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Broad spectrum screening of 463 organic contaminants in rivers in Macedonia

Draženka Stipaničev[1], Zrinka Dragun[2*], Siniša Repec[1], Katerina Rebok[3], Maja Jordanova[3]

[1]Croatian Waters, Central Water Management Laboratory, Ulica grada Vukovara 220, 10000 Zagreb, Croatia
[2]Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Biological Effects of Metals, Bijenička cesta 54, 10000 Zagreb, Croatia
[3]Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University in Skopje, Arhimedova 3, 1000 Skopje, Macedonia

* Corresponding author:
Zrinka Dragun
Laboratory for Biological Effects of Metals
Division for Marine and Environmental Research
Ruđer Bošković Institute
Bijenička c. 54, 10000 Zagreb, Croatia
Phone: xx385-1-4680216;
Fax: xx385-1-4680242;
E-mail: zdragun@irb.hr

Running head: Screening of organic contaminants by UHPLC-QTOF-MS in rivers

Abstract

Target screening of 463 organic contaminants in surface water using ultra high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) with direct injection was performed in spring of 2015 in northern Macedonia, at six sampling sites in four rivers belonging to Vardar basin: Kriva, Zletovska, Bregalnica and Vardar. The aim of the study was to differentiate between various types of organic contamination characteristic for different types of anthropogenic activities, such as mining, agriculture, and urbanization. Depending on the studied river, 9-16% of analyzed compounds were detected. The highest total levels of organic contaminants were recorded in agriculturally impacted Bregalnica River (1839-1962 ng L^{-1}) and Vardar River downstream from the city of Skopje (1945 ng L^{-1}), whereas the lowest level was found in the mining impacted Zletovska River (989 ng L^{-1}). The principal organic contaminants of the Bregalnica River were herbicides (45-55% of all detected compounds; 838-1094 ng L^{-1}), with the highest concentrations of bentazone (407-530 ng L^{-1}) and molinate (84-549 ng L^{-1}), common herbicides in rice cultivation. The main organic contaminants in the other rivers were drugs (70-80% of all detected compounds), with antibiotics as a predominant drug class. The highest drug concentrations were measured in the Vardar River, downstream from Skopje (1544 ng L^{-1}). Screening of surface water by UHPLC-QTOF-MS was proven as a practical tool for fast collection of comprehensive preliminary information on organic contamination of natural waters, which can present a significant contribution in the monitoring and preservation of good ecological status of freshwater ecosystems.

Keywords: drugs, freshwater, pesticides, organic contamination, screening

1. Introduction

Natural waters are in a great danger of getting more and more contaminated with numerous organic and inorganic contaminants, because of a use of surface waters as recipients for wastewaters; most municipal and industrial effluents, containing a large variety of contaminants (such as pharmaceuticals, surfactants, biocides, personal care products, and sweeteners), as well as their transformation products even after their treatment, end up in rivers, streams or lakes (Rodriguez-Mozaz et al., 2004). To protect the freshwater ecosystems, European Union has issued a set of environmental quality standards (EQS) in its Water Framework Directive (WFD), which for now comprises 45 priority substances in surface aquatic bodies (EPCEU, 2013). About one third of the priority substances covered by this Directive are pesticides, and although drugs are not yet properly covered by environmental regulations, they are considered as emerging contaminants, due to their toxicity in very low concentrations and continuous discharge into urban rivers (Zhou et al., 2014). The exposure to pesticides from water not only can have serious consequences for humans, but also may have ecotoxicological effects for aquatic flora and fauna (De Gerónimo et al., 2014). Similarly, water contamination with some pharmaceuticals has become a major subject of worldwide concern (Rodriguez-Mozaz et al., 2004) because they contribute to development of antibiotic-resistant bacteria (Guardabassi et al., 1998) and compromise the long-term survival of many species (Fuhrman et al., 2015). For example, intake of compounds with estrogenic properties via food or drinking water probably can cause a decrease of sperm counts and the increasing incidence of testicular cancer and other disorders regarding male infertility (Sharpe and Skakkebaek, 1993).

Currently, at European legislative level, only analytical methods focused on the target analysis of a limited number of pre-selected compounds included in the pesticide residue definition are taken into account (Villaverde et al., 2016). However, it is important to point out that monitoring of only those target compounds, which are listed in WFD, misses important site-specific and potentially ecotoxicologically relevant compounds that are not covered by the Directive. Therefore, information on the actual levels of wide range of organic contaminants, including pesticides and drugs, in the aquatic environment is fundamental for proper risk assessment and planning of risk reduction measures (De Gerónimo et al., 2014). As some of these chemicals have been shown to provoke toxic effects in fish, e.g. endocrine disruption, already at sub-nanogram levels (Hansen et al., 1998; Purdom et al., 1994), their determination requires high-sensitivity analytical methods (Rodriguez-Mozaz et al., 2004). Commonly, when assessing organic substances, numerous analytical methods may have to be used to cover a large number of known compounds (Gómez et al., 2011), which can be rather costly and time-consuming. Therefore, to overcome those difficulties, new strategies and instrumentations are needed, which will be focused primarily on fast and simple preliminary screening of samples (Allinson et al., 2015), with the general aim to identify the most problematic contaminants, which will be further on individually monitored.

To meet arising requirements of developing science, industry and society, a rapid development in the field of pesticide and other organic compound analysis occurred, including the development of different mass spectrometry detectors (triple quadrupole, ion-trap, time-of-flight, quadrupole-time-of-flight), as well as development of "ambient-ionization" mass spectrometry techniques (Botitsi et al., 2011). New screening methods were created that combine a mass-structure database with gas or liquid chromatography coupled to mass spectrometry to create a system that can screen samples for large number of compounds and give reliable indication of the presence of specific trace organic chemicals in analyzed samples (Botitsi et al., 2011; Allinson et al., 2015; Guibal et al., 2015; Stipaničev et al., 2015). Such possibilities in the field of water analysis are provided by application of high resolution quadrupole time-of-flight mass spectrometry (QTOF-MS), which enables simultaneous quantitative determination of numerous target compounds, due to its sensitivity and selectivity in full scan analysis, as well as additional qualitative analysis of other compounds included in a mass spectral library (Guibal et al., 2015). It is especially useful for analysis of transformation/degradation products of organic compounds, such as pesticides (Sevilla-Morán et al., 2014). The sensitive full spectrum acquisition and the high mass resolution and mass accuracy provided by TOF-MS make this technique especially suited for wide-scope screening in the environment, where a large number and types of organic contaminants are present (Hernández et al., 2012), and, additionally, it allows the investigation of hundreds of compounds in the same run (Díaz et al., 2012). Screening approach was already proven as a useful basis for monitoring of natural waters in several field studies, such as assessment of groundwater quality in Netherlands (ter Laak et al., 2012) and screening of several German, Dutch, Swedish, French and UK rivers (Schwarzbauer et al., 2010).

