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# Survival of *Escherichia coli* in Freshwater

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## Abstract

Experiments on the survival of *Escherichia coli* were conducted in the water of man-made Zegrzynski Reservoir. The aim of this study was to evaluate how selected biocenotic factors (predation, coliphage infection, presence of autochthonic microflora and nutrient conditions) affect this process. We observed residual living cells of *E. coli* up to about thirty days of the investigation. We found that the major factor that was responsible for mortality of *E. coli* was microflagellate grazing and exposure to an aquatic environment. Size-selective preferences of microflagellates toward *E. coli* cells were observed. No visible effect of bacteriophages on the survival of *E. coli* was detected. Better nutritional condition and the presence of native heterotrophic microflora significantly prolonged the survival time of *E. coli* in the studied environment.

**Keywords:** *E. coli* survival, freshwater, phagotrophic flagellates, grazing, coliphages, native bacteria

## Introduction

Studies of factors affecting the survival of *Escherichia coli* in natural waters are of great interest due to the importance of these microorganisms as indicators of fecal pollution in natural waters. Numerous investigators have reported that fresh water exhibits a marked bactericidal activity toward enteric bacteria [15, 31, 13, 48]. Based on a large number of laboratory studies various physicochemical factors (such as light, temperature, pH, toxicity of heavy metals) are believed to have a significant impact on bacterial mortality. Recent studies have focused especially on various biocenotic mechanisms (i.e. competition, allelopathy, parasite infection and predation) that are involved in a bactericidal phenomenon in natural waters [44, 32].

Several authors have deduced that visible light has a negative effect on *E. coli* cells in freshwater [20, 32, 4, 6, 7]. This was revealed by a decrease in the numbers of *E. coli* metabolic active cells. These studies showed that visible light provokes a decline in active transport of radiolabeled amino acids in population of enteric bacteria. Another explanation of sublethal injury induced by sunlight is accumulation of peroxides or other toxic substances due to UV-B photooxidation of organic matter [26].

McFeters and Stuart [34] observed that the survival of *E. coli* exposed to river water was inversely proportional to temperature (5 to 15°C). Similar results have been noted in estuarine water [17]. However, Soracco et al., [49] detected prolonged survival of pure *E. coli* cultures in a warm freshwater pond (> 25°C).

Generally speaking, in the presence of toxic heavy metals (Sn, Cd, Cu, Ni, Pb, Hg, Mn, Zn) the percent of surviving heterotrophic bacteria decreases with increase of metal concentration [39]. In natural conditions the existence of organic compounds, absorption of heavy metals (e.g. cadmium) on particles, formation of ligands and chelating factors may reduce their toxicity [3].

Biological interactions between allochthonous bacteria and natural microbiota are complex and poorly understood. Although competition with autochthonous bacteria for substrates and antibiosis, due to products of autochthonous organisms, may affect a reduction in the coliform population, stimulatory effects may also occur [11, 34].

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In recent years there has been considerable speculation on the role of parasites in controlling the bacterial population. Years ago Mitchel and Morris [36] reported that seawater, bactericidal to *E. coli,* contained large numbers of *Bdellovibrio bacteriovorus.* Also Guelin et. al., [25] suggested that bdellovibrios were important in the self-purification processes of polluted rivers. Recent studies have confirmed that *Bdellovibrio* invades Gram-negative bacteria, mainly *Pseudomonas* and enteric group, including *E. coli* [45].

Bacteriophages have also been considered as a biotic factor affecting removal of coliforms from natural environments. Numerous workers described a strong correlation between *E. coli* and coliphage numbers. However, several investigations have shown them to be ineffective [9, 22, **21].**

Contemporary studies have emphasized the important role of protozoa in controlling bacterial populations [31, 18, 44, 47, 22]. Gonzales et al., [22] using fluorescent labeled bacteria showed that protists, both flagellates and ciliates, ingested and digested enteric bacteria in both freshwater and marine samples. Size-selective grazing by protists has also been reported [2, 12, 37].

The aim of this paper was to study the survival of *Escherichia coli* in water of the man-made Zegrzynski Reservoir. The importance of natural microbiota for this process was investigated. This paper deals with the problem of the impact of autochthonic bacteria, coliphages, and flagellates predation on the survival of *E. coli.* The effect of nutritional conditions and temperature was also incidentally examined.

