Електроаналітичне визначення сукралози проводили також методом циклічної вольтамперометрії. Про те, що електрохімічне визначення сукралози відбулося, можна судити за поступовим, але різким зростанням значення струму у певних значеннях потенціалу. При цьому інтенсивність цього зростання залежатиме від концентрації цукрозамінника.

В якості фонового електроліту використано розчин із нейтральним рівнем pH. В якості робочого електроду використовується матеріал на основі карбону (графіт, карбонові нанотрубки).

Нами запропоновано новий метод визначення сукралози, пов'язаний із залежністю пікового значення струму при її електрохімічному окисненні від концентрації. При цьому зберігається лінійна залежність між піковим значенням струму та концентрацією цукрозамінника.

Ключові слова: сукралоза, електрохімічне визначення, циклічні вольтамперограми, мембранний електроліз, газовані напої, фоновий електроліт.

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VULNERABILITY OF FRESHWATER MUSSEL *UNIO TUMIDUS* TO WATERBORNE MIXTURE OF PSYCHOACTIVE SUBSTANCES AND MICROPLASTIC

This study investigates the effects of psychoactive substances and microplastics (MP) on the aquatic environment using swollen river mussels (*Unio tumidus*) as bioindicators. Mussels were exposed to microplastic, caffeine, chlorpromazine and their mixture for 14 days and biochemical biomarkers of stress and toxicity were analysed. All exposures caused the signs of toxicity, indicated as the loss of lysosomal membrane stability, inactivation of choline esterase, and decrease of the Zn/Cu ratio. All exposures, particularly MP, increased the glutathione level, indicating the involving of low weight cellular thiols in the stress response. Exposure to MP induced superoxide dismutase, and mixture decreased phenol oxidase activity, confirming the negative cumulative effect of the combine exposure.

Key words: pharmaceuticals, antioxidants, copper, zinc, cumulative effect.

The modern life of humankind cannot be imagined without plural pharmaceutical and personal care products [1]. Particularly, nowadays, the psychoactive substances increasingly applicate due to the prevalence of stress, anxiety, and symptoms of post-traumatic stress disorder among the victims of military activities [14]. Correspondingly, they input into the surface waters, and these so-called 'micro pollutants' expected to create the mixtures with cumulative effects on the aquatic organisms [1, 3, 6]. The psychotropic pharmaceutical chlorpromazine (Cpz) was selected for this study as one of most popular drug not only in the schizophrenia curing, but also prospective pharmaceutical due to its antibacterial effect, antitumor activity, inhibition of the replication in different viruses including SARS-CoV-2 [6, 22]. Caffeine (Caff), a central nervous system stimulant, utilized both as medicine

and food supplement, is arguably the most frequently ingested pharmacologically active substance in the world [1]. Consequently, its level in the surface waters is recommended to be the marker of anthropogenic pollution [24].

The realistic approach in the aquatic ecotoxicology of 'micro pollutants' must provide the study of chemical mixtures [16, 19]. Particular attention in their composition causes the microplastics (MP), widespread aquatic pollutant and suspected sorbent of hydrophobic substances like Cpz and Caff on the surface of particles [2]. The distortion of the responses to some pharmaceuticals in the exposures to their mixtures with MP was reported [16, 17].

The bivalve molluscs are recognized sentinel organisms in the assessment of the pollution due to the sessile habitats, filter-feeding and worldwide distribution [10, 11]. Therefore, to approximate exposure conditions to the environmental relevance, the goal of this study was to indicate whether the approved biochemical indexes of stress and toxicity in bivalves are the suitable biomarkers of vulnerability of the mussels to the combination of these substances and MP. The essential metals Zn and Cu accumulation was also included in the expertise according to the previous experience of its application [17].

Materials and methods of research

Caffeine of pharmaceutical quality was provided by PJSC SIC "Borshchahivskiy CPP", all other reagents/chemicals were of the highest analytical grade available and purchased from Sigma-Aldrich (St. Louis, MO), or Synbias (Kyiv, Ukraine).

The adult male specimens of swollen river mussel *Unio tumidus* (mean length 9.1-10.4 cm, weight 56-90 g) were collected in the summer (July) at the middle stream of the Dniester River basin (the Seret River), Ukraine. About 200 individuals were acclimated to the laboratory conditions for up to seven days after the capture as it was described earlier [15]. Then, molluscs were distributed randomly to four groups: untreated mussels (C) and treated with MPs (1 mg L^{-1}), Caff (20 µg L^{-1}), Cpz (12 ng L^{-1}) and their mixture (Mix) during 14 days at the temperature 18° C. After exposures, molluscs were immediately dissected on ice. The digestive gland tissue was dissected and utilized for study. All steps were carried out at 4°C. The single-use samples were frozen (–40 °C) until analyzed. Soluble protein concentration in the supernatant of 10% homogenate of digestive gland was measured according to the method of Lowry *et al.* (1951) [12], using bovine serum albumin as the protein standard.

