
Joint Danube Survey 3

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Non-target screening of organic pollutants

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Table of content

1	Introduction	4
2	Methods	4
2.1	Samples and sample preparation	4
2.2	UHPLC-ESI-Q-TOF-MS	5
2.2.1	Instrumentation	5
2.2.2	MS only method	5
2.2.3	AutoMSMS method	5
2.2.4	Validation qual/quant method	5
2.2.5	Software for data analysis and PCDL databases	5
2.2.6	Q-TOF-MS non-target screening workflow	6
2.3	HPLC-ESI-Q-TOF-MS	7
2.3.1	Instrumentation	7
2.3.2	Data analysis	7
2.4	Gas chromatography-mass spectrometry	7
2.4.1	Liquid-liquid extraction	7
2.4.2	Direct analysis of large volume samples	8
2.4.3	Semi-quantitative assessment	8
3	Results	8
3.1	UHPLC-ESI-Q-TOF-MS	8
3.1.1	Target analysis	8
3.1.2	Non-target analysis	9
3.2	HPLC-ESI-Q-TOF-MS	15
3.2.1	Target analysis and suspect screening	15
3.3	GC-MS	15
4	Conclusions	28
5	References	29

1 Introduction

Most of human activities (agricultural, industrial and domestic) lead to water contamination with numerous synthetic compounds of which most are not monitored in routine analyses. Although the majority of these compounds are present at low concentrations, many of them raise considerable (eco)toxicological concerns, particularly when present as components of complex mixtures. Largely unknown long-term effects on aquatic life and human health are caused by chemical pollution (Schwarzenbach et al., 2006; Kolpin et al., 2002; Richardson, 2007). The analyses of organic contaminants in different environmental compartments are predominantly based on chromatographic separations and mass spectrometric detection (Wille et al., 2012). To ensure that all contaminants with their degradation products and metabolites are detected a non-targeted approach is also required (Ferrer and Thurman, 2012). Considering the above, non-target and target screening was performed on the 68 JDS 3 water samples collected from the Danube River and its tributaries. The prerequisite for non-target analysis is a mass spectrometer sufficiently sensitive to detect and identify the compound directly, recording the full spectrum rapidly and at the same time having high mass accuracy for components present at very low concentrations. According to Krauss et al. (2010) the aim of non-target analysis is to search for as many compounds in a sample as possible with the focus on compounds not previously known to be present. Another important feature of a non-target method is that the acquired full dataset of mass spectra enables retrospective analyses of the sample. An availability of comprehensive mass spectral libraries with accurate mass fragmentation information was shown to be of importance at confirmation of the identity of detected substances (Zedda and Zwiener, 2012). During the JDS 3 ultra high performance liquid chromatography electrospray ionisation quadrupole-time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS), high performance liquid chromatography coupled with electrospray ionisation quadrupole-time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) and gas chromatography mass spectrometry (GC-MS) in three different laboratories were used for non-target screening. A specific statistical chemometric software was used to find pollution patterns of organic compounds acquired with the UHPLC-ESI-QTOF-MS.

2 Methods

2.1 Samples and sample preparation

Polycarbonate bottles containing 0,25 L (LC-MS) and 1 L (GC-MS) of surface water sample from all JDS 3 sites were shipped to the laboratories each 3-4 days during the survey and stored cool until analysis. Sampling, quality control measures (field blanks) and the way of controlling the sample temperature during the transport are described in Chapter 2. Samples were filtrated through 0,2 µm PTFE filter prior to analysis. Ultrapure laboratory water samples were always processed in parallel with the environmental water samples.

A subset of 22 samples was obtained by large volume sampling of 500 L of water sample through a series of three solid phase extraction cartridges capturing a wide range of polarity (neutral, acidic, basic) substances (for details see Chapter 2 and 26).

2.2 UHPLC-ESI-Q-TOF-MS

2.2.1 Instrumentation

The samples were analysed in Central Water Management Laboratory of Croatian Waters in Zagreb, Croatia. Chromatographic separations were carried out with the 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA, USA) using a reversed phase ACQUITY UPLC HSS T3 analytical column (150 mm x 2.1 mm, 1.8 μ m). The mobile phase gradient was from 100% water to 100% organic solvent in 20 min run and the sample injection volume was 100 μ L. The temperature of the column chamber was set at 50°C. In positive electrospray ionisation (ESI+), the mobile phase was composed of solvent A (5 mM ammonium acetate/ HAc (pH=4.7) and B (100% MeOH). Gradient elution with a flow rate of 0.4 mL/min was used. The analytes were detected using an 6550 i-Funnel Q-TOF-MS (Agilent Technologies) providing 40,000 resolving power and < 2 ppm accuracy at 4 GHz detector rate.

2.2.2 MS only method

For MS screening method the acquisition rate in MS1 mode was 2 spectra/s (4100 transients per spectrum). The measured mass range was 100-1000 m/z in the centroid and profile mode. The capillary and fragmentor voltages were 3500 V and 400 V, respectively. The sheat gas flow was 11 L N₂/min, flow of the drying gas was 18 L N₂/min while nebulizer was kept at 30 psig. The resolution power for ESI+ was 52296 at 922.009798 m/z and 21801 at 118,086255 m/z. A correction for any possible drift in the mass axis during measurement was done automatically with lock 2 mass ion software.

2.2.3 AutoMSMS method

For autoMSMS mode screening method the acquisition rate in MS1 was 2 spectra/s (4100 transients per spectrum) and measured mass range was 100-1000 m/z in the centroid and profile mode. The acquisition rate in MS2 was 3 spectra/s (2650 transients per spectrum) and measured mass range was from 50 to 1000 m/z while the data were obtained at settings of narrow width isolation. Collision energies were fixed at 10, 20 and 40 eV.

2.2.4 Validation qual/quant method

Target screening method was developed for a mixture of 168 organic substances containing pesticides and pharmaceuticals such as antidepressants, anti-epileptic, neuroleptics, opioids, benzodiazepines/hypnotics, cardiovascular medial and hallucinogens/stimulants. Calibration curve was obtained by direct injecting, in triplicate standard solutions at seven concentration levels starting from 1 to 1000 ng/L. Correlation coefficients > 0.99 were used as linearity acceptance criterion. Accuracy and the precision was calculated by analysing blank samples spiked at three concentration levels and were evaluated within-day in quintuplicate at each concentration level. Acceptance criteria were (i) recoveries of 70% and 110% for accuracy and (ii) RSD lower than 20% for precision. Once validated, the screening method has been applied to the analysis of different surface water to test its applicability.

2.2.5 Software for data analysis and PCDL databases

2.2.5.1 Software for data analysis

Analyses were conducted using the MassHunter Profinder Qualitative Analysis tools of the MassHunter Workstation Software (version B.06.00, Built 6.0.605.0, Agilent Technologies) with software tools: Molecular Formula Generator (MFG), Find by Ion, Find by Formula and Molecular Feature Extractor (MFE). Statistical analyses were conducted by using the Mass Profiler Professional software (MPP, Version 12.6.1, Agilent Technologies). Quality control in MPP was used for elimination of unreliably identified compounds or compounds not relevant for data evaluation. After

quality control in MPP a differential analysis was performed. Chemometric statistical analysis with reliable peak-finding algorithm was applied at the non-target screening in order to reduce the false positives/negatives. Comparison of samples was based on compounds (entities) determined by their full scan data.

2.2.5.2 Personal Compound Database Library - PCDL databases

Forensic toxicology, Pesticide and Metlin metabolite PCDLs, all in total with more than 65000 compounds, were used to identify drugs of abuse, medical drugs, pesticides, alkaloids, toxic reagents, and their metabolites. Information obtained in PCDLs provides compounds' name, CAS number, molecular and structural formula, neutral mono-isotopic mass, isotope pattern, retention time (optional) and MS/MS spectra generated at CID energies of 10, 20 and 40 eV.

MassHunter Forensic Toxicology PCDL ver. 4.1 contains mass spectra of 7509 compounds and MS/MS library of more than 2500+ compounds; MassHunter Pesticide PCDL ver. 4.1 contains mass spectra of 1664 compounds and MS/MS library of more than 600 compounds and MassHunter METLIN metabolite PCDL ver. 5 contains mass spectra of 64092 compounds and MS/MS library of more than 8040 compounds. All MS/MS spectra were obtained at three collision energies (10, 20 and 40 eV).

2.2.6 Q-TOF-MS non-target screening workflow

After recording full scan acquisition in Q-TOF MS, all generated mass spectrometric data were sent to MassProfiler software (cf. Section 2.4.1 above) where untargeted data mining and batch recursive feature extraction was performed. Features (unprocessed information about the compounds) extracted with recursive analysis were subjected to compound alignment and statistical analyses using MPP. Molecular Feature Generator in MPP software was used for calculation of features' accurate masses accompanied with information on molecular formula, isotopic pattern, isotopic spacing, and the difference between the theoretical exact mass of the assigned formula and the acquired accurate mass for the feature. In the final list MPP features were divided into three groups: first was the PCDL match defined by presence of the compound in PCDL database (name of the substance assigned), second was unknown (molecular formula provided), and third was total unknown (only an accurate mass and retention time defined).

Results of MPP analysis were then exported to autoMSMS method for further identification and confirmation of compounds with accurate mass, fragmentation by MS/MS, and characteristic isotope signatures and fragments. In autoMSMS method Agilent MassHunter Qualitative software with MFE, MFG and PCDL accurate mass library were used.



Figure 1 Non-target workflow used for analyses by UHPLC-Q-TOF-MS

A presence of compound's mass spectrum found in autoMSMS also in PCDL led to the provisional identification of the compound. Characteristic fragments acquired in autoMSMS were considered as sufficient additional information to fully confirm identity of the substance. An injection of standard chemical would be needed for unequivocal confirmation in cases when compound's spectral data was not present in PCDL. A workflow used for identification of unknown compounds is presented in Figure1.

2.3 HPLC-ESI-Q-TOF-MS

2.3.1 Instrumentation

The samples were analysed in Zweckverband Landeswasserversorgung (LW) Betriebs- und Forschungslabor in Langenau, Germany using high resolution LC-MS with duplicate direct injection of 100 µl water sample both in ESI+ and ESI- mode. The high performance liquid chromatography system Prominence LC20 Series (Shimadzu, Duisburg, Germany) coupled with the TripleTOF 5600 mass spectrometer (AB SCIEX, Concord (ON), Canada) was used. After electrospray ionization in positive and negative mode, the data were collected in full scan mode (m/z 100 – 1200 Da). The HPLC column Zorbax Eclipse Plus C18, 2.1 x 150 mm (Agilent, Waldbronn, Germany) and the guard column AQ C18 2.0 x 4 mm (Phenomenex, Aschaffenburg, Germany) were used. Both eluents water (A) and acetonitrile (B) contained 0.1 % formic acid, respectively. A multi-step gradient with the following parameters was applied in ESI+ and ESI-: 1 min at 2% B, within 1 min to 20% B, within 14.5 min to 100% B, hold for 5.5 min at 100% B, within 0.1 min back to 2% B and 4.9 min for equilibration at 2% B. The flow rate was constant 0.3 ml/min and the column temperature was 40°C. Nitrogen was used as drying and curtain gas. The source parameters were set to GAS 1 35 psi, GAS 2 45 psi, Curtain Gas 40 psi, temperature 550°C, ion source voltage 5500 V (-4500 V for ESI-), declustering potential of 100 V (-60 V for ESI-) and a collision energy of 10 eV (-10 eV for ESI-). In addition, an IDA-experiment (Information Dependent Acquisition) was used in which MS/MS-spectra of compounds that fulfill certain criteria were acquired (collision energy 40 eV). For instance, blank compounds as well as features which do not exceed a threshold of 100 cps were excluded. The mass spectrometer was calibrated using external calibration delivery system CDS and internal calibration with known contaminants. All systems, the HPLC and the mass spectrometer were controlled and data were acquired as well as processed by AnalystTF™ 1.6 software (AB Sciex, Concord (ON), Canada).

2.3.2 Data analysis

Data Analysis of target and suspected compounds were conducted using the qualitative analysis tool MasterView™ of the PeakView™ software (version 2.0, AB Sciex, Concord (ON), Canada). Comparisons of the Danube River samples with a blank injection and a multi component reference standard (about 315 substances) were performed. Compounds were designated as ‘identified’ if accurate mass, isotope pattern and retention time in the sample conformed to those of the reference standard. In cases where the IDA-experiments supplied reliable MS/MS-spectra, the data were additionally used for comparison.

2.4 Gas chromatography-mass spectrometry

2.4.1 Liquid-liquid extraction – method 1

Water samples (1000 mL) were placed into a glass separating funnel, spiked with 10 µl (10 ng/l) of methanolic perdeuterated phenanthrene and 10 µL (10 ng/L) of methanolic perdeuterated DDT internal standard solutions to give a final concentration of 1 µg/L and then extracted by two portions of dichloromethane (2 x 40 mL). After extraction the final combined extract was dried with anhydrous sodium sulphate and then evaporated to the final volume of 1 mL using vacuum rotary evaporator.

The GC-MS screening analysis was performed with Agilent 7890 gas chromatograph coupled to Agilent 5975 C mass spectrometric detector (MSD; Agilent Technologies, Little Falls, DE, USA). The system was equipped with the Agilent Multimode (MMI) Inlet allowing introduction of 50 µl of extract into the GC system in the solvent vent injection mode. The MMI was ramped from 70°C to 260°C (5 min) at a rate of 600°C/min. Capillary GC analysis was performed on a 30 m x 250 µm I.D., 1 µm d_f HP-5MS column (Agilent Technologies). The oven was programmed from 50°C (3 min) at 30°C/min to 200°C, at 5°C/min to 280°C and finally at 30°C/min to 310°C (5 min). Hydrogen was used as a carrier gas. The MSD was operated in the electron impact (EI) full scan mode (m/z 50–600) for all samples. Identification of compounds was performed using mass spectrum libraries Wiley 7n and NIST11, followed by manual interpretation. Molecular masses of numerous detected compounds

were additionally confirmed in the mode of positive chemical ionisation using methane as a reagent gas. A retention time index has been calculated for each detected substance based on the injection of the Kovats's mixture of alkanes for comparison with retention time indices in the NIST library and thus increasing the confidence in identification.

