New Priority Substances, Biocides and Pesticides, in the Aquatic Environment of Romania

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The biocides cybutryne, terbutryn and dichlorvos, and the pesticides aclonifen, bifenox, quinoxyfen are new priority substances of Water Framework Directive of the European Union. The article describes the development and validation of a liquid chromatographic-tandem mass spectrometry method (LC-MS/MS) for the simultaneous determination of the six organic pollutants. The proposed method included an automated solid phase extraction (SPE) step using polymeric cartridges (OASIS HLB) followed by LC-MS/MS, under multiple reactions monitoring mode (MRM). Organic contaminants were analyzed in surface water samples from the Danube River and its tributaries (Arges River, Jiu River, and Olt River). Terbutryn and dichlorvos were below the limits of detection (LODs) in all samples. The surface water samples were found to be contaminated by quinoxyfen (0.4-3.5 ng/L), aclonifen (1.21-8.4 ng/L), bifenox (1.8 ng/L), below environmental quality standards. The pesticide detected with the highest frequency was quinoxyfen. The highest concentrations of pesticides were recorded in Olt River (aclonifen 8.4 ng/L) and in Danube River in Turnu Magurele sampling point (quinoxyfen 3.5 ng/L).

Keywords: priority substances, biocides and pesticides, LC-MS/MS, Danube River

The Water Framework Directive (WFD) of the European Commission (EC 2000/60/EC) describes the monitoring of priority substances and other pollutants in the surface waters of the European Union [1]. The daughter directive 2008/105/EF of the European Parliament and the Council of the European Union has defined environmental quality standards (EQSs) for priority substances in water, with the aim to protect the aquatic environment from adverse effects of these substances [2]. The list of priority substances was recently revised by Directive 2013/39/EU [3]. New priority substances were added by the European Commission, amongst these are the biocides cybutryne, dichlorvos and terbutryn, the pesticides aclonifen, bifenox and quinoxyfen. Molecular formulas of the analytes are presented in table 1 with their environmental quality standards for maximum allowable concentration (MAC-EQS).

Terbutryn and cybutryne (Irgarol) are s-triazine compounds used as algaecides/biocides in buildings. To reach the planned effect, the biocides have to migrate from building materials into the target cells through the surface water film on the materials. During this process the biocides can be leached of or washed away by rainwater [4]. Also terbutryn is an aquatic herbicide used for control of submerged and free-floating weed and algae. Quinoxyfen is used in the formulation of fungicides. Bifenox and aclonifen are chlorinated biphenyl ethers used as herbicides [5].

The hazardous nature of priority contaminants is caused by their toxicity in combination with high chemical and biological stability, and a high lipophilicity. They accumulate in the adipose tissue of fishes and wildlife. A part of these pollutants that are released into aquatic environment will incorporate in sediments which might act as the major source of pollution to water [6].

The most widely used analytical methodologies for the analysis of priority contaminants in environmental waters are based on solid phase extraction (SPE) followed by liquid chromatography coupled to mass spectrometry (LC–MS) [4-9] or gas chromatography coupled to mass spectrometry (GC–MS) [10-12]. Growing concern for Danube water quality is mainly determined by the fact that it is an important source of drinking water for riparian population. Up to now the occurrence of pesticides in the Danube River basin has been investigated by some studies [13-14].

In this paper we proposed a method for simultaneous quantification of three biocides (cybutryne, terbutryn and dichlorvos) and three pesticides (aclonifen, bifenox and quinoxyfen) from Romanian surface water samples (Danube River and three major tributaries: Arges, Jiu, Olt) using automated SPE and LC-ESI-MS/MS. The main objective of this research was to investigate the occurrence of some priority pesticides in Romanian surface waters.

