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RESEARCH ARTICLE

Changes in protistan abundance and bacterial activity in response to the addition of eukaryotic inhibitors to natural lake water

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Abstract. Two short-term (24 h and 48 h) microcosm experiments using natural waters from three eutrophic lakes (Masurian Lake District, Poland) were performed to assess the impact of eukaryotic inhibitors (a combination of cycloheximide and colchicine) on the abundance of nanoflagellates and small ciliates in the <15 μm fraction. The results showed that eukaryotic inhibitors were not completely effective against either group of protists; however, they reduced their numbers considerably. At 24 h of the experiment, 41, 15, and 7% of nanoflagellate and 48, 23, and 3% of ciliate abundances were not lysed, depending on the lake from which water was taken. However, after 48 h of incubation, only below 7% of nanoflagellates and 33, 40, and 17% of ciliates were present in the treatments with inhibitors. Our results suggest that inhibitors may indirectly change bacterial growth and activity, but they do not definitively inhibit these processes. It was concluded that eukaryotic inhibitors are more effective against small nanoflagellates than larger nanoflagellates and ciliates. Concentrations of inhibitors higher than 200 and 100 mg l^{-1} for cycloheximide and colchicine, respectively, and an incubation time longer than 24 h also seemed to be more appropriate to achieve the complete inhibition of protists.

Keywords: Bacterial activity, Ciliates, Eukaryotic inhibitors, Eutrophic lakes, Nanoflagellates

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Introduction

Grazing pressure by protists is an important factor influencing bacterial numbers and biomass, their taxonomic, morphological, and genetic diversity, and their metabolic activity (Hahn and Höffle 2001, Pernthaler 2005). Bacterivorous microorganisms may also play an important role in removing a variety of pathogenic bacteria contaminating and/or inhabiting natural waters (Wcisło and Chróst 2000, Smith 2010, Pang et al. 2016).

One of several methods applied to estimate protistan grazing on bacterial communities in both field and laboratory experiments is eukaryotic inhibition (Sanders and Porter 1986, Sherr et al. 1986). This technique allows measuring grazing on natural bacterial populations and involves minimal water sample manipulation (Newell et al. 1983, Sherr et al. 1986). The effectiveness of eukaryotic inhibitors depends on their properties, the composition of the

protistan communities, and their cell cycles (Suhama and Hanson 1971, Sanders and Porter 1986, Sherr et al. 1986). One of the most important disadvantages of this technique may be the fact that the addition of antibiotics may increase the substrate pool for bacterial growth (Ammerman et al. 1984). In addition, soluble material leaking from cells killed by inhibitors may be a major source of nutrients and energy for surviving microorganisms (Badalucco et al. 1994). On the other hand, inhibitors may indirectly limit bacterial growth by decreasing the availability of nutrients regenerated by protozoans (Sherr et al. 1986). Generally, the use of eukaryote inhibitors is based on three assumptions: 1) the target protists are completely inhibited, 2) non-target eukaryotes (microalgae and metazoans) are not inhibited, and 3) the inhibitor has no effect on bacterial activity (Tremaine and Mills 1987, Shimeta and Cook 2011).

Sherr et al. (1986) found a mixture of cycloheximide (an inhibitor of 80S ribosomal protein synthesis) and colchicine (inhibiting microtubule polymerization) to be the most specific inhibitors from among different eukaryotic antibiotics. These authors concluded that the combination of these inhibitors caused the complete inhibition of reproduction and feeding of protozoa (both laboratory-cultured flagellates and ciliates and natural heterotrophic nanoplankton assemblage) and had no significant effect on natural bacterioplankton.

Recent studies indicate that the use of cycloheximide and colchicine in combination resulted in the lowest and almost constant heterotrophic nanoflagellate abundance and had no effect on bacterial activity in comparison to the dilution and size-fractionation methods (Adamczewski et al. 2010). Other studies show that among ten inhibitors tested, only thiram was completely effective against the natural assemblages of protists and did not kill invertebrates (Shimeta and Cook 2011). It should be emphasized that Shimeta and Cook (2011) studied organisms inhabiting marine sediments. Therefore, more detailed studies are required to assess the effectiveness of inhibitors on planktonic protists and bacteria.

