Tissue-specific accumulation of cadmium and its effects on antioxidative responses in Japanese flounder juveniles

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\textbf{ABSTRACT}

This study investigated the accumulation of cadmium (0–8 mg Cd L\textsuperscript{-1}) and its toxicological effects on oxidative stress biomarkers in different tissues of Japanese flounder juveniles. Following Cd exposure for 28 d, accumulation of Cd in fish was dose-dependent and tissue-specific, with the greatest accumulation in the liver, followed by the kidney, gill, and muscle. Although the gill and liver mounted active antioxidant responses at ≥4 mg L\textsuperscript{-1} Cd including a decrease in glutathione level and GST and GPx activities, the antioxidant response failed to prevent lipid peroxidation induction in these organs. In the kidney, increased GPx and GST activities and decreased SOD activity were observed in fish exposed to high Cd concentrations, but LPO levels did not significantly differ among the exposure concentrations. The gill was most sensitive to oxidative damage, followed by the liver; the kidney was the least affected tissue.

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1. Introduction

Cadmium (Cd) is a widely distributed metal that enters the aquatic environment via natural sources such as river run-off from Cd-rich soils and anthropogenic sources like urban run-off and industrial waste dumping (Cao et al., 2009). Cd is a highly toxic metal that can damage a variety of organ systems in exposed aquatic organisms (Livingstone, 2001; Asagba et al., 2008). For example, Cd exposure can result in serious damage to various tissues including the kidney, liver, gill, intestine, brain, and heart (Reméo et al., 2000; Atli et al., 2006; Soares et al., 2008). Thus, increasing Cd exposure in aquatic environments could also seriously threaten the safety of fish products and thus consumer health.

Generally, fish concentrate and accumulate Cd in large quantities in their tissues either through water or food consumption (Dural et al., 2006; Soares et al., 2008; Sana et al., 2009). However, the accumulated Cd is rarely distributed uniformly within the tissues of fish (Gill et al., 1992; Ranaldi and Gagnon, 2009). The pattern of Cd distribution largely depends on tissue-specific bioavailability, uptake and elimination rates, and uptake mechanisms (Lange et al., 2002; Kim et al., 2004; Dang and Wang, 2009). To date, accumulation of Cd has often been investigated in fish tissues including livers, kidney, gill,
and muscle because these tissues have been identified as the main storage sites for Cd (Miliou et al., 1998; Lange et al., 2002; Kim et al., 2004; Dural et al., 2006; Dang and Wang, 2009; Ranaldi and Gagnon, 2009; Sana et al., 2009).

Like other toxic metals, Cd may induce its toxicological effects via inhibition of enzymes, disruption of the structure and function of subcellular organelles, covalent modification of proteins, and inhibitory or stimulatory effects on the regulation of expression of various proteins (Reméo et al., 2000; Soares et al., 2008). Previous reports demonstrated that oxidative stress-induced lipid peroxidation (LPO) in fish exposed to Cd was the most pronounced and well-known biochemical effect (Reméo et al., 2000; Pandey et al., 2001; Sayeed et al., 2003; Asagba et al., 2008). Cd may trigger redox reactions that generate free radicals and reactive oxygen species (ROS) including superoxide anion radicals (O₂⁻•), hydrogen peroxide (H₂O₂), hydroxyl radicals (•OH), lipid hydroperoxide (LOOH), alkoxyl radical (RO•), and singlet oxygen (O₂) (Livingstone, 2001; Atli et al., 2006). ROS play a significant role in the signal transduction pathway and transcription factor regulation (Flora et al., 2008). Moderate amounts of ROS (particularly superoxide anion and hydrogen peroxide) may serve as second messengers in signal transduction pathway by activating the NF-kappa-B transcription factor, but high concentration of ROS can be deleterious for tissues (Valko et al., 2007). Under normal conditions, ROS and other free radicals are detoxified and removed by antioxidant defense systems including enzymatic antioxidants (i.e. catalase, CAT, superoxide dismutase, SOD; glutathione peroxidase, GPx, and glutathione S-transferase, GST) and non-enzymatic antioxidants (i.e. reduced glutathione, GSH) (Basha and Rani, 2003; Hansen et al., 2007; Asagba et al., 2008; Messaoudi et al., 2009).

If the production of ROS overwhelms the antioxidant defense system, oxidative stress will occur in various fish tissues, potentially leading to various types of intracellular damage (Pandey et al., 2001; Sayeed et al., 2003; Barata et al., 2005). Research on the mechanisms by which Cd exerts toxicological effects on fish tissues has recently begun to focus on the oxidative stress responses of fish to Cd exposure (Lange et al., 2002; Basha and Rani, 2003; Shi et al., 2005; Atli et al., 2006; Hansen et al., 2007; Xu and Bai, 2007; Asagba et al., 2008). These previous studies indicated that the antioxidative defense mechanisms that fish have developed to counteract oxidative stress are highly tissue-specific and affected by the type of exposure, the form of the metal, the dose, and the duration of exposure.

