Predicted No-Effect Concentration of Roundup in the Composition with Pharmaceutical Chlorpromazine or Heating Causes Similar Biochemical Disturbances in Bivalve Mollusk *Unio Tumidus*

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Abstract

The ability of bioindicators to reflect the specific impacts in complex exposures is unpredicted. This study aimed to track the particular effects of pesticide Roundup (Rn) and antipsychotic drug chlorpromazine (Cpz) on the mussel *Unio tumidus* at environmentally relevant mixtures. The mussels were treated by Rn ($17 \mu\text{g L}^{-1}$), Cpz ($18 \mu\text{g L}^{-1}$), mixture of Rn and Cpz at 18°C (RnCpz) as well as by Rn at 25°C (RnT). Digestive glands were examined after 14 days of exposure. The indexes of stress response (total antioxidant capacity, glutathione (GSH&GSSG), metallothioneins (MTSH and Zn-MT), protein carbonyls levels), and markers of metabolic and detoxication (CYP450 related (EROD), Glutathione *S*-transferase (GST), cholinesterase, caspase-3, citrate synthase (CS) activities), lysosomal membrane integrity, and Zn level were analyzed. Mostly common responses of mussels were indicated as the increase of oxidative stress, MTSH (except Cpz-group), EROD and CS (except Rn-group) responses. Rn-group indicated almost no-effect or abnormal for expected symptoms effect. However, under the heating Rn caused the decrease of Zn accumulation and loss of lysosomal integrity. Cpz provoked major response diverseness: a decrease in Zn and GST levels and an increase in lysosomal integrity. Thus, complex exposures abolished the individual response traits. Summarising, the application of integrated indices has benefits when evaluating the effects of complex exposures.

Highlights

1. Exposure of bivalves to Roundup (Rn) and heating (T) or chlorpromazine (Cpz) in nanomolar concentrations.
2. Antioxidants and metallothioneins represent common hormetic-like responses.
3. Specific responses to Rn and Cpz were abolished in their joint exposure.
4. RnT decreases tissue Zn and lysosome integrity indicating damage.

1. Introduction

Bivalve molluscs are recognised bioindicators of surface water chemical pollution and climate changes. Various integrative biomarkers have been utilised for characterising the health status of these organisms affected by abiotic stresses (Louis et al. 2020). However, specific responses of molluscs to certain xenobiotics are not well understood and were examined generally in the exposures to single substances at high concentrations (Giménez and Nunes 2019), which are hardly expected in the environment unless the accidental release of pollutants occurs. The demand for “evidence-based ecotoxicology” (Martin et al. 2019) includes elaborating of the environmentally realistic models for the risk assessment, including the testing of the responses to combine exposures at low environmental concentrations. This study aimed to track the particular effects of pesticide Roundup (Rn) and antipsychotic drug chlorpromazine (Cpz) on the mussel *Unio tumidus* at environmentally relevant mixtures.

The substances selected for the study, herbicide Roundup (Rn) and drug chlorpromazine (Cpz), are common aquatic pollutants. The Rn (a commercial form of glyphosate) belongs to the most frequently used pesticides globally as a weed killer (Matozzo et al. 2020). The levels of glyphosate in the freshwaters reach hundreds of $\mu\text{g L}^{-1}$ (Bonansea et al. 2018). It has been suggested that one of the possible reasons for glyphosate toxicity could be its chelating properties towards divalent metal ions that alter the bioavailability of nutrients in plants and soil microorganisms (Mertens et al. 2018). Therefore, its impact on the metal metabolism in the non-targeted species can be expected. However, the signs of its toxicity for aquatic species are mostly reported as the oxidative stress responses, endocrine, immune and histological alterations at as low as $0.1\text{--}100\ \mu\text{g L}^{-1}$ concentrations in the *in vitro*, *ex vivo*, acute and sub-chronic *in vivo* exposures (El Haj et al. 2019; Khoma et al. 2020, 2021; Matozzo et al. 2020 and references therein).

