Simultaneous uptake of Cd from sediment, water and diet in a demersal marine goby *Mugilogobius chulae*

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Abstract

The embryonic state of our knowledge regarding the simultaneous uptake of trace metals *via* multiple routes in aquatic organisms makes it difficult to accurately assess the bioaccumulation and risk of metals. This study used cadmium (Cd) and a demersal marine fish (the yellowstripe goby) as a model system to determine tissue-specific uptake of Cd under conditions of simultaneous exposure to Cd from water, sediment and diet. A triple stable isotope tracing method was used in which each exposure route was spiked by a different stable isotope (^{110}Cd, ^{111}Cd and ^{113}Cd). The results revealed that the fish took up waterborne and sedimentary Cd *via* gills and gastrointestinal tract (GT), and that of dietary Cd was *via* the GT. The gills absorbed Cd predominantly from water (77.2-89.4%), whilst the GT absorbed Cd mainly from diet (81.3-98.7%). In the muscle and carcass, Cd uptake was mainly from the diet (47.1-80.4%) and water (22.8-51.6%). Our study demonstrated that when aquatic animals were subject to simultaneous exposure through multiple uptake routes, the uptake and relative importance of each route for metal accumulation was highly tissue-specific and more complex than a single route of metal exposure.

Key words: marine fish, cadmium, demersal animals, sediment, multiple routes, isotope tracers

Graphic Abstract

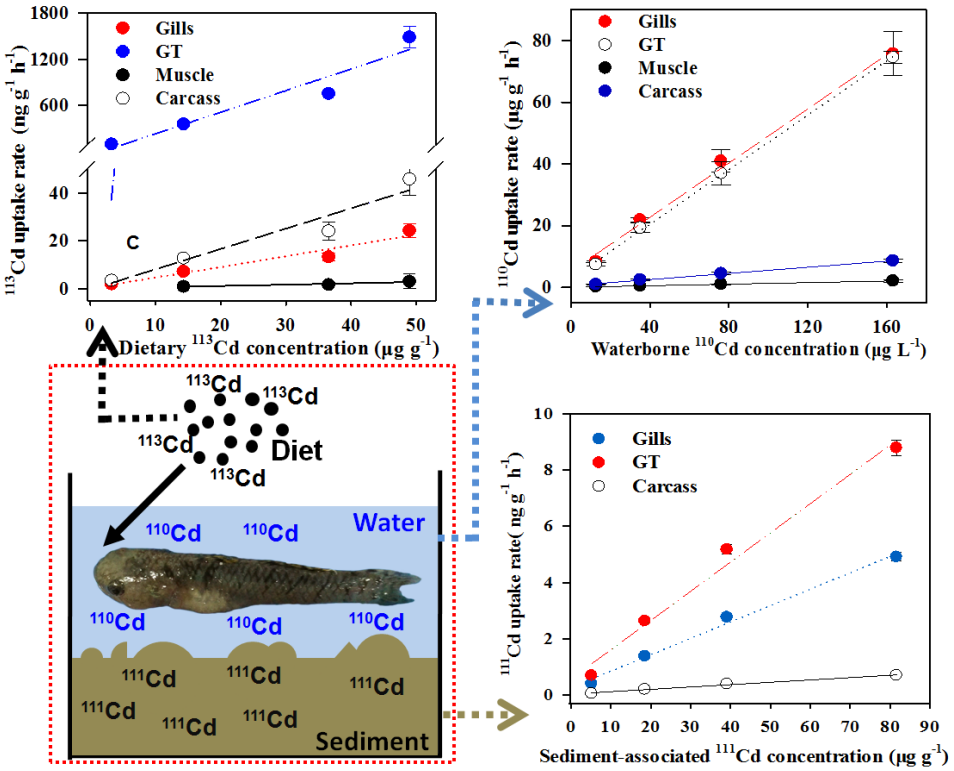

Figure Caption: The simultaneous uptake of waterborne ^{110}Cd, sediment-associated ^{111}Cd, and dietary ^{113}Cd in the different tissues (the gills, gastrointestinal tract (GT), muscle and carcass) of the yellowstripe goby (*Mugilogobius chulae*) exposed to Cd *via* three routes.

1. Introduction

The widespread exploitation of cadmium (Cd) since the second Industrial Revolution has elevated concentrations of this biologically non-essential and highly toxic trace metal in environments. The occurrence of Cd contamination was responsible for the notorious disease of Itai-itai (a bone disease characterized by fractures and severe pain caused by the excessive Cd intake) in the Toyama Prefecture of Japan in the 1950's [1]. Since then, there has been serious international concern regarding Cd pollution [2,3].

Marine ecosystems, the ultimate receptacles of the most anthropogenic pollutants, are frequently subjected Cd pollution, especially in coastal and estuarine areas [3-6]. Among aquatic organisms, the level of Cd bioaccumulation in fish has considerable significance for the health of the general population, given the fact that the consumption of fish is usually the dominant route of Cd exposure to human [7-9]. Indeed, the bulk of studies have observed high levels of Cd bioaccumulation in fish, which results in the associated high risk of Cd exposure to the general populations *via* fish consumption [10-13].

Moreover, fish often play an integral role in aquatic ecosystems, due to their key trophic niches and wide habitats, which facilitates their use as a model with high ecological and environmental relevance for the understanding of trace metal bioavailability and bioaccumulation behaviors [14]. Hence, the levels of metal bioaccumulation in fish, as an assessment endpoint, have been utilized in many previous studies assessing population health risk and ecological risk of heavy metal pollution in aquatic ecosystems [8,13]. However, there have been surprisingly few attempts made to understand the uptake of sediment-associated metals in fish in relation to benthic aquatic invertebrates (e.g., oysters, scallop, and aquatic insects [15,16]), even though sediments are well known as a major carrier of trace metals and thus are potential secondary sources of their contaminants in aquatic ecosystems. To date, the bioavailability and bioaccumulation of sedimentary metals to fish remains at the embryonic state, especially for the demersal fish that are sediment-dwelling and/or

deposit-feeding, which should have a great potential for the take up of sedimentary metals (e.g., Gobioidei such as *Rhinogobius giurinus* and *Rhinogobius cliffordpopei* [17,18]). In field studies, there have been many reports that demersal fish can accumulate high levels of metals and thus they might pose a substantial health risk to human health [8,19,20]. Consequently, a substantial need exists to characterize the uptake of sediment-associated metals in fish species, especially in demersal species, which have great significance for both population health and ecological risk assessments for heavy metal pollution in aquatic habitats.

