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Effects of waterborne Fe(II) on juvenile turbot *Scophthalmus maximus*: analysis of respiratory rate, hematology and gill histology*

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Abstract The concentration of Fe(II) is high in some groundwater supplies used in turbot culture, and the toxicity of waterborne Fe(II) is unknown. We investigated the stress responses of juvenile turbot, *Scophthalmus maximus*, exposed to Fe(II) of different concentrations (0.01, 0.05, 0.1, 0.5, 1, and 2 mg/L) for 1, 7, 14, and 28 d, under the same ambient conditions of other parameters. Changes in respiratory rate, hematological parameters, and gill structure were determined. The results show that waterborne Fe(II) did not cause severe hematological perturbation to turbot. A low-medium Fe(II) concentration (lower than 0.1 mg/L) could boost the respiratory rate, and caused no or very limited damage to fish. A high Fe(II) concentration (0.1 mg/L or higher), however, caused gill damage, such as vacuoles in branchial lamellae, epithelial necrosis, and hypertrophy of epithelial cells, and even death after extended exposure time. Therefore, excess waterborne Fe(II) and long-term exposure to Fe(II) could be responsible for poor growth and high mortality of turbot in culture. The concentration of waterborne Fe(II) in turbot culture should be kept below 0.1 mg/L.

Keyword: *Scophthalmus maximus*; waterborne Fe(II); respiratory rate; hematological parameter; gill structure

1 INTRODUCTION

Iron is one of the most important essential elements for fish (Gregorovic et al., 2008) and has a number of fundamental roles in many physiological processes, including respiratory and photosynthetic electron transport (Granger and Price, 1999). In water, soluble iron exits mainly in two forms: bivalent, Fe(II), and trivalent, Fe(III). Fish can take up both forms from water through the gills (Bury and Grosell, 2003). However, excess waterborne iron is toxic to fish (Peuranen et al., 1994; Lappivaara et al., 1999; Dalzell and Macfarlane, 1999). Fe(II) in water is more toxic to fish than Fe(III) as the former is more soluble and easily absorbed (Vuori, 1995). After absorption, Fe(II) can accelerate the formation of highly reactive free radicals, which may damage fish metabolism and disturb physiological processes (Bury and Grosell, 2003). Fe(II) concentration is generally low in most water sources. Even in a water area of high waterborne iron, the Fe(II) concentration would be low because Fe(II) is easily oxidized into Fe(III) in the presence of oxygen (Steffens et al., 1993). Toxicity of waterborne Fe(II) to fish is rarely considered and only studies about the effect of waterborne Fe(II) on Atlantic salmon (Teien et al., 2008) and common carp (Yekta et al., 2009) have been reported. However, the concentration of waterborne Fe(II) might be relatively high in some cases, such as anoxic groundwater (Teien et al., 2008) and some water areas either enriched with

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iron in sediments or polluted by waste from iron pickling (Stumm and Lee, 1961). Anoxic groundwater is frequently used in marine fish culture. In Tianjin, China, where turbot is widely cultured, Fe concentration in groundwater can reach 1 mg/L (data not present). Therefore, toxicity of Fe(II) should not be neglected.

