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Diatom diversity and ecological status of the Lasovačka and Lenovačka streams near Zaječar: consideration of WFD implementation in Serbia

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Abstract: The development and improvement of the monitoring system of rivers according to the requirements of the Water Framework Directive (WFD) is essential for all European states because assigning a wrong ecological status class (misclassification) to a water body can have significant economic consequences. In order to point out some of the key issues of WFD implementation in Serbia, we conducted a one-year study of the composition and abundance of diatoms in the phytobenthos in two streams, the Lasovačka and Lenovačka, in eastern Serbia. The physicochemical quality elements were also used in the assessment of their ecological status. The diatom community exhibited a higher species richness in the Lasovačka (88 taxa) than in the Lenovačka stream (63 taxa). Such a detailed study enabled us to record some of the rare taxa, e.g. *Caloneis budensis* in the Lasovačka, which was the first finding of this species in Serbia. The Lasovačka and Lenovačka streams were downgraded to moderate and poor ecological status, respectively, because of the high nitrogen load. Since the monitoring results based on the diatom indices resulted in the assignment of two streams to a good ecological status, the possible reason for the mismatch between the biological and physicochemical quality elements could be due to the lack of analysis of benthic invertebrate fauna or the application of diatom indices that are insufficiently sensitive to the specific environmental pressure. The results of this study show that conducting a comprehensive ecological investigation of rivers within the framework of the WFD in Serbia is urgently needed.

Key words: diatoms; WFD; new record; ecological status; streams

INTRODUCTION

From the moment the EU Water Framework Directive (WFD) was enforced, it was continuously deployed, providing ecological assessment and classification systems with the aim of achieving "good ecological status" and "good surface water chemical status" in all types of surface water bodies by 2015 [1]. As a non-EU state, Serbia took part in the implementation of this EU water policy after a delay of more than ten years. The first monitoring program according to the WFD was performed in Serbia in 2012 [2], but it has not been fully implemented [3].

Previous investigations related to the flora and fauna of the Lenovačka and the Lasovačka streams are scarce. In 2011, the diatom community of the Lenovačka spring was analyzed in order to assess its water quality [4]. A total of 19 different taxa was recorded, and according to the Diatom-Based Eutrophication/Pollution Index (EPI-D) index, water quality belonged to the second class. This was before a new

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system of monitoring based on the WFD was applied in Serbia.

The aims of this study were to analyze the diversity of diatom communities in the Lenovačka and Lasovačka streams during a one-year period, and to assess the ecological status of the streams using different chemical parameters of water and diatom indices proposed by Serbian legislation, as well as the EPI-D and the Trophic Diatom Index (TDI) which are applied in other EU countries. The achievement of the first goal will contribute to better understanding of diatom flora in similar habitats, while the second aim of the authors was to focus on some important issues of WFD application in Serbia.

MATERIALS AND METHODS

Study site and sampling plan

In the past, the Lenovačka and the Lasovačka streams were two major tributaries of the Grliška River, the left tributary of Beli Timok (the rivers Beli and Crni Timok form Veliki Timok, the right tributary of the Danube and the largest river in eastern Serbia). Both streams receive water from several small tributaries. In 1989 a dam was constructed on the Grliška River and a reservoir for water supply was formed about 16 km southwest of the city of Zaječar in eastern Serbia. A brief description of the climatic conditions and precipitation of the studied area is given in Gavrilović et al. 5].

The samples for water quality and diatom analyses were collected monthly from April 2012 to March 2013 at one site in each stream (the Lenovačka stream (43°48’42.06”N; 22°11’36.84”E, 198 m a.s.l.) and the Lasovačka stream (43°48’19.38”N; 22°12’6.84”E, 213 m a.s.l.) (Supplementary Fig. S1)). The Lenovačka and the Lasovačka streams are shallow watercourses with large differences in depth, width and water velocity. Thus, during high flow in April, the estimated velocities (the time it takes for a floating object to travel 10 m in the middle of a stream) were 0.26 m/s and 0.45 m/s, respectively. At the sampling sites during months with continuous water flow, the stream width varied from 2-6 m in the Lenovačka, and from 3-11 m in the Lasovačka stream, while the depth changed from 3-30 cm and 8-50 cm, respectively. Although the stream banks were surrounded by forest trees, for the purpose of sampling, open spots (without shade) were chosen. Since the first of the abovementioned streams dries up almost regularly every year, samples from the Lenovačka stream were not taken in August, September and October in 2012. In addition, sampling of the two streams was not conducted in December 2012 due to the high snow cover and inaccessibility of the sites.

