

Protozoans Prefer Large and Metabolically Active Bacteria

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Abstract

The aim of our studies was to verify the hypothesis that heterotrophic nanoflagellates (HNF) prefer large and metabolically active bacterial cells, and avoid small and inactive bacteria. Determined grazing rates on bacteria differing in sizes and metabolic activity and observed changes in bacterioplankton structure in samples with and without bacterial grazers indicated that HNF prefer large (but not too large) and actively metabolizing bacterial cells. Bacterial size fraction between 0.4 and 0.8 μm represented the majority of all grazed bacteria, i.e. $61.6 \pm 6.9\%$. Grazing rates on live bacteria were 1.83 times greater than grazing rates on dead bacteria. This preferential protozoan feeding strongly affects composition and activity of bacterial communities in aquatic environments.

Keywords: protozoan grazing, food selectivity, microbial loop

Introduction

Heterotrophic bacteria are the major utilizers of organic matter in all aquatic ecosystems [1]. They convert dissolved organic matter (DOM), which predominates in the total organic matter present in all natural waters, into their biomass. High-nutritional quality bacterial particulate organic matter can be efficiently utilized by bacterivorous protozoans. Heterotrophic nanoflagellates (HNF), whose cell sizes range between 1 and 10 μm , are major consumers of bacteria [2]. HNF are later consumed by larger protozoans and other planktonic animals. Thus, production of bacterial biomass and its consumption by HNF within the microbial loop represent important links between DOM and higher trophic levels. Protozoan grazing on bacteria is responsible for the transfer of a large portion of DOM assimilated by bacteria into higher trophic levels.

Determination of protozoan grazing rates on natural aquatic bacteria is necessary to estimate the amount of energy and carbon transfer and to evaluate the role of bacteria and bacterivorous protozoans in the overall carbon cycling within aquatic food-webs. The results of these estimations may be incorrect if food selectivity of protozoan grazing is not taken into consideration. It is generally known that not all bacteria are grazed with the same efficiency. Protozoa probably avoid extremely large or small bacterial cells [3, 4]. Moreover, non-active bacteria are grazed with lower rates than active bacteria [5, 6].

The aim of these studies was to verify the hypothesis that heterotrophic nanoflagellates prefer large and more active bacterial cells, avoiding small and inactive bacteria. We measured protozoan grazing rates on small, medium and large sized bacterial cells and on metabolically active and inactive bacteria. The determinant of bacterial metabolic activity was the content of visible nucleoid in their DAPI-stained and isopropanol-rinsed cells [7,

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8, 9, 10, 11]. The changes in the abundance of bacteria of different size and different nucleoid content in water samples with bacterivorous protozoans were compared to samples without bacterivorous protozoans.

Materials and Methods

Sampling

Studies were conducted in the surface layers (0-0.5 m) of ten lakes (Mazurian Lakes District, Poland) during the spring and summer from 1998 to 2001. The studied lakes represented different eutrophication conditions (Table 1). Water samples were taken at five sampling sites located along the length of the sampling lake and mixed vol/vol to obtain a representative sample for every studied lake.

Protozoan Grazing Rates

Protozoan grazing rates were measured using radio-labelling of prey bacteria from natural bacterial assemblages by [³H-methyl]thymidine [12]. Determination of protozoan grazing on bacteria involves the measurement of the radioactivity transfer from labelled bacteria (size fraction < 1 µm) to protozoans (size fraction > 1 µm). First, we prepared a suspension of radioactive bacteria. Lake water samples were filtered through 1 µm pore-size membrane filters (Poretics), supplemented with [³H-methyl]thymidine (TdR, spec. activity 90-97.5 Ci/nmol, NEN Du Pont) and incubated for 20 hours in the dark. During incubation period radioactive TdR was incorporated into bacterial DNA. ³H-DNA-labelled bacteria were harvested on 0.2 µm polycarbonate membrane filters (Poretics). Filters were gently sonicated in small volume of the filter-sterilized (0.2 µm) water from the studied lake to release labelled bacteria from the filter surface and to resuspend them in water. Concentrated suspension of labelled bacteria was filtered again through 1 µm pore-size filters (Poretics) to remove larger bacterial cells and clumps of bacteria that may have been formed during concentration procedures. A prepared suspension of the known number of labelled bacteria and their radioactivity was added to lake water samples pre-filtered through 10 µm pore-size filters (Nuclepore). Protozoan grazing on bacteria proceeded for 1 hour of incubation. Samples were then fixed with formalin (2% final conc.) and triplicate subsamples were filtered through 1 µm pore-size membrane filters i.e., protozoans were harvested on 1 µm pore-size filters. Protozoans contained consumed radioactive bacteria. Radioactivity on filters was determined in a scintillation counter. Controls of abiotic adsorption of labelled bacteria on filters and/or particulate matter were prepared in separate samples fixed with formalin before the addition of radioactive bacterial suspension. Controls were filtered through 0.2 µm and 1 µm pore size filters to determine the total radioactivity of labelled bacteria added at time zero and blank radioactivity of protozoan samples, respectively.