Another studied chemical Cpz is the first generation neuroleptic medication known as dopamine D_2 receptor antagonist (Li et al. 2016). The Cpz investigation was motivated by its popularity. Among 197 substances analysed in hospital wastewater, chlorpromazine belongs to 15 particularly hazardous chemicals present at high concentrations (mg L^{-1}) (Frédéric and Yves 2014). The persistence of Cpz in river water and strong adsorption on sediments have also been reported (Jiménez et al. 2016). According to Baresel et al. (2015), the mean Cpz concentration in Swedish sewage treatment plant effluents amounts to $11.3\ \text{ng L}^{-1}$. Recently, chlorpromazine has been found to have antiviral activity *in vitro* against the influenza virus, HIV. Actually, it is listed among "the most promising molecules for inhibiting coronaviruses in human cells" (Stip et al. 2020). The drug is known to interact with the cell membrane dynamin and block clathrin-dependent endocytosis essential for coronavirus entry into the host cell (Plaze et al. 2020). Consequently, its input into the water runoff is expected to be enhanced. The biological effects of Cpz are associated with calcium homeostasis (Xu et al. 2010) and manganese toxicity (Nelson et al. 2018). Therefore, its biochemical effects expected to be related to the involvement in metals uptake and metabolism. High toxic pressure of Cpz has been confirmed on the fish plasma model in the concentration of $36\ \text{ng L}^{-1}$ (Miller et al. 2019). However, the results of acute and chronic exposures of cladoceran *Daphnia magna* (Oliveira et al. 2015; Alkimin et al. 2020) and mussels *Mytilus galloprovincialis* (Yang et al. 2011) to Cpz in a range from 0.01 to $250\ \mu\text{g L}^{-1}$ almost did not show the changes in terms of physiological and biochemical parameters.

The elevated temperature has been shown to exacerbate the toxicity or distort effects in bivalve molluscs induced by xenobiotics including mixtures containing Roundup (Negri et al. 2013; Patra et al. 2015; Payton et al. 2016; Louis et al. 2020; Khoma et al. 2021). Mainly, metal metabolism and toxicity depend on thermal stress, and lysosomal biomarkers reflect this impact in mollusks (Izagirre et al. 2014).

Based on our own experience (Khoma et al. 2020, 2021) and aiming to provide environmentally expected limits of the impact, we used the low Rn concentration of $17\ \mu\text{g L}^{-1}$ that corresponds to $6.1\ \mu\text{g L}^{-1}$ or $36\ \text{nM}$ of glyphosate. This concentration commensurate with approximately 0.2 of Predicted No Effect Concentration (PNEC), the estimate derived from multispecies data for long-term exposures (Maycock et

al. 2012). Cpz concentration of $18 \mu\text{g L}^{-1}$ or 57 nM has been chosen to cause minor physiological effects in aquatic animals (Oliveira et al. 2015). The duration of exposure (14 days) and two temperatures (18 and 25 °C) were based on the previous experience (Khoma et al. 2021), the latter in relevance to the range of water temperatures in the sampling area at the Dniester River basin.

Due to the suspected ability of both substances to affect metal metabolism, we focused on the metallothionein-related thiols and their participation in zinc accumulation, the level of glutathione (GSH/GSSG), and lysosomal integrity. Also, we evaluated the oxidative stress and biotransformation activities (cytochrome P450-related ethoxyresorufine *O*-diesterase and glutathione *S*-transferase), metabolic activity (citrate synthase), neurotoxicity (basing on the cholinesterase activity), and apoptosis (through the activity of caspase-3, the central executive enzyme). An integrative analysis of the biomarker responses was applied for the comparison of experimental groups.

2. Materials And Methods

Methodology used is given in detail in Supplementary materials.

2.1. Chemicals

All reagents were of the Reagent grade or higher (S1 Appendix). They were obtained from Sigma-Aldrich (USA) or the Synbias (Ukraine). Roundup (Rn) formulation was Roundup MAX, Monsanto, USA, and chlorpromazine (Cpz) was of pharmaceutical grade, AMINAZINUM, Pat "Halychfarm", ATX N05A A01.

2.2. Experimental groups

Adult bivalve molluscs *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 a reference (Gnatyshyna et al. 2020). Specimens were transported to the laboratory and preacclimated to the laboratory conditions for up to seven days after the capture in the aerated, dechlorinated, softened tap water and fed 500 mg of Tropical SuperVit Basic contained beta-1.3/1.6-glucan twice a week. After that, molluscs were distributed randomly to five groups. The first group was exposed to the aquarium water only and was considered control (C). The Rn- and RnT-groups were exposed to organophosphonate pesticide Roundup MAX ($17 \mu\text{g L}^{-1}$, corresponding to $6.1 \mu\text{g L}^{-1}$ or 36 nM of glyphosate) at the temperatures 18 °C and 25 °C, respectively. The temperature was increased gradually in the RnT-group during 24 h. The Cpz-group was exposed to $18.0 \mu\text{g L}^{-1}$ or 56 nM of Cpz, and the RnCpz-group - to a mixture of Rn and Cpz at 18 °C. The duration of exposure was 14 days. Water was changed and chemicals replenished every two days. *Throughout the experiment*, molluscs were fed with the same regularity. No mortality was detected during the experimental exposures.

