Prokaryotes from Different Phylogenetic Groups in Surface Microlayer and Subsurface Water in a Eutrophic Lake

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Abstract

Prokaryotes from different phylogenetic groups were studied in surface microlayer (SM, up to 100 µm) and subsurface water (SW – 20 cm) in a eutrophic lake over three months (July, August, and October). The abundance of prokaryotes was determined by epifluorescence microscopy after DAPI staining, and phylogenetic diversity was determined by fluorescence in situ hybridization (FISH) with group-specific, fluorescently labeled oligonucleotide probes. In SW bacteria made up most of the entire community of DAPI-stained microorganisms (54-69%) and in SM bacteria made up only 33-44% of DAPI-stained microorganisms. Archaea corresponded to a small fraction of both bacterioneuston and bacterioplankton. The counts of Archaea and bacteria were significantly higher in SW than in SM. Among all proteobacteria included in the research, γ-proteobacteria represented the most abundant fraction: 42-72% in SM and 39-61% in SW. Statistical analysis revealed that the abundance of γ-proteobacteria is positively correlated with temperature and with dissolved oxygen. β-proteobacteria were the least abundant fraction.

Keywords: surface microlayer, communities, phylogenetic groups of prokaryotes, FISH

Introduction

Assessment of biodiversity, abundance, and activity of microorganisms constitutes a basis for the understanding of microbiological dynamics in the aquatic environment [1, 2]. The traditional analysis of bacterioplankton was based mainly on measurements averaged for the whole community – the so-called “black box” approach [3]. Such an approach was necessary because the morphology of bacterioplankton is mostly little diversified and a direct visual identification is possible as in the case of other plankton groups. In the past our understanding of bacteria diversity also was limited by the necessity of isolating and examining the pure cultures, whereas by applying the currently available methods, one can grow only <1% of the bacteria occurring in nature [4].

FISH is a commonly applied technique that enables a direct analysis of bacterial population structure, both in the natural environment and in the technical system [5, 6]. The FISH technique has been applied in much ecological research in order to determine the diversity of communities of microorganisms in inland waters [3, 7-9].

The surface microlayer (SM) is the most outer, very thin (100-200 µm) layer of the water reservoir, which develops in the water-air interphase. It is a special chemical and physical environment, quite different from the subsurface water [10, 11]. Due to phenomena such as absorption, diffusion, flotation, and precipitation, numerous organic substances accumulate in SM, mainly lipids, proteins, and polysaccharides, and their derivatives [12, 13].
Good oxygenation and high concentration of nutrients are favourable for the development of bacteria, and therefore an increased abundance of bacteria, called bacterioneuston, is usually observed in SM. On the other hand, however, several physicochemical factors (changes in the temperature, UV radiation, the presence of toxic substances) are responsible for the fact that SM is a very unstable environment compared with the subsurface water (SW). Therefore, bacterioneuston is more exposed to stressful ecological factors than microorganisms, which live in the pelagic zone. These factors obviously exert a considerable impact on microbiological diversity of SM.

When undertaking research on biological diversity of bacteria inhabiting the SM, the authors assumed that the composition of bacterial populations from this unique environment probably differs from populations of bacteria in the subsurface water (SW). Furthermore, it was expected that bacterial populations from SM and SW would change, more or less dynamically, according to the seasons and in the spatial system.

Materials and Methods

The water was collected from eutrophic Lake Brzeźno located in the forested area of Bory Tucholskie National Park in northern Poland. The morphometric and trophic characteristics of the lake are presented in Table 1. It is a forest lake, virtually devoid of human pressure, which enables the determination of natural differences between SM and SW.

Water samples were collected over three months: July, August, and October 2010, from 3 stations located in the pelagic zone and 3 in the littoral zone. Water of the surface microlayer (SM) was collected according to Garrett’s [14] technique, using a plexiglass plate that collects a 150 μm layer (SM) and SW.

The subsurface water (SW) was collected from a depth of 20 cm using a sterile glass pipette and an automatic Pippet-Boy pump (De Ville). The samples of SM and SW were taken in 10 replications.

The physico-chemical parameters of surface and subsurface water were measured simultaneously with collection of the samples used for microbiological analyses. pH, temperature, and redox potential were measured by a multimeter (Elmetron) and conductivity was measured by a conductometer (Slandi) equipped with a microelectrode. All these parameters were indicated in at least five replications. The light and UV radiation were measured by a PMA 2200 photometer (Solar Light Co.) in air over the water surface. The estimated values of light and UV are values that come to the water surface. The presented values of light and UV are averaged from 5 estimations conducted during 1 minute (Table 2).

For each replicate sample, 20 ml of each lake-water sample was filtered through a white polycarbonate 0.2 μm 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 the impact on the integrity and characteristics of cells.

