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Waterborne zinc pyrithione modulates immunity, biochemical, and antioxidant parameters in the blood of olive flounder

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ABSTRACT

In this study, potential immunological and hematological effects of different concentrations (0, 1, 10, and 50 μg L^{-1}) of waterborne zinc pyrithione (ZnPT) were studied in the blood of the olive flounder *Paralichthys olivaceus* over 30 days. Reduced alternative complement activity (ACH50) and lysozyme activity were measured in fish exposed to 10 and/or 50 μg L^{-1} of ZnPT for 20 days. Decreased levels of total Ig were also observed in response to 10 and/or 50 μg L^{-1} ZnPT during the exposure period. Levels of cortisol, a marker of stress, were significantly increased by 10 and 50 μg L^{-1} ZnPT from day 10, and by 1 μg L^{-1} exposure on day 30. The levels of red blood cells (RBCs) and white blood cells (WBCs) decreased following exposure to 10 and/or 50 μg L^{-1} ZnPT, while no significant change was observed in hemoglobin level. Concentrations of total protein and albumin were significantly reduced with 50 μg L^{-1} ZnPT at day 20. Alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activities were significantly increased following exposure to 10 and/or 50 μg L^{-1} ZnPT. Lipid peroxidation was induced by ZnPT, and higher concentrations (10 and 50 μg L^{-1}) significantly increased intracellular malondialdehyde levels during exposure. Regarding the subsequent antioxidant response, intracellular glutathione levels increased significantly in response to 10 and 50 μg L^{-1} ZnPT on days 20 and 30. Similarly, catalase and superoxide dismutase activity was significantly increased in response to 10 and 50 μg L^{-1} ZnPT after day 10. Taken together, changes in the studied parameters suggested the immunotoxicity of ZnPT, with modulations observed in hematological homeostasis and oxidative stress induction in the blood of olive flounder.

1. Introduction

Zinc pyrithione (ZnPT) is an organic complex containing two pyrithione ligands chelated to Zn^{2+}, and is one of the most widely used metal pyrithione booster biocides in many copper-based antifouling paints [1]. ZnPT has been produced extensively for many purposes, particularly for industrial applications, boat and ship paint, oilrigs, underwater plumbing, and aquaculture tanks [2]. In addition, due to its broad spectrum of antimicrobial activity, ZnPT has been widely used as an algaecide, bactericide, and fungicide [3]. In the past, an organotin (OT) compound, tributyltin (TBT), was applied widely as an antifouling agent in marine antifouling paints on ship hulls to control fouling organisms [4]. In recent decades, the use of TBT has been strictly banned in marine antifouling paints due to its persistence and toxic effects on non-target aquatic organisms. Since the global ban of TBT, ZnPT-based antifouling paints have been manufactured globally as a substitute for OT [1]. The high ZnPT consumption for various purposes has led to its rapid occurrence in freshwater, seawater, and sediments due to its low water solubility and high degradability characteristics [5]. In aquatic environments, ZnPT is readily photo-degraded under direct sunlight to less toxic compounds [6]. Conversely, the formation of a more stable copper pyrithione (CuPT) is possible in the aquatic environment, which is readily converted from ZnPT in the presence of Cu^{2+} [5–7].

Zinc and ZnPT are ubiquitous pollutants in the aquatic environment, and their presence and persistence are attracting increasing attention due to their potential effects on non-target organisms [6,7]. Thus,

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Table 1
Summary of studies investigating the exposure of various concentrations of ZnPT to different fish species.

Fish species	Exposure condition	Exposure	Endpoints measured	Reference
Brachydanio rerio	8–9 μg L^{-1} for 7 d	Waterborne	EC50 and embryo toxicity	[14]
Carassius auratus gibelio	0.163, 0.126, and 0.113 mg L^{-1} for 96 h	Waterborne	LC50 and tissue accumulation	[17]
Cyprinodon variegatus	400 μg L^{-1} for 96 h	Waterborne	LC50	[61]
Danio rerio	0, 0.003, 0.008, 0.016, 0.03 or 0.16 μM for 8 d	Waterborne	Morphological, histological, and molecular response	[20]
Gambusia holbrooki	40, 80, and 160 μg L^{-1} for 96 h	Waterborne	Neurotoxicity, oxidative stress, and histological changes in liver and gill	[19]
Lepomis macrochirus	0.021 μg L^{-1} for 96 h	Waterborne	LC50	[8]
Oryzias latipes	3–7 μg L^{-1} for 7 d	Waterborne	EC50 and embryo toxicity	[14]
	0.185–0.370 μg L^{-1} for 4 m	Waterborne	Survival, development, weight/length ratio, and infestation	[15]
	0, 1, and 10 μg L^{-1} for 48 h	Waterborne	Swimming behaviour, survival, respiration, morphology, and hatching rate	[16]
Oryzias melastigma	43 μg L^{-1} for 96 h	Waterborne	LC50	[10]
Oncorhynchus mykiss	3.2 μg L^{-1} for 96 h	Waterborne	LC50	[8]
Pagrus major	98.2 μg L^{-1} for 96 h	Waterborne	LC50	[18]
	3.88 mg L^{-1} for 96 h	Waterborne	Oxidative stress in gill, liver, brain, and kidney	[9]
Pimephales promelas	2.6 μg L^{-1} for 96 h	Waterborne	LC50	[8]
Salvelinus fontinalis	0.008 mg L^{-1} for 96 h	Waterborne	LC50	[8]

previous studies have found that ZnPT can be highly toxic to aquatic organisms such as algae [8,9], planktons [8,10], polychaete [11], and mollusks [8,12,13]. In fish, the ecotoxicological impacts of ZnPT have been examined by measuring endpoints such as acute toxicity, developmental toxicity, and histopathological changes (Table 1). For example, LC_{50} values have ranged from nanogram to microgram levels in numerous fish (references in Table 1). The detrimental effects of ZnPT have been extensively studied on the survival, growth, swimming behaviour, respiration, and morphology of Japanese medaka *Oryzias latipes* embryos [14–16]. Bioconcentrations of ZnPT have been observed in the liver and kidney tissues of *Carassius auratus gibelio* [17]. Moreover, ZnPt was shown to induce molecular biomarkers, neurotoxicity, oxidative stress, and histopathological changes in several fish [9,18–20]. These findings suggest that ZnPT has detrimental effects on fish; however, knowledge on the potential effects of ZnPT on immunity and blood homeostasis are unclear.

