HYDROBIOLOGY IN ENVIRONMENT PROTECTION

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silver nanoparticle, mechanism of nanosilver

Maciej K. BEŁCIK, Agnieszka TRUSZ-ZDYBEK*

EFFECT OF SELECTED SILVER COMPOUNDS ON MICROORGANISMS

Silver is well known for its antibacterial properties, which for many years were used in medicine and pharmacy. In the literature you can find information about the consumption of metallic silver as a medicine for stomach ulcers, or drinking water from the silver cups to protect against plague. In recent years, along with the development of new technologies an increasing interest in compounds of nanosilver, so silver particles with a size less than 100 nm. Components of that type have different properties of the materials from which they are generated. Reduction of particle size results in a change of the mechanical, physical and chemical properties. Effect of nanosilver activity does not causes impairment only one of the vital functions, but series of processes which causes gradual destruction of cells inter alia by covering the cell wall, penetration of the inside a cell activities of mitochondrial, and genetic material disorders. Nanoparticles destroy also fungal cells disturbing the water management and some viruses by decomposition protein-lipid capsid shells. This paper presents an overview of methods obtaining silver nanoparticles and mechanism of their action described in literature. What is more was presented results of described studies confirming the effect of nanosilver action on microorganisms.

1. INTRODUCTION

Silver is the metal with the symbol of Ag, it is characterized by thermal and electrical conductivity, plasticity and strength. It owes its properties due to the crystal structure, forming regular space lattice. The basic cell unit of silver crystals has a cubic shape. Silver properties for years were used in medicine and pharmacy. The ancient Egyptians were dipped metallic silver into water for treating stomach ulcers, Roman legionaries accelerated the healing of injuries by applying to them the silver coins. What's more silver cups protect against plague.

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Antibacterial properties possess compounds such as colloidal protargol and colargol but also lapis - commonly known as silver nitrate.

A new perspective for the bactericidal properties of silver has created a relatively new branch of science which is nanotechnology. The range of interest to researchers in this field are materials and substances built from crystals of a size not exceeding 100 nm. Nanomaterials properties has different from those with which they were formed. Small size allows to change mechanical, physical and chemical properties.

In the case of silver the biochemical activity of nanoparticles is a result of ratio their weight to the surface. The lower is mentioned ratio the higher activity have nanosilver particles [1].

2. OBTAINING A SILVER NANOPARTICLES

Among the many methods of the synthesis silver nanoparticles described in literature, chemical reduction is the most commonly used mechanism for their generation. This method involves the reduction of the silver salt with a reducing agent in the presence of stabilizer enabling to stop the process of connections the nanoparticles into larger agglomerates. These stabilizers cause inhibition of growth in particle size. The time of adding the stabilizers to the reaction mixture depends on the size of the nanoparticles which will be generated. Commonly used silver salt in chemical reduction method is silver nitrate ($AgNO_3$), other compounds might be $AgClO_4$, $AgBF_4$, $AgPF_4$ but unfortunately only silver nitrate guarantee constant reaction speed. Example reducing agents include borohydriate ($NaBH_4$), hyrazine (N_2H_4), ethanol (C_2H_5OH), sodium citrate [2, 3, 4].

Another variation of chemical reduction method is reverse micelle system. This method uses a microemulsion, where the water drops suspended in an oil are reaction medium and the size of the nanoparticles generated is limited by the size of those 'bioreactors' [3, 4, 5].

Obtaining a silver nanoparticles is also possible by a method based on photochemical reduction. This method allows to blend all substrates before initiation of reaction. Photochemical reduction takes place under the UV lamp, where prepared mixture is subjected to irradiation. Diameter of obtained nanoparticles in this method are 1-7 nm [4].

Another method of synthesis nanosilver is microwave irradiation, which is used also to obtain gold nanoparticles. Microwave method of synthesis nanosilver depend on microwave beam, which goes through the material. This radiation energy is transformed into thermal energy, which increase the material temperature [4, 6].

Electrochemical method obtaining a nanoparticles was developed in 1994. Electrochemical method allow to dissolution of silver on an anode and in that same time re-

duction of transitional slat on a cathode. Production of metal particles were stabilized by suitable salts, for example tetraalkyl ammonium salt. Potentiostatic and galvanostatic polarization of silver is based on the similar procedure to described above [3, 4].

Other method to obtain silver nanoparticles involves reactions between solid, which is suspended in liquid medium. The name of this method is laser ablation and depend on transfer solid material into surrounding liquid in a bubble form. This process is triggered by a pulsating laser beam. As a reducing liquid medium are used ethylene or diethylene glycol [3, 4].

Relatively new and not fully understood method of obtaining silver nanoparticles is green synthesis. Nanosilver in green synthesis method is based on enzymatic reaction of *Bacillus subtilis*. Bacterial cultures allows to reduction of metal ions in silver nitrate salt by the mediation of nitrate reductase [4, 7].

3. INFLUENCE OF SILVER ON MICROORGANISMS

The fact that silver compounds cause the inhibition and death of bacterial cells, fungi and spores was observed at the beginning of the nineteenth century. Studies have shown a direct toxicity of the silver ions to the cells of microorganisms. Produced nanosilver did not result in loss of these properties, on the contrary it has created a new possibilities for the use it to destroy bacteria and fungi.

Nanosilver works similar to antibiotics by affecting all the vital functions of microorganisms such as the ability to move and growth, metabolism, reproduction and adaptation skills.

The main action of nanosilver on the microorganisms is the process of surrounding them with a thin layer of metal. This process allows not only to reduce movement by blocking cells cilia and flagella. It allows also to block specific cellular organelles such as fimbria and pilus. Blocking fimbria by a nanosilver doesn't allow the cells to stick the ground. The other cannot participate in the process of conjugation for at becomes impossible to exchange information on adapting to changing environmental conditions. Cells surrounding by the nanosilver layer prevents bacterial cell division and its reproduction.

Cysteine is one of the components bacterial cells wall. This amino acid is built from carbon, hydrogen, oxygen, nitrogen and sulfur. The thiol is a highly reactive group which can be transformed by oxidation to disulfide derivative cysteine. Colloidal nanosilver may catalyze oxygen ions and particles to transform into atomic oxygen, which can react with thiol groups and prevent formation of new cell walls. What's more, this reaction can result in degradation of existing cell walls.

Silver is an excellent conductor to cause disruption of the cell membrane polarization, which disturbs the transport of nutrients to the interior of cells. On catalysis

properties result in denaturation of proteins, including enzymes by occurring free protons which cut of disulfide derivative cysteine. In addition, nanosilver also impairs the process of respiration and energy supply by disruption of respiratory chain. Death of bacterial cells is achieved by deactivation of enzymes and blocking metabolic reactions into them.

Effect of nanosilver activity does not focus only on one of the vital functions of cells, but it causes a number of processes resulting in their gradual destruction. Nanoparticles destroy also fungal cells by disrupting their water management and some viruses by decomposition in protein-lipid capsid shell [1, 2].

4. EFFECT OF NANOSILVER ON SELECTED BACTERIAL STRAINS

In many research into effect of nanosilver on microorganisms the main topic is impact of size and shape nanoparticles on antimicrobial activity. The size of silver nanoparticles effect on their surface. Lower size of particles have larger surface area to contact with the bacterial cells. Panacek et al. [9] studies presents the results of minimum concentration of silver nanoparticles causing inhibition of growth some bacterial strains. Studies showed, that 25 nm particles showed higher activity in every bacteria strain then particles with larger radius. The lowest effect of growth inhibition was showed for particles with average size 50 nm. Research show also that result of adding nanosilver into bacterial strains results not only in inhibition growth of bacteria but its kill them [8, 9].

Antimicrobial efficacy depends also on the shape of nanoparticles. Pal et al. [10] in their research showed that they achieved inhibition of growth bacteria with silver content of 1 µg in the case of triangular-shape nanoparticles. That was the most effectiveness shape of nanosilver. Other shapes needed more silver content like 12,5 µg for spherical nanoparticles and from 50 to 100 µg in case the rod-shape nanoparticles [8, 10].

In other studies Li at al. [11] investigate antibacterial mechanism of silver nanoparticles to Gram-negative bacteria – *E. coli*. Gram-negative bacteria has a membrane outside the peptidoglycan layer, which protect them from substances may cause to destroy cells. The results showed that silver nanoparticles enhanced the permeability of this membrane, what is more the researchers noticed inhibition of respiratory chain dehydrogenases as a result to addition of nanosilver. This results were also confirmed by research carried out by Sondi at al. [12] which noticed completely inhibition of growth *E. coli* on LB agar plates. The same experiment showed that silver nanoparticles immersed in a liquid medium regardless of the concentration didn't caused inhibition of growth bacteria. In result of use nanosilver in liquid medium was only delay of *E. coli* growth.

Yoon at al. [13] in their research carried out on *E. coli* and *B. subtilis* noticed that silver nanoparticles completely inhibited bacteria at the concentration higher than 70 µg/mL. In comparison with each of strains was observed greater sensitivity of *B. subtilis* then *E. coli*. Authors thinks, that explanation of more resistive *E. coli* to nanoparticles was outer membrane of Gram-negative bacteria, then Gram-positive *B. subtilis*.

5. SUMMARY

Antimicrobial activity of silver is well known for years, but along with the development of nanotechnology silver returned to the range of interests researchers. Reduction of particle to the size not exceed 100 nm allows to effective prevention of bacterial, viruses and fungi growth.

Over the last few years many studies have been conducted about methods of obtaining silver nanoparticles, also on mechanism of their action.

Although the mechanism of silver nanoparticles is not fully understood, it can be determined as an effective. The research showed that silver nanoparticles penetrate cells wall and blocking the respiratory chain of bacteria.

Currently there are studies conducted in Institute of Environmental Engineering of Wroclaw University of Technology about effect of silver nanoparticles obtained in microwave method on microorganisms found in Wroclaw drinking water.

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biofilm, bioreactor, bacterial cells

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Joanna SURMACZ-GÓRSKA[*]

EVALUATION OF THE GROWTH AND DEVELOPMENT OF BACTERIAL BIOFILMS IN LABORATORY CONDITIONS

Bacteria, due to the high genetic and physiologic variability, possess the ability to colonize different ecological niches and to adapt quickly to changing and often unfavorable environmental conditions. The planktonic bacterial cells are sensitive to a variety of adverse environmental factors, therefore, in order to increase the chances for survival they form multilayer aggregates called *biofilm*.

Settled form of life in the complex structure of biofilms provides many benefits: it increases the chances for survival, enables easy access to nutrients, allows genetic exchange between the cells and rapid transmission of information via quorum sensing.

In the environment single-species biofilms occur rarely. Typically these structures are created of a number of bacterial species, often with different requirements for life. In addition, they could include cells of algae, protozoa, insect larvae, etc. Such structures are multi-species symbiotic microenvironment, in which each species plays a specific role. It is important that biofilms are called as aqueous environment; a large part of this structure is occupied by water channels.

Due to the complexity of natural biofilms, mainly mono- and two-species structure are tested in a laboratory. This article presents the characteristics of monospecies biofilms, created by strains of *Escherichia coli*. Two methods of biofilm culturing were used: an increase in 6-well plates and cultured in Drip Flow Reactor. Characteristics of the biofilms were carried out, including, among the others: determination of CFU, XTT metabolic activity measurement, comparison of biofilm biomass, observation of developing structures using confocal microscopy, Live / Dead staining, measuring the amount of polysaccharides and proteins in the bacterial extracellular matrix. Experimental results allowed for a comparative analysis of biofilms, formed by different strains of bacteria, and showed the differences in the rate of development and form of these structures.

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1. INTRODUCTION

Biofilm is defined as a complex of microorganisms, which are embedded on a common surface and form numerous microcolonies. These structures a surrounded by an extracellular matrix (extracellular polymeric substances, EPS) [8-10]. Currently biofilm is considered the basic form of living bacteria, which allows them to survive in adverse environmental conditions. Bacteria in this structure may be even a thousand times more resistant to harmful agents than planktonic bacteria belonging to the same species [13]. Therefore, in the environment approximately 95% of the bacteria are present in the form of biofilm. The natural biofilms may also contain: fungi, protozoa, algae, plant and animal debris, and inorganic substances [8, 16].

A significant part of the biofilm structure is occupied by free spaces, so-called water channels. Therein the fluid circulates providing the oxygen and nutrients to individual cells, and receiving potentially harmful products of their metabolism. Since the water content in the biofilm can be up to 75-90% of its weight, this structure is considered an aqueous microenvironment. Extracellular matrix, which is a mixture of various polymers, is highly hydrated. The EPS include: proteins, nucleic acids, lipids, polysaccharides and neutral and acidic phospholipids [8, 16]. However, the composition of the polymeric matrix is highly dependent on environmental conditions. Extracellular polysaccharides play an important role, because they enhance biofilm integrity and they are involved in cell adhesion to the surface. Another important component are proteins, which in addition to their role in the adhesion process may also act as ligands or receptor cells. EPS may be either hydrophilic or hydrophobic. Matrix is a material binding cells together and protecting them against harmful influence of external factors [4, 5, 7, 17].

Biofilm structure provides many benefits to the bacterial cells occurring within it. First of all cells have better access to nutrients, they are largely protected from the damaging effects of the external environment, they can synchronize their metabolism through the rapid exchange of quorum sensing signals, and thus they perform multiple processes faster and more efficiently (eg. decomposition of toxic substances) [1, 5, 11, 19]. Moreover, due to proximity of cells, genetic exchange between them and acquisition of new genes is possible, which allows them better adaptation to the environment. Biofilm-forming microorganisms are also characterized by different metabolic activity levels within the membrane, which affects the resistance of the microorganisms. In the deeper layers of the biofilm cells are smaller and often remain in anabiosis state, acting as reservoir of reserve cells. Whereas cells occurring on the surface are more metabolically active and they are dividing rapidly, so that the structure can grow. Mature biofilms, composed of metabolically differentiated cells, have a large adaptability to different environmental conditions and the greatest resistance to antimicrobial agents [5, 6, 9].

The present study includes characterization of biofilms formed by *Escherichia coli* strains, purchased from the BCCM / LMBP strains collection.

2. MATERIALS AND METHODS

2.1. BREEDING OF STRAINS

For the tests two strains of *Escherichia coli* (LMBP 4547 and LMBP 5420) were used, which were purchased from the BCCM / LMBP strains collection (Ghent University, Belgium). The strains were stored in Eppendorf vials at -20 °C, with the addition of glycerol (850 mL cell suspension, 150 mL of sterile glycerol).

Two methods of cultivation of strains were employed. The first method of biofilm culturing was conducted in sterile 6-well plates. Each well contained 5 mL of culture medium (TSB; Tryptic Soy Broth), and 100 mL of 24-hour suspension of an appropriate bacterial strain. The coupons were incubated in an incubator at 37 °C.

In the second method sterile plastic containers were used to culture the biofilm. Each container contained 50 mL of nutrient broth (TSB), 1 ml of an appropriate bacterial strain and degreased glass slide. Incubation was carried out in an incubator at 37 °C.

After a suitable period of culturing biofilm was scraped into 10 mL of PBS, using a sterile scraper for this purpose.

2.2. CFU ENUMERATION

The biofilm cells concentration was determined by enumeration of the colony-forming units (CFU). Biofilms of both strains were grown on the 6-well plates, where growing area was 9.15 cm^2. The plates were incubated at 37 °C for 24 to 72 h. After this period biofilm was scraped and suspended in 5 mL PBS.

Dilutions of 10^{-10} were made and 10 µL from each dilution was seeded on TSA plates (triplicate). The plates were incubated at 37 °C for 24 h and then the grown colonies were counted. The numbers of bacteria were calculated according to the formula:

$$\text{CFU/mL} = (\text{average CFU} / \text{drop volume})(\text{dilution counted})$$

Later it was converted into CFU/ cm^2:

$$CFU/cm^2 = (CFU/mL * PBS\ volume) / growing\ area$$

so,

$$CFU/cm^2 = (CFU/mL * 5\ mL) / 9.15\ cm^2$$

2.3. DETERMINATION OF BIOFILM BIOMASS – CRYSTAL VIOLET ASSAY

Crystal violet staining was used to determine the total attached biofilm. Biofilm was grown in 6-well plates at 37 °C for 24 h to 72 h. After this time the medium was removed, the wells was washed with water and 5 mL of methanol was added. After incubation (15 minutes) methanol was removed, wells were drained and crystal violet was added. The assay was removed after 5 minutes. The wells were washed with water twice and acetic acid (33%) was added to each well. The final step was the measurement of absorbance (570 nm). The blank test was acetic acid.

2.4. DETERMINATION OF BIOFILM ACTIVITY – XTT ASSAY

XTT is a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H- tetrazolium hydroxide, which is converted to soluble products into aqueous medium . However, PMS (phenazine methosulfate) is an intermediate electron acceptor, so that the degree of XTT reduction is increased in cells [15].

Biofilm was grown in 6-well plates at 37 °C for 24 h to 72 h. After this time the medium was removed and the wells were washed with water. Next 5 mL of mixture containing 200 mg XTT/l and 20 mg PMS/L was added. The plates were shaken in the dark for 2 h (120 rpm). The absorbance was measured (490 nm). The blank test was the XTT/PMS mixture.

2.5. EPS EXTRACTION AND MEASUREMENT OF PROTEINS AND POLYSACCHARIDES

The extraction of EPS from biofilms of *E. coli* strains 4547 and LMBP 5420 LMBP was performed using ultrasonic sonicator Branson S-150, equipped with a titanium tip diameter 13 mm. Sonication was performed in an ice bath (4°C). Samples were sonicated (37 W / 10 ml sample) for 60 seconds After sonication the

sample was centrifuged at 14000 rpm for 10 min. Then supernatant was filtered through a hemicellulose acetate filter (pore diameter 0.45 um). The content of proteins and polysaccharides was analyzed in obtained filtrate.

The Dubois method (phenol – sulfuric acid method) was used for the measurement of polysaccharides. From each sample, 0.5 mL of supernatant was collected, 0.5 mL phenol and 2.5 mL of sulfuric acid VI (97%) was added. The prepared samples were centrifuged for several seconds and incubated at room temperature for 15 min. Absorbance measurements were made at 490 nm using a solution of PBS as a blank.

The protein content of the extracted EPS was determined by fluorimetric method, using Quant - iT Assays and Qubit analytical station. The working solution was prepared (1:200 Quanit - iT protein reagent A in Quanit - iT protein buffer B) and 180 mL of the working solution and 20 mL of the sample (in triplicate) to eppendorf were added. Samples were incubated at room temperature, then the measurements on Qubit Fluorometric Station - iT were performed.

2.6. THE ADHESION OF MICROORGANISMS TO HYDROCARBONS

The hydrophobicity of LMBP 4547 and LMBP 5420 strains were tested by determination of the adhesion degree of cells to hydrocarbons. 24 h bacterial suspension was prepared (incubation on a shaker, 120 rpm, 37 °C), then the cells were centrifuged (9000 rpm, 4 °C, 5 min.) The sediment was washed twice with PBS. Cells were suspended in PBS (optical density of the suspension between 0.4 and 0.6 (A_0); 600 nm). 150 mL n-hexadecane was added to 3 mL of the suspension and mixed for 1 minute. The samples were left at room temperature for 10 minutes to pellet the cells. In the final stage the suspension optical density (A_t) was again measured. The percentage of attached cells was calculated from the formula:

$$\% \text{ attached cells} = (1-At/A0)\times 100$$

2.7. CLSM OBSERVATION

Biofilm developing on basic glass was observed by using CLSM (Confocal Laser Scanning Microscopy) with Olympus Fluoview Software Ver.3.1a. UPLSAPO 60XS NA:1.3 and lasers 405 nm, 448 nm, 515 nm, 559 nm and 635 nm were used for observation. The controls were conducted every 24 h for 3 days.

2.8. LIVE/DEAD STAINING

The number of live and dead cells in biofilms of both strains in the subsequent days of culture was examined. For this purpose, Live/Dead staining was performed, using the LIVE/DEAD BacLight Bacterial Viability Kit. Observations were made using the Zeiss Fluorescent Microscope with AxioVision software.

3. RESULTS

CFU examination showed that the strain *E. coli* LMBP 4547 demonstrated an upward trend in the following days of cultivation. For 24 h biofilm the value of CFU/mL was $8{,}36\times10^{10}$, which was equivalent about $4{,}57\times10^{10}$ CFU/cm^2. At 48 h biofilm cells was $2{,}5\times10^{11}$ CFU/ml ($1{,}37\times10^{11}$ CFU/cm^2), and for 72 h was equal to 3×10^{12} CFU/mL ($1{,}64\times10^{12}$ CFU/cm^2). In the case of LMBP 5420 strain the number of cells was $4{,}84\times10^{7}$ CFU/ml ($2{,}64\times10^{7}$ CFU/cm^2) after 24 h incubation, the next day there was a slight decrease of the number of viable cells in cultures ($2{,}87\times10^{7}$ CFU/ml $1{,}57\times10^{7}$ CFU/cm^2), and after 72 h of incubation the number of cells increased again to a value of $5{,}9\times10^{9}$ CFU/ml 3.22×10^{9} CFU/cm^2).

Control of biofilm biomass, using crystal violet staining, confirmed the results of CFU. Absorbance was measured from each well, and then the results were averaged from the plate. The mean absorbance of each plate, between successive days of culture, was compared (Table 1).

Table 1. Comparison of the absorbance of biofilms *E.coli* LMBP 4547 and LMBP 5420.

	LMBP 5420	**LMBP 4547**
24 h	0,796	0,183
48 h	0,091	0,339
72 h	1,007	0,689

The results showed that *E. coli* biofilm LMBP 4547 grew in subsequent days of culture, gradually increasing their biomass. However, in the case of *E. coli* LMBP 5420 strain different situation was observed - the first day of cultures biomass of was very high, and on the second day there was a rapid decrease and re-growth after a further 24 h. From the presented values it can be concluded that the strain of *E. coli* LMBP 5420 was characterized by significantly faster rate of growth of biofilm, as a result after 48 h the aging processes of the structure was observed (the rapid reduction of the biomass, detachment the part of microcolonies and the return cells

into suspension). The next day of incubation resulted of the biofilm redevelopment (Figure 1).

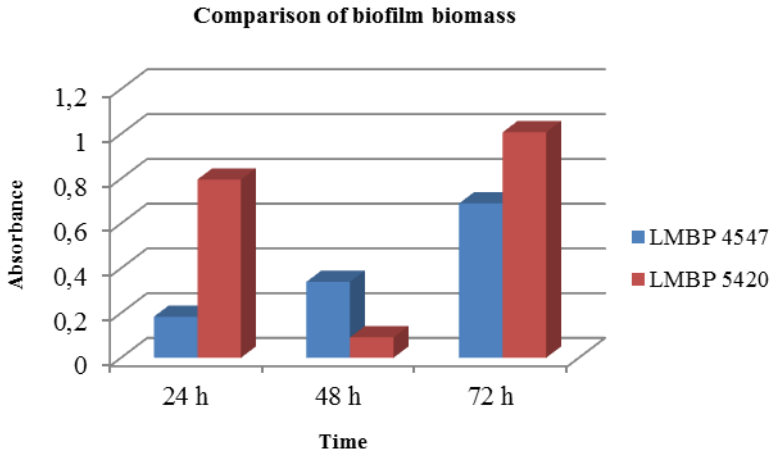

Fig. 1. Comparison of the biofilms biomass of LMBP 5420 and LMBP 4547 strains, in time-dependent cultures.

Analysis of the metabolic activity of both strains confirmed the previous results. Incubation of the samples with the addition of XTT and subsequent measurement of absorbance showed that the activity of the *E. coli* LMBP 4547 strain gradually increased on subsequent days of culture. However, the activity of LMBP 5420 strain after 24 h was very high (Figure 3), while in the next day of cultures strongly plummeted and after a further 24 h gradually increased. The analysis was based on the average results of each plate (Table 2, Figure 2).

Table 2. Absorbance of biofilm LMBP 4547 and LMBP 5420 strains after 24 h, 48 h and 72 h of cultures (490 nm), indicating their activity.

	LMBP 4547	**LMBP 5420**
24 h	0,495	1,306
48 h	0,497	0,258
72 h	0,534	0,462

Intensive coloration of the sample suggests stronger metabolic activity of bacterial cells and may be related in direct proportion to the number of viable cells in each biofilms (Figure 3).

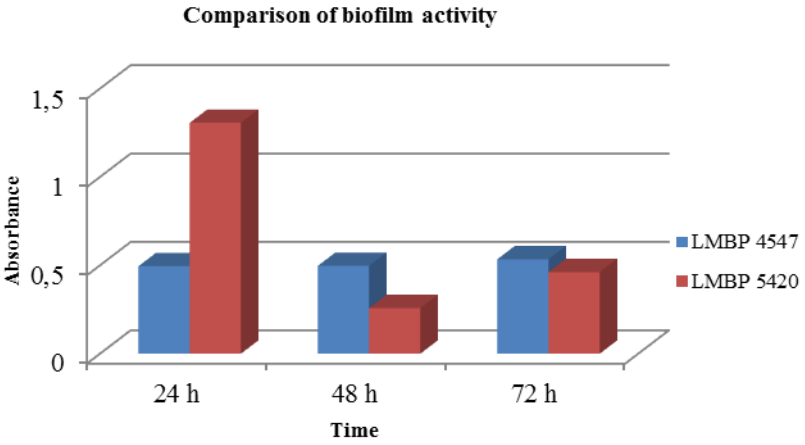

Fig. 2. The comparison of biofilms activity in time-depended cultures.

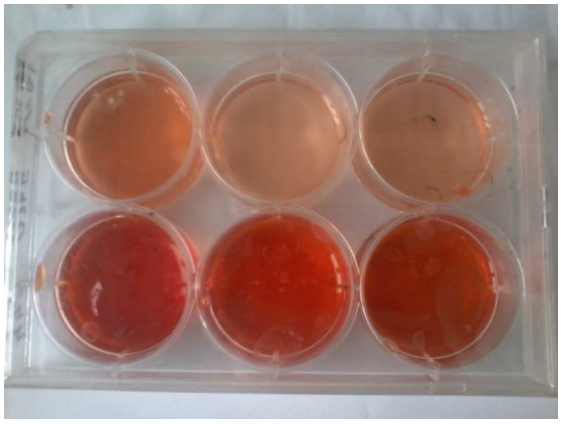

Fig. 3. LMBP 5420 biofilm with XTT addition.

EPS extraction from 24 h biofilm by sonication was conducted, then the content of proteins and polysaccharides in samples was measured. The content of polysaccharides was determined by Dubois method (phenol-sulfuric acid method), the result was compared to a standard curve of glucose concentration. The Quant-iT Assays Kit was used to determine the amount of protein. The results are shown in Table 3.

Control of cell adhesion to hydrocarbon (n-hexadecane) showed that the strain attached LMBP 5420 at 14%, while strain LMBP 4547 attached at 8%. Therefore, LMBP 5420 strain has a higher hydrophobicity than the strain LMBP 4547.

Table 3. Absorbance of biofilm LMBP 4547 and LMBP 5420 strains after 24 h, 48 h and 72 h of cultures (490 nm), indicating their activity.

	LMBP 4547	**LMBP 5420**
The average concentration of proteins in the EPS [µg/ml]	107.1	124.7
The average concentration of polysaccharides in the EPS [µg/ml]	444.8	98.9

The assessment of the development and the comparison of biofilm structures was made by using confocal microscopy. Selected images obtained in subsequent days of cultivation, are presented in Figure 4 and 5.

