



## Full length article

## Waterborne manganese modulates immunity, biochemical, and antioxidant parameters in the blood of red seabream and black rockfish

Jeong Wan Do<sup>a,1</sup>, Manoharan Saravanan<sup>b,c,1</sup>, Sang-Eun Nam<sup>b</sup>, Hyun-Jeong Lim<sup>d</sup>, Jae-Sung Rhee<sup>b,c,e,\*</sup><sup>a</sup> Aquaculture Research Division, National Institute of Fisheries Science, Busan, 46083, South Korea<sup>b</sup> Department of Marine Science, College of Natural Sciences, Incheon National University, Incheon, 22012, South Korea<sup>c</sup> Research Institute of Basic Sciences, Incheon National University, Incheon, 22012, South Korea<sup>d</sup> Aquaculture Industry Research Division, East Sea Fisheries Research Institute, Gangneung, 25435, South Korea<sup>e</sup> Institute of Green Environmental Research Center, 169 Gaetbeol-ro, Yeonsugu, Incheon, 21999, South Korea

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## ABSTRACT

Immunotoxic effects of manganese (Mn) were investigated in the blood of the economically important marine fish, red seabream (*Pagrus major*) and black rockfish (*Sebastes schlegelii*) when exposed to different concentrations of Mn (0, 0.5, 1, and 2 mg L<sup>-1</sup>) for 14 days. During exposure, the levels of alternative complement activity in both fish were significantly lowered at 2 mg L<sup>-1</sup> of Mn of exposure. Lysozyme activity was significantly decreased in black rockfish in all concentrations of Mn after 14 days, while in red seabream, the decrease was significant with concentrations of 1 and 2 mg L<sup>-1</sup> of Mn after 7 and 14 days of exposure. A significantly low level was observed only in the 2 mg L<sup>-1</sup>-exposed red seabream on day 14 of exposure. The concentrations of hemoglobin, red blood cells, white blood cells, and total serum proteins were significantly decreased in both fish under exposure to 1 and 2 mg L<sup>-1</sup> of Mn, while cortisol, alanine transferase, aspartate transaminase, and alkaline phosphatase levels were significantly increased compared to the levels of control groups. No significant change was found in serum glucose and albumin except in red seabream exposed to 2 mg L<sup>-1</sup> of Mn for 14 days. The responses of the antioxidant defense system were significantly induced in both fish after exposure to 1 and 2 mg L<sup>-1</sup> of Mn on day 7 and 14 of exposure. Taken together, alterations of these parameters suggest the immunotoxicity of waterborne Mn produced by the modulation of hematological components and the induction of oxidative stress in the blood of these marine fish.

## 1. Introduction

Manganese (Mn) is an essential micronutrient element found in all tissues of living organisms, being required for macromolecular metabolism (e.g., amino acid, lipid, protein, and carbohydrate metabolism), cellular biochemistry, and physiological functions [1]. Manganese is ubiquitous in the environment as the 12th most abundant element in Earth's crust, constituting about 0.1% of it [2,3]. Manganese has been extensively used as a raw material in many commercial products like dry cell batteries, glass, ceramics, dyes, pigments, soil and food supplements, and pesticide formulations [3,4]. Manganese from municipal wastewater discharge, sewage sludge, mining and mineral processing, emissions from alloy, steel and iron production, combustion of fossil fuels, and emissions from the combustion of fuel additives are the major

routes for Mn density increase in various segments of the aquatic ecosystem [5]. Manganese concentrations, particularly in freshwaters, have been reported to be between 1 and 200 mg L<sup>-1</sup> [2]. Higher levels of Mn can cause significant threats to the environment, harming aquatic organisms and humans [5], and is becoming a global problem as a potential emerging contaminant due to the lack of facilities for the removal of elevated Mn levels in aquatic ecosystem [6,7]. Moreover, in many countries it has been recommended that the maximum acceptable standard limit for Mn in drinking water should not exceed 0.1 mg L<sup>-1</sup> and in Korea the limit is 0.05 mg L<sup>-1</sup> [8].

Fish have been widely used as sentinel indicators of aquatic pollution because of their high sensitivity to environmental contaminants [9]. Waterborne metals can easily enter the body through the gills or other tissues and are potentially bioconcentrated in vital organs [10].

\* Corresponding author. Department of Marine Science, College of Natural Sciences, Incheon National University, Incheon, 22012, South Korea.  
E-mail address: [jsrhee@inu.ac.kr](mailto:jsrhee@inu.ac.kr) (J.-S. Rhee).

<sup>1</sup> These authors contributed equally to this work.

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The effect of Mn on aquatic ecosystems has become a serious environmental problem worldwide, but little attention has been paid to the Mn adverse effects on fish [7,11]. Alternative complement activity (ACH<sub>50</sub>), lysozyme, and immunoglobulins (Ig) are some of the major indicators for the homeostasis of the immune system in fish [9]. In toxicological studies, ACH<sub>50</sub> and lysozyme levels have been most frequently examined in serum of fish in order to monitor the potential effect of environmental changes or pollutants on fish innate immunity [12]. Lysozyme is an antimicrobial enzyme produced as a component of humoral immunity, being an important parameter of innate immunity in fish [13]. Immunoglobulins are glycoproteins that contribute both to innate and adaptive immunity in fish, protecting the host against several pathogenic invasion and diseases [9].

Hematological profiles of fish are commonly used to assess environmental pollution in aquatic ecosystems and the health status of fish [14]. Cortisol is a major corticosteroid hormone in fish and plays an important role as a primary stress response, and thus is widely used as a biomarker in toxicological studies [15,16]. In general, biochemical parameters are extensively used as sensitive biomarkers for detecting the potential adverse effects of environmental contaminants, including metals [17]. Among them, glucose, albumin, and total protein are commonly used as strong indicators of toxic stress, induced by environmental pollutants, and general health and nutritional status of fish. Proteins play a vital role in architecture, physiology, and metabolism [16]. During stressful conditions, fish typically use their body protein, converting it into energy to cope with the energy demand [18]. In this sense, proteins are considered an alternative source of energy, along with glucose, to meet the increased energy demands of fish under stress conditions [19].

Blood enzymes have also been used as valuable indicators of exogenous stress and cellular damage in fish exposed to diverse groups of toxicants. Of them, alanine transferases (ALT) and aspartate transaminase (AST) are a group of enzymes responsible for the conversion of amino acids and  $\alpha$ -ketoacids. Alkaline phosphatase (ALP) catalyzes the dephosphorylation of a range of molecules such as nucleotides and proteins, and assays of ALP activity in blood are used as indicators of hepatic tissue damage, kidney dysfunction, and bone disease [20].

Accumulated exogenous metals can induce oxidative damage by generating free radicals, such as reactive oxygen species (ROS), and/or modifying antioxidant defense systems [21]. Production of ROS is typically associated with cellular injuries due to lipid peroxidation, DNA damage, oxidation of proteins, depletion of sulfhydryls, and altered calcium homeostasis and membranes induced by metals [22,23]. Manganese is a redox active metal that acts as a ROS generator by producing hydroxyl radicals, superoxide radicals, or hydrogen peroxide [2,3]. In this case, two possible mechanisms have been suggested for the generation of metallic ROS, namely potential interference of metal-related processes and the generation of free radicals by metal ions through Haber-Weiss reactions [21]. Lipids are susceptible to oxidation and malondialdehyde (MDA) is the main product of lipid peroxidation, often being used as an indicator of oxidative damage [24]. In fish, ROS generated in tissues and subcellular compartments, and the deleterious effects of free radicals, are efficiently scavenged by the antioxidant defense system, which is mainly formed by non-enzymatic antioxidant glutathione (GSH) and endogenous antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST) [25]. These antioxidant enzymes protect cells from free radicals-triggered lipid peroxidation (LPO), protein oxidation, and DNA damage [26]. Among these, SOD and CAT play an essential role in cellular defense mechanisms against oxidative stress by catalyzing the dismutation of superoxide anions to hydrogen peroxide and molecular oxygen. This process seems to be the first line of defense against ROS in fish [25,27]. Taken together, measurements of antioxidant parameters have been commonly used as potential biomarkers of oxidative stress in fish [28].

