

Original article:

**LOW-TOXIC HERBICIDES ROUNDUP AND ATRAZINE DISTURB FREE RADICAL PROCESSES IN *DAPHNIA* IN ENVIRONMENTALLY RELEVANT CONCENTRATIONS**

Viktor Husak<sup>1</sup> , Tetiana Strutynska<sup>1</sup> , Nadia Burdyliuk<sup>1</sup> , Anzhelika Pitukh<sup>1</sup> ,  
Volodymyr Bubalo<sup>2</sup> , Halina Falfushynska<sup>3</sup> , Olha Strilbytska<sup>1</sup> , Oleh Lushchak<sup>1,4\*</sup> 

<sup>1</sup> Department of Biochemistry and Biotechnology, Vasyl Stefanyk Precarpathian National University, 57 Shevchenka Str., Ivano-Frankivsk, 76018, Ukraine

<sup>2</sup> Laboratory of Experimental Toxicology and Mutagenesis, L.I. Medved's Research Center of Preventive Toxicology, Food and Chemical Safety, MHU, Kyiv, Ukraine

<sup>3</sup> Ternopil Volodymyr Hnatiuk National Pedagogical University, M. Kryvonosa, 2, Ternopil, 46027, Ukraine

<sup>4</sup> Research and Development University, 13a Shota Rustaveli Str., Ivano-Frankivsk, 76018, Ukraine

\* **Corresponding author:** Oleh Lushchak, Department of Biochemistry and Biotechnology, Vasyl Stefanyk Precarpathian National University, 57 Shevchenko Str., Ivano-Frankivsk, 76018, Ukraine. Tel/Fax.: +380342714683; E-mail: [oleh.lushchak@pnu.edu.ua](mailto:oleh.lushchak@pnu.edu.ua)

<https://dx.doi.org/10.17179/excli2022-4690>

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>).

**ABSTRACT**

The use of glyphosate-based Roundup and triazine herbicide Atrazine has increased markedly in last decades. Thus, it is important to evaluate toxic effects of these herbicides to non-targeted organisms such as zooplankton to understand their safety toward aquatic ecosystems. In the current study, we performed *Daphnia* toxicity tests based on lethality to identify LC<sub>50</sub> that provides acute aquatic toxicity classification criteria. LC<sub>50</sub> for Roundup exposure for 24 hours was found to be 0.022 mg/L and 48 hours - 0.0008 mg/L. Atrazine showed LC<sub>50</sub> at concentrations of 40 mg/L and 7 mg/L for 24 and 48 hours, respectively. We demonstrated that exposure to ecologically relevant concentrations of Roundup or Atrazine decreases lipid peroxidation and protein thiol levels, however caused increase in carbonyl protein and low-molecular-weight thiols content. Moreover, the herbicide treatments caused increase of superoxide dismutase activity. Our data suggest that at very low concentrations Roundup and Atrazine disturb free radical processes in *D. magna*.

**Keywords:** Roundup, Atrazine, *Daphnia*, oxidative stress, toxicity

**INTRODUCTION**

The antiweed herbicides Roundup and Atrazine are among most commonly used treatments in the world. Research surrounding safety of these herbicides has been controversial since Roundup causes varied diseases from cancer to autism (Wang et al., 2019; Pu

et al., 2020), and Atrazine causes breast, ovarian, uterine cancers as well as leukemia and lymphoma (Pathak and Dikshit, 2011). However, reports provided by industry claim they have very low toxicity toward non-target organisms. An active ingredient in Roundup is

organophosphate glyphosate, N-(phosphonomethyl)glycine, in the form of its isopropylamine salt, with additional formulations containing so-called "inert" ingredients in different proportions, that are believed to be more toxic than glyphosate (Phyu et al., 2004). The mechanism of glyphosate toxicity in non-target organisms is mostly unknown, but it may cause uncoupling of oxidative phosphorylation (Peixoto, 2005). Atrazine, a triazine herbicide, that consists of a triazine ring, along with five nitrogen and one chlorine atoms, is used in the form of emulsifiable concentrate, soluble powder and granules. Investigation of the potential toxicity of Roundup and Atrazine on various model organisms may help to elucidate their toxicity and the defense pro-survival mechanisms.

The unique biological features make *Daphnia* a very suitable model system for toxicological evaluations. Different parameters of toxicity, especially those based on lethality, are mainly used in toxicological studies on *Daphnia*. Toxicity parameter, LC<sub>50</sub> is referred as a concentration that causes mortality of 50 % of individuals in a period of observation time. It can be used as the first screening method for the assessment of the lethal toxicity (Guilhermino et al., 2000). Additionally, the toxic effects of tested drugs could be assessed using enzymatic and non-enzymatic biomarkers of toxicity in concentrations below those that cause mortality. Since some toxicants may cause oxidative stress, the toxicity for *D. magna* can be assessed with an array of oxidative stress markers including activities of glutathione-S-transferase, catalase, superoxide dismutase, glutathione reductase (Dionísio et al., 2020; Nkoom et al., 2019) and oxidation-end products of lipids such as thiobarbituric acid reactive substances (Lushchak et al., 2004).

Growing evidence suggests that organismal exposure to commercial herbicide formulations may perturb free radical processes and inhibit the mitochondrial respiratory chain (Bailey et al., 2018). The molecular mechanisms of oxidative stress induction by glyphosate-based Roundup and Atrazine are well

characterized. Uncoupling of mitochondrial oxidative phosphorylation may be a major effect of herbicide intoxication (Pathak et al., 2011; Peixoto, 2005). The impaired mitochondrial function caused by glyphosate-based herbicides can be related to increased ROS generation (Bailey et al., 2018; Gomes and Juneau, 2016). Atrazine induces mitochondrial dysfunction by disruption of membrane potential and depletion of intracellular ATP levels (Liu et al., 2013).

This study aimed to evaluate the effects of Roundup and Atrazine in *D. magna*, affecting the parameters of survival. This is the first study on the effects of Roundup and Atrazine on oxidative stress indicators in *D. magna* exposed to concentrations that did not cause mortality. As reported herein, this research provided important information on the physiological and biochemical effects associated with the exposure of aquatic organisms to Roundup or Atrazine, focusing on disturbance of free radical processes caused by glyphosate-based and triazine herbicides and antioxidant response. The data obtained can be used to improve environmental risk assessments.

## MATERIALS AND METHODS

### Reagents

Phenylmethylsulfonyl fluoride (PMSF), ethylenediamine-tetraacetic acid (EDTA), xylenol orange, cumene hydroperoxide, ferrous sulphate, *N,N,N',N'*-tetramethylethylenediamine were purchased from Sigma-Aldrich Corporation (USA). Roundup and Atrazine were purchased from Bayer AG (Germany) and Shanghai Sinogreatland Industrial Co., Ltd. (China), respectively. All other reagents were of analytical grade.

### Animals and experimental conditions

*Daphnia magna* Straus is kept in the laboratory for more than 10 years. Water fleas were fed baker yeasts (0,25 million cells per L) and half of the water was changed twice a week. Water parameters were  $20 \pm 2$  °C, pH 7.6-7.8, 7.2-7.6 mg/L of O<sub>2</sub>. The photoperiod

was 16h light:8h dark. Experiments were carried out in a static mode, under the same conditions, but with the addition of the commercial herbicides Roundup which contains glyphosate [N-(phosphonomethyl)glycine] or Atrazine at a concentration of 550 or 500 g/L, respectively. Groups of forty 7-8 days old organisms were placed in 3L glass jars with different nominal concentrations of either Roundup or Atrazine herbicides: 0.1; 0.5; 1 and 5 µg/L. Herbicide concentrations used in this work were selected based on an LC<sub>50</sub><sup>24</sup> value (half-lethal dose after 24 h exposure) for Roundup (0.022 mg/L) and for Atrazine exposure (40 mg/L), which were defined according to ISO standard (OECD, 2004; Olorunsogo et al., 1979). Water fleas were treated for 24 h (no mortality was observed at the studied concentrations). Studied parameter were measured in at least four biological replicates.

