introduction of *Chlorella vulgaris* in the exposure media in the amount of about 100 thousand cells / dm^3 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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COMBINE EXPOSURES TO LOW ROUNDUPCONCENTRATION INDUCES THIOLOME RESPONSE IN THE DIGESTIVE GLAND OF BIVALVE MOLLUSK

Glyphosate is one of most popular weed killers in the word. Its toxicity to aquatic organisms was investigated mostly in the acute high experimental exposures. The aim of this study was to evaluate the effect of low, 0.5 of Predicted No Effect Concentration (PNEC), of glyphosate in the combinations to freshwater bivalve mollusks.We treated the mussels *Unio tumidus* with glyphosate-based herbicide RoundupMAX (Rn, correspondent to 16.9 µg L^{-1} or 40 nM of glyphosate) in the combination with heating 25° C (RnT) or chlorpromazine (RnCpz, 18.0 µg L^{-1} or 56 nM of Cpz) and Cpz alone during 14 days. The responses of oxidative stress were evaluated in the digestive gland. The enzyme activities were changed only by the exposures to Rn (increase of superoxide dismutase) and Cpz (decrease of catalase), whereas the elevation of total glutathione (GSH) level was indicated in all exposures except Rn, and metallothonein-associated thiols (MTSH) – in all exposures except Cpz. Lipid peroxidation was increased in all exposures by 16.6% maximally. Total balance of antioxidants versus prooxidative changes was increased in all exposures contained Rn (by ~3 times in RnT-group) and decreased in the exposure to Cpz alone. Metallothionein chromatographyc features did not indicate substantial oxidative changes in all exposed groups. Hence, combine exposures distort prominently the oxidative stress responses to xenobiotics in the freshwater mussels even in low, nanomolar concentrations. The ability of Rn to induce MTSH seems to be the decisive input in the antioxidant defence at combine exposures.

Key words: Bivalve mollusk, Roundup, Heating, Chlorpromazine, Antioxidants, Metallothioneins, Thiolome.

Formulation Roundup (commercial form of phosphonoorganic glyphosate) belongs to 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 (EPSP) 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, its utilization since 1974 had brought several negative experiences concerning its impact on the non-targeted organisms. Both its active ingredient glyphosate and the adjuvants presented in the commercial formulations were

reported to cause toxicity in different experimental models, including cancer in mammalians [15]. Different studies indicated high level of glyphosate in the environment and organisms. For example, in agricultural area at the northwest of Mexico in urine of agricultural workers 2.23 µg/L of glyphosate derivate, 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 reflected high sensitivity of freshwater mussels to the local environmental peculiarities (even depending on the location in relation to dam of power plant) [8, 11]. In the subchtronic exposure in vivo to Roundup alone (80 nM of glyphosate) and in combinations with selected pharmaceuticals and heating particular toxicity of roundup to the mussels was shown [10, 12]. Importantly, in the ex vivo exposure to roundup, the lowest concentration (40 nM), correspondent to 0.5 of Predicted No Effect Concentration (PNEF), caused the most prominent responses of stress and toxicity [12].

Therefore, the aim of this study was to evaluate the subchronic effect of 0.5 PNEF concentration of glyphosateto freshwater bivalve mollusk, and the effect of its combine exposure with other environmental challenges – heating and pharmaceutical substance. Utilised elevated temperature 25^{o} C is corresponding to the maximum water temperature detected in the Dniester bazin in the typical location of sampled mollusks (https://ukr.seatemperature.net/seas-and-rivers/reka-dnestr). Pharmaceuticals, despite comparatively low concentrations in surface waters, result in specific manifestations of toxicity in aquatic organisms [2]. The pharmaceutical substance selected for this study is chlorpromazine (Cpz), the first generation neuroleptic drug [13]. Itwas detected in the waster waters in rather high concentrations [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". The indexes of oxidative stress and metallothioneins chromatographyc behavior were selected for this study as the expected sensitive indicators of inappropriate effects [21, 22].

Materials and methods

All reagents were of the Reagent grade or higher and 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 preacclimated 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 (Rn, formulation RoundupMAX, Monsanto, USA, 16.9 µg $L^{-1,}$correspondent to 6.1 µg L^{-1} or~40 nM of glyphosate) at the temperatures 18^{o} C and 25^{o} C (RnT), to chlorpromazine (CPZ,18.0µg L-1 or 56 nM), or mixture of Rn and CPZ (Mix) at 18^{o} C during 14 days. Water was changed and chemicals replenished every two days. *Throughout the experiment*, mollusks were fed with the same regularity.

