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INHIBITORY EFFECT OF ANTIBIOTICS ON THE GROWTH OF HETEROTROPHIC BACTERIA INHABITING MARINE BEACH

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Abstract

The objective of the present study was to evaluate heterotrophic bacteria capable of growth in the presence of different antibiotics and their mixture in such dynamic ecosystem as marine beach. Mixture antibiotics had the strongest inhibitory effect on the growth of bacteria inhabiting sand of studied beach. Culturable bacteria were more resistant to ampicillin than to novobiocin and tetracycline. Inhibitory influence antibiotics on growth bacteria inhabiting studied beach were in the following order: mixture antibiotics > novobiocin > tetracycline > ampicillin.

Key words: marine beach, heterotrophic bacteria, antibiotic resistance

INTRODUCTION

Recently, there has been a growing interest in the presence of different pharmaceutical substances, mainly antibiotics, in the aquatic environment (Reinthaler et al. 2003, Schwartz et al. 2003). Antibiotics and their metabolites are discharged in various amounts into the aquatic environments as a result of indiscriminate use of those organic compounds in medical, veterinary, agriculture, animal husbandry and aquaculture practices (Lin et al. 2004, Alpay-Karaoglu et al. 2007). It had been demonstrated that antibiotics are generally, poorly absorbed by the animal and human body, and thus are extracted unchanged or transformed, via urine and feces mainly to soil and water (Gulkowska et al. 2007). In water bodies, concentration of antibiotics ranges from 0.01 to 9.5 $\mu g \cdot dm^{-3}$ in the water and from 0.1 to 10.0 $mg \cdot kg^{-1}$ in the sediment (Backhaus and Grimme 1999). Although antibiotics are discharged into the water basins in very low concentrations these pharmaceuticals are of poor degradability and showed direct toxic effect to aquatic organisms, mainly bacteria (Kümmerer et al. 2000, Batt et al. 2006, Gulkowska et al. 2007, Tamtam et al. 2008). Antibiotic substances may strongly affect quantitative and qualitative composition of bacteriocenoses in water ecosystems and may also eliminate sensitive bacteria,

which might play an important ecological role (Chelossi et al. 2003, Costanzo et al. 2005). On the other hand the number of antibiotic resistant bacteria in aquatic environments has increased dramatically as a consequence of the widespread use of these drugs by humans (Blyela et al. 2004, Matyar et al. 2007, Dang et al. 2008). The rapid increase in the number of antibiotic resistant and multi-resistant bacteria is due, in part, to the ability of these organisms to transfer antibiotic resistance agent among the bacterial population by cell to cell contact and transfer among bacteria genetic elements such as plasmids (Meirelles-Pereira et al. 2002).
In most studies on development of antibiotic resistant bacteria inhabiting lakes (Walczak and Donderski 2004), rivers (Tamtam et al. 2008) and marine environments (Hermansson et al. 1987, Nair et al. 1992, Mudryk 2005) the single disc diffusion method was applied. There are relatively few studies on the resistance of water bacterial community to antibiotics, in which these organisms were directly exposed to a variety of antibiotics (Lobova et al. 2002, 2008, Schwartz et al. 2003 and Matyar et al. 2007). Therefore an objective of the present study was to evaluate capability of heterotrophic bacteria to grow in the presence of single antibiotics and their combination in such a dynamic ecosystem as marine beach.