Fish gills are directly exposed to ambient water, and their large surface area facilitates toxicant interaction and absorption. Therefore, fish gills are vulnerable to toxic chemicals and heavy metals such as iron (Mallatt, 1985; Evans, 1987). Studies on iron toxicity to fish gills have mainly focused on total iron and Fe(III). Peuranen et al. (1994) and Fish (2009) reported that the iron could accumulate in gills as physical clogging and lead to gill damage, respiratory disruption, and fish mortality. Dalzell and MacFarlane (1999) indicated that the gills appear to be the main target for iron toxicity by observation of Fe accumulation on the gill surface when brown trout were exposed to lethal and sublethal iron concentrations. As discussed above, there have been few studies on the toxicity effect of waterborne Fe(II) on fish physiology and gill morphology. Teien et al. (2008) observed the accumulation process of different types of iron, including Fe(II) on Atlantic salmon gills and found that Fe accumulation on gills could induce toxic effects. Yekta et al. (2009) indicated that waterborne ferrous sulphate could damage common carp gills at low pH. In addition to damaging fish gills, iron can enter the body of fish (Bury and Grosell, 2003; Carriquiriborde et al., 2004). Excess iron in fish would then induce metabolic disorders and lead to changes of many physiological variables, such as hematological parameters. Hematological parameters, including red blood cell (RBC), blood hematocrit (HCT), blood glucose and plasma sodium, were determined to evaluate the toxic effects of iron in the banded tilapia, *Tilapia sparrmanii* (Wepener, 1990) and whitefish, *Coregonus lavaretus* (Lappivaara and Marttinen, 2005).

Turbot, *Scophthalmus maximus*, is an important aquaculture flatfish in coastal areas of northern China after introduction from Europe in the 1990s. Groundwater is widely used in turbot and other fish culture due to its stable temperature, especially in summer and winter seasons. The growth period of fishes cultured in groundwater is extended; however, fish cultured in groundwater sometimes present a low growth rate and a high mortality (Nyberg et al., 1995; Fish, 2009). Fish (2009) indicated that these phenomena were caused by higher concentrations of heavy metal ions, including iron, in groundwater. Under the anoxic environment of groundwater, the iron mostly exists as Fe(II), which may also be an important factor in the above phenomena (Geen et al., 2006). Therefore, it is important to determine how Fe(II) affects cultured turbot; however, to date, few studies investigating these issues have been reported. In this research, physiological changes, including changes in respiration rates, hematological parameters and gill histological structure were studied in turbot exposed to different concentrations of Fe(II). The results will contribute to determining safe concentrations of waterborne Fe(II) in turbot culture.

2 MATERIAL AND METHOD

2.1 Fish and rearing conditions

Juvenile turbot were purchased from a fish farm in Rizhao, China, and cultured in our laboratory between October–November 2008. After acclimatization for 7 d, the fish were reared at 16–20°C under natural light conditions, and were fed to satiation twice a day using commercially formulated diet. Water was refreshed entirely twice a day. In the experimental period of 28 d, the water parameters such as dissolved oxygen, pH, and salinity were measured regularly, being at 7.7–8.3 mg/L, 7.4–7.8, and 35 g/L, respectively.

2.2 Experimental design

Fish weighing 23.0±4.0 g (about 4 months old) were randomly selected and assigned to six dosage groups and two control groups [a blank control group and an ascorbic acid (AA) control group] in 16 90-L aquaria (n=21 per aquaria). Concentrations of Fe(II) were set by adding ferrous chloride to the water. AA was used to keep the waterborne Fe(II) stable, and the concentration (25 mg/L) was chosen based on initial trial experiments. The Fe(II) concentration was determined daily before and after water changes according to Viollier's method (2000) with some modification (Table 1).

2.3 Respiratory rate

Three fish were randomly selected from each aquarium and used to observe breathing on Days 1, 7, and 28. Their average respiratory rates (breaths per minute) were calculated from three measurements of 5 min each.

2.4 Hematological parameters

On Days 1, 7, 14, and 28, three fish from each aquarium were randomly chosen for hematological

Table 1 Fe(II) and ascorbic acid (AA) concentrations in control and experimental groups

Groups	Fe(II) concentration (mg/L)		AA concentration (mg/L)
	After water change	Before water change	
Blank control	-	-	0
AA control	-	-	25
0.01 mg/L	0.009±0.001	0.009±0.003	25
0.05 mg/L	0.048±0.002	0.045±0.005	25
0.1 mg/L	0.090±0.007	0.085±0.010	25
0.5 mg/L	0.484±0.011	0.463±0.033	25
1 mg/L	0.912±0.026	0.886±0.040	25
2 mg/L	1.958±0.040	1.870±0.051	25

"-" means the Fe(II) concentration was under the detection limit.

measurements. After fish were anesthetized with MS-222, blood samples from the caudal vein were quickly drawn into sterile syringes and immediately transferred into heparinized Eppendorf tubes. Hematological parameters, such as RBC count, HCT, hemoglobin (HGB) and mean cellular hemoglobin concentration (MCHC), were determined by a blood cell counter (Mindray BC-3000 Plus, China) within 2 h.