Experimental part

Chemicals and reagents

Cybutryne, terbutryn, dichlorvos, aclonifen, bifenox, and quinoxyfen with purities higher than 93% were purchased

Compound	Molecular formula	MAC-EQS/ ng/L
Aclonifen	$C_{12}H_9ClN_2O_3$	120
Bifenox	$C_{14}H_9Cl_2NO_5$	40
Cybutryne	$C_{11}H_{19}N_5S$	16
Quinoxyfen	$C_{15}H_8Cl_2FNO$	2700
Terbutryn	$C_{10}H_{19}N_5S$	340
Dichlorvos	$C_4H_7Cl_2O_4P$	0.7

Table 1
MOLECULAR FORMULA, ENVIRONMENTAL QUALITY STANDARDS FOR MAXIMUM ALLOWABLE CONCENTRATION (MAC-EQS)

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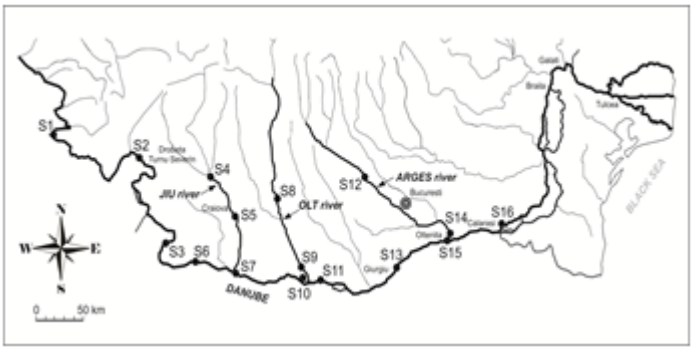


Fig. 1. Map of the study area in the Danube River basin, location and codes of the sampling points

Compound	*Polarity*	*t_R (min)*	*MRM transitions (m/z)*	*Fragmentor voltage (V)*	*Collision energy (eV)*	*Dwell time (ms)*
Bifenox	ESI +	8.721	342→310	100	5	200
			342→189	100	20	200
Quinoxyfen	ESI +	8.950	308→197	150	30	50
			308→272	150	30	50
Aclonifen	ESI +	7.916	265→248	100	18	200
			265→193	100	18	200
Cybutryne	ESI +	3.367	254→198	120	18.	75
			254→156	120	25	75
Terbutryn	ESI +	2.821	242→186	120	18	75
			242→71	100	30	75
Dichlorvos	ESI +	2.459	221→109	100	20	100
			1221→45	100	5	75

Table 2
RETENTION TIMES AND MS/MS PARAMETERS FOR THE ANALYSIS OF TARGET PESTICIDES

from Sigma-Aldrich (Steinheim, Germany). *Individual s*tandard stock solutions of compounds (around 500 mg/L) were prepared by dissolving of the solid standards in methanol. The acetonitrile, sodium hydroxide solution, methanol, LC-MS grade formic acid (98%), used for preparation of standards, mobile phase and pH adjustment were supplied by Sigma-Aldrich. Working standard mixtures were prepared by diluting the individual stock solution in acetonitrile. High purity water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Glass microfibre filters were purchased from Whatman (United Kingdom). SPE cartridges, built of a hydrophilic and a lipophilic monomer (Oasis HLB: 200 mg, 6 mL), were acquired from Waters (Milford, Massachusetts, USA).

Sampling sites and sample collections

The collection of the samples was performed in May 2015. Samples were collected from 10 locations along the Romanian part of the Danube River, as follows: Bazias (S1), Gura Vaii (S2), Calafat (S3), Rast (S6), Bechet (S7), Islaz (S10), Turnu Magurele (S11), Giurgiu (S13), Oltenita (S15), Calarasi (S16). Also, samples were collected from 2 locations from each of the main tributaries of Danube River, as follows: Jiu River, Filiasi (S4), Podari (S5), Olt River, downstream Slatina(S8), Izbiceni (S9), Arges River, 36 km upstream Bucharest (S12), upstream of the confluence with Danube River (S14), one location being close to their confluence with Danube River, as shown in figure 1. Water samples were collected in 200mL amber glass bottles, previously rinsed with water sample at the sampling site. After collection, the samples were kept at 4°C until arrival to the laboratory and pretreated by automated solid phase extraction within 48h.

