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ABSTRACT

During a survey of diatoms in the artificial sandpit lake "Peskara" in the protected zone of the Rusanda Nature Park, a large number of brackish taxa at epipsamic and epiphytic communities were recorded (e.g. *Bacillaria paxillifera, Caloneis permagna, Ctenophora pulchella, Entomoneis costata. Haslea duerrenbergiana, Navicula salinarum, Nitzschia agnita, N. filiformis, Staurophora brantii, Seminavis strigosa, Tryblionella apiculata*). One new genus *Seminavis* and 15 diatom species were noted for the first time for the diatom flora of Serbia. Dominant taxa changed over seasons without any regularity. In spring 2021, we have observed an increased abundance of centric species *Thalassiosira pseudonana* and *Stephanodiscus parvus* while in other seasons it was difficult to discern a pattern of dominance among benthic diatoms. Metabarcoding analysis of the same sample using 18S rRNA as a gene marker confirmed the dominance of centric diatoms. This study contributes to a better understanding of brackish diatom taxa found in inland habitats, many of which can be utilized as useful tools for the ecological status assessment.

Introduction

During the recent period, the number and the size of sand- and gravel-pit lakes have increased due to the increasing demand for building material. After the exploitation of materials by digging, the depressions usually gradually fill up with groundwater thus creating artificial lakes most frequently used for recreation or as commercial fishponds (Çelik 2002). Moreover, the constant presence of water maintained by the inflow of groundwater and/or surface runoff leads to the development of a stable aquatic biota (Damnjanović et al. 2019). One of the most common organisms in this type of aquatic environment are diatoms. Diatoms, silicate-shelled microalgae, are one of the most successful groups of photosynthetic, eukaryotic microorganisms which can colonize different substrates, e.g. stones, sand, sediment and aquatic vegetation (Round et al. 1990; Townsend and Gell 2005). Factors that dominantly affect the structure of diatom communities are conductivity and mineralization (Potapova and Charles 2003). Increased conductivity caused by higher concentration of different cations (e.g. sodium) and anions (for example, carbonates, chlorides, etc.) creates favourable conditions for the development of species that are characteristic for marine, brackish and coastal waters. Different studies showed that brackish areas formed naturally or anthropogenically are inhabited by a highly diverse diatom flora (Stenger-Kovács et al. 2014; Stenger- Kovács and Lengyel 2015; Żelazna-Wieczorek et al. 2015; Vidaković et al. 2019, 2020; Heudre et al. 2021; Ćirić et al. 2021a).

Sand- and gravel pit lakes are usually located in river floodplains and are generally isolated, shallow water bodies. They are characterized by high biodiversity, and diatoms are one of the most dominant taxa (Tavernini et al. 2009). Sandpit lake "Peskara" is atypical in this regard, because it is not positioned near a river, but is instead located between two shallow saline lakes. Therewith, data about organisms are scarce (Ćirić et al. 2021b; Tomović et al. 2022). The aim of this study was to investigate the diatom community from this sandpit lake with a focus on brackish species. We also made a comparison between morphological (light microscopy) and molecular identification methods. This paper provides a baseline for future research of diatom ecology.

