Relationships between bacteria and heterotrophic nanoflagellates in lake water examined by different techniques controlling grazing pressure

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ABSTRACT: A dialysis bags experiment, involving the use of natural water from eutrophic Lake Mikołajskie (Mazurian Lake District, Poland), was performed to assess the impact of grazing pressure by heterotrophic nanoflagellates (HNF; <10 µm) on bacterial abundance, their size distribution, taxonomic composition and activity measured by bacterial secondary production (BP) and the contribution of active bacteria with an intact membrane (MEM+). Three different techniques — size-fractionation (<1.0 µm pore size), dilution (10×) and selective eukaryotic inhibition (cycloheximide and colchicine) — were applied to control HNF grazing. There were significant differences in the effectiveness of grazing pressure limitation between the different techniques employed. The most distinct changes in the bacterial community were found in the control and the 10× diluted treatments; in both, a considerable increase in HNF abundance was noted. In these 2 treatments, a significant decrease in both bacterial numbers and contribution of MEM+ cells, as well as an increase in the proportion of bacterial filaments (up to 50 µm long), was observed. In contrast, the use of cycloheximide and colchicine in combination resulted in the lowest and an almost constant HNF abundance. In addition, high numbers of bacteria with a large proportion of MEM+ cells (30 to 40 %) and exponential increase of bacterial secondary production were found. These results suggest that eukaryotic inhibition is the most useful method of limiting HNF grazing pressure and involves minimal manipulation of the water sample. Close relationships between bacteria and HNF confirm the crucial role of nanoflagellates in shaping bacterial abundance, morphology and activity.

KEY WORDS: Bacteria · HNF · Grazing pressure · Dialysis bags · Filtration · Dilution · Eukaryotic inhibition

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INTRODUCTION

It is well known that protists, especially small heterotrophic nanoflagellates (HNF) in the size range of 2 to 10 µm, are the major grazers of bacteria (e.g. Fenchel 1982, Sanders et al. 1989). They can not only affect bacterial numbers and biomass, but also distinctly alter taxonomic composition, morphological structure and

activity of bacterial communities (reviewed by Peralta 2005). Within the past 2 decades, various techniques have been applied to estimate protistan grazing on bacterial communities both in field and laboratory experiments, including serial dilution (Landry & Hassett 1982, Landry et al. 1995, Dolan & McKeon 2005), size-fractionation (Wright & Coffin 1984, Kuoppaleinikki & Kuosa 1990, Jezbera et al. 2006), labeling of

bacterioplankton with [^3H]-methyl-thymidine (Koton-Czarnecka & Chróst 2002), ingestion of fluorescent latex beads (Børsheim 1984), fluorescently labeled bacterioplankton concentrated from the water (Sherr & Sherr 1993, Šimek et al. 2001, 2005), as well as selective metabolic inhibition of grazers (Sanders & Porter 1986, Sherr et al. 1986). Furthermore, fluorescence *in situ* hybridization (FISH), in combination with advanced fluorescent microscopy, enables the assessment of HNF and ciliate prey preferences by analyzing bacterial prey composition in protistan food vacuoles (Jezbera et al. 2005, 2006). It should be emphasized that the first paper documenting that protozoan grazing induces taxonomical (using FISH probes) as well as morphological shifts in bacterial communities was published in the 1990s (Šimek et al. 1997). However, these techniques are not entirely satisfactory and have specific disadvantages. Thus, it is important to consider such methods not as alternatives, but as complementary experimental techniques. For example, the dilution method changes the food concentration and, therefore, the activity of grazers (Børsheim 1984). One of the most important disadvantages of the dilution technique is the possibility that *per capita* consumption rates of microzooplankton may differ in various dilution treatments; also, grazer concentration may not be linearly related to dilution factor throughout the incubation time (Evans & Paranajpe 1992, Dolan & McKeon 2005). The size-fractionation technique allows estimation of predator–prey interactions within a specific cell-size fraction (Rassoulzadegan & Sheldon 1986). On the other hand, filtration through a 1 μm filter will remove most bacterivores, although some flagellates can pass through and bloom (Wright & Coffin 1984, Kuuppo-Leinikki & Kuosa 1990). After filtration, some protistan cells may be damaged, leading to an increase in organic matter and nutrients in the water sample (Sieburth & Davis 1982), or remain dormant and start to divide after 48 h (Sanders & Porter 1986). Moreover, the retention of particles and organisms associated with protistan cells can also cause errors in grazing estimations by altering the species assemblage towards species that prefer free-living bacteria (Sibbald & Albright 1988). The eukaryotic inhibition technique allows measuring of grazing on natural bacterial populations with minimal manipulation of the water sample (Newell et al. 1983). Inhibitors should be specific to appropriate bacterivorous groups, and prevent their feeding without affecting the growth of bacteria. However, in a natural, mixed-species assemblage, an unknown proportion of organisms may be affected in an unexpected manner by a given inhibitor, and for this reason some protistan species may survive and ingest bacterial cells (Sanders & Porter 1986, Sherr et al. 1986).

