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Biotransformation of pharmaceuticals in surface water and during waste water treatment: Identification and occurrence of transformation products

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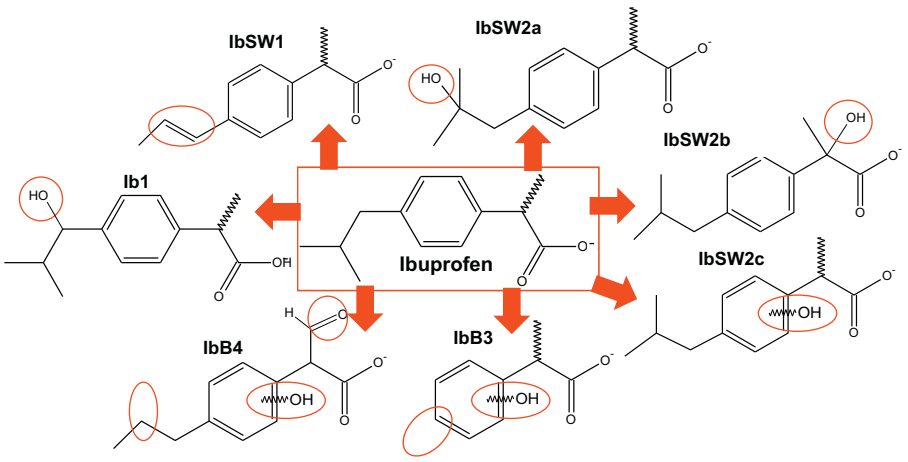
HIGHLIGHTS

- Biodegradation assays of 5 highly-consumed pharmaceuticals were performed.
- 22 TPs were identified by LC–HRMS making use of a QTOF instrument.
- 3 more TPs were found by common fragmentation pathway in effluent wastewater.
- Up to 14 TPs were detected in effluent wastewater and surface water samples.
- Some TPs were more frequently detected than their parent compound.

GRAPHICAL ABSTRACT



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ABSTRACT

Venlafaxine, gemfibrozil, ibuprofen, irbesartan and ofloxacin are highly-consumed pharmaceuticals that show considerable removal efficiencies (between 40 and 98%) in wastewater treatment plants (WWTPs). Consequently, they are expected to generate transformation products (TPs) during wastewater treatment and in surface water (SW) receiving WWTP effluent. In this work, degradation experiments for these five pharmaceuticals have been carried out with SW and WWTP activated sludge under laboratory-controlled aerobic conditions to identify their transformation products by liquid chromatography coupled to time-of-flight mass spectrometry (LC–QTOF MS). Initially, 22 pharmaceutical TPs were tentatively identified. A retrospective analysis was performed in effluent wastewater (EWW) and SW samples. All parent compounds as well as several TPs were found in some of the selected EWW and SW samples. Additionally, valsartan and 3 TPs were also detected by searching for common fragments in these waters. It is important to highlight that some TPs, such as *O*-desmethyl-venlafaxine and an oxidized gemfibrozil TP, were more frequently found than their corresponding parent compounds. On the basis of these results,

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it would be recommendable to include these TPs (at least those found in EWW and SW samples analyzed) in monitoring programs in order to gain a more realistic understanding of the impact of pharmaceuticals on water quality.

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1. Introduction

Large amounts of pharmaceuticals are used around the world and can reach the aquatic environment through urinary excretion and improper disposal [1]. Venlafaxine (antidepressant), gemfibrozil (lipid regulator), ibuprofen (anti-inflammatory), irbesartan (angiotensin II receptor antagonist) and ofloxacin (quinolone antibiotic) (Fig. 1a) are among the most highly consumed drugs [2]. As a consequence, these compounds have been found in wastewaters [3,4] and in surface waters [4–6] since incomplete elimination occurs in wastewater treatment plants. These compounds show different removal rates during wastewater treatment. According to the literature, ibuprofen is the pharmaceutical which shows the highest removal efficiency (~92%) followed by gemfibrozil (~76%), venlafaxine (~50%), ofloxacin (~48%) and irbesartan (~42%) [7–11]. This elimination can be attributed mainly to biotransformation in combination with sorption processes [12]. Therefore, it is expected that potentially persistent TPs are generated by transformation/degradation processes in WWTPs, when TPs are sufficient stable or their biotransformation rate is slower than that of a parent compound. The ecotoxicological effects of these TPs are mostly unknown, although some of them could be as, or even more, hazardous than the parent compound, potentially producing negative effects on humans and wildlife [1,13–15]. For these reasons, and considering the high consumption of pharmaceuticals, it is important to investigate the possible presence of their TPs in the aquatic environment.

Few articles have reported the degradation or biotransformation (by activated sludge) of irbesartan [16], gemfibrozil [17], venlafaxine [18,19] and ofloxacin [20]. However, ibuprofen degradation has been frequently studied [12,21–23]. Unfortunately, these studies are mainly focused on the determination of degradation rates and not on the identification of transformation compounds generated in degradation processes [24].

The vast majority of recent methods for the determination of pharmaceuticals in the aquatic environment are based on the use of liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) using triple quadrupole (QqQ) [6,25–28] or ion trap (IT) [5,29,30] analyzers. In the last few years, the presence of pharmaceuticals in environmental samples has also been investigated by LC coupled to Orbitrap MS [24,31] or time-of-flight mass spectrometry (TOF MS) [32,33]. The latter is a powerful tool for screening pharmaceuticals and their TPs in water due to the accurate mass measurements, high resolving power and high full-spectrum acquisition sensitivity [34–36]. Moreover, using a hybrid QTOF MS enables the acquisition under MS^E mode, this is, the sequential application of two acquisition functions with different collision energies in a single run. By applying low energy (LE) in the collision cell, fragmentation is minimized, and the information obtained corresponds normally to non-fragmented ions, related to the parent molecule. However, at high collision energy (HE), fragmentation will take place, resulting in abundant fragment ions. The acquisition in MS^E mode allows applying the so-called "fragmentation-degradation" methodology [37] to search for analyte-related compounds in waters based on the investigation of common fragment ions.

The goal of this work was to carry out a detailed biotransformation study of five pharmaceuticals in surface water and activated sewage sludge. This was performed under laboratory-controlled conditions and the pharmaceutical TPs were identified by LC–QTOF MS. Subsequently, a retrospective analysis was performed in effluent wastewater and surface water samples (previously analyzed by QTOF for screening of pharmaceuticals and drugs of abuse) with the aim of searching for the TPs identified in the laboratory experiments. A different strategy, based on "common fragmentation pathway", was also applied to the water samples, and allowed the further identification of three more TPs.

2. Experimental

2.1. Reagents and chemicals

See Supplementary information SI.

2.2. Activated sewage sludge

Secondary activated sewage sludge, free of heavy particulates and light fractions, was obtained on December 4th 2012 from the Amsterdam West sewage treatment plant, a plant with a capacity of approx. 800,000 person equivalents with a total average dry weather flow of 172,000 m^3/d. The sludge sample was continuously aerated and stored at room temperature for one week before use in order to reduce the amount of organic matter, and characterised by its total amount of suspended solids (TSS). This parameter was determined by gravimetric analysis ($n = 3$), as described by ESS method 240.2 [38]. Four mineral solutions (see Table 1SI), prepared in demineralised water, were used for the preparation of the mineral medium for biotransformation experiments according to OECD Guideline 301a [39]. The mineral solutions were conserved at 4 °C and inspected to be precipitate free before use.

2.3. Instrumentation

2.3.1. LC–ESI–QTOF MS

An Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) was interfaced to a QTOF mass spectrometer (QTOF Xevo G2, Waters Micromass, Manchester, UK) using an orthogonal Z-spray electrospray interface, operating in both positive and negative ion modes. The resolution of the TOF mass spectrometer was ~20,000 at full width half maximum (FWHM) at m/z 556. The LC separation was performed using an Acquity UPLC BEH C18 analytical column (100 × 2.1 mm, 1.7 μm particle size, Waters) at a flow rate of 0.3 mL/min. (For further details see Supplementary information SI).