In fish, the gills and the gastrointestinal tract (GT) are the two main routes for metal uptake [16]. In general, the gills are the main site for uptake of dissolved metals in the water phase, and the GT is the principle site for the assimilation of dietary metals. However, our previous work has suggested that the GT is also important for uptake of dissolved metals in marine fish, owing to the continuous exposure of the GT caused by the need to drink seawater for osmoregulation purposes [21-23]. Moreover, we have demonstrated the significant influence that simultaneous water borne and dietary metal exposure has on the uptake of dietary metals by the GT of marine fish [24], and we further demonstrated that metal uptake *via* the water route can be substantially affected when fish are simultaneous exposed to metal through the dietary route [23]. These results revealed that the uptake of metal from multiple routes is more complex than a single route of metal uptake, and there are tissue-specific interactions among metals from different exposure pathways [23,24]. Nevertheless, most previous studies on fish determined tissue-specific metal uptake using a single route of metal exposure (i.e. there was no dietary metal exposure in determining waterborne metal uptake and *vice versa*), which yields a very limited picture of how specific fish tissues accumulate metals under realistic conditions. This is especially the case for demersal fish, which are often exposed to metal through three routes simultaneously (i.e., waterborne, dietary and sediment-associated metals).

In the present study, we used the highly toxic element Cd and the yellowstripe goby (*Mugilogobius chulae*), a typical demersal fish with potential as a model marine

fish [24,25]), as a model system to investigate metal uptake under conditions of simultaneous exposure *via* three routes (i.e. water, sediment and diet). A triple stable isotope tracing method was used to explore to the uptake of spiked waterborne (^{110}Cd), sediment-associated (^{111}Cd) and dietary Cd (^{113}Cd). We quantified time-course bioaccumulation and influx rate of Cd in the gills, GT, muscle and carcass when the fish were simultaneously exposed to the three routes of Cd. Furthermore, the tissue-specific distribution and relative importance of Cd from the different routes was determined.

2. Materials and methods

2.1. The test organisms and Cd

The juvenile marine yellowstripe gobies (*Mugilogobius chulae*, 1.64 ± 0.12 g fish^{-1}) were provided by Guangdong Laboratory Animals Monitoring Institute (Guangzhou, China).Fish were acclimatized for 2 weeks in the laboratory aquaria before the exposure experiment. During the acclimatization, the fish were fed with oven dried peanut worms (*Sipunculus nudus*) fragmented into pieces with diameters <1 mm.

The stable isotopes ^{110}Cd, ^{111}Cd and ^{113}Cd (99.6%， International Atomic Energy Agency Office at USA, New York) were used as tracers, whilst $CdCl_2$ that contained Cd with natural isotopic ratios (Sigma-Aldrich) was used as a typical, non-tracer source of Cd.

2.2. Cd equilibration in the water and sediment

The surface sediment (~ 0-5 cm depth) was collected from Daya Bay (Guangdong Province, South China (114°40′ E, 22°40′ N)), and transported to the lab in airtight containers. The sediment was sieved (mesh size of 0.43 mm) and washed with distilled water 5 times to remove the background heavy metals. To re-establish the salinity, the sediment was further washed through with seawater (30-32 psu) 3 times. Washed sediment was settled overnight and overlaying water was siphoned off. The sediment was then oven-dried at 80 °C. The moisture and organic matter content of

the sediment was 16.3±1.12 % and 13.8±1.27 % (n= 5). The Cd content in the sediment was 0.019±0.002 μg g^{-1} dry weight (n= 5).

The Cd equilibration in the Cd-spiked sea water and sediments was determined in a preliminary experiment. Briefly, the sea water was first spiked with typical Cd. The nominal Cd concentration in the sea water was 20, 50, 100, and 200 μg L^{-1}. Then, the prepared sediment was added to the aquaria containing the Cd-spiked sea water, homogenized for 15 minutes, and shaken for 30 minutes. The equilibration of Cd between the spiked water and sediments was then investigated. The results suggested the time required to reach equilibrium was 4 weeks (STable 1 (Supporting Information of Table 1)). Thus, the Cd concentration of water and sediment at the end of the equilibration was the reference for the following Cd uptake experiments (STable 1).

2.3. Cd spiking in water, sediment and diet

Four treatments (namely T1, T2, T3 and T4) were used in this study. Seawater treatments were spiked with ^{110}Cd at concentration of 16, 44, 90, and 185 μg L^{-1} in T1 to T4 respectively (STable 1). Sediments of T1-T4 were spiked with ^{111}Cd at concentrations of 6, 22, 45, and 90 μg g^{-1} DW (STable 1) by adding a known volume of solution containing ^{111}Cd to a known mass of the prepared sediment for each concentration (1: 2 ml g^{-1}). The sediment was then, homogenized for 15 minutes, shaken for 30 minutes, and oven-dried for 48 h at 60 °C. To spike fish diets with ^{113}Cd, peanut worms were maintained for 4 weeks in seawater containing ^{113}Cd at concentrations of 15, 40, 87 and 178 μg L^{-1} for T1 to T4 respectively. The measured ^{113}Cd content in the peanut worm was 5.17±0.61, 28.45±2.46, 54.1 ±6.11, 69.2±5.03 μg g^{-1} in T1-T4, respectively.

2.4. Cd uptake experiment

The Cd uptake experiment (24 h) was conducted in 100 ml beakers. The ^{111}Cd spiked sediment was first added in the beaker, and then the ^{110}Cd spiked seawater was lightly

added. The beakers were then left to stabilize for 48 h.

Juvenile marine yellowstripe gobies were individually kept and fed in similar beakers for 1 week to acclimate to the experimental conditions, during which the fish were not exposed to Cd beyond background. Before Cd exposure, fish were starved for 48 h and then fed with ^{113}Cd spiked diet for 1 h (resulting in the ingestion of food equivalent to ca. 13.5% of body weight). Fish were then individually transferred to the prepared beaker containing the ^{111}Cd spiked sediment and ^{110}Cd spiked seawater. As the chyme evacuation time of the fish was 24 h after a single dose of dietary Cd exposure and the fish finished the dietary Cd uptake within 24 h based on our previous study, the simultaneous uptake experiment was conducted over 24 h for all routes in the present study.

During the 24 h uptake experiment, 8 fish in each treatment were sampled at 2, 4, 8, 12, and 24 h. The sediment, overlying water (< 5 cm to the sediment) and water (middle column water) was sampled at the same time intervals. Fish feces were siphoned off gently at 8, 12, 18 and 24 h.

The fish were sacrificed by overdose MS-222. The gills, gastrointestinal tracts (GT), muscle and carcass were then sampled [24] and the chyme in the stomach and intestine was carefully collected. The pore water in the sediment was immediately extracted by centrifugation at 3500 rpm for 10 min.

