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Antibiotic Sensitivity of Neustonic Bacteria in Lake Jeziorak Mały

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Abstract

This paper presents the results of research on the antibiotic sensitivity of bacteria inhabiting the surface microlayer (SM) and the subsurface water (SSW) of Lake Jeziorak Mały. It follows from the results that doxycycline, oxytetracycline and nalidixic acid are characterized by the strongest antibacterial effect. For some antibiotics, different strengths of the effect on bacteria isolated from the surface microlayer and subsurface water have been observed.

Keywords: surface microlayer, neustonic bacteria, antibiotics

Introduction

The zone of contact between water and air and the organic layer that is formed on the surface of waters constitutes a unique habitat for microorganisms. In accordance with the definition used by Norkrans [1], this organic layer is defined as the surface microlayer. Adhesive forces acting as a result of intermolecular attraction at the border of two phase centres - water and air - contribute to the existence of a surface membrane.

The physical stability of the membrane is possible thanks to the forces of surface tension. On the other hand, on account of the extreme values of temperature, salinity and doses of solar energy, this is actually an unstable environment in comparison with bulk water [2]. The nutrient conditions here are favourable for chemoautotrophs on account of the easy availability of carbon dioxide and reduced mineral compounds, and for heterotrophs on account of the concentration and variety of organic compounds [3].

Chemical research until now has indicated the richness of organic material, which achieves far higher concentrations in the surface microlayer than a few centimetres below. In the whole pool of organic material, some of the superficially active substances are not highly soluble in water, as a result of which they display a tendency to accumulate in the surface microlayer [4]. The majority of the organic substances occurring in the surface microlayer reduce the surface tension of water and hence, they can be adsorbed in the water-air interphase. On the other hand, inorganic ions increase the surface tension of the water and do not undergo adsorption but are washed into the subsurface water [5]. Daumas et al. [6] believe that organic microparticles accumulate in the narrow surface microlayer (up to 100 μm), while soluble microgens in the form of inorganic ions are accumulated in a slightly thicker layer of water (up to 1 mm). In connection with the higher concentration of organic substances in the surface microlayer, it is likely that antibiotics and other pharmaceuticals which get into the environment with sewage can accumulate in this layer, as they do in the soil. The contact of bacteria with antibiotics often leads to their building up resistance to antibiotics used in medical treatment, which is an unfavourable or even dangerous phenomenon. The presence of antibiotics in the natural environment causes the selection of micro-organisms, eliminating sensitive microflora, which might play an important ecological role. It has been shown that antibiotics brought into the

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environment in an artificial way undergo microbiological decomposition, like other organic compounds [7]. Many antibiotics contain aminoacids and simple sugars in their structure, which, after enzymatic hydrolysis, become a source of carbon and nitrogen for microorganisms. This phenomenon is one of the main factors, apart from processes of sorption, limiting the biological role of antibiotics in the natural environment [7].

Materials and Methods

Study Area

The study was conducted on Lake Jeziorak Mały. This water body lies within the town of Iława and is part of the Iława Lake District. The surface area of the water body is 26 ha, and the maximum depth is 6.4 m. This lake does not have any inlets or outlets, apart from a shallow and narrow (1.5 m) arm at its northern part, joining it to Lake Jeziorak.

Sampling

The study was conducted from May to October 2001. Samples of water for microbiological analysis were taken from three different sites (Fig. 1). Water from the surface microlayer was taken:

A) with the aid of a glass plate collecting a layer of water of 100 μm in thickness
B) with the aid of a plexiglass plate collecting a layer of water of 150 μm in thickness
C) with the aid of a Garrett net 1 with a mesh diameter of 65 μm collecting a layer of water of 250 μm in thickness
D) with the aid of a Garrett net 2 with a mesh diameter of 200 μm collecting a layer of water of 350 μm in thickness

Samples of sub - surface water from a depth of 20 cm were taken with a sterile glass pipette with the aid of an automatic Pippet-boy pump (De Ville). The samples from the microlayer and the sub-surface water were put into sterile glass bottles and transported to the laboratory in a thermoisolated container with ice at a temperature of ≈ 7°C. The time from the moment of taking the samples to their analysis did not exceed 3 hours.

