

Water Quality Assessment of Surface Waters and Wastewaters by Traditional and Ecotoxicological Indicators in Ogosta River, Bulgaria

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Abstract: Surface water samples in Ogosta River, Bulgaria and wastewater samples at the inlet and outlet of WWTP–Montana were tested for the traditional parameters and ecotoxicological effect. The river and Dam surface waters comply with category A1 of Directive 75/440/EEC for pH, EC, COD, TSS, NO_3^- , Cl^- , SO_4^{2-} , B, Ba, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, V and Zn; with category A2 for BOD_5 , NH_4^+ -N and Fe; and with category A3 for TN_b and As. The average annual concentrations of Al, Cr (III), Cr (VI) and U are lower than the set limits in the Water Framework Directive. Arsenic concentration in all the samples exceeds the maximum allowed concentration, a results from natural processes. All the levels of the studied parameters in the outlet wastewater samples are lower than the limits, set in Directive 91/271/EEC and in the complex permit of the WWTP. The results of the biotest Phytotoxkit FTM show low ecotoxicity of the water samples. Optimization of the sample pretreatment prior to this ecotoxicological test is analyzed and discussed.

Keywords: Surface Water, Wastewater, Ecotoxicity, Phytotoxkit FTM, Ogosta River.

Introduction

The world's population, living in urban areas is expected to increase from 50% in 2008 [7] to 66% by 2050 [27] mainly due to urban growth and the demographic changes in less developed countries. Due to the global economic development [22], it is expected that the Earth's population will reach 10 billion by 2050 [27]. The population growth leads to an increase in the demands for larger quantities of water with sufficient good quality to ensure life, human health, economy, optimal conditions for the reproduction and development of aquatic ecosystems, and necessitates enormous quantities of wastewater to be treated, used and subsequently discharged into the water bodies without further contaminating them. Legislation in this area derives from the Water Framework Directive (WFD) [6] and is reflected in harmonized water quality directives – Directive 91/271/EEC [4], Directive 98/83/EC [5] and Directive 75/440/EEC [3].

The WFD is the first European Directive [2, 21, 23] that focuses on water environment sustainability through an integrated and coordinated approach to water management [26]. Since its introduction in 2000, the Directive aimed at a framework establishment to reach “good status” for water bodies throughout the European Union (EU) by the Member States. The set goal by 2027 was the achievement of at least “good status” for both surface water bodies (rivers, lakes, transitional and coastal waters) and groundwater. But at the end of the first management cycle (2009-2015), surface water bodies in “good status” in EU only increased by 10% from 2009 to 2015 [28]. Similar numbers were reported for Germany – 10% of about total 9800 surface water bodies [30]. It is anticipated that only ~18% of all German river bodies are likely to achieve the WFD management objectives for “good status” by the end of the second management cycle in 2021 [18].

In order to assess the potential of biological, chemical and physicochemical stressors that influence ecosystems, it is necessary to determine the ecotoxicity of water samples. Selected bioassays are used for this purpose. One of the most widely used ecotoxicological tests for freshwater analysis is Daphtoxkit FTM [20], which counts the number of dead and immobilized newborns of the crustacean species *Daphnia magna*. The Phytotoxkit FTM biotest, originally developed for soil analysis, is also used for water samples to evaluate phytotoxicity in recent years. It uses the plant species *Sorghum saccharatum*, *Lepidium sativum* and *Sinapis alba* and measures the decrease in seed germination and root growth after 3 days of contact with the sample.

The aim of the present paper is i) to investigate the quality of surface waters and wastewaters in the catchment of Ogosta River; ii) to compare the results from traditional physicochemical water monitoring with ecotoxicological tests; iii) to evaluate the information power of a single, simple and green integrative phytotoxicological assessment of water pollution.

Materials and methods

Sampling

Surface water samples (1. Ogosta Dam, 2. Ogosta River below the Dam wall, 3. Ogosta River in the central part of the city of Montana, 7. Ogosta River after the town of Mizia, prior to its inflow in the Danube) and wastewater samples (5. WWTP-Montana inlet and 6. WWTP-Montana outlet) were collected in four sampling campaigns (Fig. 1) to cover three seasons – spring (13/05/2019), summer (16/07/2019) and autumn (03/10/2019 and 13/11/2019).

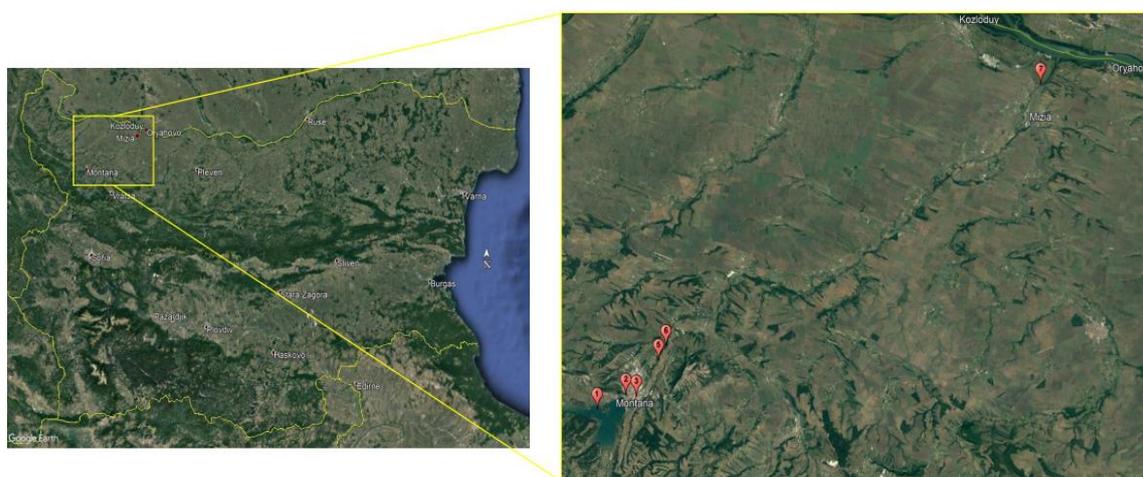


Fig. 1 Sampling map

Water samples were collected in plastic and glass bottles and stored at 4 °C prior to being transported to a laboratory.