The present study investigated the accumulation of Cd and its toxicological effects on the growth and antioxidative mechanisms of Japanese flounder (Paralichthys olivaceus), a declining commercial fish in Chinese coastal waters. In the spawning and nursery grounds of the flounder in the Bohai Sea, the average Cd concentrations were recently recorded to be 0.21 μg L⁻¹ but reached as high as 2–16.1 mg L⁻¹ in some extreme locations (Zhang, 2001; Peng et al., 2009). This study has three main objectives: (1) to investigate the accumulation behavior of waterborne Cd in the liver, kidney, gill, and muscle with different exposure concentrations and the effects of this accumulation on flounder growth; (2) to assess the extent of LPO and how antioxidants (SOD, CAT, GST, GPx, and GSH) in the gill, liver, and kidney responded to waterborne Cd exposure at 2–8 mg L⁻¹ for 28 d to counteract oxidative stress; and (3) to evaluate the combined effectiveness of using tissue-specific oxidative stress biomarkers and measurements of Cd accumulation to assess Cd toxicity in flounder juveniles.

<table>
<thead>
<tr>
<th>Table 1 – The initial weight (W₀), final weight (W₇), and specific growth rate (SGR) of Japanese flounder juveniles exposed to a range of Cd concentrations for 28 d.</th>
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<tr>
<td><strong>Treatments</strong> (mg L⁻¹)</td>
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<td>Control</td>
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<td>* P &lt; 0.05.</td>
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<td><strong>Values are presented as mean ± standard deviation, n = 40.</strong></td>
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2. Materials and methods

2.1. Experimental protocol

Four hundred flounder juveniles (9.76 ± 1.78 g in body weight, W; 11.54 ± 0.62 cm in total length, L₇) were obtained from Shunyuan Fish Hatchery Station, Rizhao, China. Fish were acclimatized in 2 m³ indoor ponds with flowing seawater and gentle aeration for one week before the experiments were initiated. During acclimation, fish were fed sand lance, Ammodotes personatus, twice a day with a light regime of 14 h light and 10 h dark. A thermostat-controlled water bathing system was used to maintain the water temperature at 17 ± 1 °C.

After acclimation, fish were fasted for 24 h prior to weighing to allow for elimination of any gut contents. They were anesthetized with 2-phenoxethanol solution and blotted with absorbent paper. Then, they were weighed to the nearest 0.01 g and measured to the nearest 0.01 cm. Prior to the experiment, a preliminary experiment on the acute toxic effect of Cd was conducted on juvenile Japanese flounder. The results revealed that the 24-h and 96-h LC₅₀ value of Cd for juvenile Japanese flounder were 81.57 (76.49–84.61) mg L⁻¹ and 26.33 (24.06–28.71) mg L⁻¹, respectively. It also revealed that a concentration of 8 mg L⁻¹ (≈1/10 the 24 h LC₅₀) did not appear to have any significant effect on the survival of the juveniles. Twelve fish of similar size were transferred into each of the 60-L experimental tanks, which were spaced randomly in an indoor pond with a constant temperature of 17 ± 1 °C. Fish size did not significantly differ among replicates or concentrations (ANOVA, P > 0.05 for each comparison; Table 1). After a 24 h acclimation period in the experimental tanks without feeding, fish were exposed in quadruplicate to one blank control (0 mg L⁻¹) and Cd solutions of 2, 4, and 8 mg L⁻¹. Each tank was filled with 50 L of filtered seawater. Water quality parameters (mean ± standard deviation) were maintained at a pH of 8.0 ± 0.1, salinity equal to 33 ± 1 ppt, and a dissolved oxygen concentration of 7.5 ± 0.2 mg L⁻¹. Cd was used in the form of CdCl₂·2.5H₂O at analytical reagent grade (purity > 99.9%; Sinopharm Chemical Reagent Co., Ltd., China). A stock solution of 100 mg Cd L⁻¹ was prepared each day with deionized water and was equilibrated for 24 h before use. The stock
solution was diluted in the experimental tanks to produce Cd solutions of each concentration. Test solutions were renewed thoroughly every day until termination of the experiment. Fish were fed sand lance to satiety twice a day. Uneaten feed and feces were removed by siphoning every day. Other rearing conditions were identical to those for acclimation. The experiment was terminated 28 d after Cd exposure began. All experiments using fish were conducted in accordance with the Guide for the Care and Use of Laboratory Animals – Chinese Version.

