norfloxacin	61	32	463	88	45	38	15	20	40	17	2	119	275	88	11
Nortriptyline	6	7	11	11	9	<mql	6	<mql	<mql	5	n.d.	n.d.	n.d.	n.d.	2
OBT	53	112	84	95	172	61	243	86	82	173	8	621	934	1082	139
PFBS	<mql	33	200	132	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<mql	2202	<mql	28	<mql
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	5	9	6	n.d.	<mql	n.d.	n.d.	2	n.d.	n.d.	16	<mql	<mql	168	7
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenytoin	315	2375	88	31	mql	110	46	88	31	110	<mql	111	1020	133	<mql
Progesterone	3	<mql	11	n.d.	n.d.	<mql	25	16	6	n.d.	<mql	64	<mql	20	<mql
Propranolol	17	13	31	28	28	8	18	18	5	14	2	9	30	16	11
Simazine	n.d.	<mql	<mql	n.d.	n.d.	n.d.	<mql	5	n.d.	<mql	1	<mql	<mql	<mql	<mql
Sucralose	638	46	<mql	n.d.	771	4111	143	125	1859	4532	52	<mql	27	<mql	1380
Sulfadiazine	<mql	20	21	5477	96	275	18	7	11	303	<mql	24	6	<mql	8
Sulfamethoxazole	1224	401	66	8963	8816	67	118	57	47	244	8	135	131	120	190
Telmisartan	471	419	1316	2	434	227	298	766	146	462	127	135	747	1208	545
Testosterone	<mql	n.d.	n.d.	n.d.	n.d.	<mql	n.d.	n.d.	<mql	n.d.	3	<mql	6	2	<mql
Trimethoprim	271	345	36	7	5843	79	17	6	7	8	2	29	61	29	28
Valsartan	180	89	416	395	375	513	412	154	315	1811	141	8063	9485	8245	<mql

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit, summ: summer

Table IX. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Bilbao in winter 2016.

Analyte	Bilbao estuary surface water (Bi)						Bilbao estuary bottom water (Bi)					
	Bi-1s	Bi-2s	Bi-3s	Bi-4s	Bi-5s	Bi-6s	Bi-1b	Bi-2b	Bi-3b	Bi-4b	Bi-5b	Bi-6b
Acesulfame	< mql	30	39	97	7	9	n.d.	n.d.	4	< mql	< mql	< mql
Acetaminophen	39	153	440	89	54	59	31	25	82	5	< mql	n.d.
Amitriptyline	n.d.	2	3	8	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	n.d.
Atrazine	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql
Bezafibrate	n.d.	n.d.	5	10	n.d.	< mql	n.d.	n.d.	< mql	n.d.	n.d.	n.d.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	45	141	247	132	127	225	60	30	75	12	8	18
Carbamazepin	1	5	7	25	< mql	< mql	n.d.	n.d.	< mql	< mql	< mql	< mql
Ciprofloxacin	n.d.	n.d.	< mql	33	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.
Clofibric acid	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Clomipramine	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diclofenac	< mql	33	47	332	5	5	n.d.	n.d.	14	39	< mql	< mql
Diuron	n.d.	< mql	6	68	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.
Eprosartan	n.d.	n.d.	16	85	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Irbesartan	n.d.	32	27	207	2	4	n.d.	n.d.	3	2	< mql	< mql
Isoproturon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.
Ketoprofen	n.d.	n.d.	n.d.	37	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Losartan	9	9	17	50	< mql	< mql	n.d.	n.d.	n.d.	< mql	n.d.	n.d.
Methylparaben	19	9	n.d.	n.d.	n.d.	n.d.	30	10	n.d.	n.d.	n.d.	n.d.
Norfloxacin	n.d.	n.d.	n.d.	7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nortriptyline	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
OBT	345	300	101	420	20	< mql	1097	1044	412	189	60	< mql
PFBS	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOA	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	n.d.	2	24	2	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenytoin	n.d.	n.d.	n.d.	15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Progesterone	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Propranolol	n.d.	< mql	< mql	9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sucralose	n.d.	30	48	212	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sulfadiazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sulfamethoxazole	< mql	35	38	92	< mql	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.
Telmisartan	2	29	30	129	2	n.d.	n.d.	n.d.	4	9	4	< mql
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trimethoprim	< mql	21	47	197	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.
Valsartan	4	27	24	80	26	n.d.	n.d.	n.d.	n.d.	< mql	< mql	< mql

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table X. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Plentzia and Urdaibai in winter 2016.

Analyte	Plentzia estuary (Pl)							Urdaibai estuary (Ur)			
	Pl-1	Pl-2	Pl-3	Pl-4	Pl-5	Pl-6	Pl-7	Ur-1	Ur-2	Ur-3	Ur-4
Acesulfame	n.d.	n.d.	< mql	6	5	n.a.	n.a.	< mql	11	30	< mql
Acetaminophen	n.d.	n.d.	6	7	27	n.a.	n.a.	25	138	321	n.d.
Amitriptyline	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Atrazine	n.d.	< mql	< mql	< mql	< mql	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Bezafibrate	n.d.	n.d.	< mql	< mql	5	n.a.	n.a.	< mql	3	8	n.d.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Caffeine	138	20	30	127	147	n.a.	n.a.	< mql	156	364	< mql
Carbamazepin	n.d.	n.d.	n.d.	< mql	5	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Ciprofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	17	n.d.
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Clomipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Diclofenac	n.d.	n.d.	< mql	5	9	n.a.	n.a.	< mql	2	5	n.d.
Diuron	n.d.	n.d.	< mql	< mql	2	n.a.	n.a.	n.d.	n.d.	4	n.d.
Eprosartan	n.d.	n.d.	n.d.	< mql	3	n.a.	n.a.	< mql	3	17	n.d.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Irbesartan	< mql	< mql	< mql	3	6	n.a.	n.a.	< mql	12	27	n.d.
Isoproturon	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Ketoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Losartan	n.d.	n.d.	n.d.	< mql	2	n.a.	n.a.	< mql	7	14	n.d.
Methylparaben	13	21	< mql	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Norfloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Nortriptyline	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
OBT	1219	210	356	218	308	n.a.	n.a.	118	336	320	< mql
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
PFOS	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Phenytoin	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Propranolol	n.d.	n.d.	n.d.	n.d.	< mql	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	5	n.d.
Sucralose	n.d.	n.d.	< mql	3	10	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Sulfadiazine	n.d.	< mql	3	9	14	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Sulfamethoxazole	n.d.	n.d.	n.d.	< mql	< mql	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Telmisartan	< mql	< mql	n.d.	4	14	n.a.	n.a.	5	22	42	n.d.
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	< mql	n.d.
Trimethoprim	n.d.	n.d.	n.d.	< mql	< mql	n.a.	n.a.	< mql	< mql	< mql	n.d.
Valsartan	n.d.	< mql	11	30	86	n.a.	n.a.	8	37	65	n.d.

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table XI. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Bilbao in spring 2016.