2.6. Cd stable isotope concentration analysis

Samples of 0.06-0.1 g were digested in 1 ml of HNO_3 (69%, ultrapure, Fisher Scientific, Geel, Belgium) for 48 h at 80 °C. The sample was pooled in the same treatment if the sample was < 0.05 g. The samples of seawater were digested by HNO_3at room temperature for 48 h (1:1). Then the content of total Cd and stable isotope (^{110}Cd, ^{111}Cd and ^{113}Cd) were quantified by inductively coupled plasma-mass spectroscopy (ICP-MS, 7700X, Agilent Technologies Inc., California, USA). The internal standard was ^{115}In and a QC sample was analysed every 20 samples during the analysis. The concentration of ^{110}Cd, ^{111}Cd and ^{113}Cd in the samples were

calculated as described by Croteau et al. [26] and Guo et al. [24] (see details in the Supporting Information).

2.7. Data calculation and statistical analysis

The influx rate of Cd (J_{in}, ng g^{-1} h^{-1}) was calculated by linear regression between the net increase of Cd in the fish and exposure time. The J_{in} was estimated from 0-12 h and 12-24 h based on the food gut pass time of this species [24].

The dietary ^{113}Cd assimilation efficiency (AEs) was calculated as: $AE = A_{24h} / A_{0h} \times 100$, where A_{24h} was the ^{113}Cd retained in the fish at 24h, and A_{0h} was the ^{113}Cd content in the fish at 0 h after feeding [24].

The differences in AEs among the T1-T4 were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey's HSD *post-hoc* test. Analysis of covariance (ANCOVA) was used to test the differences in the slope from the regression between Cd uptake rate and ambient Cd content, using ambient Cd content as the covariate.

Normality and homogeneity of data was determined using Kolmogorov-Smirnov test and Levene's test. Difference was regarded as significant when $p< 0.05$. All statistical analyses were performed by the SPSS software package (vs. 18, SPSS Inc., Chicago, USA).

3. Results

3.1. The verification of Cd content in sediment, water and chyme

3.1.1. The ^{110}Cd, ^{111}Cdand ^{113}Cdcontents in the overlying water and water

The^{110}Cd concentration in the water and overlying water was similar (SFig. 1A&D (Supporting Information of Figure 1)). The sediment-derived ^{111}Cd was detected in both in the water (0.77-5.92 μg L^{-1}, SFig. 1C), and overlying water (1.26-15.3 μg L^{-1}, SFig. 1E) of T2-T4. The dietary ^{113}Cd was detected in the in the water (0.57-1.71 μg L^{-1}, SFig. 1C) and overlying water (1.02-2.23 μg L^{-1}, SFig. 1F) of T3 and T4. However, ^{111}Cd in sediment and ^{113}Cd in diet was 5.90-31.4$\times 10^3$-fold higher than

that in the water and overlying water.

3.1.2. The ^{110}Cd, ^{111}Cdand ^{113}Cdcontents in the pore water and sediment

The water-derived ^{110}Cd concentration was between 0.73-32.7 μg L^{-1} in the pore water (SFig. 2A), and 0.77-7.16 μg g^{-1}in the sediments (SFig. 2D). The pore water ^{111}Cd was 26.6-695 μg L^{-1} in T1-T4 (SFig. 2B). The ^{111}Cd in the sediment was slightly lower than the nominal values (SFig. 2E). Dietary ^{113}Cd in the pore water was only found in T4 (0.35-0.58 μg L^{-1}, SFig. 2C).

3.1.3. The ^{110}Cd, ^{111}Cdand ^{113}Cd contents in the chyme

The water-derived ^{110}Cd increased steadily from 4-24 h in the chyme of the fish (0.22-8.97 μg g^{-1}, SFig. 3A). The sediment-derived ^{111}Cd was only detectable in the chyme of T3-T4 (0.21-1.29 μg g^{-1}, SFig. 3B), while the dietary ^{113}Cd showed a steady decrease from 2-24 h in all four treatment groups (SFig. 3C).

3.2. The tissue specific uptake of waterborne ^{110}Cd

The newly bioaccumulated waterborne ^{110}Cd increased with the exposure time in the four tissues (i.e., gills (Fig. 1A), GT (Fig. 1B), muscle (Fig. 1C) and carcass (Fig. 1D)). The gills and GT accumulated comparable amounts of ^{110}Cd, and the muscle had the lowest values (Fig. 1).

The gills and GT showed similar ^{110}Cd influx rate (J_{in}, ng g^{-1} h^{-1}), which was 8~11-fold higher than that of carcass, and 30~60-fold higher than that of muscle (Table 1). Moreover, the J_{in} of ^{110}Cd in the tissues was linearly correlated with the ^{110}Cd contents in water (Fig. 2A). The slope of the regression was significantly higher in the gills and GT compared with those in the carcass and muscle (ANCOVA, $p<$ 0.05, Fig. 2A).

3.3. The tissue specific uptake of sediment-associated ^{111}Cd

The sediment-associated ^{111}Cd contents in the four tissues also increased steadily with

the exposure time (Fig. 3). The GT showed the highest ^{111}Cd concentration, followed by gills and carcass, and the lowest values was found in the muscle (Fig. 3).

The J_{in} of ^{111}Cd in the GT was 1.7~2.0 fold higher than that in the gills, and 11.7~13.1-fold higher that in the carcass (Table 2). The slope of the regression was highest in the GT, which was significantly higher than that in the carcass (ANCOVA, $p< 0.05$, Fig. 2B).

3.4. The tissue specific uptake of dietary ^{113}Cd

All treatment groups showed similar AEs of dietary ^{113}Cd (1.35-1.74 %, ANOVA, $p> 0.05$, SFig. 4). The concentrations of dietary ^{113}Cd in the tissues displayed a quick increase during 0-12 h, and then a low increase during 12-24 h in the gills, muscsle and carcass (Fig. 4), while the^{113}Cd contents in the GT decreased steadily from 12 to 24 h (Fig. 4B).

During 0-12 h, the J_{in} of dietary ^{113}Cd was highest in the GT, which was 47~61-fold and 26~32-fold higher than that of gills and carcass respectively (Table 3). Furthermore, the slope of the regression was significantly higher in the GT than that in the other tissues (ANCOVA, $p< 0.05$, Fig. 2C).