## Materials and Methods

Experiments were conducted in surface water samples from Zegrzynski Reservoir (November - January). The studied object is the man-made, overflow reservoir (30.3 $km^2$ area, water exchange 2-3 days) with **III** class of, according to sanitary state, water quality (OECD Environmental Performances Reviews, 1995).

All experiments were carried out in sterile 3-liter flasks with 2-liter subsamples. Subsamples were obtained by filtering fresh water through 0.2, 1.0 and 3.0 µm pore-size polycarbonate membranes (Nuclepore Corp.). The incubation of inoculated subsamples was done in the dark, at 20°C or at *in situ* temperature with stirring. To each variant of the experiment an equal density of inoculum of *Escherichia coli* was added. The contribution of *E. coli* to the total number of natural bacteria was adjusted to 30-70% and was dependent on the experiment.

*Escherichia coli* strain (ATTC 35218) used in all investigations came from a stock culture that had been stored under glycerol layer (20% v/v) at -70°C. Working inoculum was prepared by melting and dilution of stock

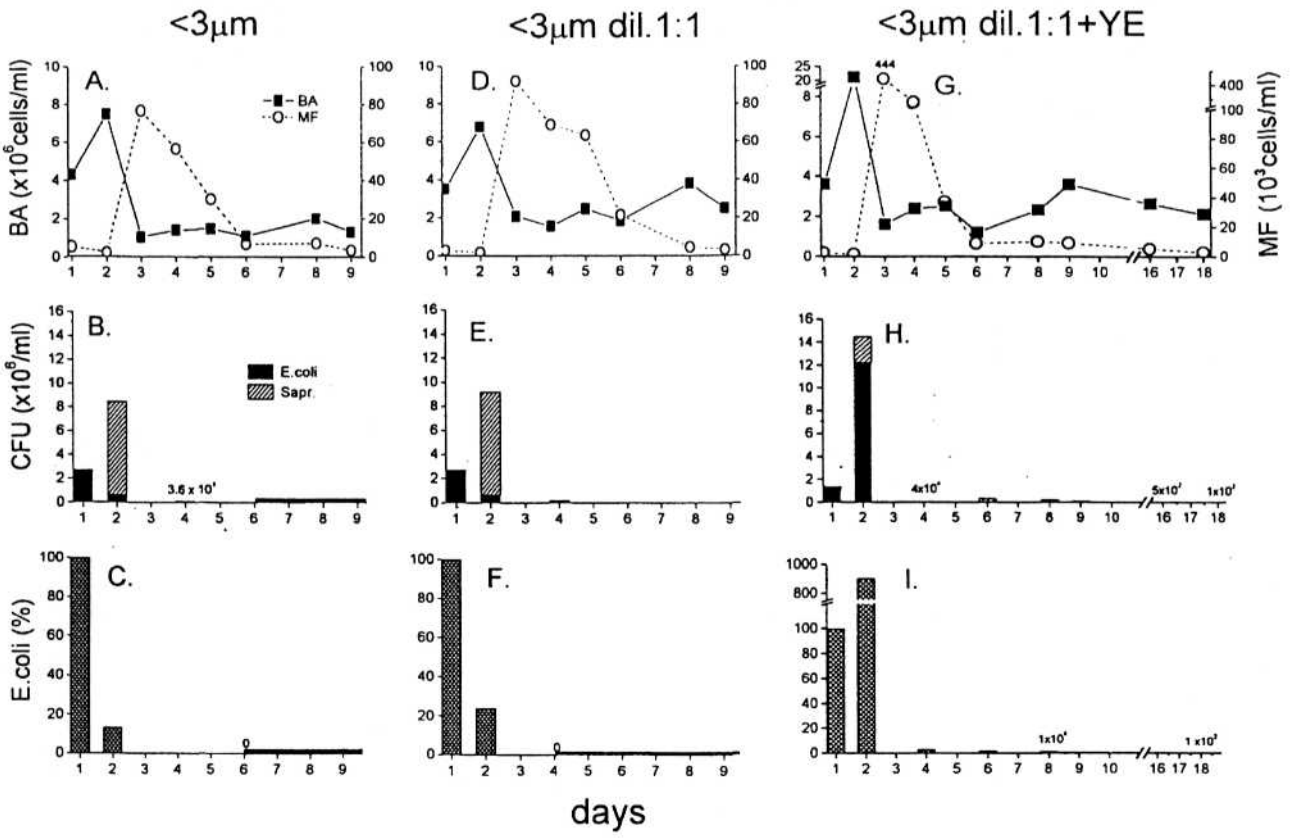


Fig. 1. Bacterial (BA) and microflagellate (MF) abundance, colony forming units (CFU) of *E. coli,* saprophytic bacteria and % of initial *E. coli* cells in water of Zegrzynski Reservoir (November 1997).
A-C: water filtrated through 0.2 µm + *E. coli;* D-F: water filtrated through 3 µm + *E. coli;* G-I: water filtrated through 3 µm + *E. coli* (on the 3-rd day water was enriched with 50 mg/l of yeast extract). — lack of viable *E. coli* cells.

culture in EB liquid medium, which contained: tryptone 1%, yeast extract 0.5%, NaCl 0.5%, dest. water 1 000 ml, pH 7.0. *Escherichia coli* was grown at 30°C for several hours. Cells from exponential phase of growth were harvested by centrifugation (4,000 x g for 10 min) and washed two-times with sterile saline solution (0.9% wt/vol) then centrifuged once again. The pellet was resuspended in a sterile saline solution. The total number of *E. coli* cells in that inoculum was counted microscopically. This final suspension was inoculated into the subsamples of the studied water to a final density of approximately $10^6$ cells/ml.

