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## Summary

1. Long-term dynamics of phytoplankton have been addressed in marine and lake systems, but rarely in rivers. Large rivers, however, are highly human-impacted, whereas global warming may further affect the functioning of phytoplankton at long-term scale.

2. In the middle section of the large European Danube River, long-term decrease in phytoplankton biomass (Chl-*a*) and increase in species diversity have formerly been revealed. The functional community composition that relates to ecosystem functioning directly has not been addressed previously. We analyze a 34-year long phytoplankton dataset from the middle river section at Göd (N-Budapest), Hungary. We focus on gradual changes in the functional composition and functional diversity components based on the functional trait and functional group approaches.

3. We hypothesized that long-term gradual changes in major environmental constraints should be followed by gradual shifts in dominance relationships among functional traits and functional groups of phytoplankton. We further hypothesized that functional shifts were highlighted by gradual changes in functional diversity components: evenness, divergence, and dispersion.

4. Water discharge of the middle Danube shifted towards the more frequent occurrence of lower values. On the other hand, high floods ( $> 3000 \text{ m}^3\text{s}^{-1}$ ) increased significantly with shortening tendency in duration and altered seasonality. The concentration of N- and P- forms, as well as total suspended solids decreased significantly. Water temperature increased significantly, especially in summer. In the phytoplankton, single-celled eutrophic centric diatoms decreased in relative abundance, but flagellated,

elongated, and filamentous forms increased. A clear functional shift was the dominance decrease of planktonic taxa and the relative abundance increase of benthic diatoms.

5. All functional diversity components increased significantly in the entire dataset, except functional evenness based on the functional group approach. At seasonal scale, all significant trends showed increases, except the functional evenness components of the functional group approach, which decreased in winter and spring significantly.

6. Long-term increase in functional diversity components alone could indicate enhanced ecosystem functioning of phytoplankton in the middle section of the Danube. However, we argue that the observed increase in functional diversity may be related to a gradual shift from high-biomass communities with the dominance of eutrophic centric diatoms towards the relative increase of several, but low-biomass elements. These include a few planktonic algae well adapted to the altered conditions, diatoms with benthic origin, and dispersed limnophilic taxa.

7. Our results provide the first evidence for a long-term phytoplankton functional regime shift in a European large river. Global warming, human impacts and oligotrophication might potentially increase the functional diversity of large river phytoplankton, but the origin and functional role of taxa should carefully be considered. The observed functional shifts in phytoplankton might also be indicative for alterations in the food web structure of the middle section of the Danube River at long-term scale.

**Key words:** climate change, Europe, functional approaches, large rivers, potamoplankton, trend analysis

## **Introduction**

Global warming alters various components of climate (IPCC, 2007). Major consequences on freshwater ecosystems are increasing water temperature, seasonally altered mixing regime of lakes (Dokulil *et al.*, 2006; Dokulil, 2014), and altered seasonal patterns in river flow (Nohara *et al.*, 2006; van Vliet *et al.*, 2013). Long-term dynamics in phytoplankton in relation to global warming have mainly been addressed in marine (Reid *et al.*, 1998; Beaugrand & Reid, 2003; Hays, Richardson & Robinson, 2005) and lake systems (Jeppesen *et al.*, 2005; Jochimsen, Kummerlin & Straile, 2013; Yang *et al.*, 2016), but rarely in rivers.

Europe's climate scenarios highlight precipitation increase in the northern, and decrease in the southern regions (IPCC, 2007). Therefore, climate change-related alterations in hydrology do occur independently of latitude. In N-European rivers, earlier ice break-up is now a clear consequence of climate change (Klavins, Briede & Rodinov, 2009); winter floods decreased in occurrence frequency in the River Elbe and Oder (Germany) (Mudelsee *et al.*, 2003); flow of the River Ebro (Spain) shows decreasing trend in its annual mean values for the last 50 years (Sabater *et al.*, 2009). European rivers therefore may face with various consequences of climate-related changes such as increasing deterioration of water quality or impairment of freshwater habitats (van Vliet *et al.*, 2013). Furthermore, European large rivers are highly human-modified due to canalization and regulation (Tockner, Uehlinger & Robinson, 2009) that may further intensify some of the climate-driven changes in river ecosystems.

In the River Danube basin, altered seasonality in water runoff has been prognosticated (ICPDR, 2013), with decrease in summer and autumn flows and increase in winter and early spring (Stagl & Hattermann, 2015). During the last decades, water temperature raised significantly in the upper (Webb & Nobilis, 2007) and also in the middle section of the river (Dokulil, 2013; Duleba *et al.*, 2014).

Climate-induced changes in environmental constraints coupled with diverse human impacts may give rise to alterations in riverine biota. Recent simultaneous invasions in European rivers - such as the Asian clams *Corbicula* spp. (Friedrich & Pohlmann, 2009; Bódis *et al.*, 2011; Pigneur *et al.*, 2011; Flourey *et al.*, 2013), the macroinvertebrate *Dikerogammarus* (Müller, Schramm & Seitz, 2002), Ponto-caspian mysids (Borza *et al.*, 2011), various vascular plants (Lukács *et al.*, 2016) or benthic algae (Puky *et al.*, 2008) - may therefore show some general consequences of such effects.

Long-term trends in phytoplankton compositions have rarely been addressed in European rivers. In the River Rhine and River Elbe (Germany), climate-related change in water discharge and underwater light climate altered spring phytoplankton blooms (Hardenbicker *et al.*, 2014). In the middle Loire (France), phytoplankton taxonomic composition was found to be responding to decreasing water discharge and to increasing water temperature (Larroudé *et al.*, 2013).

Compared to the 1960s (Szemes, 1964; Szemes, 1967; Uherkovich, 1969), the River Danube was affected by severe eutrophication in the 1980s (Garnier *et al.*, 2002). Chl-*a* and algal numbers increased in the middle river section (Kiss, 1985), whereas nutrients hardly limited phytoplankton growth (Kiss, 1996). In the 1990s, spring phytoplankton blooms still resulted in  $\sim 150 \mu\text{gL}^{-1}$  Chl-*a* peaks, with a second late summer maximum depending on underwater light conditions (Vörös *et al.*, 2000). Eutrophication of the Danube affected the phytoplankton community composition considerably (Kiss, 1994; Kiss & Schmidt, 1998).

Due to the enhanced effectiveness in sewage control in the Danube basin, the nutrient status has been improved significantly (Niemeyer, 1999; Istvánovics & Honti, 2012). The reduced P load resulted in considerable drop in algal numbers (Kiss *et al.*, 2006) and biomass (Chl-*a*, in Istvánovics & Honti, 2012), while the trophic status shifted

towards the oligotrophic range (Istvánovics & Honti, 2012). The aforementioned trends led to gradual change in the taxonomic composition of phytoplankton with increasing tendency in species diversity (Verasztó *et al.*, 2010). Taxonomic richness however has been questioned as reliable proxy of functional diversity in context of ecosystem functioning (Buckland *et al.*, 2005; Magurran *et al.*, 2010; Hillebrand *et al.*, 2017). Mechanisms potentially underlying taxonomic changes in the Danube River phytoplankton in a functional context have not been addressed previously.

Here, we analyze a long-term Danube phytoplankton dataset (1979-2012) from the middle river section, Göd (N-Budapest), Hungary. We use two complementary functional approaches: the functional group (FG) concept *sensu* Reynolds (Reynolds *et al.*, 2002), and the functional trait (FT) approach (Weithoff, 2003; Litchman & Klausmeier, 2008). Functional approaches have enabled better understanding of complex riverine processes repeatedly, such as river continuum and zonation (Abonyi *et al.*, 2012; Abonyi *et al.*, 2014), floodplain dynamics (Devercelli, 2006; Stanković *et al.*, 2012; Stević, Mihaljević & Špoljarić, 2013), and various aspects of biomass/diversity relationships (Borics *et al.*, 2014).

The FG approach assumes that species groups do occur in phytoplankton according to distinguished set of environmental conditions (Reynolds *et al.*, 2002). Applying the approach, natural and human-induced shifts in river phytoplankton compositions can be assessed (Abonyi *et al.*, 2012; Bolgovics *et al.*, 2017). Phytoplankton functional traits are morphological, physiological and behavioural characteristics of taxa (Weithoff, 2003; Litchman & Klausmeier, 2008) that potentially affect fitness (Violle *et al.*, 2007). Therefore, FTs also enable the prediction of community responses to environmental changes (Violle *et al.*, 2014).

