Biol. Stud. 2022; 16(4): 33–44 • DOI: https://doi.org/10.30970/sbi.1604.694
www.http://publications.lnu.edu.ua/journals/index.php/biology

UDC: 502/504:57(477.81) 577.47: 504.054

ACCUMULATION OF MICROPLASTICS IN THE BIVALVE MOLLUSC *UNIO TUMIDUS* UNDER EXPERIMENTAL AND FIELD EXPOSURES

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Martyniuk, V. V. (2022). Accumulation of microplastics in the bivalve mollusc *Unio tumidus* under experimental and field exposures. *Studia Biologica*, 16(4): 33–44. doi:10.30970/sbi.1604.694

Background. An increased production and widespread use of plastics have made microplastic (MP) pollution a serious environmental problem. Most of MP found in the marine environment comes from rivers, however, the freshwater pollution by MP is less studied. Filter-feeding organisms, like bivalve molluscs, are the primary target organisms for MP. Nevertheless, the studies of MP accumulation in the bivalves mainly focus on the marine species and depend on expensive equipment. The goal of this study was to detect the presence of MP in the body of freshwater bivalve mollusc *Unio tumidus* from a typical field site in Western Ukraine and under laboratory sub-chronic exposure to microplastic at a typical concentration for freshwater.

Materials and Methods. For the study, we exposed molluscs to waterborne MP (0.1–0.5 mm) in the concentration of 1.0 mg L^{-1} corresponding to ~850 items L^{-1} for 14 days and analysed the concentration of MP in the soft tissues and water every two days. The molluscs and water from the field site, confirmed as polluted one, were also analysed. To estimate the number of MP particles, we used a modified method based on the cleavage of biological materials with potassium hydroxide and hydrogen peroxide and microscopic analysis of MP after the staining with fluorescent Nile Red dye.

Results. The MP concentration in the soft tissues of the specimens from the reference site was 9.5 items per soft body and demonstrated a bell-shaped response curve throughout the 14-days exposure with maximum of 327.0 items per body on the 10th day and a negative correlation with the concentration of MP in the experimental tank that changed within the range of 590–790 items L^{-1}. The level of MP in the field specimens from the polluted area was 76.5 items per body, and in the water, it was about

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103 items L^{-1}. The maximum MP abundance factor, calculated as $CF_i = C_i/C_0$ (utilising the value 9.5 as C_0), was 83.18 and 8.05 for 10 days of exposure and field specimens, correspondingly.

Conclusion. These data indicate the high accumulative ability of *U. tumidus* towards microplastics and draw attention to the utilizing of this species for the biomonitoring of microplastics pollution and depuration of surface waters from it.

Keywords: microplastics, *Unio tumidus*, accumulation, microscopy, Nile Red

INTRODUCTION

The consumption of plastic materials worldwide is huge and constantly growing because of their versatility and durability due to their high chemical stability and low degradability (Al Rayaan, 2021; Yeung *et al*., 2021). Single-use plastic is one of the most persistent pollutants in modern society (Yeung *et al*., 2021). It is estimated that almost 60 % of solid plastic waste is disposed of in the environment or landfills worldwide due to its large production and consumption in various uses such as packaging (Zhang & Han, 2019), construction (Far & Nejadi, 2021), electrical and electronics, agriculture and healthcare.

Microplastics (MP) are plastic particles less than 5 mm in size that are formed after large plastic wastes enter the aquatic environment and are destroyed by environmental exposure, physical destruction and biodegradation (Moore, 2008; Cole *et al*., 2016). According to various estimates, annually 4.8–12.7 million tons of MP enter the World ocean, of which about 5 trillion are on the surface. MP occurs in almost all marine and freshwater environments, as well as in protected and remote areas (Eriksen *et al*., 2014), which makes its potentially detrimental effect a global problem. Importantly, 70–80 % of the total amount of MP found in the marine environment enter the environment from freshwater rivers (Desforges *et al*., 2014), however, freshwater pollution by MP has been studied to a lesser extent. Besides its own expected toxicity, MP can serve as a substrate for environmental toxins diluted in water (Nolte *et al*., 2017; Guilhermino *et al*., 2018), such as pesticides, persistent organic pollutants (POPs) and pollutants from domestic sewage (Rochman *et al*., 2015).

