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One new species of Aneumastus D.G. Mann et Stickle (Bacillariophyceae) from Krka River, Croatia

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Abstract: Karst rivers in Croatia are common phenomena and known biodiversity hotspots for many different groups of organisms. Due their importance from a natural, cultural and socio–economic point of view, most of them are protected as National Parks. Diatoms in these habitats are not well known, and recently detailed study has been started. During observations of samples from Lake Visovac on Krka River, one *Aneumastus* species with specific morphological features, has been recorded. The species is characterized by linear valves with almost parallel margins in the mid–valve, rostrate apices and striae that are partly biseriate, coarsely punctate and distantly spaced. In this study, it is described as a new species *Aneumastus visovacensis* sp. nov. Its morphology is documented with light and scanning electron microscopy, described in detail, and compared with the most similar species from *A. tusculus* species complex.

Key words: diatom, freshwater, karst river, Lake Visovac, taxonomy

Introduction

The genus *Aneumastus* D.G. Mann et Stickle was established in 1990 (Round et al. 1990) and since then many new species have been described. The first revision of the genus was performed by Lange–Bertalot (2001) with description of four new species and four new combinations. Later, Levkov et al. (2007) described eight new taxa (seven species and one variety) from Lake Ohrid. More recently Kulikovskiy et al. (2012) described nine species from Lake Baikal. Currently, the genus *Aneumastus* includes 42 species (GUIRY & GUIRY 2022), most of which occur in the Northern Hemisphere. There are only few records of this genus in the Southern Hemisphere (e.g. Cocquyt 1998; Moser et al. 1998) and in most cases it is rare. Species of the genus *Aneumastus* mostly occur in oligotrophic, calcareous waters, but few species might tolerate higher levels of trophy and salinity such as *A. rostratus* (Hustedt) Lange–Bertalot, *A. minor* Lange–Bertalot, *A. balticus* Lange–Bertalot and *A. maculosus* (Donkin) Lange–Bertalot Lange–Bertalot (2001). Many species of this genus are considered as endemic for ancient lakes such as Ohrid, Baikal and Hövsgöl. Molecular analyses of the species from Lake Ohrid showed that most of the endemic species constitute a monophyletic group and probably represent a species flock (Stelbrink et al. 2018). These findings were supported by fossil record where most of the species occurred almost at the same time in the core, indicating a process of in situ speciation due to environmental changes (Cvetkoska et al. 2021). Very likely the same radiation process happened in Lake Baikal, where morphologically similar species inhabit the lake (see Kulikovskiy et al. 2012).

In general, the genus *Aneumastus* might be divided into two main groups based on the structure of striae/areolae: 1) species with entirely uniseriate striae and 2) species with partly biseriate striae near the valve margin. Interestingly, species from both groups can be found sympatric in the ancient lakes and other calcareous freshwater habitats. There is one species, *A. aksaraiensis* Spaulding, Akbulut et Kociolek (in SPAULDING et al. 2003) with entirely biseriate striae, except the areolae bordering raphe which are slit–like. The position of latter species in the genus *Aneumastus* is not clear, and molecular analyses might provide additional support for its phylogenetic position.

Aneumastus species have been infrequently recorded on the Balkan Peninsula. Beside Lake Ohrid and Prespa (N. Macedonia, LEVKOV et al. 2007), there is a single record in Serbia (TRBOJEVIĆ et al. 2019) and in Croatia (PLENOVIĆ–MORAJ 1995). In the previous research on diatoms from Krka River two taxa have been recorded as *Navicula pseudotuscula* Hustedt and *N. tuscula* (Ehrenberg) Grunow (PLENOVIĆ–MORAJ 1995). More recently, diatom studies on Krka River reveal existence of at least four taxa and one of them possesses distinct morphological features than already known species and here is described as *Aneumastus visovacensis* sp. nov.

