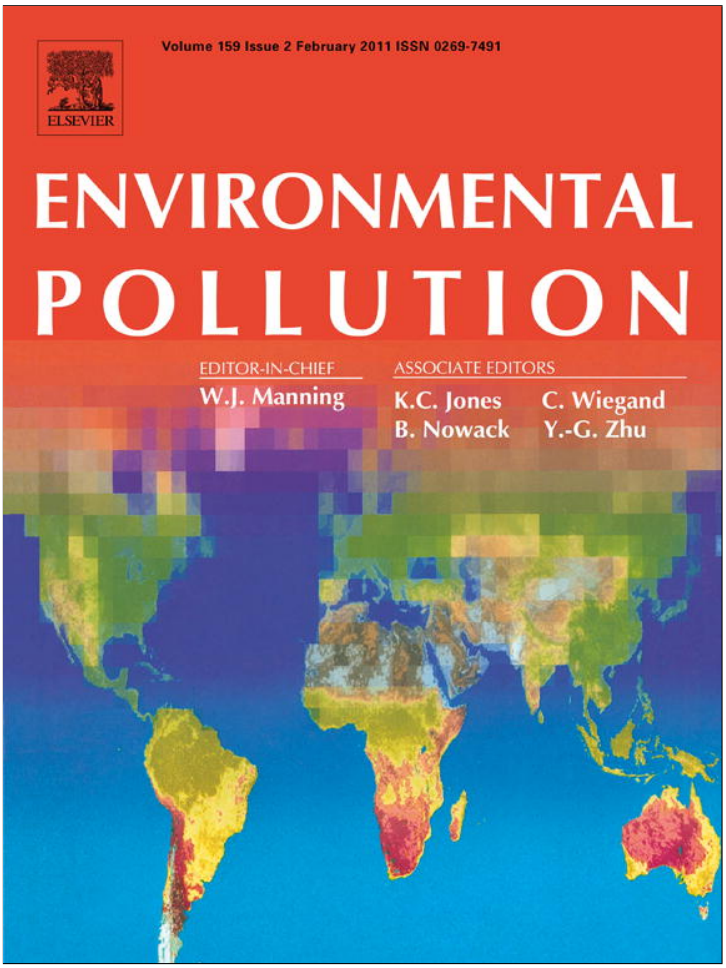

Environmental Pollution 159 (2011) 557–566

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Nontarget analysis of polar contaminants in freshwater sediments influenced by pharmaceutical industry using ultra-high-pressure liquid chromatography–quadrupole time-of-flight mass spectrometry

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Comprehensive analysis of freshwater sediments by UPLC/QTOF indicated importance of pharmaceutically-derived polar contaminants.

ARTICLE INFO

Article history:
Received 2 August 2010
Received in revised form
7 October 2010
Accepted 10 October 2010

Keywords:
High-resolution mass spectrometry
Ultra-high-pressure liquid chromatography
Polar contaminants
Sediments
Nontarget contaminants

ABSTRACT

A comprehensive analytical procedure for a reliable identification of nontarget polar contaminants in aquatic sediments was developed, based on the application of ultra-high-pressure liquid chromatography (UHPLC) coupled to hybrid quadrupole time-of-flight mass spectrometry (QTOFMS). The procedure was applied for the analysis of freshwater sediment that was highly impacted by wastewater discharges from the pharmaceutical industry. A number of different contaminants were successfully identified owing to the high mass accuracy of the QTOFMS system, used in combination with high chromatographic resolution of UHPLC. The major compounds, identified in investigated sediment, included a series of polypropylene glycols (n = 3–16), alkylbenzene sulfonate and benzalkonium surfactants as well as a number of various pharmaceuticals (chlorthalidone, warfarin, terbinafine, torsemide, zolpidem and macrolide antibiotics). The particular advantage of the applied technique is its capability to detect less known pharmaceutical intermediates and/or transformation products, which have not been previously reported in freshwater sediments.

1. Introduction

Freshwater systems throughout the world are exposed to an increasing pressure from various anthropogenic sources (Schwarzenbach et al., 2006). After the release into the natural waters, anthropogenic contaminants are subject to various compound-specific biogeochemical processes, which determine their distribution and ultimate fate in the aquatic environment. Sediments were shown to be a major sink of various organic pollutants, which may represent a significant hazard to the benthic organisms (Chen and White, 2004). The composition of ecotoxic organic compounds in contaminated sediments is very complex, which requires laborious analytical procedures for their comprehensive analysis (Biselli et al., 2005; Fernandez et al., 1992). The chemical characterization of nontarget sediment contaminants is strongly dominated by the usage of gas chromatography–mass spectrometry (GC–MS), which limits the range of identifiable toxic contaminants to volatile and semi-volatile and relatively non-polar compounds (Biselli et al., 2005; Kaisarevic et al., 2009).

Recent developments in liquid-chromatography–tandem mass spectrometry (LC–MS–MS) (Barcelo and Petrovic, 2007) facilitated measurements of different types of emerging polar contaminants in the environment (Giger, 2009; Gros et al., 2006). A large majority of LC–MS applications in environmental analysis is directed towards highly specific determination of pre-selected target compounds using selected reaction monitoring (Richardson, 2008). Numerous multi-residue analytical methodologies, encompassing several dozens of individual compounds, have been developed in the last decade, becoming a preferred tool for the determination of various compound classes such as pharmaceuticals (Gros et al., 2006; Kim and Carlson, 2005), surfactants (Gonzalez et al., 2008) and pesticides (Hernandez et al., 2005).

In contrast, application of LC–MS techniques for nontarget screening of environmental contaminants is still relatively rare (Richardson, 2008). One of the reasons is high cost of the LC–MS equipment capable of determining accurate mass of the detected compounds. Moreover, well-known difficulties associated with the structural elucidation of a true unknown from its empirical formula (Thurman et al., 2005) and a rather limited availability of comprehensive mass spectral libraries (Ferrer et al., 2006) represent important obstacles for a more widespread application of LC–MS techniques for identification purposes.

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doi:10.1016/j.envpol.2010.10.009

Nevertheless, there have been several examples in the literature on the identification of unknown water contaminants using liquid chromatography (LC) coupled to high-resolution mass spectrometry (Bobeldijk et al., 2001; Bueno et al., 2007; Ferrer et al., 2004; Hogenboom et al., 2000; Ibanez et al., 2005), mainly using time-of-flight (TOF) mass analyzers. Kern et al. (2009) developed a six-step funnelling procedure for the identification of transformation products (TPs) of organic contaminants in natural waters, based on the application of ion trap orbitrap tandem mass spectrometry, and reported on identification of 19 TPs. Most recently, Krauss et al. (2010) gave an excellent overview of the state-of-the-art and future trends of the application of high-resolution mass spectrometric techniques in the environmental analysis.

Due to the high complexity of environmental samples the efficiency and resolution of the chromatographic system, coupled to mass spectrometric detection, play an important role in the successful identification of unknowns. Compared to the conventional HPLC technique, the coupling between UHPLC and QTOFMS significantly increases the potential for the identification of nontarget polar contaminants in environmental samples by providing superior chromatographic efficiency and separation power. The resulting peaks are sharper, which significantly reduces the problem of co-eluting peaks and mass spectral overlap (Plumb et al., 2004). However it is interesting to note that the most of the reports, which employed UHPLC–QTOFMS, focused either on the quantitative target analysis (Farre et al., 2008; Gonzalez et al., 2008) or, in some cases, on a wide-spectrum screening of pre-selected environmental contaminants (Ibanez et al., 2009).

