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Acute and Chronic Effects of the Antifouling Booster Biocide Diuron on the Harpacticoid Copepod *Tigriopus japonicus* Revealed through Multi-Biomarker Determination

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Abstract: Diuron, an additive biocide in antifouling paints, is widely employed to curtail the attachment of organisms on submerged surfaces in aquatic structures. Despite the detection of diuron in aquatic ecosystems, information regarding its acute and chronic impacts on aquatic invertebrates, particularly planktonic crustaceans, remains limited. In this study, we analyzed the acute (24 h) and chronic (12 days exposure across three generations) effects of different concentrations of diuron (1/10 of the no observed effect concentration (NOEC), the NOEC, and 1/10 of the lethal concentration 50% (LC50), derived from the 24 h acute toxicity value of 1152 μg L^{-1}) on the harpacticoid copepod *Tigriopus japonicus*. The acute exposure experiment indicated that the 1/10 LC50 value of diuron significantly reduced the copepod's feeding rate and acetylcholinesterase activity. In response to the 1/10 LC50 value, the intracellular reactive oxygen species were elevated alongside increased malondialdehyde levels, while the glutathione content was depleted. The enzymatic activities of glutathione *S*-transferase, catalase, and superoxide dismutase were significantly enhanced by the 1/10 LC50 value, suggesting a proactive role of the antioxidant defense system against oxidative stress. Conversely, the activities of glutathione peroxidase and glutathione reductase enzymes were increased at the NOEC value, while their values were reduced by the 1/10 LC50 value. Chronic exposure to 1/10 NOEC and NOEC values revealed the adverse multigenerational effects of diuron. The second generation exhibited the most sensitivity to diuron, with the NOEC value notably reducing survival rate, body length, nauplius-to-adult development, neonates per brood count, and extending the reproduction period. Taken together, our findings underscore that even sublethal diuron levels can adversely impact copepod populations across generations through intergenerational toxicity.

Keywords: diuron; copepod; neurotoxicity; oxidative stress; chronic toxicity

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1. Introduction

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a phenylurea herbicide that has been widely employed as an additional antifouling booster biocide in antifouling paints [1,2]. These paints are used to coat underwater structures such as ship hulls to prevent the colonization of biofouling organisms. Additionally, diuron finds extensive applications in both agricultural settings, including fruits and vegetables, and non-agricultural areas like railway lines and home gardening, for the purposes of weed, grass, moss, and bush control [3]. The introduction of diuron into aquatic ecosystems can occur through various pathways, including cleaning processes, ship anchorage, painting, spray drift, leaching, and runoff [4]. Owing to its widespread use, easy release into the environment, and incomplete removal during wastewater treatment, diuron is frequently detected in diverse environmental settings. These include rivers, streams, estuaries, lakes, and oceans [5].

For example, environmental monitoring has revealed diuron concentrations in coastal regions of different countries, such as Sweden (100 ng L^{-1}) (Dahl and Blanck, 1996) [6], the USA (12 ng L^{-1}) [7], the UK (6742 ng L^{-1}) [8], Brazil (50–7800 ng L^{-1}) [9], Japan (2.18 µg L^{-1}) [10], and Republic of Korea (0.035–1.36 µg L^{-1}) [11].

Diuron exhibits high resistance to both hydrolysis and photolysis [4,12,13]. Its water solubility at 20 °C is 42 mg L^{-1}, and its degradation time span ranges from one month to one year due to its chemical susceptibility to environmental conditions [4,12,13]. Instances of non-degradation over a 42-day period in seawater at 15 °C have been reported. Persistence of diuron beyond 60 days has been observed in estuarine surface water environments [13,14]. Its relatively low octanol–water partition coefficient (log KOW) of 2.6 results in a limited affinity for binding to sediments and soils. This characteristic enhances its water solubility, thereby posing risks to aquatic life. Consequently, instances of diuron bioconcentration, bioaccumulation, and consequential toxicity have been documented in various aquatic animals [15]. For instance, a study in the Sungai Pulai estuary, Malaysia, reported diuron concentrations ranging from 7.7 ± 0.3 to 18.5 ± 0.8 µg kg^{-1} in three marine fish species (*Otolithus ruber*, *Polydactylus sextarius*, and *Thryssa dussumieri*) [16]. The potential for diuron to undergo biomagnification along aquatic food chains, from lower to higher trophic levels, accentuates its impact. This bioaccumulation of diuron can have adverse effects on non-target organisms in aquatic environments. Such effects include reproductive disorders with anti-androgenic potential in Nile tilapia [17] and genotoxicity in oysters [18]. Despite the widespread presence of diuron in aquatic settings and its well-documented toxicity in aquatic organisms, there remain significant gaps in our understanding of the mechanisms underlying diuron's toxicity and its chronic effects on non-target invertebrates exposed to environmentally relevant levels.

Exposure to xenobiotics, whether direct or indirect, can induce alterations in the physiological and biochemical processes of aquatic organisms [19]. Alterations in the behavior exhibited by these organisms can serve as sensitive indicators, offering early signals of the adverse effects of contaminants in aquatic environments [20]. Among the essential processes, feeding is of significant importance for survival, growth, and reproduction. In the context of the cholinergic system, acetylcholine (ACh) plays a critical role as a neurotransmitter in both neuromuscular and central synapses. The enzyme acetylcholinesterase (AChE) is responsible for breaking down ACh into choline and acetic acid within the synaptic cleft, thus influencing motor function [21]. Several studies have provided evidence that diuron exposure can induce oxidative stress [22–26]. Oxidative stress arises from an imbalance between reactive oxygen species (ROS) levels and antioxidants [27]. ROSs attack cellular membrane lipids, leading to the generation of malondialdehyde (MDA). MDA acts as an indicator of lipid peroxidation, revealing oxidative damage that occurs when organisms fail to effectively counter oxidative stress through the utilization of antioxidant defenses. Antioxidant components play a crucial role in neutralizing ROSs and mitigating the risk of oxidative damage [28]. Among these critical components, glutathione (GSH) emerges as a pivotal intracellular antioxidant thiol which is actively involved in detoxifying xenobiotics [27]. Antioxidant enzymes, including catalase (CAT) and glutathione peroxidase (GPx), function to detoxify hydrogen peroxide (H_2O_2), converting it into water (H_2O) and oxygen (O_2). Superoxide dismutase (SOD) aids in detoxifying the superoxide anion (O_2^-), converting it into H_2O_2 and O_2. Another essential enzyme, glutathione reductase (GR), facilitates the conversion of oxidized glutathione (GSSG) into two reduced GSH molecules using NADPH as a cofactor [28,29].