To our best knowledge, since information on the toxicological

effects of Mn on marine fish is scarce, more studies, using primary and secondary responses, are needed in order to determine their toxic potential. Consequently, the present investigation aimed to study the ecotoxicological impacts of Mn at different nominal concentrations (0, 0.5, 1, and 2 mg L<sup>-1</sup>) using various biomarkers in the red seabream (*Pagrus major*) and black rockfish (*Sebastes schlegelii*), two economically important marine fish species. Our results would be helpful in understanding the toxicological effects of Mn, the sensitivity of marine fish against waterborne Mn, and the physiological stress response of fish.

## 2. Materials and methods

### 2.1. Fish

All animal handling and experimental procedures were approved by the Animal Welfare Ethical Committee and the Animal Experimental Ethics Committee of the Incheon National University (Incheon, South Korea). The fish used in this study were obtained from enclosure aquaculture in Tongyeong, Gyeongnam, South Korea. The two fish species are neither endangered nor protected at this site.

Juvenile red seabream (~4 months after hatching; 10.45 ± 1.23 cm in length) and black rockfish (~5 months after hatching; 9.66 ± 1.10 cm in length) were maintained in an automated aquaculture system in artificial seawater (6.24 ± 0.53 mgO<sub>2</sub> L<sup>-1</sup>) at 20 °C under 14 h light: 10 h dark lighting conditions. The fish were maintained in glass aquaria (60 L capacity) with each aquarium accommodating up to 10 juvenile fish. They were fed frozen mosquito larvae and *Artemia salina* (< 24 h after hatching) twice a day until satiation.

### 2.2. Manganese exposure and blood sampling

Manganese (as MnCl<sub>2</sub>) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA; 95% purity) and was dissolved in ultra-pure water. Juvenile red seabream and black rockfish were exposed to different concentrations (0, 0.5, 1, and 2 mg L<sup>-1</sup>) of Mn for two weeks at 20 °C. Levels of Mn in freshwater typically range from 1 to 200 µg L<sup>-1</sup> [2]. Concentrations of dissolved Mn in natural waters that are essentially free of anthropogenic inputs can range from 10 to > 10,000 µg L<sup>-1</sup>, but dissolved Mn concentrations in natural surface waters rarely exceed 1000 µg L<sup>-1</sup> and are usually less than 200 µg L<sup>-1</sup> [2,5]. In marine environments, the level of Mn concentration was previously reported to be 0.3 mg L<sup>-1</sup> [5]. Typically, ambient Mn concentrations in seawater have been reported to range from 0.4 to 10 µg L<sup>-1</sup> [29], with an average of about 2 µg L<sup>-1</sup> [2]. However, ecotoxicological studies showed that relatively high concentrations of Mn (> 2 mg L<sup>-1</sup>) produced toxicological effects in fish exposed during short time periods (~96 h) (Table 1). We confirmed that previous reports showing the toxicological effects of Mn concentrations from 0 to 2 mg L<sup>-1</sup> are scanty in fish. Therefore, we chose nominal concentrations of 0.5, 1, and 2 mg L<sup>-1</sup> of Mn to determine the potential impact of Mn on marine fish.

A preliminary study on the toxicity of Mn revealed that concentrations above 100 mg L<sup>-1</sup> induced mortality within 2 weeks in both fish species, with exposure for 14 days causing over 14% and 19% mortality in red seabream and black rockfish, respectively. In this study, half of the test solutions were refreshed every 24 h with the addition of the corresponding concentration of Mn. The diet was continuously supplied during the experimental period under the same environmental conditions. In each of the exposed groups, 15 fish were collected on days 7 and 14, and were dissected to obtain blood samples. Fifteen fish for each group were separated into three subgroups as triplicate samples (n = 5 for each subgroup) and serum samples from each group were pooled. To collect blood, individuals from the control and exposed groups were anesthetized by immersion in a solution of MS-222 (200 mg L<sup>-1</sup> tricaine methanesulfonate, Sigma-Aldrich, Inc. St. Louis, MO, USA). The fish reached a deep stage of anesthesia after

**Table 1**  
Summary of studies on various concentrations of Mn exposure to different fish.

| Fish species                   | Exposure condition                                | Exposure method | Endpoints measured  | References |
|--------------------------------|---|-----------------|---|------------|
| <i>Argyrosomus japonicus</i>   | 5 mg L <sup>-1</sup> for 2 weeks                  | Waterborne      | Survival, growth, plasma parameters, and histopathology in gills, liver, and muscle                                       | [62]       |
| <i>Carassius auratus</i>       | 3.88 and 7.52 mg L <sup>-1</sup> for 96 h         | Waterborne      | Oxidative stress, hematological and biochemical parameters in blood   | [7]        |
| <i>Colossoma macropomum</i>    | 3.88 mg L <sup>-1</sup> for 96 h                  | Waterborne      | Oxidative stress in gill, liver, brain, and kidney  | [45]       |
| <i>Heteropneustes fossilis</i> | 3440 mg L <sup>-1</sup> for 96 h                  | Waterborne      | Blood pyruvate level  | [69]       |
| <i>Oreochromis niloticus</i>   | 0.2, 1.5, and 2.9 mg L <sup>-1</sup> for 96 h     | Waterborne      | Genotoxic, biochemical, and bioconcentration effects  | [70]       |
| <i>Rhamdia quelen</i>          | 4.2, 8.4, and 16.2 mg L <sup>-1</sup> for 15 days | Waterborne      | Mn accumulation in tissues (i.e. gill, brain, kidney, and liver), oxidative status, and hematological parameters in blood | [44]       |
| <i>Rhamdia quelen</i>          | 9.8 mg L <sup>-1</sup> for 20 days                | Waterborne      | Mn accumulation in tissues (i.e. gill, brain, kidney, and liver), oxidative status, and hematological parameters in blood | [71]       |
| <i>Rutilus caspicus</i>        | 60, 150, and 300 mg L <sup>-1</sup> for 96 h      | Waterborne      | Gill histopathology, immune indices, oxidative condition, and saltwater resistance  | [39]       |
| <i>Rutilus caspicus</i>        | 60, 150 and 300 mg L <sup>-1</sup> for 96 h       | Waterborne      | Plasma biochemical parameters   | [11]       |
| <i>Tilapia sparrmani</i>       | 4.43 mg L <sup>-1</sup> for 96 h                  | Waterborne      | Hematology and biochemical parameters   | [54]       |

approximately 2 min. Blood samples (200 µl per fish) were collected by cardiac puncture in heparinized tubes (Sarstedt, Nümbrecht, Germany) and were immediately centrifuged at 1600 × g for 10 min at 4 °C to separate the serum.

