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Food selection by bacterivorous protists: insight from the analysis of the food vacuole content by means of fluorescence in situ hybridization

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Abstract

A modified fluorescence in situ hybridization (FISH) method was used to analyze bacterial prey composition in protistan food vacuoles in both laboratory and natural populations. Under laboratory conditions, we exposed two bacterial strains (affiliated with β- and γ-Proteobacteria – *Aeromonas hydrophila* and *Pseudomonas fluorescens*, respectively) to grazing by three protists: the flagellates *Bodo saltans* and *Goniomonas* sp., and the ciliate *Cyclidium glaucoma*. Both flagellate species preferably ingested *A. hydrophila* over *P. fluorescens,* while *C. glaucoma* showed no clear preferences. Differences were found in the digestion of bacterial prey with *B. saltans* digesting significantly faster *P. fluorescens* compared to two other protists. The field study was conducted in a reservoir as part of a larger experiment. We monitored changes in the bacterial prey composition available compared to the bacteria ingested in flagellate food vacuoles. Bacteria detected by probe HGC69a (Actinobacteria) and R-BT065 were negatively selected by flagellates. Bacteria detected by probe CF319a were initially positively selected but along with a temporal shift in bacterial cell size, this trend changed to negative selection during the experiment. Overall, our analysis of protistan food vacuole content indicated marked effects of flagellate prey selectivity on bacterioplankton community composition.

Keywords: Protistan bacterivory; FISH; Reservoir; Heterotrophic nanoflagellates; Grazing

1. Introduction

A variety of mechanisms have been proposed as regulators of bacterioplankton including temperature, resources (bottom-up), grazing (top-down), or viral lysis [1–4]. Nutrient availability and more recently mainly grazing impact on bacterioplankton have received probably the most attention. Heterotrophic nanoflagellates (HNF) and ciliates have been widely accepted to be the most prominent bacterivores in most aquatic systems [5,6]. With the advent of novel techniques of molecular biology, it is now possible to examine the relative importance of these mechanisms.

The impacts of protistan grazing on bacterial communities are based on the complex interplay of several factors. These include grazing selectivity (by size and other features), differences in the sensitivity of bacterial species to grazing, differences in responses of single bacterial populations to grazing (size flexibility and physiology), as well as the direct and indirect influence of grazing on bacterial growth conditions (modifying e.g. substrate supply) and bacterial competition via elimination of competitors [7–10]. Furthermore, it appears that different protistan species can evoke different responses

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in the bacterial community composition (BCC) [11], which implies that different bacteria are probably ingested and metabolized at different rates. This may be deduced from laboratory studies of flagellate feeding which have shown significant taxon-specific differences in feeding mechanisms and selectivity [12]. Thus, each predator community may exert specific and a highly complex top-down pressure on the bacterial community shaping BCC and diversity [9,11]. However, the effects of grazers are not easily predicted. For example, when protists are the major bacterivores, both very small and large bacterial cells gain some specific size refuge [9]. Additionally, recent studies have revealed that also various non-morphological traits such as motility, physicochemical surface characteristics and toxicity affect bacterial vulnerability to protistan feeding [10].

Experiments designed to investigate the changes in BCC caused by protistan grazing have been done mainly in freshwaters [13–15], and it has been suggested that the rapid BCC changes are induced by grazing, mainly by introducing a temporal imbalance between bacterial species-specific growth and removal rates [16]. While this idea seems to be intuitively reasonable, there is no direct evidence that under typical natural conditions with relatively low prey abundance, protists do select for or against certain bacterial species or groups. Such a study, i.e. exploring the possibility to detect relative proportions of different phylotypes in bacterioplankton compared to their proportions among bacterial prey ingested in protistan food vacuoles, has not yet been conducted under in situ conditions.

Fluorescence in situ hybridization (FISH) as a tool to investigate protistan vacuoles content has been used only in a few laboratory studies with cultured organisms. Gunderson and Goss [17] investigated food vacuole contents of the ciliate *Tetrahymena*. Pernthaler et al. [18], used in situ hybridization and fluorescently labeled bacteria uptake to assess the grazing rates of protists on different subdivisions of the phylum Proteobacteria in a chemostat experiments.

Here we describe studies designed to investigate protistan food preferences by analyzing food vacuole content via FISH targeted cells inside of food vacuoles. In the first one, under the laboratory conditions, we tested the approach on different cultured protists employing several rRNA targeted probes to estimate species-specific differences in ingestion and digestion rates. The second study was performed in the canyon-shaped Římov reservoir, building on our previous work on the impacts of the top-down (size fractionation) and bottom-up manipulations, investigated by transplanting of samples and incubation in dialysis bags in different parts of the reservoir [19–22]. Natural populations were subjected to manipulations designed to provoke changes in BCC. In both the laboratory and field study, we employed a slightly modified FISH approach to examine protistan prey by comparing and quantifying the proportions of bacterial prey versus prey ingested in the food vacuoles of protists.

2. Materials and methods

2.1. Laboratory experiments

To verify our modified FISH approach (for details see below) we conducted laboratory experiments with three protists fed with a defined mixture of two different laboratory grown bacterial strains. Three bacterivorous protistan species were used – a scuticociliate, *Cyclidium glaucoma*, and two HNF, *Bodo saltans* and *Goniomonas* sp. *C. glaucoma* and *Goniomonas* sp. were isolated from the Římov reservoir by M. Macek and K. Šimek, *B. saltans* was isolated by D. Springmann, for details see [3].

Replicates of each protistan cultures were carefully cleaned by filtration through sterile filtration funnels using 1 or 2 μm polycarbonate filters (OSMONIC Inv., Livermore, USA, for details see [3,20]) to remove the vast majority of the accompanying bacteria. By dilution of our pre-cleaned protistan culture roughly 1:5 with the bacteria free bactopepton/yeast extract liquid media (20 $mg l^{-1}$), we obtained the protozoan numbers resembling those occurring in meso-eutrophic freshwaters [5], i.e. roughly 2000 flagellates ml^{-1} and 50 ciliates ml^{-1}. Then, in the bacteria-free medium we let the protists digest their food vacuole content for 2 h (after 2 h, no FISH – positive bacteria were detected in the food vacuoles) and subsequently inoculated them into the mixture of two bacterial species at a final concentration of $\sim 1 \times 10^6$ bacteria ml^{-1}. The mixture consisted of roughly one half of β-Proteobacteria – *Aeromonas hydrophila* (for reference see [23]), concentration of 5.356×10^5 ml^{-1}, and one half of γ-Proteobacteria – *Pseudomonas fluorescens* 5.215×10^5 ml^{-1}. These bacterial concentrations were similar in all treatments (±5%). In the preliminary experiments, both bacterial strains, routinely grown on bactopepton/yeast extract liquid media (20 $mg l^{-1}$), were found to support the growth of tested protists (data not shown). Average length of both bacterial strains was in the range of 0.97–1.18 μm, width 0.35–0.46 μm. All cell size measurements were done from pictures taken with a CCD camera connected to the microscope, and measured with an semi-automated image analysis system (LUCIA, Laboratory imagining, Prague, Czech Republic) after an image processing procedure (edge finding, digitalization etc.). For the three protists tested, duplicated, 500 ml experimental treatments were run in total of six 1000 ml Erlenmayer bottles at 22 °C.