The aim of this study was to investigate the potential of hybrid quadrupole time-of-flight mass spectrometry (QTOFMS) coupled to ultra-high-pressure liquid chromatography (UHPLC) for the identification of major organic contaminants present in the polar fraction of freshwater sediments influenced by wastewater discharges from the pharmaceutical industry. Unlike most of the previous reports, which usually reported on identification of few unknowns, our study demonstrates a large potential of this technique for a comprehensive characterization of contaminated sediments.

2. Materials and methods

2.1. Chemicals

All chemicals were of high purity grade. Acetonitrile (J.T.Baker, Deventer, Netherlands) and formic acid (Fluka, Buchs, Switzerland) were of LC–MS–MS grade. Hexane, dichloromethane and methanol (all from Merck, Darmstadt, Germany) were of HPLC grade. Water was purified using Elix-Milli-Q system (Millipore, Bedford, USA). Silicon dioxide (99.8%) was purchased from Sigma–Aldrich (Steinheim, Germany). Zolpidem hemitartarate (1 mg/mL; solution in methanol) was purchased from Lipomed AG (Arlesheim, Switzerland). Azithromycin (≥95%) and warfarin (>98%) were bought from Fluka (Buchs, Switzerland). Deuterated sulfamethoxazole ([$^2H_4$] SMX), erythromycin (98%), torsemide (≥98%), terbinafine hydrochloride (≥98%), chlorthalidone (pharmaceutical grade) as well as commercial mixtures of polypropylene glycols (P 400, P 1200) were purchased from Sigma–Aldrich (Steinheim, Germany). Octylbenzene sulfonate (C_8-LAS) was purchased from Fluka (Buchs, Switzerland), while a commercial mixture of linear alkylbenzene sulfonates (LAS) was donated by detergent industry Saponia (Osijek, Croatia).

2.2. Investigated site and sampling

Sediment sample, analysed in this study, was collected from the Gorjak Creek, a small water course in the area of the city of Zagreb, Croatia. The Gorjak Creek is highly influenced by wastewater discharges from pharmaceutical industry. The sample of surface sediment was taken in February 2008 at the location situated 500 m downstream from the wastewater discharge using a plastic coring device. The wet sediment sample was homogenised and an aliquot of about 1 kg was air-dried at the room temperature in a clean fume hood. Finally, the sample was pulverized using a mechanical mill.

The collected sediment sample was predominately composed of silt (60%) and contained 44% dry matter. The content of total organic carbon, nitrogen and sulphur was 6.9%, 0.65% and 0.22%, respectively.

2.3. Analysis

The diagram shown in Fig. 1 indicates main steps of the nontarget analysis of polar contaminants applied in this study. The protocol included an exhaustive sediment extraction using two different solvents, extract fractionation to isolate the polar fraction and subsequent UHPLC–TOFMS analysis providing high-performance chromatographic separation and mass spectrometric identification using the accurate mass feature.

2.3.1. Sediment extraction and fractionation

Briefly, air-dried sediment sample (50 g) was extracted employing Soxhlet apparatus, using dichloromethane (200 mL) and methanol (200 mL) as solvents in two separate cycles. In addition, the same volume of pure dichloromethane and methanol was processed identically as the sediment extract to obtain the blank sample. The methanol and dichloromethane extracts were combined and an aliquot (20%) was evaporated to dryness in the stream of nitrogen using a TurboVap system (Caliper Life Sciences, Hopkinton, MA, USA). The residue was subjected to fractionation using a simple column filled with 5 g of deactivated (15% water) silica gel (Terzic et al., 2009). Three different fractions were sequentially eluted as follows: A) nonpolar fraction – elution with 20 mL n-hexane, B) medium polar fraction – elution with 25 mL dichloromethane and C) polar fraction – elution with 25 mL methanol. An aliquot (0.5 mL) of the polar (methanol) fraction was mixed with 0.5 mL of ultrapure water and analysed using an UHPLC–QTOFMS system. The first two fractions (A and B), which contained non-polar and medium-polar compounds were analysed by gas chromatography–mass spectrometry (GC–MS), and the results were discussed elsewhere (Terzic et al., 2009).

2.3.2. UHPLC–QTOFMS analysis

The LC–MS characterization of nontarget polar contaminants was performed using ultra-high-pressure liquid chromatography (UHPLC) coupled to quadrupole-time-of-flight mass spectrometry (QTOFMS). UHPLC separation was performed using a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent delivery system and autosampler. The chromatographic separations employed a column (50 mm × 2.1 mm) filled with a 1.7 μm BEH C_{18} stationary phase (Waters Corp., Milford, MA, USA). Binary gradients were employed for the elution. In the positive ionization mode (PI) the eluents A and B were 0.1% (v/v) formic acid in water and 0.1% formic acid in acetonitrile, respectively. The

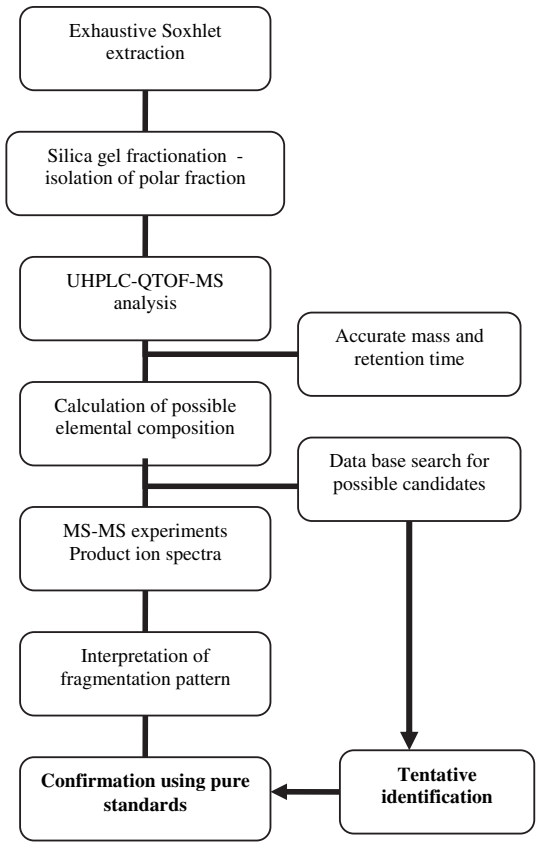

Fig. 1. Diagram of the applied analytical protocol.

gradient elution at 0.4 mL/min started at 5% B, which was kept for 1 min and then the percentage was linearly increased in several steps to reach 95% B in 19 min, and the final conditions were kept for another 2 min. The total run-time, including column conditioning to reach initial conditions was 23 min. Eluents used in the negative ionization (NI) mode consisted of (A) water and (B) acetonitrile without addition of formic acid, while the elution gradient was identical to that used in the PI ionization mode (a stepwise linear increase from 5% B to 95% B in 19 min, followed by an isocratic hold for 2 min).

The mass spectrometry was performed on a QTOF Premier instrument (Waters Micromass, Manchester, UK) using an orthogonal Z-spray–electrospray interface. Drying gas and nebulizing gas was nitrogen, while argon was used as a collision gas in MS–MS experiments. The desolvation gas flow was set to 600 L/h at a temperature of 280 °C. The cone gas flow was adjusted to 30 L/h, and the source temperature to 120 °C. The capillary and cone voltages in the PI and NI were 3500 and 3200 V, respectively, while the cone voltage in both modes was set to 35 V. The instrument was operated in V mode with TOFMS data being collected between *m/z* 100–1000, applying collision energy of 5 eV. All spectra were recorded using extended dynamic range (DRE) option in order to correct for possible peak saturations and the data were collected in the centroid mode with a scan time of 0.08 s and interscan time of 0.02 s. For each precursor ion, tentatively identified in MSTOF mode, several MS–MS runs, applying different collision energies, were performed in order to obtain a fragmentation degree suitable for further structural elucidation.