Bivalves as filters have highly developed processes of intracellular internalization of microparticles (Wesch *et al*., 2016). The level of MP in wild or cultured marine molluscs ranges from 0.9 to 10.5 particles per gram (Davidson & Dudas, 2016). The particles mainly accumulate in the digestive glands and gills (Pedersen *et al*., 2020). However, a significant amount excreted *in vivo* in the form of pseudofaeces can be identified in the gills, but some particles remain in the digestive tract (Fernández & Albentosa, 2019).

Consequently, filter-fed organisms, like bivalve molluscs, are the primary target organisms for MP and can be promising bioindicators for MP pollution. However, the studies of its accumulation in the bivalves mainly focus on the marine species and depend on expensive equipment which complicates the process of biomonitoring on a regular basis. To our knowledge, there is no available information concerning the MP level in the freshwater bodies and in molluscs in Ukraine.

The goal of this study was to elucidate the ability of freshwater molluscs *Unio tumidus* to accumulate MP in their body under conditions of the environmentally relevant exposure.

MATERIALS AND METHODS

The MP was prepared from the Polyethylene terephthalate (PET) bottles by milling with stainless steel cutter. The obtained powder of PET particles was then sieved using two laboratory sieves, pore size 0.1 and 0.5 mm. No surfactant was used to avoid any potential toxic effect of the substance used and potential change in the behavior of MP. A manual vigorous mixing was thus applied to the stock suspension before adding the final aliquot to the aquariums. An aliquot of the suspension was added to aquariums to obtain the MP concentration of 1.0 mg L^{-1} that corresponded to ~850 items L^{-1}. This concentration and the size of MP particles corresponded to the typical MP characteristics that enter primary freshwater ecosystems through surface runoff or wastewater treatment plants (Lasee *et al*., 2017). The average MP concentrations in samples collected from the lakes in urban areas can reach up to 5.51 mg·L^{-1} (Lasee *et al*., 2017).

For the exposure, the specimens of *Unio tumidus* (~6 years old, 8.5–9.9 cm length, and 67–80 g weight (**Table 1**) were collected at the end of summer (August) from the site located on the Sluch River (the Pripyat basin in the upper stream) that can be qualified as relatively pristine site due to its location in the upstream part of the river (the village of Chervony Sluch with the population of about 100 people, (49°41′53″ N, 26°20′45″ E). No industrial or household pollution was expected due to the small drainage area on this territory. Previous field studies of *U. tumidus* in this area confirmed it as a reference site (Gnatyshyna *et al*., 2020; Khoma *et al*., 2020 Martyniuk *et al*., 2022).

For the study of MP accumulation in molluscs in the field experiment, a typical polluted area was selected. It was the site in the middle portion of the Nichlava River, the Dniester River basin (the village of Chornokintcy with the population of about 1000 people, 48°58′12″ N, 26°01′48″ E), which receives its waters from a relatively big drainage area where the purification plants are absent and municipal pollution was expected. Generally, this river is the most contaminated Dniester tributary in the middle stream according to the data of the regional office of the State *Water Resources* Agency (https://www.vodgosp.te.ua/water-resources.html). Our previous studies of field populations of *U. tumidus* confirmed the chronical impact of pollution in this site (Martinyuk *et al*., 2022).

The sampling of molluscs and water (in the polluted site) was carried out simultaneously. The individuals from each site were transported to the laboratory in 25 L cages with aerated native water. The molluscs from the contaminated site were examined on the day of sampling, and the specimens from the reference site were depurated for 21 days in 80 L tanks in the aerated, dechlorinated, softened tap water (18±1 °C, pH 7.3±0.2, $CaCO_3$ level 86.8±1.0 mg·L^{-1}, dissolved oxygen level 8.67±0.51 mg·L^{-1}, ammonia (NH_3/NH_4^+) and nitrite levels below 0.1 mg·L^{-1}) and fed with 500 mg of Tropical SuperVit Basic containing beta-1.3/1.6-glucan twice a week.