In aquatic toxicology studies, blood has been used to reflect the patho-physiology of the whole body, and blood parameters are commonly used as biomarkers for diagnosing the structural and functional status of fish [21]. In immunological studies, the levels of alternative complement activity (ACH50), lysozymes, and total immunoglobulin (Ig) have been suggested to predict the health status of aquatic organisms, since changes in these parameters can reflect disease/stressful conditions; however, these parameters have received limited attention in the case of antifoulants, including ZnPT. The complement system plays an essential role in fish innate immunity, with extensive immune recognition capabilities [22]. Lysozyme activity in serum is a convenient parameter for monitoring the potential impact of environmental changes on fish innate immunity [23]. Data suggest that the responses of hormonal (e.g. cortisol), hematological (e.g. hemoglobin [Hb]; red blood cells [RBCs], white blood cells [WBCs]), and biochemical (total protein; glucose; albumin; alanine aminotransferase [ALT]; aspartate aminotransferase [AST]; alkaline phosphatase [ALP]) parameters in fish can be affected by toxicants [24,25]. ALT and AST play vital roles in protein/carbohydrate metabolism and the conversion of amino acids and α-ketoacids. Serum ALP regulates various metabolic processes, including catalyzing the dephosphorylation of nucleotides and proteins, calcium and phosphate metabolism, and phosphate group transfer [24,25]. These parameters are used widely as indicators of tissue damage in the liver and kidney.

In living organisms, environmental toxicants can lead to the production of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and/or hydroxyl radicals through direct and/or indirect mechanisms [26]. Oxidative stress results from increased ROS production and reduced antioxidant defense, or a combination of both mechanisms [27]. Oxidative stress induces severe molecular damage and leads to the dysfunction of multiple organs and systems. Malondialdehyde (MDA) is a main lipid oxidation product formed by the decomposition of unsaturated fatty acid peroxides, which are normally degenerated by ROS [28]. The MDA content can directly reflect the degree of oxidative damage induced by toxicants. Glutathione (GSH) is an important low-molecular-weight non-enzymatic antioxidant involved in cellular metabolism, ROS detoxification, and xenobiotic excretion [27]. GSH acts directly as a reductant or as a substrate for antioxidant enzymes, and ensures the reduction of oxidants and the neutralization of peroxides [29]. The antioxidant defense system performs an important function against the generated ROS. Superoxide dismutase (SOD) primarily catalyzes the transformation of superoxide anion to hydrogen peroxide (H_2O_2), whereas catalase (CAT) converts superoxide anion to H_2O and O_2 [27]. Therefore, SOD and CAT activities are a first-line of defense against oxidative stress, and these enzymes protect the cells against lipid peroxidation, protein oxidation, and DNA damage caused by ROS [28]. These parameters are routinely measured and used as valuable biomarkers to assess the impacts of environmental toxicants, even in olive flounder [30,31]. In this study, to determine whether ZnPT affects innate immunity, biochemical homeostasis, and antioxidant defense systems, the response of blood biomarkers were measured in the economically important marine fish *Paralichthys olivaceus* upon exposure to different concentrations of waterborne ZnPT (0, 1, 10, and 50 μg L^{-1}).

2. Materials and methods

2.1. Fish

The current study was performed following the guidelines of the Animal Welfare Ethical Committee and the Animal Experimental Ethics Committee of the Korea Institute of Ocean Science and Technology (KIOST, South Korea). *P. olivaceus* (average mean weight 94.3 ± 6.82 g) were obtained from the local fish farm in Tongyeong, Gyungnam, South Korea. The fish were randomly introduced to three aquaria. They were stocked in an automated aquaculture system containing filtered seawater (6.78 ± 0.71 mgO_2 L^{-1}) at 18 ± 1.2 °C. A photoperiod consisting of a 14:10 light-dark (LD) cycle was used. Fish were fed with frozen mosquito larvae and *Artemia salina* twice a day until satiation for 16 days before the experiment.

2.2. ZnPT exposure and blood collection

Round tanks (450 L) were prepared to house *P. olivaceus* during ZnPT exposure. Fish were acclimatized for an additional 5 days in the experimental tanks to reduce the potential stress. ZnPT was purchased from Sigma-Aldrich, USA. Ultra-pure water was used to prepare the ZnPT stock solution at different concentrations (0, 1, 10, and

50 μg L^{-1}). For the toxicity studies, healthy fish were taken from the stock and exposed to different ZnPT concentrations for 30 days at 20 °C. Prior to this experiment, we conducted a preliminary toxicity study to determine the final ZnPT concentrations; concentrations of ZnPT above 250 μg L^{-1} induced high mortality within 1 week. Various concentrations of ZnPT (0, 1, 10, and 50 μg L^{-1}) were added to each aquarium. Fifteen fish per group were separated into three subgroups (n = 5 per subgroup). The test water was renewed after 48 h and freshly prepared solution was added to maintain ZnPT at a constant concentration. During the experimental periods, fish were fed continuously every day under the same environmental conditions. Fish mortality/survival was recorded every 24 h, and no mortality was observed during the above study period. In addition, there was no significant phenotypic abnormality or physiological change during experiment. This was probably due to the relatively low concentrations of ZnPT and the controlled conditions in the exposure system, which can prevent exogenous infections. After 5, 10, 20, and 30 days, fish from the control- and ZnPT-treated groups were taken for further analysis. Fish were first anaesthetized (not euthanized) with 200 mg L^{-1} of MS-222 solution (tricaine methanesulfonate, Sigma-Aldrich, USA). Blood samples (> 100 μL per fish) were collected by cardiac puncture and transferred into heparinized tubes (Sarstedt, Nümbrecht, Germany). The remainder of the blood sample was centrifuged at 1600 × *g* at 4 °C for 10 min to separate the serum for further analysis.