In order to ensure maximum accuracy and reproducibility of the system, all acquisitions were carried out using an independent reference spray via the lock spray interface. Leucine enkephalin was applied as a lock mass both in PI (*m/z* 556.2771) and NI mode (*m/z* 554.2615). Besides that, two internal standards, including deuterated sulfamethoxazole (d4-SMX) and linear octylbenzene sulfonate (C_8-LAS) were added to all sediment extracts in order to provide a control of the instrument performance.

The chromatograms, recorded in the total ion current (TIC) mode, were systematically examined by manually generating mass spectra of each individual peak using background-subtraction option. Only major peaks (>10% of the full scale intensity) were subject to identification process as non-target analytes. The generated TOF mass spectra were studied in detail in order to detect key ions suitable for the structural elucidation of the detected compounds. In addition, when a tentative identification, based on TOF mass spectra, indicated the presence of contaminants, which typically occur in mixtures of related compounds such as homologues and oligomers, extracted ion chromatograms (XICs) were applied to facilitate their visualization and subsequent confirmation.

Elemental composition of the selected precursor and product ions were calculated using the Elemental Composition Calculator embedded into the MassLynx software.

The main criteria, applied in setting up search parameters for the calculation of the most probable molecular formula, followed the general rules applied in mass spectrometry, such as the assessment of the isotopic pattern and the nitrogen rule. Such an initial expert assessment was used to decide whether to exclude or include some elements (e.g. halogens) in the calculation of molecular formula and to estimate the number of carbon atoms. This proved to be essential for reducing the number of calculated elemental compositions, which greatly facilitated further steps leading to the final identification of the candidate compounds. Elements regularly considered in the calculations included C, H, N, O and S, while in some cases other elements (Cl, P and F) were also taken into consideration. Since the analytical approach aimed at identifying a broad spectrum of unknown compounds, the double bond equivalent (DBE) parameter was set at −1.5 to 50. The acceptable deviation from the theoretical *m/z* values was set up at 5 mDa.

2.3.3. Identification protocol

Identification protocol consisted of three main phases, including TOFMS experiments, MS–MS experiments and confirmation of the identifications by reference standards. The first identification step for all identified contaminants included calculation of the possible elemental composition of the selected protonated or deprotonated molecules from the mass spectra recorded in TOFMS mode and an exhaustive database search for possible candidate compounds (Fig. 1). The databases used in this study included primarily Chemspider, Merck Index and ESIS, as well as an in-house database. For some common types of contaminants, the information on accurate mass together with information on retention time was sufficient for a reliable identification, provided that reference compounds were available. For unknown or less common compounds the ultimate assignment of the identity of the nontarget compounds was achieved by tandem mass spectrometry (MS–MS) of pre-selected precursor ions, followed by comparison of the observed fragmentation patterns with the expected main product ions of the selected candidates. Finally, the confirmation of the identified nontarget compounds was achieved by comparing the key identification parameters (accurate mass of the protonated or deprotonated molecule, isotopic pattern, accurate mass of the product ions, fragmentation pattern and retention time), obtained from the analysis of the sediment extracts with those of the corresponding reference compounds.

3. Results and discussion

3.1. UHPLC separation

The chromatograms of the sediment sample recorded in both positive and negative ionization modes were rather complex, exhibiting presence of a large number of different organic compounds (Fig. 2). Despite the fact that the analysed extract contained only rather polar organic compounds (as defined by their behaviour during silica gel fractionation), the observed peaks eluted over a wide range of retention times, indicating significant variability of hydrophobic moieties in the individual molecules. Moreover, it should be taken into account that complex environmental samples typically contain hundreds of individual chemical compounds present in rather different concentrations. In fact, as a rule, the probable number of individual components in a given concentration range drastically increases with the decreasing concentration. Identification of trace components is therefore extremely difficult and they are assessed mainly using tailored target LC–MS analyses based on selected reaction monitoring (SRM) technique. On the contrary, this study was focused at identifying the major components of the extract, which were represented by distinct peaks in the recorded TIC chromatograms. The application of UHPLC, providing superior chromatographic resolution, resulted in a reduced overlap and better quality of the mass spectra of the selected candidate compounds, which was very important for the successful outcome of the identification process.

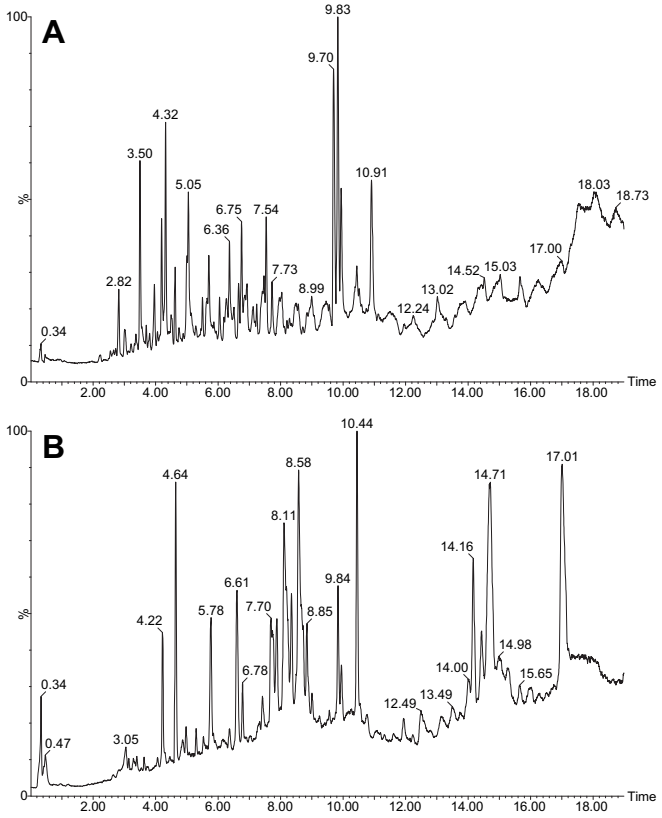

Fig. 2. UHPLC–QTOFMS total ion current chromatograms of the sediment extract from the Gorjak Creek, Croatia acquired in A) positive mode and B) negative ionization mode.

3.2. Identification of contaminants using TOFMS

Despite well-known difficulties encountered during elucidation of chemical structures of unknown (nontarget) compounds based on LC–MS analyses due to the lack of comprehensive searchable libraries of reference spectra, we were able to identify a large percentage (>80%) of the major peaks in the TIC chromatograms. Nevertheless, the prominent peaks having RT of 2.82, 4.62 and 9.83 min in the positive ionization mode and 6.78, 9.84, 9.96, 10.44 and 14.16 min in the negative ionization mode, remained unidentified. For these peaks we encountered problems either in the assignment of the molecular formula to the measured accurate mass or in linking the assumed elemental composition to a defined chemical structure.

The list of compounds, identified in positive and negative electrospray ionization mode, are presented in Tables 1 and 2. The tables show the key data used for identification, including the most probable elemental composition, comparison of experimental and theoretical mass, absolute and relative mass error and retention time. The table also contains the information on the normalised i-FIT parameter, which indicates the agreement between the measured and theoretical isotopic patterns. For the less common contaminants such as pharmaceuticals the identifications at this stage were considered only tentative. However, for some common contaminant classes, such as common fatty acids (RT = 10.02–17.13 min; Table 2) and the major anionic surfactants alkylbenzene sulfonates (RT = 7.15–9.07; Table 2), accurate mass and retention time were actually sufficient for a reliable identification. Besides that, both classes occur in the environment as homologue series, providing easily recognizable patterns in reconstructed XIC chromatograms. The most prominent LAS homologues were C_{12} and C_{13} LAS, due to the enhanced enrichment of the higher homologues in the sediment as compared to the aqueous phase (Lara-Martin et al., 2008; Terzic et al., 2008). The fatty acid pattern showed a strong predominance of palmitic and stearic acids, which was expected because they belong to the most common saturated fatty acids.

