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*Short research contribution*

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## PHYLOGENETIC DIVERSITY AND ABUNDANCE OF BACTERIA FROM SURFACE MICROLAYER AND SUBSURFACE WATER IN EUTROPHIC LAKE

**ABSTRACT:** The bacteria from different phylogenetic groups were studied in surface microlayer (SM, up to 100  $\mu\text{m}$ ) versus subsurface water (SW – 20 cm) in eutrophic lake from spring to autumn of 2007. Abundance of bacteria was determined using a combination of direct counting of 4',6-diamidino-2-phenylindole and the phylogenetic diversity was determined in fluorescence *in situ* hybridization (FISH) method with group-specific, fluorescently labeled oligonucleotide probes. The numbers of DAPI bacteria varied between 4.75 and fluorescence *in situ* hybridization (FISH) demonstrated that Eubacteria constituted the majority of the whole bacterial population and their percentage share ranged from 59 to 75%. Abundances of alpha- beta-Proteobacteria and Cytophaga-Flavobacteria groups varied across seasons, layers, and lacustrine zones. The lowest number of alpha-Proteobacteria group bacteria was observed in spring (SM –  $0.2 \times 10^6$ , SW –  $0.16 \times 10^6$  cells  $\text{cm}^{-3}$ ), whereas the highest in autumn (SM –  $0.62 \times 10^6$ , SW –  $1.6 \times 10^6$  cells  $\text{cm}^{-3}$ ). The percentage share of these groups of bacteria in the Eubacteria domain was lower in spring (20–50%) than in summer and autumn (from 65 to over 80%). No fixed difference between the composition of SM and SW bacteria was noticed. Seasonally occurred changes are similar in both layers.

**KEY WORDS:** surface microlayer, bacterial communities, biodiversity of bacteria, FISH

The surface microlayer (SM) constitutes the external very thin (around 100–200 mm) layer (depending on the used device) at the air-water interphase. SM forms a peculiar chemical and physical environment, which is different from subsurface water (SW) (Falkowska 2001, Kostrzewska-Szlakowska 2003, Hillbricht-Ilkowska and Kostrzewska-Szlakowska 2004). Due to effects of such phenomena as absorption, diffusion, flotation, or atmospheric precipitation, SM accumulates a number of organic substances, primarily lipids, proteins, as well as polysaccharides and their derivatives, which form a surface film, also called biofilm (Joint and Morris 1982, Plasquellec *et al.* 1991, Walczak and Donderski 2005); this structure separates the aquatic environment from the air.

The SM also contains a higher number of bacteria, referred to as bacterioneuston. In comparison to subsurface water, the SM is considered as a highly unstable environment. This fact is associated, primarily, with sudden changes in temperature or solar radiation reaching this layer. Bacterioneuston inhabiting the SM are more exposed to stressful ecological factors than microorganisms living deeper in the water column. Potentially

harmful factors, such as, intensive solar radiation, temperature, and the presence of toxic substances, play an important role in struggle for survival. All these factors are selective and exert a considerable influence on the microbiological composition of this environment.

In order to understand the role of bacterioplankton in this environment, it is necessary not only to determine its abundance, but also its affiliation with specific systematic groups (Cottrell and Kirchman 2000). Evaluation of biodiversity, abundance, and activity of microorganisms occurring in water lays the foundation for microbiological research of aquatic environment (Amann *et al.* 1990). Traditional analysis of bacterioplankton communities has often relied on bulk measurements averaged across the entire assemblage – the so-called “black box approach” (Pearce 2003). This approach has been necessary because the majority of bacterioplankton have very limited morphological details, preventing the direct visual identification possible with other planktonic groups (Amann *et al.* 1995, Glöckner *et al.* 1996). In the past, our understanding of bacterial diversity was limited by a necessity to observe and investigate pure bacterial cultures, while <1% of bacteria occurring in nature can be cultured with available methods (Cottrell and Kirchman 2000).

