Composition and dynamics of microeukaryote communities in the River Danube

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Abstract: The diversity of microeukaryote communities inhabiting rivers is still poorly known. Here, we have analyzed the periphytic and planktonic microeukaryote communities present in one section of the River Danube by two different methods: 18S rRNA–based terminal restriction fragment length polymorphism with fragment sequencing and microscopical analysis of the phytoplankton and periphyton. Both data sets were then related to environmental variables. Molecular fingerprinting revealed diverse communities with fluctuating composition, with the majority of sequences affiliated to the groups Bacillariophyta, Synurophyceae and Chlorophyceae. This was in accordance with microscopical data. The total number of detected T–RFs during the study period was 145, with more than half of the T–RFs being restricted to either plankton or periphyton. This suggests that the likely different natural selection regimes experienced by microeukaryotes in these two environments may promote the presence of different lineages in each of them. Significant correlations were found between phytoplankton chlorophyll–a content, phosphorus content, temperature, and the T–RFLP pattern of the planktonic microeukaryotic community, suggesting that the former environmental factors are especially important in structuring the planktonic microeukaryote communities in the River Danube. These data, together with earlier studies suggest that molecular methods are an invaluable addition in pursuit of the better understanding of the diversity and fluctuation of freshwater microeukaryotic communities.

Key words: River Danube; microeukaryotes; clone library; 18S rRNA; terminal restriction fragment length polymorphism, phytoplankton, microscopy

Introduction

Microbes play vital roles in ecosystem functioning and biogeochemical cycling in the biosphere (Falkowski et al. 2008). However, their phylogenetic diversity as well as structuring patterns still remain unclear. During the last fifteen years, molecular analyses of microbial communities have changed our view of diversity in the biosphere. Traditionally, it has been assumed that the global microbiota was composed of a relatively few cosmopolitan species (e.g. Finlay 2002). However, recent molecular studies have shown that there is tremendous microbial diversity at the genetic and metabolic level, and that there are cosmopolitan as well as endemic species (see reviews in Hughes–Martiny et al. 2006; Logares 2006; Lopez–Garcia & Moreira 2008). Many of the early conclusions were drawn from studies based on morphological traits, but now it is known that there is ample cryptic diversity among microeukaryotes (reviewed in Logares 2006) and that many microbial groups escaped detection by microscopy studies.

The initial efforts in molecular diversity research of eukaryotic aquatic microbes were

focused on marine environments (e.g. Guillou et al. 1999; Diez et al. 2001; 2004, Moon–van der Staay et al. 2001; López–García et al. 2001; Massana et al. 2004; Romari & Vaulot 2004) and subsequently, lakes started to be investigated (e.g. Lefranc et al. 2005; Lepère et al. 2006). In most cases, high phylogenetic diversity was found in these environments. However, several studies have indicated that most marine and freshwater microeukaryotes are phylogenetically distantly related due to relatively few historical marine–freshwater transitions (e.g. Alverson et al. 2007; Logares et al. 2007; Lozupone & Knight 2007; Shalchian–Tabrizi et al. 2008). So far, the microbial communities inhabiting rivers and their dynamics have been investigated little (but see e.g. Dorigo et al. 2002; Dorigo et al. 2009), and this has been the reason for us to carry out the present study. From an ecological perspective, rivers have important roles in interconnecting lake communities with each other, as well as interconnecting (microbial) communities inhabiting marine and freshwater environments. Thus, members from different lake communities as well as marine and freshwater groups have been found in rivers. In addition, the significance of sessile life forms is bigger compared to lakes and oceans, and the dynamic nature of rivers will most likely generate structuring and dynamic patterns in the microbial communities inhabiting them that are different from the ones occuring in more stable marine and lacustrine systems.

In the present study we applied molecular methods to study the composition and dynamics of microbial eukaryotes in the Hungarian stretch of the River Danube. The temporal dynamics in community composition of planktonic and benthic microeukaryotes was probed with terminal restriction fragment length polymorphism – T–RFLP of the 18S rRNA gene. In addition, representative populations were putatively identified by molecular cloning and sequencing of the 18S rRNA gene.

The River Danube is the second largest river in Europe (2780 km long, 817000 km^2 catchment area). To date, planktonic and benthic algae, and protozoa in the River Danube at Göd (river km 1669) have been extensively studied by light and electron microscopy (Bereczky et al. 1983; Kiss 1987, 1994, 2000; Kiss & Genkal 1993; Bereczky & Nosek 1994; Ács et al. 2006; Kiss et al. 2009). Sporadic studies have also characterized planktonic picoalgae (Mózes, A., personal communication). All these studies utilized microscopy techniques and morphological criteria of taxon identification, normally restricted to a few microeukaryote groups. Generally, the use of microscopy–based techniques is restrictive in terms of throughput when compared with molecular techniques. In addition, the identification of species by microscopy is often unclear, since morphospecies (species defined using morphological characters) can be in fact species complexes (Montresor et al. 2003; Kim et al. 2004). For a detailed discussion of the advantages and disadvantages of microscopy–based methods and molecular fingerprinting in the analyses of microbial eukaryotes, see Szabó et al. (2007).

The objectives of this study were to follow the temporal dynamics of benthic and planktonic microeukaryotes by using microscopical (algal species) and molecular methods (all eukaryotic groups), and to relate their diversity to environmental variables.

Methods

Study site and sample collection

Planktonic and periphytic samples were collected from the River Danube at Göd (river km 1669) between December 2005 and May 2006. Planktonic samples were collected with weekly frequency for microscopical analysis and the measurement of physicochemical parameters. Plankton samples for molecular analysis were collected at least once a month. The sampling date for molecular and microscopical analysis did not coincide in the case of a single sample in December. Periphyton samples were collected with at least monthly frequency. On two occasions (05.04.2006, 03.05.2006), benthic sample collection was not possible due to high water level of the Danube. Planktonic samples (1.5 l) were drawn from the upper 20 cms of the current line of the river into sampling bottles. Depending on plankton density, 250 to 500 ml sample was filtered onto a 0.22 μm white Millipore filter and stored in liquid nitrogen until extracted, and 500 ml was preserved with Lugol's iodine solution for subsequent microscopical analysis. For periphyton samples, 5 to 8 similar sized pebbles near the shoreline were collected. The samples were transported into the laboratory within 30 minutes of sampling, where periphyton was brushed off from the pebbles and washed into approximately 200 ml water. 100 ml of this well–mixed sample was subsequently filtered onto 0.22 μm white Millipore filters and stored in liquid nitrogen until extracted while the other 100 ml of this sample was preserved with Lugol's iodine solution for subsequent microscopical analysis.