The aim of the present study was a complex comparison of the major methods used to limit grazing pressure by bacterivorous nanoflagellates (HNF <10 μm in size) in lake water samples. Three different techniques were used to compare the effectiveness of HNF grazing limitation in water from eutrophic Lake Mikołajskie: size-fractionation, dilution and selective eukaryotic inhibition. The present study also shows that grazing pressure by HNF influences bacterial abundance, morphology, taxonomic composition, cellular activity and bacterial secondary production.

MATERIALS AND METHODS

Sampling area. The dialysis bags experiment was conducted using water samples taken from eutrophic Lake Mikołajskie (Mazurian Lake District, northeastern Poland; area 498 ha, max. depth 26 m, mean depth 11 m) during the summer stratification (10 to 14 July 2006). Natural lake water for the experiment was collected from the pelagic zone at the deepest part of the lake, from the upper trophogenic water layer corresponding to a maximum Secchi depth of 1.5 m. Water samples were collected at 0.5, 1.0 and 1.5 m, mixed (v/v) and an integrated water sample (~10 l) was transferred to polycarbonate flasks and transported to the laboratory within 4 h. Water temperature was measured with an YSI 6600 sonde (Yellow Spring Instruments). Dissolved organic carbon (DOC) concentration was determined in water samples filtered through 0.2 μm pore size polycarbonate membrane filters (Millipore) using a Shimadzu TOC 5050 carbon analyzer.

Experimental setup. Water samples were pre-filtered through a 10 μm mesh size plankton net to remove large protists, rotifers and crustaceans (Fig. 1). 50 ml of the <10 μm water sample fraction, containing both bacteria and HNF, was used as a control (control treatment). Grazing pressure by HNF was limited by means of 3 different techniques: size-fractionation, dilution and eukaryotic inhibition. For size-fractionation (Kuuppo-Leinikki & Kuosa 1990), 100 ml of the <10 μm water sample fraction was filtered through a 1.0 μm pore size polycarbonate membrane filter (Millipore) using low vacuum (<2 kPa) to eliminate HNF cells. This filtrate, without HNF, was marked as the <1 treatment. For dilution (Landry & Hassett 1982), 20 ml of the <10 μm water sample fraction was diluted (1:9 ratio) with lake water filtered through a 0.2 μm pore size polycarbonate membrane filter (Millipore), and marked as the 10 $\times$ treatment. For eukaryotic inhibition (Sherr et al. 1986), a mixture of 2 eukaryotic inhibitors composed of cycloheximide and colchicine (concentrations of 200 mg l^{-1} and 100 mg l^{-1} , respectively; Merck) was added to the <10 μm water sample fraction and

Fig. 1. Scheme of the dialysis bags experiment. For all treatments, lake water was pre-filtered through 10 µm mesh size plankton net. Triplicate dialysis bags were used for each time period (24, 48, 72 and 96 h) of the experiment in all treatments. See 'Experimental setup' for treatment definitions

incubated in the dark for 2 h. This variant, which stopped HNF grazing pressure, was marked as the INH treatment (Fig. 1). Three subsamples were collected from all treatments (0 h), while the remaining portion of the water samples was used for further procedures.

Dialysis bags (Spectra/Por, MWCO 100 000 Da, 24 mm flat width; Roth) were cut to a length of 25 cm (20 ml capacity), washed in hot tap water and then soaked for ca. 3 h in distilled water. Triplicate dialysis bags for each 24 h sampling interval were filled with water samples from all treatments and incubated in the dark for 96 h in separate aquaria (50 l capacity) with natural lake water (Fig. 1). Dark conditions were ensured to eliminate mixotrophic nanoflagellate activity and to force them to a heterotrophic mode of nutrition. Water in the aquariums was saturated using oxygen pumps. The temperature of the water was adjusted according to *in situ* temperatures with an immersion heater (Lake Mikołajskie, 24.0°C; lake water in the aquaria, 24.0 ± 0.6°C). Triplicate water samples (the entire volume of in the dialysis bags) were taken from each studied treatment at 24, 48, 72 and 96 h of the experiment for microbial parameter analyses.

