CARD-FISH and prey tracer techniques reveal the role of overlooked flagellate groups as major bacterivores in freshwater hypertrophic shallow lakes

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Summary

Heterotrophic nanoflagellates (HNF) and ciliates are major protistan planktonic bacterivores. The term HNF, however, describes a functional guild only and, in contrast to the morphologically distinguishable ciliates, does not reflect the phylogenetic diversity of flagellates in aquatic ecosystems. Associating a function with taxonomic affiliation of key flagellate taxa is currently a major task in microbial ecology. We investigated seasonal changes in the HNF and ciliate community composition as well as taxaspecific bacterivory in four hypertrophic freshwater lakes. Taxa-specific catalyzed reporter deposition-fluorescence in situ hybridization probes assigned taxonomic affiliations to 51%–96% (average ± SD, 75 ± 14%) of total HNF. Ingestion rates of fluorescently labelled bacteria unveiled that HNF contributed to total protist-induced bacterial mortality rates more (56%) than ciliates (44%). Surprisingly, major HNF bacterivores were aplasticid cryptophytes and their Cry1 lineage, comprising on average 53% and 24% of total HNF abundance and 67% and 21% of total HNF bacterivory respectively. Kinetoplastea were important consumers of bacteria during summer phytoplankton blooms, reaching 38% of total HNF. Katablepharidacea (7.5% of total HNF) comprised mainly omnivores, with changing contributions of bacterivorous and algivorous phylotypes. Our results show that aplasticid cryptophytes, accompanied by small omnivorous ciliate genera Halteria/Pelagohalteria, are the major protistan bacterivores in hypertrophic freshwaters.

Introduction

Heterotrophic and mixotrophic flagellates and ciliates are considered as the key bacterivores in aquatic environments (Sherr and Sherr, 1988, 1994; Berninger et al., 1991; Arndt et al., 2000; Jones, 2000). Heterotrophic nanoflagellates (HNF, most commonly 2–8 μm in size) represent an extremely diverse polyphyletic group of largely uncultured protists, lacking sufficient distinctive morphological features for reliable species detection (e.g. Arndt et al., 2000; Jeuck and Arndt, 2013; Adl et al., 2019). Their phylogenetic diversity is hidden behind simple, oval cells, typically owning a single nucleus and one to two flagella. This holds particularly true for the so-called ‘naked’ planktonic HNF, such as Kinetoplastea, Cercozoa, Spumella-like and other chrysophytes, or stramenopiles (Arndt et al., 2000; Boenigk and Arndt, 2002; Jürgens and Matz, 2002; Grossmann et al., 2016). Consequently, in classical grazing studies on planktonic protokaryotes, bacterivorous HNF were treated as one taxonomically undefined functional guild, which reacts uniformly to certain environmental factors (Berninger et al., 1991; Sanders et al., 1992; Gasol and Vaqué, 1993).

Although the sequencing of 18S rRNA genes brought new insights into the phylogenetic diversity of protists in planktonic environments (Mangot et al., 2013; Simon et al., 2015; Adl et al., 2019; Bock et al., 2020), the relative abundances of particular protistan phylotypes obtained by sequencing poorly correspond to their microscopic counts in the natural samples (Mukherjee et al., 2015, 2020; Piwosz et al., 2020). The rapidly growing amount of sequence data resulted in a disproportional boom of diversity research not accompanied by a corresponding progress in studies on abundance, morphology and various ecological aspects of individual
protistan groups, such as feeding modes or trophic roles in microbial food webs. This obvious imbalance in research likely represents one of the largest knowledge gaps in the field and significantly limits hypothesis-driven research on ecological traits of protists (Grossmann et al., 2016; Stern et al., 2018; Pitsch et al., 2019; Piwosz et al., 2021).

However, eukaryotic 18S rRNA encoding gene sequences of natural protistan communities provide valuable information on the distribution patterns of various protistan groups in a broad variety of aquatic habitats (e.g. Logares et al., 2012; de Vargas et al., 2015; Bock et al., 2020; Sieber et al., 2020) and for designing novel probes for catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH), targeting the major protistan groups in aquatic environments (Piwosz et al., 2021). The CARD-FISH became an indispensable tool for studying eukaryotic communities in the plankton, focusing on their ecological role and trophic interactions (Not et al., 2005, 2008; Mangot et al., 2009; Lepère et al., 2010; Unrein et al., 2014; Mukherjee et al., 2015; Piwosz et al., 2016). Current CARD-FISH protocols targeting protists in combinations with various fluorescence-labelling techniques allow determining taxonomic affiliation and feeding modes of protists at a single cell level in natural environments (Massana et al., 2006, 2009; Unrein et al., 2014; Grujić et al., 2018; Šimek et al., 2020).

The progress in applications of CARD-FISH probes to protists detection was the basis for new intriguing findings in aquatic microbial ecology, reflecting a shift from originally morphology-based approaches to their combinations with molecular methods: (i) Using specific FISH probes, novel lineages of marine Stramenopiles (MAST), such as nanoplanктonic MAST-4 and MAST-1 lineages were discovered as ubiquitous in open oceans, contributing up to 20% of all HNF and exhibiting high growth and bacterivory rates (Massana et al., 2006, 2009; Mangot et al., 2018); (ii) Several recent studies suggested that small aplastic cryptophytes (Piwosz et al., 2016; Grujić et al., 2018) can play an important role as pelagic HNF bacterivores; (iii) There is increasing evidence that Kinetoplastidae and omnivorous Katablepharidacea can be important bacterivores in a broader variety of freshwater pelagic habitats (Arndt and Mathes, 1991; Boenigk and Arndt, 2000; Domazin et al., 2003; Mukherjee et al., 2015, 2019). These findings indicated that small ‘Spumella-like’ chrysophytes, choanoflagellates and other easily cultivable groups are less important planktonic flagellated bacterivores than has been assumed on the basis of classical morphology-based microscopic studies (Arndt et al., 2000; Boenigk and Arndt, 2002; Jürgens and Matz, 2002; Jeuck and Arndt, 2013). Consequently, current concepts describing the composition and grazing activities of major freshwater bacterivorous HNF and ciliates (Šimek et al., 2019) require considerable revisions and verifications under natural conditions.

