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The Influence of Selected Antibiotics on Aquatic Microorganisms

ANTONI MIERNIK* and MARZENNA RZECZYCKA

Department of General Microbiology, Institute of Microbiology, Faculty of Biology,
University of Warsaw, Warsaw, Poland

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Abstract

This paper presents the influence of penicillin, erythromycin, oxytetracycline and streptomycin on aquatic microorganisms isolated from three reservoirs with varied extent of environmental pollution (Sulejów Reservoir, Zegrze Reservoir and Vistula River). The experiments were conducted in especially prepared microcosms (aerated 15 l aquariums). From among the examined antibiotics streptomycin showed the longest activity in the water environment (27 days), followed by oxytetracycline (22 days). Erythromycin had 13 days activity while the penicillin only 4 days. The highest number of bacteria was observed in the Vistula, fewer in Zegrze Reservoir. Sulejów Reservoir was characterized by the lowest number of bacteria. The strains isolated from Sulejów Reservoir were characterized by higher degree of biodiversity than those from Zegrze Reservoir or Vistula River. The dominant genera were *Pseudomonas* (49%), followed by *Acinetobacter* (21%), *Flavomonas* (9%), *Alcaligenes* (9%), *Vibrio*, *Moraxella* and *Oligella*. The Vistula River was dominated by *Aeromonas* (88%) and *Pseudomonas* (12%) genera. The strains isolated from Sulejów Reservoir were more sensitive to antibiotics than those from the much more polluted Vistula, this being evidenced by lower MIC and MBC values.

Key words: antibiotics in water environment, water microorganisms

Introduction

Antibiotics are an important group of pharmaceuticals in today's medicine. Besides treatment of human infections, they are used in veterinary medicine, animal husbandry and agriculture (Khachatourians, 1998). In Europe alone we consume 10 000 tons of antibiotics per year (Kümmerer, 2003). Annual estimates for antibiotics application in US agrifood industry are 8 million and 22 tons for animals and fruit trees, respectively (Gillespie, 2004).

About 90% of antibiotics used in agrifood industry are growth-promoting agents; only 10% of them are used against bacterial diseases (Troussellier *et al.*, 2002).

Extensive antibiotics application in animal husbandry from the early 50s sped up the growth and body weight increase of chicken, swine and sheep. Using small doses of these compounds (70–140 g of chlortetracycline or erythromycin per one ton of food) a body weight increase from 3 to 7% (Gillespie, 2004) was achieved. Inconsiderable amounts of these substances that might to be supplied to animal food for significant biomass increase encouraged applying them on a large scale. In 1960 the first reports on the influence of tetracycline concentrations in chicken food on the emergence of resistant staphylococci in the environment appeared. In the United States food products from farms using antibiotics were examined. It appeared that they exceeded all permitted standards by even 25%. The consumption of antibiotics in agriculture is from 100 to 1000 times higher than in medicine (Dewey *et al.*, 1997).

The appearance of new antibiotics has resulted in acquired resistance to these new compounds. This may be provoked by mutation, conjugation, transduction, transformation and transposition (Hughes and Datta, 1983). Another bacterial resistance source is plant cultivation and connected with this spraying of fields and orchards with antibiotics. In the US, for instance, consumption for the above-mentioned purpose is about 22 tons a year (Marshall and Petrowski, 1990). The most frequently used are tetracyclines and streptomycin. About 15–19 tons of antibiotics are used yearly for plant cultivation. Bacteria causing fruit trees infections (*Ervinia*) may become resistant

Corresponding author: A. Miernik, Department of General Microbiology, Institute of Microbiology, ul. Miecznikowa 1, 02-096 Warsaw, Poland; phone (48) 22 5541431; **e-mail: a.miernik@biol.uw.edu.pl**

to specified class of substances and transfer resistance genes to human pathogenic bacteria (*Salmonella*), (US Office, 1995).