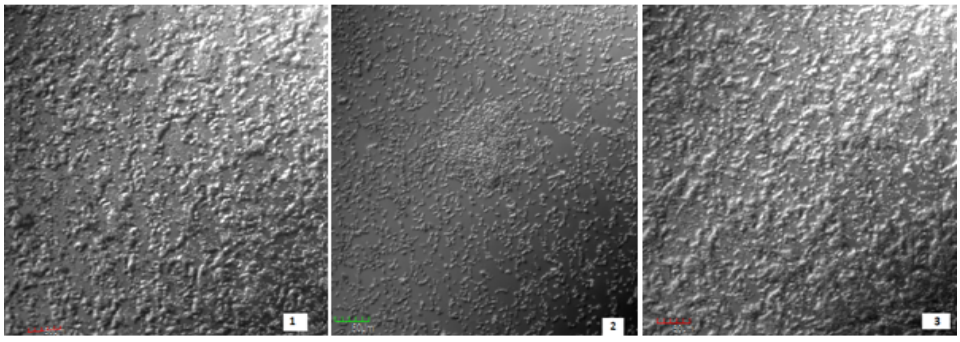

Fig. 4. The structure of LMBP 4547 biofilm (1 - 24 h biofilm; 2 - 48 h biofilm; 3 - 72 h biofilm) (objective 40×).

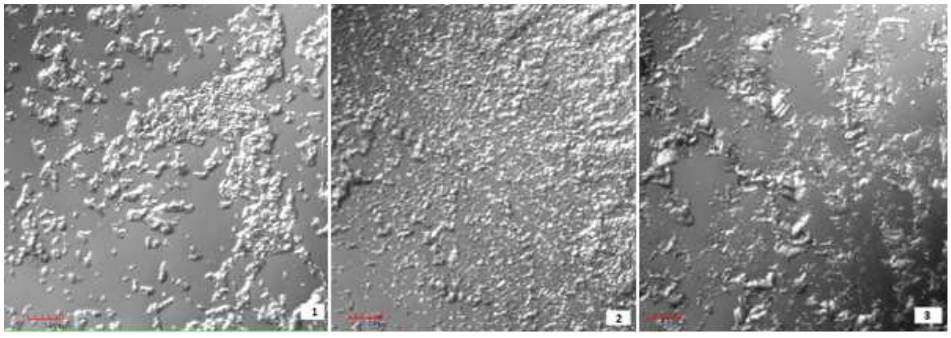

Fig. 5. The structure of LMBP 5420 biofilm (1 - 24 h biofilm; 2 - 48 h biofilm; 3 - 72 h biofilm) (objective 40×).

The Live/Dead staining allows comparison of the number of viable bacterial cells in relation to all the cells in the biofilm (Figure 6).

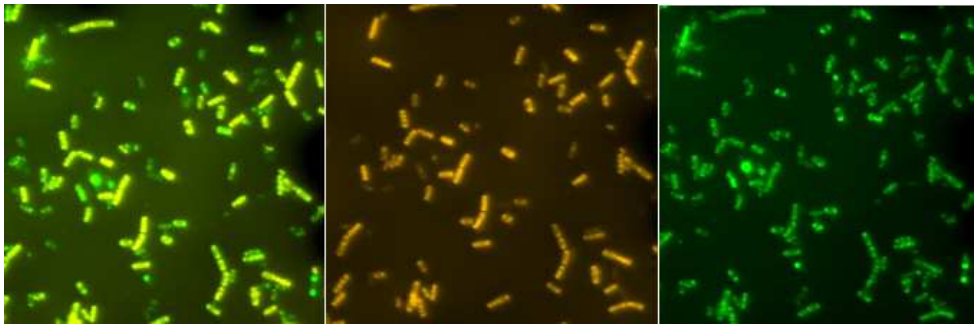

Fig. 6. The Live/Dead staining of LMBP 5420 strain, after 24 h incubation (objective 60×).

Staining of biofilms of both strains in the subsequent days of culture was performed. During each preparation approximately 20 images were obtained, from which the number of viable cells and all cells of biofilms were calculated (using ImageJ). On the basis of the images, it was found that cells number of LMBP 4547 strain was different for each day of culture. The number of cells of a strain of LMBP 5420 was smaller on each day of incubation (Table 4). In 4547 LMBP biofilm more than a half of biomass was living cells (53% - 60%). A similar result was obtained with a strain of LMBP 5420, where viable bacterial cells were from 58% to 67% of all cells. The differences between the strains are small and may be caused the subjective choice of photos.

Table 3. The average number of all cells and the average number of living cells in both strains in subsequent days of incubation.

	Number of cells			
	LMBP 4547		LMBP 5420	
	All cells	Living cells	All cells	Living cells
24 h incubation	519	303	457	308
48 h incubation	291	155	219	138
72 h incubation	414	251	168	97

4. DISCUSSION

The purpose of the study was to verify whether the two strains, belonging to the same species, form the biofilms of the same architecture and activity. Various test methods were used: CFU as the primary method of indicating the number of living cells structure [2], the crystal violet staining method in order to illustrate the amount of biomass or XTT analysis in order to compare the activity of biofilms [12, 14].

Conducted comparative analysis of two strains of *Escherichia coli* biofilms show a significant differences in the structures created by them. Although these strains belong to the same species, the rate of biofilm development and biofilm biomass differ. *E. coli* strain LMBP 4547 developed more evenly, which was determined by CFU calculation and crystal violet staining. Also the activity of the biofilm increased gradually in the subsequent days of culture. Whereas *E. coli* LMBP 5420 strain showed fast growth of the structure on the first day of culture, and then the biomass and the activity were rapidly reduced after 48 h incubation. On the following day biofilm was gradually reconstructed.

Such biofilm life cycle is a perfect example of the development and maturation processes, occurring in this structure. These processes often lead to death or separation of part of the structure, and then rebuild it again. Biofilms as a structure dynamically reacting to any changes in the environment, are exposed to temporary fluctuations of the number of cells. Based on literature data of different researchers conclusion can be drawn that these structures are characterized by a specific life cycle. This cycle consists of a stage of cell adhesion to the surface, the initial EPS production, formation of microcolonies and differentiation of the cell within them, and the aging processes of the structure. These processes are related to, inter alia, the depletion of nutrients within the biofilm, in the effect the part of the cells return to the planktonic form and populate the new ecological niches. The structure can be rebuilt when the nutrients are again supplied [3, 18].

Observations made using a Confocal Laser Scanning Microscopy showed differences in the construction of various structures of biofilms. Biofilm LMBP 4547 was more uniform and evenly distributed over the entire surface of the glass, while the LMBP 5420 strain create clearer and larger microcolonies, which significantly changed during the breeding. However, Live/Dead staining showed that the number of viable cells in the biofilm was fixed, regardless of the day of culture (the medium ranges was 55-65% of the total biomass of biofilm). All the performed experiments revealed, that the construction of biofilms within a species can be various.

5. SUMMARY

Antimicrobial activity of silver is well known for years, but along with the development of nanotechnology silver returned to the range of interests researchers. Reduction of particle to the size not exceed 100 nm allows to effective prevention of bacterial, viruses and fungi growth.

Over the last few years many studies have been conducted about methods of obtaining silver nanoparticles, also on mechanism of their action.

Although the mechanism of silver nanoparticles is not fully understood, it can be determined as an effective. The research showed that silver nanoparticles penetrate cells wall and blocking the respiratory chain of bacteria.

Currently there are studies conducted in Institute of Environmental Engineering of Wroclaw University of Technology about effect of silver nanoparticles obtained in microwave method on microorganisms found in Wroclaw drinking water.

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anfsurfactants, seawater, the Baltic Sea,
chromophoric dissolved organic matter,
chlorophylls, spectrophotometric measurements

Violetta DROZDOWSKA*, Natalie L. FATEYEVA**

SPECTROPHOTOMETRIC STUDY OF NATURAL BALTIC SURFACTANTS – RESULTS OF MARINE EXPERIMENT

Knowledge of the luminescent properties of surfactants (surface active agents) in the surface layers of the sea is extremely important for the interpretation of remote images of the sea, which in modern oceanography play a very important role. As well as, to assess the role of the organic molecules, forming the surface layers of a sea, in the gas exchange between a sea and an atmosphere. The main source of marine surfactants are marine phytoplankton exudates and their degradation products. Advanced research of Baltic surfactants is conducted in Institute of Oceanology Polish Academy of Sciences for several years. To study the spatial distribution and luminescent properties of surface active molecules of dissolved organic matter (DOM) in Baltic waters - several marine experiments, in different regions of Baltic Sea, were organized. Water samples from a surface film were collected by a glass plate. The water, from a 15 cm top layer of a sea, was collected using a wide container which was properly charged and, partially immersed in water, took on water from the layers of not more than 10-15 cm. The measurements of 3D fluorescence spectra of seawater samples were performed immediately on board of the ship.

Comparison of 3D fluorescence spectra for samples from a film and a top layer indicates that the molecules of chromophoric DOM (maximum for ex. 300-400 nm/em. 380-540 nm) are found in a greater concentration in a surface film, while the molecules of Chlorophyll *a* (Chl *a*) (ex. 425-475 nm/em. 675-685 nm) in a 15 cm top layer. The recorded differences in concentration between a film and a top layer for both CDOM and Chl *a* are very small. It can be assumed that, regardless of research area of the sea, satellite images show the contents representative for both, a film and a top layer. However there is a need to repeat this type of experiments to confirm the results.

Spectrofluorometric study of samples taken from different regions and layers of Baltic Sea allow distinguishing a chemical composition and concentration of optical active molecules contained in seawater. The measurements of 3D fluorescence spectra of seawater samples, taken from a surface film and a 15 cm top layer, were carried out during the research cruise of r/v Oceania in April 2010.

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The results of spectra analyses show that the molecules of CDOM, (maximum for ex. 300-400 nm/em. 380-540 nm) are found in greater concentration in a surface film, while the molecules of Chl *a* (ex. 425-475 nm/em. 675-685 nm) in a top layer.

1. INTRODUCTION

The sea surface is the interface between an atmosphere and a sea, where there are a variety of biological and physical and chemical processes. Their properties effect the processes of the accumulation and exchange of surface active molecules and contribute to the dynamic properties of the surface (surface wave spectrum) and fluxes (especially in gas exchange and production of marine aerosol) as well as to the formation of inherent optical properties of seawater [1-4]. Moreover, the presence of a specific organic molecules in a surface film may restrict the supply of light energy into the different depths of the sea [5].

Marine surfactants, that form a natural surface film, consist of organic and polar molecules. Their main source are marine phytoplankton exudates and degradation products [6]. What is more a significant fraction of surfactants produced in plankton cultures are not metabolic end products of excretion. These materials may be the result of rapid degradation of biopolymeric material by bacteria or enzymes of bacteria and as a result of condensation of low molecular weighted exudates to form of surface-active macromolecules [7]. While many of these particles are quite soluble (surface active), they are likely to contain hydrophobic groups (weakly surface active) [8].

Molecules of DOM are huge amount of organic compounds, chemically not entirely classified. The best tool for identifying their composition is the 3D Excitation-Emission Spectroscopy, based on the absorption and fluorescence properties of DOM molecules. As was firstly reported by Coble [15] the typical DOM components are Marine Humic-like (peak M) and Humic-like (peaks A and C) fractions with their ex./em. maxima around 310-390 nm and 260 and 310nm/450 nm, respectively.

In the first step, our study consisted of performing the spectrofluorometric measurements of the 3D fluorescence spectra of seawater samples taken from a surface film and a 15 cm top layer [9]. Then the research was to work out the obtained spectra and to analyze the results [10]. During the cruise in April 2010, studies were conducted in different regions of Baltic Sea, to assess the anthropogenic impact (estuaries, proximity to ports and shipping lanes) on the marine environment.

The primary scientific objectives were to investigate the variability of luminescent properties of CDOM (surfactant) molecules contained in the surface layers of a sea. Moreover, intention was to identify the specific CDOM molecules occurring in various regions of Baltic Sea and estimate their concentration.

2. EXPERIMENTAL

Water samples from a surface film were collected by a glass plate method. Samples from a 15 cm top layer were collected using a wide container, properly charged and partially immersed in the water. For the analysis of luminescent properties of an unknown mixture of dissolved organic compounds contained in the study samples the matrixes of Spectral Fluorescence Signature (SFS) were performed. The 3D SFS spectrum is recorded as a matrix of fluorescence intensity in co-ordinates of excitation and emission wavelengths in a definite spectral window. The characteristic spectral structure of SFS is used for substance recognition, while the intensity of fluorescence serves as a measure of substance quantity [11].

To record the 3D fluorescence spectra the spectrofluorometer FLUO-IMAGER M53b (LDI, Estonia; SCALAR, The Netherlands), operating in the UV and visible spectral range, was applied. The optical system consists of a light source (xenon lamps which emits light in the range 200-650 nm and the diffraction grating 600 l/mm) and photomultiplier (recording emissions in 205-730 nm). The spectrofluorometric measurements of seawater samples were carried out immediately on board.

The 3D fluorescence spectrum, excited every 1 nm, gives a picture of emissions peaks from the all components in a sample. These components are collected with photosynthetic pigments of phytoplankton and CDOM molecules (eg mainly humus). Automatically performed, analysis of the recorded spectra - using a library of primary photosynthetic pigments and organic components - is based on comparison of the recorded 3D spectra with the reference ones [11].

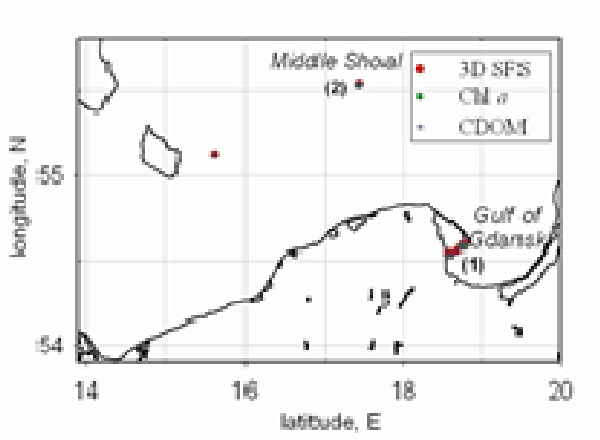

Fig. 1. Map of measuring stations during a research cruise located in (1) estuary and (2) open sea regions of Baltic Sea.

The all 3D fluorescence spectra recorded during a research cruise, carried out in the open sea (Middle Shoal) and coastal zone (Gulf of Gdansk), revealed specific features attributed to these regions. The stations 1 and 2 are chosen as the representatives for estuary and open sea waters, respectively, Fig.1.

Additionally, on every monitoring station, the standard *in situ* measurements of water salinity and CDOM and Chl *a* concentrations contained in a surface layer of a sea were performed by laboratory methods described in literature [12-14].

2. RESULTS AND DISCUSSION

How it was predictable, with the approach to coastline the water salinity decreased and the concentration of CDOM and phytoplankton increased [14-19].

Very interesting results, supporting the conclusions of the above mentioned standard studies, have provided measurements of 3D fluorescence spectra of seawater samples in UV and visible light. Fig. 2a and b shows the 3D fluorescence spectra in UV spectral range of seawater samples taken from a top layer in estuary and open waters, respectively. In the open sea the low intensity of the fluorescence signal - with one dominant maximum at wavelengths 310-320 nm (excitation)/390-410 nm (emission) - was observed, Fig. 2b. While in the coastal waters the band was much broaden and a signal intensity was much stronger, and the additional maximum - in the band 355 nm (excitation)/400-450 nm (emission) – occurred, Fig. 2a. Hence, in the samples from open waters lower concentration of organic matter molecules was observed than in the coastal waters. And in open water the lighter fraction of CDOM – with the peak shifted towards blue – appeared. While coastal water contained both fractions of CDOM: characteristic by slightly greater and smaller atomic mass as well, ie with a maximum shifted slightly toward red and blue, respectively. According to Coble classification [15] of marine CDOM molecules, in samples from open sea the Marine Humic-like fraction (peak M) dominated while in gulf waters an additional Humic-like one (peaks A and C) gave a significant contribution.

Additionally, the 3D SFS measurements of seawater samples taken from the estuary and open sea waters were performed in the visible spectral range and the results for a surface film and a top layer are presented in Figs.3a,b and 3c,d, respectively.

Concentration of Chl *a* in a surface film changed from 5.86 mg/m^3 in estuary waters to 2.9 mg/m^3 in open sea ones, while in a top layer it changed from 6.34 mg/m^3 to 2.93 mg/m^3, respectively. Firstly, a greater concentration of Chl *a* was recorded in coastal estuary waters of Gulf of Gdańsk, where mixing of seawater with fresh water masses provides a constant supply of nutrients. Secondly, a concentration of Chl *a* was a little greater in a top layer than in a surface film; this applied to both open and coastal waters. This could be explained by the morphology of Chl *a*, which

is part of the larger and heavier organisms (algae), which are living organisms, constantly moving in water, reacting to light and temperature, and therefore prefer to be in a water layer than in a surface film. Moreover the fluorescence bands of Chl *a* with a maximum in 682 nm, induced by direct excitation of Chl *a* (in 435 nm) and via another accessory pigments (in a range 460-550 nm) revealed that the same pigment composition existed in samples taken from a surface film and a top layer on one station. What is more a pigment compositions in estuary and open sea waters differed from each other.

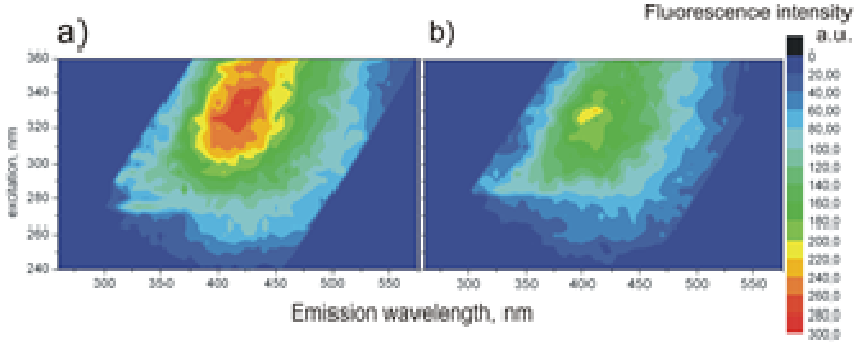

Fig. 2. CDOM in a top layer, a) coastal waters and b) open sea; stations 1 and 2.

The results of CDOM molecules concentration in a surface layers, presented in Fig. 3, showed that CDOM concentration in a surface film was a little higher in coastal waters (1.97 mg/m^3) than in open waters (1.54 mg/m^3), Fig.3a,b. As well as in a top layer a concentration of CDOM was higher in coastal waters (1.67 mg/m^3) than in open sea waters (1.16 mg/m^3), Fig. 3c, d. Hence greater concentrations of CDOM, both in the open and coastal waters of Baltic Sea, were recorded in a film than in a top layer.

It is known that one of the main sources of CDOM molecules in seawater are phytoplankton exudates and their degradation products and organic matter brought to sea with river runoff. Lighter fraction of CDOM floating in surface layers of the sea are the main component of surfactants. Then they can be moved from the sea into the atmosphere with aerosol droplets generated by a wind or transferred together with the air masses from (or to) other areas of seas or lands. However in the case of our study stations, the 3D SFS spectra revealed the same chromophoric component composition contained in samples from a surface film and a top layer. It indicated the presence of the same molecules of CDOM, i.e. their source, both in a top layer and a surface film.

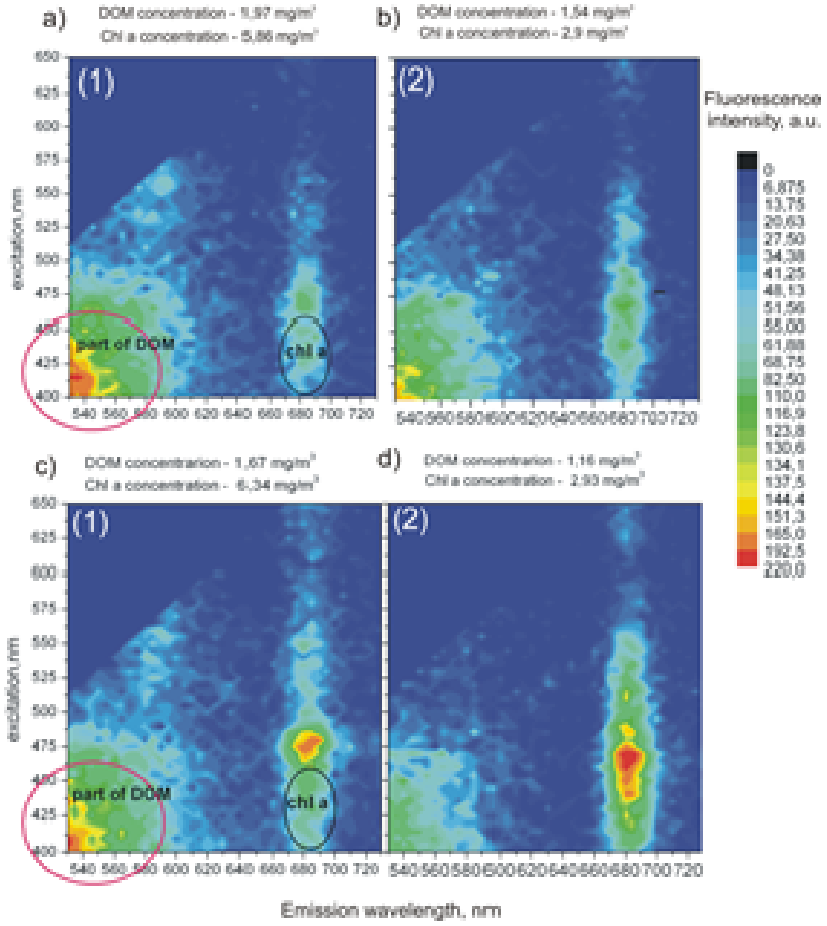

Fig. 3. 3D SFS spectra of a surface film (a and b) and a top layer (c and d) in estuary and open sea waters, stations 1 and 2, respectively; see on a map on Fig.1.

3. CONCLUSIONS

The interpretation of spectrofluorometric measurements obtained in a Baltic cruise allowed dividing the study area, according to the intensity and shapes and positions of spectral emission bands in 3D fluorescence spectra, into estuary (coastal zone) and open sea waters. The representative for these regions were stations 1 and 2, respectively.

Firstly, the spectrofluorometric measurements carried out in UV spectral range revealed, in samples from both Baltic regions, a strong and distinctive emission band of Marine Humic-like fraction of CDOM while in estuary waters an additional strong fluorescence emission band in a range 430-450 nm, excited in a range 260-360 nm. It indicated a presence in estuary waters Humic-like fraction of DOM, i.e. anthropogenic impact into marine environment.

Secondly, our results revealed that the values of CDOM concentrations were a little higher in a film than in a top layer. In terms of the Chl *a* concentration, it was lower in a film than in a top surface layer, but the differences were very small. These differences between a surface film and a top layer - both in open and coastal waters - were insignificant for remote sensing techniques that assume the homogeneousness of surface layer of seawater column. And hence we could conclude that calculated inhomogeneities of the vertical distributions of luminescing molecules in the surface layers of a sea were insignificant and did not affect the interpretation of remote images in studies of passive remote sensing sea surface.

In the next stage of study is scheduled a cruise - combined with undersatellite research that provide the spectra of light coming from the sea. Further, the larger database of the optical parameters of sea surface layers and the spectra of light registered over the sea will help to assess the effect of surfactants on the light coming from the sea.

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mercury, Salvinia natans, bacteria

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RESISTANCE OF BACTERIAL STRAINS THAT COEXIST WITH MACROPHYTES *SALVINIA NATANS* TO MERCURY (II) IONS

Mercury is one of the most toxic heavy metals, which doesn't satisfy any biological functions in living organisms. Microflora (bacteria and fungi as well) takes an active part in the transformation of mercury in the environment. Apart from the ability to methylation (eg, Pseudomonas spp) some strains are able to reduce mercury compounds to volatile form. Bacterial detoxification system encoded by *mer* operon is traced in Gram-positive and Gram-negative bacteria. Bacteria coexisting with macrophytes *Salvinia natans* were isolated on nutrient agar, potato dextrose agar and mineral glucose (0,5%) base. Then pure colonies were applied to a gradient plates (from 0,150 ppm to 0,300 ppm) and incubated for 48 hours at 22 °C. Strains resistant to high concentrations of mercury Gram were stained. This ability to detoxify mercury compounds to volatile form can be ascribed to the use of bioaugmentation of mercury-contaminated soil and support of biological water phytoremediation.

1. INTRODUCTION

Mercury, which belongs to the group of heavy metals takes the sixth position in the list of the most dangerous chemicals [2]. Its organic compounds, e.g. methylmercury, are characterized by a strong neurotoxic effect [7]. Due to its properties this metal (collectively with cadmium and lead) did not fulfill any physiological functions in living organisms.

Mercury has unique properties (it occurs in a liquid state at room temperature, the volatile elemental form) and is characterized by a high toxicity depends on the form of occurrence of heavy metal in the environment. The most toxic forms of mercury are methyl derivative compounds, the only ones that actually have the ability to become involved in the food chain. Methylmercury is characterized

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by neurotoxic, nephrotoxic and teratogenic effects, undergoes bioaccumulation and biomagnification, which increases the risk of toxicity to living organisms. The least toxic form of mercury is elemental mercury (Hg^0). Therefore, extremely important task aiming at reduction of hazardous compounds is the transformation of the mercury to volatile form.

Two major categories of mercury sources are natural resources accumulated in the earth's crust in the form of minerals (eg calomel) and anthropogenic mercury sources.

Natural processes of mercury emissions include:

- erosion of minerals containing mercury;
- processes of weathering and sedimentation;
- volcanic eruptions [4].

Anthropogenic sources include the production of lighting (mercury lamps), battery, chlorine, caustic soda, dental materials, measurement and control equipment (thermometers), processes of gold and silver amalgamation and coal combustion process [3].

In addition to the above-mentioned sources a significant role in the accumulation of heavy metal in the environment plays mercury reemission. The primarily sources of remission are: liquid and solid industrial waste collected from landfills (batteries, thermometers, barometers, cables, lamps) and sediments formed as a result of industrial discharge into surface waters [3].

The high mobility of mercury, industrial demand and high toxicity determines the need to take the necessary action to reduce emissions of mercury in the environment.

The alternative methods of cleaning the environment are phytoremediation and bioremediation [6]. Some microorganism have ability to reduction organic compound to volatile form.

Therefore, studies attempted isolation of bacteria from *Salvinia natans* resistant to mercury for later use in supporting the process of phytoremediation.

2. MATERIALS AND METHODS

The test material consisted of river pleustophyte *Salvinia natans* (Fig. 1). Floating fern was collected during the growing season from the Odra river (Wrocław).

To tests was selected healthy subjects *Salvinia natans* and following actions were taken:

1. shaking in an ultrasonic bath in physiological solution for 15 minutes;
2. shaking in an ultrasonic bath in the sterile distilled water for 15 minutes;
3. sterile homogenisation of biological material;

4. making a dilutions series (from 10^{-1} to 10^{-7}) and inoculation on the bacteriological medium;
5. making the fingerprints leaves and roots of healthy organisms on the medium.

Fig.1. *Salvinia natans* in the natural environment.

After that the plates were incubated at 22 °C for 48 hours. During the tests were examined three bacteriological medias: nutrient agar, potato agar and mineral medium with 0.5% glucose.

Fig. 2. The gradient plates (from 0,15 mg Hg/dm^3 to 0,30 mg Hg/dm^3).