2.3. Immunological parameters

ACH50 levels were estimated in fish using an established method [22] with slight modifications (buffer volume, basic instruments employed). A working buffer of ethylene glycol tetraacetic acid (EGTA)-Mg-gelatin-veronal buffer (GVB; veronal-buffered saline containing 10 mmol L^{-1} EGTA, 10 mmol L^{-1} $MgCl_2$, and 0.1% gelatin) was prepared and sheep red blood cells (SRBC; 1.5 × 10^6 cells; National Institute of Toxicological Research, South Korea) were used as targets for the assay. Then, the hemolytic activity of olive flounder serum was reconstituted by incubating SRBCs (5 μL) in 6% serum (25 μL) and 10 mM phenol red-free Hank's buffer with 5 mM Mg^{2+} and 0.15 NaCl (pH 7.3) in a 96-well plate. The plate was incubated for 90 min at 20 °C with gentle shaking. The hemolytic reaction was stopped by adding 1 mL of stop solution (working buffer containing 10 mM EDTA). Hemolysis was measured spectrophotometrically with a Varioskan Flash spectrophotometer (Thermo Fisher Scientific, USA) at 414 nm. Complete (100%) and zero hemolysis (0%) were measured by adding the washed SRBCs (25 μL) to distilled water (100 μL) and phenol red-free Hank's buffer, respectively. ACH50 was calculated as the reciprocal of the serum dilution, causing 50% lysis of SRBCs (ACH50; U mL^{-1}) based on the value of Y/1 - Y against the reciprocal of the serum dilutions on a log-log scaled graph.

Lysozyme activity was measured using a turbimetric assay [32], modified in a 96-well plate. Briefly, *Micrococcus lysodeikticus* (ATCC No. 4698; 0.3 mg mL^{-1}) (Sigma-Aldrich, USA) lyophilized in sodium phosphate buffer (0.05 M; pH 6.2) was used as a substrate for plasma lysozyme. Triplicates of test serum (diluted 1:2, 10 μL) were added to 200 μL of the *M. lysodeikticus* suspension, and the mixture was incubated at 25 °C. The reduction in absorbance at 450 nm was measured after 0.5 and 4.5 min with a Varioskan Flash spectrophotometer (Thermo Fisher Scientific, USA). One unit of lysozyme activity was expressed as the amount of enzyme that caused a decrease in absorbance of 0.001 per min.

Total Ig contents were estimated using a method performed in gilthead seabream [33] with slight modifications (buffer volume, basic instruments employed). The serum samples were diluted 100-fold with NaCl (0.85%) and the protein content of each sample was measured following the Bradford method using bovine serum albumin (BSA) as a standard [34]. Each serum sample was mixed with an equal volume of polyethylene glycol (PEG; 10,000 MW; Sigma-Aldrich, USA) solution. Subsequently, the mixed sample was incubated for 2 h and centrifuged at 5000 × *g* for 10 min at 4 °C. Supernatant from each sample was moved and diluted 50-fold with NaCl (0.85%) and the protein content was measured as described above. Differences between the protein contents of the untreated and PEG-treated sample corresponded to the total Ig content, expressed as mg mL^{-1}.

2.4. Hematological and biochemical studies

The serum cortisol level was examined using an ELISA kit (Fish Cortisol ELISA Kit, CUSABIO, TX, USA) with the enzymatic substrate tetramethylbenzidine (TMB). Color intensity was inversely proportional to the concentration of cortisol in the samples and the absorbance was read at 450 nm in a Varioskan Flash spectrophotometer (Thermo Fisher Scientific, USA). The cortisol level was expressed as ng mL^{-1}.

Hb content (g dL^{-1}) in blood was determined spectrophotometrically using the cyanmethemoglobin method at 540 nm. RBCs (10^6 dL^{-1}) and WBCs (10^4 dL^{-1}) were counted manually with a hemocytometer. The serum glucose level (Glucose Colorimetric Detection Kit, Invitrogen, CA, USA) and albumin (Fish Albumin ELISA Kit, MyBioSource, CA, USA) were examined with commercial kits, based on the manufacturer's instructions. The serum total protein level was determined according to the Bradford method. Furthermore, levels of the enzymes ALT, AST, and ALP were estimated in serum using a Fish Alanine Aminotransferase ELISA Kit, a Fish Aspartate Aminotransferase ELISA Kit, and a Fish Alkaline Phosphatase ELISA Kit (MyBioSource, USA), respectively, based on the manufacturer's instructions.

2.5. Antioxidant enzymes activity

To estimate MDA, blood samples were mixed with five volumes of Tris buffer (20 mM) and then, centrifuged at 30,000 × *g* at 4 °C for 30 min. The obtained supernatants were heat-denatured at 75 °C for 15 min. Thiobarbituric acid-reactive substances were quantified at 535 nm using a Thermo Varioskan Flash spectrophotometer (Thermo Fisher Scientific, USA), and their concentrations were examined based on a standard curve using MDA bis (dimethylacetal, Sigma-Aldrich, USA). Finally, the MDA levels were estimated based on a calibration curve and calculated as nmol of MDA per mg of total sample.

Intracellular GSH contents and SOD and CAT activity were determined as described in our previous study, with small modifications in buffer volume and the basic instruments used [25,31,35]. To measure GSH levels, pooled bloods were washed in 0.9% NaCl, and then homogenized in trichloroacetic acid at a ratio of 1:20 (w/v) with a Teflon homogenizer. Each homogenate was centrifuged at 3000 × *g* at 4 °C for 10 min. The upper aqueous layer was collected and used to assay GSH contents using an enzymatic method with a Glutathione Assay Kit (Catalog No. CS0260; Sigma-Aldrich, Inc.) based on the manufacturer's protocol. The GSH contents were measured at an absorbance of 420 nm with a Thermo Varioskan Flash spectrophotometer (Thermo Fisher Scientific) and standard curves were generated using GSH equivalents of 0, 150, and 350 μM. Intracellular GSH content was expressed as nmol per mg of the total protein.

To determine the enzymatic activities of CAT and SOD, the pooled fish were homogenized in ice-cold buffer (0.25 M sucrose and 0.5% Triton X-100, pH 7.5) at a 1:4 ratio (w/v) using a Teflon homogenizer. The homogenate was centrifuged at 3000 × *g* at 4 °C for 30 min. The upper aqueous layer was collected to analyze activity according to the manufacturer's procedures. CAT and SOD activities were measured using Catalase (Catalog No. CAT100; Sigma-Aldrich Chemie, Switzerland) and SOD Assay Kits (Catalog No. 19160; Sigma-Aldrich Chemie) at absorbances of 520 and 440 nm, respectively, at 25 °C with a Thermo Varioskan Flash spectrophotometer (Thermo Fisher Scientific), and expressed as unit (U) per mg of total protein.