2.4.2 Direct analysis of large volume samples – method 2

An aliquot of 2 ml extract corresponding to 2 L water sample obtained by LVSPE (cf. Section 2.1) was used for GC-MS screening analyses after its reconstitution into organic solvent and spiking with methanolic perdeuterated phenanthrene at concentration level of 1 µg/L. The system was equipped with the Agilent Multimode (MMI) Inlet allowing introduction of 125 µl of the extract to the GC system in the solvent vent injection mode. The rest of the analysis conditions were identical to those described in Section 2.4.1.

2.4.3 Semi-quantitative assessment

An estimation of concentrations of compounds detected in the full scan EI mode was performed. Concentration values based on comparison of the signal (relative abundance) of an unknown compound to the signal generated by the known concentration of an internal standard were estimated (Slobodnik et al., 2012). In the procedure, a signal of the quantification ion of the deuterated internal standard (m/z 188 for phenanthrene- D_{10}) was compared with the signal of its overall mass spectrum (Total Ion Current; TIC), which resulted in estimation of its relative intensity (i.e., 34% from the TIC response, RSD = 0.93%, $n = 6$). The same procedure was applied to the unknown compound (selection of the most abundant ion; determination of its intensity relative to the overall intensity (TIC) of the whole mass spectrum). The ratio between signals of quantification ions of the unknown substance to that of the known internal standard was then corrected for their percentage representativeness of the TIC and the final concentration was calculated (e.g. IF signal of 10 ng/L internal standard phenanthrene- D_{10} is 100,000 (arbitrary units), TIC corrected signal is 34,000 AND TIC corrected signal of unknown substance is 17,000 THEN the estimated concentration of unknown substance is 5 ng/L). It should be made clear that the method provides only rough indicative estimations of actual concentrations. However, additional comparisons obtained with standard compounds for large proportion of the substances usually detected in surface water samples showed that the error is usually contained within one order of magnitude (Slobodnik et al., 2012).

3 Results

3.1 UHPLC-ESI-Q-TOF-MS

3.1.1 Target analysis

Results of target screening of 68 JDS 3 samples for a wide range of pharmaceuticals and illicit drugs are presented in Figure 2. A total of 154 out of 168 studied analytes were found to be present in at least one sample. Detailed information on the occurrence and concentrations of detected compounds per sampling site is presented in the full report on the attached CD-ROM (Annex I - Report_tab_CW_QTOFMS).

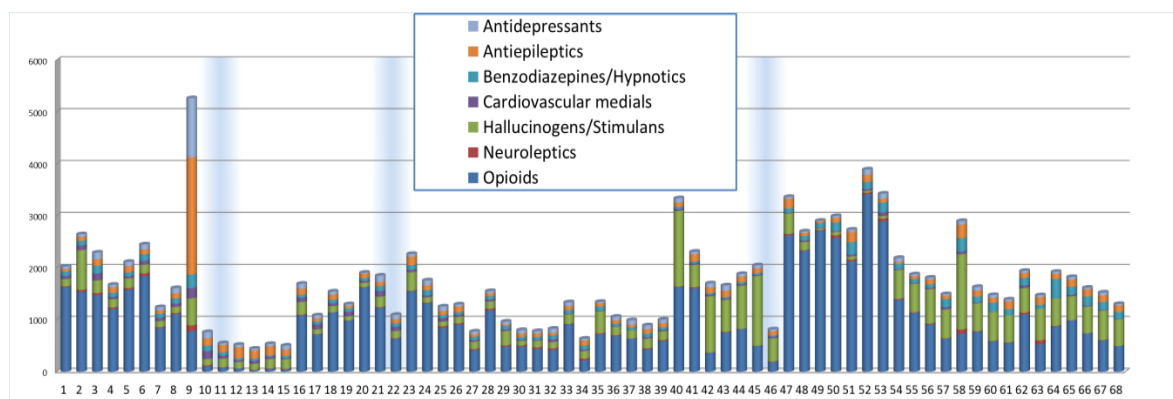


Figure 2 Occurrence profile of different groups of pharmaceuticals and illicit drugs in the 68 JDS3 samples; blue vertical lines are presenting rainy period, x-axis represents sampling stations and y-axis indicates cumulative concentrations of all determined substances (in ng/L) with a quantitative proportion of the particular group of substances (cf. different colours)

3.1.2 Non-target analysis

Initial quality control on acquired 16214 raw features in MPP with filtering by frequency, sample variability, flags, abundance, significance testing and fold change resulted in 7767 processed features that were detected in 68 JDS3 samples (Figure 3). Please, note that all target compounds (Section 3.1 above) were excluded from non-target analysis.

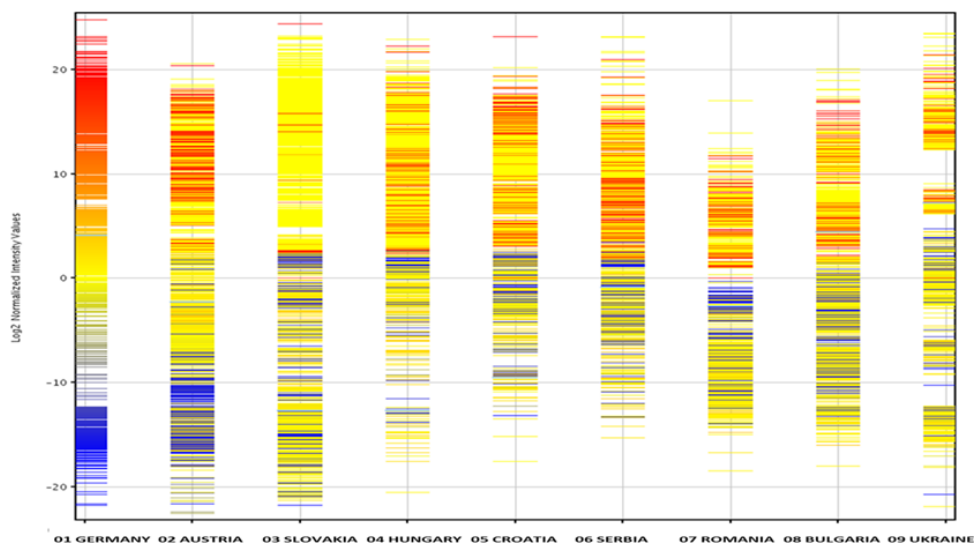


Figure 3 Distribution of 7767 different mass spectral processed features through the Danube river and its tributaries; Danube countries are shown on x-axis and normalised signal intensity values are represented on y-axis; each single feature/compound is represented by a horizontal bar at a fixed position on the chart (position given by a unique combination of retention time, accurate mass spectrum, name, molecular formula, etc.) and the intensity of signal increase is indicated by blue (low) to red (high) colour

The figure indicates that the highest number of different features (i.e. also chemical entities present in samples) with highest signal intensity was found in Germany and the least number of features/substances was identified in samples from Romania. From these 7767 processed features ID Browser recognised 3442 match compounds in the PCDL library which allowed for assigning the

compounds with a defined name, accurate mass, molecular formula, retention time, CAS number and isotopic pattern. For 3370 (unknown) compounds a molecular formula was calculated and supplemented with accurate mass, retention time and isotopic pattern, and 955 (fully unknown) compounds were defined only with accurate mass and retention time (Figure 4). Detailed information on the occurrence of all features (PCDL match compounds, unknowns, total unknowns) determined by the MPP is presented in the full report on the CD-ROM (Annex I - Report_tab_CW_QTOFMS).

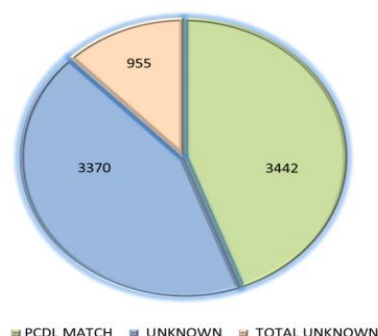


Figure 4 Overview of compounds' identification results; full scan mass chromatograms of all 68 JDS3 samples obtained by UHPLC-ESI-Q-TOF-MS were evaluated with the Mass Profiler Professional (MPP) software

The autoMSMS method was applied for all detected compounds from all 68 samples (7767 processed features resulting in assigning PCDL match compounds, unknowns and total unknowns (cf. text above), with focus on 5014 spectral data acquired with CE 10, 20 and 40 eV, which were matching those already stored in the available databases). This allowed to finally arrive to the reduced list of compounds recognised by name, high accurate mass and fragments. The autoMSMS evaluation of this large dataset is still on-going, however, the substances listed in Table 1 can already be considered as unequivocally identified, despite standard chemicals of these substances were not available for the final confirmation.

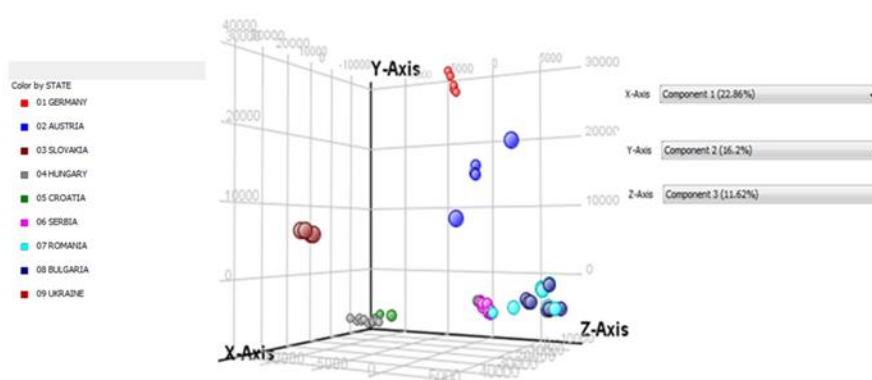


Figure 5 Similarity of pollution profiles among different Danube countries evaluated with the Principal Component Analysis (PCA) of JDS 3 non-target screening data obtained with UHPLC-ESI-Q-TOF-MS

Principal Component Analysis (PCA) was performed with the MPP software on all data sets for detection of similarities and differences in the patterns of pollution between different Danube countries discriminated by the major trends in the data. Figure 5 shows that similarities in pollution pattern exist among Serbia, Romania, Bulgaria and Ukraine and between Croatia and Hungary whereas rather unique character of pollution can be seen in the upstream countries (Germany, Austria, Slovakia).

Table 1 List of selected non-target compounds unequivocally identified by UHPLC-QTOF-MS operated in autoMSMS mode