### 2.3. Manganese analysis

The analytical measurements (e.g. extraction, derivatization, clean-up steps, and quantification) of the actual concentrations of Mn were performed using a Chemical Analysis Specialist at the Advanced Science & Technology Center, Yonsei University (Seoul, South Korea). Manganese content in each sample was quantified by a NexION300 inductive coupled plasma mass spectrometer (ICP-MS; PerkinElmer, Shelton, CT, USA). Certified standards provided by the manufacturer were used throughout Mn analyses.

### 2.4. Immunological responses

Alternative complement activity (ACH<sub>50</sub>) assays were conducted following the method conducted in gilthead seabream [30] with small modifications (e.g., buffer volume and basic instruments employed). Ethylene glycol tetraacetic acid (EGTA)–Mg-gelatin-veronal buffer (GVB; veronal-buffered saline containing 10 mmol L<sup>-1</sup> EGTA, 10 mmol L<sup>-1</sup> MgCl<sub>2</sub>, and 0.1% gelatin) was prepared as working buffer and sheep red blood cells (SRBC; 1.5 × 10<sup>6</sup> cells; National Institute of Toxicological Research, South Korea) were used as targets. The reconstitution of the hemolytic activity of red seabream and black rockfish serum was measured by the incubation of SRBC (5 µl) in 6% red seabream and black rockfish serum (25 µl), 10 mM phenol red-free Hank's buffer with 5 mM Mg<sup>2+</sup>, 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, Tewksbury, MA, USA) at 414 nm. Complete (100%) and no hemolysis (0%) were measured by adding the washed SRBC (25 µl) to distilled water (100 µl) and phenol red-free Hank's buffer, respectively. The ACH was calculated as the reciprocal of the serum dilution, causing 50% lysis of SRBC (ACH<sub>50</sub>; U mL<sup>-1</sup>) based on the value of Y/1 - Y against the reciprocal of the serum dilutions on a log-scaled graph.

Lysozyme activity was measured by turbidimetric assays [31] modified into 96-well plates. Briefly, the lyophilized *Micrococcus lysodeikticus* (ATCC No. 4698; 0.3 mg/mL; Sigma-Aldrich, Inc. St. Louis, MO, USA) in sodium phosphate buffer (0.05 M; pH 6.2) was used as a substrate for the serum lysozyme. Triplicates of the 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, Tewksbury, MA, 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 immunoglobulin (Ig) content was measured following the method conducted in gilthead seabream [32] with small modifications (e.g., buffer volume and basic instruments employed). The serum samples were diluted with NaCl (0.85%) to one hundredth and the protein content for each sample was measured with the Bradford method using bovine serum albumin (BSA) as standard [33]. Each serum sample was mixed with an equal volume of polyethylene glycol (PEG; 10,000 MW; Sigma-Aldrich, Inc. St. Louis, MO, USA) solution. Subsequently, the mixed sample was incubated for 2 h and centrifuged at 5000 × g for 10 min at 4 °C. The supernatant from each sample was moved and diluted to one-fiftieth with NaCl (0.85%) and the protein content was measured as mentioned above. The differences between protein content of the untreated and PEG treated sample corresponded to the total Ig content and were expressed as mg mL<sup>-1</sup>.

### 2.5. Hematological and biochemical analysis

The hemoglobin content of both fish was measured spectrophotometrically using the cyanmethemoglobin method at 540 nm. Red blood cell (RBC: 10<sup>6</sup> mm<sup>-3</sup>) and white blood cell (WBC: 10<sup>4</sup> mm<sup>-3</sup>) were counted manually with a hemocytometer. Glucose and albumin were measured with commercial kits or assays using the Glucose Colorimetric Detection Kit, Invitrogen, Carlsbad, CA, USA and Fish Albumin ELISA Kit, MyBioSource, Inc., CA, USA, respectively. Serum total protein levels of both fish were measured using the method described by Ref. [33].

Blood cortisol concentration was measured using an ELISA kit (Fish Cortisol ELISA Kit, detection range 0.0023 ng mL<sup>-1</sup> - 10 ng mL<sup>-1</sup>; CUSABIO, TX, USA) with enzymatic substrate tetramethylbenzidine (TMB). The intra-assay coefficients of variance for both fish samples were < 9%. The intensity of color was assumed 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, Tewksbury, MA, USA).

### 2.6. Enzymatic activity

The enzymes like alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) of both fish species were measured with commercial kits or assays (Fish ALT, AST, and ALP ELISA Kits, MyBioSource, Inc., CA, USA) according to the manufacturer's instructions.

### 2.7. Responses of antioxidant defense system

After Mn exposure, total GSH content and enzymatic activities of CAT and SOD were measured according to our previous study with slight modifications in buffer volume and basic instruments used

[34–36]. In this study, GSH concentration in supernatants was determined by the enzymatic method using the Glutathione Assay Kit (Catalog No. CS0260; Sigma-Aldrich, Inc.) according to the manufacturer's protocol. The GSH content was 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  $\mu\text{M}$ . Non-enzymatic antioxidant glutathione contents were expressed in  $\text{nmol mg p}^{-1}$ .

The levels of CAT and SOD activities were measured in the supernatants with enzymatic methods using commercial Catalase (Catalog No. CAT100; Sigma-Aldrich, Inc.) and SOD Assay Kits (Catalog No. 19160; Sigma-Aldrich Chemie, Switzerland) according to the manufacturer's protocols, respectively. Total SOD and CAT activities were then measured at absorbances of 440 and 520 nm, respectively, at 25 °C with a Thermo Varioskan Flash spectrophotometer (Thermo Fisher Scientific). The CAT and SOD activity was expressed in  $\text{U mg p}^{-1}$ .

Blood samples were mixed with five volumes of Tris buffer (20 mM) containing NaCl (150 mM), aprotinin (2  $\mu\text{M}$ ), benzamide (100  $\mu\text{M}$ ),  $\beta$ -mercaptoethanol (10 mM), and leupeptin (20  $\mu\text{M}$ ). The samples were centrifuged at 30,000  $\times g$  at 4 °C for 30 min and the resulting 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, Tewksbury, MA, USA). Thiobarbituric acid reactive substance concentrations were measured based on a standard curve using Malondialdehyde (MDA) bis (dimethyl acetal) produced by Sigma-Aldrich, Inc. (St. Louis, MO, USA). The MDA levels were measured based on a calibration curve and calculated as nM of MDA per  $\mu\text{g}$  of total sample. The MDA content was expressed in  $\text{nmol mg p}^{-1}$ .

## 2.8. Statistical analyses

The software package SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The data are expressed as means  $\pm$  S.D. Significant differences between the control and exposed groups were analyzed using one-way or multiple-comparison ANOVAs followed by Tukey's tests. Differences with  $P < 0.05$  were considered significant.

## 3. Results

### 3.1. Mn concentrations in seawater and blood samples

To measure the stability and bioconcentration of waterborne Mn in artificial seawater and fish tissues such as blood, gill, intestine, liver, and muscle, respectively, Mn concentrations were measured in days 0, 7, and 14 (Table 2). The actual concentrations of Mn were maintained during the experimental period. The measured concentrations in blood were very high in the seventh and fourteenth days of exposure. Time-dependent drastic Mn accumulation was measured in gill, intestine, and liver tissues that are putatively related with immunity, while slight increase of Mn was observed in muscle. Overall, higher levels of Mn were detected in the blood of black rockfish than in red seabream.