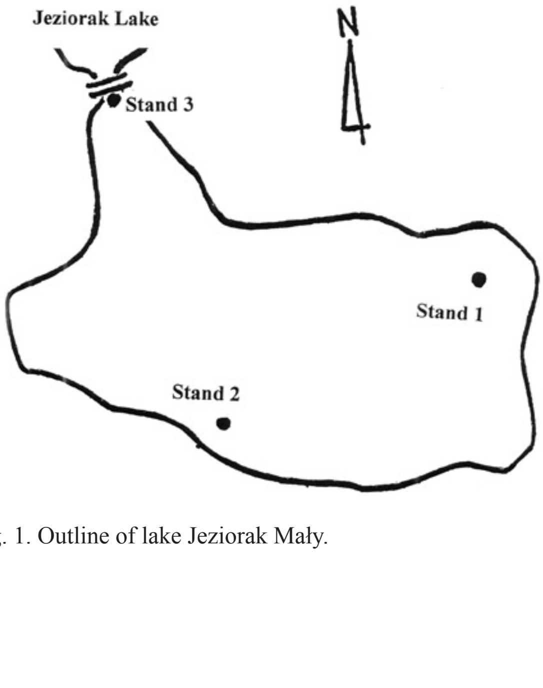

Fig. 1. Outline of lake Jeziorak Mały.

Determination of Heterotrophic Bacteria

The number of heterotrophic bacteria (CFU) was determined using the spreed plate method. Sterile buffer water was used for diluting the water samples [8]. The samples were seeded in three parallel repetitions in quantities of 0.1 ml on the surface of Plate Count Agar (Difco). After 6 days of incubation at a temperature of 20°C, the colonies of heterotrophic bacteria were counted, and then randomly transplanted onto semi-liquid iron-peptonic agar [9] to be stored for further tests at a temperature of +4°C.

Antibiotic Sensitivity of the Bacteria

The antibiotic sensitivity of the bacteria was tested using the disc (diffusion) method with BIOMED discs 6 mm in diameter. In the course of the studies, 10 different antibiotics were tested: 1) aminoglicozid antibiotics (neomycin, streptomycin), 2) macrolide antibiotics (erythromycin), 3) peptide antibiotics (colistin), 4) antibiotics from the tetracycline group (doxycycline, oxytetracycline), 5) lincozamids (clindamycin), 6) natural penicillins (penicillin G), 7) semi-synthetic penicillins (ampicillin), and 8) chinolonocarboxyl acids (nalidixic acid).

Preparation of Bacterial Inoculations

Isolated bacterial strains were seeded onto liquid iron-peptonic medium after Ferrer, Stappert, and Sokolski [9].

After 72 h incubation at 20° C, the optical density of the culture was measured at a wavelength of $\lambda = 565$ nm using a Spekol spectrophotometer (Carl Zeiss Jena) and brought to a value of 0.15. Sterile liquid iron-peptonic medium was used as a diluant.

Measurement of the Antibiotic Sensitivity of Strains

Sterile, solid medium (nutrient agar) from the company BTL was poured into sterile Petri dishes and seeded with 0.1 ml bacterial inoculation prepared earlier (1 strain - 1 dish). Discs of blotting paper soaked in five different antibiotics were placed on the seeded medium at distances no less than 2 cm. Then the dishes were stored for 30 minutes at a temperature of 4°C in order to diffuse the antibiotics from the discs to the medium, after which they were placed at a temperature of 20°C and incubated for 72 h. The degree of sensitivity or resistance of the strains was determined on the basis of the measurement of lightened zones (in mm) around the disc and comparing them with data given by the manufacturer.

Table 1. Number of heterotrophic bacteria in SM and SSW of lake Jeziorak Mały.