2.5 Gill sampling and slicing

After blood sampling, the gill arches were sampled, fixed in Bouin's fluid for 24 h and then stored in 70% ethanol. The tissues were dehydrated in an alcohol series, embedded in paraffin wax (Gurr, 1962) and then cut into 6 μm sections using a rotary microtome (Leica, Germany). The sections were stained with hematoxylin and eosin (HE), and observed under a light microscope (Nikon, Japan). Multiple sections of each specimen were prepared, and at least three slides, each with three to four sections, were used for observation.

2.6 Statistical analysis

Data were analyzed with the SPSS package for Windows (Version 15.0, SPSS, Chicago, IL, USA). Significant differences were determined using one-way ANOVA followed by Duncan's multiple comparison test at the 0.05 level of probability.

3 RESULT

Fish mortality was only observed in the 2 mg/L Fe(II) group, in which one fish died on Day 1 and the rest died within 7 d. Symptoms, such as loss of equilibrium, abnormal swimming, high breathing frequency, and color changes of the fish skin were observed before death.

The respiratory rates of fish in the experimental groups generally increased with increasing Fe(II) concentration. The values showed no significant differences on Days 1 and 7. The highest respiratory rates on Days 1 and 7 appeared in the 2 mg/L and 1 mg/L groups, respectively. On Day 28, the 0.1, 0.5, and 1 mg/L groups showed a significant increase compared with the two control groups ($P<0.05$, Fig.1).

The change of blood parameters in each group is shown in Fig.2. On Day 1, the average RBC count showed a decreasing trend as the Fe(II) concentration increased. The value in the 2 mg/L group was lower than those of the other groups but with no significant difference. On Days 7, 14, and 28, the average RBC count in the 1 mg/L group was maintained at a high level. The values of HGB and HCT had similar

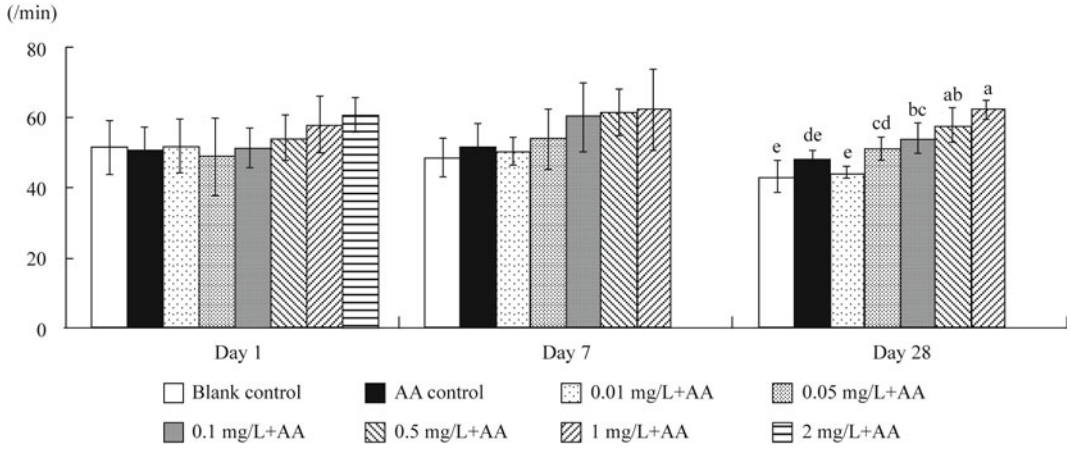

Fig.1 The respiratory rates of turbot in the blank, ascorbic acid (AA) control and experimental groups on Days 1, 7, and 28

The groups on Day 28 with the same letters indicate no significant difference, while those with different letters indicate significant difference in respiratory rates (Duncan's multiple comparison test, $P<0.05$, $n=6$).

