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Dinitroaniline herbicide pendimethalin affects development and induces biochemical and histological alterations in zebrafish early-life stages

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Abstract

Pendimethalin (PND) is a dinitroaniline preemergent herbicide widely used to control grasses and weeds. The present study aimed to evaluate the PND potential effects on the development of zebrafish early-life stages. The research focuses first on acute toxicity, followed by the integration of toxicity results through histopathology, oxidative status, and neurotoxicity evaluation of sublethal and environmentally relevant concentrations. Zebrafish larvae exposed to PND showed mortality and developed sublethal alterations including impaired fin development, lordosis, scoliosis, blood congestion, impaired blood flow, and reduced heartbeat. PND exposure (0.5 mg/L) affects musculoskeletal development leading to delayed and reduced ossification of the vertebral centra in the developing vertebral column and disruption of muscle morphology. Herbicide exposure (0.5 mg/L and 0.05 mg/L) led also to biochemical changes of antioxidant enzymes, increasing the activity of CAT, GR, and GPx, while no effects were observed on the activity of SOD and GST in zebrafish larvae. Lastly, AChE activity, a biochemical marker of neurotoxicity, was also increased in zebrafish larvae exposed to 0.5 mg/L of PND. These results confirm the developmental toxicity of PND in zebrafish early-life stages, pointing out the potential role of oxidative stress in the onset of sublethal alterations.

1. Introduction

Pesticides are chemicals, natural or synthetic, used in modern agricultural practices to control pests, weeds, and diseases in plants. Pesticides include a wide range of herbicides, insecticides, fungicides, rodenticides and nematicides. According to Bojarski and Witeska (2020) the herbicides are used in the largest quantities (47.5%), while the use of insecticides and fungicides is lower (29.5 and 17.5%, respectively) and contamination of natural waters may affect aquatic organisms (Bojarski et al., 2018).

PND (*N*-(1-ethyl propyl)-3,4-dimethyl-2,6-dinitrobenzenamine) is a dinitroaniline herbicide that inhibits chromosome separation and cell wall formation targeting on the microtubules involved in the division of plant cells (Tabassum et al., 2016; Vighi et al., 2016). The chromosomal and interphase nuclear aberrations induced by PND in plants reflect the cytogenotoxicity of this compound (Verma and Srivastava, 2018).

This chemical is predominantly applied to soil as a preplant and preemergence herbicide and has been on the market for approximately 30 years (Vighi et al., 2016). Absorbed by roots and leaves, this active substance is not translocated in plants (Danion et al., 2012a). PND is classified as "persistent-bioaccumulative slightly toxic" compound (toxicity class III) and its use has been authorized by the Directive 91/414/EEC (Danion et al., 2018a). Although the high affinity of PND to bind to soil and sediment particles it has been also found in agricultural areas where it was not applied (e.g Baltic Sea and arctic ice cores), demonstrating the ability to be subject to medium range transport through the air (1–1000 km) with consequently deposition in open waters (EFSA, 2016). PND has been detected in wet deposition in Denmark (352 ng/L), in the Huelva estuary in Spain (370 ng/L) and in rivers in Brittany, France (840 ng/L) (Danion et al., 2012a; Danion et al., 2012b). Moreover, due to the high bioconcentration factor, the PND uptake by aquatic organisms can lead to interaction with tissue and cells, having negative impacts on various biotic components of freshwater ecosystems such as phytoplankton, zooplankton, and aquatic fauna (Danion et al., 2018a). In fact, PND has been also detected in the muscle of wild brown trouts (40 ng/g) (*Salmo trutta trutta*) collected from Liri river (Italy) which receives water coming from an area with increasing farming activities, irrigation practices, and widespread use of fertilizers and pesticides (Zezza et al., 2020). Moreover, the wild brown trouts exposed to pesticides, including PND, showed higher vitellogenin levels in comparison to fish sampled from the headwater site (not impacted by wastewater effluents from sewage treatment plants), pointing out the potential rule of PND in the feminization of collected fish (Zezza et al., 2020). Disruptions of reproductive function have been also recently investigated in *Clarias batrachus* exposed to sub-lethal concentrations of PND, where an increase in male plasma vitellogenin and gonadal aromatase activity have been reported following herbicide exposure (Gupta and Verma, 2020a). The reproductive capacity of rainbow trout (*Oncorhynchus mykiss*) has been significantly affected by exposure to this herbicide and high bioconcentrations of PND have been measured in eggs and sperm of exposed fish and this

transgenerational exposure to PND impacted the pathogen susceptibility of offspring (Danion et al., 2018a; Danion et al., 2018b). PND exposure led to innate and adaptive immunity disturbances in rainbow trout chronically exposed to the active substance (alone and with adjuvant) (Danion et al., 2012a; Danion et al., 2012b). However, the developmental toxicity of PND in both mammals and aquatic organisms is still poorly understood. Several studies investigated the effects of PND exposure on the oxidative status of aquatic organisms highlighting the oxidative stress as a shared mediator involved in the adverse outcomes of PND (Danion et al., 2014; Gupta and Verma, 2020b; Tabassum et al., 2016). Oxidative stress is classically defined as an imbalance in oxidant and antioxidant species within a system in which oxidant species are predominant (Neier et al., 2015; Sehonova et al., 2019). Recent studies have reported associations between a variety of different endocrine-disrupting chemicals (EDCs), including herbicides, and oxidative stress (Gaaied et al., 2019; Matozzo et al., 2020; Moura et al., 2018; Shukla et al., 2017). Interestingly, oxidative damage has been also investigated as a crucial mechanism of toxicity of PND involved in the development of pancreatic cancer *in vitro* (Arici et al., 2020). PND exposure could raise the risks of developing some cancer types, including lung, rectal, and pancreatic cancers of the gastrointestinal system, and according to 2020–2024 Advisory Group to Recommend Priorities, this herbicide was signed as an agent with medium priority (IARC 2014; 2019).

The present study aimed to investigate the PDN potential effects on the development of zebrafish early-life stages. The first phase involved an acute toxicity test aimed to calculate toxicological endpoints and to establish, according to the sublethal alterations evaluated, a precise phenotype to use in the second phase for a more accurate study of developmental toxicity. For this purpose, in the first phase, zebrafish fertilized eggs were exposed to five concentrations of PND for 96 hours, and daily lethal and sublethal alterations were recorded. In the second phase, two concentrations of PND (0.5 mg/L and 0.05 mg/L), including environmentally relevant concentrations of human exposure, were used to investigate the PDN effects on skeletal and cartilage. Finally, the investigation of PND

toxicity was integrated by histopathology, oxidative status and neurotoxicity evaluation. These findings could help to elucidate the potential mechanism of toxicity of PND in exposed fish.

