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MICROALGAL FUNCTIONAL TRAITS

Phytoplankton biomass and functional composition in the Danube River and selected tributaries: a case study Joint Danube Survey 4

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Abstract In 2019, phytoplankton and environmental parameters were analysed monthly during the growing season from April to September at 26 sampling sites in the Danube and 10 additional sampling sites in the main tributaries as part of the Joint Danube Survey 4, organised by the ICPDR. Our results showed that both phytoplankton biomass and composition follow the River Continuum Concept on free-flowing sections, but also responds to hydromorphological changes where the largest dam Iron Gate represents the largest interruption of the river and the phytoplankton continuum. Besides longitudinal interruption, water residence time was the most important factor for phytoplankton composition, while nutrients were less relevant. The low phytoplankton biomass and its composition in the Danube support the oligotrophication trend, but this one-year study could not confirm it with certainty. Phytoplankton is the most important autotrophic component in the Upper and Middle Danube, where environmental conditions do not support the optimal growth of other river flora.

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The predominant FGs coda were A, C and D as a typical potamoplankton component, while the codon TB occurs throughout the Danube but is more prominent in the Upper reach and other river sections with higher discharge events.

Keywords Reynold's functional groups · Potamoplankton · Oligotrophication · River Continuum Concept · Hydromorphology

Introduction

The uppermost river segments, where riverine biota is primarily dependent on terrestrial carbon input (Vannote et al., 1980) phytoplankton mainly consists of tychoplanktic elements (Bolgovics et al., 2017). In contrast, large potamal rivers have characteristic high-biomass phytoplankton assemblages with characteristic planktonic taxa (Reynolds & Descy, 1996). River regulation and excessive nutrient inputs to rivers have fundamentally altered the composition and biomass of phytoplankton in rivers (Harper, 1992; Tockner et al., 2009). These changes mainly affected the large rivers such as the Danube, which crosses four capital cities and densely populated areas with 79 million people in Europe.

As far as phytoplankton is concerned, only sporadic data were available for the Danube until the 1960s (Borbás, 1879), when regular monitoring of the river began. Studies from this time (Szemes, 1964, 1967; Uherkovich, 1969) did not report an increased trophic status in the Danube, but from the early 1980s onwards, serious eutrophication began, leading to phytoplankton assemblages with large biomass, especially in the Middle reach (Kiss, 1985, 1994; Garnier et al., 2002). These quantitative changes coincided with shifts in phytoplankton composition, resulting in higher abundance of green algae (Schmidt, 1994). The results of the first JDS also showed that serious hypertrophic situations with an extremely large (150 µg l^{-1}) phytoplankton biomass could develop in the middle section of the Danube (Németh et al., 2002).

Due to global warming, remarkable changes in variables considered important for phytoplankton, such as water temperature (Dokulil, 2014) or water discharge (ICPDR, 2013; Stagl & Hattermann, 2015), have been observed in recent decades. Due to the efforts made by the countries of the Danube basin in the field of nutrient removal from incoming wastewater, oligotrophication could be observed (Istvánovics & Honti, 2012; Abonyi et al., 2018). The decreasing trends in the trophic status of the Danube have also been highlighted in the three JDS reports showing a reduction in the biomass peaks of the late summer phytoplankton assemblages in the middle Danube section (Németh et al., 2002; Dokulil & Kaiblinger, 2008; Dokulil & Donabaum, 2014). In addition to the obvious decline in the amount of algae in the Danube (Kiss et al., 2006), a long-term analysis of the size traits of microalgae in the middle section of the river showed a shift towards smaller forms (Abonyi et al., 2020b).

Since the beginning of this century, the International Commission for the Protection of the Danube River (ICPDR) and the 14 cooperating states have organised monitoring campaigns along the Danube every six years with the aim of obtaining information on water quality and biota along the entire length of the river. The first three sampling campaigns (2001, 2007, 2013) can be considered as snapshots of biological, physical and chemical variables and provided data only for the late summer periods. For biological elements with a long generation time (macrophytes, fish, aquatic macroinvertebrates), these surveys provided more reliable data than for the elements of the microbiota (phytoplankton and phytobenthos), which are subject to unpredictable changes during the year, both in terms of composition and biomass. Therefore, to improve the quality and usability of phytoplankton data during JDS4 (2019), the organisers proposed monthly sampling (April to September) at 26 sites covering the entire river and 10 additional ones on the main tributaries.

In this study, we summarise the results of this fundamental phytoplankton survey, focusing on the similarities and difference of functional properties of the phytoplankton in the Upper, Middle and Lower reaches of the Danube. The aim of this paper is to describe the environmental characteristics, phytoplankton biomass and functional composition along the Danube and in the tributaries and to identify key factors shaping the phytoplankton community and biomass, including not only physical, chemical and

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hydrological properties, but also longitudinal barriers caused by human activities.

Materials and methods

Study area

During Joint Danube Survey 4 (JDS4), 51 sampling sites were sampled, most of which are so-called Trans-National Monitoring (TNMN) sites and are part of a long-term monitoring of the river. For the study of phytoplankton, 26 sampling sites in the Danube and 10 sampling sites in side arms (Mosoni and Ráckevei-Soroksári Danube Arms) and small to very large tributaries (Morava, Váh, Hron, Ipeľ, Drava and Prut) were selected (Fig. 1). Their basic characteristics and the names of the sampling sites can be found in Table 1. The numbers of the sampling sites are identical to the original JDS4 codes. According to ICPDR (2021), sampling sites in the main river 1–14 and 40 are strongly influenced by impoundments, while sampling sites 16–31 and 41–51 are located on the free-flowing section.

The latest data on the Danube River and its catchment are described in detail in ICPDR (2021), while a detailed description of the JDS4 sampling sites can be found in the study of Liška et al. (2020).

With a length of 2,857 km, a catchment area of 803,260 km^2 and an average discharge of 6,500 m^3 s^{-1}, the Danube is the second largest river in Europe. The river basin covers the territory of 19 countries with 79 million inhabitants. Fourteen countries have an area of more than 2,000 km^2 in the river basin. Two small rivers, the Brigach and the Breg in Germany, form the Danube. During its course, the Danube flows through a wide variety of landscapes and is divided into three river sections (river reaches). The Upper Danube begins at its source and ends at rkm 1,790 and includes the Western, Eastern and Lower Alpine Foothills. The Middle reach of the Danube stretches from rkm 1,790 to rkm 943 and includes the Hungarian Danube Bend, Pannonian Plain Danube and Iron Gate Danube ending with the largest hydropower plant Iron Gate. The Lower reach of the Danube begins at rkm 943 and ends at the Danube Delta, where it divides into three main arms (Chilia, Sulina and Saint George). Besides the

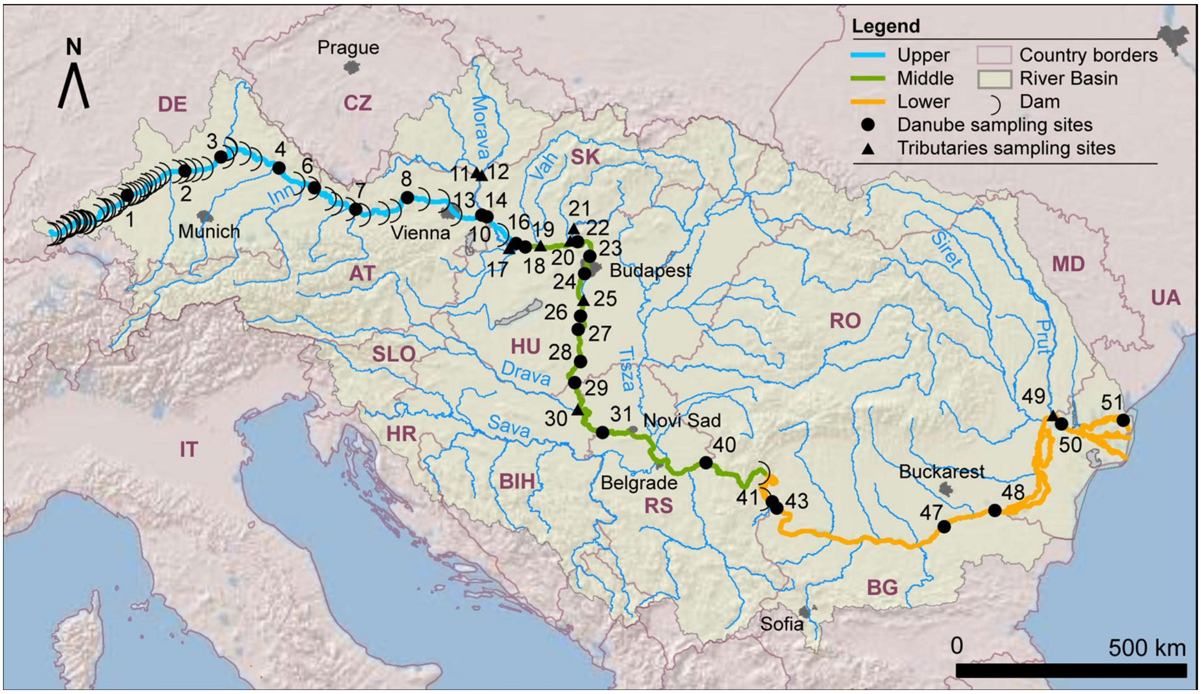


Fig. 1 Map of the study area. The numbers represent the sampling sites. See Table 1 for the names of the sampling sites

Table 1 List of sampling sites with their names, river names, countries, geographical coordinates, distances from confluences (Rkm) and River Basin size

Site number	Site name	River	Country	Latitude	Longitude	Rkm	River basin (km^2)
1	Böfinger Halde	Danube	DE	48.424	10.027	2,581	8,129
2	Bittenbrunn	Danube	DE	48.736	11.155	2,479	19,921
3	Above Klösterl—Kelheim	Danube	DE	48.918	11.866	2,415	23,029
4	Niederalteich—Mühlau	Danube	DE	48.775	13.009	2,258	47,506
6	Jochenstein	Danube	DE/AT	48.521	13.702	2,204	77,091
7	Enghagen	Danube	AT	48.240	14.512	2,113	90,931
8	Oberloiben	Danube	AT	48.388	15.523	2,008	96,357
10	Hainburg, upstream Morava	Danube	AT	48.163	16.990	1,879	130,759
11	Pohansko	Morava/Dyje	CZ	48.723	16.885	17	12,540
12	Lanžhot	Morava	CZ	48.687	16.989	79	9,725
13	Devín	Morava	SK	48.188	16.976	1	26,575
14	Bratislava	Danube	SK	48.140	17.084	1,868	131,907
16	Medveďov/Medve	Danube	SK/HU	47.790	17.660	1,806	132,168
17	Vének	Mosoni Danube Arm	HU	47.736	17.782	0.1	18,060
18	Gönyű	Danube	HU	47.743	17.844	1,790	149,840
19	Komárno	Váh	SK	47.761	18.142	1.5	19,661
20	Kamenica	Hron	SK	47.826	18.723	1.7	5,417
21	Salka	Ipeľ	SK	47.886	18.763	12	5,060
22	Szob	Danube	HU/SK	47.813	18.863	1,707	183,210
23	Budapest upstream (Megyeri Bridge)	Danube	HU	47.616	19.102	1,660	184,100
24	Budapest downstream (M0 bridge)	Danube	HU	47.388	19.004	1,630	185,000
25	Tass	Ráckevei-Soroksári Danube Arm	HU	47.034	18.978	59	850
26	Dunaföldvár	Danube	HU	46.817	18.926	1,560	187,680
27	Paks	Danube	HU	46.634	18.880	1,532	187,900
28	Baja	Danube	HU	46.201	18.924	1,481	204,140
29	Hercegszántó/Batina/Bezdan	Danube	HU/HR/RS	45.915	18.806	1,434	210,900
30	Drava mouth	Drava	HR	45.552	18.865	5	39,658
31	Ilok/Bačka Palanka	Danube	HR/RS	45.232	19.361	1,300	255,898
40	Banatska Palanka/Bazias	Danube	RS/RO	44.805	21.384	1,073	570,896
41	Upstream Timok (Rudujevac/Gruia)	Danube	RS/RO	44.261	22.685	847	577,085
43	Pristol/Novo Selo Harbour	Danube	RO/BG	44.172	22.782	837	580,100
47	Downstream Ruse/Giurgiu (Marten)	Danube	BG/RO	43.911	26.067	488	672,600
48	Chiciu/Silistra	Danube	RO/BG	44.137	27.051	375	698,600
49	Giurgiulesti	Prut	MD/RO	45.472	28.197	0.5	27,480
50	Reni	Danube	RO/UA	45.456	28.260	132	805,700
51	Vilkove—Chilia/Kilia arm	Danube	RO/UA	45.395	29.581	18	817,000

Danube Delta, it includes the Western Pontic Danube and the Eastern Wallachian Danube.