Accordingly, the main aim of our study was to perform wide-scope target screening of organic contamination of several rivers in northern Macedonia which belong to Vardar river basin by application of ultra high performance liquid chromatography (UHPLC) coupled to QTOF-MS (UHPLC–QTOF–MS). Since liquid chromatography-mass spectrometry (LC-MS) has been proven as an excellent analytical tool in the determination of pesticides (Masiá et al., 2014), the proposed instrumental system will be an ideal option for accomplishing our goal. It provides a possibility of simultaneous analysis of a large number of organic compounds, it enables fast, simple and reliable performance by direct injection of samples, it has a low detection limits (on the order of single ng L^{-1}), and it does not require sample preconcentration (e.g. solid phase extraction (SPE)) prior to analysis (Kowal et al., 2009; Yu et al., 2012). However, despite the strong potential, wide scope screening of hundreds of compounds by combined LC-TOF has been scarcely explored (Hernández et al., 2012). In our previous research, UHPLC–QTOF–MS has been already successfully applied to analysis of pharmaceuticals in raw and treated wastewater from Virovitica wastewater treatment plant in Croatia (Topić Popović et al., 2015) and to screening of water samples of the Danube River within Joint Danube Survey 3 (Stipaničev et al., 2015).

In northern Macedonia, there is a great need for characterization of ecological quality and contamination status of local rivers, since they are flowing through an area of active mining and developed agriculture, specifically rice cultivation (Andreevska et al., 2013; Ramani et al., 2014; Rebok, 2013; Stuhlberger, 2010). In addition, there are only three active wastewater treatment plants in that country: in the cities of Ohrid, Prespa and Dojran, but they are not fully completed and untreated sewage is discharged directly into rivers and/or lakes (Mitev and Mitanovska, 2011). So far, detailed analysis of physico-chemical and inorganic contamination of several rivers in north-eastern Macedonia has been performed (Ramani et al., 2014), but diffuse agricultural sources of pollution and their impact on quality of water resources were not thoroughly examined (Mitev and Mitanovska, 2011). Therefore, specific aim of our study was to analyze, by use of UHPLC–QTOF–MS, surface water samples of four rivers flowing through northern Macedonia, which are influenced by different sources of pollution, specifically by municipal and industrial wastewaters of large towns, mining effluents and agricultural runoff. Additionally, we aim to define organic contaminants which are representative for each of the studied pollution sources and areas, which will facilitate further monitoring of freshwaters in Macedonia, but also broaden the existing level of awareness on organic contamination of freshwaters throughout the world.

2. Materials and methods

2.1. Study period and area

The sampling of river water was carried out in four rivers/six sampling sites in north Macedonia in May of 2015, which falls within a typical period of pesticide application, from April to July (Laganà et al., 2002; Papadakis et al., 2015). Since so far information on organic contamination of those rivers was not available, a single sampling was performed to obtain preliminary information on types and concentrations of organic contaminants present in the surface water of selected rivers, as a basis for further study of this area.

Four rivers/six sampling sites, which are characterized in the Table 1, included (1) the Kriva River, which is the longest tributary of the River Pčinja - a left tributary of the Vardar River; (2) the Zletovska River, which is one of the most polluted tributaries of the Bregalnica River (Dolenec et al., 2005); (3) the Bregalnica River, which is the longest left tributary of the Vardar River; and (4) the Vardar River, which is the longest and major river in Macedonia. It is important to point out that the area along the course of the river Bregalnica, including the regions of Kočani, Štip, Vinica and Blatec, is known as a rice production core of the Republic of Macedonia (Andreevska et al., 2013).

2.2. Analysis of organic contaminants by UHPLC-QTOF-MS

154 *2.2.1. Sample preparation and reagents*

155 Surface water samples were collected in polycarbonate bottles (250 mL), which are better suited for
156 water sampling and storage than glass bottles, because some organic compounds tend to adsorb on
157 glass (Stan et al., 1995). The samples were frozen immediately after sampling and stored at -20°C
158 until analysis (for approximately one week). Cooling between 0-4°C (few days only) and freezing at -
159 20°C (longer time periods) are the most common preservation techniques for organic samples that
160 cannot be analyzed soon after sampling (Bogialli et al., 2014). Prior to analyses, water samples were
161 thawed and then filtrated on 0.2 μ PTFE filters. Ultrapure laboratory water samples were always
162 processed in parallel with the environmental water samples.

163 All chemicals were of high purity grade. Suprapur formic acid was from Merck (Darmstadt,
164 Germany). Water, acetone and methanol (all from J.T. Baker, Deventer, Netherlands) were of LC MS
165 grade. Ammonium formate was from Fischer Scientific (New Jersey, USA). Ammonium fluoride was
166 from Fluka Analytical (Sigma Aldrich, Steinheim, Germany). LC/MS Pesticide Comprehensive Test
167 Mix Kit Sub Mix 1-9, comprising 273 compounds, and Forensic-Tox Comprehensive Mix Kit Sub
168 Mix 1-10, comprising 138 compounds, were from Agilent Technologies ,USA. Analytical standards of
169 sulfa drugs (sulfachloropyridazine, sulfadimethoxine, sulfamethazine, sulfamethizole and
170 sulfametoxazole) were from Supelco (Sigma Aldrich, Steinheim, Germany). Ciprofloxacin,
171 erythromycin, trimethoprim, and Pharmaceuticals Mix#1 and 2#, comprising 12 compounds, and
172 Steroids and Mixed Pharmaceuticals Mix, comprising 10 compounds, were purchased from Restek
173 (Bellafonte, USA), and azithromycin was from Dr. Ehrenstorfer (Germany). 1H-benzotriazole and
174 gabapentin were purchased from Neochema (Germany) and PFOA from Sigma-Aldrich (Germany).
175 PFOS was purchased from Accustandard, Inc. (New Haven, USA).
176
177 *2.2.2. Methods and analytical procedure*

178 Organic contaminants in surface river water were determined by UHPLC–QTOF–MS, using direct
179 injection method. Analyses were performed on 1290 UHPLC system, Agilent Technologies, USA
180 (G4226A autosampler, G4220B binary pump and G1316C thermostated column). The separation of
181 analytes was done using the RP column ACQUITY UPLC, HSS T3 (150 mm× 2.1 mm, 1.8 μm). The
182 gradient was from 100% water to 100% organic solvent in 20 minutes run. The injection volume was
183 100 μL. The temperature of the column chamber was set to 50°C. In positive ESI (ESI(+)), the mobile
184 phases were composed of solvent A (5 mM ammonium formate/formic acid) and B (100% MeOH). In
185 negative ESI (ESI(-)), the mobile phases were composed of solvent A (1 mM ammonium fluoride) and
186 B (100% MeOH). Gradient elution with a flow rate of 0.4 mL min^{-1} was used. The analytes were
187 detected using a 6550 i-Funnel Q-TOF–LC/MS (Agilent Technologies, USA) in 4 GHz detector rate,
188 with a 40,000 resolving power and b2 ppm accuracy. QTOF mass spectrometer (Agilent 6550) was
189 operated in positive (ESI+) and in negative (ESI-) ion mode. Ions were generated using a dual AJS

ESI (Agilent Jet Stream) ion source. Operation conditions in ESI(+) mode were as follows: sheath gas temperature 375°C, gas temperature 125°C, heat gas 12 L N2 min^{-1}, drying gas 15 L N2 min^{-1}, capillary voltages 3500 V, fragmentor 400 V, and nebulizer 35 psig. In ESI(-) ionisation mode operation conditions were as follows: sheath gas temperature 375°C, gas temperature 125°C, heat gas 12 L N_2 min^{-1}, drying gas 15 L N_2 min^{-1}, capillary voltages 3000 V, fragmentor 400 V, and nebulizer 35 psig. The resolution power for ESI(+) was 52,296 at 922.009798 m/z and 21,801 at 118.086255 m/z. The resolution power for ESI(-) was 55,966 at 1033.988109 m/z and 22,683 at 112.985587 m/z . Correction during measuring for any possible drift in the mass axis was done automatically with lock 2 mass ion software. Analyses were performed using MS and MS/MS mode with fixed collision energy and in mass range of 50–1000 m/z. Data were further processed with Agilent MassHunter Workstation software (Quantitative Analysis version B.07.00/Build 7.0.457.0 for QTOF, Agilent Technologies, USA). Calibration curve was created using triplicate standard solutions at 7 concentration levels ranging from 1-1000 ng L^{-1}.