Shallow freshwater hypertrophic lakes are becoming the most common waterbodies worldwide (Scheffer, 2004; Meerhoff and Jeppesen, 2009), while microbial trophic interactions are largely understudied in these systems (Sommaruga, 1995; Šimek et al., 2019) with the exception of certain aspects of trophic cascading from fishes and zooplankton to primary producers and microbes (Jeppesen et al., 1998; Jürgens and Jeppesen, 2000). Notably, these hypertrophic lakes represent ideal, model ecosystems with high HNF and ciliate abundances (Arndt et al., 2000; Šimek et al., 2019) suitable for applications of CARD-FISH probes targeting HNF at different taxonomic levels, which can serve as ‘high resolution in situ laboratories’ for testing hypotheses on the role and feeding preferences of aquatic protists.

To track the temporal HNF community dynamics in four hypertrophic lakes, we used CARD-FISH probes targeting important groups containing bacterivorous representatives, while ciliate assemblages were analyzed by means of classical staining procedures (Posch et al., 2015). To assess taxon-specific and aggregated bacterivory rates of HNF and ciliates, we used fluorescently labelled bacteria (FLB) as tracers. While major ciliate bacterivorous taxa in shallow lakes have been reported (Šimek et al., 2019), much less is known about core HNF bacterivorous taxa, requiring the use of FISH probes in combination with prey tracer techniques in situ (Massana et al., 2009; Unrein et al., 2014). The previous studies and our preliminary applications of CARD-FISH probes targeting flagellates indicated high proportions of aplastic cryptophytes, kinetoplastids and katablepharids in samples from eutrophic brackish waters (Piwosz et al., 2016, 2021), a mesoeutrophic reservoir (Grujić et al., 2018; Šimek et al., 2020), and also from the investigated hypertrophic shallow lakes. These studies suggested that small aplastic cryptophytes and their Cry1 lineage might be bacterivorous and, moreover, highly abundant members of plankton communities. Also a recent finding of a significant correlation between cryptophytes and bacteria in sequencing-based study of 83 lakes on a European scale (Bock et al., 2020) seems to support this trophic coupling. Using available FISH probes, we tested whether the aplastic Cryptophyceae and their Cry1 lineage are important flagellated bacterivores in hypertrophic lakes. Furthermore, we expect that these shallow lakes, rich in organic particles with associated bacteria, such as algal and colonial cyanobacterial blooms, host large populations of bacterivorous...
Kinotoplastea, considered as grazers on particle-associated bacteria (Caron, 1987; Zubkov and Sleigh, 2000; Mukherjee et al., 2015). Members of another ubiquitous group of freshwaters flagellates, Katablepharidacea (Arndt et al., 2000; Bock et al., 2020; Sieber et al., 2020), are considered mostly as algivorous or omnivorous HNF. However, we hypothesize that they are important bacterivores in prokaryote-rich hypertrophic lakes.

Results

Basic physical, chemical and microbial characteristics of the lakes

The monthly sampling (April–September 2018) yielded 24 analyses of microbiological and chemical parameters (Figs 1–3; Supplementary Tables S1 and S2). These data indicated the hypertrophic state of the lakes, reflected in low water transparency (15–90 cm), high nutrient and Chl-a concentrations, and microbial abundances (Fig. 1). The average (±SD) abundances of the heterotrophic microbes were 21.8 ± 12.7 × 10⁶ bacteria ml⁻¹, 11.1 ± 7.2 × 10³ HNF ml⁻¹ and 265 ± 207 ciliates ml⁻¹. Low microbial abundances in the lake Rod in April and May (Fig. 1B) and enhanced water transparency >130 cm was due to a temporal clear-water phase with low Chl-a concentrations and high cladoceran abundances (180–565 individuals L⁻¹).

Microbial food web dynamics and bacterial mortality induced by protistan grazing

Marked increases in concentrations of bacteria, HNF, ciliates and Chl-a were observed during the June–September period (Fig. 1). Phytoplankton biomass fluctuated in a wide range of Chl-a values of 65–696 μg L⁻¹ (Fig. 1; Supplementary Table S2). Based on the FLB tracer technique, HNF and ciliates were the most important bacterivores while no FLB uptake was observed in plastid-bearing flagellated protists. The relative importance of aggregated HNF and ciliate bacterivory, as proportions of bacterial production grazed in the size fraction <1 μm, or proportions of bacterial standing stock grazed per day (Fig. 2A–D), accounted (average ± SD) for 53.1 ± 61% and 51 ± 60% (ranging only moderately – from 40.2% to 54.9% in the particular lakes, for details see Table 2) respectively. Average doubling times of suspended bacteria based on the thymidine uptake assay were 19 ± 10 h (median 23 h).

Fig. 1. Time-course changes in total abundance of bacteria, heterotrophic flagellates, ciliates and concentrations of chlorophyll-a in ponds Kvítkovický (A), Rod (B), Klec (C) and Dehtář (D) over the period April–September 2018. Values are means of duplicates; error bars show range of values.

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There was a large temporal variability in the contributions of HNF and ciliates to their aggregated bacterivory rates (Fig. 2A–D). On average, HNF bacterivory was slightly but insignificantly (t-test, p > 0.05) higher than ciliate grazing, accounting for 56% and 44% of total HNF and ciliate bacterivory, respectively. However, we found a consistent trend of higher proportions of HNF bacterivory in spring (66.4%–77.7%, Table 2), compared to higher ciliate contributions to the aggregated protistan grazing during summer months in all lakes (50.6%–72.6%).

Flagellate assemblage composition and grazing assessed by CARD-FISH and FLB

The four selected FISH probes targeting major bacterivorous flagellate taxa (Fig. 3; Table 1) cumulatively targeted 51%–96% (average ± SD, 75 ± 14%) of total HNF (Fig. 3E–H). Thus, the dynamics of the majority of HNF bacterivores was covered at sufficient resolution, revealing marked compositional changes in time and between the lakes (Fig. 3), but also general patterns in the representation of the probe-defined flagellate taxa. The following order of their average contributions (±SD, n = 24) to total HNF were determined: total heterotrophic Cryptophyceae (probe CryptoB, 53 ± 13%), its Cry1 lineage (Cry1-652, 23 ± 16%), Kinetoplastea (Kin516, 14 ± 12%) and Katablepharidacea (Kat-1452, 7.5 ± 5%).

While high proportions of aplastidic CryptoB were observed in all samples, their Cry1 lineage showed a more irregular distribution, accounting for the majority of all aplastidic cryptophytes in some samples, while being almost absent in some late-summer samples from Kvítkovický and Rod (Fig. 3A–D).

Kinetoplastids were present in all samples, but were more abundant during the summer period (Fig. 3A–H), along with algal and cyanobacterial blooms. This was also reflected in significant positive correlations of kinetoplastid numbers with concentrations of Chl-a and cyanobacterial biomass ($r^2 = 0.557$ and 0.408).
respectively, \( p < 0.001; \) Supplementary Fig. S1). Katablepharids were most abundant in the Rod and Dehtář lakes, particularly in summer months, while being almost absent in Klec (Fig. 3A–H).