Fluorescent in situ hybridization (FISH) based on the application of 16S rRNA probes is an extremely useful technique, which facilitates examination of structures of bacterial communities. In situ hybridization with oligonucleotide probes is a widely used technique that enables a direct analysis of microbial population structure in both natural environment and engineered systems (Anton *et al.* 1999). This technique and its application in microbial ecology were extensively described by Amann *et al.* (1995). The FISH technique has been used in numerous ecological studies with a purpose to determine biodiversity of microbial populations of inland waters (Alfreider *et al.* 1996, Glöckner *et al.* 1996, Pearce 2003, Weiss *et al.* 1996). Additionally, this technique provides a reliable measure of activity or, at least, potential activity of cells, because ribosomes undergo hybridization, and their amount depends on metabolic activity of a cell.

When undertaking research into biodiversity of bacteria inhabiting SM the authors assumed that composition of bacteria population of such unique environment (SM) would differ from bacteria of SW. Moreover, it was expected that bacteria populations of SM and SW would, more or less dynamically, undergo changes in terms of seasons as well as spatial distribution.

The water was collected from Lake Brzeżno (53°57,5'N, 17°48,6'E) located in the forested area, “Bory Tucholskie” National Park (north Poland). The lake is located 139.8 m above sea level, and covers 71.6 ha with a maximum depth of 9.7 m. The lake is eutrophic with total organic carbon content (TOC) ranging from 5 to 15 mg l<sup>-1</sup>, average total phosphorus – 0.083 mg dm<sup>-3</sup> and average total nitrogen – 1.28 mg dm<sup>-3</sup>.

Water samples were collected in three seasons: spring (May), summer (July) and autumn (October) of 2007, from two stations located in the pelagic and two in the littoral zone. Water of the surface microlayer (SM) was collected according to the Garrett (1965) technique, using a plexiglas plate that collects a 150 mm water layer.

The subsurface water (SW) was collected from a depth of 20 cm using a sterile, glass pipette and an automatic pump Pippet-boy (De Ville). The samples of SM and SW were taken in ten replications.

The physico-chemical parameters (pH, temperature, conductivity, light and UV radiation) of surface and subsurface water were measured simultaneously with collection of the samples used for microbiological analyses (Table 1).

Determination of total bacterioplankton density and in situ hybridization was following. For each replicate sample, 20 ml of each lake-water sample was filtered through a white polycarbonate 0.2 mm screen membrane filter (Millipore). Cells were fixed with 2 ml of 4% paraformaldehyde for 30 min. A gentle vacuum was then applied and cells were rinsed in 15 ml distilled water. Filters were removed from the filtration apparatus, air dried, placed on a glass microscope slide, and stored at –20°C. All preservation and hybridization conditions were selected to minimize impact on the integrity and characteristics of cells.

The following oligonucleotide probes were used (numbering in parentheses according to Brosius *et al.* (1981): (i) EUB 338, complementary to a region of the 16S rRNA (338–355) specific for the domain Bacteria (5'-GCTGCCTCCCGTAGGAGT-3'); (ii) ALF1b, complementary to a region of the 16S rRNA (19–35) conserved in the alpha-subclass of Proteobacteria and some other Proteobacteria (5'-CGTTCGYTCTGAGC-CAG-3'); (iii) BET 42a, complementary to a region of the 23S rRNA (1027–1043) specific for the beta-subclass of Proteobacteria (5'-GCCTTCCCCTTCGTTT-3'); (iv) CF 319a, complementary to a region of the 16S rRNA (319–336) conserved in the Cytophaga-Flavobacteria (CF) group (5'-TG-GTCCGTGTCTCAGTAC-3'); (v) NON338, complementary to a region of the 16S rRNA (338–355) nonsense probe – negative control (5'-ACTCCTACGGGAGGCAGC-3'). Each probe was covalently linked at the 5'-end to a single fluorescent dye molecule (CY3 or Fluorescein).