#### **Acute toxicity tests**

All experiments were performed according to the standard procedure (Olorunsogo et al., 1979) for determining 24 and 48 h LC<sub>50</sub> for *D. magna*. Water fleas were not fed during the tests. Twenty neonates (~ 24 hours old) per treatment in groups of five per 250 mL of test solution were used. Temperature and photoperiod were as described above. Oxygen concentrations and pH levels were determined at 0, 24, and 48 h time points.

Groups of twenty organisms (in quadruplicate with 5 organisms of every instars per replicate) were placed in glass jars with different nominal concentrations of Roundup or Atrazine herbicides: 0.0005; 0.001; 0.005; 0.01; 0.02; 0.03; 0.05; 0.1; 0.5; 1; 3; 5; 10; 30; 50 and 100 mg/L. The animals in a control group were maintained in the same manner but without the addition of drug into the water. Death was recognized as immobilization for 15 s after stimulation by a bright light. After exposure, animals were dried by filter paper and used for analysis.

#### **Determination of oxidative stress indices**

Lipid peroxide (LOOH) content was assayed by the FOX (ferrous-xylenol orange) method (Hermes-Lima et al., 1995). Aliquots of the supernatants were used for the assay as described previously (Lushchak et al., 2005). The content of LOOH was expressed as nanomoles of cumene hydroperoxide equivalents per gram wet mass of tissue.

Carbonyl groups of proteins (CP) were determined as described previously (Lenz et al., 1989). Animals were crashed by homogenization (1:10, w:v in 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 1 mM PMSF) and centrifuged (8,000 g, 15 min, 4 °C). Supernatants were removed and 0.25 ml aliquots were mixed with 0.25 ml of 40 % trichloroacetic acid (TCA) (final concentration 20 %) and centrifuged (5,000 g, 5 min, 21 °C). CP levels were measured in the resulting pellets by reaction with 2,4-dinitrophenylhydrazine, leading to the formation of dinitrophenylhydrazones (Lenz et al., 1989). Values are expressed as nanomoles of carbonyl groups per milligram protein (nmol mg protein<sup>-1</sup>).

Free thiols were measured spectrophotometrically by the Ellman procedure (Ellman, 1959). Total thiol concentration was measured in supernatants prepared as for the CP assays (Lushchak and Bagnyukova, 2006). For determination of low-molecular-mass thiols (L-SH), aliquots of supernatants were mixed with TCA to reach a final TCA concentration of 10 %, centrifuged (16,000 g, 5 min, 21 °C) to remove pelleted protein and the final supernatants were used for the assay. Thiol concentrations were expressed as micromoles of SH-groups per gram tissue wet mass. The high-molecular-mass or protein thiol (P-SH) content was calculated by subtracting the L-SH from the total thiol concentration.

#### **Assay of enzyme activities and protein concentration**

Supernatants were prepared as described above for CP. The activities of antioxidant enzymes including superoxide dismutase (SOD)

and catalase were measured as described previously (Lushchak et al., 2005). One unit of SOD activity was defined as the amount of enzyme (per mg of protein) that inhibited a quercetin oxidation reaction by 50 % of maximal inhibition. Inhibition values for SOD activity were calculated using an enzyme Kinetics computer program (Brooks, 1992). One unit (U) of catalase activity is defined as the amount of enzyme that consumed 1  $\mu\text{mol}$  of hydrogen peroxide per minute expressed per milligram of soluble protein.

Soluble protein concentrations were measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.

### Statistics

The mortality data were used to calculate 24 and 48 h the median lethal concentration ( $\text{LC}_{50}$ ) by Probit analysis (Stephan, 1977). Data are presented as means  $\pm$  S.E.M. Statistical analyses were carried out in Prism 7 (GraphPad Software, USA) using analysis of variance ANOVA followed by the Dunnett's test to compare multiple experimental treatments to the control value with  $p < 0.05$  as statistically significant.

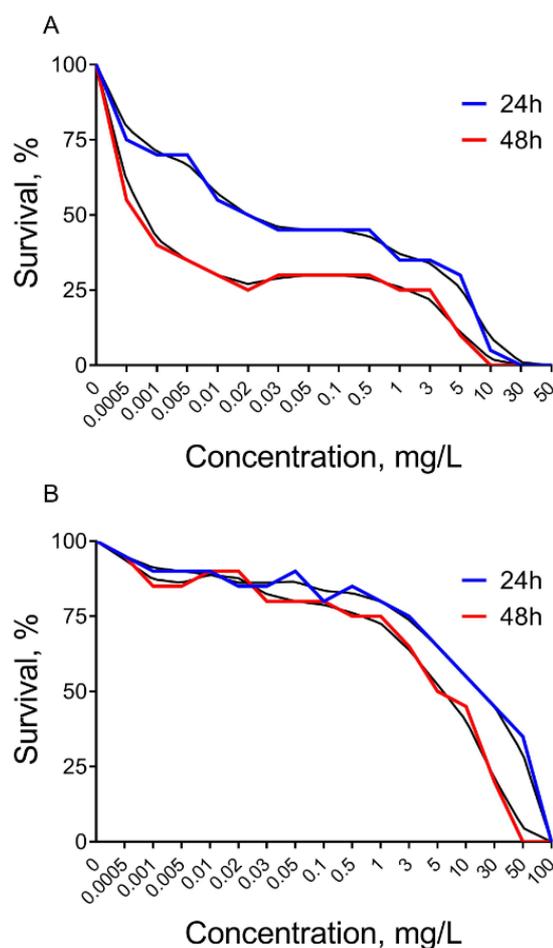
## RESULTS

### Mortality

Roundup herbicide causes *Daphnia* mortality at very low concentrations. According to a Probit test, it was found that Roundup at a concentration of 0.022 mg/L led to 50 % *Daphnia* mortality during 24 h ( $\text{LC}_{50}^{24} = 0.022$  mg/L) (Figure 1A). We observed 50 % of dead individuals after Roundup treatment for 48 hours at a concentration of 0.0008 mg/L ( $\text{LC}_{50}^{48} = 0.0008$  mg/L). We also found complete mortality at concentrations of 30 mg/L ( $\text{LC}_{100}^{24} \geq 30$  mg/L) and 10 mg/L ( $\text{LC}_{100}^{48} \geq 10$  mg/L) during 24 and 48 hours of Roundup exposure, respectively (Figure 1A).

Atrazine herbicide was found to be less toxic on *Daphnia*. According to a Probit test

50 % of *Daphnia* mortality was observed under herbicide exposure at a concentration of 40 mg/L for 24 hours ( $\text{LC}_{50}^{24} = 40$  mg/L) (Figure 1B). Atrazine kills 50 % of the *Daphnia* individuals after 48 hours at a concentration of 7 mg/L ( $\text{LC}_{50}^{48} = 7$  mg/L). Exposure to concentrations of 100 and 50 mg/L of this herbicide resulted in the complete mortality of *Daphnia* population within 24 hours ( $\text{LC}_{100}^{24} \geq 100$  mg/L) and 48 hours ( $\text{LC}_{100}^{48} \geq 50$  mg/L), respectively.