MATERIALS AND METHODS

Study area and sampling

The study was carried out on marine non-tidal sandy beach (54°43′N, 17°14′E) near Czołpino locality (southern Baltic Sea), (Fig. 1). It represents a dissipative beach type with longshore bars and troughs and have a slope of 7° and a width of the beach is about 65 m. In general, fine and medium grained and sand grains have a size between 0.125-0.250 mm. Studied beach influenced by conditions of the open sea is localized in the Slovinski National Park – World Biosphere Reserve, which characteristic protected elements are mainly the only one in Europe sandbars with moving dunes. Dunes which are moving with the speed of 5-30 m yearly are covered by "sand-loving" saltgrasses as: *Cakile maritima baltica, Honckenya peploides*, *Ammophila arenaria*, *Calammophila baltica*, *Corynophorus canescens*, *Helichrysum arenarium* and *Linaria odora.*
People rarely visit the studied beach that is localised in the national park, and so the level of human pressure is relatively low there.
Sand samples were collected at Czołpino beach during autumn 2008 from a transect marked along a profile formed perpendicularly to the shoreline, four sampling sites were located along this transect (Fig. 1). Site 1 was located approximately 1-1.5 m from the waterline into the water, at a depth of about 1 m; site 2 was situated at the waterline; site 3 lay halfway up the beach, at a 30 m distance from the shore, and site 4 lay in a sheltered place among the dunes, 60 m away from the shore.
Sand core samples were taken with a hand-operated sampler (length – 80 cm, inner diameter – 10 cm), five per site. In the field, the sampled sand cores 15 cm long were divided vertically into two sections: 0-5 cm and 10-15 cm and placed in sterile

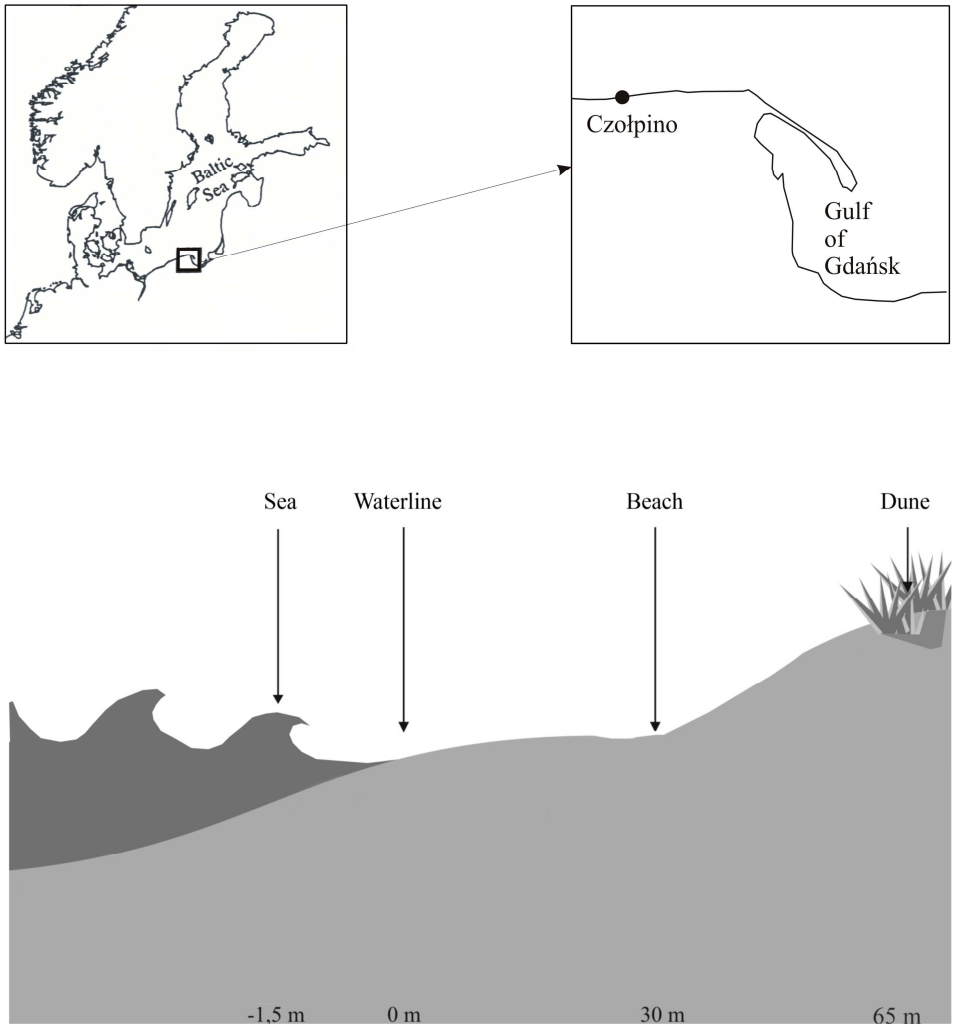


Fig. 1. Location of sampling sites on the sandy beach in Czołpino

plastic boxes which were put into containers with ice and transported to the laboratory. The time between sample collection and performance of bacteriological analyses did not usually exceed 2-3 h.