On a cell-specific basis of all HNF, flagellate grazing rates ranged from 7.6 to 39.2 bacteria flagellate \(^{-1}\) h\(^{-1}\), averaging 18.4 ± 8.5 (±SD) bacteria flagellate \(^{-1}\) h\(^{-1}\) and the mean cell volume (MCV) for the whole HNF community was 34.0 ± 11.5 μm\(^3\). However, the combination of CARD-FISH and FLB tracer techniques allowed us to estimate also taxa-specific MCV and grazing rates (Table 3) and aggregated grazing impacts of different
Table 1. Characteristics of CARD-FISH probes used in this study.

| Probe   | Target                      | Sequence 5'–3'                      | Formamide concentration (%) | Reference            |
|---------|-----------------------------|------------------------------------|----------------------------|----------------------|
| CryptoB | Cryptophyceae_1             | ACGGCCCAACTGCTCCT                  | 50                         | Metfies et al. (2007) |
| Cry1-652| CRY1 lineage of cryptophytes| TTTCACAGTWAACGATCCGC              | 30                         | Grujić et al. (2018) |
| Kat-1452| Uncultured Katablepharidae   | TCCCCGARMATCGACGGCG               | 60                         | Grujić et al. (2018) |
| Kin516  | Kinetoplastea               | ACCAGACTTGTCCTCC                  | 30                         | Bochdansky and Huang (2010) |
| EUK516  | Competitor for probe Kin516 | ACCAGACCTGCCCTCC                  | 30                         | Bochdansky and Huang (2010) |

The applied taxonomic terminology followed a review of Adl et al. (2019).

Table 2. Parameters of protistan grazing on bacteria in four shallow hypertrophic lakes: Relative proportions of bacterial standing stocks grazed by both HNF and ciliates per day and the relative contributions of HNF and ciliates to their aggregated total bacterivory rates. The values represent averages (bold) with range of values (min–max) in parenthesis for the spring period (April to June, n = 3), summer (July to September, n = 3) and the whole study period (April-September, n = 6).

| Lake      | Period       | Bacterial standing grazed per day (%) | HNF (%)          | Ciliate (%)       |
|-----------|--------------|--------------------------------------|------------------|-------------------|
| Kvítkovský| April - June | 28.5 (10.6–55.5)                     | 77.7 (66.4–84.4) | 22.3 (15.8–33.6) |
|           | July - Sept. | 51.9 (42.9–63.7)                     | 27.4 (24–35.8)   | 72.6 (64.2–77.7)  |
|           | April - Sept.| 40.2 (10.6–63.7)                     | 55.2 (24–84.4)   | 44.8 (15.8–77.7)  |
| Rod       | April - June | 47.2 (5.6–126)                       | 64.2 (56.2–68.4) | 35.8 (31.7–43.9)  |
|           | July - Sept. | 49.9 (29.7–71.9)                     | 48.4 (43.8–54.5) | 51.6 (45.5–56.2)  |
|           | April - Sept.| 48.5 (5.6–126)                       | 56.3 (43.8–68.4) | 43.7 (31.7–56.2)  |
| Klic      | April - June | 71.9 (23.4–114)                      | 71.5 (45.4–91.7) | 28.5 (8.3–54.6)   |
|           | July - Sept. | 36.8 (16.6–62.8)                     | 49.4 (39.9–60.2) | 50.6 (39.8–60.1)  |
|           | April - Sept.| 54.3 (16.6–114)                      | 60.8 (39.9–91.7) | 39.2 (8.3–60.1)   |
| Deštěř    | April - June | 51.8 (22.4–112)                      | 74.1 (70.1–77.2) | 25.9 (22.8–30.1)  |
|           | July - Sept. | 54.9 (46.5–92.4)                     | 42.6 (29.9–65.2) | 57.4 (34.8–70.1)  |
|           | April - Sept.| 53.4 (22.4–112)                      | 58.3 (29.9–77.2) | 41.7 (22.8–70.1)  |

Table 3. Cell-specific bacterial uptake rates, cell biovolumes of different probe-defined flagellate taxa and estimated range of doubling times of the flagellate groups, based on bacterial prey biovolume ingested (bacterial MCV, 0.095 μm³) per hour as related to the flagellate MCV, assuming a range of 30–40% of HNF volumetric gross growth efficiency (Strálek, 1997; Šimek et al., 2018).

| Probe | Cell-specific uptake rates (bacteria cell⁻¹ h⁻¹) | Flagellate lineage-specific cell volumes (μm³) | Estimated average doubling time (hours) |
|-------|-----------------------------------------------|---------------------------------------------|---------------------------------------|
|       | Mean ± SD range                               | Mean ± SD range                             | range                                 |
| CryptoB | 28.2 ± 11.9 | 10.6 – 55.2                                 | 53.8 ± 82.2 | 4.6 – 72.6 | 49 – 66 |
| Cry1-652| 18.3 ± 8.3 | 7.4 – 38.6                                 | 18.4 ± 9.2 | 4.5 – 41.5 | 24 – 32 |
| Kin516  | 22.4 ± 9.7 | 8.9 – 46.0                                  | 73.8 ± 64.8 | 15.0 – 368 | 83 – 111 |
| Kat-1452 | 15.6 ± 7.7 | 5.2 – 34.6                                  | 149 ± 60.2 | 48.0 – 308 | 248 – 330 |

probe-defined flagellate taxa (Figs 3I–L). Evaluating all samples (Fig. 3I–L), the average (±SD) contributions of the probe-defined groups to total HNF bacterivory were: total aplastidic cryptophytes (probe CryptoB, 67 ± 13%), its Cry1 lineage (Cry1-652, 21 ± 13%), kinetoplastids (Kin516, 14 ± 11%) and katablepharids (Kat-1452, 7 ± 1.4%). Moreover, analyses of a number of DAPI-stained bacteria in food vacuoles of CARD-FISH-targeted bacterivorous flagellates provided, independently of the tracer technique, an additional snap-shot proxy of bacterial uptake rates and individual in situ variability of the uptake data (see examples in Table 4 and Supplementary Fig. S2). This relative proxy of bacterivory indicated comparable numbers of ingested natural bacteria over summer months by Cry1 and kinetoplastids compared to all HNF (Supplementary Fig. S2[AC]), while being significantly higher in CryptoB group compared to all HNF (Fig. S2[B]). In contrast, Katablepharids showed remarkable changes in bacterial uptake over the season, with almost no bacteria ingested in the springtime, yielding a highly significant difference of this proxy compared to all HNF (Table 4 and Supplementary Fig. S2[D]).