**Figure 1:** The mortality rate of *Daphnia magna* under 24 and 48 hours of exposure to Roundup (A) and Atrazine (B) treatments 0.0005-100 mg/L

### Oxidative stress markers

Based on the  $\text{LC}_{50}^{24}$  for Roundup and Atrazine, which was defined earlier (paragraph "Mortality"), we tested the concentrations of herbicide that was significantly lower than  $\text{LC}_{50}^{24}$  and did not cause the death of *Daphnia*

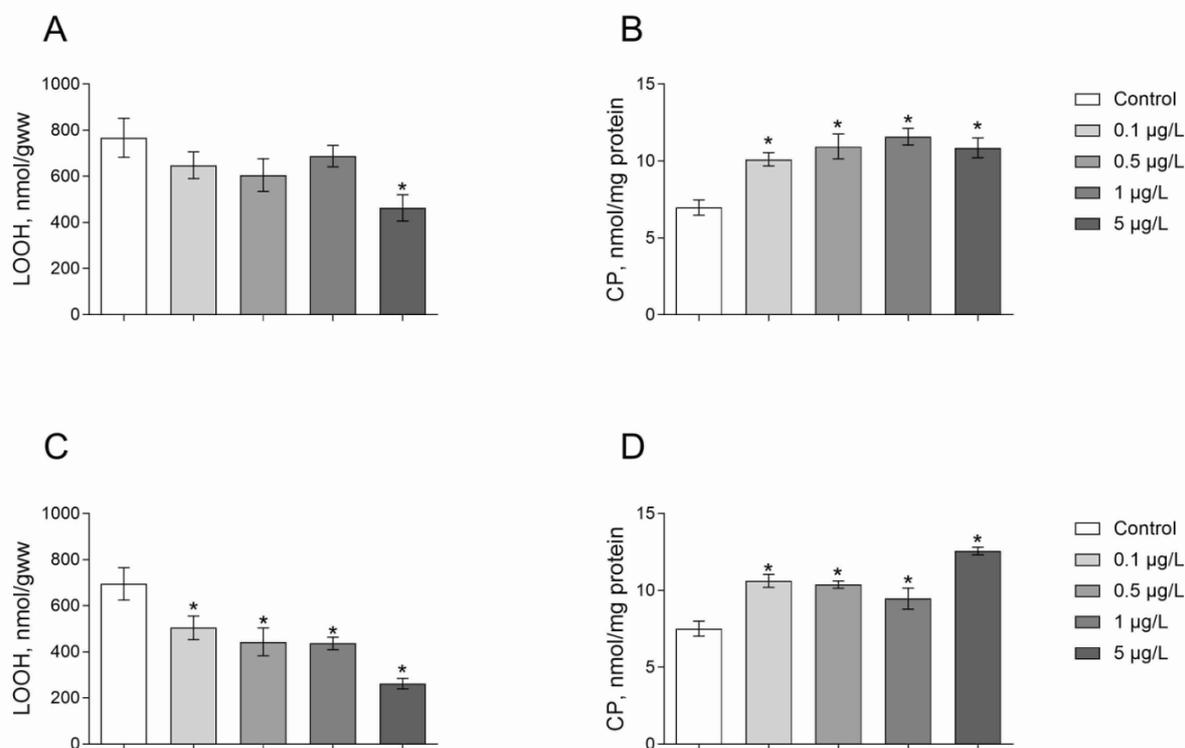
aged 7-8 days. The following concentrations of drugs were tested: 0.1; 0.5; 1 and 5  $\mu\text{g/L}$ .

We measured the level of oxidatively modified molecules including protein- and low-molecular-mass thiol-group-containing compounds, and lipid peroxide contents in *Daphnia* as a measure of oxidative homeostasis in response to different concentrations of herbicides. We found that the level of lipid peroxides (LOOH) was largely decreased under Roundup exposure. The content of LOOH decreased by 40 % under the Roundup exposure at a concentration of 5  $\mu\text{g/L}$  as compared to the control (Figure 2A; Dunnett's test,  $p = 0.015$ ). Atrazine treatment for 24 hours at all concentrations tested led to a lower LOOH level by 27-62 % as compared to animals of the control group (Figure 2B; Dunnett's test,  $p < 0.05$ ).

ROS and their derivatives can induce the formation of additional carbonyl groups in proteins (CP). Given this, CP content serves as a reliable indicator for the assessment of oxidative damage. We found that *Daphnia* treatment with Roundup led to an increase in

CP content as compared with the control group (Figure 2C). Indeed, 24 hours of exposure to Roundup at the concentrations of 0.1, 0.5, 1 and 5  $\mu\text{g/L}$  results in 45-65 % higher CP content as compared to the control (Dunnett's test,  $p < 0.002$ ). Atrazine treatment showed similar effect to Roundup on the CP. We observed 41-68 % higher CP levels in *Daphnia* exposed to 0.1, 0.5, 1 and 5  $\mu\text{g/L}$  of Atrazine as compared to the control group (Figure 2D; Dunnett's test,  $p < 0.03$ ). Probably, the treatment of *Daphnia* with these concentrations of herbicides promotes overproduction of free radicals resulting in oxidative damage of proteins.

The content of low-molecular-mass thiol-group containing compounds (L-SH) was 1.5-fold higher under the Roundup exposure at a concentration of 5  $\mu\text{g/L}$  as compared to the control group (Figure 3a; Dunnett's test,  $p = 0.013$ ). Moreover, *Daphnia* exposed to Atrazine at all tested concentrations had significantly higher levels L-SH than those in control treatments (Figure 3C; Dunnett's test,  $p < 0.04$ ).



**Figure 2:** Levels of lipid peroxides and carbonyl proteins in *D. magna* exposed to Roundup (A, B) or Atrazine (C, D) for 24 hours. Values are expressed as mean  $\pm$  S.E.M. ( $n = 4-6$ ). \*Significantly different with  $p < 0.05$  (ANOVA followed by Dunnett's multiple comparison tests)

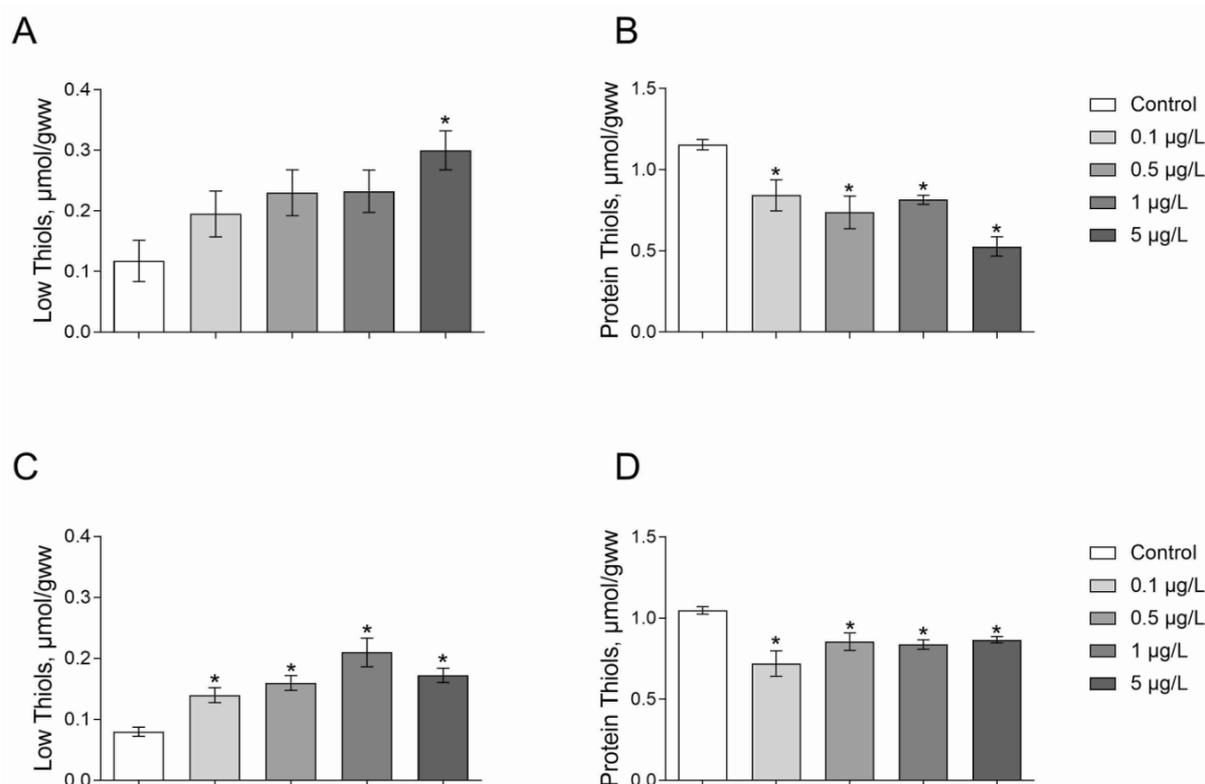
The study showed a reduced level of protein thiols (P-SH) under Roundup exposure. Indeed, we observed 28-57 % decreased content P-SH under Roundup treatment at the concentrations of 0.1-5  $\mu\text{g/L}$  as compared to the control group of *Daphnia* (Figure 3B; Dunnett's test,  $p < 0.03$ ). The level of P-SH increased 17-31 % relative to control animals under exposure to Atrazine at a range of concentrations of 0.1-5  $\mu\text{g/L}$  for 24 hours (Figure 3D; Dunnett's test,  $p < 0.05$ ).