Table 1. Trophic status of the studied lakes

Lake	Mean TSI *	Trophic status
Kuc	42.59 ± 3.40	mesotrophic
Majcz	43.10 ± 1.15	mesotrophic
Przystań	44.35 ± 2.82	eutrophic
Dargin	50.80 ± 4.98	eutrophic
Śniardwy	54.95 ± 5.10	eutrophic
Boczne	57.61 ± 8.91	eutrophic
Mikołajskie	61.87 ± 5.03	eutrophic
Ryńskie	62.11 ± 4.84	eutrophic
Tałowisko	62.68 ± 5.91	eutrophic
Szymon	63.93 ± 3.08	eutrophic
Szymoneckie	64.15 ± 5.84	eutrophic
Smolak	no data	polyhumic

* TSI (trophic state index) was calculated for each studied lake from Secchi disk visibility, chlorophyll_a concentration and concentration of total phosphorus in the spring and summer of 2000 and 2001.

Total Bacterial Number

Total number of bacteria was determined in formalin-preserved samples (2% final conc.) using epifluorescence microscopy. DAPI-stained bacterial cells were counted on 0.2 µm pore-size black polycarbonate membrane filters (Millipore) [13].

NuCC

Number of nucleoid-containing bacteria (NuCC) was determined in formalin-preserved, DAPI stained, and hot (60°C) isopropanol-rinsed samples [14].

Experimental Procedures

We made four different experiments to ascertain the preferential feeding of Protozoa towards large and active bacteria.

Experiment 1 was performed in samples from five Mazurian lakes (Kuc, Mikołajskie, Ryńskie, Szymon and Smolak) in June 1998. We compared the changes in abundance of nucleoid-containing bacteria with time in triplicate lake water samples pre-filtrated through 10 µm and 1 µm pore-size membrane filters (Poretics). Filtration through 10 µm removed metazoans and large protozoans that are regarded to be consumers of bacterivorous HNF, thus mainly effective HNF grazing on bacteria occurred in sample pre-filtrated through 10 µm pore-size filters. Filtration through 1 µm pore-size filter was made to reduce the number of bacterivorous nanoflagellates. Samples filtered through 10

μm and $1\ \mu\text{m}$ pore-size filters were incubated for 72 hours and subsamples (10 ml) were collected from each sample in definite time intervals (12 or 24 hour intervals). The total number of bacteria and the number of nucleoid-containing cells were determined in subsamples.

Experiment 2 was performed in the pelagial of Lake Mikołajskie in June 2000. We used the radioisotopic method to determine the grazing rates on heat-killed bacteria and on living bacteria. To kill bacteria we heated (at $80\ ^\circ\text{C}$) the prepared suspension of radioactive bacteria before its addition to lake water samples containing bacterivorous protozoans. To determine protozoan grazing rates on dead and living bacteria we proceeded as described above.

During **Experiment 3** we estimated the percent contribution of bacterial cells differing in size to the total bacterial number grazed by HNF. For this purpose we used radiolabelled different size fractions of bacteria ($<1.2\ \mu\text{m}$, $<0.8\ \mu\text{m}$ and $<0.4\ \mu\text{m}$) for protozoans in samples taken from Lake Mikołajskie in April 2000.

Experiment 4 was made to evaluate the shift in bacterioplankton size structure caused by protozoan grazing. We used water samples from Lake Kuc and Lake Mikołajskie taken in August 2000. Similar to Experiment 1 we pre-filtered triplicate lake water samples through $10\ \mu\text{m}$ and $1\ \mu\text{m}$ pore-size membrane filters (Poretics) and incubated them for 48 hours. Subsamples (10 ml) were collected from each sample at 12 hour intervals, preserved with formalin and filtrated through 1.0 , 0.8 , 0.6 and $0.4\ \mu\text{m}$ pore-size membrane filters (Nuclepore). We determined the total bacterial number and the number of metabolically active bacteria in each subsample.

Results

Experiment 1

The results of Experiment 1, i.e. changes in number of nucleoid-containing bacteria with time in samples with and without bacterivores are shown in Fig. 1. Standard deviations of all results did not exceed 15%. In Lake Mikołajskie (Fig. 1A) the initial contributions of nucleoid-containing bacteria (NuCC) to total bacterial number in samples with and without bacterivorous protozoans were high and similar, 86.25% and 88.35% of bacteria were active (contained visible nucleoid) in samples without bacterial grazers (pre-filtrated through $1\ \mu\text{m}$) and with grazers (pre-filtrated through $10\ \mu\text{m}$), respectively. Small differences between samples appeared after 48 hours of incubation (active bacteria constituted 96.58% in samples without grazers and 81.12% in samples with grazers) and they subsisted until the end of incubation (active bacteria constituted 98.68% in samples without grazers and 83.14% in samples with grazers) but they were not statistically different.

In Lake Kuc (Fig. 1B) the contribution of nucleoid-containing bacteria increased in samples with reduced protozoan grazing and fluctuated in samples with effective protozoan grazing. Significant differences in contri-

bution of NuCC between samples appeared after 48 hours of incubation, 83.3% of bacteria were active in samples pre-filtered through $1\ \mu\text{m}$ and only 60.5% of bacteria were active in samples pre-filtered through $10\ \mu\text{m}$. These differences disappeared after 72 hours of incubation.