Measurement of environmental parameters
Environmental variables of the river water were analysed weekly throughout the year. Nitrogen forms and soluble reactive phosphorus were determined spectrophotometrically. Nitrate content was measured with the salicilate method, according to Felföldy (1987). Ammonium content was measured according to the ISO standard (MSZ ISO 7150–1:1992). Orthophosphate content was measured with ammonium molybdate reagent and 10% ascorbic acid according to Felföldy (1987). Each measurement was carried out using filtered water (filter pore size 0.45 μm). Conductivity and pH were measured with a WTW multiline portable meter, turbidity was measured with a Lovibond PC Checkit portable meter. Oxygen concentration was measured by Winkler's method (Felföldy 1987). Chlorophyll–a was extracted from the sample by methanol, the concentration was determined according to Goodwin (1976), measuring the absorbance of the extract at 747, 666 and 653 nm.

Microscopical phytoplankton and periphyton analyses
Semiquantitative analysis (percentual ratio of taxa was calculated, as the sampled surface was unknown), and species identification of periphytic microalgae was carried out using an inverted Olympus IX70 light microscope. Algal relative abundance was determined by Utermöhl's method (1958) and analysed statistically according to Lund et al. (1958). Each cell of filamentous and coenobial algae was counted, except for cyanobacteria where filaments were treated as a single individuum. Samples for diatom analysis were treated with H_2O_2 and HCl, washed three times in distilled water, and subsequently mounted on glass slides in Naphrax. In each sample, 400 valves were counted and identified. Acid–treated diatom samples were also mounted on SEM stubs, coated with gold and analysed with a Hitachi S–2600N scanning electron microscope.

Quantitative analysis of the phytoplankton was performed using Utermöhl's method by inverted microscope (OPTON Invertoscope–D). For counting statistics and calculation errors suggestions of Lund et al. (1958) were used (400 algal specimens were counted, the calculation error is ±10%). Percentage composition of centric diatoms was determined by scanning electron microscope (HITACHI S–2600–N), published in detail by Kiss (1986).

Nucleic acid extraction
DNA extraction was carried out using the Ultraclean Soil DNA extraction kit (Mobio Lab. Inc. Carlsbad, CA, USA), according to the manufacturer's instructions for maximum yield. In this DNA extraction procedure, the entire –80 °C preserved filters are directly subjected to mechanical lysis by bead–beating which ensures that the samples are well mixed. Extracted nucleic acids were quantified and sized by agarose gel electrophoresis, ethidium bromide staining and UV transillumination (Eiler & Bertilsson 2004). DNA concentration in extracts varied between 5 and 100 ng.μl^{-1}.

T–RFLP analysis
DNA extracts were used as templates for PCR amplification of 18S rRNA genes using eukaryotic primers Euk1A (5'CTGGTTGATCCTGCCAG3') tagged with hexafluorescein at the 5' end, and Euk516r (5' ACCAGACTTGCCCTCC 3') (Diez et al. 2001). This universal eukaryotic primer pair amplifies a region between position 4 and 563 (*Saccharomyces cerevisiae* position) (Diez et al. 2001), which includes the variable regions V1 to V3 (Neefs et al. 1993). The reactions were carried out in a 50 μl reaction volume, and each reaction mixture contained 10–50 ng DNA template, 10 pmol of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 U Taq polymerase and reaction buffer (DyNAzyme II; Finnzymes OY; Espoo, Finnland). Thermocycling was carried out with a Stratagene Robocycler using an initial 5 min denaturation at 95 °C, 30 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 2 mins followed by a final 10 min extension step at 72 °C. Pooled PCR products were digested with mung bean nuclease to eliminate single stranded PCR products (Egert & Friedrich 2003), followed by purification and concentration using a Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). The PCR product concentration in the eluate was analysed by agarose gel electrophoresis and comparison to a Low DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA). Approximately 50 ng PCR product from each sample was digested with 4 units of the restriction endonuclease *Msp*I (Sigma Aldrich, St Louis, MO, USA) for terminal restriction fragment length polymorphism analysis. Reactions with PCR product, enzyme and buffer were incubated at 37 °C for 16 h according to the manufacturer's instructions. Terminal fragments were sized by electrophoretic separation and detection on an ABI 3700 96–capillary sequencer after addition of a 1000bp ROX–labelled size marker (Applied Biosystems, Foster City, CA, USA) to each sample. The size and quantity of terminal restriction fragments were analysed using GeneScanView 1.1/8 software (CRIBI, Biotechnology Centre, University of Padova, http://grup.cribi.unipd.it) as previously described (Eiler & Bertilsson 2004). T–RFs between 40 bp and 550 bp were included in the analysis. T–RF fragment sizes were rounded up or down to the nearest integer. The lower cutoff for TRFs to be included in the comparative analysis was 0.6% of the total peak area.

Cloning and sequencing
Two samples were selected for subsequent cloning and sequencing: 17.05.2006 periphyton and 03.05.2006 plankton. These two samples contained the largest

number of representative T–RFs within the periphytic and the planktonic samples, respectively. 18S rDNA for cloning was amplified by PCR as described above. Six replicate reactions were pooled, followed by purification and concentration using Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). PCR products for cloning were gel–purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The PCR product concentration in the eluate was estimated by agarose gel electrophoresis, using SybrSafe staining (Invitrogen, Carlsbad, CA, USA) and comparison to a Low DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA). 40 ng of PCR product was cloned into the pCR4–TOPO vector and transformed into chemically competent *E. coli* One Shot TOP10 using the TOPO TA cloning kit for Sequencing (Invitrogen, Carlsbad, CA, USA). In total, 96 (periphyton sample) and 56 (plankton sample) positive clones (colonies) from the two libraries were randomly picked and transferred to 96 well plates containing LB medium with 50 $\mu g.ml^{-1}$ kanamycin. Clones were grown overnight at 37 °C at 200 rpm agitation and then pelleted by centrifugation at 4000 rpm for 30 min using a microtiter centrifuge. Harvested cells were resuspended in 60 µl sterile Q–grade water and lysed by heating at 98 °C for 20 min. Lysates were directly used for PCR amplification with the vector primers M13f and M13r. A 50–fold dilution of the amplification product was used for a subsequent nested PCR reaction with the primer pair Euk1A–Hex and Euk516r. PCR conditions were as described above. PCR products were screened by T–RFLP as described above, in order to gain infomation about the quality of the clones. This screening was also necessary to obtain *Msp*I T–RF length of the clones to link sequenced clones to T–RFs observed in the environmental samples. Based on the T–RFLP results, 24 clones were selected for sequencing. Sequencing was carried out with an ABI 3700 96–capillary sequencer using Euk1A primer and the BigDye terminator kit vs. 3.1 (Applied Biosystems, Foster City, CA, USA). After chimera checking with the RDPII Chimera Check program, 4 clones were discarded as ambiguous or likely chimeras. The sequences of the remaining 20 clones were compared with those currently available in the NCBI database using BLAST (Basic Local Alignment Tool) in order to select reference sequences and obtain a preliminary phylogenetic affiliation of the clones. All sequences were deposited in GenBank under accession numbers EF649715 – EF649734.