After exposures, molluscs were dissected on ice. For lisosomal membrane stability and cholinesterase activity, the samples were utilised immediately. For all analyses except metallothioneins and caspase-3, tissues 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 (PMSF) for proteolysis inhibition. Homogenates were centrifuged at 6000g for 10 min, and the resulting supernatant was kept at -40 °C. For the analysis of cholinesterase, the 10% w/v homogenate was prepared in the same buffer without PMSF. The protein concentration was analysed in the 6000g supernatant according to the method of Lowry et al. (1951), using bovine serum albumin as the protein standard.

2.3 Assays for metallothionein and glutathione

Concentration of metallothioneins protein (MT) was assessed using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) reduction method after the ethanol/chloroform extraction from tissue 1:3 w/v homogenate in 20 mM Tris-sucrose buffer with 0.1% β -mercaptoethanol, 0.5 mM PMSF, 6 μ M leupeptine (Viarengo et al. 1997). The concentration of MT was expressed in μ g g⁻¹ tissue FW.

For the evaluation of Zn concentration in metallothioneins (Zn-MT), 70 mg of tissue per individual (to a total of 350 mg from five specimens in the group) were combined. Two replicates for each group were accomplished. The samples were homogenized in 10 mM Tris-HCl buffer, pH 8.0 containing 10 mM β -mercaptoethanol and 0.1 mM PMSF and subjected to the isolation of thermostable supernatant. The MT-contained fractions were isolated from the supernatant by size-exclusion chromatography on Sephadex G-50 with necessary adjustments needed to avoid their oxidation (Roesijadi and Fowler 1991). Low weight (approximately 7 kDa) fractions with high absorbance at 254 nm and high D₂₅₄/D₂₈₀ density ratio were identified as putative MTs-containing peak and pooled (to the total of 10 mL) for the Zn determination.

Total glutathione and oxidized glutathione (GSSG) concentrations were quantified by the glutathione reductase recycling assay (Griffith 1980) in the protein-free extract of homogenate using DTNB. Concentration was expressed as nmol g⁻¹ FW. The concentration of the reduced glutathione (GSH) was calculated as the difference between the total glutathione and GSSG concentrations. The redox-index of glutathione (RI GSH) as the ratio of concentrations GSH/GSSG was calculated.

2.4. Oxidative stress and toxicity assays

Total antioxidant capacity (TAC) was determined as ABTS radical scavenging activity (Re et al. 1999). ABTS^{•+} radical cations (ABTS^{•+}) were generated from 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) by potassium persulfate. The ascorbic acid was used as the reference compound. The reduction in absorbance of ABTS^{•+} solution was recorded at 734 nm. The result was compared with control (only ABTS^{•+} solution).

Protein carbonyls (PC) were determined as an index of protein oxidation in the sediment of 10% w/v homogenate in sulfosalicylic acid after its centrifugation by reaction with 2,4-dinitrophenylhydrazine

(DNPH) (Reznick and Packer 1994). The concentration of carbonyls was expressed in nmol PC per g FW.

Cholinesterase (ChE, EC 3.1.1.7) activity was determined in the homogenate according to the colorimetric method of Ellman et al. (1961) at 25 °C. Acetylcholine iodide (ATCh) was used with DTNB as the thiol indicator. Enzyme activity was referred to the protein content.

Lysosomal membrane integrity was determined by Neutral Red retention (NRR) test based on the lysosomes ability to concentrate the dye as it was described in El Haj et al. (2019). The tissue samples (30 mg) were incubated for 2 h with a saline solution containing NR, washed and fixed in formaldehyde (0.5% in 1% CaCl_2). After fixation, the formaldehyde impregnated tissue fragments were removed and frozen (up to one week). Dye was extracted in acid alcohol (1% acetic acid in 50% ethyl alcohol) and analyzed spectrophotometrically at 550-nm.

The ability of exposures to induce cytochrome P450 (CYP450) activity was quantified as 7-ethoxyresorufin *O*-deethylase (EROD) activity in the supernatant of 10% w/v homogenate by measuring the formation of resorufin at 572 nm (Klotz et al. 1984). The reaction was initiated by the addition of 0.5 mM NADPH. EROD activity was calculated using a molar extinction coefficient of $73.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and referred to the soluble protein concentration.