The aim of the present study was to test the hypothesis that a mixture of cycloheximide and colchicine can cause the complete lysis of natural nanoflagellates and ciliates in the fraction < 15 μm without direct effects on bacterial activity. To test our hypothesis experiments with the use of water from three eutrophic lakes of different morphometry were performed.

Study area

The microcosm experiments were conducted using water samples taken from three lakes (Masurian Lake District, north-eastern Poland) during summer stratification in July (Experiment I) and August (Experiment II). The studied lakes differ in morphometry (Table 1). Lake Szymon is a small, shallow lake; Lake Śniardwy is a large, deeper lake, while Lake Tałty is medium-sized and the deepest one. The trophic state index (TSI) of the lakes, calculated from chlorophyll *a* and total phosphorus (TP) concentrations and Secchi disc visibility (SD) according to Carlson (1977), indicated that all of the studied lakes were eutrophic (TSI from 53.4 to 59.2) (Table 2).

Material and methods

Water temperature, pH, conductivity, and oxygen concentration were measured *in situ* at 0.5 m depth

Table 1
Morphometric characteristics of the studied Masurian lakes

Lake	Area (ha)	Mean depth (m)	Max depth (m)	Mixing Type
Śniardwy	11340	5.8	23.4	Polymictic
Tałty	1160	13.5	44.7	Dimictic
Szymon	154	1.1	2.9	Polymictic

Table 2
Trophic characteristics of the studied Masurian lakes (mean values from July and August)

Lake	DOC (mg l^{-1})	Chl *a* (μg l^{-1})	TP (μg l^{-1})	SD (m)	TSI
Śniardwy	8.9	14.4	37	2.4	53.4
Tałty	11.3	24.8	42	1.2	59.2
Szymon	11.6	11.0	38	1.5	55.0

DOC – dissolved organic carbon, Chl *a* – chlorophyll *a*, TP – total phosphorus, SD – Secchi disc visibility, TSI – trophic state index

intervals with an YSI 6600-meter (Yellow Spring Instruments, USA). Chlorophyll *a* (Chl *a*), extracted with 98% acetone, was measured with a TD-700 (Turner Design, USA) fluorimeter (Arrar and Collins 1997). Total phosphorus (TP) concentration was determined spectrophotometrically according to Koroleff (1983). Dissolved organic carbon (DOC) concentration was determined in water samples filtered through 0.2 μm pore size polycarbonate membrane filters (Millipore) with a Shimadzu TOC 5050 carbon analyzer.

Natural lake water samples for the experiments were collected from the deepest parts of the lakes and from the upper trophogenic layer at 0.5 m intervals and mixed to obtain one integrated lake water sample. The experiments were conducted in l^{-1} glass bottles with water filtered through a 15 μm mesh size plankton net that contained a mixed species assemblage of nanoflagellates and small ciliates (control treatment). A mixture of two eukaryotic inhibitors composed of cycloheximide (200 mg l^{-1}) and colchicine (100 mg l^{-1}) (Sherr et al. 1986) was used to eliminate protists. Both inhibitors were added to the <15 μm filtrate and incubated in the dark for 24 h (Experiment I) and 48 h (Experiment II) at a mean temperature of 20°C, according to *in situ* temperatures. This variant was marked as the inhibition (INH) treatment. Water samples (15 ml in total) were taken before the addition of the inhibitors (T = 0) and after 4, 8, 12, 24, and 48 h of incubation for nanoflagellates and after 1, 2, 4, 8, 12, 24, and 48 h of incubation for bacterial abundance and activity (all bacterial data were used for statistical analyses, but only the abundance and activity of bacteria at the end of the experiments are shown). Ciliate abundances were determined at the beginning and the end of incubation.

Nanoflagellate (NF) samples (10 ml) were fixed with formalin (final concentration 2%), stained with DAPI (Porter and Feig 1980), filtered through 0.8 μm pore size polycarbonate membrane filters (Millipore), and enumerated using an epifluorescence microscope (Nikon Optiphot 2).

