



Bioaccumulation of cadmium bound to humic acid by the bivalve *Meretrix meretrix* Linnaeus from solute and particulate pathways

Xing Wu^{1,2}, Yongfeng Jia^{1,*}, Huijie Zhu¹, He Wang¹

1. Key Laboratory of Pollution Ecology and Environmental Engineering, Institute of Applied Ecology,
Chinese Academy of Sciences, Shenyang 110016, China. E-mail: wuxing@yahoo.cn

2. Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

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Abstract

Humic acid is an important pool of heavy metals in sediments. Generally, the presence of refractory humic matter in aquatic systems would decrease the bioavailability of Cd and hence control the bioaccumulation of Cd in benthic animals. In the present work, we applied a suspending system to determine the relative importance of particulate and dissolved fractions of humic acid as Cd sources for the bivalve *Meretrix meretrix* and to investigate the difference of bioaccumulation characteristics of Cd adsorbed on different fractions of humic acid. The results showed that the bioaccumulation characteristics of Cd from particulate and dissolved fractions of humic acid were apparently different due to biological responses of the clam to the particulates. At Cd concentration of 140 mg/kg, the accumulation of Cd from Cd associated with humic acid mixtures was mainly attributed to the dissolved fractions of humic acid. Compared to particulate fractions, Cd associated with the dissolved fractions of humic acid may be more bioavailable.

Key words: humic acid; bioaccumulation; cadmium; *Meretrix meretrix* Linnaeus

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Introduction

Most metal pollutants discharged into aquatic environments would be rapidly removed from the water column and transported to bottom sediment. The sediment was considered as a repository for trace metals. Metal concentrations in sediments are often several orders of magnitude higher than that of dissolved phases (Burton Jr, 1991). Whereas, recent studies demonstrated that sediment has become an important source of metal accumulation for aquatic animals (Farang et al., 1994; Wang and Fisher, 1999). The bioavailability of trace metals in sediments was influenced by various physical-chemical and biological factors, such as particle size, amount and type of minerals and organic matters, affinity of the contaminant to the particles, uptake route (Lee, 1992; Eggleton and Thomas, 2004; Kukkonen et al., 2004; Croisetiere et al., 2006). Among these factors, the uptake route and the speciation of metals play very important roles in bioaccumulation of metals by animals (Bryan and Langston, 1992; O'Day et al., 2000).

Marine invertebrates are exposed to metals from both dissolved and particulate phases. Non-essential toxic metals, such as Cd, may not have specific uptake mechanisms and appear to follow existing pathways for essential metals

(Roesijadi and Robinson, 1993). Metals dissolved in water may be accumulated by direct adsorption to body surfaces. The dissolved metals can be taken up across the cell membrane of permeable surfaces by one or more transport routes, including carrier-mediated transport, membrane channels, and passive diffusion of lipid-soluble (non-polar) metal forms which dissolve in the lipid bilayer (Simkiss and Taylor, 1995; Tessier et al., 1994; Rainbow, 1997). The most important sites for the absorption of dissolved Cd are gills and dissolved metals can be passively accumulated by facilitated diffusion or actively pumped across the membranes (Rainbow, 1997). Particulate metals can be accumulated by animals following ingestion and digestion of food in the gastro-intestinal tract. The adsorbed metals on the surface of particles can be released into the gastro-intestinal environment due to desorption or substrate solubilization by enzymes and non-enzymatic ligands. Comparing dissolved metals, bivalves may be more capable to control the accumulation of metals from particulate phase by regulating food distribution between extracellular and intracellular digestion processes (Decho and Luoma, 1996). The separation of these two exposure pathways is fundamental for understanding factors controlling metal bioavailability and for setting water and sediment quality criterias.