The probes used in this study are listed in Table 3. All in situ hybridizations were performed according to the procedure described by Amann et al. [19] 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⁻¹. 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 by Amann [19], 20 mMTris-HCl (pH 7.4), 0.01% SDS). The washing buffer was removed by rinsing the slides with double distilled H₂O.

Samples were simultaneously stained with 4,6-diamidino-2-phenylindole (DAPI) [20]. The slides were then rinsed briefly with double-distilled H₂O and allowed to air-dry prior to microscopic observation. The filters were placed on slides, mounted with a 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, the number of cells was 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 a EUB338 probe.

Statistical analyses were done using STATISTICA 6.0 software. Analysis of variance (ANOVA) was the main statistical method used in calculations.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of Brzeźno Lake.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
</tr>
<tr>
<td>Height of water surface n.p.m.</td>
</tr>
<tr>
<td>Area</td>
</tr>
<tr>
<td>Maximum depth</td>
</tr>
<tr>
<td>Average depth</td>
</tr>
<tr>
<td>Total organic carbon content (TOC)</td>
</tr>
<tr>
<td>Total phosphorus</td>
</tr>
<tr>
<td>Total nitrogen</td>
</tr>
</tbody>
</table>
Results

It appears from the data presented in Table 2 that in SM and SW different physicochemical conditions prevail. Differences in the intensity of the visible spectrum (p<0.05) and UV (p<0.01) were statistically significant in the studied water layers (Table 4). A significant difference in values of these parameters also was observed between samples collected in summer (in July and August) and in autumn (in October). The highest values of these parameters were recorded in August, and they were strongly correlated with each other (r=0.96). Differences of temperature, conductivity and pH values were statistically significant for individual sampling rounds (months) (p<0.01), but did not differ statistically in the studied layers (p>0.05). The differences for all the studied physicochemical parameters between zones of the lake were not statistically significant (p>0.05). This indicates that environmental conditions in different zones of Lake Brzeżno are

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>rRNA target site (position of E. coli)</th>
<th>Dye</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB 338</td>
<td>most bacteria</td>
<td>16S (338-355)</td>
<td>CY3</td>
<td>[15]</td>
</tr>
<tr>
<td>ALF1b</td>
<td>α-proteobacteria and some other proteobacteria</td>
<td>16S rRNA (19-35)</td>
<td>CY3</td>
<td>[16]</td>
</tr>
<tr>
<td>BET 42a</td>
<td>β-proteobacteria</td>
<td>23S rRNA (1027-1043)</td>
<td>CY3</td>
<td>[16]</td>
</tr>
<tr>
<td>GAM42a</td>
<td>γ-proteobacteria</td>
<td>23S (1027-1043)</td>
<td>CY3</td>
<td>[16]</td>
</tr>
<tr>
<td>ARCH915</td>
<td>Archaea</td>
<td>16S (915-934)</td>
<td>fluorescein</td>
<td>[17]</td>
</tr>
<tr>
<td>NON338</td>
<td>nonsense probe – negative control</td>
<td>16S (338-355)</td>
<td>CY3</td>
<td>[18]</td>
</tr>
</tbody>
</table>

Table 4. Analysis of variance (ANOVA) of physicochemical parameters according to sampling rounds (month) and of the investigated layers.

<table>
<thead>
<tr>
<th>Physicochemical parameters</th>
<th>Factor</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>months</td>
<td>548.78</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>layers</td>
<td>0.12</td>
<td>0.737</td>
</tr>
<tr>
<td>pH</td>
<td>months</td>
<td>10.56</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>layers</td>
<td>0.03</td>
<td>0.883</td>
</tr>
<tr>
<td>Conductivity</td>
<td>months</td>
<td>28.35</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>layers</td>
<td>0.049</td>
<td>0.829</td>
</tr>
<tr>
<td>Visible light</td>
<td>months</td>
<td>3.53</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>layers</td>
<td>7.87</td>
<td>0.023</td>
</tr>
<tr>
<td>UVB</td>
<td>months</td>
<td>14.76</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>layers</td>
<td>63.95</td>
<td>0.000</td>
</tr>
</tbody>
</table>
similar, and therefore during the subsequent data analysis the results obtained for the pelagic and littoral zones were averaged.

The total number of prokaryotes in water samples determined with DAPI was the highest in July, both in SM and SW (Table 5). The analysis of variance revealed that differences in the abundance of bacteria during individual sampling rounds (months) were statistically significant (p<0.05).

In July and in August the total number of prokaryotes was higher in MS, whereas in October the number of prokaryotes was much higher in SW – all differences were significant at p<0.001. In SW, bacteria made up most of the entire population of DAPI-stained microorganisms (Table 5) and their percentage contribution was 54-69%. Whereas in SM, Eubacteria made up only 33-44% of DAPI-stained microorganisms.