MATERIAL AND METHODS

Study area. The Krka River is a karst river, located in the Dinaric region of Dalmatia, Croatia. The river is characterized by a strong interconnection of surface and groundwater. The chemistry of groundwater is influenced by lithology, with varying degrees of karst formation and complex groundwater pathways (CUKROV et al. 2007). The Krka River is characterized by tufa formations – an unusual phenomenon: secondary calcium carbonate deposits, which are the result of calcium carbonate precipitation and biotic activity (PRIMC–HABDIJA & MATONIČKIN 2005; VILENICA et al. 2018). A lentic dilatation of the Krka River is Lake Visovac, which also belongs to a group of karstic barrage lakes (GLIGORA UDOVIČ et al. 2017). The lake is located in the lower course of the river with a maximum depth of 25 m, and surface area of 572 km^2. Lake Visovac is a monomictic system formed by the formation of the Skradinski fluvial travertine barrage. Lake Visovac has a shorter water residence time than other lakes in the Mediterranean region, creating a unique aquatic system. Due to the lack of morphometric data for the volume of Lake Visovac, the residence time of the water was not calculated. Previous studies have confirmed that the residence time is postively related to plankton density (ŠPOLJAR et al. 2005). According to the bathymetric characterization of the lake, the water level of 50% retention in Lake Visovac was 45.57% (MARASOVIĆ & JEFTIMIJA 2021). In the middle of Lake Visovac is Visovac Island, which is one of the most important natural and cultural treasures of Croatia. Since 1445, the Franciscan Monastery of Our Lady of Mercy and the Church of Our Lady of Visovac have been located here, so throughout the turbulent Croatian history, the island of Visovac has remained an island of peace and prayer. The Franciscan monastery is also a fortress of spirituality and faith, and one of the most important foundation stones for the survival of Croats and the preservation of Croatian national identity (GLIGORA UDOVIČ et al. 2011).

Field and laboratory procedures. The samples were collected on the right coast at the Visovac Island (Fig. 1) from available substrates (stones, sand, macrophytes, surface sediment) during May 2017. Surface sediment samples on depth larger than 0,5 m were collected with Van Veen dredge. Samples from stones and tufa were scrubbed using tooth–brush and rinsed with sampled water. Collected materials were then stored in plastic containers and preserved with 2–4% formaldehyde. For the physico–chemical analysis of water in situ measurements of water temperature, pH, conductivity, oxygen concentration and saturation were done with a portable multimeter (Hach HQ40d, Germany). For water chemistry, the results of other parameters were downloaded from Croatian national standard monitoring from the Croatian Waters Institution. Water chemistry samples were collected on May 24, 2017, on standard monitoring point of Lake Visovac, 30 m southeast (SE) of Visovac Island.

Diatom samples were treated with $KMnO_4$ and 37% HCl and left overnight to remove carbonates. $KMnO_4$ was added to oxidise the organic matter and the samples were boiled at 80 °C for 30 min. Distilled water was used to rinse the samples several times and then centrifuged. Diatom slides were prepared using Naphrax as mounting medium. Slides were observed under oil immersion at 1000× magnification with a Nikon Eclipse 80i microscope, equipped with Nikon Coolpix

Table 1. Environmental parameters of Lake Visovac during investigated period in May 2017. (BOD_5= biological oxygen demand, COD–Mn = chemical oxygen demand, TP = total phosphorus, TN = total nitrogen, TOC = total organic carbon, DOC = dissolved organic carbon).

Env. parameters	**May 2017**
Chl–a (μg.l^{-1})	2.52
Secchi (m)	7
Temperature (°C)	16.1
pH	8.1
Conductivity (μS.cm^{-1})	554
Suspended matter (mg.l^{-1})	1
Alkalinity ($CaCO_3$– mg.l^{-1})	200
TH ($CaCO_3$– mg.l^{-1})	285.9
Turbidity (NTU)	1.2
DO (O_2– mg.l^{-1})	10.2
Saturation DO (%)	104.2
COD–Mn (O_2– mg.l^{-1})	1.1
BOD_5 (O_2– mg.l^{-1})	0.8
Ammonia (N– mg.l^{-1})	0.004
Nitrites (N– mg.l^{-1})	0.004
Nitrates (N– mg.l^{-1})	0.19
TN (mg.l^{-1})	0.69
TP (mg.l^{-1})	0.013
TOC (C– mg.l^{-1})	0.59
DOC (C– mg.l^{-1})	0.68
SiO_2 (mg.l^{-1})	3.31