Two other classes of contaminants, alkyldimethylbenzyl ammonium (benzalkonium) cationic surfactants and polypropylene glycols (PPG), were identified using the same approach. The benzalkonium surfactants represented the most prominent peaks in the UHPLC chromatogram, recorded in the positive ionization mode (RT = 9.70–14.62 min; Table 1). These surfactants are widely used in pharmaceutical formulations owing to their excellent antiseptic properties. Due to their pronounced lipophilic moiety these compounds have a high tendency to adsorb on particles and were shown to accumulate in aquatic sediments (Li and Brownawell, 2009). Since they are positively charged at lower pH values, which were applied for UHPLC–QTOFMS analyses in the positive ionization mode, they can be easily detected as corresponding benzalkonium cations. The most prominent homologues of benzalkonium surfactants were dodecyl and tetradecyl homologues with corresponding masses of benzalkonium cations at *m/z* 304.2998 and 332.3316, respectively (Table 1). It is interesting to note that the measured accurate mass of benzalkonium cations gave only 2 hits for the corresponding elemental composition, probably due to the earlier described high positive mass defects characteristic of alkylammonium ions (Li and Brownawell, 2009).

Another very abundant group of compounds, detected in the positive ionization mode, were polypropylene glycols (PPGs; Table 1). This compound class is closely related to polyethylene glycols (PEGs), which are common constituents of municipal wastewaters (Castillo and Barcelo, 1999). However, in the Gorjak Creek PPGs strongly predominated, indicating a specific

Table 1
List of compounds identified in freshwater sediment impacted by pharmaceutical industry. Identification was made using ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) in positive polarity electrospray ionization mode.

Compound	Abbreviation	Retention time (min)	Elemental composition	Theoretical mass (*m/z*)	Experimenal mass (*m/z*)	Error		i-FIT[a]
						mDa	ppm	
Pharmaceuticals:								
Torsemide intermediate	TORI	3.50	$[M+H]^+$ $C_{12}H_{14}N_3O_2S$	264.0807	264.0813	0.6	2.3	0.0
Zolpidem	ZOLP	4.19	$[M+H]^+$ $C_{19}H_{22}N_3O$	308.1763	308.1769	0.6	1.9	0.0
Azithromycin	AZI	4.32	$[M+H]^+$ $C_{38}H_{73}N_2O_{12}$	749.5164	749.5168	0.4	0.5	0.1
Desmethyl azithromycin	DAZI	4.33	$[M+H]^+$ $C_{37}H_{71}N_2O_{12}$	735.5007	735.5004	−0.3	−0.4	0.3
Torsemide	TOR	5.05	$[M+H]^+$ $C_{16}H_{21}N_4O_3S$	349.1334	349.1331	−0.3	−0.9	0.0
Erythromycin	ERY	5.43	$[M+H]^+$ $C_{37}H_{68}NO_{13}$	734.4691	734.4706	1.5	2.0	1.4
Erythromycin anhydrous	ERY-H_2O	6.05–7.12[b]	$[M+H]^+$ $C_{37}H_{66}NO_{12}$	716.4585	716.4583	−0.2	−0.3	0.1
Terbinafine	TERB	7.24	$[M+H]^+$ $C_{21}H_{26}N$	292.2065	292.2057	−0.8	−2.7	0.0
Warfarin	WAR	7.55	$[M+H]^+$ $C_{19}H_{17}O_4$	309.1127	309.1116	−1.1	−3.6	0.0
Pharmaceutically derived compounds:								
Polypropylene glycols; $n = 3–16$[c]	PPGs	2.61–9.96						
	$n = 6$	5.02	$[M+H]^+$ $C_{18}H_{39}O_7$	367.2696	367.2685	−1.1	−3.0	0.1
	$n = 7$	5.66	$[M+H]^+$ $C_{21}H_{45}O_8$	425.3114	425.3102	−1.2	−2.8	0.2
	$n = 8$	6.27	$[M+H]^+$ $C_{24}H_{51}O_9$	483.3533	483.3526	−0.7	−1.4	0.0
	$n = 9$	6.85	$[M+H]^+$ $C_{27}H_{57}O_{10}$	541.3952	541.3940	−1.2	−2.2	1.2
	$n = 10$	7.41	$[M+H]^+$ $C_{30}H_{63}O_{11}$	599.4370	599.4368	−0.2	−0.3	0.0
	$n = 11$	7.97	$[M+H]^+$ $C_{33}H_{69}O_{12}$	657.4789	657.4785	−0.4	−0.6	0.0
	$n = 12$	8.50	$[M+H]^+$ $C_{36}H_{75}O_{13}$	715.5208	715.5209	0.1	0.1	0.0
	$n = 13$	9.01	$[M+H]^+$ $C_{39}H_{81}O_{14}$	773.5626	773.5629	0.3	0.4	0.0
Alkyl dimethyl benzyl ammonium compounds; $n = 8–18$[d]	ADBAC							
	$n = 12$	9.70	M^+ $C_{21}H_{38}N$	304.3004	304.2998	−0.6	−2.0	0.0
	$n = 14$	10.91	M^+ $C_{23}H_{42}N$	332.3317	332.3316	−0.1	−0.3	0.4
	$n = 16$	13.12	M^+ $C_{25}H_{46}N$	360.3630	360.3645	1.5	4.2	0.0
	$n = 18$	14.62	M^+ $C_{27}H_{50}N$	388.3943	388.3949	0.6	1.5	0.0

[a] Normalised i-FIT values calculated using the MassLynx software; lower the value better the fit between the theoretical and observed isotopic patterns of a given ion.
[b] Three ERY-H_2O isomers were chromatographically separated.
[c] PPG oligomers with $n = 3–16$ were detected as single-charged species, however, only the most abundant PPGs are listed in the Table.
[d] Six homologues of ADBAC were detected, only the most abundant are listed in the Table.

Table 2
List of compounds identified in freshwater sediment impacted by pharmaceutical industry using ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) in negative polarity electrospray ionization mode.

Compound	Abbreviation	Retention time (min)	Elemental composition	Theoretical mass (*m/z*)	Experimenal mass (*m/z*)	Error		i-FIT[a]
						mDa	ppm	
Pharmaceuticals:								
Chlorthalidone	CHTD	4.22	$[M-H]^-$ $C_{14}H_{10}N_2O_4SCl$	337.0050	337.0039	−1.1	−3.3	0.2
Torsemide	TOR	4.64	$[M-H]^-$ $C_{16}H_{19}N_4O_3S$	347.1178	347.1192	1.4	4.0	0.1
Torsemide intermedier	TORI	5.78	$[M-H]^-$ $C_{12}H_{12}N_3O_2S$	262.0650	262.0648	−0.2	−0.8	1.0
Warfarin	WAR	6.08	$[M-H]^-$ $C_{19}H_{15}O_4$	307.0970	307.0968	−0.2	−0.7	0.9
Linear alkylbenzene sulfonates								
	C_{10}LAS	7.27–7.42	$[M-H]^-$ $C_{16}H_{25}O_3S$	297.1524	297.1512	−1.2	−4.0	0.0
	C_{11}LAS	7.67–8.11	$[M-H]^-$ $C_{17}H_{27}O_3S$	311.1681	311.1658	−2.3	−7.4	0.2
	C_{12}LAS	8.11–8.58	$[M-H]^-$ $C_{18}H_{29}O_3S$	325.1837	325.1832	−0.5	−1.5	0.0
	C_{13}LAS	8.57–8.85	$[M-H]^-$ $C_{19}H_{31}O_3S$	339.1994	339.1999	0.5	1.5	0.0
	C_{14}LAS	9.07	$[M-H]^-$ $C_{20}H_{33}O_3S$	353.2150	353.2147	−0.3	−0.8	0.1
Fatty acids:								
Lauric acid		10.02	$[M-H]^-$ $C_{12}H_{23}O_2$	199.1698	199.1697	−0.1	−0.5	0.0
Myristic acid		12.09	$[M-H]^-$ $C_{14}H_{27}O_2$	227.2011	227.2006	−0.5	−2.2	0.0
Linolenic acid		11.86	$[M-H]^-$ $C_{18}H_{29}O_2$	277.2168	277.2174	0.6	2.2	0.3
Palmitoleic acid		12.83	$[M-H]^-$ $C_{16}H_{29}O_2$	253.2168	253.2165	−0.3	−1.2	0.0
Linoleic acid		13.54	$[M-H]^-$ $C_{18}H_{31}O_2$	279.2324	279.2321	−0.3	−1.1	0.1
Palmitic acid		14.71	$[M-H]^-$ $C_{16}H_{31}O_2$	255.2324	255.2321	−0.3	−1.2	0.0
Oleic acid		15.08	$[M-H]^-$ $C_{18}H_{33}O_2$	281.2481	281.2462	−1.9	−6.8	0.6
Stearic acid		17.01	$[M-H]^-$ $C_{18}H_{35}O_2$	283.2637	283.2639	0.2	0.7	0.0