Grazing of all aplastidic cryptophytes, targeted by the CryptoB probe, clearly dominated the aggregated protozoan (of both HNF and ciliate) bacterivory: this group alone consumed ~25%–35% of bacterial production. Note that it was quite easy to distinguish the smaller aplastidic (heterotrophic) and plastid-bearing flagellate cells (‘green’ algae such Rhodomonas or Cryptomonas...
spp.) in natural samples processed by CARD-FISH, both being targeted by the same general probe (CryptoB) for cryptophytes (Fig. 5). The autotrophic cryptophytes, such as *Rhodomonas* and *Cryptomonas* spp., have much larger cells (9–18 μm in size), keeping well preserved ovoid drop-shaped morphology in FISH preparations, with characteristically located chloroplasts and no FLB uptake. In contrast, much smaller heterotrophic cryptophytes are voracious bacterivores without chloroplasts (Figs 4 and 5).

The prominent heterotrophic bacterivores (CryptoB in Fig. 3) were morphologically diverse, with cell length from 3 to 12 μm and MCV of 54 ± 82 μm³ (Table 3, see examples in Fig. 4A–H). All these phylootypes ingested natural bacteria and FLB (Fig. 4E–H). Moreover, CryptoB displayed...
significantly higher numbers of ingested bacteria compared to all HNF present in samples, accompanied with larger uptake variability (Supplementary Fig. S2 [B]). Overall, they contributed disproportionally more to total HNF bacterivory than to total HNF abundance (Fig. 3M). For instance, in May they accounted for 80%–90% of total HNF bacterivory (Fig. 3I–L). Also aggregated proportions of the HNF targeted by the three probes, CryptoB, Kin516 and Kat-1492, contributed disproportionally more to total HNF bacterivory (84.6%–92.7%) than to total HNF abundance (68%–87.6%, for details see Fig. 3N).
Within the Cryptophyceae, members of the monophyletic lineage Cry1 (probe Cry1-652) represented highly important HNF bacterivores, with relatively uniform morphology and location of nuclei in rounded cells (2.5–5 μm diameter, MCV of 18.4 ± 9.2 μm³; Fig. 4I–N). Their cell-specific bacterivory rate (Table 3) and the variability of a number of ingested DAPI-stained bacteria were similar to those of all HNF (Table 4, Supplementary Fig. 2S[A]). The NCBI NT database (Altschul et al., 1990) revealed that the sequences targeted by the Cry1-652 probe are present in freshwater lakes in Europe, USA, Asia and Africa (Supplementary Table S3).

CARD-FISH-stained kinetoplastids were elongated-ovoid to drop-shaped flagellates (5–9 μm length, MCV of 74 ± 65 μm³), with a well distinguishable nucleus and kinetoplast after DAPI-staining (Fig. 4O–S). Their cell-specific bacterivory rates (Table 3) and numbers of ingested DAPI-stained bacteria were mostly similar or slightly higher than those of all HNF (Table 4, Supplementary Fig. 2S[C]).

Kathablepharids targeted by probe Kat-1452 were oblong cells with rounded ends (6–10 μm, MCV of 149 ± 60 μm³, Table 3) with a large posteriorly situated nucleus and two flagella. These phylotypes ingested both bacteria and small algal prey as documented in Fig. 4T–W. However, bacterial uptakes in July and September samples indicated a larger role of bacterivory during summer, compared to the negligible bacterivory (Supplementary Fig. 2S[D]) and the prominence of algivory exhibited by this group in May. Besides the studied hypertrophic lakes, the sequences targeted by Kat-1452 probe were detected in freshwater habitats in Europe and USA (Supplementary Table S4).

Ciliate assemblage composition and bacterivory

The lakes were numerically dominated by Stichotrichia (Halteria and Pelagohalteria spp.), algivorous Prorodontida (Urotricha spp. and Balanion planktonicum), and Oligotrichia (namely, Rimostrombidium spp., Fig. 6A–D). The ciliate cell-specific uptake rates ranged from 279 to 1976 bacteria ciliate⁻¹ h⁻¹, with the grazing rates reflecting the temporarily changing ciliate assemblage compositions in the lakes, that is shifts between morphospecies with distinct abilities to ingest bacteria (Fig. 6E–H). In terms of total ciliate bacterivory, the most
important ciliate bacterivores were (Fig. 6E–H): (i) *Halteria* and *Pelagohalteria* spp., (ii) in some cases Oligotrichia (namely, *Rimostrombidium* spp.), followed by (iii) the less abundant but highly efficient bacterivores from Peritrichia (genera *Vorticella* and *Epistylistis*), (iv) the more abundant *Scuticociliatia* (mainly genus *Cyclidium*), (v) the Prorodontida with negligible bacterial uptakes (only *Colesp* spp.), and (vi) unidentified ciliates mostly without bacterivorous activity (‘others’ in Fig. 6). However, at the genus level, *Halteria/Pelagohalteria* spp. were clearly the most important bacterivores in these lakes (Fig. 6E–H), comprising, on average, 56.1% of total ciliate grazing.

**Discussion**

Flagellate groups assumed as major planktonic bacterivores

Our study, based on CARD-FISH and tracer techniques applied in plankton environments, is the first one to convincingly document that aplastic cryptophytes are prominent and seasonally ubiquitous HNF bacterivores, controlling a large part of bacterial production in hypertrophic shallow lakes (Figs 2–4), which are becoming increasingly important waterbody types worldwide. This finding differs from previous studies, based on morphology-based microscopic approaches, attributing the role of major heterotrophic bacterivorous flagellates to heterokont taxa (mainly chrysomonads and bicosoecids), choanoflagellates, kataphepharids, kinetoplastids and cercozoans (Sherr and Sherr, 1994; Arndt et al., 2000; Boenigk and Arndt, 2000, 2002). From these groups, mainly easily cultivable aplastic cryptophytes, such as *Spumella*-like species or *Paraphysomonas* spp., have been assumed to be highly abundant and important bacterivorous taxa in the majority of freshwater and marine pelagic environments (Sherr and Sherr, 1994; Arndt et al., 2000; Boenigk and Arndt, 2002). This is also reflected in their frequent use as model flagellated bacterivores (Choi and Peters, 1992; Lim et al., 1999; Grossmann et al., 2016). Moreover, many chloroplast-bearing taxa among cryptophytes and cryptophytes are also capable of phagotrophy, thus benefiting from a mixotrophic lifestyle (Holen and Boraas, 1995; Jones, 2000). However, mixotrophic nanoflagellates with the detectable FLB uptake were virtually absent in the hypertrophic lakes, compared to high HNF densities, while the prominent role of aplastic cryptophytes as possible pelagic bacterivores (Fig. 3) has been overlooked until recently (Grujić et al., 2018; Šimek et al., 2020).