Analyte	Bilbao estuary surface water (Bi)						Bilbao estuary bottom water (Bi)					
	Bi-1s	Bi-2s	Bi-3s	Bi-4s	Bi-5s	Bi-6s	Bi-1b	Bi-2b	Bi-3b	Bi-4b	Bi-5b	Bi-6b
Acesulfame	< mql	9	16	33	n.a.	90	n.d.	n.d.	n.d.	< mql	n.a.	n.a.
Acetaminophen	< mql	19	17	95	n.a.	77	n.d.	n.d.	< mql	< mql	n.a.	n.a.
Amitriptyline	n.d.	< mql	< mql	15	n.a.	n.d.	n.d.	n.d.	< mql	3	n.a.	n.a.
Atrazine	< mql	< mql	< mql	< mql	n.a.	< mql	< mql	< mql	< mql	< mql	n.a.	n.a.
Bezafibrate	< mql	< mql	7	18	n.a.	5	< mql	< mql	< mql	< mql	n.a.	n.a.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Caffeine	44	47	64	171	n.a.	395	57	41	65	28	n.a.	n.a.
Carbamazepin	< mql	4	17	38	n.a.	15	< mql	< mql	3	4	n.a.	n.a.
Ciprofloxacin	n.d.	n.d.	3	540	n.a.	< mql	n.d.	n.d.	n.d.	298	n.a.	n.a.
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Clomipramine	n.d.	n.d.	< mql	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Diclofenac	n.d.	< mql	27	78	n.a.	28	n.d.	< mql	23	23	n.a.	n.a.
Diuron	n.d.	< mql	12	41	n.a.	15	n.d.	n.d.	n.d.	< mql	n.a.	n.a.
Eprosartan	n.d.	n.d.	13	36	n.a.	39	< mql	n.d.	n.d.	< mql	n.a.	n.a.
Genistein	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Genistin	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Irbesartan	n.d.	< mql	39	280	n.a.	39	< mql	< mql	7	13	n.a.	n.a.
Isoproturon	< mql	< mql	< mql	< mql	n.a.	< mql	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Ketoprofen	n.d.	n.d.	n.d.	10	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Losartan	n.d.	< mql	11	38	n.a.	16	n.d.	n.d.	< mql	6	n.a.	n.a.
Methylparaben	< mql	< mql	n.d.	< mql	n.a.	n.d.	< mql	< mql	n.d.	< mql	n.a.	n.a.
Norfloxacin	n.d.	n.d.	< mql	4	n.a.	n.d.	n.d.	n.d.	< mql	< mql	n.a.	n.a.
Nortriptyline	n.d.	n.d.	< mql	6	n.a.	n.d.	< mql	n.d.	< mql	< mql	n.a.	n.a.
OBT	267	272	273	218	n.a.	635	1434	810	553	475	n.a.	n.a.
PFBS	n.d.	n.d.	3	9	n.a.	3	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
PFOA	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
PFOS	n.d.	< mql	13	28	n.a.	13	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Phenytoin	6	100	355	1401	n.a.	10	< mql	< mql	74	84	n.a.	n.a.
Progesterone	< mql	< mql	< mql	< mql	n.a.	< mql	< mql	< mql	< mql	< mql	n.a.	n.a.
Propranolol	n.d.	< mql	< mql	10	n.a.	< mql	n.d.	n.d.	< mql	< mql	n.a.	n.a.
Simazine	< mql	n.d.	< mql	n.d.	n.a.	< mql	< mql	n.d.	< mql	< mql	n.a.	n.a.
Sucralose	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Sulfadiazine	< mql	10	19	33	n.a.	1	< mql	< mql	< mql	n.d.	n.a.	n.a.
Sulfamethoxazole	5	27	24	108	n.a.	9	n.d.	n.d.	< mql	< mql	n.a.	n.a.
Telmisartan	6	29	122	531	n.a.	57	n.d.	< mql	33	47	n.a.	n.a.
Testosterone	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.
Trimethoprim	3	18	55	485	n.a.	14	< mql	< mql	16	14	n.a.	n.a.
Valsartan	n.d.	< mql	47	89	n.a.	175	< mql	n.d.	n.d.	15	n.a.	n.a.

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table XIII. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Bilbao in summer 2016.

Analyte	Bilbao estuary surface water (Bi)						Bilbao estuary bottom water (Bi)					
	Bi-1s	Bi-2s	Bi-3s	Bi-4s	Bi-5s	Bi-6s	Bi-1b	Bi-2b	Bi-3b	Bi-4b	Bi-5b	Bi-6b
Acesulfame	n.d.	< mql	23	74	28	49	n.d.	n.d.	20	20	9	n.a.
Acetaminophen	< mql	< mql	19	27	25	43	n.d.	< mql	9	9	< mql	n.a.
Amitriptyline	n.d.	n.d.	< mql	36	n.d.	n.d.	< mql	< mql	2	6	< mql	n.a.
Atrazine	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.a.
Bezafibrate	< mql	4	9	67	14	4	< mql	< mql	10	13	4	n.a.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Caffeine	29	25	117	90	66	209	48	16	61	50	35	n.a.
Carbamazepin	< mql	5	14	93	19	14	< mql	< mql	14	18	6	n.a.
Ciprofloxacin	n.d.	< mql	4	111	4	< mql	n.d.	< mql	11	37	7	n.a.
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Clomipramine	n.d.	n.d.	n.d.	2	n.d.	n.d.	< mql	n.d.	< mql	n.d.	n.d.	n.a.
Diclofenac	1	4	10	265	17	3	< mql	< mql	14	14	3	n.a.
Diuron	< mql	7	14	75	19	7	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Eprosartan	n.d.	8	21	183	31	19	n.d.	n.d.	22	31	10	n.a.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Imipramine	n.d.	n.d.	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Irbesartan	4	18	53	494	88	28	n.d.	< mql	56	90	16	n.a.
Isoproturon	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	< mql	< mql	< mql	n.a.
Ketoprofen	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Losartan	< mql	8	24	183	37	21	n.d.	< mql	24	37	10	n.a.
Methylparaben	50	19	11	< mql	< mql	< mql	25	12	8	9	9	n.a.
Norfloxacin	n.d.	n.d.	n.d.	62	< mql	n.d.	n.d.	n.d.	< mql	25	n.d.	n.a.
Nortriptyline	< mql	< mql	< mql	6	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.a.
OBT	691	401	472	606	113	697	3977	1755	174	145	38	n.a.
PFBS	< mql	11	26	158	39	51	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
PFOA	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
PFOS	n.d.	< mql	12	24	n.d.	7	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	2	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Phenytoin	n.d.	< mql	9	39	11	10	n.d.	4	9	11	6	n.a.
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Propranolol	n.d.	< mql	4	17	4	< mql	n.d.	n.d.	4	5	n.d.	n.a.
Simazine	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Sucralose	< mql	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.a.
Sulfadiazine	6	13	31	29	22	21	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Sulfamethoxazole	< mql	< mql	< mql	11	< mql	< mql	n.d.	n.d.	< mql	< mql	< mql	n.a.
Telmisartan	10	43	126	969	238	94	< mql	< mql	126	185	44	n.a.
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Trimethoprim	< mql	< mql	4	21	5	< mql	n.d.	n.d.	4	5	2	n.a.
Valsartan	26	32	66	248	65	66	n.d.	n.d.	60	60	32	n.a.

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table XIV. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Plentzia and Urdaibai in summer 2016.

Analyte	Plentzia estuary (Pl)							Urdaibai estuary (Ur)			
	Pl-1	Pl-2	Pl-3	Pl-4	Pl-5	Pl-6	Pl-7	Ur-1	Ur-2	Ur-3	Ur-4
Acesulfame	n.d.	< mql	< mql	5	9	7	57	n.d.	< mql	41	22
Acetaminophen	n.d.	< mql	< mql	5	4	5	25	n.d.	n.d.	4	n.d.
Amitriptyline	n.d.	n.d.	n.d.	n.d.	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.
Atrazine	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.
Bezafibrate	n.d.	n.d.	< mql	< mql	< mql	< mql	3	n.d.	n.d.	< mql	n.d.
Butylparaben	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	88	86	80	39	221	83	44	28	67	103	120
Carbamazepin	n.d.	< mql	< mql	2	5	9	45	n.d.	2	14	n.d.
Ciprofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Clomipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diclofenac	n.d.	n.d.	n.d.	n.d.	< mql	< mql	< mql	n.d.	n.d.	< mql	n.d.
Diuron	< mql	< mql	< mql	< mql	2	3	13	n.d.	< mql	7	n.d.
Eprosartan	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	42	n.d.	n.d.	n.d.	n.d.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Irbesartan	n.d.	< mql	< mql	3	11	18	182	n.d.	< mql	12	n.d.
Isoproturon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.
Ketoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Losartan	n.d.	n.d.	n.d.	n.d.	< mql	< mql	26	n.d.	< mql	9	n.d.
Methylparaben	< mql	19	12	11	35	9	n.d.	5	6	5	n.d.
Norfloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nortriptyline	n.d.	< mql	< mql	n.d.	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.
OBT	23	20	17	19	18	17	1208	25	21	24	31
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	< mql	n.d.
PFOSA	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	n.d.
Phenytoin	n.d.	n.d.	n.d.	n.d.	< mql	< mql	13	< mql	< mql	20	n.d.
Progesterone	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.
Propranolol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.
Simazine	n.d.	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	< mql
Sucralose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	< mql	n.d.
Sulfadiazine	n.d.	< mql	< mql	2	3	3	2	n.d.	n.d.	< mql	n.d.
Sulfamethoxazole	n.d.	n.d.	n.d.	< mql	< mql	< mql	7	n.d.	n.d.	< mql	n.d.
Telmisartan	n.d.	< mql	2	4	10	20	83	< mql	4	14	n.d.
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trimethoprim	< mql	< mql	< mql	< mql	1	1	3	n.d.	n.d.	< mql	n.d.
Valsartan	n.d.	n.d.	n.d.	< mql	6	12	120	n.d.	< mql	149	n.d.

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table XV. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Bilbao in fall 2016.