The bacteria susceptibility to mercury was determined by backing plates with a medium *contaminated* with mercury solution 0.15 mg Hg/dm³ ($(HgNO_3)_2$). Then, to check the minimum inhibitory concentration of the growth of microorganisms the purified culture resistant to mercury inoculated to gradient plates (from 0.10 mg Hg/dm³ to 0.15 mg Hg/dm³; from 0.10 mg Hg/dm³ to 0.20 mg Hg/dm³ and from 0.15 mg Hg/dm³ to 0.30 mg Hg/dm³) (Fig. 2).

3. RESULTS AND DISCUSSION

In tested medias the best growth of bacteria was characterized by a nutrient agar medium, but for validating results it has been rejected, due to the presence of protein in the composition. For further testes mineral medium with glucose (0.5%) was selected. In this medium mercury is most likely available for microorganisms.

Both bacteria isolated from plant surfaces and the homogenate from plant showed resistance to heavy metal.

Table 1. Preliminary identification of strains resistant to mercury

Number of strain	Place of collection	Resistant to 0,15 mgHg/dm^3	Resistant to 0,30 mgHg/dm^3	Initial identification of the shape of bacteria	G+/-
1	The homogenate from the plant 1	+++	++	Cocci	+
2	The homogenate from the plant 2	+++	++	Cocii	+
3	Leaf of plant 1	+++	+	Bacilli	-
4	Root of plant 1	+++	+	Bacilli	-
5	Root of plant 2	+++	++	Bacilli	-
6	Shaking with ultrasound (plant 4)	+++	+	Bacilli	-
7(10)	Leaf of plant 4	+++	+	Bacilli	-
8	Leaf of plant 5	+++	+	Bacilli	-
9	Root of plant 3	+++	++	Bacilli	-
10(13)	Root of plant 4	+++	++	Bacilli	-

50 strains were isolated, of which 25 were characterized by resistance to mercury concentration of 0.15 mg Hg/dm³. To optimize further research the 10 most resistant

strains were selected (minimum inhibitory concentration of bacteria growth - 0.30 mg Hg/dm³. All the strains were Gram stained. 20% were Gram-positive cocci, 80% Gram-negative bacilli (tab. 1).

Because of good growth of bacteria on the gradient plates the minimal concentration of inhibition growth was selected - 0.30 mg Hg/dm³.

4. CONCLUSIONS

- 50 strains were isolated, of which 25 were characterized by resistance to mercury concentration of 0.15 mg Hg/dm³. To optimize further research the 10 most resistant strains were selected (minimum inhibitory concentration of bacteria growth - 0.30 mg Hg/dm³).
- all the bacteria resistant to the highest concentration of mercury were Gram + (80% of the bacilli), which may be associated with the cell wall structure;
- whether the bacteria isolated from the rhizosphere of plants and bacteria collected from the surface and from the homogenate *Salvinia natans* showed resistance to a given heavy metal;
- the obtained strains, after making a series of tests (identification, assessment of selected enzymatic activity) can be ascribed to an attractive material for bioaugmentation the phytoremediation of water polluted by ions mercury (II).

The study done by the MNiSW project titled "Evaluation of the efficiency of phytoremediation of polluted water of mercury (II) pleustophyte Lower Silesia Nr N° N523 612139.

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Enhanced Biological Phosphorous Removal (EBPR),
Phosphate Accumulating Microorganisms (PAO),
Denitrifying Phosphate Accumulating Microorganisms (dPAO)

Ewa GALAS, Katarzyna PIEKARSKA*

THE PHOSPHATE ACCUMULATING MICROORGANISMS

Phosphorus and nitrogen are the major factors of water eutrophication. For many years it was thought that both of those elements are equally responsible for the eutrophication process. A study conducted by Schindler showed that phosphorus is the factor limiting the growth of algae [1]. It becomes harmful for the living organisms to inhabit the aquatic environment due to excessive growth of phytoplankton organisms in the water, which is going to be unfit for human consumption. For that reason, it is crucial important to pay special attention on the reduction of phosphorus inputs into the surface water. This article provides preliminary results of research on the biodiversity of PAO's bacteria.

INTRODUCTION

Phosphorus is an important macro mineral necessary for the proper functioning of every organism. It is also the most important element responsible for eutrophication. In water it is observed that the concentration valume above 0,1 gPO_4/m^3 intensifies algae growth. The maximum concentration of phosphorus allowed in treated wastewater being introduced into the surface water is 1.0 gP/m^3 (or 90% reduction) for the population equivalent of more than 100000 inhabitants. For the comparison in Canada, the concentration of phosphorus in treated wastewater discharged to the receiver must be in the range of 0.3-0.5 gP/m^3 [2]. In order to prevent eutrophication the efficiency of wastewater treatment must be improved and the regulations of allowed concentration of phosphorus discharged to surface water should be strengthen. The phosphorus concentration in raw wastewater ranges from 5 g P/m^3 to 20 gP/m^3, lower concentrations are observed in the effluents from areas where detergents without phosphates are used. It is known that phosphate derived from

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detergent amounts from 50% to 75% of the total content of P in wastewater [3]. So, it is important to introduce a control system consisting in reducing the consumption of detergents containing phosphates or tend to their total withdrawing from the use. This system could contribute to a significant reduction of phosphorus load into wastewater.

1. PHOSPHORUS REMOVAL FROM WASTEWATER

Phosphorus compounds can be removed from wastewater by biological, physicochemical and chemical methods. Currently, biological methods are the most popular. They are based on the liveliness of activated sludge microorganisms. In conventional activated sludge methods heterotrophic bacteria use inorganic compounds, mainly nitrogen and phosphorous, as an energy sources to synthesize necessary materials as building blocks of their biomass charge from the sewage. This process is called "simple assimilation" and it allows the removal of 20 to 30% of phosphorus from wastewater. The effectiveness of biological phosphorus removal capacity can be increased by using specific heterotrophic bacteria PAO (Phosphate Accumulating Microorganisms) to excessive phosphate accumulation in their cells [4, 5].

1.1. THE ENHANCED BIOLOGICAL PHOSPHOROUS REMOVAL – EBPR

Enhanced Biological Phosphorus Removal (EBPR) is the most promising method used in wastewater treatment. This process requires alternating anaerobic-aerobic or anaerobic-anoxic conditions, which allow selection and development of specific microbes PAO. Those microorganisms are primary responsible for the EBPR process. Phosphate Accumulating Organisms accumulate excess phosphorus in the form of polyphosphate under alternating anaerobic-aerobic or anoxic conditions. Under anaerobic conditions the volatile fatty acids are absorbed and stored inside the PAO bacteria in the form of poly-β-hydroxyalkanoates (PHA). This transformation generates energy mainly from the disintegration of polyphosphate, which causes the release of phosphate from the cells and increase of its concentration in wastewater [2, 5, 6]. Under aerobic condition, PAOs are able to use their stored PHA as energy source for biomass incrementation, glycogen replenishment, phosphate uptake and polyphosphate storage (fig. 1). The intensity of this process is significant. The total amount of phosphates collected from wastewater is higher than the amount ejected in the anaerobic condition [7].

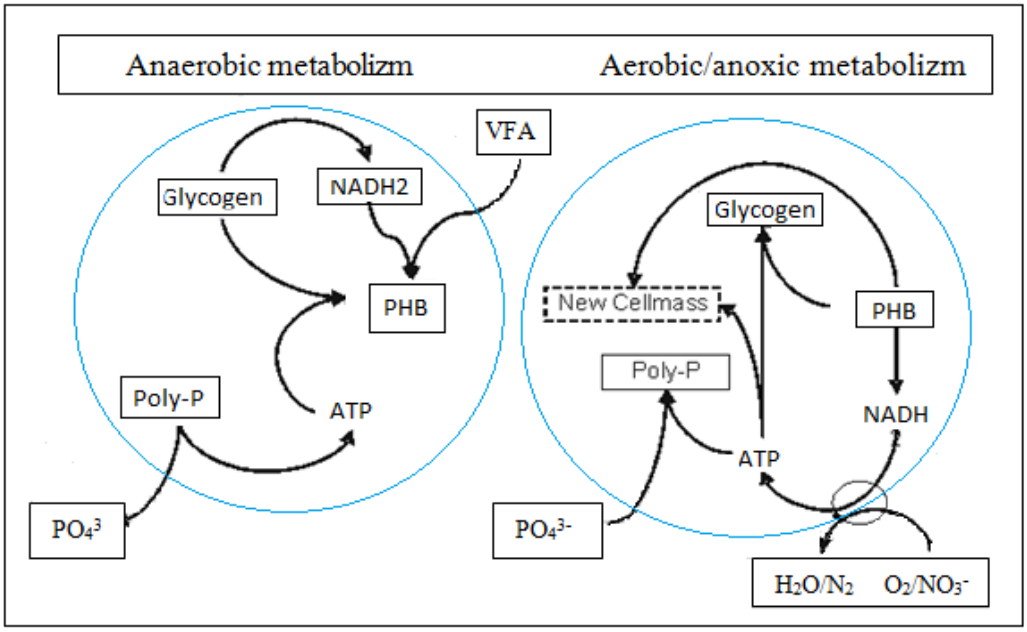

Fig. 1. Metabolism of PAO under anaerobic and aerobic/anoxic conditions, Smolders et al (1994).

2. THE PHOSPHATE ACCUMULATING MICROORGANISMS (PAO)

There are many types of bacteria able to store phosphorus as polyphosphates in special granules. For many years Scientists thought that *Acinetobacter sp.* strain, discovered by Fluhs and Chen in 1975, is solely responsible for the EBPR process. However, subsequent studies revealed that it is not the only one or even the dominant strain [8, 11].

In the last decade the identification of PAOs has proceeded through the use of molecular techniques. The use of this methods showed that a high diversity of PAO bacteria are present in lab and full-scale EBPR sludge [13].

A research performed by Eschenhagen et al. (2003) confirmed that not only one specific organism is responsible for the EBPR. During the study, they compared the PAO bacteria coming from samples of activated sludge from two laboratory plants with different methods of operation: one plant was working in anoxic/oxic condition with EBPR process without nitrification, the other in Phoredox system with EBPR, nitrification and denitrification processes. In both plants *Tetrasphaera spp.* dominated the PAO community. A small amounts of *Microlunatus spp. and Rhodocyclus group* were detected. The microbiological analysis was carried by fluorescence in situ hybridisation (FISH) and 16 S rDNA method. Authors underline that the appearance

and the dominance of the PAO community is probably controlled by the chemical and technical parameters of the wastewater treatment plants.

Bond et al. (1995) used the phylogenetic analysis of 16S rRNA to observe differences in bacterial community structure between phosphate and non-phosphate removing sludge. The *Rhodocyclus* group from subclass of the *β-Proteobacteria* represented a in the phosphate-removing community. Their conclusions were supported by the observation of an abundance of *Rhodocyclus* bacteria using FISH method in other sludges samples from good EBPR processes performance [10].

Hesselmann et al. (1999) in their research suggested that the *β-Proteobacteria* subclass should be named as *"Candidatus Accumulibacter phosphatis"* as closely related to *Rhodocyclus.* Hesselmann et al. (1999), Crocetti et al. (2000) and Liu et al. (2001) shown a highly amount of *Accumulibacter* in many research carried in lab-scale sludge. Similar results were obtained in full-scale sludge from different countries across the world. In all of studies taken by e.g. He et al. (2005), Wong et al. (2005) and Kong et al. (2004), *Accumulibacter* species was present in relative abundance from 4 to 22% of all bacteria. As a conclusion, the *Accumulibacter* was stated to be an important ospecies contributing to P removal in EBPR plants [12].

Now is known, that the bacteria able to accumulate phosphates belong to the following classes: *α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Bacteroidia, Actinobacteria.* Senior and Dawes (1973) created a list of more than 30 strains of bacteria accumulating polyphosphate. Among them some strains are considered to be able to accumulate the largest quantity of polyphosphate, which are: *Acinetobacter calcoaceticus, Areobacter aerogenes, Azotobacter agilis, etc. Acinetobacter johnsonii and Microlunatus phosphoorus* [9, 10].

The research on bacteria PAO and dPAO biodiversity should be continued to aspire to connect specific organisms to their function in the Enhanced Biological Phosphorus Removal process. The results of those observations may be useful to increase the efficiency of EBPR process.

3. PRELIMINARY STUDIES.

The aim of the study was to isolate potential PAO bacteria from activated sludge and to carry out their preliminary identification. The sludge used in the study comes from Międzybórz Wastewater Treatment Plant working in SBR system. The samples were taken from oxygen stage. To isolate potential bacteria PAO, it was used a method of early induction of excessive phosphate incorporation. Calls were grown for 7 days at 20 °C on the acetate substrate with trace elements [14]. As a result of the research, 25 different bacterial isolated strains were obtained.

The Albert's stain technique was used for identification potential PAO bacteria(- negativ, + positive). This method allows to detect the presence of polyphosphate particles inside the cell. The Gram stain method was used for determine the morphology of isolated cells. The results are shown in tab 1.

Table 1. The results of preliminary studies.

Stain number	**Albert's method**	**Gram method**	**Shape**
1'	+	Gram -	*Cocci*
2'	-	Gram -	*Cocci*
3'	+	Gram -	*Rod*
4'	+	Gram +	*Cocci*
5'	+	Gram -	*Cocci*
6'	+	Gram -	*Rod*
7'	-	Gram -	*Cocci*
8'	+	Gram -	*Cocci*
9'	+	Gram -	*Cocci*
10'	+	Gram -	*Rod*
11'	+	Gram -	*Cocci*
12'	+	Gram -	*Filamentousk*
13'	+	Gram +	*Rod*
14'	-	-	-
15'	+	Gram +	*Rod*
16'	+	Gram +	*Rod*
17'	-	Gram -	*Rod*
18'	-	Gram +	*Cocci*
19'	+	Gram +	*Cocci*
20'	+	Gram -	*Cocci*
21'	-	Gram +	*Cocci*
22'	-	Gram +	*Cocci*
23'	+	Gram +	*Rod*
24'	+	Gram -	*Cocci*
25'	-	Gram +	*Cocci*

In the figures 1 and 2 are shown the results of Albert's staining (black granules of polyphosphate against in green cytoplasm).

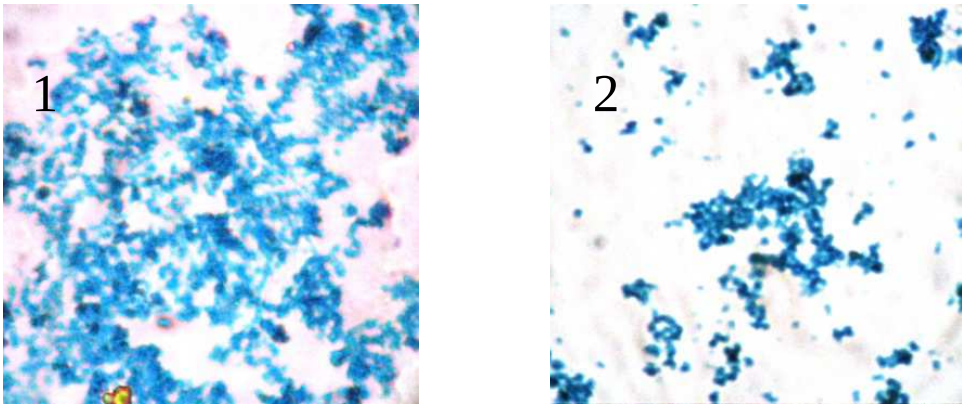

Fig. 2. Strains No. 1' and 20' contains seeds of polyphosphates.

After determining the morphological characteristics of PAO strains, the study of the ability of bacteria to bio-accumulate polyphosphate was started. The results will be compared with literature data.

CONCLUSIONS

For many years Scientists thought that only one group of bacteria PAO has the ability to excessive phosphate accumulation in cells. However, a study of the bacterial biodiversity, using genetic engineering denied this thesis. Bacteria responsible for the EBPR process may be a heterogeneous group, may be also characterized by a significant variation in biodiversity during the process. The preliminary studies confirmed that the PAO bacteria are a heterogeneous group.

Therefore, it is very important to continue the research on bacteria PAO and dPAO biodiversity and to aspire to connect specific organisms to their function in the Enhanced Biological Phosphorus Removal process. The results of those observations may be useful to increase the EBPR process efficiency, which will reduce the amount of phosphorus discharged to the receiver, and consequently, will improve the quality of water resources.

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phytoremediation, mercury, toxicity, bioaccumulation

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THE INFLUENCE OF MERCURY ON *SALVINIA NATANS*

Treatment of mercury contaminated water was carried out with the use of *Salvinia natans* derived from the artificial plant-breeding and from the environment (Oława river). The negative influence of mercury on plant organisms was evaluated on the basis of biomass growth changes in the phytoremediation process of water contaminated with 0.150, 0.200 and 0.300 mgHg/dm^3. This assessment has been confirmed by studies of photosynthesis pigments and total protein content in plants.

1. INTRODUCTION

The problem of environmental degradation caused by the pollution load tends to look for ways of limiting their negative impact on the biotic components of ecosystems and eliminating the health risks, stemming from human exposure to chemical contaminants. Metals are accumulating in the food chain, hence the organisms of higher trophic levels are particularly vulnerable to toxic effects caused by their presence. Mercury occurs in different chemical forms. It is an element of a particularly high biochemical activity, affecting its presence in various environmental components [1].

One way of limiting the migration of mercury in the aquatic environment may be the process of phytoremediation, allowing the bioaccumulation and phytoevaporation [2]. Accumulating the pollutant in the pool of autotrophic organisms and utilizing the biomass can break the cycle of metal circulation along with the matter in ecosystems. The plant species capable of bioaccumulating the elements exceeding the regulatory levels, toxic metals in particular [3-11], are used to minimize the occurrence of contaminant in the water. Among the aquatic plants in Poland, which are characterized by high speed and high efficiency of bioaccumulation of heavy metals, are pleustophytes of *Lemna sp.*, as well as *Salvinia natans*. *Salvinia natans* may be capable of releasing a volatile form of an element in the atmosphere (phytoevaporation). In view of the existing threat, which

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is mercury(II)-contamination of the waters of Lower Silesia (Czarna Woda, Kaczawa) [12,13], it is reasonable to use *Lemna* sp. And *Salvinia natans*, the vegetation of the Odra river, in the treatment of the aquatic environment. These organisms are tolerant to temperature range and the insolation during the vegetative season in Polish conditions.

2. METHODOLOGY

The preliminary research aimed to optimize the culture conditions for autotrophic organisms to the Polish climate zone in the vegetative season (average temperature 22 ± 10 °C, humidity 30 ± 2%, temperature of the water 20 ± 10 °C). The organisms from the specified species *Salvinia natans* from artificial plant-breeding, selected on the basis of preliminary morphological evaluation, were used in the study. Experimentswere also carried out on plants from Oława river (harvest point: Na Grobli in Wrocław).

Organisms were collected in the end of the vegetative season - harvest in autumn of 2011 (in the case of plants from environment, the mature plants with developed sporangia). After the selection, the plants were cultured in Hoagland medium with the following composition: KNO_3 – 1.02 g/dm^3, $Ca(NO_3)_2$×4H2O – 0.71 g/dm^3, $NH_4H_2PO_4$ – 0.23 g/dm^3, $MgSO_4$×7H_2O – 0.49 g/dm^3, $MnCl_2$×4H_2O – 1.81 mg/dm^3, $3H_3BO_3$ – 2.86 mg/dm^3, $CuSO_4$×5H_2O – 0.08 mg/dm^3, $ZnSO_4$×7H_2O – 0.22 mg/dm^3, MoO_3 – 0.09 mg/dm^3, $FeSO_4$×7H_2O (0,5%) – 0.60 mg/dm^3 [14]. Hoagland medium was made on the basis of redistilled water and contaminated with mercury salt $Hg(NO_3)_2$. The pH of pure Hoagland medium and mercury contaminated solutions was of 4-4.5.

The biological material (calculated per fresh matter) were introduced into reactors containing water contaminated with mercury at concentrations of 0.150, 0.200 and 0.300 mgHg/dm^3 and the control sample (uncontaminated medium). For each concentration, the experiment was carried out in 20-day cycles. The selected days of the experiment (3rd, 6th, 10th, 13th, 17th and 20th day) were adjusted in a separate reactor (stationary culture). Gradually, after the scheduled time, the plants were as a whole recovered from the reactor and underwent the relevant physicochemical and biochemical analysis. Biological studies (determination of photosynthesis pigments and total protein content in plants) allowed to assess the physiological condition of the plants in selected culture conditions [14-15].

Chemical and biological analysis was performed in a certified Laboratory of Toxicology and Environmental Research in the Institute of Environment Protection Engineering at the Wroclaw University of Technology.

3. RESULTS OF INVESTIGATIONS

The study of the dry matter changes of *Salvinia natans* (the artificial plant-breeding) indicates the increase in the cultures at concentrations of 0.150 and 0.200 mgHg/dm^3, similarly as in the control (uncontaminated Hoagland medium culture).

This tendency is observed until the 6th day of the experiment, whereas in the successive days the dry matter is maintained at a constant level. At the concentration of 0.300 mgHg/dm^3 no increase of dry matter was observed (Fig. 1).

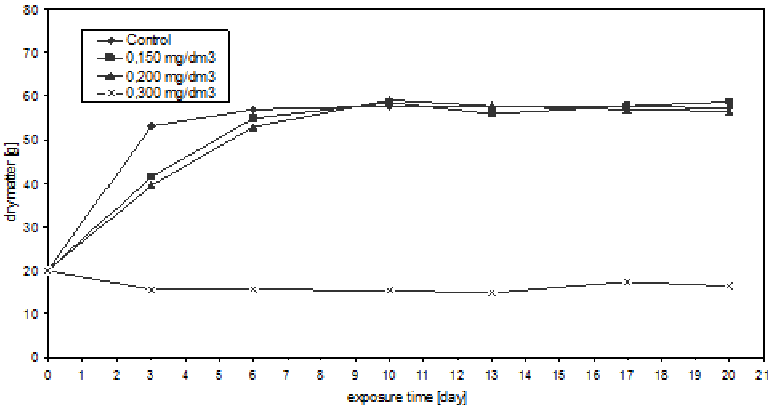

Fig. 1. Changes in the dry matter content during the phytoremediation process (the artificial plant-breeding).

Quantitative change (increase/decrease) of dry matter in relation to the initial value (Fig. 2) shows an increase in the biomass up to 2.5 g in the cultures contaminated with 0.150 and 0.200 mgHg/dm^3. In the case of 0.300 mgHg/dm^3 concentration dry matter levels did not change during the experiment.

In the case of the plants from Oława river in the mercury contaminated culture, a slight difference is observed in the increase in dry matter during the first week of the experiment (up to 0.5 g of dry matter), irrespectively of the concentration of metal in solution. Due to the maturity of *Salvinia natans* retrieved from environment, the change of the dry matter in control is also relatively small in comparison with the plants from artificial plant-breeding. In the artificial conditions plants do not develop sporangia and are able to reproduce the whole year.

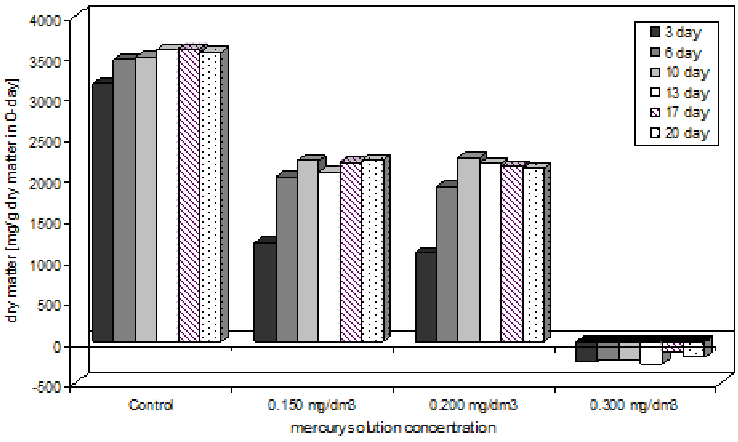

Fig. 2. The change in the dry matter of phytoremediation process (the artificial plant-breeding) with respect to day zero.

The photosynthesis pigment contents in artificial plant-breeding (Fig. 3) confirmed the negative impact of mercury not only on the growth of dry matter, but also on the content of chlorophyll type a and b in plants. However, compared to the control, the amount of chlorophyll is greater than at the beginning of the experiment (on the 3rd day of the cultivation at 0.150 mgHg/dm3 it is almost 0.25 mg chlorophyll/g of fresh matter). The decrease of pigments active in the photosynthesis process was observed only on the 20th day.

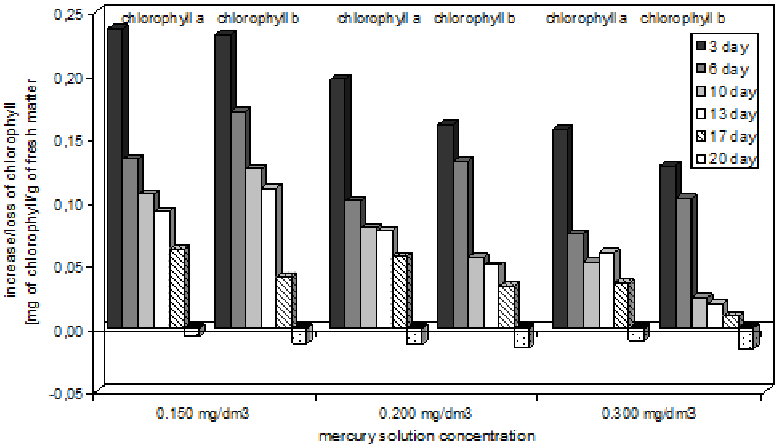

Fig. 3. Changes of the content of chlorophyll a and b in artificial plant-breeding compared to the control.

The photosynthesis pigments content in the plants from Oława river is up to 0.10 mg chlorophyll/g of fresh matter and practically does not change during the course of the experiment [16]. Compared with the control, there is a slight decrease in chlorophyll content of up to 0.05 mg chlorophyll/g of fresh matter. The level of pigments content in control is comparable to the samples contaminated with mercury, what may indicate the transition of organisms into sleep period.

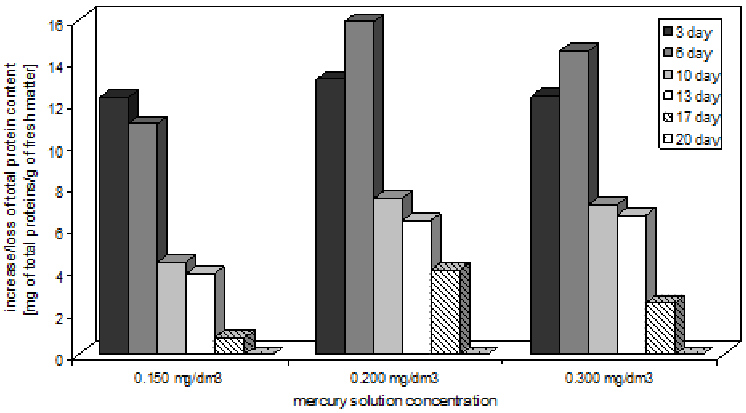

Fig. 4. Changes of total protein content in artificial plant-breeding compared to the control.

CONCLUSIONS

1. The plants with the artificial breeding adapted to the conditions created in the experiment.These plants are capable of year-round sit-breeding (continuous growth) without the transition to the sleep period. These plants lost the ability to produce sporangia.

2. The river plants generally responded worse to the changing conditions of breeding, after having been transferred from breeding to the laboratory conditions. The plants were mature organisms (harvest in the autumn of 2011) with sporangia, slowly entering the sleep period.

