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EVALUATION OF THE GENOTOXIC POTENTIAL OF SURFACE WATER OF ANTHROPOGENICALLY LOADED AREAS USING EUKARYOTIC TEST OBJECTS (ON THE ZUBRA RIVER EXAMPLE, LVIV, UKRAINE)

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Background. Rivers are important water objects in many cities in Ukraine and around the world, and their hydroecological state causes concern. One of them is the Zubra River (Lviv, Ukraine), a right tributary of the Dniester which is subject to long-term anthropogenic load from sewage and household waste, and its hydroecological state is assessed as unsatisfactory (Ivanov *et al.*, 2023). The aim of the study was to evaluate the genotoxic potential of surface water in anthropogenically loaded areas on the example the Zubra River using *Allium cepa* and *Drosophila melanogaster* as eukaryotic test objects.

Materials and Methods. Surface water samples of the Zubra River were taken in different seasons of 2023 and their chemical composition was analyzed by standard methods. The impact of the water samples on the survival of *Drosophila melanogaster* laboratory strain *Oregon R* was assessed using the larval feeding method, while their genotoxic potential was evaluated through the dominant lethal mutation test. The phyto- and cytotoxicity of water samples were assessed using the *Allium cepa* test based on the growth and mitotic activity of onion roots of the *Stuttgarter Riesen* variety. The genotoxic potential was determined using the ana-telophase method by analyzing the frequency of chromosomal aberrations.

Results and Discussions. The chemical analysis revealed an increased concentrations of ammonium, nitrite and phosphate ions in surface water samples from the Zubra River indicating its Vth quality class (very poor) and an unsatisfactory hydro-ecological state. Treatment of *D. melanogaster* with river water samples reduced their survival rate by up to 77.66–78.97 % compared to the control, and significantly increased the frequency of the dominant lethal mutations by 1.54–3.44 times. Water samples significantly decreased the growth and mitotic activity of the *Allium cepa* roots as test objects, however no genotoxic effect was detected: the frequency of chromosomal mutations in the apical meristem was not statistically different from the control.

Conclusion. For an objective assessment of the genotoxic potential of surface water of rivers in anthropogenically loaded areas, it is recommended to apply plant and animal objects that differ in their test reactions. The study based on the example of the Zubra River showed that surface water samples with an elevated concentrations of ammonium ions, nitrites and phosphates do not cause a genotoxic effect in *A. cepa* as a plant object but show a weak genotoxic potential in *D. melanogaster* by inducing a significant increase in the frequency of dominant lethal mutations and a decrease in individual survival rates.

Keywords: water of the Zubra River, biotesting, chromosomal mutations, genotoxicity, *Drosophila melanogaster, Allium cepa*

INTRODUCTION

The problem of pollution of natural water bodies, especially in anthropogenically loaded areas, is becoming increasingly urgent (Tsvilynyuk *et al.*, 2017; Loboda *et al.*, 2023). Malfunction of wastewater treatment facilities, septic tanks and, sometimes, their complete absence lead to contamination of water bodies with industrial waste, household sewage, sewage effluents, various pesticides, household waste, microplastics (Loboda *et al.*, 2021; Ivanov *et al.*, 2023). The abovementioned pollutants include substances with toxic and genotoxic effects that are dangerous for hydrobiota and human health (Doleželová *et al.*, 2011; Tsvilynyuk et al., 2017). Detection of genotoxicants both by methods of chemical analysis and biological test objects is essential for the assessment of the ecological state of water bodies (Radić *et al.*, 2010; Krainiukova *et al*., 2021; Ali *et al*., 2022). In the context of this problem, the ecological condition of the Zubra River is especially important since it is the only river with an open channel that flows within the city of Lviv (Ukraine) and is used in its economic activities (Tsvilynyuk *et al.*, 2017). The hydro-ecological state of the river has been assessed as unsatisfactory due to the significant content of biogenic substances, particularly ammonium, nitrate and phosphate ions. It has been noted that the main source of pollution of the river is a significant amount of wastewater discharged from the residential district of Sykhiv as well as sewage and rainwater runoff (Ivanov *et al.*, 2023).