The way of metal associated with various sediment

* Corresponding author. E-mail: yongfeng.jia@iae.ac.cn

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components determines to a large extent whether the metals can be absorbed by the benthic animals. Generally, the important trace metal binding substrates in oxidized estuarine sediments are organic matter, iron(III) oxyhydroxides, and little manganese(IV) oxides (Lion et al., 1982). Humic acid (HA) represents an operational defined species of organic matter that is relatively refractory to animals and microorganisms. It is an important pool for metals and other contaminants. Decho and Luoma (1994) reported that Cd and Cr bounded with humic coating particles were difficult to be absorbed by the clams *M. balthica* and *P. amurensis*. The Cd assimilation from sediment was also reduced by humic acid coating for the polychaete *Capitella* (Selck et al., 1999). A very important group of complexing agents in natural aquatic ecosystems, which is assumed to decrease the bioavailability and toxicity of metals, is dissolved organic matter (DOM) (Buffle et al., 1977; Guerrero et al., 2000). The most important components of DOM are dissolved humic and fulvic acids. They are often present at concentrations which are many orders of magnitude higher than that of the trace metals (Buffle et al., 1977; Voets et al., 2004). The presence of DOM can greatly decrease the concentration of free metal ions in solution and hence inhibit the absorption of the metals ions. Most of the results found in previous researches reported a decrease of Cd bioavailability in the presence of DOM, and quantitative results agreeing with free ion activity model have been reported for different benthic organisms (Poldoski, 1979; Van Ginneken et al., 2001; Vigneault and Campbell, 2005). Generally, the presence of refractory humic matter (as coatings of particles or ligands in solution) in aquatic systems would decrease the bioavailability of Cd and hence controls the bioaccumulation of Cd in benthic animals. To our knowledge, few researchers have investigated the effect of particulate fractions of humic acid on the bioaccumulation of Cd. However, the distribution of metals between particulate fractions and dissolved fractions of humic acid may also have a great impact on the accumulation characteristics of Cd.

In the bioaccumulation experiment, it is ideal to maintain the chemical concentration constant during the test (Arnot and Gobas, 2006). Most research works on bioaccumulation of heavy metals from dietary exposure chose species of static particle feeders (deposit feeders) as biomonitors, such as crustaceans, polychaetes, and few were reported on the bioaccumulation of heavy metals over a period of exposure time from constantly fed particulates using suspension-feeders as biomonitors.

In the present work, an important trace metal sink in sediments, i.e., humic acid, was chosen as the adsorption substrate. One of the most toxic heavy metals, Cd, was used as the trace metal contaminant. Bivalve *Meretrix meretrix* Linnaeus was selected as the biomonitor. The suspending system that can maintain the concentration of particulates constant in the container was applied. The objectives of this study were to determine the relative importance of particulate and dissolved fractions of humic acid as Cd sources for the bivalve *M. meretrix* and to

investigate the difference of bioaccumulation characteristics of Cd adsorbed on different fractions of humic acid.

1 Materials and methods

1.1 Testing animal

M. meretrix is a nearly equilateral triangular-shaped clam with highly coloured shells that inhabits sandy substrates in the lower intertidal and shallow subtidal areas of the Bohai and western Yellow Sea. At present, it is widely cultured by farmers in the Shandong Province and adjacent coastal areas of China because of its relatively high meat yield, and delicious taste. For its widely distribution and sensitivity to contaminants, it has been used as bio-indicator in environmental monitoring research (Wang et al., 2005).

The testing animal *M. meretrix* with the body length of 3–4 cm was obtained from a local open market. The bivalves were acclimated in artificial seawater at -23°C for 7 days before they were introduced into the Cd-containing particulates suspension system. During the acclimation period, only individuals that extended their siphons were used in the experiments. The culture medium used in all experiments was artificial seawater prepared by dissolving sea salt in tap water. The tap water had been dechlorinated for at least 24 hr and the initial salinity was set at 25‰. The pH of the prepared artificial seawater was 7.7.

1.2 Particulate suspending system

The suspending system was designed to maintain the seston concentration constant. It consists of a temperature-regulated barrel with a submerged pump at the bottom. The waterspout of the pump was fixed along the tangent line of the barrel and can induce a sustained spiral upwelling current. A mesh was used to hold *M. meretrix*. The system was continuously aerated from the bottom of the barrel to provide oxygen and to assist in suspending particulates.