[a] Normalised i-FIT values calculated using the MassLynx software; lower the value better the fit between the theoretical and observed isotopic patterns of a given ion.

pharmaceutical source. Indeed, PPGs are widely used in the pharmaceutical industry as inactive (low-toxic) solvents of active drug ingredients. Most of the distinct peaks, apparent in the UHPLC trace, acquired in the positive ionization mode (Fig. 2A), belong to PPGs. The most prominent ones were those having 6–11 propoxy units, which can be easily recognized in the chromatogram reconstructed using either their corresponding $[M+H]^+$, $[M+Na]^+$ and $[M+NH_4]^+$ ions. Besides that, higher PPG oligomers, having MW > 1000 were present as multiply charged ions and were also recorded in the chromatogram. The chromatographic pattern of PPGs is characterized by regularly increasing retention times of individual PPG oligomers, which were accompanied by *m/z*

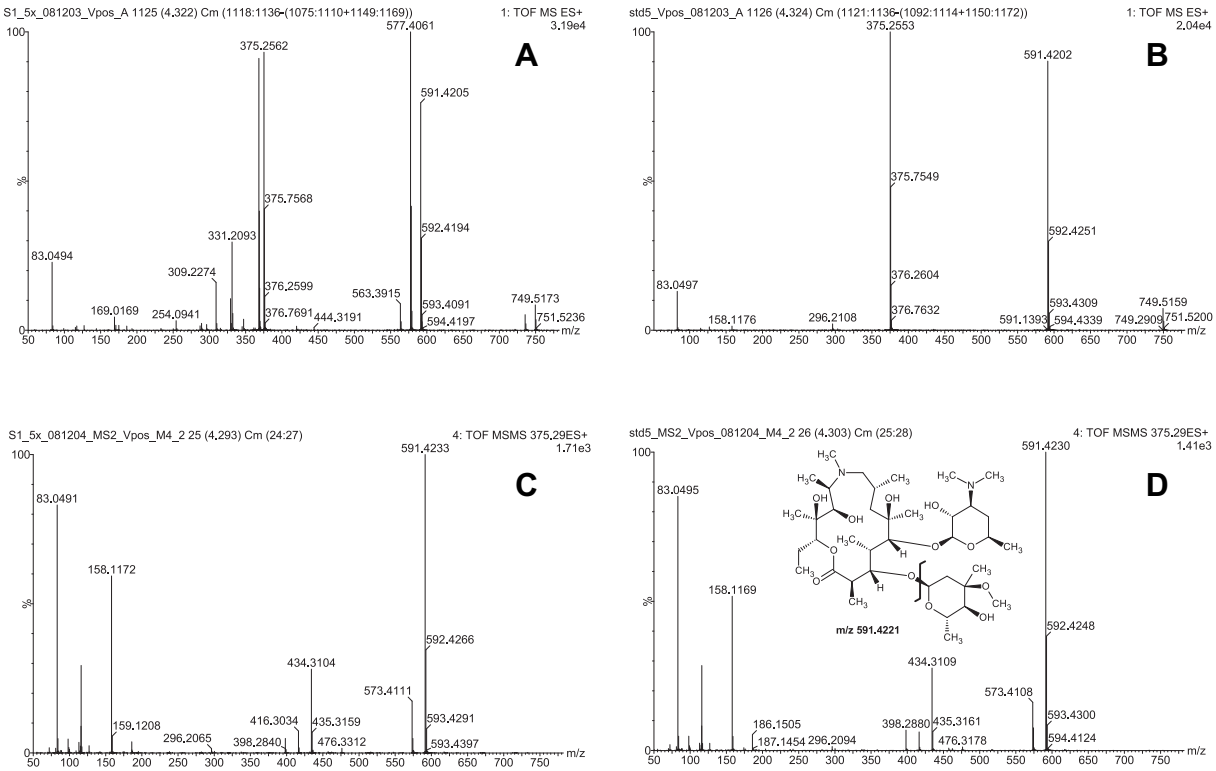

Fig. 3. Identification of azithromycin in the sediment extract from the Gorjak Creek using UHPLC–QTOFMS with electrospray ionization in positive polarity mode: A) TOF mass spectrum of the peak at 4.3 min; B) TOF mass spectrum of azithromycin standard; C) product ion spectrum of precursor ion *m/z* 375 in sediment sample; D) product ion spectrum of azithromycin standard using ion *m/z* 375 $[M+2H]^{2+}$ as a precursor.

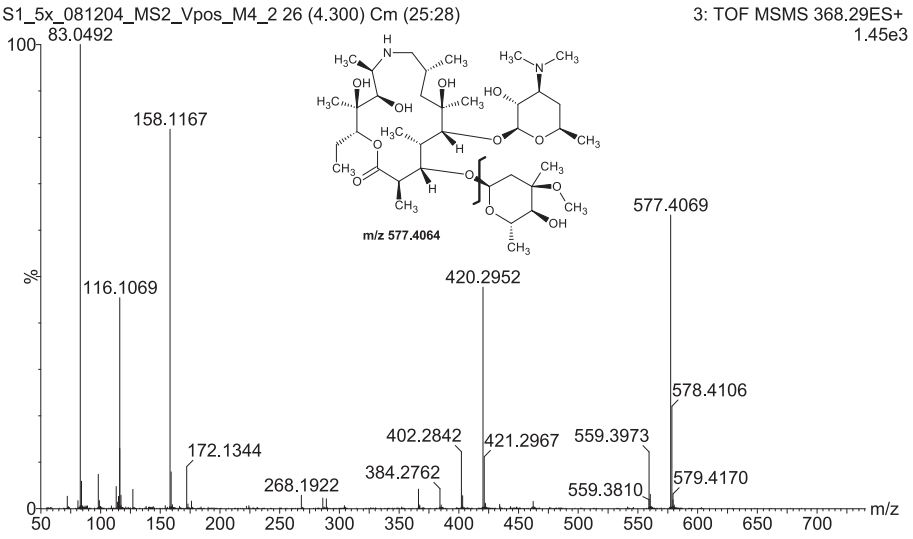

Fig. 4. Product ion spectrum of the unknown compound coeluting with azithromycin (RT 4.32 min) obtained using ion *m/z* 368 as a precursor.

values increasing at a regular interval of exactly 58.0419 Da, corresponding to propoxy unit (C_3H_6O). These features were confirmed by analysis of commercial PPG mixtures. Owing to such a clear pattern, additional MS–MS experiments on individual PPGs were not considered necessary for final confirmation.

