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MICROBIOLOGICAL COMPOSITION OF THE BIOFILM ON THE METAL SURFACES OF SEWAGE CONSTRUCTIONS

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Abstract. The microbiological composition of the biofilm, formed on the surface of sewage treatment constructions grids, and the seasonal variation in the number of its main components were examined. The strain *Ch.5* was isolated and identified from the enrichment culture of sulfate-reducing bacteria. It was classified as belonging to *Desulfomicrobium* genus according to its physiological and biochemical characteristics.

Keywords: biofilm, microbiological composition, sulfate-reducing bacteria, *Desulfomicrobium*.

1. Introduction

The sewage treatment constructions are an important part of the city infrastructure, and their stable functioning ensures the ecological safety of the hydrosphere. The peculiar feature of the sewage treatment constructions operation is biologically active medium, which causes the formation of the biofilm on the work surfaces [1]. Pursuant to [2], the biofilm can be the medium for active corrosion processes with the speed rate of up to 40 mm/year [3]. Therefore, the microbe biocorrosion is considered to be the main reason of emergencies [2]. It should be noted that the biocorrosion of concrete as the structural material of sewage treatment constructions is studied actively [3-5], while there are relatively few investigations on the corrosion of metal equipment.

The main component of the biofilm corrosive microbe community is sulfate-reducing bacteria, iron-reducing bacteria, denitrifying and ammonifying bacteria [6]. With the biofilm functioning, the number of the microorganisms constantly varies. If the number of sulfate-reducing bacteria reaches 10^6–10^7 cell/cm^2, the biofilm becomes corrosively active [2]. It has been established that the biofilm with the high titre of sulfate-reducing bacteria is formed on the grids of the sewage treatment constructions at the initial stage of sewage treatment [7]. The seasonal variation in the number of the main components of the biofilm microbe community, which is one of the factors of the stable and long-term functioning of the sewage treatment constructions, has not been studied. It is also important to classify the sulfate-reducing bacteria (the most aggressive component of the biofilm) as belonging to a certain genus in order to search for and use the inhibitors-biocides, characterized by specific functioning [8].

The aim of this paper is to examine the seasonal variation in the number of the main components of microbe community of the biofilm, formed on the metal surfaces of the sewage treatment constructions (Chernihiv), to isolate the sulfate-reducing bacteria from the biofilm and to identify them by their physiological and biochemical characteristics.

2. Experimental

The samples of the biofilm, formed on the metal surfaces of the sewage treatment constructions (Chernihiv) at the initial stage of sewage treatment (water grids), were gathered within the period from July 2016 till August 2017: sample 1 – June 2016; sample 2 – February 2017; sample 3 – May 2017; sample 4 – August 2017. The samples were in the form of grey and black suspension with a specific strong smell.

The number of the microorganisms in the gathered biofilm samples was calculated using the method of decimal serial dilution during the bacteria seeding to the correspondent liquid selective mediums: sulfate-reducing bacteria (SRB) – to Postgate “B” medium, iron-reducing bacteria (IRB) – to the Kalinenko medium, denitrifying bacteria (DNB) – Giltay medium and ammonifying bacteria (AMB) – to meat peptone broth [9].

The enrichment cultures of sulfate-reducing bacteria were obtained using the enrichment culture method in the liquid elective Postgate “B” medium from the biofilm samples.

The strain *Ch.5* of sulfate-reducing bacteria was received by numerous reseeding of certain communities

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on the dense and liquid Postgate "B" medium. The cleansing of the culture was controlled with the help of the microscope and by seeding to the medium of the following composition (g/l): peptone – 4; glucose – 10; Na_2SO_4 – 2; $MgSO_4$ – 1; agar – 15, in order to identify Clostridium bacteria, which cause fermentation. The supplements, which had been sterilized separately, were added directly before usage: Mohr's salt (5% Mohr's salt solution in 1% HCl) – 10 ml; 1% Na_2S – 2 ml.

Physiological and biochemical characteristics of the extracted strain were defined by the ability of bacteria to use different sources of carbon: alcohols, organic acids, amino acids. Organic acids and alcohols were added to the Postgate "B" medium without the yeast extract in the amount, equal by the mass to carbon in 3.5 g/l of sodium lactate, amino acids – in 2.0 g/l of sodium lactate. To define the use of electron acceptors by the bacteria, sulfate, sulfite, thiosulfate or simple sulfur at concentration of 4.5 g/l were added to the Postage "C" medium, free of sulfates. Bacterial cells preparations were stained by Gram [9]. The strain was classified to genus according to [10].