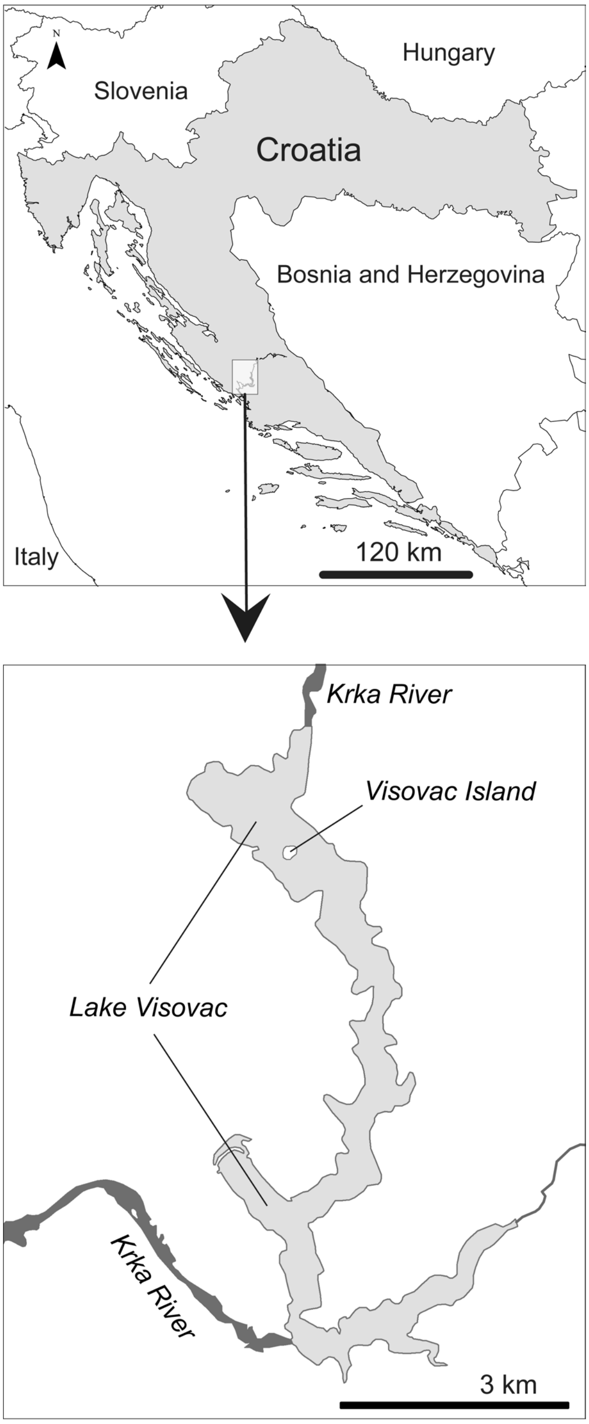


Fig. 1. Map of Krka River with location of the type locality.

P6000 digital camera. For scanning electron microscopy (SEM), the material was prepared by drying clean diatom suspension onto cover slips that were carbon tape attached to the SEM stubs and coated with gold–palladium (Polaron SC7640 sputter coater, Quorum Technologies, Ashford, UK). SEM observations were performed using a Cambridge S4 Stereoscan at 10 kV (Cambridge Instruments Ltd, Cambridge, UK) at the Friedrich Hustedt Study Centre for Diatoms (BRM), Bremerhaven, Germany and FE–SEM Mira 3 Tescan (Tescan MIRA3, Brno, Czech Republic) at 4 kV at Institute of Oceanography and Fisheries (IOR), Split, Croatia.