**Enumeration techniques:** *E. coli* and saprophytic bacteria enumerations were performed with spread plate methods. Triplicate samples (0.1 ml) were spread onto appropriate medium. The number of CFU (colony forming units) was counted.

*E. coli* was enumerated on a selective Endo medium (Bio Merieux: peptone 1%, di-potassium hydrogen phosphate 0.25%, lactose 1%, sodium sulfite, anhydrous 0.33%, fuchsine 0.03%, agar 1.25%). Plates were incubated at 37°C for 24 hours, then the number of CFU with red, greenish, metallic fuchsine sheen were counted.

Saprophytic bacteria were enumerated on nutrient agar (Bio Merieux, France). Plates were incubated at 20°C for two weeks, then CFU of saprophytic bacteria were counted.

Enumeration of somatic coliphages was done by plaque techniques. Coliphages are capable of infecting *E. coli* host strains. They produce visible plaques (clearance zones) in a confluent lawn of *E. coli.* Appropriate water sample was mixed with small volume of semi-solid nutrient medium. A culture of *E. coli* (working solution) was added and plated. After that, the plates were incubated at 37°C for 48 hours and then read for visible plaques.

Total number of bacterial cells (BA - bacterial abundance) and heterotrophic microflagellate abundance (MA) in the subsamples were determined by epifluorescence microscopy after DAPI (4'-6 diaminophenylindol) staining, following the procedure of Porter and Feig [41]. Final DAPI concentration was 5 µg/ml, staining time 10 min. Bacteria and microflagellate were counted in 30-40 fields of one preparation at lOOOx and 400x magnification, respectively.

## Results

The experiments on the survival of *Escherichia coli* cells in water of Zegrzyfiski Reservoir were carried out three times. The results of the first experiment, which was carried out in November, are presented in Fig. 1. Bacterial abundance in variant I (water < 0.2 µm supplied with *E. coli)* increased up to the end of experiment (Fig. 1A.). The growth rate (µ) of BA was 0.27 $day^{-1}$, and generation time (g) lasted 2.6 days. The number of £5-*cherichia coli* CFU decreased slowly from 1.3 x $10^6$/ml to 8 x 107ml, i.e. to 6% of the initial value (9-th day). After a month of investigation the residual number of *E. coli* - 25 CFU/ml (0.006% of initial value) was detected (data not presented).