3.3. Ultimate identification of nontarget pharmaceuticals using QTOFMS

3.3.1. Azithromycin and its intermediate

The peak at the RT of 4.32 min exhibited rather complex TOF mass spectrum with several prominent ions, including *m/z* 591.4205, *m/z* 577.4061, *m/z* 375.7568 and *m/z* 368.2492 (Fig. 3A). This indicated that either several compounds coeluted at the specified retention time and/or a substantial fragmentation of eluted compound(s) occurred in the ion source or in the TOF analyzer at the applied conditions. The isotopic patterns of the two ions at *m/z* 375.7568 and *m/z* 368.2492, showing a difference of 0.5 Da suggested that these two ions were double charged species of the two minor ions observed at *m/z* 735.5004 and *m/z* 749.5137, which correspond to single-charged species of the parent molecules. The tentative elemental composition of the ion at *m/z* 749.5137 was calculated using MassLynx software. The best match was obtained for the formula $C_{38}H_{73}N_2O_{12}$. The

Table 3
Elemental composition and accurate mass measurement of product ions of pharmaceutical compounds identified in freshwater sediment, determined by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS).

Compound	Precursor ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Product ions					
				Elemental composition	Theoretical mass	Experimental mass	mDa	ppm	i-fit
Positive ionization:									
Torsemide intermediate	264	30	22	$C_{12}H_{11}N_2$	183.0922	183.0914	−0.8	−4.4	0.0
				$C_{11}H_8N_2$	168.0687	168.0678	−0.9	−5.4	0.1
Zolpidem	308	30	23	$C_{17}H_{15}N_2O$	263.1184	263.1185	0.1	0.4	0.0
				$C_{16}H_{15}N_2$	235.1235	235.1225	−1.0	−4.3	0.8
Azithromycin	375[a]	30	12	$C_{30}H_{59}N_2O_9$	591.4221	591.4233	1.2	2.0	0.1
				$C_{30}H_{57}N_2O_8$	573.4115	573.4111	−0.4	−0.7	0.7
Desmethyl azithromycin	368[a]	30	12	$C_{29}H_{57}N_2O_9$	577.4064	577.4069	0.5	0.9	0.2
				$C_{29}H_{55}N_2O_8$	559.3958	559.3951	−0.7	−1.3	0.9
Torsemide	349	30	20	$C_{12}H_{14}4N_3O_2S$	264.0807	264.0797	−1.0	−3.8	1.4
				$C_{12}H_{11}N_2$	183.0922	183.0918	0.4	−2.2	1.1
Erythromycin	734	30	15	$C_{29}H_{54}NO_{10}$	576.3748	576.3740	−0.8	−1.4	1.5
				$C_{29}H_{52}NO_9$	558.3642	558.3651	0.9	1.6	0.9
Erythromycin anhydrous	716	30	15	$C_{29}H_{52}NO_9$	558.3642	558.3638	−0.4	−0.7	0.6
				$C_{29}H_{50}NO_8$	540.3536	540.3539	0.3	0.6	1.2
Terbinafine	292	30	12	$C_{11}H_9$	141.0704	141.0691	−1.3	−9.2	0.0
Warfarin	309	30	12	$C_{16}H_{11}O_3$	251.0708	251.0714	0.6	2.4	0.0
				$C_9H_7O_3$	163.0395	163.0387	−0.8	−4.9	0.0
Negative ionization:									
Chlorthalidone	337	25	15	$C_6H_5NO_2SC$	189.973	189.9727	−0.3	−1.6	0.2
				$C_8H_4NO_2$	146.0242	146.0234	−0.8	−5.5	0.0
Torsemide	347	25	30	$C_{12}H_{12}N_3O_2S$	262.0650	262.0658	0.8	3.1	0.9
				$C_{12}H_8N_3S$	226.0439	226.0452	1.3	5.8	2.0
Torsemide intermediate	262	25	25	$C_{12}H_9N_3$	195.0796	195.0798	0.2	1.0	1.5
				$C_{12}H_9N_2$	226.0439	226.0436	−0.3	−1.3	1.9
Warfarin	307	25	22	$C_{16}H_{10}O_3$	250.0630	250.0635	0.5	2.0	0.0
				$C_9H_5O_3$	161.0239	161.0239	0.0	0.0	0.0

[a] Double charged ions were selected as precursors for azithromycin and desmethylazithromycin.

Chemspider database offered 26 hits most of which belonged to (2R,3S,4R,5R,8R,10R,11S,12S,13S,14R)-11-(4-dimethylamino-3-hydroxy-6-methyl-tetrahydropyran-2-yl)oxy-2-ethyl-3,4,10-trihydroxy-13-(5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl)oxy-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one, which is a macrolide antibiotic, known under its common name azithromycin. A comparison of the mass spectra of the chromatographic peak eluted at the RT of 4.32 min from the sediment sample (Fig. 3A) and reference azithromycin standard (Fig. 3B) confirmed the identity of azithromycin but also revealed that ions at *m/z* 735.5004, *m/z* 577.4061 and *m/z* 368.2492 reflect the coelution of an unknown impurity. The mass fragmentation patterns of azithromycin and the coeluted impurity were rather similar (Fig. 3A), showing a mass difference of 14 Da for the single charged ions (*m/z* 735.5004 and 577.4061) and of 7 Da for doubly charged species (*m/z* 368.2492). This indicated that the two structures differed by one CH_2 group. The formula of the presumed protonated molecule $[M + H]^+$ was $C_{37}H_{71}N_2O_{12}$ (Table 1), which corresponded to protonated desmethylazithromycin. Desmethylazithromycin is an important intermediate in the azithromycin production, which to our knowledge was not reported earlier in the environment.

The final confirmation of azithromycin and its intermediate in the sediment sample was obtained in an additional MS–MS experiment using the doubly charged ions *m/z* 375 and *m/z* 368 as the precursors (Fig. 3C and D, Fig. 4) The MS–MS conditions in this experiment and accurate masses of the two major product ions are given in the Table 3.

The structures in the Figs. 3C and D illustrate the predominant fragmentation pattern of the doubly charged ion *m/z* 375. It should be noted that the product ion spectra of the sediment sample and reference compound are identical, showing no interference from the co-eluting impurity. The MS–MS spectrum of azithromycin fragmentation pattern involves the loss of the cladinose unit yielding the ion *m/z* 591.42, followed by a subsequent loss of water yielding the ion *m/z* 573.41. The transition of doubly charged ion at *m/z* 375 to the ion at *m/z* 591 is widely employed for target analysis of azithromycin by tandem mass spectrometry (Goebel et al., 2004; Senta et al., 2008).