Instrumentation

For the LC analysis, an Agilent 1260 HPLC system with a binary pump was used. This was equipped with a C18 analytical column of 100mm×2.1 mm and 3.5 μm particle size (Agilent Zorbax Eclipse Plus C18). The mobile phases, A and B, were ultrapure water with 0.1% formic acid and acetonitrile, respectively. The chromatographic separation was achieved with the following gradient: 0-4 min 40-70% B, 4-5.8 min 70-100%B, 5.8-8 min 100%B, 8.01-16 min 40%B. The column temperature was kept at 20 °C. The flow rate was constant, 0.3mL/min during the whole process and a volume of 25μL of standard solutions and sample extract was injected in every case. All the analytes were eluted within 11 min. The LC system was connected to a triple quadrupole mass spectrometer Model 6410 Agilent (Agilent Technologies, Waldbronn, Germany) equipped with electro-spray ionization (ESI) source, operating in positive and negative ion mode. The optimal MS parameters were as follows: gas temperature, 350^{0}C; gas flow, 9 L/min; nebulizer gas, 40 psi; capillary voltage, 4000 V. Nitrogen was served as the nebulizer and collision gas. The analyses were done in the positive ion mode for all compounds. For increased sensitivity and selectivity, data acquisition was performed working in Multiple Reaction Monitoring (MRM) mode. For each substance, two signals were monitored, corresponding to the transition between the precursor ion of the protonated molecule $[M+H]^+$ of the two most abundant product ions. The most abundant one was used for quantification while the other one was used for confirmation. Instrument control and data processing were carried out by means of MassHunter software from Agilent Technologies. The cell acceleration voltage (CAV) used was 7. Analyte instrumental detection limits were lowered also after a rigorous MS optimization procedure in which all MS parameters were optimized for each analyte. MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in table 2. The TIC (total ion chromatogram) MRM Chromatogram of mixture standard solution in acetonitrile (5μg/L) obtained in these conditions is presented in figure 2.

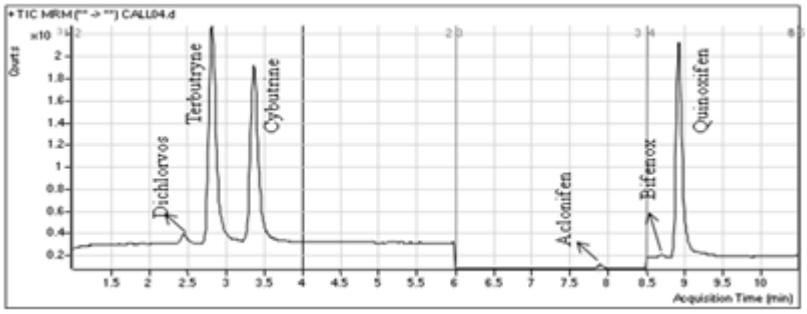


Fig. 2. The TIC MRM Chromatogram of mixture standard solution in acetonitrile (5µg/L)

Water sample preparation and SPE extraction

In order to reach the sensitivity required in this field, and to be able to determine low biocides and pesticides concentration levels possible present in surface water, a pre-concentration of 100 times has been applied. The pre-concentration applied to the water sample was adapted partially form previous literature [4, 6, 9].

All water samples were filtered using glass fiber filters to remove particles large than 0.45µm and then kept at 4^0C until analysis. The pH of the samples was adjusted to 7 using a solution of *sodium hydroxide* solution. The samples were extracted with an automated solid-phase extraction apparatus (Dionex Autotrace 280, Thermo Scientific). Oasis HLB cartridges (200 mg sorbent/6mL cartridge, Waters, USA) were conditioned with 6 mL methanol and 5 mL ultrapure water at 5 mL/min and then the sample was loaded at a flow rate of 5 mL/min. Cartridges were rinsed with 10 mL ultrapure water and then dried for 30 min under a current of nitrogen at 10 mL/min and eluted with 4 mL methanol at 5mL/min. Samples were then evaporated to almost dryness in a Turbo Vap LV (Caliper Life Sciences Inc., Hopkinton, MA, USA) under a current of nitrogen at 35^0C. The residue was reconstituted with 1mL methanol and analyzed by LC-MS/MS.

Validation study

Prior to its application, the method was validated for surface water considering the following parameters: linearity, limits of detection, intra-day and inter-day precision, accuracy and recovery. The calibration curve was obtained by analyzing standard solutions at five concentrations between 0.1 and 10 µg/L. Linearity was assumed if the correlation coefficient value was higher than 0.98. Limits of detection (LOD) and limits of quantification (LOQ) were determined as the minimum detectable amount of analyte, in the sample chromatogram, giving peaks for which the signal-to-noise ratio was 3 and 10, respectively. The estimated values of LODs were in the range from 0.4 ng/L to 1.2ng/L, whereas corresponding LOQ values were in the range of 1.3-3.9 ng/L. For *intra-day* experiments, four replicates were spiked, extracted and analyzed in the same day, whereas for *inter-day* assays, the extraction and analysis were performed for one sample, in four days. Individual recoveries for overall analytical procedure were determined by spiking surface water with working standard mixture at approximately 0.05 µg/L. Un-spiked surface water samples were previously analyzed to confirm the absence of any significant peak at the selected transitions and positive findings were subtracted from spiked samples. The whole method was considered accurate if recoveries were in the 70–120% range, and precision was satisfactory if the relative standard deviation RSD was lower than 15%.