Data of samplig	Layer of water					Average in SM	E SM/SSW
	A	B	C	D	WPP		
May	15*	11	18	30	7	18.5	2.6
July	72	19.5	39.3	38.6	8.8	42.3	4.8
October	107.2	88.8	123.8	85.2	1.2	101.2	84.3
Average	64.7	39.8	60.4	51.3	6	54.0	30.6

A - sample taken using glass plate, layer about 100 µm thick; B - sample taken using plexiglass plate, layer about 150 µm thick; C - sample taken using Garrett net 1, layer about 250 µm thick; D - sample taken using Garrett net 2, layer about 350 µm thick; SM - surface microlayer (A+B+C+D/4); SSW - sub-surface water from a depth of 20 cm; E - enrichment coefficient - SM/SSW; * - number of heterotrophic bacteria x 10^3 cells/l.

Results

Results of the research on the number of heterotrophic bacteria (CFU) in the surface microlayer and in the subsurface water of Lake Jeziorak Mały are presented in Table 1. It follows from these results that this number undergoes a seasonal fluctuation and depends on the plate or net used to take the samples.

A comparison of the number of heterotrophic bacteria in the surface microlayer (SM) and in the subsurface water (SSW), shows that in the studied months the number of heterotrophic bacteria in the subsurface water layer (SSW) was several times lower than that in the surface microlayer (SM). The maximum heterotrophic neustonic bacteria in the surface microlayer was found in autumn, in October - $101.2 \cdot 10^3$ cells/cm^3, and the minimum in spring, in May - $18.5 \cdot 10^3$ cells/cm^3. It follows from this data that in autumn the number of heterotrophic bacteria in the biofilm was about 5.5 times higher than in spring.

Analysis of the average values of the number of bacteria isolated in the microlayer, implies that the highest number of bacteria was obtained when the samples were collected with a glass plate (sample A), and the least – when using a plexiglass plate (sample B).

The data presented in Figures 2 and 3 indicate that tetracyclines (doxycycline and oxytetracycline) and nalidixic acid, one of the chinolonocarboxyl acids had the strongest inhibitory effect on the strains tested.

Among all the strains tested, 83.8% of SM bacteria were sensitive to doxycyline. The percentage of the dxycycline-sensitive strains in the SSW was even higher, reaching 96.0%. However, the resistance to this antibiotic was distinctly different among the strains isolated in SM and SSW (8.6% and 4.0% of strains, respectively). 69.5% of SM strains and 76.0% of SSW strains displayed sensitivity to oxytetracycline, and 17.1% of SM bacteria and 18.0% of SSW bacteria displayed resistance to this antibiotic. The remaining strains displayed average sensitivity to the antibiotic tested.

Bacteria from the SM and SSW reacted almost identically to nalidixic acid. 78.1% of SM strains and 76.0% of SSW strains displayed sensitivity, and 10.0% of strains in both SW and SSW displayed resistance to this antibiotic. The remaining strains displayed average sensitivity to this antibiotic.

Among aminoglycosides, streptomycin stood out as having a stronger effect than neomycin. The data presented in Figure 2 show that 46.7% of SM strains and 50.0% of SSW strains displayed sensitivity to streptomycin. However, more strains resistant to this antibiotic (31.9%) occurred among SM bacteria than in those isolated in SSW (16.0%). Among all SM bacteria, 14.3% were sensitive to neomycin, while of those isolated in SSW only 4.0% displayed this sensitivity. However, similar numbers of strains resistant to this antibiotic were found among SM (32.4%) and SSW (32.0%) strains.

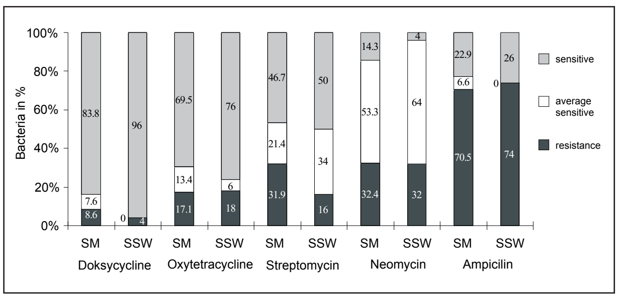

Fig. 2. Influence of antibiotics on bacterial strains isolated from SM and SSW water of lake Jeziorak Mały (average).