Usually antibiotics are sprayed on more important surfaces, such as plant raising areas. As the result of various abiotic factors (wind, waterfall, wastewaters) and biotic (insects) they may reach natural water ecosystems (Gottlieb, 1976). A consecutive reason for using antibiotics is prolonging the freshness of vegetables, fruits and flowers. During the washing process part of the antibiotics are washed away directly to the ground, part of them reaches the food chain (Levy, 1992).

As the result of current antibiotics use the presence of these substances in water environment has been detected, especially when the possibility of their penetration to natural waters exists, but data concerning antibiotics and products of their degradation concentration in this environment are rather limited. It was determined that the amount of erythromycin in rivers oscillates around 1 µg/l (Watts *et al.*, 1983). Degradation products of erythromycin or roxithromycin in water environments up to 6 µg/l were found (Hirsch *et al.*, 1999). In a stream having no contact with wastewater, 2–100% of the bacteria in it were found to be resistant to streptomycin, erythromycin, vancomycin, tetracycline or penicillin.

Antibiotics have different biodegradation times and they have been found to persist in the environment much longer than was previously supposed. Their traces have been detected in trout muscle after 77 days treatment. Fish consume only about 20% antibiotics, the remaining 80% stays in the environment. Oxytetracycline biodegradation in fish is slow; half-life is estimated at from 30 to 142 days (Samuelsen, 1989).

Antibiotics biodegradation seems to be a very significant problem in the environment. Antibiotic toxity depends upon biodegradation processes and antibiotic suspension mass (Hektoen *et al.*, 1995). Bottom sediments show detectable antibiotic concentrations even 180 days after introduction of antibiotics. The half-life of some antibiotics is estimated at 300 days (Halling-Sørensen *et al.*, 1998). The drugs enter the aquatic environment and may reach drinking water if they are not biodegraded or eliminated during sewage treatment.

Elimination of different drugs during passage through municipal sewage treatment is more than 60%, but it should be stressed that some antibiotics like erythromycin or tetracycline are not degradable in sewage treatment (Gottlieb, 1976; Ternes, 1998). Antibiotics elimination rate depends on a number of different factors (Blok and Booy, 1984; Shimp and Pfeander, 1985; Steffenson and Alexander, 1995; Wang *et al.*, 1995; Zaidi and Mehta, 1995). From the ecological point of view estimation of the toxic influence of antibiotics on soil and water bacteria seems to be very important. Antibiotics as biologically active substances are affecting quantitative and qualitative microflora composition (Kreuger, 1992; de Oliveira and van Elsas, 1995).

The aim of this study was: 1) determinination of the changes in aquatic bacteria number depending on streptomycin, erythromycin, oxytetracycline and penicillin in the water, 2) isolation and identification of the water bacteria, 3) determination of the sensitivity of isolated strains to selected antibiotics.

Experimental

Materials and Methods

Isolation of bacteria. Bacteria were isolated from: Sulejów Reservoir (Smardzewice pier), Zegrze Reservoir (500 Chain Hotel pier) and Vistula River (Warsaw, in the vicinity of the Princess Anne Gynecological Hospital). (Miernik, 2004). To isolate bacteria from the natural environment suitable samples from the above indicated sites were diluted (Gerhardt and Murray, 1981) plated and incubated for 48 h at 20°C. The number of bacteria was determined by the plate method on nutrient agar.

Antibiotic activity assay. The experiments were conducted in 15 l volume aerated (1 l per min) microcosms at the temperature of about 20°C. Microcosms were filled with water from three reservoirs with varied extent of environmental pollution (Sulejów Reservoir, Zegrze Reservoir and Vistula River). Each aquarium contained 1000 µg of penicillin, erythromycin, oxytetracycline or streptomycin per 1 liter. Control aquarium contained no antibiotics. Antibiotic activity assay was determined during 28 days experiments.