### Activities of antioxidant enzymes

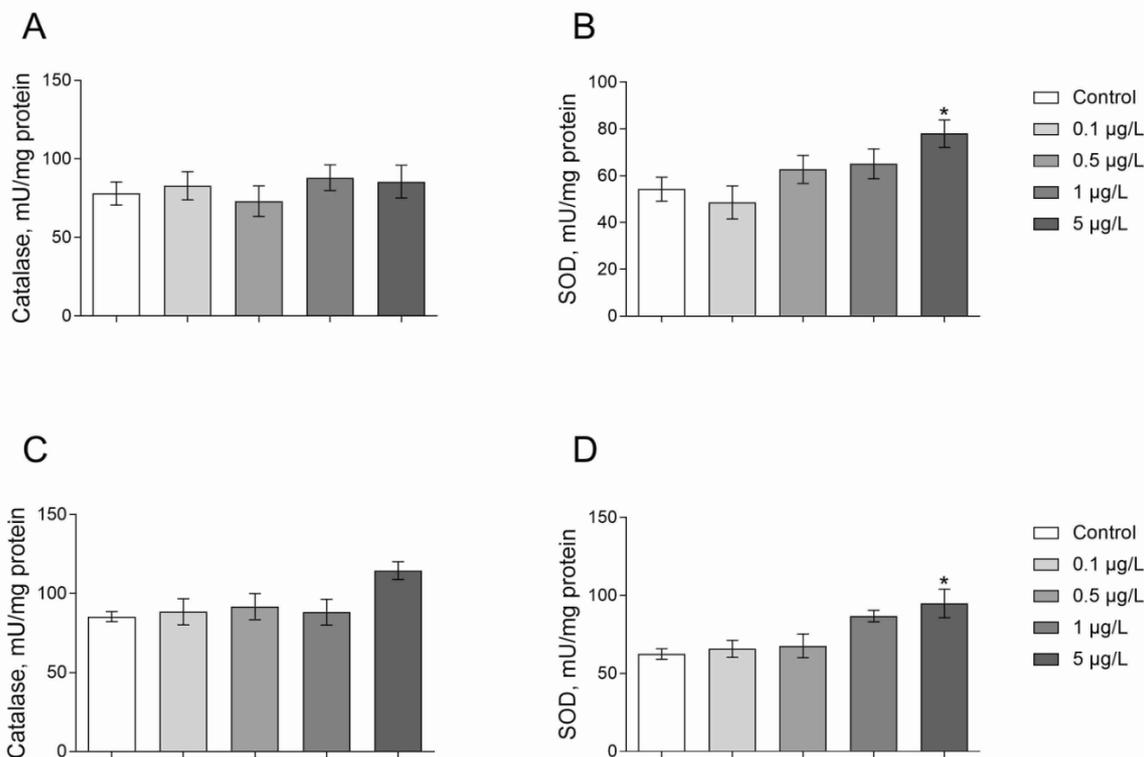
Superoxide dismutase (SOD) and catalase are considered as the most important antioxidant enzyme systems in invertebrate species.

We observed 43 % higher SOD activity in Roundup exposed *Daphnia* at 5  $\mu\text{g/L}$  for 24 h when compared with control group animals (Figure 4B; Dunnett's test,  $p = 0.048$ ). *Daphnia* exposed to Atrazine at concentrations of 1 and 5  $\mu\text{g/L}$  had greater SOD activities by 41 and 49 % respectively as compared to control (Figure 4D; Dunnett's test,  $p < 0.05$ ).

The catalase activity in *Daphnia* of the control group was  $78.0 \pm 6.5$  U/mg protein (A) and  $85.2 \pm 7.1$  U/mg protein (B) (Figure 4). Catalase activity was not affected by both Roundup and Atrazine treatments for 24 hours at a range of concentrations of 0.1-5  $\mu\text{g/L}$  (Figure 4).



**Figure 3:** The levels of low-molecular-mass thiols (A, C) and protein thiol groups (B, D) in 7-8-day-old *D. magna* exposed to Roundup (A, B) or Atrazine (C, D) for 24 hours. Values are expressed as mean  $\pm$  S.E.M. ( $n = 4-6$ ). \*Significantly different with  $p < 0.05$  (ANOVA followed by Dunnett's multiple comparison tests)



**Figure 4:** Catalase and SOD activities in *D. magna* exposed to Roundup (**A, B**) or Atrazine (**C, D**) for 24 hours. Values are expressed as mean  $\pm$  S.E.M. (number of pools  $n = 3-6$ ). \*Significantly different with  $p < 0.05$  (ANOVA followed by Dunnett's multiple comparison tests)

## DISCUSSION

The study was initiated to determine the acute toxicity of the formulated herbicides Roundup and Atrazine to the aquatic invertebrate *Daphnia magna*. Since many pesticides exert toxic effects related to oxidative stress, we also investigated biochemical responses of oxidative stress against Roundup or Atrazine and their toxicity consequences. In this study, *D. magna* showed LC<sub>50</sub> under Roundup treatment for 24 hours at a concentration of 0.022 mg/L and 48 hours at a concentration of 0.0008 mg/L. Our results are in good agreement with the previous experimental data of Sarigül and Bekcan (2009) that showed that the LC<sub>50</sub> for *D. magna* was 0.019 mg/L of Roundup (0.012 mg/L-0.024 mg/L 95 % confidence interval) for 24 hours, but the LC<sub>50</sub> was 0.012 mg/L (0.001 mg/L-0.016 mg/L 95 % confidence interval) for 48 hours (Sarigül and Bekcan, 2009). We also found complete mortality at concentrations of 30

mg/L and 10 mg/L during 24 and 48 hours of Roundup exposure, respectively. A comparative study of the acute toxicity of six different glyphosate-based herbicides on *D. magna* found an LC<sub>50</sub> value at the range of 4.2-117 mg/L (Melnichuk et al., 2007). Various environmental tests of active glyphosate-based toxic substances significantly differ in LC<sub>50</sub> value for *D. magna*. One study has reported LC<sub>50</sub> values at 13-20 mg/L (FAO, 2001), while others have reported LC<sub>50</sub> at 234 mg/L (Le et al., 2010), 780 mg/L (McAllister and Forbis, 1978), 930 mg/L (Forbis and Boudreau, 1981) or even more 200 mg/L (Pereira et al., 2009).