CAS	FORMULA	NAME	NOTES	FREQUENCY	m/z	H+	CE	fragment1	fragment2
2163-69-1	C11 H22 N2 O	Cycluron	Herbicide	68	198,1732	199,1810	20	72,0444	89,0709
134-62-3	C12 H17 N O	DEET / Diethyltoluamide	Insecticide	68	191,1310	192,1383	20	119,0491	91,0542
51235-04-2	C12 H20 N4 O2	Hexazinone	Herbicide	68	252,1586	253,1659	20	171,0877	
90-33-5	C10 H8 O3	Hymecromone	Choleretic;Insecticide	68	176,0473	177,0547	40	77,0386	68,9971
60142-96-3	C9 H17 N O2	Gabapentin	Anticonvulsant	67	171,1259	172,1332	40	67,0542	55,0178
56392-16-6	C15 H25 N O4	Hydroxymetoprolol	Beta-Blocker, metabolite	67	283,1784	284,1856	40	56,0495	74,0600
23103-98-2	C11 H18 N4 O2	Pirimicarb	Insecticide	66	238,1430	239,1503	20	72,0444	182,1288
37517-30-9	C18 H28 N2 O4	Acebutolol	Beta-Blocker	65	336,2049	337,2122	10	116,1070	98,0964
39809-25-1	C10 H15 N5 O3	Penciclovir	Antiviral	65	253,1175	254,1248	40	135,0301	110,0349
1593-77-7	C18 H35 N O	Dodemorph	Fungicide	64	281,2719	282,2791	40	98,0964	55,0542
34661-75-1	C20 H29 N5 O3	Urapidil	synthetic	64	387,2270	388,2355	40	190,1101	70,0651
33817-20-8	C22 H29 N3 O6 S	Pivampicillin	Antibiotic	63	463,1777	464,1850	10	274,1108	244,1002
298-81-7	C12 H8 O4	Ammoidin	Naturally occurring compound	60	216,0423	217,0495	40	174,0311	90,0464
13655-52-2	C15 H23 N O2	Alprenolol	Beta-Blocker	59	249,1729	250,1801	20	116,1070	72,0808
13912-80-6	C12 H17 N O3	Nicoboxil	Rubefacient	58	223,1208	224,1281	40	124,0393	78,0338
70-70-2	C9 H10 O2	Paroxypipione	Hormone	55	150,0681	151,0752	40	77,0386	
2382-79-8	C13 H15 N3 O2	Acetyltryptophanamide	Synthetic	54	245,1164	246,1237	20	159,0917	201,1022
827-61-2	C9 H15 N O2	Aceclidine	Parasympathomimetic	48	169,1103	170,1175	20	110,0964	
657-24-9	C4 H11 N5	Metformin	Antidiabetic	45	129,1014	130,1087	10	60,0556	71,0604
554-62-1	C18 H39 N O3	Phytosphingosine	PCPP, shampoo	42	317,2930	318,3003	20	60,0444	
1695-77-8	C14 H24 N2 O7	Spectinomycin	Antibiotic	40	332,1584	333,1671	10	98,0600	
633-47-6	C13 H24 N2 O2	Cropropamide	Stimulant	38	240,1838	241,1917	40	100,1121	69,0335
3485-14-1	C15 H23 N3 O4 S	Ciclacillin	Antibiotic	37	341,1409	342,1488	20	98,0964	
51338-27-3	C16 H14 Cl2 O4	Diclofop-methyl	Herbicide	37	340,0269	341,0336	20	123,0570	
99011-02-6	C14 H16 N4	Imiquimod	Immunomodulator, virustatic	35	240,1375	241,1449	20	185,0822	
1177865-17-6	C24 H35 N7	NSC 23766	Inhibitor	32	421,2954	422,3028	20	349,2135	
120162-55-2	C13 H16 N10 O5 S	Azimsulfuron (IN A8947)	Azimsulfuron-methyl	31	424,1026	425,1097	40	182,0560	139,0489
101622-51-9	C15 H18 N6 O	Olomoucine	Chemotherapeutic	28	298,1542	299,1623	40	91,0542	177,0883
1637-39-4	C10 H13 N5 O	trans-Zeatin	Naturally occurring compound	28	219,1120	220,1193	40	119,0352	136,0618
20380-58-9	C17 H23 N O2	Tilidine	Analgesic	26	273,1729	274,1809	40	155,0855	77,0386
103-33-3	C12 H10 N2	Azobenzene	Dye	25	182,0844	183,0917	40	77,0386	
75330-75-5	C24 H36 O5	Lovastatin	Anticholesteremic	25	404,2563	405,2636	10	199,1481	285,1849
224789-15-5	C23 H32 N6 O4 S	Vardenafil	Erectile Dysfunction Treatment	25	488,2206	489,2290	40	151,0853	312,1574
83-33-0	C9 H8 O	1-Indanone	Oxidation product	24	132,0575	133,0648	20	77,0386	105,0699
1704-28-5	C18 H23 N O	Aldimorph	Fungicide	24	283,2875	284,2950	40	57,0699	98,0946
15870-91-4	C14 H14 O4	Prenylamine	Vasodilator	24	329,2143	330,2216	40	91,0542	
309-29-5	C24 H30 N2 O2	Doxapram	Stimulant	21	378,2307	379,2384	40	97,0886	129,0699
14028-44-5	C17 H16 Cl N O3	Amoxapine	Antidepressant	20	313,0982	314,1055	20	271,0633	70,0651
34866-47-2	C13 H21 N3 O3	Carbuterol	Bronchodilator	20	267,1583	268,1656	20	134,0600	177,0659
2430-27-5	C8 H17 N O	Valpromide	Anticonvulsant	20	143,1310	144,1383	20	57,0699	72,0444
30344-00-4	C18 H18 N4 O2	ADMA	Naturally occurring chemical	19	202,1430	203,1503	20	70,0651	88,0869
33629-47-9	C14 H21 N3 O4	Butralin (Sutralin)	Herbicide	19	295,1532	296,1605	40	57,0699	178,0737
6552-12-1	C10 H15 O4 PS	Fenthion-oxon	Insecticide Metabolite	19	262,0429	263,0501	20	231,0239	216,0005
5355-16-8	C13 H16 N4 O2	Diaveridin	Cocciidiostatic	17	260,1273	261,1346	20	245,1033	123,0665
6452-71-7	C15 H23 NO3	1-(2-(allyloxy)phenoxy)-3-(isopropylamino)propan-2-ol	Beta-Blocker	16	265,1678	266,1751	10	72,0808	225,1359
57526-81-5	C12 H19 N O3	Prenalator	Sympathomimetic	16	225,1365	226,1438	20	72,0808	56,0495
14556-46-8	C14 H22 Cl NO2	5-Carboxybupranolol	Beta-Blocker	15	301,1081	302,1154	20	246,0528	
70374-39-9	C13 H10 Cl N3 O4 S2	Lornoxicam	Non-steroidal antiphlogistic	14	370,9801	371,9874	20	95,0604	121,0415
865318-97-4	C15 H25 N5	Ametoctradin	Fungicide	13	275,2110	276,2174	40	176,0931	149,0822
59338-93-1	C16 H21 N5 O2	Alizapride	Antihistamine	11	315,1695	316,1768	20	124,1121	148,0393
57-68-1	C12 H14 N4 O2 S	Sulfadimidine	Chemotherapeutic	11	278,0837	279,0910	40	201,0441	92,0495
81-82-3	C19 H15 Cl O4	Coumachlor	Rodenticide	10	342,0659	343,0732	20	163,0390	285,0313
51276-47-2	C5 H12 N O4 P	Glufosinate	Alt CAS: 53369-07-6	10	181,0504	182,0577	20	56,0495	136,0522
543-82-8	C8 H19 N	Octodrine	Sympathomimetic	10	129,1517	130,1590	10	57,0699	71,0855
331830-20-7	C13 H8 N2 O3	1,4-DPCA	Inhibitor	5	240,0535	241,0608	10	223,0502	
525-82-6	C15 H10 O2	Flavone	Endogenous Metabolite	5	222,0681	223,0754	40	77,0386	65,0386

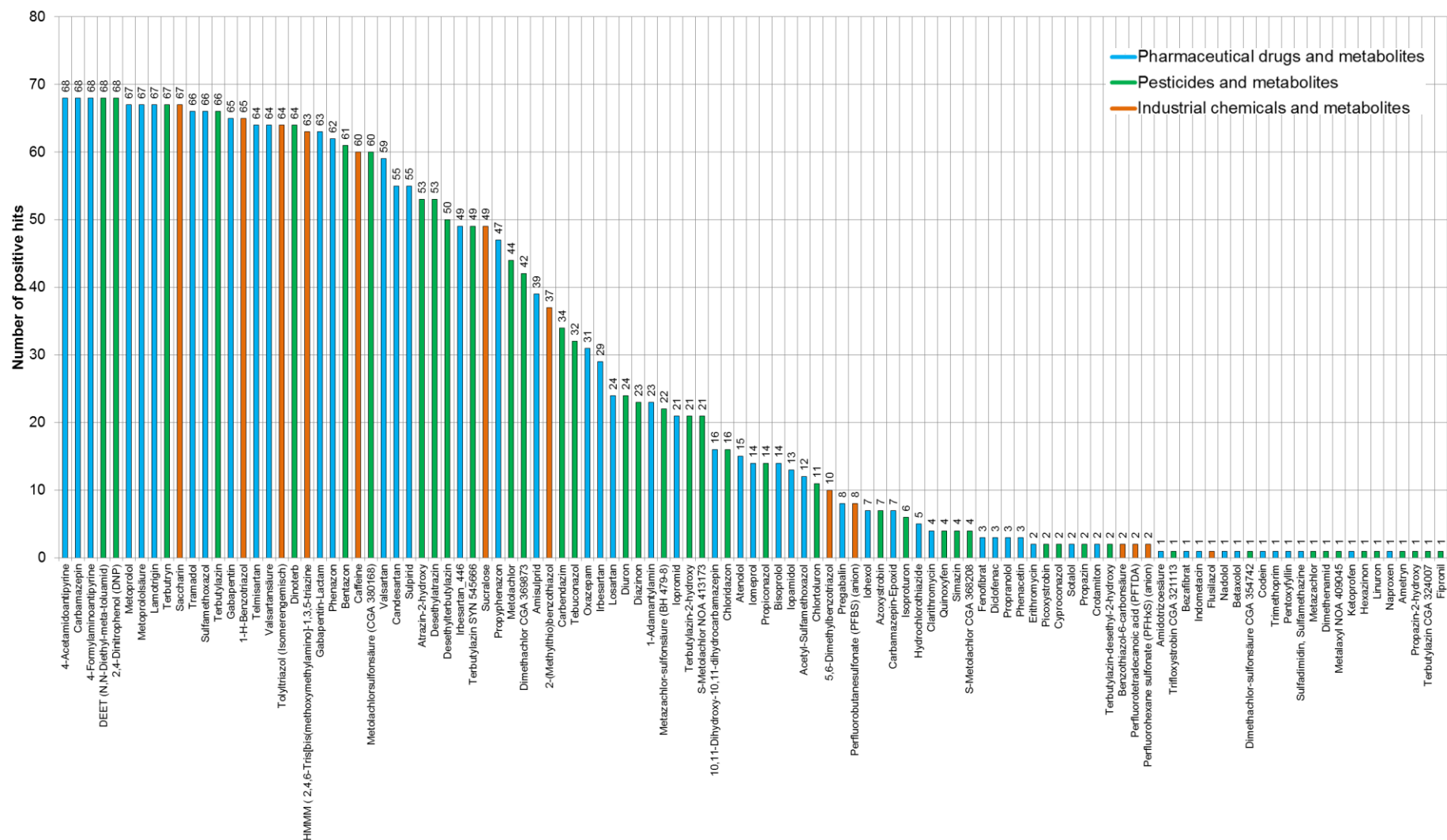


Figure 6 : Frequency of appearance of 110 'identified' suspect pollutants (315 tested) in JDS3 surface water samples; results obtained from non-target screening workflow by HPLC-ESI-Q-TOF-MS operated in ESI⁺ and ESI⁻ modes

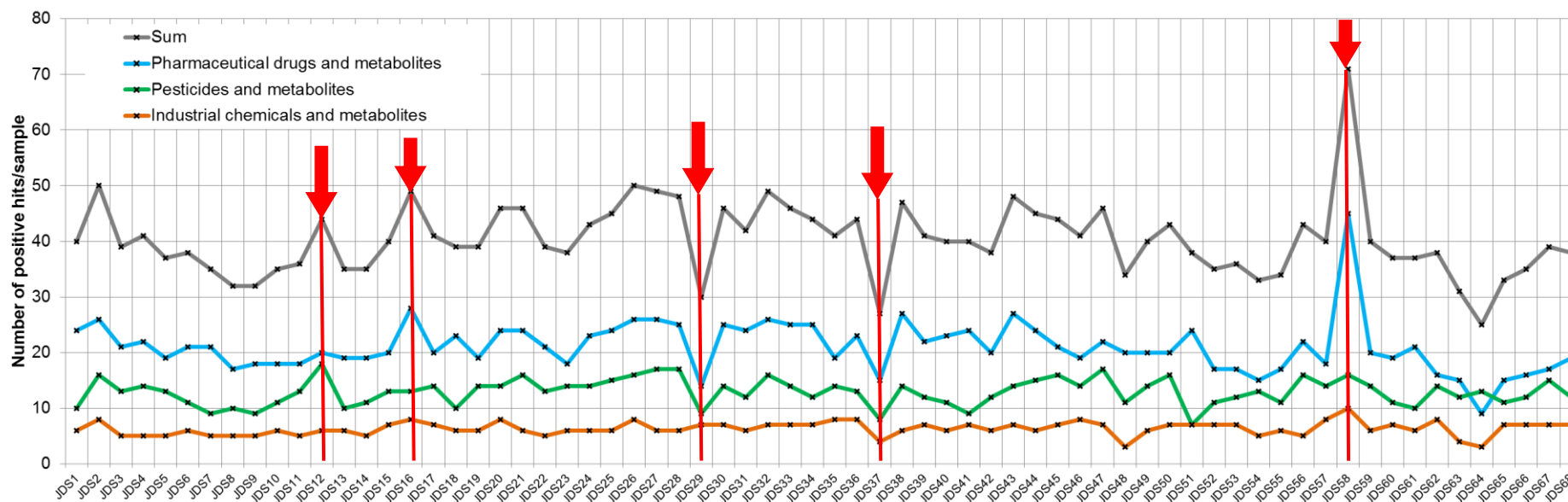


Figure 7: Frequency of appearance of 110 'identified' pollutants sorted by groups (Pharmaceutical drugs, pesticides and industrial chemicals as well as their metabolites, respectively) in JDS 3 surface water samples.

3.2 HPLC-ESI-Q-TOF-MS

3.2.1 Target analysis and suspect screening

LW laboratory conducted screening of 315 suspected organic pollutants in all 68 JDS 3 samples. The ‘suspect screening’ showed that 110 substances were detected in at least one sample (Figure 6). The substances 4-acetamidoantipyrine, carbamazepine, 4-formylaminoantipyrine, DEET and 2,4-dinitrophenol were detected in all 68 samples. Next to the evaluation of the relative signal intensities for each of the detected substances also a retrospectively obtained semi-quantitative results using a single-point calibration curve were provided for a subset of 110 compounds. Detailed results are presented in the full report on the CD-ROM (Annex II- Report _tab_pos_neg_LW).

In Figure 7 the frequency of appearance of these ‘identified’ pollutants is plotted for each single sample (JDS1 - JDS68). The pollutants were merged into three groups, namely Pharmaceutical drugs, pesticides and industrial chemicals (as well as their known metabolites). The grey line represents the sum of all detected substances. The grouping of the substances reveals interesting courses which are marked by the red arrows. For instance, the first red arrow highlights the fact that the “peak” in the sum function of sample JDS 12 is mainly caused by Pesticides (green course) while Pharmaceutical drugs and Industrial chemicals show an inconspicuous course. Furthermore, the high number of positive hits in case of JDS58 is almost only related to Pharmaceutical drugs (and Metabolites). Further interesting courses are marked in the same manner. These finding might allow the assignment of different sources of pollution which are released into the aquatic environment. On the other hand, a decrease of the sum function (arrow three and four) could possibly indicate a dilution of the surface water by less influenced inflows.

3.3 GC-MS

Altogether, 68 water samples from the Danube River and its tributaries were analysed by liquid-liquid-GC-MS and 22 LVSPE extracts of water from the Danube River were analysed by the second method. Based on the obtained spectral information, chemical structures of 298 analytes (method 1) and of 288 analytes (method 2) could be proposed (see Figure 8,9 and Table 2,3). An additional 29,2 % (method 1) and 37,7 % (method 2) compounds on the sampling site remained unidentified. For comparison, screening of 98 water samples in the JDS1 revealed the presence of 96 provisionally identified analytes and screening of 124 water samples in the JDS2 revealed the presence of 158 provisionally identified analytes. The used LVSPE sampling and concentration technique seems to be superior to that of LLE in terms of extraction efficiency of wide polarity range compounds and sensitivity allowing determinations at ng/L levels. On the other hand LLE was more selective to non-polar and volatile compounds.