3. The dry matter increase (the artificial plant-breeding) in contaminated Hoagland medium with mercury concentrated at 0.150 and 0.200 mgHg/dm^3 is comparable to the control (uncontaminated Hoagland medium culture). Changes are observed until day 6 of the experiment (the maximum increase of 2.5 g of dry matter), on the following days no dry matter changes are observed. The 0.300 mgHg/dm^3 concentration does not affect the growth of biomass, the amount of biomass is constant.

4. In the case of river plants cultivation, the mercury concentration in the water and the duration of the plants exposure to metal in the solution did not significantly alter the amount of dry matter (the maximum increase was 0.5 g).

5. The chlorophyll a and b contents in artificial plant-breeding in comparison to the control is higher in all cultures contaminated with mercury up to the 20th day of the experiment and equals a maximum of 0.25 mg chlorophyll/g of fresh matter on the 3rd day of the experiment at concentrations of 150 mgHg/dm^3. On every day of the experiment there was a gradual decrease of the pigment content active in the photosynthesis process observed in the fresh matter of plants from the artificial breeding.

6. The photosynthesis pigment content in the fresh matter of the rivers plants can be estimated at approx. 0.10 mg chlorophyll/g of fresh matter in all cultures during the experiment period, including the controls. With regard to the control, the content of the chlorophyll is reduced by 0.05 mg chlorophyll/g of fresh weight in the phytoremediation process.

7. The total protein content in the artificial plants-breeding compared to control during the experiment (except for day 20) is max. 16 mg of total proteins/g of fresh matter greater on the 6th day.

8. While assessing the influence of mercury on the plants during the course of the experiment, an increase of dry matter in the first week of culturing was observed at lower concentrations of mercury in the solution. Longer exposure of plants to mercury does not cause further changes. This may be associated with declining physiological condition of the plants, which is indicated by the results of total protein and chlorophyll content.

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phytoremediation, Lemna sp., *Salvinia natans, aquatic environment, metals*

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USE OF *LEMNA* SP. AND *SALVINIA NATANS* IN BIOREMEDIATION OF AQUATIC ENVIRONMENT CONTAMINATED WITH METALS

The reduction of contamination in aquatic environments requires the elimination of hazardous substances in an environmentally friendly way. For this purpose, the process of phytoremediation makes use of plants capable of accumulating toxins in amounts exceeding their nutritional demand. The final effect of the process is bringing the contaminated aquatic environment to a condition acceptable by legal regulations. Phytoremediation may be used for decontamination of water environments with low excess of contamination levels. This article presents the effectiveness of bioaccumulation of metals by aquatic plants of *Lemna* sp. and *Salvinia natans*, species native to the territory of Poland.

1. INTRODUCTION

Excessive water contamination is the ecological problem that affects water resources all over Poland, in particular in the industrialized regions. Phytoremediation allows the ecosystems to recover their lost values. The legitimacy of using native plant species in the phytoremediation process stems from their tolerance for the climate conditions of the particular region, thus allowing for higher efficacy of the process at relatively low financial expenses. In Poland, plants of established capabilities for bioaccumulation of numerous toxic substances, which can successfully be used for phytoremediation, include *Lemna* sp. (*Lemna minor*) and *Salvinia* sp. (*Salvinia natans*). Both pleustophytes species occur in the growing period in stagnant water (oxbow lakes, ponds) and running water, but only at the edge of reservoirs and among another aquatic vegetation. These plants prefer eutrophic reservoirs of water.

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Determination of optimum process conditions, assessment of the effectiveness of bioaccumulation of toxins and investigation of the mechanisms of hazardous substances biodegradation are important aspects of the research process. Currently, the effects obtained with the phytoremediation method are not always comparable to those achieved with physicochemical methods (extraction, chemical precipitation, ion exchange, etc). This environmentally clean and energy-saving technology may currently be a supplement of the conventional methods, with the perspective of becoming an alternative to them in the near future. The principal advantages of phytoremediation are low capital expenditure, minimum interference with the existing environmental conditions, as well as the general approval of public. Phytoremediation entirely eliminates the need to introduce chemical compounds requiring further processing or storage. A significant asset is the possibility of performing phytoremediation *in-situ*, i.e. in locations where the contamination is generated, thus reducing costs of transportation and expences related to the maintenance of the treatment plant.

Phytoremediation has significant limitations such as the requirement to use shallow water reservoirs. A significant drawback is that the slow plant growth affects the rate of water treatment. Overly excessive contamination levels may damage plants and cause the toxic metal to enter circulation in trophic chains. On the contrary, distribution of toxins in the pool of various organisms may solve the problem of local environmental pollution [1-3].

2. THE PRINCIPLE OF THE PHYTOREMEDIATION PROCESS

Phytoremediation uses the plants' natural capabilities to acquire, accumulate and biodegrade contaminants. At concentrations close to those defined in standards, toxins are easily accumulated in the walls of plant cells. Unfortunately, high emissions of various pollutants into the environment result in the natural levels of hazardous substances being significantly exceeded, increasing the harmful effect of these substances on the organic matter, and thus on the biosorption process. In order to achieve quick recultivation of the contaminated environment, plant species need be selected according to their toxin uptake capabilities. Phytoremediation should be completed at the plant development stage, when the concentration of compounds in the biomass is at its highest. Organisms used for phytoremediation must be characterized by high accumulation of toxins and tolerance to high toxin concentrations, salinity and the presence of other contaminants. In addition, they should be characterized by high biomass growth and capability of transferring toxins from the roots to the overground parts of the plant, allowing for the possibly most effective elimination of hazardous substances from the contaminated environment along with the plant material. One of the techniques of surface water phytoremediation, rhizofiltration,

makes use of plants with roots just below the water surface. It is based mostly on employing the plant roots zone in the uptake, accumulation and biodegradation of toxins within plant tissues (with limited ability to transport the absorbed ions to overground organs) [4-8]. Due to their low content of augmentative tissue, *Lemna* sp. and *Salvinia* sp. display above-average capability to accumulate toxic substances.

3. THE PROBLEM OF METAL CONTAMINATION IN POLAND AQUATIC ENVIRONMENT

The effects of toxic substances on the aquatic environment are associated with the chemical form of the toxin, its properties, its amount and water conditions (pH, hardness, dissolved oxygen concentration, solid suspensions content, presence of chelating agents and mineral acids) [9]. High importance is also attributed to the presence of other elements and substances, as interactions between these entities may lead to changes in the toxic character of the particular contaminant. Due to the advanced development and industrialization of Poland, the natural aquatic levels of contaminants, particularly heavy metals, are exceeded, leading to environmental degradation. The presence of hazardous substances in the aqueous environment may also stem from the natural presence of these compounds in geological formations.

The copper levels measured in the reservoirs of the Legnica-Głogów Copper District were reported to be above 500 mgCu/dm^3, while the natural river water content of copper is 1-2 µg/dm^3 [9]. The Voivodship Inspectorate for Environmental Protection [10] report arsenium on water conditions in Lower Silesian Voivodship contains information on high concentrations of arsenic and mercury, severely exceeding the geological background levels in the rivers Kaczawa and Czarna Woda. In 2005, the respective excesses were 63 and 123 mgAs/dm^3 and 2.01 and 1.40 mgHg/dm^3. Excessive heavy metal contamination was also observed in the river Ślęza (264 mgCu/dm^3 - background level 7 mg/dm^3; 437 mgZn/dm^3, 37 mgCr/dm^3). Arsenic, barium and copper excesses were measured in the river Odra within the borders of Lower Silesian Voivodship. Emission of metal-bearing dusts from the metallurgical industry in Lower Silesian Voivodship was 114 t/year, which leads to a significant amount of metals entering surface waters with atmospheric precipitation [9]. The levels of metal elements in surface waters are also elevated by the metallurgical, mining, paint and paper industry, together with agricultural intensification measures associated with the use of fertilizers and pesticides. A significant problem is posed by leaks and waste oozes from community and industrial waste disposal sites. Cadmium concentration of 5.2 mgCd per kg of soil was reported in the landfill vicinity areas in Ciechanów [11]. Wastewaters discharged into surface waters from various industrial plants are characterized by high heavy metal load and low content of biogenic ele-

ments, which raises difficulties in their biological decontamination. In 2005, the wastewaters from the KGHM plant (ZGW water treatment plant in Legnica) contained up to 0.25 mgHg/dm^3, with the permissible average daily limit of 0.1 mgHg/dm^3 [12]. In 2010, the Voivodship Inspectorate of Environmental Protection in Katowice presented a report on the exceeded boron (non-metallic element) levels at Potok Goławiecki measuring point (at the Mała Wisła estuary), ranging from 1.34 to 4.63 mgB/dm^3 (average of 3.317 mgB/dm^3) [13]. Significant contamination with boron was observed in the waters of the multilayer water-bearing system in the region of the dumping grounds from the then being liquidated chemical plant "Tarnowskie Góry" (up to 240 mgB/dm^3 in the Quaternary water-bearing layers and up to 116 mgB/dm^3 in Triassic layers [shell limestone]) [14]. Boron concentration in the pre-treated copper industry wastewaters (KGHM Legnica) was 3.58 mg B/dm^3 [9]. According to Lis (unpublished data), the water in Biała Przemsza and Czarna Przemsza rivers display boron concentrations of 20-590 µl/dm^3 (average of 125 µl/dm^3) [9].

4. THE EFFICACY OF AQUATIC ENVIRONMENT DECONTAMINATION BY NATIVE PLANTS *LEMNA* SP. AND *SALVINIA NATANS*

Lemna sp. and *Salvinia natans* plants are pleustophytes with high tolerance to temperature ranges and the level of insolation during the vegetative period in Poland. They are characterized by high capability to accumulate the contaminants and by fast and relatively steady growth. *Lemnacea* species are known for their capability to eliminate nutrients and organic substances [15,16], suspensions [17,18] and various trace elements [4,19-21] from water. Studies of the biosorption efficacy in Poland's conditions confirmed that *Lemna minor* was efficient in phytoremediation in relation to Cd, Pb, Zn, Cu and Ni [22]. In addition, *Lemna minor* and *Lemna gibba* proved to be good bioaccumulators of boron in waters with low concentration of that element. *Lemna minor* was efficient in decontaminating waters at concentrations of 0.01, 0.11 and 1.01 mgB/dm^3, with the maximum biosorption of 3200 µgB/g of dry matter [23]. Frick [24] showed the impact of pH on bioaccumulation of boron. At pH of 5.0, *Lemna minor* tolerated aquatic boron levels up to 20 mgB/dm^3 without symptoms of growth inhibition. Toxic effect of boron at concentration of 20 mgB/dm^3 was observed only after three days of plant exposure. At pH of 4.0 (20 mgB/dm^3) and after seven days of exposure, *Lemna minor* accumulated 0.093 mgB/g of fresh mass (148% compared to control), while at pH of 7.0 as much as 0.257 mgB/g of fresh mass (525% compared to control). Biosorpion of boron by *Lemna gibba* in waters with boron concentrations of 0.3 to 10 mgB/dm^3 proved most efficient for concentrations below 2 mgB/dm^3 [25]. At the end of the experiment, the boron content in tissues was 930 to 1900 mgB/kg

of dry matter. The maximum efficacy of bioaccumulation of boron by *Lemna gibba*, equaling 40%, was observed during the first two days of the experiment [26].

Aquatic plants of the *Salviniaceae* family are excellent hypercumulants of heavy metals compared to other plant species [4,27-32]. Similarly as in the case of *Lemna* sp., high rate and efficacy of bioaccumulation of heavy metals by *Salvinia* sp. is observed within the first week of exposure to toxins. Phytoremediation efficacy of as much as 96% was observed for copper ions on the sixth day of culturing at the exposure risk of 5 mgCu/dm^3 [28]. Maximum biosorption of copper was obtained on the eight day of culturing in waters with concentration of 10 and 15 mgCu/dm^3, and on the ninth day in waters with concentration of 20 and 25 mgCu/dm^3. Morphological changes (leaves turning yellow until partial discoloration) occurred as late as at the beginning of the second week of culturing at higher concentrations of aquatic copper (8th day, 20 and 25 mgCu/dm^3). In case of chromium, the content of this element in *Salvinia minima* biomass after seven days of exposure to 2 mgCr/dm^3 was 449.37 µgCr/g, rising to only 451.39 µgCr/g after two weeks [31,32]. *Salvinia herzogii* accumulates (mostly in roots) more than 90% of chromium present in water at concentrations of 1 to 6 mg/dm^3 (within 31 days), the uptake being most intensive on the first day of exposure [30]. Poland's native species *Salvinia natans* is a hypercumulant which, apart from eliminating high concentrations of metal ions and biogenic elements [4,33-38], is capable of phytoevaporation of the contaminants into the atmosphere following their transformation into volatile forms [3]. Studies on bioaccumulation of copper (from 0.01 to 1 mgCu/dm^3 of water) confirmed the efficacy of *Salvinia natans* in the process [33,36]. The maximum efficacy of ca. 90% was obtained at concentrations below 0.05 mgCu/dm^3 [36]. The highest dry mass increase in case of *Salvinia natans* was observed in waters with concentrations of 2 mgCu/dm^3 [38]. The recent study by Hołtra et al. [39] showed a two- or even three-fold increase in the accumulation of boron by *Salvinia natans* compared to control in cultures contaminated with concentrations of 1 and 3 mgB/dm^3, without observing any morphological changes in the plants. At higher concentrations of boron, the bioaccumulation was limited by damages occurring at the cellular level of the plants.

5. SUMMARY

Studies conducted to date confirmed the efficacy of Poland's native species of *Lemna* sp. and *Salvinia natans* in the process of rhizofiltration, especially during the first days of water decontamination process, when the efficacy of the process was highest. Biological methods allow to improve the quality of life without creating additional burden and landscape changes due to introduction of new chemicals, with un-

predictable consequences. Phytoremediation by *Salvinia natans* and by mixed systems including *Lemna* sp. may solve the problem of water treatment, even after preliminary treatment of wastewater by conventional methods. The biomass increase can significantly reduce the risk of eutrophication. The effectiveness of the phytoremediation process depends on the climatic conditions in Poland. Temperature decrease causes the inhibition of natural water treatment in the period between late autumn and early spring. A significant limitation to this method is the sensitivity of pleustophytes to the concentration of pollutants in water (this is the stress factor causing phytotoxic effect) as well as the cumulative capacity of plants at the contamination site. Phytoremediation of water also depends on the chemical speciation of toxins, pH, temperature, light intensity, the presence of organic substances, metals and nonmetals, ionic strength and oxygen level.

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Antibiotic resistance, water supply system, biofilm, microorganism

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ANTIBIOTIC RESISTANCE OF MICROORGANISMS ISOLATED FROM THE WATER SUPPLY SYSTEM IN WROCLAW

After Alexander Fleming's discovery in 1929 researchers started investigating antibiotics. Nowadays, antibiotics have become so common that they are overused, which leads to the development of resistance in microorganisms. The lack of response of microorganisms to chemotherapeutic agents may be a threat to human health.

An increasingly common phenomenon occurring in water distribution systems is the so-called biofilm, i.e. biological membrane. Microbes that live in such a system are more resistant to chemical agents (disinfectants, antibiotics). Biofilm bacteria have developed many defense mechanisms against harmful agents.

In the case of resistance to chemotherapeutics this is the conjugation process, which consists in transferring the resistance gene in microorganisms.

1. ADMISSION

Nowadays, the use of antibiotics is very widespread and popular, which increases the resistance of microorganisms to this type of medicine in all natural environments. Antibiotic-resistant microorganisms in ground and surface waters used for municipal purposes pose a serious threat to the health of people and animals. Modifications to the water treatment systems do not provide biological and chemical stability. This leads to the re-growth of microorganisms in the water supply in the form of plankton or biofilm.

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The variety in environmental conditions and the diversity of species of bacteria in biofilm can provide microorganisms with adequate protection against antibacterial agents. Microbes living in the biological membrane developed many defenc mechanisms against bactericidal substances. As a result of a shortage in food sources in the biofilm structure, active forms used dead cells as food. The slowed growth of microorganisms facilitates the activation of the enzymatic action. Damage resulting from the action of harmful compounds is repaired. Biological membrane can return to the previous state in a few hours. Therefore, antibiotics do not enter the interior of the biofilm.

The use of disinfectants or antibiotics induces point mutations in genes of bacterial biofilm. Microbes living in the biological membrane can transfer resistance genes to future generations, or, in the process of conjugation, to other species of bacteria. Progeny released into the external environment are already resistant to noxious agents.

The biofilm on the surface of the pipeline increases the size of the microorganisms in drinking water. Bacteriological contamination, including pathogenic forms resistant to antibiotics, is one of the most significant and undesirable changes to the composition of water. The amount of bacteria in the water is influenced primarily by the density of microorganisms in the biological membrane, the flow velocity and the adhesive properties of microorganisms.

The paper presents the results of research on drug resistance of microorganisms isolated from the Wroclaw tap water.

2. MATERIALS AND METHODS

Water samples were taken from selected locations in the „Na Grobli" and „Mokry Dwór" Water Treatment Plants (ZPW) – (after disinfection). The locations where samples were collected from the water supply system were selected based on the distance from the ZPW. Water was collected into sterile 1L bottles, then it was filtered using sterile membrane filters of 0,2 µm. Filters were placed in 90 ml of growth medium and subjected to 1 minute of of ultrasound in order to separate the microorganisms from the filter surface. Microorganisms were isolated by plate method using specialist microbiological media, including the R2A medium. Preliminary assessment of microbial biodiversity was conducted based on the observation of colony morphology and Gram's method of cell staining. Antibiotic resistance was assayed using disk-diffusion method. Cultures of microorganisms were plated on Muller-Hinton medium, and then discs impregnated with antibiotics were applied. After 24h of incubation, zones of inhibition were examined.

Table 1. Antibiotics used in clinical drug resistance.

Class of antibiotics		Antibiotic	Concentration	Symbol
Aminoglycosides		Amikacin	30 µg	AK
		Streptomycin	10 µg	S
β-lactam Antibiotics	Aminopenicillins	Amoxicillin	25 µg	AML
Tetracycline		Tetracycline	30 µg	TE
Synthetic chemiotherapeutics	Sulfonamides	Trimethoprim	5 µg	W

3. RESULTS

35 different strains were isolated from the tap water samples. As a result of Gram staining 33 strains of gram-positive and 2 gram-negative strains were obtained. Among the isolated strains *cylindrical forms* predominated in the shape of the cells. The obtained Gram positive strains were the following *cocci* (five strains), *bacilli* (11 strains), *cylindrical forms* (16 strains) and *yeasts* (1 strain). 2 *bacteria* were isolated from the gram-negative organisms.

Table 2. Cell morphology.

Cell shape	Number of strains	
	Gram-positive	Gram-negative
Cocci	5	—
Bacteria	—	2
Bacilli	11	—
Cylindrical forms	16	—
Yeast	1	—

To determine the resistance of microorganisms to antibiotics one of the methods developed by Bauer were used. Microorganisms were divided into the following:

- Sensitive “S”
- Intermediate “I”
- Resistant “R”

The following is a selection of photographs documenting the study of antibiotic resistance of strains.

The table 3 shows the results of the antibiotic resistance of strains.

Table 3. The results of the resistance of strains on selected antibiotics.

Number of strains	**Antibiotics**				
	AK – 30 µg	S – 10 µg	AML – 25 µg	TE – 30 µg	W – 5 µg
1	**R**	**R**	**R**	**R**	**R**
2	**R**	**R**	**R**	**R**	**R**
3	**R**	**R**	**R**	**R**	**R**
4	**R**	**R**	**R**	**R**	**R**
5	**R**	**R**	**S**	**R**	**R**
6	**R**	**R**	**R**	**R**	**R**
7	**R**	**R**	**R**	**R**	**R**
8	**R**	**R**	**R**	**R**	**R**
9	**R**	**R**	**R**	**R**	**R**
10	**R**	**R**	**R**	**R**	**R**
11	**R**	**R**	**R**	**R**	**R**
12	**R**	**R**	**R**	**R**	**R**
13	**R**	**R**	**R**	**R**	**R**
14	**R**	**R**	**R**	**R**	**R**
15	**R**	**R**	**R**	**R**	**R**
16	**R**	**R**	**R**	**R**	**R**
17	**R**	**R**	**R**	**R**	**R**
18	**R**	**R**	**R**	**R**	**R**
19	**R**	**R**	**R**	**I**	**R**
20	**R**	**R**	**R**	**R**	**R**
21	**R**	**R**	**R**	**R**	**R**
22	**R**	**R**	**R**	**R**	**R**
23	**R**	**R**	**R**	**R**	**S**
24	**R**	**R**	**R**	**R**	**R**
25	**R**	**R**	**R**	**R**	**R**
26	**R**	**I**	**R**	**R**	**R**
27	**R**	**R**	**S**	**R**	**R**
28	**R**	**R**	**R**	**R**	**R**
29	**R**	**R**	**R**	**R**	**R**
30	**R**	**R**	**R**	**R**	**R**
31	**R**	**R**	**R**	**R**	**R**
32	**R**	**R**	**R**	**R**	**R**
33	**R**	**R**	**R**	**R**	**R**
34	**R**	**R**	**R**	**R**	**R**
35	**R**	**R**	**R**	**R**	**R**

The table shows that all cultures are resistant to antibiotics. Only two strains (number 5 and 27) are sensitive to amoxicillin and strain number 23 is sensitive to tetracycline. Strain 19 exhibits intermediate sensitivity to tetracycline and strain 26 to streptomycin.

The results may seem alarming. However, for confirmation of drug resistance of the isolated strains further investigation is required (classification of strain to genus/species, examination of the gene of resistance using molecular biology techniques).

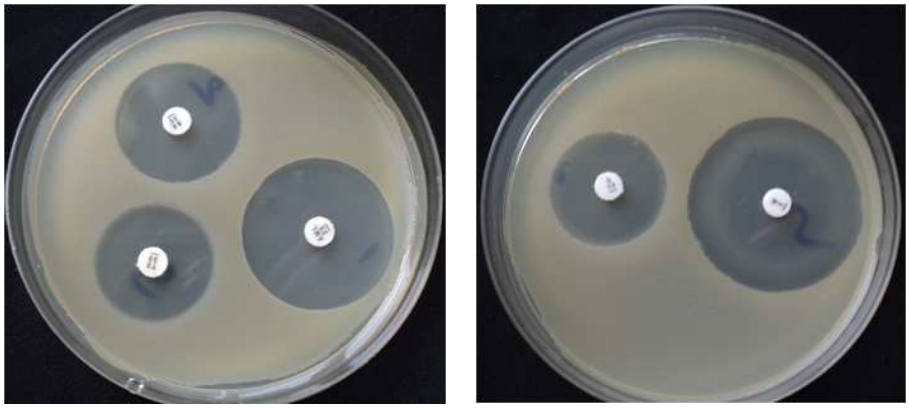

Fig. 1. Antibiotic resistance of isolated bacteria.

CONCLUSION

The article identified the sensitivity of microorganisms isolated from the Wroclaw water supply system to selected antibiotics. Only 2 out of 35 strains were sensitive, and 3 intermediate.

Antibiotic resistance of microorganisms is a serious issues, especially in water supply systems. Antibiotics allow for the extension of human life, but also, if overused, lead to an increased resistance of microorganisms. Therefore it would be advisable to reduce the use of antibiotics, or to improve the already known antibiotics, and produce vaccines. It is also important to monitor the drug-resistant bacteria in an aqueous environment.

The work is carried out with the help of MPWiK SA in Wroclaw.

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aquatic fungi, Oder River, biomass, CFU, drug

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AQUATIC FUNGI IN THE ODER RIVER, ITS TRIBUTARIES AND THE RIVERS DIRECTLY INFLOWING TO THE BALTIC SEA UNDER SUMMER HYDROCHEMICAL CONDITIONS

The aim of this study was to determine the fungal biomass in the surface water at 9 sites located along the Odra River, from the Czech Republic border to Szczecin, 10 stations on the main tributaries of Oder River and 9 on the rivers directly inflowing to the Baltic Sea. The study was conducted over two consecutive days in August 2011, at moderate water levels. Fungal biomass was calculated on the basis of quantitative chromatographic analysis of ergosterol in seston. Furthermore, the number of colony forming units (CFU/mL) was determined by screening method, with dilutions on Sabouraud's medium supplemented with chloramphenicol at 5 days of incubation. Determinations were also made with regard to chemical composition and physicochemical properties of the water samples.

The biomass of aquatic fungi most frequently ranged from 0.2 to 0.5 μg/L, and the abundance of fungi in river waters did not exceed 4000-25000 CFU/mL. These figures are average or slightly higher than those recorded in other Polish rivers. In addition, there was a statistically significant correlation between fungal biomass and the content of POC, phenols, total nitrogen, electrolytic conductivity, and pH. The abundance of fungi (CFU/mL) depended on the content of DOC and ammonium nitrogen (statistically significant relationship).

In addition, our analysis also concerned the drug resistance of fungal colonies isolated from the Odra surface waters and its main tributaries, using the disc diffusion method. The water samples collected from 10 sites contained fungi resistant to amphotericin B (10 μg), and 11 samples showed the presence of fungi resistant to the antibiotic gentamicin (10 μg). In the samples tested there was not any colony sensitive to the commonly used antimycotic clotrimazole (10 μg) and the antibiotic erythromycin (15 μg). It seems likely that aquatic fungi may acquire drug resistance as a result of horizontal transfer of a gene responsible for drug resistance from other microorganisms, or as a result of the introduction of antibiotics and antimycotics to aquatic ecosystems in wastewater.

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1. INTRODUCTION

Microfungi have been found in almost all types of aquatic environments in the world where they are actively involved in the microbial decomposition of organic matter, especially plant-based matter, preparing the bottom of the water body for settlement by other organisms, mainly invertebrates. It has been shown that after just a few hours, plant leaves decomposing in water are covered with a biofilm of microorganisms, predominantly fungi [32]. Aquatic fungi may be suspended in the water column, settling on the bottom, periphyton or entering rivers directly in terrestrial runoff. They can also be of anthropogenic origin. To date, there have been numerous literature data on the taxonomic identification of *Hyphomycetes*, a yeast-like and zoosporous fungi occuring in water [5, 6, 21]. There is, however, a lack of quantitative data, i.e. concerning biomass and abundance of fungi in the water column, especially in relation to hydrochemical conditions in aquatic ecosystems. It has also been shown that aquatic fungi actively participate in the mitigation of anthropogenic stress by participating in the biotransformation of organic xenobiotics in the aquatic environment, which can improve water quality. These features indicate a previously ignored role of this group of organisms in the self-purification of waters and a potential use as bioindicators of anthropogenic transformation of the aquatic environment [24, 35, 40].

There have been increasingly frequent reports that aquatic fungi can cause diseases in fish and other aquatic animals. The invasion of host tissues is facilitated, among others, by an acquisition of resistance to commonly used antifungal agents. In addition, aquatic fungi are also present in drinking water, with the limit of occurrence of fungi in water intended for drinking set at 100 CFU per 100 ml of water [2]. However, the importance of fungi in drinking water for water quality and human health is still unclear. Some researchers have reported that some fungal species isolated from water may cause diseases and allergies in humans [1, 9, 18]. In addition, aquatic organisms resistant to antibiotics can pass this trait to pathogenic microorganisms, e.g. through a horizontal gene transfer, which may have important implications for animal and human health. Acquisition of resistance to antibiotics by aquatic organisms may result from the entry of metabolically active antifungal agents into the aquatic environment with agricultural and urban waste water. Accordingly, aquatic fungi may then be used as indicators of anthropogenic changes in aquatic environments [31].