After exposures, mollusks were immediately dissected on ice. For all biochemical traits except metallothioneinselution, digestive glands samples were prepared from eight individual mollusks in each experimental group.Tissues were sampled at 4^{o}Cand frozen (–40 ^{o}C) until analyses. For metallothioneins detection, the combine samples from five specimmens (totally 350 mg) were prepared in triplicate. Methodology used for each biomarker given in detail in [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 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for proteolysis inhibition. Homogenates were centrifuged at 6,000 x g for 10 min, and the resulting supernatant was kept at -40^{o} 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 (NBT) 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_2O_2 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_{254}/D_{280} 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 ± SD. Shapiro-Wilk test was used for the assessment of normality. Data were analyzed 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.Index of Antioxidant/ProoxidantBalance (APB) was defined as the shift of balance beween 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 of APB equalled 4.0 in the control group. The IBM SPSS Statistics version 24 software for Windows were used.

Results and discussion

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

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 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 peak with higher molecular mass [24].

The correlations between the indices were scant: only positive correalation between SOD and CAT *($r=0.481$, $p<0.001$)* and negative correlation between GSH and SOD *($r=-0.425$, $p<0.001$)* were found forstudied parameters.

Thus, each exposure caused specific response of antioxidative enzymes. However, combine exposures distort prominently the oxidative stress responses to xenobiotics in the mussels even in low, nanomolar concentrations. The ability of Rn to induce MTSH seems to be the decisive input for the antioxidant defence activity at combine 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 molluscan metalated and common metallothioneins, usually it was evident that the part of these tiols are in the apo-form, So they can easely participate in the antioxidant response. Mooreover, the number of metallothionein-related thiols in the cells of mollusks are comparable to the concentration of glutathione (Fig. 1) [10, 11].

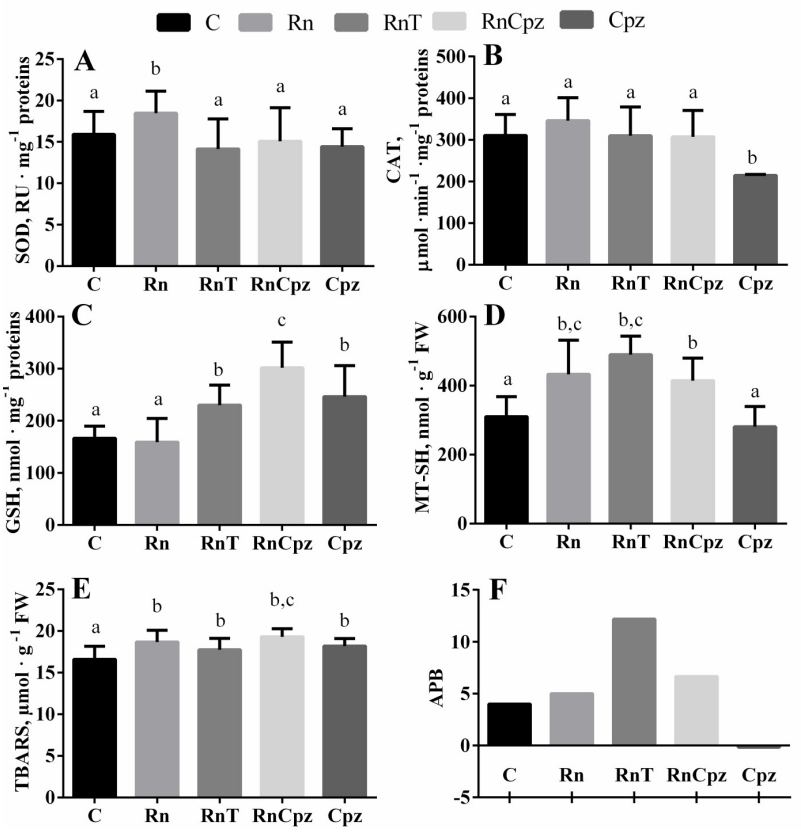

Fig. 1. Oxidative stress parameters in digestive gland of *U. tumidus* after 14 days of experimental exposures to Roundup (Rn), Roundup and heating (RnT), Roundup and Clorpromazine (RnCpz), and Clorpromazine (Cpz) during 14 days: A – SOD activity; B – Catalase activity; C – Glutathione total concentration;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