Results

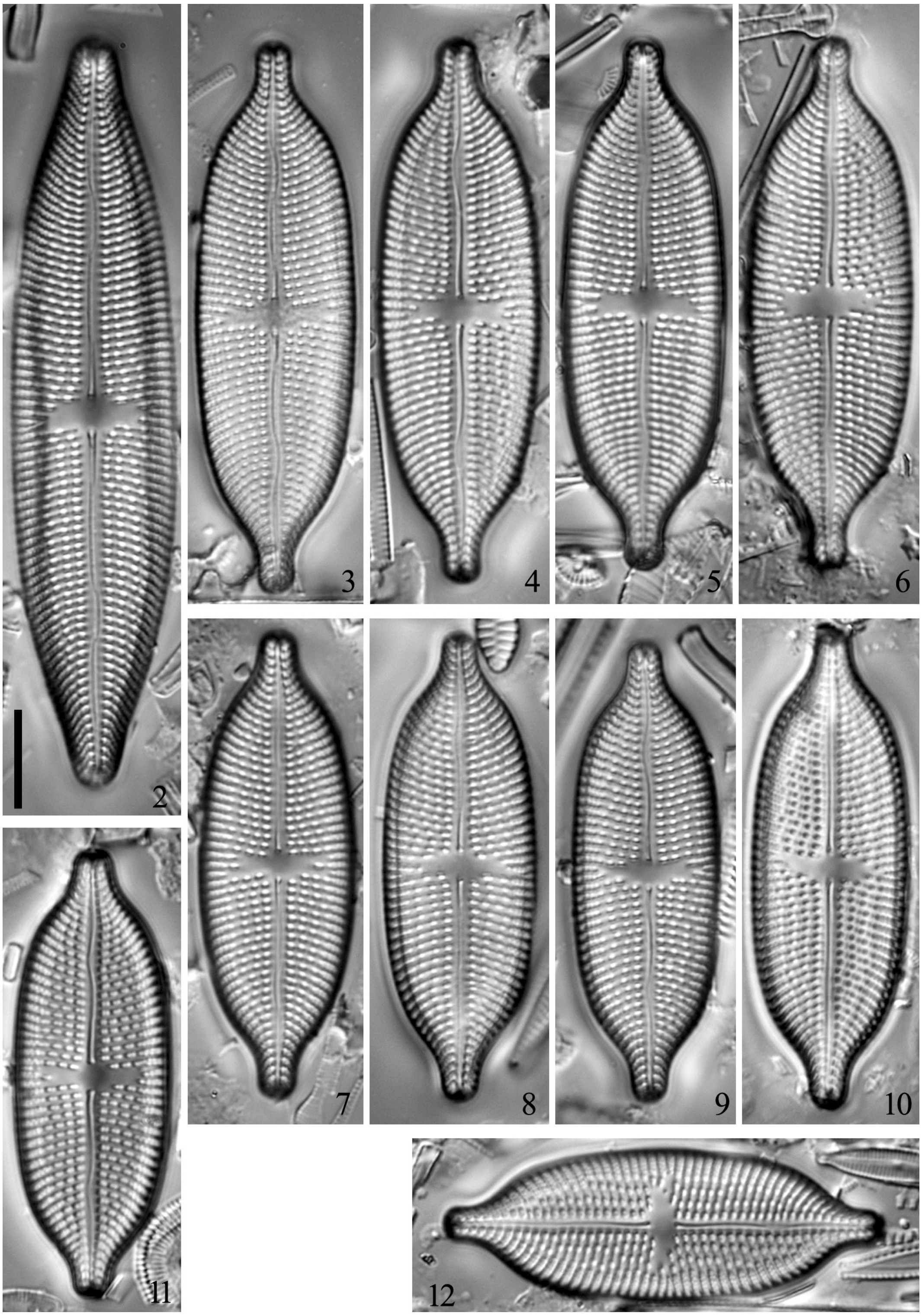
Aneumastus visovacensis **Gligora Udovič et Levkov sp. nov. (Figs 2–12: LM, Figs 13–19: SEM)**

Light microscopy (Figs 2–12): Valves linear to linear–lanceolate with almost parallel margins in mid–valve. Valve ends protracted, abruptly short–rostrate, in post–initial cells valves weakly protracted and not clearly separated from valve body. Valve length 44–77 µm, valve breadth 15–17 µm. Axial area narrow, linear. Central area moderately large, irregularly rectangular by two to three shortened striae at each side. Raphe weakly lateral with two slight undulations of the outer fissure of both raphe branches. Striae radiate throughout, 10–12 in 10 µm (usually 11 in 10 µm).

Scanning electron microscopy (Figs 13–19): Striae near raphe uniseriate composed of large elongated, bone–shaped or X–shaped areolae, becoming biseriate near valve margin and composed of small, round areolae (Figs 13–17). Areolae adjust axial area smaller and lunate (Figs 13, 15, 17). Areolae complex, occluded, opened into deep pits via sieves of small pores (Fig. 15). Raphe is undulate with proximal raphe ends slightly dilated and unilaterally deflected. Distal raphe ends hooked and extended onto valve mantle (Fig. 16). Girdle bands open, valvocopula bears single row of pores, other bands with two rows of very small round pores (Fig. 16). Internally, raphe is very narrow linear with indistinct and straight proximal raphe ends (Figs 18, 19). Distally, raphe terminates with helictoglossae (Fig. 18). Areolae in striae irregularly positioned giving the impression of longitudinal lines (Fig. 18). Striae openings round to ellipsoidal towards near axial area becoming elongate towards valve margin. Sieves of small pores present within striae (Fig. 19). Small pseudoseptum present at pole (Fig. 18).