In light of these reports, the aim of this study was to determine the biomass and abundance of fungi in the Oder River, its major tributaries, and in the rivers directly inflowing to the Baltic Sea, in relation to selected physico-chemical parameters of water and analyze their resistance to antibiotics and mycobiotics.

2. MATERIALS AND METHODS

2.1. STUDY AREA

The study included 9 stations on the Oder River course starting from the border of the Czech Republic to Szczecin and 10 stations on the main tributaries of Oder River and 9 on the rivers directly inflowing to the Baltic Sea (Fig. 1). Oder River have a regulated water course with active water transport for more than 100 years. Rega River, Lega River, Parsęta River, Łeba River and Słupia River represents rivers typical for the late glacial, moraine catchment with higher forestations and lower anthropogenic pressure on water quality than in Oder River catchment. The study material was collected from surface water (up to 1 m) in the midstream in August of 2011 and stored one day in a cool place in polyethylene bottles (1 L) before analysis. Samples collections took place during two days, at average level of river water.

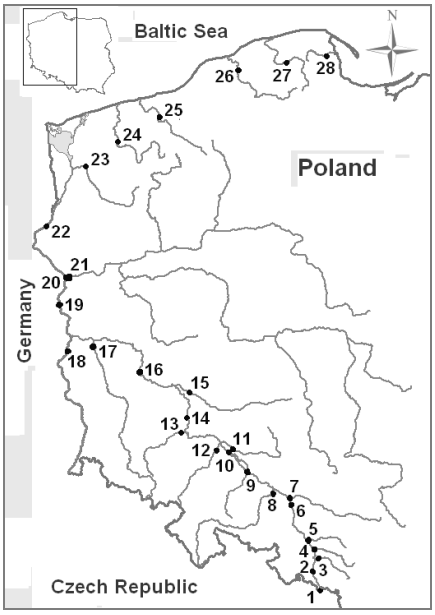

Fig. 1. Map with the river net and the location of the sampling points: 1. Oder River - Chałupki, 2. Odra River – Racibórz, 3. Ruda River- Kuźnia Raciborska, 4. Bierawka River – Bierawa, 5. Gliwicki Canal - Kędzierzyn Koźle, 6. Odra River – Opole, 7. Mała Panew River – Czarnowąsy, 8. Nysa Kłodzka River – Skorogoszcz, 9. Oława River – Oława, 10. Odra River – Wrocław, 11. Widawa River – Psary, 12. Bystrzyca River – Wrocław, 13. Kaczawa River – Prochowice, 14. Odra River – Ścinawa, 15. Barycz River – Osetno, 16. Odra River - Nowa Sól, 17. Bóbr River – Dychów, 18. Nysa Łużycka River – Gubin, 19. Odra River – Słubice, 20. Odra River – Kostrzyń, 21. Warta River – Kostrzyń, 22. Odra River – Krajnik, 23. Ina River – Goleniów, 24. Rega River – Płoty, 25. Parsęta River – Karlino, 26. Słupia River – Słupsk, 27. Łeba River – Lębork, 28. Reda River – Wejherowo.

2.2. PHYSICO-CHEMICAL ANALYSES OF WATER

Basic physiochemical water parameters electrolytic conductivity, temperature and oxygen saturation, were measured in the field by means of a HQD 9200 meter by Hach Lange. Chlorophyll *a* concentration was measured by spectrophotometric measurement after homogenisation of the suspension remaining on a GF/F filter and extraction with boiling ethanol [26, 33]. Dissolved organic carbon (DOC) was determined by the method of high-temperature catalytic combustion in a Shimatzu TOC-5050A analyser [41]. In order to determine the concentration of particulate organic carbon (POC), the colorimetric dichromate method was applied [4], involving the analysis of suspension residue on the GF/F filter. The quality of the dissolved organic matter was estimated with the application of the Specific UV Absorbance index ($SUVA=Abs._{260nm}*1000*DOC^{-1}$) [41]. Phenols concentration was determined according to Lowry's method [27]. Ammonium nitrogen and total nitrogen were determined by methods described by Hermanowicz [19].

The results were subjected to statistical analysis in SPSS 19. The Kruskal-Wallis was used to estimate the difference between means. The significance of the correlation was estimated by the Pearson coefficient. Differences of $p < 0.05$ were considered as statistically significant.

2.3. ERGOSTEROL AND CFU ESTIMATION

Unfiltered samples of water (250 µl), diluted to proportions 1:10 and 1:100, were inoculated directly onto Petri plates containing chrolamphenicol (POCh) enriched Sabouraud agar. The plates were incubated at a temperature of 25 °C for 5 days. After the incubation period the total number of colonies of fungi were determined [8].

The biomass of fungi suspended in water was calculated based on chromatographic quantitative analyses of ergosterol using the HPLC method according to Davis and Lamar [7], Mille-Lindblom and Tranvik [30] and Jorgensen and Stepanauskas [22]. The method involves analysis of the concentration of ergosterol, a component of fungal cell membrane, and its conversion to aquatic fungi biomass. One litre of water sample was filtered through GF/F filters with 0.7 µm mesh, immediately frozen in a temperature of -25 °C. During further analysis, the frozen filters were placed in test-tubes, mechanically ground into smaller fragments, and inundated in liquid nitrogen. After vaporisation, 4mL 10% KOH in methanol and 1mL cyclohexan were added. After leakproof sealing of the test-tubes, the substrate was subject to sonication in an ultrasonic cleaner for 15 minutes at a frequency of 42 kHz. The suspension was heated in water bath for 90 minutes at a temperature of 70 °C. After cooling the test-tubes to room temperature, 1mL distilled water and 2 mL cyclohexan were added. Then, the test-tubes were gently rotated 30 times for approx. 1 minute for the purpose of extracting ergosterol. At a further stage, the content

of the test-tubes was centrifuged for 5 minutes at 1000 g. After that, the cyclohexan fraction was collected and placed in a lockable glass test-tube. Further 2 mL cyclohexan was added to the suspension, and the shaking and centrifuging procedure was repeated. Both portions of the supernatant were mixed and vaporised dry by means of a stream of nitrogen at a temperature of 40 °C. The sediment was dissolved in 1mL methanol and incubated for 30 minutes at a temperature of 50 °C. After filtering the sample through a syringe filter with 0.2 μm mesh, it was stored at a temperature of 4 °C until analysis by means of HPLC. The chromatographic set consisted of the following modules: System Gold 125 Solvent Module, 166 Detector, Autosampler 502 made by the Beckman Company and a computer equipped with the System Gold The Personal™ Chromatograph program. Chromatographic separation was performed at a temperature of 30 °C on a Beckman C18 Ultrasphere ODS 5μm; 4.6 mm×25 cm column with the isocratic flow of methanol and distilled water solution at 98:2 volume proportions, set at the 1.5 mL/min level. Ergosterol was determined using a UV detector with wavelength 282 nm after elution time lasting approximately 11 minutes. Taking into account the fact that very small amounts of ergosterol were present in the river samples being analyzed, the standard addition method was used. To convert ergosterol to biomass of aquatic fungi a coefficient of 5.5 mg of ergosterol in 1 g of aquatic fungi and 35% carbon content was accepted [22]. Method validation revealed that the reclamation of ergosterol released from fungal hyphae and propagules of the fungi is 91±3%, and the method error does not exceed 0.1 ng/L.

2.4. DRUG SUSCEPTIBILITY TESTING

The assessment of sensitivity to commonly used antibiotics and anitmycotics was conducted using the disk diffusion method (Holt, 1975). Substrate YNB (yeast nitrogen base) was used for testing with the content: yeast extract 0.5%, glucose 3%, agar 1.8%. The medium was sterilized for 20 minutes at 1.5 atmospheres and its pH was established at 7. The medium was then deposited in a thin layer onto 9 cm diameter Petri plates. To assess sensitivity sterile discs impregnated with antibiotics: gentamycin (10 μg) and erythromycin (15 μg) (Oxoid) as well as antimycotics: amphotericin B (10 μg) and clotrimazol (10 μg) (Liofilchem). Fungi suspension for assessment of sensitivity was obtained from 5 day old cultures raised on Sabouraud medium, by flushing morphotypes using 9 ml 0.1% peptone saline (Oxoid CM0733). The resulting suspension was evenly spread on the surface of the YNB substrate, the excess was poured off and the top of the plate was left slightly incline at room temperature allowing drying of the substrate surface. Subsequently, onto the surface of the substrate paper disks saturated with the drugs were placed and the plates were incubated at a temperature of 25 °C. Sensitivity assessment was performed after 24 hours based on inhibition of growth according to a scale presented in Tab. 1 [20, 31].

Table 1. Interpretation of the results obtained using the disc-diffusion method of antibiotic and antimycotic sensitivity on the basis of the diameter of growth inhibition.

Drug	Sensitive	Intermediate	Resistant
Diameter of growth inhibition (in mm)			
Amphotericine B	≥15	10-14	<14
Clotrimazole	≥18	14-18	<14
Erythromycin	≥23	14-22	≤13
Gentamicin	≥15	13-14	≤12

3. RESULTS AND DISCUSSION

The average biomass of aquatic fungi was mostly within the range of 0.1-0.5 µg/L, with the number of fungi in river waters in the range 3000-25000 CFU/mL. In only one location, the Warta River in Kostrzyn, the average fungal biomass was 9.5 µg/L (Table 2). Coastal rivers had a smaller biomass of aquatic fungi than the Oder River and its tributaries. These figures are average or slightly higher than those recorded in other Polish rivers [22]. It is known that differences in fungal biomass and abundance depend on physico-chemical factors such as pH, temperature, availability of nutrients especially nitrogen and phosphorus, and organic matter [13, 24]. In a study conducted by Pascoal and Cassio [34], water hydrochemical conditions played an important role in the taxonomic differentiation of *Hyphomycetes*. Furthermore, Ferreira and Chauvet [10] showed that the occurrence of various species of aquatic fungi depended on temperature and oxygen concentration in the water. Other studies on the effects of temperature on fungi have shown that there are species that occur throughout the year.

There are also other species that are typical for a particular season. This seasonality of particular species has been demonstrated, for example, by studies on the Dudhawa dam reservoir in India. *Aspergillus niger* occurred with the greatest frequency in the winter and rainy seasons, while in summer it was not reported at all or only sporadically [37]. Growth of fungi from class *Hyphomycetes* and many species of yeast are inhibited at temperatures below 5 ºC, and hence in winter their biomass is reduced. *Oomycetes* occurs most frequently or only during cooler periods. Since noone so far has determined a factor which is most crucial for changes in fungal biomass in aquatic ecosystems, the mycological analysis of the analyzed water samples was augmented with an examination of chemical composition and the physico-chemical properties of the water.

Table 2. Average biomass, abundance and concentrations of selected physicochemical parameters in the Oder River, its main tributaries and the rivers flowing directly into the Baltic Sea ($p < 0.05$).

River	Stand	Fungal biomass [µg/L]	CFU/mL	EC [µS/cm]	Temperature [°C]	SWWT [%]	SUVA	Chlorophyll *a* [µg/L]
Oder	Chałupki	0.51	9500	308	15.0	97.00	43.3	2.9
Oder	Racibórz	0.17	3800	573	15.3	94.10	32.8	4.6
Ruda	Kuźnia Raciborska	0.20	4000	1352	13.4	86.10	23.4	12.5
Bierawka	Bierawa	0.33	4500	5470	16.0	91.50	25.4	6.1
Kanał Gliwicki	Kędzierzyn Koźle	0.17	15600	3222	17.9	89.70	25.7	32.6
Oder	Opole	0.20	16900	1018	17.1	99.40	30.8	3.6
Mała Panew	Czarnowąsy	0.21	5800	386	17.5	88.20	35.3	1.9
Nysa Kłodzka	Skorogoszcz	0.30	6400	301	18.5	92.40	25.8	7.1
Oława	Oława	0.25	4800	693	17.0	93.30	28.0	2.7
Oder	Wrocław	0.25	5800	669	18.6	96.60	35.6	5.9
Widawa	Psary	0.45	9900	649	17.7	80.50	30.2	6.3
Bystrzyca	Wrocław (Jarnotów)	0.24	5100	548	17.7	94.10	26.0	12.5
Kaczawa	Prochowice	0.39	9400	421	17.1	89.00	37.4	3.3
Oder	Ścinawa	0.31	9600	368	18.8	89.30	30.3	6.1
Barycz	Osetno	0.18	8000	630	18.8	74.00	32.0	2.1
Oder	Nowa Sól	0.12	8900	853	19.3	84.30	34.6	5.0
Bóbr	Dychów	0.32	19400	222	15.7	93.40	37.8	2.7
Nysa Łużycka	Gubin	0.31	10200	358	16.8	62.10	36.4	1.7
Oder	Słubice	0.23	11900	651	18.1	79.50	34.0	5.9
Oder	Kostrzyń	0.09	7400	652	18.7	79.80	30.8	6.1
Warta	Kostrzyń	9.43	22900	521	19.1	92.90	33.0	24.7
Oder	Krajnik	0.26	25200	586	20.0	77.00	32.3	7.5
Ina	Goleniów	0.26	21400	583	19.8	72.10	32.2	2.5
Rega	Płoty	0.25	13000	475	18.6	82.60	38.2	1.7
Parsęta	Karlino	0.30	8900	456	17.2	87.10	43.2	1.9
Słupia	Słupsk	0.11	5400	338	17.9	89.40	57.4	2.5
Łeba	Lębork	0.26	7600	355	14.9	85.00	63.3	1.3
Reda	Wejherowo	0.17	8200	394	16.5	83.50	19.9	6.3

The water temperature of the studied rivers ranged from 13 to 20 °C, and dissolved oxygen ranged from 62 to 99%. (Table 2). Within these ranges there was no statistically significant correlation between disolved oxygen or temperature vs. biomass of aquatic fungi. Our previous studies showed that an increase in the biomass of fungi can lead to oxygen depletion in the water; an inverse relationship between the average biomass of fungi and DO in the waters of Polish rivers [16].

In this present study we observed a statistically significant inverse relationship between mycoplankton biomass and water pH ($R^2 = 0.24$), which is consistent with

previous literature [24]. A significant correlation existed between electrolytic conductivity (EC) and fungal biomass (Fig. 2). An increase in EC in water to more than 600 µS/cm caused a decline in the biomass of fungi. This explains the drop in mycoplankton biomass in rivers with high mineral pollution [12, 13], which in turn leads to a reduction in biological mineralization of organic matter in rivers.

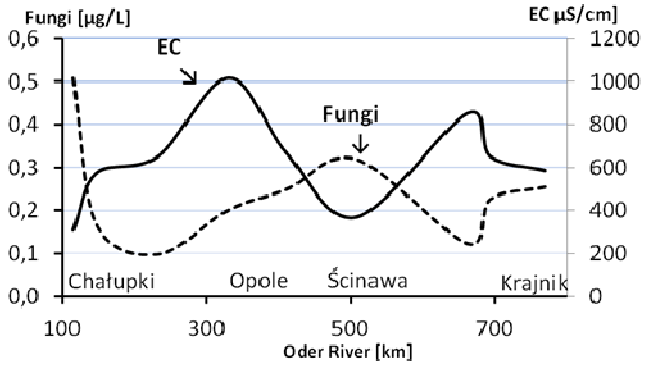

Fig. 2. Fungal biomass in the water of Oder River courses against electrolytic conductivity.

Another very important factor for the dominance of some species of fungi and the disappearance of others from ecosystems is the competition between fungi and other organisms, especially algae and blue-green algae [6, 28, 34, 38]. In this study there was no statistically significant correlation between the concentration of chlorophyll *a* and the average abundance of fungi, suggesting that in the examined river waters fungal biomass depended mainly on other factors.

There were however a statistically significant relationships between the concentrations of DOC and CFU/ml (R^2 = 0.31), and between POC and mean values of fungal biomass (R^2 = 0.21). This is consistent with literature data. Previous studies have shown that in the waters of the DOC-rich rivers in north-eastern Poland, mainly from forested catchments, there is an excessive growth of fungi and bacteria [15]. There was no statistically significant correlation between fungal biomass and quality of organic matter (estimated using SUVA).

Greater fungal biomass is associated with an increase in water pollution. There was a statistically significant positive correlation between fungal biomass and the concentration of phenols (R^2 = 0.21), which enter the water primarily with municipal and industrial wastewater. This indicates a previously ignored role of microfungi in the self-purification of water. It is known that fungi contain enzymes from the group of polyphenol oxidases that catalyze the oxidation of various compounds, which leads to a depletion of oxygen in an aqueous environment [3]. This explains an increase in fungal biomass in eutrophic waters, heavily polluted, with

an increased amount of phenols, both anthropogenic and from decaying detritus. In addition, microfungi use phenols as a carbon source [11, 29].

Table 2. Assessment of sensitivity to antibiotics and antimycotics of various fungal morphotypes isolated from the surface waters of the Oder River, its tributaries and rivers flowing directly into the Baltic Sea.

Sample number	**River**	**Station**	**Drug sensitivity**			
			AMB	**GM**	**E**	**S**
1	Oder	Chałupki	S	R	R	R
2	Oder	Racibórz	S	S	R	R
3	Ruda	Kuźnia Raciborska	S	S	R	R
4	Bierawka	Bierawa	S	S	R	R
5	Kanał Gliwicki	Kędzierzyn Koźle	R	R	R	R
6	Oder	Opole	R	R	R	R
7	Mała Panew	Czarnowąsy	S	S	R	R
8	Nysa Kłodzka	Skorogoszcz	R	S	R	R
9	Oława	Oława	S	S	R	R
10	Oder	Wrocław	R	R	R	R
11	Widawa	Psary	S	R	R	R
12	Bystrzyca	Wrocław (Jarnotów)	R	S	R	R
13	Kaczawa	Prochowice	S	S	R	R
14	Oder	Ścinawa	S	S	R	R
15	Barycz	Osetno	S	S	R	R
16	Oder	Nowa Sól	S	S	R	R
17	Bóbr	Dychów	R	S	R	R
18	Nysa Łużycka	Gubin	S	R	R	R
19	Oder	Słubice	R	S	R	R
20	Oder	Kostrzyń	R	R	R	R
21	Warta	Kostrzyń	R	R	R	R
22	Oder	Krajnik	S	R	R	R
23	Ina	Goleniów	R	R	R	R
24	Rega	Płoty	S	R	R	R
25	Parsęta	Karlino	S	S	R	R
26	Słupia	Słupsk	S	S	R	R
27	Łeba	Lębork	S	S	R	R
28	Reda	Wejherowo	S	S	R	R

GM – gentamicin (10 µg), **AMB** – amphotericin B (10 µg), **E** – erythromycin (15 µg), **S** – clotrimazole (10 µg), S – sensitive, R – resistant

With an increase in organic matter and water eutrophication, the content of nutrients increases, such as nitrogen, flowing into the water in a mineral form or in organic matter which is metabolised to provide biologically available forms of this element. In this study there was a significant positive correlation between the number of fungi (CFU/mL), and the quantity ammonium nitrogen (R^2 = 0.25), and between fungal

biomass and the level of total nitrogen (R^2 = 0.31). Ammonium is formed in the decomposition of dead plants and animals during the digestion of proteins, and also in the process of ammonification of urea. A fungal biomass increase with increasing nitrogen concentration was also found in previous studies [16, 22, 39], which indicates that this is an important factor stimulating the growth of microfungi.

Because in recent years there have been an increasing number of reports that aquatic fungi cause infections in fish and other animals, this study in the Oder River, its tributaries the rivers directly inflowing to the Baltic Sea included a test of fungal drug resistance. The analyses included two commonly used antimycotics: amphotericin B (10 µg) and clotrimazole (10 µg), and two antibiotics: gentamicin (10 µg) and erythromycin (10 µg). We found that in as many as 10 rivers some the studied fungi were resistant to amphotericin B (Table 3), a very strong antifungal polyene antibiotic. As only a small number of the tested morphotypes were resistant to this mycotic, it seems that the resistance must have been acquired. In addition to the 11 of the 28 examined sites, we identified morphotypes sensitive to gentamicin, a typical antibiotic. In none of the rivers did we find fungi sensitive to erythromycin, and more interestingly - to clotrimazole, an imidazole inhibiting the synthesis of ergosterol and with a very broad spectrum of action [17, 36].

A number of literature data (primarily on bacteria present in surface waters) reported that microbial saprophytic populations acquired resistance to antibiotics from other microorganisms, including pathogenic ones, mainly via horizontal gene transfer, as well as from exposure to antibiotics and other drugs in the aquatic ecosystems entering with wastewater [14, 23, 25. 31]. Similar mechanism of resistance to drugs may also apply to aquatic fungi (this also suggested by the fact that drug resistance occurred mainly near large cities) but it demands the further detailed researches.

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Baltic Sea sediments, Baltic sedimentary bacteria,
antibiotic residues, environmental pollution,
growth inhibition, mixture toxicity

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IMPACT OF ANTIBIOTIC RESIDUES PRESENT IN BALTIC SEA SEDIMENTS ON THE GROWTH OF SEDIMENTARY BACTERIA

The presence of antibiotic substances in the marine environment is of high concern since it potentially accelerates widespread bacterial antibiotic resistance and may have negative effects on bacteria responsible for organic matter mineralization and processes of nitrification and denitrification. Thus concentration of the antibiotic residues and its biological effects on ecosystem should be investigated. The research material was collected during the cruises of s/y Oceania in 2010-12 from the Gulf of Gdańsk and Pomeranian Bay. Nine compounds (sulfatiazole, sulfadiazine, sulfamerazine, sulfametazine, sulfachloropiridazine, sulfamethoxazole, sulfadimethoxin, trimethoprim, enrofloxacin) were identified in sediment samples using LC-MS/MS technique. The highest concentrations were found for sulfamethoxazole and trimethoprim (from ng to $\mu g \times g^{-1}$ d.w.).

The effect of sulfamethoxazole, trimethoprim, sulfadiazine, tetracycline and their mixtures (in concentrations of 0.5 $\mu g \times ml^{-1}$ and 0.5 $mg \times ml^{-1}$) on the sedimentary Baltic bacteria was tested. Seven strains isolated from sediments from Gulf of Gdańsk and Gdańsk Deep, namely *Pseudomonas fluorescens*, *Pseudomonas guineae*, *Bacillus barbaricus*, *Microbacterium oxydans*, *Pseudomonas baetica*, *Bacillus niacini* and *Shewanella denitrificans* were used in the experiments. It has been observed that higher concentrations of the antibiotics, mainly tetracycline and mixtures of all of the compounds tested, inhibit the growth of the bacteria. In the case of *Pseudomonas baetica and Bacillus niacin* the stimulation of growth with sulfamethoxazole (with higher concentrations) was observed. The results of the chemical analyzes showed contamination of coastal sediments from the Baltic Sea with antibiotic compounds. The results of microbiological assays indicate that the concentrations found in the environment can affect the bacteria in sediments and thus the functioning of the ecosystem of the Baltic Sea.

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1. INTRODUCTION

The usage of pharmaceuticals is increasing worldwide. In recent years, the occurrence and fate of pharmaceuticals in the aquatic environment has been recognized as an emerging issue. Many drugs used in veterinary and human medicine after administration enter into the aquatic environment via wastewater treatment plants effluents and surface runoff of soil [39]. Many of these bioactive compounds end up in low concentrations inland aquatic ecosystems and finally in the marine environment. Their determination has been made possible due to the development of modern analytical techniques such as high performance liquid chromatography coupled with mass spectrometry. Among a wide variety of pharmaceuticals, antibiotics show special significance due to their extensive use in humans and animals to treat microbial infections and as feed additive to promote growth of livestock animals [18]. During the past decade, investigations related to antibiotics have been published documenting their presence in river water and sediment, estuaries, coastal waters, and hospital wastewater [11, 38, 36, 34, 22, 15, 23]. Although concentrations of antibiotics in the environment were low (ng × L^{-1} lever) and many of them do not exhibit an acute aquatic toxicity to organisms, they can have a significant, cumulative effect on the metabolism of nontarget organisms and on entire ecosystems [10, 31]. They may also cause widespread of antibiotic-resistant genes [25, 28]. There has been growing concern that antibacterial residues in the aquatic environment could have a negative effects on non-pathogenic bacteria responsible for organic matter mineralization and processes of nitrification and denitrification, influencing microbial processes, with consequent effects on the whole ecosystem. The data on antibiotic residues presence in the marine environment, especially in coastal areas are few, moreover, implications of residual antibiotics in marine environment have remained poorly understood [26]. Therefore, in the present study, the first results on the concentrations of selected antibiotics (sulfonamides, fluorochinolones and trimethoprim in the baltic sediments are reported. The main objective of this study was to investigate, in the laboratory experiments, the influence of identified antibiotic residues (individual compounds and their mixtures) on the growth of bacteria isolated from Baltic sediments to assess the possible risk of the antibiotics to Baltic microbial community and to establish rationale for further research in this field.

2. MATERIALS AND METHODS

2.1. SAMPLING

Surface sediment samples were collected in 2010-2012 from the southern Baltic Sea during the cruises of r/v Oceania using a Reineck or Niemistö corers. Details of the sampling sites are shown in Fig.1. The 0÷5 cm layer of sediments was retrieved, frozen (-18 °C) in pre-cleaned glass jars and transported to the laboratory for chemical analysis. For microbiological analyses sediment samples were stored in sterile glass bottles at 4 °C and handled within 4 hours after sampling.

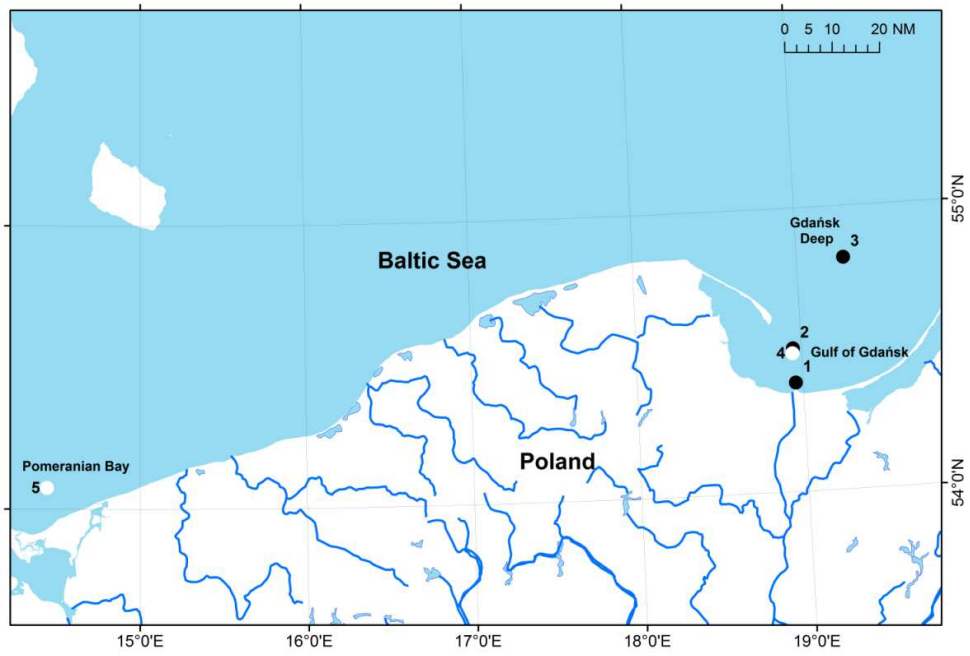

Fig 1. Locations of sampling sites.