All in situ hybridizations were performed according to the procedure described by A man n *et al.* (1995) in 20 µl of hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% SDS and variable formamide concentrations with a 2 µl probe solution. Hybridizations were carried out at 46°C for 2 h in an equilibrated sealed moisture chamber. The final probe concentration was approximately 50 ng µl<sup>-1</sup>. Subsequently, a stringent washing

step was performed at 48°C for 20 min in 50 ml of prewarmed washing solution (variable NaCl concentration as described in A man n *et al.* (1995), 20 mM Tris-HCl (pH 7.4), 0.01% SDS). The washing buffer was removed by rinsing the slides with doubledistilled H<sub>2</sub>O. Samples were simultaneously stained with 4,6-diamidino-2-phenylindole (DAPI) (Porter *et al.* 1980). The slides were then rinsed briefly with double-distilled H<sub>2</sub>O and allowed to air-dry prior to microscopic observation. The filters were placed on slides, mounted with the 4:1 mix of Citifluor (Citifluor Products, Canterbury, Kent, UK) and Vectra Shield (Vector Laboratories, Inc.) to minimize bleaching, and viewed at 1250, under oil immersion, with a Nikon Eclipse 400 epifluorescence microscope equipped with a 100-W mercury lamp and adequate photo-filters.

Enumeration results were always corrected by subtracting signals observed with the nonsense probe NON338. In all cases, cell numbers were obtained from a minimum of 10 fields of view per well and from six wells per sample. The total number of cells present was estimated by both the DAPI count and the number of cells that hybridized with probe EUB338.

Statistical analyses were done using program STATISTICA 6.0. Analysis of Variance (ANOVA) was the primary statistical method used in calculations.

Variations in temperature, conductivity, visible light, and UV were significant for

Table 1. Physico-chemical parameters of investigated surface (SM, 100 mm) and subsurface water (SW, 20 cm).

	Temperature (°C)		pH		Conductivity (µS cm)		Visible light (klx)		UVB (µW cm <sup>-2</sup> )	
	SM	SW	SM	SW	SM	SW	SM	SW	SM	SW
Spring:										
Littoral	17.1	17.7	8.00	8.00	379	379	13.10	8.33	4.42	2.05
Pelagic	17.6	17.7	8.03	8.03	379	377	15.83	11.83	5.89	2.97
Summer:										
Littoral	21.2	20.4	8.07	8.03	348	350	64.10	29.47	10.90	4.32
Pelagic	20.9	20.7	8.17	8.20	350	346	91.00	80.13	16.57	6.45
Autumn:										
Littoral	16.8	16.7	8.03	8.00	301	297	6.67	6.47	1.04	0.35
Pelagic	16.6	16.7	8.17	8.17	302	298	29.13	13.03	2.33	0.83

Table 2. Total numbers of bacteria (cells  $\text{cm}^{-3}$  as DAPI  $\pm$  SD) and EUB as % of DAPI in surface layer (SM, 100  $\mu\text{m}$ ) and subsurface layer (SW, 20 cm).

Seasons	Surface microlayer		Subsurface water	
	Littoral	Pelagial	Littoral	Pelagial
Spring	13.38 $\pm$ 1.72	10.42 $\pm$ 0.91	9.05 $\pm$ 0.56	7.14 $\pm$ 0.56
	68%	67%	71%	75%
Summer	5.77 $\pm$ 0.92	9.16 $\pm$ 0.47	4.81 $\pm$ 0.64	8.11 $\pm$ 0.71
	71%	59%	69%	67%
Autumn	5.96 $\pm$ 0.28	6.54 $\pm$ 0.48	4.75 $\pm$ 0.47	5.84 $\pm$ 0.17
	63%	61%	65%	69%