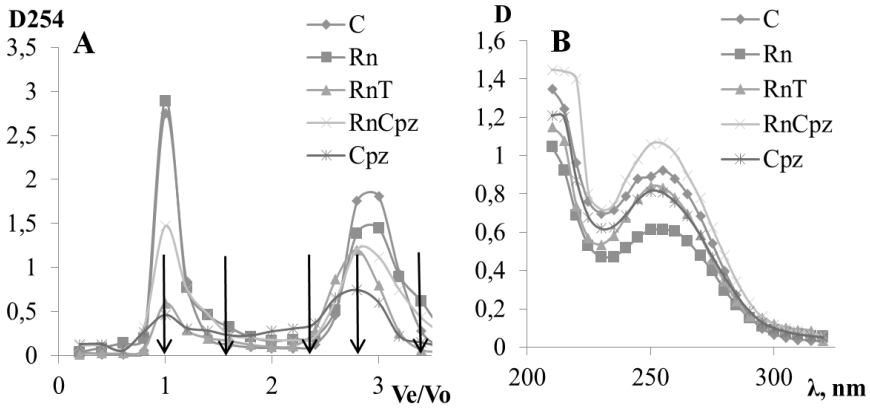

Fig. 2. Properties of low weight thermostable proteins eluted by size exclusion chromatography on Sephadex G-50 from the digestive gland of *U. tumidus* in the control (C) and groups exposed to Roundup (Rn), Roundup and heating (RnT), Roundup and Clorpromazine (RnCpz), and Clorpromazine (Cpz) during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

Comment. A. arrows 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.

When we studied the effect of twice-higher concentration of Rn alone and in the combine exposures, the decreased SOD but increased levels of GSH, MTSH and LPO were indicated [10, 11, 12]. Consequently, despite different responses of SOD, in both subchronic exposures and co-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 compare to control detecting the early stage of the injury.

In the present study it was shown the particular response to Cpz alone. It caused the decrease of CAT activity, absence of MTSH changes and total prooxidant shift. Cpz effect on the mussels was studied at the first time, according to our knowledges. CPZ is a lipophilic phenothiazine drug with antipsychotic and neuroleptic activities. For aquatic organisms, toxicity of CPZ was shown at the concentrations of µg-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 was shown that impact of combination of high Arsenic concentration (1 mgL^{-1}) and warming (21 °C versus 17oC) in Mytilus galloprovincialis caused more prominent changes in the levels of SOD, CAT, LPO, GSH that single exposures to Arsenic or warming during 14 days [4].

To summarize, according to our results, at the circumstances close to environmentally realistic, main antioxidant activities in the mussels digestive gland belong to cellular low weigh thiols. We detected that the low impact of Rn that can be aggravated in the combine exposures with heating or pharmaceutical substance.Despite the variability of responses to Rn in acute and subcronic exposures to the PNEC or 0.5 PNEC of Rn, in each case MTSH were among the most sensitive molecular targets for its effect.

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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 низької концентації гліфосату, яка становить 0,5 максимальної неефективної концентрації, в комбіновані експозиції на прісноводних двостулкових молюсків. Двостулкові молюски *Unio tumidus* піддавали впливу гербіциду на основі гліфосату Roundup MAX (Rn, вміст якого становив 16,9 мкг $л^{-1}$ або 40 нМ гліфосату) окремо, у поєднанні з підвищенною температурою води до 25°C (RnT), у поєднанні з хлорпромазином (RnCpz, 18,0 мкг $л^{-1}$ або 56 нМ Cpz) і окремо Cpz протягом 14 днів. Реакції окисного стресу оцінювали в травній залозі. Активність ферментів антиоксидантного захисту змінювалась лише після впливу Rn (збільшення активності супероксиддисмутази) та Cpz (зниження активності каталази). Підвищення рівня загального глутатіону (GSH) спостерігали у всіх експозиціях, крім Rn, а тіолів, у складі металотіонеїнів (MTSH), –у всіх групах, крім Cpz. Посилення перекисного окиснення ліпідів відбувалося в усіх випадках максимально на 16,6 %. Загальний баланс антиоксидантів у порівнянні з прооксидантними змінами збільшувався в

дослідних експозиціях, що містили Rn (у ~ 3 рази в групі RnT), і зменшувався при впливі лише Cpz. Особливості хроматографічного аналізу металотіонеїнів експерементальних 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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