The FISH method was applied to two domains: bacteria and Archaea. In the surface microlayer and subsurface water, there were much more bacteria than Archaea (Fig. 1). The total number of Archaea cells occurring in water ranged from 0.08×10⁶ to 1.7×10⁶ cells·cm⁻³, i.e. one to two orders of magnitude less than bacteria whose count ranged from 5×10⁶ to 22×10⁶ cells·cm⁻³. The abundance of Archaea was higher in SW than in SM, and this difference was statistically significant (p>0.05). The highest abundance of Archaea occurred in autumn (October). In the micrography displayed in Fig. 2, it is possible to observe the presence of Archaea cells in a “neustonic net.” The number of bacteria was higher in subsurface water compared with the surface film (p<0.05), whereas it appears from the statistical analysis that the number of bacteria did not change significantly over time.

Among proteobacteria, γ-proteobacteria had the highest relative contribution (Fig. 3). This group accounted for 42-72% of all proteobacteria occurring in SM and 39-61% in SW (Fig. 4). Statistical analysis revealed that the abundance of γ-proteobacteria is positively correlated with temperature (r=0.91) and with oxygen content in water (r=0.76). Therefore, the largest numbers of these bacteria were found in July. α-proteobacteria were yet another group with a relatively high percentage contribution, whereas β-proteobacteria were the least abundant (Fig. 3).

<table>
<thead>
<tr>
<th></th>
<th>SM</th>
<th>SW</th>
</tr>
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<tbody>
<tr>
<td><strong>July</strong></td>
<td>38.19±4.3</td>
<td>31.75±5.3</td>
</tr>
<tr>
<td></td>
<td>33%</td>
<td>69%</td>
</tr>
<tr>
<td><strong>August</strong></td>
<td>37.18±5.5</td>
<td>25.51±4.2</td>
</tr>
<tr>
<td></td>
<td>35%</td>
<td>67%</td>
</tr>
<tr>
<td><strong>October</strong></td>
<td>11.35±2.8</td>
<td>27.24±3.8</td>
</tr>
<tr>
<td></td>
<td>44%</td>
<td>54%</td>
</tr>
</tbody>
</table>

Fig. 1. Abundance of bacteria (E) and Archaea (A) in samples of the surface microlayer (SM) and subsurface water (SW).

Fig. 2. The Archaea (bright points) from subsurface water (October) and extracellular polysaccharide matrix (indicated by arrows) forming a so-called “neustonic net” – microscope Olympus BX50, magnification 1000 x, fluorescein dye.

Fig. 3. Abundance of different groups of proteobacteria in investigated samples.
The total number of bacteria, as well as their percentual contribution, fluctuated over time. The total number of prokaryotes (DAPI) in SM and in SW was at the highest level in July. The different bacterial abundances recorded in summer (July, August) and autumn (October) were significant. An increase in the count of bacteria in lake water is usually caused by intensive development of phytoplankton [23]. Also, heavy and persistent precipitation in July could significantly influence the number of bacteria in water.

Results obtained by several researchers [3, 34-36] indicate that bacteria constitute a significant percentage in the total number of DAPI-stained cells. In the presented study, bacteria made up from 33% to 69% of the total number of bacteria, which is in line with the results of studies quoted above. At the same time, there is a clear difference in the contribution of bacteria to the total pool of microorganisms in SM and SW. A smaller contribution of active bacteria (which contain active ribosomes) in relation to dormant bacteria in SM could likely be a cause of that. Various studies [37-39] indicate that bacteria in SM, especially during the day with intensive insolation, are much less active compared with bacteria living in deeper water layers.

The analysis of results on Archaea is difficult, because Archaea are not thoroughly investigated due to difficulties in their culturing and observation. In the literature there are references to the fact that Archaea constitute a rather small fraction of both bacterioplankton and bacterioplankton [36, 40, 41], which is evidenced by the presented results. Also, in the case of Archaea light and UV seem to be an important factors determining their occurrence. During the months of intensive radiation, i.e., in July and August, there were very few Archaea. The increase of their abundance took place only in October, which can be explained by lower values of UV radiation. Also, the results of the research by Pernthaler et al. [42] prove that bacterioplankton is rich in Archaea only in autumn, whereas in other seasons, its presence is hard to find. Perhaps Archaea (often anaerobic microorganisms) appear in the surface water layer owing to autumn water circulation in the reservoir, transported from bentic water layers. Several studies describe Archea as typical of deep freshwater [36, 43] and deep marine environments [44, 45].