3.5. The distribution and relative importance of Cd uptake route

3.5.1. The tissue specific distribution of Cd

The time-course percentage partitioning of newly bioaccumulated Cd among tissues was similar among T1-T4 for ^{110}Cd (SFig. 5), ^{111}Cd (SFig. 6), and ^{113}Cd (SFig. 7). Thus, the data of T1-T4 was pooled (Fig. 5). The proportion of the ^{110}Cd and ^{111}Cd was highest in the carcass, followed by the gills and GT, and it was lowest in the muscle (Fig. 5A&B). The highest ^{113}Cd contents were in the GT, followed by the carcass (Fig. 5C).

3.5.2. The relative importance of Cd

The time-course relative importance (%) of ^{110}Cd, ^{111}Cd, and ^{113}Cd in the tissues was

similar among T1-T4 (SFig. 8-11). Thus, the data of T1-T4 was pooled (Fig. 6). In the gills, ^{110}Cd from the water was dominant (77-89 %), followed by ^{113}Cd from the diet (5-18 %) and then ^{111}Cd from the sediment (4.3-5.6 %, Fig. 6A). In the GT, the contribution of the ^{113}Cd was dominant (81-99 %, Fig. 6B). In the muscle and carcass, the^{113}Cd showed slightly higher contribution than ^{110}Cd, while the proportion of ^{111}Cd was very low (0.7-2.2 %, Fig. 6C & D).

4. Discussion

4.1. The scenario of simultaneous uptake of Cd from water, sediment and diet

This study firstly detailed the scenario of time-course tissue-specific uptake of Cd from water, sediment and diet in demersal marine fish. The findings demonstrated that the bioaccumulation of waterborne ^{110}Cd in the four tissues of the fish proportionally increased with the increase of ambient ^{110}Cd levels, and also increased steadily with the exposure time. This pattern was almost same among the four treatment groups (i.e., T1-T4, Fig.1), which is consistent with our previous studies on Cd uptake in this species [24], and is similar to the findings for the marine black seabream (*Acanthopagrus schlegeli*) [21]. Moreover, we likewise found that the dietary ^{113}Cd AEs and the time-course changes in the ^{113}Cd content of the tissues were also similar among T1-T4 (Fig. 4 & SFig. 4), which corresponded with our previous studies [22,24]. Therefore, there was no unequivocal evidence that the uptake of Cd *via* one of the three routes was significantly affected by the simultaneous exposure of Cd from the other routes, at least within the range of ambient Cd concentrations used in this study.

Although our recent findings in marine fish revealed the interaction between waterborne and dietary metal uptake with a simultaneous exposure scenario (i.e. a substantial effect of waterborne Cd on the uptake of dietary Cd [23], and the suppression of dietary metal on the uptake of dissolved metal in the fish GT [24]), it is noteworthy that the presence and magnitude of the effect of a given route of Cd uptake on the another route of Cd uptake might be closely related to the ambient metal

concentrations in each route, based on the results of the present study and previous work [24]. Hence, it seems that the paradigm of simultaneous uptake of metals from multiple routes in marine fish is far more complex than that of a single route of metal uptake in both short-term [23,24]) and long-term metal exposure scenarios [27,28]. As a consequence, simultaneous uptake of metals by organisms *via* multiple routes should be addressed extensively because this scenario has a much higher environmental relevance for population health risk and/or ecological risk assessments of heavy metal pollution.

4.2. The uptake of sediment-associated Cd in the demersal fish

The present study demonstrated that the uptake of sediment-associated ^{111}Cd showed a similar pattern to waterborne ^{110}Cd (Fig. 3), suggesting that the predominant route of sediment-associated ^{111}Cd uptake is from the dissolved ^{111}Cd in the water (e.g., overlying and pore water *via* dissolved or particle-associated ^{111}Cd; SFig. 1). During the exposure in the present study, no frequent burrowing behavior was observed in the fish and the GT of the fish was not found to contain sediments. Consequently, the fish ought to have taken up a very small amount of sediment-associated ^{111}Cd *via* the ingestion of bulk of sediment (SFig. 1). The present findings were in line with several observations made under field exposure conditions [29,30]. In Lake Laflamme (Quebec City, Canada), for instance, Hare et al. [30] reported that most invertebrate taxa accumulated more than 75% of their Cd from the water column compartment (mainly from overlying water). Only those with typical burrowing and/or sediment-feeding behaviors took up amounts of Cd from the sediment compartment. These results demonstrated the great significance of sediment-derived Cd in the water compartment (e.g., overlying water and pore water), which is particularly critical in determining bioavailability and bioaccumulation of the sedimentary metals in demersal fish [29,30].

In addition, the results of the present study revealed a remarkable difference in tissue-specific Cd bioaccumulation between the sediment-associated ^{111}Cd and

waterborne ^{110}Cd. First, the uptake rate of ^{111}Cd in the gills was much lower than that of waterborne ^{110}Cd when the concentration of ^{111}Cd in overlying water was similar to that of ^{110}Cd in water (Table 1 & Table 2). In contrast, the GT showed a higher ^{111}Cduptake rate in the T4 (8.42-9.15 ng g^{-1} h^{-1}) than that for ^{110}Cd in the T1 (6.83-7.96 ng g^{-1} h^{-1}) when the ambient Cd levels in the two phases were comparable (Table 1 & Table 2). Moreover, we found that the ^{111}Cd uptake rate and concentration in the GT was much higher than that in the gills (Table 2& Fig. 3), while the two tissues had comparable ^{110}Cd uptake rates and concentrations (Table 1 & Fig. 2). This suggests that the GT played a more important role than the gills in the uptake of the sediment-derived ^{111}Cd in the water (which might be as particle-associated Cd), while the two tissues had comparable importance in the uptake of dissolved waterborne ^{110}Cd, which has been not been demonstrated previously in fish species. Indeed, the dominant role of GT in the uptake of sediment-associated metals has been previously found only in the aquatic invertebrates that ingest sediments as food resources (e.g., Oligochaetes [31]), or in the suspension-feeders that ingest metal-enriched particles (e.g., snail [32]); clam [33], oyster [34]; and mussel [35]). In the present study, therefore, we suggest that the more important role of the GT than the gills in the uptake of sediment-associated ^{111}Cd might result from the ingestion of ^{111}Cd bound to sediment particles, based on the above empirical studies [32-35]. Thus, further attempts are needed to quantitatively determine the uptake of sediment-associated Cd from the dietary phase *via* ingestion of particles.