In our study, we used CARD-FISH probes detecting different taxonomic levels, from the lineage-specific Cry1-652 to class-specific Kin516 (Kinetoplastea). Our approach represented a trade-off between reachable taxonomic resolution and the highest proportions of major bacterivorous taxa detected with the available probes of different levels of taxonomic resolution. The approach proved to be successful since the aggregated coverage of three major probes, CryptoB, Kat-1492 and Kin516, was on average ($n = 6$ samples per lake, Fig. 3N) 68%–87.6% of total HNF, while the parallel application of the FLB assay showed that these probe-targeted flagellates accounted even for 84.6% to 92.7% of total HNF bacterivory. Thus only between 7.3% and 15.4% of total HNF bacterivory could not be attributed to probe-defined flagellate groups in our study (Fig. 3N). Among the bacterivorous HNF that were not targeted by the FISH probes, chrysophytes and choanoflagellates likely played an important role (Arndt et al., 2000; Boenigk and Arndt, 2002; Bock et al., 2020). However, among chrysophytes only *Dinobryon* sp. was morphologically distinguishable bacterivore observed sporadically in samples after ice-melt in March, while our study was performed during the April–September period. Likewise rather rare in our samples, choanoflagellates were sometimes distinguishable on the basis of a visible collar in some summer samples.

Aplastic cryptophytes: a changing paradigm of the core planktonic bacterivores

Aplastic cryptophytes have not been recognized as bacterivores for a long time, as 18S rRNA sequence data do not provide information on trophic interactions and the presence or absence of chloroplasts in cells. Thus, most sequences are attributed to autotrophic cryptophyte groups (e.g. Bjorbaekmo et al., 2020), or to their known mixotrophic members described previously (Jones, 2000; Marshall and Laybourn-Parry, 2002). Moreover, recent comparative analyses (Piwosz et al., 2020) indicate that cryptophytes in general, as well as their aplastic Cry1 lineage, are underrepresented in amplicon sequencing results and thus metabarcoding data cannot precisely estimate their proportions. From whole group of heterotrophic cryptophytes currently only members of the genus *Gonioomonas* have been isolated (being moderately distant from Cry1 lineage, see a phylogenetic tree in Šimek et al., 2020), with three described species, but only one typical freshwater representative, *G. truncata* (von der Heyden et al., 2004). Generally, the diversity of aplastic species within cryptomonads has received little attention and interestingly there seems to be no evidence supporting the presence of a plastid of primary or secondary endosymbiotic ancestry in goniomonads (Cenci et al., 2018).
The Cry1 lineage of cryptophytes was originally discovered in sequence data (Shalchian-Tabrizi et al., 2008) and tentatively suggested to include bacteriovorous representatives (Piwosz et al., 2016). This was later confirmed by inspecting food vacuole contents of flagellate grazers grown in two individual samples from a mesotrophic reservoir used for laboratory experiments, manipulating bacterial prey availability by additions of different bacterial species (Grujić et al., 2018; Šimek et al., 2020). In the latter studies, the bacterial enrichments induced the most rapid growth responses of small Cry1 cells with doubling times of 10–16 h. It might also indicate an opportunistic lifestyle of these small flagellates in plankton environments, that is the ability to rapidly decimate fast-growing bacterioplankton taxa associated with particular algal blooms, which form short-lived abundance peaks (Zeder et al., 2009; Eckert et al., 2011; Salcher et al., 2013; Šimek et al., 2014).

In addition, CARD-FISH in combination with the FLB tracer techniques have clearly confirmed high rates of bacterivory in aplastidic cryptophyte taxa (Figs 3 and 4). The discovery of their high abundances in situ, their high growth potential and cell-specific bacterivory rates (Table 3), and preliminary indications of their occurrence in other aquatic ecosystems ranging in trophy and salinity (Piwosz et al., 2016; Shiratori and Ishida, 2016, Piwosz, 2019; Šimek et al., 2020, see also Supplementary Table S3) can considerably broaden the understanding of trophic relationships among prokaryotes and bacteriovorous HNF taxa. In contrast, easily distinguishable, chloroplast-bearing autotrophic cryptophytes (e.g. Cryptomonas, Rhodomonas, Fig. 5) did not ingest FLB and represent a large group of intensively studied bloom-forming algae (e.g. Sommer et al., 1986, 2012). However, progress in this field is currently severely limited by the lack of studies that combine CARD-FISH and in situ prey tracer techniques in aquatic ecosystems with a broad range of trophic states.

Considering the large biovolume of bacterial prey ingested by the small Cry1 cells, and assuming the typical HNF gross growth efficiency of 30–40% (Straile, 1997; Šimek et al., 2018), we estimated their in situ doubling times at ca. 24–32 h. Since their growth rates closely correspond to an approximately daily bacterioplankton doubling in the lakes, these taxa are apparently capable of meeting their entire carbon requirements solely on a bacterial diet, with e.g. doubling times of 10–16 h in bacteria-enriched treatments (Šimek et al., 2020). Our doubling times presented for Cry1 (Table 3) could underestimate their growth potential on suspended bacteria, as a considerable part of bacterioplankton in the shallow lakes formed small flocks, representing inaccessible prey items to the tiny (2.5–5 μm diameter) Cry1 bacterioves. However, since these small and morphologically uniform HNF lack distinctive morphological features, it is not so surprising that they have so far ‘slipped under the radar’ of microbial ecologists.

All aplastistic cryptophytes targeted by the general CryptoB probe were morphologically more diverse. The probe also targets Cry1, but >50% of the FISH-positive cells were diverse, relatively large (6–12 μm cell size; Fig. 4A–H) morphotypes of voracious bacterioves. They had significantly higher cell-specific uptake rates than the Cry1 (Table 3), reaching in some individuals up to 70–80 bacterial cell⁻¹ h⁻¹. Their aggregated bacterivory accounted for approximately 35–40% of total protist-induced bacterial mortality, thus confirming the study hypothesis regarding their important trophic role.