Product ion mass spectrum of desmethylazithromycin presented in Fig. 4 corresponded well with the fragmentation pattern

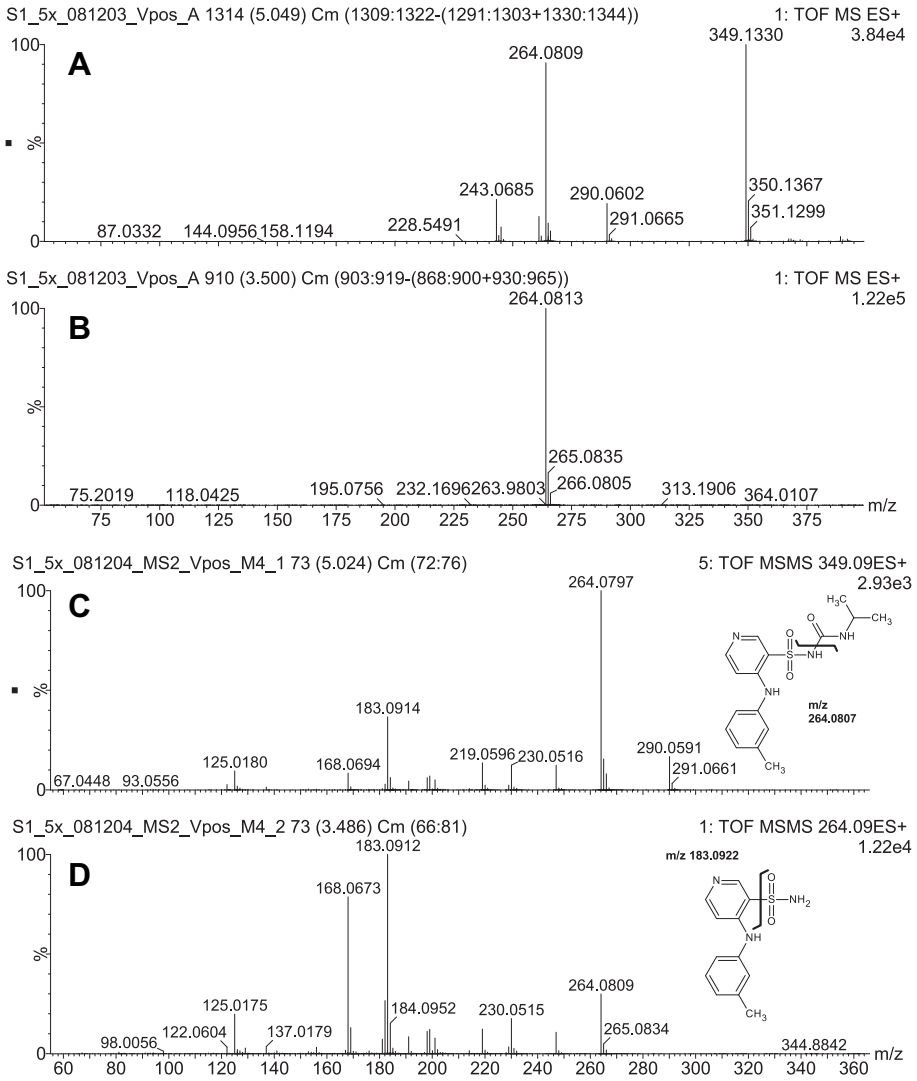

Fig. 5. Identification of torsemide and its intermediate in the sediment extract from the Gorjak Creek using UHPLC–QTOFMS with electrospray ionization in positive polarity mode: A) TOF mass spectrum of the protonated molecule at RT 5.0 min; B) TOF mass spectrum of the protonated molecule at RT 3.5 min; C) product ion spectrum of precursor ion *m/z* 349 at RT 5.0 min; D) product ion spectrum of precursor ion *m/z* 264 at RT 3.5 min).

of azithromycin (Fig. 3D). Higher product ions of azithromycin and desmethylazithromycin, which still kept the macrolide ring, differ by 14 Da as indicated above, while smaller fragments (*m/z* 158.1167, 116.1069 and 83.0492) were identical for both compounds. It should be noted that all experimentally determined product ions corresponded to the theoretical ones with absolute accuracy, being mainly below 0.001 Da (Table 3). The ultimate identification was achieved by analysis of a reference standard (not shown).

3.3.2. Torsemide and its intermediate

Similarities between the mass spectra are often a strong indication that two or more peaks could be structurally related, which can be of great help for the elucidation of transformation products for which reference standards are not available (Kern et al., 2009). The peak at the RT of 5.05 min in UHPLC–QTOFMS chromatogram acquired in the positive mode showed a relatively complex TOF mass spectrum containing two prominent ions at *m/z* 349.1330 and *m/z* 264.0809 (Fig. 5A). The most probable elemental composition of these two ions was calculated to be $C_{16}H_{21}N_4O_3S$ $[M + H]^+$ and $C_{12}H_{14}N_3O_2S$ $[M + H]^+$, respectively. The constant ratio of the two ions across the chromatographic peak indicated that the ion *m/z* 264.0809 was a fragment ion of the ion *m/z* 349.1330 rather than a coeluted compound. The database search for the formula derived from the ion *m/z* 349.1330 suggested popular diuretic torsemide as the possible candidate (molecular formula $C_{16}H_{20}N_4O_3S$). The identity of this compound was confirmed by analysis of pure reference material under the same conditions (not shown).

The final confirmation of torsemide presence in the investigated sediment sample was achieved by performing an MS–MS experiment with both the real sediment sample (Table 3; Fig. 5C) and a reference compound (not shown). The fragmentation pattern of torsemide is very similar to the fragmentation patterns of some other substituted sulphonamides (Niessen, 2006). The two major product ions, used for the identification of torsemide were *m/z* 264.0809 and *m/z* 183.0912. The first ion is formed by cleaving off the isopopropylaminocarbonyl group (C_4NOH_8), while the second one involves complex rearrangement, including further loss of SO_2 group (63.9619 Da), the attachment of the sulfonamide nitrogen to the pyridine ring (Hu et al., 2008) and subsequent loss of NH_3 (17.0265 Da). The product ion spectra of torsemide from the

