

introduction of *Chlorella vulgaris* in the exposure media in the amount of about 100 thousand cells / dm<sup>3</sup> did not show a significant corrective effect on the toxicity of pesticides for non-target species *Danio rerio*, which doesn't exclude the positive impact of algae on the functioning of the ecosystem in general and requires a more detailed analysis.

*Key words: Danio rerio, pesticides, toxicity, chlorella.*

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## **COMBINED EXPOSURES TO LOW ROUNDUP CONCENTRATION INDUCE THIOLOME RESPONSE IN BIVALVE MOLLUSK**

Glyphosate is a weed killer used worldwide. Its toxicity to aquatic organisms was investigated mostly at acute high levels of exposure. The study aimed at evaluating the effect of low glyphosate concentration in the combination with pharmaceutical or heating to freshwater bivalve mollusks. We exposed the mussels *Unio tumidus* to glyphosate-based herbicide Roundup MAX (Rn, 16.9 µg L<sup>-1</sup> or 40 nM of glyphosate, roughly a half of PNEC (Predicted No Effect Concentration) estimate derived from multispecies data), chlorpromazine (Cpz, 18.0 µg L<sup>-1</sup> or 56 nM), the combination of Rn and heating (25° C, RnT), and the combination of Rn and Cpz (RnCpz) during 14 days. The responses of oxidative stress were evaluated in the digestive gland. The enzyme activities changed only in the exposures containing Rn (increase of superoxide dismutase) and Cpz (decrease of catalase), whereas the elevation of total glutathione (GSH) level was indicated in all exposed groups except Rn and of metallothionein-associated thiols (MTSH) in all groups except Cpz. Analysis of metallothionein by means of size-exclusion chromatography did not indicate substantial oxidative changes in any group. Lipid peroxidation increased in all exposures, maximally up to 16.6%. The total balance of antioxidant versus prooxidative changes increased in all exposures containing Rn (by approx. 3 times in RnT-group), while decreased in the Cpz-group. Hence, combined exposures distort prominently the oxidative stress responses to xenobiotics in the freshwater mussels even at low, nanomolar concentrations. The ability of Rn to induce MTSH seems to be the decisive input in the antioxidant defence of the mussels affected by the mixed physical/chemical exposures.

*Keywords: Bivalve mollusk, Roundup, Heating, Chlorpromazine, Antioxidants, Metallothioneins, Thiolo-me.*

Roundup (a commercial form of organophosphorus glyphosate) belongs to the most utilised pesticides over the world as weed killer [15]. Glyphosate has not molecular targets in the animals, but it inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EC 2.5.1.19) of the shikimate pathway, which is essential for the synthesis of aromatic amino acids and of almost all other aromatic compounds in algae, higher plants, bacteria, and fungi [20]. However, glyphosate utilisation since 1974 had brought several negative experiences concerning its impact on non-targeted organisms. Glyphosate, both as the active ingredient and with the adjuvants presented in the commercial Roundup formulations, was reported to cause toxicity in different experimental models, including

cancer in mammals [3, 15]. Different studies have indicated a high level of glyphosate in the environment and organisms. For example, it has been found 2.23  $\mu\text{g/L}$  of glyphosate derivate in the urine of agricultural workers in the northwest of Mexico, and 53% of the workers showed nuclear damage [3].

The bivalve mollusks are highly recommended aquatic organisms for the bioindication of aquatic pollution due to their filter-feeding lifestyle, long life spans, and sedentary habits [16]. However, these unique features make them particularly sensitive to environmental perturbations resulting from global climate change and lead to their global decline [18]. In our previous studies, we reported high sensitivity of freshwater mussels to the local environmental peculiarities (even depending on the location of the power plant dam) [8, 11]. In the subchronic exposure *in vivo* to Roundup alone (80 nM of glyphosate) and in combinations with pharmaceuticals and heating, particular toxicity of Roundup to the mussels has been shown [10, 12]. Importantly, *ex vivo* exposure to Roundup at as low as 40 nM glyphosate concentration caused the most prominent responses of stress and toxicity [12]. This concentration corresponds to approximately a half of the Predicted No Effect Concentration (PNEC) estimate derived from multispecies data [25].