Glutathione S-transferase (GST, EC 2.5.1.18) activity was assayed using GSH and 1-chloro-2,4-dinitrobenzene (CDBN) as the substrate (Habig et al. 1974). The GST activity was expressed in $\text{nmol min}^{-1} \cdot \text{mg}^{-1}$ soluble protein.

Caspase-3 (EC:3.4.22.56) activity as the marker of apoptosis was assayed colorimetrically in 25% w/v homogenate of digestive gland tissue based on the hydrolysis of peptide acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3 that produces a colored product p-nitroaniline (pNA). The pNA was detected at 405 nm ($\epsilon_{\text{mM}} = 10.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) (Du et al. 1997). The activity of caspase-3 was expressed in $\text{nmol pNA min}^{-1} \text{ mg}^{-1}$ of soluble protein.

Citrate synthase (CS, EC 2.3.3.1) activity was measured in the 10% w/v homogenate of the digestive gland according to Flynn et al. (2015) as the maximum rate of increase in absorbance at 412 nm, caused by the production of a coenzyme A-SH and monitored by DNTB. CS enzyme activity was calculated by subtracting the background activity (negative control) from the CS enzyme activity (positive reaction) for each sample and quantified using the molar extinction coefficient of DTNB ($14,150 \cdot \text{M}^{-1} \text{ cm}^{-1}$).

2.5. Zinc concentration in the tissue and metallothioneins

The concentration of Zn was measured in the digestive gland tissue (Zn-t) and the pooled MT-containing eluate (Zn-MT) received by chromatography 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) (Wang et al. 2018). The samples were dried at 105 °C for 24 h and then digested with HNO_3 . The received ash was dissolved in 1% trichloroacetic acid, then 0.6 M 5-Br-PAPS in carbonate buffer (pH 8.9)

was added. The mixture was incubated for 30 min at 20⁰ C. Zn concentration was evaluated from the absorbance of the metal-5-Br-PAPS complex at 560 nm. Fe ions were masked with citric acid, and Cu ions were masked with salicylaldoxime, deferoxamine, and sodium citrate. The detection limit was 0.1 µg·g⁻¹ FW. Quality control was performed by the method of Standard Addition. Metal concentration in the tissue and MTs was expressed as nmol or µg·g⁻¹ FW.

2.6. Statistical analysis

Results were expressed as mean ± standard deviation. Metal in MTs analysis was repeated in four samples for each of two independent combined from five individuals replicates in a group, resulting in n = 8 for each group. For all other traits, the sample size was eight from eight individuals. Shapiro-Wilk test was used for the assessment of normality. Data were analyzed with parametric Student's t-test significant at p < 0.05. Principal component analysis (PCA) was performed to assess the relations between measured parameters utilizing the rotation method Varimax. The adequacy of data was evaluated based on the value of the KMO and Bartlett's test of sphericity. Canonical discriminant analysis was utilised for the separation of the exposed groups. The IBM SPSS Statistics version 26 software for Windows was used for calculations. Correlation was significant at p < 0.05 level (r > 0.304) and p < 0.01 (r > 0.393) (2-tailed), n = 40.

Integrated Biomarker Index (IBR) elaborated by Beliaeff and Burgeot (2002) was calculated for biomarkers (totally 14). The indices of CAP, EROD, GST, protein carbonyls (PC), GSH, GSSG, RI GSH, metallothionein concentration (MTSH), lysosomal integrity (NRR), caspase-3 activity (Cas-3), cholinesterase activity (ChE), citrate synthase (CS), Zn-metlothionein (Zn-MT) and Zn total (Zn-t) were used for the computation of data. The standardization of data was achieved by Xi calculation: $X_i = (M_i - M_t) / SD_i$. For Ai calculation, the computation of data was made as to the Xe-Xc, assuming that the changes of the value in each exposed group (Xe) in relation to control (Xc) is corresponding to the stress or toxicity responses, and the value of standardized marker Xc was adjusted to zero. IBR value for each group was calculated as $[(A_1 \times A_2) + (A_2 \times A_3) + \dots (A_n \times A_1)] / 2$. Since the value of IBR is dependent on the number of markers, the termed IBR value was given as IBR/n with n = 14.