Material and methods

Study site and sampling

Sandpit lake "Peskara" was created after commercial sand excavation and is located inside the Rusanda Nature Park, positioned between the two lakes –
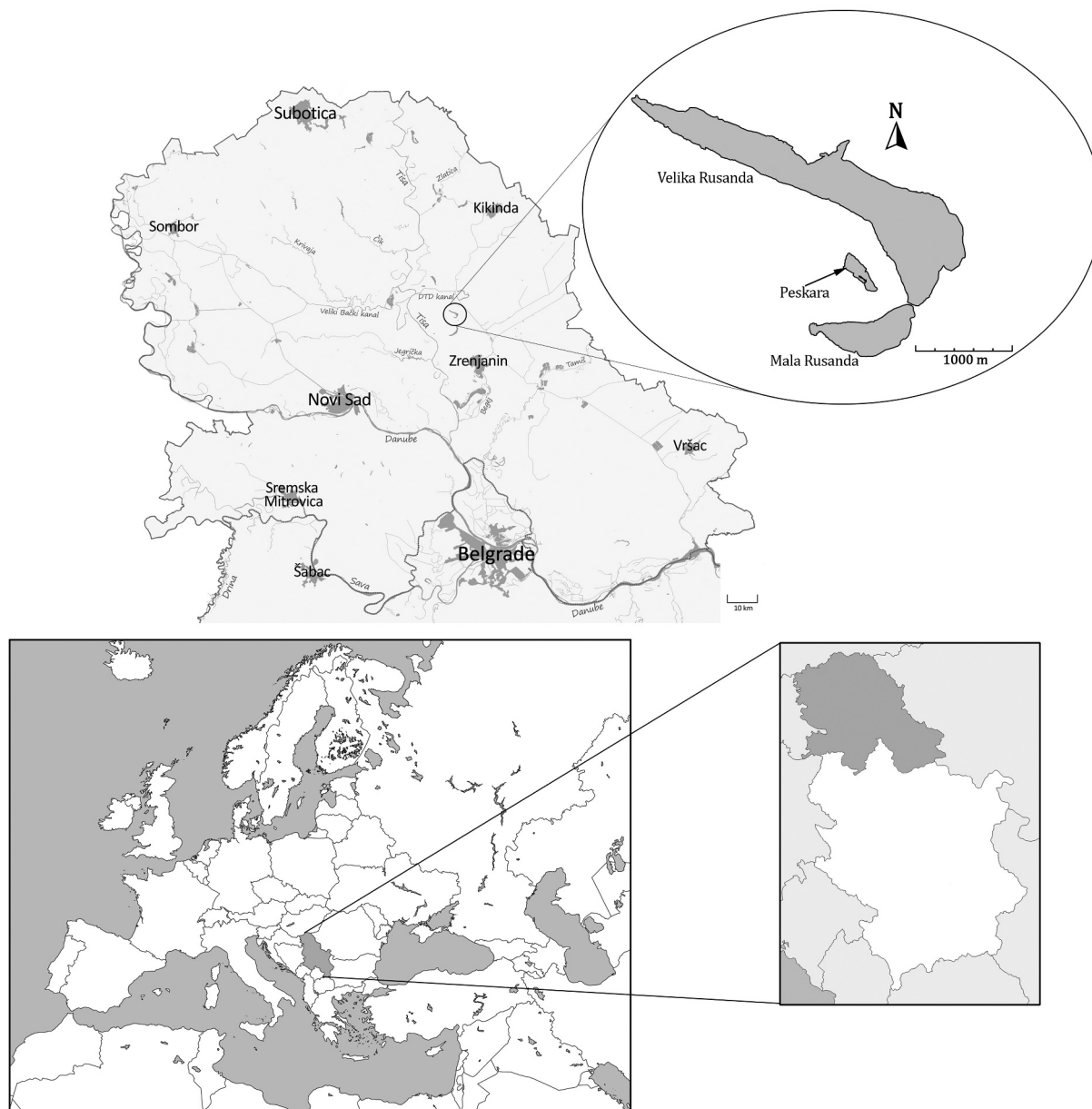
Figure 1. Location of the sampling site in the artificial sandpit lake "Peskara".

Lake Velika Rusanda and Lake Mala Rusanda (Figure 1). The sandpit lake is around 205 m long, 107 m wide, with an average depth of 4.4 m and filled with subsaline, alkaline water rich in sodium and bicarbonate ions (Ćirić et al. 2021b).

Samples for water and benthic diatom analysis were collected from the shore of the sandpit lake "Peskara" from October 2019 to April 2021 (6 sampling, 12 samples). Diatoms were collected from the sand (epipsamic) and reed (epiphytic). Samples collected in April 2021 were divided into those for morphological analysis and those for 18S rRNA metabarcoding analysis.

Measurement of physicochemical parameters

Several parameters were monitored in situ: water temperature (T), pH, and conductivity (COND) were measured using Water Multimeter 18.52.01 (Eijkelkamp

Agrisearch Equipment, Giesbeek, the Netherlands), while the dissolved oxygen (DO) was measured with a DO meter HI9147 (Hanna Instruments, Woonsocket, the USA). The determination of metal cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) was done by inductively coupled plasma optical emission spectrometry (ICP-OES) using Thermo Scientific iCAP 6500 Duo ICP (Thermo Fisher Scientific, Cambridge, the UK). Ammonia concentration (NH4+, SRPS EN ISO 14911:2009), nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}) (U.S. EPA 1997), total phosphorus (TP, SRPS EN ISO 6878:2008), total nitrogen (TN, SRPS EN 12260:2008), carbonates (CO_3^{2-}) and bicarbonates (HCO_3^-) (APHA, AWWA and WPCF, 1995a), chloride (Cl^-, APHA, AWWA and WPCF 1995b) and sulfate (SO_4^{2-}, APHA, AWWA, and WPCF 1995c) were measured in the laboratory. Salinity was calculated according to the formula given by Boros et al. (2013).

Morphological analysis

To remove organic material from diatom cells, samples were treated with $KMnO_4$ and hot conc. HCl (Taylor et al. 2007). Cleaned diatom frustules were mounted with Naphrax (Brunel Microscopes Ltd). Slide observations were performed using the light microscope Zeiss AxioImagerM.1 with DIC optics and AxioVision 4.9 software. The abundance was estimated by counting 400 valves per slide. Micrographs were digitally manipulated, and plates were created using Adobe Photoshop CS6.

Metabarcoding analysis

18S rRNA metabarcoding analysis was conducted only on epipsamic sample from April 2021.