Other CARD-FISH detected protistan groups

Kinetoplastids, in line with our expectations, were more abundant during the summer (up to 38% of total HNF) and contributed significantly more to bacterial mortality rates during this period (Fig. 3), when larger algae or colonial cyanobacteria blooms were observed. Kinetoplastids are associated with detritus particles (Caron et al., 1982; Zubkov and Sleigh, 2000), where they can feed on surface-associated bacteria (Caron, 1987; Weitere and Arndt, 2003). These flagellates were also reported from deep oxygenated lake waters during periods of high concentrations of settling organic particles (Mukherjee et al., 2015, 2019). These large ‘lake snow’ particles represent a substratum rich in organic matter, on which rapid growth of specific groups of associated bacteria was reported (Grossart et al., 2005), thus considerably enhancing the prey availability for the bacterioves specialized in feeding on surface-associated bacteria (Caron, 1987; Zubkov and Sleigh, 2000). Since we used suspended FLB as bacterivory tracers, the cell-specific bacterivory rates of kinetoplastids may be underestimated to a certain extent, because of their decreased efficiency in cropping suspended bacteria. Despite this, our estimates indicate that kinetoplastids comprised on average 13%, but up to 35% of the total HNF bacterivory during summer bloom events (Figs 1 and 3). Assuming that bacteria are the sole food source of kinetoplastids (Arndt et al., 2000; Boenigk and Arndt, 2000), their MCV and cell-specific bacterivory rates yield doubling time estimates of ∼3–4 days (Table 3). These results indicate slower growth compared to the approximately one doubling per day estimated for the small Cry1 cells and their bacterioplankton prey.

The Kat-1452 probe targeted typical omnivorous katablepharids that ingested both bacteria (Domaizon et al., 2003) and smaller algae (Fig. 4T–W). However, recent experimental results indicated that they also prey
on smaller HNF, e.g. on cells of the Cry1 lineage (Šimek et al., 2020). Thus, our current views on the complexity of trophic relationships between small, primarily bacterivorous flagellates and medium-size omnivorous-predatory HNF have been considerably revised (Piwosz et al., 2021). While previous studies demonstrated that katablepharids preferentially graze on smaller algae (Clay and Kugrens, 1999; Kwon et al., 2017; Ok et al., 2018), sometimes using a specific collective swarming strategy (Clay and Kugrens, 1999; Okamoto and Inouye, 2005), our analyses of individual bacterial uptakes (Supplementary Fig. 2S[D]) revealed a more diverse feeding strategy including a temporal shift from the dominance of largely algivorous Katablepharidaceae (with low uptake of bacteria) in spring to omnivorous phylothypes with high uptake rates of bacteria in summer. Since also bacterial abundance peaked in summer, our results indicate the need for a finer taxonomic resolution of the katablepharids as well as other HNF groups in order to detect the presence of different lineages with specific food preferences. However, taking into account the large MCV of katablepharids in general (149 μm³), the contribution of a solely bacterial diet would be insufficient to support rapid growth (Table 3). This corresponds also to their moderate bulk bacterivory, accounting for 1%–16% of total HNF bacterivory.

Ciliate bacterivory rates

In terms of bulk ciliate bacterivory and cell-specific grazing rates of particular ciliate species or morpho-species (Posch et al., 2015), our current study confirms the conclusions of the previous study conducted on hypertrophic lakes, where total ciliates bacterivory rates were similar to those of HNF (Šimek et al., 2019). Interestingly, the major ciliate bacterivores were not specialized bacterivores but omnivorous Haterlia/Pelagohalteria (Stichotrichia), feeding on a broader size spectrum of plankton particles (~0.5–6 μm, Jürgens and Šimek, 2000), while the Oligotrichia from the genus Rimostrombidium fed mainly on smaller algae (Müller and Schlegel, 1999; Posch et al., 2015; Šimek et al., 2019). These two groups accounted for, on average, ~2/3 of total ciliate bacterivory, while specialized peritrich and scuticociliate bacterivores (Beaver and Crisman, 1989; Foissner and Berger, 1996) comprised the remaining ~1/3 of the total ciliate bacterivory (Fig. 6E–G). However, even the low abundance of the efficient picoplankton grazers from peritrichous ciliate genera, such as Vorticella and Epistylis (Bickel et al., 2012; Šimek et al., 2019; Weisse et al., 2021) contributed disproportionately more to total bacterial mortality. The markedly increased contributions of ciliate bacterivory to aggregated HNF and ciliate grazing in the summer period (Table 2) was attributable to marked increases in total ciliate numbers in general, paralleled by enhanced proportions of bacterivorous peritrichs and scuticociliates (Fig. 6).

General implications of our findings

The aggregated FISH-probe hybridization rates were high, sometimes exceeding 90% of total HNF (Fig. 3). This suggests that the flagellate groups such as choanoflagellates and chrysophytes, commonly considered the prominent freshwater bacterivores (Šimek et al., 1997; Boenigk and Arndt, 2002; Jürgens and Matz, 2002) substantially contributing to protistan-related taxa in sequence-based studies (Del Campo and Massana, 2011; Bock et al., 2020; Sieber et al., 2020), likely play smaller roles as bacterivores in hypertrophic lakes. Although we did not study these groups specifically with FISH probes (appropriate ones targeting specifically their bacterivorous members are not available, Piwosz et al., 2021), this conclusion is based on our finding that only ca. 7%–15% of total HNF bacterivory can be attributed to different groups than to those targeted by the CARD-FISH-probes used in our study (Fig. 3N). Moreover, aplanodetic cryptophytes and their Cry1 lineage are also ubiquitous in other freshwater and brackish habitats (Piwosz et al., 2016; Grujić et al., 2018; Šimek et al., 2020; Šimek and Mukherjee, unpublished data), with sequences and FISH-positive cells of the Cry1 lineage being detected in aquatic habitats of trophic states from oligo- to hypertrophy (Figs 3 and 4; Supplementary Table S3). Our results also confirmed the trend of an increasing role of ciliate bacterivory towards hypertrophic habitats (Beaver and Crisman, 1989; Nakano et al., 1998; Zingel et al., 2007; Šimek et al., 2019; Weisse et al., 2021).

Our study presents the first seasonal field data on the composition of HNF assemblages with taxa-specific bacterivory rates in hypertrophic shallow lakes; however, we are currently unable to generalize these findings over a broader scale of trophic states. From the general trends of decreasing concentrations of bacteria, phytoplankton, organic particles and limiting nutrients with increasing oligotrophy (Berninger et al., 1991; Gasol and Vaqué, 1993; Arndt et al., 2000), we assume that proportions of specialized bacterivorous kinetoplastids (Caron, 1987; Mukherjee et al., 2015, 2019) and bacterivorous lineages of katablepharids might be lower in more oligotrophic systems. In contrast, proportions of mixotrophic chrysophytes and cryptophytes, profiting from the flexible lifestyle under enhanced water transparency (contrasting to the hypertrophic turbid lakes), might be higher (Holen and Boraas, 1995; Jones, 2000).
Notably, small cell size, fast growth and high bacterial ingestion rates of small aplastidic cryptophytes and their Cry1 lineage (Table 3, see also Šimek et al., 2020) could predetermine these groups to be highly competitive bacterivores even in various oligo-mesotrophic environments with considerably lower bacterial densities.