Statistical analysis

Diversity and evenness of the samples was calculated according to the Shannon–Wiener equation, using the number and relative abundance of periphytic or planktonic species, or that of the T–RFs as a proxy for OTUs, respectively: $H' = -\sum_{i=1}^{S} p_i \ln p_i$ where S is the number of T–RFs, p_i is the relative abundance of each T–RF and maximum diversity (H_{max}) is defined as lnS. Evenness is defined as the quotient of diversity (H) and maximum diversity (H_{max}) (SHANNON & WEAVER 1949).

Community fingerprints and also microscopy–based community composition of planktonic and periphytic communities were also correlated with measured physicochemical parameters using Sørensen index of similarity in a Mantel test in Statistica (Statsoft, Tulsa, U.K.).

Results

Environmental variables

The water level of the River Danube was very low in early December 2005 (data not shown). This low water level was also characteristic in January and February 2006, apart from a minor peak. A period of flooding started later in February, followed by larger floods in March and a particularly major flood in April 2006. The water level decreased quite evenly after the April flood. Despite the floods, several physicochemical river water parameters were relatively stable: pH ranged from 8.12 to 8.74; dissolved oxygen content ranged from 8.67 $mg.l^{-1}$ to 11.03 $mg.l^{-1}$ (data not shown). Nitrate content of the water was also quite stable (Fig. 1). The ammonium content of the water dropped during the April flood, peaked in early May – still during the flood – and dropped again after the flood (Fig. 1). The turbidity increased during the flood (data not shown). The inorganic phosphorus content of the water was relatively high in the winter, peaked in March, and started dropping in late April and May (Fig. 1).

Phytoplankton biomass and composition

In December 2005 and January 2006, both the phytoplankton biomass and the chlorophyll–a content was quite low due to the winter (Fig. 1 and 2). The lowest biomass (0.60 $mg.l^{-1}$) was measured on the 11th January, and chlorophyll–a content was also very low on this date (6.68 $\mu g.l^{-1}$) even though the minimum concentration was reached on the 5th April (3.472 $\mu g.l^{-1}$). Following the January low, phytoplankton biomass increased steadily until the end of February, particularly due to the rapid division of centric diatoms. Centric diatoms were dominated by *Stephanodiscus minutulus* (KÜTZING) CLEVE et MOLLER, *S. invisitatus* HOHN et HELLERMANN, *S. hantzschii* GRUNOW in CLEVE et GRUNOW, *Cyclostephanos dubius* (FRICKE)

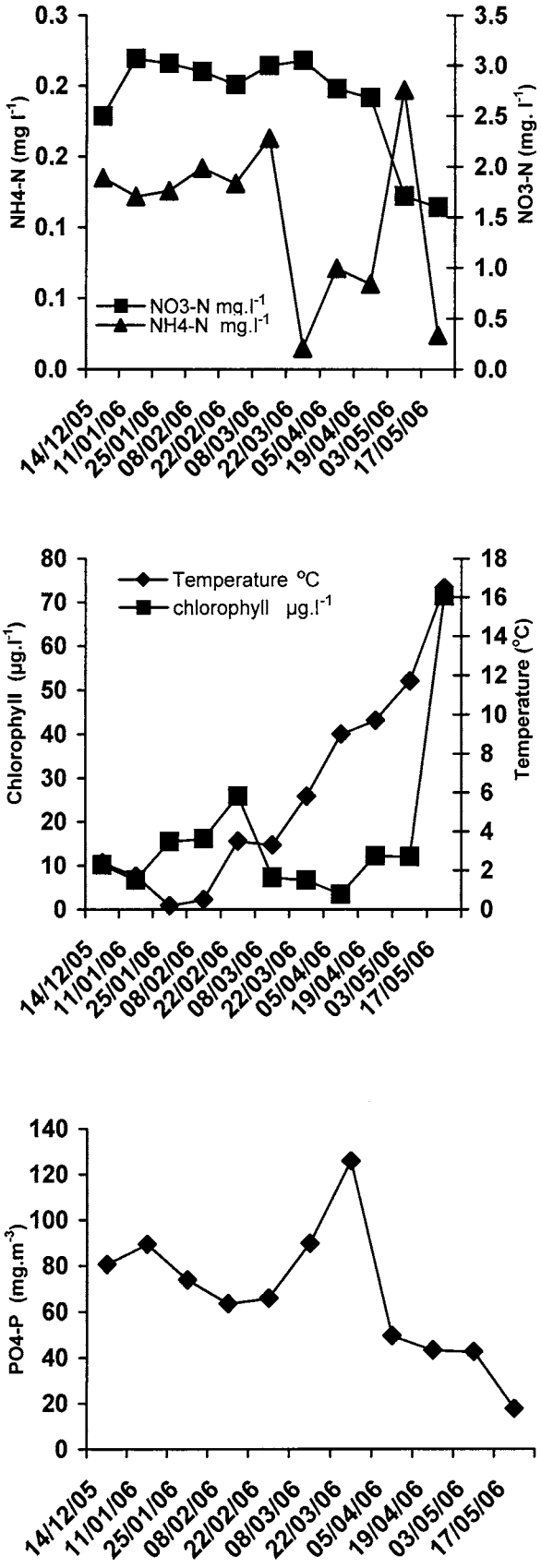


Fig. 1. Physicochemical parameters of the water at or near the sampling dates [(NO_3–N) nitrate, (NH_4–N) ammonium, (PO_4–P) inorganic phosphorus].

Round, *Cyclotella atomus* Hustedt and *C. meneghiniana* Kützing. Phytoplankton biomass decreased extensively in early March and reached its minimum during the April flood. Parallel with the decrease in water discharge from early May onwards, phytoplankton underwent rapid multiplication and by the end of the investigated period, it reached high biomass and species richness. Phytoplankton was dominated by *Chlamydomonas globosa* Snow, *C. reinchardtii* Dangeard, *Coelastrum microporum* Nägeli, *Dictyosphaerium pulchellum* Wood, *Koliella longiseta* (Vischer) Hindák, *Monoraphidium pusillum* (Printz) Komarkova–Legnerova (Chlorophyceae), *Chroomonas acuta* Utermöhl, *Cryptomonas marsonii* Skuja, *C. ovata* Ehrenberg (Cryptophyta).

