

ORIGINAL ARTICLE

Differential freshwater flagellate community response to bacterial food quality with a focus on *Limnohabitans* bacteria

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Different bacterial strains can have different value as food for heterotrophic nanoflagellates (HNF), thus modulating HNF growth and community composition. We examined the influence of prey food quality using four *Limnohabitans* strains, one *Polynucleobacter* strain and one freshwater actinobacterial strain on growth (growth rate, length of lag phase and growth efficiency) and community composition of a natural HNF community from a freshwater reservoir. Pyrosequencing of eukaryotic small subunit rRNA amplicons was used to assess time-course changes in HNF community composition. All four *Limnohabitans* strains and the *Polynucleobacter* strain yielded significant HNF community growth while the actinobacterial strain did not although it was detected in HNF food vacuoles. Notably, even within the *Limnohabitans* strains we found significant prey-related differences in HNF growth parameters, which could not be related only to size of the bacterial prey. Sequence data characterizing the HNF communities showed also that different bacterial prey items induced highly significant differences in community composition of flagellates. Generally, *Stramenopiles* dominated the communities and phylotypes closely related to *Pedospumella* (*Chrysophyceae*) were most abundant bacterivorous flagellates rapidly reacting to addition of the bacterial prey of high food quality.

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Introduction

A unique role of pelagic bacterial communities is their ability to transform dissolved organic material into particulate material making it available to higher trophic levels. Small protists, largely heterotrophic nanoflagellates (HNF) are generally considered to be the major link connecting dissolved organic material, bacteria and the grazer food chain (Jürgens and Matz, 2002; Sherr and Sherr, 2002). However, knowledge of which species or taxa of bacterioplankton are actually consumed by small protists is still quite rudimentary (Boenigk and Arndt, 2002) and available for only a few freshwater habitats with a rather limited taxonomic resolution

(Jezbera *et al.*, 2005; Pernthaler, 2005; Salcher *et al.*, 2008). Thus, the important questions of which bacterioplankton taxa represent the major link in carbon flow to the grazer food chain (Šimek *et al.*, 2006; Salcher *et al.*, 2007), and which grazer taxa mediate this process in different aquatic environments remain unanswered (Montagnes *et al.*, 2008; Nolte *et al.*, 2010; Weber *et al.*, 2012).

In a broad variety of freshwater ecosystems, bacterioplankton communities are frequently dominated by representatives of only a few phylogenetic clusters of *Betaproteobacteria* and *Actinobacteria* (Newton *et al.*, 2011). Among *Betaproteobacteria*, two groups of microbes, differing in many aspects of their lifestyles (Jezbera *et al.*, 2012), are globally distributed and abundant in a wide array of habitats — the genus *Limnohabitans* (mostly affiliated with the R-BT065 cluster, Kasalický *et al.*, 2010, Jezbera *et al.*, 2013) and *Polynucleobacter necessarius* subsp. *asymbioticus* (Hahn *et al.*, 2012).

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Limnohabitans bacteria are abundant in circumneutral or alkaline lakes (Šimek *et al.*, 2010), they display high growth rates and metabolic flexibility, with a notably tight relationship to algal-derived organic substances and algal exudates (Peréz and Sommaruga, 2006; Šimek *et al.*, 2011). Their high growth potential and a significant contribution to bulk bacterioplankton biomass are counterbalanced by a marked vulnerability to protist grazing (Jezbera *et al.*, 2005; Šimek *et al.*, 2006; Salcher *et al.*, 2008). These ecological traits, together with the fact that strains representing different lineages of the genus *Limnohabitans* have been recently isolated (Kasalický *et al.*, 2013), make this bacterial group an invaluable model for testing their role in carbon flow to higher trophic levels.

One can postulate that certain bacterial taxa, 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 given environment. Thus, the growth parameters and a biomass increase of natural HNF communities feeding on such taxa can be suggested as a measure of carbon flow from a particular bacterial group to the predator and, moreover, of the food quality of a particular bacterial prey type for HNF.

Many different methods to study bacterivory by flagellates have been proposed; however, none are appropriate for assessing the dynamics of a particular prey item abundant and present in natural bacterioplankton (Montagnes *et al.*, 2008). Most of the approaches build on ingestion of labeled bacterial prey surrogates (for example, Sherr and Sherr (1993); Montagnes *et al.*, 2008), recently improved by detection using, for example, fluorescence *in situ* hybridization (FISH)-probes targeting bacteria directly in food vacuoles (Jezbera *et al.*, 2005; Šimek *et al.*, 2007). However, ingestion of prey need not yield population growth of HNF (Boenigk *et al.*, 2004; Taraó *et al.*, 2009). Novel methodical approaches are consequently needed to examine the role of particular bacterioplankton taxa in carbon transfer to HNF. We assume that prey quality and its availability can influence the community composition of HNF (Pernthaler, 2005) and this community

composition can be now unveiled by using pyrosequencing of eukaryotic small subunit (SSU) rRNA amplicons (Massana *et al.*, 2004; Nolte *et al.*, 2010).

In this study, we exploited an innovative experimental design to address the following aims: (i) to examine growth community parameters (growth rate, lag phase and flagellate growth efficiency) of freshwater HNF assemblages feeding on bacterial strains of different food quality with focus on closely related *Limnohabitans* strains of diverse cell size and morphology compared with strains from *Actinobacteria* and *Polynucleobacter* lineages and (ii) to examine how the identity of the bacterial prey can modulate HNF community composition using pyrosequencing of SSU rRNA amplicons.

Materials and methods

Experimental organisms

Six different bacterial strains (Table 1), from three important freshwater clades (Newton *et al.*, 2011), were used as prey items for flagellate predators. Four bacterial strains affiliated with the genus *Limnohabitans* (from the R-BT065 subcluster of *Betaproteobacteria*, Šimek *et al.*, 2001; Kasalický *et al.*, 2010), of different sizes and shape, were selected: *L. parvus* (strain II-B4^T) and *L. planktonicus* (strain II-D5^T, Kasalický *et al.*, 2010), both isolated from Římov reservoir (South Bohemia, Czech Republic), and further two undescribed *Limnohabitans* strains—2KL-27 and 2KL-1 isolated from Klíčava reservoir (Central Bohemia, Czech Republic). Other two strains represented phylogenetically distinct prey items, one strain of the genus *Polynucleobacter* (*P. cosmopolitanus*, strain MWH-Molso2, *Betaproteobacteria*, Hahn, 2003; Boenigk *et al.*, 2004) and the other (MWH-Wo1, Hahn and Pöckl, 2005) from the Luna 2 cluster of *Actinobacteria*.