In Lake Ryńskie (Fig. 1C) the contribution of NuCC to the total number of bacteria was initially small in both types of samples (36.8% and 36.6% in samples pre-filtrated through $1\ \mu\text{m}$ and pre-filtered through $10\ \mu\text{m}$, respectively). During the first 24 hours the numbers of NuCC increased in both types of samples. After 48

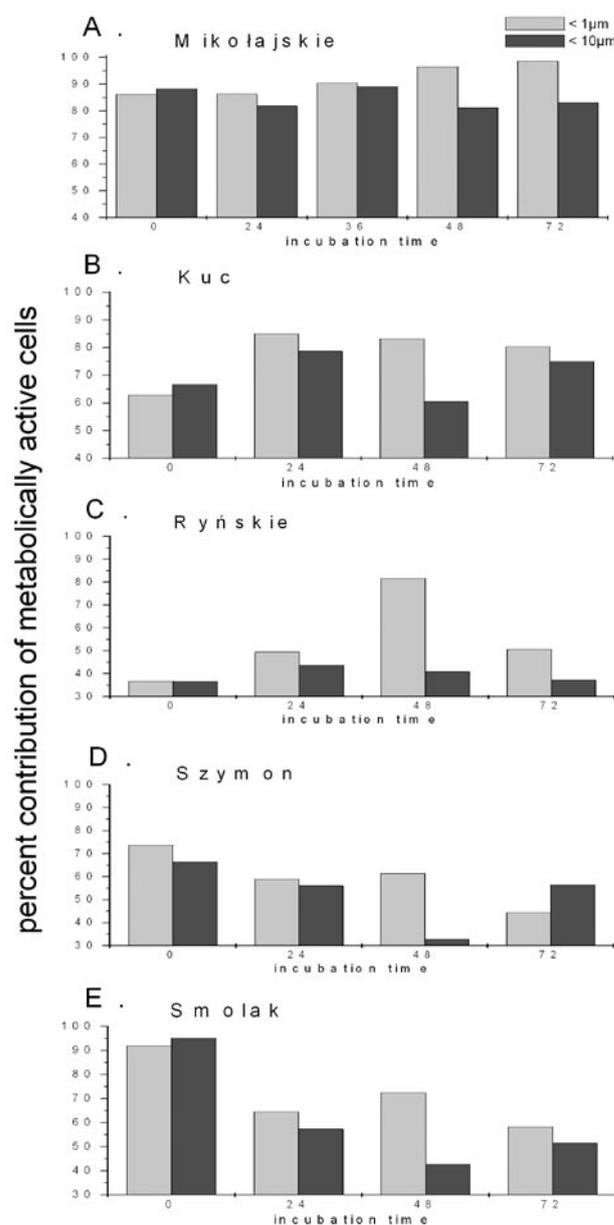


Fig. 1. Changes in the number of nucleoid-containing bacteria during incubation with bacterial grazers (in samples pre-filtered through $10\ \mu\text{m}$ pore-size filters) and without bacterial grazers (in samples pre-filtered through $1\ \mu\text{m}$ pore-size filters) in Lake Mikołajskie (A.), Lake Kuc (B.), Lake Ryńskie (C.), Lake Szymon (D.), and Lake Smolak (E.).

hours of incubation in samples pre-filtered through 1 µm we still observed the great increase of contribution of nucleoid-containing bacteria, NuCC constituted 81.8% of total number of bacteria in comparison to 36.8% at the beginning of the experiment. The number of active bacteria in these samples then began quickly to decrease, and the contribution of NuCC at the end of incubation was only 50.7%. In samples pre-filtered through 10 µm the numbers of active bacteria were slightly dropping already after 48 hours of incubation, the contribution of NuCC was 43.8% after 24 hours of incubation, 40.9% after 48 hours of incubation, and 37.7% after 72 hours of incubation. The differences between both types of samples were most distinct after the 48th hour of incubation.

In Lake Szymon (Fig. 1D) the contribution of NuCC to total bacterial abundance fluctuated in both types of samples. However, after 48 hours of incubation we noted a statistically significant difference (t-test ANOVA) in the contributions of active bacteria in samples pre-filtered through 1 µm and 10 µm and it was 61.4% and 32.9%, respectively. In the course of further incubation the difference between both samples decreased.

In Lake Smolak (Fig. 1E), initially very high contributions of nucleoid-containing bacteria in both types of samples (91.9 % in samples without grazers and 95.2% in samples with grazers) were decreasing. Contributions of NuCC to the total bacterial number was smaller in samples pre-filtered through 10 µm than in samples pre-filtered through 1 µm. Similarly to lakes Kuc, Ryńskie and Szymon the difference between samples was statistically significant after 48 hours of incubation, 72.5% of bacteria were active in samples without bacterivorous protozoans and only 42.8% of bacteria were active in samples with bacterial consumers.

Experiment 2

In Experiment 2 grazing rates on live radioactive bacteria were $14.1 \pm 1.93 \times 10^5 \text{ cells ml}^{-1} \text{ h}^{-1}$ and grazing rates on heat-killed radioactive bacteria were $7.7 \pm 1.19 \times 10^5 \text{ cells ml}^{-1} \text{ h}^{-1}$ (Fig. 2). Determined grazing rates on live bacteria were 1.83 times greater than grazing rates on dead bacteria.