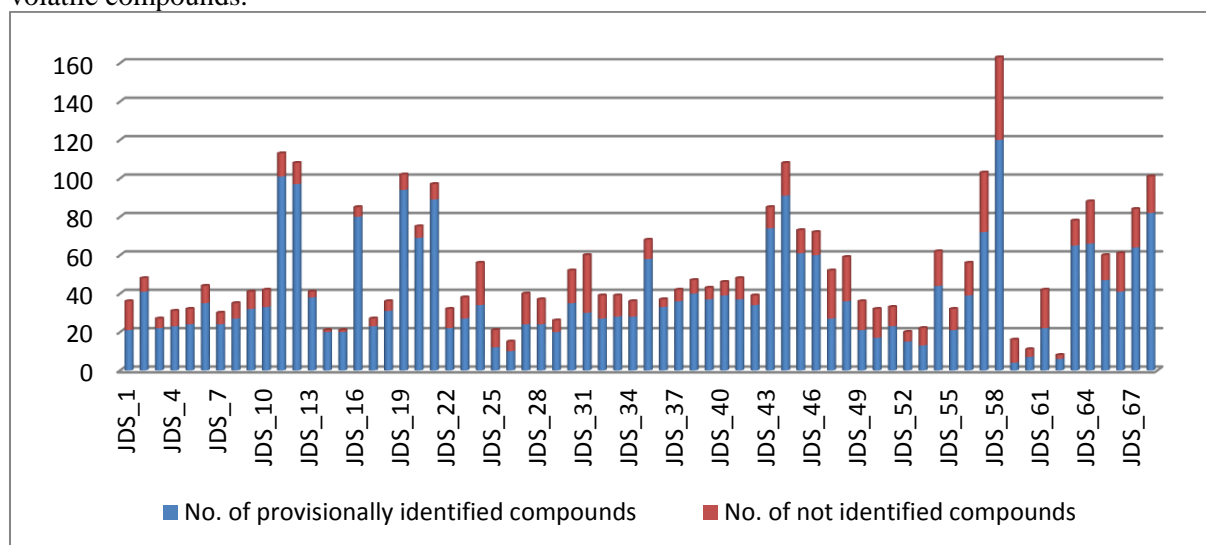


Figure 8: Number of compounds detected with LVI-GC-MS in the 22 JDS 3 surface water samples obtained with the LVSPE sampling technique

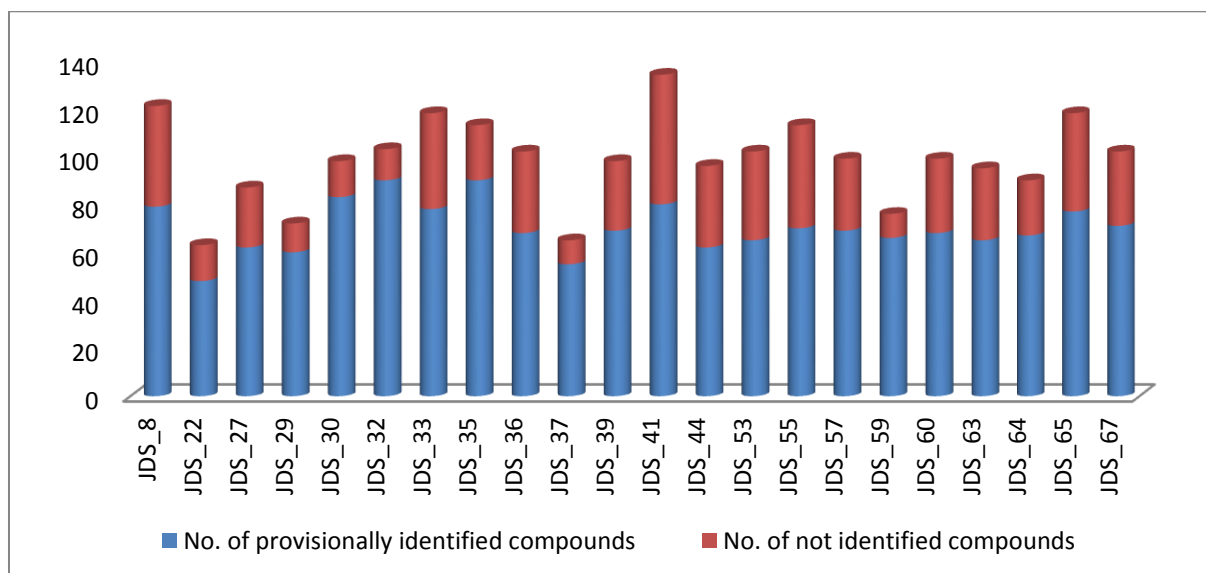


Figure 9: Number of compounds detected with LVI-GC-MS in the 22 JDS3 surface water samples obtained with the LVSPE sampling technique

Table 2 List of compounds provisionally identified in the surface water of the Danube River by the method liquid-liquid-LVI-GC/MS

Compound	CAS no.	Compound	CAS no.
1,4-Benzenediamine, N-(1-methylethyl)-N'-phenyl-	101-72-4	1-Decene	872-05-9
1,4-Cyclohexanedione, 2,2,6-trimethyl-	20547-99-3	Fluoranthene	206-44-0
1-Decanamine, N,N-dimethyl-	1120-24-7	Fluorene	86-73-7
1-Decene	872-05-9	Fluorene, X-methyl-	n/a
1-Docosanol	661-19-8	Galaxolide X	n/a
1-Dodecanamine, N,N-dimethyl-	112-18-5	Heneicosane	629-94-7
1-Hexadecanol	36653-52-4	Heptacosane	593-49-7
1-Hexanol-2-ethyl-	104-76-7	Heptadecane	629-78-7
1H-Indene, 1,3-dimethyl-	2177-48-2	Heptane, 1-(1-butenyloxy)-, (E)-	56052-80-3
1-H-Indene, X,X-dimethyl	n/a	Heptane, 3-[(ethenyloxy)methyl]-	103-44-6
1-H-Indene, X-methyl-	n/a	Heptanoic acid	111-14-8
1-Chloroundecane	2473-03-2	Heptasiloxane, hexadecamethyl-	541-01-5
1-Monolinoleoylglycerol trimethylsilyl ether	54284-45-6	Hexacosane	630-01-3
1-Nonene	124-11-8	Hexadecane, 1-bromo-	112-82-3
1-Octadecanol	112-92-5	Hexadecane, 2,6,10,14-tetramethyl-	638-36-8
1-Tetradecanamine, N,N-dimethyl-	112-75-4	Hexadecanoic acid, butylester-	111-06-8
1-Tetradecene	1120-36-1	Hexadecanoic acid, methylester-	112-39-0
1-Undecene	821-95-4	Hexadecene	n/a
2(3H)-Benzothiazolone	934-34-9	Hexachlorobenzene	118-74-1
2,2-Bis(p-acetoxypheyl)propane	192-62-8	Hexane, 1,6-diisocyanato-	822-06-0
2,3-dichlorophenyl)-1 H-imidazole-2-thiol	282730-10-3	Hexanoic acid	142-62-1
2,4-Imidazolidinedione, 3-methyl-	6843-45-4	Hexanoic acid, 2-ethyl-	149-57-5
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	719-22-2	Hexasiloxane, tetradecamethyl-	107-52-8
2,5-Diethylphenol	876-20-0	Hexa-X,X-diethylbenzene	n/a
2,6,6-Trimethyl-2-cyclohexene-1,4-dione	1125-21-9	Ibuprofen	15687-27-1
2,6,6-Trimethyl-4-hydroxy-1-cyclohexene-1-carboxaldehyde	1125-29-9	Iminostilbene	256-96-2
2,6-Dimethylphenyl isocyanate	28556-81-2	Indane	496-11-7
2-Ethyl-1-H-indene	17059-50-6	Indene	95-13-6
2H-1-Benzopyrane-2-one	91-64-5	i-Propyl 14-methyl-pentadecanoate	100033-66-2
2-Methylindene	2177-47-1	Isocurcumenol	n/a
2-n-Octylfuran	4179-38-8	Limonene	138-86-3
2-Pentenoic acid	626-98-2	Methanol, (1-amino-2-benzimidazolyl)-	156576-15-7
2-Propenenitrile, 3,3-diphenyl-	3531-24-6	Methaqualone metabolite II (hypnotic)	n/a
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	5466-77-3	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	n/a
3,4-dichloro-6-(2,4-dihydroxyphenyl)pyridazine	129287-29-2	Methyl dihydrojasmonate	24851-98-7

3-Dodecene, (E)-	7239-23-8	N,N,N,N-Tetraacetylenediamine	10543-57-4
3-Hexadecene, (Z)-	34303-81-6	Naphthalene	91-20-3
3-Isopropyl-2-methoxy-5methylbenzoic acid	n/a	Naphthalene, 1,2,3,4-tetrahydro-5-methoxy-	1008-19-1
4,7-Methano-5H-inden-5-one, 3,3a,4,6,7,7a-hexahydro-	14888-58-5	Naphthalene, 1,2-dimethyl-	573-98-8
4-Ethylbenzoic acid	619-64-7	Naphthalene, 1-methyl-	90-12-0
4-Isopropylphenylisocyanate	1000314-42-7	Naphthalene, 2,6-dimethyl-	581-42-0
4-Piperidinone, 2,2,6,6-tetramethyl-	826-36-8	Naphthalene, 2,7-dimethyl-	582-16-1
4-Propoxybenzaldehyde	5736-85-6	Naphthalene, 2-ethenyl-	827-54-3
4-tert-Octylphenol	140-66-9	Naphthalene, 2-methyl-	91-57-6
5-Isopropyl-3,3-dimethyl-2-methylene-2,3-dihydrofuran	81250-44-4	Naphthalene, X,X-dimethyl-	n/a
5-Methyl-2,4-diisopropylphenol	40625-96-5	Naphthalene, X-ethenyl-	n/a
5-Octadecene, (E)-	7206-21-5	n-Decanoic acid	334-48-5
5-Tetradecene	41446-66-6	n-Hexadecanoic acid	57-10-3
7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione	82304-66-3	Nonacosane	630-03-5
7-Hexadecene, (Z)-	35507-09-6	Nonanal	124-19-6
9H-Fluorene, X-methyl-	n/a	Nonanoic acid	112-05-0
Acenaphthene	83-32-9	Octacosane	630-02-4
Acenaphthylene	208-96-8	Octadecane	593-45-3
Acetamide, diethylamino-N-(1-phenylethyl)-	77882-83-8	Octadecane, 2-methyl-	1560-88-9
Acetophenone	98-86-2	Octadecanoic acid	57-11-4
Acetophenone, 4'-hydroxy-	99-93-4	Octadecanoic acid, butyl ester	123-95-5
Aniline	62-53-3	Octane	111-65-9
Anthracene	120-12-7	Octane, 1,1-oxybis-	629-82-3
Azulene	275-51-4	Octanoic acid	124-07-2
Benzaldehyde	100-52-7	Octanoic acid, hexadecyl ester	42231-43-6
Benzaldehyde, 3-hydroxy-4-methoxy-	621-59-0	octyl-diphenylamine	n/a
Benzamide, N,N-diethyl-4-methyl-	2728-05-4	Oxime, methoxy-phenyl	n/a
Benzenamine, 4-octyl-N-(4-octylphenyl)-	101-67-7	Pent-1-yn-3-ene, 4-methyl-3-phenyl-	65050-80-8
Benzenamine, N,N-diethyl-	91-66-7	Pentacosane	629-99-2
Benzenamine, N,N-dimethyl-	121-69-7	Pentadecane	629-62-9
Benzene (X-methyl-X-propenyl)-	n/a	Pentadecane, 2,6,10,14-tetramethyl-	1921-70-6
Benzene, (1-methyl-2-cyclopropen-1-yl)-	65051-83-4	Pentachlorobenzene	608-93-5
Benzene, 1-(1,1-dimethylethyl)-X-ethyl	n/a	Pentanoic acid	109-52-4
Benzene, 1,1'-sulfonylbis[4-chloro-	80-07-9	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutylester	100014-07-5
Benzene, 1,4-bis(1-methylethyl)-	100-18-5	Pentasiloxane, dodecamethyl-	141-63-9
Benzene, 1-ethenyl-3-methyl-	100-80-1	p-Ethyldiphenylmethane	620-85-9
Benzene, 2,4-diisocyanato-1-methyl-	584-84-9	Phenanthrene	85-01-8
Benzene, cyclopropyl-	873-49-4	Phenol	108-95-2
Benzene, isocyanato-	103-71-9	Phenol, 2,4-bis(1,1-dimethylethyl)-	96-76-4
Benzene, pentyl-	538-68-1	Phenol, 2,6-bis(1,1-dimethylethyl)-	128-39-2
Benzene, X-butenyl-	n/a	Phenol, 2,6-dichloro-	87-65-0
Benzene, X-ethenyl-X-methyl	n/a	Phenol, 2,6-dimethoxy-	91-10-1
Benzene, X-chloro-X-isocyanato-	n/a	Phenol, 3-ethyl-	620-17-7
Benzene, X-methyl-X-(X-methylethenyl)-	n/a	Phenol, 3-methyl-	108-39-4
Benzenecetonitrile	140-29-4	Phenol, 4-methoxy-	150-76-5
Benzenemethanamine, N,N-dimethyl-	103-83-3	Phenol, 4-methoxy-3-(methoxymethyl)-	59907-65-2
Benzenemethanol, alpha-methyl-	1517-69-7	Phenol, 4-methyl-	106-44-5
Benzenepropanal	104-53-0	Phenol, m-tert-butyl-	585-34-2
Benzenepropanenitrile, .beta.-phenyl-	2286-54-6	Phenol, p-tert-butyl-	98-54-4
Benzenesulfonamide	98-10-2	Phenol, X-(1,1-dimethylethyl)-X-methyl-	n/a
Benzoic acid	65-85-0	Phenol, X-(1-methylethyl)-	n/a
Benzoic acid, 2-(hydroxymethyl)-	612-20-4	Phenol, X-ethyl-Xmethyl-	n/a
Benzoic acid, 4-ethoxy, ethyl ester	23676-09-7	Phenol, X-octyl-	n/a
Benzoic acid, p-tert-butyl-	98-73-7	Phenol, x-tert-butyl-	n/a
Benzoic acid, X-methoxy-	n/a	Phthalic anhydride	85-44-9
Benzonitrile, 2-hydroxy-	611-20-1	Phthalimide	85-41-6
Benzophenone	119-61-9	Phytol	n/a
Benzothiazole	95-16-9	Piperonal	120-57-0
Bibenzyl	103-29-7	p-Isopropenylphenol	4286-23-1
Bicyclo(4.1.0)heptane, 3-methyl-7-pentyl-	41977-48-4	Propanoic acid, 2-methyl-, 3-(4-t-butyl)phenyl-	100013-18-7
Biphenylene	259-79-0	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	27367-34-3
Bisphenol A	80-05-7	Pyrene	129-00-0
Butenoic acid	n/a	Pyridine, 2-(methylthio)-	18438-38-5
Butylated Hydroxytoluene	128-37-0	Quinoline	91-22-5
Butyl-octyl-Phthalate	84-78-6	Quinoline, 1,2-dihydro-2,2,4-trimethyl-	147-47-7
Caffeine	58-08-2	Quinoline, X-methyl	n/a
Camphenol	3570-04-5	Silane, diethoxydimethyl-	78-62-6
Caprolactam	105-60-2	Silane, ethenyl dimethoxymethyl-	16134-56-8
Cotinine	486-56-6	Silicone grease, Siliconfett	n/a
Cycloheptasiloxane, tetradecamethyl-	107-50-6	Some chlorinated benzene	n/a
Cyclohexane, isocyanato-	3173-53-3	Styrene	100-42-5
Cyclohexane, isothiocyano-	1122-82-3	Tetracosane	646-31-1
Cyclohexane, X-isocyanato-X-(isocyanatomethyl)-X,X,X-	n/a	Tetradecane	629-59-4
Cyclohexanone, 4-(1,1-dimethylethyl)-	98-53-3	Tetradecane, 1-bromo-	112-71-0
Cyclohexasiloxane, dodecamethyl-	540-97-6	Tetradecanoic acid	544-63-8
Cyclohexene, 1-chloro-4-(1-chloroethenyl)-	13547-06-3	Tetraglyme	143-24-8
Cycloocta1,3,6-triene, 2,3,5,5,8,8-hexamethyl-	n/a	Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]-	n/a
Cyclopentasiloxane, decamethyl-	541-06-2	Tetrachloroethylene	127-18-4
Cyclopropane, 1-ethyl-2-heptyl-	74663-86-8	Toluene	108-88-3
Cyclotetradecane	295-17-0	Tri(2-chloroethyl) phosphate	115-96-8