Bacterial numbers, biomass and size distribution. Water samples were preserved with 37% formaldehyde (final concentration 2%). Subsamples of 1 ml were stained with DAPI (final concentration 1 µg ml⁻¹), filtered through 0.2 µm pore size polycarbonate membrane filters (Millipore) and enumerated by epifluorescence microscopy (Nikon Eclipse E 400 with UV-2A filter; Porter & Feig 1980). Bacterial biomass (BB) was calculated by converting DAPI-stained bacterial cell

volume to carbon units using the biomass conversion factor of 250 fg C µm⁻³ (Psenner 1993). Variations in DAPI-stained bacterial cell length (Pernthaler et al. 1996) were used to subdivide bacterial numbers (BN) and BB into 4 size classes (0.2–0.5, 0.5–1.0, 1.0–2.0 and >2.0 µm) according to Lebaron et al. (2001). Between 500 and 1000 DAPI-stained bacterial cells in at least 10 to 20 digital images on each filter were measured by an image analysis system according to Psenner (1993). Bacterial cell parameters of pixel measurement, i.e. volume, length and width, were obtained using formulas given automatically by LUCIA software (Laboratory Imaging).

Percentage contribution of active bacteria with intact membrane. LIVE/DEAD BacLight Bacterial Viability Kits were used to determine the numbers of active bacteria with intact membranes (MEM+) according to Schumann et al. (2003). Samples of water were preserved with 25% glutaraldehyde (final concentration 4%). A mixture of 2 stains, SYTO 9 and propidium iodide (1:1 ratio, final concentration for both dyes 0.15%), was added to 1 ml of sample, incubated for 15 min at room temperature in the dark, filtered through 0.2 µm pore-size polycarbonate membrane filters (Millipore) and enumerated by epifluorescence microscopy (Nikon Eclipse E 400, B-2A filter). The contribution of MEM+ bacteria was calculated as the ratio of MEM+ to the sum of MEM+ and MEM– cells.

FISH. Water samples (5 ml) were fixed with freshly prepared buffered paraformaldehyde (pH 7.4, final concentration 2%). Samples were filtered through 0.2 µm pore size white polycarbonate membrane filters (Millipore), rinsed 2 times with 5 ml of sterile water, dried at room temperature and stored at –20°C. Whole-cell *in situ* hybridization of sections from the polycarbonate filters was performed with the oligonucleotide probes EUB338 (Amann et al. 1990), NON338 (Wallner et al. 1993), BET42a (Manz et al. 1992) and CF319a (Manz et al. 1996), as described by Pernthaler et al. (2001). Oligonucleotides labeled with the cyanine dye CY3 were synthesized by Interactiva (Ulm). After FISH, the filters were air-dried and mounted on glass slides in a mix amended with DAPI (final concentration 1 µg ml⁻¹) (Pernthaler et al. 2002). The fractions of FISH-stained bacteria in at least 1000 DAPI-stained bacterial cells per sample were enumerated by epifluorescence microscopy (Olympus BX 51, UV-2A and G-2A filters for DAPI and CY3, respectively).

Bacterial production. Bacterial secondary production (BP) was determined with the [³H]-methyl-thymidine ([³H]TdR) incorporation method (Chróst & Rai 1994). Total BP and BP in 2 size fractions of bacterial cells, <1.0 µm (free-living) and >1.0 µm (large free-living and attached), was determined. To measure free-living BP, water samples were filtered through

1.0 μm pore size polycarbonate membrane filters (Millipore). The BP in the $>1 \mu\text{m}$ fraction was calculated as the difference between the total BP and BP in the $<1 \mu\text{m}$ fraction. Samples of water (2.5 ml) were supplemented with 500 μl [^3H]TdR (specific activity 60 Ci nmol^{-1} , final concentration [^3H]TdR in assays 16.68 nmol l^{-1} ; MP Biomedicals), incubated in the dark at *in situ* temperature for 60 min and fixed with 37% formaldehyde (final concentration 4%). Cold (0°C) 60% trichloroacetic acid (TCA) was then added to a final concentration of 6%. After 30 min of TCA precipitation at 0 to 1°C , the TCA precipitates were collected on 0.2 μm pore size cellulose nitrate membrane filters (Sartorius) and rinsed 3 times with 5 ml 5% cold TCA. The filters were placed in scintillation vials, dissolved with 5 ml of high-capacity scintillation cocktail (Rotiszint 2211) and assayed in a scintillation counter (Wallac 1400 DSA) using the external standard channel ratio model. The amount of [^3H]TdR incorporated into bacterial DNA was converted to bacterial cell production using the conversion factor of 1.25×10^6 cells pmol^{-1} TdR (Chrst & Rai 1994). Bacterial cell production was transformed to bacterial organic carbon production using a conversion factor of 19.8 fg C cell^{-1} (Lee & Fuhrman 1987).