The Danube and its tributaries are exposed to considerable anthropogenic pressure. The main pressures are water abstraction (industry, irrigation, household supply), drinking water supply, wastewater discharge (municipalities, industry), hydropower generation, navigation, dredging, gravel extraction and recreation. Most of these pressures lead to degradation of hydromorphology, which plays an important role in the functioning of the entire aquatic ecosystems. There are hundreds of significant impoundments and dozens of hydropeaking sections. Approximately 1,069 km of the river's total length is dammed, representing 37% of its length. The most significant dam is the Iron Gate 1 dam, which impairs the flow of the Danube as far as Novi Sad (about 500 km or 18% of the total length). The most important free-flowing sections are downstream of the Gabčikovo Dam in Slovakia (Fig. 1, sampling site 16) and Novi Sad in Serbia, and downstream of the Iron Gate 2 Dam (Fig. 1, sampling site 41) to the Black Sea.

The Danube has many large tributaries, the main of which are the Inn, Morava, Drava, Tisza, Sava, Iskar, Siret and Prut, in downstream order. As the Danube is a very large river, it also has many side arms. The Mosoni Danube Arm is a 124 km long meandering side arm on the margin of the alluvial talus in the Szigetköz floodplain (Guti, 2006) and Ráckevei-Soroksári Danube Arm being the second largest Danube side arm in the Hungarian section with a length of 58 km. The water flow in this side arm can be fully regulated by the Kvassay and Tassi sluices, which are located at the two ends of the branch. It is heavily polluted by wastewater (Vadadi-Fülöp et al., 2007). With a length of 725 km, the Drava is one of the largest tributaries of the Danube and connects the Alpine regions of Italy, Austria and Slovenia with the Pannonian regions of Croatia and Hungary. The Morava is 350 km long, and it springs in the Czech Republic at the foothill of the Králický Sněžník and flows into the Danube at Devín (Beránková & Ungerman, 1996). The Slovak rivers Váh, Hron and Ipeľ are influenced by the various branches of industry and agriculture (ICPDR, 2009). The Váh is 402 km long and has an average discharge of 161 $m^3 s^{-1}$. It is the largest river in Slovakia and the major Slovak tributary of the Danube. The Hron is also one of the largest Slovak rivers, with a length of 298 km and a catchment area covering about 11% of Slovakia's territory. The Ipeľ River is the largest left tributary of the Hungarian Danube with a catchment area of 5,108 km^2.

Sampling and sample analysis

Sampling and sample analysis were carried out as part of the Joint Danube Survey 4, organised by the Monitoring and Assessment Expert Group of the International Commission for the Protection of the Danube River (ICPDR). Phytoplankton samples were collected and analysed by national certified laboratories using the methodology agreed in the preparatory phase. The methodology included monthly sampling of phytoplankton from April to September 2019, together with water for basic physical and chemical variables, i.e. temperature, pH, dissolved oxygen, saturation, conductivity, alkalinity, ammonia, nitrites, nitrates, total nitrogen, soluble reactive phosphorus, biological oxygen demand, chemical oxygen demand, total suspended solids and chlorophyll *a*. Total nitrogen was not measured at sampling sites 1–10, therefore it was excluded from the analysis and used only descriptively. The analytical methods used by the national laboratories for the analysis of the basic physical and chemical properties of the water were standardised, validated methods whose performance is systematically checked within the QUALCO Danube analytical quality control testing scheme organised by the ICPDR at the basin level (Hamchevici et al., 2020). Phytoplankton samples were collected from most sampling sites in the middle of the river (thalweg), preserved with acidic or alkalic Lugol's solution depending on the national protocols and stored in the dark at a temperature between 4 and 8°C before analysis (CEN - EN 16698, 2015). Samples were counted according to Utermöehl method (CEN - EN 15204, 2006). Biovolumes were calculated by determining the average individual size of up to 30 randomly selected cells of each taxon and then multiplying by the observed species abundance or obtained from the national database. Biomass (fresh weight) was derived from biovolume and used for further analyses, where 1 $mm^3 l^{-1} = 1 mg l^{-1}$ (CEN - EN 16695, 2015). Phytoplankton taxa were assigned to functional groups (FGs) according to Reynolds et al. (2002), Borics et al. (2007) and Padisák et al. (2009). A total of 154 samples were collected in the Danube and 59 in the tributaries. A few missing samples and minor deviations from the original sampling plan are described in detail in Stanković et al. (2020).

Discharge data were obtained from the national hydrological services. Theoretical water residence time (WRT) was calculated as a function of drainage area (A_d, km^2) and discharge (Q, m^3 s^{-1}) using the equation $WRT = 0.08 \times A_d^{0.6} \times Q^{-0.1}$ (Soballe & Kimmel, 1987; Leopold et al., 1995).

Data analysis

The map of the study area was created in ArcGIS Desktop 10.7 and the layers for the map were downloaded from DanubeGIS (www.danubegis.org). Cluster analysis of environmental factors in the Danube based on Euclidean distance was done in Primer 6 software (Clarke & Gorley, 2006). The contour plot of seasonal and spatial changes of the concentration of chlorophyll *a* (Chl-a) and total phytoplankton biomass in the Danube and tributaries, and the seasonal and longitudinal differences in fitted linear trends for Chl-a in the Danube were done in Grapher™ (2019). The Spearman correlation coefficient was used to examine the correlations between phytoplankton biomass (concentration of Chl-a and total biomass) and environmental variables in the Danube and tributaries using IBM SPSS Statistics (IBM Corp. Released, 2013). We used ordinary least squares regression to assess the relationships between mean values (values averaged across all months) of nutrients (TP and TN) and Chl-a (as a proxy of phytoplankton biomass) at the site level. Pearson's correlation coefficient was used to evaluate the relationship between the mean values of the environmental parameters and Chl-a. The analyses were carried out with the R package (R Core Team, 2022).

Grapher™ (2019) was used to prepare the plot of the proportion of FGs along the Danube and tributaries. Analysis of seasonal and spatial changes in phytoplankton FG composition using PERMANOVA with two factors, months and three Danube reaches, as well as cluster analysis of FG composition in the Danube and tributaries based on Bray–Curtis similarity was done in Primer 6 software (Clarke & Gorley, 2006). Primer 6 software was also used for a one-way SIMPER analysis based on Bray–Curtis similarity carried out for the FG phytoplankton composition, with characteristic FGs in three sections of the Danube (Upper, Middle and Lower reaches).

The composition of the phytoplankton FGs was related to the environmental parameters for each river reach of the Danube using canonical redundancy analysis (RDA). The RDAs were performed using the CANOCO 5 software (ter Braak & Šmilauer, 2012). All FGs, 26 sampling sites and eight environmental variables were used. The results of the ordination were presented in correlation triplots. Phytoplankton biomass data were log-transformed. Environmental data were normalised prior to the analysis and a Draftman's plot was conducted to eliminate the variables with significant autocorrelation. Forward selection was applied to datasets with response variables and environmental descriptors as explanatory variables, and only those that appeared significant at $P \leq 0.05$ significance level (999 permutations) were used.

Results

Environmental characteristics of the Danube and tributaries

The physical and chemical properties of the water, presented as minimum, maximum and mean values for three Danube River reaches are listed in Table 2 and for each tributary separately in Table 3. Water temperature in the Danube ranged from 9.2 to 29.0°C and increased downstream. The pH was neutral to slightly alkaline in both the Danube and the tributaries. Dissolved oxygen concentration and saturation showed conditions with slightly lower oxygen content (5.5 mg l^{-1}; 58.1%), but never hypoxia, and with oversaturation (12.2 mg l^{-1}; 132.7%) in the Danube, with the greatest variation occurring in the Middle reach. Similar conditions were observed in the tributaries, except for high oversaturation (170.0%) in the Ráckevei-Soroksári Danube Arm. Conductivity showed a similar range in all three Danube sections, with slightly higher values in the Upper Danube (255–544 µS cm^{-1}).

Mean values of nutrients generally showed a decreasing trend of nitrogen compounds downstream of the Danube, with the highest values of ammonia, nitrites and nitrates in the Upper Danube and TP in the Middle Danube, e.g. nitrate values ranged from 0.365 to 2.800 mg N l^{-1}. Phosphorus was most available in the Middle Danube, reaching 0.076 mg P l^{-1}

Table 2 Minimum, maximum and average values of physical, chemical and hydrological parameters for Upper, Middle and Lower Danube from April to September 2019

Parameter with abbreviations used in the text and units	Upper		Middle		Lower	
	Min–Max	Mean	Min–Max	Mean	Min–Max	Mean
Temperature (°C)	9.2–24.0	16.1	10.8–28.1	18.7	11.0–29.0	21.8
pH	7.5–8.6	8.2	6.8–8.4	7.8	7.3–8.8	7.9
Dissolved oxygen (mg l^{-1})	6.9–12.08	9.6	5.5–11.6	8.7	6.0–12.2	7.9
Oxygen saturation (%)	81.0–111.6	99.6	58.1–132.7	93.4	66.0–125.0	89.8
Conductivity (µS cm^{-1}) 25 °C	255–544	383	274–477	345	290–452	378
Alkalinity (mg $CaCO_3$ l^{-1})	94.0–229.0	160.7	95.0–251.5	166.2	125.0–168.0	143.6
Ammonia (mg N l^{-1})	0.004–0.200	0.030	0.004–0.125	0.015	0.003–0.088	0.037
Nitrites (mg N l^{-1})	0.003–0.035	0.011	0.004–0.024	0.011	0.005–0.030	0.017
Nitrates (mg N l^{-1})	0.580–2.800	1.456	0.472–1.880	1.097	0.365–1.420	0.860
Total nitrogen [TN] (mg N l^{-1})[a]	1.120–2.350	1.522	0.500–2.500	1.466	0.250–1.884	1.215
Soluble reactive phosphorus [SRP] (mg P l^{-1})	0.003–0.065	0.018	0.009–0.076	0.033	0.014–0.069	0.044
Total phosphorus [TP] (mg P l^{-1})	0.016–0.140	0.053	0.024–0.218	0.089	0.020–0.133	0.075
Biological oxygen demand [BOD] (mg O_2 l^{-1})	0.3–3.0	1.4	0.5–7.0	3.0	0.9–4.4	2.3
Total suspended solids [TSS] (mg l^{-1})	1.5–230.0	28.3	6.0–93.0	33.5	3.0–108.0	36.7
Discharge (m^3 s^{-1})	66–3,564	1,252	1,163–8,510	2,804	1,570–12,140	5,739
Residence time [RT] (day)	10.4–46.0	31.6	44.9–104.6	59.0	90.3–135.2	108.7

[a] Values for total nitrogen for Upper reach were calculated only from existing data only for sampling sites 14 and 16

SRP and 0.218 mg P l^{-1} TP values. Among the tributaries, the Morava was the most nutrient-rich with TN up 6.800 mg N l^{-1} and TP up to 0.799 mg P l^{-1}, while the lowest values were measured in the Prut with TN up to 1.210 mg N l^{-1} and TP up to 0.113 mg P l^{-1}.