Cyclotetrasiloxane, octamethyl-	556-67-2	Triacetin	102-76-1
Cyclotrisiloxane, hexamethyl-	541-05-9	Tributyl acetylcitrate	77-90-7
Decane	124-18-5	Tributyl phosphate	126-73-8
Decanoic acid	334-48-5	Tributylamine	102-82-9
DEHP	117-81-7	Tricosane	638-67-5
Dibutylphthalate	84-74-2	Tridecane	629-50-5
Diethylphthalate	84-66-2	Triethylamine	121-44-8
Diisobutyl phthalate	84-69-5	Triphenylphosphate	115-86-6
Diisopropylnaphthalene	38640-62-9	Undecane	1120-21-4
Dimethyl phthalate	131-11-3	Vanillin	121-33-5
Dimethyl sulfone	67-71-0	X- Dodecene	n/a
Diphenyl sulfide	139-66-2	X Eicosyne	n/a
Dipropylene glycol monomethyl ether	n/a	X,X-Benzenediol, X,X-bis(1,1-dimethylethyl)-	n/a
Docosane	629-97-0	X,X-Diisopropylnaphthalene	n/a
Dodecane	112-40-3	X-Butenoic acid	n/a
Dodecanoic acid	143-07-7	X-Decenol	n/a
Drometrizole	2440-22-4	X-Ethylbenzophenone	n/a
E-15-Heptadecenal	1000130-97-9	X-Fluorene	n/a
Eicosane	112-95-8	X-Hexadecanol	n/a
Eicosanol	n/a	X-Hexadecene	n/a
Ethanol, 2-phenoxy-	122-99-6	X-Hydroxybiphenyl	n/a
Ethanone, 1-(3,4-dimethoxyphenyl)-	1131-62-0	X-Methylstyrene	n/a
Ethanone, 1-(4-methylphenyl)-	122-00-9	X-n-butyl-1H-Indene	n/a
Ethanone, 1,1'-(1,4-phenylene)bis-	1009-61-6	X-Nonenal	n/a
Ethanone, 1-[4-(1-hydroxy-1-methylethyl)phenyl]-	54549-72-3	X-Nonene	2216-38-8
Ethanone, X-(X-aminophenyl)-	n/a	X-Octadecenoic acid	n/a
Ethaqualone	7432-25-9	X-Pentanol, X-methyl-	n/a
Ethylmethylmaleimide	20189-42-8	X-Phenylene	n/a
Ethylparaben	120-47-8	X-Phenoxypropan-X-ol	n/a
Fenam	957-51-7	X-Tetradecanol	n/a
1,4-Benzenediamine, N-(1-methylethyl)-N'-phenyl-	101-72-4	X-Tetradecene	n/a
1,4-Cyclohexanedione, 2,2,6-trimethyl-	20547-99-3	X-Tridecene	n/a
1-Decanamine, N,N-dimethyl-	1120-24-7	X-Undecene	n/a

Table 3 List of compounds provisionally identified in the surface water of the Danube River by the method LVSPE-LVI-GC/MS

Compound	CAS no.	Compound	CAS no.
1,3-Cyclohexadiene, 1,2,6,6-tetramethyl-	514-96-5	Benzoic acid, 2,4-dichloro-, methyl ester	35112-28-8
1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone	7226-23-5	Benzoic acid, 2-chloro-	118-91-2
1-Eicosanol	629-96-9	Benzoic acid, 3,4-dimethyl-	619-04-5
1H-Pyrazole, 4,5-dihydro-5,5-dimethyl-4-isopropylidene-	106251-09-6	Benzoic acid, 4-acetyl-, methyl ester	3609-53-8
1H-Pyrazole, 4-ethyl-3,5-dimethyl-	7554-67-8	Benzoic acid, 4-methyl-	99-94-5
2,6,6-Trimethyl-2-cyclohexene-1,4-dione	1125-21-9	Benzonitrile, 2-hydroxy-	611-20-1
2,6-Dimethylphenyl isocyanate	28556-81-2	Benzophenone	119-61-9
2-Cyclohexen-1-one, 3,6-dimethyl-6-(1-methylethyl)-	54410-58-1	Benzothiazole	95-16-9
2-Cyclopenten-1-one, 2-methyl-	1120-73-6	Benzothiazole, 2-(methylthio)-	615-22-5
5-Azabenzimidazole	272-97-9	Benzyl alcohol	100-51-6
5-Eicosene, (E)-	74685-30-6	Benzyl chloride	100-44-7
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	82304-66-3	BHT	128-37-0
Aniline	62-53-3	Bicyclo[3.2.1]octane	6221-55-2
Benzaldehyde, 2-methyl-	529-20-4	Bisphenol A	80-05-7
Benzene, 1-methoxy-4-(1-methylethyl)-	4132-48-3	Butanamide	541-35-5
Benzenecetic acid	103-82-2	Butanoic acid, 3-hydroxy-	300-85-6
Benzenepropanenitrile, β -phenyl-	2286-54-6	Caffeine	58-08-2
Bicyclo[4.2.0]octa-1,3,5-triene, 2,4-dimethyl-	28749-81-7	Camphenol	3570-04-5
Butanamide	541-35-5	Caprolactam	105-60-2
Cyclohexanamine, N-cyclohexyl-	101-83-7	Carbamazepine	298-46-4
Cyclotetradecane	295-17-0	Carbamic acid, phenyl-, phenyl ester	4930-03-4
Decane, 1-bromo-	112-29-8	Citronellyl formate	105-85-1
Docosane	629-97-0	Cotinine	486-56-6
Eicosane	112-95-8	Cyclododecane	294-62-2
Ethanol, 2-ethoxy-	110-80-5	Cyclohexanamine	108-91-8
Ethanone, 1-(2-hydroxy-5-methylphenyl)-	1450-72-2	Cyclohexane, isocyanato-	3173-53-3
Ethanone, 1-(2-methyl-1-cyclopenten-1-yl)-	3168-90-9	Cyclohexane, isothiocyanato-	1122-82-3
Ethanone, 1-(3-methylphenyl)-	585-74-0	Cyclohexanethiol	1569-69-3
Glycerin	56-81-5	Cyclohexanol, 3,5-dimethyl-	5441-52-1
Imidazo[4,5-d]imidazole-2,5-(1H,3H)dione, tetrahydro-1,3,4,6-tetramethyl-	10095-06-4	Cyclohexene, 1,5,5-trimethyl-3-methylene-	16609-28-2
Isophorone	78-59-1	Cyclopenta[c]pentalen-3(3aH)-one, octahydro-1,2,3a,6-	120052-69-9
Isopropyl phenyl ketone	611-70-1	DEHP	117-81-7
n-Decanoic acid	334-48-5	Dibutyl phthalate	84-74-2
N-Morpholinomethyl-isopropyl-sulfide	77422-34-5	Dicyclohexyldisulphide	2550-40-5
p-Cresol	106-44-5	Diethyl Phthalate	84-66-2
Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	119-47-1	Diethylpent-4-enylamine	13173-21-2
Phenol, 2,4,6-tris(1-methylethyl)-	2934-07-8	Diethyltoluamide (DEET)	134-62-3
p-tert-Butyl catechol	98-29-3	Diisobutyl phthalate	84-69-5
Pyridine, 2-(1-methyl-2-pyrrolidinyl)-	23950-04-1	Dimethyl phthalate	131-11-3
Quinazoline, 4-methyl-	700-46-9	Dimethyl sulfone	67-71-0
Quinoline, 2,4-dimethyl-	1198-37-4	Diphenylamine	122-39-4
Quinoline, 2-methyl-	91-63-4	Dodecane	112-40-3
Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-	61050-91-7	Dodecane, 1-bromo-	143-15-7
Tetraglyme	143-24-8	Drometrizole	2440-22-4
1(2H)-Naphthalenone, 7-(1,1-dimethylethyl)-3,4-dihydro-	22583-68-2	D-Verbenone	18309-32-5
1(3H)-Isobenzofuranone	87-41-2	Ethanol, 2-(2-butoxyethoxy)-	112-34-5
1,3,5-Triazine-2,4,6-triamine	108-78-1	Ethanol, 2,2'-oxybis-	111-46-6
1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	137-89-3	Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]-	143-22-6
1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	99-86-5	Ethanol, 2-phenoxy-	122-99-6
1,3-Cyclopentanedione, 2-methyl-	765-69-5	Ethanone, 1-(1a,2,3,5,6a,6b-hexahydro-3,3,6a-	80114-25-6
1,4-Benzenediamine, N-(1-methylethyl)-N'-phenyl-	101-72-4	Ethanone, 1-(1H-pyrrol-2-yl)-	1072-83-9
1,4-Cyclohexanedione	637-88-7	Ethanone, 1-(1-methyl-1H-pyrrol-2-yl)-	932-16-1
1,4-Cyclohexanedione, 2,2,6-trimethyl-	20547-99-3	Ethanone, 1-(2,5-dimethylphenyl)-	2142-73-6
1,6-Dioxacyclododecane-7,12-dione	777-95-7	Ethanone, 1-(2-hydroxyphenyl)-	118-93-4
1,7-Dimethylxanthine	611-59-6	Ethanone, 1-(3,4-dimethylphenyl)-	3637-01-2
1-Azabicyclo[2.2.2]octane, 4-methyl-	45651-41-0	Ethanone, 1-(4-ethylphenyl)-	937-30-4
1-Butanamine, N-butyl-	111-92-2	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	2478-38-8
1-Decanol, 2-hexyl-	2425-77-6	Ethanone, 1-(4-methylphenyl)-	122-00-9
1-Decene, 4-methyl-	13151-29-6	Ethanone, 1,1'-(1,4-phenylene)bis-	1009-61-6
1-Dodecanamine, N,N-dimethyl-	112-18-5	Ethanone, 1-[4-(1-hydroxy-1-methylethyl)phenyl]-	54549-72-3
1-Eicosanol	629-96-9	Ethanone, 1-[4-(1-methylethyl)phenyl]-	645-13-6
1H-Benzotriazole, 5-methyl-	136-85-6	Ethanone, 2-amino-1-phenyl-	613-89-8
1-Hexadecanol	36653-82-4	Ethene, (2-ethoxy-1-methoxyethoxy)-	54063-18-2
1-Hexanol, 2-ethyl-	104-76-7	Ethosuximide	77-67-8
1H-Inden-1-ol, 2,3-dihydro-3,3-dimethyl-	38393-92-9	Ethylmethylmaleimide	20189-42-8
1H-Inden-1-one, 2,3-dihydro-3,3-dimethyl-	26465-81-6	Fluoranthene	206-44-0
1H-Indole-3-acetic acid, methyl ester	1912-33-0	Fluorene	86-73-7
1H-Pyrrole, 2,3-dimethyl-	600-28-2	Gabapentin	60142-96-3
1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	20189-42-8	Glycerol 1-palmitate	542-44-9
1-Chloroundecane	2473-03-2	Heptadecanoic acid, 16-methyl-, methyl ester	5129-61-3
1-Octanamine, n-octyl-	1120-48-5	Heptane, 1-(1-butenyloxy)-	56052-80-3