Physicochemical indicators

Samples are analysed for pH, electrical conductivity (EC), chemical oxygen demand (COD), biochemical oxygen demand after 5 days (BOD₅), total organic carbon (TOC), ammonium nitrogen (NH₄⁺-N), nitrates (NO₃⁻), total nitrogen (TN_b), total phosphorus as orthophosphate (TP), chlorides (Cl⁻) and sulfates (SO₄²⁻).

The determination of pH and EC was performed on a combined device SensIon+ MM734 (Hach Lange GmbH, Berlin, Germany) [10].

For the determination of the BOD₅, a standard methodology was used [19], based on the measurement of the dissolved oxygen in the sample on the first and on the fifth day. Between the measurements, the samples were stored in thermostat Friocell FC 222 (Friocell, Germany) at 20 ± 1 °C in dark. All steps of the standard procedure were followed [19].

The methods for the spectrophotometric determination used cuvette tests LCK 1414 for COD [14], LCK 380 for TOC [16], LCK 339 for nitrates (nitrate nitrogen) [15], LCK 305 for ammonium nitrogen (ammonium ion) [9], LCK 138 for TN_b [12], LCK 348 for TP [11], LCK 311 for Cl⁻ [13], and SulfaVer 4 powder reagent for the determination of SO₄²⁻ [17]; a portable spectrophotometer DR 3900 (Hach Lange GmbH, Berlin, Germany); and thermo-reactor LT 200 (Hach Lange GmbH, Berlin, Germany) for COD, TOC and TP. The detailed procedure is described elsewhere [32].

The determination method for total suspended solids (TSS) in water is based on the air-pressured filtration of the sample through glass-fibre filters and subsequent drying of the filter at 105 ± 2 °C. The mass of the particles retained onto the filter (1.5 µm) is measured by an analytical balance (RADWAG AC310/C/2, Radom, Poland) with an accuracy of 0.01 g [1].

Trace elements

For the determination of the dissolved content of the trace elements, 15 mL of the sample, intended for ICP-MS analysis, were filtered with a 25 mm PES sterile syringe filters (0.45 µm). The filtered samples are collected in new clean and sterile 15 mL centrifuge tubes and 10 µl of concentrated nitric acid is added (Fisher Chemicals, TraceMetal Grade). After homogenization, samples are stored at 4 °C prior to analysis. For the determination of Cr(VI), samples are filtered in 10 mL glass extraction tubes and immediately 500 µl 2% Aliquat 336 (trimethyloctyl ammonium hydroxide) in xylene is added and extracted for 8 min. The analysis of the upper organic layer of the extracted water samples containing the extracted complex of Cr(VI) was carried out using electrothermal atomic absorption spectrometry (ETAAS) by injecting 10 µL in a pyrolytic-graphite-coated furnace, following the temperature programme:

Drying – 120 °C (Ramp time 20 s / Hold time 10 s);

Pre-treatment – 1500 °C (Ramp time 15 s / Hold time 10 s);

Atomization – 2500 °C (Ramp time 0 s / Hold time 3 s);

Cleaning – 2700 °C (Ramp time 2 s / Hold time 3 s).

Trace elements analysis of the water samples was carried out with an ICP-MS PerkinElmer SCIEX – ELAN DRC-e (MDS Inc., Concord, Ontario, Canada). The spectrometer was

optimized (RF power, gas flow, lens voltage) to provide minimal values of the ratios CeO^+/Ce^+ and Ba^{2+}/Ba^+ as well as maximum intensity of the analytes. External calibration by a multi-element standard solution was performed. The calibration coefficients for all calibration curves were at least 0.99.

The concentrations of the analyzed elements were determined in the standard mode with the exception of As and Se. The potential polyatomic interferences caused by Ar-based polyatomic species on all of the selenium isotopes and on ^{75}As were eliminated using a quadrupole cell in a DRC (Dynamic Reaction Cell) mode with O_2 as a reaction gas [24]. The optimized oxygen flow rate and the RPq (Dynamic Bandpass Tuning parameter) value are presented in Table 1 after the isotopes of As and Se.

Table 1. Measurement conditions for ICP-MS, *Oxygen flow rate [mL/min]/RPq [V]

Instrument	Operating conditions
Argon plasma gas flow	15 L/min
Auxiliary gas flow	1.20 L/min
Nebulizer gas flow	0.90 L/min
Lens voltage	6.00 V
ICP RF power	1100 W
Pulse stage voltage	950 V
Dwell time	50 ms
Acquisition mode	Peak hop
Peak pattern	One point per mass at maximum peak
Sweeps/reading	8
Reading/replicates	1
Sample uptake rate	2 mL/min
Number of runs	6
Rinse time	180 s
Rinse solution	3% HNO_3 (v/v)
Monitored isotopes	$^{11}B, ^{27}Al, ^{51}V, ^{52}Cr, ^{54,56,57}Fe, ^{55}Mn, ^{59}Co, ^{60,62}Ni, ^{63,65}Cu, ^{64,66}Zn, ^{75}As(1.0/0.7)^*, ^{77,78}Se(1.5/0.3)^*, ^{80,82}Se(0.9/0.3)^*, ^{113,114}Cd, ^{137,138}Ba, ^{200}Hg, ^{206,208}Pb, ^{238}U$

Single element standard solutions of Al, As, B, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, V, Zn and U (Fluka, Germany) with an initial concentration of 10 $\mu g/mL$ were mixed and used for calibration after appropriate dilution to obtain the following concentrations: 0.5, 1.0, 5.0, 10.0, 25.0 and 50.0 ng/mL . All solutions were prepared with double deionized water (Millipore purification system Synergy, France).

