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Antibiotic Resistance among Bacteria Inhabiting Surface and Subsurface Water Layers in Estuarine Lake Gardno

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Abstract

Antibiotic resistance of heterotrophic bacteria isolated from the surface and subsurface water of estuarine lake Gardno was determined. The levels of resistance of bacteria to various antibiotics differed considerably. Antibiotic resistance between neustonic and planktonic bacteria and microflora inhabiting different parts of lake Gardno was nearly identical. Besides gentamycin, no differences in the antibiotic resistance between pigmented and non-pigmented bacteria were noted. Majorities of bacterial strains were characterised by resistance to 4-6 antibiotics. Bacterial resistance to antibiotics was dependent on chemical structure.

Keywords; bacterioneuston, bacterioplankton, antibiotic resistance, estuarine lake

Introduction

Many bacteria isolated from natural environments possess an important ecological quality, namely that of resistance to antibiotics, which can be picked up in the course of selective processes [25, 28]. Over recent years many studies have been carried out to investigate the occurrence and distribution of antibiotic-resistant bacteria in water basins [3, 14, 23, 24].

Antibiotic resistant microorganisms may be associated with reduced penetration of the antibiotic into the cell, or can result from active processes such as changes in the transport of those compounds into or from bacterial cells [13]. Bacterial resistance to antibiotics is located in plasmids of 1-30 megadaltons molecular weight [20]. Genes assembled in plasmids protect bacterial populations against antibiotics. It is the R plasmid that plays a substantial role in bacterial resistance to antibiotics [19, 28]. The R plasmid can be transferred between various strains of bacteria through conjugation and transformation processes [14]. There are four classic mechanisms of resistance specified by plasmids: inactivation, impermeability, bypasses and altered target site; all occur in aquatic environments [6]. Also, intracellular binding seems to be a valid mechanism for immobilising an inhibitor [9]. Resistance can also be associated with the production of enzymes that modify and inactivate antibiotics [21]. According to Hermansson et al. [13] some strains of bacteria resistant to antibiotics do not contain any plasmids. In such a case bacterial resistance to antibiotics depends on the mobile genetic elements, called transposons [14].

A large number of bacteria and actinomycetales occurring in aquatic ecosystems are capable of synthesising compounds of antibiotic nature [18, 22]. These inhibitory substances are 2,000-15,000 dalton large molecules and their concentration in water could be ca 1 $\mu g \cdot cm^{-3}$ [22, 30]. Bacteria slowly but steadily synthesise and secrete into water a number of antibiotic substances, namely phenazines, pyrrolnitrin, bacteriocins, glycolipids and bromopyrrolic compounds [1, 4, 23, 31]. All of these antibiotic substances inhibit bacterial respiration and biosynthesis of cellular structures [2, 15]. A lot of algae, mainly *Chloro-*

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phycae, Rhodophycae and *Phyophyceae* also produce substances of antibiotic character, which inhibit the growth of bacteria [11, 18]. In aquatic systems beside autochthonous source antibiotics are also anthropogenic ones. Several different kinds of antibiotics are used in fish farms to control bacterial and fungal diseases by incorporation into the feed [14,16]. The fish absorbs not all antibiotics, some are released to water and sediments as uneaten fish feed or in faeces.

Over recent years fairly much attention has been paid to the problem of bacterial resistance to antibiotics occurring in freshwater basins [14, 17] and in marine water bodies [24, 25]. However, no such studies have been undertaken in the dynamic water ecosystems such as estuaries definitely are. The aim of the present study was to determine resistance to antibiotics of bacteria isolated from the estuarine lake Gardno. Different antibiotics are considered here as an environmental selection factor.

Material and Methods

The Study Area

Studies were carried out in estuarine lake Gardno situated in Slowinski National Park (Fig. 1). The lake is very shallow (1.3 m average depth) but covers a large area (2.500 ha). The shallow depth and large area as well as the lack of shielding winds makes possible the full mixing of water in both vertical and horizontal profiles. As a result, the lake can be regarded as a polymictic basin in which no thermal or oxygen stratification is observed. The emergent macroflora covers 4% of the lake surface, forming a wide offshore belt 20-100 m wide, which constitutes a residence for many bird species. Lake Gardno is characterised by intermediate conditions between marine and inland environments. On the one hand it is supplied by water of the river Lupawa, on the other hand it is connected with the Baltic Sea, whose large volumes of sea water abundantly penetrates into the lake via a 1.3 km channel. Therefore, water of that lake, or its part acquires seawater quality, resulting in 2-5‰ salinity. Consistently, with the Venetian system lake Gardno can be classified as mixo-oligohaline type (0.5-5.0°/oo) [7].