The data station operating software was MassLynx version 4.1 (Waters).

2.4. Degradation experiments

Biotransformation experiments were performed to investigate the potential for degradation in surface water and by active sludge. The solutions used for biotransformation experiments were indi-

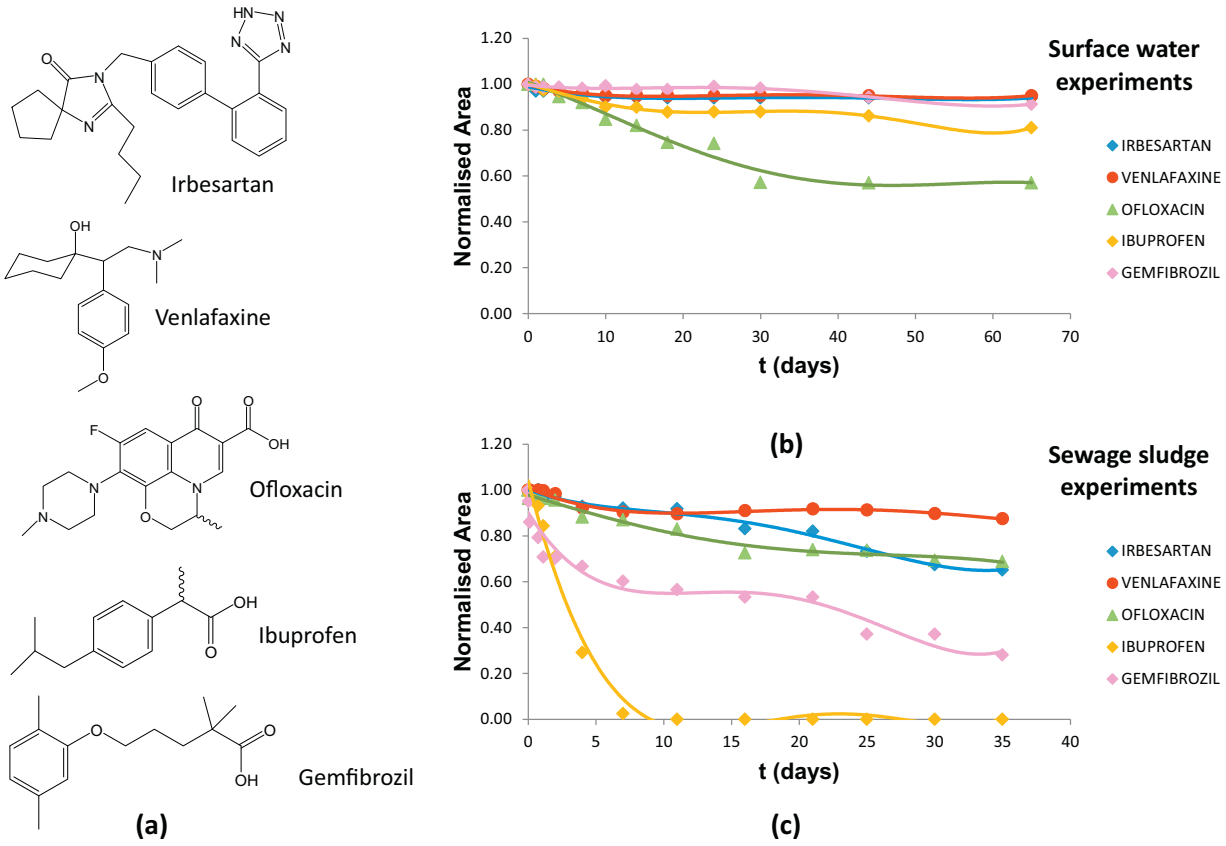

Fig. 1. (a) Structures of ofloxacin, gemfibrozil, irbesartan, venlafaxine and ibuprofen. Degradation curves after (b) 65 d in surface water and (c) 35 d with activated sludge.

vidually spiked at 0.5 mg/L with venlafaxine, irbesartan, ibuprofen and gemfibrozil, and at 0.05 mg/L with ofloxacin. These relatively high concentrations allowed better detection and identification of TPs and facilitated the detection of minor TPs. Non-spiked surface water and medium inoculated with sludge were subjected to the same conditions and used as control samples, to assure that the transformation products formed came from the degradation of the parent pharmaceutical under study. So, differential ions/chromatographic peaks between the degraded sample and the corresponding control sample would correspond, in principle, to transformation products. Of course, it was checked that the analytes were not present in the surface water.

2.4.1. Surface water

Experiments were performed in samples collected from Mijares River (Valencian Region, Eastern Spain) in 2012 (pH 8.1 ± 0.2) kept in darkness at room temperature. 2-mL aliquots were sampled at different time intervals (0, 1, 2, 4, 7, 10, 14, 18, 24, 30, 44 and 65 days after application) and immediately stored at −20 °C.

2.4.2. Activated sludge

Experiments were carried out in medium prepared according to Guideline OECD 301a [39] with pH 7.4 ± 0.2. The medium was inoculated with sludge to give a TSS of 100 mg/L. Previously, different experiments were performed at 100 and 1000 mg/L in order to check the optimum working concentration. It was observed that the degradation was very fast at 1000 mg/L and therefore the transformation products could not been easily identified. Using the lower concentration of TSS facilitated the study of the formation of TPs. Solutions were kept in darkness at room temperature in Erlenmeyer-flasks (100 mL), maintaining aerobic conditions under continuous shaking (130 rpm). Abiotic controls were maintained under identical conditions with medium sterilized by the addition of 1 mM sodium azide. 2-mL aliquots were sampled at different time intervals (0, 5 min, 1, 3, 18 and 27 h, 2, 4, 11, 16, 21, 25, 30 and 35 days after spiking) and immediately stored at −20 °C.

In both cases, extracts were centrifuged and 50 μL were directly injected into the UHPLC–QTOF MS system. The filtration step was avoided trying to minimize possible losses during this step.

2.5. Identification of TPs by MetaboLynx application manager

The general strategy followed for identification of TPs using UHPLC–ESI–QTOF MS can be found elsewhere [40,41].

MetaboLynx XS (an application manager within MassLynx v 4.1) was used to process QTOF MS data. This software compares narrow-window eXtracted Ion Chromatograms (nw-XICs) of a positive/degraded sample versus a control sample in order to detect, identify and report differential ions/chromatographic peaks which would correspond, in principle, to transformation products [40].

The TPs detected were been named as follows: the first letter(s) corresponds to the initial of the pharmaceutical (e.g., "I" for Irbesartan, "V" Venlafaxine, or "Ib" Ibuprofen), followed by the process involved ("SW" for experiments with surface water or "B" for biotransformation experiments using activated sludge). Then, a number (1, 2, 3,. . .) was added to enumerate TPs. Isomeric compounds have the same number but an additional final letter (a, b, c. . .). So, VB1a corresponds to the transformation product 1 of venlafaxine identified after biotransformation experiments with activated sludge. Specifically, it refers to isomer a.

2.6. Searching for unknown TPs by common fragmentation pathway

Assuming that most TPs share their fragmentation pathways with the parent pharmaceuticals but also with other TPs [42], spe-

Table 1
Irbesartan, valsartan and metabolites/TPs obtained in hydrolysis and biotransformation experiments by LC–ESI–QTOF MS.