Analyte	Bilbao estuary surface water (Bi)						Bilbao estuary bottom water (Bi)					
	Bi-1s	Bi-2s	Bi-3s	Bi-4s	Bi-5s	Bi-6s	Bi-1b	Bi-2b	Bi-3b	Bi-4b	Bi-5b	Bi-6b
Acesulfame	n.d.	< mql	< mql	< mql	< mql	39	n.d.	n.d.	< mql	< mql	< mql	< mql
Acetaminophen	9	21	235	85	116	237	n.d.	n.d.	150	119	< mql	< mql
Amitriptyline	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.	< mql	11	n.d.	n.d.
Atrazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Bezafibrate	n.d.	n.d.	4	17	< mql	n.d.	n.d.	n.d.	< mql	15	< mql	n.d.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	51	85	217	231	292	588	27	220	99	40	44	72
Carbamazepin	1	2	6	17	4	5	n.d.	< mql	3	13	2	2
Ciprofloxacin	n.d.	47	65	90	51	48	n.d.	n.d.	57	62	52	53
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Clomipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diclofenac	< mql	21	74	276	47	78	n.d.	< mql	39	295	21	20
Diuron	n.d.	< mql	8	24	5	4	n.d.	n.d.	4	15	3	< mql
Eprosartan	n.d.	n.d.	28	65	19	25	n.d.	n.d.	< mql	56	11	n.d.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Irbesartan	5	11	65	214	26	40	n.d.	< mql	23	181	8	6
Isoproturon	n.d.	n.d.	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.
Ketoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Losartan	n.d.	< mql	20	48	15	19	n.d.	n.d.	12	51	6	n.d.
Methylparaben	30	66	n.d.	n.d.	n.d.	n.d.	< mql	34	n.d.	n.d.	n.d.	n.d.
Norfloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nortriptyline	n.d.	n.d.	1	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	2	n.d.	n.d.
OBT	421	612	487	413	162	152	1017	1572	185	375	474	267
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	n.d.	n.d.	< mql	< mql	n.d.	< mql	n.d.	n.d.	< mql	< mql	n.d.	n.d.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenytoin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Propranolol	n.d.	< mql	< mql	5	< mql	< mql	n.d.	n.d.	< mql	3	< mql	n.d.
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sucralose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sulfadiazine	n.d.	7	27	n.d.	17	24	n.d.	n.d.	n.d.	7	n.d.	n.d.
Sulfamethoxazole	7	16	125	214	22	< mql	n.d.	n.d.	43	227	9	< mql
Telmisartan	< mql	12	52	639	19	34	n.d.	< mql	17	49	< mql	10
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trimethoprim	n.d.	18	118	405	15	< mql	n.d.	< mql	36	310	< mql	< mql
Valsartan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table XVI. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Plentzia and Urdaibai in fall 2016.

Analyte	Plentzia estuary (Pl)							Urdaibai estuary (Ur)			
	Pl-1	Pl-2	Pl-3	Pl-4	Pl-5	Pl-6	Pl-7	Ur-1	Ur-2	Ur-3	Ur-4
Acesulfame	n.d.	n.d.	n.d.	n.d.	< mql	4	9	n.d.	< mql	42	<mql
Acetaminophen	n.d.	n.d.	n.d.	n.d.	< mql	12	39	n.d.	23	72	n.d.
Amitriptyline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Atrazine	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	n.d.
Bezafibrate	n.d.	n.d.	n.d.	n.d.	< mql	2	3	n.d.	n.d.	< mql	n.d.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	212	39	37	43	74	89	182	84	362	1077	80
Carbamazepin	n.d.	n.d.	n.d.	n.d.	2	2	3	< mql	2	3	n.d.
Ciprofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.	< mql	n.d.
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Clomipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	< mql	n.d.
Diclofenac	< mql	2	1	< mql	< mql	< mql	4	n.d.	19	35	n.d.
Diuron	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7	n.d.
Eprosartan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	14	23	n.d.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Irbesartan	< mql	2	2	2	7	9	20	2	9	24	n.d.
Isoproturon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Ketoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Losartan	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	3	< mql	8	15	n.d.
Methylparaben	65	14	25	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.
Norfloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nortriptyline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
OBT	431	735	501	169	352	440	4138	17	19	23	27
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	13	n.d.
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	n.d.	< mql	8	6	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenytoin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	< mql	< mql	n.d.
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Propranolol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sucralose	n.d.	n.d.	n.d.	n.d.	< mql	58	87	n.d.	n.d.	< mql	n.d.
Sulfadiazine	< mql	< mql	< mql	2	5	5	8	n.d.	n.d.	< mql	n.d.
Sulfamethoxazole	n.d.	n.d.	n.d.	n.d.	< mql	< mql	19	n.d.	n.d.	< mql	n.d.
Telmisartan	< mql	< mql	4	2	8	17	3	< mql	7	8	n.d.
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trimethoprim	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2	n.d.
Valsartan	n.d.	n.d.	n.d.	< mql	< mql	27	51	n.d.	84	219	n.d.

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table XVII. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Bilbao in winter 2017.

Analyte	Bilbao estuary surface water (Bi)						Bilbao estuary bottom water (Bi)					
	Bi-1s	Bi-2s	Bi-3s	Bi-4s	Bi-5s	Bi-6s	Bi-1b	Bi-2b	Bi-3b	Bi-4b	Bi-5b	Bi-6b
Acesulfame	< mql	82	103	191	71	147	n.d.	n.d.	< mql	< mql	n.d.	n.a.
Acetaminophen	< mql	46	98	15	30	49	n.d.	n.d.	37	< mql	n.d.	n.a.
Amitriptyline	n.d.	n.d.	3	17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Atrazine	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.a.
Bezafibrate	n.d.	n.d.	9	30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Caffeine	65	102	372	177	163	699	108	54	71	66	45	n.a.
Carbamazepin	1	2	16	52	18	7	n.d.	n.d.	< mql	< mql	< mql	n.a.
Ciprofloxacin	n.d.	< mql	9	78	< mql	< mql	n.d.	n.d.	< mql	7	< mql	n.a.
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Clomipramine	n.d.	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Diclofenac	n.d.	n.d.	115	650	89	74	n.d.	n.d.	n.d.	47	n.d.	n.a.
Diuron	n.d.	n.d.	17	81	n.d.	< mql	n.d.	n.d.	n.d.	< mql	n.d.	n.a.
Eprosartan	n.d.	n.d.	23	115	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Irbesartan	7	12	101	438	29	33	n.d.	n.d.	11	11	5	n.a.
Isoproturon	n.d.	n.d.	< mql	< mql	< mql	< mql	n.d.	n.d.	< mql	< mql	n.d.	n.a.
Ketoprofen	n.d.	n.d.	n.d.	57	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Losartan	n.d.	n.d.	22	102	< mql	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Methylparaben	12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Norfloxacin	n.d.	n.d.	< mql	15	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	n.a.
Nortriptyline	n.d.	n.d.	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.	n.a.
OBT	1267	265	306	606	415	273	1498	2289	649	674	236	n.a.
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
PFOS	n.d.	n.d.	4	mql	< mql	< mql	n.d.	n.d.	< mql	< mql	< mql	n.a.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Phenytoin	n.d.	n.d.	n.d.	mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Propranolol	n.d.	n.d.	< mql	11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Sucralose	n.d.	63	694	330	< mql	< mql	n.d.	n.d.	n.d.	< mql	n.d.	n.a.
Sulfadiazine	< mql	9	33	49	20	28	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Sulfamethoxazole	< mql	63	227	mql	15	< mql	n.d.	n.d.	n.d.	< mql	n.d.	n.a.
Telmisartan	6	8	65	316	12	19	n.d.	n.d.	7	13	5	n.a.
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Trimethoprim	8	25	389	2046	< mql	< mql	n.d.	n.d.	20	26	n.d.	n.a.
Valsartan	n.d.	n.d.	n.d.	182	147	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table XVIII. Mean concentrations (ng/L) of the target analytes at each sampling point of the estuaries of Plentzia and Urdaibai in winter 2017.