We found that Atrazine is less toxic for *Daphnia* as compared to Roundup. The toxicity test showed LC<sub>50</sub> at a concentration of 40 mg/L and 7 mg/L for 24 and 48 hours of Atrazine exposure respectively. Previously obtained LC<sub>50</sub><sup>48</sup> values for Atrazine were 24.6 mg/L (Phyu et al., 2004), 26.9 mg/L and 36.5 mg/L for *D. carinata*, *D. magna* and *D. pulex*,

respectively (Brooks, 1992). We also found that exposure at the concentrations of 100 and 50 mg/L of this herbicide resulted in the total mortality of the *Daphnia* population within 24 hours and 48 hours, respectively.

As the consequence of the intensive use of Roundup, the glyphosate or the degrading product aminomethyl phosphonic acid (AMPA) are often detected in the aquatic ecosystems. The levels of surface water contamination by pesticides vary greatly in different countries depending on season (peak in spring/summer). Indeed, in the surface water of Netherlands the concentration of glyphosate is 0.5-1 µg/L and AMPA – 6 µg/L (IPCS, 1994). Glyphosate concentration in the lakes and rivers is < 5153 µg/L (IPCS, 1994), surface waters – 100-300 µg/L (Gottrup et al., 1976) of Canada. In USA, the glyphosate concentration is 35-1237 µg/L and AMPA concentration is 2-35 µg/L (IPCS, 1994). In Ukraine, there are no data on the content of glyphosate and AMPA in surface waters. The maximum permissible concentration of glyphosate in environmental waters is 0.02 mg/L. In many countries, after application in agricultural areas, Atrazine has been found in groundwater at levels of 0.01-6 µg/L. It has also been detected in drinking-water in several countries at levels of 0.01-5 µg/L (UNEP, 1998).

The toxicity of pesticides could be also assessed using some biochemical parameters. Since pesticides could induce oxidative stress, we suggest measurements of oxidative damage levels for toxicity evaluation in *Daphnia*. Reactive oxygen species (ROS) damage almost all cellular macromolecules including proteins, lipids, nucleic acids. Cellular proteins are primary targets for ROS attack (Lushchak, 2011). Various ROS and their derivatives cause specific types of damage to individual amino acids in the polypeptide chain via the formation of additional carbonyl groups. Hence, the determination of protein carbonyl content serves as a reliable indicator for the assessment of oxidative damage of proteins within the cells. In this study, it was

found higher CP at all experimental concentrations of either Roundup or Atrazine. Hence, the treatment of *Daphnia* even at very low concentrations of these herbicides promotes ROS generation and the body's antioxidant system is not able to neutralize them, resulting in oxidative damage of proteins. The formation of protein carbonyl groups is a virtually irreversible process that is associated with a functional decline of the target proteins, which are thought to contribute to the aging process and age-related pathogenesis (Lushchak, 2011).

Nowadays, a number of research have been devoted to studying the role of lipid peroxidation (LOOH) in the mechanisms of pathological conditions development within the body (Lushchak et al., 2004). Lipid peroxidation is a process of free radical attack of lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids. Accumulation of the LOOH products is associated with the complete destruction of unsaturated fatty acids and acetyl residues of phospholipids, disruption of the structure and function of proteins, the death of body cells. Lipid peroxidation is currently considered as one of the main causes of cell damage and death (Liu et al., 2013; Pereira et al., 2009). The results obtained for LOOH level indicated that Roundup and Atrazine exposure decreased the lipid peroxidation in *Daphnia*. However, previous studies have shown an increase in lipid peroxidation resulting from both pesticides treatment. Indeed, Blahová and colleagues (2013) reported an increase in oxidized lipids in the kidneys of zebrafish (*Danio rerio*) exposed to Atrazine (30 and 90 µg/L) compared to controls (Blahová et al., 2013). Independently, Chromcova and colleagues (2013) and Nwani and colleagues (2010) have also reported an increase in LOOH level due to the Atrazine exposure of various fish species. According to previous experimental data, Roundup exposure also led to an increase in the levels of reactive thiobarbituric acid (TBA-active products) in the silver carp tissues, which are thought to reflect the intensity of lipid peroxidation (Lushchak et al.,

2009). Lipid hydroperoxides are not only a marker for oxidative damage to lipids, they are also involved in the activation of antioxidant enzymes (Lushchak and Bagnyukova, 2006; Valavanidis et al., 2006). As a result, an enhanced antioxidant response can suppress the intensity of lipid peroxidation (Bagnyukova et al., 2007).

Glutathione is involved in the detoxification of ROS through direct interaction with them or as a cofactor for antioxidant enzymes (glutathione peroxidase and glutathione-S-transferase) as well as systems for xenobiotics binding (Lushchak, 2012). Oxidative stress, caused by xenobiotics, can deplete glutathione reserves and other low molecular weight thiol-containing compounds (Lushchak, 2011). The level of thiol group-containing low molecular mass (L-SH) compounds was higher, but the level of protein thiols significantly decreased in *Daphnia* under 24-h Roundup and Atrazine exposure. Decreased levels of high molecular weight thiol groups of peptides and proteins may indicate irreversible destructive processes of these structures. Higher levels of thiol-containing compounds were previously found under a triazine pesticide exposure, namely simazine. It has been shown to increase glutathione levels in carp liver in a chronic experiment for 14 and 28 days. However, simazine exposure of fish for 60 days led to decreased glutathione content as compared to the control group of fish (Stara et al., 2018). Oropesa and colleagues (2009) observed an increased glutathione level in fish after acute exposure to simazine.

To minimize oxidative damage to cellular components, organisms have developed antioxidant defense systems. SOD is an enzyme of the first-line defense system against reactive oxygen species (ROS) which is responsible for the utilization of one of the most common free radicals – superoxide anion. Superoxide anion radical ( $O_2^{\cdot -}$ ) is perpetually generated in normal body metabolism, particularly through the mitochondrial energy production pathway. Therefore, superoxide dis-

mutase activity is one of the markers of oxidative stress in the cell (Lushchak, 2011). We found an increase in SOD activity under Roundup and Atrazine exposure for 24 hours in *Daphnia*. Higher SOD activity may indicate that the herbicide may cause superoxide anion overproduction, which in turn, serves as a signal to increase the activity of superoxide dismutase. Similar results were obtained in the study Jin and colleagues who reported an increase in SOD activity in the liver of zebrafish (*Danio rerio*) exposed to different concentrations of Atrazine for 14 days (Jin et al., 2010). The activity of SOD was not affected in the brain, gills, liver and intestines of carp by Simazine exposure for 14, 28, or 60 days, however, SOD activity increased significantly in the muscles after 14 and 28 days of exposure to this herbicide (Stara et al., 2018). However, Paulino and colleagues reported no changes in SOD activity in the gills in neotropical freshwater fish (*Prochilodus lineatus*) during acute exposure to different concentrations of atrazine (Paulino et al., 2012).