After the period of depuration, 60 specimens were placed in a tank with the tap water that contained MP in the concentration of 1.0 mg L^{-1} which corresponded to ~850 items L^{-1}. The specimens were collected, and water was replaced every 2 days with MP content being renewed each time. The duration of exposure was 14 days.

For the analysis, molluscs were immediately dissected on ice. In each group individual's length, total soft body weight and Condition index (CI) of soft tissues was calculated as the ratio: (drained mass of soft tissues/length^3). The specimens were examined for the presence of parasites and sex under a light microscope. Only parasite-free molluscs were used for the investigation. For MP analysis, common tissues samples were prepared individually from four molluscs in each period of exposure and frozen (-40 °C) until analyses. The amount of MP in the water was calculated after its filtration and staining of the existing particles. All subsequent steps were perfomed under a fume hood, and all glassware and laboratory instruments were washed three times with distilled water before use.

To estimate the number of particles in each individual tissue sample, we used the potassium hydroxide/hydrogen peroxide alkali- digestion method based on fat saponification and tissue break down (Hurt *et al.*, 2020). We added 10 mL of 1 M KOH and 5 mL of sodium dodecyl sulfate (0.5 % wt./Vol. (approximately 5 g/L)) per g of mollusc tissue in a beaker of the required volume and covered it with aluminum foil. The beaker was placed in a water bath for at least 24 hours at a temperature of 50 °C, during which time the beaker was gently shaken several times (the tissue dissolves faster when stirred). After 24 hours of incubation, the resulting mixture was filtered through a membrane filter, 8–12 μm pore size. The filters were then placed back in the original peroxide oxidation beaker according to the standard procedure (Masura *et al.*, 2015) to digest any residual organic material. The total number of particles collected on the filter from the individual sample of tissue was calculated after staining.

The water samples were filtered without previous digestion. The volume of water for analysis was 1 L.

Staining of particles. Nile Red (NR) (9-diethylamino-5H-genso [alpha] phenoxazin-5-one) is the most promising dye for MP due to its high adsorption and fluorescence intensity, short incubation time, and good affinity for a number of polymers (Maes *et al.*, 2017; Prata *et al.*, 2019). The filters with MP particles obtained from the tissues and from the samples of water were stained with Nile Red at a concentration of 10 μg/mL, utilizing 10% dimethyl sulfoxide (DMSO) as a solvent (Kaile *et al.*, 2020). Fluorescent particles were visible under illumination. We divided the filter into four sections and calculated manually the number of all particles on the filter under a Mikmed 5 optical microscope (with additional UV (395 nm) illumination from an YATO YT-08582 lamp (40× magnification). Almost all detected MP particles were stained, so the number of misidentified particles was low. Metallographic microscope identification of MP was used to confirm this detection METAM P-1 (magnification 125×).

Statistical analysis. For all traits, the sample size was four. The analysis of each sample was conducted in two replicates. Results were expressed as mean ± SD. Data were analyzed with parametric Student's *t*-test significant at $p < 0.05$. Pearson correlation analysis was perfomed to analyze the strength and direction of linear relationship between two continuous variables. Correlation was significant at $p < 0.05$ level ($r > 0.707$) and $p < 0.001$ ($r > 0.925$) (2-tailed), n = 4. The IBM SPSS Statistics version 24 software for Windows were used for calculations.

RESULTS AND DISCUSSION

The morphological analysis of molluscs (**Table 1**) indicated the similarity of the specimens from the polluted field site and depurated specimens. Under laboratory exposure the decrease in the length of molluscs on the 8th–12th days and the reduction in their mass on the 10th–12th days were detected with the recovery by the 14th day as compared with the starting day of exposure (day 0). The CI value was significantly higher compared with control on the 8th and 12th days of exposure.