Water quality analysis

To assess the ecological status of the two streams, the following physical and chemical water parameters were monitored: pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrates (NO_3^-), total phosphorus (TP) and chlorides (Cl^-); water temperature (T), conductivity (COND) and nitrites (NO_2^-) were also measured. A water field kit MULTI 340i/SET (WTW, Weilheim, Germany) was used to record T, DO, pH and COND *in situ*. Water samples for other parameters were adequately transported (as quickly as possible at 4°C) and analyzed at the Institute of Chemistry, Technology and Metallurgy (University of Belgrade) and the Public Water Company in Zaječar. TAN, NO_2^- and TP were determined with a UV Vis spectrophotometer (PerkinElmer Inc., Waltham, USA) after direct nesslerization [6], formation of the azo dye [7] and according to the ammonium molybdate method after persulfate digestion [8], respectively. NO_3^- was measured by ion chromatography (Dionex 2020i); Cl^- was analyzed according to the argentometric method [9].

Analysis of diatom community

The samples of epilithic diatoms were taken from five stones according to the standard EN 13946 [10]. Diatom samples were treated with concentrated H_2SO_4 and supersaturated solutions of $KMnO_4$ and oxalic acid 11] in the Institute of Botany and Botanical garden “Jevremovac”. The samples were rinsed with distilled water until the pH was 6-7 and were placed on microscopic plates using Naphrax medium. Identification of the diatoms was carried out using a Carl Zeiss AxioImager.M1 microscope with AxioVision 4.8 software and DIC lens at 1600× magnification. Diatoms were determined using the literature [11-

15]. In order to calculate the relative abundance of diatoms growing on stones, 400 valves per sample were counted. According to the indicator values of the identified taxa, diatom indices were calculated in the streams using OMNIDIA 6 software. Three diatom indices, Indice de Polluo-Sensibilité (IPS), Comission for Economical Community (CEE) and EPI-D were used for ecological status assessment, while the TDI was used as an indicator of the streams' trophic state. The same software was used for Shannon-Wiener index calculation.

Data analysis

The data used in this study were monthly values of two different sets: physical and chemical parameters and diatom indices. For the first set, different percentiles were computed using an empirical distribution function with interpolation (MS Excel) method (e.g. the 10th percentile for DO and the 80th percentile for other parameters). For the second data set, basic descriptive statistics were calculated using STATISTICA 6.0. Since the recommended class limits exist only for IPS and CEE in the Serbian legislation, for the EPI-D and TDI indices we used the boundaries from some EU countries as an illustration of their applicability to our river systems [3].

RESULTS

Physical and chemical parameters of water

Physical and chemical water parameters of the Lasovačka and Lenovačka streams showed seasonal variations that are characteristic for small and medium lowland watercourses of the North Temperate Zone. In the Lasovačka stream, the highest water temperature was recorded in July (20.0°C), while in the Lenovačka the maximum was observed one month before (31.2°C). Water was the coldest in January in both streams, 4.5°C and 1.0 °C, respectively. In most samples from the Lasovačka stream, DO was above 8.5 mg/L (Table 1), except in August and September when it was 8.2 mg/L; in the Lenovačka, the maximum was recorded in January (14.6 mg/L) and minimum in June (10.3 mg/L).

Table 1. Water quality parameters in two investigated streams expressed as minimum and maximum values. Extreme and average values of measured physical and chemical water parameters in the Lasovačka and Lenovačka streams are presented.