1.3 Synthesis of Cd-adsorbed humic acid

Humic acid was purchased from Sinopharm Chemical Reagent Corporation Co., Ltd., China. In the experiments investigating the difference of Cd accumulation characteristics between Cd-humic acid mixtures (including both the dissolved and particulate fractions) and Cd-humic acid particulates, 1.5 g of humic acid was suspended in 150 mL of artificial sea water, followed by introducing certain amounts of cadmium solution to prepare the Cd-humic acid mixtures and the Cd loading concentration was set to be 140 mg/kg. In order to prepare humic acid particulates, 50 g humic acid was resuspended in 50 L artificial sea water and left to equilibrate for 2 days, and then the supernatant was discarded to remove the dissolvable fractions. This process was repeated several times to get the particulate phase of humic acid. A 1.5-g of humic acid particulates was suspended in 150 mL of artificial sea water followed by introducing certain amounts of cadmium solution. The Cd loading concentration of particulate humic acid was also set at 140 mg/kg. This is relevant to

the contaminant levels in the polluted area along the east coast of China (Fan et al., 2002). Detection of Cd remained in the artificial sea water showed that the introduced Cd was completely adsorbed onto the particulate humic acid. The Cd-adsorbed humic acid mixture (Cd-HA_m) and particulate humic acid (Cd-HA_{p-140}) were prepared and introduced everyday before the suspension was changed with fresh seawater.

In the experiments aiming at clarifying the contribution of Cd bounded with dissolved and particulate fractions of HA_m to the apparent bioavailability of Cd-HA_m, the prepared Cd-HA_m was settled down for 24 hr and the dissolved fractions were carefully isolated from the Cd-HA_m by siphonage. Every day, the dissolved fractions of Cd-HA_m (Cd-HA_d) and the remained particulate components (Cd-HA_{p-70}) were introduced separately before the suspension was changed with fresh seawater. The partition coefficient of Cd between the dissolved and particulate fractions was measured to be 0.437. The Cd level of Cd-HA_{p-70} was calculated as ca. 70 mg/kg and the Cd concentration in the Cd-HA_d bearing systems was about 8 µg/L.

1.4 Bioaccumulation experiments

Fifteen clams were placed in each suspending system containing 15 L of artificial seawater and fed with the prepared contaminants (1.5 g Cd-HA_p, 150 mL Cd-HA_d or Cd-HA_m). The clams were cultivated at 23°C for 28 days. The suspension was changed every 24 hr with freshly prepared artificial seawater and Cd-adsorbed contaminants. At various time intervals, four clam individuals were randomly sampled from three parallel running suspending systems and were transferred to clean seawater to evacuate the gut content for 3 days, then they were preserved in Teflon sealing bag at -20°C for further analysis.

1.5 Analysis

The clams were defrosted at 30°C for 5 hr, and the soft tissues were dissected and dried at 105°C for 24 hr. The shell length, shell weight, and wet weight (ww) and dry weight (dw) of the soft tissue were measured. The dried tissues were digested according to the method described in the literature (Xu et al., 1998). The concentration of cadmium was determined using a Varian AA-240 atomic absorption spectrophotometer with the detection limit of 0.05 mg/L. Reference standard samples (NIST 296 – trace elements in mussel tissue) were analyzed and the recovery percentages of certified values was (87 ± 8)% for Cd. The concentration of Cd in the body of *M. meretrix* was expressed as µg Cd/g ww and Cd uptake rate by the clams was expressed as µg Cd/(g·day) ww.