Limits of quantification. Trueness checks.

The limits of quantification (LOQ) for the trace elements in water are determined on the basis of 6 σ -criteria and are presented in Table 2. To determine the trueness of the ICP-MS measurements, a comparative analysis using ETAAS (Zeeman Perkin-Elmer 3030, Llantrisant, UK) was performed (*t*-test). The accuracy of the proposed method was checked by analyzing standard reference material NIST 1640a (Trace Elements in Natural Water). The obtained values for analytical recovery varied between 95% and 108%, which was considered as satisfactory.

Table 2. LOQ for trace elements using ICP-MS

Element	Al	As	B	Ba	Cd	Co	Cr	Cu	Fe
LOQ $\mu\text{g/L}$	0.04	0.005	0.01	0.01	0.001	0.001	0.001	0.002	0.003
Element	Hg	Mn	Ni	Pb	Se	V	Zn	U	
LOQ $\mu\text{g/L}$	0.01	0.001	0.001	0.001	0.001	0.03	0.005	0.001	

Ecotoxicological analysis

To assess the potential of the physicochemical, chemical and biological stressors to influence the ecosystems, the ecotoxicity of the collected samples was determined by using selected biotests. Part of each sample, intended for ecotoxicological analysis was filtered with a 25 mm PES sterile syringe filters (0.2 μm) and frozen. For the estimation of the effect of the sample filtration, the samples collected on 03/10/2019 were additionally filtered through filters with different pore sizes (0.2, 0.45 and 1.5 μm).

One of the most used ecotoxicological tests for freshwater analysis is the biotest Daphtoxkit FTM (MicroBioTests Inc. Ghent, Belgium), which utilizes dormant eggs (ephippia) of *Daphnia magna* [20]. A Standard Freshwater was prepared as hatching and dilution medium. The rinsed ephippia were transferred into a hatching Petri dish in 50 mL pre-aerated Standard Freshwater and incubated at 20 to 22 °C under continuous illumination of minimum 6000 lux for 3 days. The neonates were pre-fed with Spirulina powder 2 h prior to the toxicity test. Each well of the test plate was filled with 10 mL of the samples or Standard Freshwater as a control sample, and 5 neonates were transferred in each well. The number of dead and immobilized neonates was determined after 48 h incubation in darkness at 20 °C. The ecotoxicological effect (%) is calculated as a ratio between the number of dead test organisms in the studied sample and the number of alive organisms in the control sample.

The Phytotoxkit FTM biotest (MicroBioTests Inc. Ghent, Belgium) measures the change of the seed germination (SG) and the root growth (RG) of the higher plants *Sorghum saccharatum* (SS), *Lepidium sativum* (LS) and *Sinapis alba* (SA) after 3 days of exposure to the analyzed samples, in comparison to a control sample. Originally, this biotest is designed to assess the ecotoxicity of soil samples, but Wiczerzak et al. [29] applied it to liquid samples of both environmental and model origin. A 6.0 ± 0.2 g layer of cotton wool (100% pure cotton) soaked with the water sample (18 mL) was covered with a black filter paper and 10 seeds of the plant species were placed in the test area. The test was performed in duplicate for each water sample for both higher plants, used in this study (SS and SA), and distilled water was used as a control sample. After 72 h incubation in darkness at 25 °C, the germinated seeds were counted, images of test plates were taken, and the length of roots was measured using the program Image J (NIH, Bethesda, MD, USA) [25]. The SG ecotoxicological effect (%) is calculated by the difference between the average number of germinated seeds in the control sample and in the water sample, divided by the average number of germinated seeds in the control sample. The RG ecotoxicological effect (%) is calculated by the difference between the average length (mm) of the roots in the control sample and average in the water sample, divided by the average length (mm) of the roots in the control sample.

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate if the sample site and the pore size of the filter used in the sample pretreatment affect the ecotoxicity test results. This statistical method is applicable when a large set of results obtained at different experimental conditions are examined. The aim of ANOVA is to investigate the impact of one or more controllable factors on the analytical results by comparing the variance caused by the change of the

experimental factor and the unexplained variance (caused by random errors). The studied factor has an impact on the experimental results when its variance is statistically greater than the random variance. Numerical estimates of this effect are the F and P-values. A statistically significant impact of the factor is observed at high values of F and low values of P ($P < 0.05$).

Results and discussion

Surface waters

The Ogosta Dam (sampling point 1) is characterized as L14 (Large lowland reservoirs up to middle depth). Ogosta River (sampling points 2, 3 and 7) is characterized as R7 (Large tributaries of the Danube). Both the L-types were used for compliance assessment of both the water bodies with WFD. The results from the four sampling campaigns are presented in Tables 3 and 4. The concentration of nitrate nitrogen was calculated using the results obtained for nitrates and the concentration of ammonium nitrogen was calculated using the results obtained for ammonium ion. Total phosphorus (measured as orthophosphate and calculated using the results obtained) was measured in all the samples. Because of the relatively high LOQ for the method of choice, a conclusion for the surface waters' status (according to the WFD) and category (according to Directive 75/440/EEC) cannot be drawn.