This study's choice of the marine copepod *Tigriopus japonicus* as the experimental species was guided by several advantages, including its small size, short life cycle, easy maintenance, sensitivity to various contaminants, and global distribution [30]. Copepods, notably, hold a vital role in aquatic ecosystems, as they transfer energy from phytoplankton (producers) to higher trophic levels (consumers), thus underlining their significance in ecosystem functioning [31]. This pivotal role underscores the importance of copepods in maintaining an ecological balance. Several antifouling booster biocides, includ-

ing chlorothalonil, copper, copper pyrithione, Irgarol, tributyltin, triphenyltin, and zinc pyrithione, have previously been assessed for their toxicity in *T. japonicus* [32–37]. Notably, a study reported the toxicity of diuron in the adult stage of *T. japonicus*, with a 96 h LC50 value of 11,000 μg L^{-1} [33]. However, there is currently no available research on the acute and chronic toxicity of diuron in this particular species. The primary focus of this study was to examine physiological parameters in response to sublethal levels of diuron, encompassing survival and feeding rate, alongside the cholinergic biomarker AChE. Additionally, this study explored oxidative stress markers like ROS, MDA, and GSH and evaluated the enzymatic activities of antioxidant defense components such as CAT, SOD, GPx, and GR. This comprehensive approach aimed to assess oxidative stress induction, detoxification response, and potential deficiencies, along with the cholinergic effects of specific xenobiotics. Furthermore, this study extended its scope by evaluating chronic exposure to 1/10 NOEC and NOEC values, thus shedding light on the multigenerational adverse impacts of diuron. By providing insights into the toxic effects of diuron on non-target aquatic crustaceans, including copepods, the outcomes of this study contribute significantly to a more comprehensive understanding of this subject matter.

2. Materials and Methods

2.1. Copepods

Tigriopus japonicus were reared in an automatically controlled aquaculture system located at Incheon National University in Republic of Korea. These copepods were cultured under static-renewed conditions. The culture medium consisted of artificial seawater (ASW; TetraMarine Salt Pro, Tetra, Cincinnati, OH, USA) at a salinity of 32 parts per thousand (ppt). The environmental conditions were maintained at a stable temperature of 24 °C following a light–dark photoperiod of 14 h of light and 10 h of darkness. Crucial water parameters including conductivity, pH, dissolved oxygen (DO), and salinity were regularly measured using a portable Orion Star meter (model 520M-01A, Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with appropriate electrodes for each parameter. The copepods were nourished with a marine microalgae species *Tetraselmis suecica* twice a week with an algae concentration of 1×10^5 cells per milliliter. The algae were cultivated in a growth medium under controlled conditions, maintaining a temperature of 22 ± 1 °C and following a 12-h light and 12-h dark cycle. The culture medium for the algae was prepared using Guillard's (F/2) Marine Water Enrichment Solution (Sigma-Aldrich, St. Louis, MO, USA) and sterilized natural seawater that had undergone filtration and autoclaving.

2.2. Acute Toxicity Test

Diuron (≥98%) and dimethyl sulfoxide (DMSO; 98%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Filtered seawater (FSW) was prepared using a 0.22 μm filter (Corning® Top Vacuum Filter CA Membrane, Corning Co., Corning, NY, USA), and the stock solutions were formulated by dissolving diuron in DMSO. To prevent photodissociation, the stock solution was stored in conditions devoid of light until the time of treatment. Working solutions were prepared by diluting individual stock solutions in 0.22 μm-filtered ASW.

For the acute exposure assessment, a range of working solutions ranging from 0 to 500 mg L^{-1} was prepared by diluting the stock solutions in FSW. A preliminary 24 h long-range finding experiment was initiated using newborn *T. japonicus* (less than 12 h old) in 6-well plates (SPL Life Science, Pocheon-si, Republic of Korea). They were subjected to a spectrum of arbitrary diuron concentrations: 0, 0.1, 1, 10, 100, 1000, and 10,000 μg L^{-1}. Each well contained a single *T. japonicus* nauplius and was maintained under static conditions without changing the exposure medium, in accordance with the established culture conditions. Each experimental treatment consisted of five replicates. This preliminary test allowed for the identification of the diuron concentration that resulted in 100% mortality of *T. japonicus* nauplii, denoted as 4000 μg L^{-1}. Subsequently, an acute toxicity test was executed for a duration of 24 h. Groups of *T. japonicus* (with five replicates per treatment)

were subjected to diverse diuron concentrations: 0, 1, 10, 50, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, and 5000 μg L^{-1}. After a 24 h exposure period, the mortality rate of *T. japonicus* was recorded from the commencement of the exposure. The acute toxicity data, such as the NOEC and LC50 values, along with their corresponding 95% confidence intervals (CIs), were calculated using probit analysis conducted using ToxRat® Professional 2.10.3.1 software (ToxRat Solutions GmbH, Alsdorf, Germany).