The organic load measured as BOD was highest in the Middle reach and lowest in the Upper reach of the Danube with a range of 0.3–7.0 mg O_2 l^{-1}, similar to the tributaries, with the lowest value in the Váh River (0.3 mg O_2 l^{-1}) and the highest in the Ráckevei-Soroksári Danube Arm (8.0 mg O_2 l^{-1}). The mean value of TSS showed an increasing trend downstream of the Danube (28.3–36.7 mg l^{-1}), although the highest value was measured in the Upper reach of the Danube (230.0 mg l^{-1}). In the tributaries, TSS was several times lower than in the main river, from 5.0 to 66.0 mg l^{-1}, except for the Prut River with TSS up to 109.0 mg l^{-1}.

Discharge ranged from 66 m^3 s^{-1} in the Upper Danube to 12,140 m^3 s^{-1} in the Lower Danube with a WRT of 10.4 to 135.2 days. Tributaries varied largely in size with discharges ranging from 2 m^3 s^{-1} in Ipeľ River and Ráckevei-Soroksári Danube Arm, and up to 1,031 m^3 s^{-1} in the Drava, with WRT ranging from 3.1 to 25.7 days.

Cluster analysis of the Euclidean distance of the physical and chemical properties of the water (temperature, pH, dissolved oxygen, saturation, conductivity, alkalinity, ammonia, nitrites, nitrates, soluble reactive phosphorus, biological oxygen demand, chemical oxygen demand and total suspended solids) based on the average data for each sampling site showed a clear grouping of the sampling sites according to river reach with minor exceptions (Fig. 2). The sampling sites of Upper reach were grouped in one large group with two subgroups of Upper and Lower sampling sites. Most of the Middle reach sampling sites were grouped in a very similar group (18–28), while sampling sites 29 and 30 were between two subgroups of Upper reach and sampling site 40 was in the group of Lower reach sampling sites.

Phytoplankton biomass in the Danube and tributaries

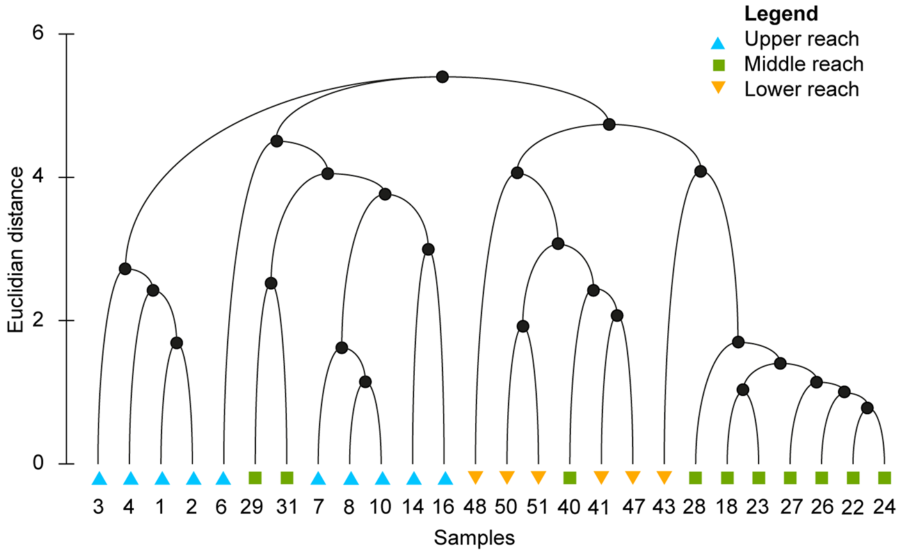
Both Chl-a and total biomass were analysed as measures of phytoplankton biomass. Both showed very similar spatial and temporal changes in the Danube and the tributaries (Figs. 3, 4, 5). In the Danube, the Chl-a concentration ranged from 0.8 to 55.7 µg l^{-1} and total biomass from 0.1 to 19.5 mg l^{-1}.

Table 3 Minimum, maximum and average values of physical, chemical, and hydrological parameters at sampling sites in the Danube tributaries from April to September 2019

Parameter with abbreviations used in the text and units	Morava (11–13)		Moson Danube Arm (17)		Váh (19)		Hron (20)	
	Min–Max	Mean	Min–Max	Mean	Min–Max	Mean	Min–Max	Mean
Temperature (°C)	9.9–26.1	19.2	10.8–23.7	18.1	10.9–24.5	19.8	9.0–24.6	19.2
pH	7.3–8.7	8.1	7.47–8.04	7.8	7.2–8.0	7.7	7.1–8.1	7.7
Dissolved oxygen (mg l^{-1})	7.1–11.4	9.1	5.7–8.8	7.0	7.3–10.7	8.3	7.4–11.7	8.7
Oxygen saturation (%) [T]	85.0–118.0	99.0	64.1–80.4	73.8	87–97.8	91.3	89.0–98.0	94.6
Conductivity (µS cm^{-1}) 25 °C	36–602	201	355–580	440	384–432	399	299–437	364
Alkalinity (mg $CaCO_3$ l^{-1})	106.0–192.0	143.9	155.0–220.0	190.3	147.0–163.0	154.5	103.0–136.0	120.2
Ammonia (mg N l^{-1})	0.010–0.120	0.053	0.005–0.090	0.049	0.020–0.070	0.043	0.020–0.220	0.083
Nitrites (mg N l^{-1})	0.003–0.058	0.023	0.005–0.048	0.023	0.017–0.031	0.021	0.009–0.042	0.022
Nitrates (mg N l^{-1})	0.050–6.000	1.461	0.913–3.822	1.647	0.920–1.720	1.237	1.330–1.840	1.615
Total nitrogen [TN] (mg N l^{-1})	0.770–6.800	2.258	1.414–4.653	2.232	1.340–2.210	1.667	1.820–2.310	2.060
Soluble reactive phosphorus [SRP] (mg P l^{-1})	0.005–0.669	0.201	0.043–0.112	0.077	0.040–0.090	0.058	0.050–0.120	0.088
Total phosphorus [TP] (mg P l^{-1})	0.038–0.799	0.279	0.078–0.244	0.145	0.090–0.160	0.123	0.090–0.190	0.158
Biological oxygen demand [BOD] (mg O_2 l^{-1})	0.9–3.8	2.3	0.7–7.0	3.9	0.4–1.5	1.1	0.8–2.4	1.5
Total suspended solids [TSS] (mg l^{-1})	5.0–66.0	18.2	11.0–48.0	22.0	10.0–18.0	12.5	7.0–42.0	15.8
Discharge (m^{3} s^{-1})	10–67	29	37–114	78	179–241	213	15,250.0	25
Residence time [RT] (day)	13.5–26.0	19.1	17.8–20.0	18.7	17.4–17.9	17.6	9.6–11.0	10.2

Parameter with abbreviations used in the text and units	Ipel (21)		Ráckevei-Soroksári Danube Arm (25)		Drava (30)		Prut (49)	
	Min–Max	Mean	Min–Max	Mean	Min–Max	Mean	Min–Max	Mean
Temperature (°C)	11.0–24.6	19.9	14.1–28.5	22.6	13.4–25.8	19.7	12.2–25.6	21.0
pH	7.3–8.7	7.9	7.6–8.1	7.9	7.5–8.3	8.0	6.8–8.2	7.7
Dissolved oxygen (mg l^{-1})	7–13.4	8.7	6.7–13.4	11.2	8.5–10.7	9.4	5.3–10.1	7.8
Oxygen saturation (%) [T]	81.0–115.0	92.7	77.1–170.0	129.3	88.8–118.9	102.9	61.7–102.2	86.6
Conductivity (µS cm^{-1}) 25 °C	428–610	517	300–360	331	254–372	300	480–644	574
Alkalinity (mg $CaCO_3$ l^{-1})	162.0–221.0	187.3	108.0–207.1	170.8	117.0–148.0	130.5	165.0–195.0	178.3
Ammonia (mg N l^{-1})	0.030–0.120	0.058	0.005–0.105	0.024	0.004–0.013	0.007	0.048–0.166	0.088
Nitrites (mg N l^{-1})	0.012–0.040	0.026	0.005–0.069	0.035	0.004–0.019	0.007	0.010–0.025	0.015
Nitrates (mg N l^{-1})	0.640–1.840	1.352	0.336–1.152	0.767	0.630–1.540	0.873	0.220–0.759	0.449
Total nitrogen [TN] (mg N l^{-1})	1.570–2.570	2.133	0.830–2.101	1.372	0.850–1.930	1.172	0.500–1.210	0.938
Soluble reactive phosphorus [SRP] (mg P l^{-1})	0.020–0.300	0.195	0.010–0.020	0.015	0.002–0.141	0.034	0.009–0.068	0.030
Total phosphorus [TP] (mg P l^{-1})	0.130–0.350	0.287	0.036–0.163	0.088	0.029–0.190	0.080	0.019–0.113	0.072
Biological oxygen demand [BOD] (mg O_2 l^{-1})	1.5–3.6	2.3	3.0–8.0	5.6	0.9–3.7	1.6	1.2–3.5	2.6
Total suspended solids [TSS] (mg l^{-1})	14.0–27.0	20.3	8.0–28.0	14.0	12.5–30.0	21.4	17.0–109.0	49.5
Discharge (m^{3} s^{-1})	2–9	4	2–52	35	326–1031	545	47–320	160
Residence time [RT] (day)	10.7–12.5	11.7	3.1–4.4	3.4	22.9–25.7	24.7	20.7–25.1	22.7

Fig. 2 Dendrogram of the cluster analysis based on the Euclidean distance of the physical and chemical properties of the water in the Danube. See Table 1 for the names of the sampling sites