Rivers are highly selective for phytoplankton (Reynolds, 1994). Selection forces act on functional characteristics of taxa (Reynolds, 2003; Huszar *et al.*, 2015). Gradual changes in environmental constraints are expected to alter the functional characteristics and so the functional diversity of river phytoplankton communities. Ecosystem functioning is directly related to functional diversity of communities (Mason *et al.*, 2005). A better understanding of long-term changes in river ecosystems therefore requires considering the functional diversity of river phytoplankton communities. Long-term changes in functional diversity may highlight shifts in the functional organization of the Danube phytoplankton in relation to climate- and human-induced changes in environmental conditions.

We hypothesize that long-term gradual changes in major environmental constraints should be followed by gradual changes in dominance relationships among FTs and FGs of the Danube phytoplankton. We further hypothesize that functional shifts are highlighted by gradual changes in major functional diversity components (Mason *et al.*, 2005): evenness, divergence, and dispersion.

We first show (i) long-term trends in major environmental constraints that potentially affect the functional composition of phytoplankton in the middle Danube; then (ii) identify gradual changes in the relative abundance of phytoplankton taxa under specific functional traits and functional groups. Finally, we highlight long-term trends in the functional diversity components of the Danube phytoplankton with potential implications for long-term changes in ecosystem functioning.

## **Material and Methods**

*The River Danube at Göd (N-Budapest, Hungary)*

The long-term phytoplankton monitoring station of the Danube Research Institute (MTA, CER) is located in the middle river section at Göd (1668 r.km, distance from the mouth), ~20 km upstream from Budapest, capital of Hungary (Fig. 1). In this middle section, the river catchment is ~185,000 km<sup>2</sup> covering a large part of Southern Germany and the Austrian Alps. Dominant vegetation types are forests (40%), grasslands (27%), and arable lands (23%) (Tóth & Bódis, 2015). Further details about the sampling can be found in Duleba *et al.* (2014); about the entire Danube basin in Sommerwerk *et al.* (2009) and Liska (2015).

#### *Phytoplankton analysis and functional approaches*

Samples were taken weekly from the middle of the streamline between 1979 and 2012, and fixed with Lugol's solution with acetic acid. Microscopic counting of phytoplankton was carried out with an Opton Invertoscope D and an Olympus IX70 inverted microscope according to Utermöhl (1958). During the count, the transect approach was mainly applied, except in case of samples with centric diatoms' bloom, when fields' counting was preferred instead of sample dilution. The sampling protocol ensured a counting accuracy of 5% according to Lund, Kipling & Cren (1958). Algal biomass was based on approximated geometrical forms at species levels (Hillebrand *et al.*, 1999); here expressed as fresh weight assuming a density of 1 in categories (see Table 1). Data consistency (taxonomic richness) over the 34-year period was tested using the ratio of estimated and observed richness from asymptotic diversity estimates based on years and individual samples (see Statistical analysis).

Phytoplankton taxa were classified into functional groups according to the functional group concept *sensu* Reynolds (Reynolds *et al.*, 2002; Borics *et al.*, 2007; Padisák, Crossetti & Naselli-Flores, 2009). Morphological, physiological and behavioural functional traits identified were based on Weithoff (2003) and Litchman & Klausmeier (2008) (Table 1). As algal taxa occurring in river plankton can originate either from the benthos or the plankton, benthic and planktonic life forms were also considered as traits.

Functional traits and groups were specified at species level and summarized in a presence-absence data frame (Supplement 1), which then was used to calculate the functional diversity components. Furthermore, the algal abundance of taxa was summed up under each specific FT and FG; then used as the functional community matrices. For time trend analysis, we calculated the relative algal abundance of taxa under each specific FT and FG.

### *Environmental variables*

Prior to analysis, we selected for environmental variables that affected significantly either the FT or the FG functional composition of phytoplankton (see Statistical analysis). These were water discharge, water temperature, total suspended solids, orthophosphate-P, ammonium-N, nitrit-N and nitrate-N. Daily water discharge was provided by the General Directorate of Water Management and was accessed online (web1). Standardized methods of chemical variables were identical to those presented in Duleba *et al.* (2014). In our study, Chl-*a* was used as a proxy for phytoplankton biomass.

### *Functional diversity metrics*

Functional diversity components calculated were functional evenness (FEve), functional divergence (FDiv) and functional dispersion (FDis) (Mason *et al.*, 2005). We used functional dispersion to replace functional richness according to Laliberté & Legendre (2010). Functional diversity components measure complementary aspects of species distributions in niche space. They represent (i) the evenness of abundance distribution in filled niche space (FEve); (ii) the abundance-weighted variance of trait values across component species (FDiv); and (iii) the mean distance in a multidimensional trait space of individual species to the centroid of all species (FDis) (Mason *et al.*, 2005). All functional diversity metrics were calculated using the ‘FD’ R package (Laliberté & Legendre, 2010; Laliberté, Legendre & Shipley, 2014).

### *Statistical analysis*

Data consistency was tested for taxonomic richness over the 34-year period based on the ratio of estimated and observed richness from asymptotic diversity estimates (see Supplement 2) in the *iNEXT* R package (Chao *et al.*, 2014; Hsieh, Ma & Chao, 2016). The analyses were performed based on incidence data for each year (function *iNEXT*, `datatype="incidence_raw"`); and based on abundance data for each sample (function *iNEXT*, `datatype="abundance"`).

The relationship between the phytoplankton FT and FG community compositions and local environmental predictors was assessed by constrained analysis of principal coordinates (distance-based RDA – ‘db-RDA’; Oksanen *et al.* (2015) based on monthly

averaged data. First we calculated a distance matrix from the Hellinger-transformed compositional data (Bray Curtis dissimilarity), and subjected its PCoA ordination scores to a distance-based redundancy analysis (function *capscale* in *vegan*; Oksanen *et al.* (2015)). Environmental variables were selected based on combined backward and forward selection. The final significance level of terms was tested by 999 Monte Carlo permutation tests in full models.

In order to reveal time trends in environmental constraints, in the relative abundance of taxa under each FT and FG, as well as in functional diversity components, we used the Seasonal Mann-Kendall – ‘SMK’ (Hirsch & Slack, 1984) and the Mann-Kendall – ‘MK’ trend tests in the *Kendall* R package (McLeod, 2011). Monthly-averaged data was used to get harmonized time intervals, to improve the significance level of trends, and to reduce temporal autocorrelation (McLeod, 2011). The only exception was the analysis of water discharge that was based on daily measurements using the Mann-Kendall trend test. For all other variables, the Seasonal Mann-Kendall test was used first including data from all seasons. Then, each variable in each individual season (winter: December-February, spring: March-May, summer: June-August, autumn: September-November) was also tested for monotonic trends using the Mann-Kendall trend analysis (McLeod, 2011). Similar approach has already been used to identify community responses to long-term changes in climate (Floury *et al.*, 2013; Larroudé *et al.*, 2013; Hardenbicker *et al.*, 2014). For all Mann-Kendall test, we used the block bootstrap method in the *boot* R package (Davison & Hinkley, 1997; Canty & Ripley, 2016) to perform bootstrap confidence interval calculations (BCIC) using 10,000 bootstrap replicates at 99% CI.

All analyses and visualizations were performed in R (R Core Team, 2015).

## Results

### *Long-term trends of water discharge in the middle Danube section*

Based on yearly median values, the Mann-Kendall (MK) trend analysis indicated a decreasing, but non-significant change in water discharge over the 34-year period in the middle Danube section (MK, tau: -0.16,  $p=0.192$ , Fig 2A). Similarly, monthly median values did not show significant changes in separate seasons (Fig 2B). Based on daily measurements, the Mann-Kendall trend analysis indicated very slight but significant decrease for the entire period (MK, tau: -0.04,  $p<0.001$ ). In separate seasons, water discharge decreased very slightly but significantly in winter, spring and summer (MK, tau: -0.07 ( $p<0.001$ ), -0.03 ( $p<0.05$ ), -0.08 ( $p<0.001$ ), respectively), while increased significantly in autumn (MK, tau: 0.03,  $p<0.05$ ). These changes resulted in a shift in the most frequent water discharge values between the two equal parts of the entire study period (before and after 1996; Fig 2D-E): the frequency of both lower discharge values ( $\sim 1800 \text{ m}^3\text{s}^{-1}$ ) and high floods ( $>3000 \text{ m}^3\text{s}^{-1}$ ) increased. High-flow water discharge also increased in values significantly over time (LM,  $p<0.001$ ). High-flow periods showed shortening tendency in duration and tended to appear seasonally either earlier (i.e. in 2003, 2004) or later (i.e. in 2002, 2007), depending on the year (Fig 2F).