Analyte	Plentzia estuary (Pl)							Urdaibai estuary (Ur)			
	Pl-1	Pl-2	Pl-3	Pl-4	Pl-5	Pl-6	Pl-7	Ur-1	Ur-2	Ur-3	Ur-4
Acesulfame	n.d.	n.d.	n.d.	< mql	12	19	46	< mql	79	126	< mql
Acetaminophen	n.d.	n.d.	< mql	10	9	34	39	< mql	56	73	n.d.
Amitriptyline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Atrazine	n.d.	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Bezafibrate	n.d.	n.d.	n.d.	n.d.	2	3	6	n.d.	n.d.	< mql	n.d.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	362	45	55	153	225	238	349	188	471	1092	76
Carbamazepin	n.d.	n.d.	n.d.	1	2	4	5	n.d.	n.d.	2	n.d.
Ciprofloxacin	n.d.	n.d.	n.d.	n.d.	< mql	< mql	< mql	n.d.	n.d.	< mql	n.d.
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Clomipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diclofenac	n.d.	n.d.	n.d.	< mql	17	19	22	< mql	19	26	n.d.
Diuron	n.d.	n.d.	< mql	< mql	3	5	5	< mql	3	9	n.d.
Eprosartan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	28	n.d.
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Irbesartan	< mql	3	4	10	20	34	62	2	10	21	n.d.
Isoproturon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Ketoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Losartan	n.d.	n.d.	n.d.	n.d.	< mql	< mql	< mql	< mql	9	16	n.d.
Methylparaben	34	23	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Norfloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nortriptyline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
OBT	1361	414	494	234	141	64	39	550	577	370	< mql
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
PFOSA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenytoin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Propranolol	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.	n.d.	n.d.
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	n.d.
Sucralose	n.d.	n.d.	n.d.	< mql	< mql	58	244	n.d.	n.d.	< mql	n.d.
Sulfadiazine	n.d.	< mql	1	8	17	29	50	n.d.	n.d.	< mql	n.d.
Sulfamethoxazole	n.d.	< mql	< mql	< mql	< mql	3	5	n.d.	n.d.	< mql	n.d.
Telmisartan	< mql	< mql	< mql	1	8	7	21	< mql	10	13	n.d.
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trimethoprim	n.d.	< mql	< mql	< mql	< mql	< mql	2	n.d.	n.d.	2	n.d.
Valsartan	n.d.	n.d.	n.d.	< mql	22	58	213	n.d.	n.d.	< mql	n.d.

n.a.= not adquired; n.d.= non detected, < mql= below method quantification limit

Table XIX. Mean concentrations (ng/L) obtained from the grab sampling (days 0th, 14th and 28th) and POCIS (1st deployment and 2nd deployment) for the estuary of Bilbao (sampling point Bi-2 and Bi-4). Grab sampling and TWA concentrations in water (ng/L) are only presented for compounds for whose Rs values were previously determined in **chapter 4.**

Analyte	Bilbao estuary (Bi-2)					Bilbao estuary (Bi-4)				
	Grab sampling			POCIS		Grab sampling			POCIS	
	0th	14th	28th	1	2	0th	14th	28th	1	2
Acesulfame	< mql	< mql	< mql	n.d.	n.d.	169	128	546	203	177
Acetaminophen	< mql	< mql	< mql	n.d.	n.d.	< mql	< mql	< mql	n.d.	n.d.
Amitriptyline	6	6	6	7	5	42	31	35	14	29
Atrazine	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	< mql	< mql	< mql	< mql
Bezafibrate	< mql	< mql	< mql	1	2	34	49	52	33	56
Butylparaben	< mql	< mql	< mql	n.d.	n.d.	< mql	< mql	< mql	n.d.	n.d.
Caffeine	82	89	133	129	74	256	142	72	341	427
Carbamazepibe	37	36	37	46	36	53	84	44	137	173
Diuron	< mql	< mql	< mql	38	26	34	49	44	224	250
Irbesartan	60	46	47	64	89	1450	1499	1024	1359	1645
Ketoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	< mql	n.d.	n.d.
OBT	40	75	123	65	59	38	39	50	76	98
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	33	< mql	< mql	36	48
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	< mql	< mql	< mql	< mql
PFOS	n.d.	n.d.	n.d.	19	15	n.d.	n.d.	n.d.	7	13
Phenytoin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5	2
Telmisartan	18	17	6	9	19	2007	1995	3040	2160	3118

n.d.= non detected, < mql= below method quantification limit

Table XX. Mean concentrations (ng/L) obtained from the grab sampling (days 0th, 14th and 28th) and POCIS (1st deployment and 2nd deployment) for the estuary of Plentzia (Pl-3) and Urdaibai (Ur-3). Grab sampling and TWA concentrations in water (ng/L) are only presented for compounds for whose Rs values were previously determined in **chapter 4**.

Analyte	Plentzia estuary (Pl-3)					Urdaibai estuary (Ur-3)				
	Grab sampling			POCIS		Grab sampling			POCIS	
	0 th	14 th	28 th	1	2	0 th	14 th	28 th	1	2
Acesulfame	< mql	22	28	27	38	51	1279	2232	433	836
Acetaminophen	< mql	< mql	< mql	n.d.	n.d.	< mql	86	66	13	21
Amitriptyline	< mql	< mql	< mql	1	2	< mql	< mql	< mql	3	2
Atrazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Bezafibrate	< mql	< mql	< mql	n.d.	n.d.	< mql	< mql	< mql	2	4
Butylparaben	< mql	< mql	< mql	n.d.	n.d.	< mql	< mql	< mql	n.d.	n.d.
Caffeine	22	174	290	416	363	95	1182	1508	932	1060
Carbamazepibe	< mql	7	6	8	8	< mql	37	47	14	22
Diuron	n.d.	n.d.	< mql	n.d.	n.d.	< mql	17	30	15	21
Irbesartan	2	43	45	17	19	3	64	130	52	100
Ketoprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3	4
OBT	150	346	459	230	530	126	63	23	32	42
PFBS	n.d.	n.d.	n.d.	16	21	n.d.	n.d.	n.d.	12	6
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	n.d.	n.d.	n.d.	6	8	n.d.	n.d.	134	87	250
Phenytoin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	< mql	8	n.d.	26	5
Telmisartan	< mql	56	94	36	67	44	1240	1660	809	1088

n.d.= non detected, < mql= below method quantification limit

Table XXI. Predicted no effect concentrations (PNEC) and chronic Risk Quotients (RQ) of the target compounds calculated for each effluent and estuary sample.

Analyte	PNEC (µg/L)	RQ effluent			RQ estuary			
		Galindo	Gorliz	Gernika	Surface Bilbao	Bottom Bilbao	Urdaibai	Plentzia
Acetaminophen	100	0.009	0.007	0.05	0.004	0.002	0.003	0.0005
Amitriptyline	10	0.01	0.002	0.004	0.004	0.001	0.0003	<mql
Atrazineb	1	<mql	<mql	0.02	<mql	<mql	<mql	<mql
Bezafibrate	0.001	132	40	101	67	15	8	11
Caffeine	0.05	2	6	1320	14	4	22	7
Carbamazepine	0.2	0.7	0.5	2	0.5	0.09	0.07	0.2
Ciprofloxacin	5	0.8	0.6	0.9	0.1	0.06	0.003	<mql
Clofibric acid	400	0.00002	<mql	<mql	<mql	<mql	<mql	<mql
Diclofenacc	0.005	382	137	386	130	59	7	4
Diuronb	0.0233	9	9	15	3	0.6	0.4	0.6
Genistein	0.013	<mql	<mql	46	<mql	<mql	<mql	<mql
Isoproturonb	0.004	1	1	<mql	<mql	<mql	<mql	<mql
Methylparaben	5	<mql	<mql	0.04	0.01	0.007	0.001	0.01
Norfloxacin	1.2	0.4	0.03	0.2	0.05	0.02	0.004	<mql
PFOS	0.1	0.09	0.02	2	0.3	<mql	<mql	0.08
Progesterone	1	0.01	0.03	0.06	<mql	<MQL	<mql	<mql
Propranolol	0.636	0.05	0.03	0.05	0.03	0.008	<mql	0.002
Simazineb	1.2	<mql	0.004	0.0009	<mql	<mql	<mql	<mql
Sulfadiazine	0.1	55	3	0.2	0.5	0.07	<mql	0.5
Sulfamethoxazole	2.4	4	0.1	0.08	0.09	0.09	<mql	0.008
Trimethoprim	3.14	2	0.03	0.02	0.7	0.1	0.001	0.002

PFOSA and genistin were not taken into consideration since they were <MQL in all the measured samples. There were no NOECs values for acesulfame, butylparaben, clomipramine, eprosartan, glycitin, imipramine, irbesartan, ketoprofen, losartan, nortryptyline, OBT, PFBS, PFOSA, phenytoin, sucralose, telmisartan, testosterone and valsartan.

Table XXII. Predicted no effect concentrations (PNEC) and acute Risk Quotients (RQ) of the target compounds calculated for each effluent and estuary sample.