To estimate protistan uptake rates, subsamples (20 ml) were taken in times 0, 5, 10, 15, 20 30 and 60 min after inoculation of protists into the experimental bacterial mixtures and fixed with 0.5% of alkaline Lugol's

solution, followed by 2% borate-buffered formaldehyde (final concentration) and within 2–3 min, several drops of 3% sodium thiosulfate were added to clear the lugol's color [24]. Sixty minutes after the beginning of the uptake experiment, 20 ml subsamples were added to and thoroughly mixed with 1980 ml of the sterile bacteria-free (filtered through 0.2 μm OSMONIC filter) bactopepton-yeast extract media in Erlenmayer bottles. Thus, the encounter probability of protists and bacterial food particles was decreased by two orders of magnitude. Then, subsamples from 0, 5, 10, 20, and 30 min after the dilution were taken (fixed as described above) to estimate the digestion rate of protists fed on both bacterial strains. The cells hybridized with the BET42a (*A. hydrophila*) and GAM42a (*P. fluorescens*) probes [25] inside of protistan food vacuoles were counted separately, making all FISH-positive cells clearly distinguishable and countable in food vacuoles by means of their relatively strong fluorescence signal (see Fig. 1) produced by targeted cells.

We calculated digestion rates of the two bacterial strains as the slope of the linear regression of log (% time zero prey per cell) based on five time points, i.e. at the times 0, 5, 10, 20 and 30 min after dilution, for details see [26]. Multiplying the slope by 100 gives an exponential digestion rate constant, K, in units of % per minute. Based on K, an expected halftime of cell content, $t_{1/2}$, was estimated by calculating the time (in minutes) required for a 50% decline in cell content [26]. Clearance rates were calculated by dividing the species-specific grazing rates by the bacterial (*A. hydrophila* or *P. fluorescens*) abundance (for details see [6]).

Since bacterial cells hybridized directly in the food vacuoles of protists are generally less bright and visible, to improve their detectability, slightly modified standard FISH protocol was used [27–29] – we prolonged the time of incubation (up to 3 h), yielding much better resolution of ingested bacterial cells, thus allowing also their relatively accurate quantification. On average, ~200 flagellate individuals were inspected for uptake of FISH-positive cells with either of the probes.

2.2. Reservoir experiments – sampling site and the design of the experiment

The field experiment was conducted in the canyon-shaped Římov reservoir (South Bohemia, 470 m.a.s.l.,

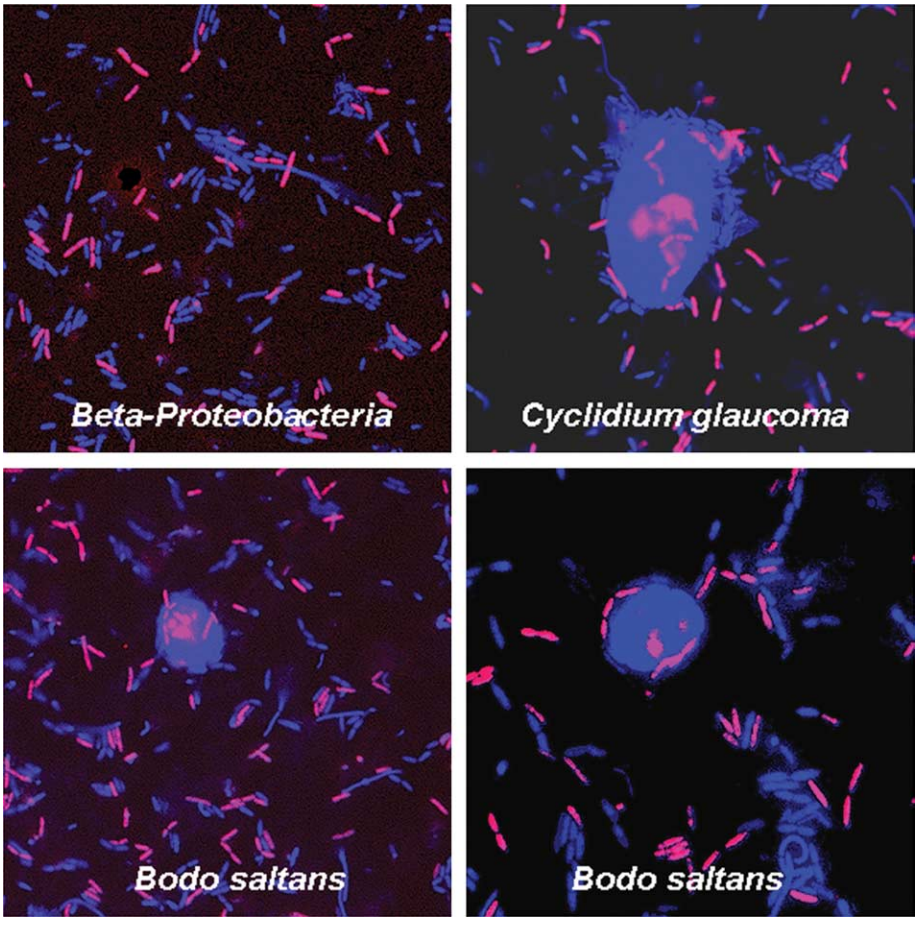

Fig. 1. Epifluorescence microscope photographs, red color – bacteria targeted with probe BET42a, blue color – DAPI stained bacteria: top left – *A. hydrophila*, top right – bacterial cells hybridized with probe BET42a inside the food vacuoles of *Cyclidium glaucoma*, lower left and right panels – bacterial cells hybridized with probe BET42a inside the food vacuoles of the flagellate *Bodo saltans*.