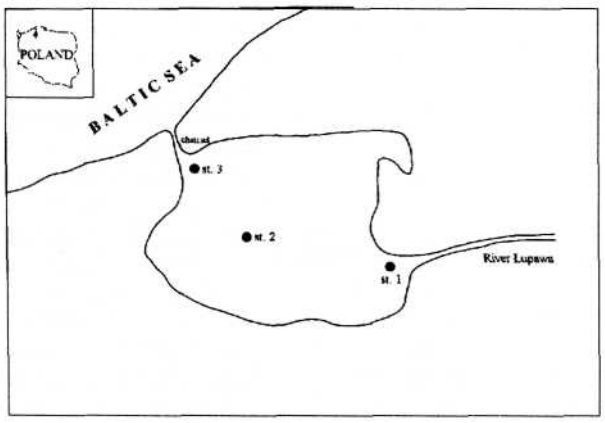

Fig. 1. Sampling station locations in lake Gardno.

Sampling

Water samples were taken during 1998 from three stations (Fig. 1) which represented different environmental conditions: one near the river inflow (freshwater zone) (station 1), one near-sea part (seawater zone) (station 3), and one in mid-lake (mixed zone) (station 2). Water samples for bacteriological analyses were taken from three layers. Film layer (FL) samples (thickness of 90 ± 17 μm) were taken with a 30 x 30 glass plate [12], surface microlayer (SM) samples (thickness of 242 ± 40 μm) were collected with a 40 x 50 cm Garrett net (24 mesh net of 2.54 cm length) [10]. Glass plate and polyethylene net were rinsed with ethyl alcohol and distilled sterile water prior to sampling. Water from subsurface layer (SUB) was taken with sterile glass pipettes at a depth of about 10-15 cm. The water samples were collected into sterile glass bottles and stored in an ice-box where the temperature did not exceed 7°C until they were taken for analysis. The time between sample collection and performance of the analyses usually did not exceed 6-8 h.

Bacteria Isolation

In order to isolate neustonic (FL and SM layers) and planktonic bacteria (SUB layer) the collected samples were diluted with sterile buffered water [5] to reach final cells frequency ranging from 10^{-1} to 10^{-4}. These, in the amount of 0.2 cm^3, were inoculated by the spread method in three parallel replicates, on iron-peptone agar (IPA) medium [8]. Incubation was carried out at 20°C for 10 days. Subsequently, about 50 bacterial colonies from each water layer were picked from the plates and were transferred to semiliquid IPA medium (5.0 g of agar per dm^3 of medium). The cultures maintained on this medium after purity control were kept at 4°C and used for further investigation in order to determine antibiotic resistance of these bacteria.

Determine Antibiotic Resistance of Bacteria

The antibiotic resistance of neustonic nad planktonic bacteria was determined by the single disc diffusion method [23, 28]. Bacteria were multiplied on agar slants (IPA) at 20°C. After 48 h they were washed off the slants with 5 cm^3 of sterile buffered water and adjusted to a turbidity of 4 on the MacFarland scale, which corresponds to 10' bacterial cells per 1 cm^3. Subsequently, 0.2 cm^3 of bacterial suspension prepared in this way was spread over iron-peptone agar plates. After 30 min of absorption time, paper discs impregnated with an antibiotic were then applied to the surface of the seeded medium. The blotting paper discs ((p 13 mm) used were manufactured by the Warsaw Serum and Vaccine Production Company and the Becton-Dickinson Company. The dishes were than incubated at 20°C for 24 h. After incubation, the diameter (in mm) of the areas where bacterial growth was inhibited by the various antibiotics was measured. Bacteria were classified as antibiotic resistant according to the manufacturer's instructions. The seventeen following antibiotics (concentration in μg per disc) were used in antibiograms: ampicil-

Table 1. Resistance to the antibiotics of bacteria isolated from different water layers (in percentage).