2.2. Sample collection and analysis

Water samples for chemical analysis were collected every other day from each experimental tank. They were filtered and acidified with a 1% (v/v) HNO₃ solution and stored at 4 °C until chemical analysis. Cd concentrations in the water samples were measured using inductively coupled plasma mass spectroscopy (ICP-MS, ELANDRC II). The absolute difference between the measured and nominal concentrations divided by the nominal concentration and expressed as a percentage was used to assess the accuracy of Cd concentrations in the test solutions.

At the end of the experiment, five fish were randomly sampled from each experimental tank. They were sacrificed and then weighed to obtain W. The specific growth rate (SGR, % d⁻¹) was calculated according to the following formula: 

\[ \text{SGR} = 100 \times \frac{(\ln W_f - \ln W_i)}{t}, \]

where W₀ and Wₜ represent the initial weight and final weight of the flounder juveniles in each experimental tank, respectively, and t is the test duration in days.

Then, the gill, liver, kidney, and muscle tissues of each sampled fish were dissected, washed in physiological saline solution (0.9% NaCl) and frozen at -20 °C until Cd accumulation quantification. For accumulation analysis, the gill, liver, and muscle samples of each individual fish were separately digested in 10 times their weight of concentrated (16 N) HNO₃ solution at approximately 80 °C for 3 h. The kidneys of the five fish from each tank were pooled as one sample for Cd accumulation quantification because the kidneys were too small to be analyzed individually. Cd concentrations were analyzed on a graphite furnace atomic absorption spectrophotometer (Model ZEEnit 600 BU, Analytik Jena AG, Germany). The accumulation factor was calculated according to the formula of Lange et al. (2002).

Another five fish were randomly sampled from each experimental tank for biochemical assays. Fish were sacrificed and weighed, and the gill, liver, and kidney tissues were dissected, washed in physiological saline solution (0.9% NaCl) and frozen in liquid nitrogen until biochemical analysis was conducted.

For the biochemical analysis, the gill and liver tissues of each individual fish were homogenized in 10 mM Tris–HCl buffer (pH 7.4) using a double glass homogenizer immersed in an ice water bath to a yield a 10% homogenate. The kidney tissues from the five fish were pooled as one sample for biological analysis for the same reason mentioned above for Cd accumulation analysis. An aliquot of the homogenate was used to determine the LPO status of the sample by measuring the thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa (1979). TBARS values were reported as nmoles malondialdehyde (MDA) per milligram protein. The remaining homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C (Eppendorf 5804R, Eppendorf AG, Hamburg, Germany). The supernatant was used for analysis of CAT, SOD, GST, GPx, and GSH.

CAT activity was determined following the method of Beers and Sizer (1952); briefly, the sample was analyzed at an absorbance of 240 nm to determine the H₂O₂ concentration. One unit of CAT activity was defined as the amount of enzyme that catalyzed the degradation of 1 μmol of H₂O₂ min⁻¹, and specific activity corresponded to the amount of H₂O₂ transformed per unit time and per mg protein (μmol of H₂O₂ min⁻¹ mg⁻¹ protein). SOD activity was assayed at an absorbance of 325 nm to indicate the inhibition of the auto-oxidation of pyrogallol, according to the protocol of Marklund and Marklund (1974). One unit of enzyme activity was defined as the amount of enzyme in a 1 ml solution that inhibited the auto-oxidation rate of 0.1 mM pyrogallol by 50%. The reduced glutathione (GSH) concentration in the tissue was determined by the method of Moron et al. (1979) by analysis of the sample at an absorbance of 412 nm with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) as the fluorescent reagent. GST activity was assayed at an absorbance of 340 nm with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to the method of Habig et al. (1974). One unit of GST activity was defined as the amount of enzyme that catalyzed the conjugation of 1 μmol of CDNB with GSH per minute at 25 °C. Glutathione peroxidase (GPx) activities were determined according to the method described by Hafeman et al. (1974); this assay is based on the detection of nonenzymatic GSH oxidation by analysis of the sample at an absorbance of 412 nm after incubation with DTNB. One unit of GPx activity was defined as the μg of GSH consumed per minute.

Protein concentrations in the gill, liver, and kidney supernatants were determined by the Bradford (1976) method using bovine serum albumin as the standard. Absorbance was recorded at 595 nm.

All of the biochemical measurements were carried out using an ultraviolet spectrophotometer (UNICO WFZ UV-2802PC/PCS; Shanghai, China) at 25 °C.