Monitoring of environmental objects is based on sanitary and ecological norms and includes toxicological, chemical and organoleptic characteristics (Arsan *et al.*, 2006). According to recommendations (World Health Organization, 1985) for reliable assessment of the ecological state of environmental objects, it is advisable to use at least two biological test systems to detect toxic and mutagenic effects of pollutants. Among the most effective ones is the *Allium*-test adapted to detecting toxic effects and assessing the potential genetic risks of pollutants in water bodies (Radić *et al.*, 2010; Tkachuk

et al., 2022). Another test object can be an animal organism, specifically *D. melanogaster*. Due to well-studied genetic control of many vital functions, *D. melanogaster* flies are used in toxicology, eco- and nanotoxicology as part of battery of tests to determine the actual and potential mutagenic activity of various factors (Chifiriuc *et al.,* 2016; Demir & Turna Demir*,* 2023).

The aim of this study was to evaluate the genotoxic potential of surface water of anthropogenically loaded areas (on the example of the Zubra River, Lviv) using *Allium cepa* and *Drosophila melanogaster* as eukaryotic test objects.

MATERIALS AND METHODS

Water samples of the Zubra River were collected in different seasons of 2023: in winter, spring and autumn at the same location (38 Khotkevycha St., near Shuvar Market, Lviv; geographic coordinates 49.795246,24.039640) according to recommendations (Arsan *et al.*, 2006). The water samples were immediately used for research. Distilled water was used in the control.

Chemical analysis of water samples. Determination of pH of water samples was carried out potentiometrically using a glass electrode (Ionometer-analyzer AI-123). The total hardness of water as well as sulfates was determined by titrimetry. Ammonium ions were determined by ion-selective potentiometry. Nitrite and nitrate ions were determined by the ion-potentiometric method. Phosphates were determined by the colourimetric method in the reaction with ammonium molybdate (Arsan *et al*., 2006). The water quality class was determined in accordance with the recommendations of the European Union Water Framework Directive (Directive 2000/60/EC) and the relevant categories given in the methodology for environmental assessment of surface water quality (Romanenko *et al*., 1998).

Treatment of *D. melanogaster* with water samples. *D. melanogaster* low-mutagenic strain *Oregon R* was used (Chifiriuc *et al*., 2016). The studied water samples in a volume of 5 mL were added directly to 40 mL of fresh typical medium (cooled to 40 °C) for larval feeding (Demir & Turna Demir*,* 2023). An equivalent volume of distilled water was used in the control. Flies (10♀ and 10 ♂) were placed in each test tube with the medium and kept at 25 °C. On the third day, the parents were removed. The number of imago that flew out was counted and their survival rate S_l (%) was determined as the ratio of the number of adults in experiment to the number of imago in the control.

The genotoxic potential (the property of chemicals to cause mutations; to detect it, DNA damage is analyzed in cells exposed to toxic substrate) was measured using the method of dominant lethal mutations (DLM-test). The latter one is aimed to detect the chromosomal aberrations that lead to the death of offspring in the early stages of embryonic development (Chifiriuc *et al*., 2023). In DLM-test, *D. melanogaster* flies were treated with the studied water samples by the method of larval feeding. The treated males were crossed with untreated virgin females, the total number of eggs laid was counted, including eggs with early embryonic death (EED) and late embryonic death (LED). The frequency of dominant lethal mutations was calculated and the obtained data were analyzed by the standard method of comparisons (Demir & Turna Demir, 2023). The mutagenicity coefficient (k) was determined as a ratio of the DLM frequency in the experiment to control.

***Allium cepa*-testing of water samples**. *A. cepa* bulbs of the *Stuttgarter Riesen* variety similar in size and weight were used in the test. The test reaction was the length of the roots of bulbs grown on the studied water samples. Twenty-five bulbs were placed in 10 mL tubes with the test water sample (distilled water in the control) and thermostated at 25 °C. On the fifth day the roots were cut off and their length (in mm) was measured (Radić *et al.*, 2010). The level of phytotoxicity (inhibition of plant growth or any adverse effect on plants caused by specific substances or growing conditions) of water samples was determined by the growth activity of the test object roots and evaluated on a five-point scale as described by Klepach *et al.* (2021).

