School of Doctoral Studies in Biological Sciences
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Faculty of Science

Differential freshwater flagellate community response to bacterial prey with a focus on planktonic Betaproteobacteria

Ph.D. Thesis

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Annotation

This thesis primarily focuses on the assumption that environmentaly important bacteria, with the major focus on those from the class of freshwater *Betaproteobacteria*, induce significant differences in growth responses and community composition of heterotrophic nanoflagellates. These prey-quality induced responses in the predator communities markedly modulate carbon flow to higher trophic levels. In contrast to many previous reports in the field of aquatic microbial ecology we combined comprehensive experiments, single-cell microscopic techniques and molecular methods, which facilitated our deeper understanding of processes and community shifts that are happening on the level of bacterial – prey and flagellate – predators.

Declaration [in Czech]

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České Budějovice, 21.2.2018 …….

Vesna Grujčić

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

1. **Grujčić V.**, Kasalický V., Šimek K. (2015). Prey-specific growth responses of freshwater flagellate communities induced by morphologically distinct bacteria from the genus Limnohabitans. Applied and environmental microbiology 81:4993–5002. doi:10.1128/AEM.00396-15.
VG was responsible for sampling and performing experiments, analyses and data evaluation, she wrote most parts of the manuscript.

2. Šimek, K., **Grujčić, V.**, Hahn, M. W., Horňák, K., Jezberová, J., Kasalický, V., Nedoma, J., Salcher, M. M., Shabarova T. (2018). Bacterial prey food characteristics modulate community growth response of freshwater bacterivorous flagellates. Limnology and Oceanography 63: 484–502. doi: 10.1002/lno.10759
VG was responsible for parts of the sampling and performing experiments, analyses and data evaluation; participated on the manuscript revision.

3. **Grujčić, V.,** Nuy, J. K., Salcher M. M., Shabarova T., Kasalický V., Boenigk J., Jensen M., Šimek K. (2018). Cryptophyta as major freshwater bacterivores in freshwater summer plankton ISME journal doi: 10.1038/s41396-018-0057-5 (in press)
VG was responsible for sampling and performing experiments, partial analyses and data evaluation, she wrote the manuscript.

Contents

1. Introduction

1.1 Microbial food webs

Dating back to the mid seventies, Pomeroy (1974) first proposed a ground breaking concept suggesting that bacteria and their grazers, namely heterotrophic nanoflagellates (HNF), are the driving forces and most important players in energy and organic carbon transfer in pelagic systems. Azam *et al.* (1983) further elaborated this novel concept and described the role attributed to bacterioplankton in transferring phytoplankton-derived organic carbon, in the form of DOM (dissolved organic matter), to heterotrophic flagellates and from them to the metazoan grazer food chain. This concept was introduced as the "Microbial loop" (Azam *et al.*, 1983) and later, due to high food web complexity with numerous trophic linkages, re-defined as "Microbial food webs". This complex trophic structure represents the most important path of transferring energy from primary producers via bacteria to higher trophic levels, which has been confirmed by numerous authors (e.g., Pomeroy and Wiebe, 1988; Ducklow, 2000; Hart *et al.*, 2000). This concept inspired research on the key microorganisms and their interactions worldwide across both marine and freshwater systems, including origin and form of DOM, rates of biomass production, transfer efficiencies and respiratory losses, etc. (e.g., Benner *et al.*, 1988; Del Giorgio *et al.*, 1999; Ducklow, 2000)

Although the microbial loop concept had been developed for marine waters it has since been applied to freshwaters. However, differences between environments may affect its relative importance for the movement of DOM to higher trophic levels. In addition, the microbial loop is more likely to dominate in oligotrophic or hypertrophic waters, rather than in eutrophic ones - there the classical plankton food chain predominates, due to the frequent fresh supply of mineral nutrients (e.g., spring bloom in temperate waters, upwelling areas).

a) Origin and forms of organic matter

Organic matter is omnipresent in inland water bodies and oceans in the form of allochthonous or autochthonous organic carbon. Organic matter is subdivided into particulate organic matter (POM) and dissolved organic matter (DOM). Generally, for simple characterization POM is assumed to be composed of living fraction (bacteria, phytoplankton and zooplankton) and

of non-living particles (aggregates, pellets and detritus, see e.g.Stramski *et al.*, 2008). DOM is one of the greatest and highly dynamic pools of organic matter in both marine and freshwater systems.

The source of DOM depends largely on the characteristics of a water body and its watershed. Generally, DOM in water bodies originates from bacterial lysis, leakage or exudation of the carbon fixed by phytoplankton (e.g., mucilaginous exopolymers from diatoms), sudden cell senescence, sloppy feeding by zooplankton, the excretion of waste products by aquatic animals, or the breakdown or dissolution of organic particles from terrestrial plants and soils (Van den Meersche *et al.*, 2004).

DOM is not directly available to most aquatic organisms but it is available to bacteria, and to a lesser extent, some osmotrophic protists or cyanobactera. Bacteria utilize this organic matter and incorporate it into their biomass. In this way, organic matter can be channeled from bacteria to their most important grazers – heterotrophic nanoflagellates (e.g., Pernthaler, 2005) and small ciliates (Šimek *et al.*, 2000). Both protozoan grazing and viral infections are the major processes balancing large bacterial growth potential (for reviews see Weinbauer, 2004; Pernthaler, 2005). Viral infection causes bacterial lysis, which releases cell contents back into the dissolved (DOM) or cell fragments to particulate organic matter (POM) pools, lowering the overall efficiency of the microbial loop (Murray and Eldridge, 1994; Wommack and Colwell, 2000). Ciliates feed on flagellates, small algae and also on bacteria and picocyanobacteria (Šimek *et al.*, 1995) thus the biomass generated within microbial food webs moves into the classical food chain (Figure 1.)

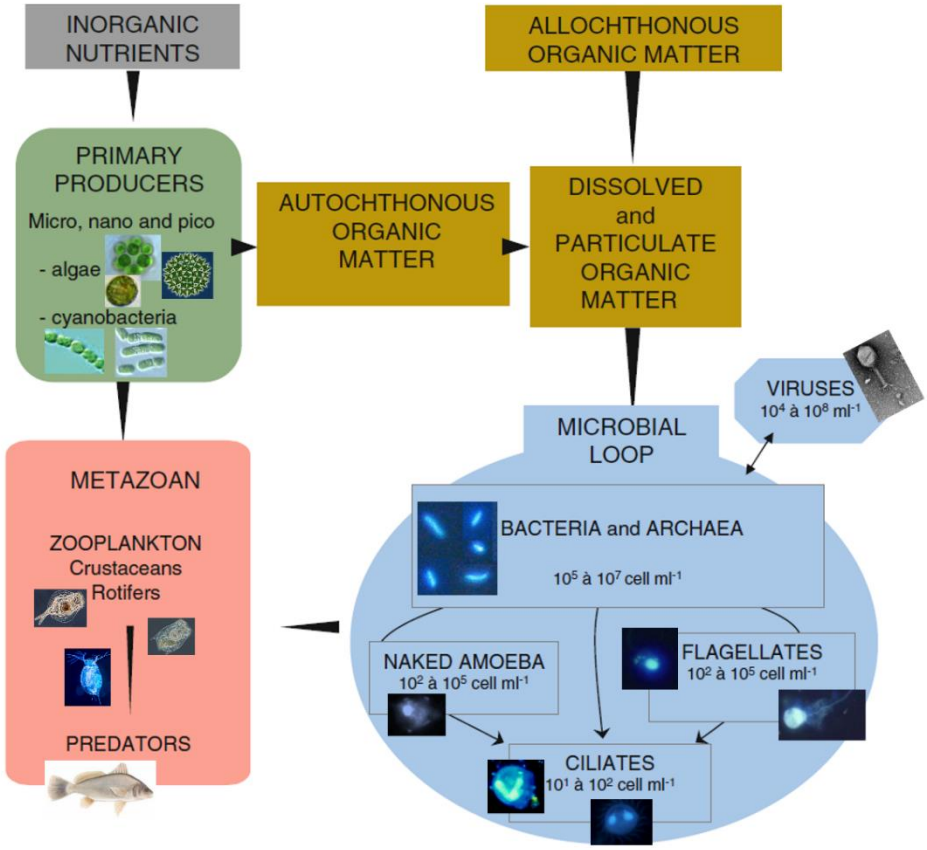

Figure 1.

A schematic flowchart of microbial loop concept and its coupling with the classical food chain. A broad range in the abundance of different planktonic microbial communities reflect different trophic levels of lakes. (Modified from Mostajir *et al.* "Microbial food webs in aquatic and terrestrial ecosystems.")

b) Biological components of microbial food webs

Abundance and diversity of microorganisms, especially in freshwaters, was for long time neglected and understudied. Only with the development of techniques that allowed easier identification and counting, like epifluorescence microscopy and molecular techniques, did it become evident how extraordinarily diverse and abundant microorganisms are. Advances in molecular biology techniques revolutionized the field of aquatic microbial ecology, discovering first immense diversity of prokaryotes followed by microbial eukaryotes. Oceans and marine habitats still remain better studied than lakes and freshwaters.

The best adaptation for life in pelagic marine and lake ecosystems is a planktonic lifestyle, or more specifically, floating. Generally, pelagic marine and lake ecosystems are the only ecosystems where the majority of biomass belongs to microorganisms. Prokaryotic and eukaryotic planktonic microorganisms may be heterotrophic, mixotrophic or autotrophic.

A practical way to study microorganisms is to divide them into size classes which are also linked to the functional role of these microorganisms in pelagic environments. Microorganisms can be classified to 5 groups according to Sieburth *et al.*, (1978) femtoplankton (0.02 – 0.2 μm), picoplankton (0.2 – 2 μm), nanoplankton (2 – 20 μm), microplankton (20 – 200 μm) and mesoplankton (0.2 – 20 mm). While these size characterizations are a simple and easy way to study planktonic organisms, it brings quite limited insights into their specific ecological roles, diversity and dynamics of these microrganisams. Consequently, the major focus of this thesis was primarily on picoplankton and nanoplankton and the relationship between these two components as the keystone trophic link within microbial food webs.

1.2 Key taxonomic groups in freshwater bacterioplankton

There is rapidly growing knowledge about key bacterial groups in freshwater bacterioplankton (Newton *et al.*, 2011). Heterotrophic bacterioplankton assemblages found in a broad variety of freshwater ecosystems are frequently dominated by representatives of a few phylogenetic clusters of *Betaproteobacteria, Actinobacteria* and *Bacteroidetes* (e.g., Lindström *et al.* 2005; Newton *et al.* 2011), which play important but quantitatively distinct roles in carbon dynamics. Among

Betaproteobacteria, two groups of bacteria differ in many aspects of their lifestyles (Jezbera *et al.*, 2012) and they are globally distributed and abundant in a wide array of freshwater habitats. Those groups are: the genus *Limnohabitans* (mostly affiliated with the R-BT065 cluster, Kasalický *et al.*, 2013) and the species-like *Polynucleobacter* C-subcluster (Hahn *et al.*, 2009). As outlined below, these bacterial groups have all the prerequisites to become invaluable models for testing specific questions related to organic carbon dynamics and its transfer to higher trophic levels in various pelagic ecosystems. Class of *Betaproteobacteria* belongs to phylum *Proteobacteria* which is composed of six classes: *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Gammaproteobacteria* and *Zetaproteobacteria*. All the bacteria belonging to this phylum are gram-negative.

The class *Betaproteobacteria* is broadly recognized for its morphological and physiological diversity. *Betaproteobacteria* are often the numerically dominant group in freshwater lakes (Buck *et al.* 2009; Glockner *et al.* 2000; Zwisler *et al.* 2003).

The genus *Limnohabitans* represents a group of environmentally important freshwater bacteria and it is currently composed of four described species (Hahn *et al.*, 2010a; Hahn *et al.*, 2010b; Kasalický *et al.*, 2010) and at least 55 distinct strains available for further investigations and detailed taxonomical affiliation (Kasalický *et al.* 2013). The genus is subdivided into five major lineages: four lineages representing so-called R-BT bacteria (i.e., those targeted with the R-BT065 probe, (Šimek *et al.*, 2001)) and one lineage (LimA) of non R-BT bacteria (i.e. those that are not hybridized with the probe, Figure 2) (Šimek *et al.*, 2013). The bacteria from this genus are known to inhabit a broad range of freshwater habitats worldwide and they can account for 5-30% of total bacterioplankton (Zwart *et al.*, 2002; Page *et al.*, 2004; Šimek *et al.*, 2010; Hahn *et al.*, 2012). They strongly prefer circum-neutral or alkaline lakes (Šimek *et al.*, 2010), except for the LimA lineage that inhabits mainly acidic, humic substances-rich habitats (Shabarova *et al.*, 2017). Notably, however, they regularly contribute unproportionally to total bacterioplankton biomass, as they generally posses larger mean cell volumes (MCV, around 0.05-0.16 μm3) than typical bacterioplankton cells (Šimek *et al.* 2006; Kasalický *et al.* 2013). In lakes they occur in neuston, epilimnion and hypolimnion, or attached to

zooplankton (Eckert and Pernthaler, 2014) which indicate their ability to live in both oxic and anoxic environments, occupying a broad bariety of distinct niches.

The R-BT lineage is known for being represented by phylotypes with opportunistic life strategies (Salcher *et al.*, 2007). These bacteria display the highest growth rates among major bakterioplankton lineages and their growth is even comparable to the growth of small HNF predators under in situ conditions (Šimek *et al.*, 2006). They also tend to accelerate their growth in experimentally manipulated treatments of "enhanced grazing pressure" (for details see Šimek *et al.*, 2006) but they are also selectively ingested by these flagellates (Jezbera *et al.*, 2006). These bacteria display high metabolic flexibility in incorporating simple organic substrates (Šimek *et al.*, 2011) and it seems that the main substrates boosting their growth are autochthonously produced algal-derived substances (Pérez and Sommaruga, 2006; Šimek *et al.*, 2008, 2011). Because of these specific ecophysiological traits, it is assumed that bacteria from this genus have a prominent role in carbon transfer to higher trophical levels mainly in pelagic environments.

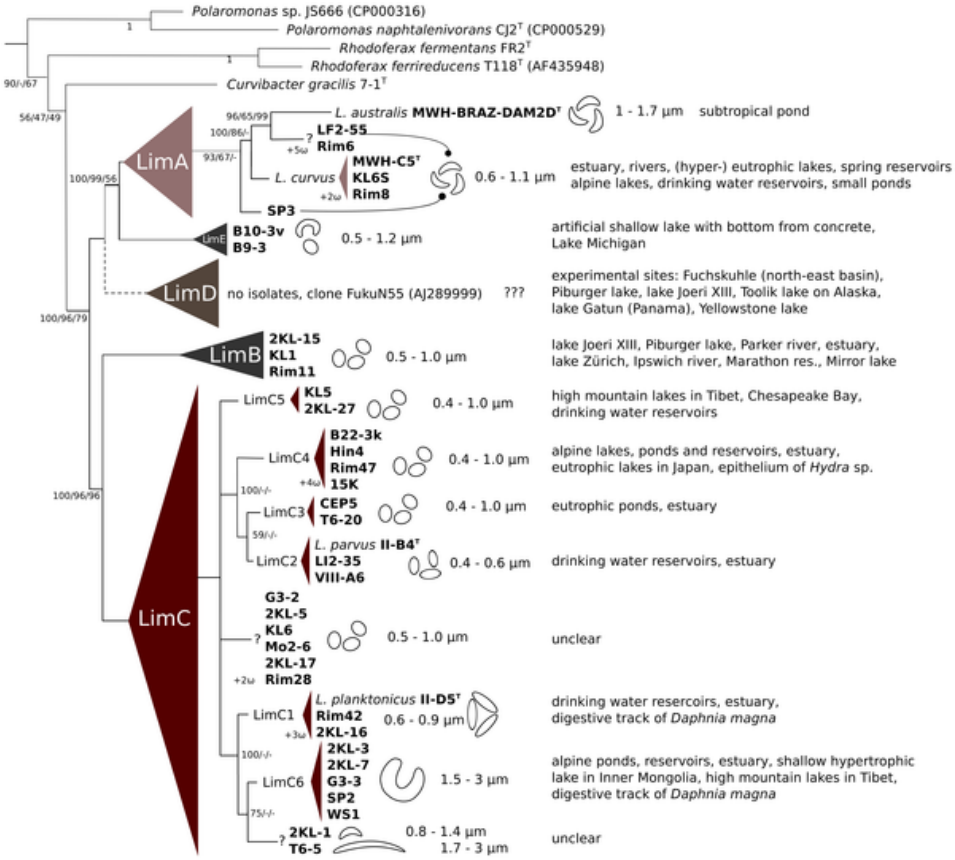

Figure 2. Microdiversity of *Limnohabitans* genus based on 40 isolated strains (taken from Kasalický *et al.* 2013)

The genus *Polynucleobacter* is the second important bacteria of the pelagic member of the *Betaproteobacteria* class. This large and diverse group of bacteria (composed of endosynbiotic and free-living bacteria, for details see e.g., Hahn *et al.*, 2012) was divided, based on phylogenetic criteria (Hahn, 2003; Wu and Hahn, 2006), into four subclusters (PnecA, PnecB, PnecC and PnecD) that are ecologically classified as oligotrophic ultramicrobactera (Salcher, 2014). In contrast to *Limnohabitans*, members of the genus *Polynucleobacter* inhabit a broad range of habitats from acidic, circum-neutral to alkaline freshwaters (Hahn, *et al.*, 2016a; Hahn, *et al.*, 2016b; Hahn *et al.*, 2017) which differ strongly in chemical, climatic and other ecological conditions (Jezberová *et al.*, 2010; Ghai *et al.*, 2011; Hahn *et al.*, 2015). Description of the whole *Polynucleobacter* genus was

specifically amended by adding revised descriptions of highly abundant free-living members of this diverse lineage (Hahn *et al.*, 2009).

The PnecC subcluster, whereof isolates were used in this research, is recognized as a cryptic species complex, i.e., their diversity cannot be resolved by 16S rRNA phylogeny (Hahn *et al.*, 2016a; Hahn *et al.*, 2016b; Hahn *et al.*, 2017). Notably, the *Polynucleobacter* bacteria of this subcluster are believed to utilized photodegradation products of humic substances and their population size therefore tends to decrease along a gradient of decreasing concentration of humic substances, ranging from 70% to almost 0% of total bacterial counts (Hahn, *et al.*, 2005; Jezberová *et al.*, 2010). These bacteria are numerically a quite abundant group but surprisingly they possess a highly passive lifestyle (for details see Hahn *et al.*, 2012). Isolated strains of these bacteria are of small to medium cell sizes and of moderate growth potential (Hahn *et al.*, 2005). The rather moderate grazing of flagellates upon PnecC bacteria is most likely regulated by their small cell size (Boenigk *et al.*, 2004; Tarao *et al.*, 2009). In contrast to *Limnohabitans* bacteria, members of the genus *Polynuleobacter* have moderate growth potential (Hahn *et al.*, 2012).

While both *Limnohabitans* and *Polynucleobacter* C-subcluster numerically represent highly successful bacterioplankton segments in particular environments (Šimek, *et al.*, 2010; Hahn *et al.*, 2012), there are also fundamental differences between them in their ecophysiological and genomic traits (as outlined above - e.g. growth potential, grazing induced mortality, substrate versatility, etc., Hahn *et al.*, 2012; Zeng, 2012).

1.3 "Black box" of heterotrophic nanoflagellates

Heterotrophic nanoflagellates (HNFs) are probably one of the most abundant eukaryotes on Earth, inhabiting freshwaters, oceans, sediments and soils (Arndt *et al.*, 2000; Pernice *et al.*, 2014; Massana *et al.*, 2015; Simon *et al.*, 2016). HNFs are unicellular, colorless eukaryotes with flagella and can vary in size from ~2-20 μm, although the majority of true bacterivorous forms are smaller than 5 μm. They are widely recognized as major bacterial grazers (Andersen and Sørensen, 1986; Fenchel, 1982b; Nakamura *et al.*, 1995; Langenheder and Jürgens, 2001), except for hypertrophic systems (Šimek *et al.*, 2000; Nakano *et al.*, 2001), and thus they serve as important nutrient remineralizers (Azam *et al.*, 1983).

Notably, as it has been mentioned in the first chapter, they also represent the most important link between DOM, bacterial carbon and higher trophic levels (Jürgens and Matz, 2002). Although their importance has been recognized in many aquatic systems, as yet we are considerably lacking in knowledge about their diversity and dynamics in natural systems. The reason is the majority of HNF are smaller than 5 μm and thus their limited morphological and behavioral characteristics, which could allow for microscopy-based differentiation of individual taxa, are largely missing. There are also some methodological problems that can bias determination of abundance, biovolume and community structure of HNF. Many flagellates could be disrupted during fixation and significant shrinkage can make biovolume estimates difficult or biased (Boenigk and Arndt, 2002).

The rise of high throughput sequencing (HTS) techniques enabled an easier taxonomic classification of these small eukaryotes (Simon *et al.*, 2015; del Campo *et al.*, 2015; de Vargas *et al.*, 2015; Debroas *et al.*, 2017), without time-consuming microscopy with the limited taxonomic resolution (Bradley *et al.*, 2016). Several studies reporting the abundance and diversity of microbial eukaryotes in lakes show that the most abundant taxa belong to *Alveolata, Stramenopiles, Cryptophyta* and fungi (Šlapeta *et al.*, 2005; Zhao *et al.*, 2011; Mangot *et al.*, 2013; Simon *et al.*, 2014). Few studies pointed to the importance of flagellates related to *Spumella* spp., which have responded most rapidly to sudden bacterial prey amendments (Šimek *et al.*, 2013) (see also Boenigk *et al.*, 2005, 2006; Grossmann *et al.*, 2016).

Despite their importance and abundance they are still mostly treated as a „black-box“ even in many current microbial ecology studies. In other words, we are looking at HNFs as functional guild, we know that they are important bacterivores and thus transfering bacterial carbon higher into food web, but which particular taxa are the main bacterivores in this process still remaines quite poorly understood and in urgent need of further studies.

1.4. Bacterivory by heterotrophic nanoflagellates and its impact on bacteria

The predator-prey relationship of protists and bacteria has coevolved billions year (Hahn and Höfle, 1999; Posch *et al.*, 1999) and is one of the oldest trophic interactions we can study in natural habitats. This long time span gave opportunity for both bacteria and protists to develop numerous adaptations and strategies (Hahn and Höfle, 1999, 2001). Bacteria develop defense strategies and protists learn how to overcome these strategies (Posch *et al.*, 1999). Studying protist-predator and bacteria-prey interactions gave rise to numerous studies which report on flagellate feeding behavior and prey selectivity (Šimek and Chrzanowski, 1992; Posch *et al.*, 1999; Boenigk and Arndt, 2000; Hahn and Höfle, 2001; Pfandl *et al.*, 2004; Pernthaler, 2005; Montagnes *et al.*, 2008).

Various bacterial prey characteristics can influence the probability of being captured and ingested by HNF, e.g. cell biochemical composition, morphology and filamentation, cell aggregation, motility, cell surface characteristics, size etc. (Chrzanowski and Šimek, 1990; Pernthaler *et al.*, 1997; Pernthaler, 2005; Montagnes *et al.*, 2008). Bacterial size is considered to be a fundamental property influencing feeding of HNFs with 'medium-sized' prokaryotes being most likely to be ingested (Posch *et al.*, 1999; Hahn and Höfle, 2001; Pernthaler, 2005; Montagnes *et al.*, 2008; Weisse *et al.*, 2016). Medium-sized prokaryotes are suggested to be metabolically more active, with high growth rates that might override the effects of enhanced HNF grazing, than very small microbes less vulnerable to the protistan grazers. When we refer to medium cell size, we are talking about the optimal size ratio between bacteria as prey and protists as predators which falls into a range of 1-4 μm, for the majority of bacteria (Hahn and Höfle, 1999, 2001; Pernthaler, 2005). The maximum size of ingestible bacterial prey in bacterivorous flagellates is normally smaller than their cell size dimeter (Hahn and Höfle, 1999). Consequently, depending on their size, bacteria are either immensely sensitive or mostly grazing protected (Figure 3).

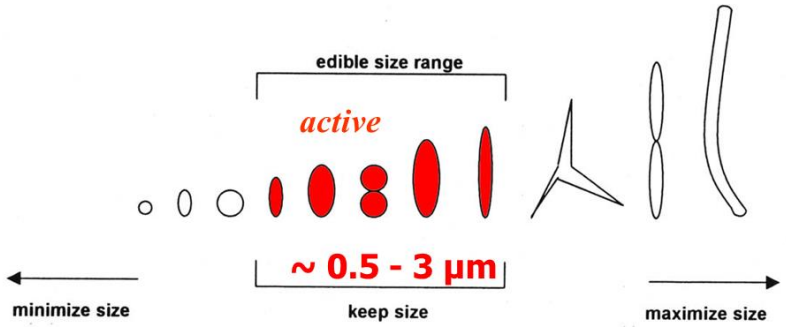

Figure 3. Size selective feeding of bacterivorus protists showing edible and grazing resistant forms of bacteria. Red stained cells are considered to be active (1 - 4 µm) and in edible size range. (Adapted from Posch *et al*., 1999)

To understand this selective behavior, it is important to recognize that even the smallest bacterivorus protists of sizes < 5 µm are highly advanced organisms with complex sensory abilities and behavioral adaptation to the predatory life style (Boenigk and Arndt, 2000; Boenigk *et al*., 2001; Boenigk and Arndt, 2002). The capture, handling and ingestion of particles by interception - feeding flagellates is a flexible multistep procedure (Jürgens and Matz, 2002). On one hand, the feeding process allows for better evaluation of food quality by protists and, on the other hand, diverse bacterial prey populations possess various anti-grazer adaptations to avoid or reduce predation (Matz *et al.*, 2002; Jürgens and Matz, 2002).

Consequently, bacteria differ in their quality as a prey, i.e. the grazing of single protistan species on various bacterial strains may yield a wide range of different growth parameters. In addition to different growth responses, different bacterial food can also distinctly modulate community composition of HNFs (Šimek *et al.*, 2013). The new insights and enhanced taxonomic resolution of highly complex bacterial prey – flagellate predator relationships undoubtedly belong to one of the most challenging topics of current aquatic microbiology. One main question, however, remains largely unanswered concerning predator-prey specificity, i.e., which bacterial groups are rapidly decimated by small HNFs and which particular HNF taxa are the main bacterivores in freshwater plankton?

1.5. Study sites

a) Řimov reservoir

Řimov reservoir is situated in the Czech Republic (South Bohemian region) and is a canyon–shaped reservoir. The parameters of the reservoir morphometry are: 2.06 km2, volume 34.5 x 106 m3, length 13.5 km, maximum depth 43 m, mean depth 16.5 m, and mean retention time 100 d. Řimov is dimictic and meso–eutrophic and is the drinking water supply for approximately half a million people in South Bohamia, including the České Budějovice region. There is a strong longitudinal gradient in nutrients, dissolved and particulate organic carbon and microbial communities between the river inflow and the dam area (Mašín *et al.*, 2003; Jezbera *et al.*, 2003).

b) Sandpit Cep

Lake Cep was created as a consequence of past sand mining in the area of the Třeboň basin, an extensive area of ponds in the South Bohemia region, which has been influenced and remodelled by people in the long run. It is a nutrient poor and oligotrophic water body.

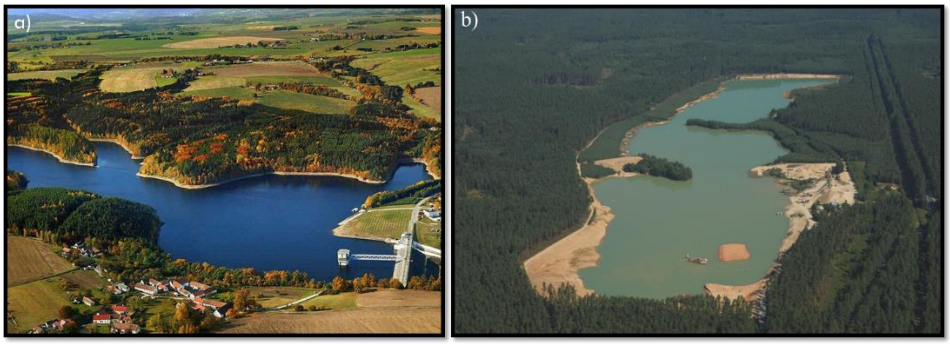

Figure 4. Photographs of a) Řimov reservoir and b) Sandpit Cep

1.6 Size fractionation experiments

In the course of this PhD work we conducted 9 total experiments during different phases of plankton succession. We tested the growth response of natural heterotrophic nanoflagellate assemblages originating from two different study sites (Římov reservoir and Lake Cep) to 12 different bacterial isolates belonging to major bacterioplankton groups (*Actionbacteria* and *Betaproteobacteria*). All of the conducted experiments were built on a very similar experimental design detailed in Figure 5.