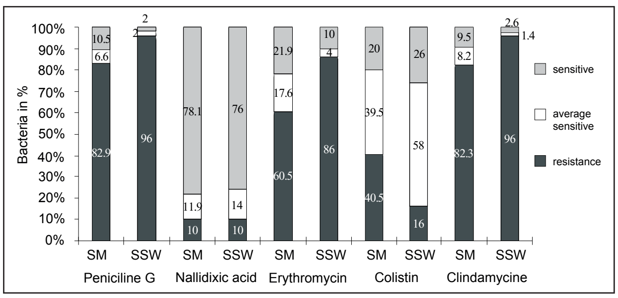

Fig. 3. Influence of antibiotics on bacterial strains isolated from SM and SSW water of lake Jeziorak Mały (average).

Penicillins (ampicillin and penicillin G) displayed different strengths in their effect on the strains tested. The presented data (Fig. 3) show that 22.9% of SM bacteria and 26.0% of SSW bacteria were sensitive to ampicillin, while only 10.5% of SM strains and merely 2.0% of SSW strains were sensitive to penicillin G. However, 70.5% of SM bacteria and 74.0% of SSW bacteria were resistant to ampicillin. From among all the strains tested, as many as 82.9% of neustonic bacteria isolated in SW and 96.0% isolated in SSW were resistant to penicillin G. Penicillin G, along with colistin and clindamycin proved to be among the weakest antibiotics.

Only 20.0% of SM strains and 26.0% of SSW strains displayed sensitivity to colistin. 40.5% of SM strains and 16.0% of SSW strains displayed resistance, while 9.5% of SM strains and 26.0% of SSW strains displayed sensitivity to clindamycin.

From among all the strains tested, 21.9% of neustonic bacteria in SW and only 10.0% of planktonic bacteria in SSW were sensitive to erythromycin. However, 60.5% of SM bacteria and as many as 86% of SSW bacteria were resistant to this antibiotic.

Among all the strains tested, no significant dependence of antibiotic sensitivity on seasonal changes was found.

Discussion

Until recently, the majority of research concerning antibiotic sensitivity was conducted on clinical materials, and there are a few papers devoted to the effects of antibiotics on neustonic bacteria.

Within the space of a few hundred micrometers from the surface membrane down, the principal ecological factors change (temperature, quantity and quality of sunlight and nutrients) and determine living conditions. These factors, (in all certainty) determine the development of bacterial life and the kinetics of biochemical metabolism, which is reflected by the number of and physiological properties of bacteria inhabiting this environment.

The results of the study presented in this paper show that the number of heterotrophic bacteria (CFU) is higher in the surface microlayer than in the subsurface water. This is in accordance with earlier research [10,11,12,13]. Also, Rheinheimer [14] found the greatest accumulation of bacteria in the surface microlayer in the Baltic sea. The maximum number of neustonic bacteria in Lake Jeziorak Mały was found in autumn (October). However, results obtained earlier [13] indicate that the greatest amount of neustonic bacteria in the surface microlayer occurs in July. The number of bacteria in the surface microlayer in the summer, in July, was on average about 5 times higher than in the subsurface water, while in autumn, in October, as much as 84 times higher.

As follows from the data reported in the present paper, the greatest number of heterotrophic bacteria occurs in the surface membrane (layer A, thickness about 100 µm) and decreases with increasing thickness of the layer. The study conducted in marine waters [15, 16, 17] also showed that the greatest number of bacteria occurred in the surface layer of the biofilm and decreased as thickness and depth increased. According to Falkowska [18], higher numbers of these bacteria in the surface layer of the biofilm are a result of greater concentration of organic material and good oxidation conditions in this layer.