Ciliates were counted on membrane filters prepared for NF. The whole filter area was inspected at 400x magnification. In addition, total ciliate abundances in all the studied lakes were determined in unfiltered water samples fixed with Lugol's solution and examined with light microscopy. These data were used to determine the percentage contribution of the fraction <15 μm to total ciliate numbers. Species composition was determined from living material in unfiltered samples and in samples that were filtered through a 15 μm mesh plankton net. Species identifications of ciliates were based mainly on Foissner et al. (1999).

Bacterial samples were preserved with formalin (final concentration 2%), stained with DAPI (final concentration 1 μg ml^{-1}), filtered through 0.2 μm pore size black polycarbonate membrane filters (Millipore), and enumerated using a Nikon ECLIPSE E 400 epifluorescence microscope (Porter and Feig 1980). Bacterial biomass 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). Bacterial secondary production (BP) was determined with the [^{3}H]-thymidine ([^{3}H]TdR) method (Chróst et al. 1988).

Data were statistically analyzed using the STATISTICA software. The non-parametric Wilcoxon signed rank test was used to analyze the

differences in NF numbers, bacterial numbers, biomass, and production between the studied treatments.

Results

Experiment I

In the control treatments with waters from lakes Śniardwy and Tałty, NF numbers decreased distinctly during the first hours of incubation, remained more or less stable during subsequent hours, and then increased markedly at the end of the experiment to values similar to those at the beginning of the experiment (Fig. 1). In the control treatment with water from the shallow Lake Szymon, NF numbers increased continuously throughout the study period, reaching values two times higher than at the start of the experiment. In all the INH treatments, the numbers of NF decreased. They decreased quite rapidly during the first 4 h of the experiment and gradually thereafter. In comparison to the control, 41, 15, and 7% of NF cells were present at the end of the experiment in the INH treatments with waters from lakes Śniardwy, Tałty and Szymon, respectively. They were represented mainly by medium-sized (5-10 μm) cells. The differences in NF numbers (both heterotrophic and autotrophic) between the

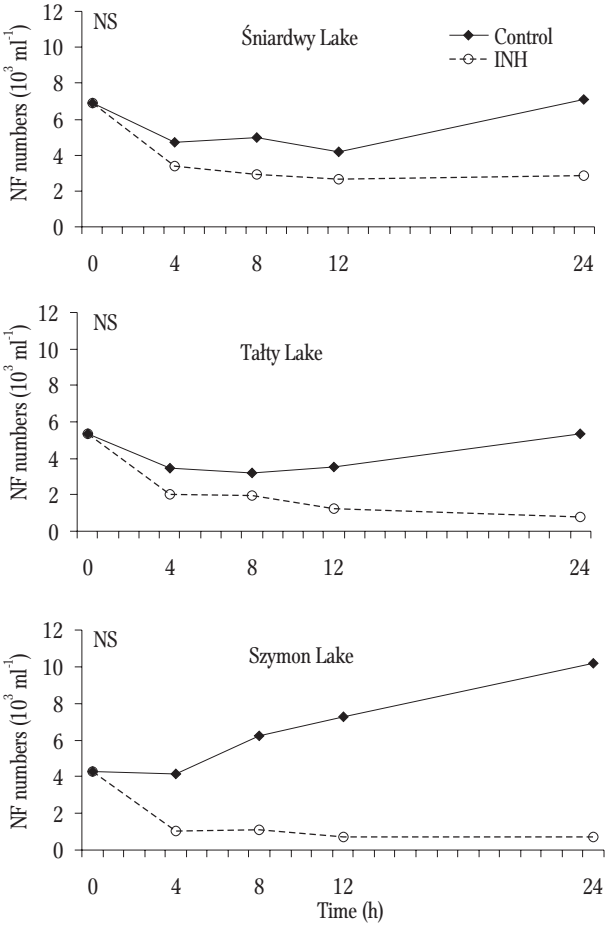

Figure 1. Experiment I. Changes in nanoflagellate abundance in control and eukaryotic inhibitors (INH) treatments with water from eutrophic Masurian lakes. NS – not significant differences between treatments at P > 0.05.