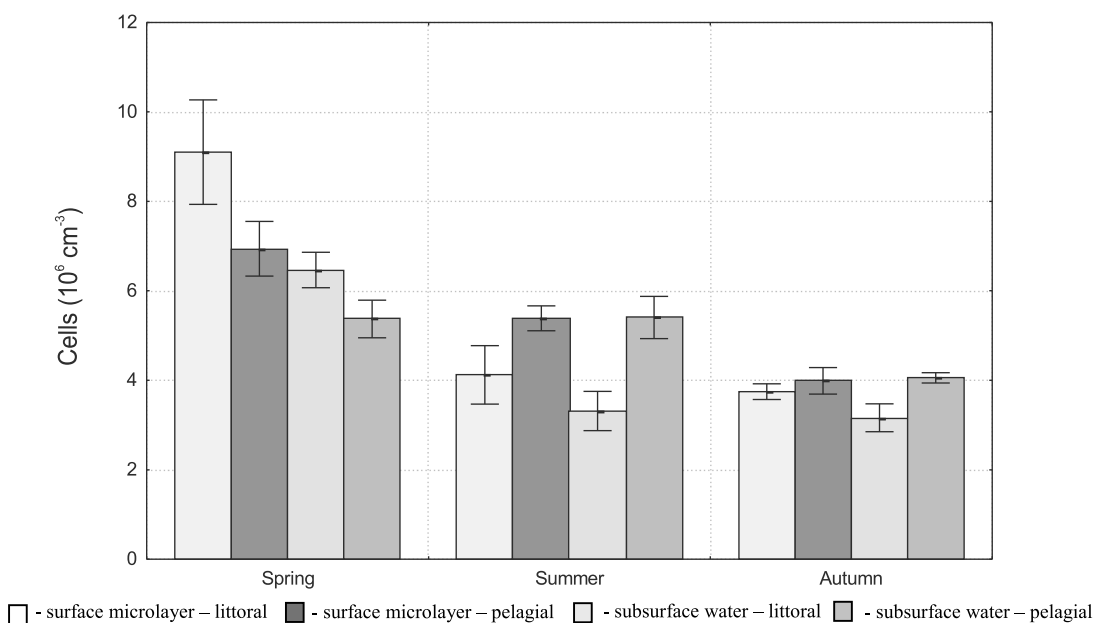


Fig. 1. Numbers of Eubacteria in investigated samples of water.

specific seasons ( $P < 0.01$ , Table 1). The lowest temperature (16.6°C) of the examined SM water was recorded in autumn, which was most probably a result of cooling by wind. The highest temperature (21.2°C) was observed in summer – water was heated up by intense solar radiation. Temperature differences between the SM and SW layers as well as differences between lacustrine zones were not significant. Electrolytic conductivity was highest in spring (377–379  $\text{mS cm}^{-1}$ ), and lowest, in autumn (297–302  $\text{mS cm}^{-1}$ ). No significant differences in conductivity were observed between examined layers and lake zones. Values of pH in all testing stations were very similar (8.00–8.20) across the entire research period. Differences in intensity of visible light and UV in examined water layers in a given season were significant ( $P < 0.01$ ) (Table 1).

The total number of DAPI bacteria in water samples was highest in spring, and lowest in autumn (Table 2). Variance analysis demonstrated that differences in abundances of bacteria in individual samples were significant ( $P < 0.05$ ) across seasons, but were not significant between water layers (SM and SW) and lacustrine zones (littoral and pelagic) (Table 2).