3. Results

3.1. Cellular soluble thiols and zinc accumulation in the digestive gland

The level of GSH increased in all exposures except Rn, particularly, by 1.8 times, in the RnCpz-group. In the latter group, the level of GSSG was also increased. As a result, the RI GSH increased compared to control in all exposed groups except the Rn-group by 36.0–48.8% (Fig. 1a-c). MT concentration increased by 33.6–57.7% in all Rn-contained exposures, mostly in the RnT-group, whereas Cpz did not change the level of MT (Fig. 1d). Zn-t concentration decreased in the RnT- and Cpz-groups compared to control value by 15.5 and 24.3% correspondingly, whereas the level of Zn-MT increased in all groups, particularly by ~53% in the RnT- and Cpz-groups (Fig. 1e and f).

3.2 Indexes of stress and toxicity

The evaluation of oxidative stress responses indicated substantial activation of the TAC by 23.4–51.3% in all exposures (Fig. 2a). The level of protein oxidation products (PC) increased compared to control in all exposures except the Rn-group (Fig. 2b). The highest increase by 32.5% was recorded in RnT-group.

The monooxygenase-related biotransformation function (CYP450) attested from the EROD activity was greatly enhanced by 2.4–3 times in all treated groups except the Rn-group (Fig. 2c). The changes of GST activity compared to control occurred in single treatments, i.e., increase by 26.2% in the Rn-group and decrease by 21.9% in the Cpz-group. In contrast, no changes were evident in the joint treatments in the RnT- and RnCpz-groups (Fig. 2d).

For the indexes of toxicity, some differences were also found (Fig. 3). ChE activity increased compared to control value in the Rn-, RnCpz- and Cpz-groups and did not differ from control in the RnT-group (Fig. 3a). The lysosomal integrity decreased in the RnT-group, increased in the Cpz-group and did not change in the rest of the groups (Fig. 3b).

The activity of the central executive enzyme of apoptosis, caspase-3, was substantially increased in the molluscs' exposures to Rn and RnCpz (by 36.4%) and was not changed in other treatment groups (Fig. 3c). The metabolic intensity was indicated by the citrate synthase activity. It enhanced compared to control in all exposures, except the Rn-group, particularly in the Cpz-related groups, by 36.5–43.0% (Fig. 3d).

3.3. Data integration

The Pearson correlation analysis revealed multiple associations among the studied indices in *U. tumidus* across all experimental groups (SI Tabl. B1). The greatest number of correlations was found for the indexes of oxidative stress and GSH. In opposite, MT and NRR did not show any correlations. The application of PCA to the datasets to identify relations between indices (Fig. 4a) shows a good distribution of the variables with a factor loading higher than |0.37|. The set of Zn-MT, TAC (ABTS*), PC, EROD, GSH, RI GSH and CS belonged positively to the first principal component (PC1) and Zn-t located opposite. The set of caspase-3, ChE and GST was related positively to PC2 and opposite to CS with factor loading higher than |0.42|. Generally, 53.6% of the variance was accounted for the first three factors.

According to Canonical discriminant analysis, all specimens in the C-, RnCpz- and Cpz-groups and 75% of specimens in the Rn- and RnT-groups were classified correctly. Fig. 4b shows that the C- and Rn-groups jointly (group centroids -7.55 and -3.96, correspondingly) are well separated in the space of discriminant variables along the axis 1 compared to located oppositely the Cpz-group with the centroid value 7.33. Subjected to combined exposures, the RnT- and RnCpz-groups were placed between these opposite positions along axis 1 and located similarly with centroids values 2.97 and 2.51 along axis 2.

The calculated values of IBR/n in relation to baseline (control) value equaled 0.54, 0.69, 1.71 and 1.84, respectively for the Rn- RnT-, RnCpz- and Cpz-groups. The highest IBR/n values were observed in the

RnCpz- and Cpz-group, indicating the particular increase in the A values for most indexes (Fig. 5). The input of Zn-MT was the highest similarly in all groups. The A values in the Cpz group were opposite to other groups for plural indexes. On the other hand, the changes compared to control in the Rn-group were lesser for all indices except Zn-MT and GSH.

4. Discussion

This study aimed to indicate whether the combine exposure or heating can distort the specific responses of bivalve molluscs to aquatic pollutants at lower possible environmental concentrations. Discriminant analysis indicated the lesser distinction from control for Rn-group, confirming the effect of concentration equalled 0.2 PNEC as almost negligible. The highest difference was detected for the Cpz-group (Fig. 4b, 5). Interestingly, the concerted exposures (RnT- and RnCpz-groups) produced similar integrative responses, distinct from each exposure to individual substance. However, a number of peculiar manifestations were indicated in each group.