T–RFLP pattern

Altogether 10 planktonic and 6 periphytic samples were analysed by T–RFLP (Fig. 3). From the 145 T–RFs identified, only 50 were classified as „major" community components (i.e. exceeded 5% in relative total peak abundance of the respective sample). The number of T–RFs in individual samples varied between 21 and 37 for the planktonic samples, and increased from 16 in December to 38 in May for the periphytic samples (Fig. 4). Many T–RFs appeared in only a single sample (60 T–RFs, these are not shown in Fig. 3) or in two samples (25 T–RFs) revealing highly dynamic communities during the sampled season.

Diversity and evenness

The Shannon diversity and evenness values of the samples were calculated using the number and relative abundance of the T–RFs, and the relative abundance of phytoplankton or periphyton taxa as a proxy for OTUs, respectively (Fig. 4). The dynamics of the diversity (H) values of the planktonic T–RFs (2.301–3.328) and that of the microscopical phytoplankton data (2.161–2.864) were very similar. By contrast, the observed richness values showed a similar tendency only until April, later the richness of the microscopically identified phytoplankton increased rapidly, whereas the richness of the planktonic T–RFs displayed a more variable pattern without any clear trend (Fig. 4).

In contrast to the pattern of the planktonic T–RFs, the diversity and richness of the periphytic T–RFs started to increase in May, after the flood

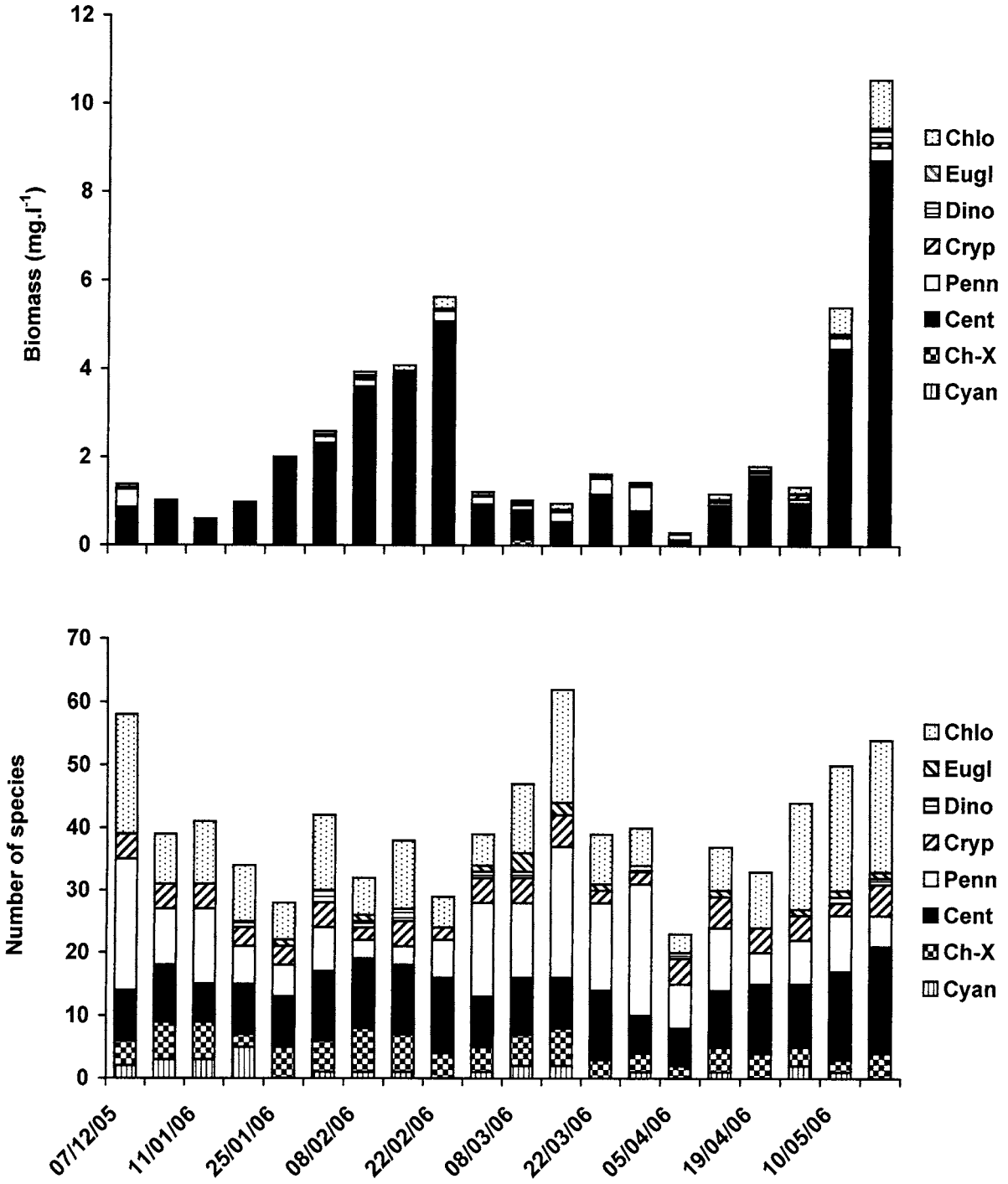


Fig. 2. Species numbers and biomass of different taxonomical groups of the phytoplankton [(Chlo) Chlorophyceae, (Eugl) Euglenophyceae, (Dino) Dinophyceae, (Crypto) Cryptophyceae, (Penn) Pennales, (Cent) Centrales, (Ch – X) Chromphyceae and Xantophyceae, (Cyan) Cyanobacteria].

had subsided. Surprisingly, the richness and diversity values of the microscopical periphyton data showed almost opposite dynamics compared to that of the periphytic T–RFs. The diversity (H=3.725) and richness (S=76) values of the former were highest in December and reached their minima in April (H=1.119, S=26), while the latter reached its minima in December (H=1.522, S=16) and their maxima in May (H=3.2, S=38).

Clones

The two samples selected for cloning and sequencing analysis (17.05.2006 periphyton and 03.05.2006 plankton) were both characterized by high Shannon diversity (3.1–3.2) and high evenness (0.9). In total, seven sequences originated from the planktonic (Fig. 3), and 13 from the periphytic sample (Fig. 3). The 5 periphytic clones EF649715–19 were diatoms (Bacillariophyta), three planktonic clones (EF649723–25) were members of the Synurophyceae group, and two periphytic (EF649720–21) and one planktonic (EF649722) clone belonged to the Chlorophyceae. These three phylogenetic groups were represented by the highest number of clones in the two clone libraries. Among the rest of the periphytic clones, two (EF649732–33) were affiliated with the Nematoda, and one clone each with the Chytridiomycota (EF649727), Dinophyceae (EF649726), Chironomida (EF649730) and Gastrotricha (EF649731). One planktonic clone (EF649734) belonged to the Bicosoecida, while one (EF649728) belonged to the Ciliophora and one (EF649729) to the Gastrotricha group.