Compound	Ionization mode	Ret. time (min)	Elemental composition	Accurate mass *m*/*z*	Mass error (mDa)	DBE	Transformation process
Irbesartan	ESI+	7.6	C25H29N6O	429.2412	0.9	14.5	
			C14H11N2	207.0920	−0.2	10.5	
			C11H19N2O	195.1504	−0.7	3.5	
			C14H10N	192.0812	−0.1	10.5	
			C13H10N	180.0808	−0.5	9.5	
			C25H28N3O	386.2242	1.0	13.5	
			C5H10N	84.0814	−0.1	1.5	
			C14H11N4	235.0982	−0.2	11.5	
ISW1a[a]	ESI+	6.6	C25H31N6O2	447.2493	−1.5	13.5	Hydroxylation
			C14H11N2	207.0930	0.8	10.5	+Hydrogenation
			C14H11	179.0867	0.6	9.5	
			C14H13N2O	225.1030	0.2	9.5	
			C6H7O	95.0484	−1.3	3.5	
			C5H10N	84.0811	−0.2	1.5	
ISW1b	ESI+	7.3	C25H31N6O2	447.2495	−1.3	13.5	Hydroxylation
			C5H10N	84.0814	0.1	1.5	+Hydrogenation
			C10H18NO	168.1390	0.2	2.5	
			C14H11N4	235.0995	1.1	11.5	
			C14H11N2	207.0922	0.0	10.5	
			C11H18NO2	196.1343	0.5	3.5	
			C13H10N	180.0814	−0.1	9.5	
			C14H10N	192.0810	−0.3	6.5	
			C14H8N	190.0659	0.2	11.5	
ISW2	ESI+	7.3	C24H27N6O	415.2236	−1.0	14.5	Demethylation
			C14H11N2	207.0927	0.5	10.5	
			C24H26N3O	372.2056	−2.0	13.5	
			C5H10N	84.0811	−0.2	1.5	
			C10H17N2O	181.1349	0.8	3.5	
IB3a[a]	ESI+	6.8	C25H27N6O2	443.2207	1.5	15.5	Oxidation
			C14H11N2	207.0920	−0.2	10.5	
			C13H10N	180.0805	−0.8	9.5	
			C14H10N	192.0803	−1.0	6.5	
			C23H25N6O	401.2075	−1.5	14.5	
			C10H14NO	164.1085	1.0	4.5	
			C5H10N	84.0814	0.1	1.5	
IB3b[a]	ESI+	7.1	C25H27N6O2	443.2205	1.3	15.5	Oxidation
			C14H11N2	207.0914	−0.8	10.5	
			C14H11N4	235.0998	1.4	11.5	
			C13H10N	180.0808	−0.5	9.5	
			C14H10N	192.0803	−1.0	6.5	
			C8H13N2O	153.1008	−2.0	3.5	
			C5H8NO	98.0599	−0.7	2.5	
			C14H8N	190.0668	1.1	11.5	
IB4[a]	ESI+	6.4	C22H23N6O	387.1948	1.5	14.5	Dealkylation (C_3H_7)
			C14H11N2	207.0919	−0.3	10.5	
			C13H10N	180.0828	1.5	9.5	
			C8H13N2O	153.1030	0.2	3.5	
			C14H10N	192.0826	1.0	10.5	
			C7H13N2	125.1061	−1.8	2.5	
			C14H11N4	235.0991	0.7	11.5	
			C5H10N	84.0815	0.2	1.5	
			C22H22N3O	344.1765	02	13.5	
			C14H8N	190.0669	1.2	11.5	
IB5[a]	ESI+	6.6	C25H27N6O3	459.2137	−0.8	15.5	Hydroxylation
			C14H11N2	207.0924	0.2	10.5	+Oxidation
			C14H13N2O	225.1043	1.5	9.5	
			C14H11N4	235.0991	0.7	11.5	
			C13H10N	180.0824	1.1	9.5	
			C14H8N	190.0667	1.0	11.5	
IB6[a]	ESI+	6.3	C25H27N6O4	475.2096	0.2	15.5	2 hydroxylation
			C14H11N2	207.0926	0.4	10.5	+Oxidation
			C14H13N2O	225.1032	0.4	9.5	
			C8H13N2O	153.1060	3.2	3.5	
			C14H11N4	235.0994	1.0	11.5	
			C24H24N5O3	430.1890	1.1	15.5	
			C13H10N	180.0820	0.7	9.5	
			C8H13N2	137.1073	−0.6	3.5	
			C14H9O	193.0670	1.7	10.5	
			C14H8N	190.0663	0.6	11.5	

Table 1 (*Continued*)

Compound	Ionization mode	Ret. time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Valsartan	ESI+	7.9	C24H30N5O3	436.2350	0.1	12.5	
			C14H11N2	207.0925	0.3	10.5	
			C14H11N4	235.0975	−0.9	11.5	
			C19H19N2O	291.1514	1.7	11.5	
			C13H10N	180.0825	1.2	9.5	
			C9H10N3O2	192.0772	−0.1	6.5	
			C14H8N	190.0648	−0.9	11.5	
			C18H20N5	306.1737	1.8	11.5	
			C24H29N5O3Na	458.2178	1.0	12.5	

[a] To our knowledge, TPs not reported in scientific literature yet.

cific nw-XICs at the expected *m/z* fragments were obtained at low (LE) and high (HE) energy from full-spectrum QTOF MS acquisitions. The presence of chromatographic peaks at different retention times (T_R) than the known pharmaceutical compound would indicate the presence of potential TPs. Using this approach, new compounds have been detected in aquatic samples, EWW and SW [37].

The TPs detected following this strategy have been named taken into account the initial of the pharmaceutical followed by a number.

2.7. Retrospective QTOF MS analysis of water samples

38 EWW samples were collected from several WWTPs of the Valencian Region (Eastern Spain) from June 2008 to December 2012. Additionally, 18 SW samples were collected from several points located in the same area in March 2010. All water samples had been previously subjected to solid phase extraction and analyzed by LC–QTOF MS for other research purposes [4,43]. Using this technique it is feasible to perform a retrospective evaluation of data at any subsequent time, due to the availability of accurate-mass full-spectrum data generated. Thus, a retrospective analysis was made investigating the presence of the target TPs using ChromaLynx XS application manager (also within MassLynx v 4.1). This software allows applying a "post-target" processing method based on selected exact masses (target list) that permits a rapid and simple reviewing by cataloguing analytes, as function of mass error and retention time deviation. Confirmation of the identity of the compounds detected was based on the accurate *m/z* of the (de) protonated molecule and at least one fragment ion, together with the agreement in retention time (deviation lower than ±2.5%) when compared with a "reference compound".

3. Results and discussion

3.1. Biotransformation rates

3.1.1. Surface water

Fig. 1b illustrates the removal curves (represented as normalised areas respect the area of each compound at $t = 0$) for all studied compounds after 65 days in surface water. Significant removal was only observed for ofloxacin which underwent a removal of around 40% after 44 days. Ibuprofen showed a slight removal (around 10%) whereas for the rest of compounds, the removal was negligible.

3.1.2. Activated sludge

In biotransformation experiments, using active sludge (AS) at 100 mg/L of TSS, pharmaceutical elimination seemed to exhibit, in general, a linear decay along the time, except for ibuprofen which followed an exponential decay curve (Fig. 1c). Total ibuprofen removal was achieved after 10 days. For gemfibrozil, a removal around 60% was obtained in 7 days, which still increased up to 70% after 35 days. Removals of around 25–30% were obtained for ofloxacin and irbesartan after 35 days. Finally, venlafaxine exhibit the lowest degradation rate. In general this is consistent with the data reported in the literature [12,17,20,22]. No removal was observed in the sterile controls, confirming that the removal was due to biotransformation and not due to sorption.