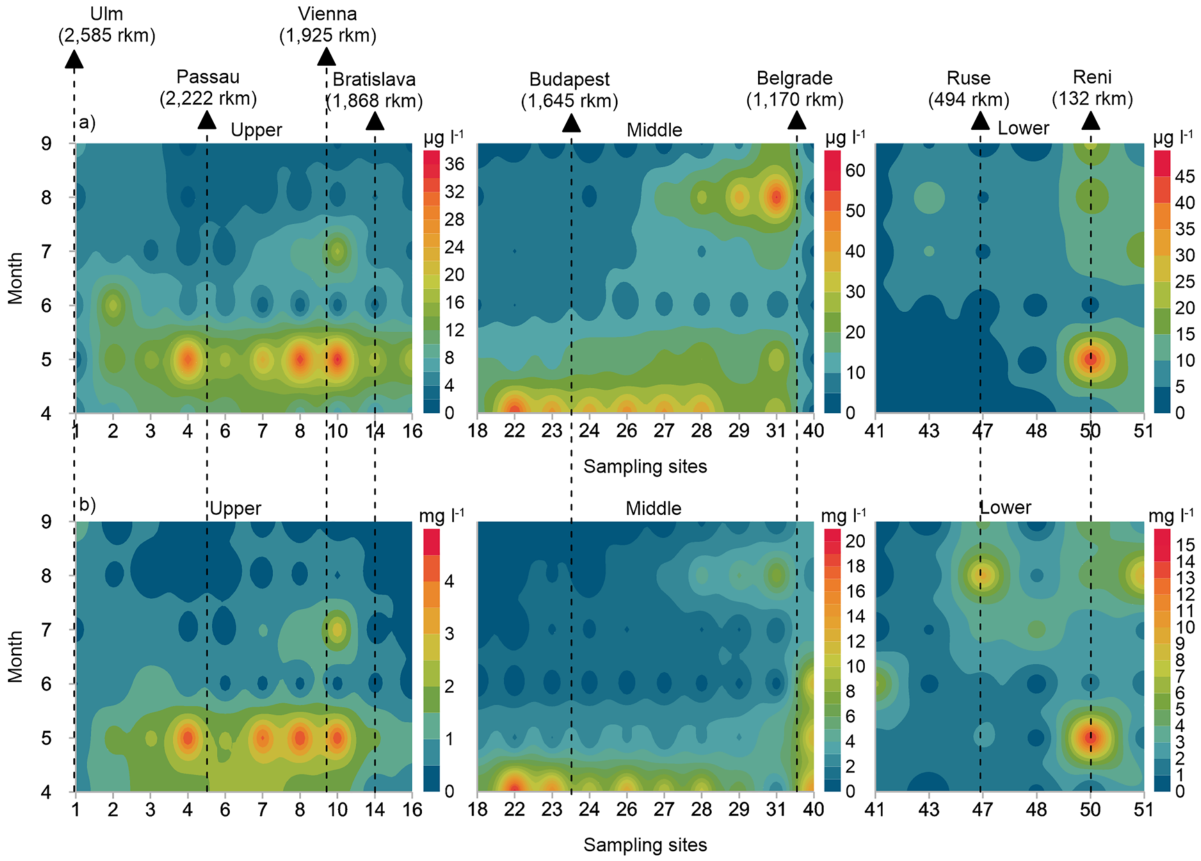


Fig. 3 Contour plot of seasonal and spatial changes of **a** chlorophyll *a* concentration and **b** total phytoplankton biomass in the Danube River at all sampling sites from April to September 2019, presented separately for the Upper, Middle and Lower reaches. See Table 1 for the names of the sampling sites. Note the different scales

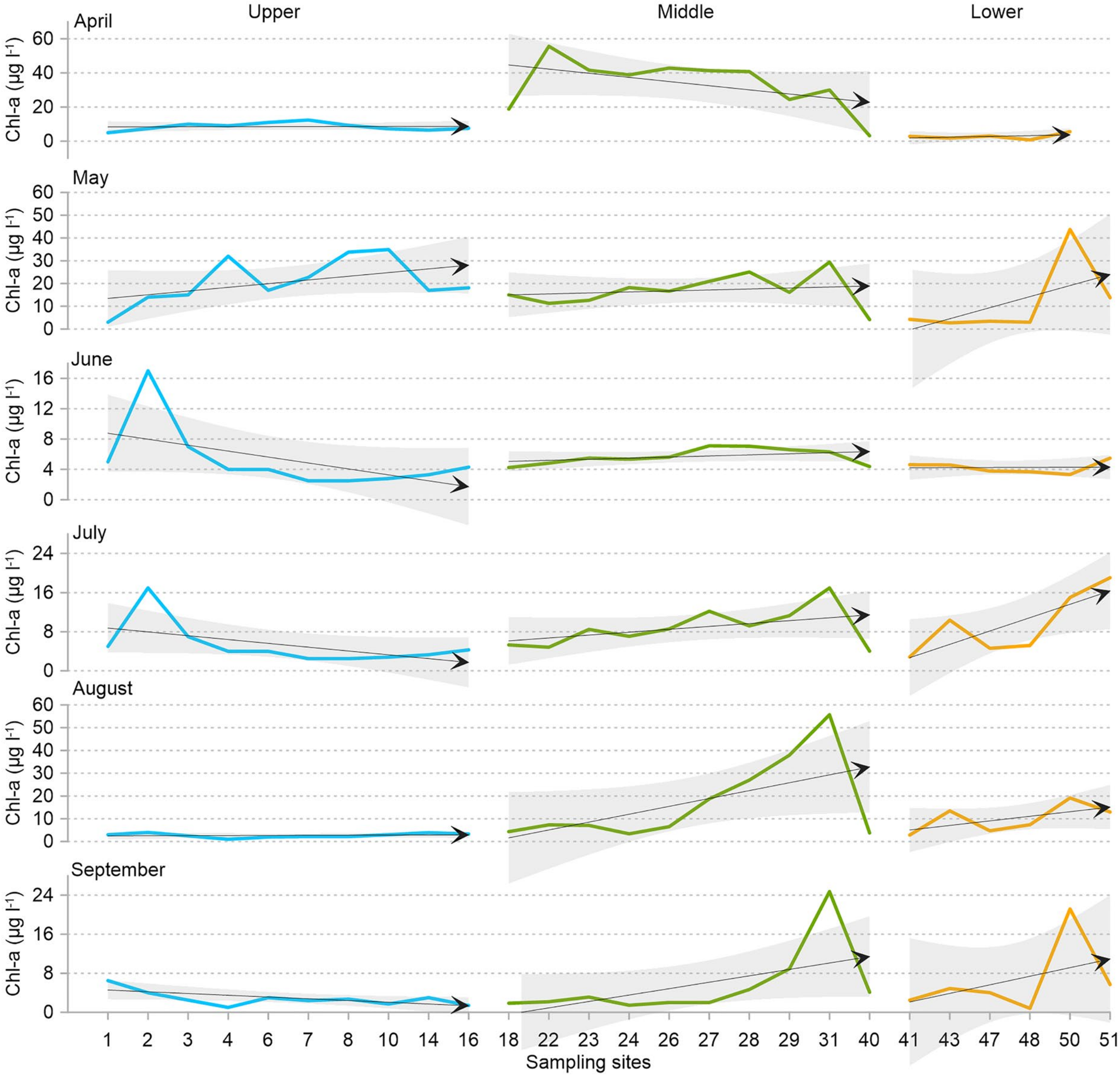


Fig. 4 Seasonal and longitudinal differences in the fitted linear trends for chlorophyll *a* (Chl-a) in the Danube at all sampling sites from April to September 2019. See Table 1 for the names of the sampling sites

We observed both spatial and temporal variations in phytoplankton biomass. The highest values were measured in the Middle reach of the Danube. In the Upper and Lower reaches, the highest phytoplankton biomass was measured in May, with occasional high values in August, while at most sampling sites in the Middle reach, it was measured in April. Trend analysis of Chl-a for each sampling month and Danube reach showed a clear longitudinal growth trend in the Middle and Lower reaches of the Danube with a decrease at the Iron Gate (Fig. 4).

In general, Chl-a levels in the tributaries were twice as high as in the Danube, ranging from 1.0 to 112.5 μg l^{-1}, while total biomass levels were slightly higher, ranging from 0.2 to 21.4 mg l^{-1} (Fig. 5). The tributaries showed remarkable differences in biomass. The rivers Morava, Ipeľ and Ráckevei-Soroksári Danube Arm (sampling sites 13, 21 and 25) showed the

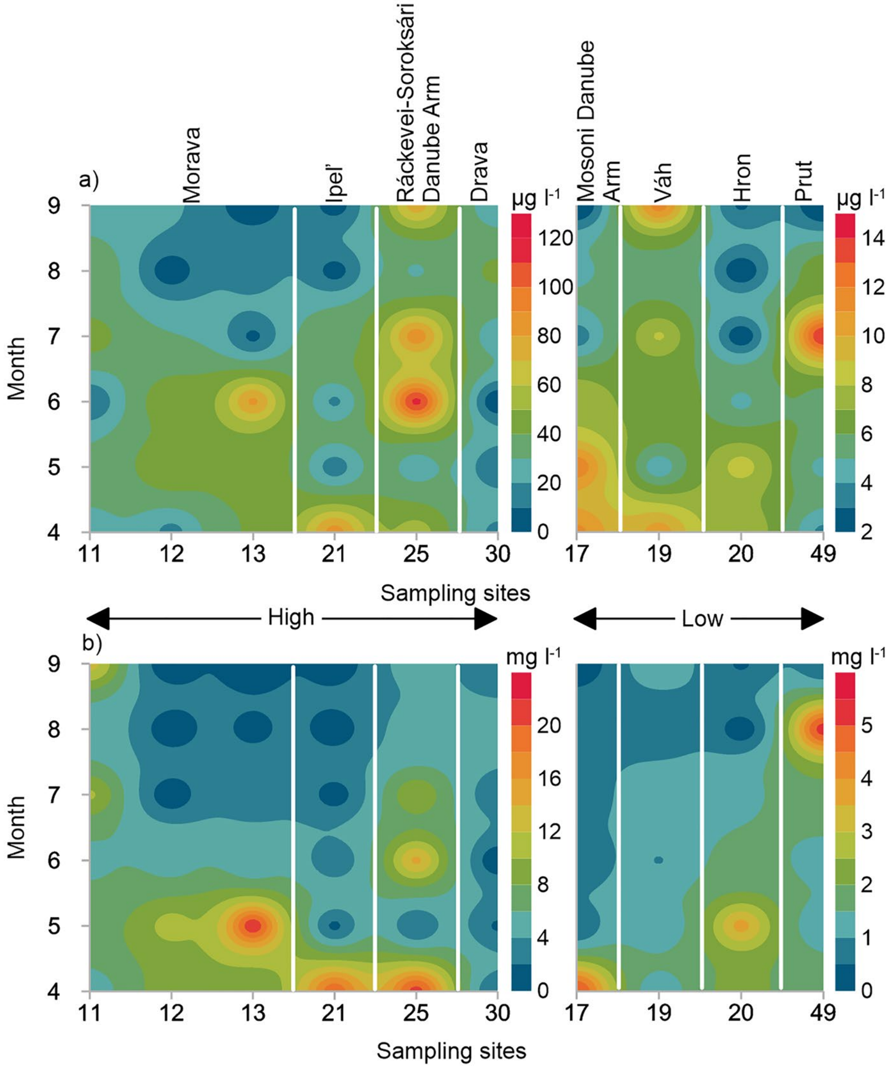


Fig. 5 Contour plot of seasonal and spatial changes in **a** chlorophyll *a* concentration and **b** total phytoplankton biomass in Danube tributaries from April to September 2019, shown separately for sampling sites with high and low chlorophyll *a* concentration and total biomass. White vertical lines separate sampling sites without continuum. See Table 1 for the names of the sampling sites. Note the different scales

highest values, while the rivers Mosoni Danube Arm, Váh, Hron and Prut (sampling sites 17, 19, 20 and 49) had Chl-a $<$ 15 mg l^{-1} and total biomass $<$ 6 mg l^{-1}. The tributaries showed similar temporal trends as the Lower Danube, with the highest values for Chl-a and total biomass in midsummer, but with larger individual fluctuations.