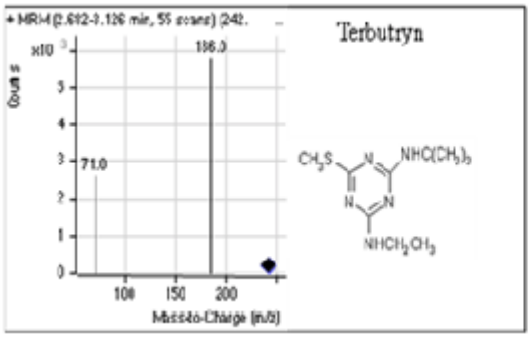
Results and discutions

MS parameters optimization

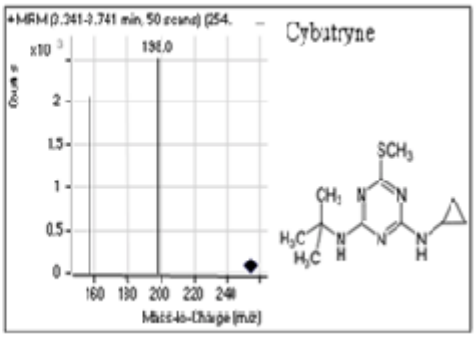
Analytes were firstly identified in full-scan mode (m/z 50-1000) by direct infusion of individual standard solutions in acetonitrile (5mg/L) at a flow rate of 0.3 mL/min, using a mixture of acetonitrile and 0.1m formic acid (80/20, v/v) as mobile phase. To optimize performance and sensitivity of MS, the fragmentor voltage was selected to produce the highest intensity for the precursor ion. After that, the collisions energy was adjusted to produce the highest intensity for the precursor ion. To select the fragmentor voltages it was varied the fragmentor from 70 to 130V. From total ion chromatogram (TIC), the corresponding $[M+H]^+$ for ESI+ were used to produce the extracted ion chromatogram (EIC) for different fragmentor voltages. Using optimized fragmentor was performed the optimization of collision energy by injecting of standard solutions of each substance (5mg/L) at constant flow of mobile phase. Each acquisition was performed in steps of 5V between 10 and 30V. The optimized collision energies were those that provide the highest signal for the precursor ion. The protonated molecule $[M+H]^+$ produced the most intense signal for all compounds and this ion was selected as the precursor ion. Once the $[M+H]^+$ signal was optimized, different collision energies were tested in order to obtain the maximum sensitivity with the highest number of product ions available. The fragmentor voltage and collision energies were optimized for each compound and ranged from 70V to 130 V and from 10eV to 30 eV, respectively. The following parameters were also optimized and finally nebulizer pressure was set at 40 psig; drying gas was set at 9 L/min and source temperature was set at 350°C. LC-MS/MS parameters for collision energy, fragmentor voltage, MRM transitions, dwell time and retention times are shown in table 2. In order to improve the sensitivity, each run was divided into four acquisition windows (time segments) and dwell time was set at 50-200 ms, depending on the time segment. The optimum setting for column temperature giving the highest resolution and the strongest analyte response was found to be 20°C. As regard the optimal flow rate, 0.3 mL/min was observed to provide best analyte separation and maximum sensitivity. The MRM spectrum of the two transitions for each compound (0.5µg/L), obtained in optimized conditions, is presented in figure 3.