The study aimed at evaluating the long-term subchronic effects to freshwater bivalve mollusks of *Unio tumidus* induced by glyphosate alone at as low as half of PNEC and jointly with other environmental challenges – heating and pharmaceutical substance. An elevated temperature corresponds to the maximum water temperature (25° C) detected in the Dniester basin in the typical location of sampled mollusks (<https://ukr.seatemperature.net/seas-and-rivers/reka-dnestr>). Chlorpromazine (Cpz), the selected pharmaceutical for this study, is the first generation neuroleptic drug [13]. Its relatively high concentration was detected in wastewater [6]. Importantly, Cpz has been found to have antiviral activity *in vitro* against the influenza virus, HIV, and, actually, it is listed among "the most promising molecules for inhibiting coronaviruses in human cells" [13]. The indexes of oxidative stress and metallothioneins' chromatographic behaviour were selected for this study as the expected sensitive indicators of toxicity manifestations [21, 22].

### Materials and methods

All reagents were of the Reagent grade or higher and were obtained from Sigma-Aldrich (USA) or from the Synbias (Ukraine). Roundup formulation was Roundup MAX, Monsanto, USA, and chlorpromazine was of pharmaceutical purity (AMINAZIN, KhSPhE "People's Health", ATX N05AA01).

Adult bivalve mollusks *Unio tumidus* Philipson, 1788 (Unionidae) (~ 6 years old, ~ 8.5 cm length, and 60–70 g weight) were collected in a river site assumed to be reference [8]. Specimens were transported to the laboratory and acclimated to the laboratory conditions for up to seven days after the capture and distributed randomly to four groups. One group was exposed to the aquarium water only and was considered control (C). Other groups were exposed to organophosphonate pesticide Roundup MAX (Rn, 16.9  $\mu\text{g L}^{-1}$ , correspondent to 6.1  $\mu\text{g L}^{-1}$  or ~40 nM of glyphosate) at the temperatures 18° C (RnC) and 25° C (RnT), to chlorpromazine (Cpz, 18.0  $\mu\text{g L}^{-1}$  or 56 nM), and a mixture of Rn and Cpz (RnCpz) at 18° C during 14 days. Water was changed and chemicals replenished every two days. Mollusks were fed with the same regularity throughout the experiment.

After exposures, mollusks were immediately dissected on ice. For all biochemical traits except metallothioneins elution, digestive gland samples were prepared from eight individual mollusks in each experimental group. Tissues were sampled at 4° C and frozen (-40° C) until analyses. For metallothioneins detection, the combined samples from five specimens (total 350 mg) were prepared in triplicate. The methodology used for each biomarker is given in detail in Khoma et al. (2020) [10, 12].

For oxidative stress assays, 6,000 x g supernatant of digestive gland tissue was prepared. The samples were homogenized (10 % w/v) in 0.1 M phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM EDTA, as well as 0.1 mM phenylmethylsulfonyl fluoride for proteolysis inhibition. Homogenates were centrifuged at 6,000 x g for 10 min, and the resulting supernatant was kept at -40° C. The protein concentration was analysed in the 6,000 x g supernatant according to the method of Lowry et al. (1951) [14], using bovine serum albumin as the protein standard.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured according to the non-enzymatic assay based on aerobic reduction of nitro-blue tetrazolium in presence of phenazine methosulphate and NADH [7]. Catalase (CAT, EC 1.11.1.6) activity was measured spectrophotometrically by monitoring the decomposition of H<sub>2</sub>O<sub>2</sub> according to Aebi (1974) [1] at 240 nm. The products of lipid peroxidation (LPO) were determined in the supernatant of 10% W/V homogenate after the sedimentation of proteins in sulfosalicylic acid as the production of thiobarbituric acid-reactive substances (TBARS) [17]. Total glutathione concentration was quantified by the glutathione reductase recycling assay [9] in the protein-free extract of homogenate using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB). Metallothioneins were isolated as the thermostable proteins by size-exclusion chromatography on Sephadex G-50 as described elsewhere [19]. Low weight (approximately 8 kDa) fractions with high absorbance at 254 nm and high D<sub>254</sub>/D<sub>280</sub> density ratio were identified as putative MTs-containing peak and pooled to the total of 10 mL for the UV-spectrum detection. Metallothionein-associated thiols (MTSH) were determined using DTNB reduction method after the ethanol/chloroform extraction from tissue homogenate [23].