Experimental design and sampling

Before the experiment, the bacterial cultures (Table 1) were pre-grown in liquid 3 g l⁻¹ NSY

Table 1 Characteristics of bacterial strains used as prey for the natural heterotrophic nanoflagellate community from the Římov reservoir

Species	Strain	Affiliation	Cell volume (in μm^3)	Cell length (in μm)	Cell shape	Origin	Reference
<i>Limnohabitans planktonicus</i>	II-D5 ^T	R-BT065 cluster, <i>Betaproteobacteria</i>	0.135 ± 0.079	1.075 ± 0.276	Large rod	Římov reservoir, Czech Republic	Kasalický <i>et al.</i> , 2010
<i>Limnohabitans parvus</i>	II-B4 ^T	R-BT065 cluster, <i>Betaproteobacteria</i>	0.052 ± 0.035	0.670 ± 0.185	Short rod	Římov reservoir, Czech Republic	Kasalický <i>et al.</i> , 2010
<i>Limnohabitans</i> sp.	2KL-27	R-BT065 cluster, <i>Betaproteobacteria</i>	0.067 ± 0.038	0.748 ± 0.185	Coccioid	Klíčava reservoir, Czech Republic	Kasalický <i>et al.</i> , 2013
<i>Limnohabitans</i> sp.	2KL-1	R-BT065 cluster, <i>Betaproteobacteria</i>	0.204 ± 0.110	1.164 ± 0.294	Large solenoid	Klíčava reservoir, Czech Republic	Kasalický <i>et al.</i> , 2013
<i>Polynucleobacter cosmopolitanus</i>	MWH-Molso2 ^T	D-subcluster, <i>Betaproteobacteria</i>	0.049 ± 0.023	0.625 ± 0.115	Short curved rods	Lake Mondsee, Austria	Boenigk <i>et al.</i> , 2004
Undescribed <i>Actinobacterium</i>	MWH-Wo1	Luna 2 cluster, <i>Actinobacteria</i>	0.071 ± 0.023	0.765 ± 0.132	Small solenoid	Lake Wolfgangsee, Austria	Hahn and Pöckl, 2005

Data on cell dimension of each tested strain represent mean value ± s.d. of >200 cells measured.

medium (Hahn *et al.*, 2004). Cells from 50 ml were concentrated by centrifugation at 5.000 g and subsequently re-suspended into 50 ml of <0.2 µm filtered and sterilized water from Římov reservoir (South Bohemia, Czech Republic). The cultures were kept on a shaker overnight to permit even re-suspension of cells and adaptation to the reservoir water. Subsequently, bacteria were enumerated as described below.

We used a natural HNF community to examine the food prey quality of the different bacteria (Table 1) and their effects on HNF community composition. The experiment was scheduled for the onset of the spring maximum of HNF in the reservoir ($\sim 4.5 \times 10^3$ cells ml⁻¹). A 10-liter water sample from Římov reservoir was collected on 18 April 2011 and then gravity filtered through 5-µm pore-size, 147-mm diameter filters. HNF in the filtered water were thus released from zooplankton grazing. After 30 h there was an approximate two-fold HNF abundance increase and a decrease in cell numbers of free-living bacteria (from 2.5 to $\sim 1 \times 10^6$ cells ml⁻¹). The vast majority of remaining bacteria was composed of small, for HNF inedible flocks or filaments.

After the 30 h pre-incubation, 250 ml of the 5-µm filtrates were further manipulated by addition of the respective bacterial strains. Triplicate experiment setup is illustrated in Figure 1. As the tested prey bacteria possessed markedly different mean cell volume (MCV, Table 1), the initial cell numbers of each bacterial strain added into experimental treatments was set to yield approximately the same initial biovolume for all six strains. Notably, the biovolume of the bacterial prey added into the HNF community represented ~ 25 -fold the background bacterial biomass (mostly composed of grazing-resistant morphotypes) 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. All treatments were incubated in the dark at 18 °C and subsamples were taken aseptically at 12–24 h intervals. At selected time points bacterial samples were collected for: (i) FISH and (ii) DNA extraction for pyrosequencing.

Bacterial abundance and sizing, flow cytometry

Bacterial abundance was measured via flow cytometry in samples stained with the fluorochrome Syto13 (Molecular Probes, Eugene, OR, USA) using the FACS-Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) as detailed in Gasol and Del Giorgio (2000). Only at t_{63} and t_{87} hours, bacteria were counted microscopically (for details see Šimek *et al.* (2007)) to quantify accurately also bacterial cells in small grazing-resistant flocks or filaments resulting from HNF grazing pressure. Bacteria (>200 cells per sample) were sized by using the semiautomatic image analysis systems (NIS-Elements 3.0, Laboratory Imaging, Prague, Czech Republic).

Catalyzed reporter deposition fluorescence in situ hybridization

Group-specific oligonucleotide probes (ThermoHybaid, Ulm, Germany) and the catalyzed reporter deposition fluorescence *in situ* hybridization protocol (Pernthaler *et al.*, 2002) were used to track: (a) time-course changes in proportions of the added prey bacteria, and (b) presence of prey bacteria in HNF food vacuoles (Jezbera *et al.*, 2005). Subsamples from control (no bacteria added) and from all bacterial prey-amended treatments were collected at times t_0 , t_{37} and t_{63} hours of the experiment. Oligonucleotide probes were employed to target: the R-BT065 cluster (probe R-BT065, Šimek *et al.*, 2001), which covers all four *Limnohabitans* strains used (Kasalický *et al.*, 2013); the whole *Polynucleobacter* cluster (probe PnecABCD-445, Hahn *et al.*, 2005); and the entire *Actinobacteria* phylum (probe HGC69a).