Physicochemical parameters

Neither Ogosta Dam nor Ogosta River is currently used for drinking water abstraction. Nevertheless, the water quality in the surface waters of the Dam and the river is compared with the set limits of the Directive 75/440/EEC (Table 3). These results confirm the findings of the Danube River Basin Directorate for a 5 year period (2013-2018) [8]. A comparison with the limits, set in the WFD is also presented.

According to the WFD, the water quality regarding pH in Ogosta River is “good” (minimal value of 7.43 and maximum value of 8.49), whilst the pH in the Ogosta Dam in July and November exceeds the set limits. As regards to the EC, the status of Ogosta River is “excellent” (minimum value 345 $\mu\text{S}/\text{cm}$) to “good” (maximum value 714 $\mu\text{S}/\text{cm}$), and in the Dam is “excellent”. No exceedings of the annual average concentrations (AACs), set in the WFD for “good status”, were recorded in 2016 [8]. Based on the results for COD, the status of the Dam and the river is “excellent” (maximum value 11.7 mgO_2/L). Data for BOD₅ is insufficient and only covers two sampling campaigns and first 3 sampling points, but the apparent trend determines the status of the surface waters as “good” and “moderate”. Although insufficient, the data can still be compared with the published results for 2016, where no exceeding of the AACs for BOD₅ was recorded. Regarding the total nitrogen, the status of the surface water in Ogosta Dam and in Ogosta River, after the Dam wall, is “excellent”, but just before the discharge of Ogosta into the Danube, the status of the river deteriorates to “moderate” (minimum value 4.07 mg/L). The same conclusion was drawn in 2017 and 2018. Nitrate nitrogen was measured and the maximum concentration was 28.2 mg/L in Ogosta River in November. According to the limits set in the WFD for nitrate nitrogen, the status of the surface waters in Ogosta Dam and in the river, after the Dam wall, is “excellent”, but just before the discharge of Ogosta into the Danube, the status of the river deteriorates to “moderate” (minimum value 4.84 mg/L). Deterioration of the surface water status in Ogosta to moderate was firstly observed in 2017 and then in 2018 [8] for the surface water samples after the town of Mizia (sample 7). As regards to ammonium nitrogen, the status of the surface waters in Ogosta Dam and in the river, after the Dam wall, is “excellent”, but just before the discharge of Ogosta into the Danube, the status of the river deteriorates to “moderate”.

Table 3. Results for surface water quality in Ogosta Dam and Ogosta river, I – mandatory value, G – guide value, E – excellent, M – moderate, * – only for lakes (sample 1)

Parameter	Sampling date	Sampling point				75/440/EEC			WFD		
		1	2	3	7	A1	A2	A3	E	Good	M
pH	13/05	8.13	7.64	7.84		6.5 –	5.5 –	5.5 –			
	16/07	8.98	7.69	8.21		8.5	9.0	9.0		6.5 –	
	03/10	8.64	7.68	7.99	8.36	(G)	(G)	(G)		8.5	
	13/11	8.07	7.43	7.79	8.49						
EC, µS/cm	13/05	196	201	216							
	16/07	172	200	208		1000	1000	1000	700	750	> 750
	03/10	186	216	205	714	(G)	(G)	(G)	650*		
	13/11	82.9	85.5	87.0	345						
COD, mg/L O ₂	13/05	7.38	6.05	6.93							
	16/07	9.22	5.97	5.90				30			
	03/10	10.8	5.89	7.66	8.27			(G)			
	13/11	9.39	7.14	11.7	9.23						
BOD ₅ , mg/L O ₂	13/05	3.44	4.16	3.45		< 3	< 5	< 7	< 2	2 – 4	> 4
	16/07	5.77	3.05	4.68		(G)	(G)	(G)			
TOC, mg/L	13/05	3.15	4.36	4.83	12.1						
	16/07	3.25	3.22	3.01	10.6						
	03/10	9.8	< 2	2.66							
	13/11	5.06	4.03	4.95							
TSS, mg/L	13/05	0.80	1.60	2.70							
	16/07	4.10	5.00	3.80		25					
	03/10	2.4	0.6	3.4	2	(G)					
	13/11	2.9	8.1	7.2	1.4						
NO ₃ ⁻ , mg/L	13/05	1.58	2.34	2.29							
	16/07	< 1	2.20	1.93		25					
	03/10	< 1	1.69	1.39	21.4	(G)					
	13/11	1.34	< 1	1.37	28.2						
NO ₃ ⁻ -N, mg/L	13/05	0.36	0.53	0.52							
	16/07	< 0.2	0.50	0.44					< 0.7	0.7 – 2	> 2
	03/10	< 0.2	0.38	0.32	4.84				0.8*	0.8 –	
	13/11	0.30	< 0.2	0.31	6.38				2*	2*	
NH ₄ ⁺ , mg/L	13/05	0.06	< 0.02	< 0.02							
	16/07	< 0.02	< 0.02	0.03		0.05	1 (G)				
	03/10	< 0.02	0.04	0.02	0.03	(G)	1.5	2 (G)			
	13/11	0.54	0.73	0.73	0.43		(I)				
NH ₄ ⁺ -N, mg/L	13/05	0.05	< 0.02	< 0.02							
	16/07	< 0.02	< 0.02	0.02							
	03/10	< 0.02	0.029	0.016	0.02				< 0.1	0.1 –	> 0.3
	13/11	0.427	0.575	0.58	0.336					0.3	
TN _b , mg/L	13/05	< 1	< 1	< 1							
	16/07	< 1	< 1	< 1		1	2	3			
	03/10	< 1	< 1	< 1	4.07	(G)	(G)	(G)	< 0.7	0.7 –	> 2.5
	13/11	< 1	< 1	< 1	6.05					2.5	
Cl ⁻ , mg/L	13/05	4.66	3.03	2.92							
	16/07	1.99	1.91	1.91		200	200	200			
	03/10	3.38	2.19	2.15	18.6	(G)	(G)	(G)			
	13/11	< 1	1.04	< 1	20.8						
SO ₄ ²⁻ , mg/L	13/05	11	12	15		150					
	16/07	12	12	12		(G)	150	150			
	03/10	12	11	12	39	250	(G)	(G)			
	13/11	11	12	12	47	(I)					