DNA extraction, PCR amplification and NGS sequencing

The total DNA from each sample (three samples) was extracted using the power Soil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) and pooled into one sample. DNA concentrations were quantified using Qubit 4 Fluorometer (InvitrogenTM), and the sample was sent for sequencing to the Novogene Sequencing Service (Cambridge, UK). The regions V4-V9 of the 18S rRNA gene were amplified using specific primers (18S V4: 528 F-706 R – 5'-GCGGTAATTCCAGCTCCAAT AG-3', 18S V9: 1380 F-1510 R – 5'-CTCTGACAA TGGAATACGAATA-3') with the barcode. PCR conditions included an initial denaturation at 94°C for 2 min, then 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, elongation at 72°C for 1 min and a final elongation at 72°C for 10 min (Visco et al. 2015). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The libraries were generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina and quantified via Qubit and Q-PCR. Sequencing was performed on a NovaSeq Sequencer (PE250, 100 K tags per sample, Q30 ≥ 75%) following the metagenomic sequencing library preparation Illumina protocol.

Sequence data processing

After quality check and primer removal, DNA reads were performed using the DADA2 Pipeline (Callahan et al. 2016). Taxonomy assignment was performed using the library 2021–06-25-Diat.barcode-release-version 10.1. xlsx (Rimet et al. 2018). Taxon assignment was performed using IDTAXA (Murali et al. 2018) with default options used for classification up to the genus level. Species-level annotation was obtained using the species assignment set generated from the above mentioned Diat barcode 10.1. In addition, amplicon sequence variants, with high abundance and ambiguous taxonomy assignment, were annotated based on the BLAST best hit in the National Center for Biotechnology Information (NCBI) nucleotide database (100% identical matches). All amplicon sequence variants not assigned to diatom algae were removed from further processing. Alignment of sequences was performed for 10 iterations and 10 refinements using DECIPHER R package (Wright 2015), while phylogeny inference was performed using FastTree 2.1.10 (Price et al. 2010). The phylogeny is presented as an unrooted tree along with a percentage heatmap. Sequence diversity within samples (alpha diversity) was estimated using the phyloseq R package (McMurdie and Holmes 2013) at the ASV level after rarefaction to even depth (sample with the lowest number of reads). Only taxa above 0.1% abundance are presented in the final diatom taxonomic list.

Results

Physico-chemical parameters

Artificial sandpit lake "Peskara" is characterized by the constant presence of water throughout the year. During the study, water temperature varied from 6.7°C in the winter 2019 to 25.1°C in the summer 2020. The measured pH values were always around 9. The conductivity was relatively high, between 1860 and 1952 µS/cm and according to Hammer's (1986) salinity classification water can be characterized as subsaline (1.5–1.6 g/L). Among all ions, sodium was the most dominant with the maximum concentration of 428.3 mg/L in May 2021. The data for anions showed the dominance of bicarbonate with the maximum concentration of 1030.9 mg/L in March 2021 (Supplementary Data 1).