2.3. Data analysis

Data were presented as the mean ± standard deviation and were checked for assumptions of normality using the Kolmogorov–Smirnov one-sample test and homogeneity of variance using the Levene test. In cases where both assumptions were met, one-way analysis of variance (ANOVA) was used to check for differences between the control and exposed groups. Data for Cd accumulation values, accumulation factors, growth (W and SGR) and biochemical parameters (CAT, SOD, GST, GPx and LPO) were analyzed by one-way ANOVAs followed by a Dunnett’s test. Differences were considered significant at P < 0.05. All statistical analysis were performed by SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).
3. **Results**

3.1. **Measured Cd concentrations in test solutions, growth and Cd accumulation in fish**

The measured Cd concentration of the control solution was 0.35±0.06 μg L⁻¹. The Cd concentrations of the 2, 4, and 8 mg L⁻¹ solutions were measured as 1.98±0.18, 3.93±0.06, and 7.13±0.14 mg L⁻¹, respectively. The errors in these concentrations ranged from 1 to 11% and all solutions fell within ±20% of their nominal targets, which is satisfactory for toxicity tests of this kind (OECD, 2000).

At the end of the test, the Wₚ of the flounder juveniles were lower in all Cd-exposed groups compared to the control group (Table 1). The differences in Wₚ from control were only statistically significant for fish exposed to the 8 mg Cd L⁻¹ solution (10.97 g) (Dunnett's test, P<0.05). The SGR was significantly decreased for fish exposed to the 4 mg Cd L⁻¹ concentration (0.92% d⁻¹; ANOVA, P<0.05) and the 8 mg Cd L⁻¹ concentration (0.41% d⁻¹; ANOVA, P<0.001) compared to the control fish (1.54% d⁻¹). No mortality occurred in any of the experimental tanks during the experiment.

Exposure to Cd resulted in a significant dose-dependent accumulation in the liver, kidney, gill, and muscle of the flounder juveniles (ANOVA, P<0.05 for each tissue-specific analysis; Table 2). At the end of the exposure period, Cd accumulations in the liver were significantly higher than control (0.48 μg g⁻¹; Dunnett's test, P<0.05 for each comparison) for all of the concentration used, with accumulation values of 9.18 (19-fold), 39.67 (83-fold), and 65.02 μg g⁻¹ (136-fold) for fish exposed to the 2, 4, and 8 mg Cd L⁻¹ concentrations, respectively. Cd accumulations in the kidney were significantly higher than control (0.37 μg g⁻¹; Dunnett's test, P<0.05 for each comparison) for all of the concentration used, with accumulation values of 7.64 (20-fold), 33.93 (89-fold), and 55.94 μg g⁻¹ (147-fold) for fish exposed to the 2, 4, and 8 mg Cd L⁻¹ concentrations, respectively. Cd accumulations in the gill were significantly higher than control (0.12 μg g⁻¹; Dunnett's test, P<0.05 for each comparison) for all of the concentration used, with accumulation values of 1.75 (15-fold), 4.11 (34-fold), and 5.60 μg g⁻¹ (47-fold) for fish exposed to the 2, 4, and 8 mg Cd L⁻¹ concentrations, respectively. Similarly, Cd accumulations in the muscle were significantly higher than control (0.02 μg g⁻¹; Dunnett's test, P<0.05 for each comparison) for all of the concentration used, with accumulation values of 0.25 (12-fold), 0.43 (21-fold), and 0.66 μg g⁻¹ (32-fold) for fish exposed to the 2, 4, and 8 mg Cd L⁻¹ concentrations, respectively.

Cd exposure showed a clear pattern of tissue-specific accumulation in flounder juveniles. Of the four tissues, the liver accumulated the highest Cd concentration, followed by the kidney, gill, and muscle (Table 2). Cd accumulation values in the liver (24–99 fold), kidney (19–84 fold), and gill (6–10 fold) were significantly higher than that of the muscle at each concentration (Dunnett's test, P<0.05 for each comparison).

The accumulation factors are presented for muscle, gill, kidney and liver at 2, 4, 8 mg Cd L⁻¹ exposure in Table 2. The accumulation factors increased as Cd concentration was elevated in all the four tissues, with factors ranging from 12.39 to 147.29 (Table 2).

3.2. **Tissue-specific oxidative stress biomarkers**

3.2.1. **Gill responses**

GSH levels (0.45–0.83 mg g⁻¹ protein) and GPx activities (36.19–52.51 U mg⁻¹ protein) in the gill of the flounder were significantly decreased at all Cd concentrations compared to those in the control (1.05 mg g⁻¹ protein, and 65.17 U mg⁻¹ protein, respectively) (Dunnett's test, P<0.05 for each comparison; Fig. 1A and B). GST activities in the gill of the flounder exposed to 4 (21.48 U mg⁻¹ protein) and 8 mg L⁻¹ Cd (17.87 U mg⁻¹ protein) were significantly reduced compared to the activity in the control (28.26 U mg⁻¹ protein; Dunnett's test, P<0.05 for each comparison; Fig. 1C). SOD activity (11.55 U mg⁻¹ protein) in the gill of flounder exposed 8 mg Cd L⁻¹ concentration was significantly lower than that in control fish (16.82 U mg⁻¹ protein; Dunnett's test, P<0.05; Fig. 1D). In contrast, the activity of CAT for Cd-exposure concentrations (4.61–4.85 U mg⁻¹ protein) did not significantly differ from that in the control (5.08 U mg⁻¹ protein; Dunnett's test, P>0.05 for each comparison; Fig. 1E). The level of LPO in the Gill of the flounder at all Cd exposure concentrations (1.95–2.9 nmol MDA mg⁻¹ protein) was significantly higher than that in the control (1.53 nmol MDA mg⁻¹ protein; Dunnett's test, P<0.05 for each comparison; Fig. 1F).