Results of validation study

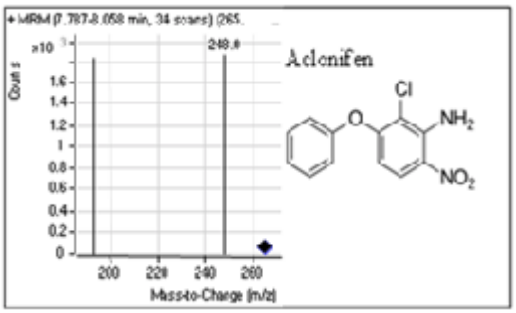
A linear working range of concentration values, ranging in the interval 0.1 to 10µg/L, with very good correlation coefficients (R^2> 0.99), was achieved. These LODs are comparable to those reported in other studies and suitable to quantification of pesticides residues in surface water [13, 14] .The calculated limits of detection (LOD) for all compounds were below 1.2 ng/L, ranging from 0.4 ng/L to 1.2 ng/L. Recoveries achieved for all target compounds were higher than 78%. Precision was expressed as a relative standard deviation (RSD) with values from 2.8 to 6.2% and from 5.3 to 11% for interday and intraday test, respectively. The performance parameters of the method (LOD, LOQ, repeatability, inter-day and intraday precisions) and all the data obtained by covering the external standard calibration methodology are presented in table 3.



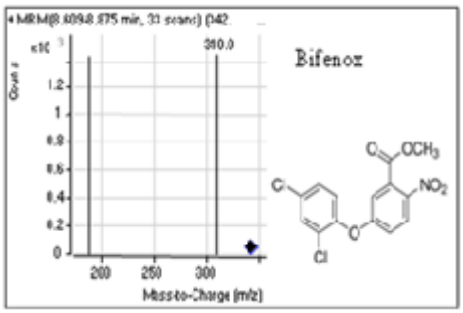
a) MRM spectrum of two transitions for terbutryn



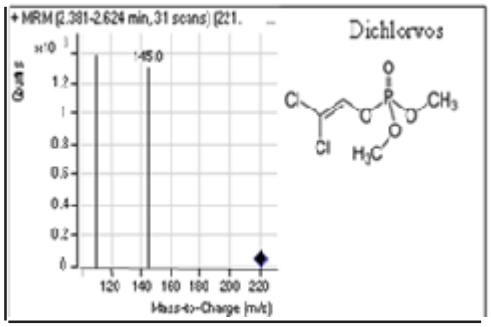
b) MRM spectrum of two transitions cybutryne



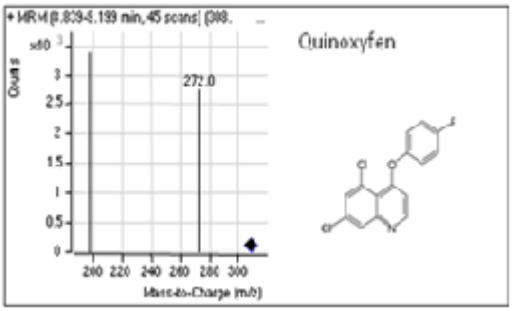
c) MRM spectrum of two transitions for aclonifen



d) MRM spectrum of two transitions for bifenox



e) MRM spectrum of two transition dichlorvos



f) MRM spectrum of two transitions for quinoxyfen

Fig. 3. MRM spectrum of two transitions for each compound (0.5µg/L)

Compound	R^2	LOD (ng/L)	LOQ (ng/L)	Recovery (%)	Intraday precision (% RSD_r)	Inter-day precision (% RSD_R)
Dichlorvos	99.97	0.6	1.9	86.3	4.1	8.1
Terbutryn	99.99	0.4	1.3	81.2	6.2	11
Quinoxyfen	99.98	0.8	2.6	88.1	4.5	9.4
Cybutryne	99.91	0.6	1.9	93.2	3.1	5.3
Aclonifen	99.96	1.2	3.9	81.4	4.0	7.4
Bifenox	99.91	1.2	3.9	78,4	2.8	5.9

Table 3
MAIN VALIDATION PARAMETERS OF METHOD: COEFFICIENT OF DETERMINATION (R^2), LOD, LOQ, RECOVERY, INTERDAY AND INTRADAY PRECISIONS FOR THE DEVELOPED METHOD

Analysis of pesticides from real water samples

The method was applied for for the determination of pollutants in sixteen real surface water samples. Results obtained are summarized in table 4. All priority pesticides and biocides detected in Danube River, Arges river, in Jiu river, and in Olt river, were found at concentrations levells below their respective Environmental Quality Standard set by the European Union, Directive 2013/39/EU [3].