The corrosion study was conducted using a gravimetric method. The steel St3ps plates (surface area 0.002 m^2), polished to the 4-5 class of accuracy, were used. Before being placed into the corrosive medium, the steel samples were cleaned by alcohol. The plates were weighed with analytical scales accurate to $5 \cdot 10^{-5}$ g. Medium Postgate "B" (pH = 7) was used as a testing corrosive medium. It was inoculated with the enrichment culture of sulfate-reducing bacteria from the biofilm (sample 1), bacteria strains *Ch.5*, *Desulfovibrio sp.* M.4.1 (gathered previously by us from corroded iron coating of the subterranean gas pipeline [11]), and *Desulfomicrobium sp.* TC4 (bought from the collection of the department of general and soil microbiology, D. K. Zabolotny Institute of Microbiology and Virology, the National Academy of Sciences of Ukraine). Strain *Desulfomicrobium sp.* TC4 was obtained from the corrosion product of brass tubes in water thermal networks. The initial titre of sulfate-reducing bacteria in the corrosive medium was 10^9 cell/ml. Samples immerse time was 240 h under 300 K (optimal temperature for the development of sulfate-reducing bacteria). Corrosion rate was calculated with the help of the formula: $k = \Delta m/(S \cdot t)$, where Δm – sample weight loss, g; S – sample area, m^2; t –time, h.

The water analysis on the sewage treatment constructions of public company "Cherhihivvodokanal" (Chernihiv), where all the city domestic and industrial wastewater fall, was conducted following the requirements of current standards [12]. The ion concentration of Fe^{+2} and Fe^{+3}, NH_4^+, PO_4^{3-} was measured with the help of a photocolorimetry method; chloride ions concentration with the Mohr method of argentometry; sulfate ions – with a complexonometrical method [13]. Biological oxygen demand (BOD_5) was measured using iodometry titrimetric analysis, while the chemical oxygen demand (COD) – a dichromate method. Suspension substances and dry matter were measured using a gravimetric method. The index of acidity was measured with pH-meter/ionomer pH/ION 340i. The concentration of hydrogen sulfide was measured by iodometric titration.

The surface analysis of the biofilm, formed on the metal samples surface, was performed with a scanning electron microscope (SEM). To fix the biofilm, the samples were immersed in a 2% glutaraldehyde solution for 1 h, dehydrated in 4 ethanol solutions (15 min each) of 25, 50, 75 and 100% concentration successively, and air dried for 24 h [14]. After fixation, the samples were examined using an electron microscopy FEIE-SEM XL 30. With the help of electron microscope the picture was taken in the mode of functioning in secondary electrons. Maximum residual pressure in the microscope pillar was no more than $6.7 \cdot 10^{-4}$ Pa under the gun current of 76 mA.

Statistical analysis of experimental data (the corrosion rate) for the reliability level 95 % and correlation analysis were carried out with the help of Microsoft Excel. The approximation coefficient (R^2) was calculated. The tests were conducted three times.

3. Results and Discussion

The number of the main microorganism physiological groups in the composition of the samples of the biofilm, formed on the metal surface of the grids of the sewage treatment constructions, is demonstrated in Fig. 1.

The titre of all the studied bacteria groups is high ($> 10^3$ cell/ml). This is attributed to the considerable level of contamination in the sewage (Table 1), including the contamination with the compounds, which are nutriments for the microorganisms (Fig. 2). Medium pH (Table 1) is also favourable for the growth of biofilm bacteria.

The number of the most aggressive component of microbe community – sulfate-reducing bacteria – is within the range of $(6.5 \cdot 10^7)$–$(3.0 \cdot 10^9)$ cell/ml (Fig. 1). In the biofilm sample 2 the sulfate-reducing bacteria prevail. The number of the iron-reducing bacteria ranges between $8.0 \cdot 10^6$ and $3.0 \cdot 10^9$ cell/ml. In the samples 1 and 4 the number of sulfate-reducing bacteria approximately equals to iron-reducing bacteria. In the sample 2 the number of IRB, DNB and AMB practically does not differ. The number of the denitrifying bacteria ranges between $1 \cdot 10^4$ and $3.0 \cdot 10^7$ cell/ml. In the sample 3 denitrifying bacteria are the least. Ammonifying bacteria are within the range of $(8.0 \cdot 10^6)$–$(3.0 \cdot 10^{10})$ cell/ml. In the samples 1 and 3 the prevailing group is ammonifying bacteria, which constitute $2.5 \cdot 10^9$ and $3.0 \cdot 10^{10}$ cell/ml, respectively. Their significant increase in the composition of the biofilm sample 3 is noticeable, which can probably be caused by the change in sewage ion concentration (Fig. 2). The comparison of the number of bacteria physiological groups and their ratio in the community in the studied

biofilm samples indicate the stable corrosively active system, maintained by trophic connections [15, 16].