Results were expressed as mean  $\pm$  SD. Shapiro-Wilk test was used for the assessment of normality. Data were analysed with parametric Student's t-test significant at  $p < 0.05$ . Pearson's correlation test for the pairs of variables was performed at a 0.05 level of significance. An index of Antioxidant/Prooxidant Balance (APB) was defined as the shift of balance between antioxidant activities (SOD, CAT, GSH, MTSH) and prooxidant manifestations (TBARS). Each index in the exposed groups was calculated as a rate of deviation from control value  $Z = (Mi - Mc)/Mc$ . The mean value of APB equalled 4.0 in the control group. The IBM SPSS Statistics version 24 software for Windows was used.

### Results and Discussion

The evaluation of oxidative stress response has indicated low sensitivity of antioxidant enzymes (Fig. 1). Indeed, SOD was activated only by Rn, CAT was inhibited only by Cpz. On the contrary, the levels of nonenzymatic antioxidants, low weight cellular thiols, GSH and MTSH were increased in all exposures, except GSH in Rn-group and MTSH in the Cpz-group. The level of TBARS increased in most exposures compared to control except the RnT-group. However, this increase was not prominent and reached only 16.6% in the combined exposure of Rn and Cpz. The calculation of the APB index demonstrated the substantial elevation of antioxidant activities in all exposures, which contained Rn, whereas prooxidative changes were predominant in the Cpz-group.

Gel-filtration of the thermostable extract from the digestive gland in each experimental group revealed the peak, which had an apparent molecular mass of 8 kDa. It was identified as a MTs-containing peak basing upon its spectral features, thermostability, and molecular weight [19] (Fig.2). The metallothioneins profile of elution and UV-spectra were not distorted in any exposure (Fig. 2), demonstrating the absence of substantial changes in the oxidative activities. Indeed, the oxidative changes of metallothioneins are accompanied by their dimerization with the appearance of peaks with higher molecular mass [24].

Statistically significant correlations between the indices were scant: only one positive correlation between SOD and CAT ( $r=0.481$ ,  $p<0.001$ ) and one negative correlation between GSH and SOD ( $r=-0.425$ ,  $p<0.001$ ) were found for studied parameters.