4.1 Can Zinc-related parameters serve as specific biomarkers of effect for low concentrations of Roundup and chlorpromazine?

The involvement of Zn in cellular defence and regulation seems to be the common mechanism that can determine the resistance to adverse impacts. Various xenobiotics and physicochemical factors have been reported to result in the impairment of Zn metabolism, and subsequently, Zn deficiency in animals (Krężel and Maret 2016). As it has been shown for several organisms, Rn can chelate metals and cause their imbalance (Tsui et al. 2005; Liz Oliveira Cavalli et al. 2013; Mertens et al. 2018). Both Rn and Cpz can affect Zn functionality indirectly via Ca-depending processes whereas the cellular entry of Zn can be realized through several types of Ca-permeable channels (Bouron and Oberwinkler 2014; Hamaguchi et al. 2014). Particularly, Rn and glyphosate selective influence L-type Ca channels and their genes in the exposures of rat testis or isolated Sertoli cells and zebrafish embryos (Liz Oliveira Cavalli et al. 2013; Gaur and Bhargava 2019). Cpz also affects many Ca-dependent processes (Xu et al. 2010; Hamaguchi et al. 2014). Indeed, we found a decrease in the Zn uptake in the RnT- and Cpz-groups (Fig. 1a), whereas Rn *per se* did not change Zn t. Importantly, at twice higher concentration, than in this study, Rn also decreased the Zn t level in the digestive gland of *U. tumidus* only in the mixture at elevated temperature (Khoma et al. 2021). Similarly, distortion of the response to Rn by warming was shown by Amid et al. (2018) in the tropical staghorn coral *Acropora formosa*. The specificity of the effect of Cpz on the molluscs is supported by the presence of a dopamine D₁-like receptor in the albumen gland of air-breathing freshwater snail *Helisoma duryi* (Planorbidae) (Mukai et al. 2004). Nevertheless, this effect of Cpz was abolished in the complex exposure of *U. tumidus* to Rn and Cpz. Thus, we confirm the ability of Rn and Cpz impact the Zn accumulation in the tissues, although for Rn only under the heating.

The elevated accumulation of Zn in MTs was the shared manifestation (Fig. 1f). MT plays a critical role in Zn homeostasis under various conditions but Zn chelating in the thiolate clusters of MT depends on the redox state of thiols (Krężel and Maret 2016). Adverse effects of different nature decrease the level of

labile Zn and metalation of MT in molluscs even when the total level of this protein is increased (Khoma et al. 2021). In the present study, the elevation of Zn-MT was well coordinated with the TAC and GSH responses (Fig. 4a). Consequently, this common response can indicate the enhancing of antioxidant activity and redox state of thiols. In opposite, the loss of MT metalation is frequently accompanied by plural signs of toxicity. For example, in the exposure to 33.6 µg/L of Rn, the depletion of Zn-MT was accompanied by the decrease of the ChE and GST activities, lysosomal integrity, and increase in the GSSG level (Khoma et al. 2021). In the present study, the signs of toxicity were almost absent. This attested the low molecular weight thiols as the successful biochemical drivers that provide the redox power in the exposures of low-level toxicity and provide the hormetic-like response.

4.2 Enzymes of biotransformation as the probable targets for Roundup and chlorpromazine

Data concerning the biotransformation of Rn and Cpz by the enzymes of phases I and II are scant and contradictory. Phosphonate-based pesticide formulations are known to cause the reduction in CYP450 enzyme levels in mammals (Mesnage and Antoniou 2017). However, the exposure of rats to Rn in the drinking water caused a decrease of EROD activity in male, but an increase in the female specimens (Larsen et al. 2014). Concerning the Cpz, it has been mentioned that a deficient number of studies were designed to investigate the environmental fate of any tricyclic antipsychotic drug (Trautwein and Kümmerer 2012). Cpz had to be classified as non-biodegradable in all of the biodegradation tests with aquatic bacteria (Trautwein and Kümmerer 2012; Jiménez et al. 2016). However, its biotransformation products have been elucidated by multiple-stage mass-spectrometry (Trautwein and Kümmerer, 2012). In the present study, the increase of EROD activity was not a specific for Cpz feature. The strong correlation between EROD and a set of indices of antioxidant and metabolic activities and Zn-MT attested this increase as a part of a well-coordinated, probably hermetic-like defensive response to low concentrations of xenobiotics (Fig. 4a).