3.2.2. **Liver responses**

GSH levels in the liver of fish exposed to 4 and 8 mg Cd L⁻¹ (0.81 and 0.65 mg g⁻¹ protein) were significantly decreased compared to the level in the control fish (0.96 mg g⁻¹ protein; Dunnett's test, P<0.05; Fig. 2A). Only the fish exposed to 8 mg Cd L⁻¹ showed significantly lower hepatic GPx activity (8.59 U mg⁻¹ protein) in comparison to the control activity (11.60 U mg⁻¹ protein; Dunnett's test, P<0.05; Fig. 2B). Hepatic GST activities were also significantly inhibited in fish exposed to 4 and 8 mg Cd L⁻¹ concentrations (21.96 and 19.62 U mg⁻¹ protein) compared to the control activity (31.96 U mg⁻¹ protein; Dunnett's test, P<0.05; Fig. 2C). In contrary, hepatic SOD activity significantly increased at 8 mg L⁻¹ Cd (15.48 U mg⁻¹ protein) compared to the control activity (10.65 U mg⁻¹ protein) (P<0.05; Fig. 2D). No significant change in CAT activity was observed for any of the Cd-exposed groups compared to the control activity level (6.78–7.73 U mg⁻¹ protein; P>0.05 for each comparison; Fig. 2E). The liver of flounder exposed to 4 and 8 mg L⁻¹ Cd demonstrated significantly higher LPO levels (1.85 and 1.98 nmol MDA mg⁻¹ protein, respectively) compared to control fish (0.87 nmol MDA mg⁻¹ protein; P<0.05 for each comparison; Fig. 2F).

3.2.3. **Kidney responses**

GSH levels in the kidney of fish exposed to different concentrations of waterborne Cd (0.91–0.96 mg g⁻¹ protein) did not significantly change in comparison to the control (1.03 mg g⁻¹ protein; Dunnett's test, P>0.05; Fig. 3A). However, significantly increased GPx (15.98 U mg⁻¹ protein) and GST (15.20 U mg⁻¹ protein) activities were measured in the kidney of fish exposed to 8 mg L⁻¹ Cd in comparison to the control values (GPx, 10.55 U mg⁻¹ protein; GST, 12.91 U mg⁻¹ protein; Dunnett's test, P<0.05 for each comparison; Fig. 3B and C). In contrast, SOD activity was significantly decreased in the kidney tissue of fish exposed to 4 and 8 mg L⁻¹ Cd concentrations (10.30 and 10.53 U mg⁻¹ protein) compared to the control (12.69 U mg⁻¹ protein).
Fig. 1 – GSH level (mg g\(^{-1}\) protein, A), GPx activity (U mg\(^{-1}\) protein, B), GST activity (U mg\(^{-1}\) protein, C), SOD activity (U mg\(^{-1}\) protein, D), CAT activity (U mg\(^{-1}\) protein, E), and LPO level (nmol MDA mg\(^{-1}\) protein, F) in gills of Japanese flounder exposed to different waterborne Cd concentrations for 28 d. Values are mean ± standard deviation, n = 20. * Indicates significant difference compared to the controls (ANOVA, P < 0.05).

Fig. 2 – GSH level (mg g\(^{-1}\) protein, A), GPx activity (U mg\(^{-1}\) protein, B), GST activity (U mg\(^{-1}\) protein, C), SOD activity (U mg\(^{-1}\) protein, D), CAT activity (U mg\(^{-1}\) protein, E), and LPO level (nmol MDA mg\(^{-1}\) protein, F) in liver of Japanese flounder exposed to different waterborne Cd concentrations for 28 d. Values are mean ± standard deviation, n = 20. * Indicates significant difference compared to the controls (ANOVA, P < 0.05).
protein; P < 0.05; Fig. 3D). The CAT activity in the kidney from fish exposed to Cd (4.68–5.69 U mg\(^{-1}\) protein) did not significantly differ from the activity in the control (5.55 U mg\(^{-1}\) protein; Dunnett's test, P > 0.05 for each comparison; Fig. 3E). No significant differences were observed in the LPO levels in the kidney tissue of fish between any Cd concentrations (1.21–1.28 nmol MDA mg\(^{-1}\) protein) and the control (1.14 nmol MDA mg\(^{-1}\) protein; Dunnett’s test, P > 0.05 for each comparison; Fig. 3F).