3.2. Identification of TPs by MetaboLynx application manager

After processing data from the biotransformation experiments using MetaboLynx, several pharmaceutical TPs were found and tentatively identified. Briefly, the identification process was the following. For all compounds detected by MetaboLynx, the accurate mass of protonated/deprotonated molecules was determined on the basis of averaged spectra obtained in the survey scan. Then, possible elemental compositions were calculated using the MassLynx elemental composition calculator with a maximum deviation of 2 mDa from the measured accurate mass. The maximum and minimum parameters were restricted considering the elemental composition of each parent compound. In order to propose a plausible chemical structure for each TP, the fragmentation pathway was studied. For calculating the elemental composition of fragment ions (obtained mainly from HE spectrum), parameters settings were restricted as a function of the calculated elemental composition of the (de) protonated molecule, while for neutral losses no restrictions were applied. In order to avoid spectrum interferences that would complicate the identification process, recognizing which ions are fragments and which are not, becomes mandatory. For this purpose, UHPLC proved valuable to identify fragment ions that are closely related to the "precursor" ion on the basis of their similar retention time and peak shape. However, and due to the high complexity of the matrix wastewater, MS/MS experiments were also performed to assure that the fragment ions which appeared in full scan experiments arose from the compound being identified, for which UHPLC played an important role. As it is shown in S.I., no significant differences were found between the MS/MS and the MS^E spectra, demonstrating the usefulness of the MS^E approach even with a very complex matrix such as wastewater (See SI, Figs. 11SI and 21SI).

Under the conditions used in these experiments, it cannot be excluded that abiotic degradation contributed to the formation of these TPs and further work would be required to elucidate the relative contributions of biotic and abiotic degradation.

3.2.1. Irbesartan

Despite the low removal rate of irbesartan in SW, three minor TPs could be detected (ISW1a, ISW1b and ISW2). A priori the relevance of these TPs could be questioned due to its low abundance. However, their (eco)toxicity (and therefore the subsequent impact in the environment) should be also assessed, particularly for ISW1b as it was found in 87% of the EWW samples analysed (see Section 3.4). In addition, five TPs were identified after biotransformation by AS (IB3a, IB3b, IB4, IB5 and IB6). ISW1a and ISW1b were the major TPs found in the AS experiments.

Table 2
Venlafaxine and metabolites/TPs obtained by LC–ESI–QTOF MS.

Compound	Ionization mode	Ret. time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Venlafaxine	ESI+	5.2	C17H28NO2	278.2117	−0.3	4.5	
			C17H26NO	260.2016	0.2	5.5	
			C15H19O	215.1440	0.4	6.6	
			C10H11O	147.0809	−0.1	5.5	
			C7H7	91.0549	0.1	4.5	
			C12H13O	173.0970	0.4	6.5	
			C3H8N	58.0654	−0.3	0.5	
			C8H9O	121.0653	−0.0	4.5	
VB1a *O*-desmethyl-venlafaxine	ESI+	3.8	C16H26NO2	264.1960	−0.4	4.5	Demethylation
			C7H7O	107.0495	−0.2	4.5	
			C11H11O	159.0790	−2.0	6.5	
			C3H8N	58.0653	−0.4	0.5	
			C6H9	81.0694	−1.0	2.5	
			C14H17O	201.1248	−3.1	6.5	
			C9H9O	133.0652	−0.1	5.5	
			C16H24NO	246.1875	1.7	5.5	
			C10H9O	145.0651	−0.2	6.5	
			C8H9O	121.0647	−0.6	4.5	
VB1b *N*-desmethyl-venlafaxine	ESI+	5.2	C16H26NO2	264.1963	−0.1	4.5	Demethylation
			C8H9O	121.0660	0.7	4.5	
			C10H11O	147.0813	0.3	5.5	
			C16H24NO	246.1857	−0.1	5.5	
			C12H13O	173.0965	−0.1	6.5	
			C15H19O	215.1437	0.1	6.5	
			C11H11O	159.0822	1.2	6.5	
			C7H7	91.0534	−1.4	4.5	
VB2[a]	ESI+	5.1	C17H28NO3	294.2055	−1.4	4.5	Hydroxylation
			C8H9O	121.0647	−0.6	4.5	
			C9H11O	135.0803	−0.7	4.5	
			C10H11O	147.0809	−0.1	5.5	
			C11H16NO	178.1254	2.2	4.5	
			C6H11O	99.0817	0.7	1.5	
			C6H9	81.0701	−0.3	2.5	
			C15H19O	215.1430	−0.6	6.5	
VB3a[a]	ESI+	2.7	C17H26NO3	292.1911	−0.2	5.5	Oxidation
			C17H24NO2	274.1814	0.7	6.5	
			C8H9O	121.0650	−0.3	4.5	
			C12H11O	171.0827	1.7	7.5	
			C11H11O	159.0788	−2.2	6.5	
			C7H7	91.0560	1.2	4.5	
			C3H8N	58.0668	−1.1	0.5	
VB3b[a]	ESI+	3.1	C17H26NO3	292.1913	0.0	5.5	Oxidation
			C17H24NO2	274.1817	1.0	6.5	
			C8H9O	121.0655	0.2	4.5	
			C8H11O2	139.0769	1.0	3.5	
			C8H14NO2	156.1029	0.4	2.5	
			C7H12N	110.0968	−0.2	2.5	
VB4[a]	ESI+	3.9	C17H24NO2	274.1834	2.7	6.5	2[a]dehydrogenation
			C8H9O	121.0652	−0.1	4.5	
			C12H11O	171.0795	−1.5	7.5	
			C14H11	179.0854	−0.7	9.5	
			C15H15O	211.1110	−1.3	8.5	
			C14H12O	196.0878	−1.0	9.0	
			C11H11O	159.0808	−0.2	6.5	
			C12H9	153.0722	1.8	8.5	
			C9H9O	133.0668	1.5	5.5	
			C7H9	93.0705	0.1	3.5	
V1	ESI+	4.1	C16H26NO2	264.1974	1.0	4.5	Demethylation
			C3H8N	58.0660	0.3	0.5	
			C16H24NO	246.1861	0.3	5.5	
			C8H11O2	139.0773	1.4	3.5	
			C7H9	93.0722	1.8	3.5	
			C14H15O	199.1134	1.1	7.5	
			C14H17O	201.1294	1.5	6.5	
			C11H9O	157.0670	1.7	7.5	
V2[a]	ESI+	4.0	C17H24NO	258.1869	1.1	6.5	Oxidation+
			C14H15O	199.1130	1.1	7.5	2 dehydrogenation
			C14H17O	201.1290	0.7	6.5	
			C11H9O	157.0664	1.1	7.5	
			C9H9O	133.0672	1.9	5.5	

[a] To our knowledge, TPs not reported in scientific literature yet.

