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ANTHROPOGENIC IMPACT ON THE DEVELOPMENT OF RESISTANCE TO ANTIBIOTICS IN MICROORGANISMS OF THE UZH RIVER (UKRAINE)

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Background. An excessive use of antibiotics in human and veterinary medicine contributes to the additional selection pressure on microorganisms and leads to the rapid spread of dangerous microorganisms with an increased ability to resist numerous classes of antibiotics. Aquatic ecosystems are among the main resistance genes pools, and therefore should be subject to mandatory control. Apparently, the spread of antibiotic-resistant microorganisms depends not only on the concentration of antibiotics entering water bodies with wastewaters, but also on other qualitative characteristics of the aquatic environment.

The aim of this work was to determine the correlation between the content of heavy metals (Cu, Ni, Zn, Cr) and nitrogen compounds (NO_3, NO_2 and NH_4) in water and the distribution of antibiotic resistant microorganisms in research sites on the Uzh River (Ukraine) that are affected by anthropogenic impact. Based on our seasonal monitoring during 2016–2017, which included determining the content of nitrogen compounds and heavy metals in the research sites, and as a result of the study on the microbiocenosis structure with a subsequent determination of antibiotic sensitivity of the dominant strains isolated from water samples, we conducted analysis of the correlation between the concentrations of these substances and the distribution of antibiotic resistant strains. This enabled us to identify the potential factors that contribute to the development of antibiotic resistance in microorganisms.

Material and methods. The relationship between the chemical parameters and the percentage of antibiotic-resistant microorganisms was determined using the linear Pearson's correlation coefficient (r). Statistical data processing was performed using the software package „Microsoft Excel". Results with a *p*-value less than 0.05 ($p<0.05$) were considered statistically significant.

Results. The relationship between the level of antibiotic resistance of microorganisms and chemical pollution has been established. A strong correlation between excessive concentrations of Zn and elevated concentrations of Ni relative to background values and increased antibiotic resistance is characteristic of the area under conditions of technogenic transformation**.** In the urbanised area, a relationship between an increase in antibiotic resistance and Ni concentrations was observed in the studied samples. In the agricultural area, a strong correlation between nitrogen compounds, namely nitrates, and an increase in antibiotic resistance of microorganisms has been established.

Conclusion. The obtained results that establish a correlation between the concentrations of chemical substances in water and the activation of antibiotic resistance in microorganisms can indicate the degree of aquatic ecosystems transformation under anthropogenic impact. Thus, a comprehensive monitoring of the environmental quality of aquatic ecosystems is essential.

Keywords: heavy metals, nitrogen compounds, antibiotics, aquatic ecosystems, microorganisms, antibiotic resistance

INTRODUCTION

The issue of antibiotic resistance of microorganisms arose in the 1950s after the first antibiotics that radically changed approaches to the treatment of infectious diseases had been discovered. This problem still persists today, posing particular threat for developing countries [8; 22]. Uncontrolled consumption and unreasonable prescriptions of antibiotics, lack of control over the circulation of antibiotic-resistant microorganisms and antibiotic resistance genes in the environment are the main social reasons for the rapid antibiotic resistance development [18, 19]. Realizing the scale of the problem, countries with a high level of development have imposed control over the presence of antibiotics in food products, in particular, poultry, livestock and bottled water. It is equally important to establish a mechanism of control over the spread of antibiotics in the environment, especially in water. Water is a key element of the substance turnover, it is included in food chains; hence it can be one of the key factors in the migration of antibiotics. After consumption by humans and animals, most antibiotics that are not fully assimilated and utilized are excreted in the feces and enter rivers, lakes and groundwater [8]. It has been proved that antibiotics used in farming are not completely assimilated by animal bodies and an estimated 25 % to 75 % of antibiotics are excreted into the environment [9]. Many studies proved that hospital effluents, wastewaters from sanitary treatment facilities and domestic effluents are virtually the main sources of antibiotics inflow into surface waters [10; 11; 16; 31].