4. Discussion

Fish exposed to Cd in this study accumulated the metal in a significant dose-dependent manner with increasing exposure concentration in all measured tissues. The accumulation factors of Japanese flounder increased with exposure concentration in muscle, gill, kidney and liver in the present study. Similarly, Lange et al. (2002) observed a significant increase of Cd accumulation factors in gills and liver of juvenile rainbow trout (Oncorhynchus mykiss) at 1.5 and 10 μg Cd L\(^{-1}\) for 28 d exposure, the highest accumulation factor was found in the liver tissue in 10 μg Cd L\(^{-1}\). On the contrary, Kim et al. (2004) reported that the accumulator of olive flounder increased with exposure period and was inversely related to the exposure contraction in gill, intestine, and muscle. Yang and Chen (1996) also found that the higher the concentration of Cd, the lower the accumulation factor at 30 and 25 °C when Japanese eels (Anguilla japonica) were exposed to 10–100 μg Cd L\(^{-1}\) for 28 d. Cd was not uniformly distributed in the tissues; rather, a preferential accumulation was observed with the following concentration gradient: liver > kidney > gill > muscle, which

![Fig. 3 – GSH level (mg g\(^{-1}\) protein, A), GPx activity (U mg\(^{-1}\) protein, B), GST activity (U mg\(^{-1}\) protein, C), SOD activity (U mg\(^{-1}\) protein, D), CAT activity (U mg\(^{-1}\) protein, E), and LPO level (nmol MDA mg\(^{-1}\) protein, F) in kidney of Japanese flounder exposed to different waterborne Cd concentrations for 28 d. Values are mean ± standard deviation, n = 4. * Indicates significant difference compared to the controls (ANOVA, P < 0.05).](image-url)
was similar to the findings observed in other marine fish such as pink snapper (Papus auratus) (Ranaldi and Gagnon, 2009), gilthead sea bream (Sparus aurata), European seabass (Dicentrarchus labrax), flathead mullet (Mugil cephalus) (Dural et al., 2006), and grunt (Terapon jarbua) (Dang and Wang, 2009). Typically, the liver and kidneys play important roles in the uptake and storage of heavy metals in fish (Hawkins et al., 1980; Ranaldi and Gagnon, 2009). This pattern is in contrast to the uptake and storage mechanisms in freshwater fish, which mainly absorb metals across the gills and accumulate them in the kidney (Hawkins et al., 1980). Like other marine fish, flounder may absorb Cd when they drink large volumes of seawater to maintain osmotic homeostasis, and thus mainly accumulate the metal in the liver and kidneys. Gill uptake by the flounder also played an important role in Cd accumulation because this is the first organ that contacted the waterborne Cd directly. Compared to the results of the present study, after bigger flounder (17.10 ± 0.11 cm in total length, 52.50 ± 0.90 g in body weight) were exposed to lower Cd concentration solutions (≤100 μg L⁻¹) for 30 d, a preferential accumulation was observed with a concentration gradient of intestine > gill > liver > kidney > muscle (Kim et al., 2004). This suggested that the gill accumulated Cd more efficiently than the liver, kidneys, and muscle. It is possible that developmental stage (fish size) and/or Cd exposure concentration may also influence the accumulation in the gill of the flounder juveniles. Accumulation of Cd in the muscle (0.25–0.66 μg g⁻¹) at each exposure concentration was only 1.0–2.7% of the accumulation in the liver (9.18–65.02 μg g⁻¹) and 1.2–3.3% of that in the kidney tissue (7.64–55.94 μg g⁻¹). The muscle tissue represented the largest fraction of the fish weight, and the Cd accumulated in this tissue was diluted into a larger total mass than other organs such as liver, kidney, and gill, which may have led to a lower Cd concentration. The highly tissue-specific accumulation of Cd in flounder suggested that they mainly absorbed Cd from drinking water and concentrated the metal most effectively in metabolically active tissues like the liver and kidney.