Heterotrophic nanoflagellate enumeration, biovolume and growth efficiency

Subsamples (1–5 ml) were stained with DAPI and HNF abundance 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 100 cells in triplicate treatments were measured manually on-screen with a built-in tool of a PC-based image analysis system (NIS-Elements 3.0, LIM, Prague, Czech Republic). Estimates of HNF growth efficiency as % based on cellular biovolume were calculated as follows:

$$\text{Growth efficiency} = \left(\frac{\text{HNF biovolume}_{\text{yield}}}{\text{bacterial biovolume}_{\text{introduced}}} \right) \times 100$$

Where HNF biovolume_{yield} is the HNF biovolume increment (that is, maximum HNF biovolume reached minus HNF biovolume present at t_0) divided by bacterial strain-specific biovolume introduced into the treatment at t_0 . The maximum HNF growth rate was calculated using log-transformed data on HNF abundance with linear regression as the slope of the best-fit line. The three consecutive time points on the HNF growth curve yielding the largest r^2 were selected for calculation. The lag 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.

Pyrosequencing of eukaryotic communities and data analysis

Extraction and purification of DNA from triplicate subsamples (50–200 ml, see Figure 1) collected at times t_0 , t_{37} and t_{63} hours of the experiment were performed as described previously (Jezberová *et al.*, 2010).

We used slightly modified broad eukaryotic PCR primers targeting the SSU rRNA genes. The primers were tagged with a 5'-tail adapter for the 454 sequencing (Table 2). The forward primer also

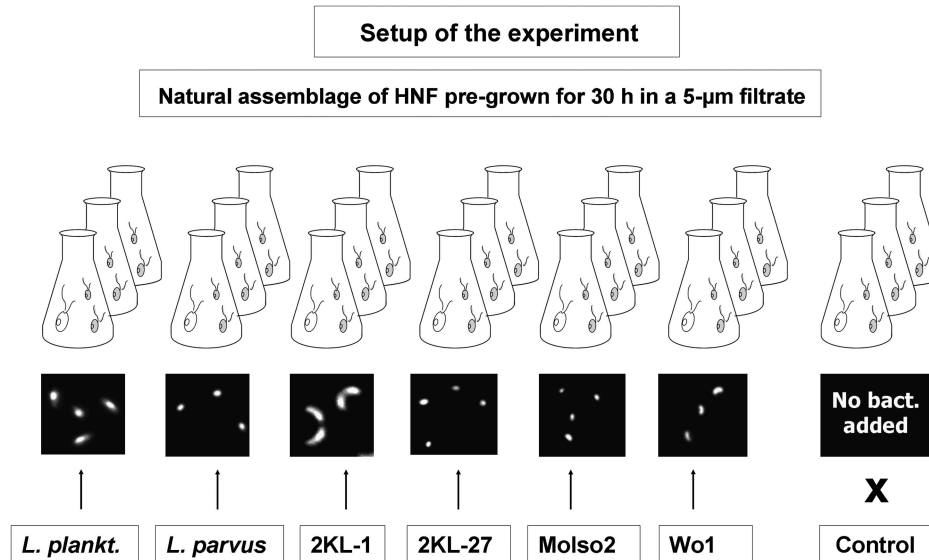


Figure 1 Experimental design: a natural HNF community in 5- μ m filtered water from Římov reservoir was pre-incubated for 30 h and then subjected to additions of different bacterial food items as the major HNF food source (for details see Methods). Note that the initial concentration of natural background bacteria was in all treatments $1.09 \pm 0.10 \times 10^6$ cells ml^{-1} (Mean \pm s.d.). The bacterial strains *L. parvus*, *L. planktonicus*, 2KL-27, 2KL-1, Molso2 and Wo1 (for cell size and morphology see the inserted microphotograph, for further details see Table 1) were added to yield ~ 25-fold natural background of bacterial biovolume present in the non-amended 5- μ m filtrate used as control. Subsamples were collected in 12–24 h intervals.

Table 2 HPLC-purified PCR primers used, which carry sequences specific for the SSU of the rRNA gene

	Sequence (5'-3')	References
Adapter A	CCATCTCATCCCTGCG TGTCTCCGACTCAG	
SSU forward primer	GTACACACCGCCCGTC	Lane, 1991; Stoeck <i>et al.</i> , 2010
SSU reverse primer	TGATCCTTCTGCAGGTTACCTAC	Medlin <i>et al.</i> , 1988; Stoeck <i>et al.</i> , 2010
Adapter B	BioTEG-CCTATCCCCTGTGTGCCTTGCCAGTCTCAG CTGAGACTGCCAAGGCACACAGGGGATAGG	

Abbreviation: SSU, small subunit.

contained a 6–10 bp tag for each of the samples inserted between the 454-adapter A and the SSU-specific part, the reverse primer was modified with a 5' BioTEG modification. The primers amplify a fragment of the SSU rRNA gene including the variable V9 region. PCR was carried out in a 20- μ l reaction with 0.2 U Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland), 200 μ M dNTPs and 0.25 pmol of each primer. The cycling profile consisted of 1 min denaturation at 95 $^{\circ}\text{C}$, followed by 30 PCR cycles (95 $^{\circ}\text{C}$ for 30 s, 60 $^{\circ}\text{C}$ for 45 s, 72 $^{\circ}\text{C}$ for 60 s) with a final extension step of 10 min at 72 $^{\circ}\text{C}$. DNA from each triplicate subsample was subjected to PCR separately. PCR was carried out eight times per sample in order to generate more products and the PCR products were pooled upon completion of the reaction.

The pooled PCR products of each sample were gel-purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and quantified on an agarose gel. We pooled 525 ng from each sample

and sequenced the pooled DNA on a 454 Roche (Durham, NC, USA) FLX sequencer (titanium chemistry). Read sequences will be deposited in the NCBI Short Read Archive (deposition in progress).

CANGS DB (Pandey *et al.*, 2011) was used for processing, adapter and primer clipping, quality filtering and grouping of sequences according to barcodes. Briefly, sequences that did not fit the following quality criteria were removed: (i) no ambiguous nucleotides (that is, no Ns in the sequence reads); (ii) quality score > 20, when averaged across the read after clipping adapters and primers; (iii) minimum sequence length of 130 bp (including PCR primers); and (iv) at least two copies of the read present in two different samples in the entire data set before clipping primers (Medinger *et al.*, 2010).