The cytotoxicity (manifested in the inhibition of cell growth and division, apoptosis activation) of water samples in the *Allium* test was determined by the mitotic activity of the root apical meristem of the test object, which was germinated for 48 hours at 25 °C (distilled water served as the control). Cytopreparations from the root meristem were prepared, the number of cells at different stages of mitosis (interphase, metaphase, anaphase and telophase), their total number, mitotic index ($MI_{\%}$), and the level of cytotoxicity were calculated as described (Klepach *et al.*, 2021). The mitotic activity of the root meristems in the experiment was expressed as a ratio of mitotic indexes in the experiment to the control (Klepach *et al.*, 2021).

The genotoxicity of water samples in *A. cepa*-testing was assessed by the frequency of chromosomal mutations in the root meristems of bulbs according to the method (Radić *et al.*, 2010). At least 500 ana- and telophases on cytopreparations were analyzed, among which cells with various types of aberrations were noted, and the frequency of chromosomal mutations was calculated as described by H. Klepach *et al.* (2021). Each assay was repeated three times.

Statistical analysis. The data of water samples chemical parameters, root length and mitotic index of the test object were compared using analysis of variance (ANOVA) to confirm the variability of the data and the reliability of the results. The data were expressed as M ± SE (mean ± standard error of the mean). P-value was assessed by Student's *t*-test to determine a significant difference between the variants in *DLM* and *Allium cepa* testing. Differences between the respective control and the experimental data were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The analysis of the chemical composition of surface water samples from the Zubra River in different seasons of the year (see **Table 1**) demonstrates a high content of biogenic substances: ammonium and nitrite ions as well as phosphates that is consistent with literature data (Ivanov *et al.*, 2023). In particular, the content of ammonium ions ranged from 4.80 to 5.90 mg/L (quality class V, very poor), the content of nitrites – 0.96–3.57 mg/L (quality class V, very poor). Notably, the content of sulfate ions was 1.2 times higher than MPC (Maximum Permission Concentration) only in the spring water sample. According to the results (see **Table** 1) and the established categories for water quality assessment (Romanenko *et al.*, 1998), the surface water samples of the Zubra River in 2023 refer to the Vth quality class (very poor).

The effects water samples from the Zubra River collected in different seasons on the *D. melanogaster* survival were assessed. According to the values presented in **Table 2,** imago survival rates in all experimental variants were significantly lower ($p \leq 0.05$) compared to the control indicating the toxicity of water samples.

Table 1. **Chemical composition of surface water samples from the Zubra River collected in different seasons**

Indicators	MPC, mg/L	Samples of water		
		winter	spring	autumn
pH	6.5–8.5	7.3±0.04	7.02±0.04	7.9±0.04
Total hardness, mmol/L	none	2.6±0.05	2.8±0.05	2.9±0.05
SO_4^{2-} (sulphates), mg/L	≤100.0	49.6±3.09	127±10	51.0±2.10
NO_3^- (nitrates), mg/L	≤40.0	16.5±0.11	14.39±0.03	14.8±0.09
NO_2^- (nitrites), mg/L	none	0.96±0.04	1.13±0.06	3.57±0.02
NH_4^+ (ammonium), mg/L	≤0.5	5.1±0.33	4.8±0.31	5.9±0.35
PO_4^{3-} (phosphates), mg/L	≤0.7	2.73±0.15	2.9±0.30	2.98±0.17

Table 2. **Imago survival rate and the frequency of DLM in *Drosophila melanogaster Oregon R* strain under the influence of the Zubra River water samples collected in different seasons**

Experimental variants	Survival rate, %	The total frequency of DLM, %	The frequency of EED, %	The frequency of LED, %
Control	100	2.08±0.10	1.10±0.05	0.98±0.04
On winter water sample				
Experiment 1	78.97*	4.98±0.25*	3.11±0.15*	1.87±0.09*
†k	-	2.39	2.83	1.91
On spring water sample				
Experiment 2	72.66	7.84±0.39*	2.81±0.14*	5.03±0.24*
†k	-	3.77	2.55	5.13
On autumn water sample				
Experiment 3	75.66*	4.81±0.24	2.99±0.15*	1.82±0.09*
†k	-	2.31	2.72	1.86

Note: * – differences are significant in relation to the control, p ≤0.05. These differences were determined by one-way ANOVA and Student's *t*-test; †k (mutagenicity coefficient)