HNF abundance, biomass and size distribution. Samples were fixed with 37% formaldehyde (final concentration 2%). Samples of 10 ml were stained with DAPI (final concentration 1 $\mu\text{g ml}^{-1}$) (Porter & Feig 1980), filtered through 1.2 μm pore size polycarbonate membrane filters (Millipore) and enumerated by epi-

fluorescence microscopy (Nikon Optiphot 2). The biovolume of HNF was calculated from measurements of cell size and their approximations to simple geometric forms. The carbon content was calculated by multiplying the biovolume with a conversion factor of 200 $\text{fg C } \mu\text{m}^{-3}$ (Brsheim & Bratbak 1987). Variations in DAPI-stained HNF cell length at 0 and 96 h of the experiment were used to subdivide their numbers and biomass into 3 size classes: small ($<5 \mu\text{m}$), medium (5–10 μm) and large ($>10 \mu\text{m}$).

Statistical analyses. Data were statistically analyzed using Origin v. 6.1 software (Origin Lab). The non-parametric Mann-Whitney *U*-test was used to analyze the differences in microbial parameters between the studied treatments. Pearson's correlation coefficients were calculated to determine relationships among experimental data. Correlations were performed for all combined treatments ($n = 20$).

RESULTS

Changes in bacterial and HNF numbers and biomass

At the start of the experiment both BN and BB were relatively high and very similar in all treatments ($\sim 17.0 \times 10^6 \text{ ml}^{-1}$ and $\sim 0.3 \text{ mg C l}^{-1}$, respectively), except the 10 $\times$ treatment in which markedly lower values were observed ($4.4 \times 10^6 \text{ ml}^{-1}$ and 0.1 mg C l^{-1} , respectively) (Fig 2A). After the first 24 h of the experiment BN showed 2 opposite trends. In the control and

Fig. 2. Changes in total numbers and biomass of (A) bacteria and (B) heterotrophic nanoflagellates (HNF) in all treatments: control, <1 , 10 $\times$ and INH. Mean values from 3 replicates $\pm$ SD. See 'Experimental setup' for treatment definitions

10 $\times$ treatments, BN decreased to minimal values, while in the <1 and INH treatments BN increased to maximal values at 48 h and then remained at the high and rather constant level until 72 h (Fig. 2A). In general, BB increased throughout the experiment in treatments with reduced HNF grazing, reaching maximal values after 48 h and then remaining quite stable (Fig. 2A). However, in the 10 $\times$ treatment after 48 h BB decreased to a value of 0.2 mg C l⁻¹. The differences in BN and BB were statistically significant in most of the possible configurations of compared data (Mann-Whitney *U*-test, $p < 0.05$).

At the beginning of the experiment, HNF were present at low densities in all treatments (0.1 to 0.4 $\times 10^3$ cells ml⁻¹), whereas HNF biomass was markedly higher in the control and INH treatments (~ 3.5 $\mu\text{g C l}^{-1}$) than in the 10 $\times$ treatment (1.9 $\mu\text{g C l}^{-1}$). The numbers and biomass of HNF showed an increasing trend in all the studied treatments (Fig. 2B). The most distinct increases both in HNF numbers and biomass were recorded in the control and 10 $\times$ treatments. In the INH treatment, maximal values of HNF numbers (2.0 $\times 10^3$ ml⁻¹) and biomass (15.1 $\mu\text{g C l}^{-1}$) noted at the end of the experiment were 2 to 4 times lower in comparison to experimental treatments in which HNF grazing was limited. Statistical analysis revealed significant differences between the studied treatments in

both numbers and biomass of HNF (Mann Whitney *U*-test, $p < 0.05$).