Antibiotics	Water layer			Average
	FL	SM	SUB	
Ampicillin	84.4	88.0	89.6	87.3
Chloramphenicol	64.8	64.6	64.6	64.7
Clindamicin	88.4	89.0	89.6	89.1
Cloxacillin	88.3	95.0	90.1	91.1
Doxycicline	70.7	71.0	71.3	71.0
Gentamycin	12.4	25.5	20.1	19.3
Kanamycin	29.3	42.4	28.1	33.3
Nalidixic acid	83.3	83.9	76.4	81.2
Neomycin	14.3	23.0	22.3	19.9
Nitrofurantion	79.6	89.3	82.7	83.9
Novobiocin	84.1	90.5	88.9	87.8
Penicillin	93.2	96.1	91.3	93.5
Rifampicin	19.8	20.3	16.0	18.7
Streptomycin	6.4	7.3	12.5	8.7
Sulfamethoxazole	91.8	96.4	90.6	92.9
Tetercycline	96.8	95.8	96.2	96.2
Trimethoprim	96.8	95.3	98.2	96.8

lin (AM, 10), chloramphenicol (CP, 30), clindamicin (CM, 2), cloxacillin (CL, 1), doxycicline (DK, 30), gentamycin (GE, 10), kanamycin (KM, 30), nalidixic acid (NA, 30), neomycin (NM, 30), nitrofurantion (NF, 200), novobiocin (NB, 30), penicillin (PL, 10), rifampicin (RF, 10), streptomycin (SM, 30), sulfamethoxazole (SU, 23.75), tetercycline (TE, 30), and trimethoprim (TM, 1.25). The antibiotics were divided into six groups (β-lactams, aminoglycosides, tetracyclines, rifampicins, sulfonamides, other) according to their chemical structure [9].

Results

The data presented in Table 1 show that bacteria inhabiting lake Gardno characterise large differences in the level of resistance to different antibiotics. About 90% of the bacterial microflora were resistant to ampicillin, clindamicin, cloxacillin, penicillin, sulfamethoxazole, tetracycline and trimethoprim, while less than 20% of the strains were resistant to gentamycin, neomycin, rifampicin and streptomycin. The results show (Tab. 1) that besides gentamycin, kanamycin, and streptomycin there were no differences between the bacteria isolated from three studied water layers in their resistance to the antibiotics used in this study.

Differences between pigmented and non- pigmented bacteria occurred in their resistance to gentamycin (Fig. 2). About 60% of pigmented bacteria were resistant to this antibiotic, whereas only 38% of the non-pigmented bacteria characterised resistance to gentamycin. In the case of other antibiotics no differences in the antibiotic resistance between pigmented and non- pigmented bacteria were noted.

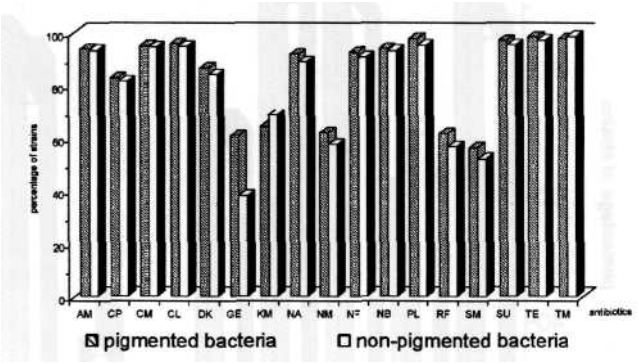

Fig. 2. Differential resistance of pigmented and non-pigmented estuarine bacteria to studied antibiotics.

The data presented in Figure 3 show that there were no differences between the bacteria inhabiting investigated parts of lake Gardno in their resistance to the antibiotics used in this study. The level of resistance of bacteria isolated from three stations, which represented different environmental conditions, was similar.

The collection of strains was analysed for multiple antibiotic resistance (MAR) (Fig. 4). About 40-60% of studied bacteria showed a 4-6 MAR pattern (i.e. resistance for 4 to 6 of the 17 antibiotics tested) and 15-20% a 7-9 MAR pattern. Only small percentages of bacteria studied showed a 0-1 and 11-17 MAR. The value of multiple antibiotic resistance neustonic and planktonic bacteria were nearly identical.

Table 2 sets data on bacterial resistance to antibiotics in relation to their chemical structure. In all water layers and stations examined strains were most susceptible to aminoglycosides and rifampicins antibiotics. While a high percentage of the bacteria were resistant to the remaining groups of antibiotics, especially to the β-lactams.

Table 2. The resistance of bacteria to antibiotic with respect to their chemical structure (in percentage).