Analyte	PNEC (µg/L)	RQ effluent			RQ estuary			
		Galindo	Gorliz	Gernika	Surface Bilbao	Bottom Bilbao	Urdaibai	Plentzia
Acesulfame	2068	0.0006	0.0006	0.006	0.00009	0.00001	0.00006	0.00003
Acetaminophen	12	0.07	0.06	0.5	0.04	0.01	0.03	0.004
Amitriptyline	0.9	0.1	0.02	0.04	0.04	0.01	0.003	<mql
Atrazine[b]	0.004	<mql	<mql	4	<mql	<mql	<mql	<mql
Bezafibrate	0.3	0.5	0.2	0.4	0.3	0.06	0.03	0.04
Butylparaben	11	<mql	0.0008	0.009	<mql	<mql	0.0002	<mql
Caffeine	0.4	0.2	0.7	150	2	0.5	2	0.8
Carbamazepine	33	0.004	0.003	0.01	0.003	0.0005	0.0004	0.001
Ciprofloxacin	7	0.6	0.5	0.7	0.08	0.04	0.003	<mql
Clofibric acid	0.3	0.02	<mql	<mql	<mql	<mql	<mql	<mql
Diclofenac[c]	0.04	43	16	44	15	7	0.8	0.5
Diuron[b]	0.0007	292	292	499	116	21	14	19
Eprosartan	0.0002	1695	2850	4394	915	280	140	210
Genistein	0.4	<mql	<mql	2	<mql	<mql	<mql	<mql
Irbesartan	0.002	637	375	470	247	91	14	91
Isoproturon[b]	0.005	0.8	1.0	<mql	<mql	<mql	<mql	<mql
Ketoprofen	0.3	1	0.05	1	0.2	<mql	0.01	<mql
Losartan	0.04	7	17	22	4	1	0.4	1
Methylparaben	91	<mql	<mql	0.002	0.0007	0.0004	0.0001	0.0007
Norfloxacin	0.04	12	1	7	2	0.7	0.1	<mql
OBT	11	0.02	0.02	0.10	0.1	0.4	0.06	0.4
PFBS	269	0.0007	<mql	0.008	0.0006	<mql	0.00005	<mql
PFOS	1	0.008	0.002	0.2	0.3	<mql	<mql	0.007
Phenytoin	9	0.3	0.01	0.1	0.15	0.01	0.00	0.00
Progesterone	6415	0.000002	0.000004	0.00001	<mql	<mql	<mql	<mql
Propranolol	0.2	0.1	0.08	0.1	0.07	0.02		0.004
Simazine[b]	0.0006	<mql	7	2	<mql	<mql	<mql	<mql
sucralose	10951640	0.0000001	0.0000004	0.0000001	0.0000001	<mql	0.00000002	0.00000002
Sulfadiazine	0.1	41	2	0.2	0.4	0.05	<mql	0.4
Sulfamethoxazole	0.5	17	0.5	0.4	0.4	0.4	<mql	0.04
Telmisartan	0.000003	438601	255286	402708	323000	61667	14000	27667
Testosterone	0.2	<mql	<mql	0.03	<mql	<mql	<mql	<mql
Trimethoprim	27	0.2	0.003	0.002	0.08	0.01	0.0001	0.0002
Valsartan	0.1	4	19	99	3	1	2	2

PFOSA and genistin were not taken into consideration since they were <MQL in all the measured sampled. There were no L(E)C50s values for clomipramine, imipramine and notriptyline.

Figure II *Compound Discoverer (2.1) workflow*

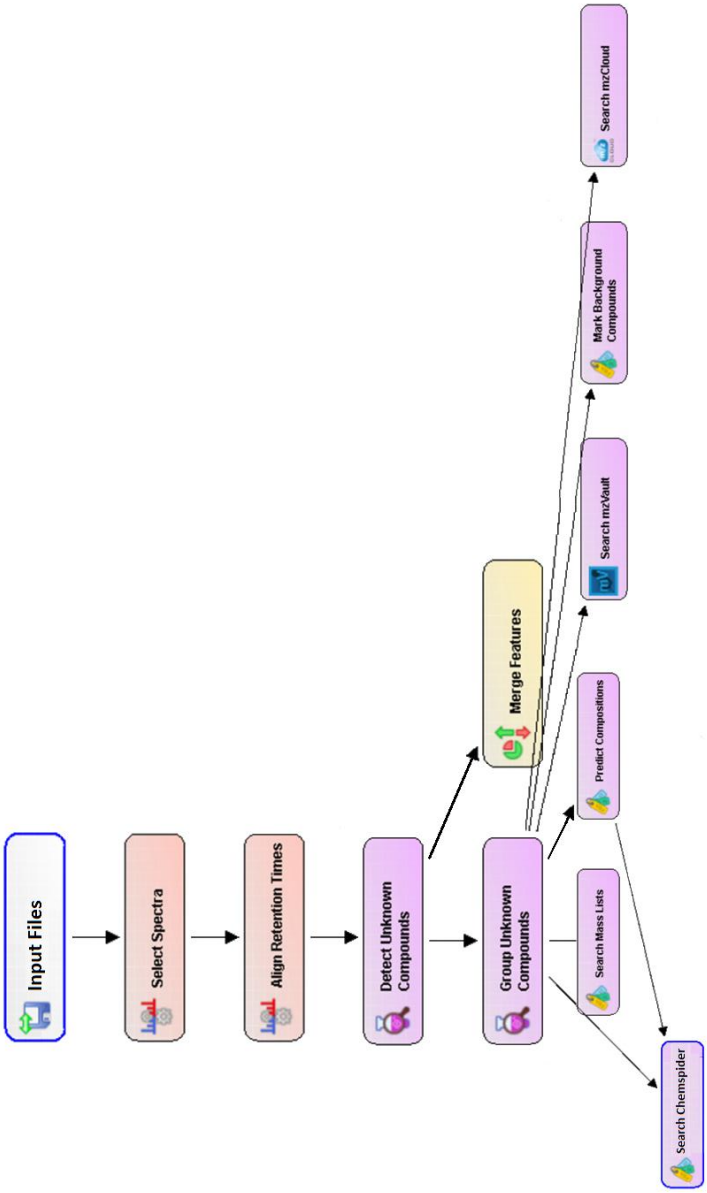


Table XXIII. Compound Discoverer (2.1) workflow settings and parameters.

1. Select Spectra	1.1 General settings	- Precursor Selection: Use MS (N - 1) Precursor - Use New Precursor Reevaluation: True - Use Isotope Pattern in Precursor Reevaluation: True - Store Chromatograms: False
	1.2 Spectrum properties Filter	- Lower RT Limit: 0 - Upper RT Limit: 0 - First Scan: 0 - Last Scan: 0 - Ignore Specified Scans: (not Specified) - Lowest Charge State: 0 - Highest Charge State: 0 - Min. Precursor Mass: 100 Da - Max. Precursor Mass: 5000 Da - Total Intensity Threshold: 0 - Minimum Peak Count: 1
	1.3 Scan event Filters	- Mass Analyzer: (not Specified) - MS Order: Any - Activation Type: (not Specified) - Min. Collision Energy: 0 - Max. Collision Energy: 1000 - Scan Type: Any - Polarity Mode: (not Specified)
	1.4 peak filters	- S/N Threshold (FT-only): 1.5
	1.5. Replacements for Unrecognized Properties	- Unrecognized Charge Replacements: 1 - Unrecognized Mass Analyser Replacements: ITMS - Unrecognized MS Order Replacements: MS2 - Unrecognized Activation Type Replacements: CID - Unrecognized Polarity Replacements: + - Unrecognized MS Resolution@200 Replacements: 60000 - Unrecognized MSn Resolution@200 Replacements: 30000
2. Align Retention times	2.1. General Settings	- Alignment Model: Adaptive curve - Alignment Fallback: Use Linear Model - Maximum Shift [min]: 2 - Shift Reference File: True - Mass Tolerance: 5 ppm - Remove Outlier: True
3. Detect Unknown Compounds	3.1. General Settings	- Mass Tolerance [ppm]: 5 ppm - Intensity Tolerance [%]: 30 - S/N Threshold: 3 - Min. Peak Intensity: 500000 - Ions: [M+Cl]-1; [M+FA-H]-1; [M+H]+1; [M+H+MeOH]+1; [M+K]+1; [M+Na]+1; [M-H]-1; [M-H-H2O]-1 - Base Ions: [M+H]+1; [M-H]-1 - Min. Element Counts: C H - Max. Element Counts: C90 H190 Br3 Cl4 F20 K2 N10 Na2 O18 P3 S5
	3.2. Peak Detection	- Filter Peaks: True - Max. Peak Width [min]: 0.8 - Remove Singlets: True - Min. # Scans per Peak: 3 - Min. # Isotopes: 1
4. Merge Features	4.1 Peak consolidation	-mass tolerance: 5 ppm - RT Tolerance 0.1 min
5. Group Unknown Compounds	5.1. Compound Consolidation	- Mass Tolerance: 5 ppm - RT Tolerance [min]: 0.5
	5.2. Fragment Data Selection	- Preferred Ions: [M+H]+1; [M-H]-1
6 Search ChemSpider	6.1. Search Settings	Database(s): ACToR: Aggregated Computational Toxicology Resource; DrugBank; EAWAG BIOcatalysis/Biodegradation Databse; EPA DSSTox; EPA Toxcast; FDA UNII-NLMBioCyc; KEGG; Mass Bank

Table XXIII. Compound Discoverer (2.1) workflow settings and parameters.