Parameter	Unit	Lasovačka stream	Lenovačka stream
pH		7.1-8.4 (7.9)	7.1-8.6 (8.1)
T	°C	4.5-20.0 (13.0*)	1.0-31.2 (11.0*)
COND	µS/cm	373-600 (507)	230-900 (647*)
DO	mg/L	8.2-11.8 (9.9)	10.3-14.6 (12.3)
TAN	mg/L	0.00-0.25 (0.11*)	0.02-0.30 (0.23*)
NO_3^-	mg/L	0.0-5.8 (1.8*)	0.4-6.8 (3.3*)
NO_2^-	mg/L	0.00-0.02 (0.01*)	0.00-0.04 (0.01*)
TP	mg/L	0.01-0.64 (0.03*)	0.01-0.15 (0.06*)
Cl^-	mg/L	1.9-9.0 (6.0*)	2.6-36.0 (13.3*)

*The mean/median values are in brackets. If a data set deviated from normality, then the median (marked with an asterisk) was used as a measure of central tendency.

T – water temperature, COND – conductivity, DO – dissolved oxygen, TAN – total ammonia nitrogen, NO3$^-$ – nitrates, NO2$^-$ – nitrites, TP – total phosphorus, Cl^- – chlorides.

When it comes to nitrogen species in the Lasovačka stream, an increased concentration of TAN was recorded at the beginning of summer (0.21 mg/L) and autumn (0.25 mg/L), and in winter (0.21-0.23 mg/L) (Fig. 1A). Nitrates were highest in 2013 and in the first two months of the study (Fig. 1B). In addition,

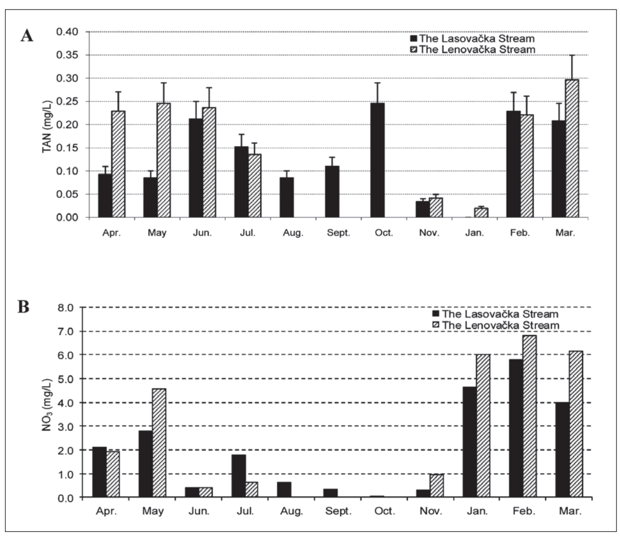

Fig. 1. Seasonal dynamics of nitrogen compounds in two investigated streams. **A** – Measured values of total ammonia nitrogen (TAN) concentrations in water. Error bars indicate uncertainty in measurements (for TAN: ±18%); **B** – Measured values of NO_3^- concentration in water. Error bars indicate uncertainty in measurements (for NO_3^-:11%). Monthly variation of nitrogen species (TAN and NO_3^-) in the Lasovačka and Lenovačka streams from April 2012 to March 2013.

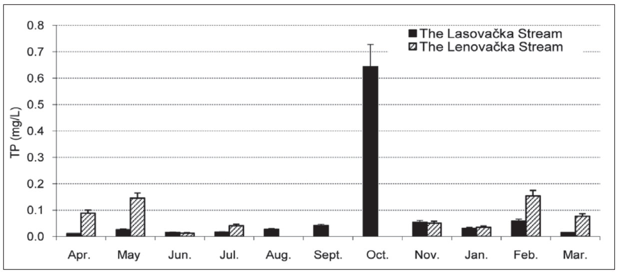

Fig. 2. Changes in total phosphorus (TP) concentrations in two streams during the research. Error bars indicate uncertainty in measurements (for TP: ±13%). Monthly variation of total phosphorus in the Lasovačka and Lenovačka streams from April 2012 to March 2013.