### *Local environmental variables predicting the functional composition of phytoplankton*

Based on monthly averages, all environmental variables affected the functional community compositions significantly; except nitrite-N for the FG composition (Fig

3). All terms were significant according to the Monte Carlo permutation tests in full models. The variation explained in the phytoplankton functional composition by local environmental predictors was 26.4% for FTs and 22.4% for FGs.

#### *Long-term trends in local environmental constraints in the middle Danube*

Based on monthly-averaged data, the Seasonal Mann-Kendall trend analysis indicated significant decreasing trend for all N- and P- forms, as well as for total suspended solids ( $p < 0.001$ , in all cases). These trends were apparent and significant in all seasons, except for nitrate-N in winter and spring (Table 2, Fig 4). Water temperature increased in the entire dataset significantly (SMK,  $p < 0.001$ ), but the positive trend in individual seasons was only significant for summer (Table 2). Between two halves of the dataset (before and after 1996), orthophosphate-P concentration decreased ~52% (from  $106.5 \mu\text{gP L}^{-1}$  to  $51.2 \mu\text{gP L}^{-1}$ ); with values appearing regularly below the P-limitation threshold of  $10 \mu\text{gP L}^{-1}$  (Reynolds, 2006) for short time periods between April and August after 1995. Similarly, nitrate-N decreased ~9% (from  $2.15 \text{ mgN L}^{-1}$  to  $1.96 \text{ mgN L}^{-1}$ ); total suspended solids ~27% (from  $28.8 \text{ mgL}^{-1}$  to  $20.9 \text{ mgL}^{-1}$ ) between the two halves of the dataset. In a similar approach, water temperature increased ~8% (from  $10.9 \text{ }^\circ\text{C}$  to  $11.9 \text{ }^\circ\text{C}$ ).

#### *Phytoplankton quantity and taxonomic richness at long-term scale*

Monthly-averaged phytoplankton biomass (Chl-*a*), species richness (S) and genus richness (G) showed significant decrease over the entire period (SMK,  $p < 0.001$  in all cases). Chl-*a* decreased in all individual seasons significantly. Species and genus

richness increased in winter and spring (not significant only for G in winter), while decreased in summer and autumn significantly (Fig 5, Table 3). Between two halves of the dataset (before and after 1996), Chl-*a* concentration decreased ~47% (from 44.6  $\mu\text{gL}^{-1}$  to 23.5  $\mu\text{gL}^{-1}$ ), while species and genus richness both ~13% (from 39 to 34 and from 30 to 26, respectively). Overall, S and G values correlated highly in the entire dataset (Pearson cor.,  $r=0.97$ ,  $n=1608$ ,  $p<0.001$ ).

#### *Long-term trends in the functional trait and functional group compositions*

Small-sized algae increased significantly over time in the entire dataset (SMK,  $p<0.01$ ), meanwhile medium and extra large ones decreased (SMK,  $p<0.001$  and  $p<0.05$ , respectively; Fig 6 and Supplement 3B). Single-celled taxa displayed a significant decreasing tendency (SMK,  $p<0.001$ ), while most of the other forms (flagellated, filamentous, elongated algae - “GAL”) showed increasing ones (SMK,  $p<0.001$ , in all cases). Taxa requiring silicon decreased in their relative abundance significantly. Other algae with supplementary pigments (phycobiliproteins) as well as vacuolated forms displayed significant increases (SMK,  $p<0.001$ ). A characteristic functional shift was the decrease in the relative abundance of planktonic and the increase of benthic taxa (SMK,  $p<0.01$ , in both cases). In individual seasons, the direction of significant trends was similar to those found in the entire dataset for almost all FTs (Fig 6B-E). The exceptions were large-sized algae and the pigment components Chl-*b* and Chl-*c*. The relative abundance of large algae increased in spring (MK,  $p<0.001$ ), but decreased in summer (MK,  $p<0.05$ ). Taxa with Chl-*b* increased in relative abundance in spring and decreased in summer (MK,  $p<0.01$  in both cases); while taxa with Chl-*c* showed opposite tendencies: decrease in spring (MK,  $p<0.01$ ) and increase in summer (MK,

$p < 0.05$ ). Most of the significant changes occurred in spring (Fig 6C), but the number of significant increasing trends exceeded the number of significant decreasing ones in all seasons (Fig 6B-E, Supplement 3B).

The relative abundance of planktonic diatoms decreased in almost all relevant FGs (SMK; **A**: i.e. *Acanthoceras*,  $p < 0.01$ ; **C**: i.e. *Asterionella*,  $p < 0.01$ , **D**: i.e. *Stephanodiscus*, *Skeletonema*,  $p < 0.001$ ) in the entire dataset. On the other hand, benthic diatoms (codon **TB**) increased significantly (SMK,  $p < 0.001$ ) (Fig 7, Supplement 3C). The decrease of planktonic diatoms was the most pronounced for single-celled eutrophic centrics (codon **D**, Fig 7A). The relative abundance of large flagellates characteristic for highly eutrophic conditions (**W1**: *Euglena*; **W2**: *Trachelomonas*, **WS**: *Synura*) decreased also over time significantly (SMK,  $p < 0.001$  in all cases). Significant increase in relative abundance occurred in both colonial (**E**: *Dinobryon*,  $p < 0.001$ ) and middle- to large-sized single-celled flagellates (**X2**: *Chroomonas*, *Chlamydomonas*,  $p < 0.001$ ; **Y**: *Cryptomonas*,  $p < 0.01$ ). Green algal taxa characteristic for deeply mixed eutrophic environments (**F**: *Dictyosphaerium*) decreased in relative abundance (SMK,  $p < 0.01$ ), while others that regularly occur in shallow well-mixed oligotrophic conditions showed increasing tendency (**X3**: *Chrysococcus*, *Koliella*,  $p < 0.001$ ). The direction of significant trends observed in individual seasons was identical to those observed in the entire dataset for all FGs (Fig 7B-E). Most of the significant changes occurred in spring and autumn. In these periods, the relative abundance increase was characteristic for diverse FGs (Fig 7C, E): flagellated forms (**X2**, **Y**), diatoms with benthic origin (**TB**), shade-adapted filamentous Cyanobacteria (**S1**: *Planktothrix*), and dispersed limnophilic elements (**K**: i.e. *Aphanocapsa*, **H1**: i.e. *Dolichospermum*, **Q**: *Gonyostomum*). Higher number of significant increasing trends occurred in winter and

spring than decreasing ones (Fig 7B-C); in summer and autumn, however, most of the significant trends were decreasing (Fig 7D-E). Significant relative abundance increase occurred for mid-sized flagellates (**X2**,  $p < 0.001$ ), elongated forms (**P**,  $p < 0.05$ ), and limnophilic taxa (**M**: *Microcystis*,  $p < 0.01$ , **L0**: *Peridinium*,  $p < 0.01$ ) in the summer phytoplankton.

### *Functional diversity components at long-term scale*

In the entire dataset, the Seasonal Mann-Kendall trend analysis indicated significant increases in all functional diversity components over time, except for functional evenness of the FG approach (Table 4, Supplement 3D). At a seasonal scale, all significant trends showed increases (Table 4, Fig 8), except functional evenness based on FGs. It showed significant decreasing tendency in winter and spring, but increased significantly in summer and autumn.

## **Discussion**

We hypothesized that functional shifts in the Danube River phytoplankton would occur in response to gradual changes in major environmental constraints at long-term scale. We showed that major environmental variables that potentially affect phytoplankton composition changed significantly over the 34-year period. In response, the functional composition of phytoplankton also altered. Single-celled eutrophic centric diatoms decreased in their relative abundance, but almost all other forms (flagellates, elongated and filamentous taxa) including dispersed limnophilic elements increased. The overall dominance of eutrophic planktonic taxa decreased, meanwhile the relative abundance

of benthic diatoms increased significantly (also in absolute values; data not shown).