area 2.06 km^2, volume 34.5×10^6 m^3, length 13.5 km, maximum depth 43 m, mean depth 16.5 m, mean retention time 100 d, dimictic, meso-eutrophic). There is a strong longitudinal gradient in nutrients and dissolved and particulate organic carbon between the river inflow and the dam area, for details see [22]. Samples were collected on May 20th, during the clear water phase from the dam area of the Římov reservoir at a standard sampling point. Water was collected from a depth of 0.5 m (20 subsamples), mixed in a pre-cleaned 50 l plastic vessel and subsamples were size-fractionated, thus representing top-down manipulation experiment. Such an approach was employed in order to yield different levels of bacterivory, so that we could induce the significant changes in microbial communities caused by different grazing pressure. In the previous studies, <5 μm treatments supported rapid growth of HNF due to the absence of grazing pressure of larger zooplankton [15,19]. To conduct the bottom-up manipulation, the different size fractions of water collected at the dam area were transferred also to the middle (moderate total phosphorus, high dissolved reactive phosphorus) and riverine parts (high total phosphorus, rather low primary production) of the impoundment and incubated there in the dialysis bags. The size fractions nominally represented: (1) a bacterivore-free treatment (<0.8 μm), filtered through 0.8 μm (assigned in figures as <0.8) Osmonic filters yielding the fraction with only bacteria and viruses; (2) a bacterivore-enhanced treatment (<5 μm) with the growing (HNF) numbers (assigned in figures as <5), with bacteria, viruses and HNF only, filtered through 5 μm pore-size filters; and (3) unfiltered sample – (assigned as UNF). For the details of filtration procedures see [19].

Since the major goal of this study was to examine selection of HNF on natural bacterioplankton, in this paper we show mainly the data from <5 μm, which repeatedly [19] yielded marked increases in HNF abundances. Overall, our experimental design subjected a bacterial community to increasing HNF grazing pressure but under four different bottom-up constraints or resource supplies. This was accomplished through incubation in different parts of the reservoir and by incubation of the same treatments in dialysis bags or bottles.

The duplicate treatments were incubated in 2.5 l (deionized water rinsed and boiled) dialysis bags (approximately 70 cm long, 75 mm diameter, molecular weight cut-off 12–16 kDa, Poly-Labo, Switzerland), and also (in the case of the dam area) in glass bottles (assigned as BOTTLE). Throughout the text and figures, the dam dialysis bag incubations are assigned as DAM < 0.8, DAM < 5, DAM UNF, and bottle incubations as BOTTLE < 0.8 and BOTTLE < 5. The transplanted samples to the middle are assigned as MIDDLE < 0.8 and MIDDLE < 5, and finally the treatments transferred to the river inflow area, assigned as RIVER < 0.8 and RIVER < 5. For the experimental design see cf. Fig. 4.

2.3. Bacterial abundance and production

Samples for bacterial counting were fixed with formaldehyde (2% final concentration wt/vol), stained with DAPI (4,6-diamidino-2-phenylindole, final conc. 0.2 wt/vol, according to [30], filtered through 0.2 μm pore-size filters and enumerated in an epifluorescence microscope (Olympus AX 70 Provis).

Bacterial production was measured using ^{3}H-thymidine incorporation with a modified method of [31]. We established empirical conversion factors (ECF) of 0.84, 1.58, 1.36 and 1.16×10^{18} cells mol^{-1} of thymidine incorporated for BOTTLE incubations, DAM, MIDDLE and RIVER dialysis bag incubations, respectively. The ECF were established on the basis of bacterial cell number increase in the 0.8 μm-filtered bacterioplankton treatments in dialysis bags [32], or vessels, using a modified derivative method [33].

2.4. Protozoan grazing and enumeration

Fluorescently labeled bacteria (FLB) prepared according to the slightly modified [6] protocol [24] were used for estimating protozoan grazing rates. Tracer was added to constitute roughly 10–15% of natural bacterioplankton abundance and during the incubation subsamples were taken at times 5, 15 and 30 min.

For HNF enumeration and tracer ingestion determinations, 50 ml subsamples were taken and fixed with the lugol-formaldehyde-decolorization technique [24]. Mostly 5 ml of subsample from the 30 min incubation for HNF (stained with DAPI on 1 μm pore-size, Irgalan black treated filters) and 20 ml of subsample from the 5 min incubation for ciliates – only DAM UNF treatment were used to determine cell specific grazing rates. Based on the three time points (5, 10 and 15 min for ciliates and 5, 15, 30 min for HNF) we checked several times during the experiment if our data are within the linear part of the FLB increase per cell with time. To estimate the total grazing rate (TGR) of protists, their cell specific grazing rates were multiplied by flagellate and ciliate in situ abundance. Since ciliate numbers were extremely low during the experiment (thus consuming only 2–5% of bacterial production daily), we do not show them specifically in the figures.

2.5. Fluorescence in situ hybridization with rRNA targeted oligonucleotide probes

Analysis of BCC in water samples as well as in the food vacuoles was performed using group specific probes [27,28]. We applied a standard FISH procedure with the modification described above for food vacuole

content analysis, as well as a recent CARD-FISH [29,34] protocol, for assessment of Actinobacteria group (employing HGC69a probe).

Six different group-specific oligonucleotide probes (ThermoHybaid, Interactiva Division, Ulm, Germany) were targeted to the domain *Eubacteria* (probe EUB338), β and γ subclasses of the class Proteobacteria (BET42a and GAM42a, respectively), to a narrow subcluster of the β-Proteobacteria; R-BT065 probe (this cluster is a subcluster of the *Rhodoferax* sp. BAL47 cluster [35]), to the *Cytophaga/Flavobacterium* group (CF319a) and Actinobacteria group (HGC69a). In the following text, the probes are assigned as EUB338, BET42a, GAM42a, CF319a, R-BT065 and HGC69a, respectively. The probes were labeled with fluorescence dye Cy3, except for the HGC69a probe, which was labeled with the horseradish peroxidase enzyme [29]. The filter sections were then stained with DAPI and the proportions of hybridized bacterial cells were enumerated using the epifluorescence microscope (Olympus AX70 Provis, 1000× magnification). Selectivity indexes were calculated by dividing the proportions of probe-targeted subgroups of ingested bacteria with their proportions available in bacterioplankton, both expressed as the percentage of EUB338. Values of 1 represent random feeding, <1 indicate negative selection, >1 indicate positive selection.

Mean cell volumes of bacterial subgroups targeted by group specific oligonucleotide probes were measured using LUCIA Imagining System, Prague. The initial values (time 0 h) for each phylogenetic bacterial group were measured for UNF and <5 treatments and then in all treatments (five values in duplicates) were sized at time 96 h. Additionally, bacteria targeted by CF319a probe, were measured at time 48 h, since the analysis of food vacuoles of flagellate predators indicated an interesting shift in food preferences during the time course of the study.