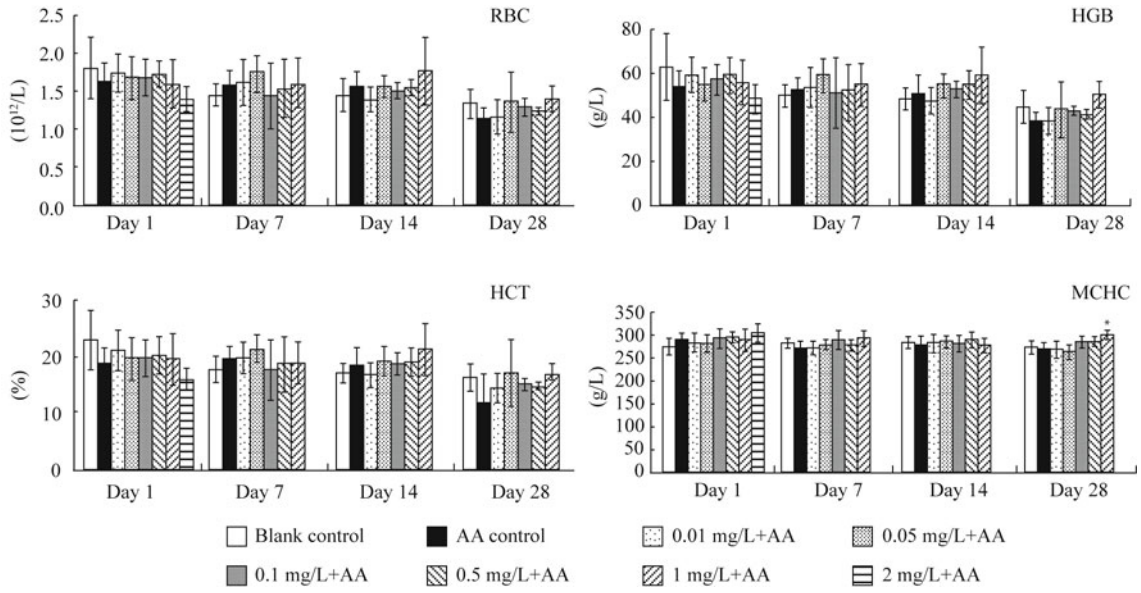

Fig.2 Blood parameters of turbot in the blank, AA control and experimental groups on Days 1, 7, 14, and 28

"*" indicates significant difference from the two control groups ($P<0.05$, $n=6$).

variation trends as the RBC count on all the sampling days. However, all three parameters showed no significant differences on each sampling day (Fig.2). The differences in MCHC among all the experimental groups were also not significant on Days 1, 7, and 14, but the values of the 1 mg/L group showed a significant increase when compared with those of the two control groups on Day 28 ($P<0.05$).