Table 3

Bacterial numbers (BN), bacterial biomass (BB), and bacterial production (BP) in control and eukaryotic inhibitors (INH) treatments with water from eutrophic Masurian lakes at 24 h (Experiment I) and 48 h (Experiment II) of the experiments. * indicates significant differences between treatments (Wilcoxon test, P < 0.05)

		Time of the experiment		
Bacterial parameters	Treatment	24 h		48 h
Lake Śniardwy				
BN (× 10^6 ml^{-1})	Control	6.28	*	5.10
	INH	6.37		8.78
BB (mg C l^{-1})	Control	0.16		0.22
	INH	0.22		0.33
BP (μg C l^{-1} h^{-1})	Control	0.92		0.76
	INH	1.84		1.15
Lake Tałty				
BN (× 10^6 ml^{-1})	Control	7.38		6.07
	INH	8.74		8.33
BB (mg C l^{-1})	Control	0.28		0.15
	INH	0.33		0.21
BP (μg C l^{-1} h^{-1})	Control	1.88	*	1.10
	INH	1.71		1.21
Lake Szymon				
BN (× 10^6 ml^{-1})	Control	5.34		2.60
	INH	7.74		7.76
BB (mg C l^{-1})	Control	0.26	*	0.07
	INH	0.23		0.25
BP (μg C l^{-1} h^{-1})	Control	1.13	*	0.84
	INH	0.63		1.27

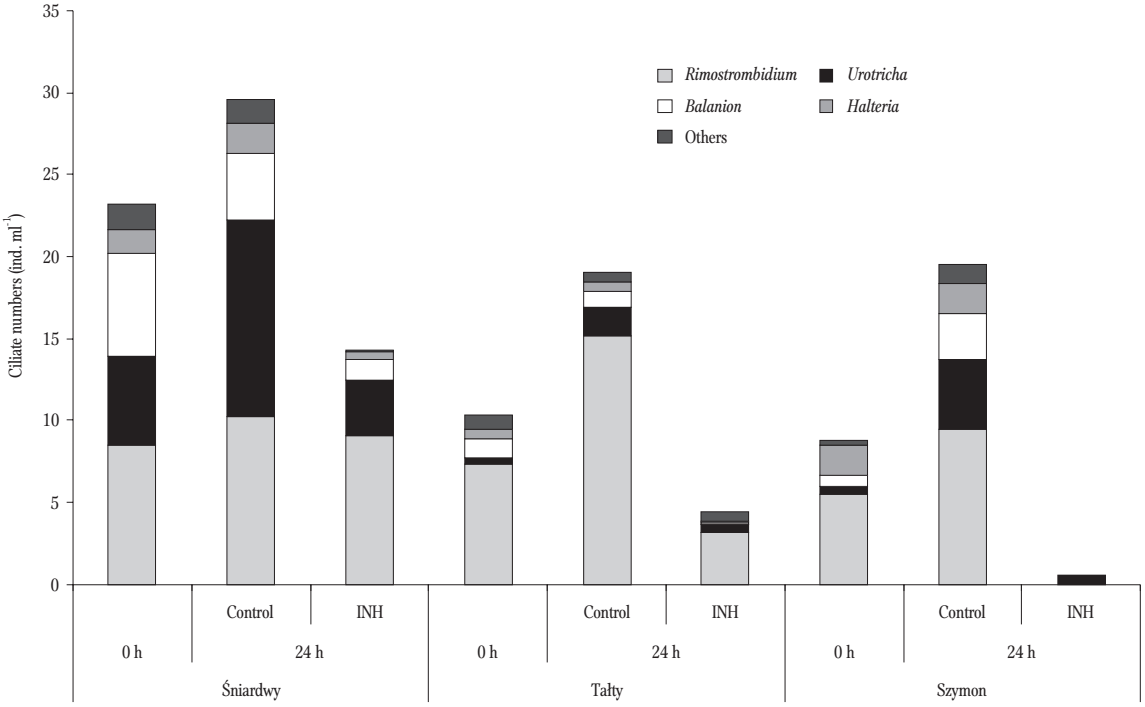

Figure 2. Experiment I. Changes in ciliate abundance, with dominant taxa marked, in control and eukaryotic inhibitors (INH) treatments with water from eutrophic Masurian lakes at 0 h and 24 h of the experiment.

treatments were not statistically significant for all the studied lakes ($P > 0.05$).