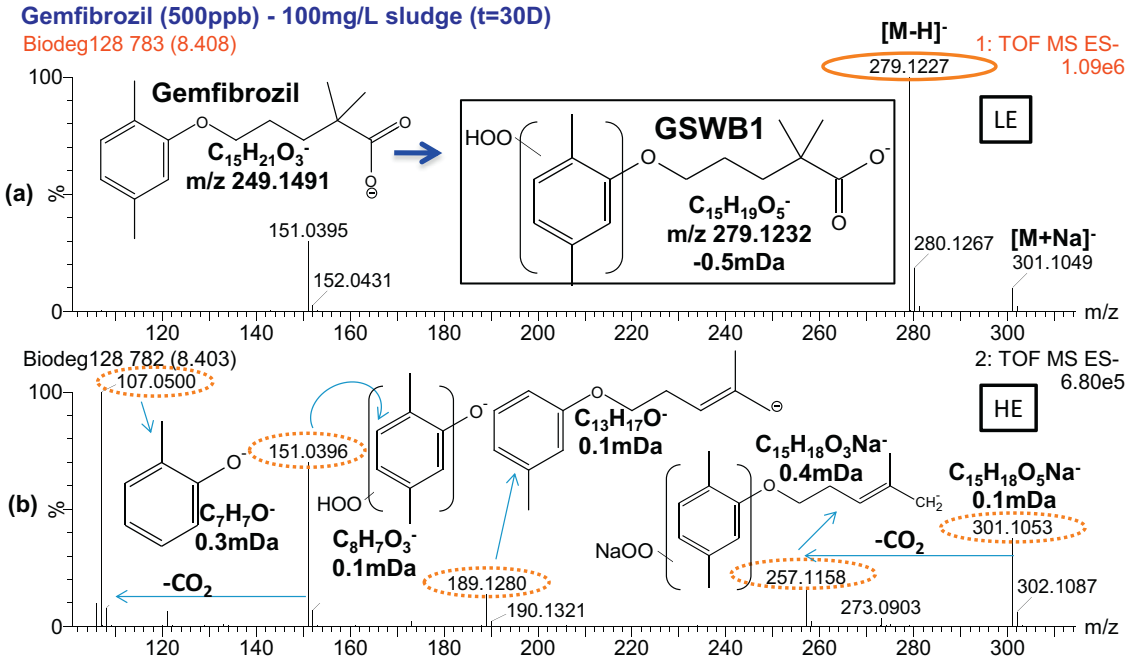

Fig. 2. Elucidation of gemfibrozil TP (GSWB1). (a) LE spectrum and (b) HE spectrum with proposed structures for its fragment ions.

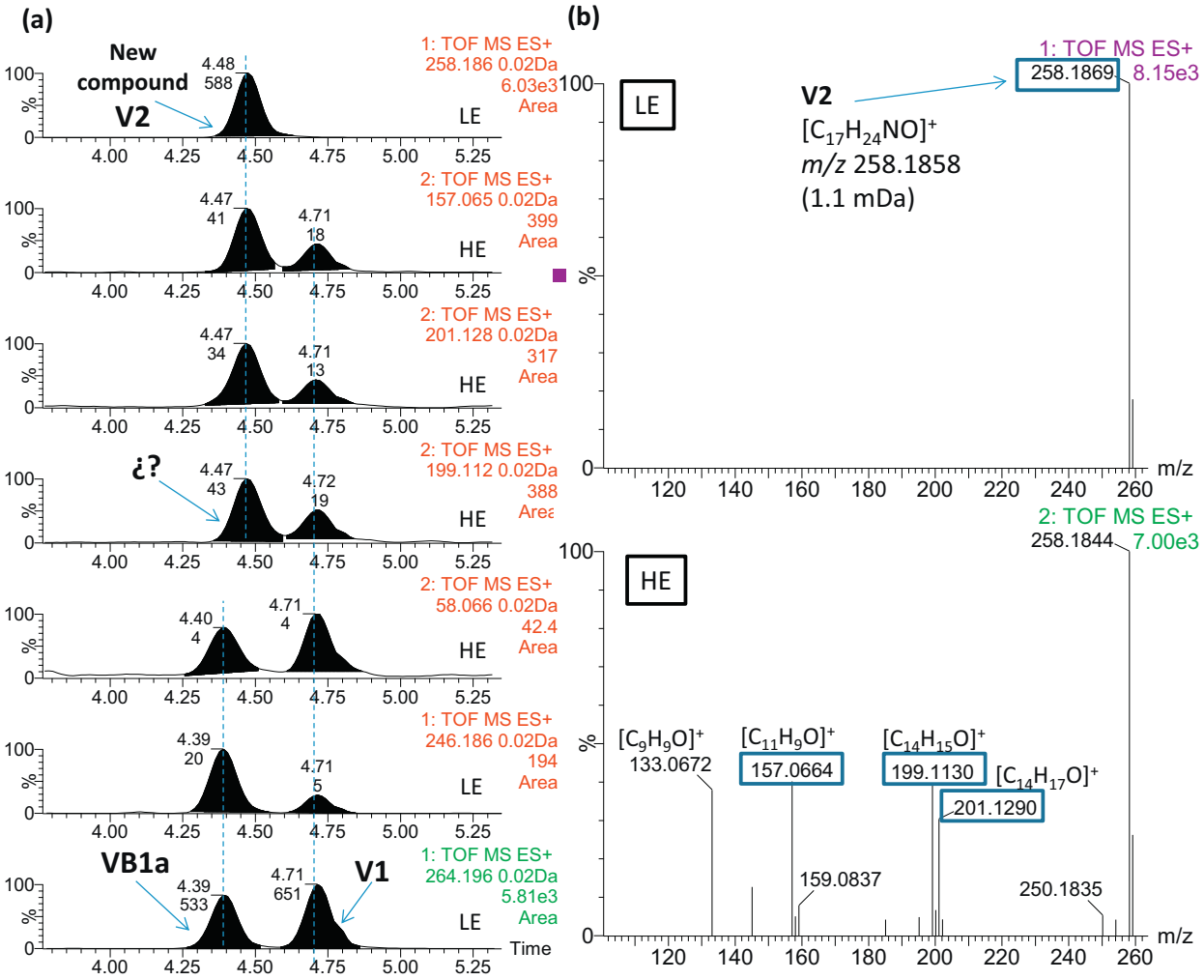

Fig. 3. Detection of V1 and V2 venlafaxine metabolites/TPs in EWW by applying the common fragmentation pathway strategy. (a) nw-XICs for $[M+H]^+$ of VB1a, V1 and V2 in LE and four fragment ions in HE. (b) LE and HE spectra of TP V2.

Table 3
Ofloxacin and the TP obtained in biotransformation experiments by LC–ESI–QTOF MS.

Compound	Ionization mode	Ret. time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Ofloxacin	ESI+	3.1	C18H21N3O4F	362.1520	−0.4	9.5	
			C17H21N3O2F	318.1615	−0.3	8.5	
			C14H14N2O2F	261.1045	0.6	8.5	
			C18H19N3O3F	344.1408	−0.2	10.5	
			C11H10N2O2F	221.0754	2.8	7.5	
			C4H8N	70.0641	−1.6	1.5	
OB1[a]	ESI+	3.8	C18H21N3O5F	378.1463	−0.2	9.5	Hydroxylation
			C17H20N3O2F	317.1518	−2.2	9.0	
			C13H12N2O2F	247.0870	−1.3	8.5	
			C18H20N3O4F	361.1422	−1.63	10.0	
			C12H15N2O3F	254.1062	−0.5	6.0	
			C13H11NO2F	232.0769	−0.5	8.5	
			C16H17N3O2F	302.1325	2.0	9.5	
			C4H8N	70.0647	−1.0	1.5	
			C15H16N2O2F	275.1210	1.4	8.5	

[a] To our knowledge, TP not reported in scientific literature yet.

Table 4
Ibuprofen and TPs obtained in hydrolysis and biotransformation experiments by LC–ESI–QTOF MS.

Compound	Ionization mode	Ret. time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Ibuprofen	ESI-	9.0	C13H17O2	205.1272	4.3	5.5	
			C12H17	161.1330	0.0	4.5	
IbSW1[a]	ESI-	6.6	C12H13O2	189.0908	−0.8	6.5	Demethylation
			C11H13	145.1018	0.1	5.5	+Dehydrogenation
			C3H5O2	73.0273	−1.7	1.5	
			C12H15O	175.1125	0.2	5.5	
IbSW2a.b.c[a]	ESI-	6.5–8.1–8.7	C13H17O3	221.1182	0.4	5.5	Hydroxylation
2-hydroxy ibuprofen (IbSW2a)			C12H17O	177.1274	−0.5	4.5	
α-hydroxy ibuprofen (IbSW2b)							
IbB3[a]	ESI-	6.2	C9H9O3	165.0545	−0.7	5.5	Dealkylation (C_4H_9) + hydroxylation
IbB4[a]	ESI-	7.1	C12H13O4	221.0816	0.2	6.5	Demethylation
			C11H13O2	177.0932	1.6	5.5	+O hydroxylation
			C10H13O	149.0948	−1.8	4.5	
			C8H6O2	134.0366	−0.2	6.0	
Ib1	ESI-	6.9	C13H17O3	221.1180	0.2	5.5	Hydroxylation
1-hydroxy ibuprofen			C12H17O	177.1272	−0.7	4.5	
			C12H15	159.1157	−1.7	5.5	
			C11H11	143.0857	−0.4	6.5	

[a] To our knowledge, TPs not reported in scientific literature yet.