2. Materials and methods

2.1. Chemicals

PND (CAS number 40487-42-1, PESTANAL®, analytical standard) was purchased from Merck Life Science, Milano, Italy. Dimethyl sulfoxide (DMSO) (> 99.9 % purity) and 3,4-dichloroaniline (> 98 % purity) from Merck Life Science (Co. St. Louis, MO). Dilution water (DW) was prepared following the OECD TG 203, Annex 2 (OECD, 1992). Formalin 37%, glycerol 99%, Tween 20 and ethanol absolute were purchased by Sigma-Aldrich (Milano, Italy).

2.2. Zebrafish maintenance and egg collection

Zebrafish early-life stages used in the experiments were obtained from the University of Teramo facility (code 041TE294). Adult wild type AB strain zebrafish were kept in 3.5 L ZebTec tanks (Tecniplast S.p.a., Buguggiate, Italy) in a recirculating water system. The parameters of the system were the following: temperature at 28°C, pH at 7±0.2, conductivity at 500±100 μS/cm and dissolved O_2 at 6.1 mg/L, ammonia 0.02 mg/L, nitrite 0.02 mg/L, nitrate 21.3 mg/L. The photoperiod was 14 h light, and 10 h dark. The spawning conditions and eggs collections were reported in Caioni et al., 2021.

2.3. Fish embryo acute (FET) toxicity tests

FET tests were performed according to OECD n. 236 (OECD, 2013). PND was tested at 0.25, 0.5, 1, 2 and 3 mg/L. These concentrations were chosen from previous dose ranging-finding tests. PND was dissolved in DMSO, because of its very low water solubility, reaching the final concentration of 0.025 % of DMSO in all experimental groups. Selected embryos were placed individually with 2 mL of test solution in each well of 24-well plates (TPP, Trasadingen, Switzerland). All test plates

had been pre-incubated (saturated) with the test solutions for at least 24 h. Subsequently, well plates were sealed with self-adhesive foil (SealPlate™ by EXCEL Scientific, Dunn, Asbach, Germany). Twenty embryos per treatment were exposed to the five concentrations of PND, and the working solutions were freshly prepared in an exposure medium every 24 h. Negative control (DW), solvent control (0.025% DMSO) and positive control (4% 3,4 dichloroaniline) were also tested. Embryos were exposed for 96 hours post fertilization (hpf) at 26 ± 1° C with14 h light and10 h of dark. Daily zebrafish early-life stages were observed up to 96 h by an inverted optical microscope (CKX 41, Olympus, Japan). Coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and absence of heartbeat, used as indicators of lethality, were recorded.,. FET tests were run in three biological replicates and toxicological endpoints including, Lethal Concentration 10, 20, and 50 (LC 10, 20, 50) and Effective Concentration 10, 20 and 50 (EC 10, 20 and 50), were calculated using TOXRAT v. 3.3 software (ToxRat Solutions GmbH, Germany). In addition to the lethal endpoints specified by OECD TG 236 any other observation was recorded as further lethal or sublethal endpoints, according to von Hellfeld et al. (2020). Common examples were reduced heartbeat or reduced blood flow, inhibited or missing pigmentation, delayed or altered development, modified movement(s), distortion of the spine, and formation of various types of oedemata. At the end of the exposure, the developmental stage did not fall into the regulatory frameworks dealing with animal experimentation, and all the experiments complied with the EU Directive 2010/63/EU.

2.4. Whole-mount Alcian blue staining

Alcian blue staining was performed according to Macaulay et al. (2017). Briefly, 5-days post fertilization (dpf) zebrafish larvae were fixed in 10% buffered formalin overnight at room temperature. The day after, zebrafish samples were washed with 1x PBS with 10% TWEEN 20 (PBST) and then stained overnight in Alcian blue solution. Next, fish were dehydrated in a graded

ethanol series (from 100% to 70%), washed in 1xPBST, digested in trypsin enzyme solution (10 mg/mL trypsin in 30% saturated sodium borate solution), and bleached with 3% H_20_2 in 1% KOH until eyes of specimens became transparent. Fish were imaged on glass depression slides in lateral and dorsal recumbency.

2.5. Alizarin red staining

Alizarin red staining was prepared according to Macaulay et al. (2017). Briefly, 5 dpf and 12 dpf zebrafish larvae were fixed in 10% buffered formalin overnight at room temperature. The day after, zebrafish samples were washed with 1x PBS with 10% TWEEN 20 (PBST) and then stained overnight in in Alizarin Red S solution and cleared in 0.5% KOH before transfer through a graded series of glycerol (from 15% to 70%). Specimens were stored at room temperature in 70% glycerol until imaging. Fish were imaged on glass depression slides in lateral and ventral recumbency.

2.6. Histopathological analyses

5 dpf zebrafish larvae (10 larvae for each experimental group: 0.025% DMSO, PND 0.5 mg/L and PND 0.05 mg/L) were fixed in 10% neutral buffered formalin (NBF) for 24 h at 4°C and then stored in phosphate buffered saline (PBS) to preserve morphology and to prevent distorsion of tissue structure. The fixed larvae were then put in biopsy bags (30 x 45 mm) and were dehydrated through a graded ethanol series by hand, to minimize artifact. The details of dehydrating steps we used are described in Table S1. After replacing water with paraffin wax, the next step was to embed the infiltrated larvae in a block of paraffin. After slowly trim away the face of the paraffin block until the first features of the fish appeared, serial 5 µm sections from all specimens were cut with a 2030 Biocut rotary microtome (Reichert-Jung, Germany), floated on a 37°C water bath and quickly mounted on glass slides (Super-Frost, Menzel-Gläser, Braunschweig, Germany). The slides were put in an incubation oven at 37° C for 24 hour and then stained manually with Haematoxylin and Eosin (HandE). The sequence and time of the staining steps are shown in Table S2. After staining,

sections were protected by mounting a coverslip over the tissue using a mounting medium to adhere the coverslip to the slide and were examined under a light microscope (DM4000 Leica). Digital photos were taken with OLYMPUS-DP12 camera for detection of histopathological alterations.

2.7. Biomarkers of oxidative stress and neurotoxicity

Five dpf zebrafish larvae were homogenized at 4 °C in 1 mL of 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA and protease inhibitor cocktail (1:10 v/v) (Sigma-Aldrich), and centrifuged at 12,000 g for 30 min at 4 °C. Supernatants (SN) were sampled and used for analyses. Total protein concentration in SN samples was also quantified (Bradford, 1976).

Superoxide dismutase (SOD) activity was measured following the method of Crapo et al. (1978). Results are expressed as U/mg protein.