2 Results and discussion

Considering that Cd may desorb from the prepared particulate humic acid during the exposure period, the Cd concentration in the dissolved phase of the system was monitored. Experimental results showed that the concentration of dissolved Cd in the suspending systems was

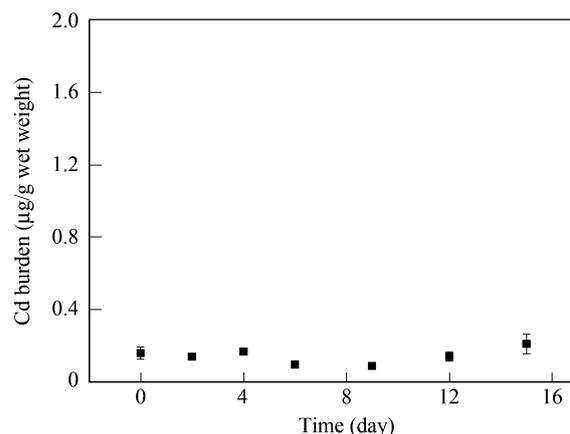


Fig. 1 Cadmium accumulation in soft tissues of *M. meretrix* from aqueous phase at the Cd concentrations of 0.5 µg/L.

below 0.5 µg/L. Further investigation demonstrated that after 15 days of exposure to 0.5 µg Cd/L solution, the uptake of cadmium by *M. meretrix* from aqueous phase was not detected (Fig. 1). Apparently, absorption from particulate phase was the dominant way of Cd assimilation in the particulate humic acid system.

The accumulation of Cd by the clams from Cd-HA_m and Cd-HA_{p-140} systems is shown in Fig. 2. At Cd concentration of 140 mg/kg, Cd was apparently assimilated in the body of the animals from both systems. Two distinct periods of accumulation were observed for Cd-HA_{p-140} system (Fig. 2a), the uptake rate was determined to be (0.022 ± 0.002) µg/(g·day) by linear fitting of the first period of assimilation ($r^2 = 0.7956$, $p < 0.0001$). The accumulation of Cd reached a steady state after 20 days of exposure for the Cd-HA_{p-140} suspensions and the internal cadmium concentration of the clams at the steady state was approximately 0.7 µg/g ww. While, for Cd-HA_m suspensions the uptake of Cd did not reach steady state after 28 days of exposure (Fig. 2b) and the uptake rate was determined to be (0.018 ± 0.001) µg/(g·day) by linear fitting of the curve ($r^2 = 0.9031$, $p < 0.0001$).

An assimilation of Cd from Cd-HA_d and Cd-HA_{p-70} is shown in Fig. 3. For the clams cultivated in Cd-HA_d systems, Cd was apparently assimilated in the body of the animals (Fig. 3a). The uptake of Cd did not reach steady state after 28 days of exposure and the uptake rate was (0.028 ± 0.003) µg/(g·day) ($r^2 = 0.8618$, $p < 0.0001$). In comparison, bioaccumulation of Cd from Cd-HA_{p-70} suspensions was not detected (Fig. 3b).

At Cd concentration of 140 mg/kg, the Cd levels in the soft-tissues of the clams increased significantly with time for both Cd-HA_m and Cd-HA_{p-140} systems. The uptake rates were comparable, whereas, the bioaccumulation characteristics were apparently different. Compared to Cd-HA_m, the accumulation of Cd through ingesting Cd-HA_{p-140} particles reached a steady state when the exposure time exceed 20 days.

As shown in Fig. 3, Cd was not assimilated from Cd-HA_{p-70} suspensions during the experimental period, indicating that the accumulation of Cd from Cd-HA_m was mainly attributed to Cd-HA_d. Marine bivalves have a