Prior to the experiments bacterial strains were pregrown in nutrient-rich liquid medium (Hahn *et al.*, 2004). Twenty-four hours before starting the experiment, bacterial strains were concentrated by centrifugation and the pellets were resuspended in 0.2 μm filtered and sterilized water from the appropriate study site (Šimek *et al.*, 2013). Samples from the study site were collected 12-24 h prior to starting the experiment and the filtration (through a 5 μm filter) was preformed in the lab. The filtration released small HNFs from grazing by bigger zooplankton such as ciliates, rotifers and cladocerans. This filtration step simplifies the trophic interactions and structure to indigenous bacteria and their grazers – HNFs. The filtered sample was left overnight in the dark to let the organisms recover from handling shock and to slightly increase the number of HNFs (released from zooplankton grazer control) and decrease the number of bacteria (getting grazed by HNF) that were naturally present in the sample. After approximately 12 hours of incubation we added each bacterial strain separately into triplicate test flasks. Because the prey bacteria differ in their mean cell volumes, the additions of morphologically distinct strains were set to yield approximately the same initial volume of biomass for all the tested strains. Furthermore, we sampled the treatments in 12-24 h intervals. At each time point we took samples for total bacteria and HNF counts, and at selected time points samples for CARD-FISH and DNA extraction. CARD-FISH (catalyzed reporter depositionfluorescence in situ hybridization) is a method used for phylogenetic staining of microorganisms. DNA from selected experiments was later isolated and we performed PCRs with primers specific for the V9 region of 18S rRNA. After approximately 60 – 90 hours, the experiment was terminated because the flagellate numbers started to drop down after the exponential growth phase.

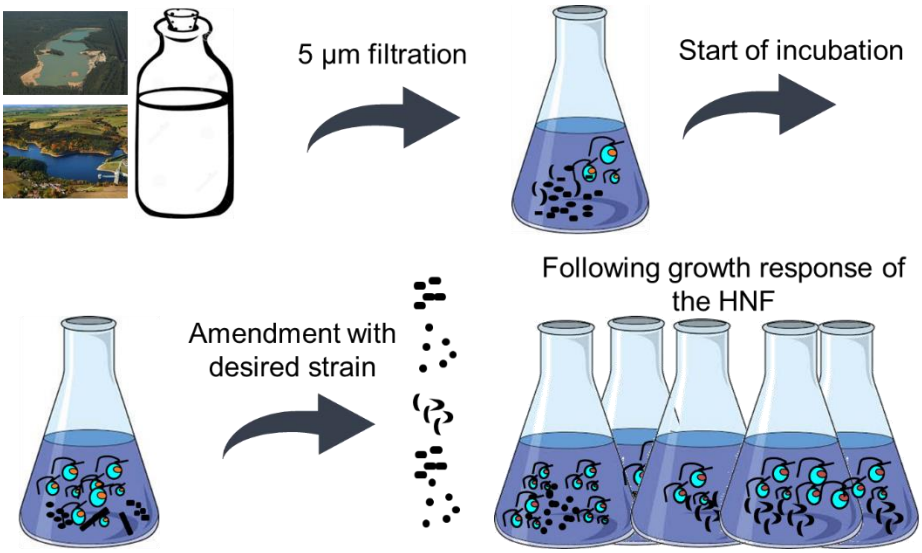

Figure 5. Shematic representation of size fraction experimental design

2. Hypotheses and Objectives

1) While *Limnohabitans* and *Polynucleobacter* C-subcluster numerically represent highly successful bacterioplankton lineages in specific freshwater environments (Šimek *et al.*, 2010; Jezbera *et al.*, 2012), there are fundamental differences in their growth potential and grazing induced mortality affecting the rate of carbon transfer from this prey to the trophic level of HNF predators.

In a series of size fractionation experiments we measured growth parameters (specific growth rate, gross growth efficiency, and length of lag phase prior reaching exponential growth phase) related to a particular prey item utilized by natural HNF communities. Those measurements can be used as a measure of the process rate of the carbon flow from a particular bacterial group to the predator and the nutritive quality of a particular bacterial prey type for HNF.

2) Different bacterial species are inducing different growth responses of natural HNF assamblages but the same prey type can induce significantly different growth responses depending on trophic status of the site and the seasonal plankton succession phases modulating differently the prey and predator communities

We performed size fractionation experiments with the same prey type in different seasons of plankton succession and from two contrasting habitats (Řimov and Cep) and followed differences in growth parameters of HNF communities.

3) While different food quality of prey bacteria induces significant differences in growth parameter of HNF grazers, shifts in size and morphology of dominant HNF groups are reflecting a shift in the HNF community composition.

Volumes of HNF at different time points of experiments are measured and changes in the sizes detected. Shifts in the community of HNFs from the time zero to the exponential phase of experiments are revealed with the use of amplicon sequencing of 18S rRNA genes and by CARD-FISH method allowing detetion of taxonomic affiliation of single microbial cells. The presence of all the prey bacteria in flagellates will be detected by means of CARD FISH with bacterial probes targeting particular prey items directly in the predator food vacuoles.

3. Results and Discussion

3.1 The most important outcomes of the thesis

The crucial contribution of the thesis to the current field of microbial ecology is the discovery that colorless Cryptophyta were major bacterivores not only in the experimental treatments but also in untreated reservoir water (**Paper III**). Further, we showed how different bacterial prey indeed induces changes in the community composition of flagellates (**Paper III)** but also in their growth parameters which are strongly season dependant (**Paper I and II**). Across all experiments conducted we observed a clear trend indicating that the nutritive quality and size appropriatness of bacterial prey were the driving forces of the HNF growth response and its dynamics in time. Thus for instance, HNFs growth dynamics showed that less suitable prey induced longer lag phases and lower gross growth efficiencies of natural HNF communities (**Paper II**). Furthermore, one of the important outcomes of this thesis was development of double hybridization technique, which facilities taxonomic affiliation of both predator and prey in the same microscopic preparation (**paper III**). Notably, these significant results initiated new cooperations with University of Duisburg-Essen (Julia Nuy) and University of Zürich (Michaela M Salcher).

3.2 Environmental relevance of *Betaproteobacterial* strains

Eleven different bacterial strains were used in prey-amendment experiments (**papers I, II, III**) and they all belong to important players in the indigenous bacterioplankton at both study sites The strains represent environmentally important linages of *Betaproteobacteria*, genera *Limonhabitans* and *Polynucleobacter*. The environmental relevance of those bacteria was tested with CARD-FISH using specific probes for both genera ((R-BT065 for *Limnohabitans* (Kasalický *et al.*, 2013) and PnecC-16S-445 for *Polynucleobacter* (Hahn *et al.*, 2005)). In the original samples used for experiments we found the following relative proportions of Limnohabitans bacteria in Římov reservoir (as % of total bacteria; mean and range of values): (1) the spring bloom phase in April (14.1%; 8.4–17.5%); (2) clear water phase in May (9.6%, 7.2–11.8%); (3) summer phytoplankton bloom in August (6.1%, 3.5–9.4%); and October period (5.9, 3.5–9.4%).

In experiments conducted in April and May, solely with *Limnohabitans* isolates in Lake Cep, the R-BT cluster accounted for 10.2% and 8.3% of total pelagic bacteria in the lake (**Paper I**).

The strains from *Polynucleobacter* C linage were used in April and August 2014 when this linage accounted for 6.6% and 3% of total bacteria in the reservoir plankton (**papers II and III**).

All the above findings well justify selecting *Limnohabitans* and *Polynucleobacter* strains as suitable experimental models. Moreover, high frequency sampling (Šimek *et al.*, 2014) discovered that Betaproteobacteria, especially R-BT linage represent fast growing bacterial taxa with doubling times *in situ* from 6-20 hours, comparable to those of small flagellates (see also the conceptual model in **Paper II)**.

3.3 Effects of a prey food quality on flagellate growth and community composition

Our results documented a large variability in HNF growth responses, with many season specific aspects which are related to temporally different community composition of bacterivorous HNFs (Domaizon *et al.*, 2003; Nolte *et al.*, 2010) (**paper II**). However, the responses of natural grazer communities to enrichment with a particular bacterial prey over different plankton succession phases have rarely been demonstrated before (**paper I, II**), which makes our study quite unique in the current field of microbial ecology.

a) Effects of prey food quality on flagellate growth

In experiments during spring bloom (April) and clear water phase (May), we fed natural HNF communities, originating from two contrasting habitats (Římov and Cep) with bigger bacterial strains from the genus *Limnohabitans*. Data from this experiments showed that higher gross growth efficiencies and faster growth rates were consistently detected in April compared to May, at both study sites. This is due to trophic structure during the algal bloom phase where larger bacteria and flagellates dominated at both study sites (**paper I**). For example, for hydrodynamic reasons, bigger bacteria have higher contact rates with flagellate cells in plankton (Boenigk and Arndt, 2000; Fenchel, 1982b). This could result in a community dominated by larger flagellate predators more suited and "preconditioned" for efficient grazing and utilization of larger bacteria.

Conversly, the lowest gross growth efficiency was consistanlty determined in Lake Cep, where low HNF abundance and the smallest flagellate cells were detected in the samples from time t_0 of the experiment (**paper I**). Generally, trends reflecting temporally distinct trophic structures were more apparent in meso-eutrophic Řimov than in oligotrophic Cep. The lake had lower bacterial and HNF abundance, lower HNF cell volumes, and increased water transparency, which indirectly indicated impact by filter–feeding zooplankton in May than in April (Šimek *et al.*, 2014; Sommer *et al.*, 2012)

In contrast to seasonal and site specific differences we did not find significant differences in HNF growth responses to prey amandments with strains from LimB, LimC, and PnecC prey categories. In some experiments we also compared growth responses of HNFs to bacteria from Luna 2 cluster of *Actinobacteria* (**Paper II)**. We decided to test *Actinobacteria* since already some previous research indicated that they are grazing resistant because of their small size and cell wall properties typical for gram positive bacteria (Pernthaler *et al.*, 2001; Tarao *et al.*, 2009). Beside the experiment with *Actinobacteria* we also included one experiment where we fed the axenic culture of *Poterioochromonas* with *Limnohabitans* bacteria from LimC linage and two strains of *Actinobacteria*. When we compared growth parameters (lag phase, growth rate and gross growth efficiency) of HNF growing on LimB, LimC, PncC and Luna2 clusters, we always found significantly lower growth rate and gross growth efficiency and significantly longer lag phase of flagellates growing on the Luna2 strain (**paper II**). This points out to less suitable characteristics of *Actinobacteria* prey to support positive population growth of HNFs. The results with axenic culture of *Poterioochromonas*, showed significantly higher gross growth efficiency values when growing on strains from LimC linage of *Limnohabitans* compared to natural mixed HNF communities growing on the same prey items (**paper II**). Thus the food quality of the LimC strains and their suitable cell sizes (falling in the middle sized bacteria) were likely primary reasons yielding the high gross growth efficiency values obtained with the predator culture.

b) Effects of prey food quality on flagellates compostion

It is important to mention that even closely related prey items can induce temporarily quite different patterns of prey-specific HNF

community shifts; a phenomenon that has rarely been documented so far. For example study perfomed in April (see Šimek *et al.*, 2013) and in August (**paper III**) where natural HNF communites were fed with closely related bacteria from the LimC lineage of *Limnohabitans* showed significant differences in the community composition of bacterivorous flagellates. Analysis of 18S rRNA amplicones, during the April experiment, showed a strong prey –specific HNF community shift to bacterivorous chrysophytes – i.e., *Pedospumella* and several *Spumella-like* related lineages. In contrast to this, the August experiment showed a different picture, according to CARD-FISH analysis (based on specific phylogenetic probes), the HNF community shifts were mediated mainly through changing proportions of bacterivorus linages of *Cryptophyta* (**paper III**). The shifts in both prey and predator communities are likely closely interconnected and occur within a time spane of aproximatelly half a day to a few days. The shifts of flagellates communities maybe be the result of rare taxa becoming dominant within changing environmental conditions (Caron and Countway, 2009; Nolte *et al.*, 2010). The rapid HNF community shift and flagellate selective feeding on fast growing or larger bacteria are changing our views on time scale at which substantial changes in carbon flow can occur in planktonic environments.

3.4 Cryptophyta – unexpected major bacterivores

One of our studies (**paper III**) documented a strong impact of prey characteristics on resulting HNF community dynamics, with remarkable shifts in the HNF community composition towards *Cryptophyta*. Furthermore, flagellates belonging to *Cryptophyta* were the most abundant bacterivores in summer plankton of Římov reservoir, which was confirmed by a tracer technique (based on additions of fluorescently labeled bacteria), which indicated that *Cryptophyta* were responsible for 70% of total HNF bacterivory and removed a total of 36.6 % of bacterial standing stock per day. This was surprising for two reasons: Firstly, amplicon sequencing results of 18S rRNA genes did not show so high abundance of Cryptophyta, since the primers we used were discriminating agains them. Secondly *Cryptophyta* have been considered mainly autotrophic or at least mixotrophic and not feeding so profoundly on bacteria like in our study. To check amplicon sequencing results we

decided to use CARD-FISH technique which revealed that the most abundant bacterivores in our experiments belonged to Chryptophyta and not to Katablepharydophyta, as the amplicon sequencing data would suggest. One clade of Cryptophyta, CRY1 was growing on all tested strains but with a profound increase on the bacterial strain Rim11 (LimC lineage of *Limnohabitans*). Cells of flagellates belonging to this clade were relatively small (~3 - 4 μm diameter), spherical and with a de-central nucleus. Cells targeted with general Cryptophyta probe had diverse morphologies and food vacuoles containing numerous bacterial preys. (**Figure 6 in paper III**).

On the other hand, Katablepharydophyta have not been observed with ingested bacteria in our experiments and their numbers increased significantly towards the end of experiment. Thus it seems quite likely that they fed on smaller bacterivorous HNF.

Furthermore, to confirm that specific groups of flagellates (Cryptophyta, CRY1, and Katablepharydophyta) ingest bacteria offered as a food in this experiment we developed a double hybridization technique. This method combines two CARD-FISH probes targeting protistan grazer as well as prey bacteria in the flagellate food vacuoles. This combination gives new insight into predator –prey interactions as it displays a unique snap-shot picture of *in situ* trophic interactions, by demonstrating directly which bacteria are preferentially consumed and which groups of flagellates are their grazers in aquatic ecosystems at a given time.

4. Conclusions and further perspectives

Our studies represent a set of unique approaches in the current field of microbial ecology since we tested not only growth response of natural HNF communities to prey bacteria from different lineages but we combined the data from different seasons of plankton succession and contrasting study sites. Moreover, the PhD thesis has brought new insight into food quality aspects of the prey bacteria and revealed both general and prey-specific trends in the growth responses of natural HNF communities. We demonstrated that even the same bacterial prey can produce significantly different HNF growth responses depending upon the season, which is likely related to marked compositional shifts in temporally evolving flagellate grazer communities. Aditionally, we detected that colorless Cryptophyta were the most abundant bacterivores not only in one of our experiments but also *in situ* in plankton of Římov reservoir. For the first time we could visualize this finding with a double hybridization method that we developed. This method allows simultaneous phylogenetic identification of both grazers and prey without additional sample manipulation. Last but not least, we demonstrated that shifts in both prey and predator communities are closely interconnected and occur within a time span of approximatelly half a day to few days.

This topic is as exciting for me as it was from the very beginning of my PhD. We have just started to finally open the „black-box“ of hetrotrophic nanoflagellates. I believe that my research and the new methods implemented considerably contributed in this line of research. Currently, there exists rising awarnes of importance of those smallest eukaryotes which is exemplified by new publications (Seeleuthner *et al.*, 2018; Carradec *et al.*, 2018) in highly prestigues journal. For understanding the role that HNFs have in planktonic envorionments it is necessary to combine epifluorescence microscopy with new molecular methods and new FISH probes designed to target particular flagellate phyla, as exemplified in our study. Furhtermore, isolation of the most important members of this taxonomically highly diverse group represents a challenging but necessary approach for further research in this field.

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6. Attahed publications

Paper 1.
Grujčić V., Kasalický V., Šimek K. (2015). Prey-specific growth responses of freshwater flagellate communities induced by morphologically distinct bacteria from the genus Limnohabitans. Appl Environ Microbiol 81:4993–5002. doi:10.1128/AEM.00396-15
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Prey-Specific Growth Responses of Freshwater Flagellate Communities Induced by Morphologically Distinct Bacteria from the Genus *Limnohabitans*

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Because their large growth potential is counterbalanced with grazing by heterotrophic nanoflagellates (HNF), bacteria of the genus *Limnohabitans*, which are common in many freshwater habitats, represent a valuable model for examining bacterial carbon flow to the grazer food chain. We conducted experiments with natural HNF communities taken from two distinct habitats, the meso-eutrophic Římov Reservoir and the oligo-mesotrophic Lake Cep (South Bohemia). HNF communities from each habitat at distinct seasonal phases, a late April algal bloom and a late May clear water phase, were each fed 3 *Limnohabitans* strains of differing cell sizes. Water samples were prefiltered (5 μm) to release natural HNF communities from zooplankton control and then amended with the *Limnohabitans* strains *L. planktonicus* II-D5 (medium sized, rod shaped), *Limnohabitans* sp. strain T6-5 (thin, long, curved rod), and *Limnohabitans* sp. strain 2KL-3 (large solenoid). Using temporal sampling and prey treatment, we determined HNF growth parameters such as doubling time, growth efficiency, and length of lag phase prior starting to exponential growth. All three *Limnohabitans* strains supported HNF growth but in significant prey-, site-, and season-dependent fashions. For instance, addition of the moderately large T6-5 strain yielded very rapid HNF growth with a short lag phase. In contrast, the curved morphology and larger cell size of strain 2KL-3 made this prey somewhat protected against grazing by smaller HNF, resulting in slower HNF growth and longer lag phases. These trends were particularly pronounced during the late May clear-water phase, which was dominated by smaller HNF cells. This may indicate a longer "adaptation time" for the flagellate communities toward the large prey size offered.

The importance of both bacteria and their protistan grazers in planktonic microbial food webs has been widely recognized (1, 2). Bacterioplankton transfers dissolved organic matter (DOM) to bacterial biomass that is consumed mainly by heterotrophic nanoflagellates (HNF) or ciliates and channeled via grazing into the metazoan grazer food chain (3–5). The amount, composition, and temporal dynamics of DOM differ widely among the diverse set of freshwater ecosystems, and this DOM distinctly modulates the growth and population dynamics of different bacterioplankton taxa (6). For instance, some bacterioplankton groups, such as *Betaproteobacteria* and *Flavobacteria*, can respond to sudden pulses in alga-derived organic carbon very rapidly, using doubling times (DTs) that vary from several hours to days (7–10).

Representatives from a few highly abundant phylogenetic clusters of *Betaproteobacteria* and *Actinobacteria* frequently dominate bacterioplankton populations in a wide variety of freshwater ecosystems (11–13). Among the *Betaproteobacteria*, *Limnohabitans* and *Polynucleobacter* have been documented as the most ecologically important genera (6, 14–18). The *Limnohabitans* genus is composed of five major lineages. Four are the so-called R-BT bacteria, which are identified via hybridization with the R-BT065 phylogenetic probe (19), and one lineage, known as LimA, does not hybridize with the probe (18). The four lineages of the R-BT cluster are represented by rapidly growing, morphologically diverse phylotypes with generally opportunistic life strategies (18, 20, 21). They also display a high metabolic flexibility in incorporating simple organic substrates (18). *Limnohabitans* bacteria from the R-BT cluster are omnipresent in a wide variety of pH-neutral or alkaline freshwater habitats (16). However, they have been also found as microflora in the digestive tract of algivorous *Daphnia magna* (22) or as epibionts in filter combs of *Daphia galeata* (23). Thus, it seems that the main substrates supporting their rapid growth *in situ* are alga-derived substances (19, 24, 25). This has also been suggested for several phylogenetically narrow lineages of *Flavobacteria* (6, 7, 9). Representatives of both the *Flavobacteria* and the *Limnohabitans* genera are dominated by larger cell sizes (9) yet are within the edible size range for HNF (26, 27). Notably, it has been shown that the high growth rates of *Limnohabitans* bacteria are counterbalanced by considerable loss rates via grazing because these bacteria are selectively grazed by flagellates (9, 28). It is because of these specific ecophysiological traits, high growth rate and high grazing pressure, that *Limnohabitans* bacteria likely have a prominent role in carbon transfer to higher trophic levels.

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TABLE 1 Morphological characteristics of the three *Limnohabitans* strains used as prey in flagellate prey enrichment experiments

Strain	Cell length, μm (range)	Cell vol, μm^3 (mean ± SE)	Cell shape	Reference
Limnohabitans planktonicus strain II-D5	1.1–1.3	0.228 ± 0.021	Rod	32
Limnohabitans sp. strain 2KL-3	2.3–3.4	0.548 ± 0.116	Large solenoid	18
Limnohabitans sp. strain T6-5	1.7–3.1	0.406 ± 0.055	Thin curved rod	18

In several lakes, remarkable changes in the relative contributions of the total R-BT bacteria or their specific genotypes to the total bacterioplankton community were found to occur during the spring phytoplankton bloom and the clear-water phase (6, 9). In addition to considerable shifts in bacterioplankton community composition, there were also changes in HNF numbers and their size class distribution. During spring bloom periods, modest top-down control of the HNF population usually allows for the development of abundant populations. Generally, large cell sizes of bacteria also prevail, with marked contributions from both *Limnohabitans* and *Betaproteobacteria* (9). This sharply contrasts to the clear-water phase, when there is strong top-down control of the plankton trophic structure by larger filter-feeding zooplankton (29). Here, HNF populations are heavily decimated and dominated by small cells, and the absolute and relative contributions of the large-cell *Betaproteobacteria* decrease, accompanied by a general shift toward smaller cells within the entire bacterioplankton (4, 9).

Notably, natural communities of bacterivorous HNF have large growth potential (20, 30), and flagellate predator-bacterium prey relationships are so flexible that sudden changes in the prey community may induce very rapid changes in the flagellate predator community (31). Feedback control of bacterial prey food quality on predator community composition and growth is potentially important but largely unstudied. This is mainly due to methodical problems and a lack of representative isolates from relevant bacterioplankton genera. Previous experiments (18) have documented rapid growth of HNF on four medium-sized but morphologically different *Limnohabitans* strains. Strains with distinct morphologies yielded differences in HNF growth and community parameters, which could not be related just to size differences among the bacterial prey (18).

In this study, we report results from experiments in which three strains of *Limnohabitans* with medium to large cell volumes were introduced into natural HNF communities collected in parallel from a meso-eutrophic freshwater reservoir and an oligo-mesotrophic sand-mining pit lake in two distinct seasonal phases (spring bloom phase versus clear-water phase). We hypothesized that different environmental scenarios and sudden pulses in availability of bacterial prey of high food quality may yield different effects on the growth parameters of bacterivorous HNF and thus also alter the rate of carbon flow into the grazer food chain. In four experiments we exploited predator-prey manipulation to address the following aims: (i) examination of the community growth parameters, such as doubling time, lag phase duration, and flagellate gross growth efficiency (GE), within freshwater HNF assemblages feeding on *Limnohabitans* strains of different cell size and morphology and (ii) examination of how the different trophic status and contrasting seasonal phases of the two study sites might modulate the growth responses of their indigenous HNF populations.

MATERIALS AND METHODS

Experimental organisms and sampling sites. Three *Limnohabitans* strains (*L. planktonicus* II-D5 [32] and two undescribed *Limnohabitans* sp. strains, 2KL-3 and T6-5 [18]), of markedly different size and shape, were used in four prey enrichment experiments with the same experimental design (details are given in Table 1 and Fig. 1).

Water samples with natural HNF communities were collected from two distinct freshwater habitats in South Bohemia (Czech Republic): Římov Reservoir (48°50′46.90″N, 14°29′15.50″E), a meso-eutrophic drinking water reservoir of circumneutral pH (for more details, see reference 23 and references therein), and Lake Cep, a sandpit lake (48°92′49.24″N, 14°88′68.11″E) created as a consequence of past sand mining in this area. The lake is generally poor in nutrients (Table 2) and is considered oligo-mesotrophic.

Design of experiments. Two experiments were conducted during two different phases of plankton succession at both study sites in parallel. The first experiment was run from 23 to 28 April 2012 (water temperatures, 16.6°C [Římov] and 17°C [Cep]), during the spring phytoplankton bloom period. The second experiment was run from 28 May to 2 June 2012 (Římov, 19°C; Cep, 21°C) and coincided with the late clear-water phase of plankton succession in both habitats. The temperature of the water at both study sites was measured on the day of water sampling. All treatments were incubated at 18°C, which closely resembled the *in situ* temperatures.

Plankton samples (10 liters) were taken from each habitat (0.5-m depth) at 24 h before the experiments started. Water (6 liters) from each habitat was gravity filtered through 5-μm-pore size filters (147-mm diameter) to release the HNF from zooplankton grazing pressure. Note that most bacterivorous HNF cells are smaller than 5 μm, and thus the filtrate

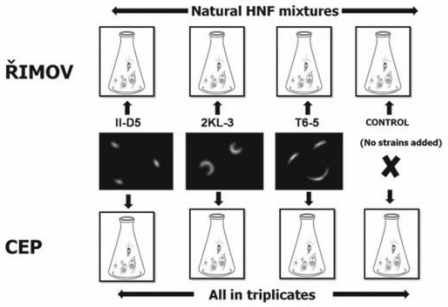

FIG 1 Schematic showing the experimental design employed using HNF populations collected in late April and late May. Plankton samples filtered through 5-μm filters were preincubated in the dark for 24 h. Subsamples were then placed in triplicate experimental vessels and immediately amended with the respective *Limnohabitans* strains (*L. planktonicus* II-D5 and *Limnohabitans* sp. strains 2KL-3 and T6-5). No bacteria were added to control treatments. The photomicrographs and cell dimensions shown in Table 1 show the typical size and morphology of the tested prey bacteria.

TABLE 2 Main chemical and microbial parameters in samples collected at t_0, showing differences between sites

Parameter[a]	Value at indicated site			
	Římov		Cep	
	April	May	April	May
TP (μg liter^{-1})	26.9	21.7	8.6	10
DRP (μg liter^{-1})	11.6	7.6	5.2	5.7
DOC (mg liter^{-1})	4.88	3.78	3.92	3.96
Chl-*a* (μg liter^{-1})	11.6	3.5	3.9	3.1
Transparency (m)	1.6	5.9	4.6	6.3
Daphnia abundance (individuals liter^{-1})	4.2	30	ND[b]	ND
HNF no. (10^3 ml^{-1})	3.529	0.47	0.872	0.371
HNF MCV (μm^3)	26.7	16.5	16.5	8.9
Bacterial no. (10^6 ml^{-1})	2.79	2.11	3.164	1.531
Bacterial MCV (μm^3)	0.0945	0.0405	0.0496	0.0415
% of R-BT065-positive bacteria (mean ± range)	14.5 ± 1.1	9.9 ± 0.6	10.8 ± 2.1	8.3 ± 0.2
% of *Betaproteobacteria* (mean ± range)	19.6 ± 0.8	16.2 ± 0.3	16.0 ± 1.1	15.3 ± 1.0

[a] TP, total phosphorus; DRP, dissolved reactive phosphorus; DOC, total dissolved organic carbon; Chl-*a*, chlorophyll *a*; MCV, mean cell volume. Percentages of FISH-positive bacteria represent mean ± range of duplicate counts.
[b] ND, not determined.

contained a representative site-specific community of HNF. The 5-μm filtrates containing the HNF communities were incubated at 18°C for 24 h. The incubation yielded approximately 2-fold increases in HNF abundance and slight decreases in the number of free-living bacteria with most remaining bacteria (~ 1×10^6 ml^{-1}) in the form of flocks or filaments inedible for these HNF populations.

All three *Limnohabitans* strains were pregrown in nutrient-rich liquid medium (3 g liter^{-1} NSY) (33). Twenty-four hours before starting the experiment, these *Limnohabitans* strains were concentrated by centrifugation (20 min, 5,000 × *g*), and the pellets were resuspended in 0.2-μm-filtered and then sterilized water from both habitats (31). This procedure did not appear to affect bacterial viability, as some of the strains grew after inoculation into experimental treatments with natural water from either habitat (Fig. 2 and 3).