The method of Aebi (1984) was used to measure catalase (CAT) activity in SN samples at 240 nm. Results are expressed as U/mg protein.

The glutathione S-transferase (GST) activity was measured at 340 nm according to Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Results are expressed as nmol/min/mg protein.

Glutathione reductase (GR) activity was evaluated measuring the (5-thio (2-nitrobenzoic acid)) TNB production at 412 nm using the method proposed by Smith et al. (1988). Results are expressed as U/mg protein.

Glutathione peroxidase (GPx) activity was measured following the decrease of NADPH at 340 nm using H_2O_2 as substrate (Lawrence and Burk, 1976). Results are expressed as nmol/min/mg protein.

The acetylcholinesterase (AChE) activity was evaluated using the method proposed by Ellman et al. (1961), following the colorimetric reaction between acetylthiocholine and 5,5’dithio-bis-2-

nitrobenzoate at 405 nm for 5 minutes using a microplate reader. The results were expressed as nmol/min/mg of protein.

2.8. Statistical analysis

The normal distribution of data (Shapiro-Wilk's test) and the homogeneity of the variances (Bartlett's test) were assessed. Results of each biomarker were evaluated using a one-way ANOVA followed by the LSD post-hoc test for pairwise comparisons. The software package Statistica 13.4 (TIBCO Software Inc.) was used. All results are expressed as means $\pm$ standard error (n=5). Statistical analysis of FET tests results was performed using ToxRat software version 3.3 (ToxRat Solutions GmbH, Germany).

3. Results

3.1 FET tests

The toxicity of PND was determined at 96 hpf as lethal concentration, and LC10, LC20, and LC50 values were 1.77, 2.07, 2.81 mg/L, respectively. Moreover, the EC10, EC20 and EC50 were 0.27, 0.35 and 0.55 mg/L, respectively (Table 1). Late coagulation and absence of heartbeat were the only lethal endpoint observed in exposed larvae. Furthermore, zebrafish larvae treated with PND developed sublethal alterations, including impaired fin development, lordosis, scoliosis, blood congestion, impaired blood flow, and reduced heartbeat. The most observed deformities were cardiac- and spine-related. The phenotype of zebrafish early-life stages treated with PND was shown in Figure 1 and 2. The number of survived embryos developing sublethal alterations increased in a concentration-dependent manner (starting from 0.5 mg/L to 3 mg/L) and in a time-dependent manner (from 72 hpf to 96 hpf). Furthermore the teratogenicity of PND was determined and calculated as teratogenic index (TI), which was defined as the quotient of LC_{50} and EC_{50}. The

greatest value (TI=5.1) reported for PND showed a wide separation between the malformation and lethaliy dose-response curves, indicating as PND causes severe malformations.

3.2. Whole-mount Alcian blue and Alizarin red staining

5 dpf zebrafish larvae exposed to PND did not show developing malformations, absence of skeletal elements and incorrect morphogenesis at the Alcian blue staining (Figure 3).

Alizarin red staining was executed at 5 dpf (immediately after the treatment with PND) and at 12 dpf. The zebrafish larvae were reared until 12 dpf in glass-tank containing clean ZebTec water (temperature: 28°C, pH: 7±0.2, conductivity: 500±100 μS/cm, and dissolved O_2: 6.1 mg/L) and DW. 5 dpf and 12 dpf zebrafish larvae exposed to 0.5 mg/L of PND showed delayed and reduced ossification of the vertebral centra in the developing vertebral column (Figure 4 and Figure 5).

3.3. Histopathological analysis

Histopathological studies are useful to evaluate the potential effects of PND in fish.

Histopathological observations indicated that PND exposure to 0.5 mg/L caused destructive effects in muscle tissues. Herein obtained results indicate that myofibrillar sarcomeric patterns were clearly altered: in particular, tissue disorganization is visible and myofibrils appeared twisted or split (Figure 6a-b). No histopathological changes were observed in the muscle of control fish (0.025% DMSO, Figure 6c). The histopathological changes may originate from the toxic effects of PND.

3.4. Biomarkers of oxidative stress and neurotoxicity

SOD activity in zebrafish larvae (Figure 7a) was not significantly affected by the PDN treatment (ANOVA: $F_{(3,16)} = 1.17$; $p = 0.35$). On the contrary, CAT activity was affected by the treatment ($F_{(3,16)} = 3.39$; $p = 0.044$), with a statistically significant increase ($p<0.05$) of the enzyme activity in fish larvae exposed to both PND concentrations tested (Figure 7b).

Similarly, the GR activity was significantly influenced by the treatment ($F_{(3,16)} = 9.83$; $p = 0.000$). In details, exposure to PND induced a statistically significant increase in GR activity at 0.05 mg/L ($p<0.001$) and 0.5 mg/L ($p<0.01$), with respect to control (Figure 7c).

GST activity was not significantly ($F_{(3,16)} = 0.90$; $p = 0.46$) affected by exposure to the herbicide (Figure 7d), whereas GPx activity was influenced significantly by treatment ($F_{(3,16)} = 3.60$; $p=0.037$) with a significant decrease in enzyme activity at 0.5 mg/L ($p<0.05$), when compared to control (Figure 7e).

Lastly, AChE activity increased significantly ($F_{(3,16)} = 2.33$; $p<0.05$) in fish larvae treated with 0.5 mg/L of PND (Figure 7f).

4. Discussion

The acute toxicity of PND was recently investigated by Meng et al. (2021) and Park et al. (2021a) that reported a LC50 of 5.409 mg/L at 72 hpf and 29.54 µM (approximatively 8.3 mg/L) at 96 hpf, respectively. In the present study the LC50 value (2.81 mg/L) calculated at 96 hpf was weakly lower compared to those reported by the other authors, but in agreement with those authors, exposure to all concentrations of PND for 24 h did not affect embryo viability.

Zebrafish larvae exposed to PND developed also sublethal alterations including impaired fin development, lordosis, scoliosis, blood congestion, impaired blood flow, and reduced heartbeat. Park et al. (2021a) showed as, the embryo exposure to 20 µM PND increased pathological outbreaks (including yolk sac and heart edema, reduced embryo length and developed a dramatic decrease of embryo heartbeats). Moreover, the developmental toxicity of PND, at the highest concentrations of exposure, increased in concentration-dependent manner with an increase of yolk sac and pericardium edema area, a reduction of the heart rate and of the body length (Meng et al., 2021). These results were in agreement with those of the present study where the number of

zebrafish early-life stages developing sublethal alterations increased in a concentration-dependent manner and also in a time-dependent manner (from 72 hpf to 96 hpf).