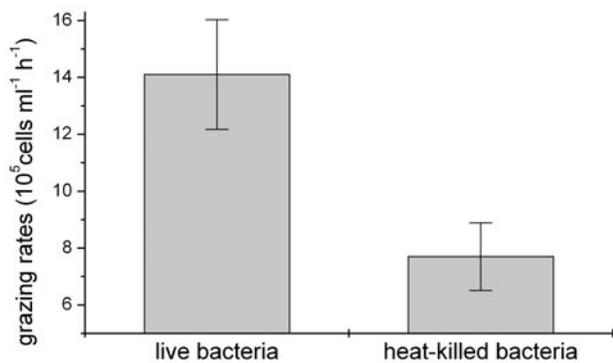


Fig. 2. Protozoan grazing rates on live and heat-killed bacteria in samples from Lake Mikołajskie (results obtained by radioisotopic method).

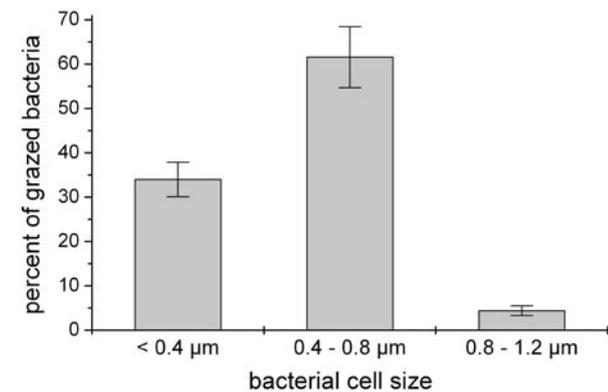
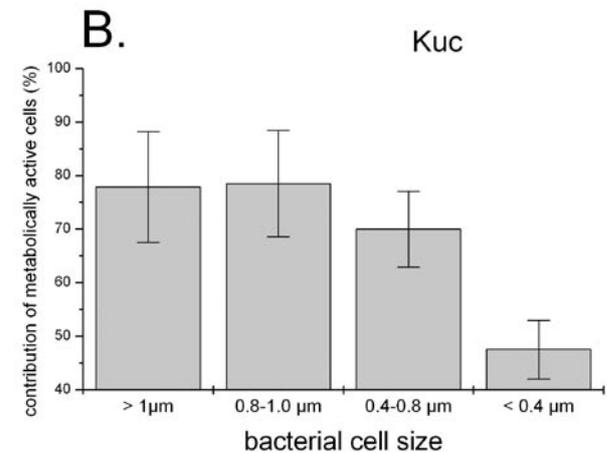
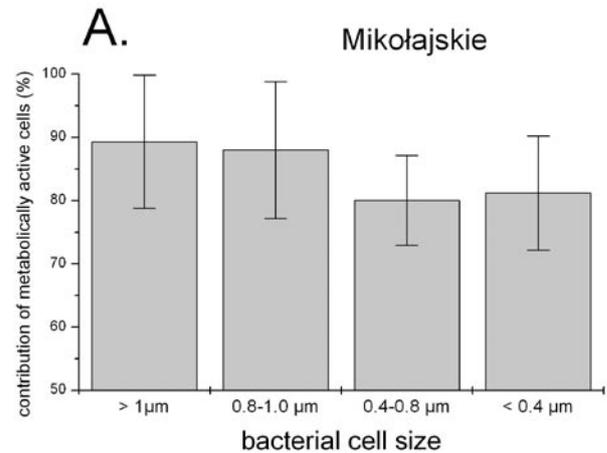


Fig. 3. Protozoan grazing on bacteria belonging to different size classes in samples from Lake Mikołajskie (results obtained by radioisotopic method).

Fig. 4. Percent contribution of nucleoid-containing bacteria to the number of bacteria belonging to the different size fractions in Lake Mikołajskie (A.) and Lake Kuc (B.)

Experiment 3

In Experiment 3 we tested the preferential feeding of Protozoa towards bacteria of defined cell size fractions (Fig. 3). We noted that the bacterial size fraction between 0.4 and 0.8 μm represented the majority of all grazed bacteria, i.e. $61.6 \pm 6.9\%$. The contributions of other bacteria to the total number of grazed bacteria were much lower, $34.0 \pm 3.9\%$ and $4.4 \pm 1.1\%$ for bacteria with cell sizes $< 0.4 \mu\text{m}$ and $0.8 - 1.2 \mu\text{m}$, respectively.

Experiment 4

In Experiment 4 we tested the hypothesis that larger bacterial cells have higher metabolic activity [6, 8]. In Lake Mikołajskie the contributions of NuCC to the total number of bacteria belonging to all four size classes were high and very similar (Fig. 4A). NuCC constituted $89.3 \pm 10.6\%$ in fraction $> 1 \mu\text{m}$; $88.0 \pm 10.8\%$ in fraction $0.8-1 \mu\text{m}$; $80.0 \pm 7.1\%$ in fraction $0.4-0.8 \mu\text{m}$; and $81.2 \pm 9.0\%$ in fraction $< 0.4 \mu\text{m}$.