As necessary, sterile, bacterium-free water from each habitat (0.2-μm filtered) was used to dilute the 5-μm fractions with growing HNF communities to obtain solutions with the same initial concentration of HNF (2×10^3 ml^{-1}). The 5-μm filtrates with natural HNF communities (300-ml aliquots) were placed in sterile 500-ml Erlenmeyer flasks. HNF populations were then amended with solutions of prey bacteria. Because the prey bacteria differed markedly in cell volume (Table 1), the additions of the morphologically distinct *Limnohabitans* strains were set to yield approximately the same initial volume of biomass for all three strains (31). Experiments were run in triplicate, and treatments were kept at 18°C in the dark. The treatments containing only natural bacteria and HNF present in the original samples from both sites served as controls compared to the prey-enriched treatments (referred to as II-D5, 2KL-3, and T6-5 throughout). Subsamples for detecting HNF and bacterial abundance and biomass were aseptically taken within a laminar flow hood at 12- to 24-h intervals. At selected time points (t_0, t_{24}, t_{48}, and t_{66}), additional samples for fluorescence *in situ* hybridization (FISH) were collected. The detailed outline of the experimental setup is depicted in Fig. 1.

Bacterial and heterotrophic flagellate enumeration and biovolumes. Samples fixed with formaldehyde (2% final concentration) were used for enumeration of bacteria (0.5- to 2-ml subsamples) and HNF (4- to 10-ml subsamples) on 0.2-μm- and 1-μm-pore-size filters (Osmonics, MN, USA), respectively. All samples were stained with DAPI (4′,6-diamidino-2-phenylindole), and the microbes were counted via fluorescence microscopy (34). Bacterial biovolume was measured by using a semiautomatic image analysis system (NIS-Elements 3.0; Laboratory Imaging, Prague, Czech Republic). To calculate mean cell volumes (MCVs) of HNF (approximated to prolate spheroids [31]), lengths and widths of 50 cells in each triplicate treatment were measured manually on-screen with a built-in tool of the PC-based image analysis system (NIS-Elements 3.0).

The net HNF growth rate was calculated based upon the equation for the exponential growth, $\mu = (\ln N_t - \ln N_0)/(t - t_0)$, where μ is the specific growth rate (day^{-1}), N_t and N_0 are HNF abundances (cells ml^{-1}) at time t (days) and time zero, respectively, and ln is natural logarithm. The three consecutive time points on the HNF growth curve yielding the largest r^2 were selected for calculation. The parameter μ allowed for calculating HNF doubling time (20) used as a growth metric throughout the text. The lag phase was calculated as the period from the time zero to the intercept between the best fit line of HNF growth and the zero-time level of HNF abundance.

Calculation of the growth efficiency of HNF were based on cellular biovolume as follows: growth efficiency = (net HNF biovolume increment/bacterial biovolume introduced) × 100, where the net HNF increment is the maximum HNF biovolume achieved minus the HNF biovolume present at t_0 and the net HNF biovolume increment is divided by the bacterial biovolume introduced into a treatment at t_0.

Additionally, relative growth rates were calculated. Rates of HNF abundance increases on different bacterial strains were related to the treatments amended with the bacterial strain T6-5, where HNF populations generally displayed the most rapid growth. Thus, the HNF abundance in T6-5 treatments (peaking at t_{66} [h]) was set as 100% for comparison of the abundance increases in other treatments.

Chemical analyses: carbon, phosphorus, and Chl-*a* concentrations. Water used for the experiments was analyzed at the time of the sample collection at the study site (Table 2). Dissolved organic carbon (DOC) was analyzed in samples filtered through glass fiber filters of 0.4-μm nominal pore size (GF-5; Macherey-Nagel) with a TOC 5000A analyzer (Shimadzu). Dissolved reactive phosphorus (DRP) (as PO_4-P) was analyzed as described by Murphy and Riley (35). Total phosphorus (TP) was determined by the molybdate method following perchloric acid digestion as described by Kopáček and Hejzlar (36). Chlorophyll *a* (Chl-*a*) concentrations were determined spectrophotometrically after the extraction with acetone (37).

CARD-FISH. The catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) protocol (38) was used with the BET42a and R-BT065 oligonucleotide probes targeting all *Betaproteobacteria* (39) and its R-BT subcluster (34), respectively. The probe R-BT065 covers all three strains from the *Limnohabitans* lineages used in these experiments (18). We examined proportions, in duplicate, of the probe-targeted bacteria in plankton of the study sites at the time of sample collection (t_0), and in all experimental treatments at times t_{24}, t_{48} and t_{66} (h). Moreover, for veri-

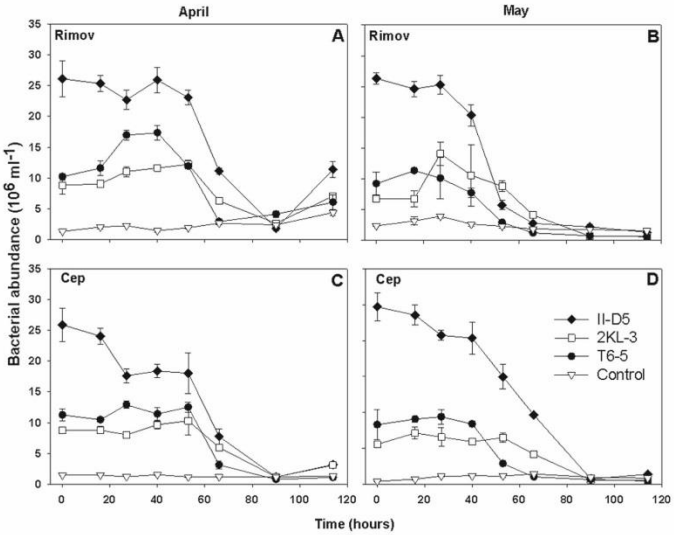

FIG 2 Temporal changes in bacterial numbers with treatments from both study sites amended with the bacterial strains (II-D5, 2KL-3, and T6-5) compared to control treatments where no bacteria were added. (A) Římov in April; (B) Římov in May; (C) Cep in April; (D) Cep in May. Values are means for triplicates; error bars show standard deviations (SD).

fying the presence of the prey bacteria in HNF food vacuoles, the CARD-FISH protocol detailed by Jezbera and coworkers (40) was applied to detect the ingested bacterial prey.

Statistical analysis. Statistical analyses were performed with JMP v.8. (2008) (SAS Institute, Cary, NC, USA). Using three-way analysis of variance (ANOVA), we analyzed the effects of two distinct phases of plankton succession, study site, and strains characteristics on HNF growth efficiency, doubling time (DT), lag phases, and percent increase in abundance of T6-5 at t_{66}. When these ANOVAs were significant, *post hoc* tests were used to determine differences among means (the Student test was used when we compared only two means, and the Tukey test was used when we compared three or more mean values). Canonical correspondence analysis (CCA) was performed with the Excel stats package. We tested whether growth parameters (doubling time, lag phase, and growth efficiency) varied with study site, month, and bacterial strain.

RESULTS

Water chemistry and Chl-*a* concentrations. Generally, phosphorus and Chl-*a* concentrations indicated that Římov was a more productive environment than oligo-mesotrophic Lake Cep (Table 2). However, during the May experiments (late clear-water phase), all chemical parameters and Chl-*a* dropped dramatically in Římov, while no obvious trends were observed in Lake Cep.

Temporal change in bacterial numbers as a response to HNF grazing. Prey availability was suddenly increased by about one order of magnitude, and thus growth responses of HNF communities were clearly attributable to the particular, introduced prey (Fig. 2 to 6). Generally, in most prey-amended treatments, bacterial numbers started to decrease markedly after approximately 40 to 66 h (Fig. 2), whereas in II-D5 treatments in Lake Cep samples, a more rapid abundance drop occurred within the first 40 h. In contrast, bacterial numbers stayed relatively stable in most T6-5 and 2KL-3 treatments between t_0 and t_{53}. However, unique trends were observed in the T6-5 (April-Římov) and 2KL-3 (May-Římov) treatments, with remarkable increases in bacterial numbers occurring mainly between t_{27} and t_{40}, which was before the onset of HNF exponential growth (Fig. 2 and 3). These data indicated growth of the treatment-added bacteria during the initial stage of the experiment. After 50 to 90 h, the growing HNF populations had decimated bacterial abundances in all treatments to levels comparable to those in the control treatments. Notably, the relative proportions of R-BT bacteria, which likely indicated a decay rate in the added prey, dropped dramatically from the initial ~94 to 98% at t_0 to treatment-specific averages ranging between 5 and 12% of total bacteria by t_{66} (data not shown). All introduced prey items were clearly detected in flagellate food vacuoles based upon their size, morphology, and hybridization with the R-BT065 FISH probe, as shown in Fig. 4. In contrast to prey-amended treatments, no obvious trends in bacterial abundance and biomass were observed in control treatments (Fig. 2 and 3).

Growth of flagellates on different *Limnohabitans* strains. Remarkable growth of HNF populations occurred in the *Limnohabitans* prey-amended treatments, with profound prey-, site-, and distinct seasonal phase-specific differences (Fig. 3). HNF abun-

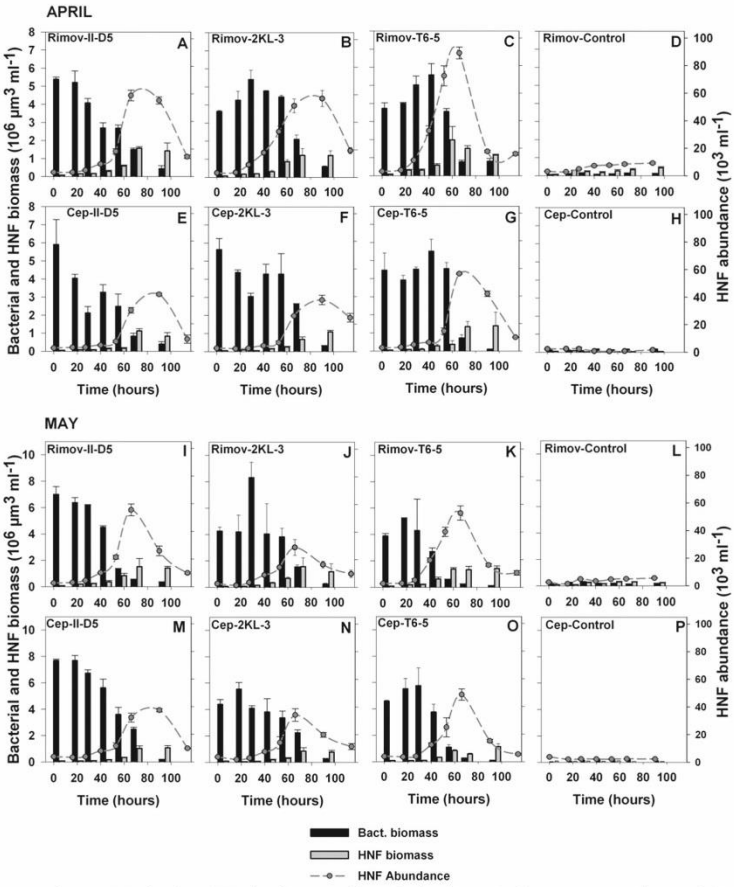

FIG 3 Time course changes in HNF abundance, HNF volume biomass, and bacterial volume biomass in all treatments compared to controls. (A to D) April, Římov; (E to H) April, Cep; (I to L) May, Římov; (M to P) May, Cep. Values are means of triplicates; error bars show SD.

dances increased from 2×10^3 cells ml^{-1} to treatment-specific maxima ranging from 30×10^3 to even 90×10^3 cells ml^{-1}, which were achieved with T6-5 (Římov-April) treatment. In most cases the maxima were recorded after 66 h, except for more slowly growing HNF in the II-D5 (Lake Cep-April) treatment. Thereafter, however, HNF numbers dramatically dropped, which tightly coincided with the rapid depletion of the introduced prey bacteria (Fig. 2 and 3). In contrast, HNF abundances remained relatively stable in the controls (Fig. 3). The temporal changes in HNF biomass generally paralleled the treatment-specific trends observed for HNF abundance.

Factors modulating flagellate growth responses. Growth efficiency (GE) ranged from 12% to 34% (Fig. 5), with an overall average value of 21% across all *Limnohabitans*-amended treatments. The length of the lag phase ranged from 14 to 42 h (Fig. 6), with pronounced differences related to plankton succession or site

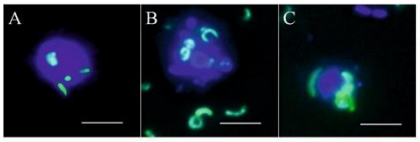

FIG 4 Photomicrographs of bacterial populations and HNF bacterivory in different bacterial treatments shown as overlay images (DAPI and fluorescein isothiocyanate [FITC] stained) of prey bacteria targeted by the R-BT065 FISH probe directly in food vacuoles of flagellates. (A) *L. planktonicus* strain II-D5; (B) *Limnohabitans* sp. strain 2KL-3; (C) *Limnohabitans* sp. strain T6-5. The scale bars show lengths of 5 μm.

(see below). In contrast, HNF doubling times (DTs) (average, 10.2 h; range, 8 to 13 h [Fig. 5]) remained within a relatively narrow range.

The population parameters of DT, length of lag phase, and GE allowed for tentative comparisons of season-, site-, and prey-specific effects modulating HNF growth responses. We found significant differences in the GEs of flagellates between phases, i.e., April versus May (three-way ANOVA, $P < 0.005$), sites ($P < 0.001$), and strains ($P < 0.001$). The highest abundances, biomasses, and GEs of HNF were recorded from treatments amended with the moderately large T6-5 strain from both April samplings. This pattern also held true for the May experiments but with generally lower values of GE (Fig. 5) than those for the April experiments. We also found the same temporal trends in other treatments, i.e., higher HNF abundance, volume biomass, and GE achieved in April than in May. There were also consistent differences in flagellate growth responses between the study sites, with the HNF populations from Římov Reservoir showing generally more robust growth then those from Lake Cep.

The DT of HNF showed two important general trends. It was shorter in April than in May, and the highest HNF growth rate was consistently detected in treatments with the medium-size T6-5 bacterium (Table 1). For instance, HNF abundance doubled within 8 h only in all the T6-5 treatments originating from Římov and from Cep in April (Fig. 5). Note that there was no obvious relationship between DT and length of lag phase of HNF, although the strains 2KL-3 and II-D5 tended to support slower HNF growth in combination with a longer lag phase before the flagellates reached exponential growth phase (compare Fig. 5 with 6A and B).

There were insignificant differences ($P > 0.05$ by three-way ANOVA) in DT between the two study sites. Furthermore, there were significant differences among HNF populations fed morphologically distinct *Limnohabitans* prey, and those corresponded to the temporal origin of the HNF population (April versus May) (Fig. 5). In all cases, HNF grew significantly faster in strain T6-5

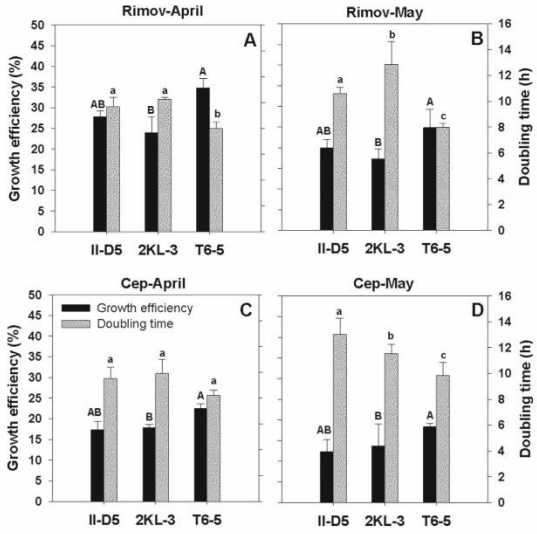

FIG 5 Growth efficiency and doubling times of HNF in all treatments amended with the bacterial strains (for details, see Fig. 1). (A) Římov in April; (B) Římov in May; (C) Cep in April; (D) Cep in May. Values are means of triplicates; error bars show SD. Different letters above bars denote significant differences between growth efficiencies (uppercase letters) and doubling times (lowercase letters). Bars with different lower- or uppercase letters within treatments from April or May are significantly different ($P < 0.05$ by three-way ANOVA followed by Tukey test).

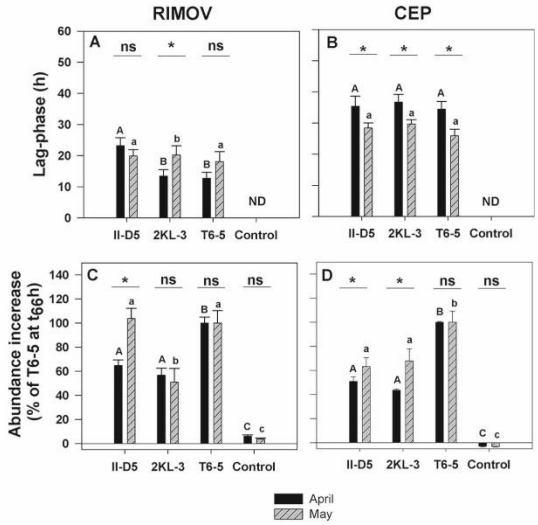

FIG 6 Duration of lag phase (A and B) and rate of HNF abundance increments (C and D) in treatments amended with different bacterial strains (for details, see Fig. 1) as related to the treatment with the strain T6-5, where the HNF abundance and biovolume increased most rapidly (set as 100% at t_{66} h; compare Fig. 4). Data are shown for Římov and Cep populations during both experiments in April and May. Lag phases for control treatments are not presented because in most cases they did not fit the exponential growth model used to calculate HNF growth parameters. Values are means of triplicates; error bars show SD. Different letters above bars denote significant differences between lag phases (A and B) and abundance increases (C and D) in different treatments. Bars with different lower- or uppercase letters within treatments from April or May are significantly different ($P < 0.05$ by three-way ANOVA followed by Tukey test). *, significant difference within the same treatment but when comparing two seasonal phases (April versus May); ns, not significant.

treatments than in other bacterial treatments (Fig. 5A to D). To further highlight this finding, the maximal increments of HNF abundance achieved in strain T6-5 treatments at t_{66} were compared to the HNF abundance increase in other treatments at the same time point (Fig. 6C and D). Notably, in all but one case the HNF abundance increase in strain T6-5 treatments was significantly higher than those in other treatments. The only exception was the strain II-D5 May-Římov treatment, which showed an HNF increment similarly robust as that with the strain T5-6 treatment (Fig. 6C). Generally, lag phase durations were significantly shorter in April than in May treatments from both study sites ($P < 0.001$) (Fig. 6A and B). Moreover, the length of lag phase was significantly inversely correlated ($r^2 = 0.521$, $P < 0.001$) with GE of flagellate communities, indicating that the longer the duration of the lag phase, the lower the GE. In fact, 52% of the variability in GE was explained by the duration of the lag phase.

HNF growth curves, GE estimates (Fig. 3), and other growth characteristics (Fig. 6) indicated that the strain T6-5 represented the best food resource for HNF in all experiments. Notably, this trend was independent of the initial flagellate mean cell volume (MCV), showing the most dramatic increases between t_0 and t_{27} (see Fig. S1 in the supplemental material).

DISCUSSION

We found that the same bacterial prey items, introduced in parallel into two aquatic habitats with differing trophic status and during two distinct phases of seasonal plankton succession, induced remarkably different, and highly specific, growth responses in the indigenous HNF communities. Most notably, sudden shifts in the bacterioplankton community were reflected in rapid shifts by the HNF community toward flagellate groups more adapted to utilize the resultant bacterioplankton groups and their morphotypes (30, 31).

***Limnohabitans* bacteria: model prey with high turnover rates in plankton environments.** *Limnohabitans* bacteria are abundant opportunistic strategists with high growth and loss rates that quickly respond to sudden pulses of organic carbon of algal origin (16, 18, 19, 21), as well as to enhanced flagellate grazing pressures (9, 28). This genus was well represented in our study sites, accounting for ~8 to 14% of total bacteria at t_0 (Table 2). Although *Limnohabitans* is a diverse genus (18), a reverse line blot hybridization technique revealed qualitative evidence that phylotypes closely related to our selected prey bacteria were present among the plankton of both study sites (41). In addition, using the cluster-specific FISH probe (R-BT065), *Limnohabitans* bacteria

are frequently detected directly in HNF food vacuoles (9, 28). Even strains of medium cell size from this genus support rapid HNF community growth (31). All the above findings well justify selecting *Limnohabitans* strains as a suitable experimental model. They are a highly dynamic and omnipresent bacterial prey in freshwaters.

Site- and season-specific responses of HNF communities to changing prey availability. The indigenous HNF communities originated from two distinct phases of plankton succession. The phases drastically differed in Chl-*a* and nutrient concentrations, in abundances and sizes of bacteria and HNF (Table 2), and in potential top-down control by large zooplankton (see, e.g., references 4 and 29). General trends reflecting temporally distinct trophic structures were more apparent in the meso-eutrophic Římov Reservoir (Table 2) than in the oligo-mesotrophic Lake Cep. The lake had lower bacterial and HNF abundances, lower HNF cell volumes, and increased water transparency, which indirectly indicated a stronger grazing impact by filter-feeding zooplankton in May than in April (9, 29). The general trends in these results were also supported by the CCA, showing negative correlation between habitats of different trophic status (Římov and Cep) and the phases of seasonal plankton succession (see Fig. S2 in the supplemental material).

Trophic structure during the algal bloom phase (Table 2) has important implications for interactions in pelagic environments (29, 42). For example, for hydrodynamic reasons, bigger bacteria likely have higher rates of contact with flagellate cells in plankton (26, 43, 44). This could cause communities dominated by larger flagellates to be more "preconditioned" for efficient predation on the larger bacteria. This was reflected in our experimental data, as significantly higher GE values (Fig. 5) were detected during the algal bloom than during the clear-water phase. Thus, during the spring bloom, larger bacterial prey are more easily ingested by larger flagellates, with shorter handling time per prey item (26, 45). Presumably, this is due to a more optimized predator-prey size ratio (27, 42, 46). Conversely, the lowest GE was consistently determined in Lake Cep (mainly in May), where low HNF abundance and the smallest flagellate cells were detected in the t_0 samples (Table 2; see Fig. S1 in the supplemental material). The size of these flagellate cells (2 to 3 μm diameter) is close to the physical size limits for ingesting large prey, and the 2KL-3 strain can exceed 3 μm in length (Table 1; Fig. 1).

General and specific trends in HNF population development. In all prey-amended treatments, after the initial HNF exponential growth phase, a dramatic drop in HNF numbers coincided with bacterial prey depletion between t_{66} and t_{90} h. The most likely explanation for this phenomenon is that the small to medium-size bacterivorous HNF (Fig. 4), governing the initial experimental phase rich in bacterial prey, were gradually preyed upon by larger raptorial HNF. The most robust shift in the MCV of HNF was generally observed after the first 16 to 27 h, e.g., from 9 to ~22 to 25 μm^3 in May in Lake Cep (see Fig. S1 in the supplemental material). Interestingly, these trends, showing marked size and community shifts among the HNF, were observed in other, similarly designed experiments (31).

Sudden, experimentally induced shifts in the prey community composition can be efficiently compensated for by a shift in natural HNF community composition (within 1 to 2 days, corresponding to our estimates of lag phase [Fig. 6]). The community composition shifts toward typical bacterivorous *Spumella*-like lineages (31, 47), most likely to optimize the prey capture efficiency of the particular bacterial prey that temporarily dominates. For instance, rapid prey size-related shifts in the predator community could be induced by very small bacteria in pelagic environments, e.g., *Polynucleobacter* spp. (48), or by the Ac1 lineage of *Actinobacteria* (9). In the latter study, smaller planktonic HNF preyed on Ac1 bacteria, whereas larger HNF and other protists preyed upon larger *Limnohabitans* bacteria. Moreover, an experimental study (31) suggested that in addition to variable growth responses, significant HNF community shifts were induced by less morphologically diverse representatives of *Limnohabitans* bacteria. These representatives were of medium to small cell size compared to the prey bacteria used here (Table 1). Interestingly, in the same experiment, an *Actinobacteria* strain from the Luna 2 cluster, which was of a size similar to that of most of the *Limnohabitans* strains tested (31), did not support growth of the same natural HNF community. This points to other important bacterial prey characteristics in addition to size and morphology, that likely modulate growth of flagellate predators (30, 49). For example, it has been documented that various representatives of the Gram-positive *Actinobacteria* are suboptimal bacterial food that barely support measurable growth of bacterivorous flagellates (27, 50).

The prey food characteristics and handling time (contact, processing, ingestion, and digestion phases) of any particular prey item represent major parameters affecting HNF growth parameters and their overall GE (26, 51). Interestingly, the significant variability in the temporal length of lag phase (15 to 43 h [Fig. 6A and B]) was unrelated to DT in our experiments. In contrast, a longer lag phase usually yielded lower GE in our treatments (Fig. 5). Moreover, the CCA data suggested that lag phase and DT were positively correlated in May, which corresponds with our results that there were always a longer lag phase and DT in May (see Fig. S2 in the supplemental material).

Prey-specific responses of HNF communities to experimental manipulations. We intentionally selected morphologically distinct bacterial strains. The II-D5 strain represented a medium cell size (1.1 to 1.3 μm long) compared to those of both the much longer T6-5 cells and the robust solenoid cells of the 2KL-3 strain (Fig. 1 and 4). Notably, filamentous cells or cells with diameters greater than 3 μm are often too large for small flagellates to ingest (27, 45, 52). Overall, cells in the intermediate size range are consumed most rapidly. This can create a "bimodal prey size distribution effect," indicating a certain level of grazing protection and thus negative selection against very small or very large bacterial cells (27).

Notably, independent of study site or experimental timing, a combination of size, food item processing time (26), and other food quality aspects of the T6-5 strain yielded the most rapid growth responses of HNF to this food amendment. Also, the CCA showed strong correlations between the bacterial strain T6-5 and the DT of HNF (see Fig. S2 in the supplemental material). Aside from this general trend, which was valid across all T6-5 treatments, there was also a strong effect of plankton succession phase on GE. It dropped significantly in all May experiments irrespective of the particular bacterial amendment (see Fig. S2 in the supplemental material). A reason for this phenomenon may be that these bacteria were still within an optimal size range (2, 27, 49, 53) for the larger HNF present in the plankton of Římov Reservoir and Lake Cep in April but were then too large for the smaller HNF present during the May clear-water phase (Table 2).

The thick, curved rod shape of the robust 2KL-3 strain (Fig. 1 and 4) could be a determining factor affecting prey processing efficiency (26), thus making them partially protected against grazing by the smallest HNF cells. This fact was likely responsible for the lower GE (Fig. 5) and the generally prolonged lag phase of HNF in our May experiments (Fig. 6A and B), where tiny flagellate cells dominated the initial communities (Table 2). When the small flagellates were suddenly exposed to the large 2KL-3 prey, a suboptimal prey/predator size ratio could induce a shift in the HNF community toward larger grazers (optimizing capture efficiency) (27), accounting for the delay in the onset of exponential growth.

In a previous experiment (31), strain II-D5 represented the best food quality resource for growth of a natural HNF community among the five *Limnohabitans* strains tested. These strains were mostly rod shaped and of medium to smaller cell size. In contrast, in the current study, strain II-D5 was not the superior prey, since HNF generally grew slower than in strain T6-5 treatments and the growth parameters were not very different from those estimated for the large cells of the 2KL-3 strain. However, one exception to this pattern should be noted. The rate of HNF abundance increase in the strain II-D5, May-Římov, treatment was slightly higher than that in the corresponding strain T6-5 treatment (Fig. 6C). This highlights the importance of prey-induced, distinct plankton succession phase- and site-specific HNF compositional shifts, which then modulate the rate at which the HNF community changes composition relative to the initial plankton sample (31).

Flagellate bacterivory significantly increases regeneration of nutrients (54), which in turn could stimulate bacterial growth and thus accelerate HNF growth. However, for simplicity we did not take this aspect into account, and our calculations of HNF growth parameters were built on a more straightforward experimental design whereby each prey item was added to yield the same initial volume of biomass. Because the added *Limnohabitans* cells were viable, the nutrient recycling effect (54), in combination with initial low HNF abundances, could also allow for bacteria growth, as observed in the nutrient-richer Římov treatments with strain T6-5 in April and with strain 2KL-3 in May (Fig. 2). Moreover, this effect should be specifically strengthened in the strain 2KL-3 treatment (Fig. 2B), where the large size seems to protect these prey from grazing by the smaller HNF cells dominating the initial phase.

Concluding remarks. Our experimental approach mimicked rapid increases in the biomass of the prey bacteria with opportunistic life strategies (typical for representatives of the *Limnohabitans* genus [18]). These prey can quickly respond to pulses of nutrients and organic matter caused by the onset of an algal bloom or by an enhanced substrate supply due to sloppy feeding of zooplankton on algae (9). Additionally, we have documented the prey-specific fashion in which sudden enrichments in bacterial prey availability modulate the trophic interactions and growth responses of natural HNF communities. Notably, the results also suggest that a combination of a longer lag phase and lower GE could be used a tool for considering the suitability of the introduced prey for the HNF community present in the original plankton sample (31, 51).