Cardiovascular effects induced by chemical exposure in zebrafish early life stages are sublethal endpoints commonly observed and with high recovery potential (Caioni et al., 2021; von Hellfeld et al., 2020). The cardiac alterations induced by PND exposure in our study (blood congestion, impaired blood flow, and reduced heartbeat) could be related to dysregulation in gene expression, particularly those genes related to cardiac development, including vmhc, nppa, tbx5a, nkx2.5, and gata4 (Meng et al., 2021). The toxicity of dinitramine (DN), an herbicide in the dinitroaniline family, was recently investigated in the zebrafish model, and along with the impaired cardiac development, zebrafish larvae treated with this pesticide showed suppressed vessel formation and angiogenesis, probably through activation of the inflammatory response (Park et al., 2021b). The reduction of blood flow reported in zebrafish larvae exposed to PND could be related to the activation of pro-inflammatory cytokines, including il6, cox2a, tnfα, cox2b, cxcl-c1c, il1b, and il2a (Park et al., 2021a). Moreover, the gene expression of vascular development factors, including nos2a, flt4, kdrl, flt1, and kdr, was disrupted in zebrafish larvae treated with PND which showed apparent regression of dorsal aorta, intersegmental vessels, dorsal longitudinal anastomotic vessel, and posterior cardinal vein (Park et al., 2021b).

Zebrafish larvae exposed to PND 0.5 mg/L showed lordosis and spinal cord malformation with notochord fragmentation. Spinal curvature was also reported by Meng et al. (2021) in zebrafish larvae exposed to 3 mg/L of PND. In the present study interestingly, the cartilage development of the head and of the notochord was not affected by PND exposure, while the mineralogenic effects were demonstrated in in the axial skeleton of the zebrafish larvae. In fact, zebrafish larvae treated with 0.5 mg/L of PND, at 5 and 12 dpf, reported reduced mineralized tissue in the head and in the vertebral centra of the developing notochord (Figure 4 and Figure 5). In zebrafish larvae, the restriction of retinoic acid activity by Cyp26b1 is required for proper timing and patterning of

osteogenesis during skeletal development (Laue et al., 2008). Cyp26b1 mutants and RA-treated wild-type fish display a reduction in midline cartilage and the hyperossification of facial and axial bones, leading to fusions of vertebral primordia (Laue et al., 2008), an opposite phenotype compared to the zebrafish larvae exposed to the highest concentration of PND. Moreover, the anabolic effect of vitamin D3 analogs and intermittent parathyroid hormone (PTH) exposure was also demonstrated in the head skeleton of zebrafish larvae at 6 dpf (Fleming et al., 2005). Therefore, the potential role of PND to disrupt the retinoid signaling and/or to interfere with vitamin D activity should be clarified. Osteotoxicity in the zebrafish model may occur after exposure to several environmental toxicants including organophosphates pesticides (Karen et al., 2001). Osteotoxicants have been classified into four categories according to the pathway involved in the transduction of the osteotoxic effects: activation/inhibition of membrane and/or nuclear receptors, alteration of redox condition, mimicking of bone constituents and unknown pathways (Fernández et al., 2018). Among nuclear receptors, estrogen and androgen receptors play a key role in bone formation and chondrogenesis (Fernández et al., 2018). Recently, chronic exposure to sublethal concentrations of PND increased plasma estradiol levels and decreased plasma testosterone leading to reproductive toxicity (Gupta and Verma, 2020a). Oxidation and reduction conditions are central events that drive skeletal development and bone maintenance. In fact, mechanosensing, a key process that tightly controls bone development/maintenance, depends on the redox balance (Fernández et al., 2018). Thus, the osteotoxic effects of PND could also be related to the imbalance of oxidative status in exposed larvae.

Zebrafish (*Danio rerio*) have proven to be a highly useful tool to assess the impact of several toxicants on skeletal muscle including pesticides (Dubińska-Magiera et al., 2016). Zebrafish larvae exposed to 0.5 mg/L of PND showed notochord degeneration and disrupted muscle fiber morphology (Fig. 6a,b). The muscular phenotype developed by zebrafish larvae treated with PND 0.5 mg/L was similar to those induced by fipronil exposure. In particular, zebrafish embryos

continuously exposed to fipronil showed reduced body length, notochord degeneration with fragmentation of notochord vacuoles and presence of fibrous material, as well as abnormal axial muscle morphology with shorter myotomes and abnormally sinusoidal muscle fibers (Stehr et al., 2006). The reduced body length and abnormal muscle fiber morphology in fipronil-exposed larvae reflect sustained bilateral contractions of the axial muscles, leading to notochord degeneration (Stehr et al., 2006). During PND exposure, the exact mechanism of action of musculoskeletal toxicity is still poorly understood and it could be related to changes in microtubule stability, which could alter the distribution and dynamic of cytoskeleton components, as recently demonstrated for glyphosate in a zebrafish model (Díaz-Martín et al., 2021). Moreover, alterations in muscle histology are usually associated to oxidative stress, which in turn could further worsen the histological injury by creating a vicious cycle (Huang et al., 2021). The teratogenic potential of PND, as confirmed by the calculation of TI, could also explain the histological alterations observed in the present study.

The antioxidant defense has been also a focus of toxicological research for the last decade as a possible mechanism of pesticide toxicity (Danion et al., 2014; Stara et al., 2020a; Stara et al., 2020b). The exposure of zebrafish larvae to ROS-generating stressful conditions may result in either induction or inhibition of antioxidant enzymes (Rodríguez-Fuentes et al., 2015; Velki et al., 2017). In the present study, PND exposure increased the activity of CAT, GR, and GPx, while no effects were observed on the activity of SOD and GST. Interestingly, the modulation of antioxidant enzymes was also observed in zebrafish larvae exposed to the environmentally relevant concentrations (e.g., CAT and GR activities). SOD is the enzyme responsible for the elimination of superoxide anion, while hydrogen peroxide can be partially eliminated by CAT and glutathione peroxidase GPx (Hoseinifar et al., 2020). Thus, the oxidative stress induced by PND exposure could be potentially related to the production of non-radical species H_2O_2 rather than superoxide anion.

In addition to CAT, hydrogen peroxide can also be neutralized by GPx in the presence of glutathione (GSH) (Hoseinifar et al., 2020). which could be potentially involved in PND oxidative stress. PND caused a reduction of GSH content in gills of rainbow trouts exposed to 800 ng/L (Danion et al., 2014). The same authors also observed a significant increase in SOD and GPx activities in the liver and a decrease of CAT activity after 28 days of exposure to all the treatment concentrations. Similarly, Acquaroni et al. (2021) observed CAT and GST decreases in the amphibian *Rhinella arenarum* larvae exposed for 96 h to the insecticide dimethoate, while the highest dose tested (1mg/L) induced an increase in SOD activity. The authors also observed an increase of TBARS levels after 96 h of exposure to both 0.5 and 1 mg/L.