The abundance of Eubacteria in all tested water samples generally corresponded to the total number of bacteria. The highest number of Eubacteria was observed in spring, and the lowest, in autumn (Fig. 1). Differences in Eubacteria abundances in individual seasons were significant ( $P < 0.05$ ). No significant differences in the number of Eubacteria were found for different water layers and lacustrine zones. Eubacteria constituted the majority

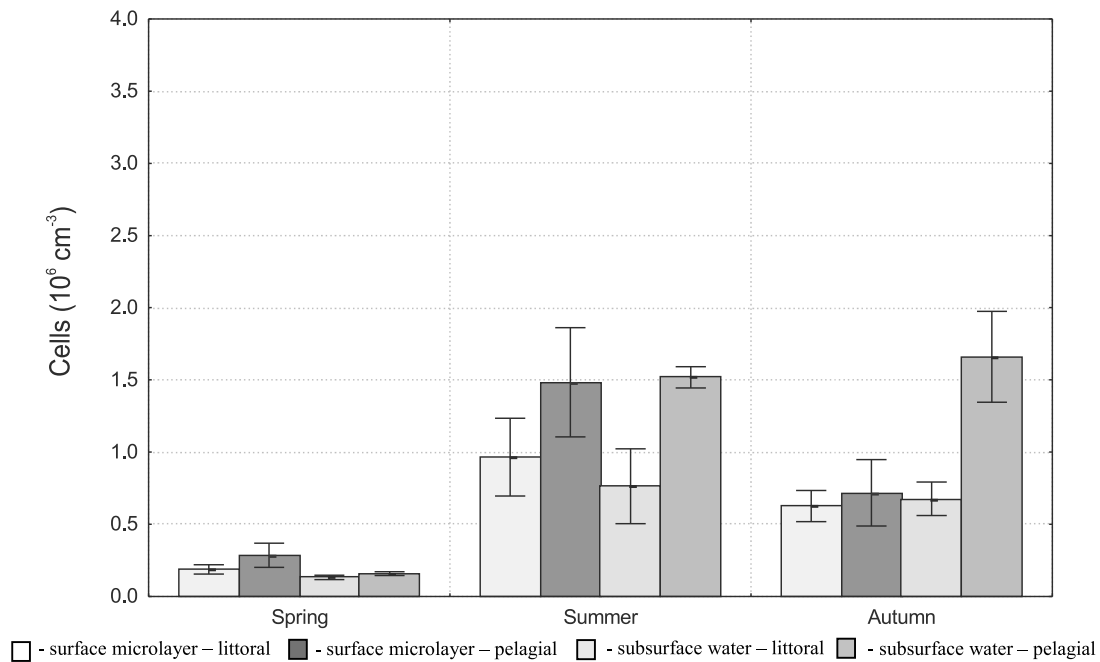


Fig. 2. Numbers of alpha-Proteobacteria in investigated samples of water.

of the whole bacterial population (Table 2) and their percentage share ranged from 59% (summer, pelagic SM) to 75% (spring, pelagic, SW).

The alpha-Proteobacteria group reached its maximum in summer and was relatively scarce in spring (Fig. 2). In the SM water, in littoral and pelagic zones, fluctuations in abundance of bacteria from this group were significant ( $P < 0.05$ ) across seasons, but were not significant between layers and zones.

In the SM water, beta-Proteobacteria were most abundant in spring, while in the subsurface water their numbers peaked in summer and autumn (Fig. 3). Recorded differences in their abundances in the SM in the littoral zone were significant ( $P < 0.05$ ). Furthermore, significant differences in abundances of beta-Proteobacteria were observed between examined layers (SM and SW) in spring in the littoral zone.

In general, bacteria from the Cytophaga-Flavobacterium group were the least common and their abundances varied only slightly across seasons (Fig. 4). The only significant difference in abundance of these bacteria was observed in the SM water in spring between the littoral and pelagic zones ( $P < 0.05$ ).

Figure 5 presents a percentage share of specific phylogenetic groups of bacteria in

examined water samples. The percentage share of alpha- and beta-Proteobacteria and Cytophaga-Flavobacteria in the Eubacteria domain was lowest in spring. In the littoral zone, the three examined phylogenetic groups constituted nearly 50% of Eubacteria in the SM water, and slightly over 20%, in the SW water. In summer and autumn, the percentage share of these phylogenetic groups in the Eubacteria group was significantly higher (from 65 to over 80%). It is also evident from the presented graph that the share of alpha-Proteobacteria in bacterial population was subject to greatest variations across seasons.