Lysosomal membrane stability (lysosomal integrity) as an index of vitality was determined in the digestive gland by the Neutral Red Retention (NRR) assay spectrophotometrically [4] and expressed in the relative units per g of FW. The elevation of the light absorption corresponded to the loss of membrane stability. For the neurotoxicity evaluation, the cholinesterase (ChE, EC 3.1.1.7) activity in the tissue homogenate was determined colorimetrically utilizing acetylcholine iodide (ATCh) as substrate and the 5,5-dithiobis-2-nitrobenzoate (DTNB) as the thiol indicator [5] and calculated using a molar extinction coefficient of $14.15 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$, and referred to the soluble protein content. The SOD (EC 1.15.1.1) activity in the soluble phase of the tissue homogenate, was determined according to the non-enzymatic assay based on aerobic reduction of nitro-blue tetrazolium (NBT) in the presence of phenazine methosulphate (PMS) and NADH [7] and expressed in the SOD units per mg of soluble protein (one unit is defined as the amount of enzyme that causes 50% inhibition of NBT reduction). The phenoloxidase-like activity (PhO, EC 1.14.18.1) was determined by recording the formation of o-quinones utilizing p-phenylenediamine (PPD) as substrate [13]. The results were expressed in international units (IU) per mg of soluble protein. One IU was defined as the amount of enzyme that catalyzes the appearance of 1 µmol of product per min using the molar extinction coefficient of PPD reaction product of 43 160 M^{-1} cm^{-1}. Total glutathione (GSH) concentration was quantified by the glutathione reductase recycling assay [8] in the protein-free extract of 10% w/v homogenate. Standards were prepared from reduced glutathione, and concentrations were expressed as µmol per g wet weight.

The concentration of Zn in the digestive gland after the digestion of samples with HNO_3 was measured utilizing the reaction of the complexation of Zn(II) with 2-(5-bromo-2-pyridylazo)-5-[N-propyl-N-(3-sulfopropyl) amino]phenol disodium salt dihydrate (5-Br-PAPS) [25], evaluated from the absorbance of the metal-5-Br-PAPS complex at 560 nm. Cu assay in the soft tissues was accomplished

by the spectrophotometric assay utilizing cuprizone (Copper Test. 1.14767 - Merck Millipore). Calculation was made utilizing the molar extinction coefficient of 16,000 M^{-1} cm^{-1} at 600 nm.

Results were expressed as mean ± standard deviation. For all traits, the sample size was eight from eight individuals. Shapiro-Wilk test was used for the assessment of normality. Data were analysed using parametric Student's t-test with Bonferroni correction significant at $p < 0.01$. The adequacy of data was evaluated based on the value of the KMO and Bartlett's test of sphericity. The IBM SPSS Statistics version 26 software for Windows was used for calculations.

Research findings and their discussion

Compared to the control, all exposures caused the increase of lysosome membranes instability (Fig. 1A). For the ChE, the prominent oppression (up to 2.6 times by MP and Cpz) was identified in all exposures (Fig. 1B).