Terbutryn and dichlorvos were below the limits of detection (LODs) in all samples. The surface water samples were found to be contaminated by: quinoxyfen (from 0.4ng/L in Jiu river and in Danube River to 3.5ng/L), aclonifen (from 1.21ng/L in Danube River, area Turnu-Magurele to 8.4 ng/L in Olt River, in Slatina area). The aclonifen values are lower than those previously reported by researchers in Sweden surface water, which reach value of 33ng/L [15].The pesticide detected with the highest frequency (56%) was quinoxyfen.

Cybutryne was detected only in one Danube water sample in Turnu-Magurele section (S11) at concentration of 1.11ng/L. The cybutryne value is similar to the previously reported in Denmark surface water (0.85ng/L) [4].

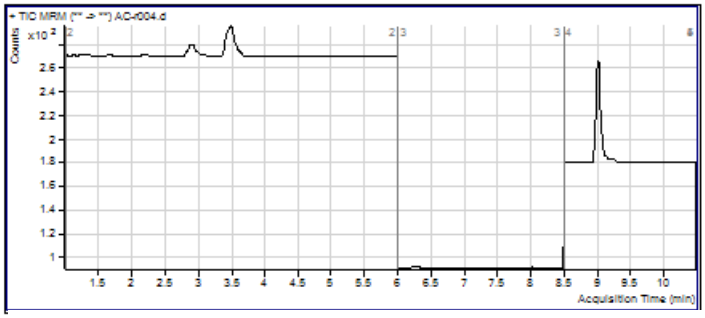


Fig. 4 LC–MS/MS chromatogram of Danube River sample in Turnu-Magurele point showing the detection of aclonifen , quinoxyfen and cybutryne

Compound/ Sampling Point	Aclonifen (ng/l)	Bifenox (ng/l)	Quinoxyfen (ng/l)	Cybutryne (ng/l)	Dichlorvos (ng/l)	Terbutryn (ng/l)
S1	<1.2	<1.2	0.67	<0.6	<0.8	<0.4
S2	<1.2	<1.2	1.07	<0.6	<0.8	<0.4
S3	<1.2	<1.2	0.81	<0.6	<0.8	<0.4
S4	<1.2	<1.2	0.42	<0.6	<0.8	<0.4
S5	<1.2	<1.2	0.4	<0.6	<0.8	<0.4
S6	<1.2	<1.2	0.41	<0.6	<0.8	<0.4
S7	<1.2	<1.2	0.61	<0.6	<0.8	<0.4
S8	8.4	<1.2	0.68	<0.6	<0.8	<0.4
S9	<1.2	<1.2	<0.4	<0.6	<0.8	<0.4
S10	1.8	<1.2	<0.4	<0.6	<0.8	<0.4
S11	1.21	<1.2	3.5	1.11	<0.8	<0.4
S12	<1.2	<1.2	<0.4	<0.6	<0.8	<0.4
S13	1.28	<1.2	<0.4	<0.6	<0.8	<0.4
S14	<1.2	<1.2	<0.4	<0.6	<0.8	<0.4
S15	<1.2	<1.2	<0.4	<0.6	<0.8	<0.4
S16	<1.2	1.8	<0.4	<0.6	<0.8	<0.4

Table 4
SUMMARY OF RESULTS FOR THE COMPOUNDS DETECTED IN SURFACE WATER FROM ROMANIA (DANUBE RIVER, ARGES RIVER, OLT RIVER, JIU RIVER)

Exemples of positive findings in Danube River, in Turnu-Magurele area shown in figure 4.

Conclusions

The LC-MS/MS developed method in the laboratory allows simultaneous analysis of three priority biocides and three priority pesticides in surface waters at low levels imposed by Water Framework Directive of the European Union. Low limits of detection obtained for all compounds (<1.2ng/L) and acceptable recovery values (>78%) show that the method is sensitive and accurate. All priority pesticides and biocides detected in Danube River, Arges river, in Jiu river, and in Olt river, were found at concentrations levells below their respective Environmental Quality Standard set by the European Union, Directive 2013/39/EU. Terbutryn and dichlorvos were below the limits of detection (LODs) in all samples. The highest concentrations of pesticides were recorded in Olt River in Slatina area (aclonifen 8.4 ng/L) and in Danube River in Turnu-Magurele sampling point (quinoxyfen 3.5 ng/L).

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