Flagellate taxonomic classification

Reads obtained from each sample were assigned to taxa defined by the NCBI taxonomy by using the

software MEGAN 4 (Huson *et al.*, 2007). The assignment to taxa is based on BLAST results. Reads were compared by BLAST (Altschul *et al.*, 1990) against the NCBI-nr database, and the resulting data sets were loaded in MEGAN. As sequence sets obtained for the different samples varied in read numbers, each sample was analyzed by using an adjusted LCA value. A basic LCA parameter of 200 was selected and LCA parameters for individual samples were normalized according to sample size. For each sample, only the number of reads assigned in a pre-run (LCA parameter set as 200) to protist taxa was considered for normalizing LCA parameters. Matching between taxon assignment by MEGAN 4 and phylogeny derived classification of reads was examined by comparing MEGAN results with a phylogenetic tree constructed with reads of a representative subset of reads. Both methods resulted in a comparable assignment of reads to taxa or phylogenetic clusters, respectively.

Results

Flagellate growth responses to bacterial prey

We tested the effects of food quality of six bacterial strains of different size, morphology and taxonomic affiliation (Table 1) on the growth and community composition of a natural HNF community from a freshwater reservoir. The bacterial strains were added in numbers (Figure 2) that compensated for their different MCVs to yield approximately the same initial total prey biovolume in all treatments (Figure 3).

HNF grazing decimated all prey types within 63 h to the abundance level of control treatment, except for the strain Wo1 (*Actinobacteria*) with a markedly smaller rate of cell decline (Figure 2). All six strains were clearly detected in HNF food vacuoles using FISH probes (Supplementary Figure 1) and all but the Wo1 strain, yielded significant growth of HNF communities, although with different growth dynamics (Figures 3 and 4). For instance, HNF numbers and biovolume peaked in the treatment amended with *L. planktonicus* at t_{37} hours, while in most other cases the HNF peak abundance appeared later at t_{50} hours. However, although cell abundance of the Wo1 strain decreased (Figure 2), no HNF growth was detected compared with control treatment (Figures 3 and 4). Bacterial cellular biovolume added into treatments compared with the net HNF biovolume increments allowed for estimates of growth efficiency of HNF on different food items (Figure 3), ranging from 25% (strain 2KL-1) to 31% (*L. parvus*).

The similar initial biovolumes of the distinct bacterial prey yielded different HNF growth dynamics (for significance see Figure 4). For instance, HNF in the *L. planktonicus* treatment achieved a population peak significantly faster (Figure 4) than in other treatments with almost no

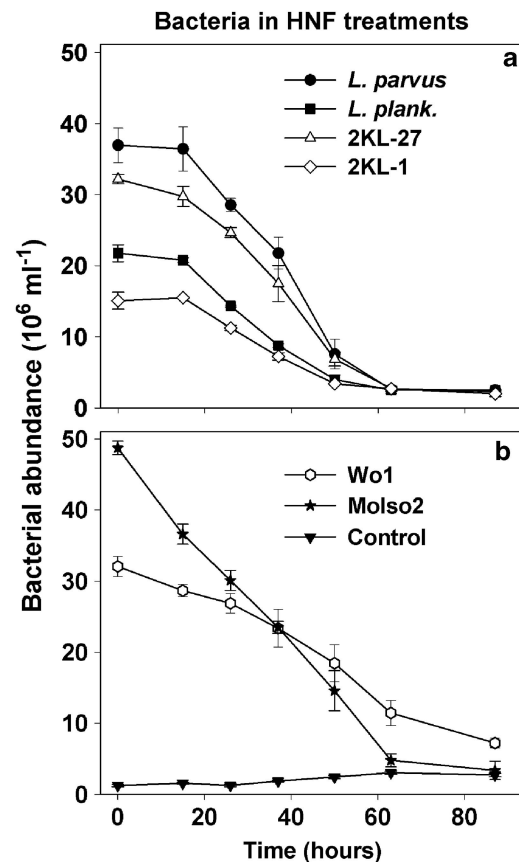


Figure 2 Time-course changes in bacterial abundance in 5- μ m treatments amended by bacterial strains of the genus *Limnohabitans* (a), that is, *L. parvus*, *L. planktonicus*, 2KL-27 and 2KL-1, and (b) with the strains Molso2 and Wo1 compared to control with no bacteria added. Values are means for triplicates; error bars show s.d..

lag phase. In contrast, HNF in the *L. parvus* treatment grew even faster but after relatively long lag phase, resembling rather growth parameters of HNF growing on strain Molso2 of a similar cell size (Table 1). In contrast, even the relatively closely related and similarly sized *Limnohabitans* strains, that is, *L. parvus* and KL-27 (Table 1 and Figure 4), gave distinct HNF growth dynamics, while the similarly sized gram-positive strain Wo1 did not support detectable HNF community growth. Note that MCVs of all the tested bacterial strains were relatively stable during the first 50 h of the experiment; that is, the coefficient of variation of MCV for each strain was within 12% of the strains' MCVs listed in Table 1 (values for t_0 hours). However, the starting prevalence of the target bacteria (tracked by FISH probes) in the treatments diminished (Figure 2) and MCVs of all bacteria present in the treatments almost doubled towards the experimental end (data not shown). It resulted from increasing proportions of larger grazing-resistant morphotypes (small flocks and filaments) that developed from the background bacteria present in the original sample as a response to increasing HNF abundance (Figure 3) and bacterivory.