Conclusions

We have shown that aplastidic cryptophytes and their Cry1 lineage are prominent bacterivores in hypertrophic lakes. Preliminary results on their abundance from other types of fresh and brackish waters (Piwosz et al., 2016, 2021) indicate that these understudied but ecologically prominent HNF taxa can represent core planktonic bacterivores, similarly to the suggested key role of the omnivorous Halteria/Pelagohalteria within ciliate assemblages in systems of higher trophic states (Šimek et al., 2019). Unfortunately, it is impossible to infer a hetero- or auto/mixotrophic lifestyle of flagellated protists only from the information carried by 18S rRNA sequences. Moreover, there are so far no isolated strains of aplastidic cryptophytes available, except members of the genus Goniomonas. Thus, before generalizing the hypothesis about the prominent role of aplastidic cryptophytes as freshwater pelagic bacterivores, investigations of a broad spectrum of freshwater bodies of different trophic states, using both CARD-FISH and sequencing approaches, will be necessary. In addition, a combination of isolation and strain sequencing would considerably refine the existing reference databases and improve the phylogenetic relationships of aplastidic members within cryptophytes. These necessary sequential steps might help detecting the environmental sequences attributable to the ‘cryptic aplastidic’ groups that have so far been considered as autotrophic or mixotrophic lineages. Building on the complementarity of sequencing and FISH approaches, such information might allow designing new probes with high taxonomic resolution that can be combined with a double CARD-FISH approach (Grujič et al., 2018) for simultaneous unveiling of phylogenetic identity of both barely distinguishable small flagellates and their preferred prey.

Experimental procedures

Study sites and sampling

We sampled four hypertrophic shallow lakes in South Bohemia (Czech Republic, Supplementary Tables S1 and S2), with area in the range of 0.22–2.28 km². These hypertrophic lakes were so far studied mainly from the point of view of fish production except for one study dealing with microbial food webs focusing on ciliate bacterivory in 10 different lakes (Šimek et al., 2019). In the current study, integrated water samples (45 L) were collected monthly, from April to September 2018, at seven sampling points along a linear transect of the open water zone from the surface layer (0–1 m) by a van Dorn sampler (Šimek et al., 2019). Two subsamples were filtered through a 200-μm mesh into sterile glass bottles for microbial activity analyses (~2 L) and into a plastic bottle for chemistry and seston analyses (5 L). Three unfiltered subsamples (50–120 ml) were fixed with Lugol’s solution for phytoplankton determination, with Lugol’s solution post-fixed by Bouin’s fluid for ciliate analyses, and with 2% formaldehyde for prokaryote and HNF quantification.

Enumeration of microbes

Duplicate formaldehyde-fixed samples were used for the enumeration of bacteria and heterotrophic protists on 0.2-μm and 1-μm pore-size filters respectively (Šimek et al., 2019). All samples were stained with DAPI (4’,6-diamidino-2-phenylindole) and microbes were counted via epifluorescence microscopy (Olympus BX53; Optical, Japan). Bacterial and flagellate cell dimensions were measured using a semiautomatic image analysis system (NIS-Elements 5.1, Laboratory Imaging, Prague, Czechia), and biovolumes were computed as detailed elsewhere (Šimek et al., 2013). Large chloroplast-bearing dinoflagellates (20–25 μm in size) occasionally occurred in the lakes, but they never ingested FLB since they prey upon other algae or protists (Arndt et al., 2000; Piwosz et al., 2021). Therefore they are not included in this study focused on bacterivorous nanoflagellates. Similarly, also other autotrophic flagellated protists with chloroplasts (e.g. Rhodomonas, Cryptomonas), which did not ingest bacteria in the lakes (Fig. 5), were not enumerated in DAPI and FISH preparations in this study focusing on bacterivorous HNF. However, we cannot entirely exclude the presence of mixotrophic flagellates with a fairly low uptake of bacteria, under detection limit of the FLB approach, thus by definition our study dealt with a subset of heterotrophic flagellated bacterivores grazing on heterotrophic bacteria.

Bacterial production

Bacterial production was measured with the thymidine incorporation assay. Triplicate 5-ml subsamples were incubated for 30 min at in situ temperature with 20 nmol L−1 [methyl-3H] thymidine (Moravek, Brea, CA, USA), then fixed with neutral buffered formaldehyde (2% final concentration), filtered through polycarbonate membrane filters (Osmonics, Livermore, CA, USA) of 0.2-μm and 1-μm porosity and extracted 10 times by 1 ml of ice-cold 5% TCA. Thymidine incorporation by free pelagic
bacteria (the 0.2–1 μm size fraction) was calculated as the difference between the radioactivity retained on 0.2-μm and 1-μm filters (Šimek et al., 2019). Cell production rates were calculated using the empirical conversion factor of \(2 \times 10^{18}\) bacterial cells per mol thymidine (Bell, 1990).

**CARD-FISH analyses of flagellate assemblages**

Samples were fixed with a Lugol-formol-thiosulphate decolourization technique (Sherr and Sherr, 1994; Jezbera et al., 2005) and filtered within 24 h. Filters were stored at \(-20^\circ\text{C}\), until further processing. The probes used were chosen after an extensive survey of natural HNF communities in the studied shallow lakes and of three oligomesotrophic and eutrophic reservoirs (Šimek and Mukherjee, unpublished data) to cover the highest proportions of total HNF with ingested fluorescently labelled or DAPI-stained bacteria. We used four oligonucleotide probes (FITC- and Alexa546-labelled, Piwosz et al., 2021), targeting the following protistan groups: all cryptophytes (CryptoB; Mettles and Medlin, 2007) and its Cry1 lineage (Cry1-652; Grujić et al., 2018), Katablepharidacea (Kat-1492; Grujić et al., 2018), Kinetoplastea (Kin516; Bochdansky and Huang, 2010), thus covering the most commonly occurring bacterivorous HNF in the studied shallow lakes. We also applied a FISH-probe Cerc193 targeting the Cerozoan novel clade 7 (Bass and Cavalier-Smith, 2004), designed for flagellated Cerozoa in a mesotrophic freshwater reservoir (Šimek et al., 2020), but we did not detect any hybridized cells in the lakes. For detailed analyses of the probe targets see Šimek et al. (2020). Moreover, updated lists of currently available sequences targeted by phylogenetically narrower FISH probes (Cry1-652 and Kat-1492), detecting the Cry1 lineage and Katablepharidacea, and their original aquatic habitats were extracted from the NCBI NT database (Altschul et al., 1990); see Supplementary Tables S3 and S4; the hybridization conditions are given in Table 1. Details of the used CARD-FISH protocol (Piwosz and Pernthaler, 2009), a troubleshooting guide addressing most frequent obstacles, and details on quality ranking and specificity tests for all FISH-probes used are given in a recent review (Piwosz et al., 2021). Hybridization was conducted at 35°C for 2–3 h for all the probes, except for the Kin516, which required 12 h of hybridization (Mukherjee et al., 2019).