		AM	LA	TET	RYF	ANT	Others
Layers	FL	24	91	86	29	89	80
	SM	28	93	84	23	93	82
	SUB	26	91	84	21	90	80
	average	**26**	**92**	**85**	**24**	**91**	**81**
Station	1	26	95	90	31	94	82
	2	27	92	87	26	90	80
	3	24	94	92	17	91	81
	average	**26**	**94**	**90**	**25**	**92**	**81**

Explanations:
AM - aminoglicosides (neomycin, streptomycin, kanamycin, gentamycin), LA - β-lactams (penicillin, ampicillin, cloxacillin), TET - tetracyclins (tetracycline, doxycycline), RYF - rifampicins (rifampicin), ANT - antimetabolic (sulfamethoxazole, trimethoprim, nitrofurantion), Others - (novobiocin, clindamicin, chloramphenicol, nalidixic acid)

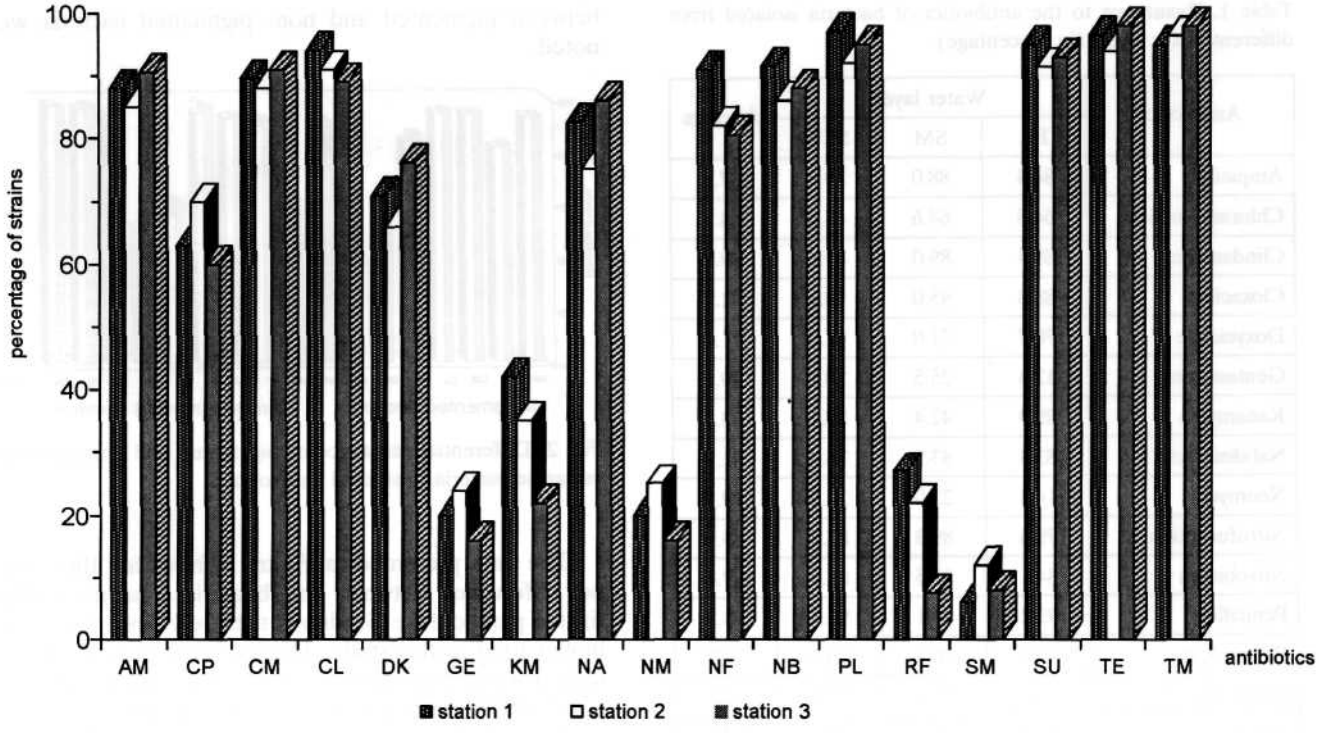

Fig. 3. Spatial distribution of resistance to different antibiotics of the studied bacteria.

Discussion

Until recently, the majority of this kind of study has been carried out on clinical material and thus, little is known of bacterial resistance to antibiotics in the natural environment [16, 19]. Hence, the role of antibiotic substances secreted into the natural environment has not been recognised in a comprehensive way and ever since has been one of the most controversial issues of microorganisms ecology [31]. Antibiotic substances may be very effective in forming the quantity and quality composition of bacteriocenoses in water ecosystems and may also play a substantial part in food competition systems [1, 23]. Studies on sensitivity to antibiotics have also been used to identify bacteria in terms of taxonomy [19].