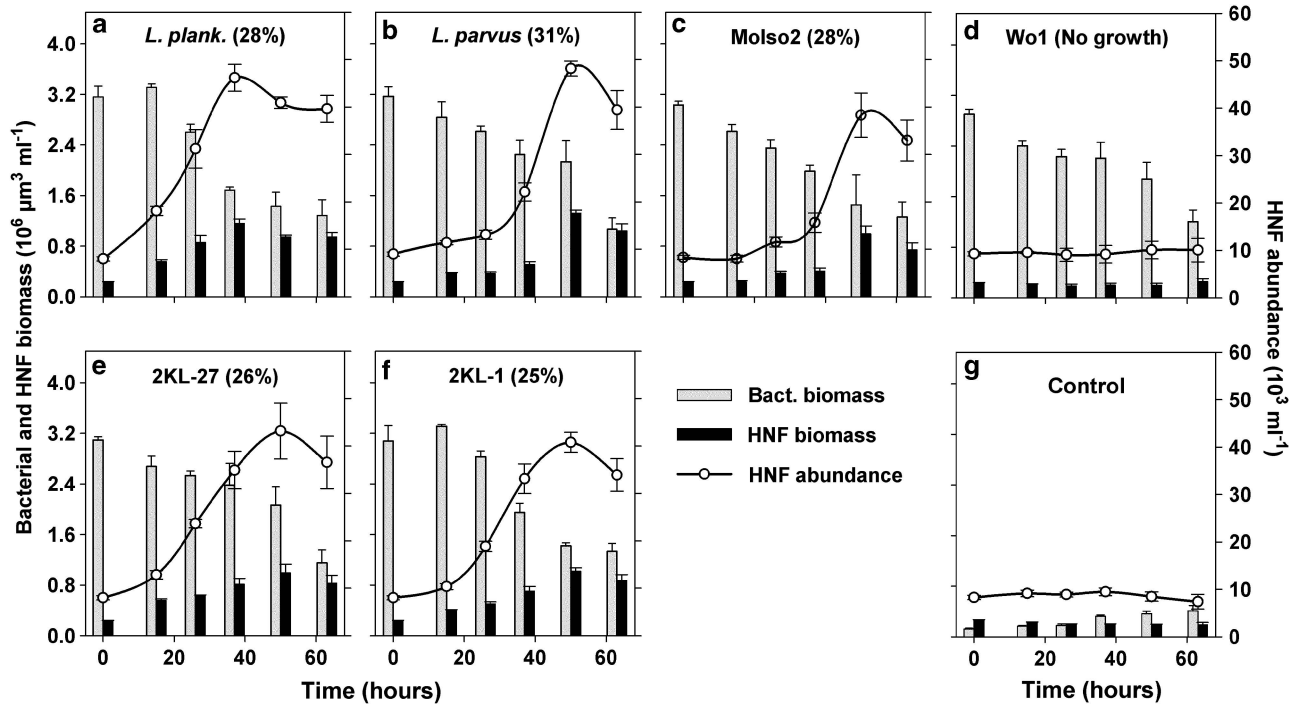


Figure 3 Time-course changes in HNF abundance and biovolume compared with bacterial biovolume in the treatments amended with respective bacterial strains (a–f, for further details see text to Figure 1 and Table 1) compared with control (g) with no bacteria added. Values are means for triplicates; error bars show s.d.. Values of growth efficiency, as % of bacterial biovolume introduced into the treatment at t_0 , are listed in parenthesis for the treatments where positive HNF growth was detected (for details see Methods).

Effects of bacterial prey quality on HNF community dynamics and composition

Prey-specific differences in HNF growth parameters were also reflected in the analysis of pyrosequenced eukaryotic SSU rRNA amplicon (in total 102 239 reads were analyzed). Among reads representing protistan taxa, *Stramenopiles* clearly dominated with 55–82% of the total numbers of reads over all treatments and the course of the study, while other groups such as *Katablepharidaceae* (0–12%), *Chonoflagellida* (6–13%) and sum of *Opisthokonta* and *Rhizaria* (6–18%) appeared either only irregularly or did not show any clear trend in the samples (data not shown). Groups of *Cryptophyta*, *Chlorophyta* and *Telonema*-related reads composed generally a negligible proportion (mostly <1%), or such reads were detectable only sporadically in control samples (data not shown).

Thus, *Stramenopiles*, including also typical bacterivorous groups of *Spumella*-like flagellates, represented the most abundant protistan groups of interest for further analysis of the bacterial prey-related responses of the HNF community. Generally, all prey types supporting HNF growth (Figure 3) induced an approximately two-fold increase in the relative proportions of the *Spumella*-like HNF (from 16 to 31–38% of total *Stramenopiles*; Figure 5) towards exponential growth phase of HNF (t_{37} hours). No such community shift was observed in Wo1 and control treatments (without HNF growth, Figure 3), in which

the overall *Stramenopiles* composition developed similarly and differed significantly from other treatments supporting HNF growth ($P < 0.01$, two-way ANOVA, followed by Bonferroni's post-test). Notably, the relative proportions of *Oikomonas* sp. (SA-2.2) and *Pirsonia* clusters significantly increased ($P < 0.01$) between t_{37} – t_{63} hours in all treatments (Figure 5).

An increase in proportions of the *Spumella*-like HNF paralleled a steep increase in total HNF abundance between t_0 and t_{37} hours (compare Figures 3 and 5) indicating high net growth rates of the *Spumella*-like HNF in all but Wo1 and control treatments. Overall, highly significant differences in growth rates of *Spumella*-like HNF were detected in the different treatments ($P < 0.0001$, ANOVA, Table 3). For instance, the *Spumella*-like HNF grew significantly faster (Table 3) in $L. planktonicus > 2KL-1 \cong 2KL-27 > L. parvus \cong Molso2 \gg Wo1$ treatment (\cong , comparable growth). In t_{37} hours, the vast majority the *Spumella*-like reads was affiliated with *Pedospumella* sp. (see phylogenetic tree, Supplementary Figure 2), with the highest proportion in $L. planktonicus$ (>95%) and lowest in Wo1 treatment (86%, Supplementary Figure 3). Overall, $L. planktonicus$ treatment yielded 2–3-fold higher abundance of *Pedospumella*-like HNF at t_{37} hours than other bacteria, however, followed by their dramatic ~7-fold decrease at t_{63} hours observed also in 2KL-1 treatment (Figure 6). In contrast, no such dramatic changes in abundance of *Pedospumella* sp.