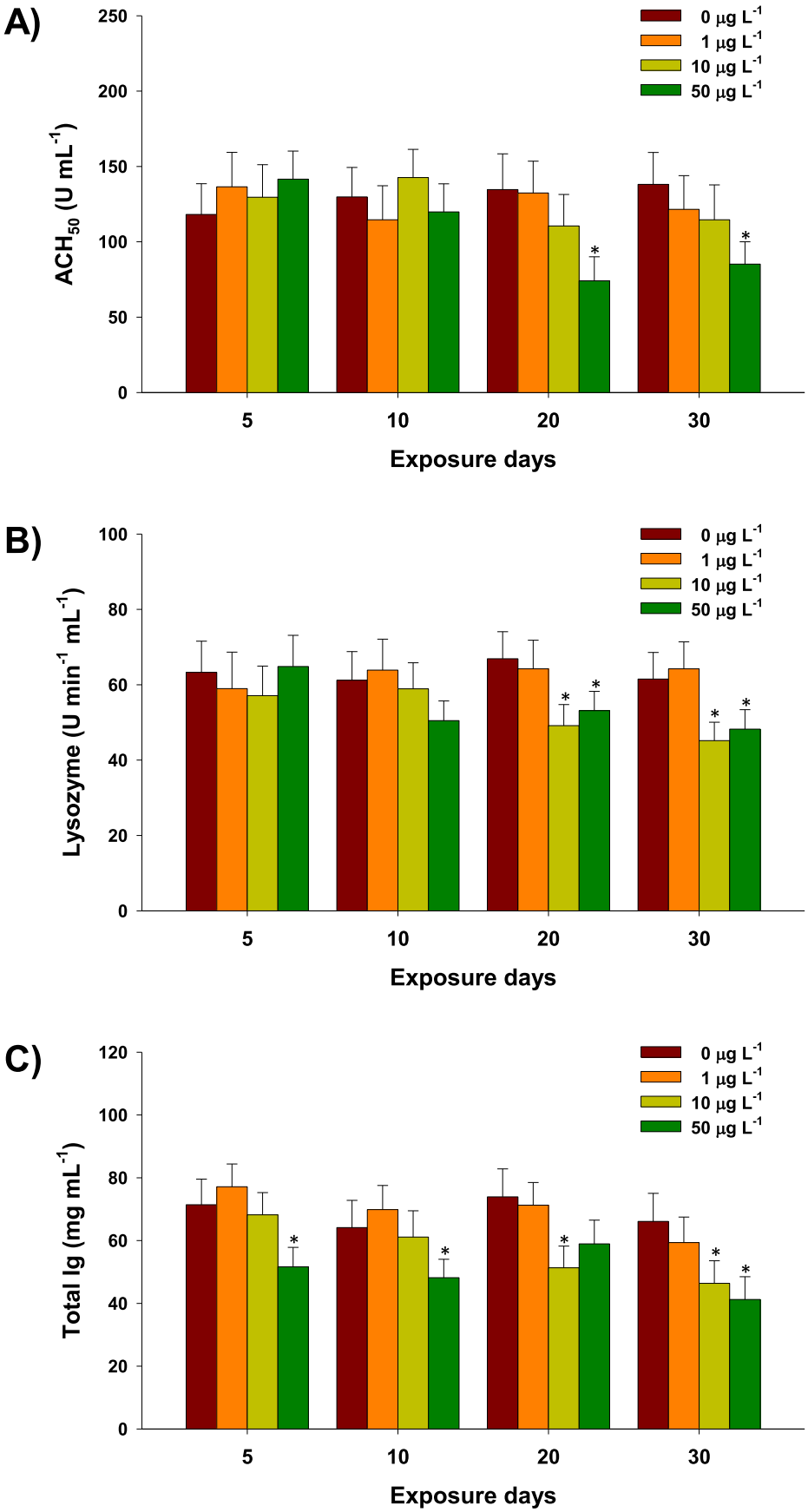

Fig. 1. Effects of 30-days exposure to different concentrations (0, 1, 10, and 50 μg L^{-1}) of waterborne ZnPT on the immunological parameters, A) ACH50, B) lysozyme, and C) total Ig in the serum of olive flounder *Paralichthys olivaceus*. Data are shown as means ± standard deviation (S.D) of three replicates. The asterisk (*) indicates statistical significance ($P < 0.05$) compared with the control value.

2.6. Statistical analyses

All data were presented as mean ± standard error of the mean (SEM) using the SPSS statistical software package (ver. 17.0, SPSS Inc., Chicago IL, USA). Two-way ANOVA was employed to investigate the interaction between ZnPT concentration and time. When significant differences were calculated via ANOVA, an additional Tukey test was used to determine significant differences between ZnPT treatment and time. A statistical probability of $P < 0.05$ was considered significant.

3. Results

3.1. Immunological response

ACH50 activity in the serum of *P. olivaceus* was significantly reduced following treatment with 50 μg L^{-1} ZnPT on days 20 and 30 compared with 0, 1, and 10 μg L^{-1} ZnPT ($P < 0.05$) (Fig. 1A). Similarly, significant decreases in lysozyme activity were measured in response to 10 and 50 μg L^{-1} ZnPT on days 20 and 30, respectively, compared with the lower concentrations (0 and 1 μg L^{-1}) (Fig. 1B). The total serum Ig contents were also significantly reduced following 50 μg L^{-1} ZnPT exposure for 30 days ($P < 0.05$) (Fig. 1C). In addition, the levels of total Ig were significantly reduced on days 20 and 30 following exposure to 10 μg L^{-1} ZnPT ($P < 0.05$).

3.2. Hematological response

The higher concentrations of ZnPT (10 and 50 μg L^{-1}) used in this study induced a significant increase in cortisol throughout the study period, except on day 5 ($P < 0.05$) (Fig. 2A). Even the lowest concentration of ZnPT used (1 μg L^{-1}) resulted in elevated cortisol levels on day 30 ($P < 0.05$). No significant change in Hb content was observed at 30 days (Fig. 2B). Exposure to 50 μg L^{-1} ZnPT significantly reduced RBC levels on days 20 and 30, and and by 10 μg L^{-1} ZnPT on day 20 ($P < 0.05$) (Fig. 2C). A significant reduction in WBC levels was observed in response to 50 μg L^{-1} ZnPT on days 10 and 30 ($P < 0.05$) (Fig. 2D). Overall, no significant modulation of hematological parameters was observed on day 5 ($P > 0.05$).

3.3. Biochemical parameters

No significant change in total protein concentration ($P > 0.05$) was observed, except for a significant decrease in samples exposed to 50 μg L^{-1} ZnPT on day 20 ($P < 0.05$) (Fig. 3A). There was no significant change in glucose concentration at 30 days with ZnPT at any concentration used ($P > 0.05$) (Fig. 3B). Exposure to 50 μg L^{-1} ZnPT significantly decreased the albumin concentration at day 20 ($P < 0.05$) (Fig. 3C), while no significant change was observed with any other concentrations used ($P > 0.05$).