Relationship between phytoplankton biomass and environmental parameters (individual data)

Spearman correlation analysis showed the strength of the relationship between Chl-a and total biomass in the Danube and its tributaries and each of the 15 environmental variables measured. In general, both measured variables showed the same relationship, but occasionally Chl-a had a more pronounced relationship with the background variables (Table 4). Temperature showed a significant negative relationship with Chl-a in the Upper and Middle Danube, while it was positive in the Lower Danube. We found a positive relationship between pH, DO, oxygen saturation and phytoplankton biomass in the whole Danube and the largest studied tributaries Ipeľ and Drava. Ammonia showed a negative correlation with Chl-a in the Upper and Middle Danube and the Morava, and a positive relationship in the Ráckevei-Soroksári Danube Arm and the Prut, while in the Upper Danube, it correlated only negatively with total biomass. Nitrates positively influence phytoplankton biomass

Table 4 Spearman's rho correlations (2-tailed) for correlations between phytoplankton biomass (concentration of chlorophyll *a* and total biomass) and environmental variables; *Correlation is significant at the $P \leq 0.05$ level; **Correlation is significant at the $P \leq 0.01$ level in bold; *n.s.* not significant; total number of samples = 213; *n* = number of cases for analyses used for environmental variables

	Danube River			Tributaries							
	Upper	Middle	Lower	Morava	MDA	Váh	Hron	Ipeľ	RSD	Drava	Prut
(a) Chlorophyll *a*	*n* = 59	*n* = 60	*n* = 35	*n* = 17	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6	*n* = 6
Temperature	**− 0.513****	− 0.279*	**0.439****	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
pH	**0.485****	**0.494****	**0.542****	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	− 0.886*
Dissolved oxygen	**0.591****	**0.832****	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Oxygen saturation	**0.444****	**0.747****	0.389*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**0.943****	n.a.
Conductivity	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**− 0.943****	n.a.	n.a.	n.a.	n.a.
Alkalinity	n.a.	n.a.	− 0.393*	n.a.	n.a.	n.a.	**− 0.943****	n.a.	n.a.	n.a.	n.a.
Ammonia	**− 0.397****	− 0.306*	n.a.	− 0.488*	n.a.	n.a.	n.a.	n.a.	0.845*	n.a.	0.812*
Nitrites	n.a.	n.a.	− 0.338*	n.a.	n.a.	n.a.	**0.943****	n.a.	n.a.	n.a.	n.a.
Nitrates	0.285*	n.a.	**− 0.546****	n.a.	n.a.	n.a.	n.a.	− 0.886*	n.a.	n.a.	n.a.
Soluble reactive phosphorus	**− 0.709****	**− 0.644****	n.a.	n.a.	n.a.	n.a.	− 0.829*	n.a.	0.883*	n.a.	n.a.
Total phosphorus	**− 0.483****	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	− 0.829*	n.a.	n.a.	n.a.
Biological oxygen demand	**0.474****	n.a.	n.a.	**0.937****	n.a.	n.a.	n.a.	0.899*	n.a.	n.a.	n.a.
Total suspended solids	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Discharge	n.a.	n.a.	n.a.	n.a.	0.886*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Residence time	n.a.	n.a.	**0.541****	n.a.	− 0.812*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
(b) Total biomass											
Temperature	**− 0.574****	n.a.	0.339*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
pH	**0.555****	**0.643****	**0.563****	0.578*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Dissolved oxygen	**0.604****	**0.698****	0.355*	n.a.	n.a.	n.a.	n.a.	**0.943****	n.a.	n.a.	n.a.
Oxygen saturation	**0.428****	**0.646****	**0.641****	n.a.	n.a.	n.a.	n.a.	**0.928****	n.a.	**1.000****	n.a.
Conductivity	0.325*	n.a.	n.a.	n.a.	n.a.	n.a.	− 0.886*	n.a.	n.a.	n.a.	n.a.
Alkalinity	0.313*	n.a.	n.a.	n.a.	n.a.	n.a.	− 0.886*	n.a.	n.a.	n.a.	**0.986****
Ammonia	**− 0.467****	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Nitrites	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.886*	n.a.	n.a.	n.a.	n.a.
Nitrates	**0.381****	n.a.	**− 0.440****	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Soluble reactive phosphorus	**− 0.647****	**− 0.566****	n.a.	n.a.	n.a.	n.a.	n.a.	**− 0.943****	n.a.	n.a.	n.a.
Total phosphorus	**− 0.701****	− 0.297*	n.a.	n.a.	n.a.	n.a.	n.a.	− 0.829*	n.a.	n.a.	n.a.
Biological oxygen demand	**0.546****	n.a.	n.a.	**0.862****	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.829*

Table 4 (continued)

	Danube River			Tributaries							
	Upper	Middle	Lower	Morava	MDA	Váh	Hron	Ipeľ	RSD	Drava	Prut
Total suspended solids	− 0.257*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Discharge	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Residence time	n.a.	0.384**	0.427*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

River short codes: *MDA* Mosoni Danube Arm, *RSD* Ráckevei-Soroksári Danube Arm

Table 5 Pearson's correlation between chlorophyll *a* and environmental variables as well as total phosphorus and total nitrogen in the Danube based on averaged site data. Significant correlation is in bold ($p \leq 0.05$ or $p \leq 0.01$)

	r	*P*	*n*
Temperature	0.1406	0.4934	26
pH	− 0.566	0.7837	26
Dissolved oxygen	0.2302	0.2579	26
Oxygen saturation	0.3096	0.1237	26
Conductivity	− 0.2476	0.2226	26
Alkalinity	0.0622	0.7627	26
Ammonia	**− 0.5689**	**0.0024**	**26**
Nitrites	− 0.3584	0.0721	26
Nitrates	− 0.1141	0.5788	26
Total nitrogen	**0.4551**	**0.0577**	**18**
Soluble reactive phosphorus	0.0659	0.7492	26
Total phosphorus	**0.5975**	**0.0012**	**26**
Total phosphorus/total nitrogen	− 0.0965	0.7031	18
Biological oxygen demand	0.1619	0.4294	26
Total suspended solids	− 0.1500	0.4646	26
Discharge	− 0.0993	0.6294	26
Residence time	− 0.0185	0.9287	26

r correlation coefficient, *P* significance, *n* number of cases

in the Upper Danube and negatively in the Lower Danube and the Ipeľ River. SRP and TP showed a significant correlation with phytoplankton biomass, which was mostly negative (Upper and Middle Danube, Hron and Ipeľ), while it was positive with Chl-a only in the Ráckevei-Soroksári Danube Arm. Organic load, expressed as BOD, showed a positive correlation with Chl-a in the Upper Danube, Morava and Ipeľ, similar to total biomass in the Upper Danube and Morava. Hydrological parameters, discharge and RT showed no stable correlation with phytoplankton biomass in the studied rivers. Discharge correlated significantly positively with Chl-a only in the Morava, while RT correlated significantly positively with Chl-a in the Lower Danube, total biomass in the Middle and Lower Danube and negatively with Chl-a in the Morava.

Relationship between phytoplankton biomass and environmental parameters (site–average data)

The average values of Chl-a showed considerable changes along the Danube (3.3–27.2 µg l^{-1}). Although this range was large enough to reveal potential relations with the relevant physical and chemical properties of water, the phytoplankton biomass showed no correlation with the majority of measured environmental variables, including the dissolved forms of nutrients (inorganic N and P) (Table 5). However, the total forms of nutrients correlated well with the Chl-a. Strong significant relationship was found between TP and Chl-a ($P = 0.0012$) while the TN/TP relationship appeared to be marginally significant ($P = 0.0577$). R-square values of linear regressions also indicated the increasing tendencies in the case of both nutrients (Fig. 6). In the case of TP the relationship was apparently nonlinear. While no change could be observed in the TP 0.03–0.6 mg l^{-1} range, a steep increase occurred in the TP < 0.6 range. The Chl-a also showed significant negative correlation with the ammonium ions.

Phytoplankton functional composition

A total of 682 taxa were identified in 213 samples from the Danube and the studied tributaries. They belonged to nine major taxonomic groups (Phylum): Bacillariophyta (249), Charophyta (23), Chlorophyta (224), Choanozoa (1), Cryptophyta (17), Cyanobacteria (77), Euglenozoa (35), Miozoa (10) and Ochrophyta (46). All taxa were assigned to 29 coda of FGs.

The composition of the FGs in the Danube showed seasonal and longitudinal changes (Fig. 7a). PERMANOVA results confirmed a significant influence of seasonal changes when months were used as a factor ($P = 0.0001$; $P < 0.05$), and a significant influence of longitudinal changes when river reach was used as a factor ($P = 0.0001$; $P < 0.05$). Cluster analysis of Bray–Curtis similarity of FG composition based on the average biomass of sampling site data showed a clear grouping of sampling sites according to river reach with minor exceptions (Fig. 7b). The sampling sites of the Upper reach were divided into three smaller groups with a similarity of 60–90%. All sampling sites of the Middle reach formed one larger group with a similarity of > 70%, except sampling site 40 that grouped together with most of the sampling sites of the Lower reach and was most similar to sampling site 50. The two Lower reach sampling sites located just downstream of the Iron Gate (40 and 41) are separated from the rest and are located between two groups of Upper reach sampling sites (3, 4, 6 and 7, 8, 10), with a similarity of > 70% to them.

The one-way SIMPER analysis, based on Bray–Curtis phytoplankton FG similarity and considering data from all 154 samples and the river reach as factors, yielded coda that contributed significantly to the similarity between samples. The Upper reach of the Danube was represented by a co-dominance of benthic (**TB**) and planktonic diatoms (**A**, **C** and **D**). Other coda that significantly contributed to the similarity between samples in the Upper reach were **X2** (9.9%), **J** (5.4%) and **Y** (5.2%). In the Middle and Lower reaches planktonic diatoms dominated, with codon D being the dominant codon, while coda **A** and **C** were co-dominant. The SIMPER analysis also showed that coda **X2** and **TB** contributed to the similarity between samples with 5.7% and 5.6% in the

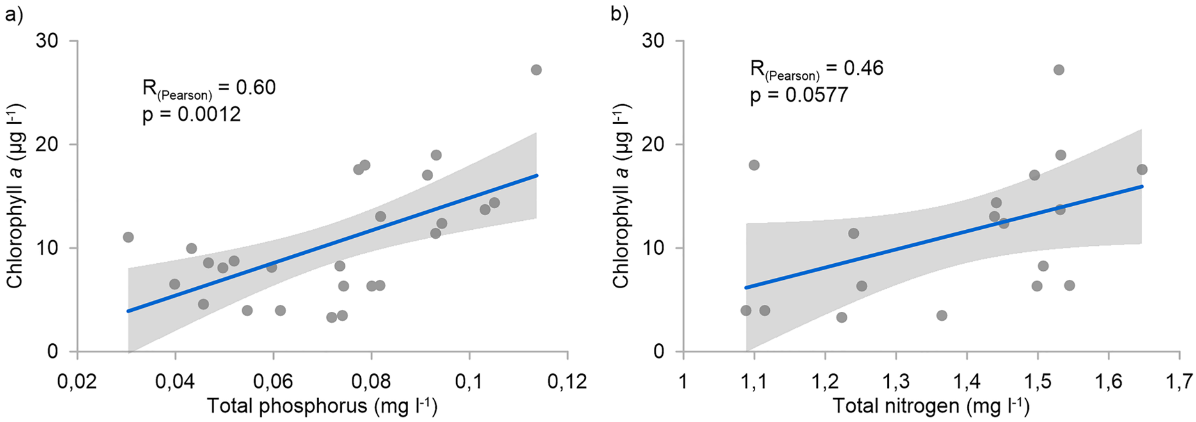


Fig. 6 Scatter plots showing relationship between phytoplankton biomass (chlorophyll *a*) and total phosphorus (left panel) and total nitrogen (right panel). Blue line indicates the ordinary least squares regression (OLS). Grey zone indicates 95% confidence level