### 3.2. Immunological parameters

The  $\text{ACH}_{50}$  activity was significantly lowered with higher concentration of Mn (2  $\text{mg L}^{-1}$ ) in both fish after 7 and 14 days compared to control ( $P < 0.05$ ; Fig. 1A and B). On day 7, the lysozyme activity in exposed red seabream was significantly decreased under concentrations of 1 and 2  $\text{mg L}^{-1}$  of Mn. Moreover, 2  $\text{mg L}^{-1}$  of Mn treatments further increased lysozyme activity at day 14 in red seabream ( $P < 0.05$ ). In black rockfish, the lysozyme activity was found to be decreased on day 14 after exposure when compared to the control group ( $P < 0.05$ ; Fig. 1C and D). Overall, most Mn concentrations tested in this study did not affect the total Ig content in either fish, although 2  $\text{mg L}^{-1}$  of Mn

**Table 2**

Aqueous Mn concentrations in the seawater and blood samples of 2  $\text{mg L}^{-1}$  Mn exposed fish during the 14 days of experiment as determined by inductive coupled plasma mass spectrometer (ICP-MS).

| Exposure (days)                                 | 0   | 7                | 14               |
|---|---|------------------|------------------|
| Mn nominal concentration ( $\text{mg L}^{-1}$ ) | Mn experimental concentration ( $\text{mg L}^{-1}$ ) <sup>#</sup> |                  |                  |
| 0.5   | 0.40 $\pm$ 0.12   | 0.89 $\pm$ 0.23  | 0.74 $\pm$ 0.19  |
| 1   | 0.88 $\pm$ 0.17   | 1.24 $\pm$ 0.43  | 2.02 $\pm$ 0.41  |
| 2   | 1.06 $\pm$ 0.24   | 2.89 $\pm$ 0.58  | 2.70 $\pm$ 0.49  |
| Mn accumulation in tissues                      |   |                  |                  |
| 2 $\text{mg L}^{-1}$ -exposed red seabream      |   |                  |                  |
| Blood ( $\text{mg L}^{-1}$ )                    | 0.23 $\pm$ 0.08   | 3.67 $\pm$ 0.88  | 5.11 $\pm$ 0.86  |
| Gill ( $\text{mg kg}^{-1}$ )                    | 0.64 $\pm$ 0.61   | 8.41 $\pm$ 2.59  | 26.57 $\pm$ 4.93 |
| Intestine ( $\text{mg kg}^{-1}$ )               | 0.38 $\pm$ 0.14   | 4.30 $\pm$ 1.22  | 9.28 $\pm$ 2.80  |
| Liver ( $\text{mg kg}^{-1}$ )                   | 0.11 $\pm$ 0.09   | 2.81 $\pm$ 0.56  | 17.12 $\pm$ 3.85 |
| Muscle ( $\text{mg kg}^{-1}$ )                  | 0.12 $\pm$ 0.07   | 1.95 $\pm$ 0.37  | 2.37 $\pm$ 0.45  |
| 2 $\text{mg L}^{-1}$ -exposed black rockfish    |   |                  |                  |
| Blood ( $\text{mg L}^{-1}$ )                    | 0.42 $\pm$ 0.11   | 5.71 $\pm$ 0.92  | 7.13 $\pm$ 1.24  |
| Gill ( $\text{mg kg}^{-1}$ )                    | 0.33 $\pm$ 0.10   | 15.28 $\pm$ 3.39 | 31.55 $\pm$ 8.22 |
| Intestine ( $\text{mg kg}^{-1}$ )               | 0.13 $\pm$ 0.06   | 2.89 $\pm$ 0.41  | 11.27 $\pm$ 2.39 |
| Liver ( $\text{mg kg}^{-1}$ )                   | 0.08 $\pm$ 0.01   | 5.25 $\pm$ 1.07  | 26.27 $\pm$ 7.34 |
| Muscle ( $\text{mg kg}^{-1}$ )                  | 0.15 $\pm$ 0.05   | 2.96 $\pm$ 0.84  | 5.49 $\pm$ 1.07  |

<sup>#</sup>Mn content in seawater was measured after 6 h from changing half of seawater with equivalent concentration of Mn.

significantly decreased the total Ig content in red seabream on day 14 after exposure ( $P < 0.05$ ; Fig. 1E and F).

### 3.3. Hematological parameters

The hematological parameters in red seabream and black rockfish exposed to different concentrations (0.5, 1, and 2  $\text{mg L}^{-1}$ ) of Mn for 14-days showed alterations when compared to control groups (Table 3). The Hb content was significantly decreased in red seabream exposed to 2  $\text{mg L}^{-1}$  of Mn after 14-days of exposure ( $P < 0.05$ ), while in black rockfish, significant decreases of Hb were measured after 14-days of exposure under 1  $\text{mg L}^{-1}$  of Mn and after 7- and 14-days of exposure under 2  $\text{mg L}^{-1}$  of Mn ( $P < 0.05$ ). In both fish, the levels on day 14 of exposure were significantly lower in groups exposed to 1 and 2  $\text{mg L}^{-1}$  of Mn than in control ( $P < 0.05$ ). In red seabream, 2  $\text{mg L}^{-1}$  of Mn also significantly decreased the RBC.

Although there was no significant change in the WBCs of red seabream, 1  $\text{mg L}^{-1}$  of Mn treatment decreased the WBCs in black rockfish after 7 and 14 days. In addition, 2  $\text{mg L}^{-1}$  of Mn significantly decreased the WBCs after 14 days. No significant changes in the levels of Hb, RBC, and WBC were detected in either fish in control or when exposed to 0.5  $\text{mg L}^{-1}$  of Mn ( $P > 0.05$ ).

### 3.4. Biochemical parameters

Treatments of 1 and 2  $\text{mg L}^{-1}$  of Mn significantly elevated the serum cortisol level in both fish on days 7 and 14 after exposure (except in red seabream exposed to 1  $\text{mg L}^{-1}$  of Mn after 7 days) compared to control ( $P < 0.05$ ; Table 3). However, total serum protein in both fish exposed to 1 and 2  $\text{mg L}^{-1}$  of Mn were significantly lowered after 14 days of exposure ( $P < 0.05$ ). In both fish, no significant results were observed in serum glucose and albumin levels in our experimental conditions ( $P > 0.05$ ).

The enzymatic activity of ALT in treated fish was significantly increased upon exposure to 1 and 2  $\text{mg L}^{-1}$  of Mn, except for black rockfish exposed to 2  $\text{mg L}^{-1}$  on day 7 after exposure (Table 3). Also, the relatively low concentration of Mn (0.5  $\text{mg L}^{-1}$ ) significantly increased ALT activity in black rockfish on day 14 after exposure ( $P < 0.05$ ). The enzymatic activity of AST was increased by exposure to 1 and 2  $\text{mg L}^{-1}$  of Mn in red seabream, whereas 2  $\text{mg L}^{-1}$  of Mn