According to the set limits in the Directive 75/440/EEC, as regards to pH, the surface water in the river is category A1, whilst in the Dam, the quality changes between A1 and A2. As regards to TN_b , the category of the surface water just before the discharge of Ogosta into the Danube is A3. Because of the way the limits are set in the Directive, for COD, the surface water category A3. There is no single result in the surface waters exceeding the limit of 25 mg/L for TSS, so the category is A1. Based on the results for the surface water quality in the Dam and the river as regards to EC, nitrates, chlorides and sulfates, the category is A1, but category A2 regarding ammonium ion.

Trace elements

It is well documented that the surface waters in Northern Western Bulgaria contain naturally elevated concentrations of As [31], which is supported by the current study. From the results obtained, the minimum concentration was 10 $\mu\text{g/L}$ in the samples after the town of Mizia (sample 7) in November, and the maximum concentration (72 $\mu\text{g/L}$) was measured in the sample from the city of Montana (sample 2) in October. Regarding Arsenic, the category of the surface water is A3. The minimum concentration of the dissolved iron was 0.25 mg/L in the Ogosta Dam in July and the maximum concentration 0.58 mg/L was measured in the sample and Ogosta River after the town of Mizia (Table 4). The category of the surface water, as regards to the dissolved iron, is A2. The concentrations of all the other measured elements (B, Ba, Co, Cr, Cu, Hg, Mn, Ni Pb, Se V and Zn) fall within the set limits in Directive 75/440/EEC and the category of surface waters is A1.

Additionally to Directive 75/440/EEC, based on the water pH, the WFD sets limits for Aluminium. The maximum allowed concentration (MAC) is set to 10 $\mu\text{g/L}$ for $\text{pH} < 6.5$ and 25 $\mu\text{g/L}$ for $\text{pH} > 6.5$. The MAC for As is 25 $\mu\text{g/L}$. The different forms of Cr, instead of the total content are set as follows – MAC for Cr (III) is 32 $\mu\text{g/L}$ and for Cr (VI) is 8 $\mu\text{g/L}$. The set limit for Uranium is 40 $\mu\text{g/L}$. And the limits for the annual average concentrations of Cu and Zn are dependent on the water hardness.

The average annual concentration of Al, Cr (III), Cr (VI) and U is measured in the surface water samples from Ogosta Dam and Ogosta River and presented in Table 5.

All the measured concentrations are lower than the limits for surface waters. Occasionally, elevated concentrations for Al were measured in Ogosta River, except in 2017 [8]. All the samples from Ogosta Dam and Ogosta River are higher than the respective annual average concentrations for As. As already discussed, such results are expected. All surface water assessment documents (2013-2018) report results above the average annual concentration for As, except in 2016. Typical concentrations that exceed the set limits were 42.9 $\mu\text{g/L}$ and 39.4 $\mu\text{g/L}$ [8]. Only for U, concentrations close to the MAC are measured in the samples obtained just before the discharge of Ogosta into the Danube. For all the other samples from all sampling points, the measured concentrations are approximately 10-fold lower.

Ecotoxicological indicators

The ecotoxicity of the surface water samples of the first sampling campaign (13/05/2019) is assessed using Daphtoxkit FTM as a test for freshwater analysis. No dead or immobilized crustaceans were observed after 48h-contact. The results obtained indicate that the water in Ogosta River is non-toxic for the neonates of *Daphnia magna*.

Table 4. Results for chemical parameters in Ogosta Dam and Ogosta River,
I – mandatory value, G – guide value