6 Search ChemSpider	6.1. Search Settings	Database(s): ACToR: Aggregated Computational Toxicology Resource; DrugBank; EAWAG BIOcatalysis/Biodegradation Databse; EPA DSSTox; EPA Toxcast; FDA UNII-NLMBioCyc; KEGG; Mass Bank
	6.1. Search Settings	- Mass Tolerance: 5 ppm - Max. # of results per compound: 100 - Max. # of Predicted Compositions to be searched per Compound: 3 - Result Order (for Max. # of results per compound): Order By Reference Count (DESC)
	6.2. Predict Composition	- Check All Predicted Compositions: True
7. Search Mass Lists	7.1. Search Settings	- Input file(s): \EFS HRAM Compound Database_OZZ.csv - Show extra Fields as Columns: False - Consider Retention Time: True - RT Tolerance : 0.5 - Mass Tolerance: 5 ppm
8.Predict Composition	8.1. Prediction Settings	Mass Tolerance: 5 ppm - Min. Element Counts: C H - Max. Element Counts: C90 H190 Br3 Cl4 F20 K2 N10 Na2 O18 P3 S5 - Min. RDBE: 0 - Max. RDBE: 40 - Min. H/C: 0.1 - Max. H/C: 3.5 - Max. # Candidates: 10 - Max. # Internal Candidates: 200
	8.2. Pattern Matching	Intensity Tolerance [%]: 30 - Intensity Threshold [%]: 0.1 - S/N Threshold: 3 - Min. Spectral Fit [%]: 30 - Min. Pattern Cov. [%]: 80 - Use Dynamic Recalibration: True
	8.3. Fragments Matching	- Use Fragments Matching: True - Mass Tolerance: 5 ppm - S/N Threshold: 3
9. Seach mzVault	9.1 Seach settings	- mzVault Library: \mzVault February 2017.db - Compound Classes: All - Match Ion Activation Type: True - Match Ion Activation Energy: Match with Tolerance - Ion Activation Energy tolerance: 20 - Match Ionization Method: True - Apply Intensity Method: true - Remove precursor Ion: true - Precursor Mass Tolerance: 10 ppm - FT Fragment Mass Tolerance: 10 ppm - IT Fragment mass tolerance: 0.4 Da - Match Analyzer Type: True - Search Algorithm: HighChem HighRes - Match factor Threshold: 50 - Max. # results: 10
10. Mark BackGround compounds	10.1 Seach settings	- Max. Sample/Blank: 5 - Max Max. Blank/Sample: 0 - Hide Background: True

Table XXIII. Compound Discoverer (2.1) workflow settings and parameters.

11. Search mzCloud	11.1. Search Settings	- Compound Classes: All - Match Ion Activation Type: True - Match Ion Activation Energy: Match with Tolerance - Ion Activation Energy Tolerance: 20 - Apply intensity threshold: True - Precursor Mass Tolerance: 10 ppm - FT Fragment Mass Tolerance: 10 ppm - IT Fragment Mass Tolerance: 0.4 Da - Search Algorithm: Cosine - Similarity Search: Similarity Forward --Library: Reference - Post Processing: Recalibrated - Match factor threshold: 50 - Max. # results per compound and spectrum: 20

Table XXIV. Information of the set compounds used to calibrate the fractionation and as reference compounds in the non-target analysis. Recovery, log P, log $D_{(pH=3)}$, C_{18} column fractionation in which each compound appear and they ionization mode and retention times in the two LC-HRMS systems used for the analysis.

Name	Recovery (%)	C_{18} fractionation			LC-HRMS	
		LogP[a]	LogD[b] (pH=3)	Fraction[c]	Ionization	RT (min)
4-Chlorophenol	65±8	2.3	2.3	F13	[M-H]-	5.63
4-hydroxytamoxifen	74±12	5.7	2.5	F14	[M+H]+	7.61
Acesulfame	62±9	-0.6	-0.8	F3	[M-H]-	0.97
Acetaminophen	64±9	0.9	0.9	F8	[M+H]+	1.23
Acetamiprid	60±6	1.1	-0.1	F8	[M+H]+	1.23
Acetochlor	48±6	3.5	3.5	F15	[M+H]+	9.77
Acyclovir	72±9	-1.0	-1.3	F2	[M+H]+	0.73
Alachlor	79±26	3.6	3.6	F16	[M+H]+	9.73
Albendazole	65±13	3.2	2.2	F13	[M+H]+	5.18
Amantadine	16±2	1.5	-1.6	F6	[M+H]+	1.56
Ambroxol	74±12	2.7	-0.6	F5	[M+H]+	3.10
Ametryn	60±12	2.6	0.6	F11	[M+H]+	4.76
Amiodarone	56±6	7.6	4.1	F16	[M+H]+	9.55
Amitriptyline	62±7	4.8	1.3	F13	[M+H]+	6.32
Amoxicillin	69±8	-2.3	-2.6	F4	[M+H]+	1.42
Ampicillin	76±6	-2.0	-2.3	F5	[M+H]+	1.42
Anastrozole	76±8	3.0	3.0	F15	[M+H]+	6.54
Atenolol	71±13	0.4	-2.8	F3	[M+H]+	0.73
Atorvastatin	74±12	5.4	5.4	F18	[M-H]-	8.04
Atrazine	89±18	2.2	1.0	F12	[M+H]+	6.40
Azelastine	79±14	4.0	0.5	F10	[M+H]+	6.14
Azithromycin	70±15	2.4	-4.6	F7	[M+H]+	2.97
Azoxystrobin	64±16	4.2	4.2	F16	[M+H]+	9.54
Bendiocarb	76±9	1.6	1.6	F13	[M+H]+	6.25
Bentazone	69±8	0.8	0.1	F9	[M-H]-	1.91
Benzethonium	73±6	-0.5	-0.5	F10	M+	10.50
Benzothiazole	54±7	2.1	2.1	F11	[M+H]+	4.85
Bezafibrate	69±6	4.0	3.9	F16	[M+H]+	7.93
Bicalutamide	75±8	2.7	2.7	F14	[M+H]+ ; [M-H]-	8.77
Bisoprolol	76±8	2.2	-1.0	F5	[M+H]+	3.48
Boscalid	61±14	4.9	4.9	F17	[M+H]+	9.43
Bosentan	73±17	4.9	4.9	F17	[M+H]+	9.31
Bupropion	82±18	3.3	0.0	F8	[M+H]+	3.38
Butylparaben	75±5	3.0	3.0	F12	[M+H]+	2.89
Caffeine	59±4	-0.5	-0.5	F7	[M+H]+	1.61
Captopril	53±5	0.7	0.7	F12	[M+H]+	2.67
Carbamazepine	76±5	2.8	2.8	F14	[M+H]+	5.76
Carbaryl	75±16	2.5	2.5	F14	[M+H]+	6.76
Carbendazim	66±12	1.8	0.7	F9	[M+H]+	1.27
Celecoxib	77±13	4.0	4.0	F16	[M+H]+ ; [M-H]-	10.54

Table XXIV. Information of the set compounds used to calibrate the fractionation and as reference compounds in the non-target analysis. Recovery, log P, log $D_{(pH=3)}$, C_{18} column fractionation in which each compound appear and they ionization mode and retention times in the two LC-HRMS systems used for the analysis.