The plot of bacterial abundance in variant II (water < 3 µm supplied with *E. coli)* was opposite to that observed in variant I (Fig. ID). Firstly BA raised from 5.4 to 7.6 x $10^6$ cells/ml, then started to decrease to 1 x $10^6$ cells/ml at the end of the experiment. Simultaneously with the decrease of total bacterial number a rapid increase of microflagellate abundance was noticed (µ estimated between 2-4 day was 1.38 $day^{-1}$, generation time = 0.5 day). The peak of microflagellate number appeared between 3rd and 4th day of investigation. At the same time *E. coli* CFU were completely reduced. No viable cells were found after the fourth day of the experiment.

Experimental variant **III** was the same as variant II, but after four days the culture was enriched with 50 mg/1 of yeast extract. An extremely high peak of total bacterial number (to 31 x $10^6$ cells/ml) was then observed (Fig. 1G). Higher abundance of microflagellate (50-60 x $10^3$ cells/ml comparing to that in variant **II** appeared from 3 to 6 day, next MA decreased to almost the same level (2-4 x $10^3$ cells/ml). After supplementing the water with yeast extract the CFU of saprophytic bacteria as well as *E. coli* significantly increased (Fig. 1H). Survival of *E. coli* was prolonged (3 days longer) and lasted up to six days.

Survival of *E. coli* cells was also examined in December (Fig. 2). We investigated variant I (water < 3 µm), and variants **II** and **III** (both contained water < 3 µm, diluted 1:1 with water filtered through 0.2 µm); however, variant **III** was additionally supplied with 15 mg yeast extract. In the first two days the bacterial abundance in variant I increased from 4.5 to 7.5 x $10^6$ cells/ml followed by its rapid decline (on the third day, Fig. 2A). The peak of total bacterial number was followed by intensive growth of microflagellate abundance to 80 x $10^3$ cells/ml. At the time of higher MA, *Escherichia coli* viable cells completely disappeared. Similarly, the number of saprophytic bacteria also significantly decreased (Fig. 2B). Dilution of sample with 0.2 µm filter-sterilized water (of reduced amount of autochtonic bacteria as well as micrograzers) did not change the tendency of fluctuation of bacteria and microflagellate abundance (Fig. 2D). However, we detected relatively higher numbers of total bacteria. Micrograzers peak lasted longer and was higher than that in the undiluted variant. *Escherichia coli* cells disappeared faster and were not present in the culture after three days.

Supplementing the water with yeast extract resulted in large multiplication of bacteria. Bacterial abundance as well as CFU of *E. coli* and saprophytic bacteria were several fold higher than in also diluted variant **II.** Consequently, microflagellate abundance was extremely high (444 x $10^3$ cells/ml). Ninety seven percent of initial *E. coli* number was reduced within the first four days but residual living cells (~ $10^2$ CFU, i.e. 0.0007% of an initial cell concentration) were observed at the very end of the experiment (to 18 day).

The third experiment was carried out in the winter January. It was conducted at *in situ* temperature (4°C). The results are shown in Fig. 3. Four versions of the experiment were performed as follows: I - autoclaved water filtered through 0.2 µm, **II** - non autoclaved water filtered through 0.2 µm, III - water filtered through 1 µm, and IV - water filtered through 3 µm.

In autoclaved samples of water we noted an almost three-fold increase of total bacterial number within three days of culture and then we did not observe any significant fluctuation of BA (Fig. 3A). Simultaneously the number of *E. coli* was reduced to 3.6 x $10^4$ CFU/ml (1.4% of initial value). On the fifth day of the experiment, only 4 x $10^2$ CFU/ml (0.0004% of initial value) were detected and after that *E. coli* disappeared completely (Fig. 3B, C).

The same version of the experiment carried out in non-autoclaved water (Fig. 3D-F) showed a bit higher, than that observed in variant I, growth of bacterial abundance (6.4 x $10^6$ cells/ml). After that it started constantly to diminish. At the time of microscopic investigation small microflagellate individuals appeared. Survival of *E. coli* extended to eleven days was noted.