Parameter	Sampling date	Sampling point				75/440/EEC		
		1	2	3	7	A1	A2	A3
As, µg/L	13/05	36	22	19		10	50	50
	16/07	23	37	39		(G)	(G)	(G)
	03/10	43	72	62	24	50		100
	13/11	37	70	61	10	(I)		(I)
B, µg/L	13/05	6.5	6.8	6.5	65	1000	1000	1000
	16/07	6.8	6.9	6.5	68			
	03/10	6.3	7.1	6.4	69			
	13/11	6.4	6.9	6.5	65			
Ba, µg/L	13/05	22	19	19	50	100	1000	1000
	16/07	16	22	15	43	(I)	(I)	(I)
	03/10	24	20	19	51			
	13/11	21	21	20	46			
Co, µg/L	13/05	0.08	0.08	0.07	0.3	20 (G)		
	16/07	0.14	0.19	0.22	0.4			
	03/10	0.15	0.19	0.11	0.4			
	13/11	0.16	0.16	0.15	0.4			
Cr, µg/L	13/05	3.3	1.2	1.3		50	50	50
	16/07	1.5	1.5	1.1		(I)	(I)	(I)
	03/10	1.6	1.6	0.9	3.9			
	13/11	1.1	1.2	0.9	2.0			
Cu, µg/L	13/05	2.7	1.0	0.7	0.9	20	50	1000
	16/07	1.2	0.6	0.8	0.8	(G)	(G)	(G)
	03/10	1.4	0.5	0.4	0.6			
	13/11	1.3	0.7	0.6	0.8			
Fe, mg/L	13/05	0.28	0.29	0.32	0.55	0.1	1	1
	16/07	0.25	0.30	0.37	0.58	(G)	(G)	(G)
	03/10	0.37	0.32	0.34	0.56	0.3	2	
	13/11	0.28	0.31	0.30	0.56	(I)	(I)	
Hg, µg/L	13/05	0.4	0.3	0.2	0.2	0.5	0.5	0.5
	16/07	0.1	0.07	0.1	0.06	(G)	(G)	(G)
	03/10	0.03	0.08	0.06	0.07	1	1	1
	13/11	0.04	0.05	0.05	0.06	(I)	(I)	(I)
Mn, µg/L	13/05	4.4	1.7	9.2	1.8	50	100	1000
	16/07	2.8	6.2	14.5	12.3	(G)	(G)	(G)
	03/10	2.6	5.6	9.5	9.5			
	13/11	2.7	3.9	9.8	5			
Ni, µg/L	13/05	0.7	0.7	0.5	1.2	20 (G)		
	16/07	0.95	1.4	1.6	7.3			
	03/10	3.0	2.8	1.1	7.4			
	13/11	0.7	0.7	0.6	1.1			
Pb, µg/L	13/05	1.1	0.5	0.03	0.03	50	50	50
	16/07	< 0.01	< 0.01	< 0.01	< 0.01	(I)	(I)	(I)
	03/10	< 0.01	< 0.01	< 0.01	< 0.01			
	13/11	0.46	0.14	0.15	0.01			
Se, µg/L	13/05	0.7	0.8	0.15	0.8	10	10	10
	16/07	0.2	0.4	0.08	0.6	(I)	(I)	(I)
	03/10	0.3	0.5	0.05	0.7			
	13/11	0.2	0.1	0.08	0.6			
V, µg/L	13/05	1.6	0.9	0.7	2.7	10 (G)		
	16/07	1.1	0.7	0.6	3.4			

	03/10	1.8	0.4	0.7	2.3			
	13/11	1.6	0.4	0.8	1.7			
Zn, µg/L	13/05	2.5	2.0	4.5	1.5	500	1000	1000
	16/07	1.5	1.3	3.4	0.53	(G)	(G)	(G)
	03/10	1.7	1.2	2.8	0.58	3000	5000	5000
	13/11	1.5	1.8	3.3	0.65	(I)	(I)	(I)

Table 5. Average annual concentrations of Al, Cr (III), Cr (VI) and U

Element, µg/L	Sample 1	Sample 2	Sample 3	Sample 7	WFD
Al	15.2	10	12.2	6	15
Cr (III)	0.2	0.07	0.05	0.2	4.7
Cr (VI)	1.7	1.3	1.0	2.0	3.4
U	0.7	0.4	0.4	5.4	5

A previous study [32] of the discriminative ability of the ecotoxicological tests towards surface water and wastewater showed that Daphtoxkit FTM and Microtox[®] are not suitable to distinguish between treated and surface waters, while Phytotoxkit FTM proved to be more sensitive using the root growth of *Sinapis alba* (SA-RG) and seed germination of *Sorghum saccharatum* (SS-SG) and *Lepidium sativum* (LS-SG). Thus the biotest Phytotoxkit FTM was used to assess the ecotoxicity of the samples from the other 3 sampling campaigns and the low ecotoxicity of the surface waters was confirmed. Moreover, most of the results indicate a negative effect, which means that the number of germinated seeds or the length of the roots is greater than those in the control sample. It is speculated that the reason is the higher content of nutrients in the surface waters. The SA-RG results of the last sample along the Ogosta River (sample 7) show the greatest ecotoxicity for the last two campaigns. This is consistent with the results obtained for the concentrations of the physicochemical parameters and trace elements, which increase along the river (except TSS, As, Cu, Pb and Zn). In addition, all the samples (except sample 1 – Ogosta Dam) showed the highest ecotoxicological effect (SA-RG and SS-RG) during the campaign on 03/10/2019. The SS-SG and LS-SG show similar ecotoxicity for all the surface water samples during the 3 sampling campaigns as the lowest effect is observed at last campaign (13/11/2019) with SS-SG showing lower results than SA-SG.

Wastewater

Physicochemical parameters and trace elements

The water quality parameters, monitored at the outlet of WWTP-Montana (98618 p.e.), according to Directive 91/271/EEC are COD, BOD₅, TSS, TP and TN_b.

No exceeding of the set limits in the Directive or in the complex permit for the discharge of WWTP-Montana, issued by the Danube River Basin Directorate (where the concentration of sulfates in the outlet is additionally set to 400 mg/L), were observed in the samples collected during the four sampling campaigns (Table 6). These results are confirmed by the data from the plant's technological monitoring for 2017 and sampling in August 2018 [32].

Ecotoxicological tests

The Daphtoxkit FTM results for the wastewater samples from the first sampling campaign showed higher mortality of *Daphnia magna* in the treated water (20%) compared to the raw water (0%) at the inlet of the WWTP. This relatively low effect and the lack of data for the other campaigns cannot lead to a definitive conclusion for the wastewater influence on

Daphnia magna but a possible reason could be found in the low nutrient content in the treated wastewaters.