No strict correlation between the logarithm of the number of a certain bacteria group and the ion concentration was identified. During the analysis of the correlation between the logarithm of the number of sulfate-reducing bacteria and the concentration of the sulfate ions, approximation coefficient was R^2= 0.31, for chloride ions R^2= 0.07. The highest approximation coefficient was defined during the analysis of the correlation between the logarithm of the number of sulfate-reducing bacteria and the pH index, R^2=0.5.

The enrichment cultures of sulfate-reducing bacteria proved to have the highest metabolic activity in summer months, which is the result of more favourable temperature conditions. For instance, for the enrichment culture from biofilm sample 1 the activity equals to 513.4 μgH_2S/ml. In winter the metabolic activity of the bacteria is much lower. For the enrichment culture from biofilm sample 2 it is only 88 μgH_2S/ml. However, it has little impact on pH of the sewage, which changed only by 0.08 pH (Table 1).

The biofilm, formed in the laboratory model experiment on a steel St3ps plate by the enrichment culture of sulfate-reducing bacteria from sample 1, is the conglomeration of bacteria, which are unequally distributed in the polymer matrix. High density of the microorganisms is observed in certain areas (Fig. 3). By their morphology the bacteria are curved sticks, appearing singly and in pairs.

The physiological and biochemical characteristics of the strain *Ch.5*, isolated from the bacteria enrichment culture, were studied. It was established that the strain bacteria are used: i) as electron acceptors: sulfate, sulfite, thiosulfate or elemental sulfur; ii) as electron donors and source of carbon: lactate, ethanol, propanol, isoamyl alcohol, formate, succinate, stearate, malate, oleate, benzoate, fumarate, arginine, tryptophane. In addition, phenol, asparagine, lysine, alanine, citrate cannot serve as substratum for the isolated strain of sulfate-reducing bacteria. The staining of bacterial cells showed that they are gram-positive. The obtained data make it possible to assume that strain *Ch.5* belongs to genus *Desulfomicrobium.*

The speed rate of steel St3ps biocorrosion in Postgate "B" medium, initiated by the strain *Ch.5* bacteria, equals to $1.75 \cdot 10^{-2}$ g/(m^2·h). The comparison of the microbe corrosion speed rate of steel St3ps, initiated by the strain *Ch.5* with M.4.1 and TC4 strains, has shown that the bacteria of *Desulfomicrobium* genus create a less aggressive medium, compared to the bacteria of *Desulfovibrio* genus (Table 2).

However, as a rule, in natural and man-caused environment, when the microorganisms are the part of the microbe community, the biocorrosion speed rate is higher. This is indicated by comparing the effects of enrichment culture of sulfate-reducing bacteria from the biofilm sample 1 and the strain *Ch.5*.

Table 1

Contamination indices of sewage dumped on treatment plants

Date	Suspension substances, mg/dm^3	Dry matter, mg/dm^3	BOD_5, mg/dm^3	BOD_{total}, mg/dm^3	COD, mg/dm^3	pH
July 2016	196	670	465.3	586.3	903.3	7.14
February 2017	244	710	490.0	617.4	776.0	7.22
May 2017	224	695	392.7	494.8	816.0	7.16
August 2017	248	549	311.3	392.3	489.3	7.51

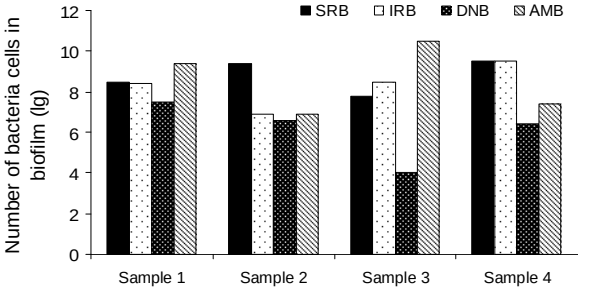


Fig. 1. The number of microorganism physiological groups of bacteria in the composition of the biofilm, formed on the metal surface (water grids)