2.3. Response to Acute Exposure

The assessment of feeding rate followed a method initially proposed for the water flea *Daphnia magna* [38], with minor modifications. In total, ninety *T. japonicus* specimens were prepared and divided into three distinct groups. Consequently, each experimental treatment comprised five replicates. The procedure was conducted under the same treatment conditions as those used in the acute toxicity test, with the addition of the food source *T. suecica* at a concentration of 1×10^5 cells mL^{-1}. These copepods were exposed to concentrations equivalent to the NOEC and 1/10 LC50 values derived from the 24 h acute toxicity test. A control group of copepods was also subjected to exposure to 32 ppt FSW for comparison. Additionally, a solvent control group was exposed to a solution containing 0.01% DMSO dissolved in 32 ppt FSW. The exposure was maintained under static conditions without changing the exposure medium, and the cultures were kept in the absence of light. Following 24 h of exposure, the contents of each beaker were thoroughly mixed to ensure the suspension of any settled algal cells. Subsequently, the algal cells were quantified using a hemacytometer. The calculation of the feeding rate was performed using Gauld's equation, originally developed for marine copepods, which takes into account the mean concentration of the food source, *T. suecica* [39].

For the assessment of AChE enzymatic activity, acetylthiocholine iodide (ATCh) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma (Sigma-Aldrich, Co.) [40]. Approximately 150 copepods were prepared for each concentration to evaluate variations in cholinergic activity. They were randomly divided into three groups, and these groups were maintained in triplicate. Following exposure to the NOEC and 1/10 LC50 values of diuron derived from the 24 h acute toxicity test, the individuals from each group were combined for the experiment. The combined samples were homogenized in ice-cold phosphate buffer (0.1 M, pH 8.0) at a ratio of 1 part sample to 5 parts buffer (*w*/*v*) using a Teflon homogenizer (Thomas Scientific, Logan Township, NJ, USA). The resulting homogenate was then centrifuged at 3000× *g* for 30 min at 4 °C. The upper aqueous layer, which contained the enzyme, was carefully collected for the AChE enzymatic assay. Subsequently, 100 μL of the supernatant was mixed with 1.3 mL of phosphate buffer (0.1 M, pH 8.0) in a 3 mL cuvette. Additionally, 50 μL of DTNB (0.01 M) and 10 μL of ATCh (0.075 M) were added as substrates. The total AChE enzymatic activity was measured using the substrate, along with a blank without ACh and a blank without the sample. This measurement was conducted for 5 min, with an absorbance recorded at 412 nm and at a temperature of 25 °C, using a spectral scanning multimode reader (Varioskan Flash, Thermo Fisher Scientific, Tewksbury, MA, USA). The recorded enzymatic activity was directly normalized to the total protein content measured in the supernatant.

2.4. Measurement of Biochemical Parameters

To assess the biochemical parameters, approximately 600 *T. japonicus* individuals were used for each concentration under investigation. These copepods were randomly divided into three groups, with each group containing approximately 200 individuals to enable triplicate measurements. Following exposure to the NOEC and 1/10 LC50 values of diuron determined from the 24 h acute toxicity test, the individuals from each group were combined for the subsequent experimental procedures.

To quantify intracellular ROS levels, we utilized 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF–DA; Sigma-Aldrich Co.) [41]. Initially, the collected copepods were rinsed with phosphate-buffered saline (PBS) and homogenized using a Teflon homogenizer

(Thomas Scientific) in a Tris-Cl buffer (composed of 50 mM Tris-Cl, 250 mM NaCl, 5 mM EDTA, and 0.5% NP-40; pH 7.4). The resulting supernatant was mixed with 40 μM of H_2DCF–DA and incubated at 37 °C for 20 min. Subsequently, we assessed the ROS level using a spectral scanning multimode reader (Varioskan Flash, Thermo Fisher Scientific) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

For the determination of intracellular malondialdehyde (MDA) content, each pooled sample underwent homogenization using a Teflon homogenizer (Thomas Scientific) in a cold buffer consisting of 20 mM Tris buffer, 100 μM benzamidine, 2 μM aprotinin, 150 mM NaCl, 10 mM β-mercaptoethanol, and 20 μM leupeptin. After centrifugation at 30,000× *g* and 4 °C for 30 min, the resulting supernatant was subjected to denaturation at 75 °C for 15 min. The level of thiobarbituric acid reactive substances was determined at an excitation wavelength of 535 nm using a spectral scanning multimode reader (Varioskan Flash, Thermo Fisher Scientific). The MDA content was quantified using a calibration curve established with malondialdehyde bis(dimethyl acetal) (Sigma-Aldrich Co.) and expressed as nanomoles of MDA per microgram of protein.

A glutathione assay kit (Catalog No. CS0260; Sigma-Aldrich Co.) was utilized to determine the GSH content. Samples pooled from each treatment were washed with 0.9% NaCl, homogenized in trichloroacetic acid (1:4, *w*/*v*) using a Teflon homogenizer (Thomas Scientific), and then centrifuged at 3000× *g* and 4 °C for 10 min. The resulting supernatant was collected, and the GSH content was measured at 420 nm following the manufacturer's instructions using a spectral scanning multimode reader (Varioskan Flash, Thermo Fisher Scientific). Standard curves were prepared using GSH equivalents of 0, 150, and 350 μM to quantify the total GSH content.

The enzymatic activities of CAT and SOD were evaluated using assay kits (Catalog No. CAT100 and 19160, respectively; Sigma-Aldrich Chemie Inc., Buchs, Switzerland). To analyze GPx and GR activities, dedicated glutathione peroxidase cellular (Sigma-Aldrich, Co.) and glutathione reductase (Sigma-Aldrich, Inc.) assay kits were used, respectively. The enzyme activities were normalized according to the total protein concentration in the samples and expressed as units per milligram of protein.