ALT activity increased significantly following exposure to 10 and/or 50 μg L^{-1} ZnPT for 30 days ($P < 0.05$) (Fig. 4A). Particularly, a significant increase in ALT activity was observed in response to 50 μg L^{-1} ZnPT at day 5. Regarding AST activity, significant increases were observed following exposure to 50 μg L^{-1} ZnPT for 10 and 30 days ($P < 0.05$) (Fig. 4B). ALP activity was significantly increased after exposure to 50 μg L^{-1} ZnPT on days 20 and 30 ($P < 0.05$) (Fig. 4C). In addition, 10 μg L^{-1} ZnPT treatment increased ALP activity on day 30 ($P < 0.05$).

3.4. Response of the antioxidant defense system

The highest concentrations of ZnPT used (10 and 50 μg L^{-1}) significantly increased the MDA levels over 30 days ($P < 0.05$) (Fig. 5A). High levels of intracellular GSH were observed in response to 10 and 50 μg L^{-1} ZnPT on days 20 and 30 (Fig. 5B). The activity of key antioxidants, namely CAT and SOD, were also significantly increased after exposure to 10 and 50 μg L^{-1} ZnPT from day 10 onwards ($P < 0.05$) (Fig. 5C and D).

4. Discussion

The highest concentrations of ZnPT used in this study significantly decreased the enzymatic activities of ACH50 and lysozyme, and the concentration of Ig. Since over-expression of the complement system is a general response to exogenous pathogens [36], the decreased ACH50 values suggest that complement components may have been depleted in *P. olivaceus* due to prolonged exposure to ZnPT. The reduced lysozyme activities and decreased Ig levels may support the trend found for ACH50. Lysozymes are mainly released from leukocytes, such as neutrophils, monocytes, and macrophages [23,37]. Thus, decreased lysozyme activity could be explained by a strong effect of ZnPT on circulating lysozyme-producing cells. Although there have been no reports on the effects of ZnPT on leukocytes in fish, Zn exposure can induce immune suppression in fish by decreasing leukocyte production, resulting in reduced lysozyme activity [38]. Since lysozymes have strong antibacterial activity, the significant decrease in lysozyme activity in the blood of *P. olivaceus* exposed to ZnPT may also indicate dysfunctional innate defense mechanisms. Reduced lysozyme activity has been reported in fish in response to biocides [39–41]. The decreased total Ig in *P. olivaceus* also supports the immunosuppressive effects of ZnPT, which may result from the reduced numbers of lymphocytes (a type of WBC) in fish [42]. Overall, the reduced immunological response indicates that *P. olivaceus* might have failed to maintain non-specific humoral immunity, and this decrease may promote susceptibility to infection or pathological challenges. A recent study suggested that Zn treatment could suppress non-specific immune responses in zebrafish [43]. Taken together, long-term exposure to high concentrations of ZnPT clearly induced immunotoxic effects in olive flounder.

Cortisol is a major circulating adrenocortical steroid hormone, which is involved in regulating osmolality, metabolism, immunity, and stress responses in fish [44]. Subsequently, cortisol has been frequently measured to estimate the potential impact of environmental pollutants on fish as a primary stress response. Significant increases in blood cortisol levels observed in *P. olivaceus* suggest that a primary adaptation response occurs when the fish are subjected to the stress of ZnPT toxicity, since increased cortisol may facilitate osmoionic balance and gluconeogenic processes to maintain homeostasis [44,45]. Although no studies have reported the effects of ZnPT on fish cortisol, Zn exposure significantly was found to increase cortisol levels in the blood of Nile tilapia [46]. No significant change in the Hb concentration was observed in the present study; however, there was a significant decrease in the levels of other essential blood components, RBC and WBC, in response to high concentrations of ZnPT in *P. olivaceus*. Previous studies have shown that Zn triggered a significant reduction in RBC levels in fish [47,48]; this can be explained by ZnPT inducing hemolysis and anemia of the RBCs by inhibiting erythropoiesis. The decrease in WBCs might be a stress response reflecting the reduced non-specific immunity, as the primary function of WBCs is the regulation of immunological function against infectious disease and pathogens in fish.

Regarding the ZnPT-induced reduction in total protein levels, this might result from reduced protein synthesis, since hypoproteinemia has been suggested as a general response in metal-exposed fish [25,49,50]. Albumin content in blood is an important indicator of liver and kidney dysfunction [51]. In fish, higher accumulation of Zn and ZnPT has been observed in the liver and kidney compared with other organs, as well as subsequent histopathological effects in tissues [17,20,47,52,53]. Embryonic exposure to ZnPT induced significant liver deformity in zebrafish [20]. Since hypoproteinemia and lowered albumin levels (hypoalbuminemia) have been suggested as a sequence of liver and kidney damage [51], we assumed that ZnPT might affect the liver and kidney. The potential accumulation of ZnPT in the liver could explain the significant increases in ALT, AST, and ALP activities observed in *P.*