To determine antibiotic activity in water the agar diffusion cylinder test was used. The following media were used: for streptomycin and erythromycin medium No 11 (Merck Cat. No 105269.0500), for oxytetracycline and penicillin medium No 2 (Merck Cat. No 105270.0500). Procedure: Petri dishes were filled with 14 ml of medium to form the base layer, and after setting this was overlayed with 4 ml of seed layer inoculated with appropriate test strain. For streptomycin this was *Bacillus subtilis* ATCC 6633, for erythromycin *Micrococcus luteus* ATCC 9341. For oxytetracycline *Bacillus cereus* ATCC 1178 was used, for penicillin *Staphylococcus aureus* ATCC 6538P. Four cylinders were placed on each ready-to-use test plate. They were filled with filtered water from the examined aquariums. Water samples were filtered (every 24 hours) using 0.2 µm filters. Petri dishes were incubated 24 h at 37°C. Measurement of inhibition zones in comparison to the control was the base for determination of antibiotic activity.

MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) determination. To determine MIC an antibiotic dilution assay technique was used (Markiewicz and Kwiatkowski, 2001). Test tubes containing serial dilutions of antibiotics were inoculated with a standard amount of the test organism and incubated 24 hours at 25°C. Following antibiotic concentrations (mg/l) were used: 0.101; 0.203; 0.406; 0.813; 1.625; 3.25; 6.5; 12.5; 25.0; 50.0; 100.0 along with the control without antibiotic. As the test microorganisms we used: *Bacillus subtilis* ATCC 6633 (for streptomycin), *Micrococcus luteus* ATCC 9341 (for erythromycin), *Bacillus cereus* ATCC 1178 (for oxytetracycline) and *Staphylococcus aureus* ATCC 6538P (for penicillin).

To determine MBC, a dilution assay technique was used (Markiewicz and Kwiatkowski, 2001).

From the appropriate dilutions (MIC) in the test tubes where growth was not observed 0.1 ml samples were plated and incubated for 48 h at 20°C. The lowest concentration not giving any colony forming units (cfu) indicated the MBC.

Identification of bacteria. Identification was based on the following diagnostic tests: microscopic observations, API-20 NE system, (bioMérieux). The API-20 NE system is a standarized micromethod combining 8 conventional tests and 12 assimilation tests for the identification of non-fastidious Gram-negative rods not belonging to the *Enterobacteriaceae.*

Effect of antibiotic on the number of bacteria. Suitable samples of examined polluted reservoirs were diluted, plated on Petri dishes filled with 12 ml of agar medium with appropriate amount of antibiotics – 50; 100; 200; 400; 600; 800 or 1000 µg/l. The dishes were incubated 10 days at 20°C.

Results and Discussion

This paper presents the influence of penicillin, erythromycin, oxytetracycline and streptomycin on aquatic microorganisms from three reservoirs with varied extent of environmental pollution. From among the examined antibiotics streptomycin showed the longest activity in the water environment (27 days), followed by oxytetracycline (22 days). Erythromycin was active for 13 days while penicillin for only only 4 days (Fig. 1). Protracted antibiotic activity is very dangerous, especially in the case of tetracyclines, which are extremely toxic (Halling-Sørensen, 2000; Hamscher *et al.*, 2000).

Antibiotics (streptomycin and oxytetracycline), which have showed the longest activity in the water environment (Fig. 1) brought about a significant decrease in the number of bacteria, correlated with increasing concentration of antibiotics (Fig. 2). It seems necessary to underline that the highest number of bacteria was observed in Vistula River (the most polluted reservoir from among the examined water bodies), followed by Zegrze Reservoir. The smallest number of bacteria was observed in Sulejów Reservoir – the least polluted of the examined reservoirs (Fig. 2). In the case of erythromycin as well as the penicillin only a slight influence on the number of bacteria was observed. Similar results were observed in the case of penicillin. It seems quite evident because the dominant bacteria in water are Gram-negative bacteria because the Gram-positive bacteria are of allochthonic source. (Donderski and Wilk, 2002; Troussellier *et al.*, 2002).

