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

B. Huerta [a], S. Rodriguez-Mozaz [a,*], C. Nannou [a], L. Nakis [a], A. Ruhí [a,c], V. Acuña [a], S. Sabater [a,b], D. Barcelo [a,d]

[a] Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain
[b] Institute of Aquatic Ecology, University of Girona, Campus de Montivili, 17071 Girona, Spain
[c] Julie Ann Wrigley Global Institute of Sustainability, Arizona State University, Tempe AZ 85287, USA
[d] Water and Soil Quality Research Group, Department of Environmental Chemistry, IDAEA, CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

HIGHLIGHTS

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

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ABSTRACT

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

1. Introduction

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

* Corresponding author.
E-mail address: srodriguez@icra.cat (S. Rodriguez-Mozaz).

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

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

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

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

2. Materials and methods

2.1. Standards and solutions

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

2.2. Sample collection and pre-treatment

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

2.3. Water extraction

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

2.4. Biofilm extraction and clean-up

Initially, sonication was pre-selected as the possible extraction method, together with pressurized liquid extraction (PLE). Four solvents were tested, including methanol, methanol/water (1:1), methanol with 0.1% EDTA, and citric buffer (pH4)/acetonitrile (1:1, v/v), all based on the authors' previous experience with pharmaceutical and EDC extraction. To reduce the number of experiments and solvent consumption, the results of one of these solvents were compared in both extraction methods to find which one had the best extraction recoveries. For sonication extraction, 200 mg of freeze-dried biofilm was placed in a 14-mL Falcon tube with 10 mL of the corresponding solvent. Extraction entailed 3 cycles of 10 min, and the supernatant was collected in a

glass vial. The extraction protocol based on pressurized liquid extraction (PLE) was performed with ASE®350 (Thermo Scientific). Biofilm (200 mg) was placed in 10 mL cells together with diatomaceous earth to fill up the rest of the cell. Samples were extracted with the corresponding solvent during 3 cycles of 5 min at 60 °C.

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

2.5. Instrumental analysis

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

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

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

2.6. Calculation of bioaccumulation factors (BAFs)

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

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

3. Results

3.1. Biofilm extraction and purification

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

3.2. Method validation

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

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

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

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

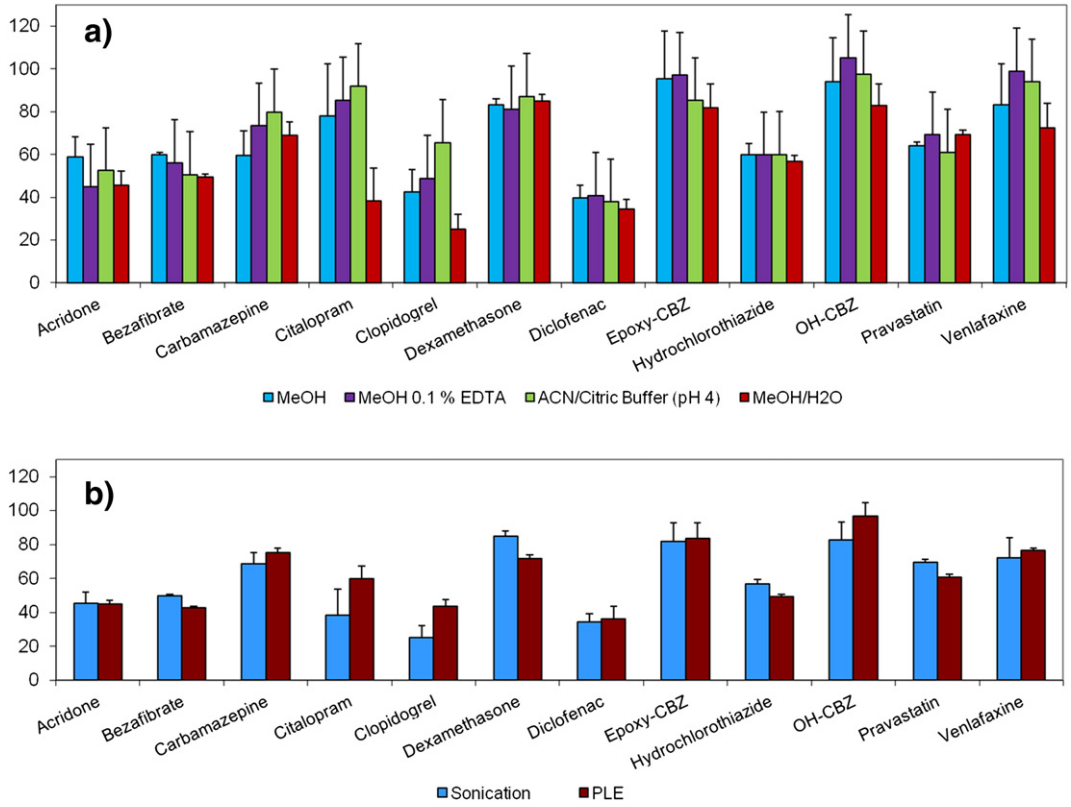

Fig. 1. Comparison of extraction efficiencies (%) for a) four different solvents by sonication and b) two extraction methods (PLE and sonication) using ACN/Citric buffer for selected pharmaceuticals.