Functional diversity components indicated long-term gradual increases both in the full dataset and in almost all individual seasons. The only exception was functional evenness of FGs, which component decreased in winter and spring significantly.

*Functional regime shift of phytoplankton in response to long-term changes in environmental constraints*

The slightly decreasing water discharge, the altered seasonality in river flow, as well as the increasing water temperature in the middle section of the Danube are all in good agreement with regional climate models (Webb & Nobilis, 2007; Sipkay *et al.*, 2012; Stagl & Hattermann, 2015). As also predicted for the Rhine River (Shabalova, van Deursen & Buishand, 2003), annual maximum discharge values seem to increase in magnitude in the middle Danube section. The more frequent occurrence of lower water discharge values is paralleled with increasing water temperature similarly to observations in other European large rivers (Moatar & Gailhard, 2006). Oligotrophication trends are now widely reported in European lakes (Jeppesen *et al.*, 2005; Morabito, Oggioni & Austoni, 2012; Pomati *et al.*, 2012) and are also observed in European rivers (Hardenbicker *et al.*, 2014; Minaudo *et al.*, 2015). In the Danube, the frequency of potentially growth-limiting conditions for phytoplankton by orthophosphate-P has increased (Istvánovics & Honti, 2012). Mechanisms that may potentially underlie such changes are increased efficiency in nutrient management (Niemeyer, 1999; Istvánovics & Honti, 2012), or historical economic changes in middle and E-Europe following the breakdown of the socialist industry (late 1980s). As one possible consequence, nutrient loads decreased ~40-50% between the 1980s and 2000s,

both at the upper and the middle sections of the Danube watershed (Weilguni & Humpesch, 1999; Schreiber *et al.*, 2005). Furthermore, dams have been built in large numbers in the upper German and Austrian Danube sections. Dams are expected to modify the transport of suspended solids (Klaver *et al.*, 2007), increase water residence time and water temperature potentially (Engel & Fischer, 2017), and may contribute to improved underwater light conditions further downstream (Kiss, 1994). In line with these, the total annual loads of suspended solids declined ~50% in the middle section of the Danube since the 1990s (Tóth & Bódis, 2015).

The most productive phytoplankton composition of large rivers occurs in middle river sections, where increased water residence time and favorable underwater light climate may enhance phytoplankton growth (Kiss, 1994; Reynolds & Descy, 1996). Under such conditions, the dominance of single-celled centric diatoms is characteristic (Kiss & Nausch, 1988; Leland, 2003; Piirsoo *et al.*, 2008) due to their resistance against the highly selective conditions (Reynolds, 1994; Reynolds & Descy, 1996). In the middle Danube, the dominance of planktonic centric diatoms (Szemes, 1967; Kiss & Nausch, 1988; Kiss *et al.*, 2012) and the co-dominance of green algae (Schmidt, Kiss & Bartalis, 1994; Várbiro *et al.*, 2007; Dokulil, 2015) have been characteristic at least since the 1960s. Here, we showed that the characteristic dominance of eutrophic centric diatoms (codon **D**) declined in the middle Danube in almost all seasons, except summer. This might be explained by the increasing summer dominance of the stenotherm *Skeletonema potamos* at this river section (Duleba *et al.*, 2014). The species is highly adapted to extreme late summer river conditions: increased water temperature, elongated water residence time and high light availability (Kiss *et al.*, 2012). Similarly to enhanced stratification in lakes, more frequently occurring lower discharge values - in parallel with gradual decrease in nutrients (especially in P) - might trigger enhanced

sedimentation of algal cells. Such mechanisms may be selecting for smaller taxa, which would at the same time benefit from enhanced nutrients' uptake due to higher surface area to volume ratio (Winder, Reuter & Schladow, 2009). While our long-term dataset did not include measurements for silicates, recent Si values (year 2017) stay above the limitation threshold of  $2 \mu\text{molL}^{-1}$  for diatoms (Egge & Aksnes, 1992) during the entire year; hence Si might have not been a limiting factor for diatoms' growth in this middle Danube section. A decreasing tendency in Si, however, is expected to occur in recent decades in rivers of the Mediterranean and the Black Sea due to nutrient retention of dams (Ludwig *et al.*, 2009). Corresponding unbalance in nutrients (i.e. relative nutrient composition like Si:P ratio) therefore might also play a crucial role in primary production, i.e. by provoking compositional shift from diatoms to non-siliceous algae (op. cit.). Size selective grazing could also be responsible for compositional shifts in river phytoplankton. While we were unable to analyse zooplankton data at long-term scale, the river section investigated can be characterized by relatively poor, nauplii dominated zooplankton, where the trophic coupling between phytoplankton and crustacean zooplankton is of only minor importance (Bothár & Kiss, 1990; Vadadi-Fülöp, 2009). Winder and Sommer (2012) argued that climate change may favor algal traits best adapted to altered environmental conditions. Such successful traits in the Danube phytoplankton could therefore be small cell size, the ability for active motion (flagellates in coda **E**, **X2**, **Y**), as well as elongated and filamentous forms (**X3**, **P**, **S1**). Seasonal shifts in water temperature and underwater light availability might also be altering green algae (**J**, **Chl-b**), which group may seem to occur earlier seasonally. Furthermore, the re-oligotrophication trend of the middle Danube is seemingly followed by pronounced functional shift from eutrophic (**D**, **F**, **W1**, **W2**, **WS**) towards the relative abundance increase of oligotrophic taxa (**X3**).

The relative abundance increase of diatoms with benthic origin (also in absolute abundance, data not shown) might be related to long-term alteration in water discharge values. Ács and Kiss (1993) highlighted that in response to lowering water discharge, benthic diatoms could increase their abundance in this middle Danube section already. Therefore, more frequent occurrence of lowering water discharge paralleled with enhanced underwater light availability could potentially enhance shoreline benthic algal growth. On the other hand, with increasing nutrient limitation, benthic algal forms might also be benefitting from direct contact to remineralized nutrients. As one example for such conditions, in the summer of the extreme low-flow year 2003, unusually low phytoplankton abundance occurred in the Danube (Kiss *et al.*, 2006). Epilithic diatoms on the other hand produced large gelatinous matrices (Ács *et al.*, 2006). Similar signs for a potential long-term shift between planktonic and benthic algae have not been reported for rivers formerly. In the Great Lakes, however, long-term decrease in nutrient concentrations and increased light availability led to similar consequences: decline in phytoplankton and increase in benthic algal production (Brothers, Vadeboncoeur & Sibley, 2016). More frequent high-flood events in spring and autumn therefore might be responsible for the recently more frequent occurrence of benthic (especially diatoms), as well as some dispersed limnophilic elements in the plankton of the middle Danube River.

Oligotrophication now prevails in the middle Danube with more frequent occurrence in lower water discharge values. These might elongate water residence time, enhance the sedimentation of both suspended solids and algae, as well as increase underwater light availability and water temperature. The observed functional changes in the Danube phytoplankton therefore could be related to climate- and human-induced long-term changes in environmental constraints. These trends might potentially be further

intensified in the near future, as regional climate scenarios prognosticate further seasonal alterations in river flow with continuous increase in water temperature (Webb & Nobilis, 2007; Dokulil, 2013; Stagl & Hattermann, 2015).

### *Functional diversity components in relation to ecosystem functioning*

The functional community structuring of the Danube phytoplankton might go towards a more dispersed state at long-term scale, based both on functional traits and functional groups. Functional diversity increase could potentially represent enhanced niche complementarity among species, either by increase in the probability of species occurrences or abundances (Laliberté & Legendre, 2010). Theoretically, increase in niche complementarity could enhance ecosystem functioning (Loreau & Hector, 2001; Naeem & Wright, 2003; Hodapp *et al.*, 2016). Here, we showed that increase in functional dispersion might be related to a long-term gradual shift from high-biomass planktonic communities towards the more frequent occurrence of dispersed elements including diatoms with benthic origin and limnophilic taxa. This conclusion might be highlighting the importance in better understanding the functional structuring of riverine phytoplankton; in context of some recently observed taxonomic diversity increase in large river phytoplankton (Verasztó *et al.*, 2010; Larroudé *et al.*, 2013).