Holotype: Slide HRNDC000046, Croatian National Diatom Collection, Faculty of Natural Sciences, University of Zagreb, Croatia.
Isotype: Slide MKNDC 011209, Macedonian National Diatom Collection, Faculty of Natural Sciences, Ss. Cyril and Methodius University, Skopje, N. Macedonia.
Type locality: Krka River, Lake Visovac, near Monastery, (43.862463 N, 15.978887 E), surface sediment collected on 27th May 2017 by Z. Levkov & M. Gligora Udovič.
Etymology: the specific epithet *visovacensis* refers to the name of the Visovac Island from where this species is described, as Visovac Island represents important natural and cultural treasures of Croatia.

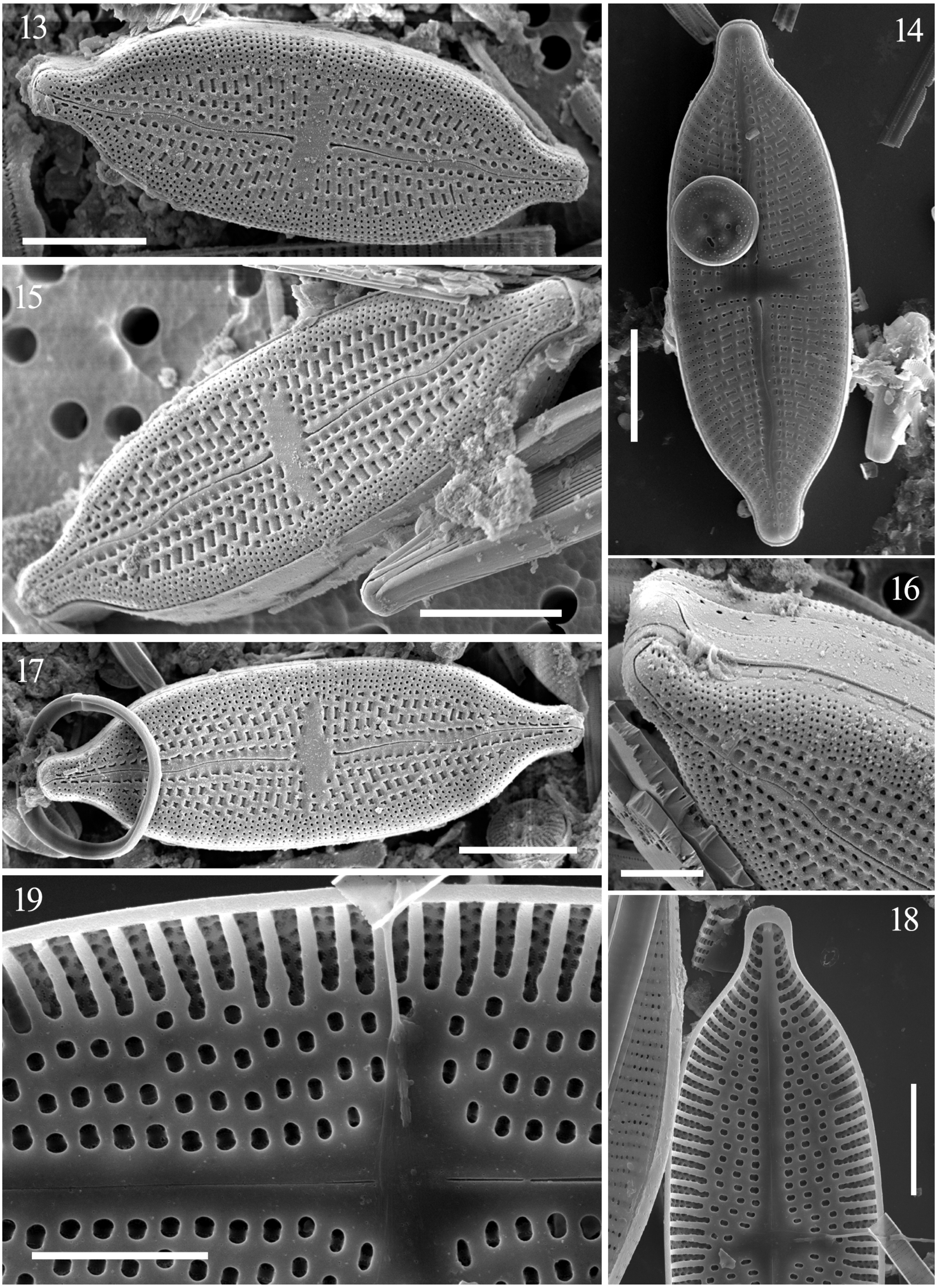
Ecology: The type locality is characterized by relatively high conductivity (517 µS.cm^{-1}) due to the presence of dissolved carbonates, high oxygen concentration (11,46 mg.l^{-1}) and oxygen saturation (125.2%), high pH values (8.42) and moderate temperature (19.7 °C). Main physicochemical parameters of Lake Visovac are given in Table 1.