The potential ability of microorganisms to survive in water is ensured by the horizontal transfer or mutation of genes [27]. Mobile genetic factors and integrons in the genomes of resistant microorganisms can be propagated and spread by transformation, conjugation, etc. [25]. In addition, contaminants such as heavy metals and detergents in the environment can promote the horizontal transfer of genes responsible for antibiotic resistance [26; 32].

The results of previous research showed that heavy metals could activate the process of gaining resistance even at low permissible concentrations. Studies on the effect of subinhibitory concentrations of Cu(II), Ag(I), Cr(VI) and Zn(II) on *Escherichia coli* proved

that they promoted transfer of antibiotic resistance genes between strains of this bacterium by conjugation [30].

The analysis of scientific literature on the determination of antibiotic resistance genes in surface waters and drinking water once again proved a high priority of this problem. Thus, according to studies conducted in Slovakia aimed to identify antibiotic-resistant bacteria in 12 rivers and 6 lakes, most of the studied strains were resistant to tetracycline, vancomycin and ampicillin [7]. Test samples collected from the Danube River in Hungary were tested for antibiotic resistance. According to the obtained results, 39 % of all isolated strains of *E. coli* and 15 % of *Klebsiella* spp. showed resistance to all tested antibiotics, except tigecycline [1]. A number of other studies conducted on surface water samples of the Danube River demonstrated a high level of prevalence of antibiotic-resistant bacteria and the respective resistance genes [12; 13; 17; 23]. A significant influence of organic substances on the development of antibiotic resistance in microorganisms in surface waters has been proved. In particular, high correlations between nitrites, nitrates and antibiotic resistance genes in microorganisms have been established [29].

The aim of this work was to determine the correlation between the content of heavy metals (Cu, Ni, Zn, Cr) and nitrogen compounds (NO_3, NO_2 and NH_4) and the amount of antibiotic resistant microorganisms in research sites on the Uzh River (Ukraine) affected by anthropogenic impact of different character.

MATERIALS AND METHODS

The results obtained during 2016–2017 seasonal monitoring of chemical parameters (nitrogen compounds, heavy metals) and microorganisms isolated from water of the Uzh River, subsequently identified and tested for sensitivity to antibiotics [4; 5; 6], were used as materials for determining the correlation between the degree of antibiotic resistance of microorganisms and the content of chemical compounds in the water samples. The strains of microorganisms isolated from water samples were identified by morphological characteristics of colonies on Hottinger agar (pH±7.2) with the subsequent differentiation on the selective media of Endo, Ploskirev, and Bismuth-sulfite agar. The genera and species of the bacteria were identified based on a set of biochemical tests Enterotest 24 and Enterotest 16 (Lachema, Czech Republic). The antibiotic sensitivity of the bacteria was determined by the Kirby-Bauer disk diffusion method on the Mueller-Hinton agar using commercial disks (HI MEDIA, India) according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) [4].

Correlation analysis was performed using a package of statistical programs “Microsoft Excel”. In particular, the relationship between the concentrations of heavy metals, nitrogen compounds and the degree of antibiotic resistance of microorganisms was determined using the linear Pearson’s correlation coefficient (r). The correlation was considered weak at *r*-value less than 0.25. If *r*-value was between 0.25 and 0.75, the correlation was considered moderate. At *r* greater than or equal to 0.75, the correlation was considered strong. Differences at $p<0.05$ were considered statistically significant.

To determine the relationship between the indicators of chemical pollution and the degree of antibiotic resistance of microorganisms, the most common strains of bacteria were selected: *Escherichia coli* A2 (n = 114) – with altered enzymatic properties – isolated from water in the area under conditions of technogenic transformation, *Escherichia*

coli A5 (n = 151) – with hemolytic activity – from the area affected by urbanization, and *Citrobacter freundii* C2 (n = 98) that dominated in the agricultural area [4].