Points 1-3 (black circles) indicate sampling of sediments in May 2012, when the sediment samples for isolation of bacteria were taken, points 4 and 5 (white circles) indicate sampling of sediments in May 2011 and 2010, when sediments for chemical analyses were taken. Sediments were taken from the southern Baltic Sea: from mouth of the Vistula River (point 1 - 54°23.098 N; 18°57.354 E, 12 m depth); the Gulf of Gdańsk (point 2 - 54°30.365 N; 18°56.985 E, 64 m depth); Gdańsk Deep (point 3 - 54°49.088 N; 19°17.148 E, 111 m depth); Gulf of Gdańsk (point 4, 54°29.317 N; 18°56.660 E, 63 m depth); Pomeranian Bay (54°04.958 N; 14°26.975 E, 12 m depth).

2.2. CHEMICAL ANALYSIS

Target compounds were selected based on their usage and literature studies. 11 target compounds were analyzed: trimethoprim, sulfonamides (sulfatiazole, sulfadiazine, sulfamerazine, sulfametazine, sulfachloropiridazine, sulfamethoxazole, sulfadimethoxin), quinolones (oxolinic acid), fluoroquinolones (enrofloxacin) and penicillin (oxacilline). The analyses were based on the procedures described by Babić et al. [1] and Białk-Bielińska et al. [2, 3]. Briefly, antibiotics were extracted from the freeze-dried sediment (2 g) in an ultrasonic bath (for 20 min) consecutively with 10 mL of methanol (Merck), 10 mL of supersaturated NH_4Cl (POCH) water solution and 2 mL of 0.1 M EDTA (POCH). The samples were centrifuged (10 min, 4000 rpm) and the supernatant was transferred to a flask, and the procedure was repeated. The combined extracts were concentrated in a rotary evaporator to a volume of about 10 mL. Residual extract was diluted in 1000 mL of Milli-Q water and the whole extract was cleaned up on a tandem SPE column (6 mL, 500 mg, Discovery DSC-SAX, Sigma-Aldrich, Germany and 6 mL, 500 mg, Oasis HLB, Waters) by using modified method described by Babić at al. [1]. The cartridges were conditioned with 2×5 mL of each methanol and water; after sample loading the cartridge was washed by 2×4 mL of 2% methanol and air-dried for about 20 min. under vacuum. Analytes were eluted with 2×5 mL of methanol and allowed to dry under a gentle stream of nitrogen. The dried extract was reconstituted in 1 mL of 10% acetonitrile. The laboratory blanks were analysed in parallel with the samples. LC-MS/MS in multiple reaction monitoring mode (MRM) was applied for determination of antibiotics in samples. Agilent 1200 Series LC system (Agilent Technologies) with Gemini C18 column (150 mm×4.6 mm, 5 μm pore size, Phenomenex Inc., Torrance, CA) were used for the chromatographic separation and HTC Ultra ion trap mass spectrometer (Bruckner Daltonics) with an electrospray ionization source (ESI+) was applied for mass spectrometric measurements. The details of chromatographic analysis were described by Białk-Bielinska et al. [2]. The limit of quantification for the applied analytical method and target antibiotics ranged from 0.5 to 1.5 $ng \times g^{-1}$ d.w. depending on individual compound.

2.3. ISOLATION OF BACTERIAL STRAINS FROM SEDIMENT SAMPLES

Viable heterotrophic marine bacteria in sediment samples were recovered using the spread plate method. Sediment samples (1 g) were resuspended in sterile Ringer solution (NaCl, 9 $g \times L^{-1}$; KCl, 0.42 $g \times L^{-1}$; $CaCl_2$, 0.48 $g \times L^{-1}$; $NaHCO_3$, 0.2 $g \times L^{-1}$) and vortexed in order to detach bacteria from the sediment particles. Serial 10-fold dilutions were prepared and aliquots (0.1 mL) were plated in triplicate onto ZoBell Agar (bacto-pepton 5 $g \times L^{-1}$; yeast extract 1 $g \times L^{-1}$; $FePO_4$ 0.01 $g \times L^{-1}$; distilled water, 250 $mL \times L^{-1}$; aged seawater 750 $mL \times L^{-1}$; agar 15 $g \times L^{-1}$). Colonies were enumerated after 2 days at 30 °C, and the Colony Forming Unit (CFU) per gram of sediment was

calculated. Distinct colonies were selected from each sediment sample and isolated as pure cultures on ZoBell Agar. Purified strains were then stocked in ZoBell medium with 15% glycerol at -70 °C. The two strains from each sampling site were selected for the further analyses.

2.4. BACTERIAL IDENTIFICATION BY 16S RRNA SEQUENCING

Selected bacterial isolates were grown in 2 mL Zobell medium. Genomic DNA was extracted using Genomic Mini Kit according to manufacturer's instructions (A&A Biotechnology, Poland). Bacterial 16S rDNA was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA primers: F27 and R1492 (Lane 1991). Polymerase chain reactions were performed using TPersonal thermocycler (Biometra, Germany) under the following conditions: denaturation 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min., 50 °C for 1 min., 72 °C for 1 min. and final extension 72 °C for 5 min. All PCR reagents were purchased from A&A Biotechnology (Poland). The amplification of 16S rDNA was confirmed by running the amplification product in 1% agarose gel in 1 × TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The PCR products (approximately 1500 bp) were cleaned up using Clean-up Kit with silica columns (A&A Biotechnology, Poland) and sequenced (Genomed, Poland). The obtained 16S rRNA gene sequences were compared to sequences in GenBank using blastn (http://blast.ncbi.nlm.nih.gov) and assigned to species using the highest-scoring sequence for which species information was available when sequence similarity was above 97 %.

2.5. TESTING THE IMPACT OF ANTIBIOTICS ON ISOLATED SEDIMENTARY BACTERIAL STRAINS

Mueller Hinton liquid medium (Oxoid, UK) was inoculated with overnight cultures of tested bacterial strains at a ratio of 1:100 and incubated at 30 °C in an air shaker for achieving an optical density at 600 nm of about 0.1 (OD 600 = 0.1). Bacterial cultures were aliquoted and selected antibiotics: sulfamethoxazole, trimethoprim, sulfadiazine, tetracycline and their mixtures were added with 50 % (v/v) methanol as a solvent. All antibiotics were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Two concentrations of the tested compounds were used: 0.5 $\mu g \times mL^{-1}$ and 0.5 $mg \times mL^{-1}$. Two kinds of control experiments were used: one culture without any additives, and the second one with methanol as a solvent in the same amount as in the experiments with antibiotics. Cultures were grown in an air shaker (30 °C) and measurements of absorbance at a wavelength of 600 nm were performed at every 30 minutes.

RESULTS AND DISCUSSION

The analyzed antibiotic residues concentrations in the collected sediment samples are presented in Table 1. The results are expressed in ng × g^{-1} dry weight. The concentrations of individual antibiotics ranged from below the limit of quantification to 1205.3 ng×g^{-1} dry weight. In sediment sample taken from station 5 (Pomeranian Bay) (Fig. 1) nine compounds were detected with highest concentration of sulfametoxazole (1205 ng×g^{-1}), sulfachloropiridine (1.7 ng×g^{-1}) and trimethoprim (1.1 ng×g^{-1}), while other antibiotic residues were present below the limit of quantification. The observed level of sulfametoxazole concentration in the collected sediment was higher than those reported for raw sewage [29, 32] and river sediments in other geographic regions [24]. In the sediment collected from deep part of the Gulf of Gdańsk (station 4) only sulfamethoxazole (4.9 ng×g^{-1}) and trimethoprim (35.7 ng×g^{-1}) were detected. Both antibiotics have been reported to occur in marine waters, but at lower concentrations [34, 26]. It has been also demonstrated that these compounds, similarly to tetracyclines, could accumulate in sediments due to strong adsorption on particles and sediments [18, 20] and were environmentally persistent in the aquatic environment [13, 14]. The presence of sulfamethoxazole and trimethoprim most probably results from applications in human medicine (in medical practice trimethoprim and sulfamethoxazole have been commonly used together in a ratio 1:5 (as co-trimoxazole) to treat wide spectrum of aerobic bacteria since late 1960s). Approximately 15% of sulfamethoxazole is excreted from the human body in its original form, while the fraction of unchanged trimethoprim amounts to about 60% [14]. It should be also mentioned that antibiotics are often incomplete eliminated during sewage treatment. Depending on wastewater treatment practices in various countries removal efficiency of sulfamethoxazole and trimethoprim ranged between 3–100% [21]. Another source of these compounds in the investigated samples may be the veterinary application, further surface runoff of soil and final discharge to the sea by rivers.

Since the presence of sulfamethoxazole and trimethoprim residues in Baltic sediments was observed, these compounds have been applied in the biological tests assessing the impact of antibiotic residues on the growth of Baltic sedimentary bacteria. Tetracycline and sulfadiazine have been also chosen for the experiments since their impact on microbial communities was previously reported [33, 16]. Two concentrations of tested chemicals have been applied in the experiments. The lower one is comparable to the concentrations noted in the environments heavily polluted by antibiotics e.g. close to aquacultures [7]. Higher concentrations were applied to facilitate the observations of the effects of the antibiotics on the bacterial growth. Hence the purpose of this study was to test the influence of selected antibiotic residues on the growth of Baltic sedimentary bacteria, the first step of the conducted biological studies was to obtain bacterial strains from Baltic sediment samples. Total

plate counts×g^{-1} (CFU×g^{-1}) of tested sediments were as follows: mouth of the Vistula River: 2.3×104 ± 1.8×103 CFU×g^{-1}, Gulf of Gdańsk: 1.8×104 ± 2×103 CFU×g^{-1}, and Gdańsk Deep 1.1×104 ± 2.5×103 CFU×g^{-1}. Two strains from each sampling points were selected and identified using their 16S rRNA gene sequences. The results of the identification are shown in Table 2. The 16S rRNA gene sequences obtained in this work were submitted to GenBank under accession numbers KC255380-KC255385.

Table 1. Concentration of antibiotic residues in sediment samples.

	Concentration of selected antibiotic residues in sediment samples [ng × g^{-1} d.w.] [RSD% n=3] at :	
	Station 4	Station 5
sulfatiazole	n.d.	b. LOQ*
sulfadiazine	n.d.	b. LOQ*
sulfamerazine	n.d.	b. LOQ*
sulfametazine	n.d.	b. LOQ*
sulfachloropiridazine	n.d.	1.7 ± 2.6
sulfamethoxazole	35.7 ± 5.8	1205.3 ± 4.1
sulfadimethoxine	n.d.	b. LOQ*
trimethoprim	4.9 ± 4.2	1.1 ± 2.9
enrofloxacin	n.d.	b. LOQ*

b. LOQ* -below the limit of quantification
n.d. - not detected

Table 2. Bacterial strains used in this study and their closest phylogenetic relatives.

Strain number	Origin of the strain	Closest phylogenetic relative, 16S rRNA sequence similarity (accession nr)
33	Mouth of the Vistula River	*Pseudomonas fluorescens* NBRC 15834, 99% (AB680973.1)
34	Mouth of the Vistula River	*Pseudomonas guineae* LMG 24017, 99 % (AM491811.1)
37	Gulf of Gdańsk	*Bacillus barbaricus* FR1_162, 99 % (EU373540.1)
40	Gdańsk Deep	*Pseudomonas baetica* a393, 99 % (FN678353.1)
42	Gulf of Gdańsk	*Microbacterium oxydans* TPR04, 99 % (EU373400.1)
43	Gdańsk Deep	*Bacillus niacini* RB-113, 99 % (JQ085399.1)

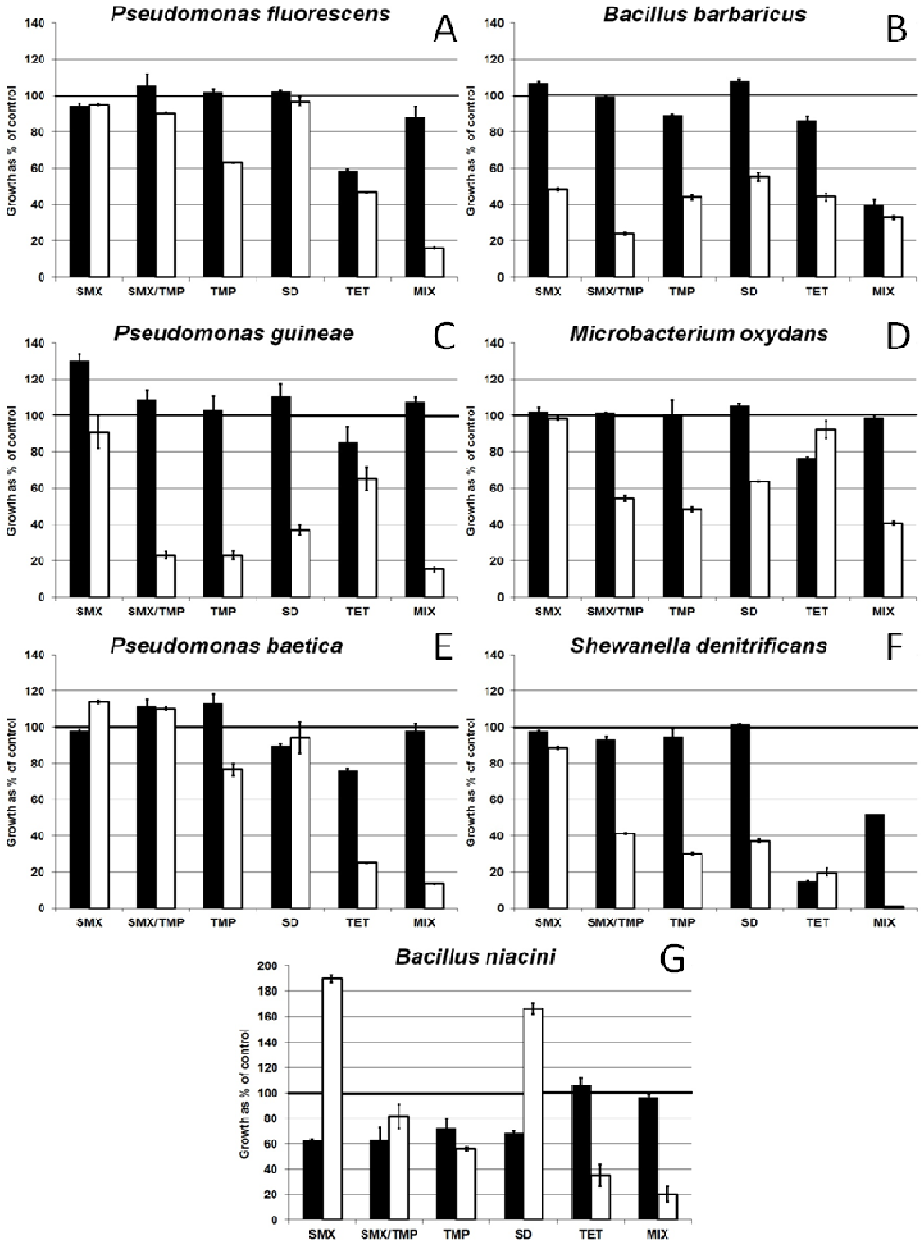

Fig. 2. Growth relative to the control (in %) upon influence of tested antibiotics: sulfamethoxazole (SMX), sulfamethoxazole with trimethoprim (SMX/TMP), trimethoprim (TMP), sulfadiazine (SD), tetracycline (TET) and mixture of all antibiotics mentioned (MIX) in concentrations: 0.5 $\mu g \times mL^{-1}$ (black columns) and 0.5 $mg \times mL^{-1}$ (white columns). Bars indicate average values (n = 3), error bars corresponds to ± standard deviation. Results after 6 hours of exposure are shown.

In the presented study lower concentrations of tested antibiotics (except of tetracycline) revealed in general little or no effect on the growth of sedimentary bacteria used in the experiments. Among four antibiotics tested tetracycline had the strongest inhibitory effect on the growth of bacteria strains at both concentrations tested (even at lower concentrations tested). The tetracyclines, which were discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites [8]. Similar effects of tetracycline on heterotrophic marine bacteria were previously mentioned by Perliński and Mudryk [30] and on soil and activated sludge bacteria [12]. Since *Shewanella denitrificans* strains are vigorous denitrifiers, and thus could have contributed to transformations of nitrogen forms in the Baltic Sea [5] we decided to include *Shewanella denitrificans* strain IOMB 364 (previously isolated by Moskot et al. [27]) to our study. Growth of this strain was only inhibited by higher concentrations used, the strongest effect was seen when mixture of antibiotics was used. In the case of *Pseudomonas baetica* and *Pseudomonas fluorescens* strains, the relative low potency of all compounds tested may be explained by the fact that strains belonging to the genus *Pseudomonas* are often naturally resistant to a number of antimicrobial agents [17]. However, even for *Pseudomonas baetica* strain the stimulation of growth with sulfamethoxazole was observed, when high concentrations of the chemical were used. Stimulation effect of sulfonamides (sulfamethoxazole and sulfadazine) on growth of *Bacillus niacini* strain were also observed. The strongest inhibitory effects was noted when mixture of all compounds was applied in the tests. The obtained results agree with those reported by other researchers, that mixtures of antibiotics had stronger inhibitory effect on the growth of bacteria than single antibiotics [9, 30, 6]. Christensen et al. [6] using different test organisms (freshwater algae *Pseudokirchneriella subcapitata*, activated sludge microorganisms, and luminescent bacteria *Vibrio fischeri*) concluded, that regardless of the tested species, combined effects were higher than predicted based on the assumption of concentration addition. Antibiotics comprise a group of pharmaceuticals specifically designed to disrupt microbial biochemical processes, and might therefore in particular have detrimental effects on microbial communities in the environment. Many of the research on effects of antibacterial compounds in the environment had focused on their influence on microorganisms, primarily in spreading of bacterial resistance to these compounds [25, 28], but also on antibiotics toxicity and other negative effects on microbial communities. Kotzerke et al. [16] reported inhibition of nitrification by sulfadiazine at 100 $mg \times kg^{-1}$. Toth et al. [35] reported that sulfadimetoxine (belonging to sulfonamides) blocked soil iron reduction and inhibited nitrification. Authors stated that antibiotics at environmentally relevant concentrations can disrupt soil microbial processes, although the detection of such impact may

be antibiotic and/or process specific. Thiele-Bruhn and Beck [35] concluded that pharmaceutical antibiotics (such as sulfonamides and tetracyclines) could exert a temporary selective pressure on soil microorganisms even at environmentally relevant concentrations. Costanzo et al. [10] indicate that antibiotics entering aquatic systems pose a potential threat to the ecosystem. Many other organisms (such as algae, plants or zooplankton) were also tested in order to reveal the effects of antibiotic residues in the environment. For example Brain et al. [4] tested effects of twelve different classes of antibiotics to *Lemna gibba* duckweed. In their study only members of fluorochinolone, sulfonamides (with sulfamethoxazole being the most toxic) and tetracycline classes of antibiotics displayed significant phytotoxicity. Acute and chronic tests performed on freshwater crustacean *Daphnia magna* with sulfadiazine, tetracycline and oxytetracycline showed no acute effects at environmental relevant concentrations but reproductive effects were observed. However, sulfadiazine at some tested concentrations demonstrated a statistically significant stimulation of the reproductive output [37].

In conclusion, the results of performed chemical analyses show contamination of coastal Baltic sediment by target antibiotic residues and the results of biological tests indicate that these contaminants may affect sedimentary microbial community. The studies will be continued to establish more extensive chemical database on antibiotics spatial and temporal distribution in the southern Baltic Sea area. The more detailed ecotoxicological studies should be also developed to assess more accurately ecological risks related to the presence of antibiotics residues in the marine ecosystem. The evaluation of the long-term changes caused by continuous exposure to antibiotics occurring in low concentrations and as a mixture of compounds seems to be necessary. This challenge is especially important in the case of marine sediments constituting large reservoir of contaminants and at the same time the environment of several ecosystem important processes like organic matter mineralization and eutrophication.

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Escherichia coli, surface waters, survival rate, temperature

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SURVIVAL RATES OF *ESCHERICHIA COLI* O157:H7 IN SURFACE WATER

1. INTRODUCTION

Microorganisms occurring in surface waters are divided in respect of their origin and possibility of development, into autochthonous organisms, for whom water is the natural place of living, and allochthonous, that is deposited, getting to water from other environments. Spring waters contain the smallest amounts of microorganisms, whereas a considerable development of microflora occurs in waters rich in organic substances. Microorganisms develop most numerously in surface waters, which are not the natural environment for pathogenic microflora [1]. Pathogenic microorganisms penetrate into surface waters together with faeces of people and animals. The main sources of water pollution
with pathogenic microorganisms are insufficiently treated sewage introduced to water reservoirs [2]. Organisms pathogenic for people and animals can get to lake waters with surface flows of rain and melting snow, or uncontrolled sewage disposal [3]. The following species of pathogenic bacteria are the most frequently isolated from surface waters: pathogenic strains of *Escherichia coli*, *Campylobacter* spp., *Salmonella* spp., *Mycobacterium tuberculosis, Vibrio cholerae, Clostridium perfringens*. Bacteria *Escherichia coli* are often used as indicators in the evaluation of surface water quality, also including those used for recreational purposes [4].

Reduction in surface water pollution by pathogenic microorganisms is important from the point of view of health protection of people and animals. In Poland water intended for human use is subjected to a number of physicochemical and bacteriological analyses. To 2011 bathing waters were microbiologically controlled in respect of the occur-

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rence of bacteria of the genus *Salmonella* due to a large risk of incidences of salmonelloses in Poland. However, in accordance with the Regulation of the Ministry of Health of 2011 [5], taking into consideration the quality of water used for recreation, it should be subjected to systematic analyses for the presence of some groups of indicator microorganisms, which include *Escherichia coli* and enterococci [6]. Marked out test bacteria may indicate the occurrence of other pathogenic bacteria in bathing waters.

In the natural environment occurs a very wide reservoir of *Escherichia* bacilli, the fact of occurring those microorganisms in people and animals is particularly dangerous. Bacteria *Escherichia coli* may also occur in the organisms as commensal species, performing a positive role, among other things by the synthesis of vitamins of groups B, K and C and colonization of mucous membranes preventing pathogenic bacteria from adhesion. Among *Escherichia coli* strains there are also pathogenic bacteria belonging to enteric pathogens, which include enteropathogenic strains EPEC, enterohaemorrhagic EHEC, enterotoxic ETEC, enteroinvasive EIEC, enteroaggregative EAEC as well as diffusely adherent DAEC. Parenteral strains include: uropathogenic UPEC, MNEC inducing meningitis and SEPEC causing sepsis [7]. Those bacteria are characterized by the occurrence of many genes of virulence, which are specific only for the given pathotype, and genes characteristic for the other pathogenic strains of *E.coli*. The indicators of pathogenicity of those bacteria include adhesins as well as entero- and exotoxins. Kuhnert and Boerlin [8] emphasize that an important marker of pathogenicity of *E.coli* strains is the occurrence of fimbriae of type 1 responsible for colonization and contributing to the development of colibacteriosis. An essential exotoxin determining pathogenicity of *E.coli* strains is hemolysin E located within plasmid Hly causing the lysis of erythrocytes.

The presence of pathogenic bacteria in surface waters used for recreation is particularly dangerous for epidemiological reasons. Many people have access to infected water during the holiday season, which poses a very large threat to them due to the fast spread of bacteria [9].

Although surface water reservoirs are characterized by a low content of bacteria of the genus *Escherichia*, when bacilli get to the alimentary tract together with contaminated water, they become dangerous to the organism. Additionally, those bacteria are characterized by a high survival rate in the water environment. They are also resistant to environmental stressors and fluctuations of abiotic conditions, which makes them highly hazardous for people [10]. Survival rates of the bacteria *Escherichia coli* depends mostly on the temperature, pH and the activity of water environment antagonistic microflora. Also different processes and physicochemical factors can have an effect on survival rates of *Escherichia coli* bacilli in surface waters. The bacteria are sensitive to the action of gamma and beta radiation and the action of chlorine, lactic acid and other disinfectants [4].

The aim of this study was to assess the survival of *Escherichia coli* O157:H7 in lake surface waters and to estimate the effect of different temperatures on behaviour of the tested bacteria.

2. RESEARCH METHODS

Samples of surface water for laboratory analyses intended for the evaluation of survival of *Escherichia coli* O157:H7 bacilli were collected from a lake situated in the Kuyavian-Pomeranian voivodeship, the district of Bydgoszcz. The area of the total drainage basin, coinciding with the direct drainage basin of the lake, is 3.5 km^2. The structure of drainage basin utilization, where agricultural areas predominate, is one of the major factors negatively affecting lake waters [11]. Material for performing microbiological analyses was collected in spring 2011 from the surface layer of water at a depth of 30 cm directly to sterile bottles with a volume of 5000 ml. Water was poured to 2 separate bottles intended for microbiological analyses. Water samples were collected and transported according to the standard PN-EN ISO 5667 [12]. The standard strain *Escherichia coli* O157:H7 was used to the study. Bacteria taken from the cryobank were passaged twice on the tryptone-soya agar at 37 °C for 24 hours. Suspension was prepared from the grown colonies, introducing 12 ml sterile demineralised water to the ampoule. Using the optical densitometer, the number of bacterial cells of *Escherichia coli* O157:H7 was determined in the inoculums in the form of suspension on a level of 10^{10} cells in 1 ml. At the next stage of the study, 5 ml of the tested bacteria suspension was inoculated to a container with water with a volume of 5000 ml. Then the samples were left at room temperature for 1 hour. After this time, the number of bacteria *Escherichia coli* was determined in 1 ml of inoculated water sample with the Most Probable Number method (MPN). Containers were placed at 4 and 20 °C. Water collected from the lake with inoculated bacteria *Escherichia coli* was systematically subjected to microbiological analyses in order to determine the survival rate of the tested bacteria in a time unit. To do that, the method of decimal dilutions on the liquid lactose medium McConkey broth with bromocresol purple (incubation for 24 hours, temperature 37 °C) was applied. After the incubation, the colony was isolated with the method of surface inoculation on solid selective Lactose TTC Agar media with tergitol, lactose, bromothymol blue and sodium sulphate. Plates with inoculations were incubated at 37 °C for 24 hours. Bacteria *Escherichia coli* grow on the Lactose TTC medium in the form of round, smooth yellow-orange colonies with the characteristic yellow crown around. The presence of at least one such a colony was assumed as a positive result. Final identification involved making biochemical tests (API 20E). The number of bacteria was determined based on the Most Probable Number (MPN). Statistical calculations of the obtained results were made using the packet OpenOffice.org Calc. version 3.3.

3. RESULTS AND DISCUSSION

The results of microbiological analyses concerning the survival rates of *Escherichia coli* bacilli in surface water were presented in tables 1-3 and in figures 1-2.

From the analyses made it follows that *Escherichia coli* O157:H7 bacilli showed similar and even elimination rate in water samples stored at 4 °C and 20 °C. On the first day of the study after inoculating water with tested bacteria their number amounted to 4.5×10^7 MPN·ml^{-1} in both cases (table 1 and 2). After nine days, the number of bacteria in water stored at 20 °C decreased rapidly to the level of 9.5×10^2 MPN·ml^{-1} (tab. 1).

Table 1. Number of *E. coli* in water at 20 °C.