GR activity is required for the regeneration of reduced GSH, previously oxidized by the action of GPx (Parolini et al., 2019), while to make these compounds more soluble in water, glutathione S-transferase (GST) catalyzes the conjugation of reduced GSH to several substrates in the presence of selenium (Hoseinifar et al., 2020). The increased activity of GR supports the hypothesis of the involvement of GSH in PND oxidative stress action. In zebrafish, Gaaied et al. (2019) observed a significant decrease of GST activity in larvae exposed for 96 h to the 2,4 D herbicide. Furthermore, an increase of CAT activity at 0.8 mg/L and of GPx activity at both 0.02 and 0.051 mg/L was observed. In addition, a generalized increase of lipid peroxidation levels in all treated animals was recorded Gaaied et al. (2019).

Oxidative stress induced by the excessive production of ROS following pesticides exposure could also lead to DNA, proteins, and membranes damages. Recently, the genotoxicity of PND was evaluated in freshwater fish *Clarias batrachus* (linn.) through comet assay, and the oxidative stress was potentially investigated as the mechanism of DNA damage (Gupta and Verma, 2020b). Moreover, several authors investigated using comet assay as an indirect measure of oxidative stress, the DNA damage caused by PND, confirming the valuable contribution of the above technique to

detect PND genotoxicity (Ahmad and Ahmad, 2016; Ahmad et al., 2018; Gupta and Verma, 2020b). Genotoxic effects are considered among the most important of the possible side effects of agricultural chemicals. If a chemical reacts with nuclear DNA, it may be mutagenic and carcinogenic to the exposed organisms (Ahmad and Ahmad, 2016). Marked ROS accumulation can also lead to cell apoptosis. In fact, the exposure to PND increased the number of apoptotic cells in the heart of zebrafish larvae and induced up-regulation of pro-apoptotic genes (*P53, FoxO1, Bax, Casp-9, Casp-3*) (Meng et al., 2021).

Interestingly, it has also been reported that oxidative stress also plays a role in the regulation and activity of AChE (Rodríguez-Fuentes et al., 2015).

The cholinergic system, which had acetylcholine (ACh) as a neurotransmitter, is involved in cognitive processes, through the activation of metabotropic muscarinic and ionotropic nicotinic cholinergic receptors (Richetti et al., 2011). The reaction responsible for the maintenance of levels of ACh is catalyzed by two cholinesterases (ChE): acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE), which is absent in teleost fish (Richetti et al., 2011). The evaluation of AChE activity is recognized as a useful biomarker to investigate the potential neurotoxicity of environmental chemicals, and usually, its activity is inhibited following exposure to numerous pollutants, including pesticides (Kais et al., 2015). Interestingly zebrafish larvae exposed to 0.5 mg/L of PND showed an increased AChE activity. These results are in agreement with those obtained by Sobjak et al. (2017) that observed a significant increase of AChE activity after 12 h, 48 h and 72 h of exposure of *Rhamdia quelen* to 6.5 mg/L of glyphosate fish larvae. The neurotoxicity of this herbicide was recently demonstrated in the zebrafish model (Wang et al., 2022). We hypothesized that increased AChE activity observed in the present study could be related to the oxidative stress induced by PND. Moreover, in the zebrafish model, the brain AChE activity increased following the induction of a hyperglycemic state which led to free radical production and

consequent oxidative stress in the different brain regions Capiotti et al. (2014) and Park et al. (2021a) demonstrated the ability of PND, probably through the oxidative stress-mediated mechanism, to impact the electron transport chain (ETC) activity and mitochondrial complexes in zebrafish larvae. The authors showed that this herbicide induced metabolic changes with a shift of the energy phenotype from energetic to quiescent after PND exposure (Park et al., 2021a). These metabolic changes could be also responsible for the increased AChE activity in zebrafish larvae exposed to PND. Conversely, Tabassum et al. (2016) reported a decrease in AChE activity in the brain of *Channa punctata* specimens exposed for 96 h to both 0.5 and 0.8 µg/L of pendimethalin. In addition, the authors observed a reduction of SOD, CAT and GST activities, and an increase of oxidative damage to lipid and proteins at the concentration tested. However, further studies are needed to elucidate the role of PND in metabolic disruption.

5. Conclusions

The results of the present study confirm the developmental toxicity of PND in zebrafish's early stages.

Displaying malformations such as impaired fin development, lordosis, scoliosis, blood congestion, impaired blood flow, and reduced heartbeat and the high value of TI show as the PND primarily affects the cardiovascular system and confirm the PND as teratogen to zebrafish embryos.

Oxidative stress could be implicated in the development of sublethal alterations. Previous studies evaluated the toxicity of this dinitroaniline herbicide in the zebrafish model, but without considering the effect of environmentally relevant concentrations. Zebrafish larvae exposed to PND 0.05 mg/L did not develop any sublethal alterations or effects at cartilage and bone levels, however, the antioxidant enzymes were affected by the herbicide treatment. Further studies are warranted to characterize the toxicological mechanisms of action of PND, and chronic studies on adult zebrafish are needed to evaluate the long-term effects of this herbicide.