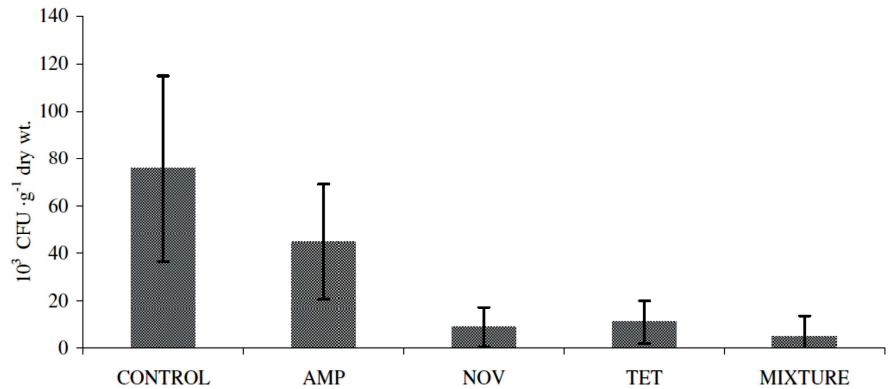
Determination of antibiotics inhibitory effect on heterotrophic bacteria growth

In order to determinate inhibitory effect of antibiotics on heterotrophic bacteria growth, 5 g sand in 45 cm^3 sterile seawater were sonificated in sonificator (Bandelin electronic sono plus 70W, 20 kHz) for 60 s in order to desorb the microorganisms normally stuck to sand grains. Following 30 min sedimentation, the supernatant was serially diluted with sterile seawater to reach final concentration ranging from 10^{-2}

to 10^{-5}. Those, in the amount of 0.2 cm^3, were in triplicate inoculated by the spread plate method onto ZoBell agar (ZB) prepared with old brackish water (salinity 8‰) (Rheinheimer 1977) supplemented with antibiotics at a concentration of 50 μg cm^{-3} according to Lobova et al. (2008). The investigation involved three antibiotics used widely in the medical practice: ampicillin (AMP – an inhibitor of the cell wall synthesis), novobiocin (NOV – a disintegration of DNA structure), tetracycline (TET – an inhibitor of protein synthesis) and their mixture. Antibiotics were first diluted in sterile distilled water and than mixed with molten agar at approximately 40-45 °C. Also, ZoBell agar without antibiotics was used as control. Plates were incubated 7 days at 20 °C, after which the number of microorganisms was counted and the results were calculated as colony forming units (CFU) per 1 gram of the dry weight of sand. Bacteria growing on medium containing one antibiotic were regarded as antibiotic resistant and mixture of antibiotics were regarded as multi-resistant. An additional sample of 10 g of sand was weighed (RADWAG WPS30S) and dried at 105 °C in order to determine the dry weight of the sand.

RESULTS

In Figure 2 are presented data about the effect of ampicillin, novobiocin, tetracycline and their mixture on the growth of heterotrophic bacteria inhabiting sand of marine beach near Czołpino. These data indicate that mixture of three studied antibiotics had stronger inhibitory effect on growth of heterotrophic bacteria than single antibiotics. Multi-resistant bacteria capable of growing on ZoBell agar supplemented with antibiotics mixture accounted only 6% ($4.7 \cdot 10^3$ CFU g^{-1}dry wet) of the total culturable heterotrophic bacteria when compared to the control ($75.7 \cdot 10^3$ CFU g^{-1}dry wet). In presence of novobiocin and tetracycline in ZoBell medium capable to grow were 12-14% ($8.8\text{-}11.0 \cdot 10^3$ CFU g^{-1}dry wet) of heterotrophic bacteria inhabiting sand of studied beach. The lowest inhibitory influence on growth of studied bacteria showed



I* – vertical bars indicate standard deviation

Fig. 2. Number of heterotrophic bacteria in the sandy beach in Czołpino capable of growth in the presence of single antibiotics and their mixture (data of all sites and sand layers)

ampicillin. The ampicillin resistant bacteria accounted 59% ($45.0 \cdot 10^3$ CFU g^{-1}dry wet) of total culturable heterotrophic bacteria.