The results demonstrate that the total number of bacteria, as well as abundances of Eubacteria and certain phylogenetic groups were subject to seasonal fluctuations. The total numbers of bacteria (DAPI) in the SM water was at the highest level in the spring, and differences in comparison to the numbers recorded in summer and autumn were significant. In the subsurface (SW) water, the total number of bacteria was also highest in the spring, but the differences compared to the other seasons were significantly smaller. An increase in bacterial abundances in lacustrine water in spring is usually caused by intense development of phytoplankton (Zwisler *et al.* 2003); however, such a substantial increase

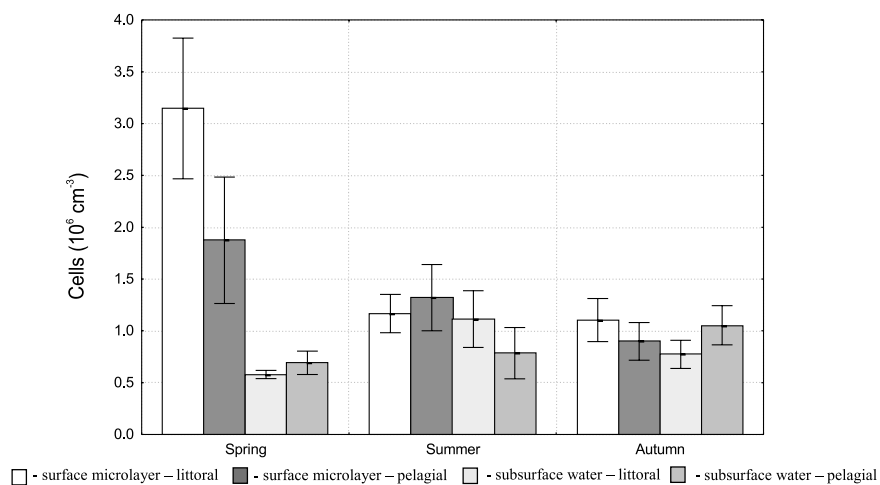


Fig. 3. Numbers of beta-Proteobacteria in investigated samples of water.

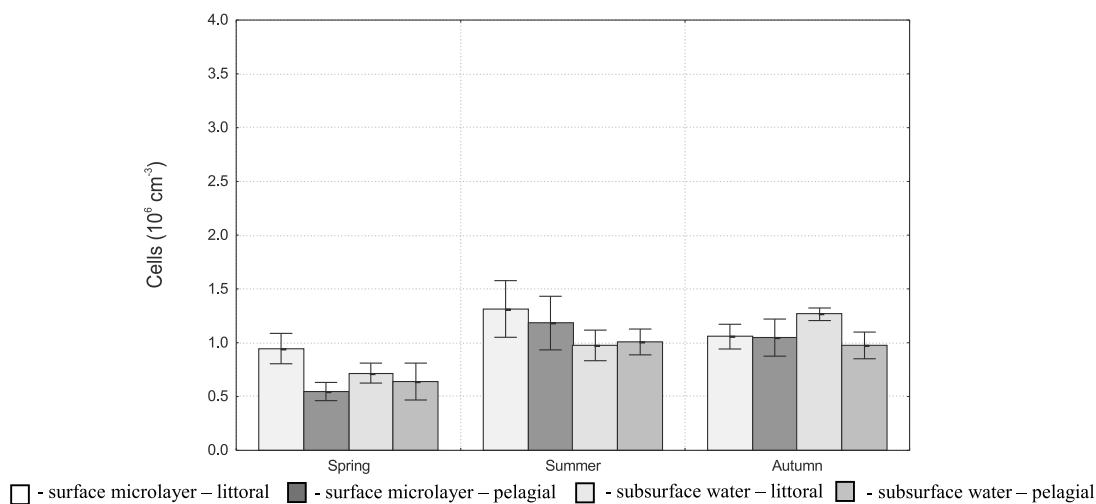


Fig. 4. Numbers of Cytophaga-Flavobacterium in investigated samples of water.