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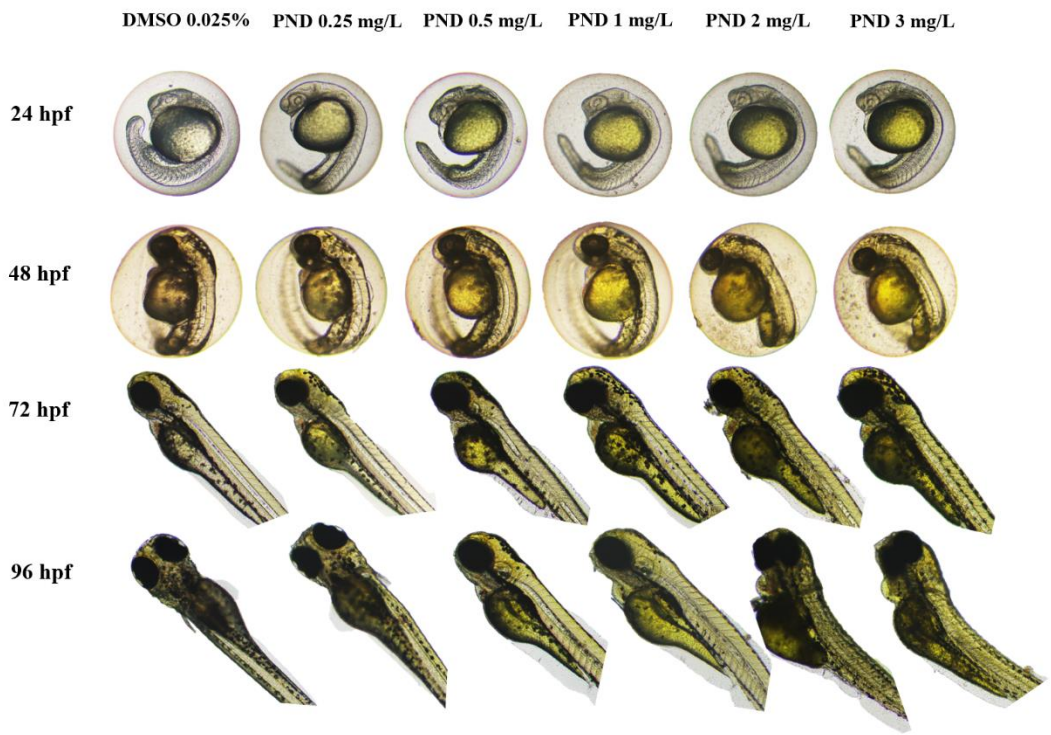

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630 **Figure 1.** Zebrafish early-life stages exposed to different concentrations of PND during FET tests.

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632 **Figure 2.** Zebrafish spinal malformations in 5 dpf zebrafish larvae.

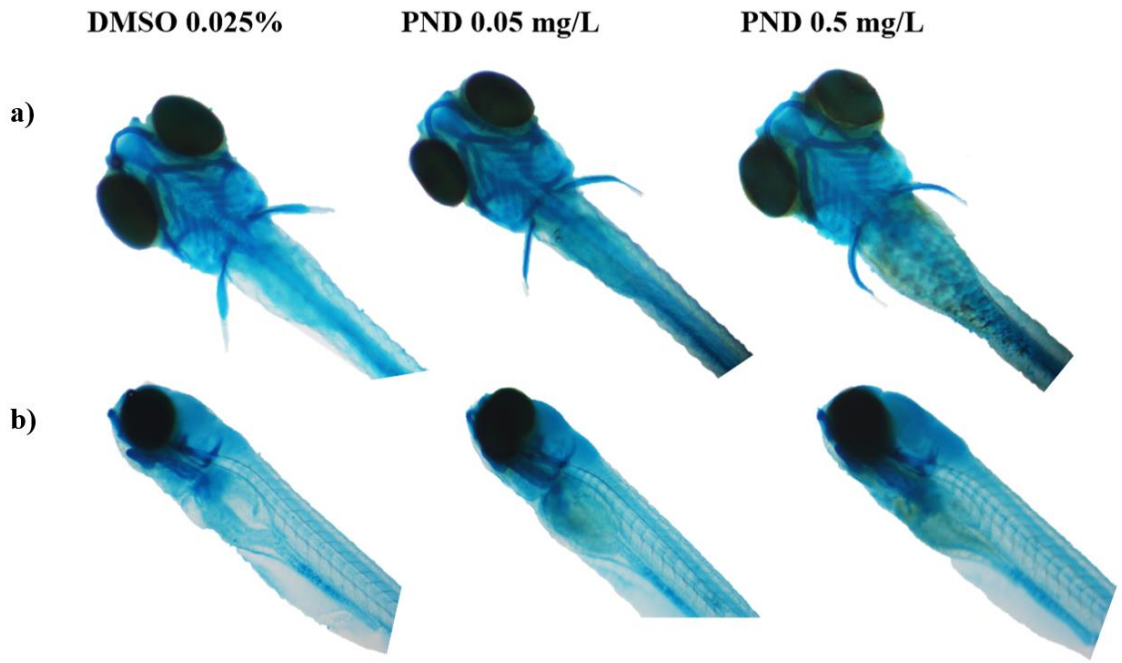
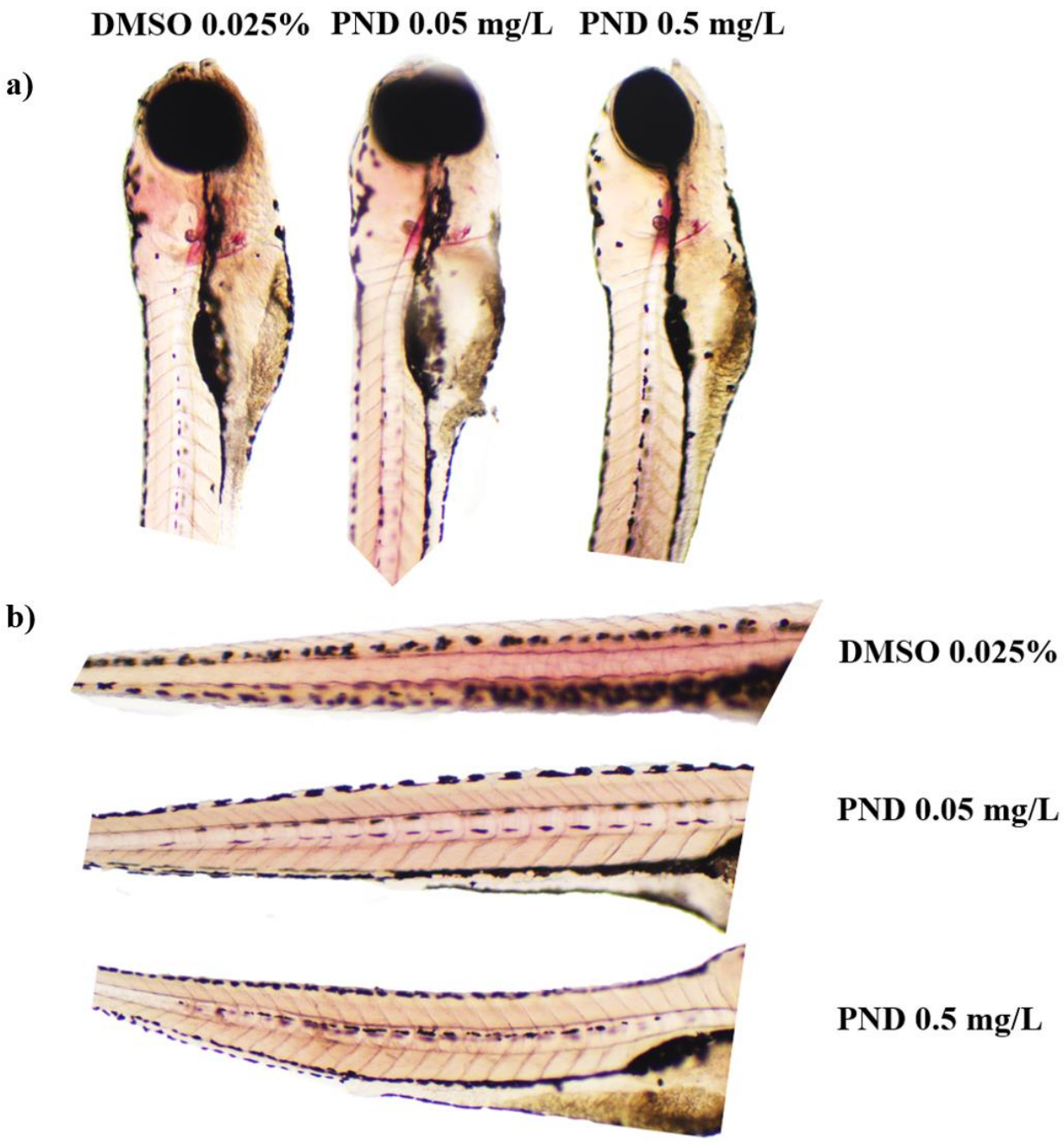