Long-term increase in functional evenness might also potentially indicate more effective resource use due to more evenly occupied niche space by species. This could lead to increase in productivity and decrease the opportunity for invaders (Mason *et al.*, 2005). Besides trends in the relative community structuring, we observed long-term increase in species number and absolute abundance of benthic diatoms in spring communities (data not shown). This phenomenon, paralleled with the relative

abundance decrease in several eutrophic planktonic elements might also contribute to functional evenness decrease in spring communities. In summer, however, besides some well-adapted taxa to altered conditions (i.e. coda **X2**, **P**, **S1**), the long-term relative abundance increase in dispersed limnophilic elements (coda **H1**, **K**, **M**, **L0**) might be explaining the increase of functional evenness. The functional diversity increase alone, however, might be misleading in context of ecosystem functioning until it is largely related to taxa with benthic and limnophilic origins.

Increased functional divergence can be indicative for higher degree of niche differentiation, and therefore lower resource competition. This may basically coincide with more efficient resource use and lead to increase in ecosystem functioning (Mason *et al.*, 2005). We showed that changes in environmental conditions in the middle Danube were diverse: more frequent lower discharge values, fast high flood events, decline in nutrients and increase in light availability and water temperature. These might also affect the divergence of FTs and FGs in multiple ways. Taxa with adaptive traits to altered conditions (i.e. smaller size, flagellates) may increase their relative abundance (Reynolds, 1994; Winder & Sommer, 2012). Also, diatoms with benthic origin may be sharing very similar trait compositions. Sudden disturbance events such as high floods then might be responsible for assembling taxa with diverse origin and with diverse adaptive features from side arms, para- and paleopotamal lentic habitats. Such mechanism could also lead to increase in the occurrence of limnophilic elements in river plankton (Mihaljević & Stević, 2011; Stević, Mihaljević & Špoljarić, 2013), and therefore increase the functional divergence of river phytoplankton communities.

### *Conclusions and outlook*

Environmental variables that can affect river phytoplankton altered significantly over the 34-year period studied in the middle Danube section. These climate- and human-related alterations seem to be affecting the functional composition of phytoplankton. The dominance of productive planktonic elements (mainly eutrophic centric diatoms) decreased, while benthic diatoms occur more frequently in the middle Danube plankton recently. The planktonic community responded to changes in the environment with the increasing dominance of taxa with highly adaptive functional traits. On the other hand, the functional community composition diversified due to the relative abundance increase in benthic, as well as in dispersed limnophilic elements.

Long-term increase in functional diversity components would alone indicate enhanced ecosystem functioning for the middle Danube phytoplankton. However, without considering long-term changes in the functional composition of phytoplankton, this conclusion alone might be misleading. Our results may be the first to demonstrate a long-term phytoplankton functional regime shift in a European large river due to global warming, human impacts and oligotrophication. Our findings may also potentially indicate altered food web structure in the middle section of the Danube River.

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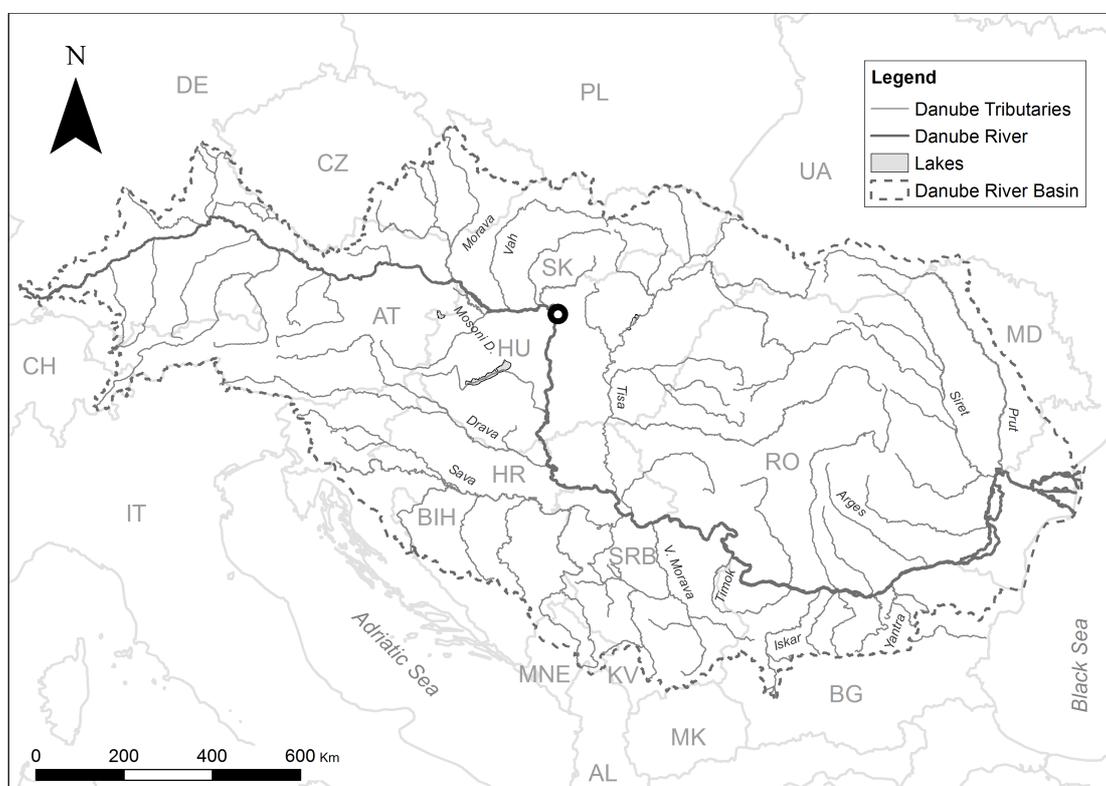
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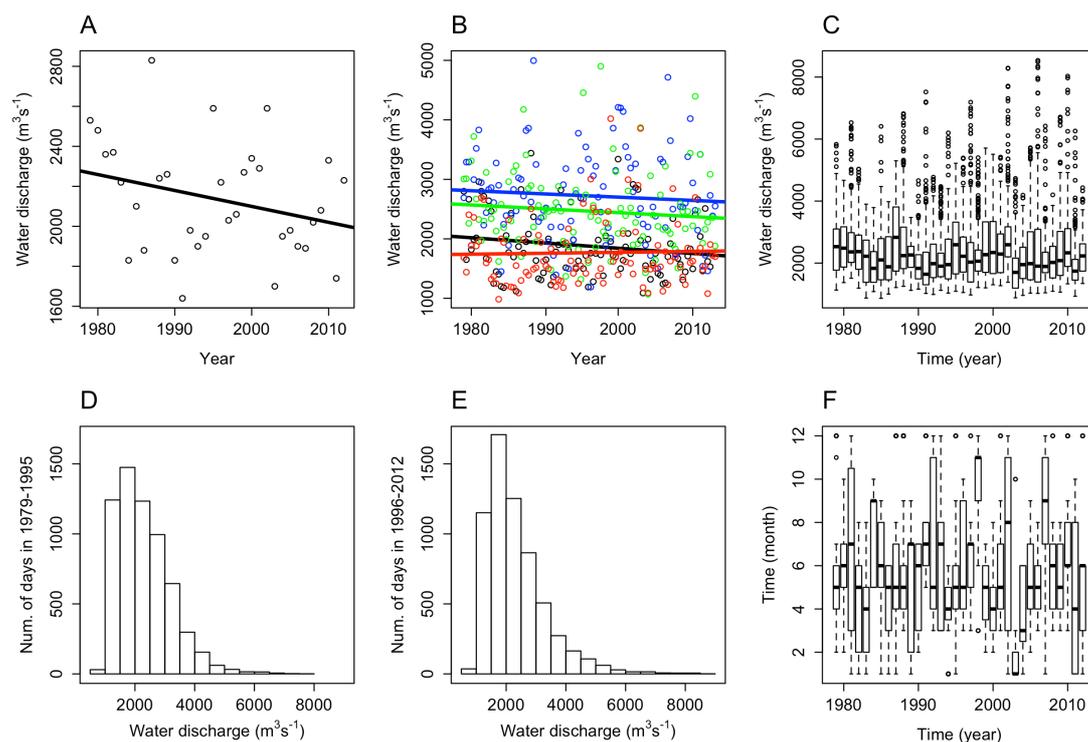
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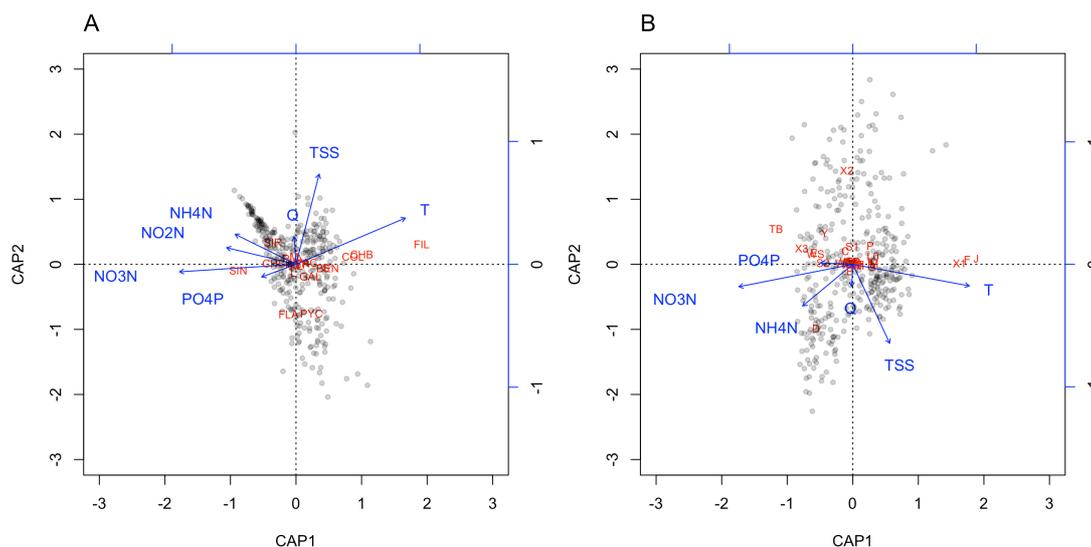
## Legends for Figures