To determine if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus, the method blank was applied (US EPA 1694, 2007). Method blank was an aliquot of reagent water (LC/MS grade water, J.T. Baker®) that was treated exactly as the samples, including exposure to all equipment, solvents and reagents that are used for sample handling.

For correction of matrix effects, we have used simple and effective method of standard addition based on recovery calculations, according to Shaw et al. (2014). By testing blank samples (LC/MS grade water, J.T. Baker®) spiked at three concentration levels, accuracy and precision were calculated, and for each concentration were evaluated in quintuplicate. Considering that direct injection does not include any other pretreatment procedure (such as extraction or clean up), the matrix effects can be assessed by comparing the signals of the analytes in spiked and original water samples, since standard addition involves the addition (spiking) of an analyte or a mixture of analytes into the sample, and measuring the analyte concentrations before and after spiking. Acceptance criteria for accuracy were recoveries between 70% and 110% and for repeatability relative standard deviations lower than 20%. We have also used high resolution for removal of interferences.

The sensitivity of the method was estimated by establishing the limits of detection (LODs) and quantification (LOQs). LODs were calculated using standard solutions prepared in spiked LC/MS water and in surface water samples. The LOQs were also determined in pure solvent and in surface water samples. The LODs were determined as the lovest analyte concentration whose qualifier presented a signal-to-noise ratio S/N≥3, and for LOQs S/N ≥10. Analytes were quantitied in full MS scan mode by accurate mass measurement and isotope pattern matching. All Ions MS/MS mode (collision energies 0, 20, 40 V) was used as QC for further confirmation by matching compounds MS/MS spectrum and retention time to reference standards. LODs, LOQs, retention times and transitions are presented for all analyzed compounds as supplementary information (Tables S1-S12).

227 *2.3. Data processing and statistical analyses*

228 Statistical program SigmaPlot 11.0 for Windows was applied for creation of graphs. All calculations
229 were performed in Excel 2007.
230
231 **3. Results and discussion**

232 The samples of surface water taken at six sampling sites from four rivers in northern Macedonia were
233 examined for presence of 463 organic compounds by use of UHPLC-QTOF-MS, to differentiate
234 between various levels and types of anthropogenic influences. The list of all analyzed compounds, as
235 well as all individual measured data, is presented as the supplementary information (Tables S1-S12).
236 In all studied rivers number of detected compounds ranged between 43 and 74 (or 9 to 16%), with the
237 lowest number of organic compounds detected in the mining impacted Zletovska River and the highest
238 in the Vardar River, downstream from the city of Skopje. Total concentrations of detected compounds
239 at six sampling sites ranged from 989.0 ng L^{-1} to 1961.6 ng L^{-1}, with the highest levels recorded at
240 both sites of agriculturally impacted Bregalnica River and in the Vardar River downstream from the
241 city of Skopje. The lowest concentration was again found in the Zletovska River (Fig. 1a).

242 The analyzed compounds could be classified in three major categories: pesticides (insecticides,
243 herbicides, and fungicides), drugs and other miscellaneous compounds. Looking at each
244 river/sampling site separately, we were able to make a conclusion about its main contaminants (Fig.
245 1a). At four out of six sampling sites, i.e. three out of four studied rivers (Kriva, Zletovska and
246 Vardar), the main organic contaminants were drugs, which contribution to total measured
247 concentrations in the surface river water amounted to approximately 70-80%. Contrary, at both sites in
248 the Bregalnica River, the highest contribution referred to herbicides, amounting to 45-55%, whereas
249 drugs were second most abundant contaminant (30-35%).
250
251 *3.1. Pesticides (insecticides, herbicides and fungicides)*

252 Out of 115 analyzed insecticides only 2-8 were detected (2-7%), depending on the sampling site, and
253 the ranges of their individual concentrations are given in Table 2. The highest total concentrations of
254 insecticides were measured in the Bregalnica River at sampling site Teranci, followed by Vardar River
255 downstream from Skopje and the Bregalnica River at the sampling site Kežovica (Fig. 1b). Total
256 concentrations of insecticides at those three sampling sites ranged from 63-102 ng L^{-1}, with
257 flonicamid, benfuracarb and methomyl being the most abundant compounds, respectively. The
258 remaining three sampling sites had total concentrations of insecticides below 40 ng L^{-1}, and the most
259 abundant insecticide was diethyltoluamide (DEET).

260 Out of 109 analyzed herbicides, 8-17 were detected (7-16%), depending on the sampling site, and the
261 ranges of their individual concentrations are given in Table 2. Herbicides are widely used in

agricultural production systems for the suppression of unwanted plants (weeds) before and during crop growth (Allison et al., 2014). Similar to Australia, where herbicides are top ranked category of sold pesticides, with more than twice higher sales compared to insecticides and five times higher than fungicides (Allinson et al., 2014), and Greece where herbicides were the most frequently detected pesticides in the river water and exhibited higher concentration values (Papadakis et al., 2015), in northern Macedonia herbicides were also found in much higher concentrations in the river water than the other two category of pesticides. They were especially high in the Bregalnica River (Fig. 1a), where total herbicide concentrations amounted to 838 and 1094 ng L^{-1} at sampling sites Teranci and Kežovica, respectively, being 4-5 times higher compared to all the other sampling sites (Fig. 1c).

Acid herbicide bentazone and thiocarbamate molinate were the most abundant herbicides in the Bregalnica River (Table 2), both of them known for their high leachability index and high risk for water contamination (Papa et al., 2004). Furthermore, acid herbicides have high aqueous solubility and low tendency for sorption to organic matter at neutral pH (Comoretto et al., 2007). Acid herbicides belong to the most used herbicides for control of broad-leaved weeds and other vegetation in the US and Europe, due to their relatively low cost and high potency even at low concentration (Laganà et al., 2002). Especially, intensive usage of acidic herbicides, particularly bentazone, is characteristic for maize, grain and rice cultivation (Laganà et al., 2002; Comoretto et al., 2007). In 2006, bentazone was ranked as third most relevant pesticide in Germany, causing problems in drinking water supply from bank filtration (Bach et al., 2010). Although European Water Framework Directive (EU WFD) currently does not specify environmental quality standard for bentazone in surface water, the International Commission for the Protection of the Rhine (ICPR) proposed a concentration limit of 0.1 µg L^{-1} as a target value for bentazone in the surface river water, to protect the quality of ground water (Bach et al., 2010). At both sites in the Bregalnica River, bentazone was detected in considerably higher concentrations (407-530 ng L^{-1}) compared to proposed concentration limit, as well as to bentazone concentrations reported for Main River in Germany (290 ng L^{-1}) (Bach et al., 2010). Bentazone was also found in high concentrations in the Ebro River in Spain, in the area where the main economic activity is agriculture, specifically rice cultivation (Köck et al., 2010), which is also a dominating culture in the region of the Bregalnica River (Andreevska et al., 2013). Although its concentration in the Ebro River water decreased from 95,466 ng L^{-1} in 2005 (Barata et al., 2007) to 1,042 ng L^{-1} in 2008 (Köck et al., 2010), it is still considerably above the values measured in the Bregalnica River. High bentazone concentrations were also detected in the other European countries: up to 27 µg L^{-1} in the tributaries of the Tiber River in Italy in spring of 2001 (Laganà et al., 2002) and up to 1.6 µg L^{-1} in 2004 in Vaccarès lagoon system in France, where major pesticide input also originated from rice cultivation (Comoretto et al., 2007).