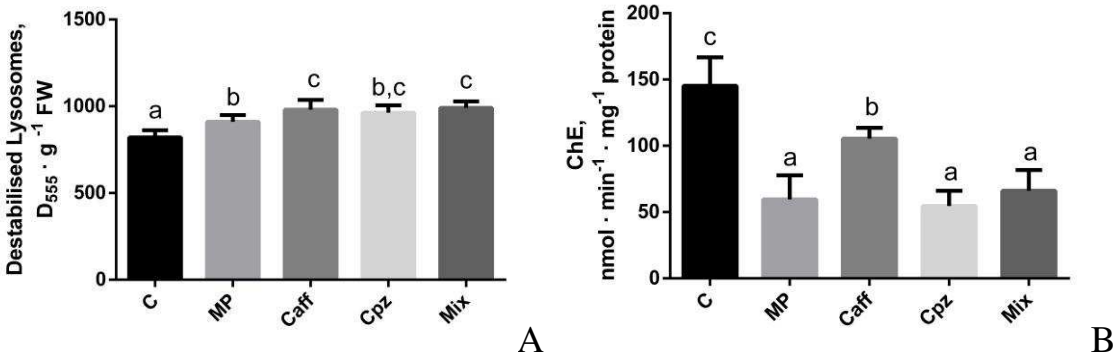
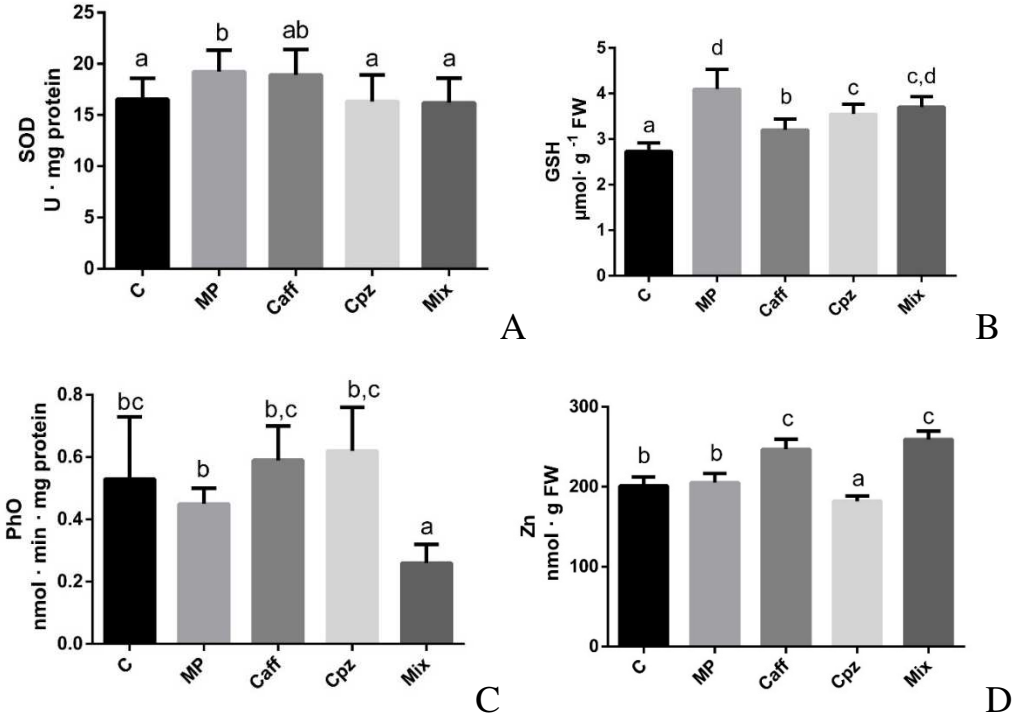


Fig. 1. Toxicity markers in the digestive gland of bivalve mollusc *U. tumidus* exposed to microplastics (MP), caffeine (Caff), chlorpromazine (Cpz) and their mixture during 14 days, *M±SD, N=8.* A - lysosomal membrane instability (NRR test); B - cholinesterase (ChE) activity. Different letters indicate the difference between the exposed and control groups, P-value < 0.01.

The SOD was significantly activated only by MP and did not change against control in other exposures (Fig 2A). The level of GSH was more sensitive and increased in all exposures, particularly by MP (Fig. 2B). The activity of PhO was oppressed substantially (by ≈2.0 times against control) only under the exposure to Mix and did not differ significantly in all other exposures (Fig. 2C).



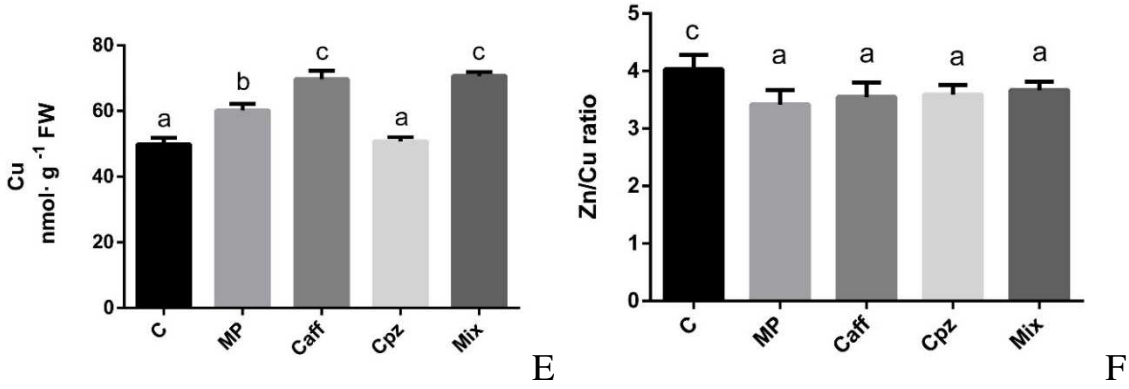


Fig. 2. The markers of stress and essential metals level in the digestive gland of bivalve mollusc *U. tumidus* exposed to microplastics (MP), caffeine (Caff) and chlorpromazine (Cpz) and their mixture during 14 days, *M±SD, N=8.* A - superoxide dismutase (SOD) activity; B - total glutathione (GSH) level; C - phenoloxidase (PhO) activity; D - concentration of Zn; E - concentration of Cu; F - the ratio of Zn/Cu concentrations. Different letters indicate the difference between the exposed and control groups, P-value < 0.01.