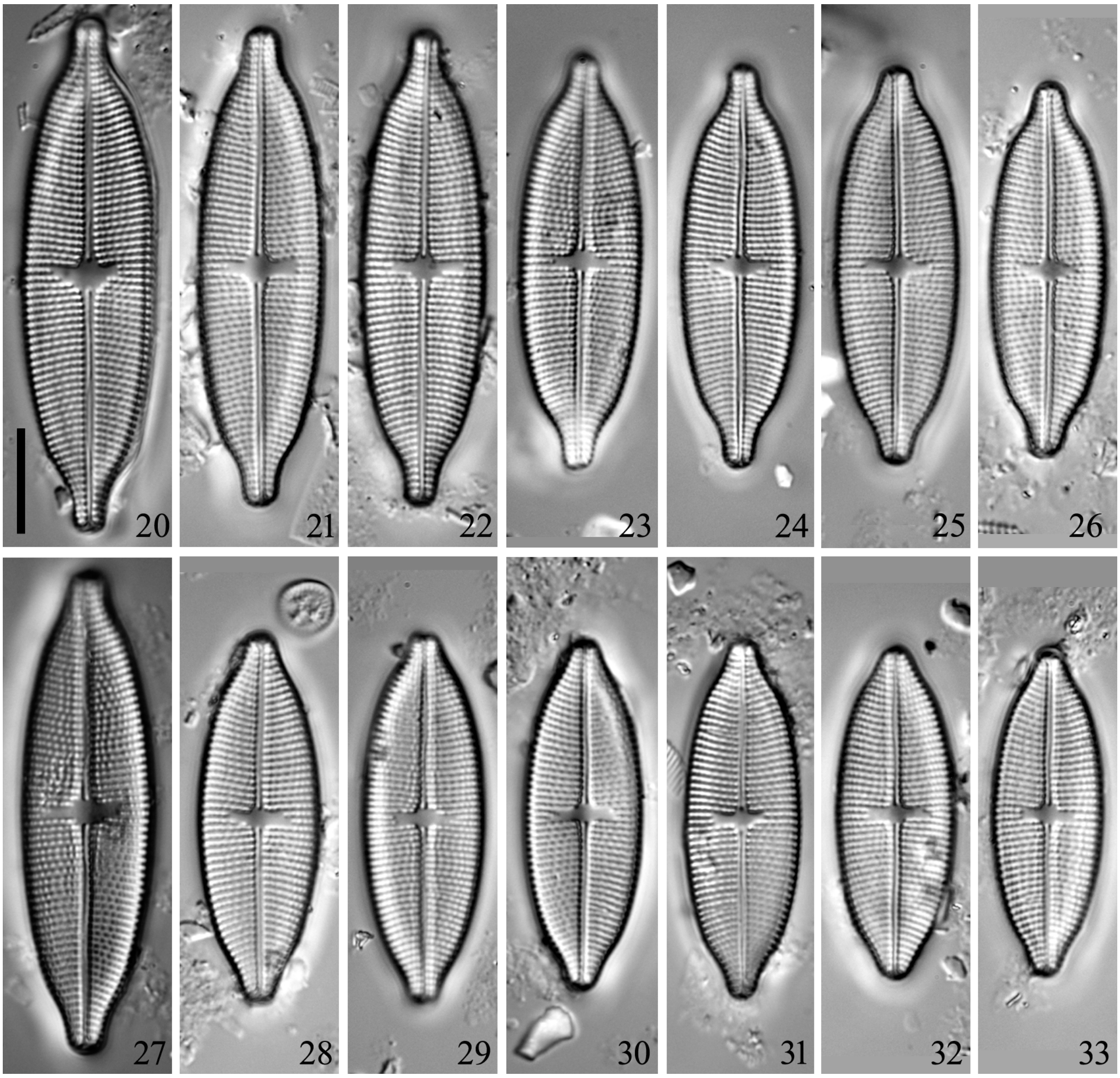
Figs 2–12. LM photographs of *Aneumastus visovacensis* Gligora Udovič et Levkov sp. nov. type population (Acc. No. HRNDC000046) showing the size range of the species. Scale bar 10 µm.