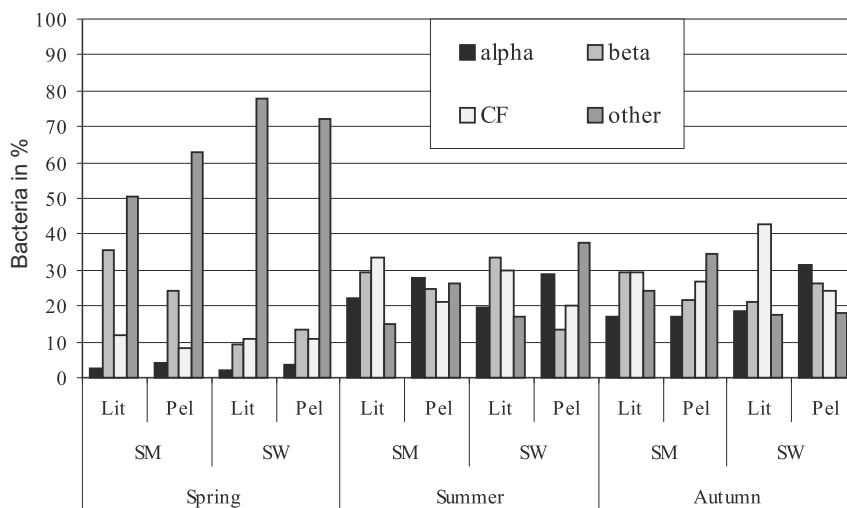


Fig. 5. Percentage abundance of alpha-, beta-Proteobacteria and Cytophaga-Flavobacterium in Eubacteria domain. SM – surface microlayer, about 100 mm, SW – subsurface water – 20 cm in littoral (lit) and pelagial (pel).

in bacterial numbers in the SM also suggests a considerable impact of an influx of organic matter from the atmosphere, for example, a fall of pollen inhabited by bacteria. Another argument suggesting that the atmospheric fall could have a significant influence on the number of bacteria in the SM water was the fact that bacteria were the most abundant in the littoral zone, that is, in the direct proximity of terrestrial vegetation.

Observed fluctuations in the total number of bacteria coincided with abundances of Eubacteria (Table 2, Fig. 1), which was a result of a similar proportion of hybridized cells with EUB 338 probe. In this study, Eubacteria constituted between 58 and 75% of the total number of bacteria. Available data demonstrate that from 30 to 80% of the total number of bacteria undergo hybridization (Ammann 1995). Differences are caused, inter alia, by relationships between the ratios of viable cells to dead cells, and active cells, containing active ribosomes, to inactive cells. The results describing a proportion of Eubacteria in the total number of bacteria coincided with data obtained by Pearce (2003), who reported the percentage of hybridization ranging from 46 to 70%. Similar results, obtained by many other researchers (Pernthaler *et al.* 1998, Glöckner *et al.* 1999, Šimek *et al.* 2001), demonstrate that Eubacteria a significant proportion of the total number of bacteria.

Fluctuations in Eubacterial abundance across seasons corresponded relatively well to variations in numbers of microbes from the beta-Proteobacteria group. The abundance of microbes from this bacterial group also peaked in spring, and differences in their numbers in specific water layers roughly corresponded to fluctuations of Eubacterial abundances. Similar results, which signify a correlation between the total number of bacteria and abundances of the beta-Proteobacteria group in inland waters, have been recorded previously (Zwisler *et al.* 2003, Schweizer *et al.* 2001, Brümmer *et al.* 2000, Šimek *et al.* 1999). In spite of the substantial share of this bacterial group in the whole bacterial population, the information regarding the genus composition of this group is still scarce. According to many studies, diversity of beta-Proteobacteria is low, and it is often the case that two or three gen-