These TPs are isomeric compounds (*m/z* 447.2508, $[C_{25}H_{31}N_6O_2]^+$) and appeared as a result of irbesartan hydroxylation and hydrogenation in different parts of the molecule (See SI, Fig. 4SI and 5SI). ISW2 corresponds to desmethyl-irbesartan (*m/z* 415.2246, $[C_{24}H_{27}N_6O]^+$). Table 1 summarizes elemental compositions, retention times, fragment ions, mass errors, DBEs and transformation processes for irbesartan and its TPs. Fig. 1SIa shows the profile (in a semiLog-linear plot) of the five main irbesartan TPs detected in AS during 35 days. IB3a and IB3b are also isomeric compounds (*m/z* 443.2192, $[C_{25}H_{27}N_6O_2]^+$) showing an oxidation of the irbesartan. IB4 (*m/z* 387.1933 $[C_{22}H_{23}N_6O]^+$) corresponds to a dealkylation of irbesartan. TPs IB5 (*m/z* 459.2145, $[C_{25}H_{27}N_6O_3]^+$) and IB6 (*m/z* 475.2094, $[C_{25}H_{27}N_6O_4]^+$) are formed after oxidation of the parent pharmaceutical and subsequent hydroxylation/s, respectively from irbesartan. ISW1b seems to correspond to one of the three TPs reported by Shah et al. [16], as both compounds share up to six fragment ions (235.0984, 207.0922, 196.1338, 192.0810, 180.0813 and 168.1388). A plausible chemical structure for each TP is given in S.I.

3.2.2. Venlafaxine

No TPs were found in SW experiments but six were identified in AS. The formation profiles of the most abundant venlafaxine TPs are shown in Fig. 1SIb. VB1a and VB1b were isomeric compounds (*m/z* 264.1964, $[C_{16}H_{26}NO_2]^+$), showing a demethylation of the venlafaxine molecule. It seems that VB1a corresponds to *O*-desmethyl-venlafaxine, as it still shows a fragment ion at *m/z* 58.0657 ($C_3H_8N^+$). Therefore VB1b might be assigned to N-desmethyl-venlafaxine. Both TPs had been previously reported by Kern et al. [18] in EWW; O-desmethyl TP was also observed in surface water by de Jongh et al. [24]. The subsequent acquisition of reference standards allowed us to confirm the identities of VB1a and VB1b, as retention times and mass spectra were in agreement with *O*- and *N*-desmethyl-venlafaxine, respectively. VB3a and VB3b, also isomeric compounds (*m/z* 292.1913, $[C_{17}H_{26}NO_3]^+$), appeared as a result of venlafaxine oxidation in different parts of the molecule. VB2 (*m/z* 294.2069, $[C_{17}H_{28}NO_3]^+$) corresponds to a hydroxylation of venlafaxine. Finally, VB4 (*m/z* 274.1807, $[C_{17}H_{24}NO_2]^+$) is a venlafaxine di-dehydrogenation product. Elemental compositions, transformation processes, retention times, fragment ions, mass errors and DBEs are summarized in Table 2.

3.2.3. Ofloxacin

No TPs were found after ofloxacin degradation in SW. Regarding biotransformation by AS, only one TP (OB1) was observed. Its formation during degradation of ofloxacin is illustrated in Fig. 1SIc. This TP corresponded to a hydroxylation of ofloxacin (*m/z* 378.1465 $[C_{18}H_{21}N_3O_5F]^+$). Elemental compositions, transformation process,

Table 5
Gemfibrozil and TP obtained in hydrolysis and biotransformation experiments by LC–ESI–QTOF MS.

Compound	Ionization mode	Ret. time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Gemfibrozil	ESI-	9.5	C15H21O3	249.1486	−0.5	5.5	
			C8H9O	121.0656	0.3	4.5	
			C15H20NaO3	271.1328	1.8	5.5	
			C7H11O2	127.0751	−0.8	2.5	
			C7H6O	106.0420	0.1	5.0	
GSWB1[a]	ESI-	8.4	C15H19O5	279.1241	0.9	6.5	Hydroxylation + Oxidation
			C15H18O5Na	301.1053	0.1	6.5	
			C7H7O	107.0500	0.3	4.5	
			C14H18O3Na	257.1158	0.4	5.5	
			C8H7O3	151.0396	0.1	5.5	
			C13H17O	189.1280	0.1	5.5	
			C8H6O3Na	173.0213	−0.2	5.5	

[a] To our knowledge, TP not reported in scientific literature yet.

retention times, fragment ions, mass errors and DBEs are summarized in Table 3.

3.2.4. Ibuprofen

This pharmaceutical was broken down yielding four TPs in SW (IbSW1 and IbSW2a,b,c) and two TPs in AS (IbB3 and IbB4) (Fig. 1SId). The TP IbSW1 was the result of a demethylation and dehydrogenation of the ibuprofen structure (*m/z* 189.0916 $[C_{12}H_{13}O_2]^-$). IbSW2a,b,c are isomeric compounds at *m/z* 221.1178 $[C_{13}H_{17}O_3]^-$, eluting at different retention times (6.5, 8.1 and 8.7 min). These compounds resulted from hydroxylation in different parts of the ibuprofen molecule. Two previously reported hydroxylated metabolites (1-hydroxy and 2-hydroxy ibuprofen) [21,23] might correspond to two of these TPs IbSW2(a,b,c). After tentative identification of these TPs, three reference standards were acquired (1-hydroxy ibuprofen, rac α-hydroxy ibuprofen and rac 2-hydroxy ibuprofen) for confirmation of their identity. The retention times and mass spectra of rac 2-hydroxy and rac α-hydroxy ibuprofen were in agreement with those of IbSW2a and IbSW2b, respectively. However, in the case of 1-hydroxy ibuprofen, the retention time was not the same as that of IbSW2c.

Moreover, AS biotransformation experiments showed IbB3 at *m/z* 165.0552 (-0.7 mDa) with an elemental composition of $[C_9H_9O_3]^-$ obtained after a dealkylation of the butyl group and subsequent hydroxylation. Unfortunately, no fragment ions were observed in the HE spectrum, maybe because it is a relative small and stable molecule, and therefore the hydroxyl group could not be located. Finally, TP IbB4 was found to have a *m/z* 221.0814 $[C_{12}H_{13}O_4]^-$. This compound shares the nominal mass with TPs IbSW2a,b,c (*m/z* 221), but they have different exact masses as well as retention times. The resolving power and mass accuracy of the QTOF MS allowed differentiation of these compounds. The elemental composition for IbB4 suggests a demethylation, hydroxylation and oxidation of the ibuprofen molecule. Elemental compositions, transformation processes, retention times, fragment ions, mass errors and DBEs are summarized in Table 4.

3.2.5. Gemfibrozil

The elimination of this lipid regulator gave a minor TP (GSWB1) in SW. This TP was also observed as a result of biotransformation in AS, although its concentration was almost 15-fold higher in activated sludge **(**Fig. 1**S**le**)**. According to its exact mass (*m/z* 279.1232, Table 5) the elemental composition of the deprotonated molecule was assigned to $[C_{15}H_{19}O_5]^-$ (+0.9 mDa), which would imply a hydroxylation and oxidation of the gemfibrozil molecule. Fig. 2 illustrates the LE and HE spectra for TP GSWB1 with the proposed structures for the fragment ions. On the basis of the fragment ions observed, it might be expected the oxidation to take place in one of the methyl groups of the benzene ring of gemfibrozil.