Growth impairment by sublethal Cd exposure is commonly observed in fish. Some examples include the growth reduction observed in juvenile guppies (Poecilia reticulata) at 0.5–1.5 mg Cd L⁻¹ for 30 d exposure (Miliou et al., 1998), larval red sea bream (Pampus major) at 0.8–1.8 mg Cd L⁻¹ for 4 d exposure (Cao et al., 2009), larval toadfish (Atherinops affinis) at 0.05–0.1 mg Cd L⁻¹ for 14 d exposure (Rose et al., 2006) and juvenile bull trout (Salvelinus confluentus) at 0.786 μg Cd L⁻¹ for 55 d exposure (Hansen et al., 2002). In the present study, the Cd-exposed groups exhibited decreased appetite and locomotion activity with a corresponding growth reduction. Flounder exposed to high Cd concentrations (≥4 mg Cd L⁻¹) usually demonstrated sluggish swimming and feeding behavior during the test. When food was provided, flounder in the control group would quickly leave the tank bottom and actively feed. In contrast, flounder exposed to high Cd concentrations tended to stay at the bottom of the tanks and were reluctant to feed. Consequently, much more uneaten food was left in these tanks, which indicated their low food intake during the test. Although the present study did not quantify the food intake of the flounder, judging from what we observed of the feeding activity and uneaten food amounts, it is reasonable to assume that the growth reduction in the flounder exposed to high concentrations might be partially caused by their low food intake and consequent malnutrition. Fish growth depends on the combined factors of feeding, assimilation, and energy trade-off. Cd exposure might have forced the flounder to divert energy from food assimilation to detoxify toxicants or counteract oxidative stress, which may have led to a decrease in the energy available for development and growth.

In general, fish utilize enzymatic and/or non-enzymatic defense mechanisms to counteract oxidative stress due to Cd exposure (Basha and Rani, 2003). SODs are metalloenzymes that represent one important line of defense against oxygen-derived free radical, catalyzes the dismutation of superoxide into molecular oxygen and H₂O₂ (Fridovich, 1995). Thereafter, H₂O₂ is detoxified by CAT, which facilitates the removal of H₂O₂ by decomposing H₂O₂ to molecular oxygen and water (van der Oost et al., 2003). The SOD–CAT system thereby constitutes the first line of defense against oxidative toxicity. Various response levels of SOD and CAT activities have been observed in the tissues of fish exposed to Cd. For example, Cd exposure increased the activity of SOD and decreased the activity of CAT in the liver of goldfish (Carassius auratus) at 5 mg L⁻¹ (Shi et al., 2005). After brown trout (Salmo trutta) were exposed to Cd for 15 d, SOD activity increased in the gill and liver but remained unchanged in the kidney tissue, whereas CAT activity increased in the gill but did not change in the liver and kidney tissues (Hansen et al., 2007). In the present study, SOD activity was significantly inhibited in the gill and kidney of flounder by high Cd exposure concentrations (8 mg Cd L⁻¹ in the gill, >4 mg Cd L⁻¹ in the kidney). On the other hand, SOD activity in the liver was significantly induced at 8 mg Cd L⁻¹ exposure. Because the liver is the major site for Cd detoxification and accumulation in fish (Messaoudi et al., 2009), a high Cd concentration in this tissue might lead to induction of SOD to eliminate the ROS generated. A simultaneous response in the activities of SOD and CAT was expected because these enzymes are linked functionally and act in tandem. However, no significant differences in CAT activity were observed in any of the tissues measured from fish exposed to any Cd concentration. Asagba et al. (2008) reported a similar finding using catfish and attributed it to the fact that CAT is not the only enzyme involved in H₂O₂ catabolism; GPx activity may also regulate the level of H₂O₂ catabolism. This result suggested that CAT might not be an effective biomarker of oxidative stress for flounder exposed to Cd.

GSH plays an important role in scavenging ROS, and a change in GSH levels can be used as an indicator of the detoxification capacity of an organism (Vijayavel et al., 2006). GSH content in the tissues of fish exposed to heavy metals was reported to be reduced or depleted in the liver of tilapia (Oreochromis niloticus; at 3 mg Cd L⁻¹ for 40 d; Xu and Bai, 2007) and in the gill of snake-headed fish (Channa punctata; a mixture of Cu, Cd, Fe and Ni for 7–30 d; Pandey et al., 2008). However, there are several other examples reported that, for liver tissue following Cd exposure, shown no change in GSH (e.g. Thomas and Wofford, 1993), increased GSH levels (e.g. Thomas et al., 1982; Tort et al., 1996), or a transitory pattern (e.g. Lange et al., 2002). In the present study, GSH levels in the gill (for all Cd exposure concentrations) and liver (≥4 mg Cd L⁻¹) of the
flounder were significantly reduced compared to control levels. However, the GSH level in the kidney tissue of the flounder did not significantly change with Cd exposure concentration. The decrease in the GSH level of the flounder may be due to the high Cd accumulation in the cells because the metal could have reacted with GSH to form GS-metal complexes, which may have led to decreased GSH content.