Mantel test

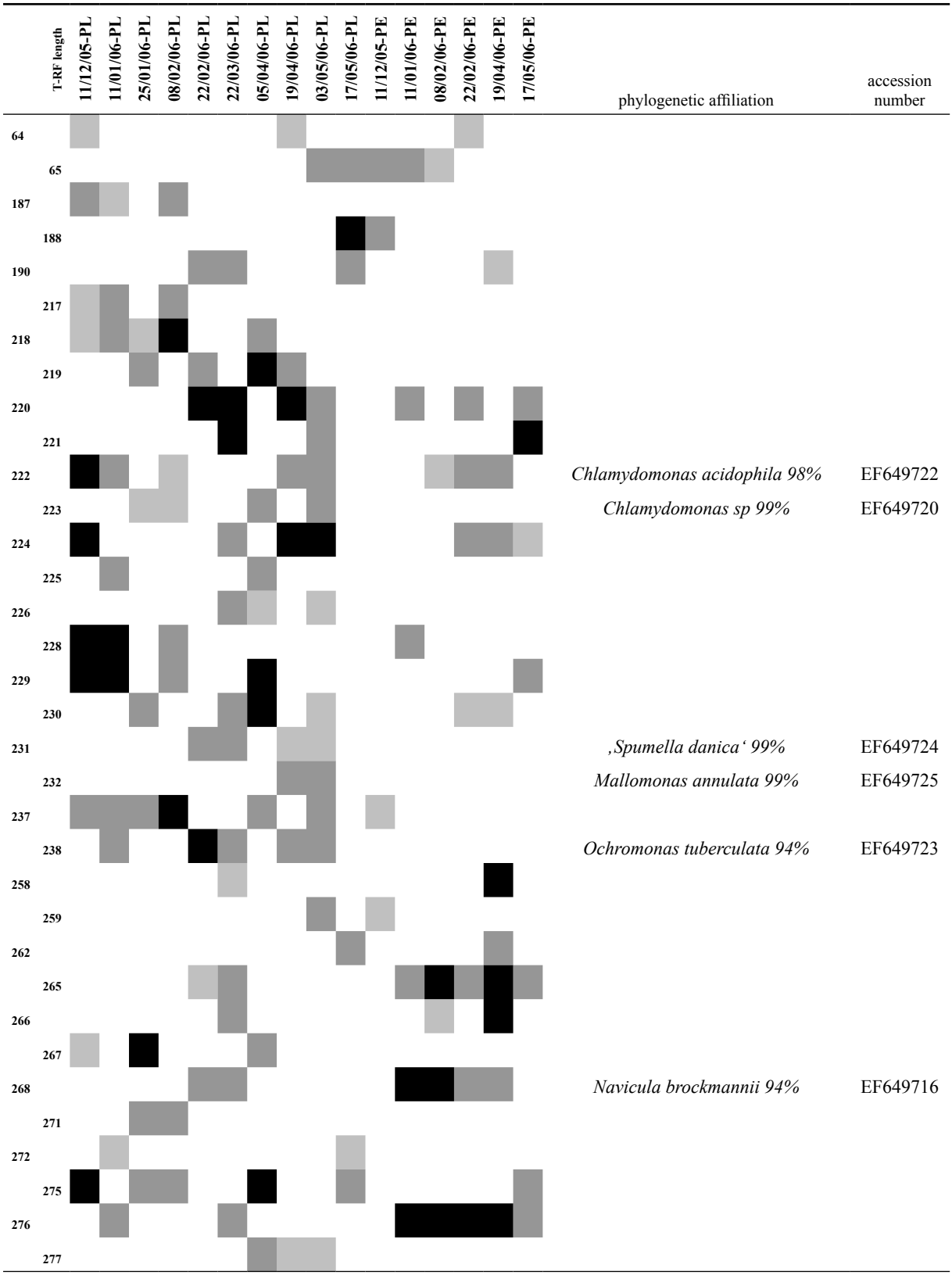
Mantel tests showed significant correlations (p value ≤ 0.05) between the T–RFLP patterns of the planktonic and periphytic communities and chlorophyll concentration, phosphorus content and water temperature (Fig. 5). No correlation was found with inorganic nitrogen content, dissolved oxygen content, turbidity and pH. Surprisingly, no significant correlation was found between environmental parameters and microscopical community composition.

Discussion

Microeukaryotes play central roles in rivers and other aquatic ecosystems, as primary producers, detritivores and predators. Knowing their community composition and the principal environmental driving variables affecting different populations is a first step to obtain information about their ecological roles and significance in the ecosystem. Hence we investigated the composition and seasonal dynamics of planktonic and benthic microeukaryote communities present in the River Danube by using microscopical traits and 18S rRNA gene variation.