The sampling sites with the highest indicators of anthropogenic pollution were taken into account. The concentrations of heavy metals and nitrogen compounds recorded in areas under different types of anthropogenic impact were analyzed. The research sites were located in three areas:

- an area under conditions of technogenic transformation (100 m downstream from the confluence of the Domoraj and the Uzh River, within the town of Perechyn, where the Perechyn forest chemical plant is located, 48°43′37.1″N, 22°28′42.1″E);
- an area affected by urbanization (on the outskirts of the city of Uzhhorod, 48°37′10.2″N, 22°15′26.4″E);
- an agricultural area (located in the proximity of the village Storozhnytsia, where agricultural lands and farms are concentrated, 48°36′11.7″N, 22°12′18.3″E) (**Fig. 1**).

Fig. 1. The map of the research sites on the Uzh River: No 1 – 100 m downstream from the confluence of the Domoraj and the Uzh River (area under conditions of technogenic transformation); No 2 – on the outskirts of the city of Uzhhorod (area affected by urbanization); No 3 – in the proximity of the village Storozhnytsia (agricultural area)

Рис. 1. Картосхема досліджуваних ділянок річки Уж: № 1 – 100 м нижче впадіння струмка Доморадж (техногенно-трансформована територія); № 2 – за м. Ужгород (урбанізована територія); № 3 – за с. Сторожниця (аграрна територія)

RESULTS AND DISCUSSION

The data on the spatial distribution of heavy metals in the Uzh River enabled us to identify areas with high concentrations of pollutants. Among them was the area under conditions of technogenic transformation, where exceeded threshold limit values of Cu, Cr, Zn were observed. In the urbanized and agricultural areas, Cr and Cu concentrations slightly exceeded the normal levels [6]. Regarding the contamination with nitrogen compounds, the territory under conditions of technogenic transformation was identified as the most affected area where the concentration of ammonium nitrogen exceeded 18 times the threshold limit values for fisheries (9.19 mg/L July). Similarly, the indicators of nitrite ions concentrations exceeded eight times the normal values (0.63 mg/L July) in the summer. Less polluted was the urban area, where small concentrations of nitrogen compounds were recorded, and the content of nitrates was within acceptable limits. In the agricultural area, higher concentrations of nitrogen compounds compared to urban areas were found. Although the content of nitrates there did not exceed the threshold limit values for fisheries, it was the highest compared to other areas [5]. Probable sources of pollutants in the Uzh River are the forest chemical plant and domestic wastewaters, as well as agricultural lands and farms located on the banks of the river [14, 20].

The entry of Cu and Zn into natural waters can possibly be due to food additives used in animal husbandry. Now that antimicrobial growth promoters have been banned, the latter are in great demand, provoking the development of antibiotic resistance [3]. Excessive concentrations of both organic and inorganic substances can be the triggering factors for stress reactions in microorganisms, which in turn activate the mechanisms of resistance or increase susceptibility to antibiotics. In the natural environment, many microorganisms form complexes, especially with such metals as Zn, Cu and Ni, but increased concentrations of metals act as stressors, being toxic to microorganisms. As a result, bacteria activate various protective and adaptive mechanisms that can trigger cross-resistance mechanisms. Metals can destroy microorganisms in the same ways as antibiotics, therefore, the mechanism of developing resistance to antibiotics is activated in bacteria as a protective reaction [2].

The results of correlation analysis indicate a close relationship between the concentrations of Zn and an increase in antibiotic resistancy of the microorganisms isolated along the entire length of the Uzh River. In the area under conditions of technogenic transformation, a particularly strong positive correlation was observed between the concentrations of Zn and antibiotics that inhibit nucleic acid synthesis. Thus, it can be assumed that Zn cations affect the RNA-polimerase activity by substituting manganese ions in the active center of the enzyme, thus affecting the level of transcription of bacterial cell genes in the case when these ions are in excess [24] (**Fig. 2**). The cultivation of *E. coli* in chemostats on appropriate media with a low content of inorganic phosphate allowed for maximum bioavailability of zinc and, with the addition of increased concentrations of zinc, made it possible to determine the effect of zinc on the regulation of 64 genes expression in response to stress. The latter included operons responsible for the formation of antibiotic resistance [15].