GST and GPx are two GSH-dependent antioxidant enzymes that use GSH as a substrate to respond to oxidative stress. GST catalyzes the transformation of a wide variety of electrophilic compounds to less toxic substances by conjugating them to GSH (van der Oost et al., 2003). GPx plays an important role in catalyzing the reduction of LOOH to stable LOH, which involves a concomitant oxidation of reduced GSH (Nordberg and Arnér, 2001). In this way, GST and GPx work together with GSH to decompose hydrogen peroxide or other organic hydroperoxides (Meister, 1988). Therefore, changes in GPx and GST activities are generally accompanied by changes in the level of GSH (Barata et al., 2005). Decreased GSH levels were observed in conjunction with significantly decreased GST and GPx activities in the gills and liver of flounder exposed to high Cd concentrations. This finding agrees with a previous report (Padmanni and Rani, 2009) of decreased GST and GPx activities in the liver of grey mullet (M. cephalus) exposed to heavy metal pollution in estuaries. Similarly, Messaoudi et al. (2009) also observed a significant decrease in GPx activity in the liver of Salarias basiliscus exposed to 2 mg Cd L−1 for 28 d. In contrast, experiments exposing silver catfish (Rhamdia quelen) to 236 and 414 μg Cd L−1 for 7 d indicated that the liver GST activities were induced (Pretto et al., 2011). In the present study, however, the activities of GST and GPx were significantly increased in the kidney of flounder exposed to 8 mg Cd L−1, although GSH levels in the kidney tissue did not change significantly with increasing Cd concentration. Basha and Rani (2003) demonstrated increased activities of GST and GPx in the kidney tissue of tilapia exposed to a sublethal Cd concentration. The increased GST and GPx activities in the kidneys may have resulted from a fast, adaptive response of the redox-defense system to Cd entrance into this tissue.

In fish, LPO is increased when oxidative stress occurs due to Cd exposure. The LPO level is primarily determined by the balance between the production of oxidants and the removal and scavenging of those oxidants by antioxidants (Filho, 1996). In the present study, after 28 d of waterborne Cd exposure, the flounder juveniles developed tissue-specific LPO and antioxidative responses in the gill, liver, and kidney. The gill is the first target of direct waterborne Cd and plays a significant role in metal uptake, storage and transfer. Moreover, its antioxidant potential is weak compared to that of other tissues (Pandey et al., 2001). Therefore, the gill is more susceptible to oxidative damage compared to the liver and kidney of flounder exposed to Cd. Although the antioxidants actively responded to oxidative stress in this tissue, they clearly failed to protect the gill from biological damage, which was evident in the high LPO observed in the gill tissue of the flounder exposed to the lowest Cd concentration (2 mg L−1). After absorption across the gills, Cd is transferred to the liver. The liver is thought to be a major site for Cd accumulation as well as for detoxification, as the liver is endowed with a strong antioxidant defense system to protect this tissue from oxidative stress caused by metal exposure (Basha and Rani, 2003). The decreased GSH content and GPx and GST activities and the increased SOD activity in the liver of the flounder indicated an active response of the antioxidants to oxidative stress in this tissue. However, the liver is also vulnerable to oxidative damage, especially when its antioxidative capacity is exceeded by a high production of free radicals. The LPO level in the liver of the exposed flounder was significantly increased when the Cd exposure concentration was over 4 mg L−1, even though the antioxidant response increased at those exposure levels. The kidney tissue was the least affected by oxidative stress due to Cd exposure in this study. GPx and GST activities were significantly induced in the kidney, and SOD activity was inhibited in the kidney only at the 8 mg Cd L−1 concentration. This result suggested that as an excretory organ at the end of the Cd transfer chain, the kidney had antioxidant defense systems that effectively scavenged ROS and prevented oxidative damage. Cd also may have been detoxified in the liver and subsequently transferred as less toxic Cd complex to the kidney; therefore, Cd might have exerted a less toxic effect on the antioxidative system of the kidney.

5. Conclusions

After 28 d of waterborne Cd exposure, the growth of the flounder was noticeably reduced at high Cd concentrations. Accumulation of Cd in the flounder was dose-dependent and tissue-specific with accumulation as follows: liver > kidney > gill > muscle. The flounder had tissue-specific antioxidative responses in the gill, liver, and kidney. The gill tissue was the most sensitive to oxidative damage among these tissues. Cd exposure at a higher concentration significantly increased LPO production in the gills and liver of the flounder. The level of GSH and the activities of SOD, GPx, and GST were significantly decreased in these two tissues except for SOD activity in the liver, which was significantly induced in fish exposed to the highest Cd concentration. Although significantly increased GPx and GST activities and decreased SOD activities were observed in the kidney tissue of fish exposed to high Cd concentrations, LPO status was not significantly altered in the kidney tissue of fish exposed to Cd. These results suggest that the flounder expressed tissue-specific antioxidative defense mechanisms to counteract oxidative stress due to Cd exposure. The antioxidants, i.e., SOD, GST, and GPx in the gill, liver, and kidney, and GSH in the gill and liver, were sensitive to Cd exposure and may potentially be used as biomarkers in evaluating Cd toxicity in flounder.

Conflict of interest

The authors declare that there are no conflicts of interest.

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