4.3. The tissue-specific relative importance of Cd from multiple routes

In the present study, we found that fish accumulated most Cd from dietary and waterborne routes, and the contribution of sediment-associated ^{111}Cd to the total Cd in the fish was very small in all four tissues (less than 5.6 %, Fig. 6). This indicated that only small amounts of sediment-associated Cd could be directly taken up by the demersal fish without ingestion of bulk of sediments. Previous studies have reported a small contribution of sediment-associated Cd to uptake in zebra mussels (5-8% [35]),

and aquatic oligochaetes (9.8% [36]). Under the simultaneous exposure of Cd from the three routes, our findings shed new light on the relative importance of Cd taken up directly from the sediments in demersal marine fish. Results suggested that when conducting population health and/or ecological risk assessments in benthic fish species, there need be little extra concern over the direct uptake of sediment-associated metals in benthic fish species in comparison with the dietary and waterborne Cd bioaccumulation.

4.4. The successful application of the triple Cd stable isotope tracing method

The inductively coupled plasma-mass spectrometry (ICP-MS) technologies have been fully developed in the past decades, which allows the accurate and cost-effective measurement of the low abundance stable isotopes. Manipulation of stable isotope ratios was thus quickly developed as a particularly useful tool in determining trace metal uptake in aquatic animals, such as snails, clams, mussels and fish [23,24,26,35,37]. In relation to the traditional gamma emitting radioisotopes, the stable isotope tracing method has several significant advantages, including the lack of handling/disposal hazard materials, the low healthy risk to researchers, relatively inexpense of the pure stable isotopes, commercial availability of stable isotopes for most metals and so forth [37]. Moreover, most elements have 2 or more stable isotopes, which combined with low detection limits by ICP-MS, allows the high potentiality to determine the simultaneous uptake of ambient trace metals through different routes [35,37]. The present study developed a triple Cd stable isotope tracing method (^{110}Cd, ^{111}Cd and ^{113}Cd) to successfully determine Cd uptake from water, sediment and diet in a demersal fish. We strongly recommended the use of multiple stable isotope tracing methods as they proved highly useful in the study of simultaneous uptake and interaction of the different exposure routes in aquatic animals. Such investigations reflect realistic exposure scenarios in which organisms in contaminated environments are simultaneously exposed metals through multiple routes, but few such studies are reported in the literature.

In conclusion, using a triple stable isotope tracing method, the present study successfully demonstrated the tissue-specific simultaneous uptake of Cd from water, sediment and diet sources in the demersal marine fish. The results revealed that the uptake of Cd by each of the three routes was not apparently affected by the simultaneous exposure to Cd from other routes. Moreover, we found that the relative contribution of sediment-derived Cd to the total Cd in the fish was very small (less than 5.6 %). In demersal fish species, therefore, we suggested the further attempts are required to evaluate the importance of trophic transfer of dietary metals derived from sediments, as secondary contaminated sources of sedimentary metals in aquatic ecosystems.

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557

Figure Captions

Fig. 1. The time-course Cd bioaccumulation by the yellowstripe goby of waterborne ^{110}Cd contents (ng g^{-1}) in the gills (panel A), gastrointestinal tracts (GT, panel B), muscle (panel C) and carcass (panel D) in treatments T1 to T4 under conditions of simultaneous exposure to Cd *via* of three routes. The concentration of waterborne ^{110}Cd was 15 (T1), 40 (T2), 87 (T3) and 178 μg L^{-1} (T4). Values of each point are means of 4-8 replications (the data was not included when there was the unsuccessful measurement due to the insufficient amount of sample/low concentration of ^{110}Cd). Error bars are 1 standard deviation.

Fig. 2. The regression of waterborne ^{110}Cd uptake rate (J_{in}, ng g^{-1} h^{-1}) with the ^{110}Cd contents in water (panel A), sediment-associated ^{111}Cduptake rate with the ^{111}Cd contents in sediment (panel B), and dietary ^{113}Cduptake rate with the ^{113}Cd contents in diet (panel C) when the yellowstripe goby was simultaneously exposed Cd from water, sediment and diet. Values of each point are means of 4-8 replications. Error bars are 1 standard deviation.

Fig. 3. The time-course Cd bioaccumulation by the yellowstripe goby of sediment-associated ^{111}Cdcontents (ng g^{-1}) in the gills (panel A), gastrointestinal tracts (GT, panel B), muscle (panel C) and carcass (panel D) in treatments T1 to T4 under conditions of simultaneous exposure to Cd via of three routes. The concentration ofsediment-associated^{111}Cd was 6 (T1), 22 (T2), 45 (T3) and 90 μg g^{-1} (T4) in DW. Values of each point are means of 3-8 replications (the data was not included when there was the unsuccessful measurement due to the insufficient amount of sample/low concentration of ^{111}Cd). Error bars are 1 standard deviation.

Fig. 4. The time-course Cd bioaccumulation by the yellowstripe goby of dietary ^{113}Cdcontents (ng g^{-1}) in the gills (panel A), gastrointestinal tracts (GT, panel B), muscle (panel C) and carcass (panel D) in treatments T1 to T4 under conditions of

587 simultaneous exposure to Cd via of three routes. The concentration of dietary ^{113}Cd
588 was 5.17 (T1), 28.5 (T2), 54.1 (T3) and 69.2 (T4) μg g^{-1} in DW. Values of each point
589 are means of 4-8 replications (the data was not included when there was the
590 unsuccessful measurement due to the insufficient amount of sample/low concentration
591 of ^{113}Cd). Error bars are 1 standard deviation.
592
593 **Fig. 5.** The time-course percentage of the newly bioaccumulated waterborne ^{110}Cd
594 (panel A), sediment-associated ^{111}Cd (panel B), and dietary ^{113}Cd (panel C) among the
595 gills, gastrointestinal tracts (GT), muscle and carcass of yellowstripe gobies
596 simultaneously exposed to Cd via three routes. Values are means of pooled data of
597 T1-T4 in each sampling time. Error bars are 1 standard deviation.
598
599 **Fig. 6.** The time-course relative importance (%) of newly bioaccumulated waterborne
600 ^{110}Cd, sediment-associated ^{111}Cd, and dietary ^{113}Cd in the gills (panel A),
601 gastrointestinal tracts (GT, panel B), muscle (panel C) and carcass (panel D) of
602 yellowstripe gobies simultaneously exposed to Cd via three routes. Values are means
603 of pooled data of T1-T4in each sampling time. Error bars are 1 standard deviation.

Table 1 The estimated waterborne ^{110}Cd influx rate (J_{in}, ng g^{-1} h^{-1}) in the gills, gastrointestinal tracts (GT), muscle and carcass of the yellowstripe goby simultaneously exposed to Cd from water, sediment and diet. The concentration of waterborne ^{110}Cd was 15 (T1), 40 (T2), 87 (T3) and 178 μg L^{-1} (T4). The "ud" was the undetectable data because of the unsuccessful measurement due to the insufficient amount of sample/low concentration of ^{110}Cd (Fig. 1).