Figure 3 shows the differences in turbot gill structure among the experimental and control groups. In the 0.01, 0.05 mg/L, and AA control groups, the structure of gills was scarcely changed compared with those in the blank control group (Fig.3a1, a2). Edema at the end of a few branchial lamellae was only observed in the fish of these groups on Day 28 (Fig.3c1, c2). In the 0.1 mg/L group, the histological structure of gills on Day 1 was normal except for occasional edema at the ends of branchial lamellae. After 7 d of exposure, mild degenerative changes were observed in gills, and vacuoles between the epithelial cell layer and the capillary vessel appeared in some branchial lamellae (Fig.3b1, b2). Vacuoles were present in nearly all the gill branchial lamellae of fish in the 0.1 mg/L group after 28 d of exposure (Fig.3c3, c4). Edema at several sites at the end of branchial lamellae was also observed in gills of the 1 mg/L group on Day 1, and the number of chloride cells (CCs) of gills increased (Fig.3a3, a4). Vacuoles were apparent in most branchial lamellae on Day 7. After 28 d of exposure, hypertrophy, fusion, and rupture occurred in some of the epithelial cell layers (Fig.3c5, c6). In the 2 mg/L group, gills showed severe damage, such as increase of CCs, formation of vacuoles, and fusion of most branchial lamella on Day 1 (Fig.3a5, a6). Up to 7 d exposure, necrosis appeared in many epithelium regions in the nearly dead turbot (Fig.3b3, b4); a similar phenomenon was also found in the newly dead turbot. In addition, tan depositions were observed under a stereomicroscope only on the gills in the 2 mg/L group on Days 1 and 7, while no morphological change was found on the gills of other groups.

4 DISCUSSION

The results of this study showed that the structure of gills was damaged when waterborne Fe(II) concentration was more than 0.1 mg/L. This is similar to what has been observed in Atlantic salmon (Teien et al., 2008), brown trout (Peuranen et al., 1994) and common carp (Yekta et al., 2009). It has been reported that accumulation of Fe in the gill epithelium would lead to gill damage, respiratory dysfunction and even death. The AA was used to keep the waterborne Fe(II) stable because it is a potent and water-soluble antioxidant that prevents Fe(II) from being oxidized (Moore and Dubach, 1951; Frei et al., 1989; Hsieh and Hsieh, 1997). The respiration rates and hematological parameter values of turbot in the AA control group were not affected at the AA concentration of 25 mg/L. The histological structure of gills in these groups was also similar to that in the blank group except that edema at the end of a few branchial lamellae was observed in the gills of a few fish on Day 28. Hence, AA is suitable