A weak correlation between Ni concentrations and antibiotic resistant microorganisms was observed in the study areas, except the urbanized one where Ni concentrations are closely correlated with resistance to antibiotics that inhibit bacterial wall synthesis: ampicillin $r = 0.78$, $p<0.05$; ceftriaxone $r = 0.87$, $p<0.05$; ampicillin/sulbactam $r = 0.67$, $p<0.05$; meropenem $r = 0.88$, $p<0.05$, $N = 10$) (**Fig. 3**).

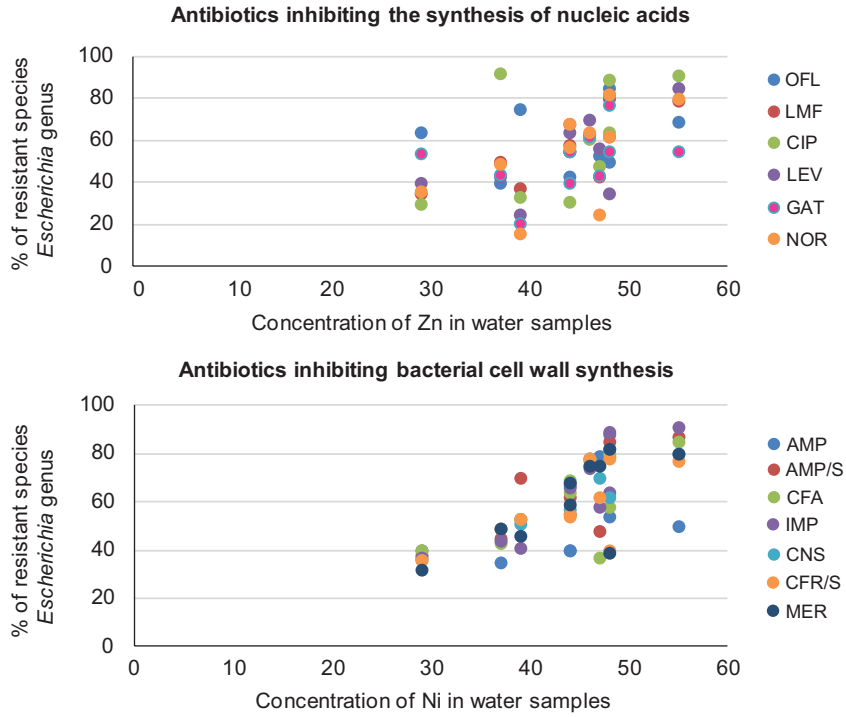

Fig. 2. Correlations between concentrations of heavy metals and the degree of antibiotic resistance of the strains of microorganisms isolated from the surface waters of the Uzh River in the area under conditions of technogenic transformation (n = 10; p<0.05). AMP – ampicillin; AMP/S – ampicillin/sulbactam; CNS – cefuroxime; CFR/S – cefoperazone/ sulbactam; MER – meropenem; IMP – imipenem; CFA – ceftriaxone; OFL – ofloxacin; LMF – lomefloxacin; CIP – ciprofloxacin; GAT– gatifloxacin; NOR – norfloxacin; LEV – levofloxacin

Рис. 2. Кореляційні взаємозв'язки між концентраціями важких металів і ступенем антибіотикорезистентності штамів мікроорганізмів, виділених із поверхневих вод річки Уж на техногенно-трансформованій території (n = 10; p<0,05). AMP – ампіцилін; AMP/S – ампіцилін/сульбактам; CNS – цефуроксим; CFR/S – цефоперазон/сульбактам; MER – меропенем; IMP – іміпенем; CFA – цефтріаксон; OFL – офлоксацин; LMF – ломефлоксацин; CIP – ципрофлоксацин; GAT – гатіфлоксацин; NOR – норфлоксацин; LEV – левофлоксацин

As previously established, elevated concentrations of nickel activate adaptation mechanisms in bacteria, for example in *E. coli* they contribute to the formation of a biofilm by means of the induction of the appropppriate genes transcription [21]. However, a negative correlation was found with respect to Cu, Cr and Pb, which might be a consequence of both the direct impact and a complex interaction in the hydroecosystem.