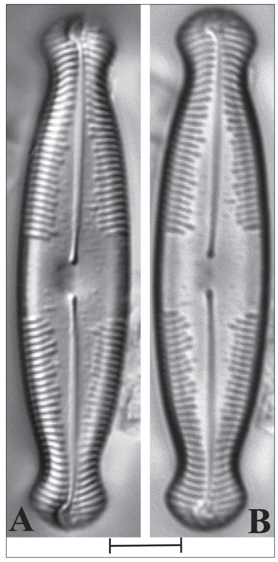

Fig. 3. LM micrograph of *Caloneis budensis* (x1600). **A** – Image taken with Differential Interference Contrast (DIC) microscopy (valve view); **B** – Image taken with Bright Field (BF) microscopy (valve view). Scale bar=10 μm.

the low levels of TP recorded in the first half of the research period were followed by a noticeable peak in October (Fig. 2).

In the Lenovačka stream, the highest concentration of TAN was recorded in March (0.30 mg/L), while the minimum was recorded in January (0.02 mg/L). The NO_3^- patterns for the two streams were similar, although the Lenovačka had mostly higher concentrations of nitrates during the study (Fig. 1B). Finally, the maximum of TP in the Lenovačka stream was in winter (0.15 mg/L).

Diversity of diatoms

Higher species richness was recorded in the Lasovačka stream than in the Lenovačka. A total of 88 different diatom taxa from 38 genera were identified in 11 samples taken from the Lasovačka. The highest number of taxa belonged to the genera *Gomphonema* (9) and *Navicula* (7). A total of 63 taxa from 26 genera were identified in 9 samples from the Lenovačka stream. The highest number of taxa belonged to the genera *Navicula* (8) and *Nitzschia* (7).

During the floristic analysis of diatoms in the Lenovačka stream, *Caloneis budensis* (Grunow) Krammer was recorded. As far as we know, this is the first finding of this taxon in Serbia. *Caloneis budensis* (Grunow) Krammer (Fig. 3); Basionym: *Navicula budensis* Grunow; Synonym: *Pinnularia appendiculata* var. *budensis* (Grunow) Cleve; Heterotypic synonym: *Caloneis macedonica* Hustedt [11] (p. 19).

We recorded a single specimen with lanceolate valve and distinctly wide, capitate apices. Valve length was 36.43 μm, breadth 8.1 μm. The axial area is lanceolate and very narrow at the apices, widening towards the center to form a transverse fascia, wide towards the margin. Raphe is lateral, arched, with external proximal raphe ends dilated. Striae are radiate at the center, becoming convergent towards the apices. Striae are crossed by distinct longitudinal lines on either side of the axial area. Striae number 20/10 μm. *C. budensis* was recorded only in a sample collected in January in conditions of very low temperature, alkaline pH, moderate electrolyte concentrations and high nitrate concentration (Fig. 1B, Table 1).

The composition of the community that developed on gravel in the Lasovačka stream bed showed pronounced seasonal variations. In the Lasovačka stream, the diversity of diatoms peaked in January 2013 (H=3.79) and was minimal in October 2012 (H=1.15). The diversity of diatoms in the Lenovačka showed the highest value in April 2012 (H=3.46), while the minimum was recorded in January 2013 (H=1.93).

When it comes to seasonal dynamics of diatom communities, the two investigated streams showed slightly different patterns of dominant species shifts. In the Lasovačka stream, *Achnanthidium minutissimum* var. *minutissimum* prevailed in April 2012 (30.7%), while in the early summer of the same year *Amphora pediculus* and *Gomphonema tergestinum* were the most abundant diatoms, with more than 30% contribution to the total abundance. In August, more than half of the diatom species growing on stones in this stream belonged to *Rhoicosphenia abbreviata*. In the following months, in autumn and the beginning of winter (January 2013), the epilithic diatom community was almost exclusively dominated by *A. pediculus* (32.3-85.0%). Finally, in the two last winter months of the study, only *Humidophila contenta* and *Meridion circulare* var. *circulare* had more than one-fifth contribution to the total abundance. Similar to the Lasovačka stream, in the Lenovačka in April, individuals of *A. minutissimum* var. *minutissimum* were the most abundant (18.7%), but in this stream *A. minutissimum* var. *minutissimum* was followed by several codominant species: *Navicula reichardtiana* (17.7%), *Nitzschia dissipata* (10.5%) and *N. fonticola* (16.7%). During the remaining study period, *A. pediculus* was the most frequently found diatom in the Lenovačka stream, with 53-71% of the total abundance.