Ciliates in the <15 μm fraction were represented by small species of the order Prostomatida (*Balanion planctonicum* Foissner, Berger and Kohmann, *Urotricha* spp. with one and two caudal cilia, mainly *U. furcata* Schewiakoff), Oligotrichida (*Rimostrombidium humile* Petz and Foissner, *Halteria grandinella* Dujardin), Haptorida (*Mesodinium acarus* Stein), and Scuticociliatida (*Cyclidium glaucoma* Müller). They constituted 48, 66, and 74% of the total ciliate numbers in the studied lakes. In all the control treatments, ciliate numbers increased slightly in treatments with water from Lake Śniardwy, while they increased distinctly in the two other treatments (Fig. 2). In the INH treatments with waters from the two deeper lakes (Śniardwy and Tałty), ciliate numbers decreased about two times, while in the treatment with water from shallow Lake Szymon they decreased 15 times. In comparison to the control, 48, 23, and 3% of ciliate cells were present in lakes Śniardwy, Tałty, and Szymon, respectively.

In all the control and INH treatments, small *Rimostrombidium* dominated at the beginning and the end of the experiment, constituting 49–80% of the total ciliate numbers. Only in the control treatment with water from Lake Śniardwy and in the INH treatment with water from Lake Szymon, species of the genus *Urotricha* prevailed or even were the only species identified at 24 h of incubation (Fig. 2).

At 24 h of the experiment in all lakes, bacterial numbers (BN) were higher in the INH treatment than in the control (Table 3). The highest differences between treatments were observed in Lake Szymon. Bacterial biomass (BB) was higher in the INH treatment than in the control in lakes Śniardwy and Tałty, whereas it was slightly lower in Lake Szymon. Bacterial production (BP) in Lake Śniardwy was two times higher in the INH treatment than in the control, while in the other two lakes (especially in the shallow Lake Szymon) it was lower in the INH treatments than in the control. The differences between the studied treatments in BN were statistically significant for Lake Śniardwy ($z = 2.02$, $P = 0.043$), in BB for Lake

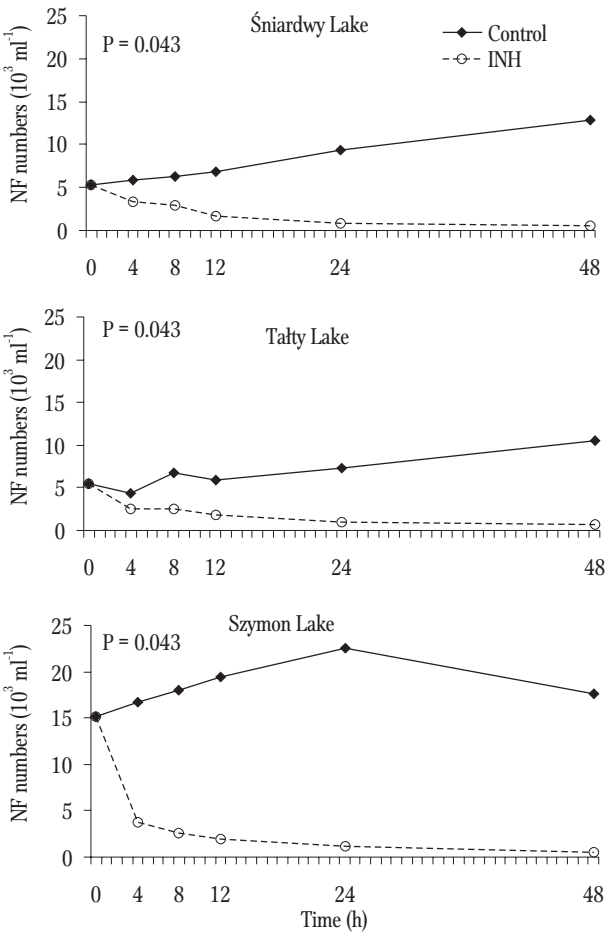

Figure 3. Experiment II. Changes in nanoflagellate abundance in control and eukaryotic inhibitors (INH) treatments with water from eutrophic Masurian lakes. P – values determined with the Wilcoxon test.