The results of variants III and IV of the experiment differed negligibly (Fig. 3G-L). The total number of bacteria in both of them displayed two maxima 13 and 10 x $10^6$ cells/ml (Fig 3G, J.). In water samples filtered through 3 μ^i we observed faster appearance and higher peak, compared to that in water filtered through 1 μm, of microflagellate abundance. The peaks appeared on the fifth and seventh day, respectively. In both cases relatively fast and analogous *E. coli* CFU reduction was detected (33.0, 12.0, 2.0, and 0.0007% of initial value). After nine days of the experiment only residual viable *E. coli* cells (50-100/ml) were observed.

## Discussion

Our experiments showed that the number of living cells of *E. coli* decreased rapidly after inoculation into natural water. On the second day of each experiment the number of living cells that were cultivable by standard Endo medium varied between 20-40% of their initial value. There are several explanations of this fact. Enteric bacteria after their transfer from optimal environments (medium or organism) to natural water may stay viable, die or evolve toward a non-cultivable stage. The term viable has been used to refer to bacterial counts in standard culture media (synonymous with cultivable or CFU).

The significant decrease of *E. coli* CFU at the beginning of the experiment may be a result of the death of bacterial cells caused by osmotic and temperature stress as well as centrifugation-resuspension procedure used to prepare the bacterial culture inoculum. Another explanation is that part of the introduced enteric bacteria became uncultivable. Several authors suggested that uncultivable cells are still viable but cannot be cultured by standard microbiological methods [7, 38]. Therefore, it is possible that in our studies a part of them was counted by the DAPI staining procedure but was not recognized as *Escherichia coli* colony forming units. Those cells are called after Roszak and Colwell [43] *somnicells.* Moreover, Grimes et al., [24] underline the importance of this