2.5. Multigenerational Response

To evaluate the long-term effects of diuron across multiple generations, focusing on lower concentrations, specifically 1/10 of the NOEC and the NOEC values, a comprehensive assessment of life-cycle parameters was carried out. In this context, thirty *T. japonicus* individuals, each less than 24 h old, were randomly distributed into three groups. These groups were exposed to the NOEC and 1/10 of the NOEC values of diuron, as established from the 24 h acute toxicity test, for a duration of 12 days under semi-static culture conditions. The culture medium underwent bi-weekly renewal while maintaining consistent concentrations of diuron. Additionally, the newly generated offspring were transferred daily to ensure the continuation of subsequent generations. Throughout the experimental period, the organisms received daily feedings of *T. suecica* daily, with a concentration of 1×10^5 cells per milliliter.

The monitoring and measurement of life-cycle parameters, including survival, body length, development, and reproductive traits, were conducted using a Nikon SMZ25 stereomicroscope (Nikon, Tokyo, Japan) equipped with a high-resolution camera, Nikon DS-Fi3. For the measurement of body length, a process involving three *T. japonicus* individuals was undertaken per replicate. Each copepod was gently placed on a glass slide using 100 μL of ASW. The entire bodies of the copepods were recorded for 1 min. Subsequently, the recorded video files were meticulously reviewed using the Nikon imaging software, NIS-Element D ver. 4.60. During this review process, the body lengths of the copepods were visually assessed and automatically calculated.

2.6. Statistics

Statistical analyses were carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The data were reported as mean values accompanied by their respective standard deviations. To assess significant differences among the experimental groups, a one-way analysis of variance (ANOVA) was conducted, followed by post hoc Tukey and Dunnett's multiple comparison tests. A significance level of $p < 0.05$ was chosen for all statistical evaluations.

3. Results

3.1. Responses to Acute Exposure

The mortality rate of *T. japonicus* displayed an upward trend with increasing concentrations of diuron and longer exposure durations (Figure 1). The calculated LC50 values for diuron exposure were 1152 μg L^{-1} for 24 h. Correspondingly, the NOEC values were 21.2 μg L^{-1} for 24 h. Exposure to the solvent control did not result in any significant mortality. In response to a concentration of 1/10 LC50, a significant reduction in the consumption of *T. suecica* by copepods was observed ($p < 0.05$), while the NOEC concentration did not induce a significant impact ($p > 0.05$) (Figure 2A). Treatment with 1/10 NOEC and LC50 values resulted in a significant decrease in AChE activity compared to the control group ($p < 0.05$) (Figure 2B), whereas the DMSO treatment did not lead to a significant alteration in AChE activity ($p > 0.05$).

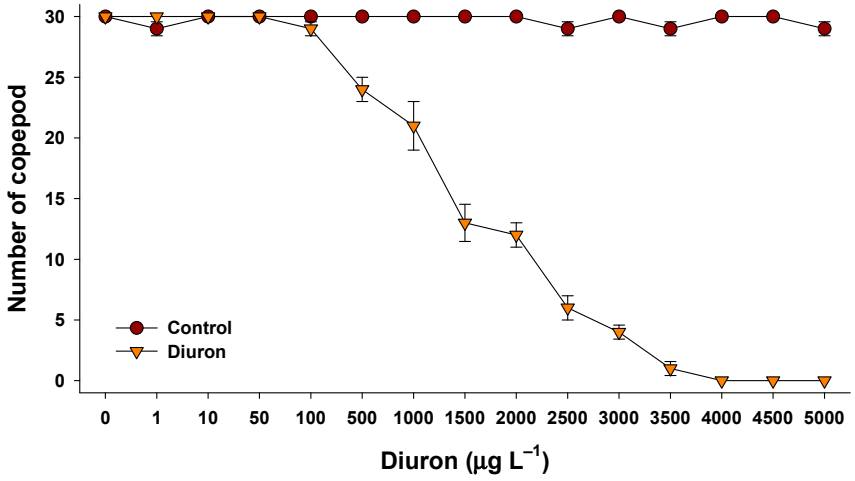

Figure 1. Survival rate of *Tigriopus japonicus* after exposure to different concentrations of diuron for 24 h. Bars indicate the standard deviation of the mean.

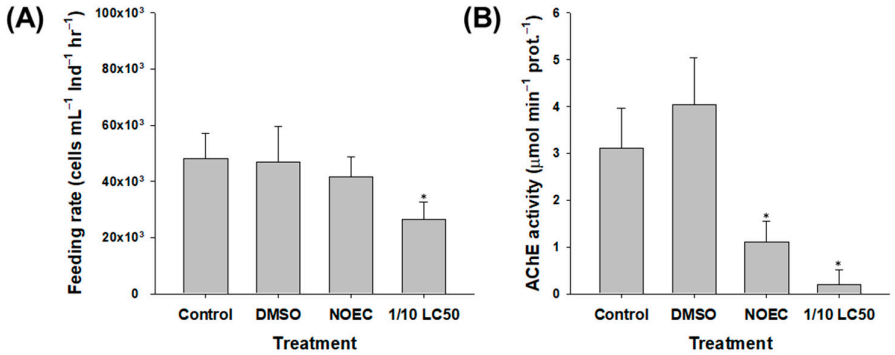

Figure 2. Measurements of (**A**) feeding rate and (**B**) AChE activity of *Tigriopus japonicus* treated with DMSO and NOEC and 1/10 LC50 values of diuron for 24 h. Bars indicate the standard deviation of the mean. Values that are significantly different from the control are marked with an asterisk * ($p < 0.05$).

3.2. Oxidative Stress and Response of Antioxidant Defense System

The intracellular ROS level displayed a notable increase upon exposure to a concentration of 1/10 LC50 diuron at both 12 and 24 h ($p < 0.05$) (Figure 3A). Additionally, a significant alteration was observed in response to the NOEC concentration at 24 h ($p < 0.05$). The MDA content exhibited a noteworthy increase at both 12 and 24 h with the NOEC and 1/10 LC50 values of diuron ($p < 0.05$) (Figure 3B).