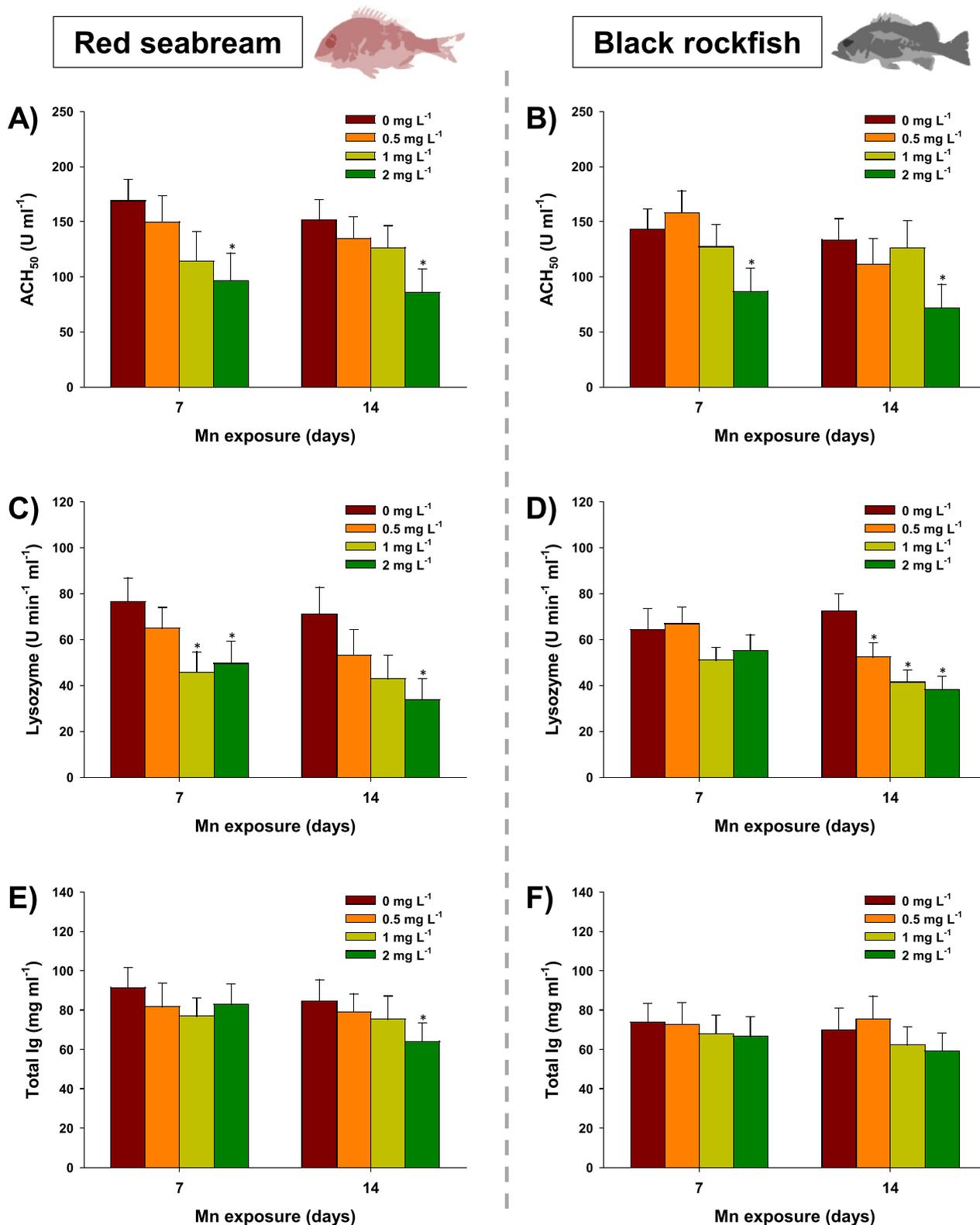


Fig. 1. Effects of exposure during 14 days to different concentrations (0, 0.5, 1, and 2 mg L<sup>-1</sup>) of waterborne Mn on the immunological parameters A) and B) ACH<sub>50</sub>, C) and D) lysozymes, and E) and F) total Ig in red seabream *Pagrus major* and black rockfish *Sebastes schlegeli*, respectively. Data are shown as means ± S.D of three replicates. The asterisk (\*) indicates statistical significance ( $P < 0.05$ ) compared with control value.

elevated the AST activity in black rockfish ( $P < 0.05$ ). The ALP activity was also increased by exposure to 1 and 2 mg L<sup>-1</sup> of Mn in both fish after 14 days ( $P < 0.05$ ).

### 3.5. Antioxidant defense system

The MDA content was significantly increased by exposure to 1 and 2 mg L<sup>-1</sup> of Mn in both fish on days 7 and 14 after exposure compared

**Table 3**

Hematological and biochemical parameters in the red seabream *Pagrus major* and black rockfish *Sebastes schlegeli* after 14 days exposure to different concentrations (0, 0.5, 1, and 2 mg L<sup>-1</sup>) of Mn.