Treatments	Gills		GT		Muscle		Carcass	
	J_{in}	S.E.	J_{in}	S.E.	J_{in}	S.E.	J_{in}	S.E.
T1								
0-12h	9.11	0.16	6.83	0.70	ud	ud	0.82	0.09
12-24 h	7.67	0.45	7.96	0.45	0.15	0.004	1.11	0.09
T2								
0-12h	23.75	0.167	17.51	1.74	ud	ud	2.12	0.17
12-24 h	20.06	1.10	21.06	1.42	0.34	0.009	2.83	0.31
T3								
0-12h	42.77	1.04	34.93	3.81	ud	ud	3.85	0.21
12-24 h	39.02	6.24	38.99	3.69	0.83	0.08	5.08	0.42
T4								
0-12h	79.44	4.79	69.95	2.59	2.57	0.57	7.06	0.47
12-24 h	71.98	9.43	79.17	1.56	1.63	0.22	10.14	0.85

Table 2 The estimatedsediment-associated^{111}Cd influx rate (J_{in}, ng g^{-1} h^{-1}) in the gills, gastrointestinal tracts (GT), muscle and carcass of the yellowstripe goby simultaneously exposed to Cd from water, sediment and diet. The concentration of sediment-associated ^{111}Cd was 6 (T1), 22 (T2), 45 (T3) and 90 μg g^{-1} (T4) in DW. The "ud" was the undetectable data because of the unsuccessful measurement due to the insufficient amount of sample/low concentration of ^{111}Cd (Fig. 3).

Treatments	Gills		GT		Muscle		Carcass	
	J_{in}	S.E.	J_{in}	S.E.	J_{in}	S.E.	J_{in}	S.E.
T1								
0-12h	0.42	0.02	0.69	0.04	ud	ud	-	-
12-24 h	0.39	0.03	0.70	0.03	ud	ud	0.06	0.001
T2								
0-12h	1.48	0.06	2.75	0.08	ud	ud	-	-
12-24 h	1.29	0.08	2.53	0.09	ud	ud	0.21	0.006
T3								
0-12h	2.99	0.08	5.26	0.21	ud	ud	0.42	0.01
12-24 h	2.54	0.09	5.10	0.11	ud	ud	0.37	0.01
T4								
0-12h	5.49	0.19	9.15	0.32	ud	ud	0.72	0.03
12-24 h	4.34	0.11	8.42	0.23	0.07	0.003	0.69	0.02

Table 3 The estimated dietary ^{113}Cd influx rate (J_{in}, ng g^{-1} h^{-1}) in the gills, gastrointestinal tracts (GT), muscle and carcass of the yellowstripe goby simultaneously exposed to Cd from water, sediment and diet. The concentration of dietary ^{113}Cd was 5.17 (T1), 28.5 (T2), 54.1 (T3) and 69.2 (T4) μg g^{-1} in DW. The "ud" was the undetectable data because of the unsuccessful measurement due to the insufficient amount of sample/low concentration of ^{113}Cd (Fig. 5).

Treatments	Gills		GT		Muscle		Carcass	
	J_{in}	S.E.	J_{in}	S.E.	J_{in}	S.E.	J_{in}	S.E.
T1								
0-12h	1.97	0.02	92.78	5.48	ud	ud	3.47	0.01
12-24 h	0.46	0.01	-28.57	1.12	0.10	0.01	1.08	0.03
T2								
0-12h	7.20	0.12	355.93	9.45	0.91	0.02	12.73	0.36
12-24 h	1.66	0.02	-63.09	2.05	0.51	0.03	2.90	0.11
T3								
0-12h	13.31	0.55	752.40	8.14	1.69	0.03	24.06	0.67
12-24 h	3.18	0.08	-86.96	3.14	0.98	0.04	5.56	0.28
T4								
0-12h	24.23	0.78	1490.31	15.99	2.98	0.05	45.77	0.87
12-24 h	7.05	0.12	-148.24	6.241	1.33	0.06	12.97	0.54

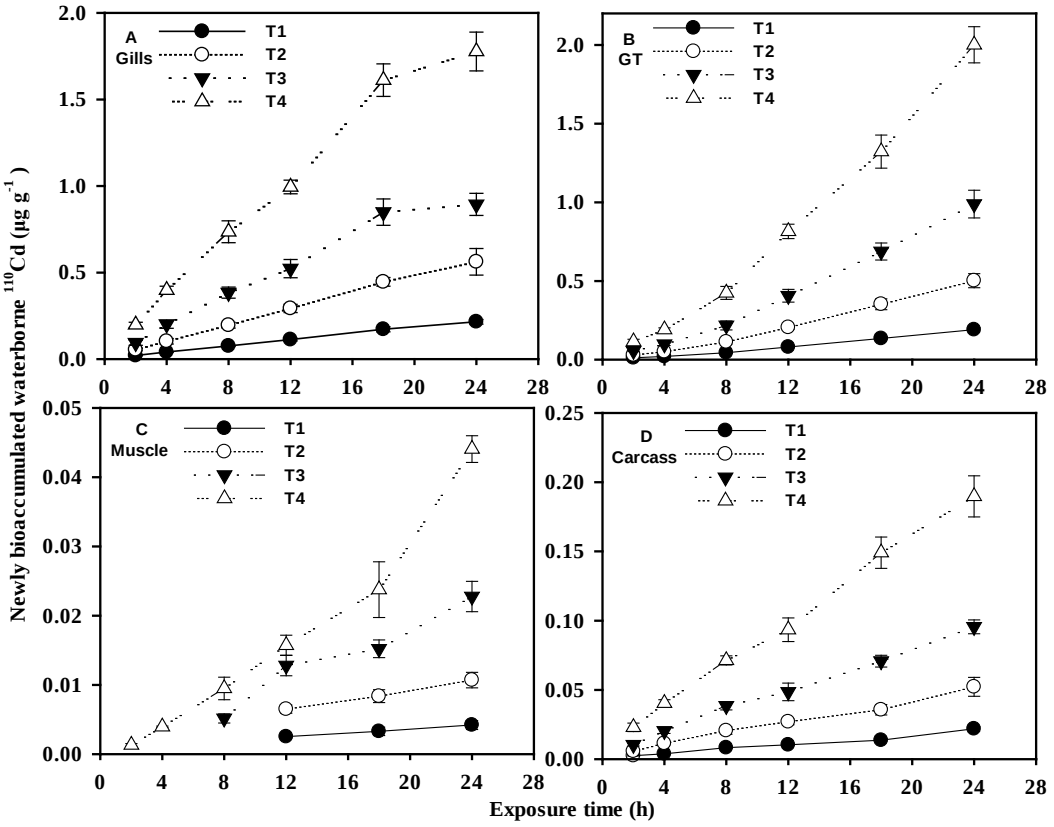


Fig. 1.