Changes in bacterial and HNF size distributions and cell morphology

At the start of the experiment in all treatments, small (0.2–0.5 μm) bacterial cells dominated total BN, constituting 50 to 58% (Fig. 3A), whereas bacteria of 0.5–1.0 μm and 1.0–2.0 μm cell size prevailed distinctly in total BB (36 to 40%; Fig. 3B). At 96 h, bacterial cells in the 1.0–2.0 μm size class composed the major part of total BN in the 10 $\times$ treatment, while 0.5–1.0 μm cells, mainly cocci, dominated in both the <1 and INH treatments. In the control treatment, small cells dominated throughout the experiment. More pronounced shifts at 96 h were observed in BB, which in most of the studied treatments was dominated by large cells >2.0 μm (40 to 62%). Taking into account bacterial cell length, the most evident changes at the end of the experiment were observed in the control and 10 $\times$ treatments, where large filamentous bacteria >10 μm (even up to 50 μm) constituted an important part of the total BB. Statistical differences in the proportion of particular size classes of bacteria were not significant in most of the possible configurations of the compared data (Mann Whitney *U*-test, $p > 0.05$).

Fig. 3. Changes in distribution of (A) bacterial numbers and (B) biomass in different size classes (0.2–0.5, 0.5–1.0, 1.0–2.0 and >2.0 μm cell length; percentage values of total numbers and biomass) in all treatments: control, <1, 10 $\times$ and INH. Mean percentage values from 3 replicates. See 'Experimental setup' for treatment definitions

The size distribution of HNF did not differ among the studied treatments at the beginning of the experiment. Small HNF cells (<5 µm in size, unidentified, free-swimming with 2 flagella) dominated in the total number (59 to 78%; Fig. 4A), whereas medium-sized cells (5–10 µm) prevailed in the total biomass (66 to 91%). At 0 h, no DAPI-stained HNF cells were found in the <1 treatment (Fig. 4B). After 96 h, large HNF cells (>10 µm, attached to bacterial and/or detrital aggregates) were observed in all the studied treatments.

Fig. 4. Changes in distribution of heterotrophic nanoflagellate (HNF) (A) numbers and (B) biomass in different size classes (<5, 5–10 and >10 µm cell length; percentage values of total numbers and biomass) in all treatments: control, <1, 10x and INH at 0 h (left panel) and 96 h (right panel) of the experiment. Mean percentage values from 3 replicates. See 'Experimental setup' for treatment definitions

These cells constituted from 12% (INH treatment) to 53% (control treatment) of the total HNF biomass, whereas their percentage share in total HNF numbers was negligible (Fig. 4).

Changes in bacterial taxonomic composition (FISH)

The most visible changes in the proportion of bacteria targeted by universal EUB338 probe for the domain *Bacteria* were noted in the INH treatment (Fig. 5). Hybridized cells increased from 49% at 0 h to 78% after 96 h. In the control treatment, we observed a slight decrease from 59 to 50% in the proportion of *Bacteria*. There were statistically significant differences in the proportion of the EUB338 probe among the 10x and both the control and <1 treatments (Mann Whitney *U*-test, $p < 0.05$). Among the bacterial groups detected by different FISH probes, *Betaproteobacteria* (probe BET42a) constituted the greatest proportion of bacterial cells in all treatments both at the beginning and at the end of the experiment, except the <1 treatment at 0 h where *Alphaproteobacteria* were the most abundant. The strongest increase in *Betaproteobacteria* was recorded in the 10x treatment (from 24 to 50%). The differences among the studied treatments were not statistically significant ($p > 0.05$). The contribution of *Alphaproteobacteria* (probe ALF968) increased by 5 to 7% in all treatments and was significantly different ($p < 0.05$) only for the control and remaining treatments. The lowest percentage share was for bacteria targeted by the CF319a probe (*Cytophaga-Flavobacterium* cluster), which decreased in the control and <1 treatments (from 8 to 3%), while increasing from 2 to 7% and from 6 to 13% in the 10x and INH treatments, respectively (Fig. 5). The differences in the proportion of the CF319a probe-detected bacteria among all the studied treatments were not statistically significant ($p > 0.05$).

Fig. 5. Changes in phylogenetic composition of the bacterial community determined by fluorescent *in situ* hybridization with different probes (EUB338 for *Bacteria*, ALF968 for *Alphaproteobacteria*, BET42a for *Betaproteobacteria* and CF319a for *Cytophaga-Flavobacterium* cluster) in all treatments: control, <1, 10x and INH. Mean percentage values (related to total DAPI-stained bacteria) from 3 replicates. See 'Experimental setup' for treatment definitions

Changes in bacterial activity

At the beginning of the experiment, the percentage contribution of MEM+ cells was similar in all treatments, constituting about 25% of BN (Fig. 6). The contribution of MEM+ cells in all treatments with limited grazing pressure increased to 33–50% within 24 to 48 h (the highest value was observed in the 10× treatment); after 72 h their percentage clearly declined to values below 15%. By the end of the experiment (96 h) the percentage of active bacterial cells had decreased in all treatments. In the INH treatment, however, the share of MEM+ cells remained at a relatively high level (28% after 96 h of the experiment). On the contrary, in the control, the contribution of MEM+ cells decreased throughout the experiment to reach a level of 10% at 96 h. There were significant differences in the percentage contributions of MEM+ cells among most of the studied treatments (Mann Whitney *U*-test, $p < 0.05$).