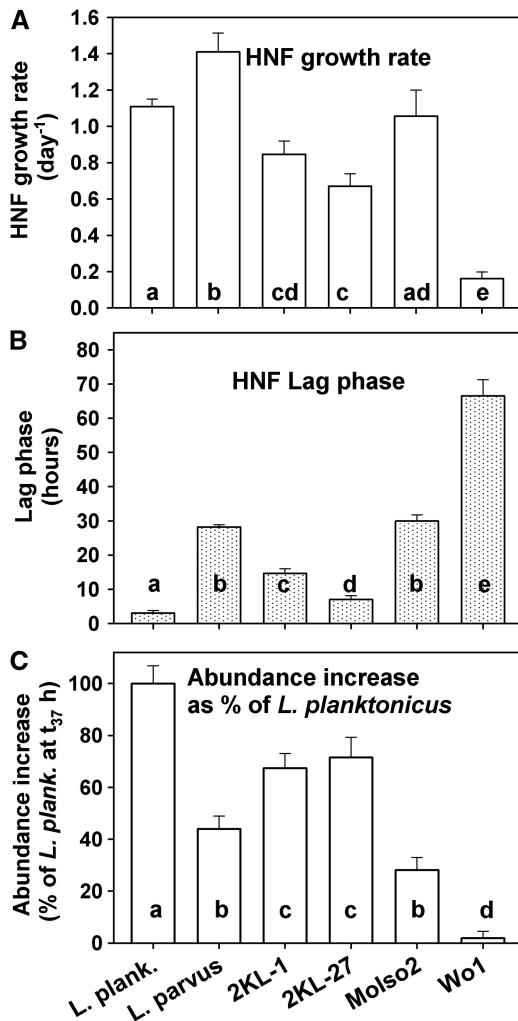


Figure 4 Comparison of HNF maximum growth rates (A), length of their lag phase after the treatment amendment (B) and the rate of HNF abundance increase growing on different bacterial strains (for further details see text to Figure 1 and Table 1) as related to the treatment with *L. planktonicus* (C) where the HNF abundance and biovolume peaked already at t_{37} hours (compare Figure 3). Values are means for triplicates; error bars show s.d. Different letters indicate a significant difference ($P < 0.05$, ANOVA, followed by Tukey's multiple comparison tests) between treatments amended with different bacterial strains.

between t_{37-63} hours were observed in *L. parvus*, 2KL-27 and Molso2 treatments.

Another two groups related to *Spumella*-like HNF, that is, cluster D (related to the so far undescribed *Spumella*-like flagellate strain JBC27) and E2 (related to the undescribed *Spumella*-like flagellate strains JBM08 and JBM18), rapidly increased both their relative and absolute numbers in some treatments (compare Figure 6 and Supplementary Figure 3), however, in distinct prey-specific fashions. Their growth rates differed significantly with the prey type ($P < 0.0001$, ANOVA, Table 3). Overall, in *L. planktonicus*, *L. parvus* and 2KL-1 treatments similar relative proportions of the major *Spumella*-like flagellate groups were found at t_{63} hours,

whereas significantly lower proportions ($P < 0.01$, ANOVA, followed by Bonferroni's post-test) and absolute numbers of the *Spumella*-like Cluster D (related to the JBC27 strain) were detected in 2KL-27 and Molso2 treatments. Interestingly, while no growth of total HNF can be seen in the Wo1 treatment (similar to control in this aspect, Figure 3), this prey addition induced some increase in relative proportions and abundance of *Spumella*-like Cluster D at t_{63} hours (Figure 6 and Supplementary Figure 3).

Discussion

Major findings of the study

This study demonstrated the suitability of *Limnohabitans* bacteria as prey for flagellates and clear prey-specific community growth responses of HNF communities to the different food quality of the tested bacteria. To the best of our knowledge, this the first study clearly documenting strong prey-specific effects of even closely related bacteria on HNF community composition. This is an ecological aspect that has been long under debate (for example, Boenigk *et al.*, 2004; Pernthaler, 2005; Montagnes *et al.*, 2008) but without any direct evidence concerning natural HNF assemblages.

Notably, the fast growing *Limnohabitans* bacteria (Šimek *et al.*, 2006; Jezbera *et al.*, 2012) likely represent a high quality resource supporting rapid growth of natural HNF communities. We are confident in this conclusion as recently four more tested strains from other *Limnohabitans* lineages (Kasalický *et al.*, 2013) supported fairly rapid growth of HNF communities in another two freshwater ecosystems (Šimek, unpublished data). Our results lend solid support to previous speculations based on preliminary indirect evidence on the key role of these bacteria in carbon flow from alga-derived substrates (Šimek *et al.*, 2010, 2011) to the plankton grazer food chain (Jezbera *et al.*, 2005). The important role of the *Limnohabitans* sharply contrasts to that of the Wo1 actinobacterial strain. Although cells of Wo1 were ingested (Supplementary Figure 1) this prey did not support any HNF community growth, likely because of limited digestibility of the bacterium for flagellates (Tarao *et al.*, 2009).

Methodical aspects—examining growth parameters of flagellates

In this study, an innovative combination of methods such pyrosequencing and FISH-targeting of prey bacteria directly in HNF food vacuoles were exploited, with the experimental design building on prey-depletion approach (Montagnes *et al.*, 2008) in ~3-day incubations with the HNF community established after 30-h in 5- μ m filtrate. Thus, in pelagic environments even more profound shifts in HNF communities (Figure 5 and Supplementary

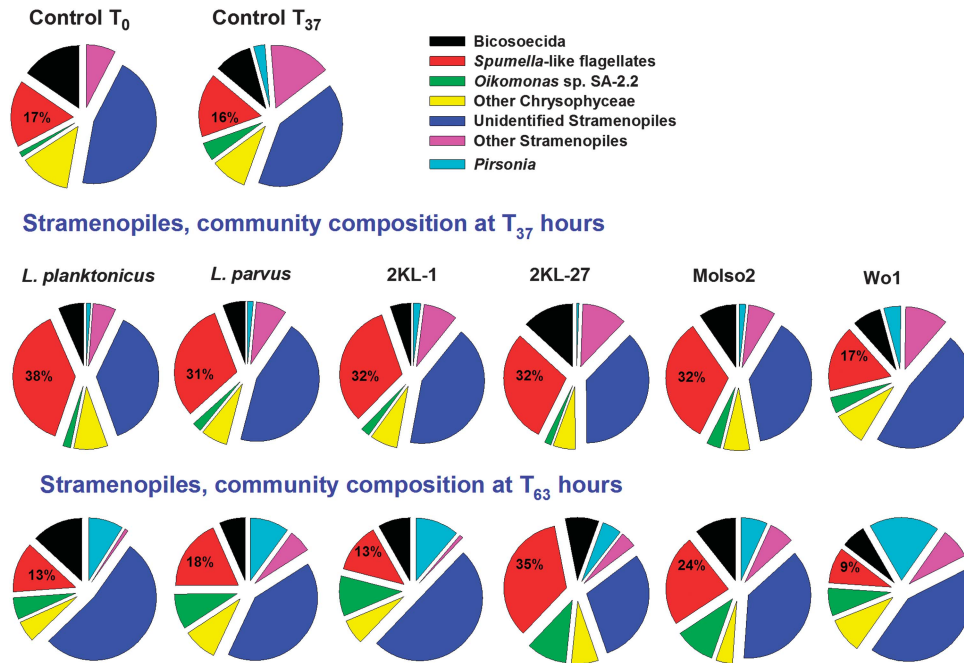


Figure 5 A pie chart of the relative proportion (as %) of different subgroups of *Stramenopiles* within total *Stramenopiles* (accounting for 58–82% of the potentially bacterivorous flagellate groups) in treatments amended with different bacterial strains at times t_{39} and t_{63} compared with control treatments at t_0 and t_{39} hours. For further details regarding the bacterial strains see text to Figure 1 and Table 1.