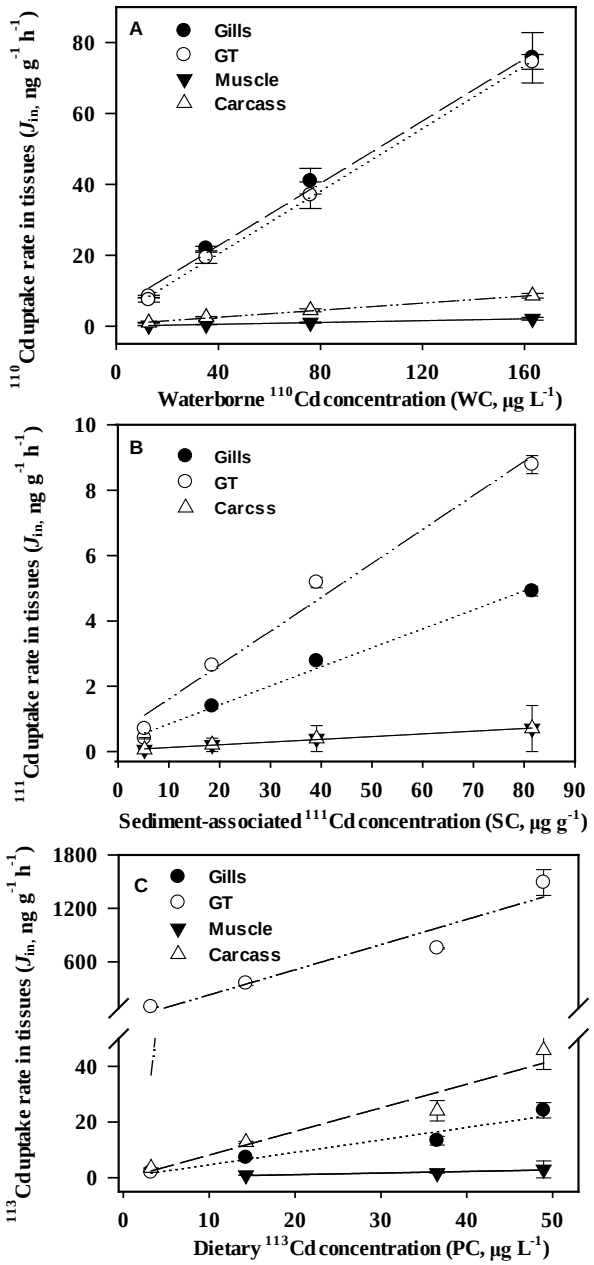
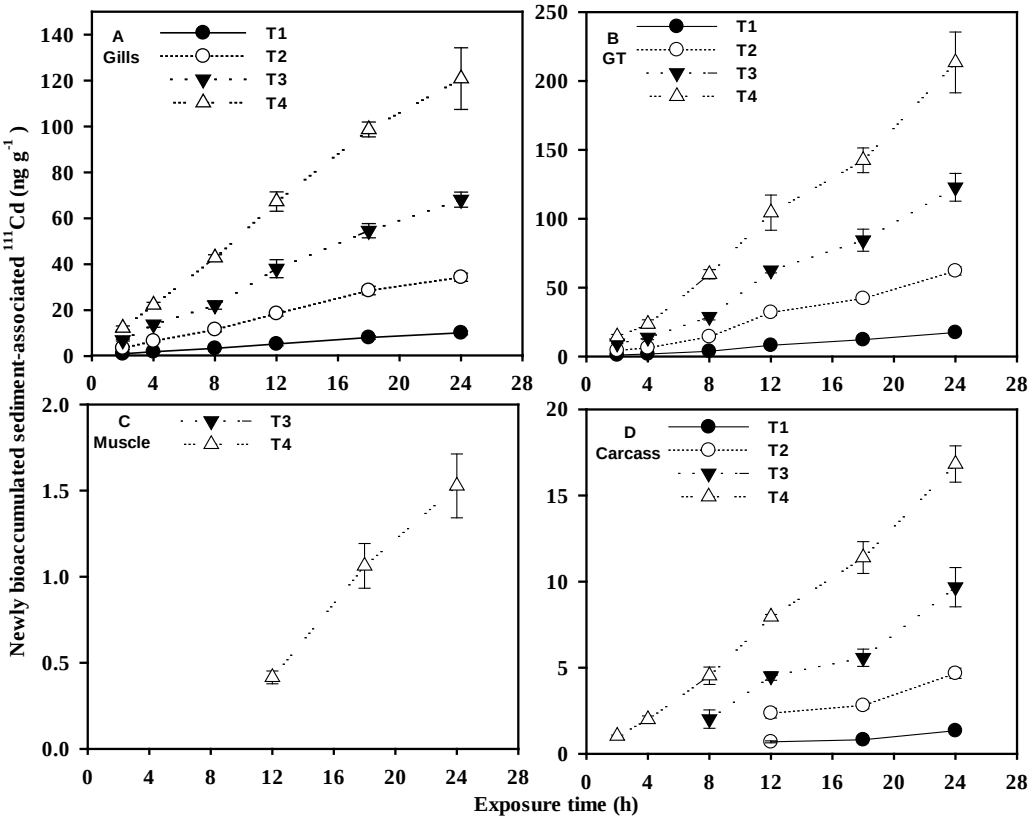


Fig. 2.