Other species present in the type material are *Cyclotella ocellata* Pantocsek, *Encyonopsis subminuta* Krammer et E.Reichardt, *Cyclotella distinguenda* Hustedt, *Cymbella subhelvetica* Krammer, *Fragilaria perdelicatissima* Lange–Bertalot et Van de Vijver, *Cymbella parva* (W.Smith) Kirchner, *Pseudostaurosira brevistriata* (Grunow) D.M.Williams et Round and *Asterionella formosa* Hassall.

According to HANŽEK et al. (2021) the assessment of the phytoplankton ecological status based on the morphological approach and eDNA metabarcoding revealed a good ecological status of Lake Visovac.



Figs 13–19. SEM photographs of *Aneumastus visovacensis* Gligora Udovič et Levkov sp. nov. type population from sample Acc. No. HRNDC000046: (13–15, 17) external view of the whole valve; (16) detail of the valve apex showing the distal raphe endings, open girdle bands and valvocopula bearing a single row of large pores; (18, 19) internal valve view showing the proximal raphe ends and complex areola structure. Scale bar 10 μm (13–15, 17, 18), 5 μm (16, 19).



Figs 20–33. LM photographs of two morphodemes of *Aneumastus stroesei* from Krka River: (20–26) morphodeme with distinctly protracted and subcapitate and (27–33) slightly protracted apices.

According to the trophic status class boundaries (OECD 1982, BRETTUM 1989) and according to the proposed trophic status class boundaries in Lake Visovac using Chlorophyll–a concentration for rapid bioassessment on a weekly basis (ŠIMUNOVIĆ et al, in press), Visovac Lake is assessed as mesotrophic.

DISCUSSION

Aneumastus visovacensis belongs to the species complex around *A. tusculus* (group 2). The latter species was observed and typified by LANGE–BERTALOT (2001) based on original material of EHRENBERG (1840) from Santa Fiora, Italy. However, in his treatment of *A. tusculus*, LANGE–BERTALOT (2001, fig 113: 5–9) illustrated several other valves from other localities that probably do not belongs to *A. tusculus* sensu stricto (MALTSE et al. 2019). *Aneumastus visovacensis* slightly resembles *A. tusculus* sensu LANGE–BERTALOT pro parte (2001, fig. 7) having linear valves with almost parallel valve margins and shortly rostrated apices. It is not known the origin (locality) of this specimen. However, clear differences between *A. tusculus* sensu stricto and *A. visovacensis* might be noticed in the valve shape (broadly elliptical with strongly convex valve margins in *A. tusculus*), proximal raphe ends (hook–shaped in *A. tusculus*) and *Aneumastus mongolotusculus* Maltsev, Andreeva et Kulikovskiy recently described from Lake Hövsgöl, Mongolia (MALTSEV et al. 2019, figs 1–16), have similar valve size and stria structure as *A. visovacensis*. It is characterized by broadly linear–elliptic valves, 42–61 µm long, 16–19 µm wide with 21–25 striae in 10 µm. Differences between *A. mongolotusculus* and *A. visovacensis* can be noticed in the valve shape (broadly linear–elliptic valves with strongly convex margins in *A. mongolotusculus*) and stria density (21–25 in 10 µm vs. 10–12 in 10 µm).

Aneumastus tusculus f. *angulatus* (Hustedt) Lange–Bertalot has broadly elliptic valves with undulate margins and distinctly capitate valve. These features make differentiation of *A. visovacensis* and *A. tusculus* f. *angulatus* straightforward. *Aneumastus maculosus* have similar valve shape (linear–lanceolate with almost parallel margins) as *A. visovacensis*, but differences can be noticed in the striae morphology (composed of 2–3 elongated areolae) and shape of the central area (elliptical to round). *Aneumastus rostratus* (Hustedt) Lange–Bertalot is frequently observed in calcareous rivers with higher electrolyte content (Hofmann et al. 2018). It is characterized by elliptic–lanceolate valves with rostrate apices, relatively small, irregularly–shaped central area and strongly radiate striae, and valve size comparable with *A. visovacensis* (length 35–65 µm, width 16–20 µm). Main differences between these two taxa might be seen in the valve shape and the size and shape of the central area.