Table 3 Estimates of maximum growth rates (mean value based on triplicate treatments) of the whole group of *Spumella*-like HNF and of three most abundant subgroups of the *Spumella*-like HNF detected in sequence analysis (compare absolute numbers and relative proportions of the target HNF groups in Figures 3, 5 and 6 and Supplementary Figure 3) between t_0 and t_{37} of the experiment

Flagellate group	Flagellate growth rate on different bacterial strains (per day, mean \pm s.d.)					
	<i>L. planktonicus</i>	<i>L. parvus</i>	2KL-1	2KL-27	Molso2	Wo1
Whole <i>Spumella</i> -like group	1.70 \pm 0.05 ^A	1.01 \pm 0.10 ^B	1.37 \pm 0.08 ^C	1.40 \pm 0.08 ^C	0.86 \pm 0.11 ^B	0.07 \pm 0.04 ^D
<i>Pedospumella</i> sp.	1.69 \pm 0.08 ^A	0.98 \pm 0.09 ^B	1.33 \pm 0.08 ^C	1.36 \pm 0.04 ^C	0.84 \pm 0.13 ^B	–0.01 \pm 0.03 ^D
<i>Spumella</i> -like Cluster D	2.88 \pm 0.09 ^A	1.88 \pm 0.10 ^B	2.73 \pm 0.08 ^A	2.27 \pm 0.08 ^C	1.59 \pm 0.11 ^D	2.20 \pm 0.09 ^C
Cluster E2	2.28 \pm 0.07 ^A	1.91 \pm 0.10 ^B	2.57 \pm 0.10 ^C	2.77 \pm 0.13 ^C	1.88 \pm 0.11 ^B	1.32 \pm 0.07 ^D

Different superscripted letters indicate a significant difference between flagellate growth rates in different treatments ($P < 0.0001$, one-way ANOVA, followed by Tukey's multiple comparison tests).

Figure 3) could be expected as a response to, for example, phytoplankton succession related bacterial blooms (Eiler and Bertilsson, 2004) lasting for few days or weeks.

In our experimental design exploited, the HNF growth responses could be directly attributed to a particular prey item dominating the prey assemblage. Moreover, due to enhanced grazing pressure in 5- μ m treatments the less abundant naturally occurring bacteria, present after 30-h incubation were mostly composed of grazing-resistant flocks and filaments (Jürgens and Matz, 2002), that is, of bacterial biomass that was likely irrelevant to the prey-specific growth responses of HNF.

Most importantly, our approach yielded ecologically meaningful prey-specific flagellate growth parameters. For instance, the combination of a high growth rate and short lag phase of the HNF communities indicates a marked suitability of a

bacterial prey for flagellates present in the assemblage, that is, demonstrating a minimum acclimatization time to the prey offered. Notably, *L. planktonicus* yielded almost immediate exponential HNF growth with minimum lag allowing for the most rapid accumulation of an HNF population peak among all the strains (Figure 3). Such results indicate that the strain represents a high quality prey. In contrast, for all the other strains a significantly prolonged lag phase were observed (Figure 3), thus indicating much longer acclimatization time and likely selection of flagellates capable to exploit more efficiently the prey offered.

Prey-specific aspects of HNF growth responses

The prey-specific HNF growth responses were reflected in different patterns and timing of the HNF community shifts (Figures 3 and 5 and

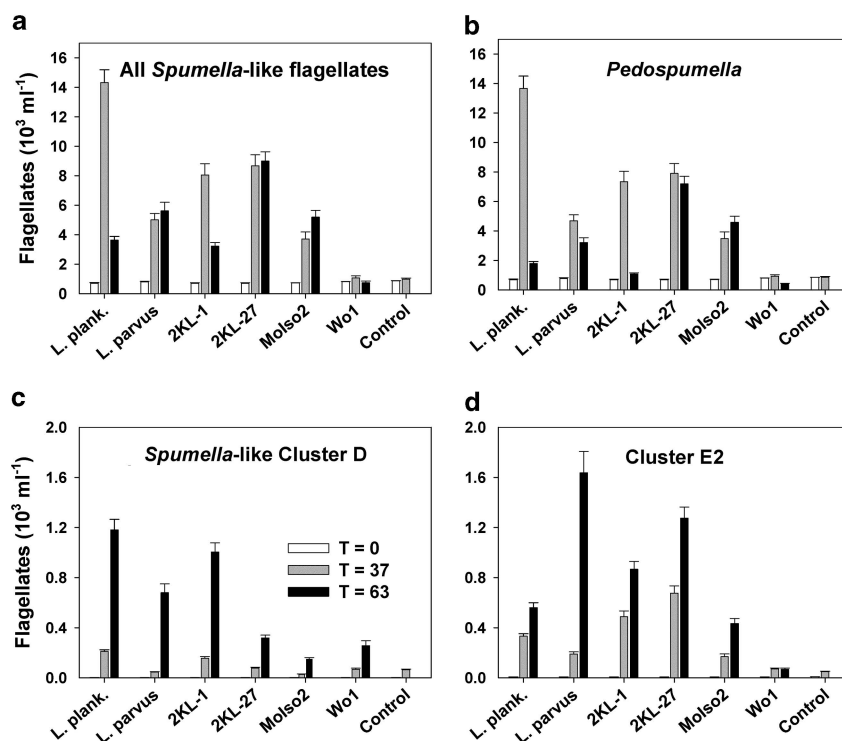


Figure 6 Absolute numbers of all *Spumella*-like flagellates (**a**) and of their three most abundant subclusters—*Pedospumella*, *Spumella*-like cluster D and Cluster E2 (**b–d**, respectively) in treatments amended with different bacterial strains at times t_{37} and t_{63} compared with control treatment. These data are based on the relative proportion of these subclusters within the target protistan groups (Figure 5 and Supplementary Figure 3) related to total HNF numbers (Figure 3). Note different y axis scaling for c, d. Values are means for triplicates; error bars show s.d.. For further details regarding the bacterial strains see text to Figure 1 and Table 1.