| Indices                                 | Mn (mg L <sup>-1</sup> ) | Red seabream   |                | Black rockfish |                |
|---|--------------------------|----------------|----------------|----------------|----------------|
|   |                          | 7 day          | 14 day         | 7 day          | 14 day         |
| Cortisol (ng mL <sup>-1</sup> )         | 0                        | 26.58 ± 4.86   | 29.14 ± 5.52   | 22.17 ± 4.26   | 26.94 ± 5.11   |
|   | 0.5                      | 24.93 ± 6.95   | 33.82 ± 6.29   | 24.29 ± 5.28   | 29.17 ± 6.93   |
|   | 1                        | 28.62 ± 5.94   | 69.95 ± 11.81* | 45.85 ± 10.11* | 66.93 ± 12.14* |
|   | 2                        | 56.93 ± 13.11* | 84.91 ± 15.47* | 36.41 ± 8.94*  | 82.94 ± 16.57* |
| Hemoglobin (g dL <sup>-1</sup> )        | 0                        | 10.51 ± 1.23   | 10.97 ± 1.11   | 9.14 ± 0.94    | 10.08 ± 0.91   |
|   | 0.5                      | 11.85 ± 1.52   | 11.14 ± 1.05   | 9.39 ± 1.07    | 9.27 ± 0.96    |
|   | 1                        | 10.07 ± 1.09   | 9.85 ± 0.97    | 8.91 ± 0.91    | 7.71 ± 0.82*   |
|   | 2                        | 10.12 ± 1.11   | 8.29 ± 1.20*   | 7.89 ± 0.85*   | 7.23 ± 0.71*   |
| RBC (10 <sup>6</sup> dL <sup>-1</sup> ) | 0                        | 1.96 ± 0.16    | 2.05 ± 0.22    | 1.75 ± 0.16    | 1.88 ± 0.21    |
|   | 0.5                      | 1.89 ± 0.15    | 1.89 ± 0.19    | 1.86 ± 0.19    | 1.81 ± 0.23    |
|   | 1                        | 1.92 ± 0.18    | 1.61 ± 0.17*   | 1.69 ± 0.17    | 1.55 ± 0.19*   |
|   | 2                        | 1.88 ± 0.19    | 1.69 ± 0.19*   | 1.71 ± 0.19    | 1.52 ± 0.18*   |
| WBC (10 <sup>4</sup> dL <sup>-1</sup> ) | 0                        | 1.52 ± 0.12    | 1.59 ± 0.16    | 1.31 ± 0.15    | 1.29 ± 0.13    |
|   | 0.5                      | 1.48 ± 0.13    | 1.51 ± 0.12    | 1.22 ± 0.14    | 1.31 ± 0.14    |
|   | 1                        | 1.56 ± 0.10    | 1.43 ± 0.13    | 1.08 ± 0.11*   | 0.89 ± 0.11*   |
|   | 2                        | 1.44 ± 0.13    | 0.82 ± 0.12*   | 1.16 ± 0.13    | 0.81 ± 0.09*   |
| Total protein (g dL <sup>-1</sup> )     | 0                        | 3.96 ± 0.29    | 4.24 ± 0.46    | 3.81 ± 0.31    | 4.11 ± 0.42    |
|   | 0.5                      | 3.28 ± 0.35    | 3.68 ± 0.49    | 3.77 ± 0.44    | 3.95 ± 0.48    |
|   | 1                        | 3.56 ± 0.47    | 3.26 ± 0.42*   | 3.69 ± 0.51    | 3.22 ± 0.53*   |
|   | 2                        | 3.11 ± 0.28    | 2.99 ± 0.43*   | 3.07 ± 0.46    | 3.10 ± 0.49*   |
| Glucose (mg dL <sup>-1</sup> )          | 0                        | 75.14 ± 8.12   | 70.11 ± 7.66   | 73.69 ± 8.63   | 78.64 ± 7.41   |
|   | 0.5                      | 78.69 ± 7.27   | 81.58 ± 9.24   | 69.14 ± 9.34   | 72.22 ± 7.08   |
|   | 1                        | 82.48 ± 9.61   | 76.26 ± 8.55   | 68.99 ± 9.99   | 82.67 ± 9.61   |
|   | 2                        | 83.16 ± 8.39   | 74.19 ± 7.61   | 76.91 ± 7.28   | 76.27 ± 9.88   |
| Albumin (mg dL <sup>-1</sup> )          | 0                        | 2.85 ± 0.38    | 2.67 ± 0.29    | 2.66 ± 0.35    | 2.86 ± 0.44    |
|   | 0.5                      | 3.10 ± 0.41    | 2.92 ± 0.33    | 2.91 ± 0.33    | 2.26 ± 0.40    |
|   | 1                        | 2.29 ± 0.36    | 1.98 ± 0.30    | 2.25 ± 0.41    | 2.37 ± 0.38    |
|   | 2                        | 2.31 ± 0.32    | 2.23 ± 0.37    | 2.12 ± 0.56    | 2.16 ± 0.33    |
| ALT (U dL <sup>-1</sup> )               | 0                        | 26.58 ± 4.28   | 31.25 ± 5.26   | 30.51 ± 6.23   | 34.28 ± 5.64   |
|   | 0.5                      | 29.22 ± 4.83   | 41.85 ± 6.25   | 39.58 ± 5.77   | 56.84 ± 8.25*  |
|   | 1                        | 45.28 ± 6.91*  | 62.80 ± 8.14*  | 49.91 ± 6.95*  | 75.25 ± 9.44*  |
|   | 2                        | 68.97 ± 7.88*  | 57.93 ± 6.91*  | 38.25 ± 5.28   | 69.14 ± 8.42*  |
| AST (U dL <sup>-1</sup> )               | 0                        | 258.6 ± 27.0   | 274.2 ± 31.2   | 311.3 ± 44.3   | 341.6 ± 42.8   |
|   | 0.5                      | 229.6 ± 25.8   | 294.4 ± 39.2   | 361.5 ± 46.9   | 329.5 ± 46.4   |
|   | 1                        | 341.6 ± 39.2*  | 395.2 ± 41.9*  | 342.0 ± 48.2   | 389.2 ± 45.1   |
|   | 2                        | 386.0 ± 44.5*  | 378.9 ± 40.1*  | 386.2 ± 49.3*  | 446.9 ± 40.1*  |
| ALP (U dL <sup>-1</sup> )               | 0                        | 144.0 ± 16.2   | 157.3 ± 20.3   | 166.9 ± 18.4   | 171.2 ± 19.7   |
|   | 0.5                      | 161.3 ± 18.9   | 168.9 ± 18.6   | 151.1 ± 19.3   | 189.6 ± 22.6   |
|   | 1                        | 169.9 ± 19.6   | 185.6 ± 19.9*  | 186.3 ± 20.6   | 220.0 ± 26.8*  |
|   | 2                        | 185.7 ± 21.1*  | 197.2 ± 26.6*  | 191.7 ± 23.1*  | 206.7 ± 22.3*  |

The asterisk (\*) indicates the statistical significance ( $P < 0.05$ ) compared to the control values.

Abbreviation: alanine aminotransferase, ALT; alkaline phosphatase, ALP; aspartate aminotransferase, AST; red blood cell, RBC; white blood cell, WBC.

to the control, except for 7 days of exposure to 2 mg L<sup>-1</sup> of Mn in red seabream ( $P < 0.05$ ; Fig. 2A and B). A significantly higher level of GSH content was measured in both fish in response to 1 and 2 mg L<sup>-1</sup> of Mn on day 7 after exposure ( $P < 0.05$ ). Also, 0.5, 1, and 2 mg L<sup>-1</sup> of Mn treatments increased GSH levels on day 14 after exposure, except for 14 days of exposure to 2 mg L<sup>-1</sup> of Mn in black rockfish ( $P < 0.05$ ; Fig. 2C and D). The enzymatic activity of CAT was increased by exposure to 1 and 2 mg L<sup>-1</sup> of Mn on day 14 after exposure ( $P < 0.05$ ; Fig. 3A and B). A significant increase was observed in SOD activity in red seabream exposed to 1 and 2 mg L<sup>-1</sup> after 7 days and 2 mg L<sup>-1</sup> of Mn after 14 days ( $P < 0.05$ ). The enzymatic activity of SOD in black rockfish was significantly increased by all concentrations of Mn during the exposure periods, except for exposures of 0.5 mg L<sup>-1</sup> of Mn after 7 days (Fig. 3C and D).

#### 4. Discussion

Serum ACH<sub>50</sub> and lysozyme activities are important indices of innate immune systems [37,38]. In this study, a significant reduction in ACH<sub>50</sub> activity was found in both fish species. Regarding the results of ACH<sub>50</sub>, the mode of action of waterborne Mn on ACH<sub>50</sub> reduction is not clear in fish [39]. However, we assume that the decrease of ACH<sub>50</sub> might have resulted from liver damage, as the complement proteins are mainly produced in the liver [39,40], which is considered the main target organ for the accumulation and detoxification of various types of metals including Mn [41–45]. Similarly, a significant decrease in serum ACH<sub>50</sub> activity was observed in the Caspian roach (*Rutilus caspicus*) when treated with different concentrations of Mn (i.e., 60, 150, and 300 mg L<sup>-1</sup>) [39]. Although there is limited information on Mn effects, other metals such as copper and cadmium also decrease ACH<sub>50</sub> activity