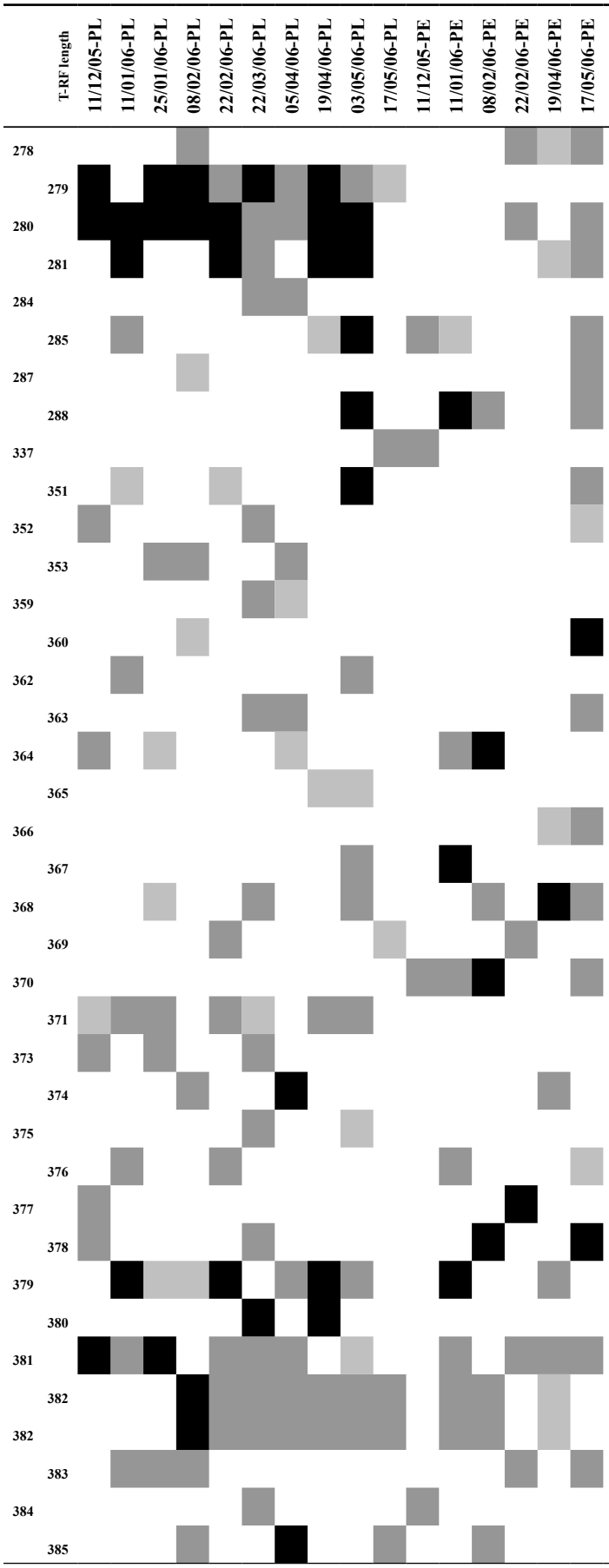
Like in previous years, the supply of inorganic nitrogen was high throughout the study period (Fig. 1). Mantel tests showed significant correlations (p value ≤ 0.05) of the T–RFLP patterns to phosphorus content, chlorophyll concentration and water temperature (Fig. 5), suggesting that phosphorus content and water temperature may be especially important in structuring community composition in the River Danube. Unlike inorganic nitrogen, the supply of phosphorus varies widely in the River Danube (between 17 and 126 $mg.m^{-3}$ in the present study period). Similarly, the temperature of the water fluctuates between extremes during the year (i.e. between near 0 to 16.5 ^{o}C in this study). T–RFLP patterns also correlated with chlorophyll–a content of the water, suggesting that periods of high and low chlorophyll levels harbor contrasting microeukaryote communities. The often complicated correlation between algal abundance and chlorophyll–a in the plankton and the periphyton has also been observed in microscopy–based studies (Aponasenko et al. 2006; Buczkó & Ács 1997; Kruskopf & Flynn 2006). No correlation was found between T–RFLP pattern and either pH, turbidity, conductivity or inorganic nitrogen content, which suggests that these factors are less important in structuring microeukaryotic community composition in the River Danube. Inorganic nitrogen is continuously abundant in the Danube, and the fluctuations of the pH are only minor. In contrast, fluctuations in phosphorus supply and large changes in water temperature likely act as structuring forces and favor communities adapted to these particular conditions. No correlation was found between environmental parameters and microscopy–based planktonic or periphytic community composition. This result was unexpected but could be due to technical constraints, as this study followed the dynamics of the plankton and periphyton for a few months only. Also, correlations between microscopy based data and environmental parameters may be diminished due to inaccuracies of microscopy based identification owing to factors detailed in the introduction (e.g. cryptic species, differentiating markers only visible under EM, etc.). In contrast, existing databases of molecular species identification are smaller but more accurate. The surprising lack of correlation between microscopical data and environmental parameters further underlines the complementary nature of microscopical and molecular methods. In this context it should also be mentioned that the

Fig. 3. T–RFLP pattern of the samples and affiliation and accession number of the sequenced clones. Only T–RFs that were observed in more than one sample are shown. The columns represent the samples (PL=plankton, PE=periphyton), the rows represent T–RF length. Light grey represents T–RF relative abundance of less than 1% in the sample, dark grey represents relative abundances between 1 and 5%, and black represents relative abundances greater than 5%. The closest affiliation and the accession number of the clones is represented. Note that phylogenetic affiliation does not represent the nearest sequence matches found by BLAST search, but the closest match with available phylogenetic information.



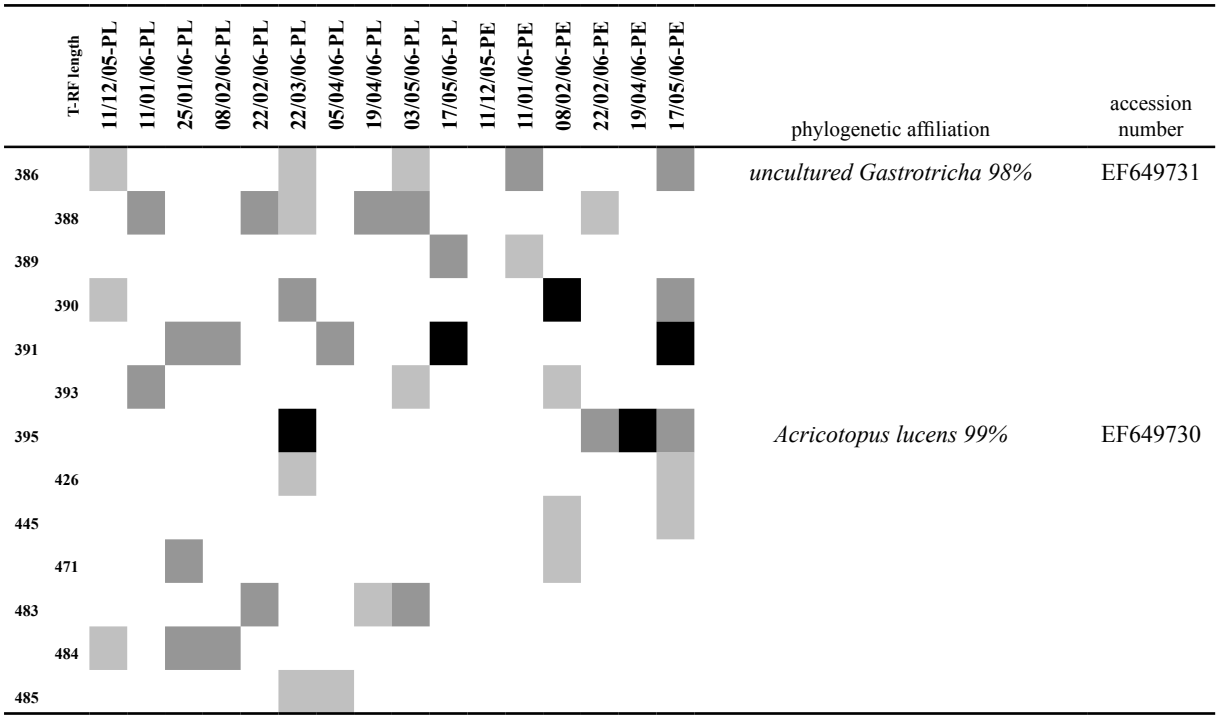
phylogenetic affiliation	accession number
Chlamydomonas acidophila 98%	EF649722
Chlamydomonas sp 99%	EF649720
‚Spumella danica' 99%	EF649724
Mallomonas annulata 99%	EF649725
Ochromonas tuberculata 94%	EF649723
Navicula brockmannii 94%	EF649716