Diatom community

During observation of the samples collected from the sand and reed from 2019 to 2021, 139 diatom taxa belonging to 47 genera were recorded (Supplementary Data 2). As many as 16 diatom species (*Diploneis calcicolafrequens, Entomoneis costata, Fragilaria vauchaeraetica, Gomphonema campodunense, G. jadwigiae, Haslea duerrenbergiana, Luticola rotunda, Nitzschia bergii, N. costei, N. perspicua, Rhoicosphenia lacustris, Staurophora brantii, Seminavis strigosa, Thalassiosira pseudonana,* and *Ulnaria grunowii*) were noted for the first time for the diatom flora of Serbia (marked with asterisk in Supplementary Data 2 and presented in Figure 2). The genus *Seminavis*, represented by *S. strigosa*, was recorded for the first time. Of the total number of identified taxa, more than 30 are characteristic of brackish waters: *Bacillaria paxillifera, Caloneis permagna, Ctenophora pulchella, Cylindrotheca gracilis, Entomoneis paludosa, Halamphora dominici, H. coffeaeformis, H. kevei, Haslea spicula, Mastogloia elliptica, Navicula erifuga, N. rostellata, N. salinarum, N. vandamii, N. veneta, Nitzschia agnita, N. dubia, N. filiformis, N. frustulum, N.*
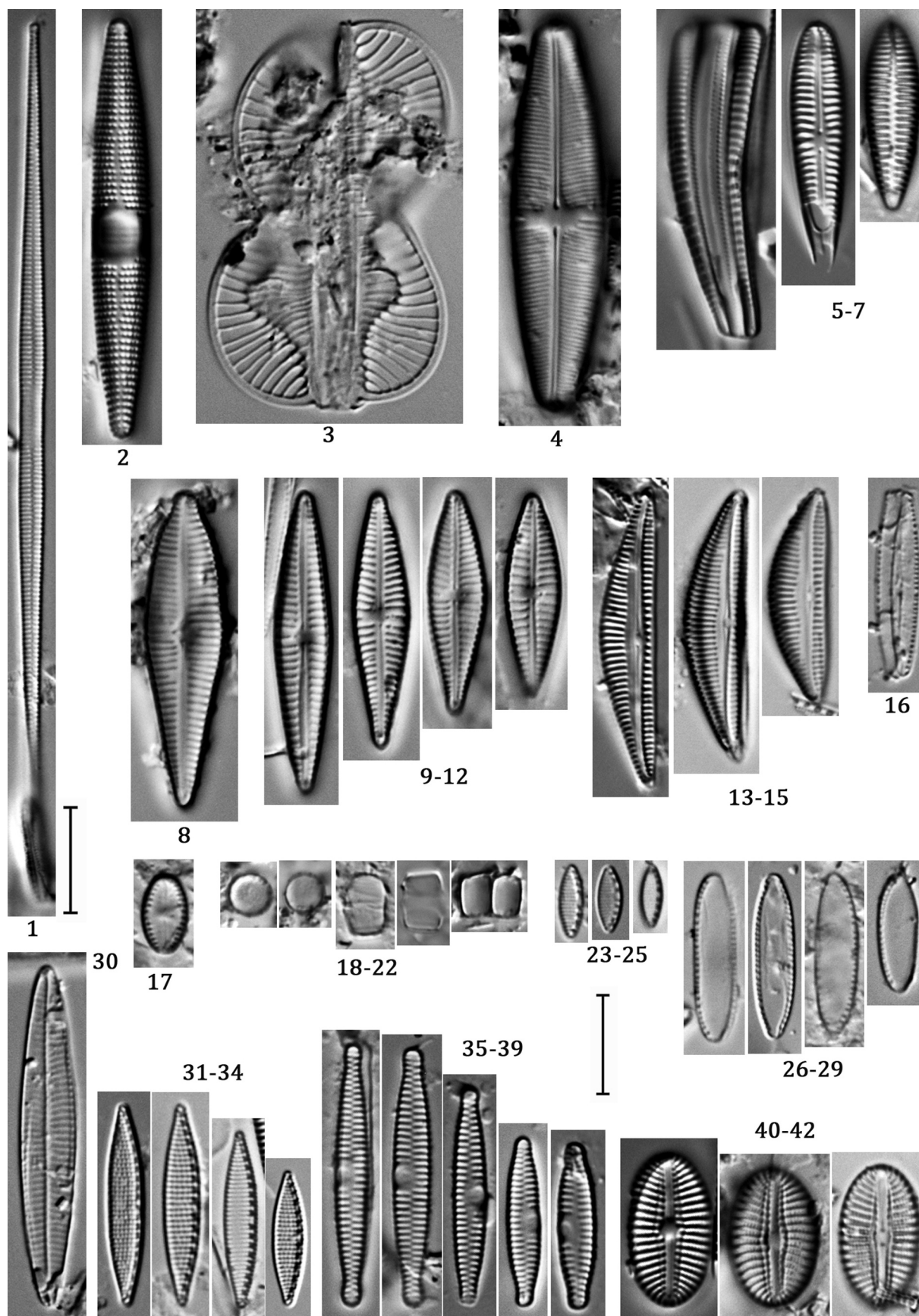
Figure 2. Light microscopy (LM) micrographs of new and some brackish taxa in Serbia. 1 *Ulnaria grunowii*; 2 *Ctenophora pulchella* 3 *Entomoneis costata*; 4 *Staurophora brantii*; 5–7 *Rhoicosphenia lacustris*; 8 *Gomphonema jadwigiae*; 9–12 *Gomphonema campodunense*; 13–15 *Seminavis strigosa*; 16 *Nitzschia perspicua*; 17 *Luticola rotunda*; 18–22 *Thalassiosira pseudonana*; 23–25 *Nitzschia inconspicua*; 26–29 *N. bergii*; 30 *Haslea duerrenbergiana*; 31–34 *Nitzschia costei*; 35–39 *Fragilaria vauchaeraetica*; 40–42 *Diploneis calcicolafrequens*. Scale bar = 10 µm.
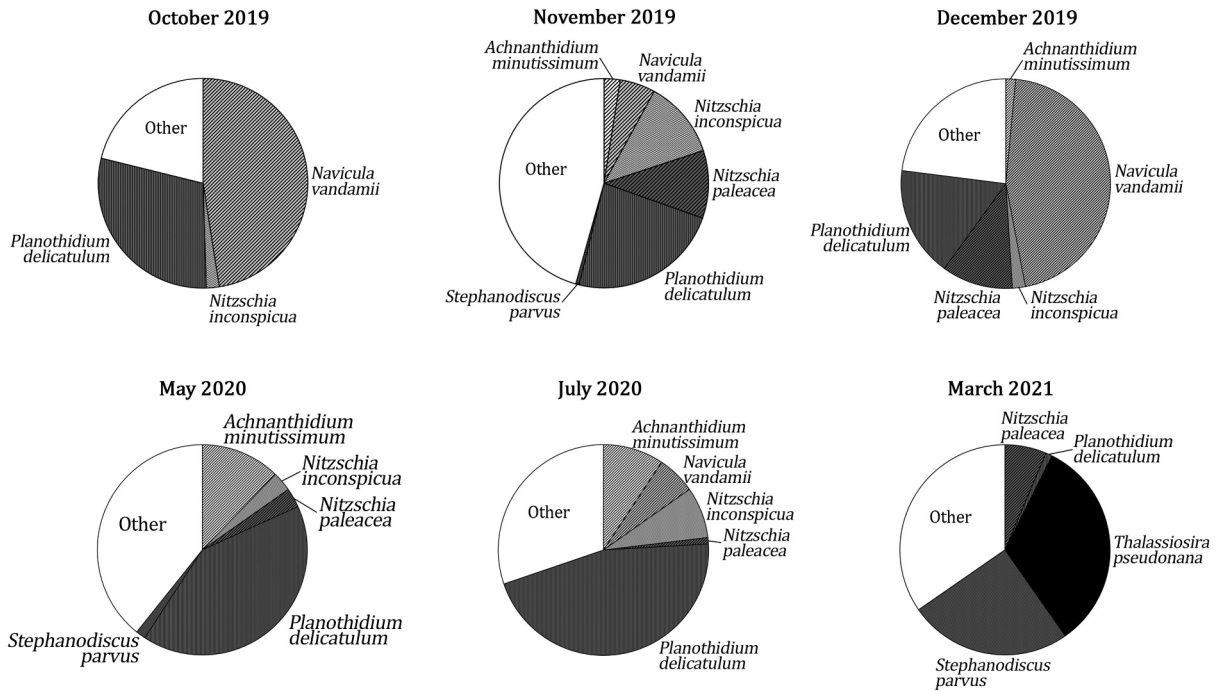
Figure 3. Dominant taxa in the epipsamic diatom community in the artificial sandpit lake Peskara.