At the second stage of biotesting the genotoxic potential of water samples was investigated in the DLM test. According to the data (see **Table 2**), the total frequency of DLM in embryos was significantly higher (p ≤0.05) compared to the control in all experimental variants. The highest frequency of DLM was noted under the influence of the spring water sample (7.84±0.39 %), which is 3.77 times higher than the control. Moreover, this sample induced a more intense increase in the frequency of DLM than winter and autumn samples. The latter ones induced DLM 2.39 and 2.31 times more frequently compared to the control. The increase in the total frequency of DLM under the influence of these samples was due to the frequency of EED rather than LED. Such differences in the results of the studies can be explained by the unequal intake of pollutants in different seasons of the

year among which there are biogenic compounds (see **Table 1**) known for their toxicity (Doleželová *et al.*, 2011). In view of their presence in concentrations higher than the MPC as well as the cumulative effect, it is possible to explain the increase in the level of DLM at different stages of embryonic development of *D. melanogaster*. Mutagenicity coefficients (k) indicate (see **Table 2**) a weak mutagenic potential of the surface water of the Zubra River in spring (k = 3.77) and very weak – in winter (k = 2.39) and autumn (k = 2.31) periods.

The results of water samples phytotoxicity measuring demonstrate (see **Table 3**) a significant decrease in the length of the test object roots in all experimental variants (by up to 74.89–89.55 %) compared to the control. The phytotoxicity indices indicate an average level of phytotoxicity of the autumn sample (IP 25.11 %) and a weak level of winter (IP 10.45 %) and spring (IP 15.77 %) water samples.

Table 3. **Growth and mitotic activity, the frequency of chromosomal aberrations in the meristem of the roots of *Allium cepa* bulbs grown on water samples from the Zubra River collected in different seasons and their phyto- and cytotoxicity levels**

Experimental variant	Water samples of different seasons		
	winter	spring	autumn
Length of the roots, mm			
Control	19.91±0.74	16.74±0.67	19.87±0.74
Water samples	17.83±0.65*	14.10±0.76*	14.88±0.58*
Growth activity of the roots, %			
Water samples	89.55 %	84.23 %	74.89 %
Indices ($IP_{\%}$) and levels of phytotoxicity			
Water samples	10.45 %	15.77 %	25.11 %
	weak	weak	medium
Mitotic indices of the meristem, %			
Control	67.50±3.48	67.80±3.37	45.69±1.97
Water samples	75.30±3.83*	80.76±4.09*	61.11±3.63*
Mitotic activity of root meristem compared to the control, %			
Water samples	89.64*	83.95*	74.77*
Indices ($IC_{\%}$) and levels of cytotoxicity			
Water samples	10.36	16.05	25.23
	weak	weak	medium
Frequency of chromosomal aberrations, %			
Water samples	8.0±0.89‡	2.7±0.47‡	5.2±0.46‡
Control	5.08±0.98	1.92±0.22	4.3±0.67
k†	1.57	1.41	1.21

Note: * – differences are significant in relation to the control, p ≤0.05; ‡ – differences are not significant in relation to the control, p ≥0.05. These differences were determined by one-way ANOVA and Student's *t*-test; †k – mutagenicity coefficient

The results of water samples cytotoxicity measuring demonstrate (see **Table 3**) that the autumn sample causes a significant decrease in the meristem $IM_{\%}$ and suppresses its mitotic activity by up to 74.77 %, which indicates an average level of its cytotoxicity (cytotoxicity index ($IC_{\%}$) 25.23). Winter and spring water samples also significantly reduced the mitotic activity of roots by up to 89.64 % and 83.95 % respectively compared to the control, indicating a low level of their cytotoxicity ($IC_{\%}$ 10.36 and 16.05, respectively). Thus, the results of the *Allium* test indicate that in different seasons of the year the surface water of the Zubra River may have medium (autumn) or weak (spring and winter) cytotoxicity due to different content of pollutants.

The genotoxic potential of water samples from the Zubra River was studied by ana-telophase method. This method allows detecting chromosomal mutations such as deletions and translocations at the anaphase and telophase stages of mitosis (Ali *et al*., 2022). The results show (see **Table 3**) that the frequency of chromosomal aberrations in all experimental variants does not differ significantly ($p \geq 0.05$) from the control, which indicates the absence of genotoxic potential of multi-seasonal samples of surface waters of the Zubra River.