Name	Recovery (%)	C_{18} fractionation			LC-HRMS	
		LogP[a]	LogD[b] (pH=3)	Fraction[c]	Ionization	RT (min)
Cetirizine	74±14	0.9	0.4	F10	$[M+H]^+$; $[M-H]^-$	6.55
Chloridazon	68±15	1.1	1.1	F12	$[M+H]^+$	3.22
Chloroxuron	67±16	3.4	3.4	F15	$[M+H]^+$	8.92
Chlortoluron	60±15	2.4	2.4	F13	$[M+H]^+$	6.43
Ciprofloxacin	32±3	-0.8	-1.7	F5	$[M+H]^+$	1.93
Clarithromycin	55±8	3.2	-0.3	F10	$[M+H]^+$	6.21
Clofibric acid	60±7	2.9	2.7	F11	$[M-H]^-$	3.10
Clomazone	54±8	2.9	2.9	F14	$[M+H]^+$	7.80
Clomipramine	54±6	4.9	1.4	F14	$[M+H]^+$	6.99
Clonidine	89±12	2.5	0.1	F8	$[M+H]^+$	1.38
Clopidogrel	62±15	4.0	2.3	F14	$[M+H]^+$	8.16
Clozapine	69±17	3.4	-1.0	F6	$[M+H]^+$	3.71
Cortisone	64±21	1.7	1.7	F13	$[M+H]^+$	5.12
Cotinine	76±3	0.2	-0.7	F5	$[M+H]^+$	0.73
Crotamiton	82±9	3.1	3.1	F15	$[M+H]^+$	7.60
Cyclophosphamide	60±16	0.1	0.1	F9	$[M+H]^+$	3.98
Cyproterone	79±17	3.2	3.2	F15	$[M+H]^+$	8.85
DEET	67±23	2.5	2.5	F14	$[M+H]^+$	6.63
Desloratadine	79±6	4.0	-0.4	F5	$[M+H]^+$	2.76
Dexamethasone	81±5	1.7	1.7	F13	$[M+H]^+$	5.95
Diazepam	55±19	3.1	2.8	F14	$[M+H]^+$	7.84
Dichlorvos	69±7	1.4	1.4	F13	$[M+H]^+$	5.35
Diclofenac	80±8	4.3	4.2	F17	$[M+H]^+$	9.52
Didecyldimethylammonium	80±23	4.0	4.0	F16	$[M+H]^+$	10.25
Diflufenican	72±5	5.1	5.1	F18	$[M+H]^+$	14.10
Dimethachlor	15±2	2.6	2.6	F14	$[M+H]^+$	7.67
Dimethoate	42±3	0.3	0.3	F10	$[M+H]^+$	3.53
Diphenhydramine	16±2	3.7	0.2	F9	$[M+H]^+$	5.00
Diuron	74±12	2.5	2.5	F14	$[M+H]^+$	7.03
Dodemorph	60±12	5.3	1.8	F14	$[M+H]^+$	6.70
Domperidone	56±6	2.9	-0.5	F7	$[M+H]^+$	4.38
Drospirenone	62±7	3.4	3.4	F15	$[M+H]^+$	8.87
Duloxetine	69±8	4.2	1.0	F12	$[M+H]^+$	6.30
EDDP	76±6	4.6	1.1	F12	$[M+H]^+$	5.85
Efavirenz	76±8	4.5	4.5	F18	$[M+H]^+$	10.19
Eprosartan	61±13	3.8	4.0	F15	$[M+H]^+$	4.88
Erythromycin	74±12	2.6	-0.9	F6	$[M+H]^+$	5.05
Ethion	29±3	3.9	3.9	F18	$[M+H]^+$	22.74
Exemestane	79±14	3.9	3.9	F16	$[M+H]^+$	8.41
Fenoxycarb	70±15	3.3	3.3	F15	$[M+H]^+$	10.37

Table XXIV. Information of the set compounds used to calibrate the fractionation and as reference compounds in the non-target analysis. Recovery, log P, log $D_{(pH=3)}$, C_{18} column fractionation in which each compound appear and they ionization mode and retention times in the two LC-HRMS systems used for the analysis.

Name	Recovery (%)	C_{18} fractionation			LC-HRMS	
		LogP[a]	LogD[b] (pH=3)	Fraction[c]	Ionization	RT (min)
Fenpropidin	64±16	5.4	1.9	F13	$[M+H]^+$	7.25
Fenpropimorph	56±9	5.2	1.7	F13	$[M+H]^+$	7.40
Fenthion	16±2	3.8	3.8	F17	$[M+H]^+$	12.18
Finasteride	74±12	3.1	3.1	F15	$[M+H]^+$	7.86
Fluconazole	60±12	0.6	0.5	F11	$[M+H]^+$	2.70
Flufenoxuron	56±6	6.1	6.1	F19	$[M-H]^-$	22.21
Flumequine	62±7	2.4	2.4	F13	$[M+H]^+$	6.31
Flusilazole	69±8	4.7	4.6	F17	$[M+H]^+$	9.75
Flutamide	76±6	3.3	3.3	F15	$[M-H]^-$	9.15
Fluvoxamine	76±8	2.8	-0.3	F10	$[M+H]^+$	5.95
Furosemide	61±13	1.7	1.7	F12	$[M-H]^-$	3.33
Gabapentin	24±4	-1.3	-2.0	F3	$[M+H]^+$	1.15
Gemfibrozil	59±18	4.4	4.4	F17	$[M-H]^-$	9.63
Genistein	79±14	3.1	3.1	F14	$[M+H]^+$	6.21
Genistin	70±15	0.8	0.8	F9	$[M+H]^+$	3.04
Glibenclamide	64±16	3.8	3.8	F16	$[M+H]^+$	9.86
Glimepiride	76±9	3.1	3.1	F15	$[M+H]^+$	10.06
Glycitin	16±2	0.3	0.3	F8	$[M+H]^+$	2.17
Hexazinone	74±12	1.4	1.3	F13	$[M+H]^+$	4.78
Hydroxychloroquine	60±12	2.9	-2.0	F6	$[M+H]^+$	0.73
Ifosfamide	56±6	0.1	0.1	F12	$[M+H]^+$	3.98
Imatinib	62±7	4.4	0.0	F3	$[M+H]^+$	2.97
Imazalil	69±8	3.8	3.2	F5	$[M+H]^+$	5.88
Imidacloprid	76±6	0.9	-1.5	F9	$[M+H]^+$	3.57
Iminostilbene	76±8	3.8	3.8	F7	$[M+H]^+$	10.15
Imipramine	61±13	4.3	0.8	F14	$[M+H]^+$	6.03
Indometacin	74±12	3.5	3.5	F6	$[M+H]^+$;$[M-H]^-$	9.60
Iprodione	59±18	2.3	2.3	F17	$[M+H]^+$	10.21
Irbesartan	79±14	5.4	4.4	F12	$[M+H]^+$	6.53
Isoproturon	70±15	2.6	2.6	F15	$[M+H]^+$	6.85
Ketoconazole	64±16	4.2	2.6	F4	$[M+H]^+$	6.13
Ketoprofen	76±9	3.6	3.6	F15	$[M+H]^+$	17.04
Lenacil	16±2	1.8	1.8	F16	$[M+H]^+$	5.77
Lidocaine	74±12	2.8	-0.6	F14	$[M+H]^+$	1.90
Linuron	60±12	2.7	2.7	F14	$[M+H]^+$	8.51
Lorazepam	56±6	3.5	3.5	F17	$[M+H]^+$	6.59
Losartan	62±7	5.0	4.2	F12	$[M+H]^+$	6.59
Mebendazole	69±8	3.3	2.4	F7	$[M+H]^+$	5.38
Mebeverine	76±6	4.9	1.4	F14	$[M+H]^+$	6.49
Meclocycline	76±8	-4.8	-4.8	F15	$[M+H]^+$	4.68

Table XXIV. Information of the set compounds used to calibrate the fractionation and as reference compounds in the non-target analysis. Recovery, log P, log $D_{(pH=3)}$, C_{18} column fractionation in which each compound appear and they ionization mode and retention times in the two LC-HRMS systems used for the analysis.