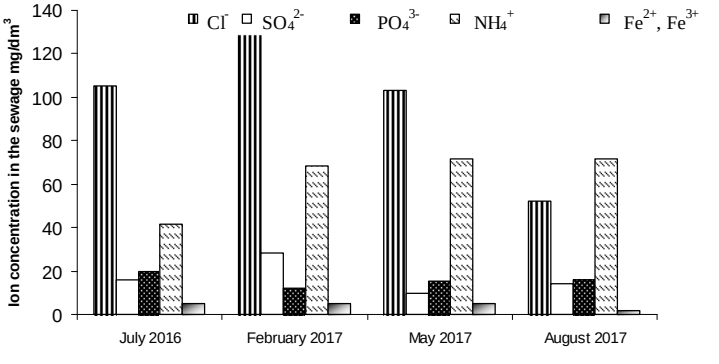
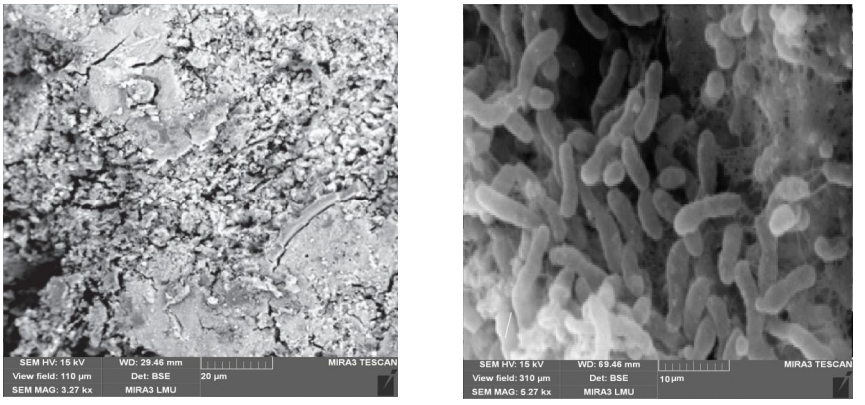


Fig. 2. Ions concentration in the sewage



a) b)

Fig. 3. FE-SEM images of the biofilm formed on St3ps steel surface after exposure to Postgate "B" medium with sulfate-reducing bacteria culture. Magnification of 1000× (a) and 8000× (b)

Table 2

Speed rate of microbe corrosion of steel St3ps, induced by the enrichment culture and certain strains of sulfate-reducing bacteria

Strain	Enrichment culture	*Desulfomicrobium sp. Ch.5*	*Desulfovibrio sp. M.4.1*	*Desulfomicrobium sp. TC 4*
k, g/(m^2·h)·10^2	1.97	1.75	3.68	2.44

4. Conclusions

It has been demonstrated that on the metal surfaces of sewage treatment constructions (Chernihiv) at the initial stage of sewage treatment process a corrosive biofilm is formed. The number of sulfate-reducing, iron-reducing, denitrifying and ammonifying bacteria in this biofilm varies, nevertheless, staying within the range which corresponds to corrosively active system.

It has been defined that the strain *Ch.5*, isolated from the biofilm enrichment culture of sulfate-reducing bacteria, can be classified as the one, belonging to the genus *Desulfomicrobium* according to its physiological and biochemical characteristics. This fact is important for ensuring the anti-corrosive protection of the metal constructions, which are under the effect of corrosively active enrichment cultures of the biofilm, with the help of inhibitors-biocides. The obtained data should be taken into account for the search of new inhibitors with the biocidal action.

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МІКРОБІОЛОГІЧНИЙ СКЛАД БІОПЛІВКИ МЕТАЛЕВИХ ПОВЕРХОНЬ ОЧИСНИХ СПОРУД

Анотація. *Досліджено мікробіологічний склад біоплівки, яка формується на поверхні решіток очисних споруд, та сезонну зміну чисельності головних її складових. З накопичувальної культури сульфатвідновлювальних бактерій ізольовано та ідентифіковано штам Ch.5, який за фізіолого-біохімічною характеристикою віднесено до роду Desulfomicrobium.*

Ключові слова: *біоплівка, мікробіологічний склад, сульфатвідновлювальні бактерії, Desulfomicrobium.*