CONCLUSION

Biotesting of surface water samples collected in different seasons from anthropogenically loaded territory (on the example of the Zubra Rive) with two eukaryotic test systems was carried out. *A. cepa* tests revealed weak and medium phytotoxicity of the studied samples, low and moderate cytotoxicity and no genotoxic effect. However, the latter one was detected in the testing on *D. melanogaster*. The results point to the importance of using at least two test-systems for an objective assessment of the state of surface water in anthropogenically loaded areas.

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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.

AUTHOR CONTRIBUTIONS

Investigation, [H.N.; K.H.; M.K.; S.H.]; visualization, [H.N.; S.H.]; validation, [H.N.; M.K.]; software, [H.N.; K.H.; K.H]; writing original draft, [H.N.; K.H.; K.H], conceptualization, [H.N.; K.H]; data curation, [H.N.; K.H]; writing – writing – review & editing, [K.H.; K.H]; project administration [H.N.; K.H.].

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АНАЛІЗ ГЕНОТОКСИЧНОГО ПОТЕНЦІАЛУ ПОВЕРХНЕВОЇ ВОДИ АНТРОПОГЕННО НАВАНТАЖЕНИХ ТЕРИТОРІЙ (НА ПРИКЛАДІ РІЧКИ ЗУБРА, м. ЛЬВІВ, УКРАЇНА) ЗА ВИКОРИСТАННЯ ЕУКАРІОТИЧНИХ ТЕСТ-ОБ'ЄКТІВ

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Обґрунтування. Важливими водними об'єктами багатьох міст України та світу є річки, гідроекологічний стан яких викликає занепокоєння. До їхнього числа належить річка Зубра (м. Львів, Україна) – права притока Дністра, яка зазнає тривалого антропогенного навантаження каналізаційними стоками та побутовими відходами, а її гідроекологічний стан оцінено як незадовільний (Ivanov *et al.*, 2023). Метою дослідження є оцінити генотоксичний потенціал поверхневої води антропогенно навантажених територій на прикладі річки Зубра за використання *Allium cepa* та *Drosophila melanogaster* як еукаріотичних тест-об'єктів.

Матеріали та методи. Зразки поверхневої води річки Зубра відбирали у різні сезони 2023 року й аналізували їхній хімічний склад стандартними методами. Визначали вплив зразків води на виживаність особин лабораторної лінії *Oregon R D. melanogaster* методом личинкового згодовування, а генотоксичний потенціал – у тесті на домінантні летальні мутації. Фіто- і цитотоксичність зразків води оцінювали у *Allium cepa*-тесті за ростовою й мітотичною активністю корінців цибулі сорту *Stuttgarter Riesen*, а генотоксичний потенціал – в ана-телофазному методі за частотою хромосомних аберацій.

Результати і їхнє обговорення. Хімічний аналіз зразків води виявив підвищені концентрації іонів амонію, нітритів і фосфатів, що свідчило про V клас якості (дуже поганий) та незадовільний гідроекологічний стан р. Зубра. Обробка особин *D. melanogaster* цими зразками знижувала їхню виживаність до 72,66–78,97 %,

порівняно з контролем, і достовірне зростання в 1,54–3,44 рази частоти домінантних летальних мутацій. За їхнього впливу фіксували достовірне зниження ростової й мітотичної активності корінців *A. cepa*, однак генотоксичного впливу виявлено не було: частота хромосомних мутацій у апікальній меристемі тест-об'єкта була близькою до контролю.

Висновки. Для об'єктивної оцінки генотоксичного потенціалу поверхневої води річок антропогенно навантажених територій доцільно застосовувати рослинні та тваринні об'єкти, які різняться тест-реакціями. На прикладі річки Зубра показано, що зразки поверхневої води, маючи підвищений вміст іонів амонію, нітритів і фосфатів, не спричиняють генотоксичного ефекту стосовно *A. cepa* як рослинного тест-об'єкта, однак виявляють слабкий генотоксичний потенціал стосовно мух *D. melanogaster*, індукуючи у них достовірне зростання частоти домінантних летальних мутацій і зниження виживаності особин.

Ключові слова: вода річки Зубра, біотестування, хромосомні мутації, генотоксичність, *Drosophila melanogaster*, *Allium cepa*

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