In Lake Kuc the percent contributions of nucleoid-containing bacteria were also high but differences between size classes were more distinct (Fig. 4B). We determined that $77.9 \pm 10.3\%$ of bacteria contained nucleoid in fractions of the largest cells ($> 1 \mu\text{m}$). The same number of bacteria contained nucleoid in fractions between 0.8 and $1 \mu\text{m}$ ($78.5 \pm 9.9\%$). In fraction $0.4-0.8 \mu\text{m}$, NuCC constituted $70.0 \pm 7.1\%$ of bacterioplankton. Clearly less nucleoid-containing bacteria were in the smallest fraction of cells ($< 0.4 \mu\text{m}$) – they constituted only $47.5 \pm 5.4\%$ of all bacteria.

Fig. 5 presents changes in the abundance of bacteria belonging to different sized fractions in time in samples with and without grazers taken from Lake Mikołajskie. Bacteria larger than $1 \mu\text{m}$ were more abundant in samples without grazing after 6, 12 and 24 hours of incubation (Fig. 5A, 5E) despite the fact that their initial number in these samples was 0.0 due to the pre-filtration through $1 \mu\text{m}$ pore-size filter. After 36 hours of incubation the numbers of bacteria larger than $1 \mu\text{m}$ were similar in both types of samples and in the end of incubation number of the largest bacteria in samples with grazing slightly exceeded number of these bacteria in samples without grazing.

Analysis of changes in number of bacteria with cell sizes ranging from 0.8 to $1 \mu\text{m}$ showed that they were more abundant in samples with bacterial grazers except 36 hours of incubation (Fig. 5B, 5F). We noted a permanent decrease in the number of bacteria belonging to fraction $0.8-1 \mu\text{m}$ in samples with effective grazing, while their number fluctuated in samples without grazing.

An opposite pattern was observed in fraction of bacteria with cell size ranging from 0.4 to $0.8 \mu\text{m}$ (Fig. 5C, 5G). Generally, these bacteria were more abundant in samples without grazing (except 6 hours of incubation). Their numbers slightly fluctuated in both types of samples.

The number of bacteria smaller than $0.4 \mu\text{m}$ in size was higher in samples without grazers during the whole incubation time but the differences between samples were in most cases statistically not significant (Fig. 5D). The contributions of these bacteria to the total bacterial abundance fluctuated in both types of samples (Fig. 5H) and their average contribution was very similar in both types of samples, $47.9 \pm 14.6\%$ in samples without grazing and $47.9 \pm 10.3\%$ in samples with grazing.

Changes in the abundance of bacteria belonging to the different size classes and their contribution to the total bacterial number during the incubation in samples from Lake Kuc pre-filtrated through 1 and $10 \mu\text{m}$ are shown in Fig. 6.

The number of bacteria larger than $1 \mu\text{m}$ in size and their contribution to the total bacterial number (Fig. 6A, 6E) clearly increased in samples without grazing (pre-filtrated through $1 \mu\text{m}$) during the whole incubation time while they were more constant in samples with bacterivores (pre-filtrated through $10 \mu\text{m}$).

Bacteria with cell size ranging from 0.8 to $1 \mu\text{m}$ were generally more abundant in samples without grazing (Fig. 6B, 6F). The number of bacteria of this cell size increased in samples without bacterivores and fluctuated during the whole period of incubation without clear tendency in samples containing bacterial grazers.

The number of bacteria with cell size ranging from 0.4 to $0.8 \mu\text{m}$ and their contribution to the total bacterial number was much higher in the samples with reduced protozoan grazing than in the samples with effective protozoan grazing on bacterioplankton during practically the entire incubation period (Fig. 6C, 6G).

The number of bacteria smaller than $0.4 \mu\text{m}$ in samples with bacterivorous protozoans was at first rising (in the 24 hour of incubation averaged $9.96 \pm 0.91 \times 10^6$ cells ml^{-1} in comparison to $3.79 \pm 0.65 \times 10^6$ cells ml^{-1} in the beginning of the experiment) and after 24 hours of incubation it rapidly dropped (at the end of the experiment it averaged $6.24 \pm 0.61 \times 10^6$ cells ml^{-1}) (Fig. 6D). In samples with reduced number of bacterial grazers the abundance of bacteria belonging to this size fraction increased during the whole experiment. It averaged $3.18 \pm 0.43 \times 10^6$ cells ml^{-1} at the beginning of incubation and $10.67 \pm 1.84 \times 10^6$ cells ml^{-1} at the end of the incubation period.

Discussion

Natural bacterial assemblages in all aquatic environments are not homogeneous. Bacterial cells have different morphologies, sizes and metabolic activity. All these features affect the protozoan grazing rates on bacteria, this being confirmed in our studies.

Similarly to other authors [15], we observed that protozoan grazing rates on live bacteria were almost two times higher than on dead bacteria. That is one of the disadvantages of several methods measuring protozoan grazing rates with the use of killed bacterial preys, for example the FLB (fluorescently labelled bacteria) method. Results obtained by these methods underestimate real grazing rates.