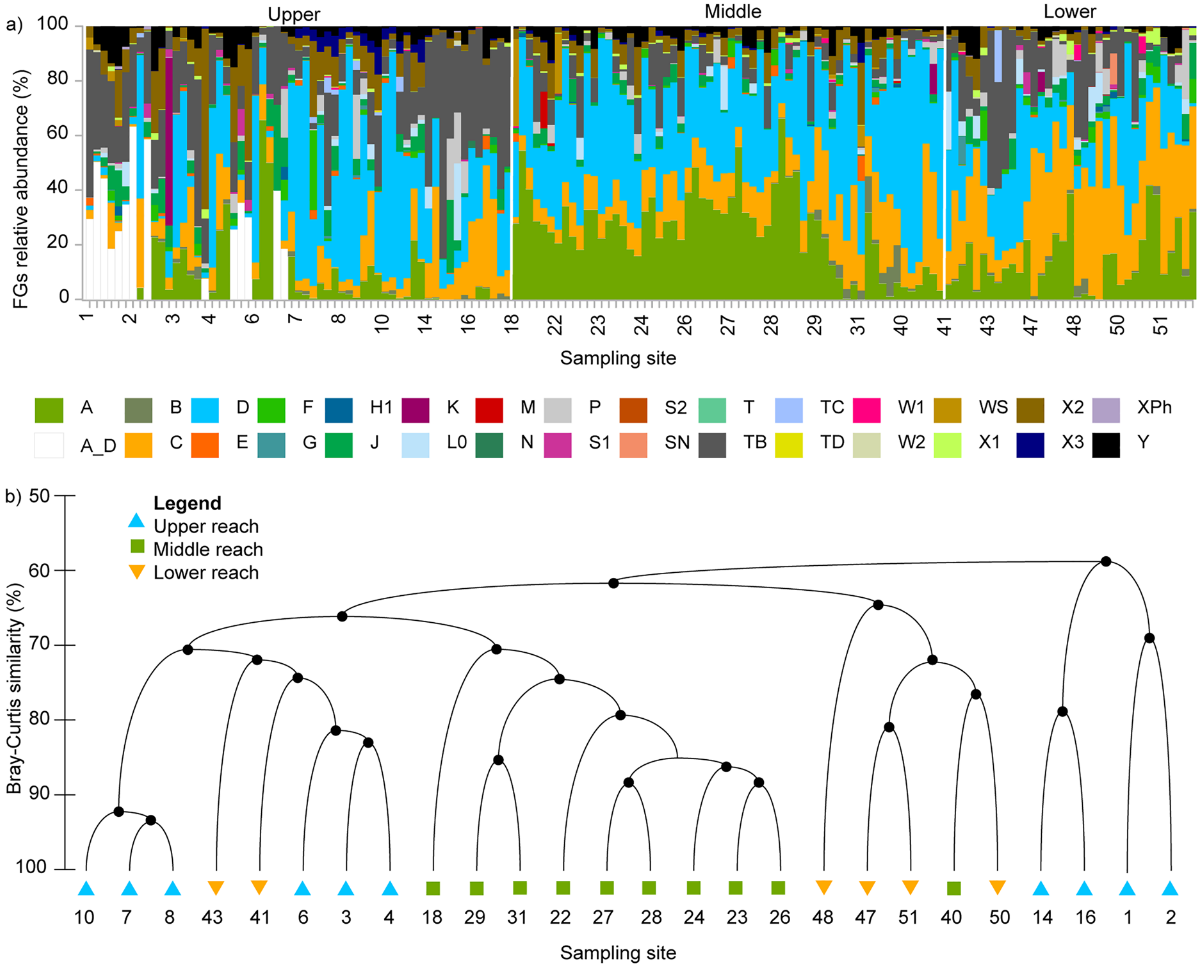


Fig. 7 **a** Relative biomass of phytoplankton functional groups in the Danube at all sampling sites during the study period from April to September 2019. The thick marks on the *x*-axis represent the months of sampling for each sampling site, starting with April from the left side at the position of the site number; **b** Dendrogram of cluster analysis based on the Bray–Curtis similarity index of phytoplankton FG composition. See Table 1 for the names of the sampling sites

Middle reach, while coda **TB** and **J** contributed to the similarity in the Lower reach of the Danube with 15.7% and 2.2%.

It is difficult to generalise about all tributaries because they differ in terms of catchment size and geographical location, but in most samples, the diatom FGs dominated there too. The composition of the FGs in the tributaries is shown in Fig. 8a. The SIMPER analysis shows in most tributaries, the coda **TB**, **C** and **D** as dominant or jointly dominant. In the Mosoni Danube Arm and the Ráckevei-Soroksári Danube Arm there was less codon **TB** and more codon **A**. A very specific assemblage could be observed at sampling site 11 in the Morava, where besides codon **TB**, codon **M** with cyanobacteria *Microcystis* spp. and codon **J** with green algae, which prefer highly enriched systems, contributed most to the similarity between the samples. The SIMPER analysis revealed that the codon $\mathbf{S_N}$ is not significant, although *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno reached a high relative abundance of > 72% in September. Other FGs that contributed to the similarity between samples in tributaries with > 5% were coda **P** (Morava (11), RSD), **J** (Morava (12), Ipeľ, Prut), **X2** (Mosoni Danube Arm, Ráckevei-Soroksári Danube Arm, Drava), $\mathbf{L_O}$ (Váh), **W2** (Ipeľ) and **Y**

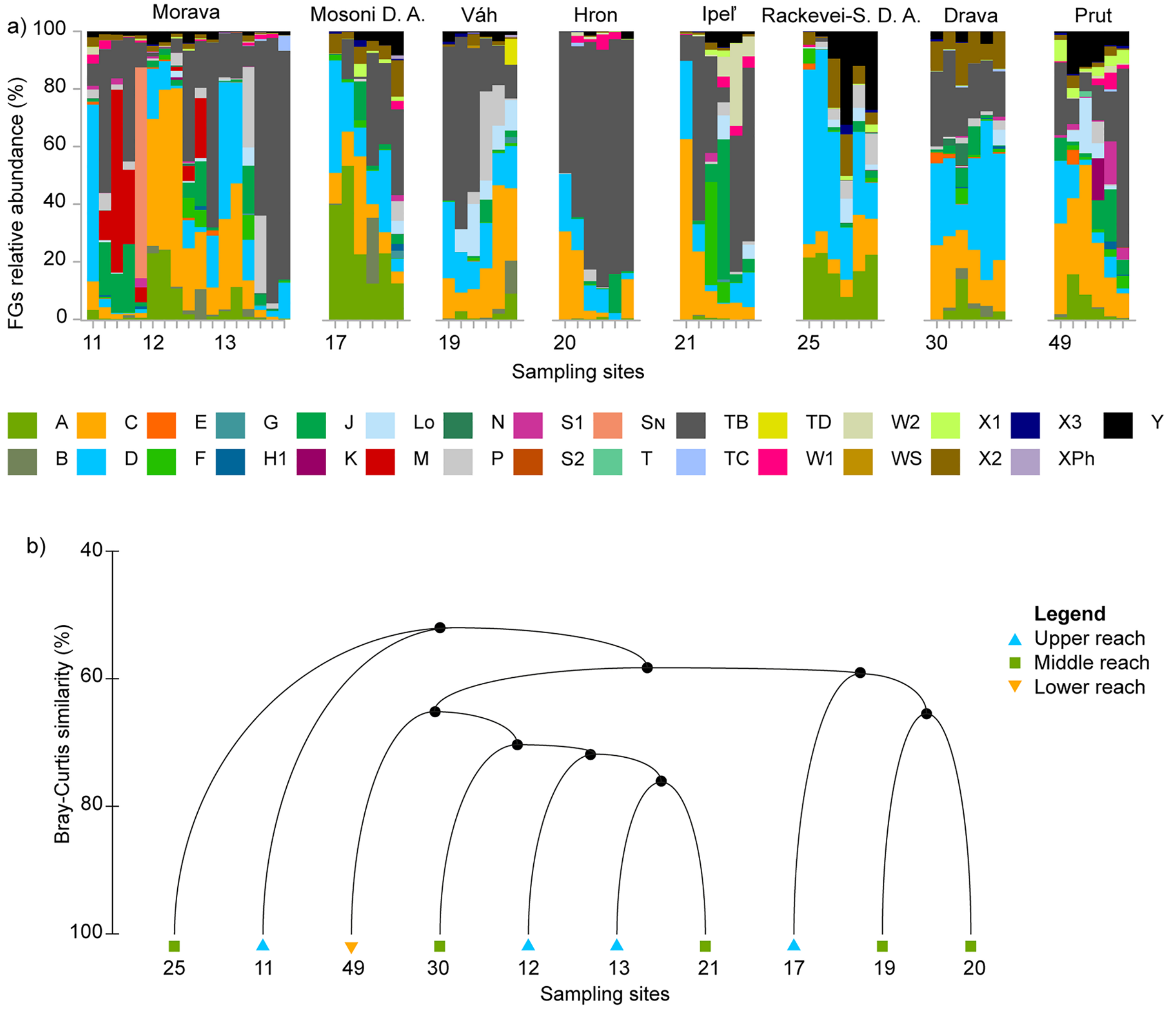


Fig. 8 **a** Relative biomass of phytoplankton functional groups in the Danube tributaries during the study period from April to September 2019. The thick marks on the *x*-axis represent the months of sampling for each sampling site, starting with April from the left side at the position of the site number; **b** Dendrogram of the cluster analysis based on the Bray–Curtis similarity index of phytoplankton FG composition

(Ráckevei-Soroksári Danube Arm). Cluster analysis of Bray–Curtis similarity between the composition of FGs based on the average biomass of the sampling sites' data showed a clear separation of Ráckevei-Soroksári Danube Arm and sampling site 11 in the Morava River with a specific phytoplankton community, a higher proportion of cryptophytes and cyanobacteria, respectively. River clusters of higher and lower trophic status clearly separated from each other. Exception was the river Prut, which although had a low trophic status, positioned closer to the rivers with higher trophic status (Fig. 8b).

To better understand the composition of the phytoplankton FGs in the Danube and its tributaries, the frequency of occurrence in the samples and the proportion of maximum biomass were analysed to identify the dominant FGs (Table 6). The results were similar to the SIMPER analysis. In the Danube, the dominant coda with high occurrence rate and high biomass were **A**, **C**, **D**, **TB** and **X2**, co-dominant coda with high occurrence rate but lower biomass were **B**, **F** and **Y**, while codon **K** was a codon with low occurrence rate and occasionally with high biomass. Codon **Y** was present in

Table 6 Composition, occurrence rate and biomass contribution of phytoplankton FGs; n = number of samples used for occurrence rate analysis