Fig. 3 Cont.



phylogenetic affiliation	accession number
Cyclotella meneghiniana 99%	EF649718
Pteromonas angulosa 96%	EF649721
Synedra ulna 99%	EF649715
Nerada mexicana 93%	EF649734
Navicula phyllepta 99%	EF649719
Melosira varians 98%	EF649717
Chromadorina germanica 93%	EF649732
Chromadorina germanica 93%	EF649733
Rimostrombidium lacustris 95%	EF649728
Rhizophydium sp 97%	EF649727
Gymnodinium beii 92%	EF649726
Chaetonotus sp 97%	EF649729

Fig. 3 Cont.



two methods targeted different sets of organisms. Using microscopy, we mainly focused on autotrophic protists, i.e. algae and cyanobacteria. Algal community composition is expected to correlate with inorganic nutrient supply. However, during floods, algal diversity is generally low even if nutrient supply is not limiting. Using T–RFLP patterns in accordance with widely used primers, we analyzed all eukaryotic organisms and some of these groups, e.g. protozoa, may correlate with different parameters of the water such as organic nutrient content and saprobity.

The richness and Shannon diversity of the T–RFLP profiles was relatively high in the samples, even though T–RFLP analysis only detects the most abundant taxa in the samples. The diversity of the planktonic T–RFs was especially high during the spring flood period, which was probably caused by the large influx of taxa originating from upstream sources (Kiss & Schmidt 1998). The diversity of the periphytic T–RFs showed the opposite tendencies and decreased during the spring flood period, probably due to the abrasive effect of the flood, which results in the enrichment of shear–resistant taxa and the drop of the abundance of more losely attached taxa. Surprisingly, the patterns of the T–RF based diversity and richness values were in sharp contrast to the diversity and richness values calculated based on microscopical methods in the case of the periphyton, suggesting that taxa undetected by either the microscopical analysis or T–RFLP but revealed by the other method may have contributed to a large extent to the dynamics of the community.

The large number of OTUs (operational taxonomic units) found by molecular fingerprinting analysis – despite the relatively low resolution of T–RFLP fingerprinting methods – suggests that microbial eukaryotes present in rivers such as the Danube are highly diverse. This parallels similar findings in marine and lacustrine environments (see review in López–Garcia & Moreira 2008). Several phylogenetic lineages appear to be restricted to either the plankton or the periphyton. The total number of detected T–RFs during the study period was 145, with only 54 of these found both in planktonic and periphytic samples. The separation of planktonic and periphytic samples is apparent in the mosaic graph of T–RFs (Fig. 3). Certain OTUs were stable, and appeared predominantly in the plankton, such as the unidentified T–RFs 237 and 280, or the OTUs tentatively affiliated with *Pteromonas angulosa* (Carter) Lemmermann (T–RF 281) or *Nerada mexicana* Cavalier–Smith et Chao (T–RF 371). Certain T–RFs, such as the ones tentatively affiliated with *Spumella danica* (Bruchmüller, Mylnikov, Juergens, Weisse

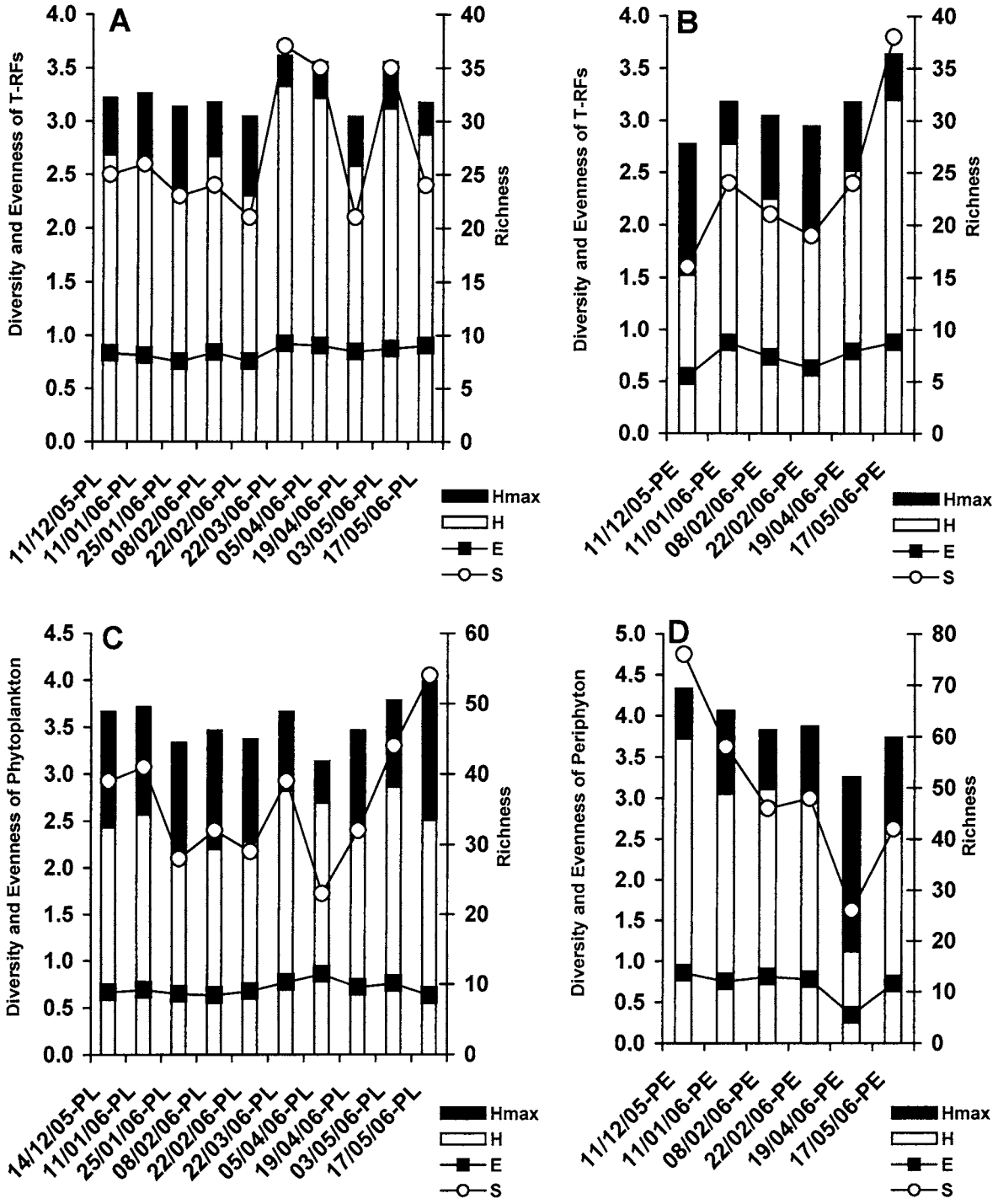


Fig. 4. Shannon diversity, maximum diversity, evenness and richness values of the planktonic and periphytic samples calculated using the number and relative abundance values of the T–RFLP (A, B) and the microscopical data (C, D), respectively [(H) Diversity, (H_{max}) maximum diversity, (E) evenness, (S) richness, (PL) plankton, (PE) periphyton].