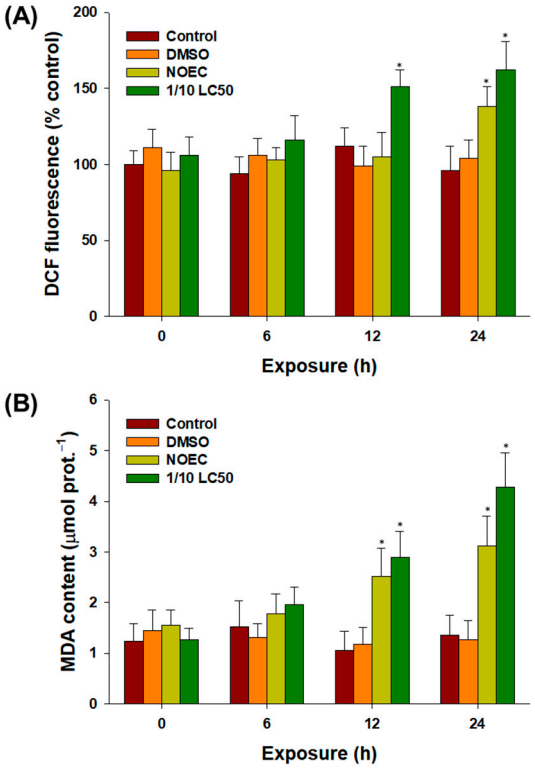

Figure 3. Time-course analysis of (**A**) intracellular ROS and (**B**) MDA levels of *Tigriopus japonicus* after exposure to DMSO and NOEC and 1/10 LC50 values of diuron for 24 h. Bars indicate the standard deviation of the mean. Values that are significantly different from the control are marked with an asterisk * ($p < 0.05$).

In terms of the GSH levels, a significant increase was recorded at 12 h with the NOEC concentration, while a significant depletion was noted at 24 h with the 1/10 LC50 concentration ($p < 0.05$) (Figure 4A). The enzymatic activity of GST showed a remarkable elevation at 24 h with the NOEC concentration and at both 12 and 24 h with the 1/10 LC50 concentration ($p < 0.05$) (Figure 4B). The enzymatic activity of CAT exhibited a significant increase at both 12 and 24 h for 1/10 LC50 concentrations ($p < 0.05$) (Figure 4C). In the case of SOD, its enzymatic activity demonstrated an increase at 24 h with both the NOEC and 1/10 LC50 concentrations ($p < 0.05$) (Figure 4D). The GPx activity was notably elevated at both 12 and 24 h with the NOEC concentration, and at 6 h with the 1/10 LC50 concentration ($p < 0.05$) (Figure 4E). However, its activity was significantly inhibited at both 12 and 24 h in response to the 1/10 LC50 concentration ($p < 0.05$). As for GR, its enzymatic activity exhibited a significant increase at both 12 and 24 h with the NOEC concentration ($p < 0.05$), while it displayed a significant elevation at 6 h and a subsequent decrease at 24 h with the 1/10 LC50 concentration ($p < 0.05$) (Figure 4F).