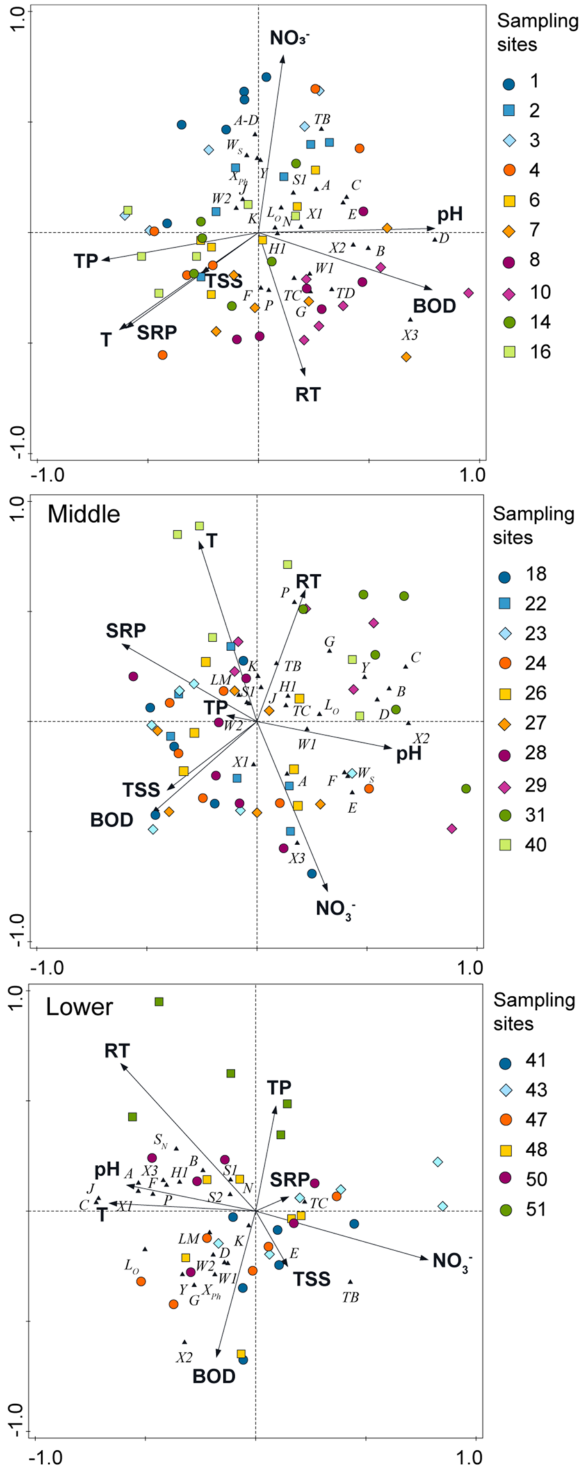
Functional groups (FGs)	Danube ($n = 154$)		Tributaries ($n = 59$)	
	Occurrence rate/%	Max. biomass proportion/%	Occurrence rate/%	Max. biomass proportion/%
D	98.70	85.14	100.00	63.07
TB	98.70	82.69	96.61	92.17
X2	98.70	66.07	94.92	17.09
C	98.05	64.98	96.61	68.82
J	94.16	31.47	98.31	48.62
Y	85.71	16.32	81.36	32.41
A	84.42	66.34	84.75	53.30
X1	83.77	6.02	91.53	7.48
B	77.92	14.46	86.44	24.72
F	70.78	29.17	84.75	35.81
P	59.09	25.37	69.49	30.94
X3	53.25	10.06	61.02	3.32
E	38.96	9.15	30.51	5.08
L_O	38.96	28.18	55.93	19.23
S1	35.06	9.74	47.46	14.90
W1	14.94	6.22	45.76	5.86
K	12.99	61.43	20.34	14.60
G	10.39	10.33	16.95	2.69
H1	10.39	4.71	22.03	2.71
TC	9.74	19.02	23.73	5.38
A–D	9.09	63.32	–	–
W_S	7.14	31.87	1.69	0.71
W2	5.84	1.96	30.51	28.55
M	4.55	13.43	13.56	63.50
X_{Ph}	3.90	1.12	6.78	1.20
S_N	2.60	11.37	1.69	73.20
N	1.95	2.66	15.25	7.70
TD	1.95	0.62	1.69	8.90
S2	0.65	0.09	1.69	0.17
T	–	–	3.39	2.00

most samples with a relative biomass of up to just over 16%. Only three coda appeared with a maximum biomass of < 1% in the Danube (**TD**, **S2**, **T**) and two in the tributaries (**WS** and **S2**). Dominant coda with high occurrence rate and high maximum relative biomass in the tributaries were identical to those in the Danube, excluding codon **X2**, which was very abundant with 94.9% of occurrence rate, but with low biomass up to only 17.1% and not stable between samples, as were coda **B** and **X3**. Coda with co-dominant features were **F** and **P**, while occasional dominant coda with low occurrence rates and sporadically high biomass were **M** and **S_N** with maximum relative biomass up to 63.5% and 73.2%, respectively.

Relationship between phytoplankton FGs and environmental parameters

The ordination results of the phytoplankton FGs and the environmental data of the RDA in the Upper, Middle and Lower reaches of the Danube are shown in the F1 × F2 ordination plots (Fig. 9).

For the Upper Danube, the first two canonical axes explained 96.1% of the FGs–environment relationship. The environmental variables pH, TP and BOD



		Axis 1	Axis 2
Axis summary statistics and variance in species data[a]			
Eigenvalues		0.46	0.034
FGs-environment correlations		0.80	0.640
Cumulative percentage variance			
Of FGs data		46.0	49.3
Of FGs-environment relation		89.6	96.1
Correlations of environmental variables and redundancy axes[b]			
Variable			
T		-0.502	-0.281
pH	p = 0,002	**0.637**	0.012
NO_3^-		0.090	**0.513**
SRP		-0.479	-0.277
TP	p = 0,004	-0.566	-0.080
BOD	p = 0,002	**0.627**	-0.166
TSS		-0.206	-0.118
RT		0.168	-0.415

		Axis 1	Axis 2
Axis summary statistics and variance in species data[a]			
Eigenvalues		0.144	0.084
FGs-environment correlations		0.769	0.747
Cumulative percentage variance			
Of FGs data		14.4	22.8
Of FGs-environment relation		41.1	65.2
Correlations of environmental variables and redundancy axes[b]			
Variable			
T	p = 0,002	-0.202	**0.611**
pH		0.471	-0.091
NO_3^-	p = 0,002	0.247	-0.576
SRP	p = 0,002	**-0.471**	0.263
TP		-0.105	0.020
BOD		-0.367	-0.313
TSS	p = 0,004	-0.313	-0.233
RT	p = 0,004	0.169	0.446

		Axis 1	Axis 2
Axis summary statistics and variance in species data[a]			
Eigenvalues		0.161	0.043
FGs-environment correlations		0.842	0.653
Cumulative percentage variance			
Of FGs data		16.1	20.4
Of FGs-environment relation		51.6	65.4
Correlations of environmental variables and redundancy axes[b]			
Variable			
T		-0.559	0.023
pH		-0.492	0.077
NO_3^-	p = 0,002	**0.658**	-0.144
SRP		0.126	0.042
TP		0.078	0.311
BOD	p = 0,02	-0.150	**-0.432**
TSS		0.122	-0.164
RT		-0.515	**0.437**

had a significant influence on the composition of FGs. Axis 1 had the highest correlation with pH and BOD, while axis 2 had the highest correlation with nitrites. Among the dominant FGs, benthic diatoms in codon **TB** and planktonic diatoms in codon **A** and **C** favoured lower RT, TP and temperature, and higher

◂**Fig. 9** Results of the redundancy analysis (RDA) between FGs and abiotic parameters for the Upper, Middle and Lower reaches of the Danube; correlation triplots are on the left, while the numerical data of the analysis can be found in the tables on the right; [a]Axis summary statistics of the two extracted canonical axes and the percentage of variance explained by the RDA ordination; [b]Correlation of the environmental variables with the ordination axes; explanatory variables at $P \leq 0.05$ significance level (999 permutations) in the forward selection are in bold with *P*-value; codes of variables: *T* water temperature, *pH* pH, NO_3^- nitrates, *SRP* soluble reactive phosphorus, *TP* total phosphorus, *BOD* biological oxygen demand, *TSS* total suspended solids and *RT* residence time

nitrate concentrations. Codon **D** preferred conditions similar to **X2** with higher pH, lower TP and T and moderate RT and nitrate concentrations. At the same time, BOD was elevated. TP generally had a negative impact on most FGs, except for coda **F** and **P**, which preferred longer RT and moderate TP. The samples of the uppermost river section positioned along the higher nitrates, samples little downstream were next to the longer RT and higher pH and BOD, while the lower sampling sites had higher temperature and higher concentration of TP.

For the Middle Danube, the first two canonical axes explained 65.2% of the FGs–environment relationship. The environmental variables water temperature, nitrites, SRP, TSS and RT had a significant influence on the composition of FGs. Axis 1 had the highest correlation with SRP, while axis 2 had the highest correlation with water temperature. In this part of the Danube, the dominant codon **D** preferred conditions with higher pH and longer RT under conditions with lower SRP, TSS and BOD. Similar conditions were preferred by the co-dominant coda **TB**, **C** and **X2**. Codon **A** associated with higher nitrate concentrations and shorter RT. The longitudinal order of the samples on the graph shows that most samples from the beginning of this reach preferred higher SRP, T and BOD, while samples from the end of this reach associated to higher pH, higher temperature and longer RT.

For the Lower Danube, the first two canonical axes explained 65.4% of the FGs–environment relationship. The environmental variables nitrites and BOD had a significant influence on the composition of FG. Axis 1 had the highest correlation with nitrates, while axis 2 had the highest correlation with BOD and RT. The dominant codon D preferred conditions with moderate RT, pH and temperature when BOD was slightly elevated. Co-dominant coda **A** and **C** favoured higher pH, higher temperature and longer RT, while higher nitrate concentration was favoured by codon **TB**. Sampling sites at the beginning of the Lower reach, just after the Iron Gate, grouped near higher nitrate concentrations and TSS and are associated with the codon **TB**. Sampling sites near the Danube Delta had the highest concentration of TP and the longest RT.

Discussion

In this paper, we report the results of a comprehensive phytoplankton survey in the Danube and selected tributaries performed by the Joint Danube Survey 4 (JDS4). This survey included phytoplankton sampling throughout the growing season (April–September) and along the entire Danube from the Böfinger Halde at 2,581 rkm to the Danube delta at Vilkove on the Chilia arm at 18 rkm (Liška et al. 2020). JDS4 is unique because phytoplankton was also analysed during the previous JDS1-3 expeditions (Németh et al., 2002; Dokulil & Kaiblinger, 2008; Dokulil & Donabaum, 2014), but sampling was done only once at the end of summer at each sampling site, so temporal changes are missing. Compared to all other previous studies of phytoplankton in the Danube, it has been analysed on many occasions, but never to this extent. Historical studies started more than 100 years ago (Brunnthaler, 1900), but even in later years only parts of the river were analysed (Kiss, 1994, 1997), only certain sampling sites were analysed (Abonyi et al., 2018) or the data from temporally and spatially different studies were combined to obtain a more complete dataset (Rusanov et al., 2022).

Longitudinal connectivity of the Danube

It would be expected that the Danube, as a large 10th order river (Tockner et al., 2009) follows the principles of the River Continuum Concept (RCC) (Vannote et al., 1980). Following this principle, the Danube was officially divided into Upper, Middle and Lower reaches (Moog et al., 2006; ICPDR, 2021), which was also confirmed by clustering our environmental data and phytoplankton composition data, with minor and very similar exceptions between the two datasets. Our results suggest that the clustering of sampling site Banatska Palanka/Bazias at 1,073 rkm (40) as the last sampling site in the Middle reach with the sampling sites in the Lower reach is to be expected as it is under the influence of the largest longitudinal barrier, the Iron Gate (ICPDR, 2021). The first two sampling sites of the Lower reach (41 and 43) were clustered with sampling sites of the Upper reach, which typically have more benthic diatoms (Stanković et al., 2012; Abonyi et al., 2014), as the proportion of tychoplanktic diatoms belonging to the codon **TB** is higher than in other sampling sites of the Lower reach. We conclude that there could be two possibilities for the origin of the tychoplanktic diatoms: either from the Iron Gate itself, which generates strong turbulence and thus lifts benthic diatoms out of the periphyton, or from smaller turbulent sidearms in this area, which artificially endow this part with certain characteristics of lower-order rivers (Borics et al., 2007).

The values of the environmental parameters also did not agree with the RCC. The increase in water temperature and the decrease in oxygen concentration along the river followed the RCC, while parameters such as nitrates and ammonia, which were highest in the Upper Reach did not. Water temperature clearly depends on the natural river order and is difficult to disturb (elevation, colder tributaries in the Upper reach), as does dissolved oxygen, which depends mainly on water temperature, dissolved solids and organic degradation (Wetzel, 2001). However, nutrients can vary considerably depending on anthropogenic influence, which is very high in the Danube catchment (ICPDR, 2021).

In agreement with RCC (Vannote et al., 1980), one would expect phytoplankton biomass to increase longitudinally, but the results of our study showed the highest values in the Middle reach instead of the Lower reach, forcing us to find other specific factors that can explain this paradox. Although our results are consistent with those of previous studies systematically analysed by Dokulil (2015), we do not consider the matter closed. A more detailed analysis of Chl-a on seasonal and longitudinal scales reveals that Chl-a not only shows an almost unpredictable behaviour in the Upper reach with its dozens of impoundments, but also shows a growing longitudinal trend in the Middle and Lower reaches in the free-flowing sections with a large break in between, the Iron Gate. The Chl-a results indicate that the phytoplankton settle upstream of the Iron Gate and then continue to develop after this large obstacle has been passed and its influence minimised. A similar decline in algal biomass was found by Sabater et al. (2008) in the Ebro River and by Istvánovics et al. (2010) in the Tisza River. The latter were not able to link zooplankton grazing to this Chl-a decline. We were also not able to link these two, as zooplankton was only sampled once in JDS4 (Kiss & Zsuga, 2020), but according to the literature, zooplankton in the Danube has little or no influence on phytoplankton biomass (Dokulil, 2015).