3. Results

3.1. Laboratory experiments

Our modified FISH protocol yields a sufficiently strong and distinguishable fluorescence signal allowing relatively precise quantification of FISH-targeted cells inside food vacuoles of protists (Fig. 1). Fig. 2 shows the results of ingestion and digestion experiments with three protistan species feeding on the mixture of bacterial strains – *A. hydrophila* and *P. fluorescens* targeted by probes BET42a and GAM42a, respectively. Based on grazing rates over the first 30 min when uptake of all three protists was roughly linear, calculated clearance rates (in nl individuals^{-1} h^{-1}) are presented in Table 1. Clearance rates estimates were similar to those estimated

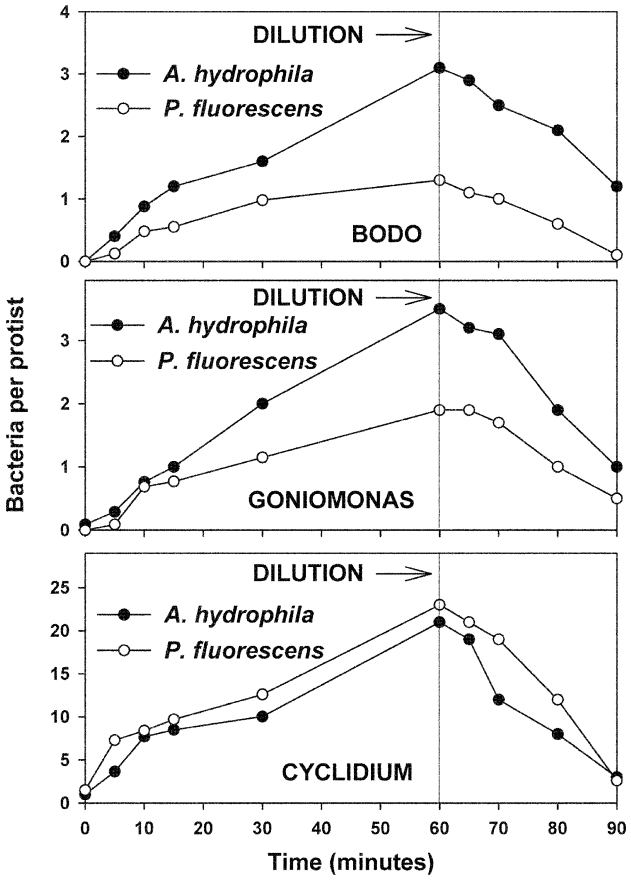

Fig. 2. Upper panel – grazing and digestion rate of *B. saltans* on *A. hydrophila* and *P. fluorescens*. Middle panel – grazing and digestion rate of *Goniomonas* sp. on *A. hydrophila* and *P. fluorescens*. Lower panel – grazing and digestion rate of *Cyclidium glaucoma* on *A. hydrophila* and *P. fluorescens*. Vertical line represents dilution with the media.

in previous studies for natural populations of *Cyclidium* [6] and flagellates [3]. At time 60 min the flagellate *B. saltans* reached the grazing rate of ~3 and 1 bacteria per individual for *A. hydrophila* and *P. fluorescens*, respectively. Uptake rates of *Goniomonas* sp. reached similar values (1.9 and 3.5 bacteria individuals^{-1} h^{-1}, for *P. fluorescens* and *A. hydrophila*, respectively. In *C. glaucoma*, both bacterial strains were processed almost identically, ingested at rates from 21 to approximately 23 bacteria per ciliate per hour. For the flagellates, despite the fact that the two bacterial prey species were similar in size, the clearance rates were markedly higher for *A. hydrophila* for both flagellate species.

Different stages of digestion of bacterial cells in protistan food vacuoles were distinguishable in the digestion part of the experiment (see Fig. 2). Slopes of the exponential declines for *A. hydrophila* and *P. fluorescens* in the protistan food vacuoles (Fig. 3) were not significantly different (ANCOVA, *F*-test, $P < 0.05$) for *Cyclidium* and *Goniomonas* species, the only significant difference ($P < 0.001$) could be found between *A. hydrophila* and *P. fluorescens* strains exposed to grazing of

Table 1
Summary from duplicated digestion experiments, where ~200 individuals predators were inspected per sample and each time point

Predator	Prey item	Clearance rate	Cell contents	R^2	K	Prey $t_{1/2}$
Bodo	*A. hydrophila*	57.9	3.1	0.932**	1.323	52
	P. fluorescens	24.9	1.3	0.854*	3.511	20
Goniomonas	*A. hydrophila*	67.1	3.5	0.936**	1.833	38
	P. fluorescens	36.4	1.9	0.932**	2.011	34
Cyclidium	*A. hydrophila*	392.1	21	0.965***	2.797	25
	P. fluorescens	441.0	23	0.867*	2.837	24

Clearance rate (nlcell^{-1} h^{-1}), Cell contents are the average number of prey per predator cell at time 60 min of ingestion just prior to diluting the sample for digestion part of the experiment. R-values are based on the linear regression of log (% t_0 cell content decrease) versus time, probability levels given as ***$p = 0.001$, **$p = 0.01$, *$p = 0.05$. K: digestion rate in log % min^{-1}, Prey $t_{1/2}$: estimated half-life in minutes of the predator food vacuole content.

B. saltans. Cells of *P. fluorescens* were digested significantly faster ($P < 0.001$) by *Bodo* than cells of *A. hydrophila*. Moreover, regression slopes of digestions of *A. hydrophila* and *P. fluorescens* by *Cyclidium* were steeper than by *Bodo* and *Goniomonas* except for *P. fluorescens* digested by *Bodo* (cf. Fig. 3 and prey $t_{1/2}$ parameter in Table 1). Correspondingly, digestion rate parameters given in Table 1 indicate the longest half-time of the *Bodo* vacuole content for *A. hydrophila* strain (52 min), compared to the markedly shorter half-times estimated for *Cyclidium* (25 and 24 min for *A. hydrophila* and *P. fluorescens*, respectively) and *Goniomonas* (38 and 34 min for *A. hydrophila* and *P. fluorescens*, respectively).

3.2. Reservoir experiments – protozoan and bacterial dynamics in different experimental treatments

Microbial communities, transplanted upstream from the DAM area to the MIDDLE and RIVER sites showed differences in time courses compared to communities that were incubated at the dam area (see Fig. 5, left panels). Strong metazooplankton (largely daphnids, data not shown) grazing pressure at the beginning of the experiment yielded typical, well developed clear water phase at the dam area with water transparency of about 7 m. It resulted also in very low initial HNF in the dialysis bags. At least 150–200 HNF individuals had to be inspected for the FISH analysis of food vacuole content per sample. Thus, we could not investigate HNF selection of different groups of bacteria because of low