unpublished), *Mallomonas annulata* (D.E. Bradley) K. Harris, *Ochromonas tuberculata* D.J. Hibberd and *Cyclotella meneghiniana* only appeared in planktonic samples. The T–RFs 223, affiliated with a *Chlamydomonas* sp., and T–RF 373, affiliated with *Navicula phyllepta* Kützing, were also restricted to the plankton. Overall, approximately 40% of the T–RFs were unique to the planktonic samples and 24% of the T–RFs were unique to the periphyton samples. Stable, predominantly periphytic OTUs were the unidentified T–RFs 265 and 276, and the OTU tentatively affiliated with *Navicula brockmannii* Hustedt (T–RF 268), although these OTUs also

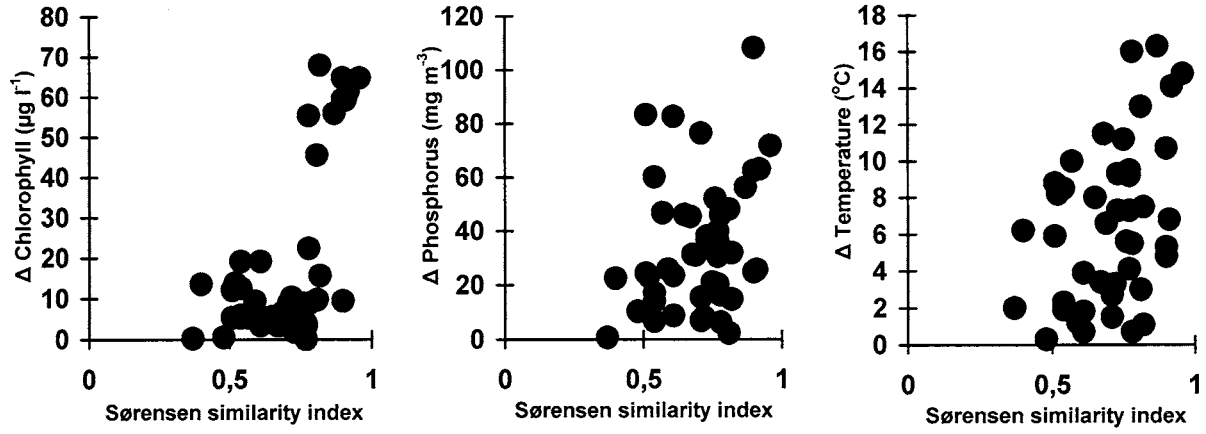


Fig. 5. Mantel plots based on Sørensen similarity matrices of the T–RFLP data and the physicochemical parameters of the water.

appeared in some of the planktonic samples. The different natural selection regimes that are experienced in the plankton and periphyton are likely selecting for different phylogenetic lineages in each type of environment. However, there is still a fraction of microeukaryotes that appear to be generalists in exploring periphytic and planktonic lifestyles, and this is supported by some of the OTUs that were stable, and appeared in both the plankton and periphyton such as the unidentified T–RFs 220, 224 and 379, or the clones tentatively affiliated with *Chlamydomonas acidophila* NEGORO (T–RF 222), *Chromadorina germanica* BÜTSCHLI (T–RF 381), *Rimostrombidium lacustris* (FOISSNER, SKOGSTAD et PRATT) PETZ et FOISSNER (T–RF 382) and *Rhizophydium* sp. (T–RF 382). Although some of the OTUs – like the examples given above – occurred stably during the winter and spring months as well, other taxa appeared to be more temperature–restricted. To mention a few, the T–RFs 271 and 484 appeared only in the winter. On the other hand, the T–RFs 277, 365, 366 and 485 only occurred in the spring samples. The T–RF richness increased in the spring, especially in the periphyton sample in May, indicating the appearance of species with a warm temperature preference.

For phylogenetic identification, one periphyton and one plankton sample were selected for 18S rRNA clone library analysis and sequencing. Among these, the Bacillariophyta group was represented by five clones, the chrysophyte class Synurophyceae and the green alga class Chlorophyceae were represented by three clones each. All other taxonomic groups were represented by a single clone (Dinophyceae, Chytridiomycota, Ciliophora, Chironomidae, Bicosoecida) or two clones (Gastrotricha, Nematoda). *Spumella* and other Synurophyceae (*Mallomonas annulata* and *Ochromonas tuberculata* affiliated clones) appeared in several plaktonic samples and seemed to be abundant members of the Danube's plankton. T–RFs related to these three clones appeared in the majority of the plankton samples, and were often present in high relative abundance (Fig. 3). *Spumella*–like flagellates are widespread and abundant members of freshwaters and represent a phylogenetically diverse group (BOENIGK et al. 2005). A number of chrysophyte species are notoriously difficult to identify by microscopy, and records of these species often depend on the availability of electron microscopes, and the use of refined sample preparation techniques such as the shadow cast technique (MØESTRUP & THOMSEN 1980). Single–celled chlorophyte species such as *Chlamydomonas* sp. are almost impossible to identify by microscopy methods – especially without extensive culturing, however, they are known to be abundant and stable members of the Danube's plankton and peripyhton (KISS 1994), and this was corroborated by our 18S rRNA–based analyses.

Our study also shows that both high–frequency sampling efforts over time and more in depth analysis of samples under scrutiny will be necessary in order to obtain a more complete characterization of the River Danube's microeukaryotes and their role in the ecosystem.

Acknowledgments
The authors would like to express their thanks to Judit Fábián for skillful assistance in the laboratory and to Maurice Taylor for English revision. The help of Paul O.B. Itor in the laboratory is acknowledged. Many thanks to Sara Barreto for useful communications on Chrysophytes. Microscope accessories funded by the instrument grant GVOP–3.2.1–2004–04–0151/3.0 were used in the research. This study was supported by a Bolyai János postdoctoral fellowship, an Eötvös Scholarship of the Hungarian State (K.É.Sz.), and grants from the Swedish Research Council and the Swedish Research Council for Environment, Agriculture and Spatial Planning (S.B.).

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Received April 25, 2009
Accepted August 11, 2009