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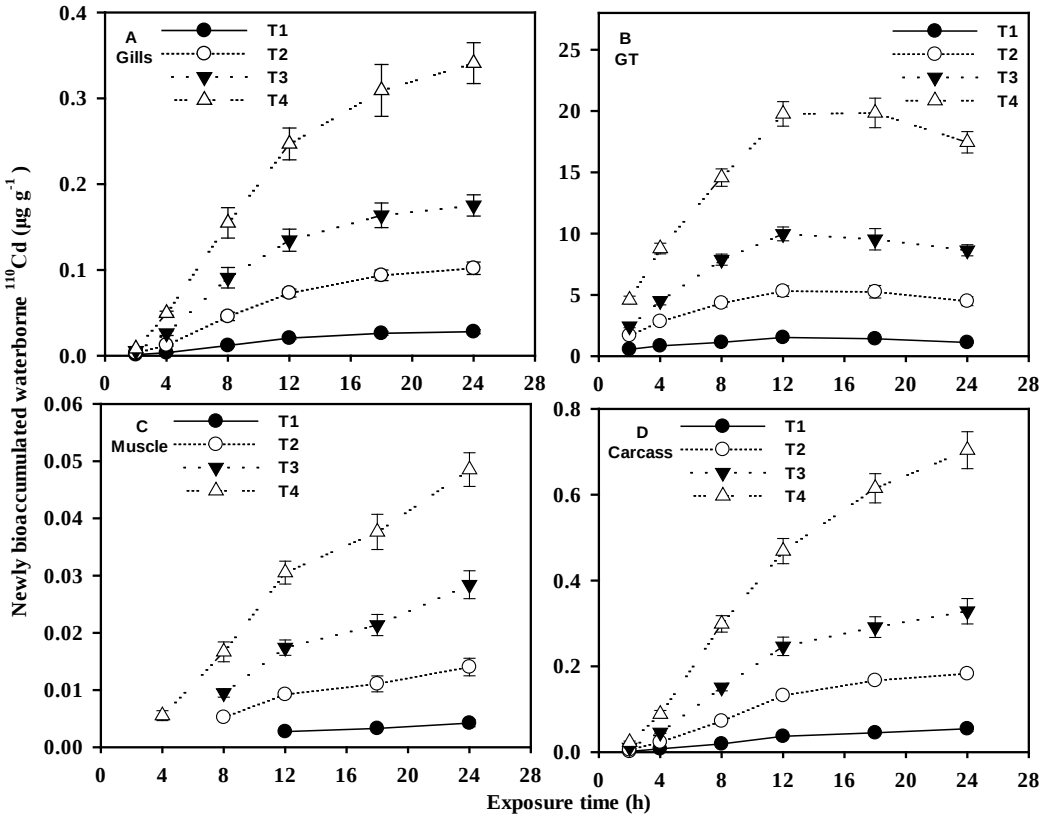

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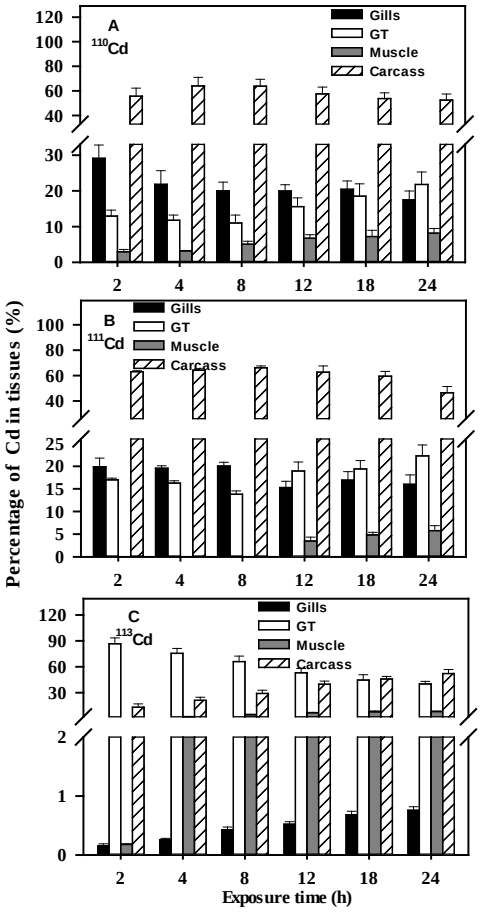

Fig. 5.

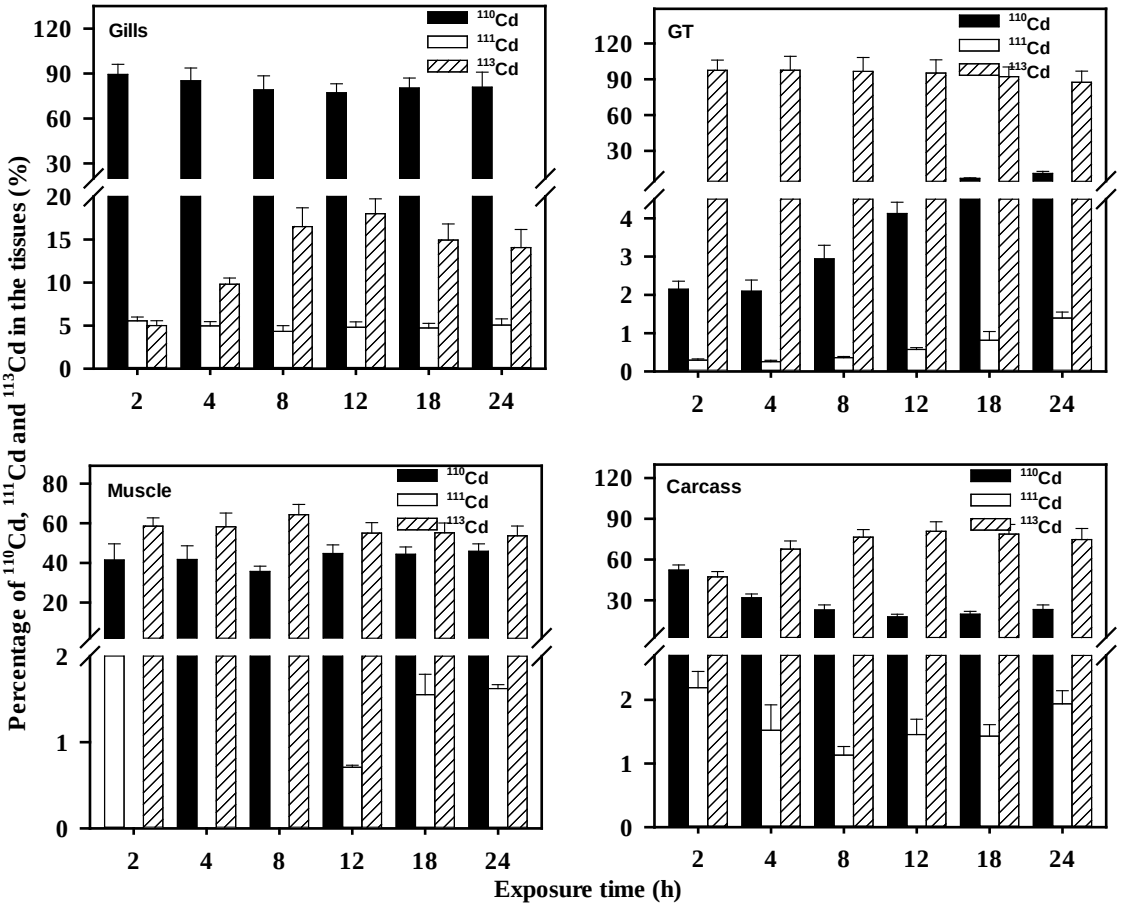

Fig. 6