Day of study	Number of bacteria	
	[MPN·ml^{-1}]	[log MPN·ml^{-1}]
1	$4{,}5 \cdot 10^7$	7.65
9	$9{,}5 \cdot 10^2$	2.97
10	$4{,}5 \cdot 10^1$	1.65
11	$9{,}5 \cdot 10^1$	1.97
12	$1{,}5 \cdot 10^1$	1.17
13	$0{,}3 \cdot 10^1$	0.47
14	0	0

In the case of samples of water stored at 4 °C (table 2), a considerable decrease in the number of *Escherichia coli* O157:H7 bacilli was recorded on 23 day of the study, their number amounted to 1.5×10^2 MPN·ml^{-1}, and then it lowered systematically to their complete elimination.

The results of the conducted experiment were subjected to transformation and statistical analysis (table 3). In both cases the values ranged from 0 to 7.65 logMPN·ml^{-1}, since after inoculation there was the same number of cells of the tested bacteria in the containers. The average number of bacterial cells in samples of water stored at 20 °C was 2.27 logMPN·ml^{-1} and was considerably smaller than in the case of samples of water stored at 4 °C, which amounted to 3.30 logMPN·ml^{-1}. Standard deviation describing the distribution of the studied feature around its mean was similar in both samples. In the case of water stored at 20 °C it amounted to 2.56 logMPN·ml^{-1}, whereas for samples of water placed at 4 °C it assumed a value of 3.27 logMPN·ml^{-1}. The study conducted by Olszewska [13] indicated that the survival of indicator bacteria in water is mainly affected by the temperature. The author proved that *Escherichia coli* bacilli survive in water environment from 35 weeks at 4 °C to about 70 weeks at 20 °C. According to Himathongkham et al. [14], the time necessary in slurry to reach 90% elimination of *E.coli* O157:H7 amounts to 21.5 days at 4 °C and 14.8 at 20 °C. Moreover, the authors emphasize a very important fact that the time of survival of *Salmonella bacilli* is shorter by about 4 days. Moreover, the sanitary risk connected

with the presence of pathogenic bacteria in surface waters is substantial and it disqualifies the usefulness of such basins for recreation and bathing purposes [3]. In the present study, the actual survival time of the *Escherichia coli* bacilli was respectively 28 days at 4 °C, whereas at a higher temperature those bacteria were identified for 13 days (table 1 and 2). Owing to that, the present study confirms the fact that at 4 °C lowering of metabolism occurs in the tested bacteria, which had a more stabilizing effect on the bacteria of *Escherichia coli* O157:H7. *Salmonella* bacilli in liquid animal faeces also show high resistance to temperature factors [15]. Wcisło and Chróst [16], in turn, in the study on *Escherichia coli* bacilli survival in fresh water proved that survival of bacteria depends on the way of their transferring from the optimal conditions to fresh water, after which some of the bacteria were capable to further culture, some show correct metabolic processes but assumed non-culturable forms. The other microorganisms did not survive under such conditions, whereas they could die as a result of osmotic or temperature stress.

Using the obtained results of the study, the course of regression lines was determined and the maximal survival time of *Escherichia coli* bacteria was estimated (fig. 1 and 2). In the experiment carried out for bacteria present in water at 4 °C this time amounted to 28 days, whereas in samples of water stored at 20 °C it was lower and assumed a value of 14 days (table 3). The theoretical maximal time of their survival at both temperatures differed by 14 days.

Table 2. Number of *E. coli* in water at 4 °C.

Day of study	Number of bacteria	
	[MPN·ml^{-1}]	[log MPN·ml^{-1}]
1	$4{,}5 \cdot 10^{7}$	7.65
9	$4{,}5 \cdot 10^{6}$	6,65
16	$4{,}5 \cdot 10^{5}$	5.65
23	$1{,}5 \cdot 10^{2}$	2.17
27	$0.9 \cdot 10^{1}$	0.95
28	0	0
29	0	0

Data presented in figures 1 and 2 indicate that differences connected with the rate of decrease in the number of tested bacteria were recorded in both studied samples. Allochthonous microorganisms, undergoing hunger stress, decrease their size and are not detectable with standard culture methods, but they can be observed in direct preparations. This phenomenon was called viable but nonculturable (VBNC) or dormant, that is sleeping. This is a state which is characteristic of live but not cultured cells. Turning into VBNC state can constitute a genetically programmed response of the bacteria, ensuring survival in unfavourable conditions. This does not change the fact that bacteria in water environment, being in the VBNC state, pose an undoubted threat to public health, since they show some metabolic activity,

and they also keep their virulence, whereas they are not detected in routine microbiological analyses of waters, based on the culture method [4].

Table 3. Indexes characterizing inactivation dynamics of *E. coli* bacilli in water at 4°C and 20 °C.

Temperature	Range	Mean	Sx	Variance	R^2	Index		Survival time of bacteria
	log MPN·ml^{-1}					a	b	Days
20°C	0 - 7,65	2,27	2,56	6,58	0,98	-0,5902	8,1704	14
4°C	0 - 7,65	3,30	3,27	10,71	0,91	-0,3211	9,029	28

In the present study it was observed that the main factor determining the survival of *Escherichia coli* w in the studied surface water is the temperature, which is clearly shown by the results of the experiment.

The temperature values indicate a relationship between the rate of decrease in the number of bacteria and their survival rate. After the first week of the study, the number of bacteria placed in water with a temperature of 20 °C decreased, which indicates a lower tolerance of such temperatures by the studied bacteria. In water stored at 4 °C a rapid decrease in the number of the tested bacteria was not recorded, but it was decreased systematically, which prolonged their survival time in the studied water samples. A similar relationship was recorded by Vang and Dolle [17] who reported that in water at 4 °C bacteria of *E.coli* O157:H7 survived definitely longer in comparison with water samples stored at 21 °C.

According to the study carried out by Chandran and Hath [18], *Escherichia coli* bacilli survive longer at a lower temperature, which can be connected with a decrease in their metabolism rate. Different results were obtained in the present study, where the daily inactivation rate of *Escherichia coli* recorded in the studied water samples amounted to 0.59 log at 20 °C and 0.32 log in water with a lower temperature of 4 °C (tab.3). The results of the study by Olszewska [13] indicate that a weekly elimination rate of those bacteria in water environment was 0.24 log (20 °C) and 0.12 (4 °C).

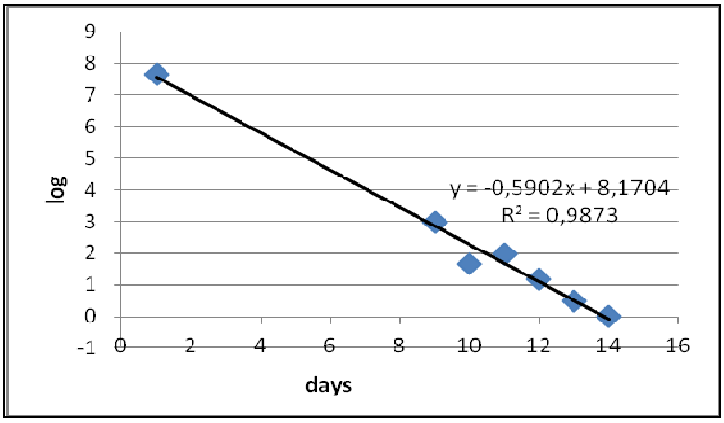

Fig. 1. Regression line of *E. coli* survival in water at 20 °C.

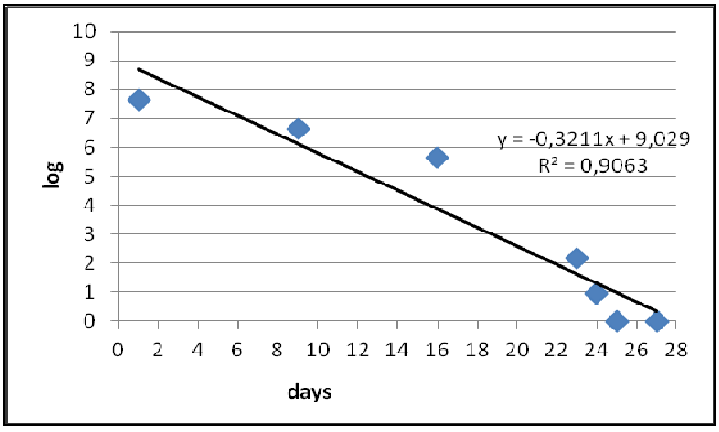

Fig. 2. Regression line of *E.coli* survival rate in water at 4 °C.

Longer time of survival of microorganisms in water environment results from the phenomenon of hunger stress, which leads to a decrease in the size of bacterial cells, which are not detectable with standard cultural methods but can be observed in direct preparations. This phenomenon was called viable but nonculturable (VBNC) or dormant, that is sleeping. This is a state characteristic of live but not for culture cells. Turning into the state VBNC may constitute a genetically programmed reply of bacteria ensuring survival in unfavourable conditions. However, bacteria in the water environment, remaining at the state VBNC, pose a unquestionable threat to public health, since they show a certain metabolic activity, but also they retain virulence, and they are not detected in routine microbiological analyses of waters, based on the culture method [3].

Summing up the results of conducted analyses it can be concluded that the bacilli of *Escherichia coli* show a long time of survival in the studied environment of surface water (14 and 28 days), which may pose a significant threat to people using this basin. Also the content of humic substances can exert a significant effect on the degree of microbiological pollution of surface waters. According to Vrede et al. [19], a higher number of bacteria were isolated in samples of water coming from a humic lake, whereas the frequency of their occurrence ranged between 9 and 43% and it was of seasonal character. Owing to that, in the summer period at higher temperatures of atmospheric air and surface waters, there is a higher probability of survival in them of pathogenic bacteria of the genus *Escherichia coli*, which also follows from the present study.

4. CONCLUSIONS

- The conducted study indicated that the temperature of surface water is one of factors which affect the elimination rate of *Escherichia coli* bacilli in this environment.
- The survival time of the bacteria Escherichia coli in water with a temperature of 20 °C was 14 days, whereas in the case of water with a temperature of 4 °C it was longer and assumed a value of 28 days.

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biosorption, heavy metals

Magdalena ZABOCHNICKA-ŚWIĄTEK*

EFFECTS OF CLINOPTILOLITE ON BIOSORPTION OF SELECTED HEAVY METALS BY *CHLORELLA VULGARIS*

The effects of natural clinotpilolite on biosorption of selected heavy metals (chromium, nickel, zinc) by the biomass of freshwater algae *Chlorella vulgaris* from the synthetic wastewater were determined. The study was conducted in mono- and multimetallic systems. The results showed that sorption of a single metal was influenced by the presence of the other metal in the multimetallic systems. Chromium(VI) was sorbed with higher efficiency from the wastewater by algal biomass from the multimetallic system was of 43% in C_0 = 10 mg/L, after 10 min. of contact time. The highest uptake of nickel(II) reached by clinoptilolite was 94% in C_0 = 10 mg/L, after 10 min. The obtained results indicate that the highest uptake of zinc(II) by clinoptilolite was 78% at the lowest C_0 = 10 mg/L, after 30 min. The sorption of metal ions using microorganisms and natural zeolites from wastewater is a very promising technology.

1. INTRODUCTION

Heavy metal pollution has become one of the major concern today. Because of their toxicity, bioaccumulation, persistence and non-biodegradability a number of technologies for removal of heavy metals have been recently developed [1]. The majority of technologies used as the most effective means for municipal sewage treatment are based on biological sorption [2, 3].

Algae belong to the plants that show fast and continuous growth at minimum environmental requirements and irrespective from seasons [4]. Microalgae can bind metals through processes of biosorption. The process of biosorption by algae is mostly due to the properties of a cell wall.

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The removal of heavy metals by clinoptilolite has an ion-exchange and sorption nature and can supports the biosorption process of heavy metals by microalgae. Clinoptilolite also exhibits excellent adsorption capacities as an independent sorbent for heavy metal [5-8]. Moreover, clinoptilolite could be also used as a very good flocculant for harvesting of algae.

Chromium, nickel and zinc are common heavy metals in industrial wastewater streams. The type and the concentration of heavy metals in the municipal wastewater depend on the level of urbanization of the catchment, sewer system and mainly on the share of industrial wastewater. Heavy metals are present in different forms depending on the physico-chemical composition of the wastewater. Municipal wastewater transport heavy metals in ion form, hardly soluble precipitates and organometallic complexes [9]. For example, the industrial waste water collected in Kowary settler (located near Zielona Góra) contains Cr(VI), Ni(II) and Zn(II) at the following concentrations: 0.06÷1.1 [mg/l], 10.4÷32.7 [mg/l] and 4.7÷17.0 [mg/l], respectively [10]. The river Biała Przemsza in the lower course is also polluted by heavy metals. The main source of this pollution is the wastewater produced by zinc smelter and mining and processing plants of Zn-Pb ores. Cr(VI), Ni(II) and Zn(II) ions are present in Biała Przemsza at the following concentrations: < 0.003 [mg/l], 0.004÷0.005 [mg/l] and 0.315÷0.622 [mg/l], respectively [11].

The passive removal of heavy metals by biomaterials requires from the substrate to display high metal uptake and selectivity. According to the literature, there is no information on the influence of natural clinoptilolite on biosorption of heavy metals by microalgal biomass.

The aim of this study was to assess the effects of natural zeolite – clinotpilolite on biosorption of selected heavy metals (chromium(VI), nickel(II) and zinc(II)) existed in industrial wastewater by the biomass of freshwater algae *Chlorella vulgaris.* The study was conducted in mono- and multimetallic systems. The effects of pH, concentrations of heavy metals, removal selectivity and contact time on removal efficiency were analyzed, and the optimum values were determined from experimental studies.

2. EXPERIMENTAL

The materials:

a) *Chlorella vulgaris* biomass was used. The algae was cultured in 250 ml flasks at 25±0.5 ºC on 12:12-h light: dark cycle. Cells in the exponential growth phase were used for all experiments. The algae were harvested by filtration. Before use, algae were washed with distilled water. The biomass was dried in an oven at 60 ºC for 24h. The biomass concentration was of 0.5%.

b) Natural zeolite (clinoptilolite) of granulation 0.0÷0.2 mm was used. Studies were carried out using clinoptilolite in untreated, powdered form. The concentration of clinoptilolite was of 1%.

c) The synthetic wastewater solution was prepared in accordance with the Polish Standard PN-72/C-04550.09 [12].

d) The heavy metals ($K_2Cr_2O_7$, $Ni(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$) solutions were prepared in the forms of salts to obtain concentration of 10, 50 and 100 [mg/L] in suitable container. Mono- and multimetallic system were used. The experiment was performed in triplicates.

e) The heavy metals solutions dissolved in the synthetic wastewater were added to the samples containing:

1. *Chlorella vulgaris* biomass - 0.5%
2. *Chlorella vulgaris* biomass - 0.5% and clinoptilolite - 1%
3. Clinoptilolite - 1%

Analyses. Based on the preliminary study, current investigations were conducted for 10 and 30 min. of contact time. After 10 and 30min the pH and heavy metal contents measurements were made.

The pH determination in wastewater was done using the pH-meter. Heavy metal concentrations was determined by the emission spectrometry with inductively coupled plasma ICP-OES according to the standard procedure PN-EN ISO 11885:2001 [13].

Metal removal from the solution was calculated according to the formula (1):

$$removal\ (\%) = \frac{C_0 - C_k}{C_0} \cdot 100 \tag{1}$$

Where C_0 and C_k are the initial and final metal concentrations (mg/L).

3. RESULTS AND DISCUSSION

3.1. EFFECT OF PH

Many studies have shown that pH is an important factor affecting biosorption of heavy metals [14-17]. During the experiment, the removal of heavy metals by some biomass reached a high plateau at around pH 4÷5 or higher. It is well known that pH affects the protonation of the functional groups on the biomass as well as the metal chemistry [18].

The ion-exchange adsorption of the metals by clinoptilolite is also depending on the pH. When the pH decreases the metal adsorption decreases. It is due to the competition of hydrogen ions [19]. The concentration of hydrogen protons at low pH value (below 5) is higher than the concentration of metal ions because

of the competition of these protons with metal ions. The decrease of competing effect between metal ions and hydrogen protons is observed with the increase in pH value. The metal ions, that are positively charged took up the free binding sites. The increase in sorption capacity at pH > 5 was described by many researchers [20-24]. The pH values obtained during the current experiment were > 5.21.

Hexavalent chromium is used in the industry in the form of chromate ion CrO_4^{2-} above pH = 6 and dichromate ion $(Cr_2O_7)^{2-}$ below pH = 6. Redox potential and pH influence on forms and sorption of chromium. In high salinity water of pH ~8 chromium could be found as cation Cr^{4+}. Forms: Cr^{6+} and Cr^{2+} are unstable. [25].

Nickel is essential for different organisms but in higher concentration it can be toxic. In wastewater nickel can be found as organic chelates that are easy available for plants. Predominantly nickel occurs in the divalent form Ni(II) [26]. Different organisms (eg. algae) and minerals (eg. clinoptilolite) can sorb nickel. Liming and phosphorus fertilizers limit assimilation of nickel for plants. Phytoassimilation of nickel significantly decreases at pH > 6. [25]. In the pH 4.0÷4.5 the solubility of that metal increases and nickel can be sorbed easier [26]. As the pH increases, the negatively charged surface of *Chlorella vulgaris* biomass and clinoptilolite becomes available for nickel cations [27].

In the absence of inorganic and organic ligands, the free Zn(II) ion with water molecules coordinates to form the octahedral $Zn(H_2O)_6^{2+}$. In freshwater at pH = 4-7 Zn(II) exists as the positively charged ion Zn^{2+}. At pH = 6 $ZnSO_4$ and free ion dominates [26, 20]. At pH > 9 anionic forms $Zn(OH)_2$, $Zn(OH)_3^-$ and $Zn(OH)_4^{2-}$ dominates [28]. PH and ratio of Ca/Zn determines level of assimilation of zinc by plants. Plants can absorb zinc as Zn^{2+}, organic chelates as well as hydrated ions. [25].

3.1.1. MONOMETALLIC SYSTEM

During the experiment there was observed only a little change (< 0.1) in the pH values after 10 and 30 minutes of contact time. A significant decrease in pH values as the initial metal concentration values increased was found in all samples.

Figure 1 presents changes in pH values with time during removal of chromium(VI) by *C. vulgaris* biomass, clinoptilolite and the mixture of *Chlorella vulgaris* biomass and clinoptilolite from synthetic wastewaters.

The pH range from 5.46 to 6.16 implies that dichromate ion $(Cr_2O_7)^{2-}$ dominates [28]. The maximum pH = 6.16 was observed after 10 min. of contact time for the sample containing algal biomass, at the initial metal concentration of 10 mg/l. The lowest pH = 5.46 was found in the sample with clinoptilolite at the initial metal concentration of 100 mg/l after 10 min. of contact time.

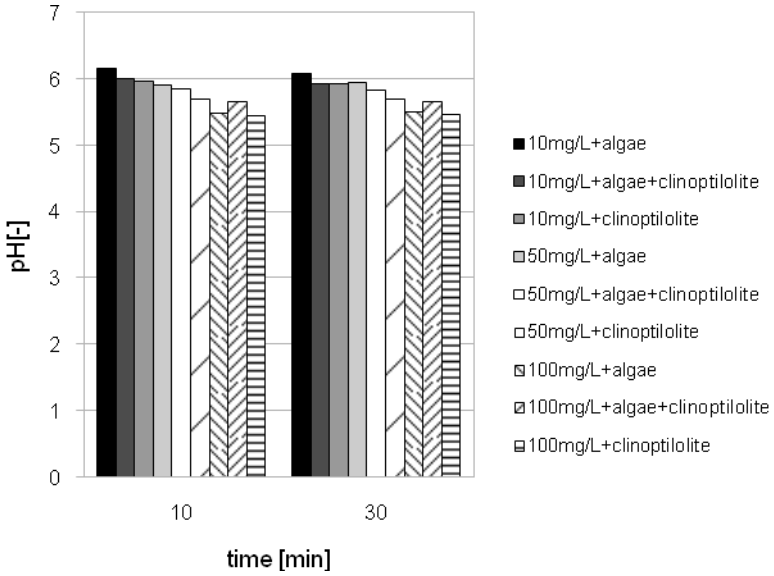

Fig. 1. pH values observed during the sorption of chromium(VI).

Figure 2 presents changes in pH values with time during removal of nickel(II) by *C. vulgaris* biomass, clinoptilolite and the mixture of *Chlorella vulgaris* biomass and clinoptilolite from synthetic wastewaters.

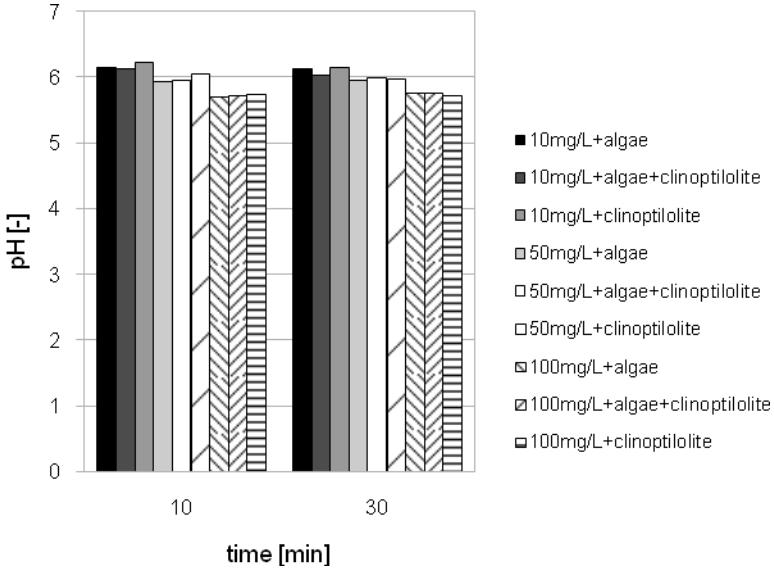

Fig. 2. pH values observed during the sorption of nickel(II).

The maximum pH = 6.22 was present after 10 min. of contact time for the sample containing clinoptilolite, at the initial metal concentration of 10 mg/l. The lowest pH = 5.70 was found in the sample with *Chlorella vulgaris* at the initial metal concentration of 100 mg/l after 10 min. of contact time.

In the obtained pH range nickel ions could be sorbed. According to the literature, nickel ions may be removed to a great extent at pH from 4.00 to 4.50 by algae [26, 20]. Nevertheless, the pH increase enhances the sorption capacity of *Chlorella vulgaris* biomass and clinoptilolite [27].

Figure 3 presents changes in pH values with time during removal of zinc(II) by *C. vulgaris* biomass, clinoptilolite or the mixture of *Chlorella vulgaris* biomass and clinoptilolite from synthetic wastewaters.

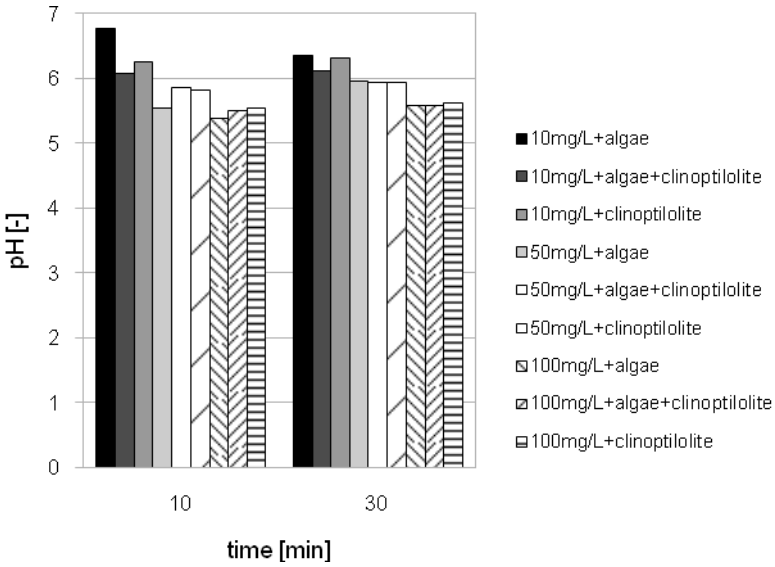

Fig. 3. pH values observed during the sorption of zinc(II).

The increase in pH values occured for the samples with *Chlorella vulgaris* biomass at the initial metal concentration of 50 and 100 mg/l from 5.55 to 5.95 and from 5.39 to 5.60, respectively. The decrease in pH from 6.78 to 6.35 was observed for the sample containing *Chlorella vulgaris* biomass at the initial metal concentration of 10 mg/l.

The maximum pH = 6.78 was present after 10 min. of contact time for the sample containing *Chlorella vulgaris* biomass, at the initial metal concentration of 10 mg/l. The lowest pH = 5.39 was found in the sample with *Chlorella vulgaris* biomass at the initial metal concentration of 100 mg/l after 10 min. of contact time.

In the pH range from 5.39 to 6.78 zinc(II) occurs mainly as an Zn^{2+}. The cationic form can be sorbed by negatively charged sorbents as *Chlorella vulgaris* biomass and clinoptilolite [20-24, 26, 29].

3.1.2. MULTIMETALLIC SYSTEM - CR(VI), NI(II) AND ZN(II)

Figure 4 presents changes in pH values with time during removal of heavy metals by *C. vulgaris* biomass, clinoptilolite or the mixture of *Chlorella vulgaris* biomass and clinoptilolite from synthetic wastewaters.

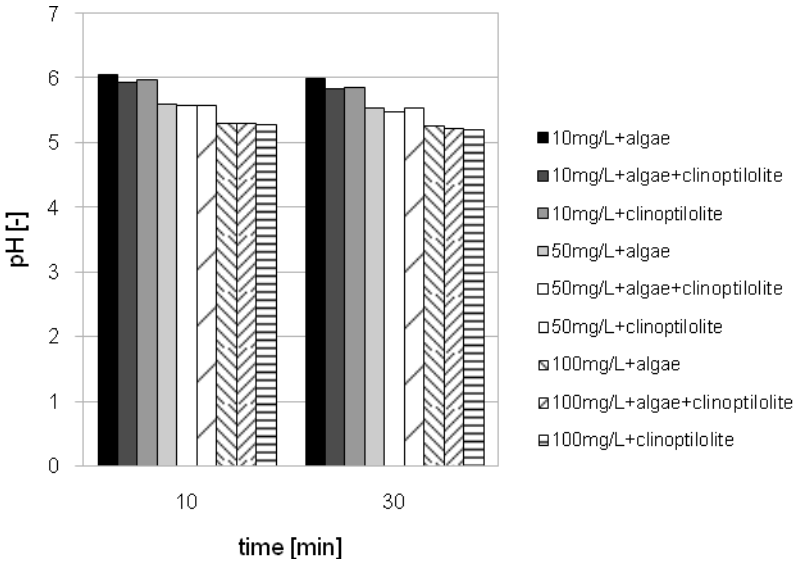

Fig. 4. pH values observed during the sorption of mixture of heavy metals (chromium, nickel, zinc).

During the experiment there was observed only a little change (< 0.1) in the pH values after 10 and 30 minutes of contact time. During the experiment pH range was from 5.21 to 6.04. The maximum pH = 6.04 was present after 10 min. of contact time for the sample containing *Chlorella vulgaris*, at the initial metal concentration of 10 mg/l. The lowest value of pH = 5.21 was found in the sample with clinoptilolite at the initial metal concentration of 100 mg/l after 30 min. of contact time.

At that pH range chromium (VI) ions are not effectively removed because anionic complexes are predominant, and those complexes cannot be sorbed due to the chemical structure [28, 30]. The anionic complexes could also affect the low pH values. The removal of nickel(II) and zinc(II) ions holds a good potential. However, the heavy metals removal could be more efficient if the time of contact would be longer [19-24, 26].