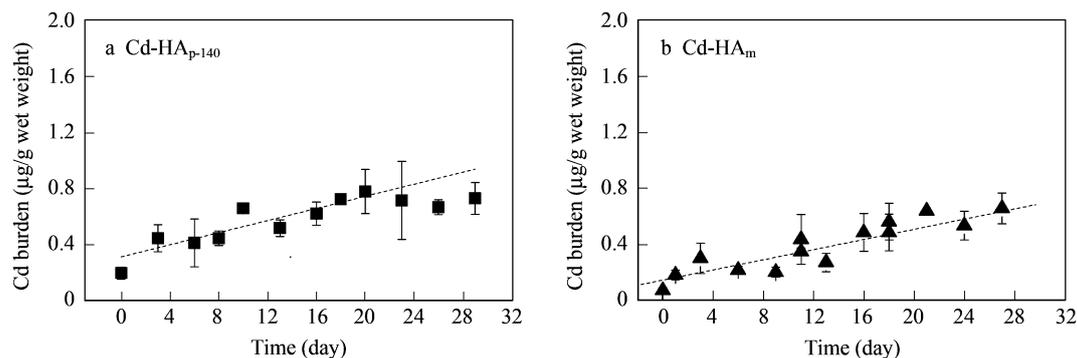


Fig. 2 Uptake curves of Cd in *M. meretrix* under 140 mg/kg Cd burden to particulate humic acid (Cd-HA_{p-140}) (a) and humic acid mixture (Cd-HA_m) (b). Each point represents (arithmetic mean \pm 1) \times standard error of four clams. The dash line represents linear regression for the uptake rate.

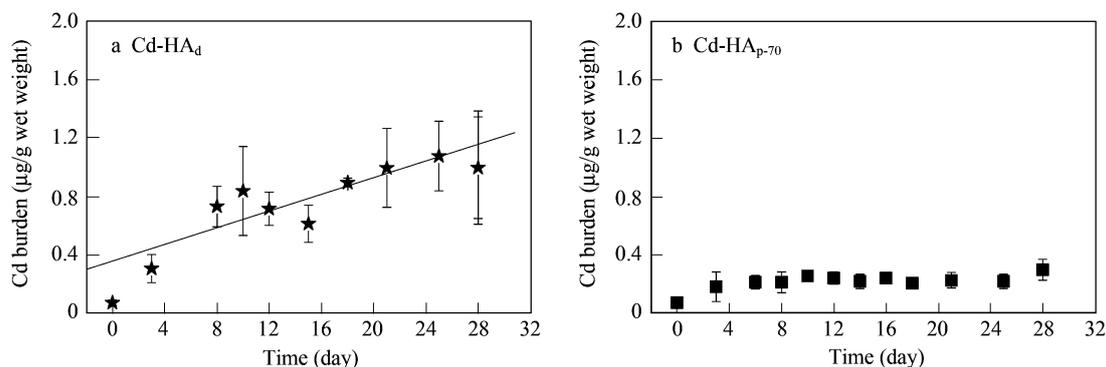


Fig. 3 Uptake curves of Cd in *M. meretrix* separately from solute and particulate phases of Cd-HA_m at Cd concentration of 140 mg/kg. (a) Cd bound to dissolved fractions (Cd-HA_d); (b) Cd associated with particulate fractions (Cd-HA_{p-70}). Each point represents (arithmetic mean \pm 1) \times standard error of four clams. The dash line represents linear regression for the uptake rate.

flexible digestion strategy which enables them to control the accumulation of food-borne metals. Particles ingested by bivalves are usually processed by two-phase digestion. The first digestion phase occurs in the stomach and intestine and is termed as “intestinal digestion” or “extracellular digestion”. In the second phase, a proportion of these food particles are further sorted into the digestive gland for more intensive digestion and this process is termed as “intracellular digestion”. The adsorbed cadmium on the surface of the particles can be released into the gastrointestinal environment due to the desorption or substrate solubilization by enzymes and non-enzymatic ligands. Under high levels of contamination, the animals will reduce the proportion of particles processed by intracellular digestion and hence reduce the assimilation efficiency of trace metals (Decho and Luoma, 1996). In our experiment, with an increasing Cd accumulation from Cd-HA_{p-140}, the clams would repulse Cd-HA_{p-140} particles from entering the intracellular digestion process. The extracellular digestion process, mean while, may not be capable to release Cd from the humic acid substrate. Thus, the accumulation of Cd was controlled by clams and reached a steady state. Compared to the Cd in particulate phase, water-borne Cd is partially absorbed by passive processes (Simkiss and Taylor, 1995). The bivalves were less available to regulate the accumulation of Cd from dissolved phases and hence the assimilation of Cd from Cd-HA_m was not controlled by the clams.