1-Phenyl-1-butene	824-90-8	Heptane, 3-[(ethenyloxy)methyl]-	103-44-6
1-Piperazinecarboxaldehyde	7755-92-2	Hexadecane	544-76-3
1-Propanol, 3,3'-oxybis-	2396-61-4	Hexadecane, 1-bromo-	112-82-3
2(3H)-Benzothiazolone	934-34-9	Hexadecanoic acid, methyl ester	112-39-0
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (±)-	79-50-5	Hexachlorobenzene	118-74-1
2,5-Diethylphenol	876-20-0	Hexanoic acid, 2-ethyl-	149-57-5
2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	14035-34-8	Hexanoic acid, 3,5,5-trimethyl-	3302-10-1
2-Chlorobenzoic acid, 2-ethylhexyl ester	59986-39-9	Ibuprofen	15687-27-1
2-Indolinone, 1-methyl-	61-70-1	Iminostilbene	256-96-2
2-Propanone, 1,1-diphenyl-	781-35-1	Indole	120-72-9
2-Propen-1-one, 1-phenyl-	768-03-6	Levomenthol	2216-51-5
2-Propenenitrile, 3,3-diphenyl-	3531-24-6	Methyl stearate	112-61-8
2-Propenoic acid, 2-methyl-, 1,2-ethanediylbis(oxy-2,1-	109-16-0	Methyl tetradecanoate	124-10-7
2-Pyrolidinone, 3-hydroxy-1-methyl-5-(3-pyridinyl)-	34834-67-8	Methylvinylmaleimide	21494-57-5
3,4-Dichloropropiophenone	6582-42-9	N,N,N',N'-Tetraacetylenediamine	10543-57-4
3,5-di-tert-Butyl-4-hydroxyacetophenone	14035-33-7	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	119-47-1
3,5-di-tert-Butyl-4-hydroxyphenylpropionic acid	20170-32-5	Naphthalene	91-20-3
3,6,9,12-Tetraoxahexadecan-1-ol	1559-34-8	N-Benzyl-2-phenethylamine	3647-71-0
3-Ethylbenzophenone	66067-43-4	n-Decanoic acid	334-48-5
3-Hexadecene, (Z)-	34303-81-6	n-Hexadecanoic acid	57-10-3
3-Hexanone, 2,4-dimethyl-	18641-70-8	Nicotine	54-11-5
3-Hydroxydiphenylamine	101-18-8	Nonane	111-84-2
4,4'-(Hexafluoroisopropylidene)diphenol	1478-61-1	Nonanoic acid	112-05-0
4-Acetylbenzoic acid	586-89-0	Octadecane	593-45-3
4-Ethylbenzoic acid	619-64-7	Octadecane, 2-methyl-	1560-88-9
4-Methyl-5H-furan-2-one	6124-79-4	Octadecanoic acid	57-11-4
4-Piperidinone, 2,2,6,6-tetramethyl-	826-36-8	o-Hydroxybiphenyl	90-43-7
4-Propoxybenzaldehyde	5736-85-6	p-Cresol	106-44-5
5-Acetyl-2-methylpyridine	42972-46-3	Pentadecane, 1-bromo-	629-72-1
5-Azabenzimidazole	272-97-9	Pentachlorobenzene	608-93-5
5-Hexene-1-ol, acetate	5048-26-0	Pentanamide	626-97-1
5-Tetradecene	41446-66-6	Phenol	108-95-2
6-tert-Butyl-2,4-dimethylphenol	1879-09-0	Phenol, 2,4-bis(1,1-dimethylethyl)-	96-76-4
7-Hexadecene, (Z)-	35507-09-6	Phenol, 2,5-dichloro-	583-78-8
7-Methoxy-2,5-dimethyl-1,2,3,4-tetrahydropyrimido[3,4-a]-indole	30689-25-9	Phenol, 2,6-dichloro-	87-65-0
8-Pentadecanone	818-23-5	Phenol, 2,6-dimethoxy-	91-10-1
Acenaphthylene	208-96-8	Phenol, 2-ethyl-	90-00-6
Acetamide, N-(4-aminophenyl)-N-methyl-	119-63-1	Phenol, 2-methoxy-	90-05-1
Acetamide, N-acetyl-N,N'-1,2-ethanediylbis	137706-80-0	Phenol, 3,4,5-trimethyl-	527-54-8
Acetic acid, (benzoylamino)hydroxy-	16555-77-4	Phenol, 3-ethyl-	620-17-7
Acetophenone	98-86-2	Phenol, 3-methyl-	108-39-4
Acetophenone, 4'-hydroxy-	99-93-4	Phenol, 4-(ethylamino)-	659-34-7
Acridine, 9-methyl-	611-64-3	Phenol, 4-(methylthio)-	1073-72-9
Anthracene	120-12-7	Phenol, 4-(phenylamino)-	122-37-2
Atrazine	1912-24-9	Phenol, 4-amino-2,5-dimethyl-	3096-71-7
Atropic acid	492-38-6	Phenol, 4-methoxy-	150-76-5
Benzaldehyde	100-52-7	Phenol, 4-methoxy-3-(methoxymethyl)-	59907-65-2
Benzamide	55-21-0	Phenol, 5-methoxy-2,3-dimethyl-	34883-01-7
Benzenamine, 2-methoxy-	90-04-0	Phthalimide	85-41-6
Benzene, (2-methoxyethyl)-	3558-60-9	Piperazine	110-85-0
Benzene, [(2-propenyloxy)methyl]-	14593-43-2	p-Isopropenylphenol	4286-23-1
Benzene, 1,2,3,5-tetramethyl-	527-53-7	p-Toluenesulfonamide	70-55-3
Benzene, 1,2,4,5-tetramethyl-	95-93-2	p-Xylene	106-42-3
Benzene, 1,2,4-trimethyl-	95-63-6	Pyrene	129-00-0
Benzene, 1,3-bis(1-methylethenyl)-	3748-13-8	Pyridine, 2-(methylthio)-	18438-38-5
Benzene, 1,3-dichloro-	541-73-1	Pyridine, 3-methoxy-	7295-76-3
Benzene, 1-ethenyl-4-methoxy-	637-69-4	Quinoline, 1,2-dihydro-2,2,4-trimethyl-	147-47-7
Benzene, 1-ethyl-3,5-dimethyl-	934-74-7	Quinoline, 2,3-dimethyl-	1721-89-7
Benzene, 1-chloro-3-isocyanato-	2909-38-8	Silane, diethoxydimethyl-	78-62-6
Benzene, 4-ethenyl-1,2-dimethyl-	27831-13-6	Spiroxamine	118134-30-8
Benzene, isocyanato-	103-71-9	Surfynol 104	126-86-3
Benzeneacetic acid	103-82-2	Terbutylazine	5915-41-3
Benzeneacetic acid, 2-hydroxy-, methyl ester	22446-37-3	Tetradecane	629-59-4
Benzenemethanamine, N,N-dimethyl-	103-83-3	Tetradecane, 1-bromo-	112-71-0
Benzenepropanenitrile, β-phenyl-	2286-54-6	Tetradecanoic acid	544-63-8
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-,	6386-38-5	Thiazolidine, 3-methyl-	52288-89-8
Benzenesulfonamide	98-10-2	Tributyl phosphate	126-73-8
Benzenesulfonamide, N-butyl-	3622-84-2	Triclosan	3380-34-5
Benzhydryl isothiocyanate	3550-21-8	Tridecane, 1-bromo-	765-09-3
Benzofuran, 2,3-dihydro-	496-16-2	Triethyl citrate	77-93-0
Benzoic acid	65-85-0	Triphenylphosphine oxide	791-28-6
Benzoic acid, 2-(hydroxymethyl)-	612-20-4	Tris(2-chloroethyl) phosphate - TCEP	115-96-8
Benzoic acid, 2,4-dichloro-	50-84-0	Tris(chloro-2-propyl) phosphate - TCPP	13674-84-5

The observed pollution is generally matching with the results of previous surveys (JDS1 and 2). Phthalates, alkylated polyaromatic hydrocarbons, alkylated phenols, alkanes and fatty acids belong to the most ubiquitous compounds detected (see Table 4).

Table 4 List of twenty most frequently detected compounds provisionally identified in the surface water of the Danube river by the LVSPE/LVI-GC-MS and LLE/LVI-GC-MS methods

LVSPE/LVI-GC-MS		LLE/LVI-GC-MS	
Compound	Frequency of identification	Compound	Frequency of identification
DEHP	22/22	Dibutyl phthalate	42/68
Benzoic acid	22/22	Diethyl phthalate	41/68
Triphenylphosphine oxide	22/22	Naphthalene, X-methyl- (isomer)	39/68
Phenol, 2,4-bis(1,1-dimethylethyl)-	22/22	1-H-Indene, X-methyl (isomer)	36/68
Diethyl phthalate	22/22	1-H-Indene, X,X-dimethyl (isomer)	36/68
Acetophenone	21/22	X,X-Diisopropyl-naphthalene	35/68
Caffeine	21/22	Indene	33/68
Metilox	21/22	1-Tetradecene	33/68
Ethanone, 1-[4-(1-methylethyl)phenyl]-	21/22	x-Xylene (isomer)	28/68
Diisobutyl phthalate	21/22	Caprolactam	28/68
Dibutyl phthalate	21/22	Ketosisophorone	28/68
Phthalimide	21/22	Caffeine	28/68
Cyclohexane, isocyanato-	20/22	Toluene	27/68
Ethanone, 1,1'-(1,4-phenylene)bis-	20/22	Phenol	27/68
Heptane, 3-[(ethenyl)oxy]methyl-	20/22	Hexanoic acid	27/68
Caprolactam	20/22	Aniline	27/68
Heptane, 1-(1-butenyloxy)-	20/22	Phenol, x-methyl (isomer)	25/68
Phenol	19/22	Naphthalene, X,X-dimethyl- (isomer)	25/68
1,4-Benzenediamine, N-(1-methylethyl)-N'-phenyl-	18/22	Hexadecanoic acid, methylester-	24/68
Cyclohexane, isothiocyanato-	18/22	Linear alkyl benzene (LAB; isomer)	22/68

Liquid-liquid-LVI-GC/MS

The characteristic pattern of pollution based on the obtained spectral information of identified compounds showed the presence of non-polar and semi-polar organic compounds. Substantial part of the identified substances were various derivatives of alkanes, alkenes, alkynes, esters, aldehydes, ketones, siloxanes, aromates and phthalates.

A significant presence of personal care products, compounds of daily used, indicators of wastewater pollution or poor efficiency of wastewater treatment plants, phosphorus flame retardants (PFRs), herbicide, was identified in samples. Among the detected compounds were:

- *Sun-screen agents*: 4-ethylbenzophenone, acetophenone and benzophenone;
- *Fragrances and musks*: limonene, vanillin, isobornyl acetate, dihydro methyl jasmonate, galaxolide and ketosisophorone;
- *Herbicide*: fenam;
- *Food additive*: triacetin;
- *PFRs*: triphenylphosphate, tri(2-chloroethyl) phosphate, tributyl phosphate;
- *Other cosmetic ingredients*: glycols, tributyl acetylcitrate, linear alkyl benzenes (LABs) and ethylparaben.

Other relevant information provided by screening was semi-quantification. Roughly estimated concentrations of detected compounds used either to compare these values with derived PNEC values for that substance or as starting values in the process of prioritization, in case of unidentified compounds. Existing PNEC values were compared with roughly estimated concentrations and in these

cases values were exceeded: *alkanes* - hexadecane at 22 ng/L (PNEC = 8,1 ng/L), heptadecane at 74 ng/L (PNEC = 1,0721 ng/L), nonadecane at 20 ng/L (PNEC = 0,12485 ng/L), eicosane at 187 ng/L (PNEC = 0,05063 ng/L), docosane at 103 ng/L (PNEC = 0,00826 ng/L); *phthalates* - dibutylphthalate at 3020 ng/L (PNEC = 600 ng/L); *endocrine disrupting compounds* - bisphenol A at 890 ng/L (PNEC = 200 ng/L); *alkaloids* - caffeine at 1464 ng/L (PNEC = 100 ng/L); *volatile organic compounds* - styren at 377 ng/L (PNEC = 1,2 ng/L); *PAHs* - acenaphthene at 109 ng/L (PNEC = 100 ng/L); *organic acids* - n-hexadecanoic acid at 338 ng/L (PNEC = 9,6223 ng/L); *amines* - 1-dodecanamine, N,N-dimethyl- at 694 ng/L (PNEC = 27,604 ng/L) and *phosphates* - triphenylphosphate at 252 ng/L (PNEC = 30 ng/L).

LVSPE-LVI-GC/MS

Usage of three types of sorbents resulted in the extension of polarity range of detected compounds containing N-, S-, O, Cl-, Br atoms. Identified compounds can be divided into several groups.

Phthalates - occurrence profile the most frequently detected ones is given in Figure 10. Diethyl phthalate, diisobutyl phthalate, dibutyl phthalate and DEHP were presented in all 22 samples. The highest estimated concentration reached diethyl phthalate at Reni (132 km, 665 ng/L). The highest estimated concentration of DEHP as WFD PS were observed in samples from Downstream Novi-Sad (1252 km) at 254 ng/L and Velika Morava (1103 km) at 240 ng/L.