Molinate, which is usually reported as a predominant contaminant at rice growing area (Cerejeira et al., 2003), was also found in high concentrations in the Bregalnica River (84-549 ng L^{-1}). Molinate is

298 commonly applied in rice cultivation, under rice-flooded conditions, and has very high water solubility
299 (McBean, 2012). It has been classified as highly hazardous pesticide by Pesticide Action Network
300 International (PAN, 2015). In the Ebro River in Spain, water contamination with molinate increased
301 from 2005 (331 ng L^{-1}; Barata et al., 2007) to 2008 (554 ng L^{-1}; Köck et al., 2010). In Portugal, in
302 monitoring period from 2002 to 2008, molinate was found as one of the most frequently used and the
303 most abundant herbicides in rivers flowing through agricultural areas occupied with rice (Mondego
304 River: median 60 ng L^{-1}; Sado River: median 140 ng L^{-1}; Silva et al., 2015). The similar situation was
305 reported for northern Greece, where molinate concentrations sometimes exceeded even 1 µg L^{-1}, with
306 the highest concentrations observed in May/June period, right after pesticide application (Papadakis et
307 al., 2015), which coincided with the sampling period of our study.

308 The remaining four sampling sites in this study had total concentrations of herbicides below 200 ng L^{-1},
309 and the most abundant quantified herbicide was triazine herbicide ametryne. It is common for some
310 herbicides to be used generally in urban areas or in gardens and orchards by private users, whereas in
311 some countries urban use of pesticides also includes additives to facades and flat roofs (Botta et al.,
312 2012). One of the main herbicides applied in the urban areas is glyphosate (Botta et al., 2012), which
313 was also detected in our study, as the main herbicide contaminant next to ametryne in all non-
314 agricultural areas. However, due to lack of adequate standard solution, it was determined only
315 semiquantitatively, and was approximately in the range of ametryne.

316 For the time being, neither bentazone and molinate nor ametryne have been listed as priority toxic
317 substances in EU WFD (EPCEU, 2013). On the other hand, atrazine and simazine, which were listed,
318 continue to be observed in water samples in Europe, despite the ban on their use in many countries
319 (Allinson et al., 2014; Silva et al., 2015). For example, simazine was found in the Rhône river waters
320 at concentration level of 0.4 µg L^{-1} in spite of its ban of use in France since September 2003
321 (Comoretto et al., 2007). In studied rivers in Macedonia, atrazine was not detected at all, but its
322 metabolites were found in low concentrations at both sites of the Bregalnica River (1.07-1.21 ng L^{-1}),
323 and in Vardar downstream from Skopje (1.38 ng L^{-1}). In addition to ban of its use, the reason for low
324 occurrence of atrazine can be found in the fact that it was generally applied for maize, and not rice
325 cultivation (Papadakis et al., 2015; Silva et al., 2015). Simazine, on the other hand, was detected only
326 in the Bregalnica River at sampling site Kežovica, in very low concentration of 0.23 ng L^{-1}.
327 Compounds which are often used instead of simazine and atrazine, such as terbuthylazine and
328 terbutryn, were also not detected in the surface water of rivers in Macedonia.

329 Moreover, out of 74 analyzed fungicides 5-10 were detected (7-14%), depending on the sampling site,
330 and the ranges of their individual concentrations are given in Table 2. Same as herbicides, the highest
331 total concentrations of fungicides were measured at both sites of the Bregalnica River, but the
332 differences compared to the other rivers were not that pronounced (Fig. 1d). Total concentrations of
333 fungicides at those two sampling sites ranged from 125-137 ng L^{-1}, with tebuconazole and

carbendazim being the most abundant compounds. The remaining four sampling sites had total concentrations of fungicides below 75 ng L^{-1}, and the most abundant fungicide was tebuconazole.

The highest concentrations of insecticides, herbicides and fungicides at both sampling sites of the Bregalnica River are in accordance with the fact that this river flows through area of very intense rice production (Andreevska et al., 2013) and that herbicides can enter as contaminants into stream, rivers or lakes directly from drainage of agricultural lands (Laganà et al., 2002). Additional specificity of rice cultivation is that agrochemicals are either directly applied on water, or on soils which will be flooded after treatment, and therefore the contaminated rice field water must be contemplated as a direct source of pesticide emission to the surrounding waters (Comoretto et al., 2007).

Since for the most of the pesticides found in the freshwaters there are no environmental quality standards proposed by the EU WFD (EPCEU, 2013), comparison is commonly made with the permissible limits established for the drinking water in Europe (100 ng L^{-1} for each pesticide individually and 500 ng L^{-1} for the sum of all pesticides found in the river water sample) (Papadakis et al., 2015; CEC, 1998). In our study, only the sampling points at Bregalnica River surpassed the limit of 500 ng L^{-1} of total pesticides (sum of all detected insecticides, herbicides and fungicides) in the surface river water, amounting to 1065 and 1294 ng L^{-1} at Teranci and Kežovica, respectively, and mostly referring to herbicides. In Kriva and Vardar rivers, total pesticide concentrations were below 300 ng L^{-1}, and in the Zletovska River even below 200 ng L^{-1}. For a comparison, total pesticide concentration exceeded 5 μg L^{-1} in draining channel of the Ebro River in Spain (Köck et al., 2010). Individual pesticide analyses indicated that, except for already discussed herbicides bentazone and molinate, only ametryne slightly surpassed the limit of 100 ng L^{-1} at three sampling points (Kriva River, Bregalnica Teranci and Vardar upstream from Skopje). In addition, if a comparison is made, for instance, with Italian regulations which set maximum residue levels of herbicides in the river water in a range from 50 to 100 μg L^{-1} (Laganà et al., 2002), none of measured values would be considered as higher than acceptable limit. However, although the limits were mostly not surpassed, it should be considered that toxic effects can occur at much lower concentrations if chemicals are present as mixtures (Baas et al., 2009).

3.2. Drugs

According to EU WFD, water contamination with pharmaceutical residues is an emerging environmental concern (EPCEU, 2013). Although environmental quality standards and lists of priority substances in this category are not yet issued, it is important to monitor frequency and concentration level of drugs occurring in natural waters, to get a general idea on their outspread and abundance worldwide. Out of 162 analyzed compounds in this category 25-45 were detected (15-28%), depending on the sampling site, and the ranges of their individual concentrations are given in Tables 3-4.