Thomann (1981) proposed a bioenergetics-based kinetic

model describing the accumulation of contaminants by aquatic animals, assuming that chemicals are available from both the dissolved and particulate phases:

$$dC_A/dt = (k_u \times C_w) + (AE \times IR \times C_f) - (k_e + g)C_A \quad (1)$$

where, $(k_u \times C_w)$ describes the absorption from aqueous phase; $(AE \times IR \times C_f)$ describes the absorption from particulate phase; $(k_e + g)C_A$ expresses the efflux and dilution of absorbed Cd. The assimilation efficiency (AE) is a critical factor determining the uptake rate of Cd from dietary uptake route. Bonding strength between Cd and substrates, to a great extent, influences the assimilation efficiency since desorption is a key process determining Cd assimilation in bivalves (Wang and Fisher, 1996).

Humic acids are a complex mixture of partially “decomposed” and otherwise transformed organic materials. These substances have medium molecular size and their molecular weight is around 5000 to 100,000 Dalton (Aiken et al., 1985). Humic acids have sufficient negative surplus charge on their surfaces and a substantial fraction of the mass of humic acids is in carboxylic acid functional groups. The carboxyl (COOH) group reacts readily with metals and gradually dissociates between pH 2.5 and 7 to form the carboxylate (COO⁻) group (Spark, 1997), which endow these molecules with greater ability to chelate positively charged multivalent ions, e.g., Cd²⁺. Previous research demonstrated that compared to manganese oxides, humic acid particles shows a similar sorption capability and bonding strength of Cd (Aualiitia and Pickering,

1987). However, it was observed in the present work that the Cd uptake rates from humic acid particles were significantly lower than that from manganese oxides (data published elsewhere).

The assimilation of Cd is controlled not only by the bonding strength of the adsorbed Cd with substrates, but also by some biological factors such as the solubility of substrate in gastro-intestinal environment, the gut retention time, and the distribution coefficient between intracellular and extracellular digestive processes. Manganese oxide tends to be solubilized due to the Mn-reducing potentials of gastro-intestinal enzymes (Turner and Olsen, 2000). Compared with manganese oxide, humic acid is reported to be refractory for the benthic animals (Qiu et al., 2007). Hence, the contaminants associated with manganese oxides would be considerably more bioavailable.

Our results showed that the accumulation of Cd from Cd-HA_m was mainly attributed to Cd-HA_d. Whereas, Cd uptake rate from Cd-HA_d ($(0.028 \pm 0.003) \mu\text{g}/(\text{g}\cdot\text{day})$) was higher than that from Cd-HA_m ($(0.018 \pm 0.001) \mu\text{g}/(\text{g}\cdot\text{day})$). Compared to Cd-HA_d system, humic acid particles present in the Cd-HA_m suspensions may induce secretions of mucus which may help to transport filtered particles in gills (Beninger et al., 1992). A primary function of mucus, composed of water, high molecular weight protein-polysaccharide complexes and inorganic salts, is to isolate the gills of bivalves from its environment (Davies et al., 1998). This will to a certain extent inhibit the accumulation of Cd from the dissolved phases. Harvey and Luoma (1985) reported that ingested iron oxides particles scavenged Ag in the digestive tract of feeding clams *Macoma balthica* and hence reduce the uptake of Ag from solutions as compared to that for unfed clams. The zero point charge of humus was found between 1.2 and 1.8 (Gjessing, 1976). Therefore, HA will be able to retain metals at very low pH levels, e.g., in the gill microenvironment. In our experiment, humic acid particles aggregated at gills of the clam may capture free Cd ions released in the gill microenvironment and reduced the accumulation of Cd from dissolved phase.

3 Conclusions

At Cd loading concentration of 140 mg/kg, the accumulation of Cd from Cd-HA_m system was mainly attributed to the dissolved fractions of Cd-HA_m. The bioaccumulation characteristics of Cd were significantly different between Cd-HA_m and Cd-HA_{p-140}, generally resulted from differences in uptake routes and the biological responses of the clam to the particulates. Regulation of the digestive processes and the interactions of dissolved Cd with filtered particles at the gills may play an important role in the Cd bioaccumulation. Compared to the particulate fractions, Cd associated with the dissolved fractions of humic acid may be more bioavailable.

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