Although species-specific flagellate predator-bacterial prey interactions have obvious fundamental consequences for the flow of carbon from DOM through particular bacterial groups to higher trophic levels (9, 31), limited attention has been paid to this important ecological process. Thus, in future studies the role of various species, representing the core bacterioplankton groups in carbon flow to higher trophic levels, should be examined in environments with contrasting DOM sources.

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Bacterial prey food characteristics modulate community growth response of freshwater bacterivorous flagellates

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Abstract

Different bacterioplankton species represent different food quality resources for heterotrophic nanoflagellate (HNF) communities, potentially affecting HNF growth, community dynamics and carbon flow to higher trophic levels. However, our knowledge of such dynamics is still very limited. Here, we describe the results of 11 experiments with natural HNF communities from distinct seasonal phases in two freshwater habitats. The HNF communities were released from predation pressure of zooplankton and incubated with 16 distinct ecologically relevant prey bacterial strains from important *Betaproteobacteria* genera (*Limnohabitans*, *Polynucleobacter*, and *Methylopumilus*) and one *Actinobacteria* strain from the Luna 2 cluster. We observed remarkable prey- and season-specific variability in community HNF growth parameters, i.e., doubling time, volumetric gross growth efficiency (GGE), and length of lag phase. All strains, except for the actinobacterium, supported rapid HNF population growth with an average doubling time of 10 h and GGE of 29%. Our analysis revealed that 59% of the variability in flagellate GGE data was explained by the length of lag phase after prey amendments. This indicates a considerable "adaptation time," during which the predator communities undergo compositional shifts toward flagellate bacterivores best adapted to grow on the offered prey. Importantly, the rapid HNF growth detected on various bacteria tightly corresponds to doubling times reported for fast growing bacterioplankton groups. We propose a conceptual model explaining the tight linkages between rapid bacterial community shifts and succeeding HNF community shifts, which optimize prey utilization rates and carbon flow from various bacteria to the microbial food chain.

In freshwater systems, the trophic interactions of protists and prokaryotes regulate the flow of dissolved organic carbon and limiting nutrients to higher trophic levels (Jürgens and Matz 2002; Sherr and Sherr 2002). Heterotrophic nanoflagellates (HNF), ciliates, and in nutrient poor systems, mixotrophic flagellates (e.g., Domaizon et al. 2003; Weisse et al. 2016), are considered to be major protistan bacterivores. Trophic interactions are well characterized from the perspective of the top-down control of bacteria. Various size-related grazing-resistant strategies, but also non-morphological traits of prokaryotes such as motility, cell surface properties, and the effect of bacterial toxicity on their vulnerability to protistan grazing are well documented (Hahn and Höfle 2001; Jürgens and Matz 2002). However, how these interactions may regulate consumer success (Boenigk and Arndt 2002; Corno et al. 2013; Chrzanowski and Foster 2014) and, in turn, also the community composition of the bacterivores (Arndt et al. 2000; Šimek et al. 2013) is much less understood.

The amounts, composition, and temporal dynamics of organic and inorganic resources differ remarkably both seasonally and among various freshwater bodies. These resources ultimately modulate the growth and population dynamics of various planktonic prokaryotes (Eiler and Bertilsson 2007; Salcher et al. 2013; Salcher 2014). Detailed insights into bacterioplankton community composition and substrate preferences are increasingly becoming available (e.g., Newton et al. 2011; Salcher et al. 2013). While these represent useful "snapshots" of the community at a given time point, they are not informative regarding turnover rates of major bacterioplankton groups.

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Such investigations require fine temporal resolution reflecting typical doubling times of planktonic prokayotes as well as protistan predators (Eckert et al. 2012; Šimek et al. 2014). Thus, the currently available data on bacterioplankton composition in situ cannot be translated into growth rates and carbon fluxes from the bacteria to the grazer food chain.

It has been suggested that the driving force for high bacterial production in pelagic environments is mediated by frequent resource shifts supporting short-lived peaks of rapidly growing bacterial lineages (Eckert et al. 2012, 2013; Salcher 2014). For instance, some *Betaproteobacteria* and *Flavobacteria* respond to sudden pulses in algal-derived organic carbon with very short doubling times from several hours to days (Zeder et al. 2009; Salcher et al. 2011; Neuenschwander et al. 2015). These short-lived bacterial peaks are rapidly succeeded by peaks of bacterivorous HNF (Eckert et al. 2012; Šimek et al. 2014). Thus, it has been hypothesized that small grazers rapidly adapt to the shifts in prey communities owing to their growth potential in situ (Šimek et al. 2006; Weisse et al. 2016). Pelagic HNF populations are usually severely top-down controlled by zooplankton grazing (e.g., Jürgens et al. 1996), while bacterial concentrations in mesoeutrophic systems do not appear to be a key factor limiting HNF growth (Jürgens 1992; Gasol and Vaqué 1993). In contrast, the trophic position of bacteria at the bottom of the food chain suggests that they are more strongly resource-limited (McQueen et al. 1986).

Different bacterial strains appear to have different nutritional value to consumers (Boenigk et al. 2006; Tarao et al. 2009; Šimek et al. 2013; Chrzanowski and Foster 2014) and this characteristic may vary even for the same prey during different seasonal phases within the same ecosystems (Grujčić et al. 2015). Moreover, the same bacterial species may not have similar nutritional quality for all members of the flagellate community (Chrzanowski and Foster 2014). Thus, seasonal shifts in prey availability likely induce very different, prey-specific growth responses of the HNF grazers, measured as growth rate and gross growth efficiency (GGE). These findings suggest that shifts in bacterial community structure (Newton et al. 2011; Salcher et al. 2013), with concomitant shifts in the quality of bacteria as food, likely cascades upward, inducing shifts in the bacterivore community (Šimek et al. 2013; Chrzanowski and Foster 2014). Many different methods to study HNF bacterivory have been proposed; however, none are appropriate for assessing the specific role of a naturally abundant prey in carbon flow dynamics (Montagnes et al. 2008). So far only a few experimental studies have focused on the growth responses of natural HNF assemblages to bacterial prey amendments using strains of relevant prokaryotic taxa that are those originating from the same systems as the consumers (Šimek et al. 2013; Grujčić et al. 2015). The lack of such data severely limits the possibility to generalize preliminary evidence on a tight coupling between prey community shifts and variable growth responses of HNF associated with very rapid shifts in the grazer community (Šimek et al. 2013), the phenomena that can be modulated by seasonally evolving trophic structure of plankton environments (e.g., Grujčić et al. 2015).

Thus in a large series of 11 flagellate predator-bacterial prey manipulation experiments, we tested the hypothesis that HNF communities rapidly respond to prey availability shifts in prey-specific fashion, with growth rates comparable to those of pelagic bacteria. Natural HNF communities in plankton samples, originating from different seasonal phases in two distinct ecosystems, and an axenic culture of *Poterioochromonas* sp. were used as flagellate bacterivores. Treatments with flagellate grazers were manipulated by the addition of 16 different strains from relevant and abundant bacterioplankton taxa (Newton et al. 2011; Jezbera et al. 2012). The aim of this study was to investigate general net effects of bacterial prey quality, specifically, the maximum growth rate, the volumetric GGE, and the length of the lag phase in different flagellate taxa. Based on our results and the existing literature we propose a conceptual model explaining the tight linkages between the rapid shifts in the bacterial community and the consequent shifts in HNF community, that optimize prey utilization rates and biomass transfer from various bacteria to higher trophic levels.

Materials and methods

Experimental organisms

For the bacterial prey manipulation experiments we used 16 representative strains (*see* Table 1 for the full list of the strains, their cell size, morphology, and origin) from several important lineages of planktonic bacteria:

1. Genus *Limnohabitans* of *Betaproteobacteria*—one strain from the lineage LimA (Rim8), one strain from the lineage LimB (Rim11), and nine strains of diverse size and morphology from the lineage LimC (II-D5^T, II-B4^T, 2KL-27, 2KL-1, 2KL-3, T6-5, Rim28, Rim47, and 15K). Two of the strains, designated II-B4^T and II-D5^T (16S rRNA gene accession numbers FM165536 and FM165535, respectively) represent type strains of the species *L. parvus* and *L. planktonicus* (Kasalický et al. 2010) while the other *Limnohabitans* strains represent so far undescribed species. Reconstructions of the phylogenetic positions of all these strains based on 16S rRNA gene and ITS sequences were presented elsewhere (Kasalický et al. 2013). Seven out of 11 *Limnohabitans* strains were isolated from the surface layer of the freshwater mesoeutrophic Římov Reservoir in South Bohemia (48°50′56″N, 14°29′26″E), three strains from the mesoeutrophic Klíčava Reservoir in Central Bohemia (50°3′58″N, 13°55′55″E), and one strain from the eutrophic Lužnice pond T6 in South Bohemia (48°50′0.453″N, 14°55′40.324″E, *see* Table 1). All strains from the B and C lineages of the genus *Limnohabitans* belong to the R-BT065 subcluster of *Betaproteobacteria* (for

Table 1. Characteristics of bacterial strains used in this study.

Species, lineage affiliation	Strain	MCV ± SD (μm^3)	Cell shape	Origin	Reference	Experiment Identifier
LimA lineage, *Limnohabitans, Comamonadaceae, Betaproteobacteria*						
Limnohabitans sp.	Rim8	0.107 ± 0.008	Solenoid	Římov Reservoir, Czech Republic	Kasalický et al. (2013)	XI
LimB lineage, *Limnohabitans, Comamonadaceae, Betaproteobacteria*						
Limnohabitans sp.	Rim11	0.056 ± 0.009	Short rod	Římov Reservoir, Czech Republic	Kasalický et al. (2013)	II, VII, VIII, X
LimC lineage, *Limnohabitans, Comamonadaceae, Betaproteobacteria*						
Limnohabitans planktonicus	II-D5^T	0.162 ± 0.045	Large rod	Římov Reservoir, Czech Republic	Kasalický et al. (2010)	IA, IB, II, III, IV, V, VI, VII, VIII
Limnohabitans parvus	II-B4^T	0.055 ± 0.006	Short rod	Římov Reservoir, Czech Republic	Kasalický et al. (2010)	IA, IB, II, VII, VIII
Limnohabitans sp.	2KL-27	0.067 ± 0.038	Coccoid	Klíčava Reservoir Czech Republic	Kasalický et al. (2013)	IA, IB
Limnohabitans sp.	2KL-1	0.204 ± 0.110	Large solenoid	Klíčava Reservoir Czech Republic	Kasalický et al. (2013)	IA, IB
Limnohabitans sp.	2KL-3	0.548 ± 0.116	Large solenoid	Klíčava Reservoir Czech Republic	Kasalický et al. (2013)	III, IV, V, VI
Limnohabitans sp.	T6-5	0.411 ± 0.045	Thin curved rod	Lužnice pond T6 Czech Republic	Kasalický et al. (2013)	III, IV, V, VI, X
Limnohabitans sp.	Rim28	0.052 ± 0.013	Coccoid	Římov Reservoir, Czech Republic	Kasalický et al. (2013)	II, VII, VIII
Limnohabitans sp.	Rim47	0.080 ± 0.021	Coccoid	Římov Reservoir, Czech Republic	Kasalický et al. (2013)	VII, VIII, X
Limnohabitans sp.	15K	0.054 ± 0.006	Ovoid	Římov Reservoir, Czech Republic	Kasalický et al. (2013)	II
PnecC lineage, *Polynucleobacter, Burkholderiaceae, Betaproteobacteria*						
Polynucleobacter sp.	czRimov8-C6*	0.058 ± 0.013	Small solenoid	Římov Reservoir,	Undescribed	IX, X
	czRimov-FAMC1*	0.049 ± 0.010	Small solenoid	Czech Republic	Undescribed	IX, X
PnecD lineage, *Polynucleobacter, Burkholderiaceae, Betaproteobacteria*						
Polynucleobacter cosmopolitanus	MWH-Molso2^T	0.049 ± 0.023	Short curved rods	Lake Mondsee, Austria	Hahn et al. (2010)	IA, IB
Methylopumilus, Methylophilaceae, Betaproteobacteria						
"*Ca. Methylopumilus turicensis*"	MMS-10A-171	0.042 ± 0.004	Short rod	Lake Zurich, Switzerland	Salcher et al. (2015)	XI
Luna-2 subcluster, *Microbacteriaceae, Actinobacteria*						
Actinobacterium Undescribed	MWH-Wo1	0.061 ± 0.021	Small solenoid	Lake Wolfgangsee, Austria	Hahn and Pöckl (2005)	IA, IB, II, XI.

MCV, mean cell volume.
* *Polynucleobacter* strains czRimov8-C6 and czRimov-FAMC1 share identical 16S rRNA gene sequences.

the probe targets, *see* Šimek et al. 2001), while the lineage Lim A is detectable in environmental samples with a double hybridization approach using a novel 23S rRNA FISH-probe (Shabarova et al. 2017).

2. Genus *Polynucleobacter* of *Betaproteobacteria*—we used two undescribed strains from PnecC lineage—czRimov8-C6 (accession number FN429658, Jezbera et al. 2011) and czRimov-FAMC1, isolated from the Římov Reservoir (Table 1) with identical 16S rRNA sequences. Furthermore, one *Polynucleobacter* strain from the PnecD lineage, *P. cosmopolitanus* (MWH-MoIso2[T]), isolated from Lake Mondsee in Austria (Hahn et al. 2010), was used in our experiments.
3. Genus *Methylopumilus*, *Methylophilaceae*, *Betaproteobacteria*—we used one strain *Ca. M. turicensis* (MMS-10A-171) isolated from Lake Zurich in Switzerland (Salcher et al. 2015).
4. Luna 2 cluster of *Actinobacteria*—we used one undescribed strain (MWH-Wo1) isolated from Lake Wolfgangsee (Hahn and Pöckl 2005).

The mixotrophic flagellate predator *Poterioochromonas* sp. strain DS was isolated from Lake Constance (accession number of 18S rRNA gene sequence AM981258, Tarao et al. 2009). The axenic flagellate culture was maintained in dim light and fed twice a month with heat-killed bacteria (60°C, *L. planktonicus* strain; pelletized aliquots stored frozen in −20°C) as described previously (Hahn et al. 1999).

Experimental design and sampling

The majority of the strains used as prey for flagellates were isolated from our major study site (the Římov Reservoir, Table 1). Before each experiment, the bacteria were pregrown in the nutrient rich liquid 3 g L^{-1} NSY medium (Hahn et al. 2004) to avoid possible effects of nutrient-deficient prey on flagellates selectivity and grazing and thus to standardize the experimental start point regarding prey food quality related to the nutrient content.

Altogether 11 experiments (for an overview *see* Table 2) were conducted during 2011–2015, spanning different seasonal phases at two natural sites—the Římov Reservoir and oligomesotrophic sandpit Lake Cep (48°92′49.24″N, 14°88′68.11″E, South Bohemia, Czech Republic). We used natural HNF communities (experiments IA, II–XI; Table 2) or the axenic *Poterioochromonas* culture (experiment IB) to examine effects of different food quality of different bacteria (Table 1) on growth parameters of the flagellate communities. Both grazer populations (HNF and *Poterioochomonas* sp.) were amended in parallel by adding exactly the same total bacterial biovolume of the bacterial strains (experiments IA and IB, Table 2).

During the exponential growth phase, bacterial cells (50 mL) were concentrated by centrifugation at 5000 × *g* and subsequently re-suspended into 50 mL of 0.2-μm filtered and sterilized water from the Římov Reservoir (experiments I–III, V, and VII–XI, *see* Table 2), or water from Lake Cep (experiments IV and VI). The cultures were kept on a shaker overnight to facilitate even re-suspension of cells and adaptation to the reservoir or lake water as detailed elsewhere (Šimek et al. 2013; Grujčić et al. 2015). *Ca. M. turicensis* was grown in 2 L setups in autoclaved water from the Římov Reservoir amended with 1 mM methanol, 100 μM methylamine, and concentrated by centrifugation. Prior to being added to experimental treatments, bacteria were enumerated via fluorescence microscopy as described below.

All experiments with natural HNF communities from the two planktonic systems (Table 2) as well as with the *Poterioochromonas* culture were carried out in a very similar fashion. A 10-L water sample from the Římov Reservoir or Lake Cep was collected and then gravity-filtered through 5-μm pore-size, 147-mm diameter filters (for more details *see* Šimek et al. 2013; Grujčić et al. 2015). The HNF in filtered water were thus released from zooplankton predation and the samples were pre-incubated for 10 h to recover from the handling shock in samples with relatively high HNF abundance (1–4 × 10^3 mL^{-1}) or up to 30 h in samples from May and October with low HNF abundance (< 0.7 × 10^3 mL^{-1}; Table 2). The longer pre-incubation resulted in a marked increase in HNF numbers, yielding time zero abundance within the range of 1.5–3.5 × 10^3 mL^{-1}. Moreover, during the pre-incubation period the abundance of the natural background bacteria decreased to levels of ca. 1 × 10^6 mL^{-1}, and the majority of remaining bacteria were either small flocks or filaments (i.e., likely HNF grazing-resistant morphotypes). After the pre-incubation, triplicate treatments of 250–500 mL of the 5-μm filtrates were manipulated by addition of the respective bacterial strains. The scheme of major steps of the experimental setup has been described elsewhere (Fig. 1 in Šimek et al. 2013). Small ciliates may in some cases pass through 5-μm pore-size filters (Nakano et al. 2001) and prey upon flagellates. However, we checked all samples from the exponential phase used to calculate HNF growth parameters (largely time points between 12 h and 70 h) and did not find any ciliates.

Six days before starting the experiment 1B (Table 2), we stopped the feeding of the axenic *Poterioochromonas* culture by heat killed food bacteria. Notably, during this period almost all fed bacteria were consumed by the flagellate (approximately 5 × 10^3 flagellates mL^{-1}) and thus they could not interfere substantially with bacterial food amendments. Moreover, the flagellate culture was further diluted by the bacteria-free inorganic IBM medium (Hahn et al. 2004) to yield a starting flagellate concentration of 1 × 10^3 mL^{-1}. Six bacterial prey types were then added to the flagellate culture (experiment IB) and their same biovolume also to the natural HNF community (experiment IA). The differences in the numbers of bacteria added in particular experiments, ranging from 15 to 45 × 10^6 cells mL^{-1}, reflect the fact that the prey bacteria differed markedly in mean cell volume and morphology (Table 1). The initial cell number

Table 2. Overview and timing of bacterial prey manipulation experiments (numbered in bold) conducted in the Římov Reservoir and Lake Cep in different seasonal phases during the period 2011–2015. Main chemical and microbial parameters are shown for samples collected for the experiments.

Experiment/timing (in situ temperature)	Site Bacterivore	HNF (10^3 mL^{-1})	HNF MCV (μm^3)	Bacteria (10^6 mL^{-1})	Bacteria MCV (μm^3)	Chl *a* (μg L^{-1})	TP (μg L^{-1})	DRP (μg L^{-1})	Bacterial strains used as prey
IA. 21. 4. 2011–24. 4. 2011 (15°C)*	Římov - HNF	0.967	12.4	4.496	0.043	11.5	33.8	1.2	II-D5^T, II-B4^T, 2KL-27, 2KL-1, MWH-Molso2^T, MWH-Wo1
IB. 21. 4. 2011–24. 4. 2011 (cultured at 18°C)	*Poteriochromonas*	The same prey bacteria as in the experiment **IA** were fed to an axenic culture of *Poteriochromonas* sp.							II-D5^T, II-B4^T, 2KL-27, 2KL-1, MWH-Molso2^T, MWH-Wo1
II. 10. 10. 2011–13. 10. 2011 (16°C)	Římov - HNF	0.658	15.5	3.944	0.056	10.2	28.2	6.7	II-B4^T, Rim11, Rim28, 15K, MWH-Wo1
III. 23. 4. 2012–27. 4. 2012 (16°C) †	Římov - HNF	3.529	26.7	2.79	0.094	11.6	26.9	11.6	II-D5^T, T6-5, 2KL-3
IV. 23. 4. 2012–27. 4. 2012 (16°C) †	Cep – HNF	0.872	16.5	3.164	0.050	3.9	8.6	5.2	II-D5^T, T6-5, 2KL-3
V. 28. 5. 2012–1. 6. 2012 (18°C) †	Římov - HNF	0.470	16.5	2.111	0.040	3.5	21.7	7.6	II-D5^T, T6-5, 2KL-3
VI. 28. 5. 2012–1. 6. 2012 (19°C) †	Cep – HNF	0.371	8.9	1.531	0.041	3.1	10.0	5.7	II-D5^T, T6-5, 2KL-3
VII. 22. 4. 2013–25. 4. 2013 (15°C)	Římov - HNF	1.554	33.1	2.072	0.061	7.7	34.8	9.1	II-D5^T, II-B4^T, Rim11, Rim28, Rim47
VIII. 26. 8. 2013–29. 8. 2013 (21°C)	Římov - HNF	1.345	22.1	3.522	0.056	8.9	20.5	3.1	II-D5^T, II-B4^T, Rim11, Rim28, Rim47
IX. 22. 4. 2014–26. 4. 2014 (16°C)	Římov - HNF	1.319	24.4	3.626	0.055	5.1	16.1	2.2	czRimov8-C6, czRimov-FAMC1
X. 18. 8. 2014–22. 8. 2014 (21°C)	Římov - HNF	1.236	24.6	2.960	0.067	12.1	19.9	3.8	czRimov8-C6, czRimov-FAMC1, Rim11, Rim47, T6-5
XI. 25. 5. 2015–29. 5. 2015 (18°C)	Římov - HNF	1.795	32.3	2.054	0.061	5.7	16.3	1.8	Rim8, MWH-Wo1, MMS-10A-171

TP, total phosphorus; DRP, dissolved reactive phosphorus; Chl *a*, chlorophyll *a*; MCV, mean cell volume.
* Selected data from the experiment IA have been used in the previous study (Šimek et al. 2013).
† Selected data from the experiments III–VI have been used also in the study of Grujčić et al. (2015).

of each bacterial strain added was set to yield approximately the same initial biovolume for all strains within the same experiment. This biovolume (1.5–5.5 × 10^6 μm^3 mL^{-1}) represented 10- to 20-fold the background bacterial biomass present in the pre-incubated HNF solution. A 5-μm filtrate containing the same starting HNF community but with no bacteria added was used as control. Thus the differences in growth responses of HNF in the amended treatments could be attributed to the effects induced by the prey added. Experiments were run for 66–100 h (in case of a longer lag phase) to cover the HNF exponential growth phase, usually till their numbers started to decrease (for details *see* Supporting Information Figs. S1–S8). All treatments were incubated in the dark at 18°C (within ± 3°C of the in situ temperature; Table 2), and subsamples were taken aseptically at 12–24 h intervals. At time 0 h, 24 h and at the time point corresponding to exponential growth phase of HNF communities, additional samples were collected for fluorescence in situ hybridization (CARD-FISH, *see* below).

Timing of the experiments fell into four different plankton successional phases: April (spring phytoplankton bloom), May (clear water phase), August (late summer phytoplankton bloom), and October (decaying algal bloom phase). Since many strains were used repeatedly in the different seasonal phases at both sites (Table 2) we also tested how the same bacterial strain affects the growth of temporally different HNF communities. For such statistical testing the data from the same prey amendments falling into the same seasonal phase were pooled together from both study sites (for details *see* Supporting Information Table S1).

The impacts of prey amendments on the HNF community composition in experiment marked as IA in Table 2 have been evaluated separately in Šimek et al. (2013) and the comparisons of season- and site-specific aspects of HNF growth responses to addition of three identical strains in experiments marked as III–VI (Table 2) are detailed in Grujčić et al. (2015). However, the data from these experiments were used also in this comprehensive study in a broader context to unveil the overall trends of HNF growth responses and their possible community shifts induced by prey amendments with a far broader variety of relevant planktonic bacteria used, moreover, across different plankton seasonal phases.

Bacterial abundance and sizing

In experiments IA, IB, and II, bacterial abundance was quantified via flow cytometry in samples stained with the fluorochrome Syto13 (Molecular Probes, Eugene, Oregon, U.S.A.) using the FACScalibur flow cytometer (Becton

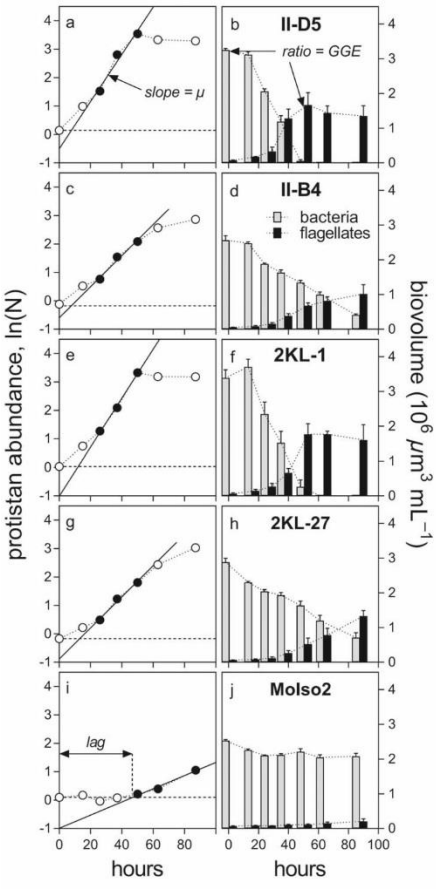

Fig. 1. Examples of time course changes in abundance (**a**, **c**, **e**, **g**, **i**) and biovolume of the mixotrophic bacterivorous flagellate *Poterioochromonas* sp. in comparison to bacterial biovolume (**b**, **d**, **f**, **h**, **j**) in the treatments amended with respective bacterial strains in experiment IB (further details *see* in Tables 1, 2). The arrows in panel (**b**) highlight the data points selected to calculate volumetric GGE values. Full symbols in panels (**a**, **c**, **e**, **g**, **i**) highlight the time points selected to calculate the maximum flagellate growth rate (slope = μ, *see* the arrow in panel **a**). The length of lag phase was calculated as the period from the time zero to the intercept between the best-fit line of the flagellate growth and the zero-time level of its abundance as depicted in panel (**i**). Values are means of triplicates; error bars show SD. Data for the strain MWH-Wo1 and control treatments are not shown as no flagellate growth was detected.

Dickinson, Franklin Lakes, New Jersey, U.S.A.) as detailed in Gasol and Del Giorgio (2000). However, after approximately 60 h of the experiments with growing abundance of grazers, enhanced proportions of flocks and filaments (apparently developing from natural background bacterioplankton cells) appeared in the samples and thus bacteria were counted via epifluorescence microscopy (Šimek et al. 2001). This also allowed accurate quantification of bacterial cells in small bacterial aggregates. In the follow up experiments, i.e., III–XI, bacterial abundance was quantified only via the microscopy. Bacteria (> 200 cells per sample) were sized by using the semiautomatic image analysis systems (NIS-Elements 3.0, Laboratory Imaging, Prague, Czech Republic).

Heterotrophic flagellate enumeration, growth, and cell size

Subsamples (1–5 mL) were stained with DAPI (4′,6-diamidino-2-phenylindole) and HNF abundance (eukaryotic cells with a visible nucleus, flagella, and typical cell shape), was determined via epifluorescence microscopy as described elsewhere (Šimek et al. 2001). To calculate mean volumes of HNF cells (approximated to prolate spheroids), lengths and widths of >50 cells in triplicate treatments were measured manually on-screen with a built-in tool established in the software NIS-Elements 3.0 (LIM, Prague, Czech Republic). Estimates of GGE of HNF as percent based on cellular biovolume were calculated as the ratio between bacterial biovolume introduced and net HNF biovolume yield in the treatment (Šimek et al. 2013), thus representing the volumetric GGE, not carbon-based GGE values. The maximum HNF growth rate was calculated using ln-transformed data on HNF abundance with linear regression as the slope of the best-fit line. The length of lag phase was calculated as the period from the time zero to the intercept between the best-fit line of HNF growth and the zero-time level of HNF abundance (Šimek et al. 2013). To illustrate the data selection and calculations used, commented examples of time-course changes in flagellate abundance, biovolume, and bacterial biovolume for the experiment IB are given in Fig. 1.

Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH)

The CARD-FISH protocol (Pernthaler et al. 2002) and oligonucleotide probes (ThermoHybaid, Ulm, Germany) were employed to target the following bacterial lineages: the R-BT065 cluster (probe R-BT065, Šimek et al. 2001), that includes all *Limnohabitans* strains used from the lineage LimB and LimC (Kasalický et al. 2013); the LimA lineage of the *Limnohabitans* genus (probe LimAE-1435, Shabarova et al. 2017); the entire cluster of *Polynucleobacter* (probe PnecABCD-445, Hahn et al. 2005); the PRD01a001B lineage of *Methylophilaceae*, *Betaproteobacteria* (probe PRD-732, targeting *M. turicensis*, Salcher et al. 2015); and the entire *Actinobacteria* phylum (probe HGC69a). We examined proportions of the probe-targeted bacteria in plankton of the study sites at the time zero (t_0), and in all experimental treatments at times t_{24} h and during HNF exponential growth phase (mostly within t_{48} to t_{66} h). Moreover, for verifying the assumed bacteria-flagellates carbon transfer, the presence of the prey bacteria in HNF food vacuoles was detected (Jezbera et al. 2005).

Phosphorus and chlorophyll *a* (Chl *a*) concentrations

Water used for experiments was analyzed to determine concentrations of (Table 2): Dissolved reactive phosphorus (Murphy and Riley 1962), total phosphorus (the molybdate method detailed in Kopáček and Hejzlar 1993), and Chl *a* determined spectrophotometrically after the extraction with acetone (Lorenzen 1967).

Statistical analysis

Statistical analyses were performed with Statistica v. 13 (Dell). Using an appropriate design of ANOVA we tested differences in growth parameters of flagellates (growth rate, volumetric GGE, and length of lag phase) associated with grazer type (natural planktonic HNF community vs. the mixotrophic flagellate *Poterioochromonas* sp.), season (April, May, August, or October), and bacterial strain or lineage used as prey, or a combination of these factors. We used one-way ANOVA for testing of effects of single factors, two-way ANOVA for testing effects of combinations of two factors; in the case of incomplete design we used Effective hypothesis decomposition. The Unequal N HSD multiple comparison post-hoc tests were used to determine differences between groups.

Results

Environmental relevance of bacterial strains used as prey for natural HNF communities

Sixteen different bacterial strains (Table 1) were used in prey-amendment experiments (an overview in Table 2). We used mainly strains from different lineages of *Limnohabitans* and *Polynucleobacter* genera isolated from the epilimnion of Římov Reservoir (nine bacterial strains out of 16, Table 1), or from the *Limnohabitans* lineages detected at this site by different methods over a seasonal cycle (Šimek et al. 2008, 2014; Jezberová et al. 2017).

Environmental relevance of *Limnohabitans* bacteria was tested with the use of the R-BT065 FISH probe, targeting the R-BT cluster of the genus *Limnohabitans* (covering the Lim B, C, D, and E lineages, Kasalický et al 2013). In the original samples used for experiments (Table 2) and in seasonal studies of the reservoir bacterioplankton (Šimek et al. 2008, 2014) we found the following relative proportions of *Limnohabitans* bacteria (as % of total bacteria; mean and range of values): (1) the spring bloom phase in April (14.1%; 8.4–17.5%); (2) clear water phase in May (9.6%, 7.2–11.8%); (3) summer phytoplankton bloom in August (6.1%, 3.5–9.4%); and October period (5.9, 3.5–9.4%). In experiments IV and VI, conducted in April and May solely with *Limnohabitans* isolates in Lake Cep (Table 2),

the R-BT cluster accounted for 10.2% and 8.3% of total pelagic bacteria in the lake, respectively.

The strains from the *Polynucleobacter* C lineage were used in April and August 2014 (experiments IX and X, Table 2) when this lineage accounted for 6.6% and 3.0% of total bacteria in the reservoir plankton, respectively. The probes targeting the LimA lineage of the genus *Limnohabitans* (probe LimAE-1435) and *M. turicensis* (probe PRD-732), whose representative strains were used only in May 2015 experiment (XI, Table 2), showed that both these bacterial phylotypes formed approximately 2% of total reservoir bacterioplankton.

Growth responses of different flagellate grazers

We employed a virtually identical experimental design in all of the experiments (overview in Table 2) to estimate maximum growth rate (or doubling time), volumetric GGE and length of lag phase after prey amendment of the treatment. Examples of calculations of the parameters are given in Fig. 1 (*see* five prey-amended treatments and the explanatory text), showing time course changes in abundance and biovolumes of the *Poterioochromonas* sp. flagellate related to the rates of decrease in biovolumes of six different prey bacteria added (experiment IB, Table 2). The data for the strain MWH-Wo1 (*Actinobacteria*) and control treatments are not plotted in Fig. 1, as no flagellate growth was detected and thus the growth parameters could not be calculated (*see* also Fig. 2, Supporting Information Fig. S1).

To compare the growth responses of the single species flagellate culture to a mixed community of planktonic HNF (experiments IA and IB in Table 2), the same six prey items were simultaneously added at the same time point to a natural HNF community sample collected from the Římov Reservoir (Fig. 2). The comparison of growth parameters showed significant differences ($p < 0.05$, two-way ANOVA, Fig. 2a,c) in response to amendments of the distinct bacterial preys. In most cases, growth rate and GGE parameters differed significantly for the same prey items utilized by different grazer type (Fig. 2). These parameters were generally higher in the axenic *Poterioochromonas* culture (e.g., the highest GGE values of 49%, 47%, and 44% detected in the II-D5, 2KL-1, and 2Kl.-27 treatments, respectively) compared to the mixed HNF community (Fig. 2b). Only the Molso2 treatment, where smaller cells of the bacterial strain (MCV - 0.049 μm^3, Table 1) were fed to the relatively large flagellate *Poterioochromonas* sp. (cell diameter of 5–6 μm), showed an opposite trend, with significantly lower growth rate and GGE compared to the corresponding natural HNF community. Notably, the strain MWH-Wo1 (Luna 2 cluster, with larger cell volume than that of Molso2, *see* Table 1), did not support any *Poterioochromonas* growth and also induced very limited growth of HNF with GGE values not exceeding 2.5% and lag phase longer than 70 h (Fig. 2a–c).

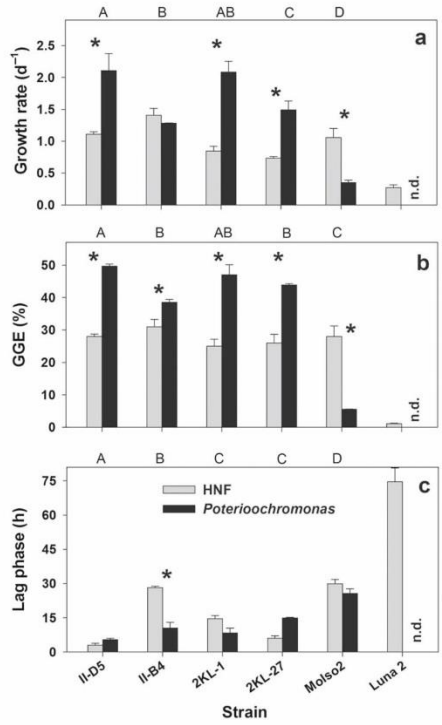

Fig. 2. Growth parameters of HNF communities vs. *Poterioochromonas* sp. Maximum growth rate (**a**), gross growth efficiency (GGE, **b**), and length of lag phase after the treatment amendment (**c**) of a natural planktonic HNF community (Římov Reservoir) in comparison to a culture of the bacterivorous flagellate *Poterioochromonas* sp. amended by additions of the same biovolume of different bacterial prey. The prey bacteria were four strains of the genus *Limnohabitans*, i.e., *L. planktonicus* (II-D5), *L. parvus* (II-B4), 2KL-27 and 2KL-1, and the strains Molso2 and Luna 2 (the MWH-Wo1 strain, for details *see* Tables 1, 2). Values are means for triplicates; error bars show SDs. Different capital letters indicate a significant difference ($p < 0.05$, two-way ANOVA, followed by Unequal N HSD multiple comparison post-hoc test) between treatments amended with different bacterial strains. Stars above bars denote significant difference in the growth responses of the HNF community vs. *Poterioochromonas* sp. growing on the same prey item. n.d. – no growth of *Poterioochromonas* on Luna 2 (the MWH-Wo1 strain) was detected.

Compared to the growth rate and the GGE, the lag phase differed significantly only in one case (the strain II-B4) between the two grazer types.

Prey-specific differences in flagellate growth responses

To examine general trends in bacterial prey-specific growth responses across all experiments conducted with natural HNF communities from the Římov Reservoir and Lake Cep (*see* Table 2 for details), we first pooled all available triplicate data obtained from the same bacterial prey amendments independent of the season during which the experiments were conducted (Fig. 3). Note that some strains were used repeatedly during different plankton successional phases from April to October, reflected in large strain-specific variability in growth data (Fig. 3, e.g., the strain II-D5 used in seven triplicated experiments that yielded a sum of 21 treatments). Some other strains were used only in one particular experiment with HNF (thus representing only one set of triplicate treatments, e.g., 2KL-1, 2KL-27; *see* Tables 1, 2).

Though the boxplots shown in Fig. 3a,c,e indicated very large variability in HNF growth parameters, these strain-specific data for growth rate, GGE and the length of lag phase significantly differed (for details *see* Supporting Information Table S2) among all bacterial strains tested ($p < 0.001$, one-way ANOVA, followed by Tukey multiple comparison test). Thus for instance, strain 2KL-27 supported significantly slower HNF growth rate ($p < 0.05$, Tukey test) compared to other eleven strains from *Limnohabitans* and *Polynucleobacter* lineages. The food characteristics of the strains 2KL-3 and of *M. turicensis* yielded significantly smaller GGE ($p < 0.001$ and $p < 0.05$, respectively) compared to other seven strains. Interestingly, significant differences ($p < 0.05$, Supporting Information Table S2) were found even among closely related strains from the LimC lineage in growth rate (2KL-27 vs. II-B4, II-D5, Rim28, T6-5, 2KL-3, and Rim47) and GGE (e.g., 2KL-3 vs. II-B4 and Rim28 strains, Supporting Information Table S2). However, prey amendments with the strain MWH-Wo1 (Luna 2 cluster) clearly yielded the most distinct HNF growth responses. They yielded frequently significantly longer lag phase, in eight cases they differed significantly in combination of two parameters from other prey amendments (most frequently long lag phase coupled with low GGE; Fig. 3b,c), or even in all three HNF growth parameters simultaneously (Supporting Information Table S2).

The data in Fig. 3a,c,e revealed typical range of values in net HNF maximum growth rate, volumetric GGE and lag phase after prey amendments for each strain. For instance, generally all strains, with the exception of the Luna 2 strain, supported relatively rapid growth with considerably high GGE values. The mean and median values for all growth measures detected for the strain MWH-Wo1 (Luna 2 in Fig. 3a,c,e) indicated a low nutritional value of this prey for HNF communities. In most cases, these treatments yielded rather low growth rate, GGE, and fairly long lag phase, reflecting a generally long adaptation period before the HNF communities displayed any measurable growth.

Box plots in Fig. 3b,d,f present variability and mean and median values of the growth parameters across all strains tested (a sum of 129 pooled treatments originating from 43 triplicate prey-amendment experiments). This gives estimates of growth of planktonic HNF with mean and median growth rates of 1.61 d^{-1} and 1.66 d^{-1} (community doubling times of 10.3 h and 10.1 h, respectively). For comparison, Fig. 3b shows also variability in growth rate of HNF communities growing in planktonic samples from the Římov Reservoir filtered through 5 μm-pore-size filters (removing HNF predators) and incubated in dialysis bags in situ without any bacterial prey amendments (21 treatments taken from Šimek et al. 2006). These treatments, where the growing HNF populations grazed only on indigenous bacterioplankton supplied by nutrients coming from an ambient environment through a dialysis membrane, yielded quite similar mean and median values of 1.75 d^{-1} and 1.59 d^{-1} (corresponding to doubling times 9.5 h and 10.5 h, respectively).

Most volumetric GGE values ($5^{th}/95^{th}$ percentile) ranged between 16% and 38%, with very small differences between the mean and median values (28.4% and 29.6%, respectively; Fig. 3d). However, some strains yielded either very high mean GGE values over 41% (strain Rim8 from the LimA lineage of the genus *Limnohabitans*), or very low mean GGE of < 12% detected for the strain MWH-Wo1 from the Luna 2 cluster. Also the length of the lag phase before the onset of HNF growth ranged considerably even for a given strain. Across all strains tested most of the lag phase duration values ($5^{th}/95^{th}$ percentile) ranged between 0.5 h and 35 h, with similar mean and median values (16.6 h and 16 h, respectively; Fig. 3f). The Luna 2 cluster (MWH-Wo1) strain was again an outlier, with the longest lag phase (Fig. 3e).

Relationships between growth parameters of flagellates

Regardless of the high variability in growth parameters (Figs. 1–3), some general trends were also obvious. High GGE values for particular treatments were usually accompanied by a short lag phase and relatively high values of maximum growth rate (*see* e.g., the strains II-D5 and 2KL-1 in *Poterioochromonas* treatments, Fig. 2). In contrast, the treatments amended with the Luna 2 cluster (MWH-Wo1) strain exemplify an opposite trend, with slow HNF growth or no growth (*Poterioochromonas* culture), in combination with low GGE values and fairly long lag phase (Figs. 2, 3a,c,e).

To confirm these trends statistically, we used data from all experiments conducted with both predator types and plotted mean values (48 triplicate treatments; Fig. 4). The linear regression analysis indicated a highly significant inverse relationship between the length of the lag phase on one side, and the maximum growth rate and GGE on the other side (Fig. 4a,b). Approximately 22% and 59% of variability in maximum growth rate and GGE, respectively, were explained by the variability in the length of lag phase. In contrast, GGE values were significantly positively correlated with maximum growth (Fig. 4c).

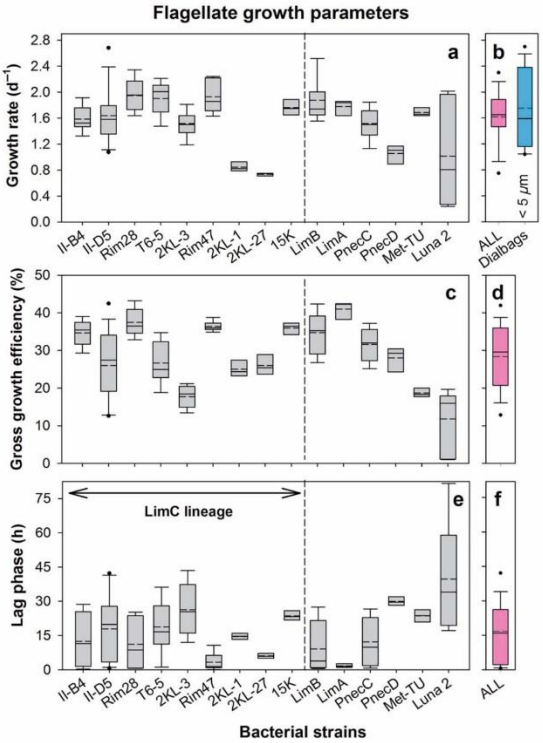

Fig. 3. Overall variability in bacterial prey-specific responses across all experiments conducted with natural HNF communities from Římov Reservoir and Lake Cep amended by additions of different bacterial strains. A total of 16 different bacterial strains were tested as HNF food, with some of them being used repeatedly during different plankton phases from April to October (for details *see* Table 1; Fig. 5). Data variability of triplicate treatments in HNF maximum growth rate (**a**), volumetric GGE (**c**), and lag phase (**e**) are shown in box plots with 5th/95th percentile (full symbols are outliers, full and dashed lines, median and mean value, respectively). The box plots representing the data for bacterial strains labeled by the strain codes (Table 1), situated to the left of the vertical dashed line in panels (**a**, **c**, **e**), are affiliated to the LimC lineage of the genus *Limnohabitans*. Other six prey categories plotted right side of the dashed line belong to different bacterial lineages and are largely represented by only one bacterial strain: *Limnohabitans* lineages—LimB (strain Rim11) and LimA (Rim8); *Polynuclobacter* lineages—PnecC (two strains with identical rRNA sequences, czRimov8-C6 and czRimov-FAMC1) and PnecD (strain Molso2); Met-TU (*M. turicensis*, strain MMS-10A-171); and Luna 2 cluster of *Actinobacteria* (strain MWH-Wo1). Significant differences between growth parameters of natural HNF communities growing on the different bacterial prey categories are shown in Supporting Information Table S2. Panels (**b**, **d**, **f**) show variability in the growth parameters for pooled data of all tested strains (ALL, pink boxplots). Panel (**b**) shows also variability in growth rate of HNF communities growing in plankton samples from Římov reservoir filtered through 5 µm-pore-size filters (removal of HNF grazers) and incubated in dialysis bags in situ without any bacterial prey amendments (Dialbags, the blue boxplot presenting the data taken from Šimek et al. 2006, for details *see* the text).

Interestingly, very high volumetric GGE values were detected in the *Poterioochromonas* predator treatments (~ 40–49.6%, red symbols in Fig. 2b) amended by four strains from the LimC lineage of *Limnohabitans* that form a separate cluster in Fig. 4a. In contrast, nine bacterial strains from this lineage used in 29 treatments with natural HNF communities (Table

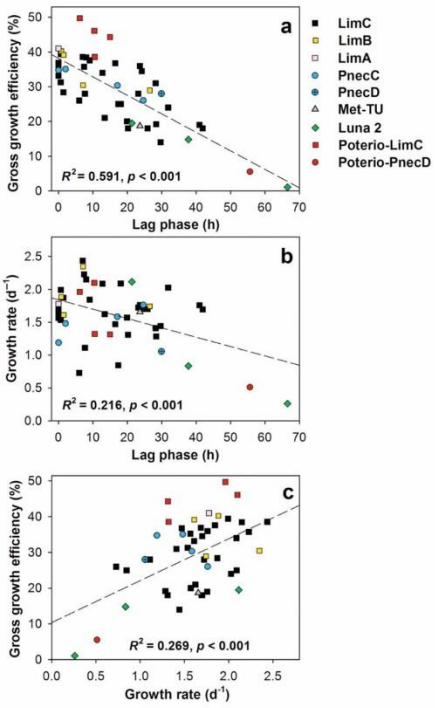

Fig. 4. Relationships between flagellate growth parameters with data pooled from all experiments (*see* Table 2) conducted with samples from Římov Reservoir, Lake Cep and with the *Poterioochromonas* sp. culture amended by different bacteria: (**a**) Gross growth efficiency (GGE) and maximum growth rate (**b**), related to length of the lag phase after the treatment was amended by different prey bacteria, and (**c**) GGE related to maximum growth rate fitted by linear regression. R^2 is the coefficient of determination of the regressions between the pairs of the parameters ($n = 48$). Bacterial strains affiliated to lineages LimA, LimB, LimC, PnecC, PnecD and the strains *Methylopumilus turicensis* (Met-TU) and MWH-Wo1 (Luna 2) were plotted separately in different symbols and colors. Note that the prey category LimC represents data gained with nine different strains in 29 treatments (compare Fig. 3; Table 1). Each data point represents the mean values from triplicate treatments. The data for the growth parameters of *Poterioochromonas* sp. on LimC and PnecD lineages are plotted as red symbols (Poterio-LimC and Poterio-PnecD) but they are involved in the overall regression analysis depicted in panels (**a**–**c**).

2) did not show similarly high GGE values (Fig. 4a), yet the growth rates were comparable for both predator types (Fig. 4b). Generally, the results obtained with the strains from the LimC, LimB, and PnecC lineages, as well as for the Luna 2 cluster showed a rather broad variability in growth parameters (Figs. 3, 4). Moreover, the data for the Luna 2 strain suggested a low nutritional value of this prey for HNF except for one data point. Moreover, we detected no growth of *Poterioochromonas* on this strain even after 112 h of the experiment (data not shown). Thus this "zero growth" point could not be used in the regression analysis while it clearly indicated inappropriateness of the prey for the predator. In contrast, in all other cases we detected measurable flagellate growth already within 66 h of the experiment, as shown in Fig. 4a,b.

Lineage- and season-specific differences in HNF growth responses to prey amendments

For further testing we selected only treatments where the same strain or strains affiliated to the same bacterial lineage were used in at least two seasonally different experiments in Římov Reservoir or Lake Cep. Thus, four lineage-specific data sets were assembled (Supporting Information Fig. S9): (1) Luna 2, the strain MWH-Wo1 was used in four experiments; (2) LimB, strain Rim11 was used in four experiments; (3) LimC, nine distinct strains were used, some of them tested repeatedly in ten experiments; and (4) PnecC, two strains used in two experiments (*see* Table 2). The differences in HNF prey-specific growth responses were first tested among these four prey groups independent of the season. The data characterizing growth responses of HNF to the strain from the Luna 2 cluster significantly differed in all growth parameters from the other three bacterial prey categories ($p < 0.05$, Supporting Information Fig. S9). In contrast, we did not find significant differences ($p > 0.05$) in HNF growth responses to prey amendments with strains from LimB, LimC, and PnecC prey categories.

To reveal season-specific aspects of growth responses of HNF communities to the four bacterial prey categories defined previously (*see* Supporting Information Fig. S9), we tested separately the data from experiments conducted in April, May, August, and October (Fig. 5; Supporting Information Table S1). Independent of the season the data for the Luna 2 cluster strain always differed significantly (two-way ANOVA, $p < 0.05$) in the growth parameters from other three bacterial prey categories. However, overall variability in HNF growth rate (Fig. 5a–d), as a response to amendments with the four prey categories, did not differ significantly over the four seasonal phases ($p > 0.05$). Significant season-specific differences ($p < 0.05$) were detected for GGE values in May (rather low values, Fig. 5f) and August (generally high GGE, Fig. 5g) that both differed from April and October data (Fig. 5e,h). However, the latter two data sets for GGE did not significantly differ one from another. The shortest lag phase (below 2.6 h) and significantly higher GGE values were detected for August compared to other seasons. Overall, the combination of growth parameters detected in August treatments, i.e., the highest GGE and shortest lag phase

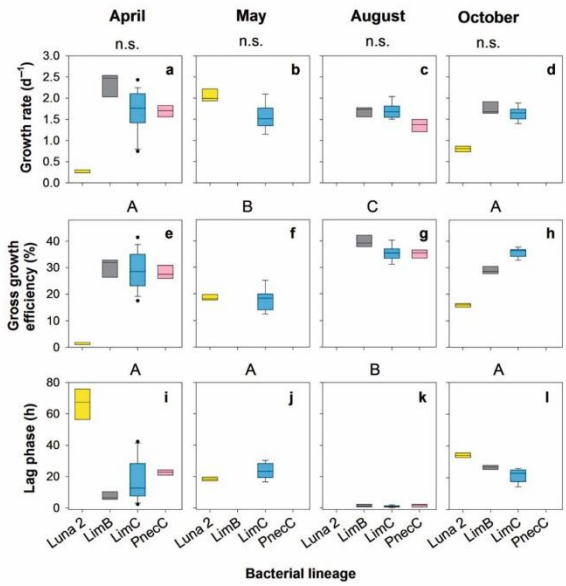

Fig. 5. Comparison of season-specific variability (experiments conducted in April, May, August, and October in Římov Reservoir or Lake Cep, for details *see* Table 2) in growth responses of natural HNF communities amended by additions of the same prey group, i.e., Luna 2 cluster (strain MWH-Wo1, yellow bars), LimB lineage (strain Rim11, grey bars), and the pooled data for the closely related strains belonging to LimC (blue bars) and PnecC (pink bars) lineages. Data variability is shown in box plots with $5^{th}/95^{th}$ percentile (full symbols are outliers, full line shows median value) of flagellate maximum growth rate (panels **a**–**d**), GGE (panels **e**–**h**), and lag phase (panels **i**–**l**). Overall, independent of the season, the data for the Luna 2 cluster always differed significantly (Two-way ANOVA, Effective hypothesis decomposition, $p < 0.05$) in the growth parameters from other three bacterial prey categories. The season-specific significant differences (Unequal N HSD multiple comparison test, $p < 0.05$) in the HNF growth responses to the added prey items are indicated by capital letters on the top of panels (**e**–**l**); n.s. – not significant (panels **a**–**d**).

(Fig. 5g,k), indicated the minimum time needed for the indigenous HNF communities to adapt to particular prey amendments (Table 2; Supporting Information Figs. S5, S7).

We also tested season-specific differences in median cell volume of HNF (Supporting Information Fig. S10). Only in the May samples (clear water phase) the flagellate cells were significantly smaller (median 10.5 μm^3, $p < 0.001$) compared to other phases with median values around 20 μm^3. Notably, in samples used in experiments done in May, also the median and mean values of HNF cell volume were almost identical, thus indicating a rather uniform small cell size of HNF. This fact was partially reflected in the combination of generally longer lag phase and lower GGE values in May, when the small flagellate cells were suddenly exposed to generally large bacteria from LimC lineage, such as the strains T6-5 and 2KL-1 (Tables 1, 2). Interestingly, however, only in late May (experiment XI, Table 2) the offered smaller cells of the Luna 2 strain (Table 1) induced more rapid HNF growth (Fig. 5b,f,j).

Discussion

Major findings

We examined the growth potential and biomass transfer efficiency of natural HNF communities feeding on a very broad spectrum of relevant planktonic bacterial groups so far not employed in previous investigations (cf. Šimek et al. 2013; Grujčić et al. 2015; Weisse et al. 2016). Moreover, our study has brought new insights into food quality aspects of the prey bacteria and revealed both general and prey-specific trends in the growth responses of natural HNF communities to changing prey food quality represented by 16 distinct

bacterial strains (Figs. 1–4). We found many significant differences in the responses of HNF communities to the prey amendments by distinct, but also by closely related bacterial strains (Supporting Information Table S2). Moreover, we demonstrated that even the same bacterial prey can produce different HNF growth responses depending upon the season (Fig. 5), being likely related to marked compositional shifts in temporally evolving flagellate grazer communities (Domaizon et al. 2003; Mangot et al. 2013).

Effects of prey food quality on flagellate growth

Our results document a large variability in HNF growth responses (Figs. 3, 4) with many season-specific aspects (Fig. 5) related to different temporal community dynamics of bacterivorous HNF that have been previously described (e.g., Šimek et al. 1997; Domaizon et al. 2003; Nolte et al. 2010). However, the responses of the natural grazer communities to enrichment with particular prey over different plankton successional phases have rarely been demonstrated (Grujčić et al. 2015). Additionally, comparisons of the same prey amendments in samples from the reservoir and the lake revealed significant differences in HNF growth parameters that can be related to different seasonal succession of plankton and different trophic status of the lakes (experiments III–VI, for a detailed analysis *see* also Grujčić et al. 2015). Thus, e.g., prey amendment with relatively large bacterial cells, such as those of the 2KL-3 strain (Table 1), can paradoxically support rather low growth rate and GGE values during clear water phase with small flagellate cell sizes present (cf. Supporting Information Fig. S10), likely due to a shift to suboptimal predator–prey size ratio (Hansen et al. 1994; Boenigk et al. 2004). It can even significantly reduce the transfer efficiency from such a prey (Fig. 3; Supporting Information Table S2) and so also the significance of the bacteria-HNF trophic link.

To our knowledge, none of the strains used in this study displayed any detectable morphology-related traits of grazing-resistance such as flock- or filament-formation (Hahn and Höfle 2001; Jürgens and Matz 2002). Moreover, using group-specific FISH-probes all bacterial prey types were observed in flagellate food vacuoles, as exemplified in Šimek et al. 2013 (Supporting Information Fig. S1 therein) and Grujčić et al. 2015 (Fig. 4 therein). In all cases, we also observed a prey abundance decrease during the course of the experiments (*see* examples in Fig. 1). This holds even true for the gram-positive MWH-Wo1 strain (Luna 2 cluster), which supported no (*Poterioochromonas*) or frequently only very limited growth of flagellate predators (Figs. 2, 3). However, in one experiment only (XI, Table 2) this apparently less utilizable prey (MWH-Wo1 strain, *see* also Tarao et al. 2009) of medium cell size (Table 1) supported considerably elevated HNF growth rate with GGE of $\sim$ 19% (Fig. 5b,f), which clearly points to the significance of the initial composition of the grazer community used in the experiment. We anticipate that natural HNF assemblages contain also bacterivorous flagellates that can relatively efficiently utilize gram-positive *Actinobacteria* considered to be partially grazing-protected (Pernthaler et al. 2001). Members of the Luna 2 cluster are assumed to be grazing resistant due to specific surface structures of their cell walls (Tarao et al. 2009). Notably, all strains were likely at least partially utilizable by the flagellates, although the different prey food quality results in the large prey-specific variability in HNF growth parameters (Figs. 3–5; Supporting Information Table S2). An intriguing question then arises: how much time does it takes before the HNF community composition shifts to efficiently utilize the available bacterial prey? Notably, many strains supported relatively rapid growth and hence the differences in lag phase seem to be related to the "adaptation time" needed to optimize the grazer community composition to perform well on the available bacterial prey (Šimek et al. 2013).