The analysis of nitrogen compounds indicators (nitrates, nitrites, ammonium nitrogen) revealed high correlations between nitrates concentrations and the degree of antibiotic resistance of the studied microorganisms in the area affected by urbanization and the agricultural area (**Fig. 4**). In the agricultural area, strong links were observed between concentrations of nitrites, ammonium nitrogen and strains resistant to antibiotics that inhibit bacterial wall synthesis and nucleic acid synthesis (ofloxacin r = 0.77, p<0.05; lomefloxacin r = 0.82, p<0.05; ciprofloxacin r = 0.89, p<0.05, N = 10). According to the results, the highest concentrations of nitrogen compounds were recorded in this part of the water body [5].

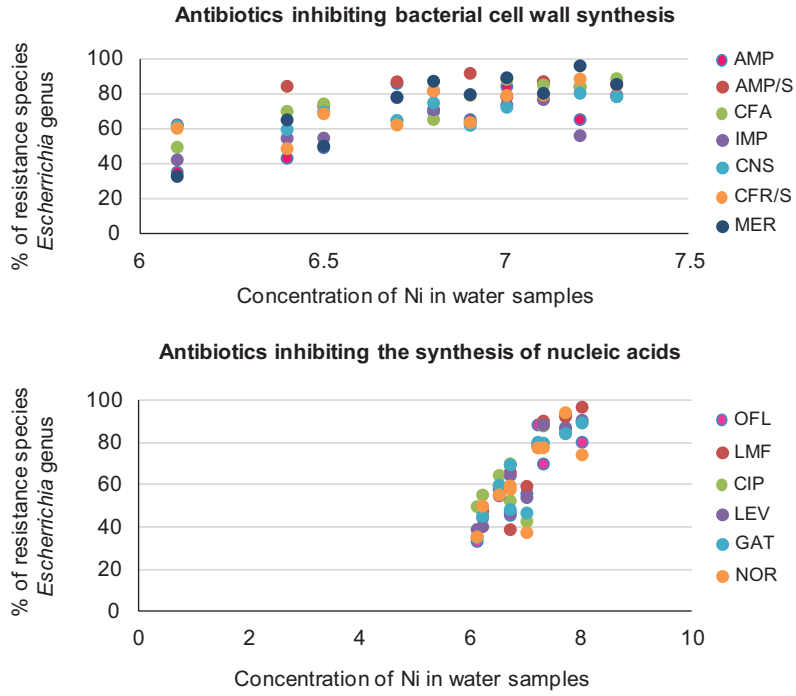

Fig. 3. Correlations between concentrations of heavy metals and the degree of antibiotic resistance of the strains of microorganisms isolated from the surface waters of the Uzh River in the area affected by urbanization (n = 10; p<0.05). AMP – ampicillin; AMP/S – ampicillin/sulbactam; CNS – cefuroxime; CFR/S – cefoperazone/sulbactam; MER – meropenem; IMP – imipenem; CFA – ceftriaxone; OFL – ofloxacin; LMF– lomefloxacin; CIP – ciprofloxacin; GAT– gatifloxacin; NOR – norfloxacin; LEV – levofloxacin

Рис. 3. Кореляційні взаємозв'язки між концентраціями важких металів і ступенем антибіотикорезистентності штамів мікроорганізмів, виділених із поверхневих вод річки Уж на урбанізованій території (n = 10; p<0,05). AMP – ампіцилін; AMP/S – ампіцилін/сульбактам; CNS – цефуроксим; CFR/S – цефоперазон/сульбактам; MER – меропенем; IMP – іміпенем; CFA – цефтріаксон; OFL – офлоксацин; LMF – ломефлоксацин; CIP – ципрофлоксацин; GAT – гатіфлоксацин; NOR – норфлоксацин; LEV – левофлоксацин

The high degree of correlation between concentration of nitrates and multidrug-resistancy of microorganisms might be due to common sources of pollution, since the main routes for nitrates to enter aquatic ecosystems are uncontrolled domestic wastewater discharges, mineral fertilizers from agricultural lands and farms located on the banks of the river. The peculiarities of relief of the water body could have contributed to its pollution, since the river in the studied areas acquires a flat type, with a slower water flow and an increased level of siltation. The influence of antibiotics on the internal processes occurring in the water body is important. For example, it was proved that the presence of antibiotics can inhibit the processes of denitrification in a water body, which in turn causes a number of environmental problems [28]. Thus, both the increased content of nitrates in water resulting from the inhibition of their decomposition and the high content of antibiotic-resistant microorganisms may have a common cause, namely the intensive use of antibiotics.