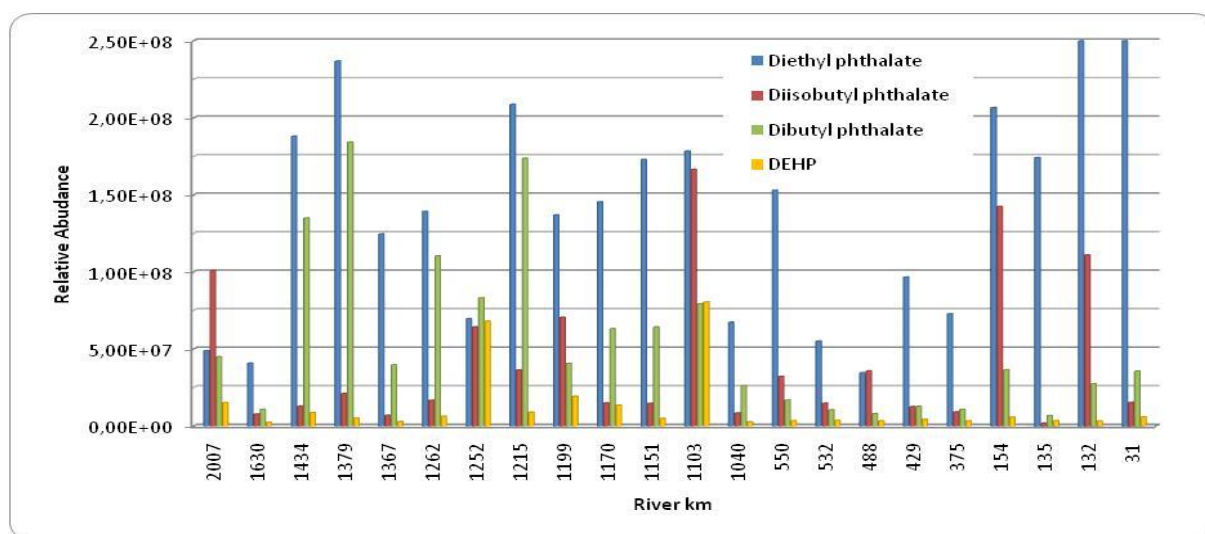


Figure 10: Occurrence profile of phthalates in the JDS3 surface water samples

Substances of daily use - occurrence profile the most frequently detected ones is given in Figure 11. *Sun-screen agents*: acetophenone, benzophenone and drometrizole were found in samples. Acetophenone was 21 times positively identified.

N,N-Diethyl-m-toluamide, abbreviated DEET, is the most common active ingredient in *insect repellents*. The highest presence was registered in samples from Tisa (rkm 1.0) (1215 km) at 48 ng/L. Sampling point Tisa (rkm 1.0) (1215 km) contained also the second highest concentration of ketoisophorone belongs to category *flavour and fragrance agent*, and benzoic acid that signal was reduced 10 times for better illustration. Benzoic acid represents *food preservative agent* that was presented in all samples, the highest concentration was registered at sampling point Downstream Drava (657 ng/L). The sampling site Downstream Arges, Oltenita (429 km) was polluted with an

antibacterial and antifungal agent - triclosan (1.9 ng/L), which was identified using retrospective analysis.

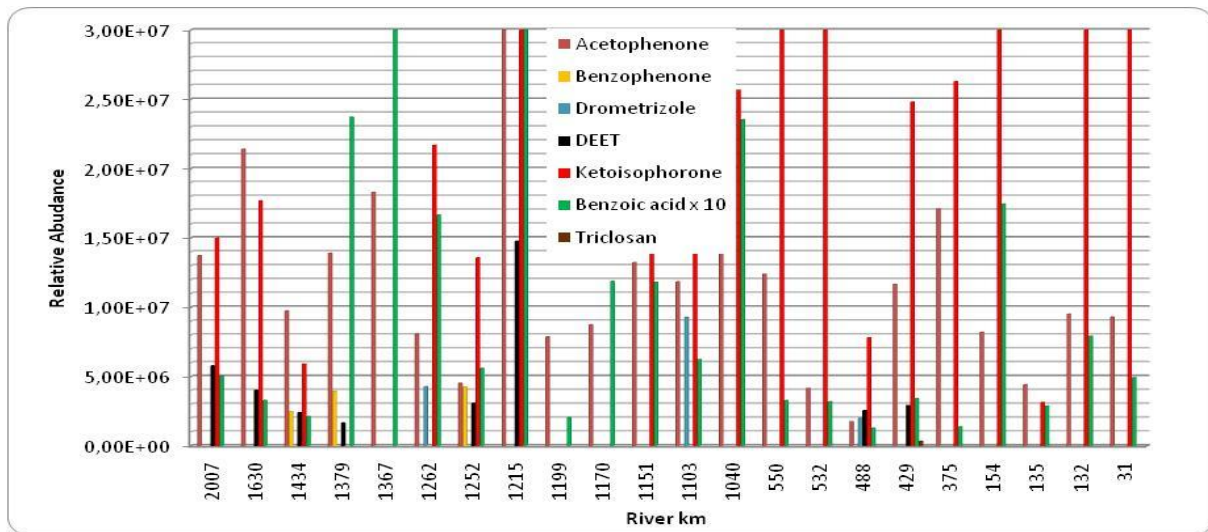


Figure 11: Occurrence profile of substances of daily use (sun-screen agents, repellent, fragrance agent and food preservative) in the JDS3 surface water samples (benzoic acid its response is 10 times reduced)

Phosphorus flame retardants (PFRs), which have already been used for over 150 years are considered as suitable alternatives for BFRs. Organophosphates are used for two reasons: the halogenated ones as FRs, while the non-halogenated ones are mostly used as plasticizers. triphenylphosphine oxide belongs to group of the non-halogenated organophosphates, was presented in all samples and its relative abundance was reduced 10 times for better illustration (the highest concentration at Downstream Zimnicea/Svishtov, 158 ng/L). The group of halogenated organophosphates was represented by tris(2-chloro-1-methylethyl) phosphate that was the most frequently identified in samples. The occurrence profile of all detected organophosphates is presented in Figure 12.

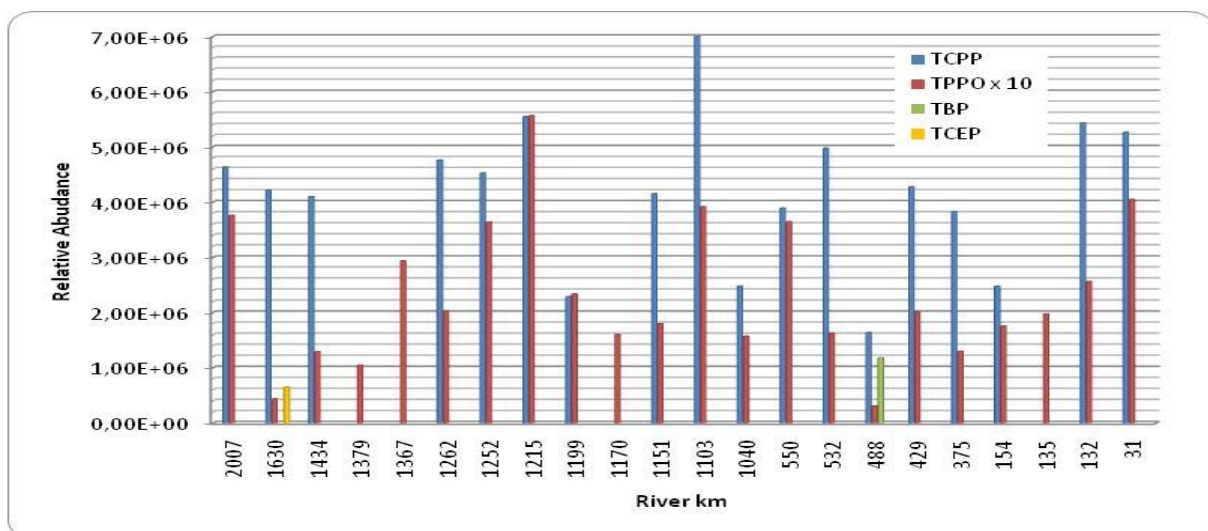


Figure 12: Occurrence profile of phosphorus flame retardants in the JDS3 surface water samples (TPPO its response is 10 times reduced)

Screening revealed two types of *pesticides*: (i) *herbicides* were represented by atrazine and terbuthylazine, and (ii) *fungicides* were represented by spiroxamine. Atrazine is on the list of WFD priority substances with its AA-EQS of 0.6 $\mu\text{g/l}$ was detected three times in samples from Downstream Arges, Oltenita (429 km) at 2.0 ng/L, Reni (132 km) at 2.2 ng/L and Sulina - Sulina arm (31 km) at 1.3 ng/L, respectively. Terbuthylazine was detected three times in samples from Oberloiben (2007 km) at 5.4 ng/L, Downstream Ruse/Giurgiu (488 km) at 1.8 ng/L and Downstream Arges, Oltenita (429 km) at 3.6 ng/L, respectively. The most frequently identified pesticides was spiroxamine and its the highest presence was registered in sampling site Hercegszanto (1434 km) at 86 ng/L. Remarkable was the presence of p toluenesulfonamide utilized as the starting material in the synthesis of biocide compound - chloramin T (see Figure 13).

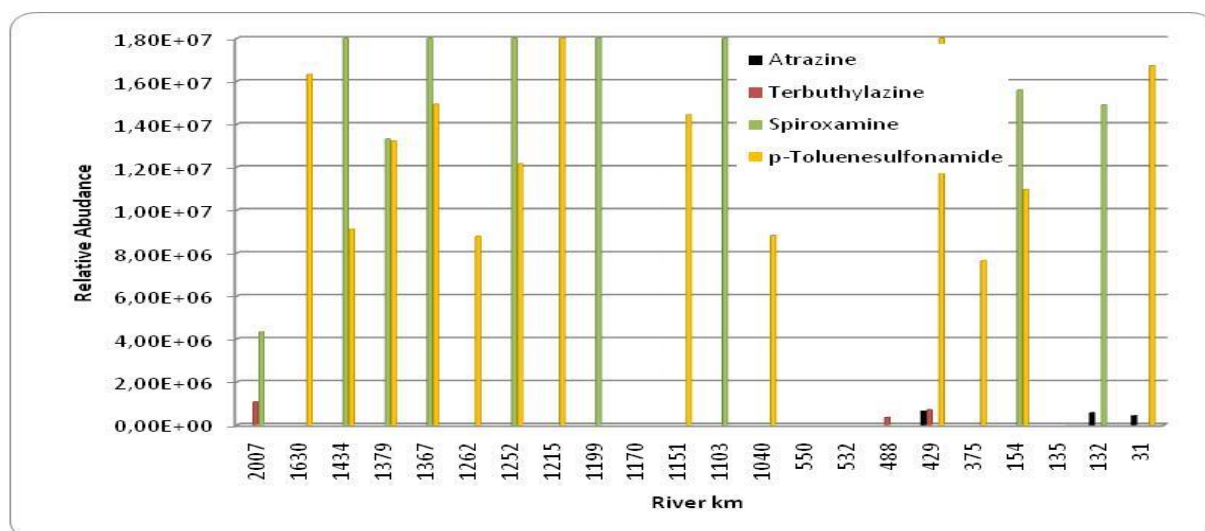


Figure 13: Occurrence profile of pesticides in the JDS3 surface water samples

Pharmaceuticals - three types of drugs were presented in LVSPE extracts (see Figure 14). The most frequently identified was gabapentin - an anticonvulsant and analgesic drug, with its the highest concentration in a sample from Budapest downstream - M0 bridge (1630 km) at 18 ng/L. Carbamazepine - an anticonvulsant and mood-stabilizing drug, was detected five times with the highest abundance in sample from Velika Morava (1103 km) at 2.2 ng/L. Ibuprofen is a nonsteroidal anti-inflammatory drug, was presented in two samples from Velika Morava (1103 km) at 7.8 ng/L and Downstream Arges, Oltenita (429 km) at 6.3 ng/L, respectively.

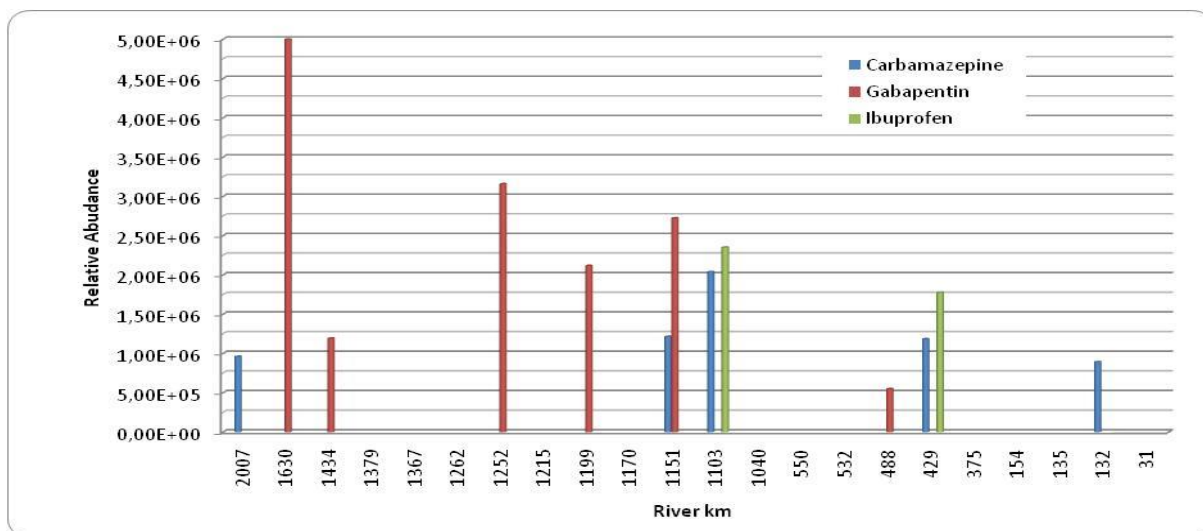


Figure 14: Occurrence profile of pharmaceuticals in the JDS3 surface water samples

Alkaloids - a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms were represented by four most frequently with found compounds namely caffeine, xanthine structurally related to caffeine, nicotine, cotinine found in tobacco and is also a metabolite of nicotine. The occurrence profiles of identified alkaloids shows that the samples from Velika Morava (1103 km) and Downstream Arges, Oltenita (429 km) contained all four ones (see Figure 15). Caffeine was identified in 21 of 22 samples.