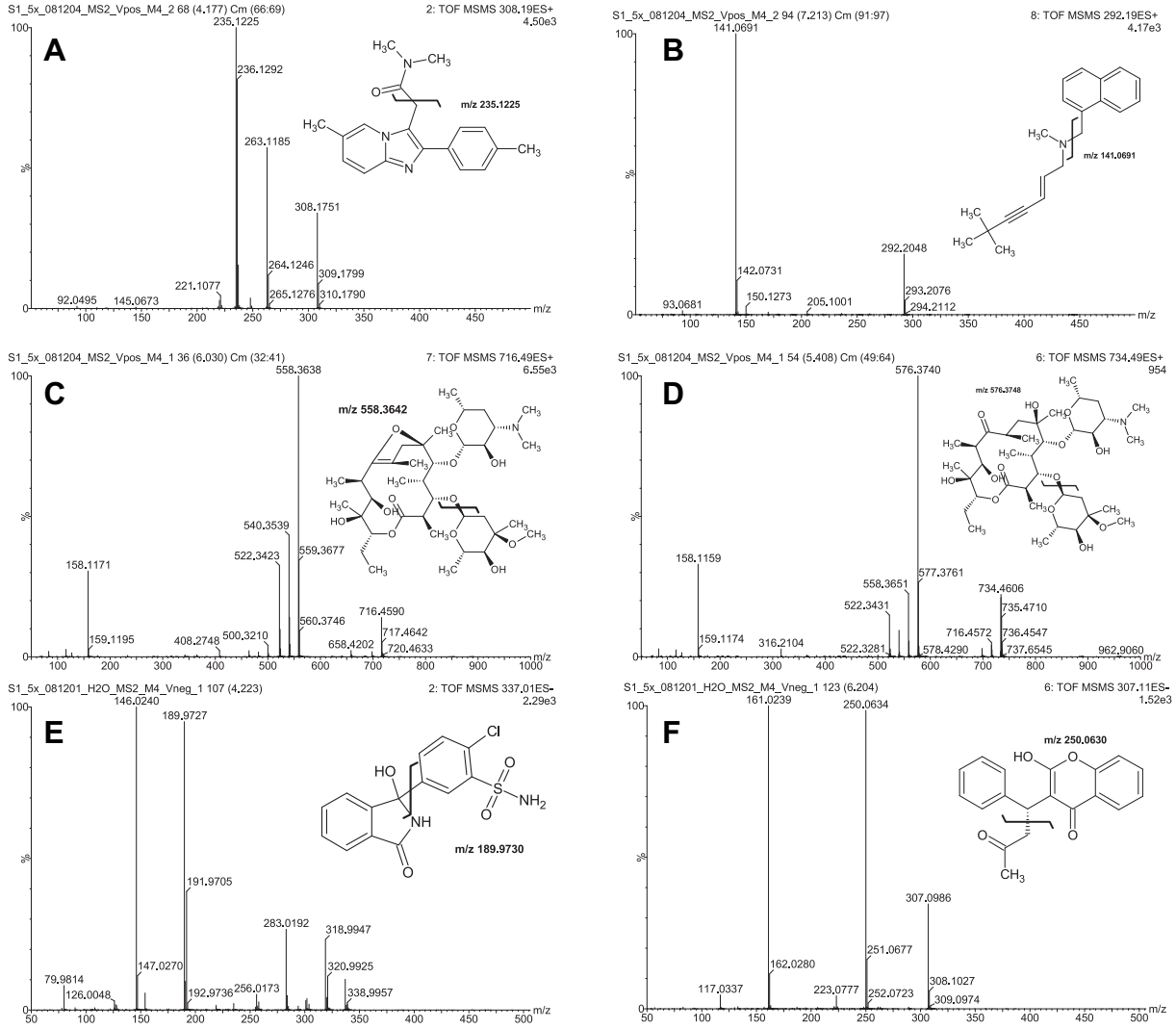

Fig. 6. Product ion mass spectra of pharmaceuticals detected in the sediment extract from the Gorjak Creek using UHPLC–QTOFMS: A) zolpidem - precursor ion *m/z* 308; B) terbinafine - precursor ion *m/z* 292; C) dehydrated erythromycin - precursor ion *m/z* 716.5; D) erythromycin - precursor ion *m/z* 734.5; E) chlorthalidone - precursor ion *m/z* 337; F) warfarin - precursor ion *m/z* 307. A–D: positive electrospray ionization; E–F: negative electrospray ionization.

sediment extract and from the reference standard (not shown) were identical and fully confirmed the tentative identification based on accurate mass and RT.

A thorough inspection of the chromatogram (Fig. 2A) showed that a prominent peak eluting at the RT of 3.5 min had a relatively simple TOF mass spectrum, containing just one prominent ion at *m/z* 264.0813 (Fig. 5B). The *m/z* value of this ion was almost identical to a previously detected product ion of torsemide (Fig. 5A) and, consequently the corresponding elemental compositions were identical ($C_{12}H_{14}N_3O_2S$ $[M + H]^+$). This indicated that the peak eluting at 3.5 min might be structurally related to torsemide. This assumption was tested by applying the MS–MS experiment using the ion at *m/z* 264 as a precursor ion. The product ion spectrum of the unknown (Fig. 5D) and that of torsemide (Fig. 5C) were almost identical, leading to the conclusion that the unknown might be 4-(3′-methylphenyl) amino-3-pyridinesulfonamide ($C_{12}H_{14}N_3O_2S$ $[M + H]^+$), which is one of key intermediates used in torsemide synthesis.

It should be mentioned that both torsemide and its intermediate can be analysed in the negative ionization mode (NI), with major ions being analogous to those shown for the positive mode (Table 3), however the retention time of the intermediate was significantly shifted under neutral pH applied in NI (RT = 5.79 min) as compared to acidic conditions applied in PI (RT = 3.5 min) (Tables 1 and 2). To the best of our knowledge this is the first time that these two compounds were identified in aquatic sediments.

3.3.3. Other identified pharmaceuticals

In addition to torsemide and azithromycin some other pharmaceutical compounds were also identified in the investigated sediments (Tables 1 and 2). Like for the previous compounds, the identification was performed using described tiered approach, including the final confirmation by reference materials. Compounds identified in the positive ionization mode included psychiatric drug zolpidem (RT 4.19 min), antifungal agent terbinafine (RT 7.24 min) and two additional macrolides, erythromycin (RT 5.43 min) and dehydroerythromycin (RT 6.05–7.12 min). In the negative ionization mode we were able to identify the anticoagulant warfarin (RT 6.08 min) and diuretic chlorthalidone (RT 4.24 min). Fig. 6 shows product ion spectra of selected protonated and deprotonated molecules, illustrating the most likely fragmentation patterns of identified compounds, while Table 3 gives the accurate masses of the two selected product ions, their elemental composition and the conditions of the MS–MS experiments.

Except for erythromycin, which was reported both in wastewater, surface water and sediments (Terzic et al., 2009, 2008; Xu et al., 2009), only few reports dealt with the environmental occurrence of terbinafine, warfarin, zolpidem and chlorthalidone (Barron et al., 2008; Fick et al., 2009). To the best of our knowledge these compounds have not been reported in aquatic sediments before.

The concentration levels of identified pharmaceutical compounds were roughly estimated using external calibration curves and the results are presented in Fig. 7. The estimated concentrations varied from 0.8 μg/g for erythromycin to 20.1 μg/g for chlorthalidone. Since the concentrations were not corrected for the extraction recovery and matrix effect, the real concentrations might be much higher. Besides that, dehyroerythromycin (ERY-H_2O) may be formed from erythromycin (ERY) during the analytical procedure (Yang and Carlson, 2004), which additionally affects the reliability of the semiquantitative estimates of these two compounds. The analyses of their standard solutions in positive ionization mode, using acidic eluents, indicated that approximately 10% of ERY was transformed to ERY-H_2O. Nevertheless, the estimated concentrations in the Gorjak Creek determined in this study

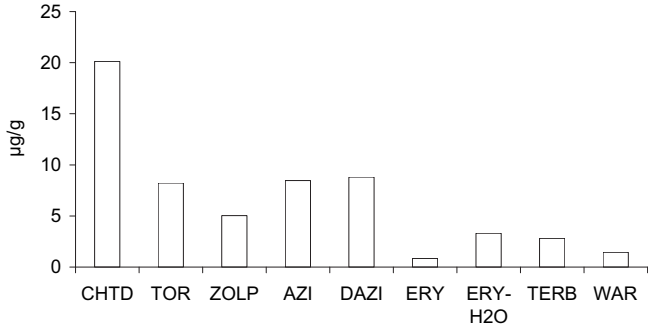

Fig. 7. Estimated concentrations of identified nontarget pharmaceuticals in freshwater sediments of the Gorjak Creek (CHTD-chlorthalidone; TOR-torsemide; ZOLP-zolpidem, AZI-azithromycin; DAZI-desmethylazithromycin; ERY-erythromycin; ERY-H_2O-dehydrated erythromycin; TERB-terbinafine; WAR-warfarin).

are still more than one order of magnitude higher than the levels reported in some other contaminated urban streams (Feitosa-Felizzola and Chiron, 2009; Tang et al., 2009), indicating that the streams receiving direct wastewater discharges from the pharmaceutical industry should be regarded as important hotspots, which warrant detailed chemical and ecotoxicological studies.

4. Conclusions

The UHPLC–QTOFMS coupling showed a high potential for the comprehensive identification of nontarget polar contaminants in complex environmental matrices. The proposed approach proved to be a useful tool for the initial assessment of contaminated hotspots by providing a basis for the selection of the most critical contaminants to be monitored using target analysis.

The finding of a significant presence of pharmaceutical compounds in freshwater sediments raises several concerns regarding their fate in the aquatic environment and potential environmental risks. All these compounds were designed to have a high biological activity however their impact on non-target organisms such as aquatic life is largely unknown. This study shows that extending analytical window to more polar compounds is important prerequisite for the detection of emerging classes of aquatic contaminants, which might be essential for a better ecotoxicological assessment of polluted freshwater sediments.

Acknowledgements

This work was financially supported by the Ministry of Science, Education and Sports of the Republic of Croatia through the project on Organic contaminants as molecular markers of the anthropogenic impact on the environment (Project No: 098-0982934-2712). Technical assistance of Nenad Muhin is also gratefully acknowledged.

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