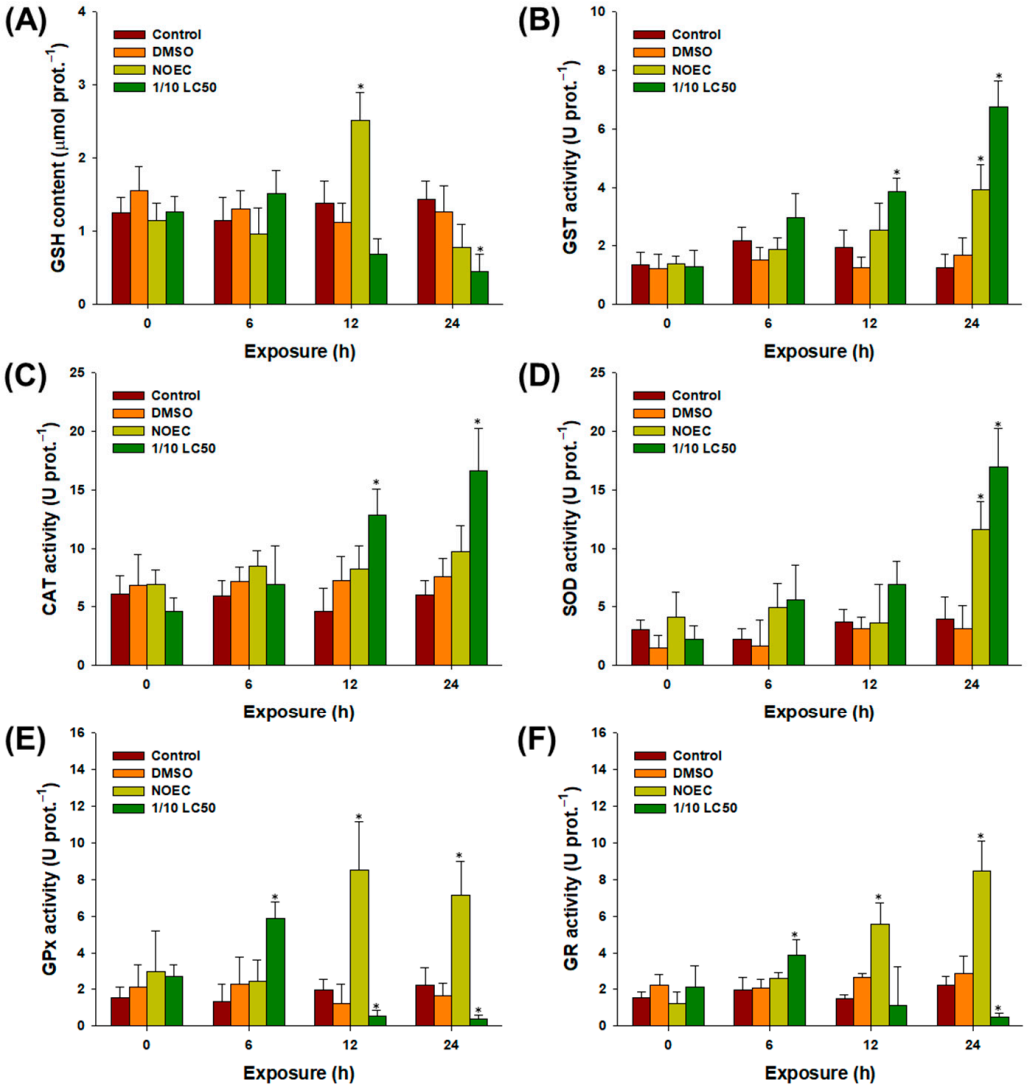

Figure 4. Time-course analysis of (**A**) GSH content, (**B**) GST activity, (**C**) CAT activity, (**D**) SOD activity, (**E**) GPx activity, and (**F**) GR activity of *Tigriopus japonicus* after exposure to DMSO and NOEC and 1/10 LC50 values of diuron for 24 h. Bars indicate the standard deviation of the mean. Values that are significantly different from the control are marked with an asterisk * ($p < 0.05$).

3.3. Response to Chronic Exposure

To comprehend the multigenerational impacts of diuron, lower concentrations, specifically 1/10 of the NOEC and the NOEC values, were assessed. This decision was made considering the observed mortality in groups exposed to the 1/10 LC50 value during chronic exposure. Notably, a significant reduction in the survival rate was apparent in the second generation (F2) exposed to the NOEC value of diuron ($p < 0.05$) (Figure 5A). The body length displayed a retardation with increases in developmental stages in the first generation (F1) and/or F2 due to the exposure to the NOEC value of diuron ($p < 0.05$) (Figure 5B–D). Reproductive parameters, specifically the nauplius count per brood and the initiation of reproduction, were significantly altered by exposure to the NOEC value of diuron in the F1 and F2 generations ($p < 0.05$) (Figure 5E,F).

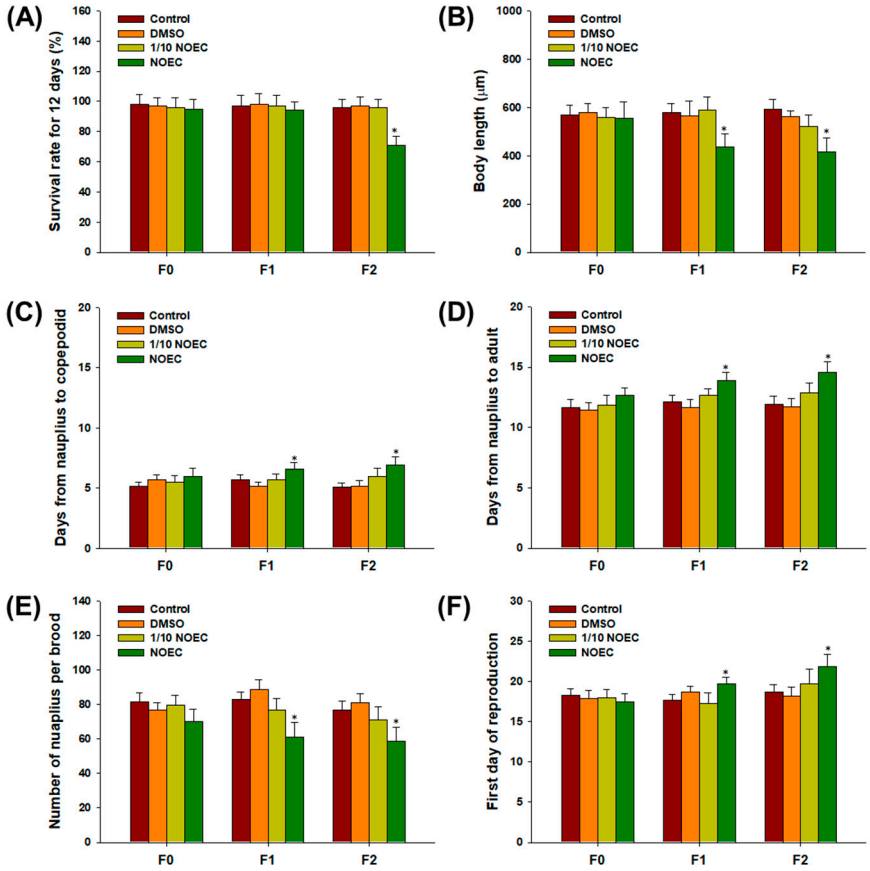

Figure 5. Multigenerational effects of diuron on (**A**) survival rate, (**B**) body length, (**C**) days from nauplius to copepodid, (**D**) days from nauplius to adult, (**E**) first day of reproduction, and (**F**) number of neonates per brood of *Tigriopus japonicus* exposed to DMSO and NOEC and 1/10 LC50 values of diuron. Bars indicate the standard deviation of the mean. Values that are significantly different from the control are marked with an asterisk * ($p < 0.05$).

4. Discussion

In the context of copepods, *T. japonicus* is often considered a promising marine invertebrate for ecotoxicity assessment [30]. Given the global distribution of the genus *Tigriopus* in coastal regions, it makes them suitable candidates for assessing the risk of diuron, as many antifouling booster biocides are released into aquatic environments during ship maintenance and treatment of submerged surfaces in aquatic structures.