The concentrations of Zn and Cu in the digestive gland were changed comparing to control in most exposures. The level of Zn decreased in the Cpz-group and increased by Caff and Mix (Fig 2D). The concentration of Cu increased in all exposures, except Cpz, up to 40% (Fig. 2E). Correspondingly, the Zn/Cu ratio decreased against control from 4.0 up to 3.6 in all exposures (Fig. 2F).

According to our results, the signs of toxicity were detected in each exposure. The most prominent signs of toxicity were evident as the ChE depletion, confirming the worth of this marker to indicate the impact of environmentally relevant concentrations of diverse xenobiotics in bivalve molluscs [18]. Concerning the Cpz, the inability to utilize acetylcholine can occur, distorting the nervous system coordination [21]. The NRR test is approved in the plural studies as the index of cell viability [20]. This is explained by the lysosomes property to be the specific target for the accumulation of different pollutants. Our study confirms this response even for the extremely low concentrations of contaminants, particularly for Cpz. Similarly, the decrease in the lysosomal membrane stability was confirmed for the effect of MP, Caff and their mixture, and Cpz on the marine mussel *Mytilus galloprovincialis* [9, 10].

Comparing the manifestations of stress, it can be assumed that the responses of GSH were most prominent, indicating the activation of antioxidant response, particularly by MP. The important role of GSH in the adaptation of bivalve mollusc to MP and pharmaceuticals was shown previously [16].

The particular sensitivity of PhO, the marker of inflammation, was the most valuable sign of the vulnerability of defence forces and the negative cumulative effect of MP, Caff and Cpz mixture. The decrease of immune response as the major target for environmentally relevant mixtures of pharmaceuticals in molluscs was described earlier [3] and consistent with our results.

At last, the impact of all exposures was evident from the Zn/Cu concentrations assessment. Importantly, our results confirm that the ratio of these essential metals in the mussels can be utilized for the assessment of their health status. Meanwhile, phenothiazines inhibit the binding of Ca to Ca-dependent proteins [22], and the interaction of Ca and Zn transport particularly for mussels is well known [23]. Hence, this effect can be a specific target for the phenothiazine Cpz.

Summarizing, the freshwater mussels *U. tumidus* can provide early warning of emerging environmental health hazards caused by psychoactive substances and their combination with MP. The depletion of Zn/Cu ratio in molluscs can be recommended as the simple index of environmental health disturbance, and the phenol oxidase activity for the indication of multi-stress impact.

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ЧУТЛИВА РЕАКЦІЯ ПРІСНОВОДНИХ МОЛЮСКІВ *UNIO TUMIDUS* НА ДІЮ СУМІШІ ПСИХОАКТИВНИХ РЕЧОВИН ТА МІКРОПЛАСТИКУ

Надходження психоактивних речовин у поверхневі води зростає через їх широке застосування у лікуванні та підтриманні емоційного стану людини. Двостулкові молюски є чутливими організмами для оцінки забруднення води. Проте вплив сполук, які змінюють поведінку та когнітивні функції людини, на нецільові організми досліджений недостатньо. Крім того, присутність мікропластику у водному середовищі може змінювати його біологічний вплив. Тому, щоб наблизити експериментальні умови до реальних, метою цього дослідження було визначити, чи є біохімічні маркери стресу та токсичності у двостулкових молюсків відповідними для оцінки одноразового та комбінованого впливу психоактивних речовин і мікропластику.

Протягом 14 днів прісноводних молюсків *Unio tumidus* піддавали дії МР (1 мг $л^{-1}$, розмір 35-50 мкм), кофеїну (Caff, 110 пМ), хлорпромазину (Cpz, 37 пМ) або їх суміші (Mix). Оцінено набір біомаркерів токсичності та концентрації цинку (Zn) і міді (Cu) у травній залозі.

Усі впливи викликали ознаки токсичності, що виражалися як втрата стабільності лізосомальної мембрани, інактивація холінестерази та зниження співвідношення Zn/Cu. Усі впливи, особливо МП, підвищували рівень глутатіону, що вказує на залучення клітинних тіолів низької ваги у відповідь на стрес. Вплив МР індукував супероксиддисмутазу, а суміш знижувала активність фенолоксидази, підтверджуючи негативний кумулятивний ефект комбінованого впливу. Рекомендуємо використовувати зниження співвідношення Zn/Cu в молюсках як оптимальний показник для оцінки порушень в екосистемі, а також оцінювати активність фенолоксидази для виявлення комбінованого впливу стресових факторів.

Ключові слова: фармацевтики, антиоксидантні ензими, мідь, цинк, кумулятивний ефект.

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