Szymon (z = 2.37, P = 0.018), and in BP for lakes Tałty and Szymon (z = 2.20, P = 0.028).

Experiment II

In all the control treatments, NF numbers increased markedly, reaching two times higher values at the end of the experiment than at the start (Fig. 3). In the INH treatments with waters from lakes Śniardwy and Tałty, the numbers decreased gradually throughout the experiment. In the treatment with water from Lake Szymon, a drastic decrease was noted during the first 4 h and then a gradual decrease was observed. Statistically, the differences in NF numbers between both treatments were significant for all the studied lakes (z = 2.02, P = 0.043). At the end of the experiment, only 4, 7, and 3% of the nanoflagellates in lakes Śniardwy, Tałty, and Szymon, respectively, were present in comparison to the control. These cells were represented, similarly to Experiment I, by HNF in the 5-10 μm size range.

The taxonomic composition of ciliates in the <15 μm size fraction was very similar to that noted in Experiment I. Ciliates in this fraction constituted 61, 70, and 73% of the total ciliate numbers. After 48 h of incubation, ciliate numbers increased in all the control treatments (Fig. 4). The highest increase was observed in the control treatments with water from Lake Szymon (3.5 times), while the lowest was noted in water from Lake Tałty (1.7 times). In all the INH treatments, only slight decreases in ciliate numbers were observed, even so 33, 40, and 17% of ciliates were present in comparison to the control.

The ciliate dominance structure differed in the studied lakes. Species of the genus *Urotricha* dominated in Lake Śniardwy (41 and 60% of the total ciliate numbers, at the start and end of the experiment, respectively), *Mesodinium acarus* in Lake Tałty (45 and 39%) and *Rimostrombidium humile* in Lake Szymon (66% at both the start and end of the study) (Fig. 4). In the INH treatments with waters from lakes Śniardwy and Tałty, shifts in the taxonomic structure were observed, and at the end of the incubation *Rimostrombidium humile* prevailed in all the lakes, constituting 87, 62, and 82% of the total numbers.

At the end of Experiment II (48 h), BN, BB, and BP were higher in the INH treatment than in the controls in all the lakes (Table 3). The greatest differences (3 times) in BN and BB between treatments were observed in Lake Szymon. Bacterial production in lakes Śniardwy and Szymon was 1.5 times higher, while in Lake Tałty it was only slightly higher in the INH treatment than in the control. However, differences in BN and BB were not statistically significant in any of the lakes ($p > 0.05$), while in BP they were significant for Lake Szymon only (z = 2.03, P = 0.043).