BP was measured in 2 treatments: control and INH. In the control treatment, BP increased during the first hours of the experiment, reaching a maximal value ($2.2 \mu\text{g C l}^{-1} \text{ h}^{-1}$) after 48 h, and then decreased to the low level of $0.8 \mu\text{g C l}^{-1}$ at the end of the experiment (Fig. 7). In the INH treatment, BP rates grew exponentially to $3.8 \mu\text{g C l}^{-1}$. There were no statistical differences in the BP between the studied treatments (Mann Whitney *U*-test, $p > 0.05$). The contribution of attached bacterial cells ($>1.0 \mu\text{m}$) to total BP predominated throughout the experiment (control, 59 to 98%; INH, 63 to 87%); thus the differences between the control and INH treatments were not statistically significant ($p > 0.05$).

Fig. 6. Changes in percentage contribution of active bacteria with intact membrane (MEM+) related to the sum of MEM+ and MEM– cells in all treatments: control, <1, 10× and INH. Mean percentage values from 3 replicates. See 'Experimental setup' for treatment definitions

Fig. 7. Changes in total bacterial production (BP) and the percentage contribution of 2 fractions of bacterial cells: free-living ($<1.0 \mu\text{m}$) and attached ($>1.0 \mu\text{m}$) to total BP in (A) control and (B) INH treatments. Mean values from 3 replicates $\pm$ SD for total BP. See 'Experimental setup' for treatment definitions

DISCUSSION

Comparison of different methods applied to limit HNF grazing

In the present study, we investigated the impact of heterotrophic nanoflagellates $<10 \mu\text{m}$ in size on bacterial communities isolated by applying 3 different techniques: size-fractionation, dilution and metabolic inhibition. The results of our experiment showed marked differences in the effectiveness of HNF grazing pressure limitation with the different techniques employed.

The size-fractionation technique allows, from the theoretical point of view, the estimation of predator–prey interactions between various groups of organisms within specific cell size. In practice, flexible flagellates identical or even twice as large as the pore size can pass through the filter, leading to underestimation of grazing values (Rassoulzadegan & Sheldon 1986,

Wright & Coffin 1984, Kuuppo-Leinikki & Kuosa 1990). In the present study, after water sample filtration, no HNF cells were found in the $<1.0\ \mu\text{m}$ filtrate, indicating that all cells were retained on a $1.0\ \mu\text{m}$ pore size filter. Most of the bacterial cells in water samples ($<10.0\ \mu\text{m}$ fraction) passed through the $1.0\ \mu\text{m}$ pore size filter and constituted 96 % of total BN and 86 % of total BB. From 24 h until the end of the experiment, a rapid increase in the HNF numbers and biomass was observed in the <1.0 treatment (Fig. 2B). To separate flagellates from water samples, we used filtration through $1.2\ \mu\text{m}$ pore size filters. Some smaller cells ($<1.2\ \mu\text{m}$) could pass through the filters and possibly developed in that treatment in the course of the experiment. Therefore, in many recent studies, $0.8\ \mu\text{m}$ pore size filters have been used as a grazer-free treatment in experiments with the application of the size-fractionation method (e.g. Šimek et al. 2001, 2005). Both lack of large grazers (e.g. ciliates, rotifers and crustaceans) and high bacterial numbers (6.7 to $16.1 \times 10^6\ \text{ml}^{-1}$) might have directly caused an increase in total HNF numbers in the <1 treatment during the second half of the incubation. It is documented that HNF assemblages without top-down control in $5\ \mu\text{m}$ -filtered treatments grew significantly faster than any bacterial group targeted by FISH probes, except for a small, but abundant, R-BT065 cluster of *Betaproteobacteria* in $0.8\ \mu\text{m}$ -filtered treatments without grazers and incubated *in situ* in dialysis bags (Šimek et al. 2006). In our experiment, lake water was filtered through $10\ \mu\text{m}$ mesh size plankton net to remove large zooplankton. However, this filtered sample contained medium-sized HNF that fed on smaller HNF. Moreover, some small ciliates such as the prostomatids *Balanion planctonicum* and *Urotricha* spp. feeding on bacteria and flagellates could pass a filter of this porosity. That is why the $5\ \mu\text{m}$ filtrate containing the vast majority of bacterivorous HNF is used in many studies (Šimek et al. 2001, 2005, 2006).