Data on antibiotics' inhibitory effect on growth of heterotrophic bacteria isolated from different parts of the beach are given in Table 1. On all studied sites mixture of three studied antibiotics had the strongest inhibitory effect on heterotrophic bacteria`growth. Between 0.2-0.3% (waterline, beach, dune) and 27% (sea) of the present multi-resistant bacteria grew on media supplemented with antibiotics mixture. The strongest inhibitory effects of novobiocin and tetracycline on bacteria growth were noted on waterline (1.3-2.2%) and dune (5.0-7.9%) and the lowest on sea (32.3-33.2%). In the case of ampicillin the strongest inhibitory influence on bacteria growth was noted on dune (37.2%) and the lowest on waterline (94.8%).

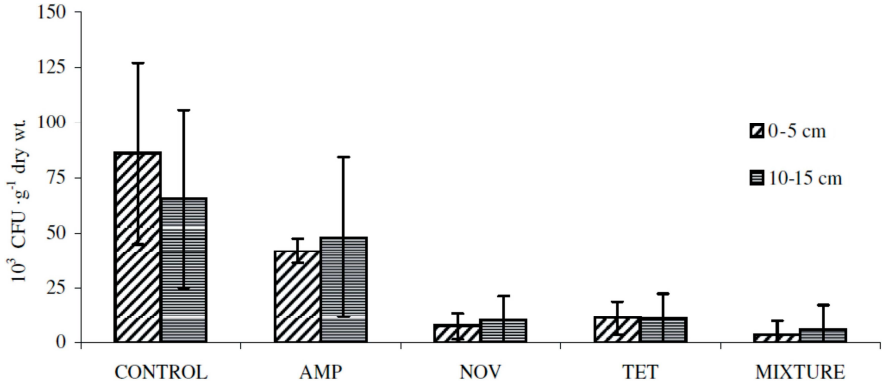
Inhibitory effect of different single antibiotics and their mixture on growth of heterotrophic bacteria inhabiting surface (0-5 cm) and subsurface (10-15 cm) sand layers are demonstrated in Figure 3. These data indicate that, generally inhibitory influ-

Table 1

Numbers of heterotrophic bacteria inhabiting sand of studied beach capable of growth in the presence of single antibiotics and their mixture (data of both sand layers)

Medium	Number of heterotrophic bacteria ($10^3 \cdot g^{-1}$ dry wt.)			
	sea	waterline	beach	dune
ZB	65.9	75.1	43.2	118.8
ZB+AMP	34.9 (53.0)*	71.2 (94.8)	29.8 (68.9)	44.1 (37.2)
ZB+TET	21.9 (33.2)	1.7 (2.2)	11.1 (25.7)	9.3 (7.9)
ZB+NOV	21.3 (32.3)	1.0 (1.3)	7.1 (16.4)	6.0 (5.0)
ZB+AMP+TET+NOV	18.3 (27.4)	0.1 (0.2)	0.1 (0.3)	0.4 (0.3)

* percentage values are given in the brackets



I* – vertical bars indicate standard deviation

Fig. 3. Effect of antibiotics and their combination on growth of heterotrophic bacteria inhabiting surface and subsurface sand layers