To the authors' best knowledge, several of these TPs, specially 6 irbesartan TPs, 5 of venlafaxine, 4 of ibuprofen and one of gemfibrozil and ofloxacin, have not yet been reported in scientific literature. TPs not reported have been marked with an "*" in the Tables 1–5. Those new TPs found for the first time in EWW and SW are also highlighted in Table 6.

3.3. Searching for unknown TPs by common fragmentation pathway

Two unknown compounds (V1 and V2) were detected by common fragmentation pathway with venlafaxine TPs in effluent wastewater. These compounds might be associated with venlafaxine metabolites or to other TPs not found in our biotransformation experiments. V1 shared the exact mass (*m/z* 264.1964) and three fragment ions (*m/z* 58.0657, 93.0704 and 246.1858) with VB1ab TPs, but eluted later (at 4.71 min). Retention times of V1 and V2 were re-calculated, since different gradient conditions were used to analyze these samples. For this purpose, T_R of six TPs were measured at each gradient conditions and two equations obtained after their graphical representation, with the correlation coefficients above 0.99 (For further details see Section 1.3. in SI).

On the other hand, V2 was detected by common fragmentation with V1. As an example, Fig. 3 shows five narrow-mass window extracted ion chromatograms (nw-XICs) for the fragment ions of V1; a new peak appeared (V2) at 4.47 min (the T_R is different than in degradation experiments as they were acquired with different gradients) sharing three of these fragment ions (*m/z* 199.1123, 201.1279 and 157.0653). After investigating the LE function at this retention time, the accurate mass was assigned to *m/z* 258.1869, corresponding to an elemental composition of $[C_{17}H_{24}NO]^+$ (1.1 mDa). Fig. 3 shows the LE and HE spectra and the elemental composition assigned to each fragment ion.

As noted above, venlafaxine V1 could have been previously reported by [18], corresponding to *O*-desmethyl-venlafaxine and not to *N*-desmethyl-venlafaxine as it shows a fragment ion at *m/z* 58.0657, $C_3H_8N^+$. Therefore, VB1a and V1 could be explained as a pair of epimer compounds.

Regarding irbesartan, an unknown compound was found by common fragment ions searching in EWW. As can be seen in Fig. 2SI, after performing the HE nw-XICs for the fragment ions of irbesartan (at *m/z* 207.0922, 180.0813 and 192.0913) a new peak was observed at 7.94 min. The LE spectrum of the chromatographic peak at this retention time provided the accurate mass of the protonated molecule at *m/z* 436.2350. According to this mass, an elemental composition of $[C_{24}H_{30}N_5O_3]^+$ (0.1 mDa) was assigned. This chem-

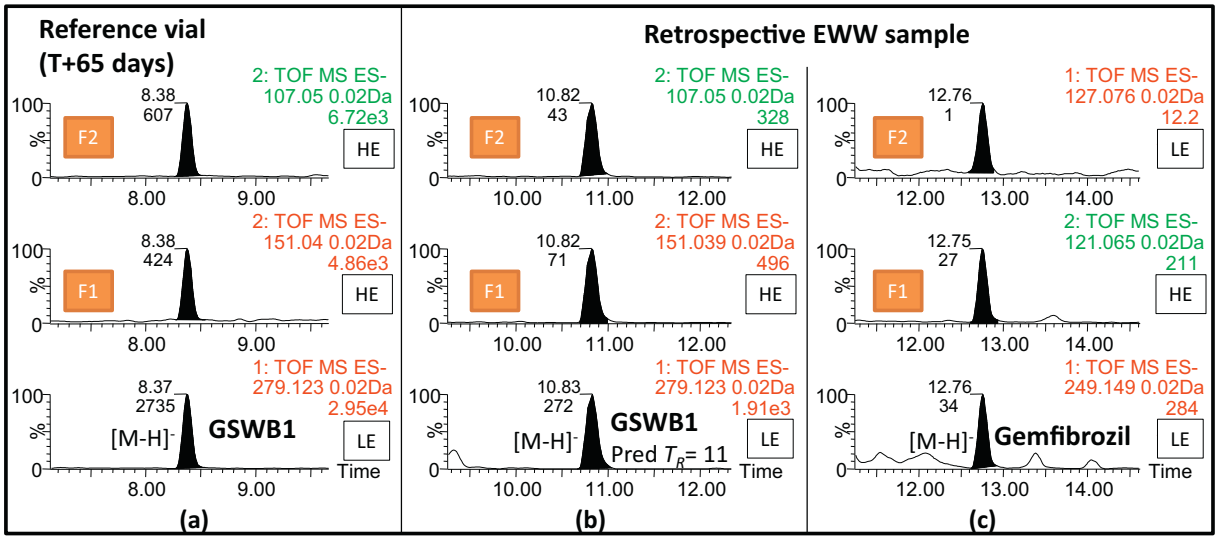

Fig. 4. Nw-XICs for $[M-H]^-$ in LE and 2 fragment ions (F1, F2) in HE for (a) reference sample vial for gemfibrozil TP GSWB1 obtained in the surface water experiments, (b) positive finding of gemfibrozil TP GSWB1 in EWW. (c) Positive finding of gemfibrozil in the same EWW sample.

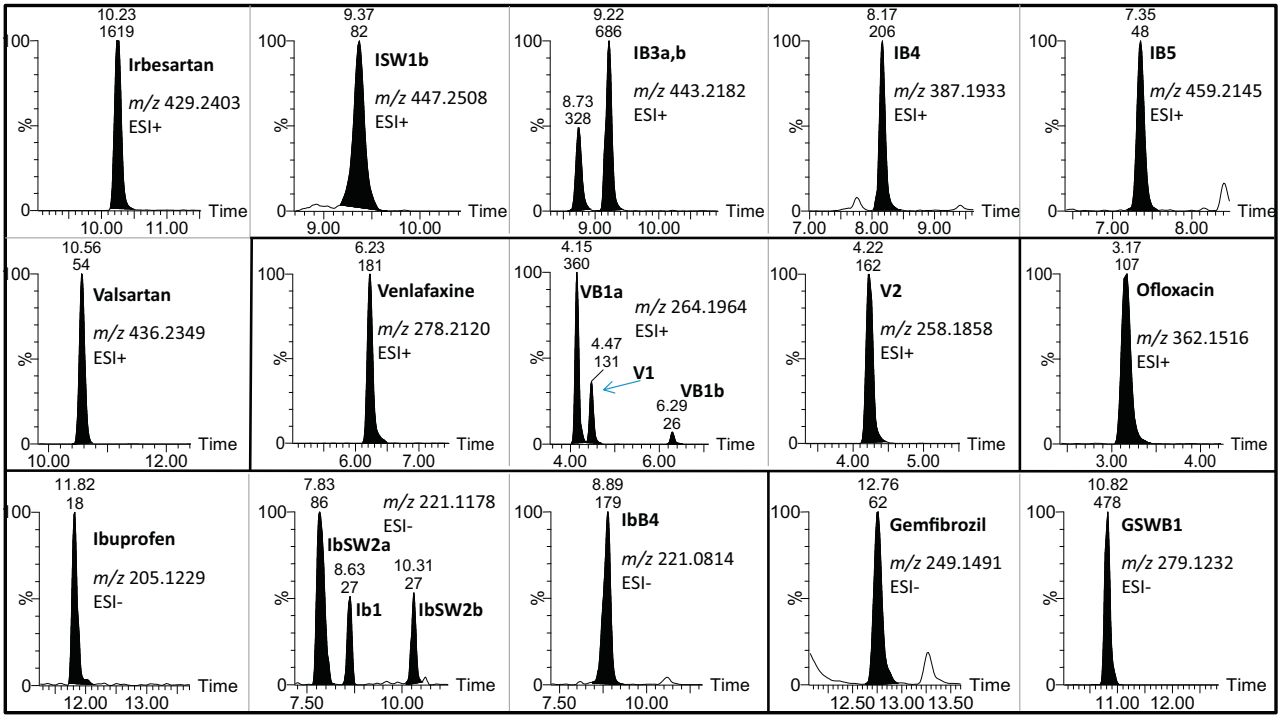

Fig. 5. Positive findings of the pharmaceuticals selected and their metabolites/TPs in different EWW samples.

ical formula corresponds to valsartan, a compound also belonging to the angiotensin receptor blockers class. Its identity was confirmed by its retention time, as the reference standard was available at our laboratory (Table 1). This reveals that common fragments may be shared not only by TPs but also by other chemically-related compounds, like in this case.