Supplementary Figure 3). Among *Stramenopiles*, mainly the typical bacterivorous *Spumella*-like flagellate taxa (Boenigk *et al.*, 2004, 2005) approximately doubled their relative proportions in the community and dramatically increased absolute numbers (Figure 6) during the exponential HNF growth (t_{37} hours) in all, but Wo1 treatment. This clearly points to the key role of bacterial food quality characteristics as the Wo1 strain was the only one that did not support HNF community growth (see also Tarao *et al.*, 2009). Interestingly, throughout the course of the study and among experimental treatments, there was no bloom of choanoflagellates (considered as bacterivores, Boenigk and Arndt, 2002) as this group generally comprised relatively stable low proportions of total protistan reads (data not shown).

During the stationary HNF growth phase (t_{63} hours) the bulk *Spumella*-like cluster dropped in both relative and absolute numbers in all but 2KL-27 and MoIso2 treatments, for which quite unique trends were observed (Figures 5 and 6). Interestingly, cell size and morphology of 2KL-27 resembled closely *L. parvus* or MoIso2 *Polynucleobacter* strains (compare Figure 1 and Table 1), but induced significantly different HNF growth responses (Figure 4). It indicates that even within closely related *Limnochabitans* bacteria non-morphology related traits have an important role in regulating exploitation of particular

bacteria (Boenigk and Arndt, 2002; Jürgens and Matz, 2002). Interestingly, the very small cells of the ultramicrobacterial ($<0.1 \mu\text{m}^3$) strain MoIso2, supported only slow growth of several *Poteriospumella* spp. genotypes in laboratory experiments (Boenigk *et al.*, 2004). However, in this study after longer lag phase, indicating ‘HNF community acclimatization time’, this prey yielded rapid doubling times of different members of the *Spumella*-like cluster (Table 3). This together with highly prey-specific HNF community shift in MoIso2 treatment points to the role of predator–prey species-specific interactions supporting the idea of clear niche partitioning even among closely related flagellate species (Boenigk *et al.*, 2004).

Our study documents, at different taxonomic levels, the strong impact of prey on the resulting HNF community dynamics over different phases of population growth. Aside from a common trend of decreasing HNF numbers at t_{63} hours (Figure 3), their MCVs increased (data not shown) in parallel with significantly higher proportions ($P < 0.01$, ANOVA, Bonferroni’s post-test) of sequences matching with the *Oikomonas* sp. SA-2.2 cluster or those loosely related to *Pirsonia* (Figure 5). Although the *Oikomonas* is considered a bacterivore (Boenigk and Arndt, 2002), the *Pirsonia* genus has been reported as a diatom feeder in brackish waters (Schnepf and Schweikert, 1997; Kühn *et al.*, 1996). This overall

community shift may reflect an increasing proportion of larger flagellates within *Stramenopiles* (Figure 5) preying upon smaller and highly abundant *Spumella*-like HNF that become food limited at t_{63} hours (Figure 2). However, this shift to larger flagellates, in contrast with the bulk decline in HNF abundance (Figure 3), could partially be related to zooplankton removal involved in our experimental design. Generally, in plankton environments larger HNF are efficiently limited by zooplankton grazing (Jürgens and Matz, 2002) and thus smaller HNF, such that *Spumella*-like bacterivorous chrysomonads frequently dominate freshwater plankton (Šimek *et al.*, 1997; Nolte *et al.*, 2010).

The bacterial prey of high quality (except for Wo1 strain) supported growth of the bulk HNF community with doubling time of 11–23 h (Figure 3), but in the range of 5.8–16 h for the representatives of rapidly growing flagellates (Table 3) affiliated with the *Spumella*-like subclusters D (related to the undescribed flagellate strain JBC27) and E2 (related to the undescribed *Spumella*-like flagellate strains JBM08 and JBM18). The rapid flagellate growth mainly on *L. planktonicus*, 2KL-1 and 2Kl-27 strains is comparable to that found for typical *Spumella*-like flagellates cultured under optimal food conditions (Boenigk *et al.*, 2007). This together with high HNF growth efficiency (Figure 3, compare Fenchel (1986)) is evidence of the food quality of most *Betaproterobacteria* strains used in this study.

Although the *Spumella*-like flagellates from the clusters D and E2 were practically absent in the control at t_0 hours, due to their high growth potential (compare *Pedospumella* sp. in Table 3) they significantly increased in relative and absolute numbers toward the end of the experiment, mainly in *L. planktonicus*, *L. parvus* and 2KL-1 treatments. Moreover, the flagellates affiliated with the *Spumella*-like cluster D were the only predator group, which grew in the presence of the Wo1 strain. Although being considered as poor quality prey (Tarao *et al.*, 2009), Wo1 did yield a certain prey-specific HNF community shift.

Concluding remarks

This study clearly demonstrated that different food properties of even closely related bacterial prey can modulate differently growth dynamics of flagellates and in turn also the overall HNF community composition. During the exponential growth phase the driving force of such a shift was the *Pedospumella* cluster (likely due to the 'inoculum size' at t_0) that overgrew other bacterivorous flagellate groups and comprised ~15–40% of total HNF numbers. Notably, our approach facilitated determining bulk parameters of HNF community growth while revealing many significant differences related to bacterial prey quality (Figure 3). Moreover, short lag phase and rapid growth responses of major bacterivorous

HNF taxa to a particular prey type might indicate its high food quality. Our experimental design combined with pyrosequencing of the grazer community could provide important insights regarding the question which bacterial strains are active in carbon transfer to the grazer food chain in a particular aquatic system and which flagellate groups are the key players in the trophic transfer.

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