incognita, *N. lorenziana, N. sigma, N. thermaloides, N. valdecostata, N. vitrea, Planothidium delicatulum, Pleurosigma salinarum, Tabularia fasciculata, Tryblionella apiculata, T. calida, T. hungarica*, etc.

Dominant taxa

By comparing diatom communities in different periods of time, we were unable to establish a pattern of taxa dominance/subdominance between the two communities (epipsamic and epiphytic) (Figure 3 and Figure 4). In the epipsamic community, *Planothidium delicatulum* was dominant in all samples (17–45.7%) while *Achnanthidium minutissimum sensu lato, Navicula vandamii, Nitzschia inconspicua*, and *Nitzschia paleacea* alternated. The largest deviation was observed in spring 2021, when centric diatoms *Thalassiosira pseudonana* (32.92%) and *Stephanodiscus parvus* (25.06%) were recorded as the most abundant. In the epiphytic community, an even greater number of

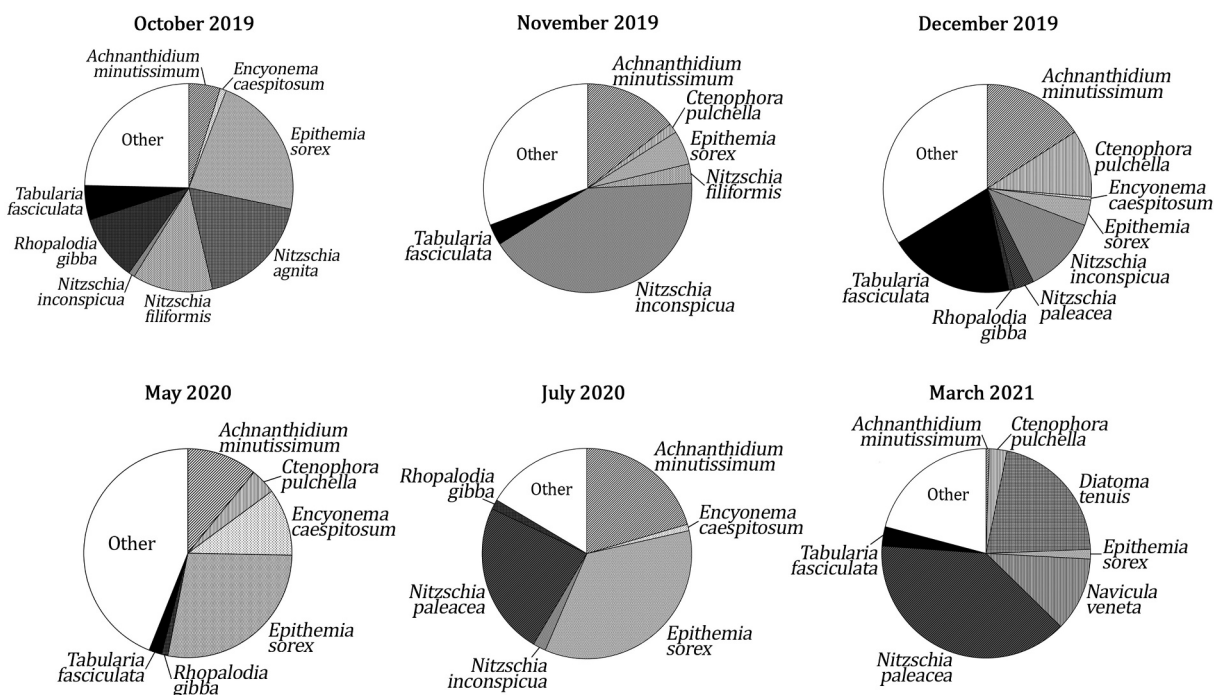**Figure 4.** Dominant taxa in the epiphytic diatom community in the artificial sandpit lake Peskara.