The GST activity did not correlate with EROD in this and other studies on aquatic animals from polluted areas or exposed to several substances (Domingues et al. 2010). The decrease or absence of GST response has been reported in the exposures of molluscs to higher Rn or glyphosate concentrations (Matozzo et al. 2019; Khoma et al. 2021). In the present study, GST activation can be explained by the low-strength Rn impact. GST activation has been also indicated in the testes of rats exposed to Rn (Liz Oliveira Cavalli et al. 2013). For the Cpz, the decrease in GST activity was one of the most particular reactions observed in this study. The inhibition of GST has also been shown for the effect of Cpz *in vitro* (Türkan et al. 2020). Moreover, among four tested drugs, Cpz has been found to be the best inhibitor for the GST enzyme (Oliveira et al. 2015). Hence, the two manifestations confirmed in our study, Zn and GST levels' decrease, might be effect-specific for this drug. However, both of them were abolished in the combined RnCpz-exposure.

In the present study, the GST activity was coordinated with those of the ChE and caspase-3 (SI Tabl. B1, Fig. 4a). The responses of GST and ChE are frequently analysed jointly as the prospective biomarkers in molluscs (Domingues et al. 2010). For the organophosphates acute effect, the expected response is the

depletion of ChE (Li et al. 2018; Matozzo et al. 2019), whereas the upregulation of ChE in molluscs is seldom detected and discussed (Domingues et al. 2010). In this study, upregulation of ChE can be a sign of the non-toxic level of exposure to Rn, that is consistent with other manifestations in Rn-group. In the vertebrates, the simultaneous increase in ChE and caspase-3 activities has been indicated (Hu et al. 2009), and ChE has been proposed as a marker for apoptosis (Zhang and Greenberg 2012). This common regularity has been detected for the neurons of insect *Locusta migratoria*, where the addition of the ChE inhibitors reduced apoptotic cell death (Knorr et al. 2020). In the freshwater mussels *Unionidae*, simultaneous increase of ChE and caspase-3 activities has been indicated under the effect of pesticides, pharmaceuticals and heating (Khoma et al. 2021). Nevertheless, the common regularity in alterations of GST, ChE and caspase-3 can not be explained unambiguously basing on the current study data. Hence, we found that EROD activation was a constituent of the common, probably hermetic-like, stress response, while the set of GST, ChE and caspase-3 activities was more exposure-specific.

4.3 How can we attest the severity of injury?

The analysis of each studied biomarker confirmed that we used low enough concentrations of xenobiotics, which did not provoke obvious toxicity. The mobilisation of antioxidants and alterations of metabolic activity correspond to low dose stimulation or hormetic response (Lefcort et al. 2008). This kind of response was evident from TAC and CS activation. A significant, but not a prominent increase of protein carbonylation can be regarded as the triggering signal for the antioxidant enhancement. Similarly, the EROD activation can induce antioxidants (He et al. 2017). Particularly, low-weight thiols seem to be of decisive importance. High inter-correlations were shown in the set of TAC (ABTS*), Zn-MT, GSH, RI GSH, CS and EROD (Fig. 4a).

GSH is on the first line of antioxidant defence as a radical scavenger. Plural examples show the involvement of GSH in the antioxidant activity in the bivalve molluscs (Regoli et al. 2011; Gnatyshyna et al. 2020; Khoma et al. 2021). In these examples and precisely under the exposures to Rn at different models, the responses of GSH&GSSG are highly different (Larsen et al. 2014; Melo Tarouco et al. 2017) depending on the severity of impact. In any case, GSH and MTSH (last except Cpz-group) activation can support the redox balance and provide the Zn-chelating in the present study's model exposures.

In this study, the joint exposure to Rn and heating can be qualified as the most disturbing. Only in this group, the lysosomal integrity was decreased. Similar observation has been indicated previously at higher concentration of mixture included Rn and heating (Khoma et al. 2021). The loss of the lysosomal integrity is an approved sign of toxicity and thermal stress in molluscs (Negri et al. 2013; Izagirre et al. 2014; Koagouw and Ciocan 2018). However, the opposite effect on the lysosome integrity was indicated in the Cpz-group. This unusual effect has also been observed for the Ca channel blocker nifedipine (Khoma et al. 2021). Both these pharmaceuticals can be involved in the Ca-related functions of lysosomes, that explain the similarity of the lysosomal manifestation. Indeed, it has been shown that the pH homeostasis in lysosomes/late endosomes is rather stable in the presence of cationic amphiphilic drugs and strictly maintained even after a longer treatment with Cpz (Hamaguchi et al. 2014).