3.2. THE INFLUENCE OF SORBENTS ON CR(VI), NI(II) AND ZN(II) CONCENTRATIONS IN MONO- AND MULTIMETALLIC SYSTEMS

Multi-layered microfibrillar framework of the algal cell wall is interspersed with amorphous material consisting of glycoproteins [31]. Proteins bonded to polysaccharides are the main components of the cell wall of green algae [32]. The metal binding on algal cells occurs due to the uronic acids that are present on the cell wall [31]. Metal biosorption by biomass is also dependent on the other components on the cell and the spatial structure of the cell wall. The most important active sites involved in the metal removal include: carbonyl, carboxyl, sulfhydryl, sulfonate, thioeter, amine, secondary amine, amide, imine, imidazole, phosphonate and phosphodiester group [33].

Brinza et al. (2007) reviewed some marine micro and macroalgal species as biosorbents for heavy metal ions. One of the microalgae mentioned in the rewiev was *Chlorella vulgaris.* It was reported that this algae is able to absorb K, Mg, Ca, Fe, Sr, Co, Cu, Mn, Ni, V, Zn, As, Cd, Mo, Pb, Se, Al and other ions with good metal removal capacity [34].

The binding of metal ions can be altered by modification of the cell wall. To enhance the metal binding capacity various physical and chemical treatments could be used [35]. Also the degradation of nonviable cells and destruction of the cell membranes offer a larger available surface area and more binding sites which are important in biosorption process [36].

For clinoptilolite, the most abundant natural zeolite, sorption includes three stages: the adsorption on the microcrystals' surface (fast process- first 30min), the inversion stage and the moderate adsorption in the microcrystals' interior. The part of heavy metal is taken up at the first stage and adsorption of the rest of metal amounts lasts about 67h [19]. "Molecular sieving" is the ability of clinoptilolite to selectively adsorb cations. According to the equilibrium studies, the selectivity sequence can be given as $Pb^{2+}>Cd^{2+}>Cu^{2+}>Co^{2+}>Cr^{3+}>Zn^{2+}>Ni^{2+}>Hg^{2+}$ [37]. This selectivity of clinoptilolite depends on the capacity of the hydrated cation to move in the channels and pores. That depends on the function of the Si/Al ratio in clinoptilolite. In summary, natural zeolites hold great potential to remove cationic heavy metal species from industrial wastewater [37].

The mechanism for Cr(VI) removal may differ from other metal ions. In algal biomass pretreatment of the cells or the change from hexavalent to trivalent chromium form is essential to increase the removal efficiency [38, 39].

Cr^{6+} and Cr^{2+} are the most available forms but they are less stable in soils. Dissolved forms of chromium such as CrO_4^{2-} and $Cr(OH)_3$ could be bioaccumulated, but phyto- and zooplankton are able to absorb of chromium in each form. Chromium sorption by plants is passive. Absorption and transport of chromium in plants is related to iron concentration in plants, hence ratio of Cr/Fe in tissue of plants is almost constant [25].

With decreasing pH (below 6), the sorption of chromium increases due to the higher degree of protonation. Then, apart from the ion exchange there are also the reduction processes observed and sorption of Cr^{3+}. Maximum reduction was observed at pH = 2 [40]. Sorption of chromium by clinoptilolite was possible after Cr^{6+} reduction to Cr^{3+}.

Figure 5 and Figure 6 present changes in concentration of chromium(VI) with time in the mono- and multimetallic systems.

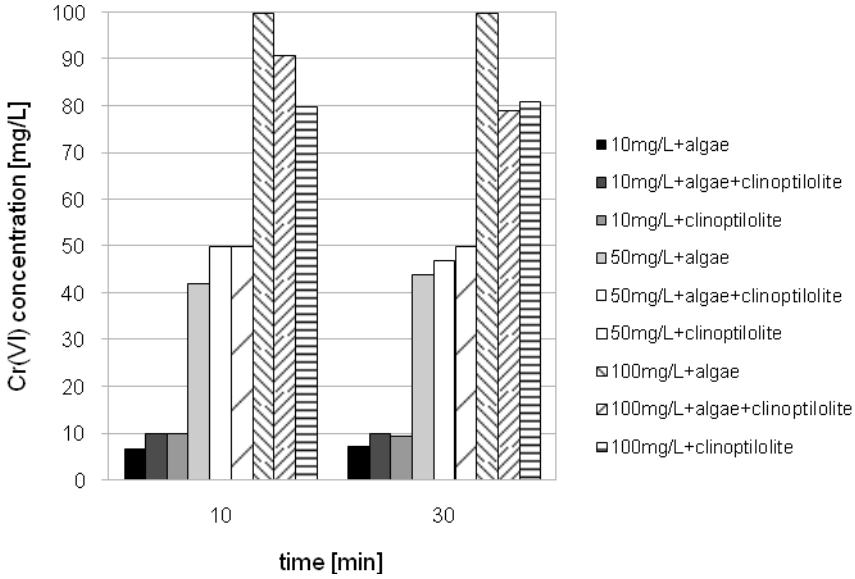

Fig. 5. Chromium(VI) concentration in the monometallic system.

Since Cr^{6+} forms are unstable [25] then sorption of chromium by used sorbents was pH dependent. No change or only a little change in heavy metal concentration for monometallic system was observed for the samples with the mixture of *Chlorella vulgaris* biomass and clinoptilolite and with clinoptilolite at the initial metal concentrations for both samples of 10 mg/l and 50 mg/l. This also occured for the sample with *Chlorella vulgaris* biomass at the initial metal concentration of 100 mg/l. It was observed for the samples with *Chlorella vulgaris* biomass at the initial metal concentration of 10mg/l and 50mg/l and after 30min heavy metal concentrations equaled 7.29 [mg/l] and 44.00 [mg/l], respectively. Only for the sample with the mixture of *Chlorella vulgaris* biomass and clinoptilolite at the initial metal concentration of 100mg/l after 10 and 30min of contact time the decrease from 91.00 [mg/l] to 79.00 [mg/l] was observed. In multimetallic system no change or only a little change in heavy metal concentration occured for the samples with the mixture of *Chlorella vulgaris* biomass and clinoptilolite and with clinoptilolite at the initial

metal concentration of 10 mg/l. This also was observed for the sample with the mixture of *Chlorella vulgaris* biomass and clinoptilolite at the initial metal concentration of 50 mg/l. The heavy metal concentration values in other samples decreased after 10min of contact time and the further change was insignificant.

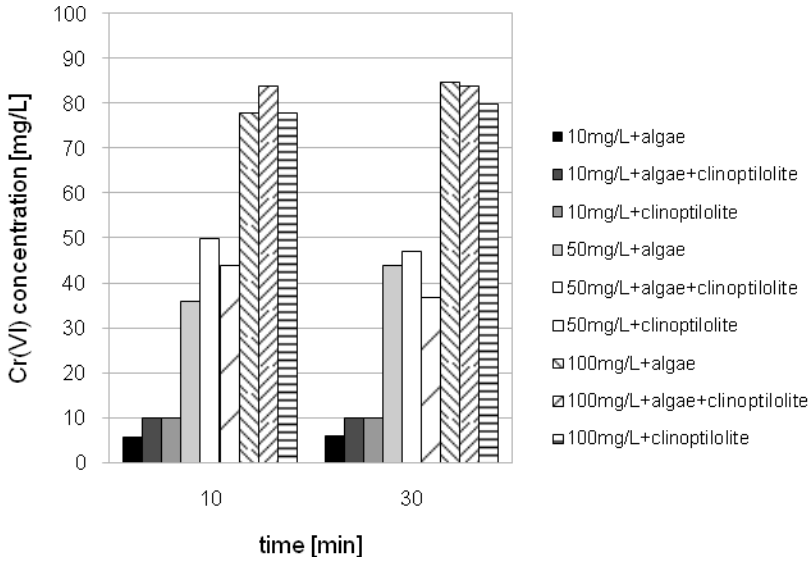

Fig. 6. Chromium(VI) concentration in the multimetallic system.

Figure 7 and Figure 8 present changes in concentration of nickel(II) with time in the mono- and multimetallic systems.

The decrease in metal concentration after 10 min. of contact time and the further decrease after 30 min. of contact time was found in all the solutions at the initial metal concentration of 10 mg/L as well as of 50 mg/l. After 30 min of contact the heavy metal concentration reached values: 25 [mg/l] in the sample with *Chlorella vulgaris* biomass and 21 [mg/l] for the samples with *Chlorella vulgaris* biomass and clinoptilolite and with clinoptilolite. At the initial metal concentration of 100 mg/l after 10 and 30 min. of contact time the decrease from 76 [mg/l] to 72 [mg/l] was found in the sample with *Chlorella vulgaris* biomass and from 55 [mg/l] to 53 [mg/l] was observed in the sample with the mixture of *Chlorella vulgaris* biomass and clinoptilolite.

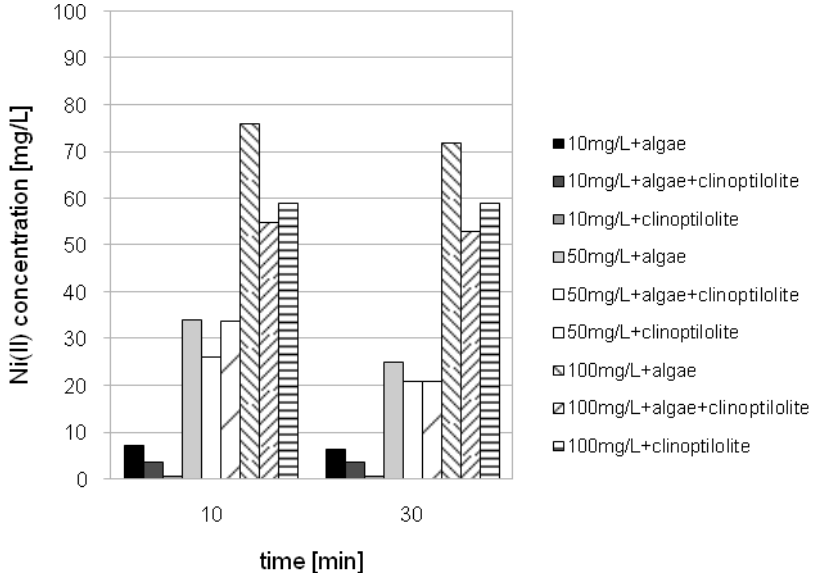

Fig. 7. Nickel(II) concentration in the monometallic system.

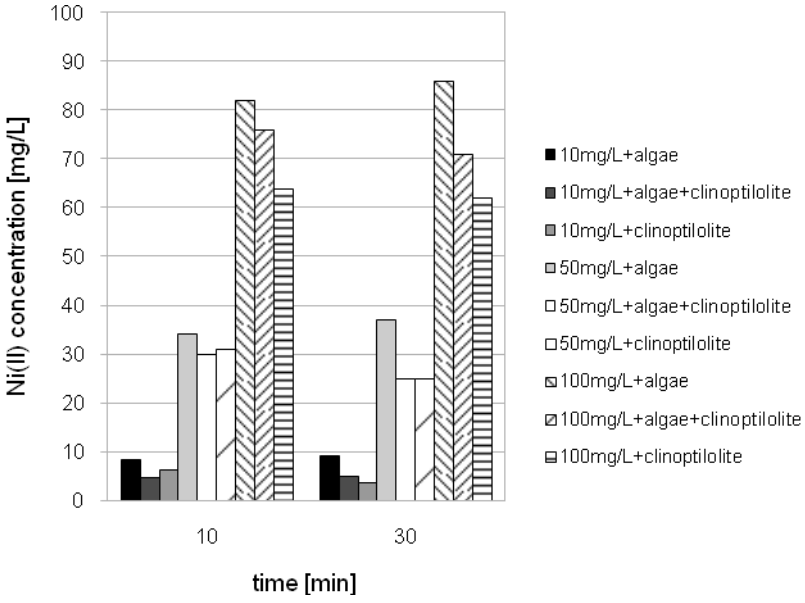

Fig. 8. Nickel(II) concentration in the multimetallic system.

In the multimetallic system metal concentrations values of all the samples at the initial metal concentration of 10 mg/l decreased after 10 min. of contact time and the further change was insignificant. For the samples at the initial metal concentration of 50 and 100 mg/l the values of heavy metal concentration have changed after 10 and 30 min of contact. After 30 min of contact at the initial metal concentration of 50 mg/l the decrease to 37 [mg/l] was found in the sample with *Chlorella vulgaris* biomass and to 25 [mg/l] for the samples with *Chlorella vulgaris* biomass with clinoptilolite and clinoptilolite. At the initial metal concentration of 100 mg/l metal concentration values reached 86 [mg/l], 71 [mg/l] and 62 [mg/l], respectively for the samples with *Chlorella vulgaris* biomass, *Chlorella vulgaris* biomass with clinoptilolite and clinoptilolite.

Figure 9 and Figure 10 present changes in concentration of zinc(II) with time in the mono- and multimetallic systems.

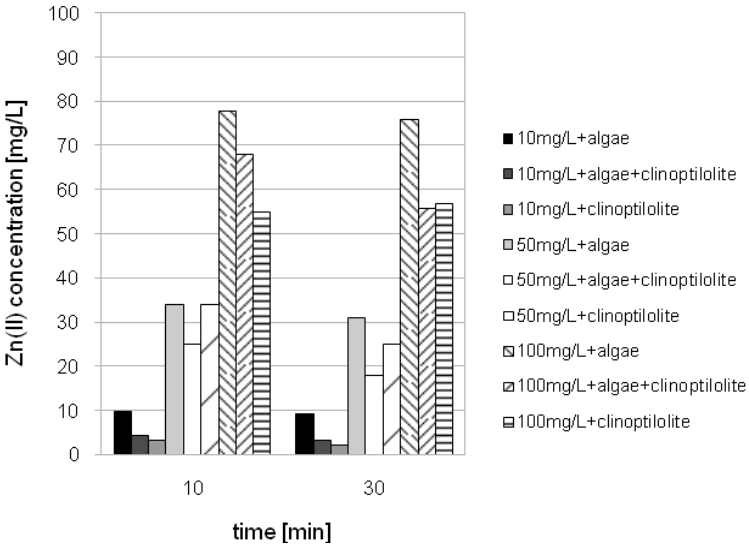

Fig. 9. Zinc(II) concentration in the monometallic system.

There was found a little decrease of Zinc (II) concentration in monometallic system in the sample containing *Chlorella vulgaris* biomass and the further change was insignificant.. The decrease in metal concentration after 10 min. of contact time and the further decrease after 30 min. of contact time was also found in all other solutions. After 30 min. of contact at the initial metal concentration of 10 mg/l the concentration reached values: 3.2 [mg/l] for the sample with *Chlorella vulgaris* biomass and

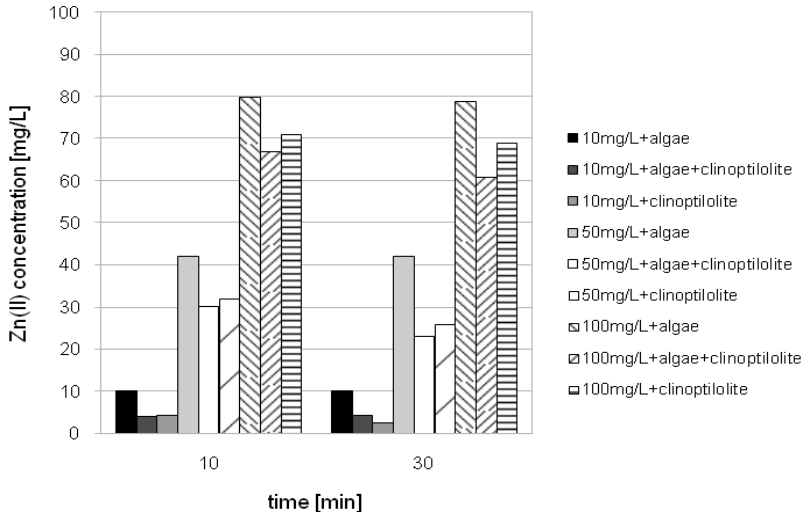

Fig. 10. Zinc(II) concentration in the multimetallic system.

clinoptilolite and 2.2 [mg/l] for the samples with clinoptilolite. At the initial metal concentration of 50 mg/l after 30 min. of contact time the decrease to 31 [mg/l] was found in the sample with *Chlorella vulgaris* biomass, to 18 [mg/l] was observed in the sample with the mixture of *Chlorella vulgaris* biomass and clinoptilolite and to 25 [mg/l] occured in the sample with clinoptilolite. At the initial metal concentration of 100 mg/l after 30 min. of contact time the decrease to 76 [mg/l] was found in the sample with *Chlorella vulgaris* biomass, to 56 [mg/l] was observed in the sample with the mixture of *Chlorella vulgaris* biomass and clinoptilolite and to 57 [mg/l] occured in the sample with clinoptilolite.

In multimetallic system metal concentration value of the sample containing *Chlorella vulgaris* biomass at the initial metal concentration of 10 mg/l did not change the concentration value. For the sample with *Chlorella vulgaris* biomass and clinoptilolite at the initial metal concentration of 10 mg/l and with *Chlorella vulgaris* biomass at the initial metal concentration of 50 mg/l heavy metal concentration values decreased after 10 min. of contact time and the further change was insignificant. For the other samples the values of heavy metal concentration have changed after 10 and 30 min. of contact. After 30 min. of contact at the initial metal concentration of 10 mg/l the decrease to 2.6 [mg/l] was found in the sample with *Chlorella vulgaris* biomass. At the initial metal concentration of 50 mg/l metal concentration values decreased to 23 [mg/l] for the samples with *Chlorella vulgaris* biomass with clinoptilolite and to 26 [mg/l] for clinoptilolite. At the initial metal concentration of 100 mg/l metal concentration values reached 79 [mg/l], 61 [mg/l] and 69 [mg/l], respectively for the samples with *Chlorella vulgaris* biomass, *Chlorella vulgaris* biomass with clinoptilolite and clinoptilolite.

4. REMOVAL OF METALS

Based on the conducted investigations, chromium(VI) was sorbed with higher efficiency from the wastewater by algal biomass from the multimetallic system (Tab. 1). The highest removal level of Cr(VI) reached by algae was in the metal concentration of 10 mg/L and 50 mg/L. At the highest initial concentration of Cr(VI)=100 mg/L removal by algal biomass was neglible in monometallic system and 22% and 15% in the multimetallic system, after 10 and 30 min. of contact time, respectively. After 30 min. of contact time the removal decreased. The removal of chromium reached by algae+clinoptilolite was the highest in C_0 = 100 mg/L, in C_0 = 10 mg/L and in C_0 = 50 mg/L was neglible in the monometallic system and of 6% in the multimetallic system.

When the anionic complexes are present the efficiency of the process could be significantly diminished [28, 30]. Hexavalent chromium is present as an anionic form which has little affinity for negatively charged ion-exchange sites in clinoptilolite. Therefore Cr(VI) was not effectively removed. Cr(VI) could be effectively removed only if it is previously reduced to trivalent form [19].

The mechanism for Cr(VI) removal by algal biomass differs from other metal ions. Algal biomass pretreatment of the cells or the change from hexavalent to trivalent chromium form is essential to increase the removal efficiency [38, 39].

The highest removal of nickel(II) reached by clinoptilolite was 94 in C_0 = 10 mg/L (Tab. 1). The selectivity series for Ni(II) was of clinoptilolite>algae+clinoptilolite>algae in the monometallic system and of algae+clinoptilolite>clinoptilolite>algae in the multimetallic system. The selectivity series at C_0 = 50 mg/L was following: algae+clinoptilolite>clinoptilolite>algae. At C_0 = 100 mg/L the selectivity series were following: algae+clinoptilolite>clinoptilolite>algae in the monometallic system and clinoptilolite>algae+clinoptilolite>algae in the multimetallic system after 10 min. and 30 min. of contact time.

Due to the competition of ions lower removal efficiency was observed in the multimetallic system [19]. The nickel(II) ions sorption decreases as the pH value decreases < 7 [19]. In the current experiment the highest pH values were observed at the initial metal concentration of 10 mg/l and the lowest for 100 mg/l. The decrease in pH values influenced the sorption. Only for the samples where *Chlorella vulgaris* biomass was used as a sorbent the highest removal values occured at the initial metal concentration of 50 mg/l. As the pH increases above 4.5, the negatively charged surface of *Chlorella vulgaris* biomass becomes available for nickel cations [27]. *Chlorella vulgaris* biomass was added to the solution at the concentration of 0.5% and clinoptilolite at 1.0%. It can be concluded that biomass concentration was insufficient for effective sorption.

Table 1. Percentage removal of Cr(VI), Ni(II) and Zn(II) by investigated sorbents.

Contact time	**10 min.**			**30 min.**		
C₀	**10 mg/L**	**50 mg/L**	**100 mg/L**	**10 mg/L**	**50 mg/L**	**100 mg/L**
Removal, %, Cr (VI)						
algae (monometallic)	33	16	0	27	12	0
algae+clinoptilolite (monometallic)	0	0	9	0	6	21
clinoptilolite (monometallic)	0	0	20	6	0	19
algae (multimetallic)	43	28	22	41	12	15
algae+clinoptilolite (multimetallic)	0	0	16	0	6	16
clinoptilolite (multimetallic)	0	12	22	0	26	20
Removal, %, Ni(II)						
algae (monometallic)	27	32	24	37	50	28
algae+clinoptilolite (monometallic)	65	48	45	64	58	47
clinoptilolite (monometallic)	94	32	41	94	58	41
algae (multimetallic)	16	32	18	8	26	14
algae+clinoptilolite (multimetallic)	52	40	24	51	50	29
clinoptilolite (multimetallic)	38	38	36	64	50	38
Removal, %, Zn(II)						
algae (monometallic)	3	32	22	8	38	24
algae+clinoptilolite (monometallic)	56	50	32	68	64	44
clinoptilolite (monometallic)	68	32	45	78	50	43
algae (multimetallic)	0	16	20	0	16	21
algae+clinoptilolite (multimetallic)	59	50	33	57	54	39
clinoptilolite (multimetallic)	57	36	29	74	48	31

The pH range in the current experiment for the adsorption on clinoptilolite is appropriate, although in the first stage of the ion-exchange (during the first 30 min.), nickel ions are not easily sorbed. This process requires the longer time of contact (up to 67h) [19].

The obtained results indicate that the highest removal of zinc(II) by clinoptilolite was 68% at the lowest C_0 = 10 mg/L (Tab. 1). The lowest removal of zinc was found by algal biomass, especially 0% of removal was found at C_0 = 10 mg/L in the multimetallic system. Addition of clinoptilolite resulted in increase of removal.

At C_0 = 10 mg/L the selectivity series were following: clinoptilolite>algae+clinoptilolite>algae. For that samples also the highest pH values occured during the current experiment. The selectivity series at C_0 = 50 mg/L as well as at C_0 = 100 mg/L was following: algae+clinoptilolite>clinoptilolite>algae after 10 and 30 min. of contact time.

It was observed the competition of ions, although not as high as in the case of nickel(II). Only nickel(II) sorption decreases significantly due to the competition [19, 37]. According to the literature, the biosorption of zinc(II) ions by algae is the most efficient after longer time of contact (> 1h). After 30 min. of contact only 50% of ions can be sorbed [41].

The obtained results also indicated the selectivity series for removal of investigated heavy metals from wastewater (Tab. 2).

Table 2. Selectivity series of algal biomass and clinoptilolite towards Cr(VI), Ni(II) and Zn(II) in the multimetallic systems.

C_0	**10 mg/L**		**50 mg/L**		**100 mg/L**	
Contact time	**10 min.**	**30 min.**	**10 min.**	**30 min.**	**10 min.**	**30 min.**
algae	Ni>Cr>Zn(0%)	Cr>Ni>Zn(0%)	Ni>Cr>Zn	Ni>Zn>Cr	Cr>Zn>Ni	Zn>Cr>Ni
algae+ clinoptilolite	Zn>Ni>Cr(0%)	Zn>Ni>Cr(0%)	Zn>Ni>Cr(0%)	Zn>Ni>Cr(6%)	Zn>Ni>Cr	Zn>Ni>Cr
clinoptilolite	Zn>Ni>Cr(0%)	Zn>Ni>Cr(0%)	Ni>Zn>Cr	Ni>Zn>Cr	Ni>Zn>Cr	Ni>Zn>Cr

There is found some differences in selectivity of heavy metals by algal biomass. The initial concentration of heavy metals and contact time influenced the selectivity series by algal biomass. The addition of clinoptilolite influenced the stabilization of selectivity towards heavy metals that then became independent of the initial concentration of heavy metals and contact time. To sum up, presence and concentration of other competing cations influenced the selectivity removal of heavy metals.

5. CONCLUSIONS

The results of investigation indicate that sorption by mixture of *Chlorella vulgaris* biomass and clinoptilolite is an efficient method for removal of Cr(VI), Ni(II) and Zn(II) from synthetic wastewater. The removal of heavy metals by clinoptilolite or the mixture of *Chlorella vulgaris* and clinoptilolite was the most efficient at the initial metal concentration of 10mg/l. The study suggests that addition of clinoptilolite to *Chlorella vulgaris* biomass results in higher removal efficiency of heavy metals at initial concentration of heavy metals of 10 mg/L. Hence, low quantity of biomass could be used in such system.

The better removal efficiency was observed for nickel(II) and zinc(II) ions from the monometallic solution.

The biosorption of Ni(II) and Zn(II) by *Chlorella vulgaris* biomass was the most efficient of 32% at the initial metal concentration of 50mg/l, after 10 min. of contact time and after 30 min. of contact time for Ni(II) and Zn(II) the removal of 50% and 38% was found, respectively. The mixture of *Chlorella vulgaris* biomass and clinoptilolite enhanced the removal of nickel and zinc in C_0 = 50 mg/L and C_0 = 100 mg/L.

The highest removal of nickel(II) reached by clinoptilolite was 94% in C_0 = 10 mg/L, after 10 min. of contact time. The results obtained indicate that at pH range from 5.70 to 6.22 Ni(II) ions could be sorbed, nevertheless based on the literature, it was hypothesized that a greater removal efficiency could be obtained at pH from 4.00 to 4.50 for algae and after a longer contact time (up to 67h) for clinoptilolite.

The obtained results indicate that the highest removal of zinc(II) by clinoptilolite was 78% at the lowest C_0 = 10 mg/L, after 30 min. of contact time. At pH range from 5.39 to 6.78 Zn(II) was present mainly as a positively charged Zn^{2+} ion and its sorption could occur. However, biosorption of zinc(II) by *Chlorella vulgaris* biomass requires longer time of the contact.

It was observed that when the substances that form anionic complexes are present as Cr(VI), the *Chlorella vulgaris* biomass was the most efficient sorbent. Chromium(VI) was sorbed with less efficiency than zinc and nickel. The higher efficiency removal for chromium from the wastewater by algal biomass from the multimetallic system was of 43% and monometallic system of 33% in C_0 = 10 mg/L, after 10 min. of contact time. There was found that clinoptilolite and the mixture of algae+clinoptilolite didn't sorb chromium(VI) or the removal of this element was lower (max up to 26%) than sorption by algal biomass.

To sum up, the biosorption of metal ions using microalgae and addition of natural zeolites such as clinoptilolite could be a very promising technology for the recovery of metal ions as well as removal of toxic heavy metal ions from wastewater, but the appropriate pH conditions as well as the adequate time of the contact and adequate dosage of sorbents are needed to make the process effective. The presence and concentration of other competing cations influenced the selectivity removal of heavy metals by algal biomass and clinoptilolite. Moreover, clinoptilolite could enhance the sedimentation process.

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