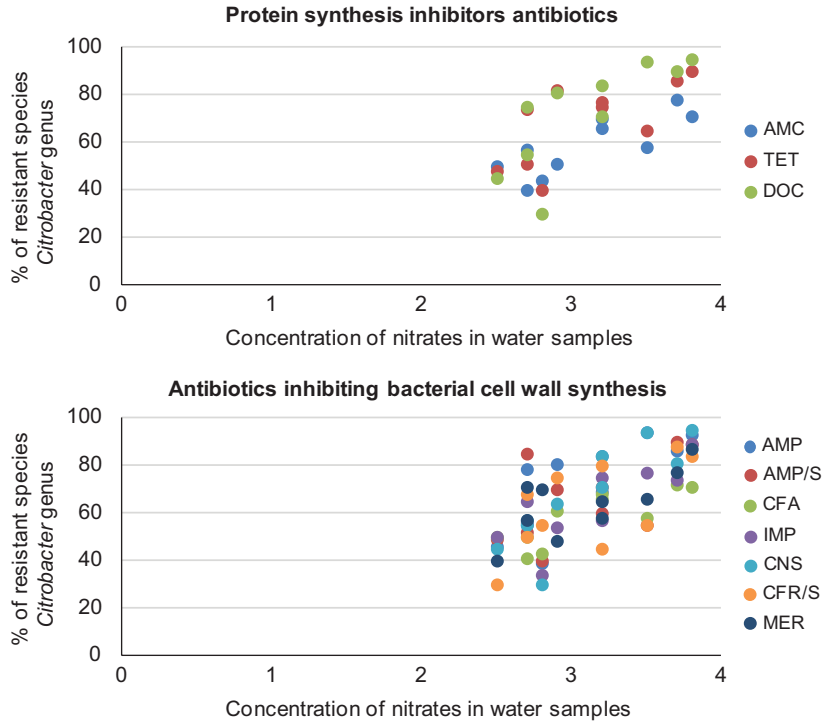

Fig. 4. Correlations between concentrations of nitrates compounds and the degree of antibiotic resistance of the strains of microorganisms isolated from the surface waters of the Uzh River in the agricultural area (n = 10; p<0.05). AMP – ampicillin; AMP/S – ampicillin/sulbactam; CNS – cefuroxime; CFR/S – cefoperazone/ sulbactam; MER – meropenem; IMP – imipenem; CFA – ceftriaxone; AMC – amikacin; DOC – doxycycline; TET – tetracycline

Рис. 4. Кореляційні взаємозв'язки між концентраціями нітратів і ступенем антибіотикорезистентності штамів мікроорганізмів, виділених із поверхневих вод річки Уж на аграрній території (n = 10; p<0,05). AMP – ампіцилін; AMP/S – ампіцилін/сульбактам; CNS – цефуроксим; CFR/S – цефоперазон/сульбактам; MER – меропенем; IMP – іміпенем; CFA – цефтріаксон; AMC – амікацин; DOC – доксицилін; TET – тетрациклін

The study has estabilised clear relations between the development of antibiotic resistance and the concentrations of metals and nitrogen compounds in the water body. Such dynamics can be a consequence of both common sources of pollution and the direct influence of pollutants on the development of mechanisms of resistance.

CONCLUSIONS

The mechanisms of the formation and spread of antibiotic resistance of microorganisms in the environment are important areas of research. A close correlation has been established between Zn ions concentrations and antibiotic resistance of bacteria in all research sites. However, the highest degree of correlation was observed in the area under conditions of technogenic transformation, where high concentrations of this element were recorded. Strong links between metals (Ni, Zn) and antibiotic resistance indicators were also found in the study area affected by urbanization. A close relation between the concentrations of nitrogen compounds, namely nitrates, and an increasing

antibiotic resistance in microorganisms is characteristic of the agricultural area. This might be the result of the excessive use of mineral fertilizers combined with the peculiarities of the relief of the area.