The culture of *Poterioochromonas* showed mostly significantly higher GGE values when growing on strains from the LimC lineage of *Limnohabitans* compared to the HNF community growing on the same prey items (Fig. 2a). However, even the high GGE values of 39–49% are well within the range of data obtained in laboratory experiments with various protistan cultures (Straile 1997). Thus the food quality of the LimC strains and their suitable cell sizes (Table 1) were likely the primary reasons yielding the high GGE values obtained with the predator culture (experiment IB). In contrast, the smaller cell size of the bacterial strain MoIso2 (PnecD lineage, Table 1) and its specific food quality aspects compared to the strains from LimC lineage (used in both experiments IA and IB, Table 2) likely limited the growth of this flagellate culture. However, the natural HNF community, composed of a mixed community of flagellate grazers of various sizes, grew on the strain MoIso2 at rates comparable to those achieved by HNF growing on the strains from the LimC lineage of *Limnohabitans* (experiment IA, Fig. 2).

Estimates of HNF growth rate and growth efficiency

The prey-amended natural HNF communities yielded mean community doubling time of 10 h and volumetric GGE around 29% (Fig. 3b,d). Also some previous studies (Jürgens and Matz 2002; Weisse et al. 2016 and references therein) reported very rapid doubling times of HNF communities in situ, comparable to our growth results (Fig. 3b). Moreover, our GGE estimates fit quite well the literature values of GGE reviewed in Straile (1997), based on numerous studies dealing with growth efficiency of both macro- and microzooplankton groups including laboratory cultures of small protists.

Notably, in situ studies where no bacterial prey was added into < 5 μm treatments (removal of zooplankton predators of HNF) and samples were incubated in dialysis bags in the Římov Reservoir (Jezbera et al. 2006; Šimek et al. 2006), showed a relatively similar range of HNF growth rates (Fig.

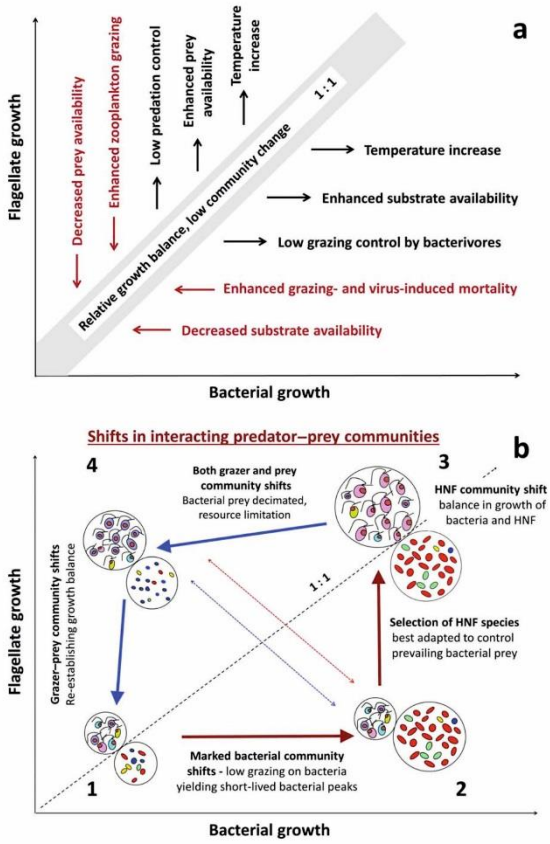

Fig. 6. A general model based on the idea that major groups of both bacterioplankton and bacterivorous HNF have comparable growth potentials in pelagic systems. Panel (**a**)—the gray area represents relative growth balance between bacteria and bacterivorous HNF, yielding an approximate 1 : 1 ratio at which a relative stability of both bacterial and HNF community compositions and growth are assumed. The black text and arrows represent factors that may stimulate either bacterial or flagellate growth; the red text and red arrows represent factors leading to decreases in bacterial and flagellate growth (for details *see* the text). Panel (**b**) illustrates four model phases labeled as 1–4, with colors and sizes of drawings and circles indicating shifts in bacterial and flagellate cell and community sizes. Phase 1—balance in relatively low growth of both bacteria and HNF. Phase 2—a marked (e.g., bottom-up induced) bacterioplankton community shift toward rapidly growing bacterial species of large cell size, yielding a temporal imbalance in bacterial prey community and their grazers that results in low grazing control of the bacteria and short-lived bacterial peaks. Phase 3—the bacterial outgrowth stimulates a flagellate predator population increase and its community shift, resulting in temporal re-establishment of the prey–predator balance at high growth rates. Phase 4—resource limitation of the prevailing bacterial prey constrains bacterial population growth and thus further growing grazer populations decimate the bacteria and in consequence resource limitations and community shifts in both predator and prey assemblages are assumed. The arrow connecting phases 4 and 1 suggests re-establishment of the predator–prey balance at low growth rates at the original start of the model cycle. However, two bi-directional dashed arrows in the middle of the picture indicate that there are many transient stages from imbalance to temporal growth balance between the predator and prey communities.

3b). In fact, these dialysis bag incubations in the reservoir can be considered as a measure of "carrying capacity" of this plankton environment in terms of the carbon pool available for growth of indigenous bacteria that fueled the HNF community growth in temperatures of 13–24°C (Šimek et al. 2006). In both types of experimental incubations, the HNF maximum growth rates and abundance peaks were mostly achieved in 36–72 h (compare examples of HNF growth curves in Supporting Information Figs. S1–S8). Thus our experimental prey amendments mimicked quite well, at least in terms of organic carbon introduced in bacterial biomass (I–XI, Table 2), the amount and rates of biomass transfer from bacteria to HNF in the reservoir plankton.

Theoretically one should assume faster growth potential of prey bacterial communities because they are dominated by cells with approximately two orders of magnitude smaller cell volumes compared to their flagellate grazers (Hansen et al. 1994; Boukal 2014). However, since bacteria are at the bottom of the food chain of a complex pelagic environment they are likely to be more strongly bottom-up than top-down controlled (McQueen et al. 1986; Gasol and Vaqué 1993), apart from being selectively top-down controlled by protistan grazing and viruses (Jürgens and Matz 2002; Weinbauer 2004). Thus it is not surprising that the same bacterial phylotypes (targeted by FISH probes) grow more slowly in situ than representative bacterial isolates from the same taxon do grow in substrate-optimized pure culture conditions (Kasalický et al. 2013). In contrast, small bacterivorous flagellates in our meso- and eutrophic study systems, with bacterial densities of $1.5–4.5 \times 10^6$ cells mL^{-1} (Table 2), were likely close to the saturation prey levels and thus HNF grew very rapidly, close to their maximum growth rates (Jürgens 1992; Arndt et al. 2000). However, HNF are usually top-down regulated by micro- and macrozooplankton (Jürgens et al. 1996; Zöllner et al. 2003; Šimek et al. 2014), which can explain the lack of a simple coupling between the abundance of bacterivorous HNF and their bacterial prey in some pelagic systems (Gasol and Vaqué 1993).

Rapid shifts in interacting flagellate predator-bacterial prey communities

Our estimates of HNF growth rates (Fig. 3) resemble tightly the maximum growth rates detected in rapidly growing bacterioplankton groups considered as "algal bloom specialists," such as those of *Limnohabitans*, *Fluviicola* sp. and species-like tribes of *Flavobacteria* (Zeder et al. 2009; Eckert et al. 2012; Neuenschwander et al. 2015). Their short-lived peaks, co-occurring with various phytoplankton taxa, last usually for a few days only and are frequently terminated by enhanced HNF abundance and bacterivory (Zeder et al. 2009; Eckert et al. 2012; Šimek et al. 2014). The latter studies, based on the use of specific FISH-probes, indicated that abundances of rapidly growing bacterial taxa double within 6–20 h.

Thus importantly, the major taxa of bacterioplankton prey (e.g., Zeder et al. 2009; Eckert et al. 2012) as well as predator communities (Arndt et al. 2000; Boenigk and Arndt 2002; Jürgens and Matz 2002) possess high growth potential that apparently contributes to their relative growth balance in situ as suggested in a simplified conceptual model (Fig. 6). However, a broad array of major bottom-up and top-down controlling factors, such as shifts in resource availability or in major bacterial mortality factors (Fig. 6a), can either accelerate or slow down the growth of both prey and predator populations (Gasol and Vaqué 1993). Rapid shifts in major top-down and bottom-up regulating factors can either result in temporal growth imbalance (phases 2 and 4 in Fig. 6b) in the predator–prey assemblages, or re-establishment of the growth balance but already at different, either low or high rates (Fig. 6b, *see* phases 1 and 3, respectively). Then, for instance, a sudden pulse in nutrient availability can induce the outgrowth of rapidly dividing bacterial species (predicted by phase 2 in Fig. 6b) that would then result in species-specific short-lived peaks of particular bacterial species (Zeder et al. 2009; Eckert et al. 2012; Šimek et al. 2014). Such an environmental scenario (observed mainly during spring bloom phases, e.g., Šimek et al. 2014 and references therein), diverts the predator–prey system to temporal growth imbalance with higher bacterial cell production than bacterial loss rates (Gasol and Vaqué 1993) till more abundant, or rapidly growing and likely distinct flagellate predator groups appear (phase 3, Fig. 6b). Such rapid flagellate community shifts induced by changing prey food quality and availability have already been demonstrated in the experiment IA (Šimek et al. 2013) and experiment X (Grujčić and Šimek, unpubl.; *see* also the text below).

We are aware that the proposed model oversimplifies the complexity of this trophic linkage and thus cannot reflect all naturally occurring predator–prey interactions. However, we hypothesize that major driving forces that fine tune these trophic interactions are not just changes in abundance, but mainly marked community shifts at both prey (Jürgens and Matz 2002; Šimek et al. 2006) and predator levels (Šimek et al. 2013), as proposed in our model (Fig. 6b). The rapid and significant community shifts optimize survival strategies and growth responses at both trophic levels. Disturbances at either side of the trophic link ((due to, e.g., resource depletion for bacteria (Gasol and Vaqué 1993), or enhanced zooplankton predation on flagellates (Jürgens et al. 1996)) induce marked responses that facilitate temporal re-establishment of the relative growth balance at different growth rates, however, already with differentially composed predator–prey communities (Fig. 6a,b).

The model predictions are supported by evidence from both field and laboratory studies. For instance, specific analyses of flagellate food vacuole contents clearly demonstrated both positive selections for, and negative selection against, certain bacterial taxa in plankton samples (Jezbera et al. 2006; Šimek et al. 2014). Feedbacks of bacterial food quality on predator community composition and growth are important but unfortunately rarely studied (Weisse et al. 2016). Notably, sequence data demonstrated that significant prey-specific shifts in the HNF predator communities were induced by

sudden shifts in bacterial prey availability (experiment IA, for details *see* Šimek et al. 2013). Moreover, in the light of our data, we assume that these shifts can be strongly season-specific (Fig. 5). For instance, we used closely related *Limnohabitans* strains from the LimC lineage in two experiments with samples from the Římov Reservoir, scheduled during different seasons (IA and X, Table 2). In the spring experiment IA (April 2011), an analysis of eukaryotic 18S rDNA sequences showed strong prey-specific HNF community shifts within *Stramenopiles*, being reflected at higher taxonomic resolution mainly through changing proportions of bacterivorous chrysophytes—i.e., *Pedospumella* and several *Spumella*-related lineages (for details *see* Šimek et al. 2013). In the summer experiment X (August 2014, Table 2), 18S rDNA sequencing and quantification of some major groups of bacterivorous flagellates by specific FISH probes resulted in a different initial HNF community, dominated by colorless members of the phagotrophic *Cryptophyta*, its CRY1 lineage (Piwosz et al. 2016) or phagotrophic *Katablepharidophyta* (Grujčić, unpubl.). Notably, prey-specific HNF community shifts in this experiment were mediated mainly through changing proportions of bacterivorous lineages of *Cryptophyta* and *Choanoflagellida* (Grujčić, unpubl.). This comparison illustrates that even closely related prey items can induce temporarily quite different patterns of prey-specific HNF community shifts; a phenomenon that has rarely been documented so far.

The shifts in both prey and predator communities are likely closely interconnected and occur within a time span of approximately half a day to a few days. The shifts in flagellate communities may be the result of rarer taxa becoming dominant with changing environmental conditions (Caron and Countway 2009; Nolte et al. 2010). In this study, we demonstrated that such rapid adaptations of the predator community have fundamental importance for the efficiency of organic matter transfer to the grazer food chain. The rapid HNF community shifts (Fig. 6b) and flagellate selective feeding on fast-growing or larger bacteria (thus cropping bacterial production rather than the standing stocks, Sherr and Sherr 2002; Jezbera et al. 2005) are changing our views on time scales at which substantial changes in carbon flow can occur. However, to document these processes at high taxonomic resolution in situ, there is an urgent need to establish novel detection techniques, such as CARD-FISH with highly specific probes (Massana et al. 2009; Piwosz and Pernthaler 2010; Mangot et al. 2013) that would allow us to precisely quantify major freshwater flagellate bacterivores without sample manipulation.

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Cryptophyta as major bacterivores in freshwater summer plankton

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ABSTRACT

Small bacterivorous eukaryotes play a cardinal role in aquatic food webs and their taxonomic classification is currently a hot topic in aquatic microbial ecology. Despite increasing interest in their diversity, core questions regarding predator-prey specificity remain largely unanswered, e.g. which heterotrophic nanoflagellates (HNFs) are the main bacterivores in freshwaters and which prokaryotes support the growth of small HNFs. To answer these questions, we fed natural communities of HNFs from Římov reservoir (Czech Republic) with 5 different bacterial strains of the ubiquitous betaproteobacterial genera *Polynucleobacter* and *Limnohabitans*. We combined amplicon sequencing and catalysed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) targeting eukaryotic 18S rRNA genes to track specific responses of the natural HNF community to prey amendments. While amplicon sequencing provided valuable qualitative data and a basis for designing specific probes, the numbers of reads was insufficient to accurately quantify certain eukaryotic groups. We also applied a double-hybridization technique that allows simultaneous phylogenetic identification of both predator and prey. Our results show that community composition of HNFs is strongly dependent upon prey type. Surprisingly, Cryptophyta were the most

abundant bacterivores although this phylum has been so far assumed to be mainly autotrophic. Moreover, the growth of a small lineage of Cryptophyta (CRY1 clade) was strongly stimulated by one *Limnohabitans* strain in our experiment. Thus, our study is the first report that colorless Cryptophyta are major bacterivores in summer plankton samples and can play a key role in the carbon transfer from prokaryotes to higher trophic levels.

Keywords: *Heterotrophic nanoflagellates, Cryptophyta, eukaryotic community composition, flagellate bacterivory, freshwater food webs, next-generation sequencing, double-hybridization technique.*

INTRODUCTION

Heterotrophic nanoflagellates (HNFs) undoubtedly belong to the most abundant eukaryotes on Earth, inhabiting freshwaters, oceans, sediments and soils (Arndt *et al.*, 2000; Pernice *et al.*, 2014; Massana *et al.*, 2015; Simon *et al.*, 2016). They are particularly abundant in planktonic communities, acting as primary prokaryotic grazers and thus playing an essential role in nutrient cycling (Boenigk and Arndt, 2002b; Weisse, 2002; Worden *et al.*, 2015; Caron *et al.*, 2016, 2017). They also represent the most important link between dissolved organic matter (DOM), and its transfer through growing bacterial cells to higher trophic levels (Matz *et al.*, 2002; Pernthaler, 2005; Azam and Malfatti, 2007). Despite their importance and abundance they have received less attention than prokaryotes (Debroas *et al.*, 2015; Simon *et al.*, 2015) and their diversity has been generally less investigated in freshwaters (Nolte *et al.*, 2010; Debroas *et al.*, 2017) than in oceans (Logares *et al.*, 2014; Pernice *et al.*, 2014; Massana *et al.*, 2015 del Campo *et al.*, 2015). Furthermore, knowledge of which species or taxa are the most important bacterivores in freshwaters and which bacteria are actually consumed by these small protists still remains poorly understood (Jezbera et al. 2005, Pernthaler 2005; Šimek et al 2013). Some studies however, pointed to the importance of flagellates related to *Spumella* spp., that rapidly respond to sudden bacterial prey amendments (Šimek *et al.*, 2013; see also Boenigk *et al.*,

2005, 2006; Grossmann *et al.*, 2016a), implying that these flagellates are significant bacterivores.

Furthermore, small size and inconspicuous morphology of HNFs makes them hard to be identified via classical epifluorescence microscopy but the advance of high-throughput sequencing (HTS) facilitated an easier taxonomic classification of these smallest eukaryotes (Simon *et al.*, 2015; del Campo *et al.*, 2015; de Vargas *et al.*, 2015; Debroas *et al.*, 2017). While HTS represents an efficient tool for an identification of different taxa in a sample, one of the main problems of this approach is how well the number of reads obtained by HTS corresponds to the real cell abundance (Wintzingerode *et al.*, 1997; Hong *et al.*, 2009; Giner *et al.*, 2016). A method enabling microscopic visualization and thus providing a more accurate quantification of specific cells, by using oligonucleotide probes as phylogenetic markers, is CARD-FISH. While there are many publications exploiting HTS (Lepère *et al.*, 2007; Mangot *et al.*, 2013; Taib *et al.*, 2013; Debroas *et al.*, 2017) or CARD-FISH approaches (Not *et al.*, 2002, 2005; Lefèvre *et al.*, 2005; Mukherjee *et al.*, 2015; Piwosz *et al.*, 2013, 2015, 2016) to analyze microbial eukaryotic communities, a combination of both methods has rarely been used (Giner *et al.*, 2016).

Contrasting to flagellates, abundance and diversity of bacteria in freshwaters is well documented, indicating the dominance of a few ubiquitous phylogenetic lineages of Alpha- and Betaproteobacteria, Actinobacteria and Bacteroidetes (Newton *et al.*, 2011). Among

Betaproteobacteria, the genera *Limnohabitans* (Kasalický *et al.*, 2013) and *Polynucleobacter* (Hahn *et al.*, 2009) are very abundant members of freshwater plankton(i.e. those to be most likely met in planktonic environments by flagellates). Previous research showed that some bacteria of the genus *Limnohabitans* induced prey-specific differences in flagellate growth parameters (Grujčić *et al.*, 2015), which influenced the community composition of flagellates (Šimek *et al.*, 2013) While *Limnohabitans* and *Polynucleobacter* are both highly abundant in a broad array of habitats, they exhibit contrasting lifestyles (Jezbera *et al.*, 2012). *Limnohabitans* have high growth rates and limited morphological versatility *in situ* (Šimek *et al.*, 2001, 2006) which makes them highly vulnerable to protistan grazing (Jezbera *et al.*, 2005; Šimek *et al.*, 2006; Salcher *et al.*, 2008). They possess generally larger genomes (2.5 - 4.9 Mb (Zeng, 2012, Kasalický *et al.*, 2017)), a high metabolic flexibility (Shabarova *et al.*, 2017; Kasalický *et al.*, 2013), and larger mean cell volumes compared to other planktonic prokaryotes (Šimek *et al.*, 2006, 2014; Kasalický *et al.*, 2013). In contrast to *Limnohabitans*, members of the *Polynucleobacter* genus have medium-sized genomes (2.0 - 2.4 Mbp; Hoetzinger *et al.*, 2016; Hahn *et al.*, 2017), a generally smaller cell size, and a more passive lifestyle relying on photodegradation products of humic substances (Hahn *et al.*, 2012). However, data on *in situ* grazing-induced population turnover rates of these bacteria is still missing (Hahn *et al.*, 2012). All the

above mentioned characteristics of the two bacterial groups makes them suitable models for testing carbon flow to higher trophic levels.

We can assume that certain bacterial taxa, especially those with high growth and grazing induced mortality rates, should have a prominent role in carbon flow (acting as 'link', Sherr *et al.*, 1987) to higher trophic levels in a particular environment. Thus, the growth parameters of natural HNF communities feeding on such taxa can be used as a measure of carbon flow from a specific bacterial group to grazers and, furthermore, of the food quality of a particular bacterial prey for HNF. It has already been demonstrated that not all bacteria stimulate the growth of HNF in the same way and their growth efficiencies directly affect the carbon flow to higher trophic levels (Šimek *et al.* 2013). We thus assume that prey quality and availability can severely influence the community composition of HNF.

In this study, we conducted short-term manipulation experiments by the addition of different strains of planktonic Betaproteobacteria to a natural HNF population. Since bacterivorous flagellates and bacteria grow with approximately the same high growth rates in plankton environments (approx. 10 h doubling time, Šimek *et al*, 2017, in press) short-term experiments with high sampling frequency allowed us to efficiently track major trends in growth and community responses of HNF amended by different prey. We combined amplicon sequencing of 18S rRNA genes and CARD-FISH with newly designed probes based on amplicons to

quantify and visualize major freshwater flagellate bacterivores. We also applied a double hybridization technique, developed by Massana *et al.*, (2009) and advanced in this study to verify taxonomic affiliations of both grazers and prey at the same microscopic preparation. This approach is, to our knowledge, rarely used in current microbial ecology. With these techniques we intended to address the following aims: (a) to investigate the effects of different bacterial prey characteristics on the growth of natural freshwater bacterivorous flagellates, (b) to examine which flagellate taxa are key bacterivores in experimental treatments, based both on abundances and specific grazing rates of prominent HNF lineages, (c) and finally to examine the quantitative match between HTS and CARD-FISH targeting prominent flagellate bacterivores in our prey-amended treatments.

MATERIALS AND METHODS

Experimental design

We applied a similar experimental design to that detailed in Šimek *et al.* (2013) and Grujčić *et al.* (2015). Plankton samples were collected from 0.5 m depth from the meso-eutrophic Římov reservoir, South Bohemia, Czech Republic (48°50’46.90"N, 14°29’15.50"E, for more details see Šimek *et al.*, 2008) at the late summer phytoplankton bloom on August 18th 2014 (water temperature 20.3^{o}C). Water was gravity filtered through 5 μm pore-size filters to release the flagellate community from grazing

pressure of zooplankton and larger predatory flagellates and ciliates. The 5-µm treatment represented a simplified prokaryote-HNF food chain supposedly dominated by small, primarily bacterivorous nanoflagellates (Šimek *et al.*, 2001). Samples were preincubated at 18º C for 12 hours, which resulted in approximately two-fold increases in HNF abundance, and slight decreases in the number of free-living bacteria (~ 1 x 10^6 ml^{-1}). Our experimental set-up was composed of five different treatments, each of them separately amended with distinct bacterial prey: two with strains of *Polynucleobacter* lineage PnecC (PnC6 and PnC1, for details see Table 1) and three with strains belonging to different lineages of *Limnohabitans* spp. (T6-5, Rim47 and Rim11, Table 1) (Kasalický *et al.*, 2013). These bacteria differed markedly in cell shape and size (Table 1). All five bacterial strains were pre-grown in nutrient- (i.e. CNP) rich liquid medium (3 g L^{-1} NSY) (Hahn *et al.*, 2004), pelleted by centrifugation, washed and resuspended in 0.2 µm filtered and sterilized water from Římov reservoir as detailed in Šimek *et al.* (2013) and Grujčić *et al.* (2015). Treatments were separately amended with solutions of prey bacteria added at approximately 10 times higher concentrations compared to natural background bacterial abundances. Since the prey bacteria differed in cell sizes (Table 1), the additions of the strains was set to yield approximately the same initial biovolumes for all strains (Šimek *et al.* 2013, Grujčić *et al.* 2015). The experiments were run in triplicates and treatments were kept at 18ºC in the dark, since the target bacterivorous grazers were purely

heterotrophic nanoflagellates. The treatments containing only natural bacteria and protists present in the original samples served as controls compared to the prey enriched treatments (referred to as PnC1, PnC6, T6-5, Rim-47 and Rim-11 throughout the text, see Table 1). Subsamples for detection of HNF and bacterial abundances and biovolumes were aseptically taken in a laminar flow hood at 12-24 hours intervals. Additional samples were taken at selected time points for fluorescence *in situ* hybridization followed by catalysed reporter deposition (CARD-FISH; t_0, t_{40}, and t_{66}), and for collecting DNA for sequencing (t_0, and t_{40}).

Table 1. Morphological characteristics of bacterial strains used as a prey for natural HNF communities in the experiments.

Species	**Strain**	**Lineage**	**Volume (μm^3)**	**Length (µm)**	**Cell shape**
Polynucleobacter sp.	PnC1 (czRimovFAM-C1)	PnecC	0.057	0.88	small solenoid
Polynucleobacter sp.	PnC6 (czRimov8-C6)	PnecC	0.049	0.58	short rod
Limnohabitans sp.	Rim11	LimB	0.051	0.63	short rod
Limnohabitans sp.	Rim47	LimC4	0.055	0.66	coccoid
Limnohabitans sp.	T6-5	LimC	0.472	2.21	thin curved rod

Enumeration and biovolume estimation of bacteria and HNFs

Samples (15-20 ml) fixed with formaldehyde (2% final concentration) were used for the enumeration of bacteria (0.5-2 ml subsamples) and HNF (4-10 ml subsamples) on 0.2-µm and 1-µm pore-sized filters (Osmonics, Inc., Livermore, CA), respectively. All samples were stained with DAPI (4', 6-diamidino-2-phenylindole, at a final

concentration of 1 μg ml^{-1}) and microbes were counted via epifluorescence microscopy (Olympus BX 60). Bacterial biovolumes were measured by using a semiautomatic image analysis system (NIS-Elements 3.0, Laboratory Imaging, Prague, Czech Republic). To calculate mean cell volumes of HNF (approximated to prolate spheroids, Grujčić *et al.* 2015), lengths and widths of 50 cells in each triplicate treatment were measured manually on-screen with a built-in tool of the image analysis system (NIS-Elements 3.0).

A treatment-specific HNF cell number increase was used to calculate maximum HNF growth rate, doubling time (DT), length of lag phase, and relative growth rate as detailed in Grujčić *et al.* (2015). Briefly, maximum HNF growth rate was calculated based upon the equation for exponential growth, lag phase was calculated as the period from t_0 to the intercept between the best fit line of HNF growth and the zero-time level of HNF abundance. Volumetric gross growth efficiency (GGE) was based on comparisons of HNF versus bacterial biovolumes (for details see Grujčić *et al.*, 2015; Šimek *et al.*, in press). Relative growth rates were derived from relating the HNF time course data from all treatments to the treatment where the most rapid growth of HNF was recorded.

Illumina sequencing of eukaryotic communities and data analysis

Genomic DNA was extracted from biomass collected on 0.2 μm-pore-size filters (47 mm diameter, Osmonics) employing a phenol-chloroform extraction and subsequent ethanol precipitation. DNA was

extracted from triplicates collected at t_0 and t_{40} hours of experiment. PCR amplification was conducted with indexed primers targeting an amplicon of 450 bp in the hypervariable V9 region of the SSU and the ITS1 region of the eukaryotic rRNA gene. Forward and reverse primers used are Euk1391F 5´GTA CAC ACC GCC CGT C´3 (Lange *et al.*, 2015) and ITS2 5´ GCT GCG TTC TTC ATC GAT 3´ (White *et al.*, 1990). Amplification was performed with a BioRad T 100 cycler with a 25 µl mix containing 2 U Phusion High Fidelity Polymerase (Finnzymes, Oy, Espoo, Finland), 5 µl of 5x HF buffer, 0.25 pM of each primer, 200 µM of each desoxyribonucleosidtriphosphate, 0.5 µl DNA template and 17.25 µl water. Concentration of DNA template ranged between 12 – 60 ng μl^{-1}. The amplification protocol was performed with 30 s initial denaturation at 98° C followed by 35 PCR cycles comprising 98° C for 10 s, 57° C for 20 s, 72° C for 35 s and a single final elongation step for 10 min at 72° C. The amplification of each sample was performed in five replicates to increase the total concentration per sample. The pooled and indexed samples were paired end sequenced by Eurofins (Eurofins Genomics, Germany, Ebersberg) with an Illumina MiSeq instrument using V3 chemistry.

Raw sequence reads were demultiplexed, quality filtered, clustered and assigned to taxonomy according to Lange *et al.*(2015) with the following modifications: Low quality tails were removed, reads with an average Phred quality score <25 were trimmed (Masella *et al.*, 2012).