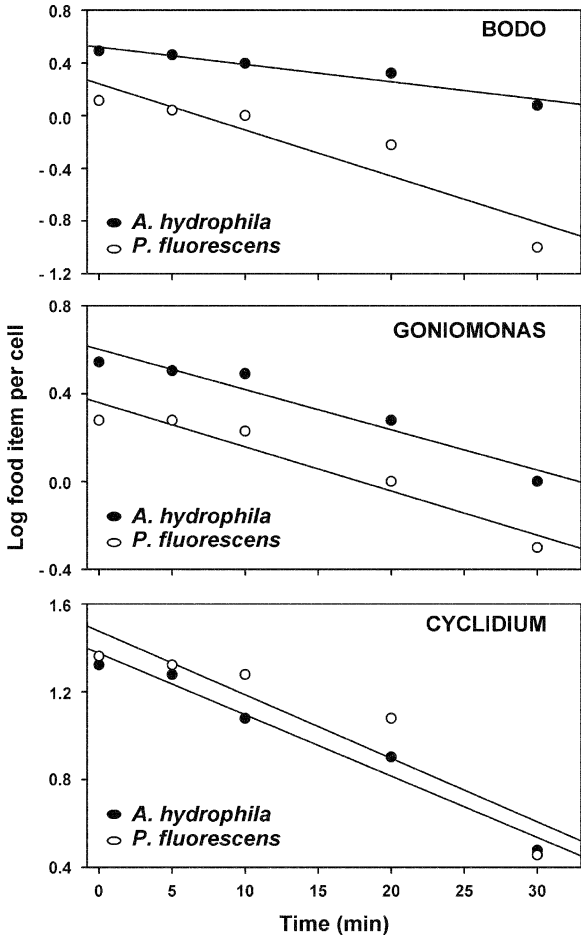

Fig. 3. Digestion part of laboratory experiments with *B. saltans*, *Goniomonas* sp., and *Cyclidium glaucoma*. Values are expressed as logarithms of food item per cell.

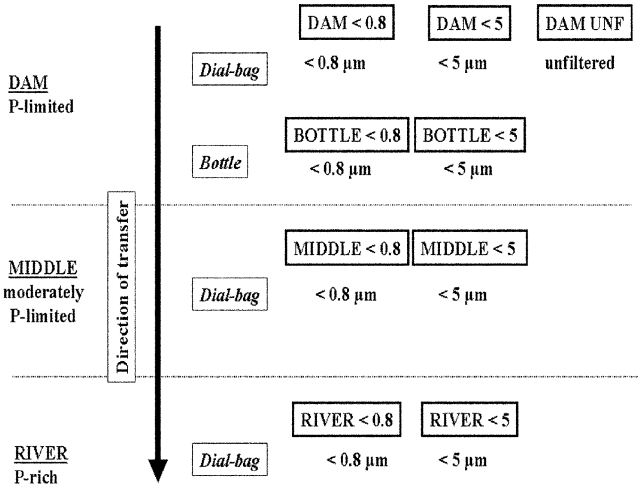

Fig. 4. Design of the reservoir transplant experiment. Symbols in bold frames represent labeling of various treatments used throughout the text. Dot lines virtually divide different parts of the reservoir.

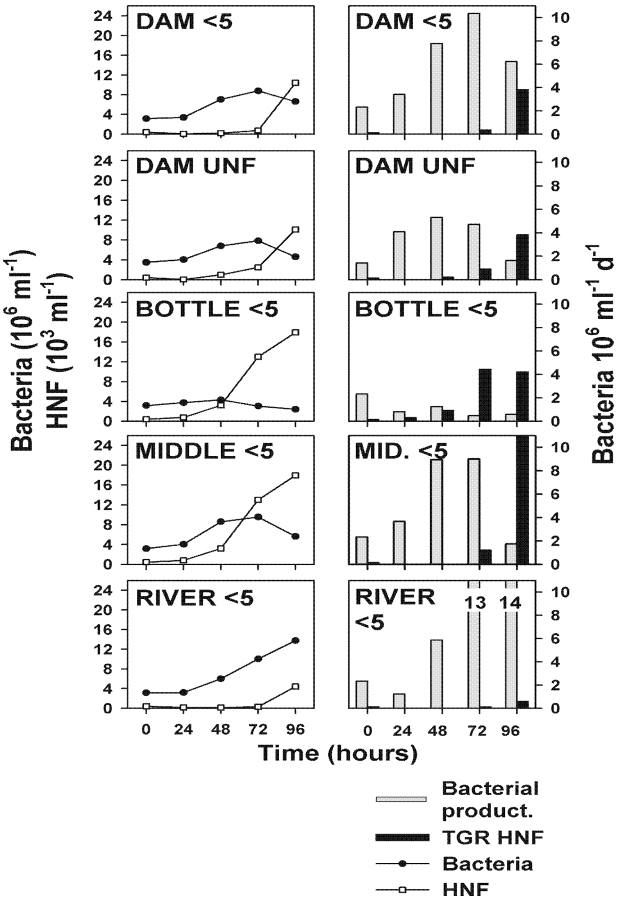

Fig. 5. Left panels – Abundances of bacteria (full dots) and HNF (empty dots) in various treatments. Right panels – Bacterial production (grey columns) and total grazing rate (TGR, black columns) in various treatments.

HNF numbers (<50 ml^{-1}) during the first two days of the experiment. Significant HNF bacterivory and sufficient concentrations for vacuole analysis appeared only during the last two days of the experiment. Therefore we show here the results of FISH only for the data from 48 to 96 h. The whole data set from bacterioplankton FISH analysis will be presented elsewhere [36].

HNF numbers rose from about 50 individuals per ml to 12, 17, 20 and 4×10^3 per ml at DAM, BOTTLE, MIDDLE and RIVER treatments at time 96 h, respectively. In the RIVER < 5 treatment, HNF grazing was low, whereas in all other treatment it reduced bacterial abundances. A similar trend occurred in all but the RIVER < 5 treatment (see Fig. 5, left panels), where this trend was delayed.

Bacterial and HNF time-course changes in concentration were reflected in rates of bacterial production and flagellate grazing (see Fig. 5, right panels). Bacterial production ranged from 0.9×10^6 $ml^{-1} d^{-1}$ in the BOTTLE treatment to 14×10^6 $ml^{-1} d^{-1}$ at the station RIVER. Protistan bacterivory increased mainly during the last two days of the experiment when HNF were abundant. The last day, grazing impact exceeded bacterial production in most of the treatments, the most markedly in the MIDDLE < 5 treatment by a factor of 6. In the treatment RIVER < 5, flagellate bacterivory was negligible due to the low HNF abundance, accounting for only ~3% of total bacterial production. In the BOTTLE < 5 treatment, all microbial parameters were generally lower (except for HNF abundance), including also much less pronounced changes in bacterial numbers (Fig. 5). In contrast, station RIVER incubations displayed the highest values of bacterial production likely as a consequence of enhanced phosphorus availability and practically no detectable impact of HNF bacterivory on steadily increasing bacterial abundance.