In our investigations we determined only 12 bacterial genera (Table I). This seems quite obvious because bacteria were isolated by the plate method on

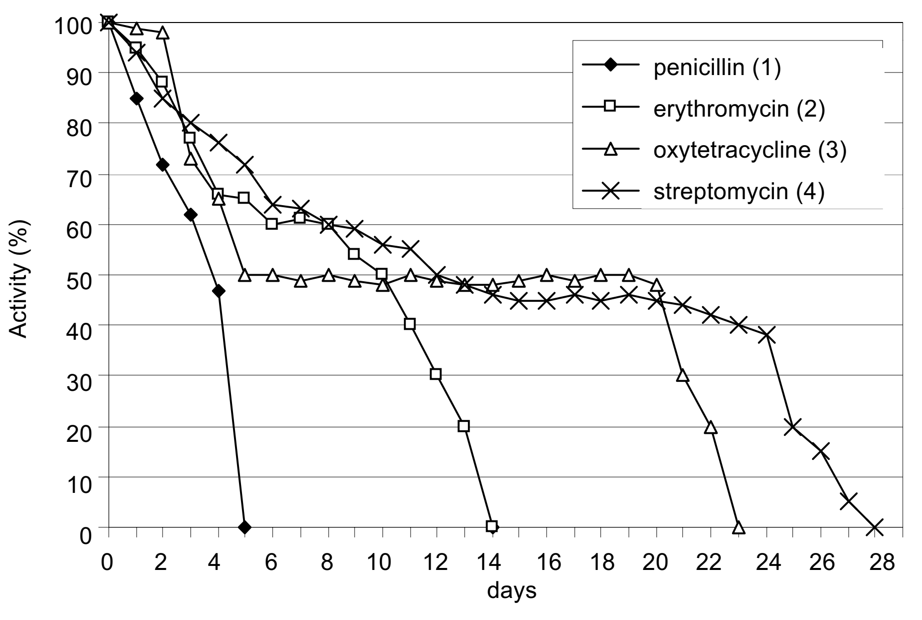

Fig. 1. Changes in activity (%) of penicillin (1), erythromycin (2), oxytetracycline (3) and streptomycin (4) during 28 days of incubation in microcosms (initial concentration of antibiotic 1000 µg/l)