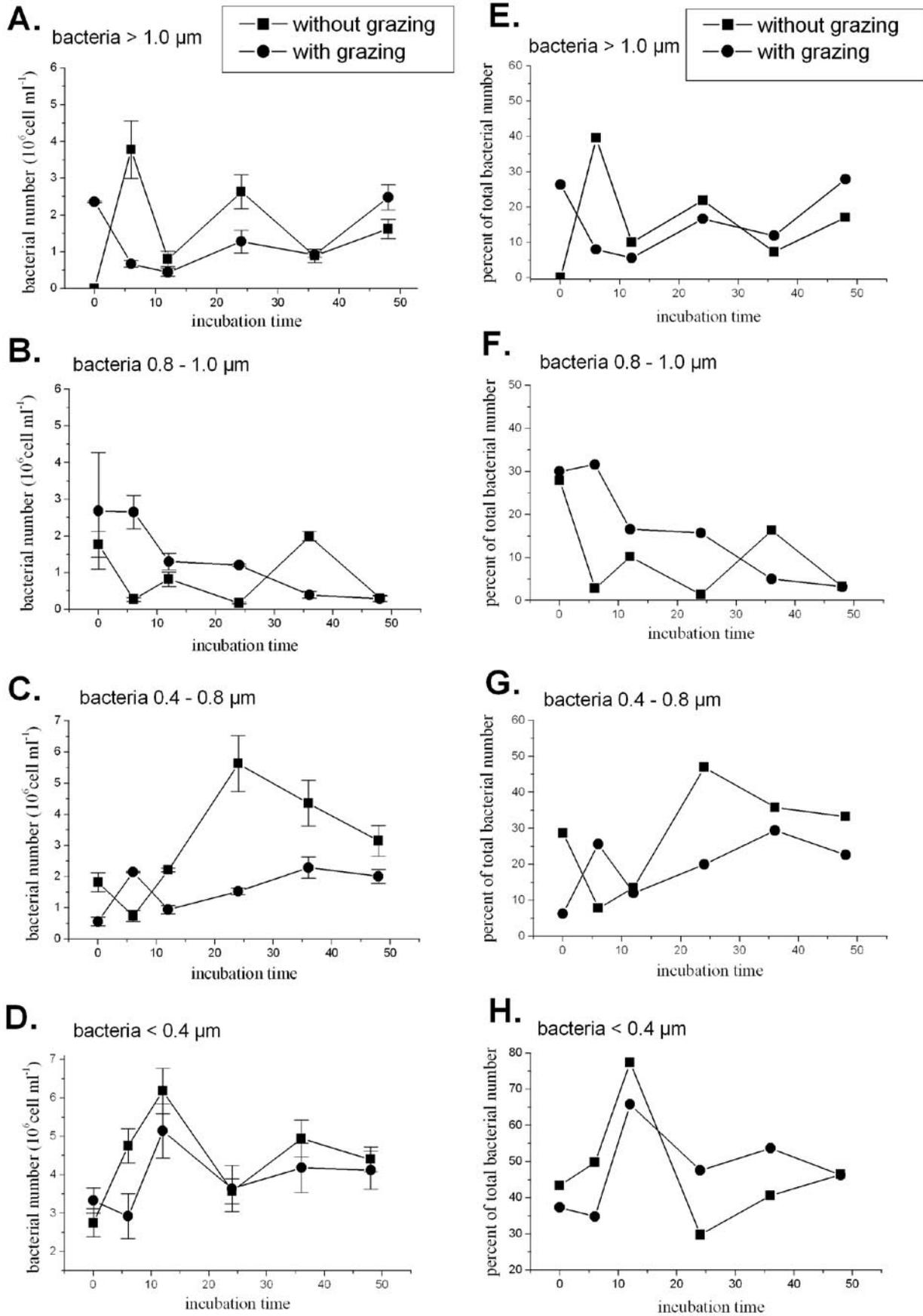


Fig. 5. Changes in bacterioplankton size structure in samples from Lake Mikołajskie during incubation with bacterial grazers (in samples pre-filtered through 10 μm pore-size filters) and without bacterial grazers (in samples pre-filtered through 1 μm pore-size filters).