taxa attained large abundances. We did not observe dominance shifts since 12 taxa with high abundances altered over seasons: *A. minutissimum, Ctenophora pulchella, Diatoma tenuis, Encyonema caespitosum, Epithemia sorex, Navicula veneta, Nitzschia agnita, N. filiformis, N. inconspicua, N. paleacea, Rhopalodia gibba,* and *Tabularia fasciculata.*

Metabarcoding analysis

Only one pooled sample (epipsamic community) was investigated for metabarcoding analysis in spring 2021. After bioinformatic processing, a total of 125,193 reads were generated in 90 amplicon sequence variants (ASVs), all of which belong to the phylum Bacillariophyta (Table 1). The alpha diversity indices showed moderate richness and diversity. In terms of taxonomic assignment, among the 90 ASVs 54 (60%) were assigned to a species level, 34 (37.78%) to a genus level, and 18 (20%) to a family level.

After removal of all of the amplicon sequence variants that were not assigned to diatoms, a total of 32 identified species were obtained, belonging to 27 genera. According to the percentage of the reads, the most abundant taxa were those from the family Stephanodiscaceae (56.30%), which could not be identified to the species level, and *Thalassiosira pseudonana* (19.11%) (Figure 5).

Although more taxa were recorded with a standard microscopic analysis (52 species belonging to 31 genera) as well as the Shannon diversity index was higher (3.01), results of the morphological analyses complied with the metabarcoding data when it comes to the dominance of two centric diatom taxa – *Thalassiosira pseudonana* and *Stephanodiscus parvus.* However, nine taxa were detected only based on sequences: *Amphora libyca, Craticula cuspidata, Fallacia monoculata, Fistulifera saprophila, Nitzschia commutata, N. thermalis, Hantzschia amphioxys, Pinnularia brebissonii,* and *P. substreptoraphe.*

Table 1. Alpha diversity at the ASV, genus, family and phylum level.

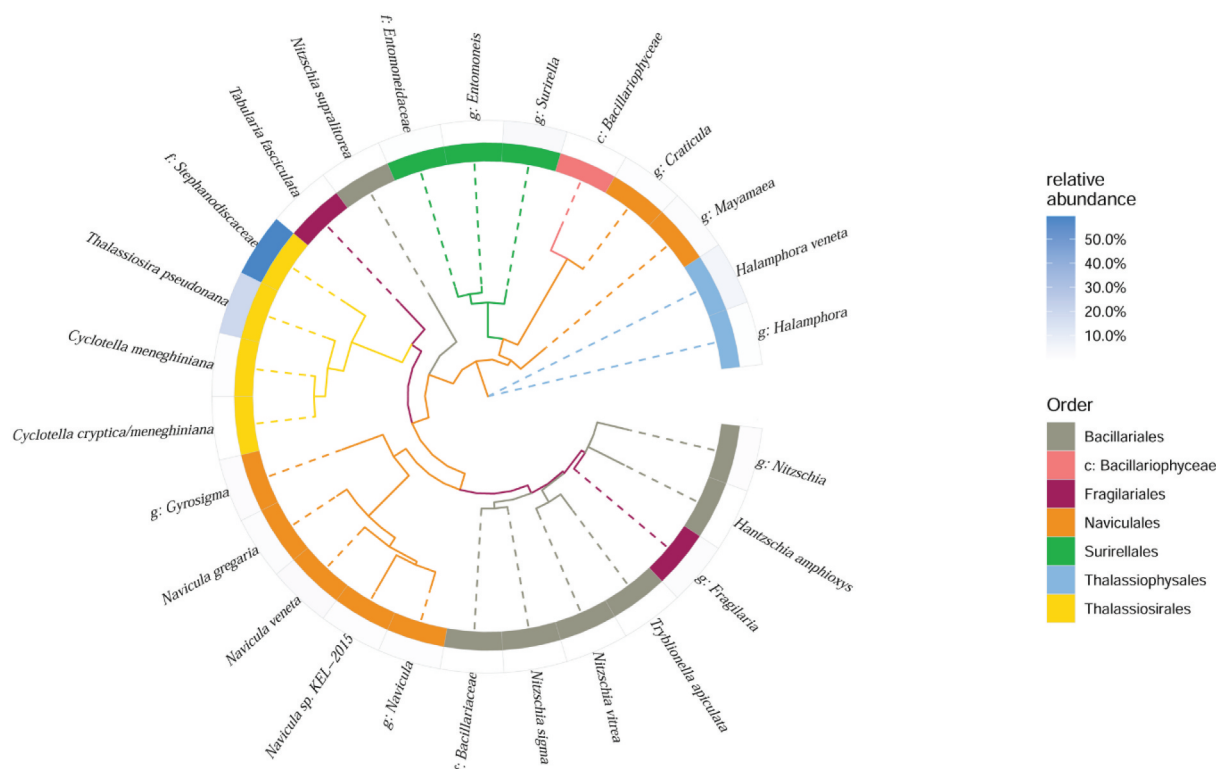| OBS, Chao1 & ACE	SE.Chao1	SE.ACE	Shannon	Simpson	InvSimpson	Fisher	Level
90	0	1.96	1.80	0.64	2.78	9.49	ASV
54	0	1.39	1.58	0.61	2.58	5.37	Species
34	0	0.99	1.48	0.61	2.57	3.22	Genus
18	0	0.97	1.38	0.60	2.50	1.60	Family

Figure 5. Phylogenetic tree based on diatom ASVs. 18S representative sequences included in the tree were extracted from Diat.barcode date base (Rimet et al. 2018).