370 The analyzed drugs could be classified according to their application in 14 categories, with following
371 order of abundance (ng L^{-1}): antibiotics (24.7-511.9) > hormones (275.2-362.1) > stimulants (97.3-
372 294.6) > analgesics (24.9-201.0) > cannabinoids (0-100.8) > antiepileptics (2.6-44.2) > hypnotics
373 (11.5-33.8) > opioids (0-27.5) > anticholesteremics (1.8-19.7) > antidepressants (1.6-13.7) >
374 antiseptics (4.1-5.9) > neuroleptics (0-2.5) > cardiovascular medicals (0-3.7) > diuretics (0). Similar to
375 our results, where antibiotics were the most abundant drug category and constituted 4-33% of all drugs
376 depending on the studied river, Topić Popović et al. (2015) reported that antibiotics comprised 14%
377 and 27% of all studied pharmaceuticals in the wastewater of Virovitica wastewater treatment plant in
378 Croatia, before and after treatment, respectively. However, they reported much higher concentrations
379 of antibiotics in raw and treated wastewaters (15,367.45 ng L^{-1} and 7,715.39 ng L^{-1}, respectively;
380 Topić Popović et al., 2015) compared to the river water of studied rivers in Macedonia (up to 512 ng
381 L^{-1}). This is in agreement with observation made by Kuster et al. (2008) that effluent waters of sewage
382 treatment plant contain higher levels of personal care products and analgesics than river waters.

383 The highest total concentrations of drugs were measured in Vardar downstream from Skopje and in
384 the Kriva River (Fig. 1e). Total drug concentrations at those two sampling sites were 1544 ng L^{-1} and
385 1064 ng L^{-1}, respectively. The remaining four sampling sites had total concentrations of drugs in the
386 range from 594-807 ng L^{-1}. However, not all analyzed drug classes exhibited the same spatial
387 distribution. The highest concentrations in the Kriva River and Vardar downstream from Skopje were
388 found only for antibiotics, analgesics, and cardiovascular medicals (Fig. 2 a,d,m).

389 In our study, antibiotics were mainly represented by sulfamethoxazole and ciprofloxacin, analgesic by
390 ibuprofen, and cardiovascular medicals by verapamil and strophanthidin. The highest
391 sulfamethoxazole concentrations were found in the Vardar River upstream from Skopje (233 ng L^{-1})
392 and in the Kriva River (170 ng L^{-1}), which is consistent with the finding of Loos et al. (2010) that
393 sulfamethoxazole was detected along the whole Danube River, and not like some other drugs only
394 downstream from big city wastewater discharges. The highest concentrations that Loos et al. (2010)
395 have found in the Danube River were much lower than our results and amounted to only 28 ng L^{-1},
396 probably due to high water discharge and dilution capacity of Danube River; however, they have
397 found higher levels in Danube tributaries, especially in Velika Morava in Serbia (85 ng L^{-1}) and in
398 Arges in Romania (204 ng L^{-1}), which were comparable to Vardar and the Kriva River. The highest
399 concentrations of ibuprofen were measured in Vardar downstream from Skopje (201 ng L^{-1}) and in the
400 Kriva River (185 ng L^{-1}). Lower ibuprofen concentrations were detected in the Danube River in
401 Austria and Slovakia, amounting between 5 and 10 ng L^{-1}, whereas in Danube downstream from
402 Beograd they were between 9 and 27 ng L^{-1}, due to the input from Velika Morava River, which had
403 somewhat higher ibuprofen concentration of 34 ng L^{-1} (Loos et al., 2010).

404 Several drug classes were found increased only in Vardar downstream from Skopje, i.e. hallucinogens
405 (stimulants), cannabinoids, antiepileptics, opioids and antidepressants (Fig. 2 c,e,f,h,j), indicating their

406 predominant urban use. Hallucinogens (stimulants) were mainly represented by caffeine and nicotine,
407 cannabinoids by CP 47,497-C8 homolog, antiepileptics by lamotrigine, opioids by hydrocodone and
408 antidepressants by *o*-desmethylvenlafaxine. Antiepileptic lamotrigine was present in the Vardar River
409 downstream from Skopje in concentration of 16 ng L^{-1}, whereas in the other studied rivers we have
410 mainly detected carbamazepine in concentrations of 3-8 ng L^{-1}. In the Danube River near Budapest
411 antiepileptic carbamazepine was found in concentration of 66 ng L^{-1} (Loos et al., 2010), which was
412 somewhat higher than either carbamazepine or lamotrigine concentrations in the Vardar River.
413 However, similar observation was made regarding the location of antiepileptic highest concentration,
414 which was, same as in our study, around the big city and therefore probably caused by untreated or
415 insufficiently treated urban effluents (Loos et al., 2010). It is interesting to notice that in addition to
416 concentration differences between rivers and sampling sites, differences also referred to specific drugs
417 within each drug class which were predominantly used in more and in less urban areas; it was
418 especially characteristic for antiepileptics (lamotrigine vs. carbamazepine), opioids (hydrocodone vs.
419 *o*-desmethyltramadol) and antidepressants (*o*-desmethylvenlafaxine vs. fluoxetine).

420 Few drug classes did not show clear spatial trend (hormones, hypnotics, anticholesteremics, antiseptics
421 and neuroleptics; Fig. 2 b,g,i,k,l). Among hormones, which were the second most abundant drug
422 category, xenoestrogens, such as *p*-nonylphenol, 4-octylphenol and bisphenol A, were much more
423 abundant in all studied rivers than natural hormones, such as estrone, progesterone and testosterone.
424 Based on human daily excretion and other physicochemical parameters such as dilution factor and
425 sorption to solid matter, natural estrogens are expected to be present in aqueous environmental
426 samples at the ng L^{-1} level (Baronti et al., 2000). Accordingly, in Llobregat river basin in Spain,
427 estrone, as one of the most ubiquitous sex steroids, has never surpassed 1.7 ng L^{-1} (Kuster et al., 2008),
428 which in terms of estrogenic activity should not pose a high risk to aquatic organisms (Petrovic et al.,
429 2004). Similarly, in our study, the highest observed estrone concentration amounted to 4.2 ng L^{-1}.
430 Contrary, xenoestrogens were present in much higher concentrations. The most abundant among them,
431 *p*-nonylphenol, was present in all rivers in comparable concentrations of 196-260 ng L^{-1}, probably due
432 to its widespread use in manufacturing of antioxidants, lubricating oil additives, laundry and
433 dish detergents, emulsifiers and solubilizers. These concentrations were close to the annual average
434 concentrations of 300 ng L^{-1} suggested in EU WFD as acceptable, but much lower than maximum
435 allowable concentration of 2.0 µg L^{-1} (EPCEU, 2013). For comparison, nonylphenol was detected in
436 several tributaries of Danube River in concentrations of 250-1300 ng L^{-1}, with highest levels found in
437 Arges River in Romania (Loos et al., 2010). Similarly, common use of bisphenol A resulted in
438 ubiquitous presence of this xenoestrogen in the river water; it is even under discussion to be added to
439 the priority substance list of the WFD (Oehlmann et al., 2008). In our study bisphenol A was found in
440 concentrations of 23-72 ng L^{-1}, depending on the studied river, which is somewhat lower compared to
441 its commonly reported concentrations (e.g. 295 ng L^{-1} in the Llobregat River in Spain, Rodriguez-

Mozaz et al., 2004; up to 490 ng L^{-1} in the Arges River in Romania and up to 246 ng L^{-1} in the Sava River in Croatia, Loos et al., 2010). It is interesting that, contrary to personal care products and analgesics, hormones were reported to be higher in the river waters than in the effluents of sewage treatment plants (Kuster et al., 2008), indicating their general application and diffuse contamination, as confirmed in our study by comparable hormone levels observed at all sampling sites (Fig. 2b).

3.3. Other miscellaneous compounds

Out of 3 analyzed compounds in this category, which included one corrosion inhibitor (1H-benzotriazol) and two surfactants (PFOA and PFOS), 1-3 were detected (33-100%), depending on the sampling site, and the ranges of their individual concentrations are given in Table 5. Their highest total concentrations were measured in the Kriva River (Fig. 1f), but differences between sites were rather inappreciable, with total concentrations of these compounds found in range from 74-173 ng L^{-1}.

