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Uptake pathways and subcellular fractionation of Cd in the polychaete *Nereis diversicolor*

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Abstract Polychaetes have often been utilized as indicator species to investigate the impacts of pollutants, such as heavy metals. The uptake of Cd by the polychaete *Nereis diversicolor* was determined at varying Ca concentrations and with pre-exposure to Ca ion channel blockers and metabolic inhibitors in simulated sea water over 1 week period. The supply of Ca in simulated sea water inhibited Cd uptake and increased Ca concentration in *N. diversicolor* after 10 μM Cd exposure. Pre-exposure to a Ca-channel blocker (Lanthanum) significantly inhibited Cd uptake, suggesting that the uptake of Cd was exerted at a Ca channel. *N*-ethylmaleimide, which specifically binds to sulfhydryl groups, inhibited Cd uptake at 10 μM, implying that the transport of Cd is carrier-mediated by proteins or other SH-containing compounds. Subcellular Cd distribution analysis showed that more than 60% of the total Cd associated with the cytosolic fraction. The presence of higher concentration of Ca in simulated sea water did not impact the proportional subcellular distribution of Cd in *N. diversicolor*. Nevertheless, the supply of Ca could significantly lower Cd concentration in cytosol and cellular debris. The present study provides evidence that Cd transport by *N. diversicolor* was mediated mainly through lanthanum- sensitive Ca ion channels and accumulated by SH-containing compounds. These results help to understand the uptake mechanism and subcellular distribution of Cd in polychaetes.

Keywords Cd · Uptake mechanism · Metal accumulation · *Nereis diversicolor*

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Introduction

Heavy metals, such as cadmium (Cd), copper (Cu), lead (Pb) and arsenic (As) could enter coastal waters as runoff with intensive anthropogenic activities, including industrial, agricultural and/or urban development. As a result, the contamination of heavy metals increasingly occurred in the coastal regions and their neighboring estuaries of China (Zhou et al. 2004). Among the invertebrate species in estuaries, the sediment-dwelling polychaete *Nereis diversicolor* has been characterized as an ecologically keystone species (Zhou et al. 2003; Mermillod-Blondin et al. 2005). Due to its rapid population growth and high tolerance to natural (e.g., salinity) as well as anthropogenic (e.g., chemical contaminants) stresses, this polychaete species is often abundant in polluted estuaries and has been proved to be a sentinel organism for the evaluation of the toxic substances, including metals (Rainbow 1995; Díez et al. 2000). *N. diversicolor* has been also recommended as a bioindicator for environmental biomonitoring and ecosystem management programs (Díez et al. 2000; Durou et al. 2005; Galloway et al. 2004).

Cadmium is usually mined and extracted from zinc ores and has been of environmental interest due to its high

toxicity to organisms (Mirsal 2004). As a non-essential element, Cd has been assumed to be taken up by transporters for essential elements as a consequence of lack of specificity of the transporters. It has been demonstrated that Cd follows Ca transport pathways, and Cd–Ca interactions have been reported previously (Rainbow 1995; Franklin et al. 2005; Kiewiet and Ma 1991; Li et al. 2008a). In polychaetes, there are few studies on the involvement of Ca channels in transporting metals.

Polychaetes may be exposed to sediment Cd at least in two ways: absorption through the skin and oral ingestion of contaminated sediment particles, while the exposure of polychaetes to water-bourne toxicants has allowed a better understanding the mechanisms of Cd uptake. To allow for some control in the experimental set-up, research with polychaete *N. diversicolor* has been performed in aqueous environments in this study. Pharmaceuticals that interact with ion channels specifically, and with high-affinity, can be applied both in vivo and in vitro to elucidate the role of an ion channel in a particular physiological process (White 1996).

In the present study, to test if Ca ion channel, Na^+–K^+ ATPase and SH-binding ligands were involved in Cd transport, the uptake of Cd was quantified with pre-exposure of the worms to Ca ion channel blockers (verapamil, lanthanum as $LaCl_3$) or metabolic inhibitors (ouabain, 2,4-dinitrophenol and *N*-ethylmaleimide). Coexisting cations or other metal ions may affect the bioavailability and toxicity of heavy metal and their subcellular distribution in organisms. Little information is available by now with regard to the effect of Ca supply on the distribution of Cd in polychaete *N. diversicolor* at subcellular level. In this study, not only the total internal Cd concentration in the worms was determined, but also the concentrations in three subcellular fractions. We aimed at elucidating the mechanisms of Cd uptake in the polychaete *N. diversicolor*, as well as the effects of Ca supply on the subcellular partitioning of Cd subsequent exposure of the polychaetes to Cd in simulated sea water.