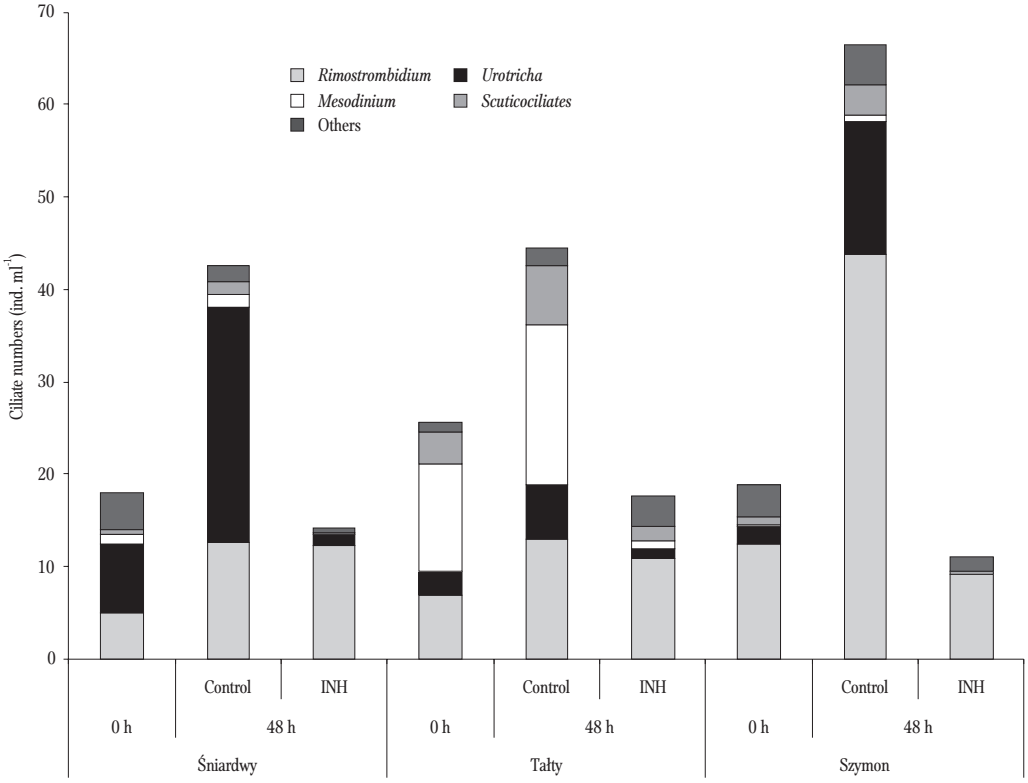

Figure 4. Experiment II. Changes in ciliate abundance, with dominant taxa or taxonomic groups marked, in control and eukaryotic inhibitors (INH) treatments with water from eutrophic Masurian lakes at 0 h and 48 h of the experiment.

Discussion

Protozoa in the size range of 2 to 20 μm have been identified as major grazers of bacteria in freshwater ecosystems (Sanders et al. 1989, Weisse 1990, Šimek et al. 2004). This group of protozoa is dominated by heterotrophic nanoflagellates. However, ciliates with cell diameters of <20 μm can be a substantial fraction of the nanoplankton biomass, and they may compete with nanoflagellates for bacteria (Šimek et al. 2000). There is also evidence that some freshwater autotrophic/mixotrophic flagellates within the >2 μm size class are able to consume bacteria (Sanders et al. 1989, Hitchman and Jones 2000). This complex assemblage of protists has a different impact on bacterial communities (Jezbera et al. 2006) depending on the season and lake trophic status (Sanders et al. 1989). The importance of individual groups among nanoplankton is difficult to evaluate because of the problem in their physical separation. In most studies, filtration through 5.0 μm pore size filters is used to estimate grazing pressure by protists on bacteria (e.g., Šimek et al. 2005, 2006, 2018). However, this method removes other potential bacterial grazers such as small ciliates, attached choanoflagellates, and medium-sized HNF. That is why the present study took the approach of filtering lake water samples through a 15 μm mesh size plankton net to separate nanoplankton from larger zooplankton. According to Sherr et al. (1986), this plankton net is better than 20 μm mesh net and has no effect on changes in bacterial and NF abundances. Our results showed that this filtration removed all large-sized protists, rotifers, and crustaceans, while leaving the small ciliates that were present in relatively high abundances and constituted from 48 to 74% of the total ciliate numbers.

Three eutrophic lakes with different morphometry were selected for the experiments because lake morphometry has a major impact on bacterial production (Cimbleris and Kalff 2003) and plankton community structure (Weithoff et al.

2010), and it also determines food-chain length (Post et al. 2000). Our results showed that while the taxonomic structure of the ciliate assemblages in the studied eutrophic lakes were very similar regardless of the study period, their numbers and dominance structures differed. Among identified species, the small-sized oligotrichs and haptorids that dominated in our two experiments may be efficient bacterial grazers (Carrias et al. 1996, Šimek et al. 2000, Zingel and Ott 2000, Comte et al. 2006).

The trophic responses of the microbial food web to eukaryotic inhibitors may change with the season (DeLorenzo et al. 2001). Furthermore, the inhibitory effects of various inhibitors on natural protozoan communities can depend on the incubation time (Sanders and Porter 1986, Tremaine and Mills 1987, Chabaud et al. 2006, Shimeta and Cook 2011). These facts promoted us to decide that in all lakes our experiments were performed twice (in July and August) at different incubation times (24 h and 48 h).