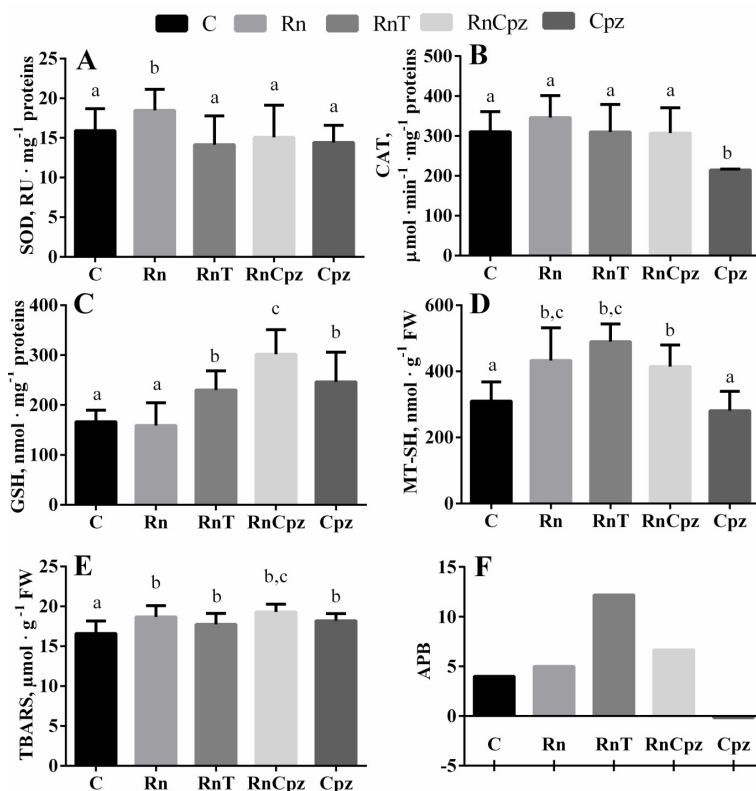


Fig. 1. Oxidative stress parameters in the digestive gland of *U. tumidus* after 14 days of exposures to Roundup (Rn), Roundup and heating (RnT), Roundup and chlorpromazine (RnCpz), and chlorpromazine (Cpz) during 14 days: A – SOD activity; B – catalase activity; C – glutathione total concentration; D – metallothionein-associated thiols; E – TBARS production; F – index of antioxidant/prooxidant balance (APB). Data (A–E) are presented as means ± SD (n = 8). Different letters above the columns indicate significantly different values (P < 0.05).

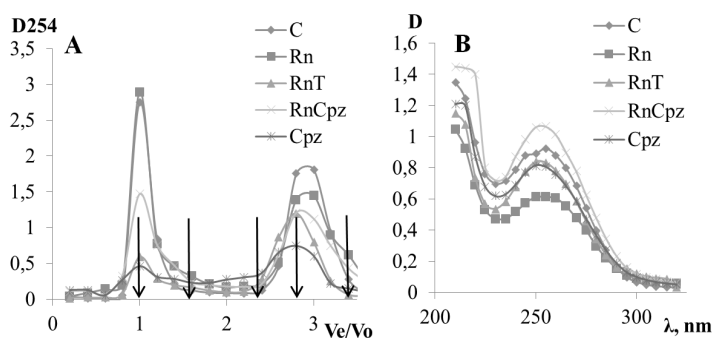


Fig. 2. Properties of low weight thermostable proteins eluted by size exclusion chromatography on Sephadex G-50 from the digestive gland of *U. tumidus* in the control (C) and groups exposed to Roundup (Rn), Roundup and heating (RnT), Roundup and chlorpromazine (RnCpz), and chlorpromazine (Cpz) during 14 days: the elution profiles on Sephadex G-50 (A), UV-spectra of low molecular weight peak (B).

Comment. Arrows (A) highlight the elution volume of markers: 25.8 kDa, 17.0 kDa, 12.3 kDa, 8.4 kDa, 3.4 kDa appropriate to 1.02; 1.6; 2.35; 2.8, 3.4 Ve/Vo correspondingly; Ve, elution volume; Vo, void volume of the column.

Thus, each exposure caused a specific response of antioxidative enzymes. However, combined exposures distorted prominently the oxidative stress responses in the mussels even at low, nanomolar concentrations. The ability of Rn to induce MTSH seems to be the decisive input for the antioxidant defence activity at combined exposures. Specific response of low weight cellular thiols metallothioneins (MTs) to transitional metals is well known [22, 24]. Their targeting by other than metals exposures of the organism is studied less [10]. However, when we compared the quantity of metallated and common metallothioneins in the digestive gland, it was evident that the part of these thiols is in the apo-form, therefore they can easily participate in the antioxidative response. Moreover, the number of metallothionein-related thiols in the tissue is comparable to the concentration of glutathione (Fig. 1 and [10, 11]). When we related the effects induced by the concentration of Rn alone with the two times lower Rn concentration used in combined exposures, the decreased SOD but increased levels of GSH, MTSH and LPO were indicated [10–12]. Consequently, despite different responses of SOD, in the exposures to Rn, the same main role of MTSH was shown. The chromatographic properties of MTs were also non-disturbed in the exposure to 80 nM of Rn alone and in combinations [10]. Only in the acute *ex vivo* exposure, the similar to utilized here about 40 nM concentration of glyphosate (Rn) caused the decrease of MTSH concentration (by ~ two times) in coordination with the depletion of total antioxidant activity [10, 11, 12]. However, the level of TBARS was not changed compared to control detecting the early stage of the injury.