An analysis of the strains as regards antibiotic sensitivity shows that particular antibiotics used in the research act with differing strengths and to different degrees on the tested strains. The effect of the antibiotics depends on the mechanism of their activity, i.e. on which cellular structures the antibiotic acts. These are usually cellular structures or enzymatic systems fulfilling a significant function for the growth and multiplication of the cells, like expression of genetic information, cellular synthesis, transport processes. The second element that influences the strength of the antibiotic's activity is the sensitivity of individual bacteria to the antibiotic applied, resulting from the functioning of the defence mechanisms, e.g. the ability to metabolize antibiotic substances or the presence of resistance as a result of earlier contact with that antibiotic [19].

The strongest acting group of antibiotics analyzed in this paper was that of tetracyclines - doxycycline and oxytetracycline. Among them, doxycycline stood out as having a wider range of activity. This results from the fact that this antibiotic does not have a hydroxyl group, in contrast to oxytetracycline, which results in an increased ability of this substance to penetrate through the cellular membrane [7, 19, 20]. Doxycycline impedes the synthesis of protein as a result of binding with bacterial ribosome 30 S [19]. This mechanism is one of the most effective in its action, which is confirmed by the results obtained in this paper. According to Podlewski [19], doxycycline is one of the strongest acting antibiotics in the tetracycline group and acts strongly on rickettsiae, anaerobic bacteria, mycoplasmas, spirochaete, actinomycete, streptococci, staphylococci and also enterococci. When it is applied, selection of resistant strains occurs more rarely. However, completely opposite results were obtained by Mudryk and Skórczewski [21], conducting research on the estuary lake Gardno. Among the bacteria isolated from this lake, almost 9% of strains displayed resistance to tetracycline. The results obtained in the present paper also do not confirm those reprted for the other water bodies [22, 23]. It may be supposed that the effect of the action of this antibiotic depends on the trophy of the water body and the site from which the bacteria used for the research were isolated.

The results presented in this paper demonstrate that from among the aminoglycoside antibiotics tested, streptomycin stood out as acting stronger than neomycin. As Korzybski et al. [24] write, this antibiotic, produced by some strains of *Streptomyces griseus*, has various effects on bacteria cells: it impedes the synthesis of protein, influences the penetrability of the cellular membrane, causes an interruption in RNA, interferes in the process initiating the synthesis of protein and triggers an irreversible blockade of the ribosomal cycle. Streptomycin also affects on the ribosomal subunit 30 S [25].

On the other hand, the mechanisms of the action of neomycin impede the synthesis of protein in systems containing cytoplasmic and mytochondrial ribosomes, causing a false reading of the genetic code, enabling a direct translation of DNA [26, 27].

The wider spectrum of the activity of streptomycin is probably a result of the efficiency of the mechanisms of this activity. The results of the research on the effects of streptomycin on neustonic bacteria obtained in this paper confirm the data presented by Mudryk and Skórczewski [21] and the results from studies conducted on fresh-water bodies [22, 23].

Analysis of data presented concerning the antibiotic sensitivity of neustonic bacteria to β-lactam antibiotics (penicillins - ampicillin and penicillin G), shows that they do not have a wide spectrum of activity, which is probably a result of their sensitivity to β-lactamase produced by various microorganisms.

Mudryk and Skórczewski [21] demonstrated that from among the antibiotics from this group tested by them, ampicillin belonged to the group of antibiotics characterized by the weakest action. Among all strains of bacteria isolated from Lake Gardno, as many as 90% were resistant to this antibiotic (in the case of the studies conducted in this paper, there were 70% of such bacteria). The activity of ampicillin towards Gram-negative microorganisms is similar to that of tetracycline and chloramphenycol [24] and this may be the reason why this antibiotic stands out as having a slightly wider spectrum than penicillin G, which acts most often by impeding reactions of transpeptidation in the biosynthesis of peptydoglykan of bacteria cell walls [24].