Generally, the calculated IBR-indices gave the possibility to analyze both specific and common traits of responses, and detect their severity (Fig. 5).

5. Conclusions

The applied low subchronic exposures to common aquatic pollutants allowed indicating hormetic-like mobilisation of antioxidant and metabolic responses in the bivalve molluscs with certain specificity in the Roundup- and chlorpromazine-exposed groups. Complex exposures abolished this specificity, and heating enhanced the toxicity of almost no-effect concentration of Roundup to molluscs. Discriminant analysis and IBR calculation of the battery of biomarkers highlighted the benefits of multi-marker expertise in identifying of response strength and specificity.

Declarations

Ethical Approval and Consent to Participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent to Publish

Not applicable.

Authors Contributions

Oksana Stoliar contributed to conception and design, acquisition, analysis, interpretation, drafted manuscript, and gave final approval. Levonas Manusadžianas contributed to conception, design, critically revised manuscript, and gave final approval. Lesya Gnatyshyna contributed to design, analysis and interpretation and gave final approval; Vira Khoma contributed to experimental analysis, statistic preparation and gave final approval; Viktoria Martinyuk contributed to sampling, analysis and gave final approval; Tetyana Matskiv contributed to analysis, draft preparation and gave final approval; Vitaliy Baranovsky contributed to statistic preparation, acquisition and gave final approval, Mykola Gladiuk contributed to sampling, draft preparation and gave final approval; Brigita Gylytė contributed to draft preparation and gave final approval.

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Competing Interests

The authors declare that they have no conflict of interest. Informed consent was obtained from all individual participants included in the study.

Availability of data and materials

The datasets generated and/or analysed during the current study (experimental data, calculation of IBR, and the applied methods) are available in the Mendeley Data repository by the following link <http://dx.doi.org/10.17632/365bfpsz4w.3>

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Figures

Figure 1

Concentrations of soluble thiols and Zn in the digestive gland of *U. tumidus* exposed to Roundup (Rn), Roundup and heating (RnT), Roundup and chlorpromazine (RnCpz), and chlorpromazine (Cpz) during 14 days. (a) GSH. (b) GSSG. (c) Redox index of glutathione (RI GSH). (d) Metallothionein (MT). (E) Zn in the tissue (Zn-t). (F) Zn in metallothioneins (Zn MT). Data are presented as means $\pm$ standard deviations ($n = 8$). Different letters above the columns indicate significantly different values ($P < 0.05$)

Figure 2

Oxidative stress and biotransformation parameters in the digestive gland of *U. tumidus* after 14 days of experimental exposures to Roundup (Rn), Roundup and heating (RnT), Roundup and chlorpromazine (RnCpz) and chlorpromazine (Cpz) during 14 days. (a) Total antioxidant (ABTS radical scavenging) activity (ABTS*). (b) Protein carbonyls production (PC). (c) 7-EROD activity; (d) GST activity. Data are presented as means $\pm$ standard deviations ($n = 8$). Different letters above the columns indicate significantly different values ($P < 0.05$)

Figure 3

Cytotoxicity and metabolism parameters in the digestive gland of *U. tumidus* exposed to Roundup (Rn), Roundup and heating (RnT), Roundup and chlorpromazine (RnCpz) and chlorpromazine (Cpz) during 14 days. (a) Cholinesterase (ChE) activity. (b) Lysosomal membrane stability (NRR). (c) Caspase-3 activity. (d) Citrate synthase (CS) activity. Data are presented as means $\pm$ standard deviations ($n = 8$). Different letters above the columns indicate significantly different values ($P < 0.05$)

Figure 4

The results of Principal Component Analysis applied to reveal the relations between measured parameters (a) and scatter plots of the canonical values on the first and second canonical discriminant axes to discriminate the groups of molluscs (b). a: The abbreviations see in the text. B: control group (1); groups exposed to Roundup (2), Roundup and heating (3), Roundup and chlorpromazine (4), and chlorpromazine (5) during 14 days

Figure 5

Biomarker star plots (Ai variables) of multiple biomarker responses of *U. tumidus* exposed to Roundup (Rn), Roundup and heating (RnT), Roundup and chlorpromazine (RnCpz), and chlorpromazine (Cpz) during 14 days compared to control values considered as zero values (C)

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