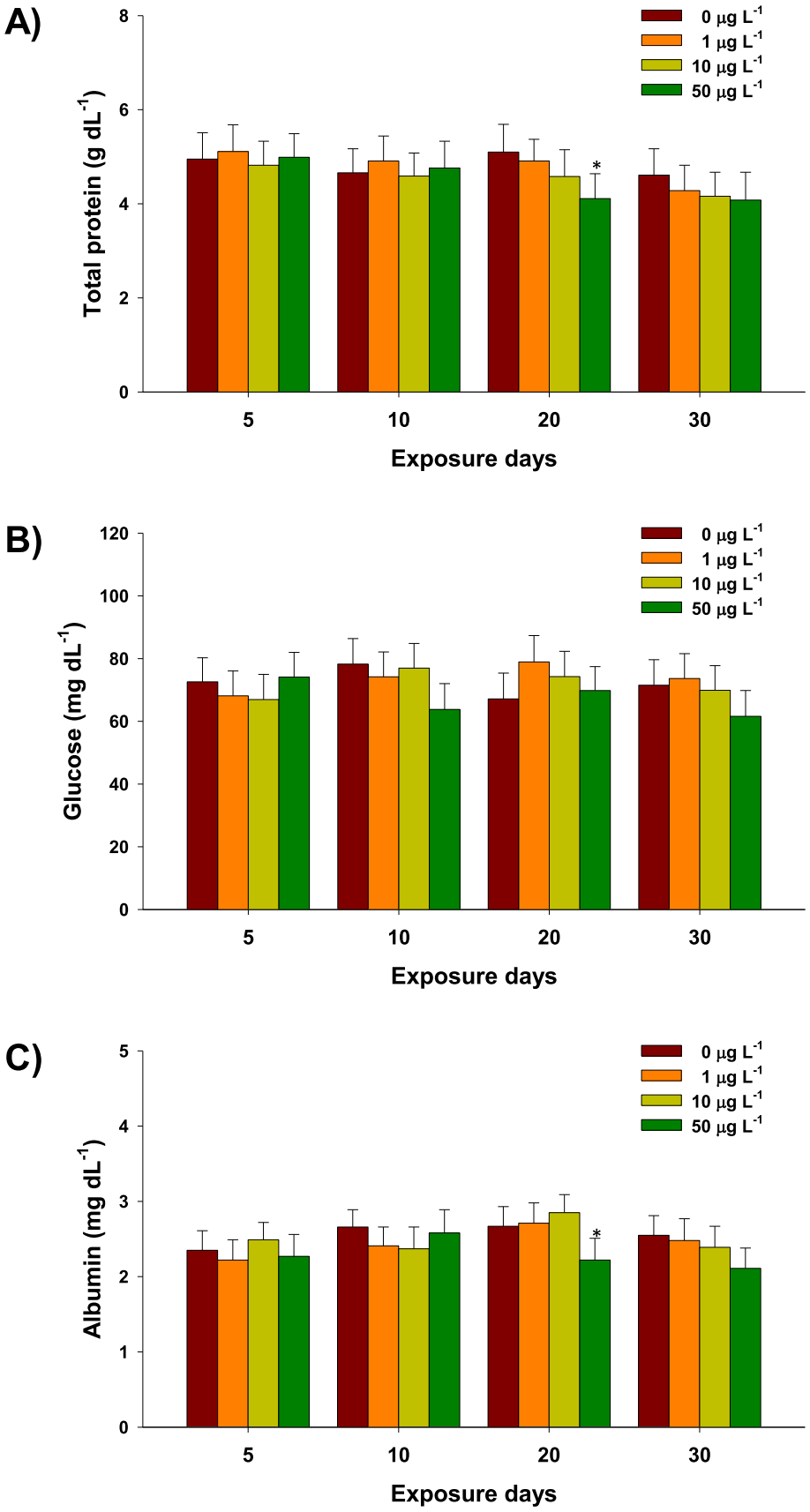

Fig. 2. Effects of 30-days exposure to different concentrations (0, 1, 10, and 50 μg L^{-1}) of waterborne ZnPT on hematological parameters, A) cortisol, B) Hb, C) RBC, and D) WBC in the serum of olive flounder *P. olivaceus*. Data are shown as means ± S. D of three replicates. The asterisk (*) indicates statistical significance ($P < 0.05$) compared with the control value.

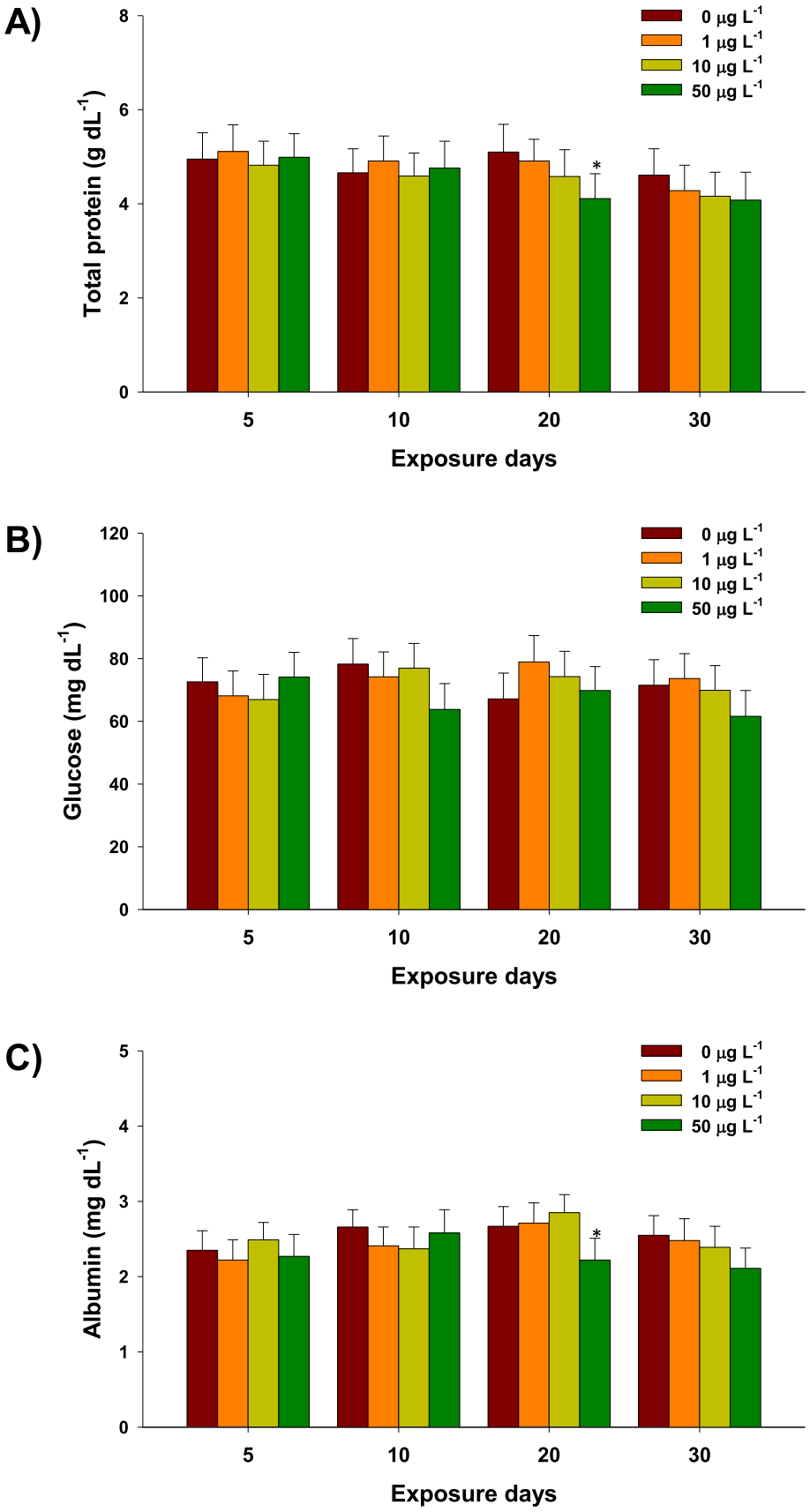

Fig. 3. Effects of 30-days exposure to different concentrations (0, 1, 10, and 50 μg L^{-1}) of waterborne ZnPT on biochemical parameters, A) total protein, B) glucose, and C) albumin in the serum of olive flounder *P. olivaceus*. Data are shown as means ± S. D of three replicates. The asterisk (*) indicates statistical significance ($P < 0.05$) compared with the control value.