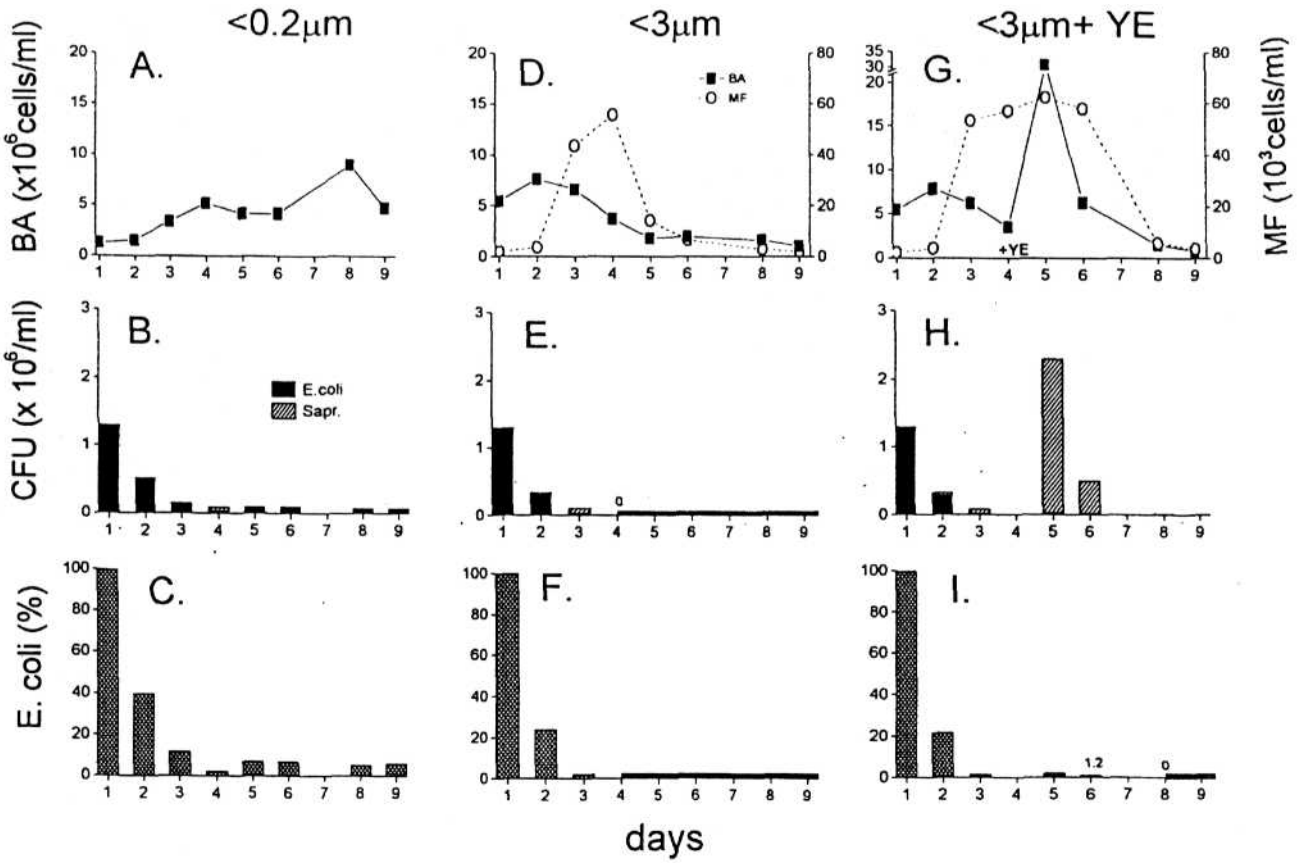


Fig. 2. Bacterial (BA) and microflagellate (MF) abundance, colony forming units (CFU) of *E. coli* and saprophytic bacteria and % of initial *E. coli* cells (December 1997).

A-C: water filtrated through 3 μm + *E.* coli; D-F: water filtrated through 3 μ^i (diluted 1:1 with water filtered through 0.2 μ^i) + *E. coli;* G-I: water filtrated 3 μm (diluted 1:1 with water filtered through 0.2 μm and enriched with 15 mg/1 of yeast extract) + *E. coli* . — lack of viable *E. coli* cells.

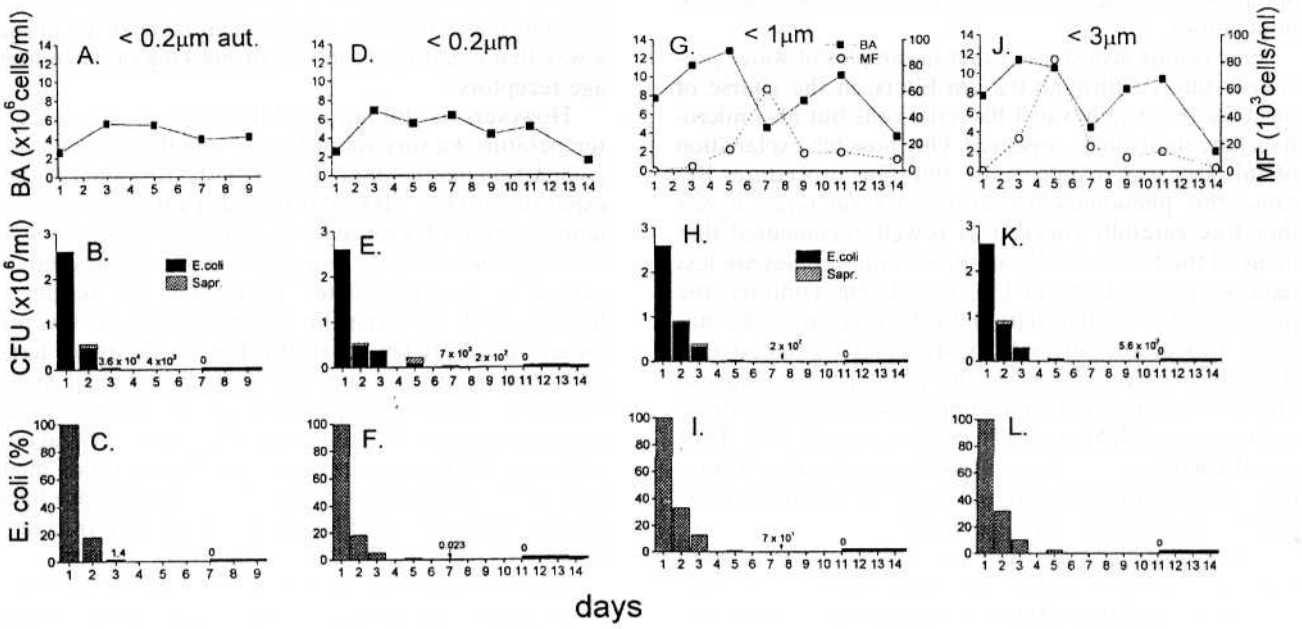


Fig. 3. Bacterial (BA) and microflagellate (MF) abundance, colony forming units (CFU) of *E. coli* and saprophytic bacteria and % of initial *E. coli* cells in water (January 1998). A-C: water filtered through 0.2 µm (autoclaved) + *E. coli;* D-F: water filtered through 0.2 µm + *E. coli;* G-I: water filtered through 1 µm + *E. coli;* and J-L: water filtered through 3 µm + *E. coli.* — lack of viable *E. coli* cells.