Table 1. **Morphological characteristics of molluscs exposed to MP in the laboratory and field conditions, M±SD, N = 4**

Days of exposure	Length, cm	Mass of soft tissues, g	CI, %
Experimental exposure			
0 day (control)	9.63±0.22	26.98±0.82	3,03±0.14
2 days	9.73±0.22	26.30±2.05	2.86±0.17
4 days	9.85±0.26	25.78±0,88	2.71±0.29
6 days	9.65±0.24	25.55±1.80	2.85±0.21
8 days	9.08±0.13*	26.83±0.31	3.59±0.12*
10 days	8.60±0.18*	20.93±0.41*	3.29±0.16
12 days	8.58±0.17*	21.33±0.88*	3.38±0.08*
14 days	9.55±0.21	26.35±0.71	3.04±0.28
Field exposure			
r Nichlava	9.65±0.24	26.28±0.84	2.93±0.16

Comment: * – the difference compared with day 0 is significant, P <0.05

The validity of MP examining in the water and tissues was indicated by the comparison of two modes of detection (**Fig. 1**). The quantitative results are represented in **Table 2**.

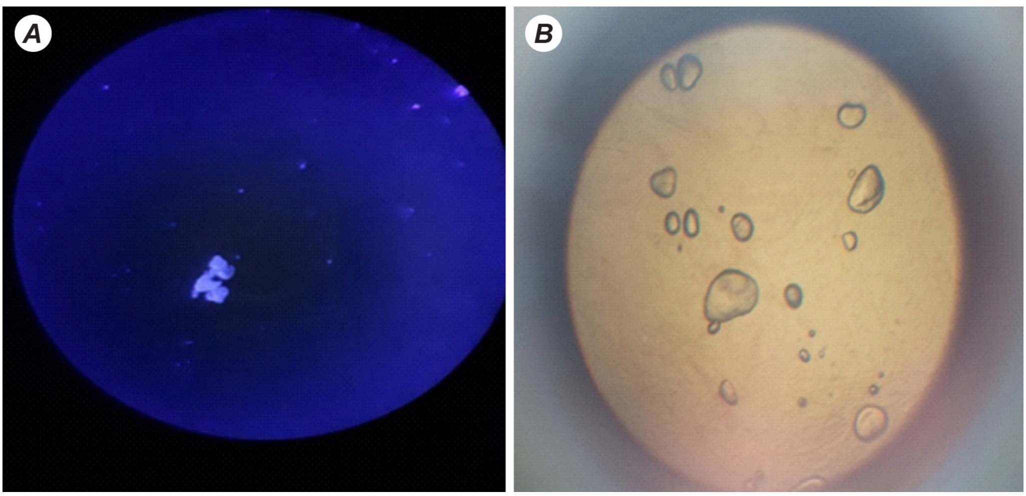

Fig. 1. Detection of microplastics in the experimental environment (1 mg L^{-1}), ***A*** – observed under an optical microscope with a fluorescent lamp (magnification 40×), plastic particles stained with Nile Red are visible as a bright structure; ***B*** – plastic particles under a metallographic microscope (at 125× magnification)

During the exposure, the number of MP particles in the tissues of molluscs and in the water changed simultaneously with the opposite regularity (**Table 2**, **Fig. 2**). The number of particles in the tissues was negligible on day 0 (without the addition of MP) and increased sharply by the 2-nd day. Generally, during the 14 days of exposure, a bell-shaped response curve for MP accumulation with the maximum correspondent to 15.6 items g^{-1} FW on the 10th day was indicated. The maximum accumulation of MP relating to the length of molluscs was also detected on days 8–10 compared with the start of exposure, and it remained significantly elevated on the 14th day.

The number of particles in the water changed throughout the 2th–14th days of exposure in the range of 590–790 items L^{-1}, decreasing from the highest level on the 2nd day to the lowest level on the 10th day of exposure by 1.3 times, but increased again by the 14th day. Correspondingly, the most prominent accumulation of MP from water was detected on the 10th day of exposure. The dynamics of the accumulation of MP in the soft tissues (in relation to total in tissues) and in the water had significant negative correlation (r = -0.799, P <0.05).