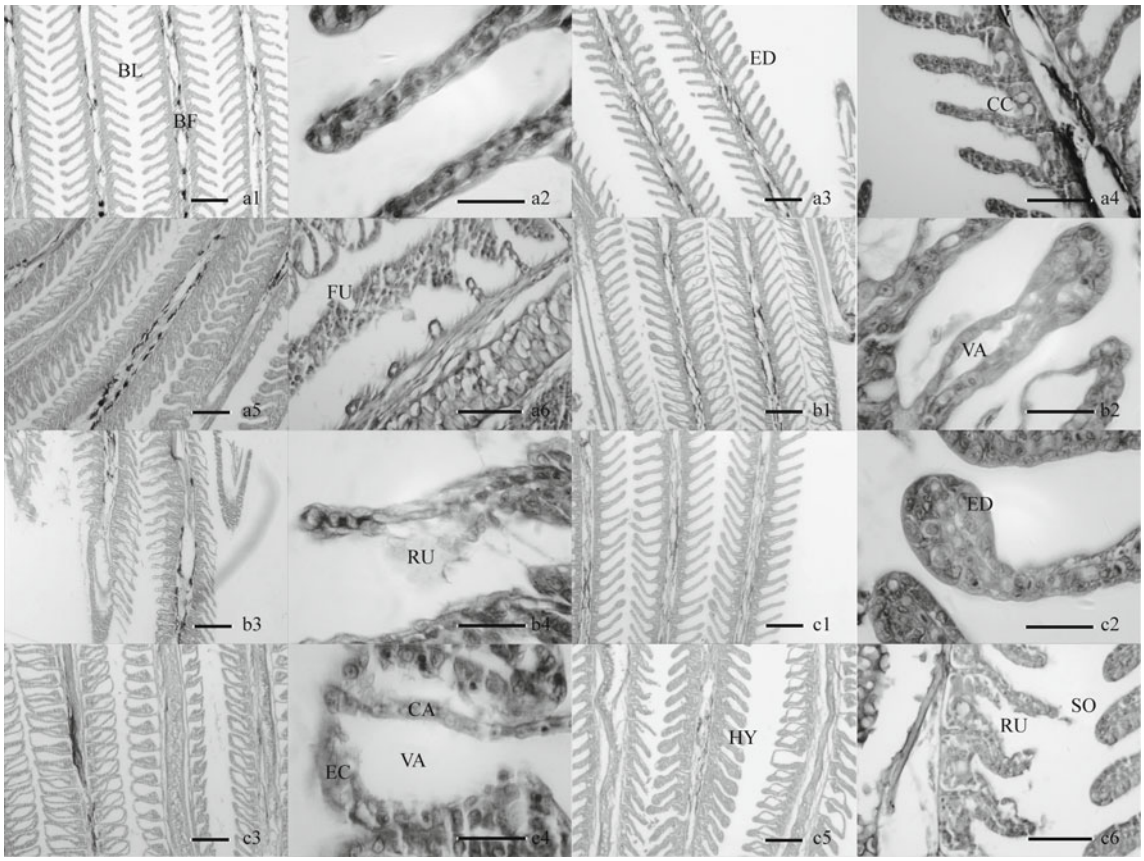

Fig.3 Histological structure of fish gills in the blank, AA control and experimental groups on Days 1, 7, and 28

a1 and a2. blank control group; a3 and a4. 1 mg/L group on Day 1; a5 and a6. 2 mg/L group on Day 1; b1 and b2. 0.1 mg/L group on Day 7; b3 and b4. 2 mg/L group on Day 7; c1 and c2. AA control group on Day 28; c3 and c4. 0.1mg/L group on Day 28; c5 and c6. 1 mg/L group on Day 28. Gill sections were of 4–5 μm thick. BF, branchial filament; BL, branchial lamella; CC, chloride cell; ED, edema; VA, vacuoles; EC, epithelial cell; CA, capillary vessel; HY, hypertrophy; SO, slough; RU, rupture; FU, fusion; Scale bar=10 μm in a1, a3, a5, b1, b3, c1, c3, c5; 5 μm in a4, a6, c6; 2 μm in a2, b2, b4, c2, c4.

to maintain the stability of waterborne Fe(II) at the given concentration.

The respiratory rate is an indicator of oxygen consumption of fish and is regarded as one of the stress responses to environmental changes. For example, the respiratory rate of the neotropical fish, *Hoplias lacerdae*, was elevated in hypoxic conditions (Campbell et al., 2009). Waterborne Fe(III) also induced a heightened respiratory rate in brown trout when exposed to lethal iron (Dalzell and Macfarlane, 1999). During the present experiment, the respiratory rates of turbot rose with the increase of waterborne Fe(II) concentration, and this trend was more notable with prolonged exposure time. Usually, a high respiratory rate represents low respiratory efficiency; thus, our results implied that Fe(II) lowered the respiratory efficiency of turbot. Two reasons could explain this phenomenon, the reduction of oxygen transport in fish blood, and damage of the gill structure. The exact underlying mechanism requires further study.

In fish, most blood O_2 is chemically bound to hemoglobin within red blood cells. An elevated RBC and HGB would result in an increase in blood O_2 carrying capacity. In addition, O_2 carrying capacity can be increased either acutely or chronically via elevation of HCT (Perry et al., 2009). When exposed to iron, banded tilapia had an increased RBC to transport more oxygen to tissues (Wepener, 1990). Our results showed that there were no significant differences of most hematological parameters among all the groups. Minor increases in RBC, HGB, and HCT were observed in the 1 mg/L group on Days 14 and 28. Meanwhile, the MCHC in the 1 mg/L group on Day 28 was significantly higher compared to that in other groups, which could be explained by a feedback regulation to increase transport efficiency and gain more O_2. It also indicates that the efficiency of O_2 transportation in turbot was slightly enhanced to resist the stress of excess waterborne Fe(II).