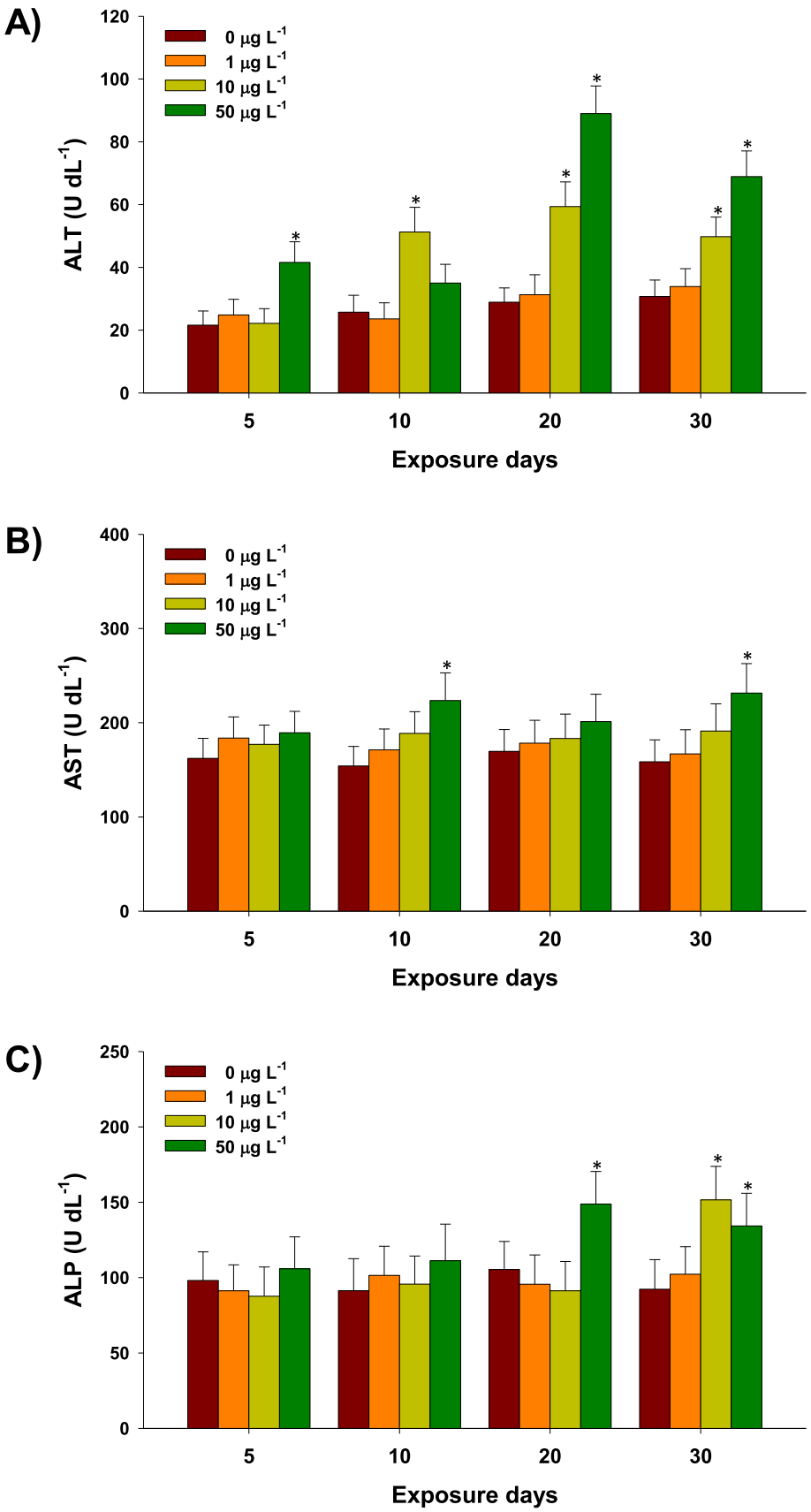

Fig. 4. Effects of 30-days exposure to different concentrations (0, 1, 10, and 50 μg L^{-1}) of waterborne ZnPT on the enzymatic activities of A) ALT, B) AST, and C) ALP in the serum of olive flounder *P. olivaceus*. Data are shown as means ± S. D of three replicates. The asterisk (*) indicates statistical significance ($P < 0.05$) compared with the control value.