Finally, another ibuprofen-related unknown compound was found in EWW. A new chromatographic peak (Ib1) sharing the same exact mass (*m/z* 221.1178) and two fragment ions (*m/z* 177.1279 and 159.1174) with the ibuprofen TPs IbSW2(abc) was observed at a different retention time (6.9 min). According to its accurate mass, an elemental composition of $[C_{13}H_{17}O_3]^-$ (0.2 mDa) was assigned. Its identity was unequivocally confirmed after injecting the 1-hydroxy ibuprofen reference standard, as retention time and mass spectrum were in agreement with Ib1.

Analysis by LC-QTOF under MS^E mode, combined with MetaboLynx application manager and/or common fragmentation pathway strategy, has proven to be a valuable tool for identification of pharmaceutical TPs in waters. The potential of this technique for tentative identification of TPs has been demonstrated, as the subsequent acquisition of reference standards has allowed the unequivocal confirmation of the suggested identity. It would be interesting to perform additional HPLC-NMR experiments to unequivocally identify the structure of these transformation products. Although this technique has proven to be very promising in the structural elucidation of organic compounds, it has not been possible to apply due to the unavailability of this equipment in our

lab. Anyway, its modest sensitivity, which is in general three orders of magnitude lower than that of HPLC-MS, might also hamper the application to these types of experiments [44].

3.4. Retrospective search in EWW and SW samples

In order to test whether the TPs identified in this work were present in aquatic samples, a retrospective evaluation of accurate-mass full-acquisition data acquired by QTOF MS was performed. For this purpose, 38 EWW and 18 SW samples, previously analyzed by LC–QTOF MS, were re-processed using ChromaLynx XS software. The database of the compounds investigated contained the elemental composition, fragment ions and retention times of the 6 parent pharmaceuticals, the 22 TPs resulting from the biotransformation experiments and the 3 TPs detected by common fragmentation pathway.

As shown in Table 6, both parent compounds as well as some TPs were found in EWW and SW. Irbesartan was detected in 92% of the EWW and in 39% of SW analyzed. Regarding its TPs, ISW1b, IB3a and IB3b were identified in more than 80% of the EWW samples. The biotransformation product IB5 was also detected in a large number of samples (79%); however, its confirmation was not possible, as no fragment ions that supported its identification were observed. As expected, fewer positive findings were found in SW. As an example, TP IB3a was detected in 84% of the EWW samples and in 22% of the SW samples. The pharmaceutical valsartan was found in 79% and 33% of the EWW and SW samples analyzed, respectively.

Venlafaxine was present in 87% of EWW and 22% of SW samples. It is important to notice that its biotransformation products VB1a and VB1b (*O*- and *N*-desmethyl-venlafaxine, respectively) were more frequently detected in effluent wastewater than parent venlafaxine itself. However, VB1b could not be confirmed with fragment ions, presumably due to its low concentration level. Regarding the compounds detected by common fragment ions, V2 was identified in 87% of the EWW samples and V1 in 58%. The frequencies of detection notably decreased in SW (6–11%).

Ofloxacin was detected in 82% of EWW and 17% of SW samples, but its only TP identified in degradation experiments was not detected in any of the samples.

Regarding ibuprofen, the TP most frequently found in both EWW and SW was IbB4 (34 and 50%, respectively), followed by 1-hydroxy ibuprofen (Ib1, 21% in EWW and 6% in SW) and 2-hydroxy ibuprofen (IbSW2a, 16% in EWW and 11% in SW). Interestingly, all these TPs were more frequently detected than ibuprofen itself.

Similarly, the gemfibrozil biotransformation product GSWB1, was more frequently detected (71% in EWW and 33% in SW) than its parent compound (24% in EWW and 22% in SW). Fig. 4 shows a positive finding of gemfibrozil and its TP GSWB1 in EWW. The retention times were re-calculated for samples analyzed under different gradient conditions (for more details, see section 1.3 in SI). In this case, the predicted retention time for this TP under the gradient conditions used in the analysis of the water samples was 11 min, close to 10.82 min observed for this peak. In addition, two fragment ions eluted at the same retention time, supporting the identity of the compound.

Some examples of positive findings are depicted in Fig. 5, where selected nw-XICs are shown for irbesartan and 5 of its TPs, valsartan, venlafaxine and 4 TPs, ofloxacin, ibuprofen and 4 TPs, and gemfibrozil and GSWB1 in EWW.

Although experiments performed under controlled-laboratory conditions will never reproduce exactly the real world conditions, the usefulness of this type of experiments is supported by the fact that several of the degradation products identified in the lab experiments have been detected in the environmental samples. This shows that the TPs discovered in this work are actually present in the aquatic environment.

Table 6
Pharmaceuticals and metabolites/TPs detected in EWW and SW samples after retrospective search in QTOF MS data.

	Positive finding (%)	
	EWW(n = 38)	SW(n = 18)
Irbesartan	92	39
ISW1b	87	6
IB3a	84	22
IB3b[b]	89	22
IB4[b]	32	11
IB5[b]	79[a]	22[a]
Valsartan	79	33
Venlafaxine	87	22
VB1a	92	17
VB1b	92[a]	17[a]
V1	58	6
V2[b]	87	11
Ofloxacin	82	17
Ibuprofen	11	6
IbSW2a	16	11
IbSW2b	8[a]	0
IbB4[b]	34	50
Ib1	21	6
Gemfibrozil	24	22
GSWB1	71	33

[a] Only the $[M+H]^+$/$[M-H]^-$ was observed.
[b] To our knowledge, TP not reported in scientific literature yet.

4. Conclusions

This work reports the degradation of five pharmaceuticals (ibuprofen, ofloxacin, venlafaxine, irbesartan and gemfibrozil) in experiments with surface water and activated sewage sludge under laboratory conditions. A total of 22 TPs were detected and tentatively identified by LC–QTOF MS. Additionally, 2 further venlafaxine TPs and 1 ibuprofen TP were found after applying the strategy based on common fragmentation pathway in effluent wastewater. After tentative identification of the TPs reported in this article, reference standards were acquired (when commercially available) to unequivocally confirm the identity of these compounds. Retrospective evaluation of accurate-mass full-spectrum acquisition data from water samples previously analyzed by QTOF MS showed the presence of parent pharmaceuticals but also 14 transformation products. It is important to highlight that, in some cases, TPs were more frequently detected than the corresponding parent compound. This was the case of ibuprofen degradation products IbSW2, IbB4 and Ib1, and the TP of gemfibrozil, GSWB1. In the light of data reported in this work, it would be recommended to include the most relevant TPs (at least those that have been found in the effluent and surface waters analyzed), in addition to the parent compounds, in future monitoring programs to gain a more realistic insight of the impact of the presence of pharmaceuticals in the aquatic environment. The environmental relevance of the TPs discovered should be addressed in a further step with studies directed towards their toxicological effects, and not only their abundance in the water ecosystem. Obviously previous synthesis of the suggested TPs would be also required, at least of those compounds that have been found in water samples. Data reported in this paper, will facilitate the future development of analytical methodologies for accurate quantification of these TPs in waters (e.g., making use of LC–MS/MS with triple quadrupole).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.09.053.

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