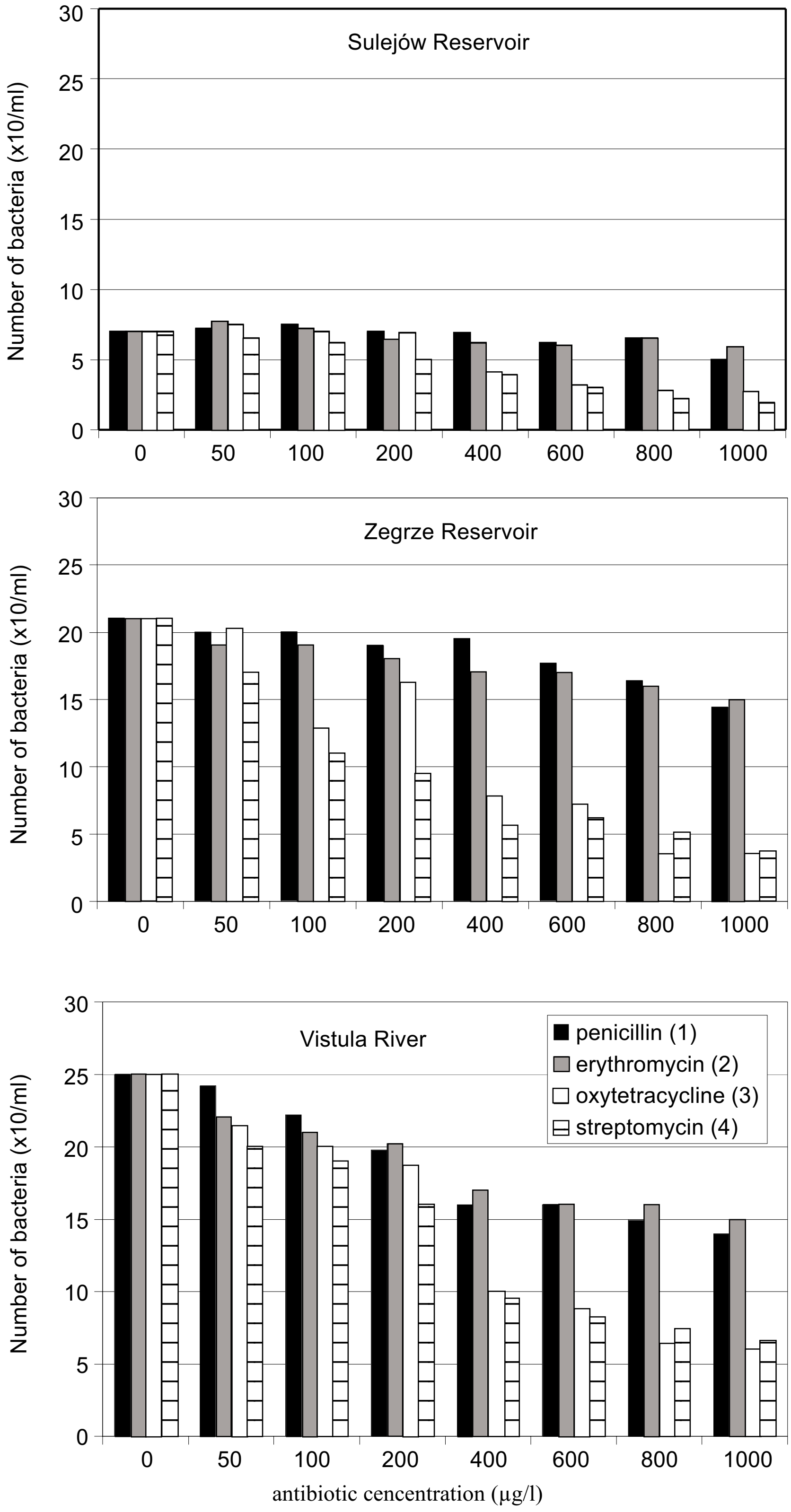

Fig. 2. Changes in the number of bacteria in Sulejów Reservoir, Zegrze Reservoir and Vistula River depending on different concentrations of penicillin (1), erythromycin (2), oxytetracycline (3) and streptomycin (4).

nutrient agar which is a better medium for allochthonic bacteria than for autochthonic that dominate in water environments.

The pollution degree of water reservoir impacts the diversity of bacterial genera. The most polluted Water-Vistula River was characterized by low diversity of genera. The dominant bacteria were *Aeromonas sp*. and *Pseudomonas* sp. (Table I), bacteria characteristic for wastewater microflora (Bahlaoui *et al*., 1997). In general *Aeromonas* sp. pathogenic for human and animals persists in polluted waters, where it is by a few orders of magnitude more frequent than in the first order water. *Aeromonas* may be responsible for gastroenteritis, skin and soft tissue infections, this genera is also present in other European rivers, such as Arga (Spain) and Garonne (France) (Goňi-Urriza *et al*., 2000). The less polluted waters – Zegrze Reservoir and Sulejów Reservoir were characterized by higher diversity of bacterial genera. Dominant genera in Sulejów Reservoir were *Pseudomonas* (49%), *Acinetobacter* (21%), *Flavomonas* (9%), *Alcaligenes* (9%), followed by *Vibrio*, *Moraxella* and *Oligella* (Table I). Sulejów Reservoir, the least polluted of the examined sources of water was characterized by 10 genera of bacteria, in more polluted Zegrze Reservoir there were 5 genera, in the most polluted Vistula River only two dominant genera of bacteria – *Aeromonas* and *Pseudomonas* were observed. A similar relation was observed in the case of microorganisms exposed to heavy metal pollution (Skowroński *et al*., 2002) and wastewaters from nitrogen fertilizer industry (Rzeczycka *et al*., 1987).