(usually unknown) fraction of enteric bacteria because of the fact that it can maintain its infectiveness, which constitutes a health risk.

The experiments have also demonstrated the close coupling between *E. coli* survival time and the number of heterotrophic flagellates. During the first experiment carried out in late autumn (November) *E. coli* cells survived much longer in sample without microflagellates (Fig. 1A-C). We noted that the time required for 50% reduction of the initial *E. coli* number lasted under two days, however, after a month of the experiment residual *E. coli* cells were also observed. High microflagellate abundance (60 x $10^3$ cells/ml) was observed between 3-6 days and was dependent upon experimental conditions (Fig. 1D-I). Supplementation of water with 50 mg/1 yeast extract prolonged both the survival time of *E. coli* and microflagellate abundance peak. At the same time the total number of bacteria (BA) and their saprophytic compartment raised rapidly. Microflagellate abundance was comparable to that observed in water without yeast extract supplementation but lasted two days longer. This may suggest that flagellate grazing on *E. coli* is more effective than on smaller autochtonic bacterial cells.

The next two experiments carried out in winter samples of water from Zegrzynski Reservoir also focused on the coupling between survival of *E. coli,* microflagellate grazing and the influence of supplementation of water with organic matter. Both of them confirmed the strong dependence of *E. coli* survival time on microflagellate abundance. We noted that rapid *Escherichia coli* CFU as well as total bacterial number (Fig. 2, Fig. 3) decrease followed by fast multiplication of grazers. That close coupling caused that *E. coli* disappeared completely after five days in water filtered through 3 µm pore size filters (Fig. 2A-C) and even after three days in diluted samples (Fig. 2D-F.). Dilution of microflagellate populations resulted in final higher, compared to that without dilution, abundance of micrograzers and significant prolonging of their numbers. It is possible that diluted population of grazers had better feeding conditions (twice as many *E. coli* cells), especially while nutritive competition was restricted.

Our results are in agreement with numerous observations that the initial density of micrograzers is an important factor controlling the bacterial population in aquatic environments [51, 29]. Size fractionation of microflagellate compartment through 1 µm and 3 µm pore size filters (Fig. 3G-J) showed significantly higher and much faster peak of MA in samples filtered through larger pore size filters. Microscopic examinations displayed that microflagellate compartment consisted of generally the same shape, unicellular, one-flagella individuals. The only changes we observed during all experiments were the size (0.5-3 µm) and number of microflagellates. We noted that *E. coli* cells which entered the natural water started to shrink until their dimensions were more susceptible to being grazed. Such an observation is in agreement with Stevenson's [50] and Baker's et al., [5] data, they noted a significant (15-300 fold) decrease in cell volume of another pathogenic bacterium *Vibrio cholerae* at initial exposure to organic compound-free conditions. Our experiments also confirmed the data of Anderson et al. [2] and Gonzalez et al. [22, 23] on predator-prey size preferences. Phagotrophic microflagellates selectively fed on larger bacterial cells. Therefore *Escherichia coli* cells seemed to be more prone to grazing pressure than smaller autochtonic bacteria. The kinetics of flagellate grazing on *E. coli* were carefully considered by Menon et al. [35]. The authors after several mathematical assumptions suggest that the relative grazing rate can be predicted to be

about twice the grazing rate on autochthonous bacterial populations.