Duplicate CARD-FISH preparations were analyzed by epifluorescence microscopy at 1000× magnification. Note that only aplastic protists were considered in this study, while e.g. plastidic cryptophytes with no FLB uptake, easily distinguishable by the presence of chloroplasts in both Alexa546- and FITC-staining (Fig. 5), were not enumerated. Chloroplasts produce bright red autofluorescence in the appropriate Chl-a excitation filter setting that does not overlap with FITC staining and only slightly overlaps in Alexa546 staining excited at different wavelengths (Piwosz et al., 2021). The flagellate taxaspecific MCVs were calculated based on the measurements of hybridized cell width and length (Šimek et al., 2020), using an image analysis system (NISS-Elements 5.1).

**Estimates of aggregated flagellate and ciliate grazing rates**

Protistan bacterivory rates were estimated using FLB (Sherr et al., 1987) prepared from a mixture of isolated strains representing the core bacterioplankton genera in lakes: *Limonhabitans planktonicus* (short rods, MCV of 0.135 μm³) and *L. parvus* (MCV of 0.055 μm³) and one undescribed *Polynucleobacter* strain (czRimov8-C6, short curved rods, MCV of 0.054 μm³, Šimek et al., 2019). The cells were mixed at a similar numerical ratio that yielded MCV ± SD of the mixture cells of 0.092 ± 0.021 μm³, which matched well the typical MCV of bacteria in the lakes (0.095 ± 0.027 μm³, median 0.089 μm³). For grazing experiments, 300-ml samples were dispensed into 1-L flasks and incubated at *in situ* temperature. The FLB tracers were added to constitute 8%–16% and 3%–7% of total bacteria for HNF and ciliate uptake estimates respectively, depending upon the season and prevailing protistan bacterivores (Šimek et al., 2019; Šimek and Sirová, 2019). We determined ciliate grazing rates in 5- and 10-min subsamples, and flagellate grazing rates in 30-min subsamples (yet within a linear increase in FLB uptake per flagellate), with greater FLB amounts added during colder water periods. The samples were fixed using a Lugol-formol-thiosulphate decolourization technique (Sherr and Sherr, 1994; Jezbera et al., 2005). Duplicate subsamples of 2–6 ml (flagellates) or 4–30 ml (ciliates) were stained with DAPI, passed through 1 μm black filters, and inspected via epifluorescence microscopy to count both HNF and ciliates as detailed elsewhere (Šimek et al., 2014, 2019). At least 100–200 ciliate and 200 HNF individuals were inspected for FLB ingestion in each sample. To estimate total protistan grazing, we multiplied average bacterial uptake rates of ciliates and HNF by their *in situ* abundances.

**Estimates of taxa-specific grazing rates**

Samples incubated with FLB were fixed, concentrated and stored frozen at \(-20^\circ\text{C}\), until further processing with the CARD-FISH protocol (see above). To examine taxaspecific uptake rates of the probe-targeted flagellate groups, their cells (>100 per sample) were inspected for
FLB uptake (see Fig. 4) using a combination of optical filter sets for excitation of Alexa546-stained (orange-red colour), or FITC-stained flagellates (green-yellow), DTAF-labelled FLB (yellow) and DAPI-stained nuclei or bacteria (blue). To obtain a proxy of the differences in bacterial uptake rates in different HNF taxa in situ (independent on FLB tracer technique), the CARD-FISH-positive cells were localized and an average number of DAPI-stained bacteria ingested per FISH-positive flagellate was counted (>50 cells per sample). Then on the same microscopic slide, the average number of DAPI-stained bacteria was also counted in food vacuoles of all randomly inspected DAPI-stained HNF (>50 cells per sample).

Ciliate abundance and assemblage composition

Ciliate abundances and a community structure were evaluated by combining epifluorescence microscopy and quantitative protargol staining as described (Foissner and Berger, 1996; Posch et al., 2015). This method combination allowed determining most of the ciliates to the genus or morphotype level and, if possible, to species level, using identification guides (Foissner and Berger, 1996; Foissner et al., 1999). To identify ciliates by fluorescence microscopy, additional criteria such as ciliate cell sizes, the position and size of nuclei, and prey characteristics were used (Foissner and Berger, 1996; Šimek et al., 2019). Between 4% and 13% of the ciliates, however, could not be identified.

Imaging of protistan cells with different prey items ingested

Multicolour z-stacks of images of protists with ingested bacterial/FLB or algal prey (Figs 4 and 5) were obtained with a motorized fluorescence microscope Nikon Eclipse 90i (Nikon, Tokyo, Japan) equipped with a monochromatic digital camera Andor Clara (Andor Technology, Belfast, UK), controlled by the software NIS-Elements 5.11 as detailed elsewhere (Šimek et al., 2019).

Environmental parameters and chlorophyll-a determination

Dissolved organic carbon was determined with the FormacsHT Analyzer (Skalar, Analytical B.V., Netherlands). For details on total and dissolved reactive phosphorus, total nitrogen and chlorophyll-a analyses see Kopáček and Hejzlar (1993) and Šimek et al. (2014).

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Author Contributions

K. Šimek conceived and designed the grazing experiments, analyzed ciliate communities and grazing, analyzed a part of FISH samples and wrote the paper with input from all authors. J. Nedoma, J. Jezerová and C. C. P. Paula were involved in the sampling, sample processing and data analyses. J. Nedoma elaborated an approach of image acquisition to document feeding modes of hybridized flagellates. I. Mukherjee processed samples for FISH analyses and with D. Sirová they contributed to data analyses and writing of the paper. J. Vrba coordinated the field sampling program and contributed to writing of the paper.

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