In our dialysis bags experiment, a 1:9 dilution ratio was used to reduce the grazing pressure of bacteria by HNF. Both bacterial as well as nanoflagellate numbers were decreased by only a factor of 4. Dilution should result in bacterial and HNF numbers about 10-fold lower, but only theoretically. In practice, microorganisms in water samples due to their heterogeneity (random distribution) could not be evenly distributed after dilution, thus this may be a disadvantage of the dilution assay. Microspatial heterogeneity in the distribution of aquatic microorganisms is easily observed by placing a small drop of water on a slide and examining it under a microscope. Clouds of bacteria surround organic aggregates (lake snow) composed of algae and animal material (Taylor & Berger 1980, Grossart et al. 1998). As shown in Fig. 2, both the numbers and biomass of HNF increased markedly throughout the

experiment, yielding no distinct changes in bacterial abundances. Bacteria present in the $10\times$ treatment (0.9 to $5.1 \times 10^6\ \text{ml}^{-1}$) were sufficient to supply food for a few thousand flagellates (Jürgens 1992). Moreover, the high percentage of MEM+ cells (50 % of total bacterial numbers) found in the $10\times$ treatment indicate that bacterial activity was probably extremely high, and flagellates increased both their numbers and biomass due to intensive grazing on active bacteria. Subsequently, only small decreases in bacterial abundances from 48 h of the experiment were observed because most newly produced cells were immediately removed.

The results of our study on the use of cycloheximide and colchicine confirmed that eukaryotic inhibition could be a useful tool to study the relationship between bacterial and HNF communities. As shown by Sherr et al. (1986), a cycloheximide-colchicine mixture was an effective inhibitor of protistan reproduction and feeding, and had no direct effect on either population growth rates or cell-specific incorporation of [^3H]-methyl-thymidine into DNA during log-phase growth of bacterioplankton. In comparison to other treatments (control, <1 , $10\times$), addition of the 2 inhibitors caused the smallest increase in HNF numbers and their biomass throughout the experiment (Fig. 2B), and the final number of HNF was 2 to 4 times lower compared to the other experimental treatments. The effectiveness of eukaryotic inhibition depends simultaneously on the inhibitor's features and on protistan community composition and their cell cycles. A visible increase in the share of active bacteria (MEM+; Fig. 6) and bacterial secondary production (Fig. 7) observed in the present study confirmed the findings by Sherr et al. (1986) that cycloheximide and colchicine in combination had no negative effect on bacterial growth and activity. The high rates of BP in the INH treatment could have occurred due to a combination of grazing inhibition and enhanced bacterial growth because of the inadvertent introduction of substrate (antibiotics) in excess. One of the more important disadvantages of this technique may be the fact that addition of antibiotics may increase the substrate pool available for bacterial growth, because substrate availability dramatically affects bacterial size and growth rate (Ammerman et al. 1984). However, there is no clear evidence for such explanation. In our experiment, dialysis bags (cutoff $100\ \text{kDa}$, width $24\ \text{mm}$) allowed the exchange of substrate resources between dialysis bags and lake water in the aquariums. Thus the concentration of DOC remained at a similar level during the entire experiment ($12.9 \pm 2.2\ \text{mg C l}^{-1}$ in lake water in aquariums; $13.1 \pm 0.2\ \text{mg C l}^{-1}$ in Lake Mikołajskie).

In conclusion, taking into account the differences found in HNF numbers and bacterial numbers, biomass and activity among the INH and remaining treat-

ments in which grazing pressure was limited, it seems that an experimental design with the eukaryotic inhibitors cycloheximide and colchicine may be useful for studying grazing pressure by HNF. However, further experimental studies with the use of filtration through 0.8 μm pore size filters (bacterivore-free treatment) to remove all bacterivores and filtration through 5.0 μm pore size filters (zooplankton-free treatment) to remove HNF predators (Šimek et al. 2006) are required to assess the effectiveness of the size-fractionation technique, because we acknowledge the fact that in our experiment the 'grazer-free' treatments did not work as anticipated.