Most researches indicates that usually microorganisms from the group of proteobacteria dominate in different aquatic communities [34, 42]. Among proteobacteria, β-proteobacteria are regarded as the most ubiquitous group in fresh waters [46] and are often dominant in communities of lakes [40, 42, 47-50]. β-proteobacteria were present in this study, but γ-proteobacteria were the dominant group.

Also, a relatively high number of α-proteobacteria was recorded in the studied samples. This is consistent with the research results obtained by Olapade and Leff [51], Araya et al. [52], and Manz et al. [53], who also prove that α-proteobacteria are one of the most numerous groups of bacteria in the inland waters. However, the studies by Walczak and Swientek-Brzezinska [54], conducted in the same lake just 3 years earlier, produced completely different results. Gucht et al. [55] proved that composition of bacterial formations might be different, and it depends on the amount of

Discussion

The surface microlayer, due to its physicochemical properties, creates a specific zone of life, both in fresh and oceanic waters. Specific conditions prevailing in this zone lead to the development of a community of organisms – neuston, which occurs on the surface of all water reservoirs. During the last 10 years efforts have been undertaken to characterize this formation and to determine its importance in the ecosystem. Therefore, it is extremely important to investigate bacterioneuston in quantitative terms, but also the diversity of groups contained therein [4, 21]. Molecular techniques were the best choice for this purpose, including FISH – fluorescent in situ hybridization [19, 22]. The unquestionable advantage of this technique is the possibility of conducting the research in situ in order to detect the microorganisms. Moreover, the method is very sensitive – with the use of specific probes, 40-80% of cells can be detected in a studied sample [23].

Due to high dissimilarity of environmental conditions and unique nature of SM, it is believed that bacteria communities developing at the surface microlayer differ in taxonomic and physiological terms from bacterioplankton occurring in subsurface water. Most of the researchers tend to conclude that there are more bacterial cells in the surface microlayer, in all water reservoirs, than in the water below that layer [24-26]. However, data obtained in this study show that in SM, Bacteria were less abundant than in SW. UV radiation seems to be the main determining factor. Samples were collected at noon when insolation is the strongest. Both light intensity and UV radiation were significantly higher in MS than in SW. Ultraviolet radiation (which is part of solar radiation) on the one hand exerts an adverse influence on all living organisms [27, 28], but on the other hand it induces photolytic decomposition of organic matter, thereby increasing the pool of readily available matter for heterotrophic microorganisms [29-32]. Research on the impact exerted by solar radiation, including UV, on the activity of intra- and extracellular enzymes of bacteria in the surface microlayer revealed that depending on the radiation intensity, not only the total number of bacteria changes, but also the total number of bacteria with an active system of electron transport [33].
available biogenic elements and the food web structure. The difference in the dominance of microorganism groups in particular years of the conducted research may result from different meteorological conditions, and in particular from the exceptionally large amount of precipitation, which took place during the described research cycle. Precipitation, and at the same time the increased surface runoff, could lead to a large amount of organic matter delivered to Lake Brzeźno from the drainage area. Zwisler et al. [23] ascertained that α-proteobacteria prefer habitats with a predominance of unstable organic matter and smaller amounts of solid particles. Moreover, bacteria from this group are incapable of active colonization of organic matter aggregates [47, 56]. This is evidenced by pictures of α-proteobacteria presented in this paper (Fig. 5) — microorganisms are distributed relatively evenly in the microscopic preparation. Whereas in the pictures of γ-proteobacteria one can see that these bacteria form conspicuous clusters.

The shape of these clusters is associated with the shape of aggregates of organic matter, which is gradually mineralized by bacteria (Fig. 6). Furthermore, Langenheder and Jürgens [57] discovered that α-proteobacteria are more abundant when Daphnia is absent and protozoa occur in large numbers. Development of a food web and the fact that some phylogenetic groups of bacteria are more readily eaten up than others could constitute an important factor influencing the population of both bacterioneuston and bacterioplankton.

At the same time, the presented data do not confirm the observations by Alonso-Sáez et al. [58] that α-proteobacteria are more sensitive to UV radiation than β- or γ-proteobacteria. In August, when the highest values of UV radiation were recorded both quantitatively and in percentage terms, α-proteobacteria were more abundant in SM than in SW.

Conclusions

The abundance of bacteria and Archaea is different for SM and SW, and at the same time their seasonal variability was observed. Light intensity and UV are the most important environmental factors differentiating the SM and SW. Seasonal variation probably also is exerted by other environmental factors (temperature, pH, conductivity). Bacteria are the dominant phylogenetic group in the SW prokaryote community. Significant differences in the percentage representation of individual proteobacteria groups in SM and SW were not found. The occurrence of individual proteobacteria groups in the water reservoir may considerably vary in time (in subsequent years) and probably depends on external factors (meteorological conditions, runoff from the catchment area).

Acknowledgements

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