In the present study, from 3 to 48% of small ciliates were present, relative to the controls, after 24 h or 48 h of incubation, which suggests that the mixture of cycloheximide and colchicine at concentrations of 200 and 100 mg l^{-1}, respectively, was only partially effective against them. Similar conclusions are reported by Sanders and Porter (1986) and Taylor and Pace (1987). According to Shimeta and Cook (2011), only fumagillin, neutral red, and thiram were 100% effective in inhibiting ciliates within 24 h of application, while anisomycin inhibited ciliates completely during 48 h. Some studies show that cycloheximide was effective in controlling protozoan growth and predation, but considerably higher concentrations ranging from 500 mg l^{-1} to 2500 mg l^{-1} were required (McCambridge and McMeekin 1980, Kota et al. 1999). In both of the present experiments, the inhibition of ciliates was less complete in the lakes with the highest initial ciliate numbers, i.e., Lake Śniardwy – 23.2 ind. ml^{-1} (Experiment I) and Lake Tałty – 25.6 ind. ml^{-1} (Experiment II). The above facts suggest that inhibitors could be more effective at higher concentrations than those applied in our study or that lower concentrations should be added several times at regular intervals during experiments. In addition, the effectiveness of inhibitors probably depends on initial ciliate numbers. Our experiments showed that different ciliate species reacted differently to the inhibitors used. For example, in lakes Śniardwy (both experiments) and Tałty (Experiment II), the numbers of *R. humile* remained almost unaffected. According to Müller and Schlegel (1999), *Rimostrombidium* is very sensitive to changes in the chemical composition of the culture medium, and it frequently does not survive transfers to fresh medium. On the other hand, *Rimostrombidium* can adapt well to harsh under-ice environmental conditions (Sonntag et al. 2006, Kalinowska et al. 2017). Our results demonstrated that this small species showed not only a characteristic adaptation to new experimental conditions, but it was also better adapted to inhibitor stress than other, more sensitive taxa such as *Mesodinium*, *Urotricha*, or *Halteria*.

Although NF numbers were considerably lower in the INH treatment than in the control after 24 h of incubation, even 41% of cells were present in Lake Śniardwy and only 15% and 7% were present in the two other lakes. After 48 h of the experiment, only less than 7% of cells were found in all the studied lakes. These were mainly larger heterotrophic forms in the 5–10 μm size class. This may indicate that smaller cells were more sensitive than larger forms. Although the duration of the experiment seems to have no importance in the case of ciliates, it should be longer than 24 h in the case of nanoflagellates. Similarly, Tremaine and Mills (1987) demonstrated that smaller protozoans continued to swim actively for up to 36 h, and all activity ceased after 48 h. In opposite to our results, these authors found that large protozoans were inhibited more quickly than were smaller cells.

At the end of both experiments, all the studied bacterial parameters, especially bacterial secondary production, which is a sensitive measure of bacterial activity, were higher in treatments with the addition of eukaryotic inhibitors than in the controls. However, clear differences between treatments were observed after 48 h of the experiment. For instance, bacterial production was up to 2 fold higher in the

INH treatment than in the control. Thus, our results suggest that the used inhibitors may indirectly change bacterial growth and activity, but they did not definitively inhibit these processes. It is also possible that the 24 h incubation time is too short to detect the effect of inhibitors on bacterial activity.

In conclusion, the numerical responses of planktonic nanoflagellates and ciliates to the eukaryotic inhibitors were specific to species or size groups. Among ciliates, *Rimostrombidium* was less sensitive to inhibitors than were other species. Nanoflagellates in the size of <5 μm were much more strongly lysed than were larger cells, which indicated that the inhibition method was useful to study small nanoflagellates predation pressure. Higher concentrations of inhibitors than those used in the present study and incubation times exceeding 24 h and even 48 h are required to achieve the complete inhibition of small protists.

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Author contributions. K.K. identified protists and drafted the manuscript, K.J.-K. counted bacteria and measured bacterial secondary production; R.J.Ch. designed the study and revised a draft version of the manuscript. All authors read and approved the final manuscript.

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