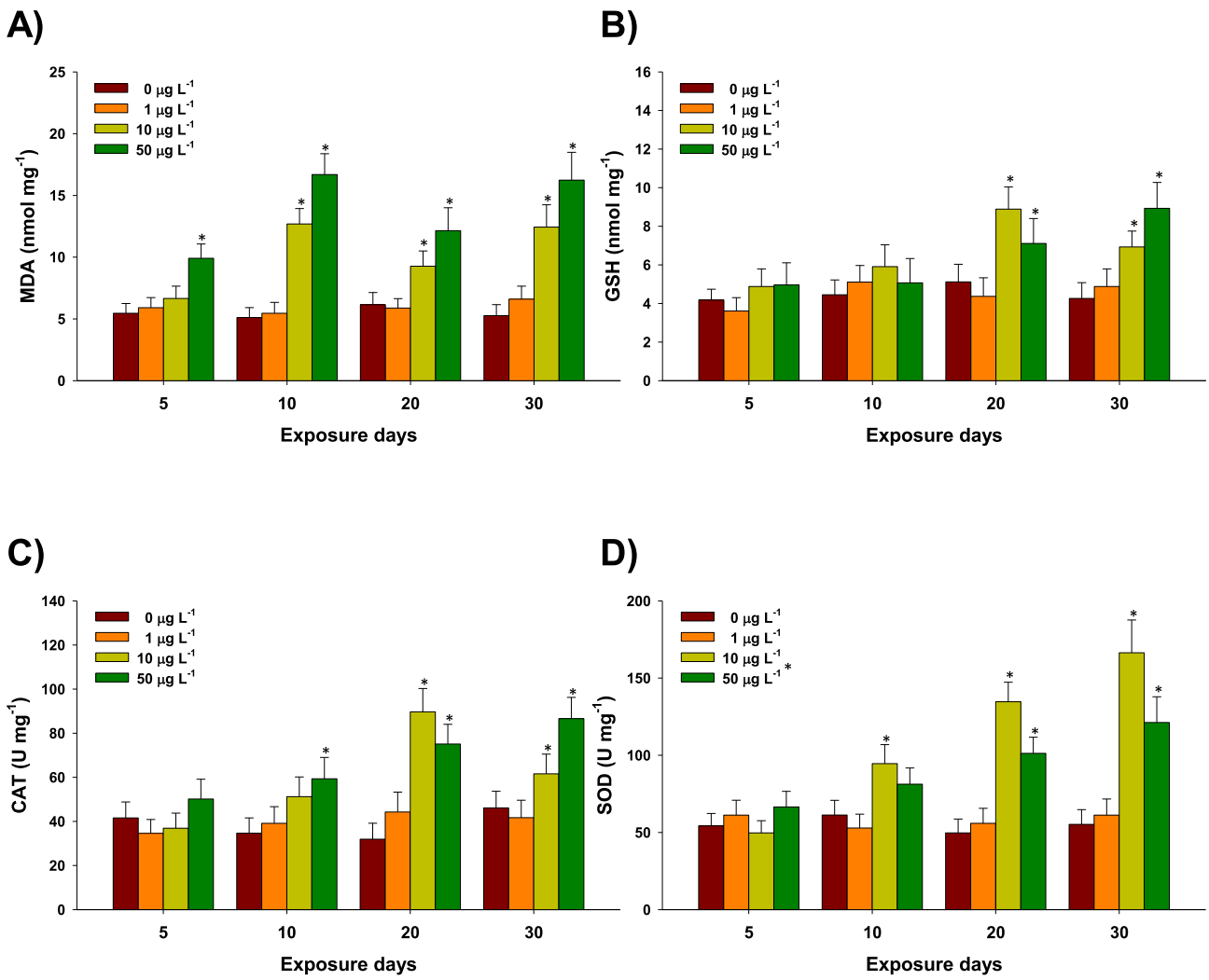

Fig. 5. Effects of 30-days exposure to different concentrations (0, 1, 10, and 50 μg L^{-1}) of waterborne ZnPT on the antioxidant defense system, A) MDA, B) GSH, C) CAT, and D) SOD in the serum of olive flounder *P. olivaceus*. Data are shown as means ± S. D of three replicates. The asterisk (*) indicates statistical significance ($P < 0.05$) compared with the control value.

olivaceus. Several studies have provided evidence of Zn- or ZnPT-induced cell rupture and/or tissue damage in the liver and subsequent elevations in ALT, AST, and ALP activity in fish [12,17,19,20,46,47,53]. Since the liver is the main target organ for detoxification, further studies on the potential effects of ZnPT on liver-specific drug metabolism and detoxification capacity can strengthen to understand the mode of action of ZnPT in fish.

Pyrithione is membrane-permeable and can directly facilitate Zn^{2+} entry into intracellular pools, including mitochondria [54]. Zn^{2+} can induce intracellular ROS generation in fish [53]. The high levels of MDA observed in the blood of *P. olivaceus* might indicate ZnPT-induced oxidative stress. Exposure to ZnPT has been shown to induce significant oxidative damage, as well as DNA damage and apoptosis in aquatic organisms [12,13,55]. Previous studies have suggested that ZnPT induces oxidative stress by increasing the amount of superoxide anions in cells [56,57]. Antioxidant defense systems play critical roles in protecting cells from the toxicity of xenobiotic electrophiles, and maintaining redox homeostasis by consuming GSH. In general, decreased GSH level suggests higher conjugation activity of GSH with certain compounds. Subsequently GSH depletion induces GSH synthesis for maintaining cellular homeostasis through a negative feedback mechanism by the activity of γ-glutamylcysteine synthetase (γ-GCS). Since prolonged depletion of intracellular GSH can induce apoptosis, cells allow for the generation of significant quantities of GSH. Thus, increased GSH level might be an adaptive response of cells to the increased oxidative stress. Increased GSH levels have been associated with elimination of ZnPT toxicity through reduction of excess ROS production and inhibition of cell death [58]. Subsequently, the first-line of the antioxidant defense system, CAT and SOD, could be stimulated to diminish ZnPT-induced oxidative stress in *P. olivaceus*. Previous reports have shown the co-induction of CAT and SOD enzymes in response to Zn exposure in fish [19,59].

Flounders are economically important fish for marine culture. The most aquaculture infrastructures are coated with antifouling agents, as infrastructure components provide surfaces for biofouling. Besides, the antifouling agents released from aquaculture system may increase stress and decrease immunity, resulting in vulnerability to disease. Previously, the toxic organotin TBT reduces resistance to infection in flounder and other flatfish [60]. However, the environmental effects of ZnPt are not fully understood in fish including flounders, as the chemical has been assumed to be environmentally neutral. Thus, our results provide some insights into the non-target effects and risks of ZnPt on immunity and homeostasis of fish.

5. Conclusion

In conclusion, long-term exposure to waterborne ZnPT significantly modulated immunological and hematological homeostasis with

induction of oxidative stress in the economically important fish *P. olivaceus*. Our results also suggested that even microgram levels of ZnPT could be toxic when the fish were exposed to it consistently. Successively measuring parameters in whole blood showed the potential target of ZnPT in this fish. However, in the environment, *P. olivaceus* can be impacted by boating and may develop resistance or compensatory mechanisms by employing these components against ZnPT contamination. Thus, our data indicate a threshold effect of *P. olivaceus* upon ZnPT exposure. Since blood components are important for maintaining general physiological and innate immunity in fish, each parameter should be developed as a biomarker for ZnPT contamination. Finally, these data on ZnPT exposure in olive flounder strengthen existing reports, and are important for understanding the response on the fate and occurrence of ZnPT in aquatic environments due to a lack of available research.

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