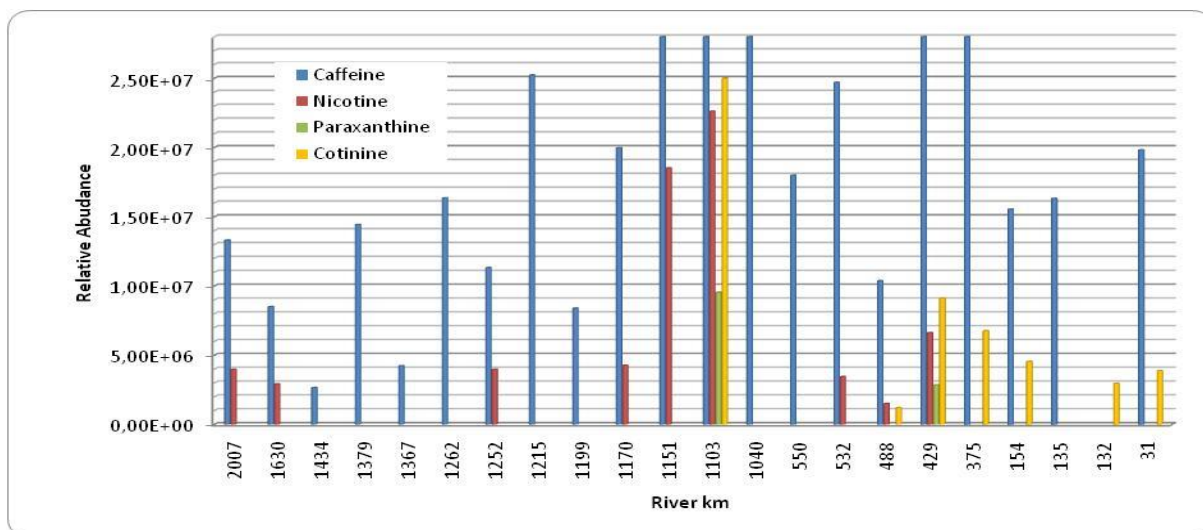


Figure 15: Occurrence profile of detected alkaloids in the JDS3 surface water samples

Screening basically offers two groups of detected compounds, one that after application of special software tools contains compounds with proposed identification and second, that even after application of special software tools contains compounds remaining unknown. Occurrence profile depicted in Figure 16 is labelled as *synthetic compounds* because it deals with compounds that are difficult to classify. Among the many compounds that have been identified such as various substituted ethers, alcohols, esters, amines, amides, glycerols, tiols, aldehydes and ketones must be pointed out at least two of them. Caprolactam is the precursor to Nylon 6 with an approximate annual production 4.5

billion kilograms and it was detected in 20 samples and its abundance was reduced 10 times for better illustration. Another interesting compounds is Metilox (Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy -, methyl ester) determined in 21 samples. The sampling point with the highest concentration of caprolactam was Tisa (rkm 1.0) (1215 km) at 561 ng/L and Metilox was presented at the highest concentration (47 ng/L) in sample from Siret (rkm 1.0) (154 rkm).

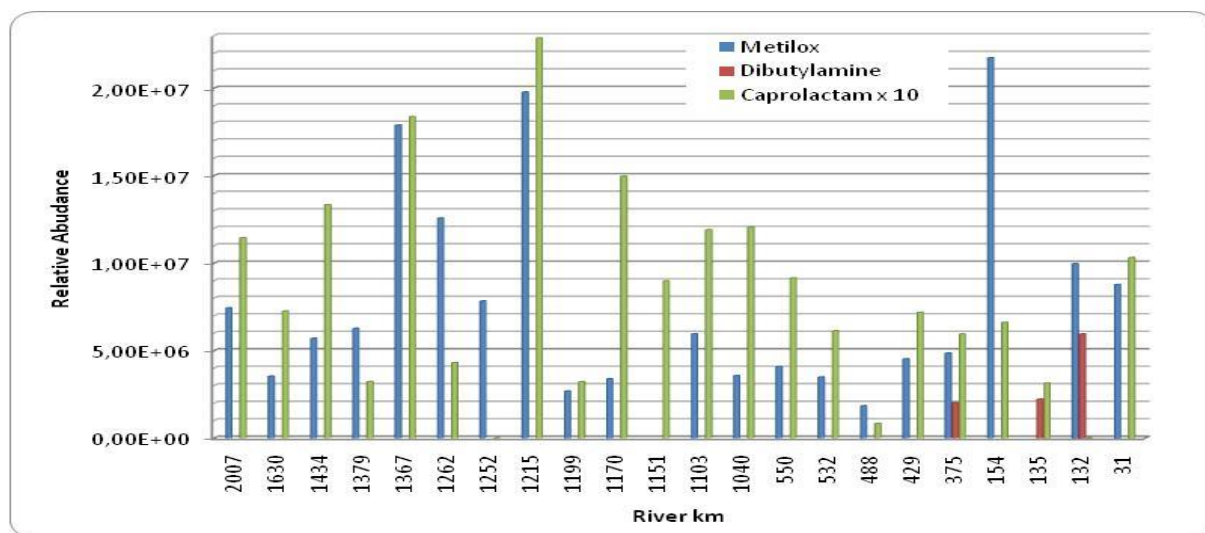


Figure 16: Occurrence profile of synthetic compounds in the JDS3 surface water samples (Caprolactam its response is 10 times reduced)

Metilox is the addition product of 2,6-di(tert-butyl)phenol and methylacrylate. It is used as an intermediate in the synthesis of phenolic antioxidants mainly for polymers. Other usages known are as an additive in motor oils, hydraulic fluids and as a lubricant and in fragrances/perfumes/deodorisers/flavouring agents. The total production volume within OECD member states by the major producer (Ciba) amounted 23,500 tonnes in 1992.

Data archivation

The chromatographic and spectral information for all the detected compounds for both approaches was stored in Data Collection Templates (Annex III - Report_tab_LLE and Report_tab_LVSPE).

3.3.1 Retrospective analysis

Full scan EI mass chromatograms containing all spectral information from GC-MS screening of JDS3 samples was stored (digital sample banking) in order to allow for its retrospective analysis. The approach was tested with substances popping out from LC-MS analyses of the same samples, which were not detected using the routine GC-MS workflow. Here, only substances amenable to GC were considered and in the process all chromatograms were manually re-checked using specific ions of the suspect substances previously 'hidden' in the background.

The retrospective analysis of JDS3 chromatograms was surprisingly successful leading to identification of several compounds such as 1H-benzotriazole, p-toluenesulfonamide, carbamazepine, atrazine, diethyltoluamide (DEET), 2-(methylthio)benzothiazole, tetraglyme, triglyme, terbutylazine,

cotinine, triethylcitrate, triclosan and nicotine. An example of retrospective identification of biocide triclosan is shown in Figure 17.

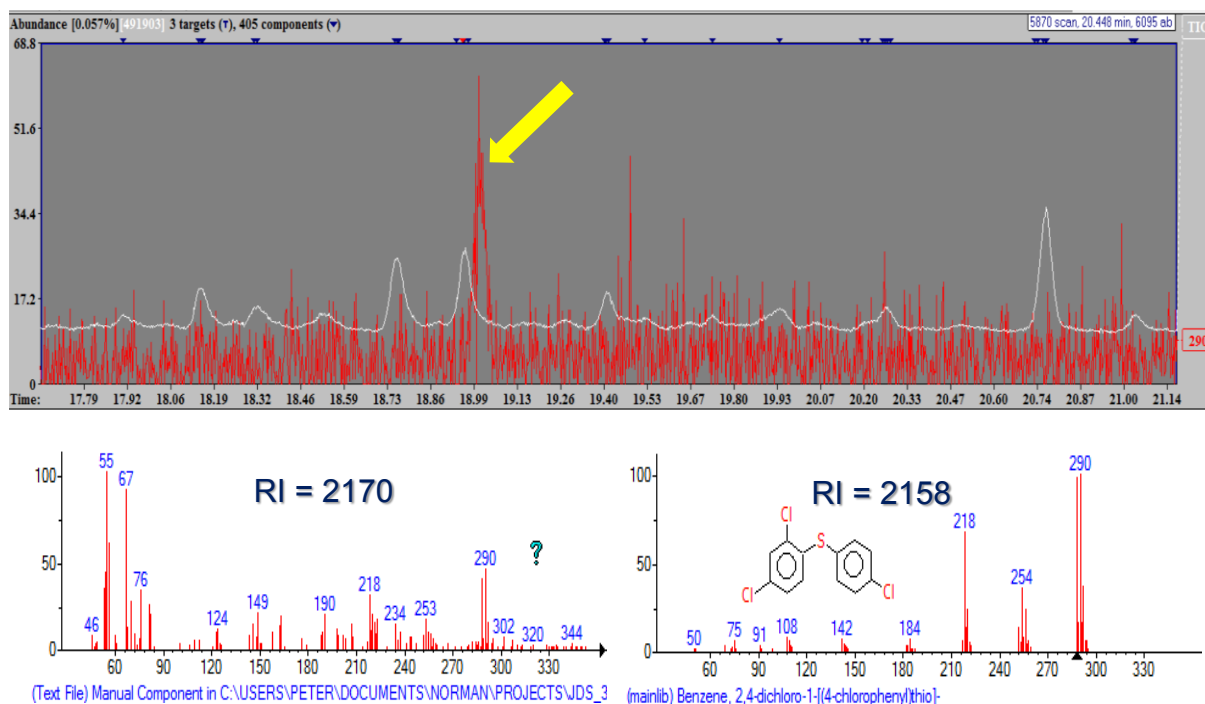


Figure 17: An example of retrospective analysis: upper window - AMDIS software did not label a component marked with yellow arrow after manual deconvolution; left window - manually processed mass spectrum of the detected compound with calculated value of retention index; right window - library mass spectrum of triclosan (C₁₂H₇Cl₃O₂) with reference RI value

4 Conclusions

Analysis of the Danube surface water samples at a basin-wide scale was conducted for the first time with two liquid chromatography-high resolution mass spectrometry instruments (UHPLC-QTOF-MS and LC-HR-MS). Target, suspect and non-target screening was performed with the major goal to search for as many compounds as possible while focusing on compounds not previously known to be present in the Danube river and its tributaries. Target analysis of 168 substances by UHPLC-QTOF-MS showed that 154 of the studied analytes were found to be present in at least one sample. Initial results from non-target screening by UHPLC-QTOF-MS revealed presence of more than 3370 different organic compounds listed by name (PCDL match). The follow up evaluations with autoMSMS method resulted in unequivocal identification of 56 substances dominated by pesticides, pharmaceuticals and personal care products. The rest of tentatively identified suspect compounds, unknowns (proposed molecular formula) and total unknowns (only accurate mass and retention time available) still need to be investigated and those results can be expected in the near future.

The ‘suspect screening’ by LC-HR-MS showed that 110 out of 315 ‘searched for’ substances were determined in at least one sample and 50 compounds were present in more than 20 samples. A semi-quantitative analysis was performed for 110 analytes. Despite the lists of target/suspect substances in two LC-MS laboratories differ, there is a good agreement on the overlapping compounds, e.g. DEET found by both laboratories in all 68 samples and gabapentin in 67 vs. 65 samples with LC-QTOF-MS and LC-HR-MS, respectively.

Both of the techniques could achieve low-ng/L detection limits of wide range substances with direct injection of the water sample, which is significantly reducing the need for laborious sample preparation. The statistical software at LC-QTOF-MS allowed for analysis of differing pollution patterns for the river stretches and countries within the basin. Combination of high resolution technique with different algorithms and the availability of comprehensive mass spectral libraries with accurate mass fragmentation information was shown to be important at the detected compounds’ identification. A Danube river basin mass spectral library linked to/or being part of existing international databases equipped with various structure elucidation tools, such as NORMAN MassBank (Schulze et al., 2012, NORMAN Association, 2014), would be of great benefit for identification of present and future emerging substances.

The GC-MS results were complementary to those obtained by LC-MS. Chemical structures of 298 and of 288 substances in 68 and 22 samples collected by two different methods (LLE and LVSPE) could be proposed. Still, up to 38% detected substances remained unidentified. A rough estimation of the compounds’ concentrations was made based on the comparison of their ion signal with that of the internal standard, which allowed for establishment of their pollution profiles across the basin and preliminary risk assessment by comparing the concentration data with available PNECs. A retrospective analysis of ‘digital sample banking’ GC-MS data proved to be successful. The presence of several pollutants, which would otherwise stay undetected, was revealed.

Obviously, spot sampling such as in the JDS 3 does not allow for assessment of trends and variations in pollution pattern of the Danube river and its tributaries. Therefore additional one year sampling during four seasons would be recommended to register pollution by e.g. pesticides and their transformation products, virucides and antibiotics. A more intense sampling (e.g. one week; 24 h sample) at selected sites would be needed to capture pollution by e.g. illicit drugs used mainly during the weekend (Karolak et al. 2012).

Non-target screening is a powerful tool at the identification of the RBSPs. Present MS systems generate vast amounts of data and therefore there is a need for strategy to reduce the amount of detected (thousands of) substances in a single sample to ‘workable’ numbers (top 10 – 100 substances). One of the possible ways out is prioritisation of non-target screening data being currently developed by the NORMAN Working Group on Prioritisation (www.norman-network.net) using the principles outlined in the recent paper by Schymanski et al. (2014) and NORMAN prioritisation

framework (2012). Presented results clearly indicates that for the assessment of the presence of organic compounds and for detection of environmental contamination in sufficiently early stage new sensitive quantitative target and non target analysis are needed. Detection of local environmental contamination in different environmental compartments at the right time prevents global spread of pollution and also a series of harmful effects that pollutants have on plant and animal organisms, including humans.

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