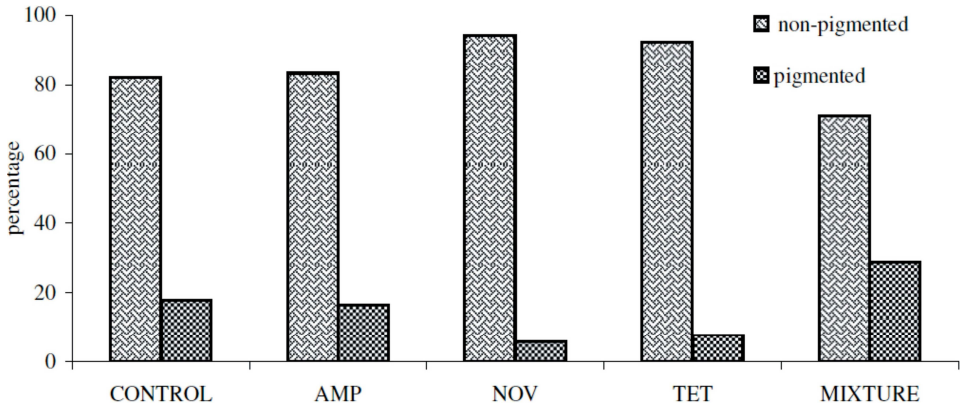


Fig. 4. Differential influence of tested antibiotics and their mixture on the growth of pigmented and non-pigmented bacteria (percentages derived from the pooled data of all sites and sand layers)

ence of antibiotics and their mixture on the growth of bacteria isolated from both studied sand layers was similar. Figure 4 presents the results of the studied influence of three antibiotics and their combination on growth pigmented and non-pigmented bacteria isolated from sand of the Czołpino beach. These data indicate that in presence of ampicillin in ZoBell medium percentage of pigmented and non-pigmented were similar when compared to the control sample. Novobiocin and tetracycline inhibited growth of chromogenic bacteria whereas mixture of antibiotics reversely – stimulated the growth of chromogenic bacteria in sand of studied beach.

DISCUSSION

According to Backhaus and Grimme (1999) and Costanzo et al. (2005) antibiotics kill or inhibit growth of bacteria inhabiting aquatic environments. However, many bacteria have developed several different and very efficient mechanisms for tolerating antibiotics (Schwartz et al. 2003). The mechanisms by which microorganisms exhibit resistance to antibiotics include mainly drug inactivation or modification of target site alteration, alteration in the metabolic pathway and reduced drug accumulation (Hermansson et al. 1987, Alanis 2005, Liasi et al. 2009).

The studies carried out by Sturtevant et al. (1971) and Guardabassi et al. (2002) showed that antibiotics mixture had stronger inhibitory effect on the growth bacteria than single antibiotics. Our observations agree with those of previous workers on influence of different antibiotics combination on the growth of aquatic bacteria. The fraction of multiresistant bacteria oscillated from 0.2 to 27% of the total culturable heterotrophic bacteria isolated from sand marine beach.

Among three antibiotics tested in this study novobiocin and tetracycline had stronger inhibitory effect on growth of heterotrophic bacteria than ampicillin. Similar results

were obtained by Blyela et al. (2004) in river, Akinbowale et al. (2006) in aquaculture and Ahmed et al. (2008) in lake.

Hermansson et al. (1987) have studied antibiotic resistance in bacteria isolated along the Swedish west coast – they reported that 0.1-11% bacterial isolates were resistant to novobiocin. Similar observations were made in this study. Only 12% of heterotrophic bacteria inhabiting sand of studied beach were resistant to novobiocin. This antibiotic is one of aminocoumarin antibiotics that were produced by the actinomycetes and is effective chiefly against Gram-positive bacteria (Barret-Bee et al. 1994). Novobiocin inhabit bacterial DNA biosynthesis by targeting the enzyme DNA gyrase, inhabits DNA topoisomerases and blocks adenosine triphosphatase activity (Downs et al. 1985).

According to the results of this study, it is apparent that bacteria isolated from studied beach beside to neomycin also low (14%) level of resistance showed to tetracycline. This means that tested bacteria were not capable of detoxifying that antimicrobial agent. Several studies (Chelossi et al. 2003, Miranda et al. 2003, Mudryk 2005, Akinbowale et al. 2006, Oliveira and Pinhata 2008**)** focused on a low level of resistance to tetracycline in marine isolates. Tetracycline broad-spectrum antimicrobial agent with activity against a wide range of Gram-positive and Gram-negative bacteria (Walczak and Donderski 2004, Alpay-Karaoglu et al. 2007, Dang et al. 2008). Tetracycline inhibits protein synthesis by preventing the attachment of aminoacyl tRNA to the ribosomal acceptor (A) site (Chopra and Roberts 2001, Chelossi et al. 2003). According to Batt et al. (2006) and Brown et al. (2006) tetracycline residues tend to be sorbed by organic matter and they can accumulate in range of several hundred micrograms per kilogram of soil and aquatic sediments as sand of marine beach.