Water quality assessment

The two tributaries of the Grlište Reservoir are not listed in the official guidelines for surface and groundwater bodies [16] because they can be considered small streams. Nevertheless, they are very important as the main water source of the aforementioned drinking water reservoir, and according to their characteristics they can be classified as type 3 water bodies. Another reason why we think that these streams should be classified as type 3 water bodies is because the reservoir was created on the river of type 3 water body.

Assessment based on physical and chemical water parameters

As can be seen in Table 2, the Lasovačka and the Lenovačka streams can be assigned to the third and fourth classes, respectively, based on national legislation [17], which is in compliance with the WFD. Although the Lasovačka and Lenovačka streams could be allocated to a high or good class based on DO, TP and Cl^-, the high nitrogen loading downgraded the quality of the water bodies to a moderate and poor status.

Assessment based on diatom indices

Two diatom indices (IPS and EPI-D) showed a good ecological status (second class), while the CEE indicated first class in both streams (Table 3). In addition, the TDI index pointed to moderate to elevated nutrient levels in the Lasovačka stream (TDI: 52.0-79.3), while in the Lenovačka, the TDI values suggested an even higher trophic status (TDI:64.7-80.8). However, there were some differences in seasonal dynamics between the IPS and the other two indices used for ecological status assessment. While the values of CEE and EPI-D were repeatedly inside the boundaries of one class (CEE – first class, EPI-D – second class), the IPS fluctuated across the upper and lower limits of "good" class.

Table 2. Determination of WFD classes based on the 10th percentile for DO and the 80th percentile for other parameters in two investigated streams. The ecological status assessment of the Lasovačka and Lenovačka streams was based on the physicochemical quality elements.

Parameter	Unit	Lasovačka stream	Lenovačka stream
pH		-*	-*
DO	mg/L	II	I
TAN	mg/L	III	III
NO_3^-	mg/L	III	IV
TP	mg/L	II	II
Cl^-	mg/L	I	I
Final		III	IV

DO – dissolved oxygen, TAN – total ammonia nitrogen, $NO3^-$ – nitrates, TP – total phosphorus, Cl^- – chlorides. Water quality class (WFD): I – high, II – good, III – moderate, IV – poor, V – bad.

* – according to the national legislation I, II, III and IV classes have the same upper and lower pH limits (6.5-8.5), so this parameter was omitted from the assessment of the ecological status because the 80th percentile for pH in both streams fell into this range.

Table 3. Determination of WFD classes based on diatom indices in two investigated streams. Ecological status assessment of the Lasovačka and Lenovačka streams was based on the biological quality elements (diatom indices).

Parameter	Lasovačka stream	Lenovačka stream
IPS	II	II
CEE	I	I
EPI-D	II	II

The classes were based on the average index values for the study period. Ecological status class (WFD): I – high, II – good, III – moderate, IV – poor, V – bad.

IPS – Specific Pollution Sensitivity Index, CEE – European Economic Community Index, EPI-D – Diatom-based Eutrophication/Pollution Index.

DISCUSSION

A detailed one-year analysis of physicochemical parameters and phytobenthic communities was conducted for the first time for two tributaries of the Grliška River. The Lasovačka and Lenovačka streams belong to the type 3 water body, i.e. streams of mean depth at an altitude of up to 500 m [17]. Based on the examined chemical parameters and according to the WFD, the Lasovačka stream had a moderate (i.e. third class water quality), while the Lenovačka stream had a poor ecological status. Although for both streams the concentrations of DO and TP assigned them to second class quality, TAN and NO_3^- concentrations were elevated, which lowered their water quality. Kumar and Oumen [18] state that nitrate increase may be due to an anthropogenic impact, agricultural activity and increased precipitation; since the streams are located in the vicinity of agricultural land, it is possible that this was the reason for the high nutrient load. Finally, the values of the TDI index indicated a nutrient-rich environment in both streams and a higher trophic status for the Lenovačka stream than for the Lasovačka, which was in agreement with the results of chemical analyses of the water. Thus, for further WFD implementation in Serbia this index could be taken into consideration for the assessment of a river's trophic state.