In the present study, it was shown the particular response to Cpz alone. This lipophilic phenothiazine drug with antipsychotic and neuroleptic activities caused the decrease of CAT activity, the absence of MTSH changes, and total prooxidant shift. To our knowledge, Cpz effect on the mussels was studied for the first time. For aquatic organisms, the toxicity of Cpz was shown to be at the concentrations of  $\mu\text{g L}^{-1}$  to  $\text{mg L}^{-1}$  in the terms of immobility and population metrics [5], and its molecular effects are almost unknown.

The input of warming is attested by the indication of oxidative stress. For example, it has been shown that exposure of *Mytilus galloprovincialis* to high arsenic concentration ( $1 \text{ mgL}^{-1}$ ) and warming ( $21^\circ \text{C}$  versus  $17^\circ \text{C}$ ) causes more prominent changes in the levels of SOD, CAT, LPO, and GSH than does single exposure to arsenic or warming during 14 days [4].

To summarize, at the circumstances close to environmentally realistic, the main antioxidant activity in the mussels' digestive gland belongs to cellular low weight thiols. We detected that the weak Rn impact can aggravate in the co-exposure with heating or pharmaceutical substance. Despite the variability of responses to Rn in long-term subchronic exposures and combined exposures at the PNEC or half of the PNEC, metallothionein-associated thiols were among the most sensitive molecular targets of its action.

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## КОМБІНОВАНА ДІЯ НИЗЬКОЇ КОНЦЕНТРАЦІЇ РАУНДАПУ НА ДВОСТУЛКОВОГО МОЛЮСКА АКТИВУЄ ТІОЛОВІ СПОЛУКИ У ТРАВНІЙ ЗАЛОЗІ

Гліфосат –це один із найпопулярніших засобів боротьби з бур'янами. Його дію для водних організмів досліджували здебільшого під час гостротоксичних експериментальних умов. Метою цього дослідження було оцінити вплив низькоїконцентрації гліфосату, яка становить 0,5 максимальної неефективної концентрації, в комбінованій експозиції на прісноводних двостулкових моллюсків. Двостулкові моллюски *Unio tumidus* ми піддавали впливу гербіциду на

основі гліфосату Roundup MAX (Rn, вміст якого становив  $16,9 \text{ мкг л}^{-1}$  або  $40 \text{ нМ}$  гліфосату) окремо, у поєднанні з підвищеною температурою води до  $25^\circ\text{C}$  (RnT), у поєднанні з хлорпромазином (RnCrz,  $18,0 \text{ мкг л}^{-1}$  або  $56 \text{ нМ}$  Crz) і окремо Crz протягом 14 днів. Реакції окисного стресу оцінювали в травній залозі. Активність ферментів антиоксидантного захисту змінювалась лише після впливу Rn (збільшення активності супероксиддисмутази) та Crz (зниження активності каталази). Підвищення рівня загального глутатіону (GSH) спостерігали у всіх експозиціях, крім Rn, а тіолів, у складі металотіонеїнів (MTSH) – у всіх групах, крім Crz. Посилення перекисного окиснення ліпідів відбувалося в усіх випадках, максимумом на  $16,6\%$ . Загальний баланс антиоксидантів у порівнянні з прооксидантними змінами збільшувався у дослідних експозиціях, що містили Rn (у  $\sim 3$  рази в групі RnT), і зменшувався при впливі лише Crz. Особливості хроматографічного аналізу металотіонеїнів експериментальних груп, не відобразили ознаки суттєвих окисних змін цих протеїнів. Отже, комбінований вплив ксенобіотиків, навіть у низьких наномолярних концентраціях, помітно змінює реакції окисного стресу в організмі. А здатність Rn індукувати MTSH, схоже, є вирішальним фактором системи антиоксидантного захисту у відповідь на комбінований вплив екологічних чинників.

*Ключові слова:* двостулкові молюски, раундап, нагрівання, хлорпромазин, антиоксиданти, металотіонеїни, тіоли.

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