Apparently, the acquisition of antibiotic resistance in bacteria is a consequence of complex processes in the aquatic ecosystem. Therefore, the investigation of complex genetic and biochemical mechanisms that determine various factors of antibiotic resistance in microorganisms is necessary. It is essential to establish the dominant mechanisms of collective selection of bacterial antibiotic resistance, detect the concentrations of antibiotics that can affect biogeochemical processes, and continuously monitor the level of antibiotic-resistant microorganisms in aquatic ecosystems.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Animal Rights: This article does not contain any studies with animal subjects performed by the any of the authors.

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ВПЛИВ АНТРОПОГЕННОГО НАВАНТАЖЕННЯ НА РОЗВИТОК АНТИБІОТИКОРЕЗИСТЕНТНОСТІ МІКРООРГАНІЗМІВ РІЧКИ УЖ (УКРАЇНА)

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Вступ. Надмірне використання антибіотичних речовин у медицині та ветеринарії сприяє додатковому селекційному тиску на мікроорганізми та призводить до стрімкого поширення небезпечних резистентних мікроорганізмів, що мають підвищену здатність протистояти численним класам антибіотиків. Водні екосистеми є

одними з основних резервуарів генів стійкості, тому й мають підлягати обов'язковому контролю. Ймовірно, поширення антибіотикорезистентних мікроорганізмів залежить не лише від концентрації антибіотиків, що потрапляють у водойму зі стічними водами, але й від інших характеристик якості водного середовища. Метою роботи було визначення взаємозв'язку між вмістом важких металів (Cu, Ni, Zn, Cr) і сполук азоту (NO_3, NO_2 і NH_4) у воді та поширенням антибіотикорезистентності мікроорганізмів, на прикладі різних ділянок річки Уж (Україна), що мають особливості антропогенного навантаження. На основі проведеного нами посезонного моніторингу протягом 2016–2017 років, що включав визначення вмісту сполук азоту, важких металів і дослідження структури мікробіоценозу з подальшим визначенням чутливості до антибіотиків домінантних штамів у зразках води, проведено аналіз кореляційної залежності, між концентраціями цих речовин і рівнем поширення антибіотикостійких штамів, який дав змогу визначити ймовірні чинники сприяння розвитку антибіотикостійкості мікроорганізмів.

Матеріали і методи. Визначення зв'язку між хімічними показниками й відсотком антибіотикостійких мікроорганізмів проводили за допомогою лінійного коефіцієнта кореляції Пірсона (*r*). Статистичну обробку даних проводили з використанням пакету програм "Microsoft Exel". Статистично значущими вважали дані з достовірністю $p<0,05$.

Результати. Встановлено взаємозв'язок між рівнем антибіотикостійкості мікроорганізмів та хімічним забрудненням середовища. Для техногенно-трансформованої ділянки характерний посилений зв'язок між наднормовими концентраціями Zn і підвищеними відносно фонових концентрацій Ni та зростанням стійкості мікроорганізмів до антибіотиків. На урбанізованій території простежується зв'язок між зростанням резистентності та концентрацій Ni у досліджуваних зразках. Тоді як для місцевості, що підпадала під вплив сільськогосподарської діяльності, встановлено посилений зв'язок між сполуками азоту, а саме нітратами, та зростанням резистентності мікроорганізмів.

Висновки. Встановлено, що взаємозв'язок між концентраціями хімічних речовин у воді та посиленням антибіотикорезистентності мікроорганізмів може слугувати індикатором ступеня трансформації гідроекосистеми під впливом антропогенного навантаження. Це доводить необхідність запровадження більш комплексного контролю у водних екосистемах.

Ключові слова: важкі метали, сполуки азоту, антибіотики, водні екосистеми, мікроорганізми, антибіотикорезистентність

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