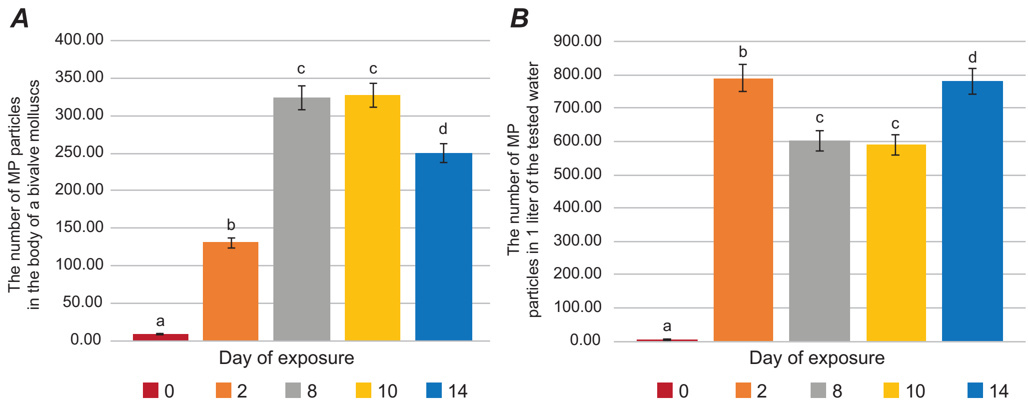

Fig. 2. The total number of MP particles in the tissues of molluscs (***A***) and in the water (***B***) under the experimental exposure to 1 mg MP L^{-1} during 14 days. Data are presented as means ± SD (N = 4). Different letters above the columns indicate significant differences between the days of exposure (P <0.05)

The MP concentration in the soft tissues of field specimens from the polluted area was higher than in the depurated molluscs, and the level of MP in the river water was about 103 items L^{-1} (**Table 2**). When we compared the ability of molluscs to accumulate MP from water (number of items per g tissues/number of paticles per L of water), its higher effectivity was indicated for the field group (~75 %). In the short experimental exposure of the molluscs from the reference site, this function was also rather high (~55 % after 10 days of exposure). Moreover, the depurated molluscs from the reference area were not cleaned totally from MP and showed the presence of these particles in the body on day 0 of exposure despite their almost total absence from water.

When we compared our data with the available information, that is mostly devoted to the marine species, the detected ability of molluscs to accumulate MP was rather high and was correspondent to or even higher than the level of MP in the tissues of mollusc in the maximally polluted marine field site (11.0±8.2 items g^{-1}) detected in Iran among 22 analyzed countries (cited from Ding *et al*., 2022).

Overall, the comparison of the field and exposed molluscs has shown the remarkable potential of molluscs to accumulate MP in comparison with the environmentally relative conditions. Importantly, Davidson and Dudas (2016) did not find any important differences in MP consumption between wild and cultured bivalves. Consequently, our results offer a perspective on utilizing of *U. tumidus* introduced in cages into the polluted areas (like waster purification plants) for the indication of pollution and cleaning of water from MP.

Table 2. **The number of MP particles in the mollusc organisms and in the experimental medium M±SD, N = 4**

Days of exposure	Items per soft body	Items g^{-1} FW	Items per length (cm^{-1})	Items in water L^{-1}	Rate of MP bioaccumulation (items per soft tissue/ items L^{-1} water), %
Experimental exposure					
0 day (control)	9.50±0.58 [a]	0.35±0.03[a]	0.99±0.06[a]	5.25±0.96[a]	184.58±27.67[a]
2 days	130.50±13.53[b]	5.01±0.86[b]	13.42±1.44[b]	790.75±4.57[b]	16.51±1.74[b]
4 days	208.25±16.21[c]	8.09±0.80[c]	21.14±1.50[c]	670.75±4.35 [c]	31.05±2.42[c]
6 days	265.0±41.43[d]	10.36±1.28[d]	27.40±3.63[d]	630.50 ±4.20[d]	42.04±6.61[d]
8 days	323.75±9.43[e]	12.07±0.27[f]	35.67 ±0.78[g]	600.75±8.30[e]	53.89 ±1.56[e]
10 days	327.0±20.46[e]	15.62±0.68[e]	38.00±1.68[e]	590.25±6.55[e]	55.40±3.42[e]
12 days	270.0±9.97[f]	12.68±0.72[f]	31.50±1.49[f]	661.25±8,54[c]	40.84±1.60[f]
14 days	250.75±25.41[g]	9.52±1.01[d]	26.27±2.84[d]	779.50±3.42[f]	32.17±3.28[c]
Field exposure					
r Nichlava	76.50±4.04	2.91±0.10	7.92±0.25	103.00±0.82	74.30±4.45