Of the examined antibiotics streptomycin and oxytetracycline showed the longest activity in water environment. Therefore MIC and MBC for the above-mentioned antibiotics were determined (Fig. 3, 4). In the less polluted waters of Sulejów and Zegrze Reservoirs (Fig. 3) the MIC of streptomycin varied from 0.1–3.2 or 0.4–25.0 mg/l for 80–90% bacteria. In the most polluted Vistula River (two dominant genera

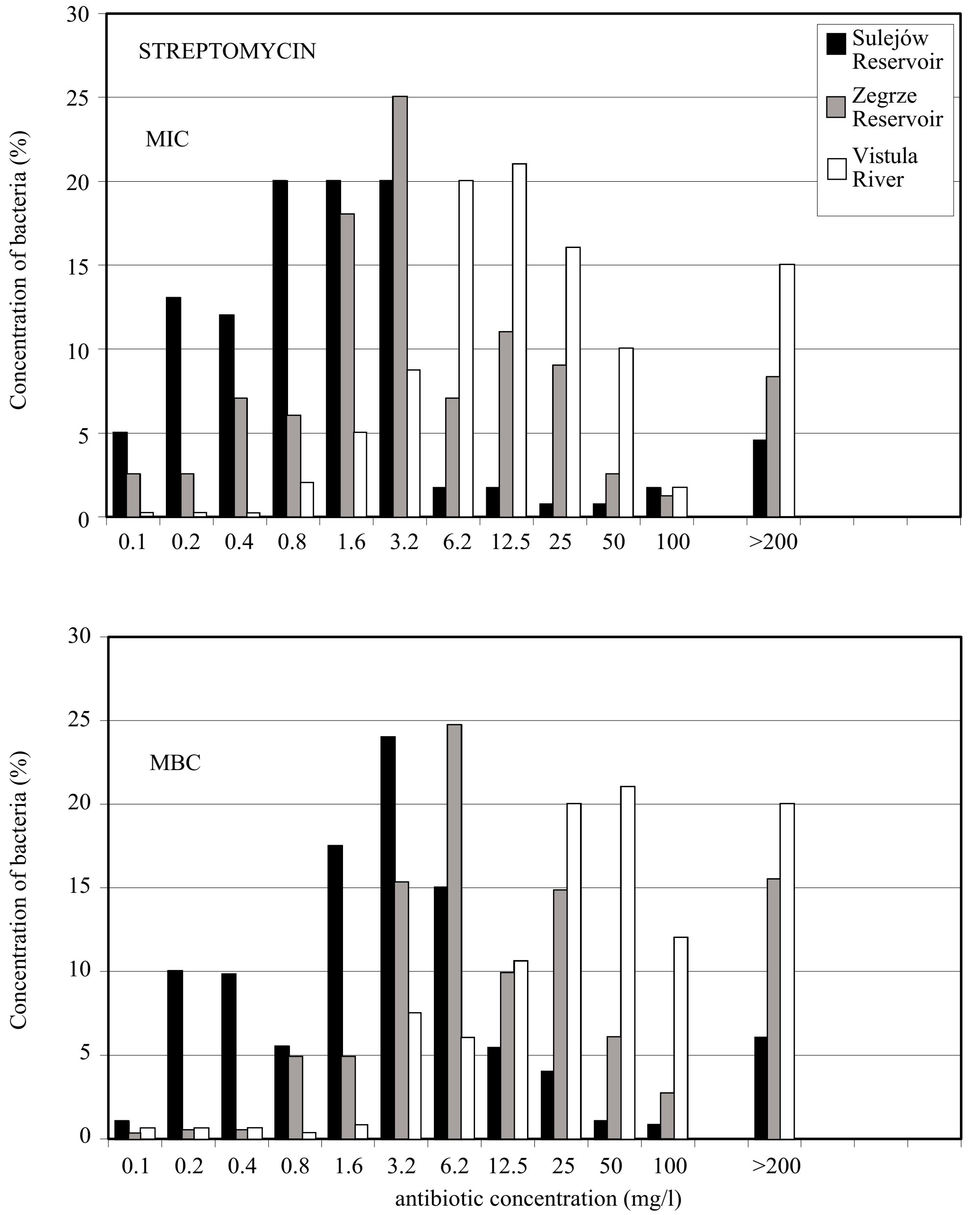

Fig. 3. Streptomycin minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for bacteria (%) from Sulejów Reservoir, Zegrze Reservoir and Vistula River.