3.3. Changes in the composition of bacterial prey available in bacterioplankton and prey ingested in flagellate food vacuoles

Time course changes in the relative importance of different groups of bacteria detected by FISH are shown in Fig. 6 (values are expressed as percentage of cells targeted by probe EUB338). Based on the data in Fig. 6, selectivity index for each phylogenetic group studied was calculated, comparing directly proportions within ingested prey to proportions in the bacterioplankton (Fig. 7).

Bacteria targeted by probe BET42a constituted most of the hybridized cells, ranging from 30% to 62% of EUB338 positive cells in the ambient water, and from 15% to 60% in the food vacuoles of HNF. A considerable increase in the proportion of bacterial cells hybridizing with probe BET42a in plankton occurred in DAM < 5 and DAM UNF treatments with time. Lower proportions of BET42a-positive cells ingested by HNF were detected mainly at the end of the experiment, at time 96 h. However, no clear trend could be identified that was common to all treatments.

Cells targeted by probe R-BT065 were grazed less than their relative proportions in the water in all but the BOTTLE treatment, where a positive selection occurred (cf. Figs. 6 and 7). Interestingly, this treatment was also characterized by presence of small HNF (average length 2.27 μm, average volume 7.3 μm^3) compared to the other treatments (10.1μm^3). In water in the MIDDLE and RIVER sites, the R-BT065 phylotypes nearly doubled compared to the proportions found in the reservoir, from 14% to 44 % of probe EUB338. Their relative importance peaked at time 72 h in the natural community, and were positively correlated with those targeted by the BET42a probe.

With regard to GAM42a, a slight decrease in the proportions of GAM42 in plankton was apparent during the study (Fig. 7). The proportions of GAM42a within EUB338 positive cells ranged from 4% to 32%, averaging on ~15%. The treatment DAM UNF was the only

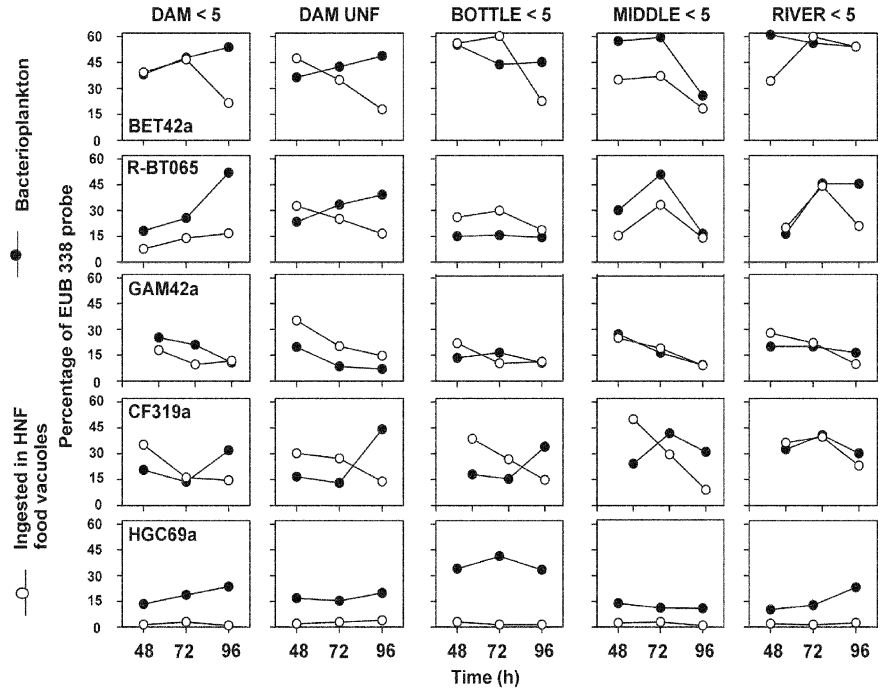

Fig. 6. HNF ingestion of bacteria targeted by five group specific probes – BET42a, GAM42a, CF319a, R-BT065, and HGC69a (empty dots) versus their proportions available in the plankton (black dots) in various treatments in time 48, 72 and 96 h. All values are expressed as percentage of EUB338-positive bacteria.

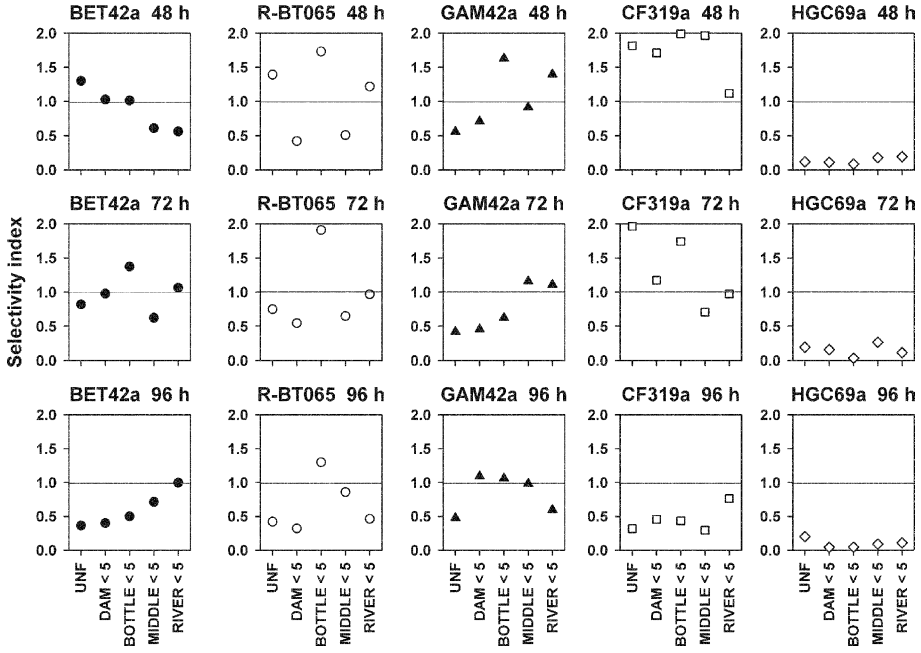

Fig. 7. Selectivity indexes of various groups of bacteria targeted by oligonucleotide probes calculated by dividing the proportions of ingested bacteria expressed as the percentage of EUB338 with the proportions of the probe-defined subpopulations present in the bacterioplankton. Values of 1 represent random feeding, <1 stand for a negative selection (avoidance), >1 present positive selection.

one in which there was a marked difference between ingested and available planktonic bacteria (Figs. 6 and 7), with HNF selecting for GAM42a-positive cells. Except for DAM UNF and DAM < 5 treatments, cells targeted with this probe were grazed in proportion to their occurrence in the surrounding water.