Name	Recovery (%)	C_{18} fractionation			LC-HRMS	
		LogP[a]	LogD[b] (pH=3)	Fraction[c]	Ionization	RT (min)
Mecoprop	81±13	3.0	2.9	F16	$[M-H]^-$	3.10
Medroxyprogesterone	74±12	3.7	3.7	F13	$[M+H]^+$	8.94
Mefenamic acid	89±18	5.4	5.3	F13	$[M+H]^+$; $[M-H]^-$	10.52
Memantine	39±4	2.1	-1.0	F3	$[M+H]^+$	3.20
Metalaxyl	70±15	2.1	2.1	F14	$[M+H]^+$	6.77
Metamitron	64±16	0.4	0.2	F16	$[M+H]^+$	2.92
Metazachlor	76±9	3.0	2.9	F18	$[M+H]^+$	7.75
Metconazole	16±2	3.6	3.6	F5	$[M+H]^+$	9.56
Metformin	24±2	-0.9	-5.7	F13	$[M+H]^+$	0.70
Methiocarb	60±12	3.1	3.1	F8	$[M+H]^+$	8.37
Methotrexate	56±6	-0.2	-0.2	F14	$[M+H]^+$	1.07
Methylparaben	62±7	1.7	1.7	F15	$[M+H]^+$	4.21
Methylpirimiphos	69±8	3.0	1.3	F1	$[M+H]^+$	10.52
Metolachlor	56±6	3.5	3.5	F15	$[M+H]^+$	9.47
Metoprolol	66±8	1.8	-1.5	F5	$[M+H]^+$	2.45
Metribuzin	81±13	2.0	1.9	F11	$[M+H]^+$	5.48
Miconazole	74±12	6.0	5.4	F16	$[M+H]^+$	8.66
Mirtazapine	79±18	3.2	-1.3	F15	$[M+H]^+$	2.36
Montelukast	79±14	8.5	8.3	F15	$[M+H]^+$; $[M-H]^-$	22.12
Myclobutanil	70±15	3.7	3.6	F4	$[M+H]^+$	8.97
Mycophenolic acid	64±16	3.5	3.4	F11	$[M+H]^+$; $[M-H]^-$	7.29
Naproxen	76±9	3.0	3.0	F18	$[M+H]^+$; $[M-H]^-$	6.15
Nitrofurantoin	16±2	0.1	-2.5	F6	$[M+H]^+$; $[M-H]^-$	2.71
Norfloxacin	47±6	-1.0	-1.8	F20	$[M+H]^+$	1.75
Norgestimate	87±8	4.1	3.8	F15	$[M+H]^+$	11.11
Nortriptyline	16±2	4.4	1.2	F15	$[M+H]^+$	6.06
OBT	74±12	2.5	2.5	F14	$[M+H]^+$	4.10
Omeprazol	60±12	2.4	1.1	F15	$[M+H]^+$	3.15
Ondansetron	56±6	2.3	1.3	F7	$[M+H]^+$	3.53
Oryzalin	62±7	2.3	2.3	F10	$[M+H]^+$	10.35
Oxazolam	69±8	3.9	3.1	F6	$[M+H]^+$	3.10
Oxybutynin	76±6	4.4	0.9	F16	$[M+H]^+$	6.66
Parathion	76±8	3.3	3.3	F12	$[M+H]^+$	12.39
Paroxetine	71±13	3.1	-0.1	F11	$[M+H]^+$	5.70
Pendimethalin	74±12	4.8	4.8	F12	$[M+H]^+$	19.30
Pentoxifylline	69±18	0.2	0.2	F20	$[M+H]^+$	3.04
PFBS	79±14	2.6	0.3	F17	$[M-H]^-$	4.74
PFOA	70±15	5.1	1.6	F12	$[M-H]^-$	6.72
PFOS	64±16	5.4	3.1	F12	$[M-H]^-$	8.47
PFOSA	76±9	4.9	4.7	F15	$[M-H]^-$	11.73

Table XXIV. Information of the set compounds used to calibrate the fractionation and as reference compounds in the non-target analysis. Recovery, log P, log $D_{(pH=3)}$, C_{18} column fractionation in which each compound appear and they ionization mode and retention times in the two LC-HRMS systems used for the analysis.

Name	Recovery (%)	C_{18} fractionation			LC-HRMS	
		LogP[a]	LogD[b] (pH=3)	Fraction[c]	Ionization	RT (min)
Phenytoin	16±2	2.1	2.1	F16	$[M+H]^+$	6.00
Pindolol	74±12	1.7	-1.5	F8	$[M+H]^+$	1.84
Pipamperone	60±12	1.9	-4.4	F19	$[M+H]^+$	1.50
Pirimicarb	56±6	1.8	0.1	F8	$[M+H]^+$	2.28
Pravastatin	62±7	1.6	1.6	F18	$[M-H]^-$	3.81
Prednisone	69±8	1.7	1.7	F15	$[M+H]^+$	4.97
Primidone	76±6	1.1	1.1	F11	$[M+H]^+$	2.44
Prochloraz	76±8	3.6	3.5	F13	$[M+H]^+$	8.23
Progesterone	81±13	4.1	4.1	F5	$[M+H]^+$	9.95
Propachlor	64±12	2.4	2.4	F4	$[M+H]^+$	7.49
Propamocarb	19±3	0.8	-2.7	F10	$[M+H]^+$	1.08
Propanil	69±14	3.1	3.1	F12	$[M+H]^+$; $[M-H]^-$	7.95
Propiconazole	70±15	4.3	4.3	F13	$[M+H]^+$	9.96
Propofol	64±16	4.2	4.2	F11	$[M-H]^-$	10.83
Propranolol	76±9	2.6	-0.7	F15	$[M+H]^+$	7.95
Propyphenazone	16±2	2.3	2.3	F16	$[M+H]^+$	6.18
Propyzamide	74±12	3.2	3.2	F13	$[M+H]^+$	9.10
Prosulfocarb	60±12	4.2	4.2	F3	$[M+H]^+$	13.70
Pyrantel	56±6	2.0	-0.5	F15	$[M+H]^+$	2.12
Pyrazophos	62±7	3.1	3.1	F17	$[M+H]^+$	12.67
Quinmerac	69±8	2.7	2.7	F16	$[M+H]^+$	2.97
Quinoxyfen	76±6	5.0	4.1	F6	$[M+H]^+$	12.49
Raloxifene	76±8	5.7	2.6	F13	$[M+H]^+$; $[M-H]^-$	5.58
Ranitidine	71±13	1.0	-2.5	F15	$[M+H]^+$	0.74
Remifentanyl	74±12	1.5	-1.9	F17	$[M+H]^+$	4.00
Risperidone	79±18	2.6	-1.0	F6	$[M+H]^+$	3.64
Ropinirole	79±14	3.1	-0.4	F16	$[M+H]^+$	2.04
Roxithromycin	70±15	3.0	-0.6	F11	$[M+H]^+$	6.50
Sertraline	64±16	5.1	1.9	F16	$[M+H]^+$	6.87
Simazine	76±9	1.8	0.6	F14	$[M+H]^+$	4.92
Sotalol	16±2	-0.4	-3.2	F2	$[M+H]^+$	0.73
Spiroxamine	74±12	4.4	0.9	F4	$[M+H]^+$	7.05
Sulfadiazine	60±12	0.4	0.3	F7	$[M+H]^+$	1.85
Sulfamethazine	56±6	0.7	0.6	F7	$[M+H]^+$	1.85
Sulfamethoxazole	62±7	0.8	0.8	F6	$[M+H]^+$	3.95
Sulfapyridine	69±8	1.0	1.0	F13	$[M+H]^+$	2.01
Sulfathiazole	76±6	1.0	0.9	F10	$[M+H]^+$	1.95
Tamoxifen	76±8	6.4	2.9	F1	$[M+H]^+$	9.24
Tebuconazole	81±13	3.7	3.7	F12	$[M+H]^+$	9.12
Telmisartan	74±12	6.1	5.2	F9	$[M+H]^+$	6.62

Table XXIV. Information of the set compounds used to calibrate the fractionation and as reference compounds in the non-target analysis. Recovery, log P, log $D_{(pH=3)}$, C_{18} column fractionation in which each compound appear and they ionization mode and retention times in the two LC-HRMS systems used for the analysis.

Name	Recovery (%)	C_{18} fractionation			LC-HRMS	
		LogP[a]	LogD[b] (pH=3)	Fraction[c]	Ionization	RT (min)
Terbinafine	89±18	5.5	2.0	F7	$[M+H]^+$	7.65
Terbuthylazine	79±14	2.5	1.3	F9	$[M+H]^+$	8.04
Terbutryn	70±15	2.9	0.9	F11	$[M+H]^+$	6.02
Testosterone	64±16	3.4	3.4	F9	$[M+H]^+$	7.36
Thiabendazole	76±9	2.3	1.4	F11	$[M+H]^+$	1.48
Thiacloprid	16±2	2.1	2.0	F15	$[M+H]^+$	5.11
Thiamethoxam	74±12	1.3	1.0	F16	$[M+H]^+$	2.56
Thymol	60±12	3.4	3.4	F18	$[M-H]^-$	8.62
Tramadol	56±6	2.4	-1.1	F13	$[M+H]^+$	2.56
Triadimenol	62±7	3.3	3.2	F14	$[M+H]^+$	7.64
Triethylphosphate	69±8	1.2	1.2	F12	$[M+H]^+$	3.55
Trimethoprim	76±6	1.3	-0.2	F15	$[M+H]^+$	1.62
Triphenylphosphate	76±8	5.1	5.1	F11	$[M+H]^+$	12.22
Valsartan	61±13	5.3	5.3	F13	$[M+H]^+$; $[M-H]^-$	5.50
Verapamil	64±12	5.0	1.5	F11	$[M+H]^+$	6.49

a) LogP has been calculated with the JChem free software in excel.
b) LogD at pH = 3 (the pH of the fractionation step mobile phase) has been calculated with the JChem free software in excel.
c) If one compound appears in more than one fraction, only the mayor fraction has been included.