Catalase mitigates oxidative stress to a considerable extent by destroying cellular hydrogen peroxide to produce water and oxygen. Catalase is a constant component of cellular structures because it neutralizes hydrogen peroxide, which is a product of SOD. That is why catalase is considered as an additional oxidative stress marker (Bartosz, 2005). However, we found that catalase activity was not affected by either Roundup or Atrazine exposure for 24 hours at 0.1-5  $\mu\text{g/L}$ . Gluszcak and co-authors (2007) also did not show changes in catalase activity in the liver of *Rhamdia quelen* under Roundup exposure at concentrations of 0.2 and 0.4 mg/L for 96 hours. Catalase activity in the liver and kidneys of silver carp was higher under Roundup exposure for 96 hours as compared to the control and was not affected in the brain (Lushchak et al., 2009). An increase in catalase activity was documented in the liver of *Prochilodus lineatus* which was exposed to 10 mg/L Roundup for 24 hours (Langiano and Martinez, 2008). An increase in catalase ac-

tivity due to the action of atrazine was observed in the other animal models. Indeed, Jin and colleagues (2010) reported an increase in catalase activity in the liver of zebrafish after 14 days of exposure to Atrazine at a concentration of 1000 µg/L. Paulino and colleagues (2012) reported no changes in catalase activity of gill in neotropical freshwater fish *Prochilodus lineatus* after acute exposure to different concentrations of Atrazine. However, triazine-induced inhibition of catalase activity was previously reported (Stara et al., 2018). Blahova and colleagues (2013) observed a significant decrease in catalase activity in all experimental groups of zebrafish exposed to Atrazine. Such differences in catalase activity indicated that it cannot be used as a reliable biomarker for Roundup toxicity. Although the activity of the catalase in *Daphnia* under Roundup treatment was not affected in current research, the hypothesis about potential Roundup involvement in ROS production, antioxidant defense responses and oxidative damage in aquatic invertebrates cannot be rejected.

Roundup concentrations that resulted in behavioral effects and induction of oxidative stress in the current study may be caused by the alteration of metabolic pathways via an effect on the gut microbiota in *Daphnia* (Suppa et al., 2020). Roundup and its components appear to act through disruption of bioenergetic functions of mitochondria. Disruption of mitochondrial membrane potential is associated with high levels of reactive oxygen species and can be correlated with activation of caspases, which is harmful to a cell due to the high risk of apoptosis. Atrazine was shown to interfere *in vivo* life parameters by oxidative stress-induced retrogression and ecdysteroid biosynthetic pathway in *Tigriopus japonicus* (Yoon et al., 2019). A significant increase in the intracellular ROS level was previously reported in *T. japonicus* in response to a high concentration (20 mg/L) of Atrazine (Yoon et al., 2019). Many studies are indicating about atrazine induced oxidative stress in various aquatic animals (Falfushynska and Stolyar, 2009; Jin, 2010; Stara et al., 2012). In this

study, it has been found that the antioxidant enzyme SOD maintains a balance in cells of *Daphnia* and protects them from oxidative damage after exposure to Roundup and Atrazine at a range of concentrations 0.1-5 µg/L. Hence, some changes in antioxidant enzyme activities could occur as an adaptation mechanism to these stressful conditions.

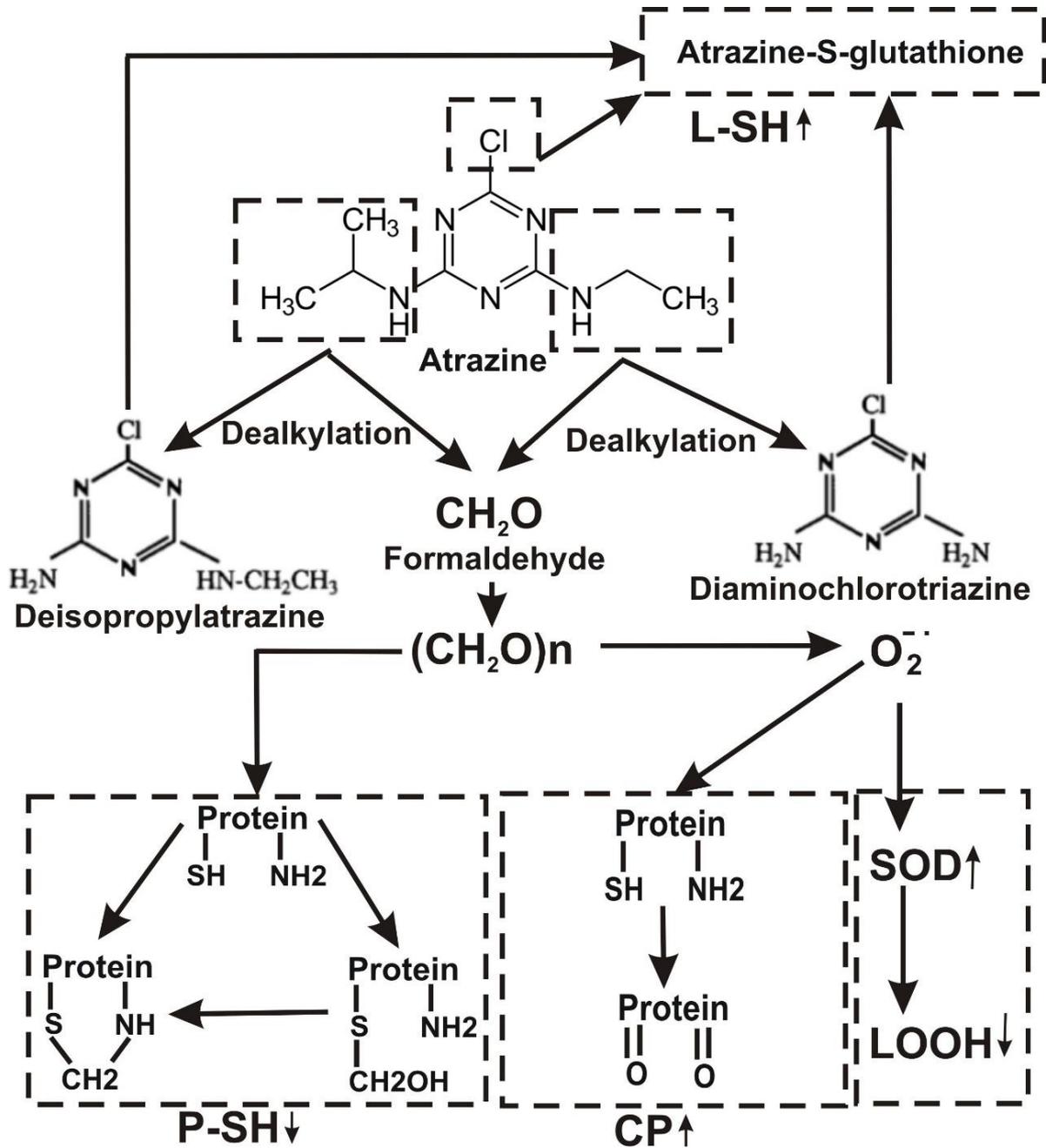
The metabolism of a pesticide may play an important role in the determination of its toxicity. The major pathway of Atrazine decay involves alkyl-oxidation followed by dealkylation associated with formaldehyde formation as a by-product (Figure 5). The primary step involved in glyphosate degradation pathway is catalyzed by glyphosate oxidoreductase, which synthesizes the glyoxylate and AMPA. Degradation pathway of AMPA includes its breakdown to phosphonoformaldehyde via transaminase enzyme with subsequent transformation to formaldehyde (Figure 6). Meanwhile, the enhancement of the glyoxylate cycle contributed to the production of ROS.