Bacteria detected by probe CF319a exhibited an interesting pattern: across all treatments at the time

48 h CF319a-positive bacteria appeared in food vacuoles in frequencies higher than their proportions in the reservoir bacterioplankton. At time 72 h, this pattern disappeared, and finally at the time 96 h, there was a remarkable switch to negative selection of CF319a phylotypes. This switch in food vacuole content was observed in almost all treatments (see Fig. 6) as reflected in the selection indexes presented in Fig. 7. This phenomenon might be explained by formation of filamentous, grazing resistant bacterial morphotypes among the CF319a phylotypes. Cell volume of CF319a targeted bacteria increased conspicuously during the time course of the study, as shown in Table 2 for times 0, 48 and 96 h.

Bacteria detected by probe HGC69a (Actinobacteria group) were clearly discriminated against in terms of grazing (Fig. 7), since they consistently contributed to only between 0% to 5% of ingested prey across all treatments, while accounting for 10% to 43% of EUB338 detectable bacteria in the ambient water environment (Fig. 6). The highest proportions in the surrounding water were observed in the BOTTLE treatment (ranging from 15% to 43%) with the excessively high grazing pressure (cf. Fig. 5) during the last two days. In other treatments no distinct trends in the time courses of HGC69a were apparent.

Results of one-way ANOVA (if significant, followed by Tukey's HSD test), testing the significance of differences between proportions of particular phylogenetic groups of bacteria in plankton with those detected in HNF food vacuoles in various treatments, are shown in Table 3. All the differences in HGC69a proportions were significant, so that the negative selection of the Actinobacteria group by protozoans was quite evident. On the other hand, GAM42a labeled Proteobacteria were not generally selected from the bacterial communities present in the reservoir. Bacteria targeted by the CF319a probe were positively selected at time 48 h and then they were more represented in the water than in protistan food vacuoles, probably as a consequence of the development of grazing-resistant morphotypes. Correspondingly, we found significant differences mainly for CF319a at times 48 and 96 h, except for the RIVER treatment with the negligible development of HNF populations (cf. Fig. 5). No common pattern can described for differences in BET42a and R-BT065 proportions, but more frequently the significant differences were observed at time 96 h with BET42a less ingested than available, namely in DAM and BOTTLE incubations.

4. Discussion

Oligonucleotide rRNA targeted probes have been used rarely to detect bacteria in food vacuoles. They were used in laboratory experiments for assessing selective HNF bacterivory [18] and for grazing of ciliates [17,37]. Recently, Diederichs et al., [38] documented the possibility of employing FISH to detect food vacuole content of benthic ciliates harvested from the natural environment in combination with measurements performed on the pure culture of *Tetrahymena pyriformis*.

To our knowledge this study presents the first successful application of FISH on bacteria directly in food vacuoles of flagellates from a natural environment combined with the specific ingestion/digestion experiments done under laboratory conditions.

4.1. Laboratory experiments

Our laboratory experiment showed clear differences in the uptake of two bacterial strains, by two different flagellates, *B. saltans* and *Goniomonas* sp. Both flagellate species showed an uptake rate 2.5–3 times higher on *A. hydrophila* than on *P. fluorescens* targeted bacteria. Most of the planktonic HNF species are known to be interception feeders that can potentially select particular prey. In contrast, the ciliate *C. glaucoma* used in the laboratory experiment is thought to be a typical suspension feeder, filtering small, dispersed bacteria [6,39]; consequently it may show almost no prey preferences among small prey cells. Correspondingly, both bacterial strains offered to *C. glaucoma* were processed almost identically both in the uptake as well as the digestion parts of the experiment. The only marked difference in the digestion

Table 2
Average volume of different phylogenetic bacterial groups (μm^3) targeted by oligonucleotide probes at the beginning and end of the study

Probe:	EUB338		BET42a		R-BT065		GAM42a		CF319a			HGC69a	
Treatment (h):	0	96	0	96	0	96	0	96	0	48	96	0	96
DAM UNF	0.116	0.336	0.130	0.205	0.163	0.219	0.222	0.326	0.126	0.146	0.658	0.085	0.069
DAM < 5	0.114	0.328	0.128	0.268	0.157	0.229	0.221	0.329	0.125	0.293	0.789	0.084	0.063
BOTTLE < 5		0.275		0.213		0.090		0.365		0.211	0.949		0.079
MIDDLE < 5		0.303		0.173		0.155		0.458		0.161	0.732		0.059
RIVER < 5		0.238		0.210		0.145		0.435		0.157	0.687		0.054

Note that at time 0 h, only two values were measured – unfiltered samples from the dam area and samples filtered through 5 μm filters. Since large shift in MCV of bacteria targeted by probe CF319a was detected, moreover they were measured also at time 48 h.

Table 3
Results of one-way ANOVA, followed by Tukeys HSD test, between individual treatments at t_{48}, t_{72}, and t_{96}

Probe (percentage from EUB338 probe)	Treatments, comparison of prey available versus prey ingested	t_{48}	t_{72}	t_{96}
BET42a	DAM < 5	ns	ns	<0.01
	DAM UNF	ns	ns	<0.05
	BOTTLE < 5	ns	<0.05	<0.05
	MIDDLE < 5	<0.05	<0.05	ns
	RIVER < 5	<0.05	ns	ns
R-BT-T065	DAM < 5	ns	ns	<0.01
	DAM UNF	ns	ns	<0.05
	BOTTLE < 5	ns	<0.01	ns
	MIDDLE < 5	<0.01	<0.05	ns
	RIVER < 5	ns	ns	<0.01
CF319a	DAM < 5	<0.05	ns	<0.05
	DAM UNF	<0.05	ns	<0.05
	BOTTLE < 5	<0.05	ns	<0.05
	MIDDLE < 5	<0.05	ns	<0.05
	RIVER < 5	ns	ns	ns
GAM42a	DAM < 5	ns	<0.05	ns
	DAM UNF	ns	<0.05	<0.05
	BOTTLE < 5	ns	ns	ns
	MIDDLE < 5	ns	ns	ns
	RIVER < 5	ns	ns	ns
HGC69a	DAM < 5	<0.05	<0.05	<0.01
	DAM UNF	<0.01	<0.05	<0.01
	BOTTLE < 5	<0.01	<0.01	<0.01
	MIDDLE < 5	<0.05	<0.01	<0.01
	RIVER < 5	<0.05	<0.05	<0.05

Tested were differences in the relative contributions (as percentage of EUB338 probe) of prey available versus prey ingested by HNF of five phylogenetic groups of bacteria targeted by the probes BET42a, R-BT-T065, CF319a, GAM42a, and HGC69a. ($p < 0.05$, $p < 0.01$, ns – not significant).

part of the experiment, among the flagellates and the ciliate was in *B. saltans*, where estimated half-life time for *A. hydrophila* was almost double of that found for *P. fluorescens*. These results indicate that each HNF species can markedly differ in its ability to ingest but also to digest the same bacterial prey, so that it is not surprising that each flagellate predator can induce highly species-specific response in the BCC [11,40].