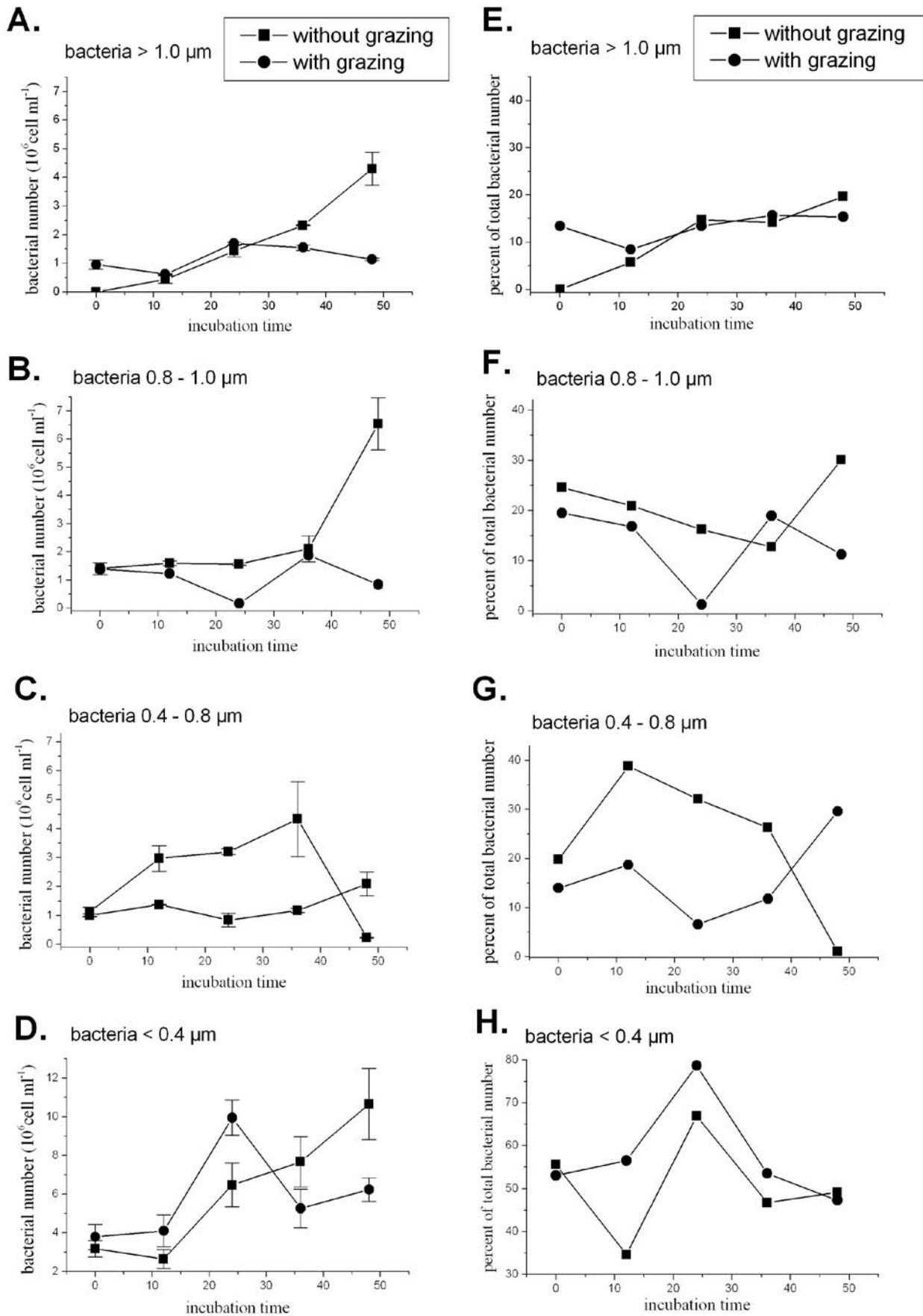


Fig. 6. Changes in bacterioplankton size structure in samples from Lake Kuc during incubation with bacterial grazers (in samples pre-filtered through 10 μm pore-size filters) and without bacterial grazers (in samples pre-filtered through 1 μm pore-size filters).

The results of our Experiment 1 obtained for all five studied lakes (Fig. 1) suggested that dead bacteria were not the only ones avoided by protozoans. They grazed reluctantly on the whole fraction of metabolically inactive bacteria which seem not to contain visible nucleoid [7, 8, 9, 10, 11] (dead, dormant, or slowly growing bacteria), clearly preferring nucleoid-containing and metabolically active bacteria. This preferential feeding was observed as a great decrease of the percent contribution of nucleoid-containing bacteria to the total bacterial number in samples containing bacterivorous protozoans. In several cases the numbers of NuCC also dropped in samples without bacterial grazers. This can be explained by the activity of bacteriophages present in all samples, or by the starvation of bacteria at the end of a long incubation period.

Thus, our results are in good agreement with other authors. del Giorgio et al. [6] demonstrated that protozoan grazing on metabolically active bacteria was four times greater than on dead or dormant bacteria. Gonzales et al. [5] showed that Protozoa selectively consume rapidly growing bacterial cells. Geider [16] using the radiolabelled tracers demonstrated that bacteria effectively metabolizing glucose are submitted to greater grazing pressure than the rest of the bacterial population. Thus, protozoan grazing probably eliminates mainly newly-produced bacterial cells rather than cell standing stock. Protozoa show chemotaxis towards chemical compounds released by actively metabolizing bacteria [17], or they distinguish between active and inactive bacterial cells because of their surface properties [18, 19]. They probably select growing and dividing bacteria because these cells are much richer in nutrients [20].

The small percent contribution of metabolically active bacteria number to total bacterial abundance observed in many environments might be caused not only by food limitation but also by strong grazing pressure and inactivation might be a defensive strategy of grazed bacteria [3, 6, 21]. Therefore, grazing regulates the abundance of aquatic bacteria and, perhaps to an even greater extent, the ratio of metabolically active bacteria to inactive bacteria number and the productivity of bacterioplankton biomass [5, 8, 22].