## CONCLUSION

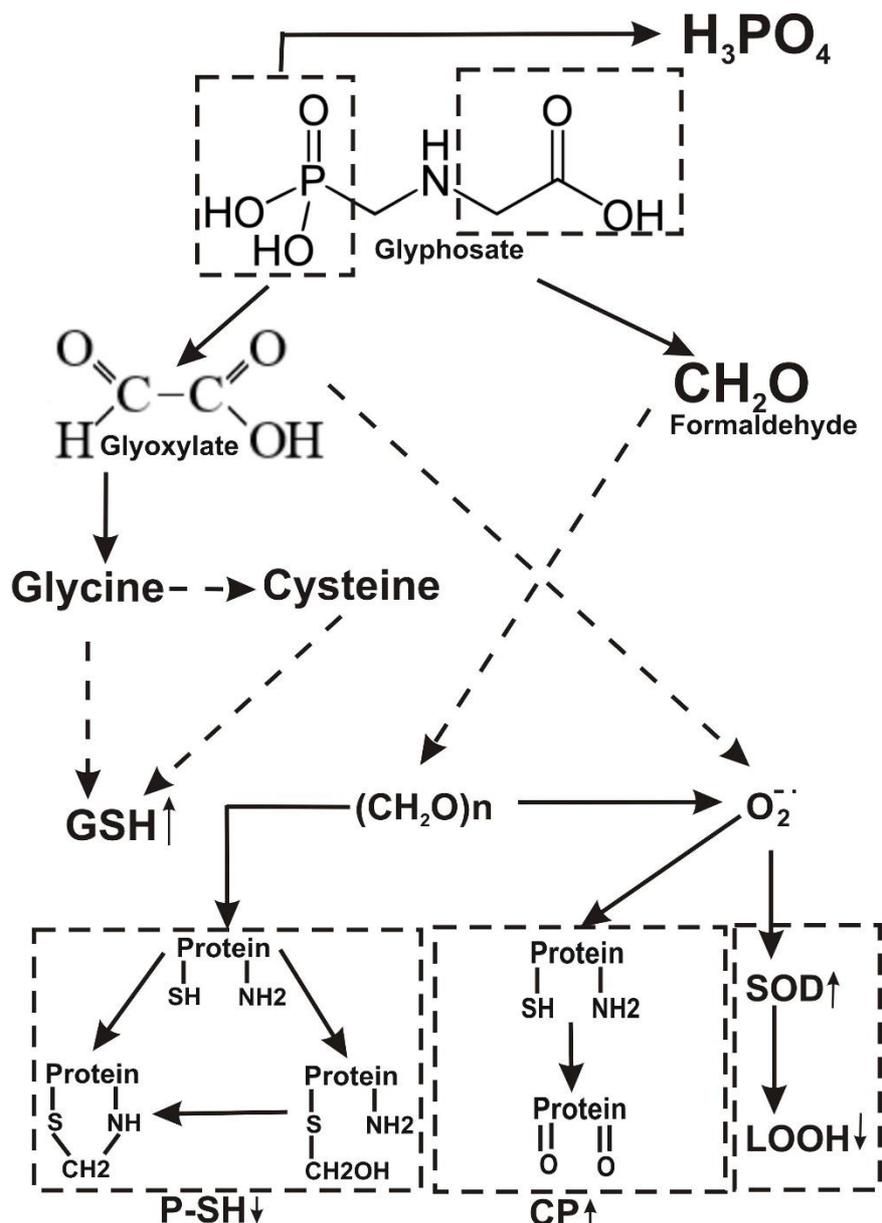
Overall, our results show that exposure to either Roundup or Atrazine causes embryonic developmental failure and causes contaminant-related induction of oxidative stress damage resulting from excessive ROS production in *Daphnia magna*. This is the first report that compares the effects of both herbicides using markers of oxidative stress and the activities of antioxidant enzymes. The common by-product of Atrazine and Roundup metabolism, formaldehyde, is considered to be contaminating chemicals that disturb free radical processes.

This study is important for understanding antioxidant response to the herbicides, the contribution of herbicides to *Daphnia* health, the risk assessment of pesticides to *Daphnia*, and reduction of pesticide threat to aquatic organisms. Since *Daphnia* is a key indicator of aquatic ecosystem health and serves as an important filter-feeder in aquatic ecosystems the

glyphosate-based Roundup and triazine herbicide Atrazine can negatively influence water quality.



**Figure 5:** Hypothetical representation of mechanisms responsible for atrazine-induced stress in *Daphnia magna*. CP, carbonyl protein; L-SH, low molecular mass thiols; P-SH, high molecular mass thiols; LOOH, lipid peroxides; SOD, superoxide dismutase.



**Figure 6:** Hypothetical representation of mechanisms responsible for glyphosate-induced stress in *Daphnia magna*. CP, carbonyl protein; GSH, glutathione; P-SH, high molecular mass thiols; LOOH, lipid peroxides; SOD, superoxide dismutase.

#### Author contributions

Conceptualization, OL and HF; methodology, VH; validation, VH; formal analysis, VB; investigation, AP, VH; data curation, OL and VH; writing—original draft preparation, OS; writing—review and editing, OL and OS; supervision, NB and TS. All authors have read and agreed to the published version of the manuscript.

#### Funding

This work was partially supported by the grant from the National Research Foundation of Ukraine #2020.02/0270.

#### Institutional Review Board Statement

Not applicable.

#### Informed Consent Statement

Not applicable.

#### Conflict of interest

The authors declare no conflict of interest.

## REFERENCES

- Bagnyukova TV, Lushchak OV, Storey KB, Lushchak VI. Oxidative stress and antioxidant defense responses by goldfish tissues to acute change of temperature from 3 to 23 °C. *J Therm Biol.* 2007;32:227-34.
- Bailey DC, Todt CE, Burchfield SL, Pressley AS, Denny RD, Snapp IB, et al. Chronic exposure to a glyphosate-containing pesticide leads to mitochondrial dysfunction and increased reactive oxygen species production in *Caenorhabditis elegans*. *Environ Toxicol Pharmacol.* 2018;57:46-52.
- Bartosz G. Food toxicity contributes to the beneficial effects of calorie restriction. *J Theor Biol.* 2005;233:451-2.
- Blahova J, Plhalova L, Hostovsky M, Divisova L, Dobsikova R, Mikulikova I, et al. Oxidative stress responses in zebrafish *Danio rerio* after subchronic exposure to atrazine. *Food Chem Toxicol.* 2013;61:82-5.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-54.
- Brooks SP. A simple computer program with statistical tests for the analysis of enzyme kinetics. *BioTechniques.* 1992;13:906-11.
- Chromcova L, Blahova J, Plhalova L, Zivna D, Stepanova S, Praskova E, et al. The effects of atrazine exposure on early life stages of common carp (*Cyprinus carpio*). *Neuro Endocrinol Lett.* 2013;34 (Suppl 2):95-101.
- Dionísio R, Daniel D, Alkimin GD, Nunes B. Multiparametric analysis of ciprofloxacin toxicity at ecologically relevant levels: Short- and long-term effects on *Daphnia magna*. *Environ Toxicol Pharmacol.* 2020;74:103295.
- Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys.* 1959;82(1):70-7.
- Falfushynska HI, Stolyar OB. Responses of biochemical markers in carp *Cyprinus carpio* from two field sites in Western Ukraine. *Ecotoxicol Environ Saf.* 2009;72:729-36.
- FAO. FAO specifications and evaluations for plant protection products: Glyphosate N-(phosphonomethyl)glycine. Rome: Food and Agriculture Organization of the United Nations, 2001.
- Forbis AD, Boudreau P. Acute toxicity of MON0139 (Lot LURT 12011) (AB-81-074) to *Daphnia magna*: Static acute bioassay report no. 27203. Unpublished study document from US EPA Library, 1981.
- Gluszcak L, Miron DS, Moraes BS, Simoes RR, Schetinger MRC, Morsch VM, et al. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). *Comp Biochem Physiol C Toxicol Pharmacol.* 2007;146:519-24.
- Gomes MP, Juneau P. Oxidative stress in duckweed (*Lemna minor* L.) induced by glyphosate: Is the mitochondrial electron transport chain a target of this herbicide? *Environ Pollut.* 2016;218:402-9.
- Gottrup O, O'Sullivan PA, Schraa RJ, Vanden Born WH. Uptake, translocation, metabolism, and selectivity of glyphosate in Canada thistle and leafy spurge. *Weed Res.* 1976;16:197-201.
- Guilhermino L, Diamantino T, Silva MC, Soares AM. Acute toxicity test with *Daphnia magna*: an alternative to mammals in the prescreening of chemical toxicity? *Ecotoxicol Environ Saf.* 2000;46:357-62.
- Hermes-Lima M, Willmore WG, Storey KB. Quantification of lipid peroxidation in tissue extracts based on Fe(III) xylenol orange complex formation. *Free Radic Biol Med.* 1995;19:271-80.
- IPCS, International Programme on Chemical Safety. Glyphosate. Geneva: WHO, 1994. (Environmental Health Criteria; no. 159).
- Jin Y. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). *Chemosphere.* 2010;78:846-52.
- Jin Y, Zhang X, Shu L, Chen L, Sun L, Qian H, et al. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). *Chemosphere.* 2010;78:846-52.
- Langiano VC, Martinez CB. Toxicity and effects of a glyphosate-based herbicide on the Neotropical fish *Prochilodus lineatus*. *Comp Biochem Physiol C Toxicol Pharmacol.* 2008;147:222-31.
- Le TH, Lim ES, Lee SK, Choi YW, Kim YH, Min J. Effects of glyphosate and methidathion on the expression of the Dhb, Vtg, Arnt, CYP4 and CYP314 in *Daphnia magna*. *Chemosphere.* 2010;79:67-71.
- Lenz AG, Costabel U, Shaltiel S, Levine RL. Determination of carbonyl groups in oxidatively modified proteins by reduction with tritiated sodium borohydride. *Anal Biochem.* 1989;177:419-25.
- Liu J, Li W, Piao X, Zhang J, Zhang D, Wei N, et al. Icariside II reduces testosterone production by inducing necrosis in rat Leydig cells. *J Biochem Mol Toxicol.* 2013;27:243-50.