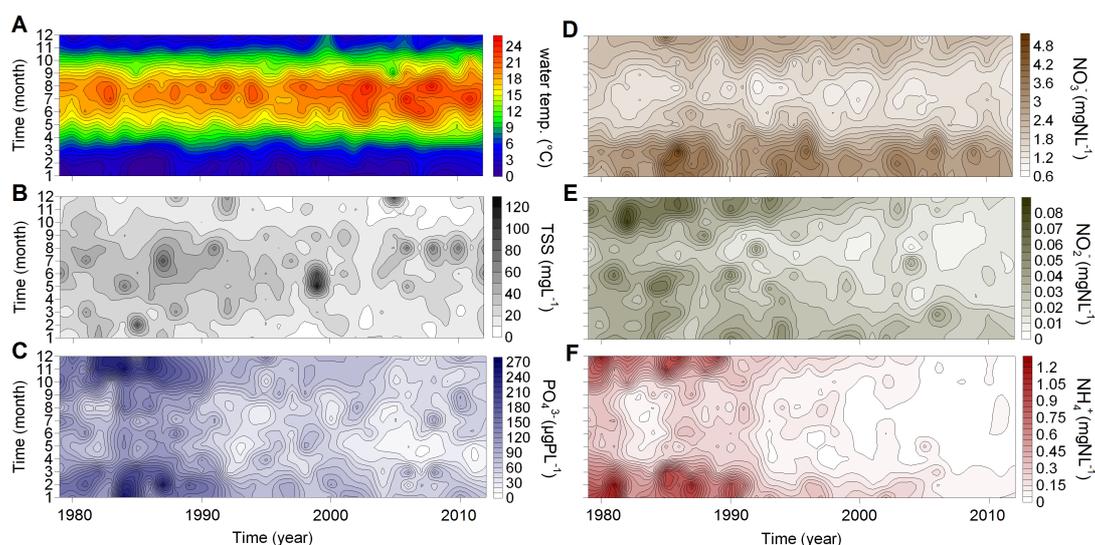
**Fig. 1** Location of the long-term phytoplankton monitoring station of the Danube Research Institute (MTA, CER) in the middle section of the Danube River, Göd (N-Budapest), Hungary.



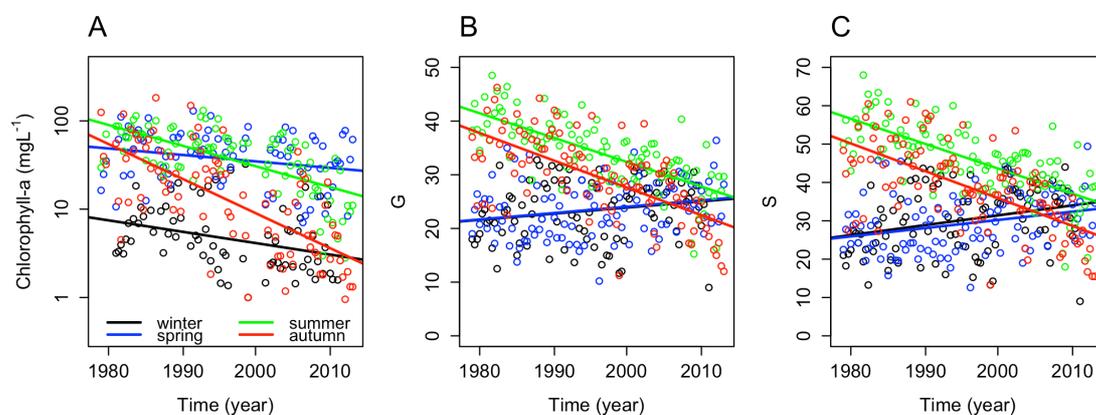
**Fig 2.** Long-term changes (1979 and 2012) of water discharge in the middle Danube section at Göd (N-Budapest) Hungary for (A) median values in each year with fitted linear trend line (MK, tau: -0.16, p=0.192); (B) monthly median values in each season with fitted linear trend lines (MK, tau in winter: -0.10 (black), spring: -0.06 (blue), summer: -0.10 (green), autumn: 0.02 (red); n.s. in all cases); (C) boxplot of daily water discharge values; (D) frequency distribution of daily water discharge values for the period of 1979-1995 and (E) for the period of 1996-2012; (F) seasonal distribution of high flood events ( $>3000 \text{ m}^3 \text{ s}^{-1}$ ).