3.3. Environmental samples

3.3.1. Water concentrations of PhACs and EDCs

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

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

Table 1
Mean percent recoveries (n = 3) of 13 EDCs at three spiking levels, and method detection and quantification limits (MDL, MQL) in biofilm (ng/g, dry weight).

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

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

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

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

3.3.2. Biofilm concentrations of PhACs and EDCs

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

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

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

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

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

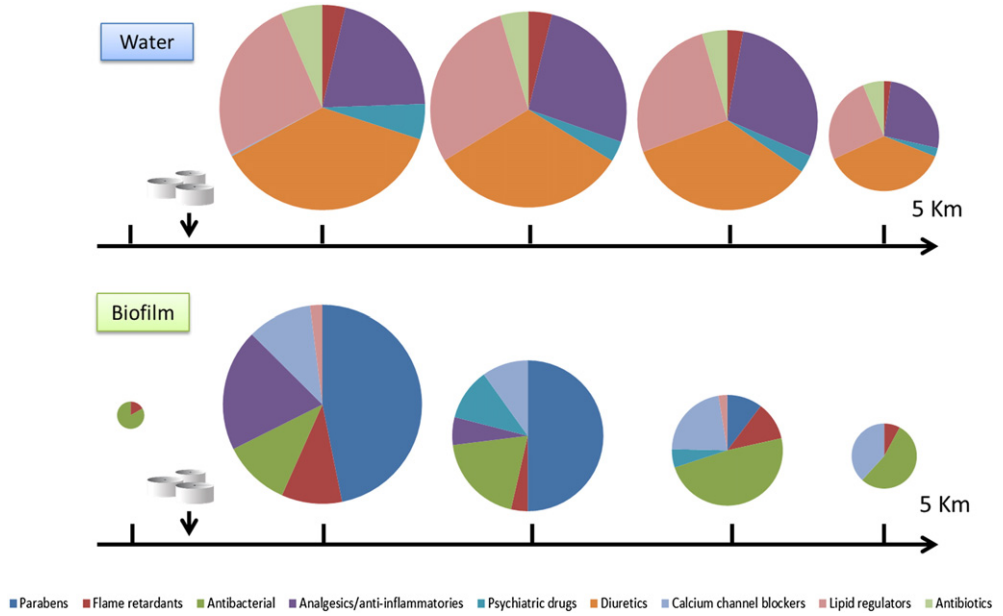

Fig. 2. Relative presence of the groups of contaminants detected in both water and biofilm along 5 km after the discharge of a WWTP.

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

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

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

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

3.3.3. Calculation of BAFs

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

4. Discussion

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

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

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

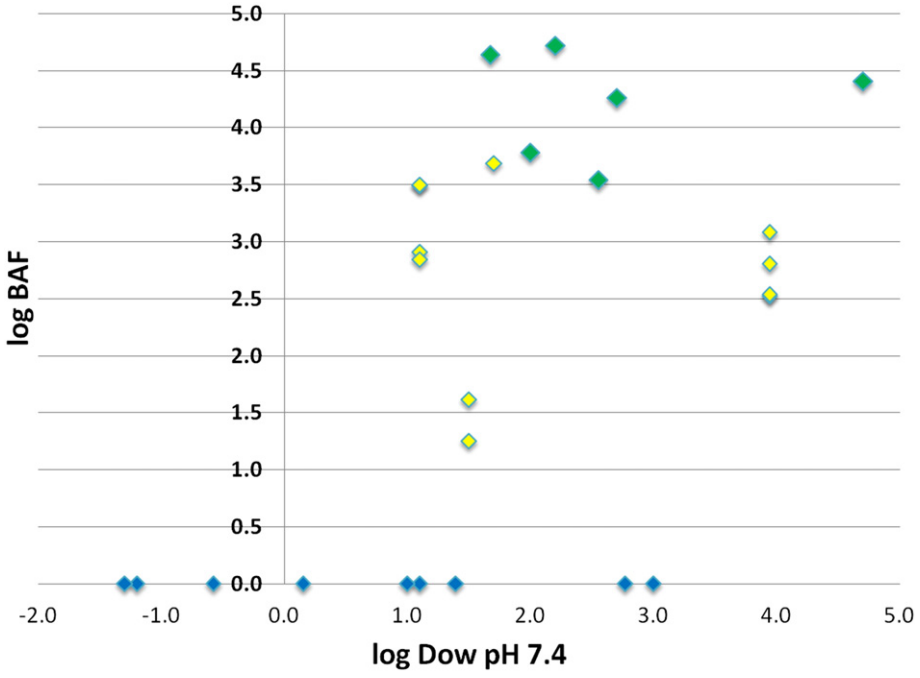

Fig. 3. Correlation between log BAFs and log Dow (at pH 7.4) for the compounds detected in water (in blue), biofilm (in green), or both (in yellow). BAFs of the compounds detected only in biofilm (green), were calculated using MDLs in water as water concentrations to avoid giving BAF values = 0.

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

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

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

5. Conclusions

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

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Appendix A. Supplementary data

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

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