The fish gill is an organ involved in a wide variety of basic functions, including oxygen uptake,

carbon dioxide release, osmoregulation, acid-base regulation, nitrogen excretion, hormone metabolism and sensing (Evans et al., 2005). The large surface area combined with short diffusion distances makes fish gills well suited for gas exchange. Nevertheless, these properties make fish gills easily damaged by environment substances. After exposure to Fe(II), many histological changes were observed in the gills, including edema, epithelial necrosis, fusion of secondary lamellae, hypertrophy of epithelial cells and sloughing off of the epithelial surface. The separation of the gill branchial epithelium observed in this study is one of the most natural responses to water pollution in fish (Mallatt, 1985; Haaparanta et al., 1997). Other histological changes, such as epithelial necrosis and rupture of the gill epithelium, are also typical metal-induced histological lesions. These histological lesions can be attributed to decreasing antioxidant activity accompanied by increasing lipid peroxidation (LPO) in metal-exposed gills (Mallatt, 1985). It is suggested that by adapting to apparently pathological symptoms, such as lifting of the epithelium and lamella fusion, fish may be able to survive the effects of pollution (Evans et al., 2005). The increase in the number of CCs in fish gills is also a common response to water pollution. The CCs may be responsible for the excretion of excess absorbed metal, such as administered arsenic (Oladimeji et al., 1984) and cadmium (Wong and Wong, 2000). In the present study, CC numbers were increased in turbot gills in the 1 and 2 mg/L groups on Day 1. With extended exposure time, the gill structure was severely damaged, and the CCs could not be clearly observed. In addition, the toxicity of Fe(II) on gill structure showed overall dose-cumulative and time-cumulative effects. With the increase in Fe(II) concentration and exposure time, gill injuries became more serious. This means that exposure to relatively low Fe(II) concentrations for longer exposure times could also lead to severe gill damage. For example, necrosis was observed on the gills of nearly dead turbot in the 2 mg/L group on Day 7, and similar lesions were also observed in fish exposed to 1 mg/L Fe(II) for 28 d. In this study, Fe(II) caused severe damage to gills at very low concentration (0.1 mg/L). This concentration is even lower than that in the groundwater used in some fish farms in China (data not present). Excess waterborne Fe(II) and long-term exposure may, therefore, be important reasons for low growth rates and high mortality of turbot in culture. It is worth mentioning that the gill structure changes of turbot exposed to different concentrations of Fe(II) were in accordance with the changes in respiratory rate. Considering there was no obvious variation in most of the hematological parameters during the experiment, it may be the damage to gill structure that lead to a decline of respiratory efficiency by causing difficulties in acquiring oxygen from water. Turbot may, however, be able to make some kind of compensation to gain more oxygen, such as increasing respiratory rate. Although hematological parameters were not significantly altered in this study, other associated parameters, such as gene expression may be changed under high Fe(II) concentrations. In fish, the reactive oxygen species induced by heavy metals would then up-regulate the expression of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) to resist the adverse effects (Farombi et al., 2007; Lopes et al., 2001). Some other genes, such as metallothionein would also be induced (Klaverkamp et al., 1984). However, the exact regulatory mechanism to manage waterborne Fe(II) still needs further study.

5 CONCLUSION

Waterborne Fe(II) in seawater can harm juvenile turbot. The toxicity of waterborne Fe(II) was shown to have obvious time- and dose-cumulative effects. With increased concentration of waterborne Fe(II) and extended exposure time, the respiration and gill structure of turbot were affected. The stress response, including a raise of respiratory rates, may be caused by the damaged gill structure. The gill was even damaged by the low waterborne Fe(II) concentration of 0.1 mg/L; therefore, the waterborne Fe(II) concentration in turbot culture should not exceed 0.1 mg/L. It is noteworthy that the Fe(II) concentration in the groundwater of some fish farms in China is higher than 0.1 mg/L. How to effectively decrease Fe(II) concentration to a safe concentration should be further studied.

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