Relations between bacteria and heterotrophic nanoflagellates

HNF are a heterogeneous assemblage: most are size-selective and able to consume preferentially small- and medium-sized active bacterial cells (Sherr & Sherr 1991, Šimek & Chrzanowski 1992, Pernthaler et al. 1996), that is why they are responsible for keeping bacterial numbers at a rather constant level in aquatic ecosystems (Chróst et al. 2000). In the present study, we found both positive and negative correlations among the total numbers of HNF and different bacterial parameters. These relationships may suggest 2 different modes of changes in bacterial cell numbers under strong HNF grazing pressure. On the one hand, negative correlations between total HNF numbers and both total BN ($r = -0.48$, $p < 0.05$) and medium-sized (0.5–1.0 μm) bacterial cells ($r = -0.49$, $p < 0.05$) may confirm effective predator–prey coupling. On the other hand, positive correlations with 2 large size classes (1.0–2.0 and >2.0 μm) of bacterial cells ($r = 0.52$, $p < 0.05$ and $r = 0.58$, $p < 0.01$) suggests that the distinct increase in BN may be a consequence of increasing HNF abundance. At the end of the experiment, large grazing-resistant filaments (>10 μm in length) constituted a large fraction of bacterial cells in the control and 10 $\times$ treatments. In addition, HNF numbers were positively correlated both with bacterial cell length ($r = 0.62$, $p < 0.01$) and width ($r = 0.60$, $p < 0.01$), indicating the crucial role of HNF in shaping bacterial cell morphology.

In the present study, *Betaproteobacteria* was clearly the most abundant group in all treatments throughout the experiment. It appears that strong grazing pressure (especially in the 10 $\times$ treatment) might have stimulated faster cell division rates of these bacteria and caused, in consequence, an increase in their numbers without cell size variations. Jezbera et al. (2006) and Šimek et al. (2007) demonstrated that *Betaproteobacteria* and its R-BT065 cluster were pref-

erentially ingested by HNF. We hypothesized that the development of *Betaproteobacteria* could be a kind of adaptation to new conditions, as many *Betaproteobacteria* are well known to be fast-growing, opportunistic strategists (Šimek et al. 2006). Members of the *Cytophaga-Flavobacterium* cluster (CF319a probe) were not numerous and showed a small range of fluctuations. Similarly to the results of Jürgens et al. (1999) and Lebaron et al. (2001), we observed that very large and filamentous bacteria hybridized with the CF319a probe at the time of high HNF numbers in the control and 10 $\times$ treatments. Hahn et al. (1999) and Pernthaler et al. (2004) suggested that some members of the CF319a group have a defense strategy against grazing which allows preservation of the diversity of bacterial species in natural freshwater systems. A lack of significant correlations among HNF numbers and different phylogenetic groups of bacteria may suggest that changes in bacterial taxonomic composition in all treatments were caused by an indirect effect as a result of chemical excretions released by flagellates, as well as in response to exudates related to grazing (chemically induced phenotypic plasticity) (Corno 2006, Corno & Jürgens 2006). On the other hand, the shift in bacterial community composition is more related to the direct effect of HNF selection. The negative correlation between total HNF numbers and the contribution of MEM+ cells ($r = -0.61$, $p < 0.01$) in our experiment may indicate that active bacteria were controlled by flagellates. Several studies suggest that the grazing rates on metabolically active bacteria may be 2 to 4 or more times higher than on inactive ones (del Giorgio et al. 1996, Chróst et al. 2000, Koton-Czarnecka & Chróst 2003). Our results indicate that cycloheximide and colchicine did not trigger any negative effect on BP (Fig. 7). The changes in BP were positively related to changes in the HNF biomass ($r = 0.69$, $p < 0.05$). We suggest that small and size-selective HNF, which dominated total HNF numbers, might have a significant impact on BP in the control treatment at the beginning of the experiment through strong grazing pressure on small and active bacteria (Pernthaler et al. 1996, Lebaron et al. 2001). In both studied treatments, particle-attached, large bacteria (>1.0 μm) dominated BP throughout the experiment due to their much higher enzymatic and metabolic activities as opposed to free-living bacteria (Chróst et al. 2000).

In conclusion, the results of the present study, and those available in the literature, suggest that a combination of 2 methods—eukaryotic inhibitors and carefully conducted size-fractionation—can result in the most realistic estimates of the impacts of protistan grazing pressure on bacterial community structure and dynamics.

Acknowledgements. This work was financially supported by the State Committee for Scientific Research grants: BW No. 1720/3, PBZ-KBN-087/P04/2003 and N304 016 32/0959 from the Ministry of Higher Education and Science. We thank anonymous reviewers for their helpful comments, which greatly helped us to improve the manuscript.

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Editorial responsibility: Karel Šimek,
Ceske Budejovice, Czech Republic

Submitted: October 8, 2009; Accepted: March 22, 2010
Proofs received from author(s): May 27, 2010