Metabolic activity is strongly associated with bacterial cell sizes – usually more active bacteria are larger than less active bacteria [6, 8]. That pattern was clearly demonstrated in our Experiment 4 in Lake Kuc (Fig. 4B). Thus, if bacterivorous protozoans prefer actively metabolizing bacterial cells, they should prefer larger ones. In fact, there is a lot of evidence confirming this hypothesis [3, 22, 23, 24]. However, preys that are too large are also avoided by HNF because most bacterivorous protozoans are able to graze efficiently on particles which are three times smaller than themselves [25]. They are not capable of grazing on bacterial filaments and on bacterial aggregates [26, 27]. That is why bacterivorous protozoans graze preferentially on medium size bacteria (from 0.4 μm to 0.8 μm) as found in Experiment 3 (Fig.

3). The presence of extremely small and extremely large bacterial cells in populations can be an effective defence mechanism against intensive protozoan grazing. Bacteria that have genetic possibilities to produce small and large cells are best adapted to environments with permanent or temporary strong grazing pressure [28]. Changes in bacterioplankton structure during the increasing number of HNF and intensive protozoan food activity were repeatedly observed in natural environments. Several authors noted that free-living small coccus and short rods, which initially predominated in bacterial assemblages were replaced by long bacterial filaments, large bacterial clumps and bacteria attached to the organic particle [3, 23, 29, 30, 31, 32, 33, 34]. These morphological transformations are caused by changes in taxonomical composition of bacterioplankton, i.e. elimination of certain bacterial species and replacement by others or by genetic properties and phenotypic plasticity of existing types of bacteria [23]. Specific chemical compounds released by grazed bacteria or by grazers might stimulate the rapid formation of long bacterial filaments [4, 35]. But the formation of filaments need not be relevant to chemical signals. It might depend on the bacterial growth rate, i.e. higher growth rate causes an increase of filament number. In this case protozoan grazing influences filament formation directly by augmenting bacterial growth rates [34]. Because of their large sizes (length up to 200 μm) bacterial filament can compose even 70% of total bacterial biomass in periods of intensive protozoan food activity [33]. Compounds released by feeding Protozoa also are suspected to stimulate the formation of bacterial aggregates. They might release specific compounds as chemical signals, changing the bacterial behaviour or as basic materials which facilitate assembling [17].

A somewhat different pattern was observed during Experiment 4 (Fig. 5, Fig. 6). In Lake Mikołajskie HNF grazed on bacteria larger than 1 μm and on bacteria with cell sizes from 0.4 μm to 0.8 μm (their abundance in samples with HNF was smaller than in samples without HNF during practically the whole incubation period). HNF probably also grazed on bacteria with cell sizes from 0.8 μm to 1 μm (their abundance in sample with HNF clearly dropped during the incubation). In Lake Kuc we observed, according to our predictions, effective protozoan grazing on bacteria belonging to the fractions from 0.8 μm to 1 μm and from 0.4 μm to 0.8 μm (their number in samples with HNF was lower than in samples without HNF for most of the incubation time). We can suppose that protozoans from Lake Kuc also grazed on bacteria larger than 1 μm because their number was almost constant in sample with HNF while it showed a distinct increase in samples without HNF. Based on our results from Experiment 3 and those reported elsewhere [36] we expected that protozoans would not efficiently consume bacteria larger than 1 μm . However, they grazed on these large bacteria both in Lake Mikołajskie and Lake Kuc. Perhaps protozoan assemblages existing in these ecosystems in August (when we carried out Experiment 4) were somewhat different

than in April (time of Experiment 3). They can differ in taxonomical composition or in physiological states and their selectivity depends on these features [37, 38]. Protozoa could change their sizes to adjust to the available stock of preys as *Paraphysomonas imperforata* had done in the experiment of Goldman & Caron [39]. Bacterivorous protozoans in Mazurian Lakes were probably much larger in the summer than in the spring and they were able to graze on a wider size spectrum of preys. A similar pattern, i.e. effective protozoan grazing on the largest bacteria was observed by Posh et al. [40] – in their experiment heterotrophic flagellate *Bodo saltans* and mixotrophic flagellate *Ochromonas* sp. eliminated almost all large bacterial cells ($> 1.5 \mu\text{m}$) and caused the predominance of the smallest cells at the end of experiment. The total bacterial number remained constant but bacterial biomass dramatically dropped.

We did not observe effective grazing on bacteria smaller than $0.4 \mu\text{m}$ either in Lake Mikołajskie (there were no differences in bacterial number between samples with and without HNF) or in Lake Kuc (their number in sample with HNF was greater or similar to their number in samples without HNF for most of the incubation time). Bacteria smaller than $0.4 \mu\text{m}$ are too small for most protozoans and they are probably less active, so we are not surprised that protozoans did not graze on them (similar to 4).

As the literature data indicate, protozoans display selectivity towards free-living or attached bacteria [41, 42] and towards motile or non-motile bacteria [43] (which had not been tested in our experiments). All these types of preferential protozoan feedings are a good explanation for the coexistence of bacteria differing in cell sizes, morphotypes, mobility, and metabolic activity in natural aquatic ecosystems [44]. Strong grazing pressure sustains the taxonomical and metabolic diversity of bacterial assemblages and enables the pool of inactive or slowly growing bacteria to exist permanently [22, 26, 45].

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