Figure 3. Representative 5 dpf zebrafish larvae stained with Alcian blue (ventral (a) and lateral (b) views of zebrafish larvae exposed to solvent control (DMSO 0.025%), PND 0.05 mg/L and PND 0.5 mg/L.



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643 **Figure 4.** Representative 5 dpf zebrafish larvae stained with Alizarin Red (lateral view (a) and
644 vertebral column (b) of zebrafish larvae exposed to solvent control (DMSO 0.025%), PND
645 0.05 mg/L and PND 0.5 mg/L).

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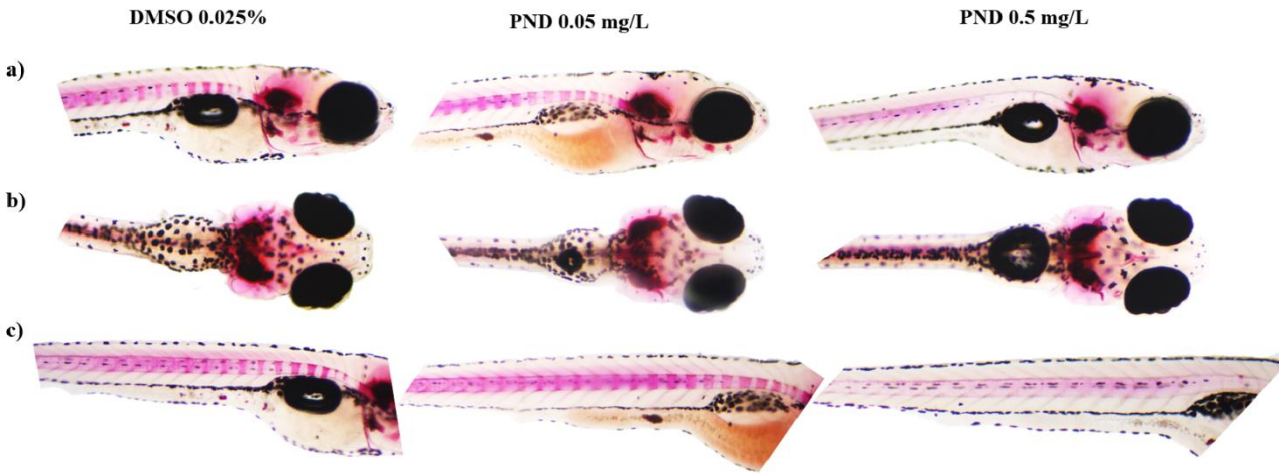

Figure 5. Representative 12 dpf zebrafish larvae stained with Alizarin Red. Lateral (a) and dorsal (b) views and vertebral column (c) of zebrafish larvae exposed to solvent control (DMSO 0.025%), PND 0.05 mg/L and PND 0.5 mg/L.

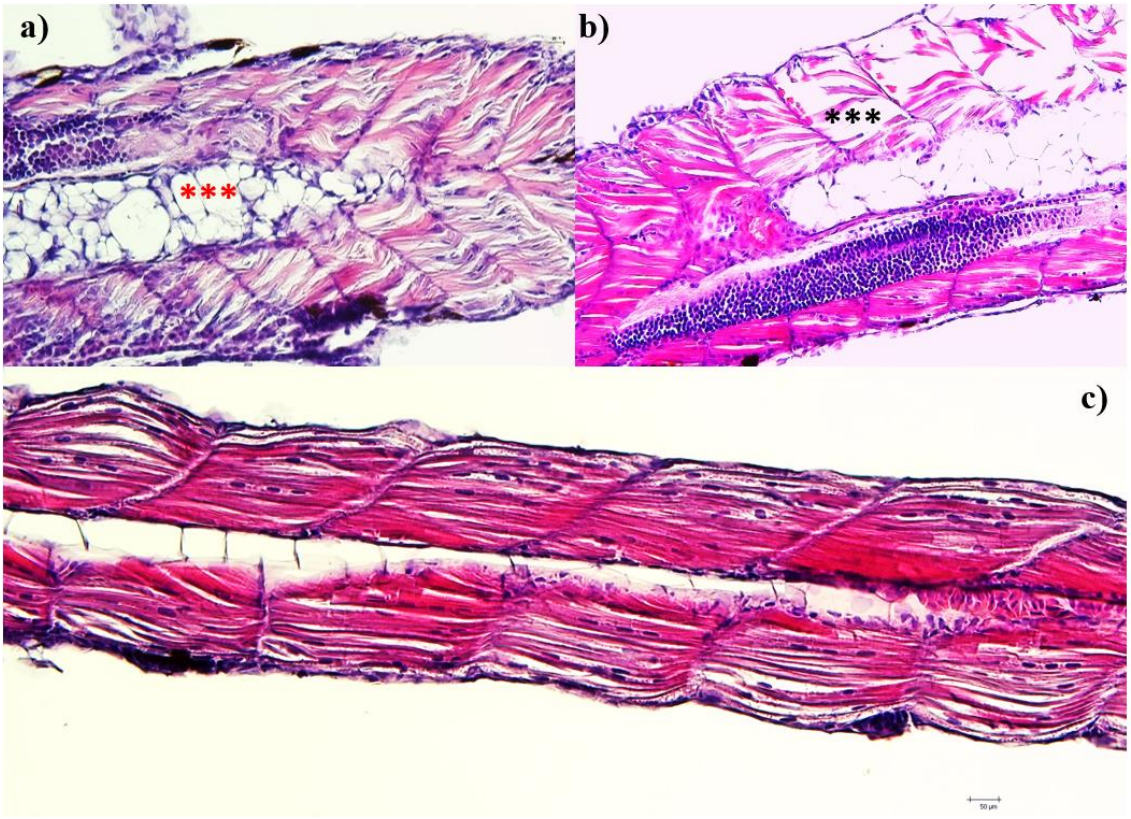

Figure 6. Histology sections of notochord and muscle of 5 dpf zebrafish larvae. a-b)Histopathological changes in notochord (red asterisk) and muscle (black asterisk) of zebrafish exposed to PND

0.5 mg/L. Tissue disorganization is visible, notochord appeared degenerated, and myofibrils appeared twisted or split. EandE 20x.