Table 6. Results for the physicochemical parameters in the inlet and the outlet samples of WWTP–Montana according to Directive 91/271/EEC (a) and complex permit (b)

Parameter		13/05/2019	16/07/2019	03/10/2019	13/11/2019	Limits
COD, mg/L O ₂	5. WWTP inlet	91.3	149.0	78.2	127.0	
	6. WWTP outlet	9.3	9.7	28.3	15.1	125 (a)
BOD ₅ , mg/L O ₂	5. WWTP inlet	44.23	79.80			
	6. WWTP outlet	2.62	7.05			25 (a)
TSS, mg/L	5. WWTP inlet	37	56.5	53	42	
	6. WWTP outlet	2	2.4	3.6	2.9	35 (a)
TN _b , mg/L	5. WWTP inlet	30.40	17.00	17.3	14.3	
	6. WWTP outlet	7.76	5.27	4.87	6.81	15 (a)
TP, mg/L	5. WWTP inlet	2.69	2.64	2.78	3.31	
	6. WWTP outlet	1.04	1.10	1.08	1.14	2 (a)
SO ₄ ²⁻ , mg/L	5. WWTP inlet	33	30	34	36	
	6. WWTP outlet	23	22	27	31	400 (b)
Fe, mg/L	5. WWTP inlet	0.38	0.4	0.45	0.46	
	6. WWTP outlet	0.28	0.29	0.29	0.25	5 (b)
Mn, µg/L	5. WWTP inlet	68	71	80	53	
	6. WWTP outlet	49	50	44	17	800 (b)
Cu, µg/L	5. WWTP inlet	1.8	0.9	0.8	0.9	
	6. WWTP outlet	0.4	0.3	0.3	0.4	500 (b)
Zn, µg/L	5. WWTP inlet	358	969	220	237	
	6. WWTP outlet	103	562	217	105	10000 (b)
Ni, µg/L	5. WWTP inlet	1.9	3.8	4.9	3.4	
	6. WWTP outlet	0.7	3.4	4.8	1.7	500 (b)
As, µg/L	5. WWTP inlet	14	15	21	22	
	6. WWTP outlet	10	12	18	18	200 (b)
Cd, µg/L	5. WWTP inlet	0.05	0.22	0.23	0.02	
	6. WWTP outlet	< 0.001	< 0.001	< 0.001	< 0.001	20 (b)
Cr, µg/L	5. WWTP inlet	1.4	1.1	1.4	1.7	
	6. WWTP outlet	1.2	1.2	1.3	1.4	100 (b)
Pb, µg/L	5. WWTP inlet	0.4	0.1	16	6	
	6. WWTP outlet	0.1	< 0.01	1.7	1	200 (b)

The use of Phytotoxkit FTM shows that the roots of both studied plants are longer (lower ecotoxicity) for the inlet water samples compared to the outlet water samples for all the sampling campaigns (except *Sinapis alba* in 13/11/2019). It is speculated that this finding is due to the higher content of nitrogen and phosphorus in the inlet samples, which concentrations are reduced during the wastewater treatment process in the WWTP–Montana. Similarly to the surface water samples, the ecotoxicological indicator seed germination shows an equal effect for all the wastewater samples during the 3 sampling campaigns, with SS-SG again being lower than SA-SG.

Influence of the sample pretreatment on the results obtained by Phytotoxkit FTM

Since Phytotoxkit FTM biotest is standardized for soil samples, there is no mandatory sample pretreatment for water samples. However, due to the accepted protocol, the sample pretreatment prior to the analysis includes filtration through sterile 25 mm PES syringes using filters with a pore size of 0.2 µm. The lack of data for analysis of water samples using

Phytotoxkit FTM, as well as the natural conditions in the ecosystems, provoke the interest of analyzing the impact of the sample filtration on the results of the biotest.

In autumn of 2019 (03/10/2019), a total of 7 samples from surface water (samples 1, 2, 3 and 7), wastewater (samples 5 and 6), as well as from the Parta channel in the industrial part of the city of Montana (sample 4) were obtained. A part of each one of the samples was filtered through filters with pore size 0.2, 0.45 and 1.5 μm . The obtained filtrates, as well as the unfiltered samples, were analyzed for the ecotoxicological effect (%) using *Sinapis alba* and *Sorghum saccharatum*.

In order to assess the effect of the different pore size on the ecotoxicity results (SA-RG and SS-RG), variance analysis (ANOVA) was applied. The results show that the variance due to the pore size is not statistically higher than the random variance for both plants (SA-RG: $F = 1.166$; $P = 0.332$; SS-RG: $F = 0.643$; $P = 0.591$). Therefore, different filtration does not affect the Phytotoxkit FTM biotest results. Box-plot diagrams of the ecotoxicological effect (%) using SA-RG and SS-RG, depending on the filter pore size, are presented in Fig. 2.