Nalidixic acid, which belongs to the group of chinolonocarboxyl acids, stood out as having the widest spectrum of activity, along with tetracycline. Other authors [21, 22, 23] obtained similar results. Mudryk and Skórczewski [21] demonstrated that, from among the strains isolated from Lake Gardno studied by them, less than 20% displayed resistance to this antibiotic. However, the results obtained in this paper indicate that, from among the strains studied, only 10% displayed resistance to this antibiotic. As Podlewski [19] writes, the mechanism of the activity of this antibiotic involves the disturbance of the replication of DNA of bacteria as a result of impeding the activity of gyrase of DNA.

As follows from the data on the influence of erythromycin, this antibiotic does not display a wide spectrum of activity. Depending on the concentration and kind of micro-organism, erythromycin, most often obtained from the culture of *Streptomyces erythreus*, acts as a bacteriostatic or bacteriocide. The mechanism of its activity depends on the disturbance of the synthesis of protein in bacteria as a result of binding with ribosome 50 S [24, 19]. It has a strong effect on streptococci and slightly less on staphylococci; moreover, it acts on *Mycoplasma pneumoniae, Listeria monocytogenes, Corynebacterium diphtheriae, Neisseria spp., Legionella pneumophila, Haemophilus influenzae,* spirochaete, rickettsiae, *Chlamydia spp*. It does not, however, act on anaerobic bacteria and Gram-negative intestinal bacilli. Erythromycin displays resistance to the other macrolide antibiotics, lincomycin and clindamycin [19].

The narrow range of erythromycin's activity on the strains tested probably results from the fact that a significant proportion of the bacteria inhabiting the surface microlayer of water bodies belong to the Gram negative bacillus group [29] to words which this antibiotic is very weak or ineffective.

The data in this paper indicate that colistine (peptide antibiotics, polymyxin group) is one of the weakest antibiotics. This antibiotic is a mixture of peptides obtained from the culture of the strain *Bacillus polymyxa var. colistinus*. It acts as a bacteriocide on Gram negative micro - organisms (including *Pseudomonas aeruginosa, Escherichia coli, Enterobacter spp., Salmonella spp.*) [19].

Clinadmycin, which belongs to the lincozamide group, is a synthetic derivative of lincomycin. The mechanism of its activity is similar to that of erythromycin. This antibiotic acts as a bacteriostatic or bacteriocide (depending on the concentration) on aerobic Gram positive bacteria. The data obtained in this paper indicate that this antibiotic is the weakest of all those tested. From among the strains tested over 90% show resistance to this antibiotic. Mudryk and Skórczewski [21] obtained similar results in their study.

The antibiotic resistance of micro-organisms may be linked with the reduced penetration of the antibiotic into the cell or may be a result of active processes, like changes in the transport of compounds to or from the bacterial cell [30]. The resistance of bacteria to antibiotics is located in the plasmids. It can be supposed that the resistance of bacteria studied in this paper might result from their numerous presence in the cells of the tested strains. As Klech and Lee [31] and Silva and Hofer [32] write, plasmid R plays an enormous role in antibiotic resistance. Plasmid R can be transferred between different strains in the processes of conjugation and transformation [33]. There are four basic mechanisms of resistance conditioned by these plasmids. These are inactivation, creation of substitute metabolic paths (bypass), inpenetrability of cytoplasmatic membranes and a change in the target site [34]. Resistance can also be connected with the production of enzymes that modify inactivate antibiotics [35]. Where the bacteria do not contain plasmids, antibiotic resistance is conditioned by a mobile genetic element called a transposome [30].

The study carried out by Jones et al. [36] in Lake Michigan, and by Hermansson et al. [30] along the western Swedish coast, demonstrated that bacteria inhabiting the surface water displayed a higher degree of resistance to antibiotics than those isolated from sub-surface waters. Similar results have been obtained in this paper as regards such antibiotics as doxycycline, neomycin, streptomycin, penicillin G, erythromycin, colistin and clindamycin. The data concerning the resistance of environmental strains to antibiotics are still fragmentary and incomplete. For this reason, further research is necessary concerning the interaction of bacteria and antibiotics. It is particularly important to answer the question whether strains from natural environments gain resistance mainly by way of adaptation or whether it is transmitted mainly by resistance plasmids.

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