The most abundant among these three compounds was perfluorooctanoic acid (PFOA). PFOA is mainly used as a polymerization aid in the production of fluoropolymers, and therefore originates mainly from direct industrial emissions (McLachlan et al., 2007). However, it is also used as a surfactant in mining wells. Accordingly, it was found in the highest concentrations in two mining impacted rivers, Kriva and Zletovska (134 ng L^{-1} and 107 ng L^{-1}, respectively). Those concentrations were somewhat higher compared to PFOA level of 60 ng L^{-1} detected in the Inn River, the Danube River tributary in Germany (Loos et al., 2010), but still lower compared to levels found in the Po River in Italy and the Tennessee River in USA (up to 337 ng L^{-1} and up to 513 ng L^{-1}, respectively) (Hansen et al., 2002; Loos et al., 2008).

4. Conclusion

Application of UHPLC-QTOF-MS for screening of 463 organic contaminants in surface water of four rivers in northern Macedonia enabled discernment between various anthropogenic influences, revealing obvious and pronounced herbicide contamination of agriculturally impacted Bregalnica River, predominant drug contamination of the Vardar River downstream from the city of Skopje, which is the capital of Macedonia, as well as altogether the weakest organic contamination in the mining impacted Zletovska River. Screening approach also facilitated identification of the most problematic compounds in each studied area, which presents a basis for future targeted survey of these rivers and introduction of necessary protection measures. Furthermore, many of compounds analyzed in this study can be considered as emerging contaminants and information about them is still rather scarce. A large dataset on organic contamination of freshwaters in Macedonia, which was gathered for the first time in the course of this study, can certainly significantly contribute to broadening of existing knowledge on outspread and degree of organic contamination in the world.

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Table 1. Characteristics of rivers and sampling sites chosen for screening of organic contaminants in surface water by UHPLC-Q-TOF-MS.

River	Sampling site	Coordinates	Location	Sources of pollution
Kriva	**Kriva Palanka**	N 42°11'39" E 22°18'34"	the exit of town Kriva Palanka, 15 km downstream from active Pb/Zn mine Toranica	urban and mining influence, mild agricultural runoff (orchards and gardens)
Zletovska	**Zletovo**	N 40°58'54" E 22°14'10"	2.5 km downstream from town Zletovo, 6 km downstream from active Pb/Zn mine Zletovo, and 15 km downstream from town Probištip	mining influence, battery factory
Bregalnica	**Teranci**	N 41°51'45" E 22°20'58"	11 km downstream from town Kočani	runoff from rice fields
	Kežovica	N 41°43'55" E 22°10'27"	35 km downstream from the mouth of the Zletovska River into the Bregalnica River, and 3 km downstream from town Štip	runoff from rice fields, urban and industrial influences (textile and meat industry, poultry and pig farms)
Vardar	**upstream from Skopje**	N 42°00'22" E 21°19'57"	0.5 km upstream from the city of Skopje	industrial, municipal and traffic influences of the capital of Macedonia
	downstream from Skopje	N 41°57'45" E 21°32'42"	2 km downstream from the city of Skopje	

Table 2. Targeted analysis of pesticides by UHPLC-Q-TOF-MS in the samples of surface water of several rivers in Macedonia belonging to Vardar basin, which were collected in May, 2015.

	Number of detected pesticides	Concentration range of individual detected pesticides / ng L^{-1}	Pesticides found in the highest concentrations
Insecticides, molluscicides, nematicides, and insect growth regulators (n=115)			
Kriva River	5	1.64-20.47	DEET (Diethyltoluamide)
Zletovska River	3	6.55-11.44	DEET (Diethyltoluamide)
Bregalnica - Teranci	5	3.53-41.67	Flonicamid > Pymetrozine > DEET
Bregalnica - Kežovica	8	1.30-31.17	Methomyl
Vardar - upstream	2	1.82-15.88	DEET (Diethyltoluamide)
Vardar - downstream	7	1.56-64.21	Benfuracarb
Herbicides and their metabolites, defoliants, and plant growth regulators (n=109)			
Kriva River	8	1.01-157.0	Ametryne
Zletovska River	8	1.30-48.31	Ametryne > Chloramben
Bregalnica - Teranci	12	1.07-530.2	Bentazone > Ametryne > Molinate
Bregalnica - Kežovica	17	0.07-549.3	Molinate > Bentazone > Ametryne
Vardar - upstream	16	1.12-119.0	Ametryne > Aclonifen
Vardar - downstream	15	0.95-56.20	Ametryne > Chloramben
Fungicides and their breakdown products (n=74)			
Kriva River	6	1.00-43.40	Tebuconazole > Hexaconazole
Zletovska River	5	3.01-36.31	Tebuconazole > Ipconazole
Bregalnica - Teranci	8	2.89-41.91	Tebuconazole > Diethofencarb > Carbendazim
Bregalnica - Kežovica	6	2.66-99.11	Carbendazim > Myclobutanil
Vardar – upstream	10	1.04-14.79	Tebuconazole > Carbendazim
Vardar - downstream	5	1.21-41.80	Tebuconazole > Carbendazim

Table 3. Targeted analysis of 89 drugs (11 antibiotics and chemotherapeutics, 8 hormones and xenoestrogens, 19 hallucinogens and stimulants, 4 analgesics, 6 cannabinoids and synthetic cannabinoids, 8 antiepileptics, and 33 hypnotics, anticonvulsants and anesthetics) by UHPLC-Q-TOF-MS in the samples of surface water of several rivers in Macedonia belonging to Vardar basin, which were collected in May, 2015.