Hydromorphological degradation is usually the strongest pressure in very large rivers (Petts, 1984; Nilsson et al., 2005) and biological quality elements (BQEs) such as macroinvertebrates and fish were traditionally the ones that provided a reliable response for water management (Urbanič et al., 2020). However, thanks to this comprehensive study, the results strongly suggest that phytoplankton respond to hydromorphological degradation in the Upper section and at the Iron Gate, giving a new perspective to this particular BQE, which has been mainly associated with eutrophication (Bellinger & Sigee, 2015). We found no evidence in our study to support the regional species pool theory of Rusanov et al. (2022). As mentioned above, our results suggest that hydrological characteristics, artificial barriers and other environmental parameters play a key role in shaping the biomass and composition of phytoplankton in the Danube.

Factors regulating phytoplankton biomass

Phytoplankton biomass is controlled by environmental factors (Reynolds, 1988a), which is confirmed by our correlation analysis. Although water temperature showed a significant correlation with Chl-a in all three river reaches, it was opposite in the Lower reach to that in Upper and Middle reaches, suggesting that it is not a critical element but rather a seasonal factor. Although phosphorus and nitrogen concentrations exceeded the values considered characteristic for mesotrophic and eutrophic conditions, their values fell in the range where they can be potentially limiting for phytoplankton (Dodds, 2006; Poikane et al., 2022). Higher nitrate concentration and inhibition by ammonia, already observed in previous reports (Kang et al., 2020), were predictors of phytoplankton biomass growth in the Upper reach. As well studied

(Salmaso & Braioni, 2008; Salmaso & Zignin, 2010), a lower flushing rate, expressed by a longer residence time (RT), had a positive influence on phytoplankton biomass in the Middle and Lower reaches. River hydrology not only has a direct impact on phytoplankton by allowing algae to reproduce, but it also dilutes phytoplankton by increasing flushing rates while bringing nutrients with it, leading to a negative correlation between nutrients and biomass (Reynolds, 1988b, 1994), as shown by the results of this study.

The more frequent increase in water temperature and decrease in water discharge in European rivers (Moatar & Gailhard, 2006) would lead us to conclude that to date not the eutrophication is the most important pressure in the Danube. Recent studies demonstrated that oligotrophication is starting, both in lakes (Morabito et al., 2012; Pomati et al., 2012) and in rivers like in the Danube itself (Abonyi et al., 2018). This trend is consistent with the results of our study, in which we measured a Chl-a concentration much lower than in previous studies, where it sometimes reached up to 150 µg l^{-1} (Dokulil, 2015). Low biomass values suggests that nutrient control measures in the Danube catchment (WFD, 2000) are working, and although lower phytoplankton biomass data were published earlier for only certain sections of the river (Dokulil, 2015), this study demonstrates that oligotrophication affects the entire Danube River. However, based on the data from one vegetation period and the hydromorphological influence on the decrease of biomass in the Lower Danube, we do not yet know whether oligotrophication is present and/or a permanent process. Therefore, this should be one of the priorities in the following Joint Danube Surveys.

The results of our study show that not all environmental parameters influence phytoplankton biomass. Today, organic pollution of large rivers, including the Danube, originates from human activities such as wastewater discharges, agriculture and industry (ICPDR, 2021) and negatively affects humans and ecosystems (Wen et al., 2017). In countries along the Upper Danube, such as Germany and Austria, almost 100% of municipal wastewater is treated by a tertiary treatment plant and discharges of BOD are close to 0. The percentage of less well treated or completely untreated wastewater is increasing downstream, together with a high emission of BOD discharges to the Danube (ICPDR, 2021). This is consistent with our findings that phytoplankton biomass is positively correlated with BOD only in the Upper reach, suggesting that planktonic algae are the dominant organic component in the water and that most of the oxygen used for BOD in the Upper Danube is due to algae and not to an external source of organic pollution. The positive relationship between dissolved oxygen and phytoplankton biomass in the Upper and Middle reaches suggests that phytoplankton is the most important autotrophic component there (Dodds, 2006), where, due to high turbidity and often steep river banks, the poorly represented phytobenthos and macrophytes cannot play a significant autotrophic role (Stanković et al., 2014; Stanković & Bubíková, 2020).

However, the negative nutrient/Chl-a relationships are not exceptional in rivers. Reviewing nutrient/Chl-a relationships in lotic systems, Bennett et al. (2021) found that 21% of the cause–effect pairs in the literature appeared to be negative because the response of phytoplankton to nutrients is influenced by other factors such as residence time (Mischke et al., 2011) and cumulative daily radiation (Várbíró et al., 2018) or turbidity, discharge and grazer density (Bennett et al., 2021).

The correlation pattern of mean values of environmental variables and Chl-a appeared to be different from that found for individual data. The lowest Chl-a values (3.3 µg l^{-1}) fell in the oligotrophic (<10 µg l^{-1}), while the highest (27.2 µg l^{-1}) fell in the upper part of the mesotrophic range (Dodds et al., 1998). The mean values of the nutrients fell within the range where they could potentially limit algal growth (i.e. TP <100 µg l^{-1} and TN $<1{,}700$ µg l^{-1} (Phillips et al., 2008). Therefore, both TN and TP had an effect on the growth of algae in the Danube. The TN/Chl-a relationship was only marginally significant, but we note here that TN values for the uppermost river section were missing and this lack of data reduced the breadth of our analysis.

These results support the view that phytoplankton is a good indicator of nutrient loading and can therefore be successfully used as a BQE for the assessment of ecological status (WFD, 2000) in very large European rivers (Mischke et al., 2018).

Phytoplankton FGs composition and factors that regulate it

Joint Danube Survey 4 is a unique baseline research that reveals the seasonal and longitudinal composition of phytoplankton FGs in the Danube and the factors that predict it, proving once again that the FG concept is irreplaceable in phytoplankton studies (Borics et al., 2007; Stanković et al., 2012; Abonyi et al., 2014, 2020a). This study showed that the FG composition of the phytoplankton exhibited the typical structure of riverine potamoplankton with dominant coda **A**, **C**, **D** and **TB** (Reynolds, 1994) with occasional co-occurrence of other typical coda like **X2**, **P** or **F**. Besides a fine response to seasonal and longitudinal changes, the composition of the phytoplankton FGs also showed a response to environmental parameters, providing a clearer understanding compared to the pure phytoplankton biomass.

Most rivers, including the Danube, are subject to frequent flow fluctuations (Reynolds, 2000) and are not limited by nutrients (Dodds, 2006). The results of our study have shown that nutrients play a minor role in the longer RT preferred by the light-harvesters, such as the dominant coda **C** and **D**. Finally, phytoplankton organisms tend to stay in the water column as long as possible and collect as much light as possible, so the longer RT was also preferred by most other coda (Reynolds, 2006). The codon **TB**, as a tychoplanktic component and more abundant in rithral rivers, was naturally pronounced in the Upper Danube, and also in the conditions of higher discharge in the Lower Danube (Borics et al., 2007). Phosphorus proved to be a significant parameter, but with opposite effect to RT and did not correlate positively with the dominant coda, as the nutrient flushing effect after heavy rainfall increases phosphorus concentration and reduces biomass (Borics et al., 2007; Stanković et al., 2012).

Grazing on zooplankton as the highest pressure on larger cryptophytes of codon **Y** is not pronounced in the Danube (Kiss & Zsuga, 2020) and together with their adaptability to different habitats makes them ubiquitous but never dominant in the Danube (Reynolds et al., 2002). Codon **X3** might indicate oligotrophication of the Danube (Abonyi et al., 2018), but although it is present in half of the samples and was more abundant in the Upper Danube with good wastewater treatment (ICPDR, 2021), its abundance was never high, so concerning this FG definite conclusion cannot be drawn. However, it showed a negative correlation with phosphorus in all RDA triplots, suggesting that it should be considered as an indicator when monitoring oligotrophication of the river in the future.

Tributaries

In most of the studies on phytoplankton in the Danube or in other large rivers, tributaries have often been neglected, although they can be important sources of phytoplankton biomass and composition in the main channel (Reynolds & Descy, 1996). In many studies focusing on tributaries (Beránková & Ungerman, 1996; ICPDR, 2009; Stanković et al., 2012), and it was shown that these tributaries cannot be neglected during the study of the main channel (Vadadi-Fülöp et al., 2007). The results of the JDS4 also confirmed that the large sampling effort applied, provides valuable information on how they shape the phytoplankton in the river Danube. Concentrations of the Chl-a in the tributaries were almost twice as high as in the Danube, and although they were significantly diluted after entering the main channel, they contributed considerably to the phytoplankton of the lower segments. The peak value of Chl-a in our study at the sampling site shortly after the Mosoni Danube Arm in April and in most samples at the sampling site after the confluence of the Drava once again underlines that the tributaries of the Danube are very important factors shaping the phytoplankton community (Rusanov et al., 2022).

The small dataset per sampling site in the tributaries may explain the small number of significant correlations of environmental parameters with phytoplankton biomass, but some conclusions can be drawn from these results. In those tributaries—like in the Morava River—that are affected by many wastewater discharges without phosphorus removal (Beránková & Ungerman, 1996), the phosphorus concentration can be so high that significant correlation with phytoplankton biomass cannot be observed. However, the composition of the phytoplankton, i.e. the presence cyanobacteria as the dominant taxonomic group with potentially toxic strains in FG coda **M** and $\mathbf{S_N}$ clearly indicates the anthropogenic loads and the potential threats to aquatic organisms and humans (Istvánovics, 2009). Fortunately, the composition of most tributaries resembled that of the Upper reach of the Danube with typical potamoplanktic centric diatoms and a higher proportion of benthic diatoms due to lower residence time and greater mixing by the current

(Reynolds, 1994). The positive correlation of phytoplankton biomass and oxygen in the largest tributaries such as Drava and Ipeľ indicates that phytoplankton is the dominant autotrophic component here, as well as in the Upper and Middle reaches of the Danube (Dodds, 2006).

Conclusions

The results of this spatially and temporally uninterrupted study filled knowledge gaps, raised new questions and allowed for more informed conclusions compared to previous studies that were limited in time and space. This study revealed the extraordinary role of the Iron Gate, which extremely slows down the river and disrupts the processes described by the River Continuum Concept, affecting the river in many ways both upstream and downstream and showing that phytoplankton can also be an indicator of hydromorphological degradation. Longer residence time and hydrological obstacles shaped the phytoplankton community more than nutrients that were sufficient for undisturbed growth. However, a clear positive correlation between phytoplankton biomass and nutrients (TP and TN) based on average site data proved once again that phytoplankton is a good biological quality element for the assessment of the ecological status of the Danube. The unusually high Chl-a concentration in the Middle Danube compared to the Lower Danube was caused by the disruption of longitudinal connectivity, which stopped the natural growth of phytoplankton along the river. Compared to previous studies, the low maximum values of Chl-a in this study together with the composition of the phytoplankton FGs prove the oligotrophication of the Danube, but long-term trends in both nutrients and biological response are needed to determine if it is a continuous and permanent process. The concept of FGs coda was a meaningful practical help to aggregate the long taxa list to the functional properties of phytoplankton and allows a better understanding of phytoplankton composition and its relationship to environmental parameters, as well as contributing to the understanding of basic processes in river ecosystems, e.g. oligotrophication.

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Data availability Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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