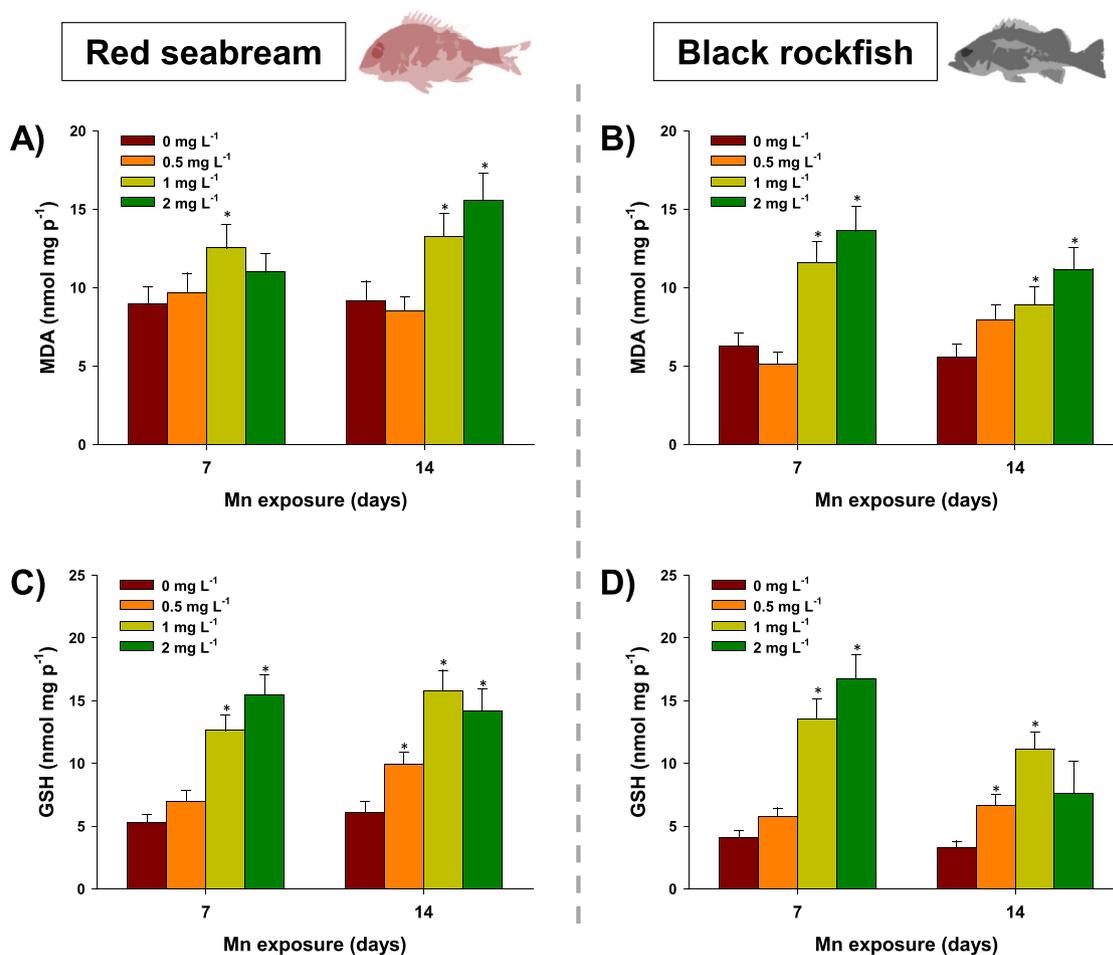


Fig. 2. Intracellular contents of A) and B) MDA and C) and D) GSH after 14 days of exposure to different concentrations (0, 0.5, 1, and 2 mg L<sup>-1</sup>) of waterborne Mn in red seabream *Pagrus major* and black rockfish *Sebastes schlegelii*, respectively. Data are shown as means  $\pm$  S.D. of three replicates. The asterisk (\*) indicates statistical significance ( $P < 0.05$ ) compared with control value.

in the Javanese carp *Puntius gonionotus* [46] and gilthead seabream *Sparus aurata* [47], respectively.

In both fish species, the lysozyme activity was significantly decreased in response to Mn exposure, suggesting that Mn treatment potentially suppressed the innate immune system of fish. Similarly, a dose-dependent inhibition in lysozyme activity was reported in *R. caspius* when treated with different concentrations of Mn [39]. Lysozyme, having antibiotic properties, is an important defense molecule in fish and can be used as a valuable indicator of innate immune response against numerous stresses [38,48]. The WBCs such as neutrophils and macrophages release the lysozyme, which in fish occurs mainly in neutrophils, monocytes, and, in smaller amounts, macrophages [9]. Thus, considering we observed lowered levels of WBCs in both fish, the decrease in serum lysozyme in both fish might have resulted from the reduction of these circulating lysozyme-producing cells [49]. Several reports suggest that metals, such as inorganic mercury and copper, have potentially detrimental effects on lysozyme activity in the European plaice *Pleuronectes platessa* [50] and Javanese carp *Puntius gonionotus* [46]. In addition, our results suggest that the level of lysozyme in fish serum could be a suitable biomarker for monitoring the potential impact of metals on fish innate immunity.

Serum Ig is a major component of the adaptive immune response of the humoral immune system and it has been recognized as a useful tool to evaluate fish immunity and homeostasis [51]. In this study, total Ig was significantly lowered by Mn exposure in red seabream, suggesting a potential immunosuppression effect of Mn by inhibiting Ig production. Since there was no significant inhibition observed in black rockfish, the

response of Ig probably depends on fish species and exposure periods. Further studies may be conducted in black rockfish with the incorporation of long periods of exposure and different concentrations of Mn. A significant decrease in total Ig levels was found in the rainbow trout *Oncorhynchus mykiss* when exposed to Cd and Hg [52] and in the Javanese carp *Puntius gonionotus* when treated with Cu [46]. Overall, the observed decreases in most immunological parameters tested in marine fish might be attributed to the immunosuppressive effects of Mn.

Hematological parameters have been extensively used as valuable biomarkers to assess the toxic stress induced by environmental contaminants (including metals). Cortisol is the predominant glucocorticoid in fish and is synthesized by interrenal cells of the head kidney. It regulates osmolality, metabolism, immune system, and stress associated responses in fish [15,16]. Although there are many reports available on cortisol induction in fish species, studies on Mn exposure are scarce. In this study, significantly higher levels of cortisol were found in both fish species after exposure to waterborne Mn. A similar trend was observed in cortisol levels of *Carassius auratus* when exposed to different concentrations of Mn [7]. Higher cortisol levels in fish indicate a primary response to cope with the toxic effects of Mn, as cortisol is normally elevated during stressful conditions [53]. In particular, the Mn-triggered decrease of WBCs observed in both fish is strongly correlated with cortisol level, as the decrease in WBCs may be due to increased secretion of corticosteroids as a non-specific physiological response of fish to environmental stressors [54].