As the 3´ends were of overall low quality, we decided to trim the reads to 89 nucleotides, and all reads with at least one base with a Phred quality score of <15 were removed. As the reverse reads had significantly lower quality than the forward reads, we decided to analyze only the single end reads to avoid quality based biases of reverse reads in the community analysis. The single-end reads were quality filtered using PANDASeq version 2.7. Reads with uncalled bases were discarded. Chimeras were identified and discarded using UCHIME. The remaining sequences were clustered to OTUs with SWARM (swarm v2.1.6, Mahé *et al.*, 2014) and assigned to taxonomic information using BLAST 2.2.30+ (Altschul *et al.*, 1990) requiring 85% identity and an evalue cutoff of $1e^{-12}$. Heterotrophic flagellates were selected by definitions of Boenigk et al. (2015) including only groups which are known to be mostly heterotrophic and to possess flagella. The amplicon data used in this study are accessible in the sequence read archive (SRA) of the NCBI database as BioProject PRJNA385800.

Phylogenetic tree reconstruction and design of novel oligonucleotide probes

Representative amplicons of the 30 most abundant OTUs were aligned with the SINA aligner (Pruesse *et al.*, 2012) and imported into ARB (Ludwig *et al.*, 2007) using the SILVA database SSURef_NR99_123 (Pruesse *et al.*, 2007). Alignments were manually refined and a maximum likelihood tree (1000 bootstraps) including their

closest relatives was constructed on a dedicated web server (Stamatakis *et al.*, 2005). Oligonucleotide probes targeting all Katablepharidophyta and the CRY1 lineage of Cryptophyta (K. Piwosz *et al.*, 2016) were designed in ARB using the tools probe_design and probe_check and evaluated with the web tool mathFISH (Yilmaz *et al.*, 2011). A similar probe for the CRY1 lineage was also designed by Piwosz *et al*. (2016), targeting exactly the same 18S rDNA sequences and being equal in terms of coverage and specificity.

Catalyzed reporter deposition fluorescence in situ hybridization (*CARD-FISH*).

The CARD-FISH protocol (Pernthaler *et al.*, 2002) was used with specific oligonucleotide probes targeting all Betaproteobacteria (BET42a, Amann and Fuchs, 2008), all *Limnohabitans* strains used in this study (R-BT065, Šimek *et al.*, 2001; Kasalický *et al.*, 2013) and *Polynucleobacter* lineage PnecC (PnecC-16S-445, Hahn *et al.*, 2005) , respectively. Fluorescein-labeled tyramides (Invitrogen Corporation, Carlsbad, CA) were used for signal amplification and an epifluorescence microscope (Olympus BX 60 microscope) for visualization. We checked for ingestion of prey bacteria in HNF food vacuoles (Jezbera *et al.*, 2005) in all experimental treatments at times t_{40} and t_{66} h.

Moreover, CARD-FISH was applied for HNF following a previously published protocol (Piwosz and Pernthaler, 2010). We used the general probes Euk516 targeting all eukaryotes (Amann *et al.*, 1990;

Beardsley *et al.*, 2005), CryptB targeting Cryptophyta (Metfies and Medlin, 2007) and two newly designed probes specific for the CRY1 lineage of Cryptophyta (CRY1-652) and Katablepharidophyta (Kat-1452, for details see Table 2). Probe CryptB covers >80 % of all Cryptophyta, including the CRY1 lineage, but does not target Katablepharydophyta. Probe Euk516 (Amann *et al.*, 1990) targeted on average 89.6% of DAPI stained eukaryotes. The newly designed probes were tested with different formamide concentrations in the hybridization buffer until highest stringency was achieved at 30% and 60% for probes CRY1-652 and Kat-1452, respectively. After signal amplification with fluorescein-labeled tyramides (Invitrogen Corporation, Carlsbad, CA), filters were counterstained with DAPI and evaluated in an epifluorescence microscope (Olympus BX 60).

Bacterial probes R-BT065 (Šimek *et al.*, 2001) and PnecC-16S-445 (Hahn *et al.*, 2005), and eukaryotic probes CryptB (Metfies and Medlin, 2007), CRY1-652 and Kat-1452 (Table 1) were also used for a double hybridization of prey and grazers in parallel (Massana *et al.* 2009) followed by amplification with fluorescein (probe R-BT065) and Alexa546 (probes CryptB, CRY1-652 and Kat-1452) labelled tyramides (Invitrogen Corporation, Carlsbad, CA), respectively.

Table 2. Details of CARD-FISH probes used in this study. See Figure S3 for details in the phylogenetic positioning of probes Cry1-652 and Kat-1452.

Probe name	Target	Sequence (5'-3')	% Formamide	Reference
Euk516	All eukaryotes	ACCAGACTTGCCCTCC	20%	Amann *et al.*, 1990
CryptB	Cryptophyta	ACGGCCCCAACTGTCCCT	50%	Metfies and Medlin, 2007
Cry1-652	CRY1 lineage	TTTCACAGTWAACGATCCGCGC	30%	this study
Kat-1452	Katablepharidophyta	TTCCCGCARMATCGACGGCG	60%	this study

Bacterivory rates of heterotrophic nanoflagellates at T_0

Grazing rates of the HNF community present in the unfiltered reservoir sample used for the experiment (T_0) were examined by using fluorescently labelled bacteria (FLB, Sherr *et al.*, 1987) prepared from a mixture of *Limnohabitans* sp. from the LimC lineage (Kasalický *et al.*, 2013) and two strains from the PnecC lineage of *Polynucleobacter* isolated from the reservoir. HNF bacterivory was determined in short-term FLB direct-uptake experiments in combination with fluorescence microscopy as detailed in Šimek *et al.*, (2001). To estimate total HNF grazing, we multiplied the average uptake rates of HNF by their *in situ* abundance at T_0.

Additionally, we quantified the average number of DAPI-stained bacteria, as well as bacteria targeted by the general probe EUB I-III (Daims *et al.*, 1999), in food vacuoles of bacterivorous HNF targeted by different CARD-FISH probes in the unfiltered samples from T_0. We applied the

general probe for Eukaryotes (Euk516) and compared it to the food vacuole contents of HNF targeted by probes for Cryptophyta and its CRY1 lineage. The combination of these methods allowed estimating cell-specific (expressed as number of bacteria ingested per flagellate cell) and bulk bacterivory rates of total HNF compared to different flagellate lineages. The proportion of bacterial standing stock removed per day was used as a proxy of the significance of the total grazing impact of the different flagellate groups in untreated reservoir water (see Table 3 for details).

Table 3. Grazing characteristics of different flagellate groups at time T_0 from Rimov reservoir. IGR – individual cell-specific grazing rate and TGR - total grazing rate calculated for the whole HNF community and of its FISH-probe defined subgroups (Crypto and CRY1 lineage). Bact $flag^{-1}$ represents average number of bacteria stained with general EUB I-III probe per group of flagellate.

	HNF $10^3 ml^{-1}$	**HNF (%)**	**Bact $flag^{-1}$**	**IGR at T_0 bac HNF^{-1}*h**	**TGR per day $10^6 ml^{-1}$*d**	**Bact standing stock grazed per day(%)**	**% of total TGR of HNF**
All HNF	5,4	100	2,9	13,7	1,78	54,2	100
All Crypto	3,38	63	3,1	14,8	1,2	36,6	70
CRY1 lineage	0,1	1,8	1,8	8,5	0,02	0,6	1,1

Statistical analysis.

Statistical analyses were performed with the Excel stats package (Microsoft Office Professional Plus 2010, Version 14.0.7128.5000). We analyzed the effects of strain characteristics on HNF gross growth efficiency, doubling time (DT), lag phases, and relative growth rates by

two-way analysis of variance (ANOVA) followed by post hoc Tukey tests. The same analysis was applied for comparing differences in percentage of hybridized flagellate cells between time t_0, t_{40} and t_{66} and differences in percentages of flagellate reads between treatments. T-tests were used for identifying differences between percentages of hybridized cells with CryptB and Kat-1452 probes and percentages of reads belonging to the same groups.

RESULTS

Time-course changes in bacteria and HNF

We tested growth responses of natural HNF communities to amendments with five different bacterial strains. While the strains differed in size, morphology and taxonomic affiliation (Table 1), they all were swiftly consumed by the grazer HNF community (Supplementary Figure 1) and thus also supported significantly more rapid growth of natural HNF communities compared to the control (Figures 1 and 2). Numbers and biomasses of bacteria and HNF remained relatively stable in control treatments, except for a slight increase of HNF within the first 16 h (Figure 1 and Supplementary Figure 1). Temporal changes in biovolumes of HNF and bacteria roughly corresponded to the treatment specific trends observed for abundances (Figure 1).

Bacterial numbers and biovolumes started to decrease markedly after 16 h in most of the prey amendments, except for treatments T6-5 and

Rim47, where bacteria decreased already shortly after the beginning of the experiment (Supplementary Figure 1). Notably, bacterial numbers and biomasses slightly increased before the onset of HNF growth in two treatments (PnC6 and Rim11, 0-16 h, Figure 1 and Supplementary Figure 1). HNF abundances increased from the initial ~5 × 10^3 cells ml^{-1} to approximately 30-50 × 10^3 cells ml^{-1} in treatment-specific fashions (Figure 1). Generally, maxima were achieved at t_{27} h, except for treatments PnC1 and PnC6 (the *Polynucleobacter* strains) where peaks occurred later (t_{40} h), followed by a subsequent decrease (Figure 1).

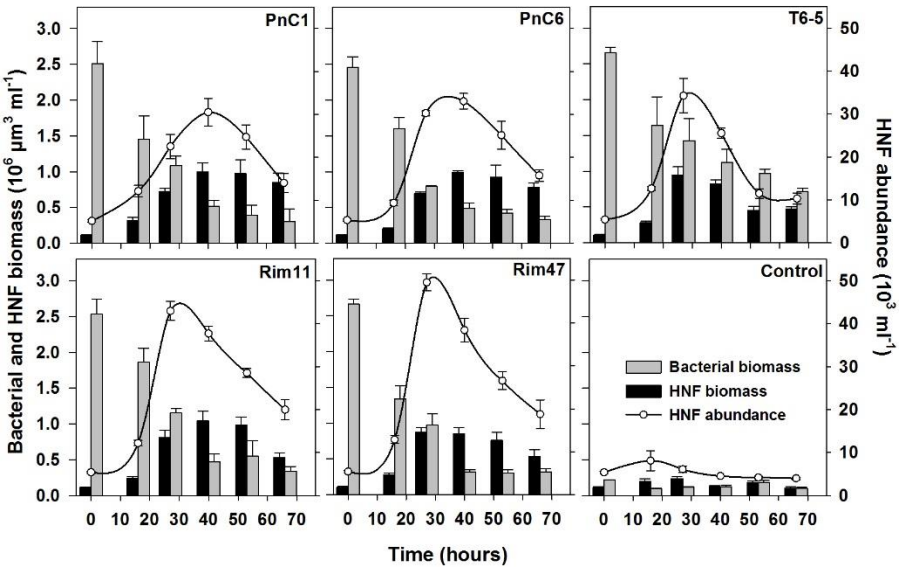

Figure 1 Time course changes in HNF abundances, HNF and bacterial biovolumes in all treatments. Values are means of triplicates; error bars show SD.

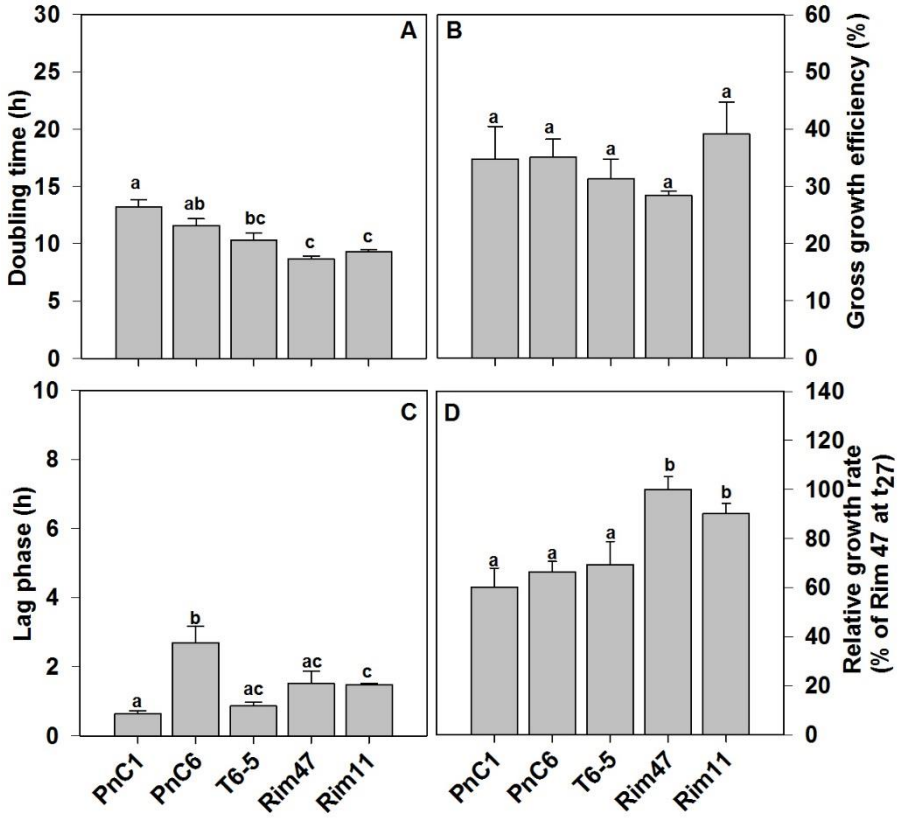

Figure 2 Doubling times (A), gross growth efficiencies (B), lag phases (C) and relative growth rates (D) of HNFs in all treatments amended with bacterial strains. Values are means of triplicates, error bars show SD. Different letters above bars denote significant differences (Two way ANOVA followed by post hoc Tukey test).

HNF growth parameters

Similar initial biovolumes of the distinct bacterial strains yielded different HNF growth dynamics. The fastest growing HNFs were those feeding on *Limnohabitans* strains Rim47 (DT=8.6 h) and Rim11 (DT=9.3 h). Doubling times (DTs) of these flagellates were significantly different ($P<0.001$, one way ANOVA followed by Tukey test) from DTs of HNFs

growing in treatments PnC1 (DT=13 h) and PnC6 (DT=11.5 h). HNF growth in treatment T6-5 (DT=10.3 h) was significantly different only from that in treatment PnC1 (Figure 2A). Lag phases of HNFs were relatively short (0.6 – 3 h) with treatment PnC6 having significantly longer DT than all other treatments ($P<0.005$). HNFs in treatment PnC1 had significantly shorter lag phase than HNFs in treatment Rim11. However, there was no significant difference in length of lag phase between treatments containing the three *Limnohabitans* strains (T6-5, Rim47 and Rim11; Figure 2C). Volumetric gross growth efficiencies (GGE) of flagellates ranged from 28% - 39% (Rim47 and Rim11, respectively) and showed no significant difference between different prey items (Figure 2B).

Relative growth rates related to the increase of HNF numbers in treatment Rim47 (the most rapid cell number increase at t_{27} h, set as 100%) were significantly lower in treatments PnC1, PnC6 and T6-5 than in Rim11 and Rim47, suggesting that the latter two strains represented the best food supporting rapid HNFs growth in combination with the shortest DTs (Figure 2D).

Effects of bacterial prey on the composition of HNF

The 18S rRNA gene amplicon dataset comprised 3,527,902 reads that were filtered for bacterivorous flagellate groups. A total of 1,576,480 reads related to flagellates were analyzed, with the most abundant group

belonging to Katablepharidophyta, accounting for 35% - 85% in the different treatments (Figure 3).

We compared relative proportions of reads assigned to heterotrophic flagellate groups at t_0 h (control t_0) of the experiment to treatments after 40 h of the experiment (Figure 3). The initial sample from Římov reservoir was composed of 47% Cryptophyta, 44% Katablepharidophyta, and low percentages (<2%) of Chrysophyceae, Bicosoecida, other Stramenopiles, Cercomonadida, Cercozoa, Choanoflagellida and Haptophyta. After 40 hours of experiment (Control t_{40}), the initial sample changed significantly ($p<0.001$), with Cryptophyta decreasing to 6% and Katablepharidophyta increasing to 84%. Chrysophyta accounted for 4% and Choanoflagellida for 2% of the flagellate reads while other groups stayed more or less stable or almost disappeared (Figure 3).

In the treatment amended with strain PnC1, Katablepharidophyta dominated the analyzed sample with 85% of reads, while only 3% belonged to Cryptophyta, 7% to Choanoflagellida and 3% to Chrysophyta. Relatively similar shifts in major flagellate groups occurred also in treatments PnC6 and T6-5, while other groups such as other Stramenopiles and Cercozoa accounted for <1% (Figure 3). Treatments Rim11 and Rim47 displayed more marked changes with a significant increase ($p>0.001$) in the proportions of flagellates representing typical bacterivorous groups such as Chrysophyta and Choanoflagellida.

Treatment Rim47 had 68% of reads belonging to Katablepharidophyta, 4% to Cryptophyta, 13% to Choanoflagellida, 9% to Chrysophyta, and ≤2% to Bicosoecida, Cercomonadida and Cercozoa. In contrast, treatment Rim11 was most distinct (Figure 3), with 35% of reads belonging to Katablepharidophyta, 32% to Cryptophyta, 15% to Choanoflagellida, 9% to Chrysophyta, 4% to Cercozoa and ≤2% to other Stramenopiles, Bicosoecida and Cercomonadida.

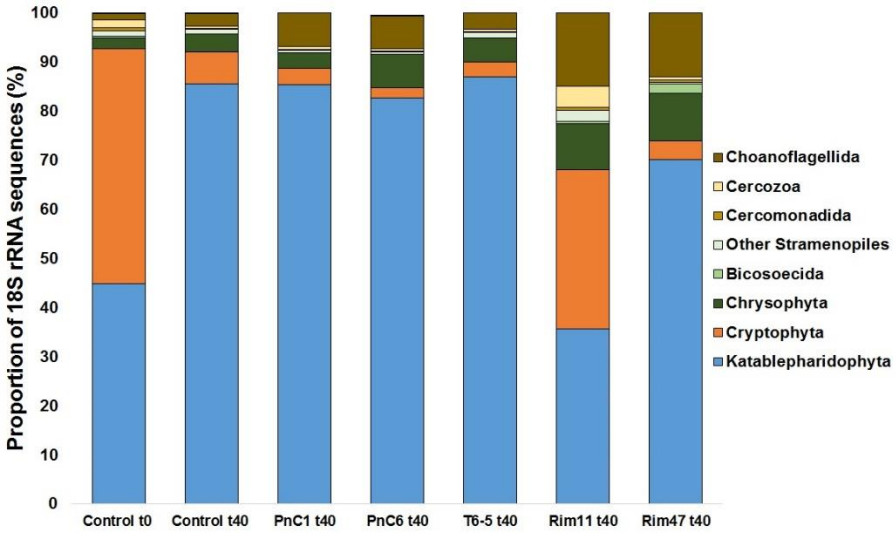

Figure 3 Percentage of reads belonging to different taxonomic groups of protists in all treatments at different time points. Control t0 represents the starting community from the reservoir. Control t40 represents the control after 40 hours of experiment, PnC1 t40, PnC6 t40, T6-5 t40, Rim47 t40 and Rim11 t40 are treatments amended with different bacterial strains after 40 hours of experiment. Values expressed as percentages are means of triplicates.

Percentages of flagellates targeted by specific CARD-FISH probes revealed highly significant differences compared to the proportions derived from amplicon sequencing (Figures 4, 5, and Supplementary Figure 3). Relative abundances of flagellates belonging to Katablepharidophyta were 1.5% at time t_0. These flagellates increased significantly ($p<0.001$; from 6.3 to 11.8%) until the end of the experiments (t_{66}) in most treatments, except for the Rim11 and control, where they represented relatively stable proportions (Figure 4). Flagellates affiliated to Cryptophyta accounted for 62.5% of all HNFs at time t_0 (Table 2). After 40 hours, their proportion increased significantly ($p<0.001$) to >70% in treatments PnC1, PnC6, T6-5, and Rim11, while they slightly decreased in treatment Rim47 and in the control. At t_{66} h, the proportions significantly decreased in PnC1 and PnC6 treatments, while in other treatments their proportions remained stable or slightly decreased compared to t_0. Relative abundances of flagellates belonging to the CRY1 clade of Cryptophyta were 1.8% at t_0 and after 40 hours this proportion significantly increased to 20.5% in Rim11 ($p<0.001$) and also slightly rose in all other treatments. At the end of the experiments, proportions of CRY1 significantly decreased in all treatments to 0.3 – 1.8% (Figure 4).

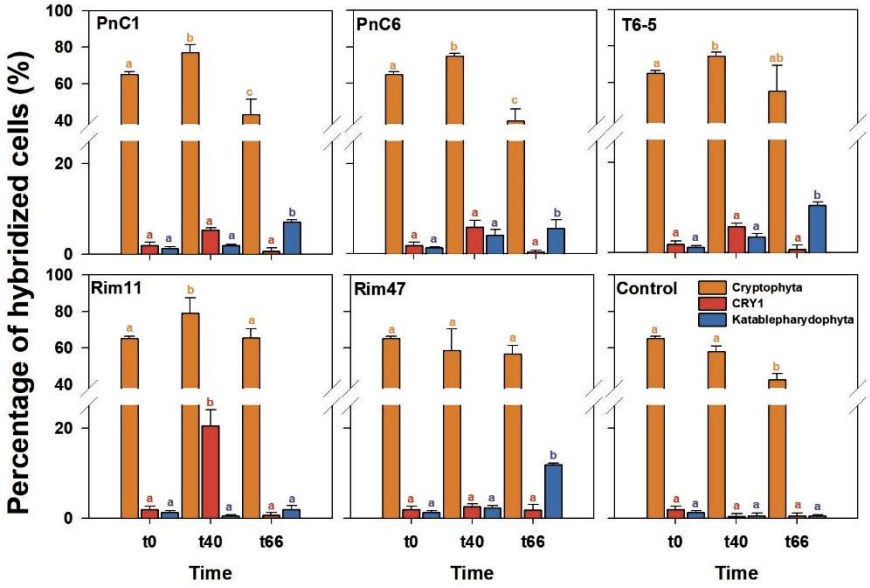

Figure 4 Relative abundances of cells hybridized with probes targeting all Cryptophyta, lineage CRY1, and all Katablepharidophyta at three different time points: t_0, beginning of experiment, representing the starting community from the reservoir; t_{40} and t_{60} represent proportions after 40 and 60 hours of experiment. Different letters above the columns indicate significant differences between different times of the experiment within one treatment targeted with one probe (post hoc Tukey test). Values are means of triplicates, error bars show SD.

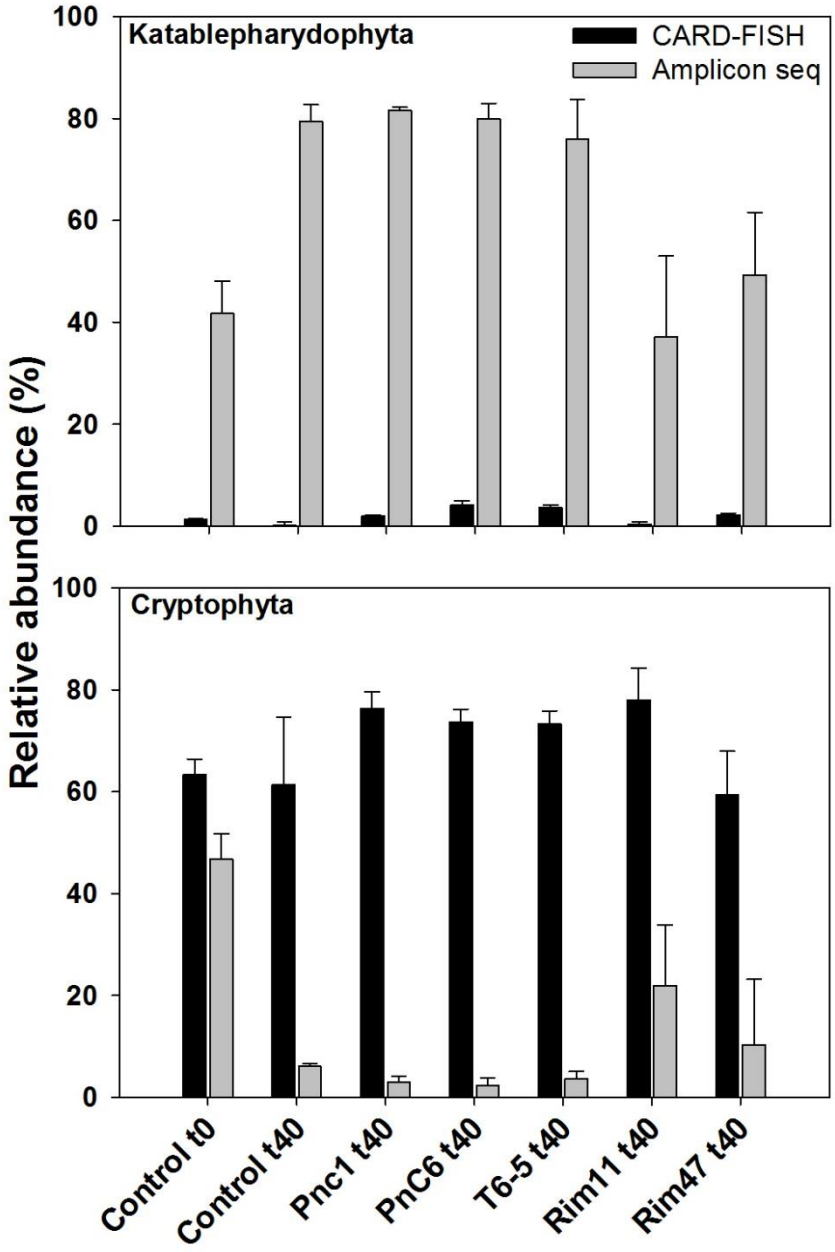

Figure 5 Comparison of relative abundances of 18S rDNA amplicon reads and relative abundance of cells get by CARD-FISH. Differences were significant for all treatments (t-test $p<0.001$). Values are means of triplicates, error bars show SD.

The pronounced growth of Cryptophyta was also visible in cell numbers (Supplementary Figure 4) where they increased from the initial 2.4 x 10^3 cells ml^{-1} to 19 - 29 × 10^3 cells ml^{-1} in treatment specific fashions (Supplementary Figure 4). Representative images of Cryptophyta, Katablepharydophyta and CRY1 lineage with ingested bacterial prey are presented in Figure 6.

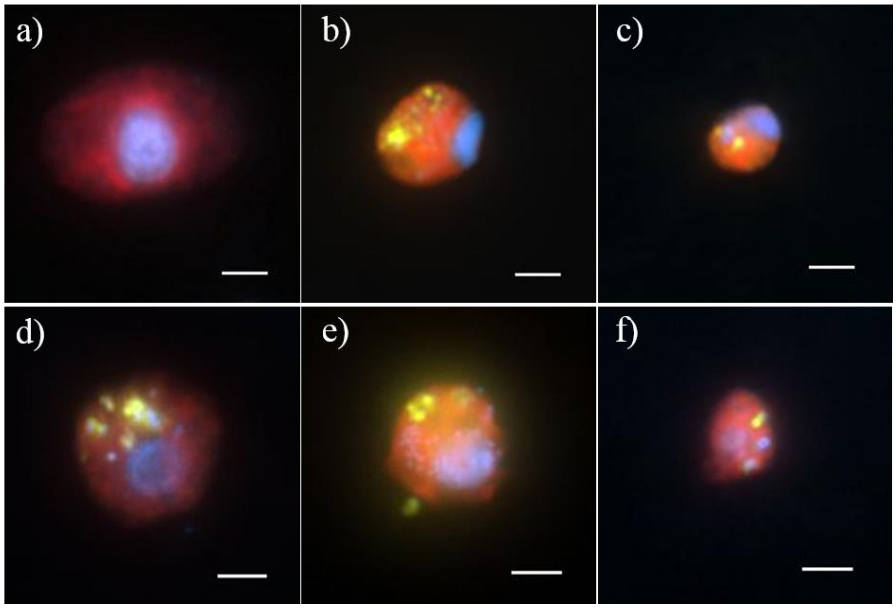

Figure 6 Double hybridization of bacterial prey and HNF predator. Each image is an overlay of three pictures of the same HNF cell observed under ultraviolet excitation (showing the blue nucleus after DAPI staining), green light excitation (red color corresponding to different HNF groups labeled with Alexa546 using CARD-FISH) and blue light excitation (yellow-green color corresponding to fluorescein labeled *Limnohabitans* spp. or *Polynucleobacter* cells in food vacuoles after CARD-FISH with probe R-BT065 or PnecC-16S-445 respectively). Scale bar is 2 µm. a) HNF hybridized with probe Kat-1452 targeting all Katablepharydophyta, b) bacteria and HNF hybridized with probes R-BT065 targeting *Limonhabitans* and CRY1-652 targeting the CRY1 lineage of Cryptophyta , c) bacteria and HNF hybridized with probes PnecC-16S-445 targeting *Polynucleobacter* and CRY1-652 targeting the CRY1 lineage of Cryptophyta to d) and e) bacteria and HNF hybridized with probes R-BT065 targeting *Limonhabitans* and CryptB targeting all Cryptophyta f) bacteria and HNF hybridized with probes PnecC-16S-445 targeting *Polynucleobacter* and CryptB targeting all Cryptophyta

Grazing impact of HNF community at time zero

The cell-specific bacterivory rate, averaged for all HNF in the reservoir, was 13.7 bacteria HNF^{-1} h^{-1} at the experimental start (T_0), corresponding to a removal of 54.2 % of the bacterial standing stock per day (Table 3). Based on the number of ingested bacteria in food vacuoles targeted by a general bacterial probe, the cell specific uptake rate of colorless Cryptophyta targeted by probe CryptB was even slightly higher (14.8 bacteria HNF^{-1} h^{-1}) than the average for the total HNF (13.7 bacteria HNF^{-1} h^{-1}). Notably, due to their high proportion of total HNF, Cryptophyta were also the most important bacterivores in the reservoir plankton at T_0, accounting for ~ 70% of total HNF bacterivory (Table 3). In contrast, flagellates affiliated to the CRY1 lineage had markedly lower uptake rates and abundances, and thus also contributed correspondingly less to the bulk HNF bacterivory.