In general, group 2 (species with partly biseriate striae) is less diverse than group 1 (species with entirely uniseriate striae). *Aneumastus macedonicus* Levkov (in Levkov et al. 2007), has rhombic–lanceolate valves with shortly protracted and narrowly to acutely rounded apices and small round to elliptical central area. These features easily differentiated it from *A. visovacensis*.

Aneumastus visovacensis might be easily differentiated by the species from group 1 (entirely unisieriate striae) by the stria structure. In few samples from Krka River, *A. visovacensis* was observed together with *A. stroesei* (Østrup) D.G.Mann. Beside differences in the stria structure, both taxa can be differentiated by the valve shape (elliptic with weakly protracted and broadly rounded valve ends in *A. stroesei*), stria density (14–16 in 10 µm). Lange–Bertalot (2001), beside lectotype specimen (op. cit, fig. 1) illustrated several other valves from various localities that might represent different species. Differences in the valve shape and size of *A. stroesei* sensu lato have been also observed in Krka River, where two morphodemes might be differentiated: first with distinctly protracted and subcapitate apices, (Figs 20–26) and the second with shortly protracted and narrowly rounded apices (Figs 27–33). However, *A. visovacensis* can be easily distinguished from *A. stroesei*, but the shape, stria morphology and stria and areola density. *Aneumastus humbodtianus* Lange–Bertalot et Miho (in Lange–Bertalot 2001) has comparable shape and size as *A. visovacensis*, but differences between these two species can be noticed in the stria structure (unseriate in *A. humboldtianus*) and areola density.

Comprehensive study on biogeography of genus *Aneumastus* was given in Glushchenko et al. (2017). Most of the species within genus are known from Holarctic. Some recent findings of rear species in South Hemisphere (Mosser et al. 1995; e.g. Cocquyt 1998; John 2012) require additional examination. Some species described from Holarctic region are widely distributed, including *A. tusculus, A. stroesei* and *A. minor* from Krka River. Nevertheless, many species of *Aneumastus* have restricted distribution such as those endemic described only in ancient lakes Baikal, Ohrid and Prespa. Distribution of some species are also restricted to fossil deposits, North America, Mongolia, and Turkey. Recently studies on diatom diversity on Balkan Peninsula indicated distribution of *A. visovacensis* only in Lake Visovac.

Based on German Red List (Hofmann et al. 2018), ecological groups within genus *Aneumastus* in Europe encompass species in oligotrophic calcareous habitats, halophil or halobiont and species preferring mesotrophic to polytrophic ecological conditions. Within seven species of genus *Aneumastus* in Europe, three of them inhabit Krka River: *Aneumastus tusculus* and A. *stoesei* prefer oligotrophic habitats on carbonate substrata and *A. minor* prefer mesotrophic to polytrophic conditions. Hofmann et al. (2018) indicating three "rear" and "endangered" species within genus *Aneumastus* in Europe, from which two species are found in Krka River. Conservation status and rarity of species within genus *Aneumastus* emphazies the importance of protection of fragile ecosystem of karst Krka River. In the Red list of Hungary (Németh 2005) several *Navicula* sensu lato species are listed, but none of them belong to the genus *Aneumastus*. Also, in the Red Data Book of Estonia, *Aneumastus* species are not included (Lilleleht et al. 2008). However, in the Red list algae in Poland (Siemińska et al. 2006) four species (*A. albanicus* Lange–Bertalot et Miho, *A. balticus* Lange–Bertalot, *A. stroesei* and *A. tusculus*) are listed and all of them are considered as rare species.

A new species *A. visovacensis* described herein was so far found only in sediments of Lake Visovac on the Krka River. However, some additional observations of samples from other karstic rivers (e.g. Plitvice/Korana) might reveal its existence in other sites in Croatia and Bosnia and Hercegovina. For instance, *Planothidium fonticolanceolatum* (Lange–Bertalot et Schimanski) Lange–Bertalot (in Lange–Bertalot & Krammer 1989) has been recorded only from karstic rivers in this region. These rivers are considered as vulnerable and fragile karst systems (Šiljeg et al. 2020), but also biodiversity hotspot with many newly described and potentially endemic species (e.g. Gligora et al. 2009; Gligora Udovič et al. 2018, 2022). On the other side, these habitats are under various climate and anthropogenic pressures such as droughts, hydrological, (dam construction), abstraction of surface and groundwater, water pollution and invasive species (Gligora Udovič et al. 2022). In this moment, *A. visovacensis* is considered as a rare species and potentially endemic.

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