In this study, 14 days of exposure to Mn negatively affected

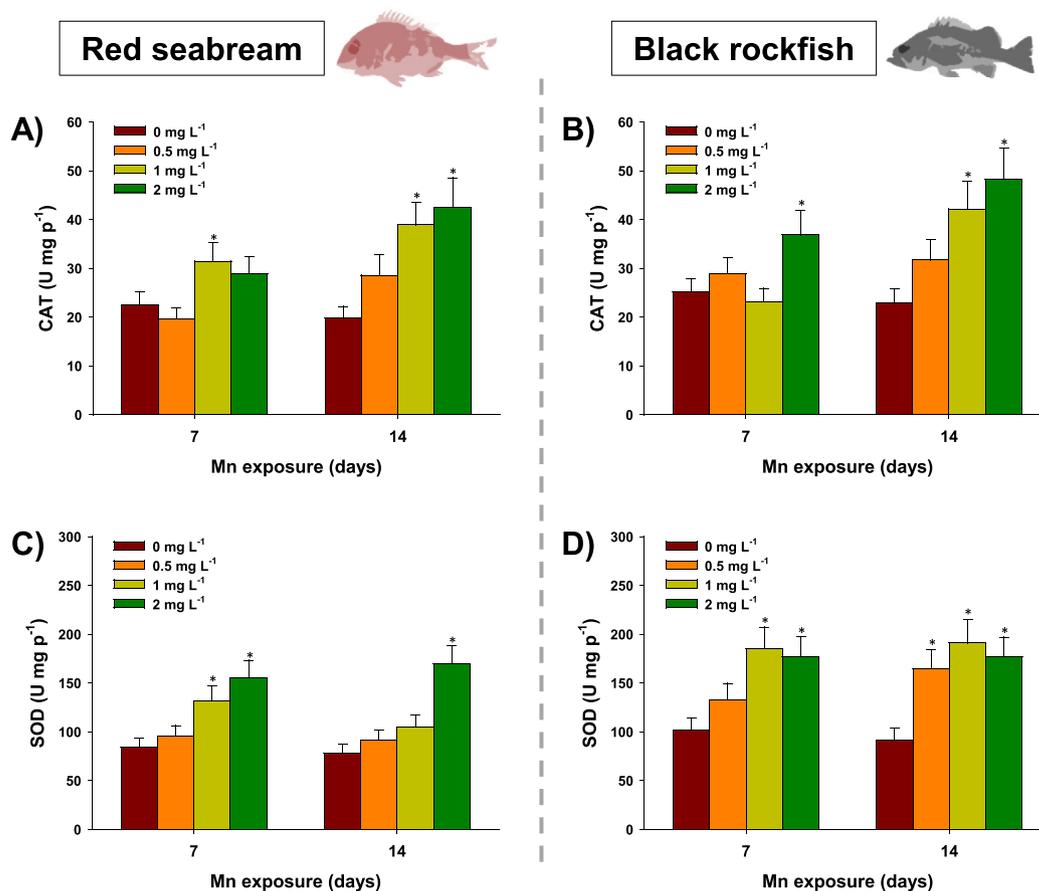


Fig. 3. Enzymatic activities of A) and B) CAT and C) and D) SOD after 14 days of exposure to different concentrations (0, 0.5, 1, and 2 mg L<sup>-1</sup>) of waterborne Mn in red seabream *Pagrus major* and black rockfish *Sebastes schlegelii*, respectively. Data are shown as means  $\pm$  S.D. of three replicates. The asterisk (\*) indicates statistical significance ( $P < 0.05$ ) compared with control value.

hematological status in both fish. Lower levels of Hb and RBC might be attributed to the inhibition of erythropoiesis and lysing of RBCs due to the toxic effects of Mn on the erythropoietic tissues. A similar result was observed in the RBC concentration of banded tilapia *Tilapia sparrmanii* when exposed to 4.43 mg L<sup>-1</sup> of Mn [54] and giant gourami *Colisa fasciatus* when exposed to different concentrations of Mn [55]. Manganese can induce cell death in the intestinal mucosa and kidney of fish, resulting in anemic conditions produced by internal hemorrhaging and loss of hematological components [54]. Manganese can also induce distorted/abnormal shape of RBCs and subsequent RBC destruction and degeneration [7]. Ions of transition metals, including Mn, can induce toxic effects via the stimulation of ROS production and cell death [21]. Since we also observed strong oxidative stress induction by Mn treatment in both fish, Mn probably activated the oxidative stress-mediated cell death process of RBC. Although we were not able to analyze cell death parameters in this study, Mn treatment induced apoptosis by apoptotic DNA fragmentation in the cells of hematopoietic tissues in the Norway lobster *N. norvegicus* [56], a process that might have contributed to the decrease of RBCs observed in the current study.

One of the major roles of WBCs in fish is the regulation of immunological functions against infectious diseases and pathogens. In our study, Mn treatment decreased WBCs in both fish, suggesting immunotoxic effects of Mn that could lead to lymphopenia and/or lymphocytopenia since lymphocytes contribute to total WBCs in circulation. Since leucopenia was observed in the banded tilapia *T. sparrmanii* when exposed to 4.43 mg L<sup>-1</sup> of Mn [54], the lower levels of WBCs observed in marine fish might be due to non-specific toxic effects of Mn on hematopoiesis or WBC maturation. As described, the potential correlation between WBCs and the level of corticosteroids could be

evidence for the Mn-triggered WBCs inhibition.

Total serum protein content was significantly decreased by Mn treatment in both fish. Hypoproteinemia was suggested as a general response in metal-exposed fish [57] and might result from reduced protein synthesis due to the higher affinity of metal ions towards the amino acid residues of proteins [58]. Since we observed Mn accumulation in both fish, higher accumulation may result in the immobilization of proteins to fulfil increased energy demands and/or impaired protein synthesis. A similar Mn-triggered decrease of total protein content was observed in *C. auratus* when exposed to Mn [7]. Although limited information is available on Mn effects, several reports suggested metal-triggered hypoproteinemia in fish [57,59,60]. A recent study also showed that accumulation of metals such as Cr, Cd, and Pb significantly decreased total protein levels in different tissues (i.e. gills, kidney, intestine, liver, muscle) of common carp (*Cyprinus carpio* L) [61].

The enzymes ALT, AST, and ALP play vital roles in protein and carbohydrate metabolism, and also act as indicators for tissue damage and cell rupture [62]. These enzymes are mainly present in the liver, and if any damage occurs in the liver they are synthesized and released into the blood stream. In this study, the enzymatic activities of AST, ALT, and ALP were significantly increased by Mn exposure. Similar, Mn effects on these enzymes were observed in the Caspian roach (*Rutilus rutilus caspicus*) [11]. These results potentially indicate that Mn may induce hepatic damage [41,43–45], as Mn has high potential to accumulate in fish liver [63]. In addition, transaminases can be highly produced and released into the blood by metal exposure [64].

The MDA levels were significantly increased in both fish in response to Mn exposure. The elevated MDA level is considered a reflection of oxidative stress induced by Mn and depletion of antioxidants. Similarly,

Mn exposure increased lipid peroxidation in the goldfish *C. auratus* [43] and Caspian roach *R. caspicus* [39]. Glutathione, a tripeptide non-enzymatic antioxidant, is the most abundant low molecular weight thiol compound synthesized in cells and plays critical roles in protecting cells from oxidative damage and the toxicity of xenobiotic electrophiles, maintaining redox homeostasis [65]. The significant elevations of GSH observed in both fish suggest a protective role of GSH against Mn-triggered oxidative stress, as GSH is rapidly consumed and synthesized during antioxidant responses. In general, higher level of intracellular GSH can increase resistance to metal-triggered oxidative stress by consuming GSH, while its lowered levels such as GSH depletion can increase susceptibility [66]. Organisms can overcome GSH depletion by increasing GSH synthesis through a negative feedback mechanism involving  $\gamma$ -glutamylcysteine synthetase (GCS) activity [67]. Although there is no result on GSH depletion in this study, the GSH level putatively decreased at early exposure period, as the concentration of Mn tested in this study induced no mortality in both fish. The concomitant elevations in the GSH content suggest that GSH plays a prominent role in neutralization of the induced oxidative stress and the generation of free radicals. Increases of the enzymatic activities of CAT and SOD strongly support Mn-triggered induction of oxidative stress and subsequent antioxidant responses in both fish. In fact, both enzymes function together and constitute the first line of defense against oxidative stress [25,68]. Accumulations of Mn in fish generate superoxide anions and activate SOD to scavenge superoxide radicals [7,45]. Similar studies on the induction of antioxidant defense systems also support the oxidative effects of Mn in fish [7,39,43–45].

## 5. Conclusion

In conclusion, waterborne Mn treatment in marine fish significantly modulated immunological and hematological homeostasis with induction of strong oxidative stress. Although each fish species presented slightly different bioconcentration potency and sensitivity of several parameters under Mn concentrations and exposure duration, the overall responses in both were comparable. Because blood is critical to maintaining physiology and innate immunity in fish, defense capacity and sensitivity are crucial to maintaining homeostasis. Finally, we highlight that the response of each parameter suggests a basis for the application of plasma components as biomarkers for Mn pollution.

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