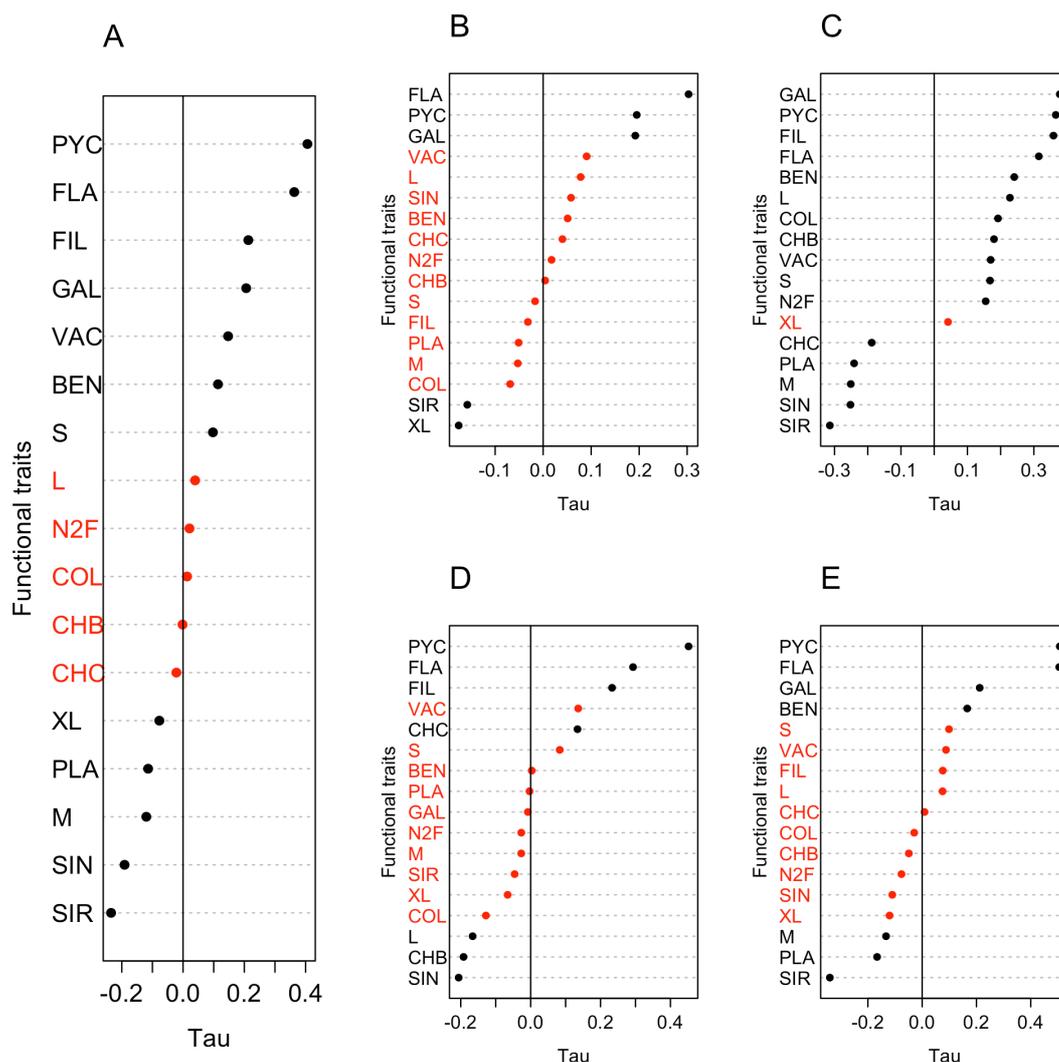
**Fig. 3** Distance-based Redundancy Analysis (db-RDA) predicting (A) the phytoplankton functional trait composition; (B) the phytoplankton functional group composition *sensu* Reynolds from local environmental predictors based on monthly-averaged data between 1979 and 2012 in the middle Danube, Göd (N-Budapest, Hungary). Abbreviations: T: water temperature, Q: water discharge, PO4P: orthophosphate-P, NO3N: nitrate-N, NO2N: nitrite-N, NH4N: ammonium-N, TSS: total suspended solids. Abbreviations of FTs (A) are detailed in Table 1; abbreviations of FGs (B) represent alphabetic letters of functional groups according to Reynolds *et al.* (2002).



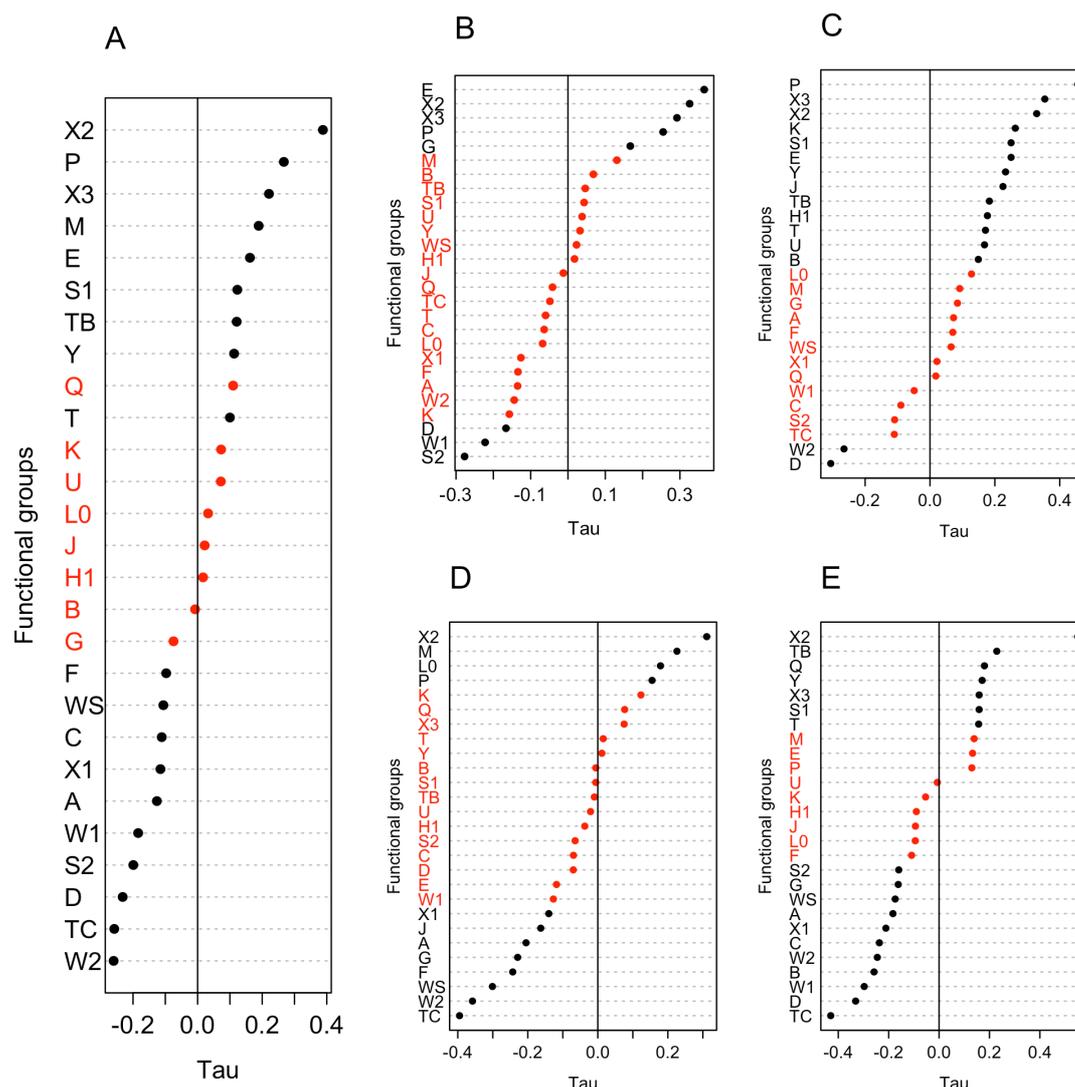
**Fig 4** Long-term changes in (A) water temperature ( $^{\circ}\text{C}$ ), (B) total suspended solids (TSS,  $\text{mgL}^{-1}$ ), (C) orthophosphate-P ( $\mu\text{gP L}^{-1}$ ), (D) nitrate-N ( $\text{mgN L}^{-1}$ ), (E) nitrite-N ( $\text{mgN L}^{-1}$ ), and (F) ammonium-N ( $\text{mgN L}^{-1}$ ) between 1979 and 2012 from the middle Danube section, Göd (N-Budapest), Hungary based on monthly average values.



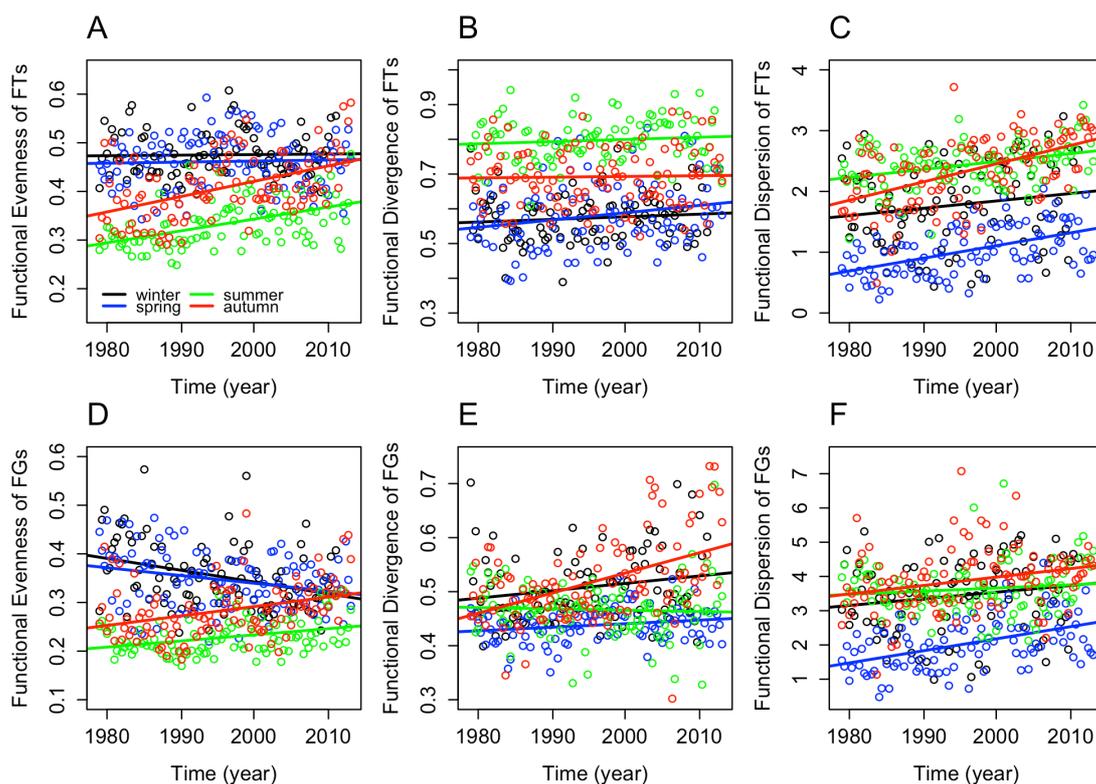
**Fig 5** Seasonal differences in fitted linear trends for (A) Chlorophyll-*a*; (B) genus richness (G); and (C) species richness (S). Figures summarize data between 1979 and 2012 from the middle Danube section, Göd (N-Budapest), Hungary based on monthly average values.