	River	Number of detected drugs	Concentration range of individual detected drugs / ng L^{-1}	Drugs found in the highest concentrations
Antibiotics and chemotherapeutics (11)	**Kriva River**	6	0.49-170.1	Sulfamethoxazole > Ciprofloxacin
	Zletovska River	2	11.70-53.13	Sulfamethoxazole > Ciprofloxacin
	Bregalnica - Teranci	3	1.71-94.75	Sulfamethoxazole
	Bregalnica - Kežovica	3	1.18-14.16	Ciprofloxacin
	Vardar - upstream	4	1.44-232.8	Sulfamethoxazole
	Vardar - downstream	6	1.60-448.1	Ciprofloxacin > Sulfamethoxazole
Hormones and xenoestrogens (8)	**Kriva River**	7	1.96-260.2	*p*-Nonylphenol > 4-Octylphenol
	Zletovska River	4	2.49-208.8	*p*-Nonylphenol > 4-Octylphenol
	Bregalnica – Teranci	6	1.52-199.6	*p*-Nonylphenol > 4-Octylphenol
	Bregalnica – Kežovica	5	1.69-213.5	*p*-Nonylphenol > 4-Octylphenol
	Vardar – upstream	6	1.54-196.1	*p*-Nonylphenol > Bisphenol A
	Vardar – downstream	6	1.04-196.8	*p*-Nonylphenol > Bisphenol A
Hallucinogens and stimulants (19)	**Kriva River**	5	2.97-72.67	Caffeine > Nicotine
	Zletovska River	6	1.15-83.53	Nicotine > Cotinine
	Bregalnica - Teranci	7	0.50-34.89	Caffeine > Nicotine
	Bregalnica - Kežovica	8	1.06-73.04	Nicotine > Cotinine
	Vardar - upstream	7	1.59-35.37	Caffeine > Nicotine
	Vardar - downstream	6	1.45-135.6	Caffeine > Nicotine
Analgesics (4)	**Kriva River**	2	3.78-184.7	Ibuprofen
	Zletovska River	2	1.80-93.81	Ibuprofen
	Bregalnica - Teranci	2	1.69-100.2	Ibuprofen
	Bregalnica - Kežovica	2	1.38-44.51	Ibuprofen
	Vardar - upstream	3	1.06-22.26	Ibuprofen
	Vardar - downstream	1	201.0	Ibuprofen
Cannabinoids and synthetic cannabinoids (6)	**Kriva River**	1	63.90	CP 47,497-C8 homolog
	Zletovska River	0	-	-
	Bregalnica - Teranci	0	-	-
	Bregalnica - Kežovica	1	64.58	CP 47,497-C8 homolog
	Vardar - upstream	2	2.14-66.04	CP 47,497-C8 homolog
	Vardar - downstream	3	2.78-93.33	CP 47,497-C8 homolog
Antiepileptics (8)	**Kriva River**	2	2.22-6.68	Carbamazepine
	Zletovska River	2	1.60-6.71	Primidone
	Bregalnica - Teranci	2	3.25-10.81	Primidone
	Bregalnica - Kežovica	1	2.61	Carbamazepine
	Vardar - upstream	1	3.62	Carbamazepine
	Vardar - downstream	5	4.74-16.10	Lamotrigine
Hypnotics, anticonvulsants, and anesthetics (33)	**Kriva River**	3	4.10-12.67	Gabapentin
	Zletovska River	4	1.46-4.66	Clorazepate
	Bregalnica - Teranci	2	4.61-29.16	Gabapentin
	Bregalnica - Kežovica	2	3.29-10.56	Gabapentin
	Vardar - upstream	3	3.51-10.59	Gabapentin
	Vardar - downstream	4	1.43-16.75	Gabapentin

Table 4. Targeted analysis of 73 drugs (27 opioids and their metabolites, 1 anticholesteremic, 22 antidepressants and their metabolites, 2 antiseptics, 13 neuroleptics, 7 cardiovascular medicals, and 1 diuretic) by UHPLC-Q-TOF-MS in the samples of surface water of several rivers in Macedonia belonging to Vardar basin, which were collected in May, 2015.

	River	Number of detected drugs	Concentration range of individual detected drugs / ng L^{-1}	Drugs found in the highest concentrations
Opioids and their metabolites (27)	**Kriva River**	5	0.55-2.13	*o*-Desmethyltramadol
	Zletovska River	0	-	-
	Bregalnica - Teranci	3	1.56-2.05	Morphine
	Bregalnica - Kežovica	1	1.77	*o*-Desmethyltramadol
	Vardar - upstream	3	2.13-11.26	*o*-Desmethyltramadol
	Vardar - downstream	5	3.42-8.35	Hydrocodone
Anticholesteremic (1)	**Kriva River**	1	10.14	Gemfibrozil
	Zletovska River	1	15.34	
	Bregalnica - Teranci	1	17.03	
	Bregalnica - Kežovica	1	4.24	
	Vardar - upstream	1	19.71	
	Vardar - downstream	1	1.80	
Antidepressants and their metabolites (22)	**Kriva River**	1	2.15	*o*-Desmethylvenlafaxine
	Zletovska River	2	1.23-5.58	Fluoxetine
	Bregalnica - Teranci	1	1.55	Venlafaxine
	Bregalnica - Kežovica	1	3.00	Fluoxetine
	Vardar - upstream	2	2.07-2.36	*o*-Desmethylvenlafaxine
	Vardar - downstream	5	1.31-4.60	*o*-Desmethylvenlafaxine
Antiseptics (2)	**Kriva River**	1	5.14	Triclosan
	Zletovska River	1	5.33	Triclosan
	Bregalnica - Teranci	1	4.14	Triclosan
	Bregalnica - Kežovica	1	5.02	Triclosan
	Vardar - upstream	1	4.26	Triclosan
	Vardar - downstream	1	5.89	Triclosan
Neuroleptics (13)	**Kriva River**	1	1.31	*N*-Desmethylclozapine
	Zletovska River	1	1.09	Risperidone
	Bregalnica - Teranci	0	-	-
	Bregalnica - Kežovica	0	-	-
	Vardar - upstream	2	1.22-1.34	*N*-Desmethylclozapine
	Vardar - downstream	1	2.52	Flupentixol
Cardiovascular medicals (7)	**Kriva River**	1	1.10	Verapamil
	Zletovska River	0	-	-
	Bregalnica - Teranci	0	-	-
	Bregalnica - Kežovica	0	-	-
	Vardar - upstream	0	-	-
	Vardar - downstream	1	3.67	Strophanthidin
Diuretic (1)	**All rivers**	0	-	-

Table 5. Targeted analysis of 3 miscellaneous chemicals (corosion inhibitor and surfactants) by UHPLC-Q-TOF-MS in the samples of surface water of several rivers in Macedonia belonging to Vardar basin, which were collected in May, 2015.

	Number of detected chemicals	Concentration range of individual detected chemicals / ng L^{-1}	Chemicals found in the highest concentrations
Kriva River	2	39.12-133.5	PFOA > 1H-benzotriazol
Zletovska River	2	16.04-106.7	PFOA > 1H-benzotriazol
Bregalnica - Teranci	3	0.07-91.11	1H-benzotriazol > PFOA
Bregalnica - Kežovica	1	74.02	PFOA
Vardar - upstream from Skopje	2	18.55-62.95	PFOA > 1H-benzotriazol
Vardar - downstream from Skopje	2	46.90-72.30	1H-benzotriazol > PFOA

Figure legends

Figure 1. Concentrations of organic contaminants (ng L^{-1}) in surface water of selected rivers belonging to Vardar river basin in north Macedonia sampled in May of 2015 and measured by UHPLC-QTOF-MS (Agilent Technologies, USA): a) all compounds; b) insecticides; c) herbicides; d) fungicides; e) drugs; f) other chemicals. For all compounds presented at figure a), the results are shown as vertical stacked bars, with different contaminant categories shown with different shades. For five main contaminant categories, the results are presented as simple vertical bars. River/sampling site legend: KR – Kriva River; ZR – Zletovska River; BT – Bregalnica River, sampling site Teranci; BK – Bregalnica River, sampling site Kežovica; VAR1 – Vardar River, sampling site upstream from the city of Skopje; VAR2 – Vardar River, sampling site downstream from the city of Skopje.