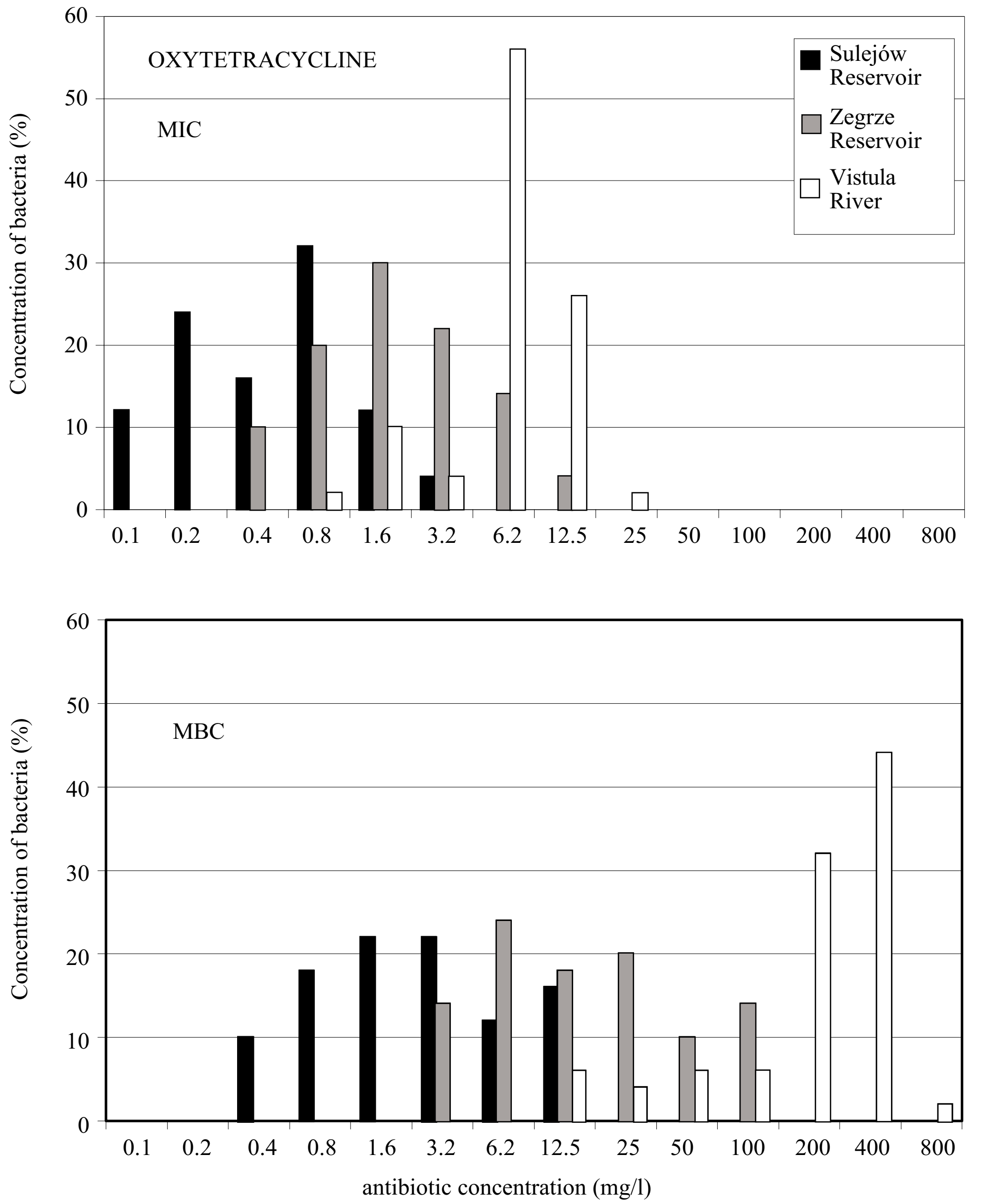

Fig. 4. Oxytetracycline minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for bacteria (%) from Sulejów Reservoir, Zegrze Reservoir and Vistula River.