**Fig 6** Tau values of (A) Seasonal Mann-Kendall trend tests for the relative abundance of each phytoplankton functional trait in the entire dataset (see abbreviations in Table 1); (B-E) Mann-Kendall trend tests for the relative abundance of phytoplankton functional traits in individual seasons: (B) winter, (C) spring, (D) summer, (E) autumn in the middle Danube section, Göd (N-Budapest, Hungary) between 1979 and 2012. Significant (black dots) and non-significant (red dots) trends are summarized. Significance level of trends and bootstrap confidence interval calculations for the Mann-Kendall tests are detailed in Supplement 3B.



**Fig 7** Tau values of (A) Seasonal Mann-Kendall trend tests for the relative abundance of each phytoplankton functional group (FG) *sensu* Reynolds in the entire dataset (see alphabetic letters of FGs in Reynolds *et al.* (2002), Borics *et al.* (2007), and Padisák, Crossetti & Naselli-Flores (2009)); (B-E) Mann-Kendall trend tests for the relative abundance of phytoplankton FGs in individual seasons: (B) winter, (C) spring, (D) summer, (E) autumn in the middle Danube section, Göd (N-Budapest, Hungary) between 1979 and 2012. Significant (black dots) and non-significant (red dots) trends are summarized. Significance level of trends and bootstrap confidence interval calculations for the Mann-Kendall tests are detailed in Supplement 3C.



**Fig. 8** Seasonal differences in fitted linear trends for phytoplankton functional diversity components: functional evenness (A, D), functional divergence (B, E), and functional dispersion (C, F) based on the functional trait (A-C) and functional group (D-F) approaches. Figures summarize data between 1979 and 2012 from the middle Danube section, Göd (N-Budapest), Hungary based on monthly averages.

### Legends for Tables

**Table 1** Morphological, physiological and behavioral traits of phytoplankton according to Weithoff (2003) and Litchman & Klausmeier (2008); as well as major life forms of algae.

Traits	Categories	Abbreviation
<i>Morphological traits</i>		
Biovolume	<100 $\mu\text{m}^3$ , 100-1,000 $\mu\text{m}^3$ , 1,000-10,000 $\mu\text{m}^3$ , >10,000 $\mu\text{m}^3$	S, M, L, XL

Greatest Dimension (GALD) Organization	Axial Linear	< 35 µm or > 35 µm single-celled, colonial, filamentous	GAL SIN, COL, FIL
<i>Physiological traits</i>			
N <sub>2</sub> -fixation Si requirements Pigment composition		yes/no yes/no Chl- <i>b</i> , Chl- <i>c</i> , phycobiliproteins	N2F SIR CHB, CHC, PYC
<i>Behavioral traits</i>			
Motility		presence/absence of flagella	FLA
Vacuolated		yes/no	VAC
<i>Life forms</i>			
Benthic Planktonic		yes/no yes/no	BEN PLA

**Table 2** Seasonal Mann-Kendall and Mann-Kendall trend analysis of major environmental variables in the middle Danube section, Göd (N-Budapest, Hungary) between 1979 and 2012. Significant positive (+), significant negative (-), and non-significant (n.s.) trends are summarized. Tau and significance levels, as well as bootstrap confidence interval calculations for the Mann-Kendall tests are given in Supplement 3A.

Parameter	GLOBAL (Seasonal M-K)	WINTER (Mann-K)	SPRING (Mann-K)	SUMMER (Mann-K)	AUTUMN (Mann-K)
Water temperature (°C)	+	n.s.	n.s.	+	n.s.
Total suspended solids (mg L <sup>-1</sup> )	-	-	-	-	-
Ammonium-N (mgN L <sup>-1</sup> )	-	-	-	-	-
Nitrite-N (mgN L <sup>-1</sup> )	-	-	-	-	-
Nitrate-N (mgN L <sup>-1</sup> )	-	n.s.	n.s.	-	-
Orthophosphate-P (µgP L <sup>-1</sup> )	-	-	-	-	-

**Table 3** Seasonal Mann-Kendall and Mann-Kendall trend analysis of Chl-*a*, species richness (S) and genus richness (G) in the middle Danube section, Göd (N-Budapest,

Hungary) between 1979 and 2012. Tau and significance levels (in brackets) are given, as well as bootstrap confidence interval calculations (BCICs) for the Mann-K tests based on 10,000 bootstrap replicates at 99% CI (in second line). Significant trends are bold and italic.

Variable	GLOBAL (Seasonal M-K)	WINTER (Mann-K)	SPRING (Mann-K)	SUMMER (Mann-K)	AUTUMN (Mann-K)
<b>Chl-a</b>	<b><i>-0.38 (***)</i></b>	<b><i>-0.24 (**)</i></b> <b><i>-0.72, -0.25</i></b>	<b><i>-0.15 (*)</i></b> <b><i>-0.55, -0.06</i></b>	<b><i>-0.47 (***)</i></b> <b><i>-1.26, -0.62</i></b>	<b><i>-0.51 (***)</i></b> <b><i>-1.33, -0.70</i></b>
<b>S</b>	<b><i>-0.14 (***)</i></b>	<b><i>0.17 (*)</i></b> <b><i>0.09, 0.60</i></b>	<b><i>0.22 (**)</i></b> <b><i>0.26, 0.62</i></b>	<b><i>-0.51 (***)</i></b> <b><i>-1.30, -0.72</i></b>	<b><i>-0.48 (***)</i></b> <b><i>-1.24, -0.67</i></b>
<b>G</b>	<b><i>-0.19 (***)</i></b>	0.11 (n.s.) -0.02, 0.46	<b><i>0.17 (*)</i></b> <b><i>0.17, 0.51</i></b>	<b><i>-0.54 (***)</i></b> <b><i>-1.38, -0.79</i></b>	<b><i>-0.49 (***)</i></b> <b><i>-1.27, -0.68</i></b>

**Table 4** Seasonal Mann-Kendall and Mann-Kendall trend analysis of functional evenness (FEVE), functional divergence (FDIV) and functional dispersion (FDIS) based on phytoplankton functional traits and functional groups *sensu* Reynolds in the middle Danube section, Göd (N-Budapest, Hungary) between 1979 and 2012. Significant positive (+), significant negative (-), and non-significant (n.s.) trends are summarized. The tau value and significance level of trends, as well as bootstrap confidence interval calculations for the Mann-Kendall tests are detailed in Supplement 3D.

FD metric	GLOBAL (Seasonal M-K)	WINTER (Mann-K)	SPRING (Mann-K)	SUMMER (Mann-K)	AUTUMN (Mann-K)
<i>Functional traits</i>					
<b>FEVE</b>	+	n.s.	n.s.	+	+
<b>FDIV</b>	+	n.s.	+	n.s.	n.s.
<b>FDIS</b>	+	+	+	+	+
<i>Functional groups</i>					
<b>FEVE</b>	n.s.	-	-	+	+
<b>FDIV</b>	+	+	n.s.	n.s.	+
<b>FDIS</b>	+	n.s.	+	n.s.	+