Comment: In each column, different letters indicate significantly different values between the days of exposure ($P < 0.05$)

To compare the obtained results with the data of other authors, we utilized the microplastic abundance factor (CF_i) (Ding *et al*., 2022). It is calculated as $CF_i = C_i/C_{0;}$ which is the quotient of MP abundance in molluscs (C_i) to the minimal value (C_0). The value of ideal C_0 was recommended as 0.040 items/g) (Ding *et al*., 2022). In our study, utilizing the value 9.5 as C_0, CF_i was 83.18 and 8.05 for 10 days of exposure and field specimens correspondingly. Another recommended index is the pollution load index (*PLI*), calculated as √CF, (Ding *et al*., 2022). *PLI* is regarded as a standardized rule for monitoring the degree of pollution between different areas. In the field studies it was indicated that bivalve molluscs from Iran contained the highest amount of microplastics ($PLI_{country}$ = 9.6), followed by Greece (9.5), China (6.5), the UK (5.9), India (5.6), Tunisia (5.5), Norway (4.9), France (3.2), Thailand (3.0), and Germany (3.0) (Ding et al., 2022). The corresponding *PLI* in our study was 7.0 and 2.2 for 10 days of exposure and field specimens correspondingly, compared to C_0 detected in our experiment.

In any case, the values of the accumulation of MP in the soft tissues of molluscs examined here were correspondent to those reported in the reference data or even higher (in comparison with the marine areas) (**Table 3**). The decrease in MP accumulation after the 10-th day of exposure can be explained by the adjusted equilibria between ingestion and elimination of particles. For example, the exposure of bivalve *Anodontites trapesialis* to MP of 55–100 µm in size (75 mg L^{-1} during 3–192 h) had shown that both accumulation and elimination of MP was at a rather high levels of 78 % in gill tissues, and 90 % in the eliminated material (Moreschi *et al*., 2020). Nevertheless, the number of items in the tissues of molluscs remained rather high.

Table 3. **Occurence of microplastic in the field according to the reference literature**

Geographical regions	Sample phylum/ class	Microplastic accumulation	Reference
England	Mussel	12.6 items/individual	Catarino *et al*., 2017
China	Bivalve molluscs	4.3–57.2 items/individual, 2.1–10.5 items/g	Li *et al*., 2015
Korean waters	Bivalve molluscs	2.19±1.20 items/individual	Cho *et al*., 2021
San Francisco Bay	Bivalve molluscs	3.0±2.4 items/individual	Klasios *et al*., 2021
Portugal lagoons	Bivalve molluscs	18.4±21.9 MP items/g	Cozzolino *et al*., 2021
Middle-Lower Yangtze River Basin	Mussel	from 0.3–4.9 items/g	Su *et al*., 2018
Coasts of Asia	Mussel	0–10.5 MPs/g	Danopoulos *et al.,* 2020

Concerning the consequences of MP accumulation for the mollusc health status, it was shown that the physical ingestion of microplastic by organism leads to blockage of the intestinal tract, inhibition of gastric enzyme secretion, reduction in feeding stimuli, decrease in steroid hormone levels, delay in ovulation and lack of reproduction. Notable histological changes and a strong inflammatory response were observed in molluscs after the exposures to extremely high concentrations of MP particles, for example, in the exposures to 2.5 g L^{-1} (Von Moos *et al*., 2012) or 0.1 g L^{-1} (Wegner *et al*., 2012). In the present study, even the comparatively low concentration of MP, 1.0 mg L^{-1}, caused the morphological signs of toxicity, namely an increase in morphological index CI with the relevance to the period of the highest MP accumulation. However, it was shown that the specific effect of MP in a single exposure to the applied concentration was almost negligible after 14 days of exposure, particularly, in the adapted to pollution population from the Nichlava River (Martinyuk *et al*., 2022).

CONCLUSION

To summarize, the comparison of our own and reference data has proven that *U. tumidus* has rather high ability to accumulate MP. Based on our results, we can recommend utlising molluscs for the depuration of the waterbodies from MP without substantial impact on their health. Hence, our results demonstrate that the freshwater bivalve can be an effective bioindicator and accumulator of MP in the polluted environment. The next steps of our study will be the comparison of the accumulative ability of *U. tumidus* to MP of smaller size and the experiments with transplanted molluscs to study their ability to purify polluted aquatic bodies.