DISCUSSION

Our study demonstrates that bacterial prey characteristics differently affect growth and community dynamics of natural freshwater bacterivorous flagellates. This was evident from both prey-specific HNF growth parameters and taxonomic shifts in flagellate communities (Figures 1, 2, 3 and 4). We are aware of the fact that the concentrations of offered bacterial prey were far higher than the typical *in situ* concentrations, which could accelerate HNF growth and thus also

influence growth parameters of HNF. However, even this increase in prey availability induced comparable doubling times of HNF as those detected in dialysis bag experiments conducted directly *in situ* in the reservoir plankton with natural HNF and bacterial concentrations (Šimek *et al.*, 2006; Šimek *et al.*, in press).

Growth parameters of HNF related to bacterial food quality

Very short lag phases of HNF (less than 3 hours) in all treatments imply that the indigenous HNF community from the reservoir responded almost immediately to the offered bacterial prey, which is not always the case (compare the data in Grujčić *et al.*, 2015 and Šimek *et al.*, in press). Further, size ratios between offered cell size of bacteria and the size of natural HNF grazers indicated that all prey items were well within the edible size range for the grazers (Šimek and Chrzanowski, 1992; Pernthaler, 2005; Pfandl *et al.*, 2004; Hahn *et al.*, 1999).

A combination of short lag phase and rapid doubling time has been suggested as indication of high food quality of certain bacterial prey for natural HNF communities (Šimek *et al.*, 2013). In our experiment such a combination was well exemplified by the significantly shorter doubling times and short lag phases detected in Rim11 and Rim47 treatments (Figure 2A and 2C). Moreover, the relative growth rates of flagellate feeding on these medium-sized *Limnohabitans* strains were also significantly higher compared to the three other bacterial strains (Figure 2D). On the other hand, large variability in triplicates for GGE estimates

(28 - 39%) did not yield clear significant differences among treatments. However, a significant inverse relationship between the length of lag phase and GGE of flagellate communities was evident in a large data set with more diverse HNF communities and bacterial prey-specific characteristics (Grujčić *et al.*, 2015, Šimek *et al.*, in press).

Mismatch between 18S rRNA amplicon data and cell abundances quantified by CARD-FISH

High throughput sequencing (HTS) allowed deeper and more detailed insights in the diversity of aquatic eukaryotes (Grossmann *et al.*, 2016; Amaral-Zettler *et al.*, 2009; Boenigk *et al.*, 2005) which, however, may not necessarily reflect an accurate estimation of the abundance of specific groups (Bachy *et al.*, 2012; Medinger *et al.*, 2010). Our study confirmed these concerns since relative abundances of reads belonging to Cryptophyta and Katablepharidophyta did not match at all with the relative abundances of cells detected by CARD-FISH in the same samples (Figures 3, 4 and 5). Such discrepancies could be explained by PCR biases of molecular approaches targeting single genes resulting in over- or underestimations of some groups (Wintzingerode *et al.*, 1997; Hong *et al.*, 2009; Quince *et al.*, 2009; Mukherjee *et al.*, 2015). In this study, we also used a high number of PCR cycles (i.e., 35), which is at the upper range of recommended values, however, yet being within the normal range. This methodical aspect might perhaps partially contribute to the high

discrepancy between amplicon and CARD-FISH results. Further, some hypervariable regions of 18S rRNA, like V4 or V9, have been shown to be better for the estimation of certain groups (Giner *et al.*, 2016).

In our study, proportions of reads affiliated to Katablepharidophyta were drastically overestimated compared to CARD-FISH counts, which could be related to high numbers of rRNA operon copies in this group (Kahn *et al.*, 2014). Copy numbers of 18S rRNA genes can vary among different protistan taxa depending on the cell and genome size (Prokopowich *et al.*, 2003). Phylogenetic position of Katablepharidophyta is still under debate and for long time they have been considered as a part of either Cryptophyta or Alveolata (Reeb *et al.*, 2009), with the latter group being known to possess very high copy numbers of 18S rRNA genes (Medinger *et al.*, 2010). However, few phylogenetic analyses confirmed their position as a sister group to Cryptophyta (Okamoto and Inouye, 2005; Okamoto *et al.*, 2009). We can exclude a taxonomic mis-assignment of short reads from amplicon sequencing, as two of the most abundant OTUs were clearly affiliated to Katablepharidophyta (Supplementary Fig. 3) and the sequence of our newly designed CARD-FISH probe targets the V9 region that is present in all reads (Table 2).

On the other hand, numbers of Cryptophyta were drastically underestimated with HTS, which might be due to primer bias as some publically available sequences for Cryptophyta have mismatches with

primers that we used in this study (for more details see Supplementary Table 1).

Although we found large mismatch between HTS and CARD-FISH results, these two methods combined provide a powerful tool to detect diversity and abundance of certain groups. Amplicon sequencing can be especially useful for identifying taxa present in a large set of samples and facilitates designing of new CARD-FISH probes. The application of group specific primers (Mukherjee *et al.* 2015), or the carefull design of new primers can decrease certain biases in amplicon sequencing. CARD-FISH on the other hand, is a very valuable method for a more accurate estimation of abundance of specific lineages since it is possible to visualise and thus directly quantify target organisms. However, also CARD-FISH has its downsides and limitations. It is very laborious and limited in the number of taxon specific probes that could be applied at the same time (Pernthaler *et al.*, 2002). Further, it is not possible to accuratelly estimate the abundances of rare taxa with CARD-FISH, while HTS can still detect them.

Cryptophyta – unexpected major bacterivores

Our study documents a strong impact of prey characteristics on resulting HNF community dynamics, with severe shift in HNF community composition towards Cryptophyta (Figure 3). Furthermore, flagellates belonging to Cryptophya were the most abundant bacterivores in summer plankton of the Římov reservoir, which was confirmed by high cell-

specific grazing rates making them responsible for 70% of total HNF bacterivory (Table 3). Additionally, they undoubtedly grew and feed on all the tested bacterial strains in our experiments as documented in the double hybridization of grazers and prey (Figure 6).

In the past decade, numerous studies suggested that the most important bacterivores in freshwaters belong to small colorless chrysomonad flagellates, so called '*Spumella*-like' flagellates (Boenigk *et al.*, 2005; Šimek *et al.*, 2013; Grossmann *et al.*, 2015; Boenigk and Arndt, 2002b; Montagnes *et al.*, 2008). The term '*Spumella*-like' is mostly used when addressing morphology of these flagellates as recently it has been shown that they are in fact polyphyletic, belonging to different groups of the class Chrysophyta (Grossmann *et al.*, 2016). Chrysophyta reads accounted for >2% of the flagellates collected *in situ* (t_0 h) and increased to 3-9% after 40 h of experiment. A significant increase in two treatments, Rim47 and Rim11, indicated efficient growth of chrysomonad flagellates on these two strains (Figure 3). Since we did not use a specific CARD-FISH probe for this group we cannot confirm agreement with the abundance estimates based on amplicon reads. Interestingly, those results partly contrast to a similar study conducted by Šimek *et al.*, (2013) scheduled to the spring bloom phase (late April) in the Římov reservoir, where the majority of reads were associated with different lineages of '*Spumella*-like' flagellates. Our experiment was conducted in late summer, suggesting that seasonal aspects strongly modulate the

community of eukaryotes and that different flagellate taxa are likely to be major bacterivores in different seasons (Šimek *et al.*, 2014; Šimek *et al.*, in press). The most abundant bacterivores in our experiments, according to CARD-FISH results, were affiliated to Cryptophyta (Figure 4 and Supplementary Figure 4). The recently discovered CRY1 clade of Cryptophyta (Shalchian-Tabrizi *et al.*, 2008) appears to be an important bacterivore in our study, growing on all tested strains but with a profound increase only on the bacterial strain Rim11. Cells of flagellates belonging to this clade were relatively small (~3-4 μm length), spherical and with a de-central nucleus (Figure 6). All observed cells were purely heterotrophic with no chloroplasts (as previously suggested by Piwosz *et al.*, (2016)) but having ingested bacteria in their food vacuoles.

Since phylogenetic resolution of amplicon sequencing is low and most Cryptophyta were considered to be either phototrophic or mixotrophic (Taib *et al.*, 2013; Simon *et al.*, 2015), the CRY1 clade and other heterotrophic cryptophytes (Table 3) were so far largely overlooked as potentially bacterivorous. However, abundances of other Cryptophyta cells not belonging to the CRY1 lineage, yet being targeted by the general Cryptophyta probe CryptB (Metfies and Medlin, 2007) were unexpectedly high (Figures 4, 5, 6 and Supplementary Figure 4). Relative abundances up to 70% of all eukaryotic cells, with, moreover, high cell-specific uptake rates (Table 3), suggest the existence of additional heterotrophic bacterivorous clades among this phylum. This is in agreement with a

recent study by Debroas *et al.*, 2017, which reported several unknown lineages of Cryptophyta. Cells targeted by the general Cryptophyta probe had diverse morphologies and food vacuoles containing numerous bacterial prey (Figure 6C-D). Notably, prior to the experiment (T_0), Cryptophyta had higher uptake rates compared to total HNF and to the CRY1 lineage (Table 3). Thus, they were the most abundant bacterivores already *in situ*, which was not reported before. Chloroplasts were never observed in these flagellates although we cannot confirm their obligate heterotrophy since the strong signal of the probe might slightly interfere with the chlorophyll *a* excitation. However, the majority of HNFs in our experiment were aplastidic, as almost no chloroplast bearing flagellates (<2%) were observed in DAPI stained preparation.

On the other hand, Katablepharydophyta have not been observed with ingested bacteria in our experiments (Figure 6A) and their numbers increased significantly only towards the end of the experiment (Figure 4 and Supplementary Figure 4). Thus we cannot exclude the possibility that they fed on smaller bacterivorous HNF. This would correspond to the fact that some species of Katablepharidophyta are known to prey on a large prey such different types of algae (Clay and Kugrens, 1999) and a peculiar way of feeding by forming swarms was observed in some cultures (Okamoto and Inouye, 2005; Clay and Kugrens, 1999).

Double hybridization as a powerful method for studying bacterivory

Our study demonstrated that the combination of high throughput amplicon sequencing with the design of specific CARD-FISH probes can serve as a powerful tool for estimating diversity and quantifying abundance of prevailing distinct eukaryotic groups. Moreover, we applied a new method (see also Massana *et al.*, 2009) for examining bacterivory by combining two probes at different trophic levels, targeting protistan grazers as well as prey bacteria in their food vacuoles (Figure 6). This combination gives new insights into predator-prey interactions as it displays a unique picture *in situ*, by demonstrating directly which bacteria are preferentially consumed and which groups of flagellates are their grazers in aquatic ecosystems at a given time.

Conclusions

The design and application of novel eukaryotic probes for CARD-FISH has been fundamental to our study, as we could quantify and elucidate the trophic mode of the CRY1 clade of Cryptophyta, discovered by Shalchian-Tabrizi *et al.* (2008). This group appeared to be an important bacterivore in summer plankton communities, feeding and growing well on several betaproteobacterial strains, but most profoundly on one strain of *Limnohabitans* in our experiment (Figure 4). To our best knowledge the CRY1 group has so far not been observed with ingested bacteria nor has their bacterivory ever been quantified. Thus, our study clearly evidenced the key role of bacterial food as carbon source fueling growth of these

small protists as suggested earlier (Piwosz *et al.* 2016). Further, flagellates targeted by a general Cryptophyta probe were the most abundant bacterivores not only in all our prey-amended treatments but also *in situ* in Římov reservoir (Table 3). For the first time we could visualize this finding via a double hybridization method that allowed for a simultaneous phylogenetic identification of both grazers and prey without additional sample manipulation (Fig. 6). Moreover, we could also demonstrate that a quantification based solely on numbers of reads by HTS is insufficient to accurately estimate abundances of certain groups, as exemplified for Katablepharidophyta and Cryptophyta. Last but not least, our study clearly demonstrated species-specific effects of the prey quality on the resulting community composition of HNF.

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Supplementary information is available at the ISME Journal website

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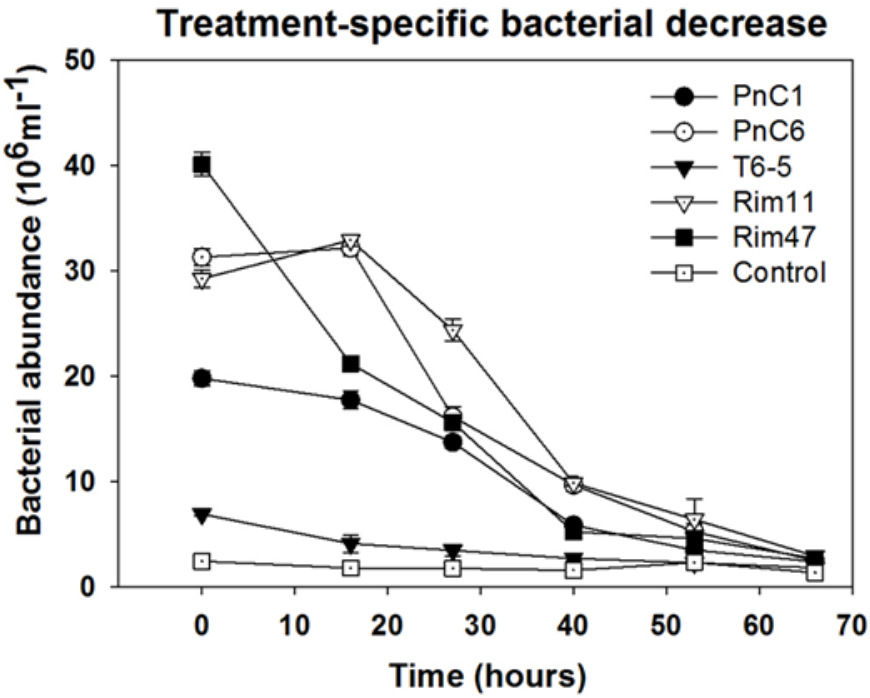
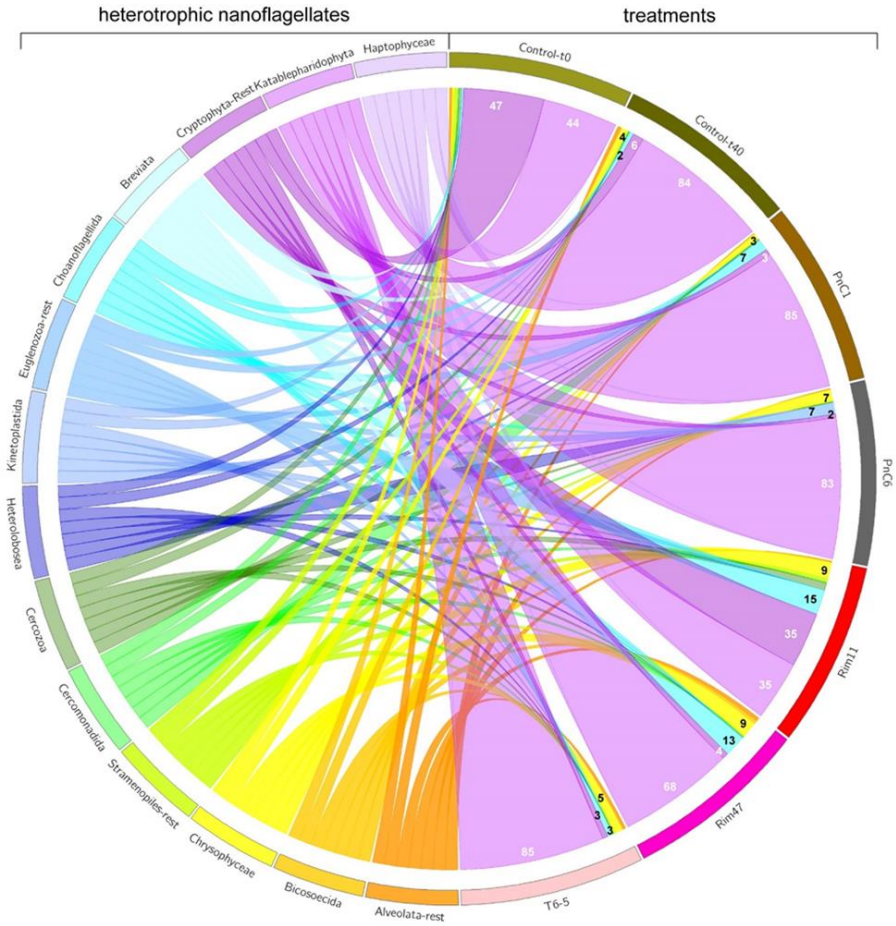
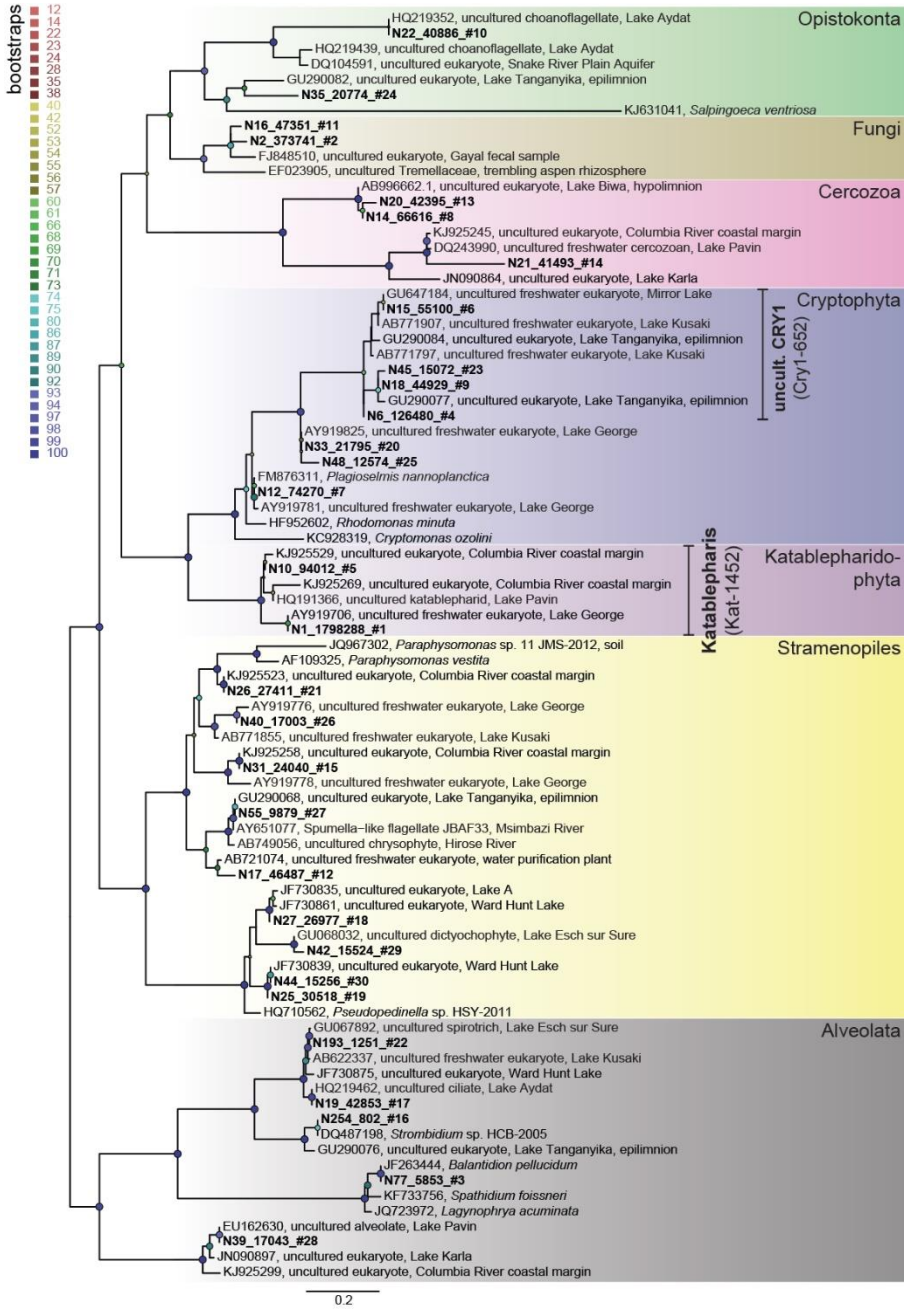
Supplementary material



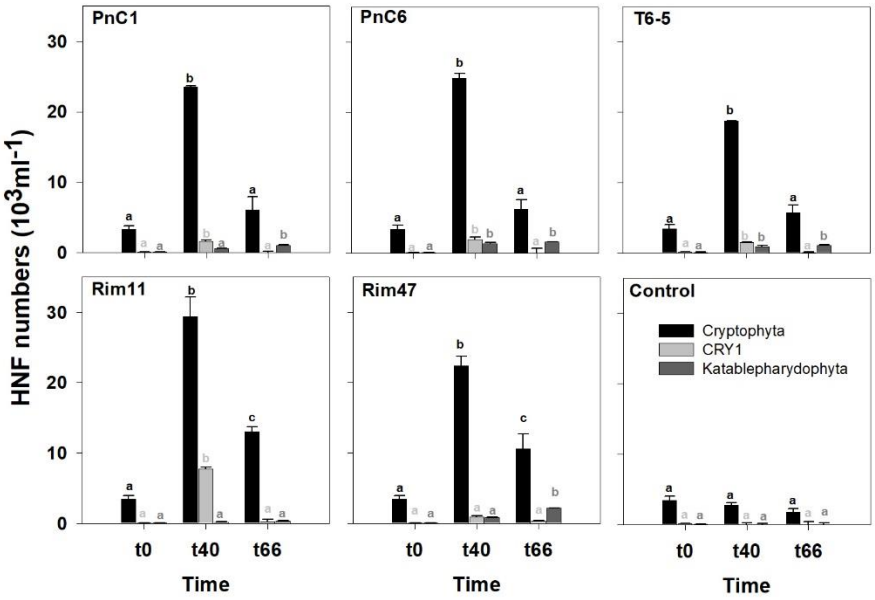
Supplementary Figure 1. Bacterial numbers in experimental treatments amended with bacterial strains (PnC1, PnC6, T6-5, Rim11 and Rim47) compared to the control where no bacterial strains were added at different times of the experiment. Values are means of triplicates. Error bars depict standard deviations.



Supplementary Figure 2. Percentages of reads affiliated with particular taxonomical groups of protists in different treatments and time points. The left half of the circus plot shows the occurring flagellate groups; the right half shows the treatments. Control t0 represents the starting community from the reservoir. Control t_{40} represents the control treatment after 40 hours of experiment, PnC1, PnC6, T6-5, Rim47, and Rim11 show the protistan community in the bacterial prey-amended treatments after 40 hours of experiment. Values are means of triplicates expressed as percentages.



Supplementary Figure 3. Bootstrapped maximum likelihood tree of eukaryotic 18S rRNA genes including representative sequences of the 30 most abundant OTUs from the amplicon dataset (marked in bold; OTU rank is indicated by #). Sequences targeted by the newly designed probes Cry1-652 and Kat-1452 are shown in brackets. Bootstrap values are indicated by differentially colored circles on nodes, the scale bar at the bottom applies to 20% sequence divergence.



Supplementary Figure 4. Absolute abundances of HNF cells hybridized with probes targeting all Cryptophyta, lineage CRY1, and all Katablepharidophyta at three different time points: t_0, beginning of experiment, representing the starting community from the reservoir; t_{40} and t_{60} represent percentage after 40 and 60 hours of experiment. Different letters above the columns indicate significant differences between different times of the experiment within one treatment targeted with one probe (post hoc Tukey test). Values are means of triplicates.

7. Curriculum Vitae – Vesna Grujčić

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STUDIES

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Biology Centre of AS CR, Institute of Hydrobiology,
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PhD topic: Planktonic Betaproteobacteria as prey and carbon source for heterotrophic nanoflagellates

2010 – 2013

Master of Science, Graduate Program in Biology
- knowledge and skills necessary for quality and competent management of national or nature parks, fish-farms, botanical and zoological gardens, parks in general, biological laboratories, etc. Furthermore, holders of a master's degree are qualified to become members of a research team of professionals conducting research in the field of natural, biotechnical and biomedical science

University J. J. Strossmayer in Osijek, Croatia
Department of Biology
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Thesis performed at: Biology Centre of AS CR, Institute of Hydrobiology, Czech Republic.
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2007 – 2010

Bachelor (Baccalaurea) of Biology
Biology

- knowledge and skills which qualify them to work in a laboratory as an associate
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PUBLICATIONS
Journal Papers

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Šimek, K., Grujčić, V., Hahn, M. W., Horňák, K., Jezberová, J., Kasalický, V., Nedoma, J., Salcher, M. M., Shabarova T. (2018) Bacterial prey food characteristics modulate community growth response of freshwater bacterivorous flagellates. Limnology and Oceanography 63.1 484–502 doi:10.1002/lno.10759.

Grujčić V., Nuy J. K., Salcher M. M., Shabarova T., Kasalický V., Boenigk J., Jensen M., Šimek K. Cryptophyta as major freshwater bacterivores in freshwater summer plankton. ISME J (in press). doi:10.1038/s41396-018-0057-5

GRANTS AND FELLOWSHIPS

National Grants as Collaborator

2013 – 2017, (GA ČR No. 13-00243S): "Unveiling life strategies of important groups of planktonic Betaproteobacteria in relationship to carbon flow to higher trophic levels"

2016, student project, (013/2016/P), Grant agency of the University of South Bohemia in České Budějovice, "How prey quality modulates community structure of bacterivorous freshwater flagellates: insight from next-generation sequencing"

2017-2018 Joint research project between Kyoto University, Japan and Institute of Hydrobiology CAS, Biology Centre, Institute of Hydrobiology CAS, The Czech Academy of Sciences "Unveiling flagellate and bacterial community dynamics and trophic interactions in two deep freshwater ecosystems by a unique methodological combination"

2017-2019, (GA ČR No. 13-009310S): "Fishponds as models for exploring plankton diversity and dynamics of hypertrophic shallow lakes"

CONFERENCES WITH ABSTRACT PUBLICATION

2013-13th Symposium on Aquatic Microbial Ecology - Stresa, Italy
2014-33rd Annual meeting of the German Society for Protozoology - Essen, Germany
2015 -14th Symposium on Aquatic Microbial Ecology - Uppsala, Sweden
2016 - A New age of Discovery for Aquatic Microeukaryotes - Heidelberg, Germany
2017 - International Congres of Protistology 2017, Prague, Czech Republic
2017 - 15th SAME (Symposium on Aquatic Microbial Ecology)

TEACHING EXPERIENCE

2014 and 2015. Microbiology practical at University of South Bohemia. (60 hours)

Research interest and skills:

- Composition, structure and functioning of the microbial communities in aquatic systems, with special emphasis on the freshwater environments. In particular, carbon flow from major linages of Betaproteobacteria (Limonahbitans, Polynucleobacter) into heterotrophic flagellates.
- Responses of microbial communities to changes in nutrients availability and predation pressure, protistan bacterivory and selective grazing
- Exploring a variety of molecular techniques (amplicon sequencing, fluorescence in situ hybridization) and combining them with fluorescence microscopy
- Isolation and cultivation approaches to access diversity and dynamics of microbial eukaryotes (protists) in freshwaters
- Manipulation experiments with protists and bacteria
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