- Lushchak OV, Kubrak OI, Storey JM, Storey KB, Lushchak VI. Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. *Chemosphere*. 2009;76:932-7.
- Lushchak VI. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol*. 2011;101(1):13-30.
- Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. *J Amino Acids*. 2012;2012:736837.
- Lushchak VI, Bagnyukova TV. Temperature increase results in oxidative stress in goldfish tissues: 1. Indices of oxidative stress. *Comp Biochem Physiol C Toxicol Pharmacol*. 2006;143:30-5.
- Lushchak V, Bagnyukova T, Lushchak O. Indices of oxidative stress. 1. TBA-reactive substances and carbonylproteins. *Ukr Biochem J*. 2004;76:136-41.
- Lushchak VI, Bagnyukova TV, Husak VV, Luzhna LI, Lushchak OV, Storey KB. Hyperoxia results in transient oxidative stress and an adaptive response by anti-oxidant enzymes in goldfish tissues. *Int J Biochem Cell Biol*. 2005;37:1670-80.
- McAllister W, Forbis A. Acute toxicity of technical glyphosate (AB-78-201) to *Daphnia magna*. Study reviewed and approved 8-30-85 by EEB/HED US EPA, 1978. <https://archive.epa.gov/pesticides/chemicalsearch/chemical/foia/web/pdf/103601/103601-200.pdf>.
- Melnichuk SD, Sherban EP, Lokhanskaya VI. Estimation of toxicity of glyphosate-based herbicides by biotesting method using cladocera. *Hydrobiol J*. 2007;43:80-91.
- Nkoom M, Lu G, Liu J, Yang H, Dong H. Bioconcentration of the antiepileptic drug carbamazepine and its physiological and biochemical effects on *Daphnia magna*. *Ecotoxicol Environ Saf*. 2019;172:11-8.
- Nwani CD, Lakra WS, Nagpure NS, Kumar R, Kushwaha B, Srivastava SK. Toxicity of the herbicide atrazine: effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa punctatus* (Bloch). *Int J Environ Res Public Health*. 2010;7:3298-312.
- OECD. OECD-202 Guideline for testing of chemicals: *Daphnia* sp. acute immobilization test. Geneva: OECD, 2004. <https://doi.org/10.1787/9789264069947-en>.
- Olorunsogo OO, Bababunmi EA, Bassir O. Effect of glyphosate on rat liver mitochondria *in vivo*. *Bull Environ Contam Toxicol*. 1979;22:357-64.
- Oropesa AL, Garcia-Camero JP, Soler F. Glutathione and malondialdehyde levels in common carp after exposure to simazine. *Environ Toxicol Pharmacol*. 2009;27:30-8.
- Pathak RK, Dikshit AK. Various techniques for atrazine removal. In: 2011 International Conference on Life Science and Technology. IPCBEE, Vol.3 (pp 19-22). Singapore: IACSIT Press, 2011.
- Paulino MG, Souza NE, Fernandes MN. Subchronic exposure to atrazine induces biochemical and histopathological changes in the gills of a neotropical freshwater fish, *Prochilodus lineatus*. *Ecotoxicol Environ Saf*. 2012;80:6-13.
- Peixoto F. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere*. 2005;61:1115-22.
- Pereira J, Antunes SC, Castro BB, Marques CR, Goncalves AMM, Goncalves F, et al. Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient. *Ecotoxicology*. 2009;18:455-63.
- Phyu YL, Warne MJ, Lim RP. Toxicity of atrazine and molinate to the cladoceran *Daphnia carinata* and the effect of river water and bottom sediment on their bioavailability. *Arch Environ Contam Toxicol*. 2004;46:308-15.
- Pu Y, Yang J, Chang L, Qu Y, Wang S, Zhang K, et al. Maternal glyphosate exposure causes autism-like behaviors in offspring through increased expression of soluble epoxide hydrolase. *Proc Natl Acad Sci U S A*. 2020;117:11753-9.
- Sarıgül Z, Bekcan S. Acute toxicity of the herbicide glyphosate on *Daphnia magna*. *J Agric Sci (Turkey)*. 2009;15:204-8.
- Stara A, Machova J, Velisek J. Effect of chronic exposure to simazine on oxidative stress and antioxidant response in common carp (*Cyprinus carpio* L.). *Environ Toxicol Pharmacol*. 2012;33:334-43.
- Stara A, Kouba A, Velisek J. Biochemical and histological effects of sub-chronic exposure to atrazine in crayfish *Cherax destructor*. *Chem Biol Interact*. 2018;291:95-102.
- Stephan CE. Methods for calculating an LC<sub>50</sub>. Washington, DC: U.S. Environmental Protection Agency, 1977. EPA/600/J-77/050 (NTIS PB274425).
- Suppa A, Kvist J, Li X, Dhandapani V, Almulla H, Tian AY, et al. Roundup causes embryonic development failure and alters metabolic pathways and gut microbiota functionality in non-target species. *Microbiome*. 2020;8(1):170.

UNEP, United Nations Environment Programme: Report of the Governing Council on the work of its 5th special session, 20-22 May 1998. New York: UN, 1998.

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol Environ Saf.* 2006;64:178-89.

Wang L, Deng Q, Hu H, Liu M, Gong Z, Zhang S, et al. Glyphosate induces benign monoclonal gammopathy and promotes multiple myeloma progression in mice. *J Hematol Oncol.* 2019;12(1):70.

Yoon DS, Park JC, Park HG, Lee JS, Han J. Effects of atrazine on life parameters, oxidative stress, and ecdysteroid biosynthetic pathway in the marine copepod *Tigriopus japonicus*. *Aquat Toxicol.* 2019; 213:105213.