ACKNOWLEDGMENT

This work has been granted by the Ministry of Education and Science of Ukraine to Oksana Stoliar (Projects No M-70/2021 and No M-84/2021 under the Lithuanian-Ukrainian and French-Ukrainian Cooperation Programs). The author is grateful to PhD supervisor Prof. Oksana Stoliar for her valuable comments, Dr. Vitaliy Mocharsky from the Ternopil Ivan Puluj National Technical University for the assistance in the metallographic microscopy and post-graduate student Kateryna Yunko for the technical assistance.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The author declares 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 author.

AUTHOR CONTRIBUTIONS

The author confirms sole responsibility for the following: study conception anddesign, data collection, analysis and interpretation of results, and manuscript preparation.

The author has read and agreed to the published version of the manuscript.

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АКУМУЛЯЦІЯ МІКРОПЛАСТИКУ ДВОСТУЛКОВИМ МОЛЮСКОМ *UNIO TUMIDUS* ЗА ЕКСПЕРИМЕНТАЛЬНОГО ВПЛИВУ ТА ПОЛЬОВИХ УМОВ

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Вступ. Зростання виробництва та широке використання пластику призвели до того, що забруднення мікропластиком (МП) визнано серйозною екологічною проблемою. Більшість МП, який міститься у морському середовищі, потрапляє у нього з річок, однак забрудненню прісних вод МП приділяється недостатня увага. Організми, що живляться за допомогою фільтрації, такі як двостулкові молюски, є первинними організмами, що зазнають впливу МП у водному середовищі. Разом із тим, дослідження його накопичення у двостулкових молюсках зосереджені в основному на морських видах та засновані на використанні дорогого обладнання. Метою дослідження було виявити наявність МП у тілі прісноводного двостулкового молюска *Unio tumidus* із типової місцевості у Західній Україні та в лабораторних умовах субхронічної експозиції за екологічно реальної для прісних водойм концентрації у середовищі.

Матеріали і Методи. Для дослідження ми експонували молюсків до МП (0,1–0,5 мм) у концентрації 1,0 мг/л, що відповідає ~850 одиниць $л^{-1}$ протягом 14 діб, і кожні дві доби аналізували концентрацію МП у м'яких тканинах і воді. Також були проаналізовані молюски та вода з ділянки водойми, визначеної як забруднена. Для оцінювання кількості частинок використовували модифікований метод, згідно з яким біологічний матеріал руйнується гідроксидом калію та пероксидом водню, а МП визначається за допомогою світлового мікроскопа після фарбування флуоресцентним барвником нільським червоним.

Результати. Концентрація МП у м'яких тканинах екземплярів із референтної місцевості становила 9,5 одиниць у розрахунку на один зразок і демонструвала криву дзвоноподібної форми протягом 14-денної експозиції з максимумом, що відповідає 327,0 одиниць на зразок, на 10-ту добу експозиції, у негативній кореляції з концентрацією МП в експериментальному середовищі, яка змінювалася в межах 590–790 одиниць $л^{-1}$. Рівень МП у польових зразках зі забрудненої території становив 76,5 одиниць на організм, а у воді – близько 103 одиниць $л^{-1}$. Максимальний коефіцієнт накопичення МП, розрахований як $CF_i = C_i/C_0$ (використовуючи значення 9,5 як C_0), становив 83,18 і 8,05 для 10 днів експозиції та польових зразків відповідно.

Висновок. Отримані результати відображають високу акумулюючу здатність *U. tumidus* стосовно мікропластика і привертають увагу до використання цього виду в біомоніторингу забруднення мікропластиком і для очищення поверхневих вод від нього.

Ключові слова: мікропластик, *Unio tumidus*, акумуляція, мікроскопія, Ніл Червоний

Received / Одержано	Revision / Доопрацьовано	Accepted / Прийнято	Published / Опубліковано
27 October, 2022	21 November, 2022	26 December, 2022	28 December, 2022