Aeromonas and *Pseudomonas*) the MIC of streptomycin was 1.6–50.0 mg/l and higher for 80% of bacteria (Fig. 3). In other European Rivers such as the Arga and Garonne, the MIC for streptomycin varied from 4.0 to 32.0 mg/l for 57% of *Aeromonas* strains (Goňi-Urriza *et al*., 2000). In the less polluted waters – Zegrze Reservoir and particularly Sulejów Reservoir a far lower contribution of bacteria for which the MIC of streptomycin was higher than 200 mg/l was observed (Fig. 3).

Values of streptomycin MBC for bacteria from all investigated environments were: 0.2–12.5 mg/l for 87% of bacteria (Sulejów Reservoir), 0.8–50.0 mg/l for 80% of bacteria (Zegrze Reservoir and 3.2–100.0 mg/l for 77% of bacteria (Vistula River) (Fig. 3).

For 96% of bacteria from less polluted Sulejów Reservoir MIC of oxytetracycline varied from 0.1 to 1.6 mg/l. For much more polluted Zegrze this value varied from 0.4 to 6.2 mg/l. In the case of Vistula River we observed 1.6–12.5 mg/l (Fig. 4). The highest oxytetracycline MIC values were observed in the case of Vistula River, the most polluted of the investigated waters. It is worth stressing that even the high MIC values for Vistula are not very impressive in comparison to values from fish farms situated along the Danish stream Vejle, where values of MIC varied from 32 to 256 mg/l for 69% of isolated *Aeromonas* (Schmidt *et al*., 2000).

Values of MBC for oxytetracycline for 100% bacteria from all investigated environments were:

Table I
Composition of bacterial microflora (genera expressed as % of the total isolates) in Sulejów Reservoir, Zegrze Reservoir and Vistula River

Genera	Sulejów Reservoir	Zegrze Reservoir	Vistula River
Pseudomonas sp.	49	54	12
Aeromonas sp.	2	0	88
Sphingomonas sp.	0	18	0
Flavomonas sp.	9	20	0
Stenotrophomonas sp.	0	4	0
Acinetobacter sp.	21	4	0
Agrobacterium sp.	2	0	0
Alcaligenes sp.	9	0	0
Vibrio sp.	2	0	0
Moraxella sp.	2	0	0
Shewanella sp.	2	0	0
Oligella sp.	2	0	0

Table II
MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) of streptomycin and oxytetracycline for 80–90% of bacteria isolated from Sulejów Reservoir, Zegrze Reservoir and Vistula River

Environment	Streptomycin (mg/l)		Oxytetracyline (mg/l)	
	MIC	MBC	MIC	MBC
Sulejów Reservoir	0.1–3.2	0.2–12.5	0.1–1,6	0.4–12.5
Zegrze Reservoir	0.4–25	0.0–50	0.4–6.2	3.2–100
Vistula River	1.6–50	3.2–100	1.6–12.5	12.5–400

0.4–12.5 mg/l (Sulejów Reservoir), 3.2–100.0 mg/l (Zegrze Reservoir) and 12.5–400.0 mg/l (Vistula River) (Fig. 4).

In our studies we showed the effect of chosen antibiotics on aquatic microorganisms from three reservoirs with varied extent of environmental pollution. In the view of the above, we conclude that the degree of water pollution impacts the diversity of bacterial genera. The most polluted Vistula River was characterized by low diversity of genera. The strains isolated from Sulejów Reservoir were characterized by highest degree of biodiversity. They were more sensitive to the examined antibiotics than those from the much more polluted Vistula, this being backed by lower MIC and MBC values (Table II). From among the examined antibiotics streptomycin shows the longest activity in the water environment, followed by oxytetracycline, erythromycin and penicillin.

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