The present study has displayed that bacteria isolated from lake Gardno have a high degree of resistance to most of the antibiotics under investigation. Such a high level of resistance might result from the fact that about 50% of estuarine bacteria have plasmids where antibiotic-fighting genes are assembled, which protect the microorganisms from being affected by antibiotics [20]. In lake Gardno, the highest bacterial resistance was noted in the cases of ampicillin, clindamicin, cloxacillin, penicillin, sulfamethoxazol, tetracycline, and trimethoprim, while at the same time the bacteria were most sensitive to gentamycin, neomycin, rimfapicin and streptomycin. Also in other water bodies (both freshwater [27, 29] and marine ones [24, 26]) a high level of bacterial resistance to ampicillin, penicillin and tetracycline was noted with a simultaneous high sensitivity to gentamycin and streptomycin.

The studies carried out by Jones et al. [17] in lake Michigen, and Hermansson et al. [13] along the Swedish

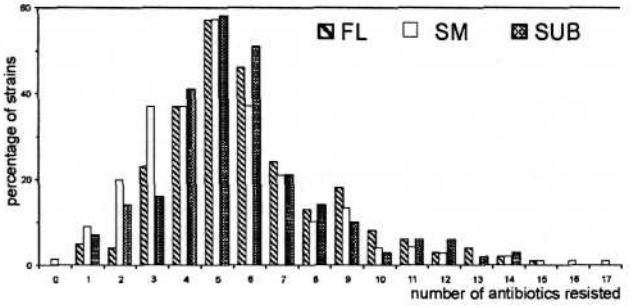

Fig. 4. Multiple antibiotic resistance bacterial strains inhabiting surface and subsurface waters of lake Gardno.

west coast showed that bacteria inhabiting the surface layers were much more resistant to antibiotics than those isolated from subsurface water. By contrast, the results obtained in the present study do not show nearly any differences in antibiotic resistance between neustonic nad planktonic bacteria, which goes along with the results obtained by Mudryk and Skorczewski [24] in Gdansk Deep. According to Klech and Lee [19], and Nemi et al.[26] the antibiotic resistance of bacteria depends on their taxonomic position rather than their origin because particularly taxonomic groups of bacteria have similar genetic information with codes for antibiotic resistance.

Nair et al. [25] determined that pigmented bacteria were more resistant to antibiotic than non-pigmented ones, whereas Mudryk and Skorczewski [24] discovered a contradictory principle. In this study besides gentamycin

no differences in the antibiotic resistance between pigmented and non-pigmented bacteria were noted.

Bacteria inhabiting many water basins are characterised by multiple antibiotic resistance. Multiple resistance is a phenomenon whose mechanisms have not yet been well recognised. According to Herwig et al. [14] multiple resistance may be coded on plasmids, mutational events or on even smaller and mobile genetic elements called transposons. Transposons are able to move between plasmids and bacterial chromosomes. The present study shows that bacterial strains inhabiting the waters of lake Gardno are characterised by multiple antibiotic resistance. Most of the microrganisms were resistant to 4-9 antibiotics used in this study. This indicates that estuarine bacteria are perfectly capable of detoxicating these antibacterial substances.

According to Foster [9] and Nair et al. [25] bacterial resistance to antibiotics is dependent on their chemical structure, which is well confirmed by the results of the present investigation. Neustonic and planktonic bacteria isolated from Gardno lake were most susceptible to aminoglycosides and rifampicins. These antibiotics interfere with bacterial ribosomal translation and consequently inhibit the synthesis of protein. Mudryk and Skorczewski [24] obtained identical results during their investigations of bacterial resistance to antibiotics in the Gdansk Deep.

A high proportion of strains inhabiting the water of lake Gardno were resistant to β-lactam antibiotics, which inhibit enzyme activity taking part in the biosynthesis process of cell walls [9]. Those results point to the fact that bacteria are capable of detoxifying these antimicrobial agents. The resistance of bacteria to β-lactam antibiotics lies in their ability to synthesise three extracellular enzymes; β-lactamase, acylase and penicillinase. These biocatalysts limit the permeability of the cytoplasmatic membrane to those antibiotics or transforms these compounds by hydrolysis of the β-lactam bond into antibiotically inactive penicilloic acid [13, 14].

The results presented in this paper have proven that antibiotics are a significant selection factor and probably are important in regulating the composition of bacterial communities in estuarine environments. Hence, further studies are necessary to establish the role of antibiotic substances in control of estauarine bacterial populations.

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