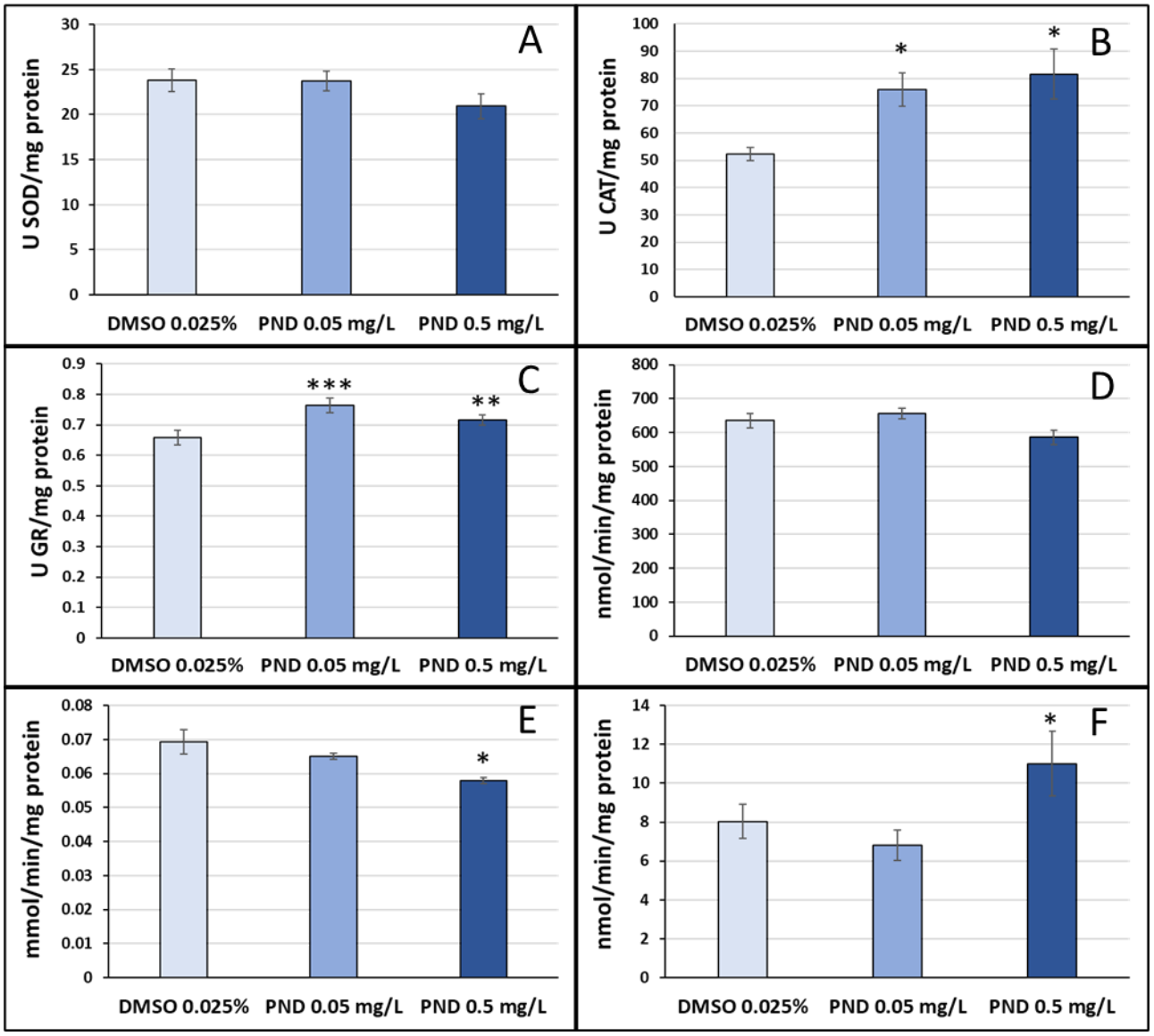

Figure 7. SOD activity, expressed as U SOD/mg protein (A), CAT activity, expressed as U CAT/mg protein (B), GR activity, expressed as U GR/mg protein (C), GST activity, expressed as nmol/min/mg protein (D), GPx activity, expressed as nmol/min/mg protein (E), and AChE activity, expressed as nmol/min/mg protein (F). The values are mean ± SE (n = 5). The asterisks indicate significant differences in comparison with control: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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666 **Table 1.** Toxicological endpoints of PND derived from zebrafish FET tests at 96 hpf.

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Time (hpf)	LC10 (95% CL) mg/L	LC20 (95% CL) mg/L	LC50 (95% CL) mg/L	EC10 (95%CL) mg/L	EC20 (95%CL) mg/L	EC50 (95%CL) mg/L
24	3.78 (1.63-n.d.)	n.d.	n.d.	3.78 (1.63-n.d.)	n.d.	n.d.
48	3.78 (1.63-n.d.)	n.d.	n.d.	3.02 (1.43-n.d.)	n.d.	n.d.
72	3.78 (1.63-n.d.)	n.d.	n.d.	0.69 (0.12-1.08)	0.89 (0.25-1.33)	1.44 (0.82-2.42)
96	1.77 (1.41-1.99)	2.07 (1.78-2.27)	2.81 (2.57-3.18)	0.27 (0.22-0.32)	0.35 (0.29-0.4)	0.55 (0.49-0.62)

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669 *CL: confidence limits; n.d.: not determined*

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author Contributions Statement

C. Merola: Methodology, Validation, Investigation, Writing – Original Draft. J. Fabrello: Formal analysis, Investigation. V. Matozzo: Visualization, Supervision. C. Faggio: Writing – Review & Editing. A. Iannetta: Formal analysis, Investigation. A. Tinelli: Formal analysis, Investigation. G. Crescenzo: Writing - review & editing. M. Amorena: Visualization. M. Perugini: Resources, Visualization, Supervision, Project administration.