The studies carried out by Lobova et al. (2002), Chelossi et al. (2003), Schwartz et al. (2003), Walczak and Donderski (2004), Akinbowale et al. (2006) in different water basins showed high bacteria resistance to ampicillin. High level of bacteria resistance to ampicillin indicated that the ampicillin resistance gene may be widely present in the gene pool of bacteria inhabiting aquatic environments (Lin et al. 2004). Also in the present study from three studied antibiotics the least inhibitory effect on the growth of the studied bacteria inhabiting sand of marine beach showed ampicillin. The ampicillin resistant bacteria accounted 59% of total culturable heterotrophic bacteria. This means that nearly 60% of tested bacteria were capable of detoxifying that antimicrobial agent. According to chemical structure, ampicillin is β-lactam antibiotic. β-lactams are the most widely used (approximately 50% of global antibiotic consumption) because they have low toxicity and are used to treat a broad range of infections (Livermore 1996, Liasi et al. 2009). These antibiotics inhibit bacterial cell – wall synthesis by interrupting the transpeptidation process that links the peptidoglycan components of the bacterial wall to each other (Roberts 1998, Backhaus and Grimme 1999, Alanis 2005). The resistance of bacteria to β-lactam antibiotics is due to their ability to synthesise three extracellular enzymes: β-lactamase, acylase and penicillinase, which can limit the permeability of cytoplasmic membranes to those antibiotics (Hermansson et al. 1987, Herwig et al. 1997). The primary bacterial resistance mechanism is enzymatic inactivation through the cleavage of the β-lactam ring by β-lactamases (Aminov and Mackie 2007).

The studies carried out by Mudryk (2005) at marine sandy beach in Sopot showed that bacteria isolated from surface sand layers were more resistant to nearly all tested antibiotics than bacteria from the subsurface sand layers. In the present study, generally no level differences in antibiotic resistance between bacteria inhabiting surface and subsurface sand layers of marine beach near Czołpino were found.
From the results of this study, it is apparent that antibiotics have influence on the level resistance of pigmented and on non-pigmented bacteria inhabiting sand of marine beach. This corresponds with the results obtained by Hermansson et al. (1987) along the Swedish west coast, Nair et al. (1992) in different regions of the Arabian Sea and by Mudryk and Skórczewski (1998) in the Southern Baltic.

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INHIBITOROWY WPŁYW ANTYBIOTYKÓW NA WZROST HETEROTROFICZNYCH BAKTERII ZASIEDLAJĄCYCH PLAŻĘ MORSKĄ

Streszczenie

W pracy przedstawiono wyniki badań dotyczących określenia hamującego wpływu różnych antybiotyków i ich mieszaniny na wzrost heterotroficznych bakterii zasiedlających piasek plaży morskiej zlokalizowanej na terenie Słowińskiego Parku Narodowego w rejonie Czołpina. Próby piasku na tej plaży pobierano w profilu horyzontalnym z czterech stanowisk (morze, strefa brzegowa, środkowa część plaży, wydma), a w profilu wertykalnym na każdym stanowisku z dwóch (0-5 cm, 10-15 cm) głębokości. Badania te wykazały, że mieszanina antybiotyków w podłożu hodowlanym wywierała bardziej hamujący wpływ na wzrost bakterii zasiedlających badaną plażę niż pojedyncze antybiotyki. Wśród testowanych antybiotyków neomycyna i tetracyklina wykazywały znacznie większy niż ampicylina hamujący wpływ na wzrost bakterii. Wykazano, że hamujący wpływ antybiotyków i ich mieszaniny na wzrost bakterii zasiedlających powierzchniowe i podpowierzchniowe warstwy piasku był podobny. Stwierdzono, że testowane antybiotyki i ich mieszanina miały wpływ na wzrost chromogennych i achromogennych bakterii zasiedlających piasek badanej plaży morskiej.