4.2. Field experiments

It is often very difficult to quantify all ingested bacteria in food vacuoles stained only with DAPI, as all DNA, that of the predator as well as of bacterial prey, is labeled masking vacuole contents. Consequently, we expressed vacuole contents in the field experiment as a percentage of the bacteria targeted by the universal EUB338 probe. Since we employed five different other probes targeting various bacterial subgroups, food vacuoles of HNF usually contained only between 1 and 3 ingested, probe-positive bacteria, making their quantification quite precise and feasible.

Based on the previous studies exploiting dialysis bags technique and size fractionation [19], filtration through 5 μm membrane filters removes virtually all HNF predators, thus allowing the rapid HNF growth. The same held true for our study, since HNF mostly reached numbers between 4 and 20×10^3 individuals ml^{-1} during the study course, except for the RIVER treatment, where the initial, extremely low HNF abundances remained for a long time almost unchanged, which can be related to markedly lower river water temperature. The large peak in HNF abundance did appear in this treatment, but as late as 144 h after the beginning of the experiment (data not shown). Consequently, bacterial numbers decreased in all but one incubation site (RIVER) as a result of HNF predation mainly at time 96 h.

The FISH conducted directly in food vacuoles of HNF indicated several important trends concerning HNF selective ingestion. Two patterns were clear when comparing proportions of genetically distinguishable bacterial prey in plankton versus those ingested by protists. Apparent feeding behavior of HNF was either consistent or variable with time. For example, during the initial part of the experiment, bacteria of the *Cytophaga/Flavobacterium* group (CF319a probe) were positively selected by bacterivorous protists. In the course of the study, however, this preference switched to avoidance likely due to the increased proportions of grazing resistant bacteria with a large mean cell volume within the CF319a phylotypes (cf. Table 2).

A consistent negative selection was found for the bacteria targeted by the HGC69a probe – Actinobacteria group. A few actinobacterial strains are known to be characterized by a small cell volume, though it does not hold true for all actinobacterial strains [41]. In our study they were small cells (mean cell volume of HGC69a probe targeted bacteria was ~0.072 μm^3, cf. Table 2) and they were able to grow and divide fast with the doubling times of 18–26 h at the temperature of 18 ° C, as estimated from an increase in HGC69a-positive cells in a bacterivore-free (<0.8 μm) treatment run in parallel in all study sites. This class of Gram-positive bacteria with a high genomic G + C content comprises a great variety of validly described species and environmental isolates [41]. However, their ecological role in the bacterioplankton remains still unknown [42].

Comparative analysis of 16S rRNA genes indicates that members of the class Actinobacteria are ubiquitous in lakes of various trophic status, size or geographic location [42]. Until recently, a direct visualization of these bacteria in environmental samples by FISH with oligonucleotide probes was not easy due to their relatively small cell size and supposedly a thick Gram-posi-

tive cell wall [43]. Employing specific isolation techniques, they are found in the smallest microbial fraction [41] and are, therefore, probably also low in total ribosome content, which limits their detectability by means of FISH with directly fluorochrome labeled oligonucleotides. It is also noteworthy, that the cells targeted by probe HGC69a were the only phylogenetic group in this study that did not positively correlate with the proportions of high nucleic content bacteria as assessed by flow cytometry. This raises an interesting question as to why these bacteria might be successful in the water column of lakes. Along with the suboptimal size for most bacterivores HNF and ciliates, one reason could be the specific cell wall properties that can protect Actinobacteria from being digested by protistan bacterivores, e.g. HNF. It is believed that such bacteria do not represent innocula from terrestrial sources, but are rather an autochthonous component of freshwater microbial assemblages [35,42].

In our study, bacteria detected by probe HGC69a were always negatively selected, which was best demonstrated in the BOTTLE treatment, where after time 48 h, the difference between the proportions of cells ingested versus those available was most prominent. Small size, (mostly C-shaped morphology bacteria, <0.1 μm^3, Hahn et al. [41] or other features probably allow this group to coexist with other bacterial groups and withstand high grazing pressure, which is undoubtedly present in systems with high numbers of phagotrophic flagellates. For example, one group of Actinobacteria became a dominant group in a continuous culture system along with extremely high grazing pressure of *Ochromonas* sp., eliminating most of bacterial competitors present in bacterivore-free treatment (Pernthaler et al. [11]. Moreover, Hahn et al. [41] performed grazing experiments with a single Actinobacterium strain isolated from the freshwater habitat, and observed complete grazing resistance against predation by *Ochromonas* sp. Overall, this diverse group of bacteria profit from relatively fast growth rate, limited vulnerability to protistan grazing when cohabiting with other ecologically important bacterial groups the environments with strong grazing pressure of highly complex HNF assemblages.

The R-BT065 probe targeted bacteria, on the other hand, showed a different pattern. At times 72 and 96 h were negatively selected in almost all but one treatment – BOTTLE (cf. Fig. 7), where smaller species of HNF dominated, consequently exerting a different feeding and selection mode on these bacteria (cf. species-specific impact of protistan bacterivores on BCC in [11]. R-BT065 targeted bacteria did not grow in the BOTTLE treatment likely as a consequence of phosphorus limitation and the strong positive selection by HNF (cf. Fig. 7). Thus, the selectivity of R-BT065 is probably treatment-specific, supposedly influenced by bottom-up factors, by the composition of the remaining bacterial community, and specifically targeted grazing pressure of the bacterivores.

HNF grazing on γ-Proteobacteria (bacteria targeted by probe GAM42a) showed no clear pattern across all treatments, suggesting hence no special adaptation of these bacteria to protistan predation. The only exception was DAM UNF treatment with the presence of large zooplankton, where γ-Proteobacteria were always negatively selected. While we did not detect any significant differences in MCV of γ-Proteobacteria among our treatments (cf. Table 2), the HNF community composition and so that also its grazing were likely different under the specific top-down control by large zooplankton (cf. [14]).

In this study, we attempted to directly measure protistan food preferences employing a molecular method – fluorescence in situ hybridization. Results obtained from this study suggest possibility of using this method as a qualitative and quantitative measure of protistan predation on bacteria. This method could serve as a powerful tool for field studies on microbial food webs and presents an important innovation in the field since the usage of fluorescently labeled particles. We also propose using this method for further analysis of protozoan preferences especially when assessing bacterial groups such as Actinobacteria. There is growing evidence, that this group of bacteria forms a substantial part of natural bacterial communities [42], which is ecologically different from Gram-negative bacteria, while its ecological role is largely unknown in freshwaters and therefore is in need of further study.

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