The survival rate clearly demonstrates exposure time- and dose-dependent toxicity within this species. The 24 h LC50 value observed in *T. japonicus* nauplius (1152 μg L^{-1}) when exposed to diuron was notably comparable to the 96 h LC50 value documented for the adult stage of this species (11,000 μg L^{-1}) [33]. This difference could be attributed to varying tolerance levels at different developmental stages, as nauplii may exhibit greater sensitivity compared to adults. This heightened sensitivity in nauplii might be due to factors such as immature metabolism, lower detoxification capacity, and reduced energy reserves for maintaining homeostasis during the early stages of copepod development [42,43]. Similar trends of increased susceptibility in juvenile stages compared to adults have been observed in aquatic invertebrates exposed to environmental stressors [43–47]. Likewise, previous studies have reported higher sensitivities in early stages of aquatic crustaceans and polychaetes exposed to antifouling booster biocides [36,48–51]. The acute toxicity value observed in *T. japonicus* when exposed to diuron aligns with the 24 h LC50 value recorded in the marine copepod *Paracalanus parvus* (Copepoda, Calanoida) [52]. Notably, *T. japonicus* displays higher sensitivity to diuron compared to *P. parvus* (24 h LC50 1968 μg L^{-1}) [52]. In water fleas, the 48 h EC50 and 96 h LC50 values were measured at

12.8 mg L^{-1} and 17.9 mg L^{-1} in *D. magna* [53] and *D. pulex* [54], respectively. The range of toxicity values seems to be wide in aquatic invertebrates, as the 96 h LC50 value was reported to be 1.1 mg L^{-1} and 19.4 mg L^{-1} in mysid *Mysidopsis bahia* [55] and amphipod *Hyalella azteca* [54], respectively. Among aquatic organisms, it appears that invertebrates, including copepods, tend to be more sensitive to diuron than marine fish [56]. The 48 h LC50 values for diuron have been shown in fish species to range from 4 to 42 mg L^{-1} [4]. The variability in mortality can be attributed to a range of factors, including the chemical's specific formulation, the developmental stage of the exposed species, uptake mechanisms, the potential for bioaccumulation, its detoxification capacity, and the efficiency of diuron excretion [4]. It is noteworthy that the effects of diuron metabolites [e.g., 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU), and 3,4-dichlorophenyl-N-methylurea (DCPMU)] should also be carefully investigated, as diuron can undergo different metabolic pathways, including demethylation, dechlorination, ring hydroxylation, N-oxidation, and conjugation to glucuronic and sulfate acids in animals [4,57,58].

In *T. japonicus*, exposure to a sublethal concentration of diuron has shown a significant impact on crucial physiological processes. Feeding, a fundamental activity for organism survival, directly affects various physiological aspects such as metabolism, sensitivity against xenobiotics, growth, and reproduction in copepods [59,60]. Disruption of feeding can lead to starvation and eventual mortality. Therefore, the potential risk posed by diuron to the physiology of *T. japonicus* is evident, with the potential to compromise health and ultimately lead to mortality. Changes in feeding behavior, which are closely linked to essential functions like movement, cholinergic response, and food digestion, might underlie this phenomenon in copepods [61].

The cholinergic system plays a pivotal role in essential physiological processes, particularly feeding behavior. The direct relationship between AChE activity and phenomena such as twitching, muscle cramps, and weakness suggests its critical function in aquatic invertebrates [21]. In *T. japonicus*, exposure to both NOEC and 1/10 LC50 values of diuron has led to a significant decrease in AChE activity. This finding is in line with initial observations in fish, where diuron was found to notably inhibit brain AChE activity in goldfish *Carassius auratus* [62]. The disruption in AChE activity can result in the accumulation of ACh within cholinergic synapses, leading to abnormal nervous system functioning. Consequently, any disturbance in AChE activity can translate into impaired movement, potentially causing starvation and eventual mortality due to compromised feeding behavior. This phenomenon has been corroborated in the copepod *Tigriopus brevicornis* [63]. While information on the effects of diuron on aquatic invertebrates is limited, notable decreases in AChE activity were observed at a concentration of 5000 μg L^{-1} in newly hatched and 48 h-old nauplii of the brine shrimp *Artemia salina* during a 24 h exposure [64]. This disruption in feeding behavior induced by diuron exposure can initiate a cascade of detrimental consequences, ultimately compromising the overall homeostasis and survival of the copepod population. Taken together, the findings concerning AChE activity underscore the neurotoxic implications of diuron on the cholinergic system of *T. japonicus* during acute exposure.

Diuron exerts its influence on the redox equilibrium by elevating the production of ROS, consequently inducing a state of oxidative stress that can oxidize various macromolecules such as lipids, proteins, and DNA [65]. Oxidative stress signifies an imbalance between the generation and elimination of intracellular ROS, stemming from disruptions in the interplay between prooxidants and antioxidants within aquatic organisms [66]. This imbalance leads to an excessive accumulation of ROS within marine organisms [27,29]. In *T. japonicus*, exposure to NOEC and 1/10 LC50 values of diuron significantly increased intracellular ROS levels. Studies on the Pacific oyster *Crassostrea gigas* have indicated that diuron-induced ROS production is associated with toxicity and developmental irregularities [24]. Diuron at 0.20 mg L^{-1} has been shown to produce ROS in the liver of gilthead sea bream [22,23]. Hence, in this species, diuron unmistakably exhibits its lethal impact at the cellular level, functioning as a potent inducer of ROS. This oxidative stress triggered by diuron plays a pivotal role in its toxicity, initiating processes such as lipid peroxidation, as

shown in the MDA levels measured in this species. The significant increase in ROS and MDA content, as observed in the rotifer *Brachionus koreanus* exposed to diuron concentrations of 1, 5, 10, and 20 mg L^{-1} for 24 h, supports our findings regarding the similar elevation of these parameters [26].