Discussion

Due to its depth and a perennial character, a stable diatom community has developed in the artificial sandpit lake "Peskara". Specific physicochemical conditions, such as an elevated concentration of electrolytes contributed to the development of a community composed of freshwater diatoms (e.g. *Achnanthidium minutissimum sensu lato*, *Gomphonema parvulum*) and diatoms characteristic for brackish and coastal waters, such as *Bacillaria paxillifera*, *Entomoneis paludosa*, *Haslea duerrenbergiana*, *H. spicula*, *Nitzschia inconspicua*, *Planothidium delicatulum*, *Pleurosigma salinarum*, *Staurophora brantii*, *Seminavis strigosa*, *Tabularia fasciculata* etc. According to the study made by Heudre et al. (2021) in the French part of the Rhin-Meuse basin species *B. paxillifera*, *E. paludosa*, *N. inconspicua*, *Halamphora coffeaeformis*, *Haslea spicula*, *Nitzschia frequens*, and *Nitzschia thermaloides* are commonly found in brackish waters, but can also be present in high electrolyte-rich inland waters. Many of these species were noticed in different saline inland waters throughout Hungary (Stenger-Kovács and Lengyel 2015). In Serbia, diatom communities in saline inland waters are mainly composed of *Navicula* and *Nitzschia* taxa (Vidaković et al. 2019; Ćirić et al. 2021a). This makes artificial sandpit lakes even more interesting and significant from the aspect of biodiversity, especially from the standpoint of brackish species. The new genus for Serbia is *Seminavis*, with the species *S. strigosa*. *Seminavis* is a marine and brackish genus established by Mann (Round et al. 1990) to accommodate semi-lanceolate species previously assigned to *Amphora* Ehrenberg. The main characteristics of the genus are uniseriate lineolate striae, slit-like areolae, and two girdle-appressed plastids of unequal size (Danielidis and Mann 2003). *Seminavis strigosa* is a mesohaline species, and so far, observed on various macroalgae in brackish and marine environments (Danielidis and Mann 2003; Wachnicka and Gaiser 2007). In the Messolonghi lagoon (Greece) *S. strigosa* occurred with the abundance between 5–7% at temperatures 21–31 °C, pH 8.4–8.9, P-PO$_4$$^{3-}$ 0.007–0.027 mg/L, and NO$_3$$^-$ 0.295–0.730 mg/L (Danielidis and Mann 2003). In the "Peskara" sandpit lake, this taxon was observed in both communities with the abundance of 0.5–3.23%. Ranges in environmental factors were: temperature 6.7–25.1 °C, pH around 9, P-PO$_4$$^{3-}$ <0.02–47.4 mg/L, and NO$_3$$^-$ <0.5–0.8 mg/L. In March 2021 when the concentration of phosphorus was high (47.4 mg/L) *S. strigosa* was present in low abundance, and a similar situation was in December 2019 when the water temperature was 6.7 °C. *Entomoneis costata*, *Haslea duerrenbergiana*, *Nitzschia bergii*, *Staurophora brantii*, and *Thalassiosira pseudonana*, species characteristic for marine, brackish or inland saline waters, are new for the diatom flora of Serbia (Krammer and Lange-Bertalot 1986; Alverson et al. 2011; Stenger-Kovács and Lengyel 2015). One relatively new taxon for science, *Fragilaria vaucheriaeraetica*, was described from ultraoligotrophic waters with very low conductivity and very low nitrate concentrations (Cantonati et al. 2019). This species occurred with 0.99–4.47% abundance in our samples, which indicates that *F. vaucheriaeraetica* can occupy a much broader ecological niche than that originally described. *Gomphonema jadwigiae* is also new for Serbia; this species is considered fairly rare within the range of its distribution. Ács et al. (2019) observed this species for the first time in Hungary in some bomb crater ponds. When firstly described in 1996 (Lange-Bertalot and Metzeltin 1996) the *G. jadwigiae* was characterized as an oligotrophic indicator. However, our results show that it can tolerate high conductivity and elevated nutrient concentrations as well. Until now, the species was known from the Nielba River (Poland), eutrophic Lake Dojran and Vardar River (North Macedonia), and meso-eutrophic–eutrophic Orangery Lake (Belgium) (Ács et al. 2019). Of the 15 new diatom taxa for Serbia, 7 are freshwater species observed mainly in waters with elevated nutrient concentrations (*Diploneis calcicolafrequens*, *Gomphonema campodunense*, *Luticola rotunda*, *Nitzschia costei*, *N. perspicua*, *Rhoicosphenia lacustris*, *Ulnaria grunowii*) (Levkov et al. 2010, 2016; Lange-Bertalot et al. 2017).