Our results also showed that in samples of water previously filtered through 0.2 μm filters, in the course of incubation not only small bacterial cells but also microflagellate individuals appeared. One possible explanation of this fact was inappropriate filtration procedure. Because this phenomenon occurred several times it was therefore carefully checked. It is well documented that many of the bacterial cells in the natural samples are less than 0.2 μm in diameter [33, 43]. On the contrary, the presence of microflagellate individuals seems to be unclear. Rassulzadegan and Sheldon [42] estimated that flagellates twice as large as the pore size can squeeze through the filter. Similar assumptions were also made by Fuhrman and McManus [19] and Cynar et al., [14]. They found bacteriovores even in 0.6-0.4 μm filtrates. Therefore interpretation of data obtained from long-term experiments should be done with care.

Another interesting question is: how accompanying bacteria present in water can affect the survival of *E. colli* Biological interactions between allochthonous bacteria (including *E. coli)* and natural microbiota are complex and poorly understood. Based on our results of survival of *E. coli* in autoclaved and in unaltered water we can conclude that in samples with living autochthonic microflora *E. coli* survived four days longer (Fig. 3A-F). This may suggest some kind of stimulation (proto-cooperation) relationship. This is with agreement with the data of Anderson et al. [1].

Our studies confirmed that *E. coli* and also other heterotrophic bacteria in the studied environment are limited by organic substrates. Enrichment of water samples with labile organic matter (yeast extract) resulted in rapid cell multiplication and prolonging of *E. coli* survival time. A similar conclusion appeared in variants with dilution of microflora (Fig. 2A-F). It is possible that reduced population not only of *E. coli,* but also other microheterotrophs might have used a bigger substrate pool and found better nutritional condition. Consequently a more numerous bacterial (prey) compartment was followed by higher microflagellate (predator) abundance.

Early studies on bacterial survival in estuarine and seawater suggested that bacteriophages may be responsible for the decline of enteric bacteria as well as native bacteria [30, 46]. We were surprised that our examinations did not show any significant multiplication of *E. coli* phages during the experiments. Average coliphage number during every experiment was negligible, stable and was in the range of 0-2 plaque forming units/ml. We expected higher virus-mediated *E. coli* mortality. Studies of Koc [27] and Kulianin [28], that were simultaneously carried out during our experiments did not exhibit any significant differences with *E. coli* (native or strain ATTC 35218) bacteriophage susceptibility, which might have explained the low phage abundance in every experiment. Therefore we agree with the suggestion of Enzinger and Cooper [16] that phages require actively growing hosts. This requirement is rarely met when coliforms are introduced into aquatic environments. Another possible explanation of the negligible impact of phages on *E. coli* mortality in natural waters is modification of the sensitivity of host cells that are grown in low nutrient environment. Chai [10] suggested that the cell envelope composition of these microorganisms might be changed in such a way that could result in loss or masking of bacteriophage receptors.

However, we did not carefully study the influence of temperature on survival of *E. coli,* and therefore a rough comparison may be done. Based on the first and the third experiments (Fig. 1B,C and Fig. 3E,F) it seems that low temperature (4ºC) caused a considerable decrease of the survival time of *E. coli.* Our results from samples without numerous microflagellates confirmed the results of Soracco et al., [49] that the survival of bacteria was prolonged at higher temperatures. But on the other hand, according to other data [34] grazing pressure of microflagellates at lower temperatures is less effective, which consequently may be a reason for the longer survival time of *E. coli.* Generally speaking, two factors which might account for differences in experimental results are interactions with the autochthonous microbiota (including predation) and also environmental stress.

In conclusion, our experiments showed that exposure to an aquatic environment and microflagellate grazing were the major factors responsible for the disappearance of *Escherichia coli* in the studied ecosystem. We noted that the time required for 50% reduction of initial *E. coli* numbers was less than two days. Size-selective grazing of flagellates on bacteria was also observed. Relatively large (0.5-2 μm) *Escherichia coli* cells were much more prone to microflagellate prey than small autochthonic bacteria. The experiments demonstrated that the possible effect of bacteriophages on the survival time of *E. coli* was insignificant. The results confirmed the assumption that *E. coli,* as well as a part of other heterotrophic bacteria in aquatic ecosystems, were limited by organic substrates. The stimulatory effect of native microflora on viability and survival time of *E. coli* was observed. The impact of temperature on the survival process was not univocal. We suggest that it also depends on other environmental factors.

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