Organisms have evolved an intricate antioxidant defense system through evolution to withstand exogenous stress and prevent cellular damage caused by excessive ROS [67,68]. Induction of oxidative stress has the potential to disrupt this defense system by affecting the content or activity of antioxidant components such as GSH, CAT, SOD, GPx, and GR [27]. GSH serves various roles in cellular processes, including cell differentiation, proliferation, and apoptosis, making it a pivotal mediator [69]. Furthermore, the non-enzymatic properties of GSH enable it to function as a scavenger for ROS through its active involvement in the detoxification process [27,66]. Thus, the observed increase in GSH levels in response to the NOEC value of diuron at 12 h signifies an augmented antioxidant capacity and enhanced resistance to oxidative stress. In contrast, the depletion in GSH levels following exposure to the 1/10 LC50 value of diuron at 24 h suggests a compromised ability of *T. japonicus* to counter oxidative stress. Persistent GSH depletion can amplify susceptibility to oxidative stress. Notably, an increase in GSH content after 1 h of exposure to diuron in zebrafish larvae at concentrations of 2, 5, and 10 mg L^{-1} has been documented [70]. Similarly, exposure to 500 µM of diuron significantly elevated GSH levels in the nematode *Caenorhabditis elegans* [71]. These findings underscore diuron's robust impact as a trigger of oxidative stress at the cellular level, with GSH playing a pivotal role in furnishing primary protection against the accumulation of ROS.

In *T. japonicus*, the significantly increased activities of antioxidant enzymes indicate the active involvement of all antioxidant components in mitigating diuron-induced ROS. The collaboration between CAT and SOD is crucial, as they work together to convert ROS into H_2O, serving as the primary defense against oxidative stress [27,66]. Their induction clearly suggests that diuron indeed acts as an inducer of antioxidant activity by generating ROS. Several studies have also reported interactions between diuron and antioxidant components. For example, in zebrafish embryos and larvae, diuron exposure significantly increased the mRNA expression of *gpx1a* and *gsr*, while the expression of *cat* showed fluctuations over the exposure period [25]. Similarly, exposure to diuron concentrations of 5, 10, and 20 mg L^{-1} for 24 h led to increased enzymatic activities of GST, CAT, SOD, and GPx in the rotifer *B. koreanus* [26]. GST and GPx enzymes were found to be strongly involved in the biotransformation of diuron in the Nile tilapia *Orechromis niloticus* [58]. Hence, the increased activity of CAT and SOD enzymes observed in diuron-exposed *T. japonicus* is intrinsically linked to the activation of antioxidant mechanisms in response to ROS-mediated oxidative stress. The induction of GPx and GR activities further suggests that diuron induces oxidative stress, prompting these enzymes to participate in eliminating intracellular ROS utilizing GSH [27,66]. However, decreased activities of GPx and GR were observed in *T. japonicus* exposed to the 1/10 LC50 value of diuron at 12 and/or 24 h. This result is closely associated with the concurrent GSH depletion observed under the same exposure conditions, as GR catalyzes the reduction of oxidized GSSG to reduced GSH [19].

To gain insights into the chronic effects of sublethal concentrations of diuron, *T. japonicus* was exposed to 1/10 NOEC and NOEC values across three generations. These concentrations mirror levels encountered in aquatic environments; a notion corroborated by various studies [6–11]. The pronounced decline in the survival rate of *T. japonicus*, observed in the F2 generation exposed to the NOEC value, indicates diminished xenobiotic tolerance/fitness and the potential accumulation of multigenerational toxicity. Regarding generational sensitivity, the F2 generation of *T. japonicus* exhibited heightened susceptibility to diuron compared to the parent generation (F0). In the rotifer *B. koreanus*, exposure to 20 mg L^{-1} diuron led to a significant survival rate decrease over eight days [26]. Remarkably, the F1 and F2 generations exposed to this NOEC value showed significant hindrance in body length and noticeable developmental delays from nauplius to adults in this species. These observations underscore that diuron may elicit adverse effects (e.g., reduced fitness)

through chronic exposure spanning multiple generations. Evidence of vertical transmission of DNA damage to offspring was evident in oyster genitors subjected to short diuron exposures (two 7-day pulses) at environmental concentrations (0.3 μg L^{-1}) during gametogenesis [72]. Similarly, in the marine medaka *Oryzias melastigma*, parental exposure to 0.5 or 5 μg L^{-1} diuron for six months led to diminished hatchability and abnormal ovarian development in subsequent medaka generations [73]. This underscores the possibility of diuron-induced damage being transmitted from F0 and F1 generations, impacting the F2 generation.

Reproduction and fecundity have long served as biomarkers for assessing stress and damage in aquatic organisms. The F1 and F2 generations of *T. japonicus* exhibited a noteworthy rise in initial reproduction, accompanied by a decrease in the number of neonates per brood. This trend signifies potential reproductive toxicity induced by diuron. Comparable findings of impaired fecundity due to diuron exposure have been documented in the rotifer *B. koreanus* at concentrations of 10 and 20 mg L^{-1} [26]. In the Pacific oyster *C. gigas*, diuron altered the mRNA expression of genes associated with gametogenesis and reproduction [18]. Though direct evidence of diuron's endocrine-disrupting effects in aquatic invertebrates is currently lacking, its potential to cause reproductive endocrine disruption has been proposed in vertebrates [17,74–76]. Considering that many biocides are recognized as endocrine disruptors in crustaceans [77,78], a thorough investigation is needed to comprehend how diuron impacts the endocrine systems of invertebrates. The delayed reproduction observed in the F2 generation of diuron-exposed *T. japonicus* could be linked to disruptions in metabolism and impairments in reproduction. Collectively, our findings underscore that prolonged exposure to sublethal diuron concentrations can have adverse effects on copepod populations, likely stemming from the oxidative stress induced by ROS production. Given the critical role of copepods in the aquatic food chain, the persistent release of diuron into the environment has the potential to disrupt the delicate balance of ecosystems.

In summary, these findings emphasize the harmful consequences of acute exposure to diuron on the behavior and physiology of *T. japonicus*, with potential implications for neurotoxicity. The research provides strong evidence of diuron-induced oxidative stress and the active involvement of antioxidant components in countering ROS toxicity. Additionally, the observed long-term effects on the F1 and/or F2 generations point to reproductive vulnerability, possibly due to consistent accumulation or diuron's potential role as an endocrine disruptor. From a risk assessment perspective, the sensitive biomarkers identified in both acute and chronic diuron exposure scenarios necessitate further investigation in ecologically relevant environments to better understand their implications.

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