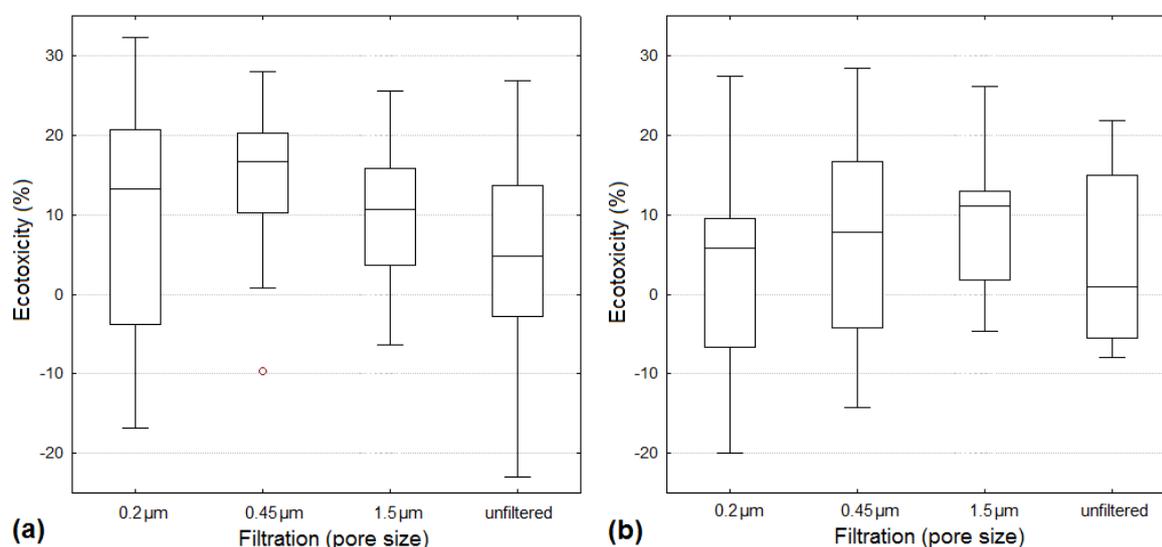


Fig. 2 Box-plot diagram of the ecotoxicological effect (%) using SA-RG (a) and SS-RG (b) depending on the filter pore size

It is interesting to notice that even filtration does not statistically impact the ecotoxicity results, the medians of the unfiltered samples are the lowest ones for both plant species. This means that roots are longer, probably due to the higher nutrient content, absorbed on the suspended particles, readily available for the organisms in the real-life unfiltered water samples.

Additionally, variance analysis is performed in order to assess if the sampling point has an impact on the root growth of *Sinapis alba* and *Sorghum saccharatum*. The results show that all 7 samples are not statistically different (SA-RG: $F = 1.063$; $P = 0.398$; SS-RG: $F = 1.604$; $P = 0.166$). This is an expected result considering the low ecotoxicity of the water samples as a result of the low concentrations of the physicochemical parameters and trace elements. The corresponding box-plot diagrams are presented in Fig. 3.

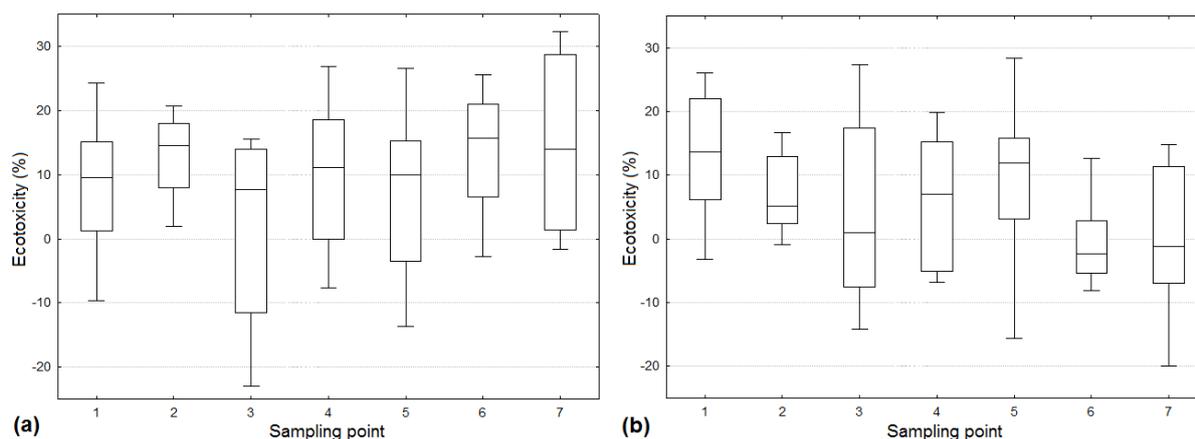


Fig. 3 Box-plot diagram of the ecotoxicological effect (%) using SA-RG (a) and SS-RG (b) depending on the sampling point

Conclusion

In four sampling campaigns (May, July, October and November of 2019), a total of 28 samples from surface water (Ogosta Dam, Ogosta River by the city of Montana and after the town of Mizia) and wastewater (at the inlet and outlet of WWTP–Montana) were tested for physicochemical, chemical and ecotoxicological indicators of water quality. The surface water in the Dam and in the river comply with category A1 of Directive 75/440/EEC as regards to pH, EC, COD, TSS, NO_3^- -N, Cl^- , SO_4^{2-} and dissolved forms of B, Ba, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, V and Zn; with category A2 as regards to BOD_5 , NH_4^+ -N and dissolved Fe; and with category A3 as regards to TN_b and As.

The average annual concentrations of Al, Cr (III), Cr (VI) and U in the samples from Ogosta Dam and Ogosta River are lower than the maximum allowed concentrations, set in the WFD for surface waters. Arsenic concentration in all the samples exceeds the maximum allowed concentration, which is a result of natural processes.

All the levels of the physicochemical and chemical parameters in the samples from the outlet of the WWTP–Montana are lower than the limits set in Directive 91/271/EEC and in the complex permit. The treatment process reduces substantially the COD, BOD_5 , TSS, Cd and Pb content, approximately four times the TP and Mn content and moderate to no reduction for Cl^- , SO_4^{2-} , As, Cr, Cu, Fe, Ni and Zn.

The results from the classical approach were confirmed by the ecotoxicological analyses, which show a low ecotoxicity of all the studied samples. The Daphtoxkit F™ is not indicative and sensitive enough for unpolluted samples. The use of the ecotoxicological test Phytotoxkit F™ necessitates procedure optimization because of the considerable efforts needed for 0.2 μm pore size filtration, which can be substituted by filtration with normal filters that can speed up the analysis. The ANOVA results show that there is no statistical difference in the ecotoxicological effect due to the different pore size of the filters used in the sample pretreatment. However this result is obtained on small data set of samples with low TSS content, thus further researches are needed. Further investigations should include also more ecotoxicological assays that would help to develop reliable water quality monitoring battery of tests.

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