The species recorded in the Lasovačka and Lenovačka streams are widely distributed in freshwater ecosystems [15]. The most numerous taxa of *Gomphonema* and *Navicul*a are common in streams of Europe and Serbia [3,19]. During the study, *C. budensis* was recorded in the Lasovačka River, and in the diatom flora of Serbia for the first time [20]. In AlgeBase it is reported as a taxon with cosmopolitan distribution [21], nowhere found in large numbers [11], and is also regarded as a rare taxon in the German Red List for diatoms [22]. Our finding of *C. budensis* in the Lasovačka stream is in agreement with Bahls [23], who found this taxon in similar ecological conditions.

In order to assess the ecological status of the Lasovačka and Lenovačka streams, IPS [24], CEE [25] and EPI-D [26] indices were used. The calculated indices did not fluctuate so much during the investigated period and were almost continually inside the boundaries of one class. This is in accordance with earlier findings that although climate may affect community structure, diatom indices are not subject to marked seasonal variation, which means that samples can be collected throughout the year 27]. According to Serbian regulations, IPS and CEE indices are used for the assessment of the ecological status and they are indicators of eutrophication and organic pollution. In the Lasovačka and Lenovačka streams, IPS pointed to the second, while CEE to the first class of ecological status. Therefore, both streams had a good ecological status, i.e., they belonged to the second class of ecological status. Based on the chemical parameters, the Lasovačka and Lenovačka streams had a moderate (medium) water quality. The index reference values used in Serbia are taken from other regions, and since environmental factors vary, each region should have its own indicator list that would more accurately reflect the level of contamination of a given aquatic ecosystem [28].

There was a discrepancy between the physicochemical and biological quality assessments in our study. There are at least two main explanations for this. Firstly, it seems that "the established ranges for the physicochemical quality elements are more stringent than is necessary to ensure the functioning of the ecosystem" [29]. However, in our opinion this was not the case. Secondly, the applied and recommended indices are insufficiently sensitive to changes, e.g. water pollution. For instance, in order to assess the ecological status of streams in Serbia, IPS and CEE indices calculated by OMNIDIA software were selected. The characteristics of indicator species inhabiting surface water bodies in Serbia (their sensitivity/tolerance to different pressures) to our knowledge were not taken into account when the aforementioned indices were implemented for the first time in our country. In other words, the list of diatom species with new indicator values (sensitivity and tolerance), refined according to the nutrient content (e.g. total phosphorus) in Serbian waters is still missing. This is one of the reasons why OMNIDIA software and associated diatom indices has only a limited application in Croatia where experts initiated the creation of a national list of diatom species with improved indicator values and their own multimetric diatom-based indices [30]. Our study poses an urgent appeal for further comprehensive work on full WFD implementation in Serbia.

CONCLUSIONS

The Lasovačka and Lenovačka streams belong to third and fourth classes of water quality, respectively. Although for the both streams some chemical parameters (DO and TP) and all diatom indices pointed to the second class, because of the high concentration of nitrogen (TAN and NO_3^-) the quality of the water was reduced. Both streams were characterized by diversified diatom communities, with genera *Navicula* and *Gomphonema* as the most numerous. The presented data increase our knowledge of the ecological status of the Lasovačka and Lenovačka streams; however, further investigations are necessary since diatom flora of southeastern Europe have been insufficiently studied.

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Supplementary Data

Supplementary Fig. S1. Map and location of sampling sites in the two investigated streams.
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Ciric%20et%20al_2840_Supplementary%20Fig.%201S.jpg