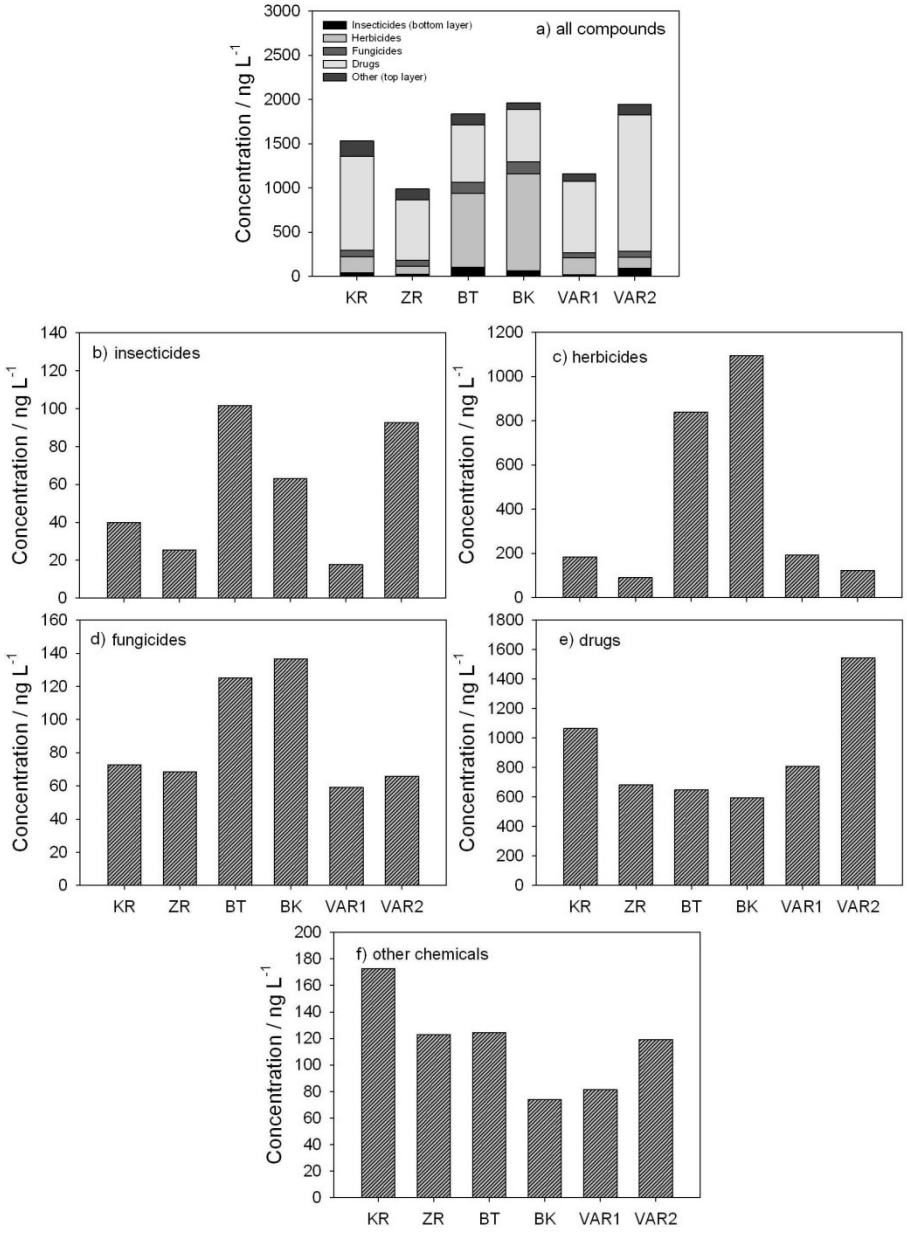
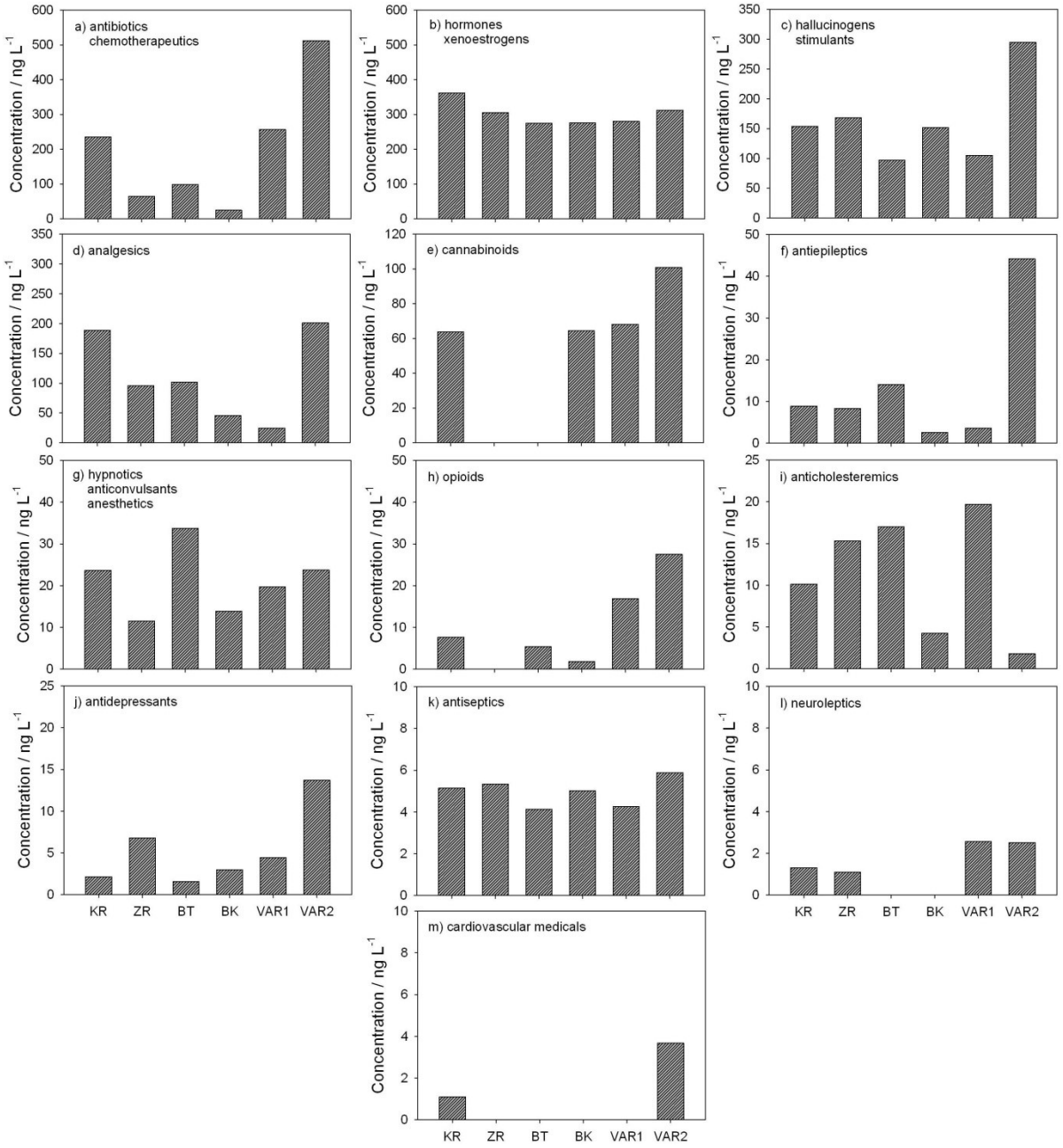
Figure 2. Concentrations of drugs (ng L^{-1}) in surface water of selected rivers belonging to Vardar river basin in north Macedonia sampled in May of 2015 and measured by UHPLC-QTOF-MS (Agilent Technologies, USA) presented separately for 13 drug classes: a) antibiotics, chemotherapeutics; b) hormones, xenoestrogens; c) hallucinogens, stimulants; d) analgesics; e) cannabinoids; f) antiepileptics; g) hypnotics, anticonvulsants, anesthetics; h) opioids; i) anticholesteremics; j) antidepressants; k) antiseptics; l) neuroleptics; m) cardiovascular medicals. The results are presented as simple vertical bars, with the same site legend as in the Fig. 1.