Planothidium delicatulum was one of the most dominant species in the diatom community (17–45.7%). According to the literature data, in Serbia it was observed in the Pek River by Obušković (1984, recorded as *Achnanthes delicatula*) and in the Ponjavica River near Omoljica (unpublished data from personal observations). *Planothidium delicatulum* can be present in both marine and freshwater environments (Riaux-Gobin et al. 2018). In the Hel Peninsula (southern Baltic Sea), *P. delicatulum* was one of the dominant taxa in sandy sediments and according to autecological preferences it is a representative of eutraphentic species (Witak et al. 2020). With the abundance exceeding 40% *P. delicatulum* was recorded in salt marshes near Łęczyca (Central Poland) (Żelazna-Wieczorek et al. 2015). *Nitzschia inconspicua* is also considered as a taxon with a preference for higher conductivity and is regarded as an inhabitant of brackish waters (Heudre et al. 2021). In

Serbia, this species was recorded in different types of habitats with different conductivities. Due to similarities, *N. inconspicua* is often confused with *N. soratensis*, which is defined as a strictly freshwater species (Kelly et al. 2015). *Nitzschia paleacea*, characteristic for eu- to polytrophic freshwaters with medium and high electrolyte content (Lange-Bertalot et al. 2017), was noticed as one of the dominant taxa in the artificial sandpit lake "Peskara" (0.98–38.81%). Establishing a pattern of dominance in the epiphytic community was difficult because 12 taxa changed over seasons. Half of our dominant taxa (*Ctenophora pulchella, Navicula veneta, Nitzschia agnita, N. filiformis, N. inconspicua*, and *Tabularia fasciculata*) are characteristic of brackish waters (Lange-Bertalot et al. 2017).

In spring 2021, the most abundant were centric taxa, *Thalassiosira pseudonana* (32.92%) and *Stephanodiscus parvus* (25.06%). *Thalassiosira* species grow primarily in marine waters, while some can also be found in estuaries, high conductance waters and rivers, polluted ponds, and other aquatic systems that have been impacted by human activities (Spaulding and Edlund 2008). Species of the other centric genus, *Stephanodiscus*, are typically pelagic or found in the littoral or benthos of fresh and brackish waters (Houk et al. 2010). Metabarcoding analysis of the spring sample mostly showed a similar observation, but with a one "gap". The relative abundance of the corresponding ASVs represented by the number of HTS reads (sequences) for the family Stephanodiscaceae was 56.30% and for *Thalassiosira pseudonana* it was 19.11%. Based on the conducted morphological analyses, we assumed that the sequence with the abundance of 56.30% belongs to the *Stephanodiscus parvus*. Also, for the sequences identified at the family level in the NCBI nucleotide database it was confirmed that they belong to the genus *Stephanodiscus*, but the sequences were associated with several different taxa (*S. parvus, S.* cf. *akanensis, S. hantszchii*) with 100% similarity. Therefore, according to the 18S rRNA gene, it is hard to conclude the exact species level and other gene analysis should be involved for better differentiation. The reverse dominance of taxa is evident when both approaches are compared. The very small frustules of *Thalassiosira pseudonana* (4–9 μm in diameter) are sometimes difficult to spot by a light microscope. On the other hand, frustules of dead planktonic specimens of *Stephanodiscus parvus* might have been collected and observed under the microscope, but the species was not detected by molecular analyses since no DNA was present. Some brackish taxa *e.g.* from the genera *Entomoneis, Halamphora* and *Nitzschia* were not assigned to the species level. Different studies showed that many diatom taxa identified with light microscopy could not have been detected with metabarcoding techniques because their sequences were not present in the database (e.g. Zimmermann et al. 2015; Rivera et al. 2018); considering the large biodiversity of diatoms, much work remains to be done. Diat.barcode is an open-access reference library dedicated to diatoms and has been maintained since 2012 (Rimet et al. 2018). Even in such a large database, there is an obvious lack of sequencing data related to brackish species. In our study, nine taxa were not noticed with the morphology-based approach. Their sequences were represented by less than 0.1% of abundance, indicating that those taxa were very rare in the sample. Therefore, they had been omitted during microscopy inspection or were present just as remnants of DNA in the sample.

In conclusion, our study contributes to the new knowledge of brackish diatom species in artificial sandpit lakes. Brackish diatom taxa from artificial sandpit lakes are poorly illustrated in literature and filling this gap is of great importance, not only for classical diatom taxonomy but also for future molecular investigations based on next-generation sequencing techniques.

Author contributions

Danijela Vidaković: Investigation; morphological analysis; writing. Jelena Krizmanić: Morphological analysis; revision and editing of the manuscript. Bojan Gavrilović: Investigation; methodology; revision and editing of the manuscript. Biljana P. Dojčinović: Chemical analysis; revision and editing of the manuscript. Zlatko Levkov: Morphological analysis; revision and editing of the manuscript. Ivica Dimkić: Molecular analysis; revision and editing of the manuscript. Miloš Ćirić: Investigation, methodology; revision and editing of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author (s).

Data availability statement

The data that support the findings of this study are available from the corresponding author [DV] upon reasonable request.

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