era constitute a clear majority (>50%) among all beta-bacteria (Pernthaler *et al.* 1998, Höfle *et al.* 1999). It is also evident from this study that the percentage of beta-Proteobacteria in the whole bacterial community was relatively constant in test samples across the entire research period, regardless of the season (23–29%). Their percentage was significantly smaller only in the spring in the SW (9–11%) and in the summer in pelagic zone also in SW (13%) (Fig. 5).

Variation in abundances of alpha-Proteobacteria was entirely unrelated and independent of changes in Eubacteria abundances. In spring, when both the total number of bacteria and the number of Eubacteria reached the highest levels, abundances of alpha-Proteobacteria were minimal. But in the summer, the number of alpha-Proteobacteria significantly increased and remained at a similar level until autumn (Fig. 2). Seasonal fluctuations in abundances of bacteria from this group were significant in the SM water in both littoral and pelagic zones; however, no significant differences were found between the SM and SW layers and between pelagic and littoral zones. Results of previous studies indicate that alpha-Proteobacteria constitute one of the most numerous bacterial groups in inland waters (Araya *et al.* 2003; Manz *et al.* 1999; Olapade and Leff 2004); however, this study demonstrated that in the spring, the percentage of alpha-Proteobacteria in the examined lake did not exceed 5% of Eubacteria. Similar data was presented by Zwisler *et al.* (2003), who indicated that in certain samples collected from lake, alpha-Proteobacteria constituted *ca.* 10% of Eubacteria and only 2.5–4.6% of cells stained with DAPI. In contrast, the results obtained in this study demonstrated that in summer and autumn, percentage of alpha-Proteobacteria in the Eubacteria domain was indeed significant, and ranged from 16 to 29% in the SM water, and from 18 to 32% in SW. It is noteworthy that in general higher numbers of these bacteria occurred in the pelagic zone, although differences between zones were not significant. This observation, however, could confirm earlier reports that demonstrated that the alpha-Proteobacteria group preferred habitats with predominating labile organic matter and lower quantities of particle matter (Zwisler

*et al.* 2003). Bacteria from this subclass are incapable of active colonization of aggregates of organic matter (Schwiezer *et al.* 2001, Knoll *et al.* 2001), which commonly occur in the littoral zone.

The abundance of bacteria from the Cytophaga-Flavobacteria group was subject to very small fluctuations, which were not significant (Fig. 4). The highest abundances of bacteria from this group were observed in summer and autumn, irrespective of the analyzed water layer. The obtained data confirmed the results of previous studies, which demonstrated that bacteria from the Cytophaga-Flavobacteria F group grew in water with accumulated resistant-to-decompose organic matter, such as natural biopolymers (Zwisler *et al.* 2003, Pernthaler *et al.* 1998). This type of matter originates from dead phytoplankton cells, which occur in large numbers in spring and early summer (Araya *et al.* 2003). The only significant difference in abundances of Cytophaga-Flavobacteria observed in this study occurred in spring in the SM water between littoral and pelagic zones with substantially higher abundances in littoral zone. This result suggested that at least part of these bacteria "settled" on the lake surface with falling organic matter, such as, pollen, or used falling organic matter as a food source. It is a well-known fact that bacteria from the Cytophaga-Flavobacteria group inhabiting water bodies often use nutritional matter that originated from outside sources for their development (Kirchman 2002). Additionally, these bacteria develop particularly well in the presence of polymers, such as, chitin or cellulose (Kirchman 2002). The percentage share of Cytophaga-Flavobacteria bacteria increased from spring to autumn in both SM and SW layers. Furthermore, the share of Cytophaga-Flavobacteria group in Eubacterial abundance was generally higher in the littoral zone than in the pelagic zone.

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