Materials and methods

Test organisms and exposures

The polychaete species *N. diversicolor* with the lengths ranging from 2.0 to 2.5 cm were collected from Rongcheng Bay, Yantai, China. The animals were then maintained in the laboratory at 15°C (salinity of 28 ppt) for 1 week prior to experimentation.

All tests in this study were performed in a chemically defined seawater medium. The seawater medium (at 28 ppt, pH 8.0) contained: 0.32 M NaCl, 22.5 mM Na_2SO_4, 7.3 mM KCl, 1.87 mM $NaHCO_3$, 42.4 mM $MgCl_2$ and 0.34 mM H_3BO_3. All chemicals used were analytical reagent grade or higher and purchased from Sigma-Aldrich Shanghai Trading Co., Ltd (Shanghai, China). The containers used in these experiments were cleaned with diluted HNO_3 and thoroughly rinsed with deionized water. The water was aerated for 6 h and then filtered through 0.2-μm polycarbonate membranes before used as the dilution water to make a concentration of Cd^{2+} (as $CdCl_2$). The solution pH was measured daily with an Orion 868 pH meter.

Effect of Ca on Cd accumulation in *N. diversicolor*

Due to the similar ionic radius, competitive effect of Ca with Cd^{2+} for the same site (i.e., Ca ion channel) on surface of the polychaetes *N. diversicolor* may impact Cd uptake. The effect of Ca on Cd uptake was tested at three different Ca concentrations (2.0, 5.0 and 10.0 mM Ca) but at the same salinity (28 ppt) in artificial seawater that was amended with additions of Ca. The low (2 mM Ca) treatment was used as a control for this experiment. The change of ionic strength after the addition of Ca may also affect Cd uptake. Nevertheless, on using the chemical equilibrium model Visual MINTEQ (Windows version of MINTEQA2) (Allison et al. 1991), it can be calculated that the activity coefficient for the Cd (f_{Cd}) decreased of only 3% with the range of Ca concentration from 2.0 to 10.0 mM in simulated sea water.

As the difference in sizes (Rozman and Klaassen 2001) and life stage (Ma 2004) of worms may affect metal uptake, polychaetes with similar size and life stage were selected, rinsed with reagent grade water. Tests were performed by exposing two polychaetes in a plastic cup containing 100 ml test solution. The mean weight of individual worms is comparable between different treatments (811 ± 70 mg wet weight). For each metal exposure, three replicates were used. Worms exposed to a Cd free test solution were used as controls. The experimental animals often showed an avoidance response when exposed to the higher Ca concentrations. A lid with some small holes was employed to avoid the worms escaping from the containers and to prevent extensive moisture loss. Tests were carried out at 20 ± 1°C in complete darkness, and test solutions were renewed daily. After exposure, the polychaetes were rinsed with reagent grade water, freeze-dried and weighed for subsequent metal analysis. The worms were rinsed with dilute EDTA solution in a preliminary study and the result showed that hardly any metal was bound on the outside of the worms. Thus, in this study the worms were not washed with EDTA solution prior to further treatment.

Effect of pre-exposure to Ca ion channel blockers and metabolic inhibitors on Cd accumulation

To test if Ca ion channel, Na^+–K^+ ATPase and SH-binding ligands were involved in metal transport, the uptake of Cd at concentrations of 10 μM was quantified with pre-exposure of the worms to a Ca ion channel blocker (verapamil, lanthanum as $LaCl_3$) or metabolic inhibitors (ouabain, 2,4-dinitrophenol and *N*-ethylmaleimide). The control samples were pre-exposed in pharmacological agents free medium at the same Cd concentrations. All blockers were obtained from Sigma-Aldrich Chemical (Sigma-Aldrich Shanghai Trading Co., Ltd, Shanghai, China) and were dissolved in the solution described above. Concentrations were 20 μM for verapamil, 50 μM for $LaCl_3$, 20 μM for ouabain, and 10 μM for *N*-ethylmaleimide. All these concentrations were selected based on previous literature reports showing that these concentrations are physiologically relevant (Wang and Fisher 1999). The worms were pre-exposed to the channel blockers and inhibitors for 12 h then transferred to simulated sea water (containing 10.0 mM Ca) containing 10 μM Cd for an additional 7 days. The channel blockers and inhibitors were not present in the sea water during the Cd exposure. After exposure, the polychaetes were rinsed with reagent grade water, freeze-dried and weighed for subsequent metal analysis.

Subcellular distribution of Cd

To investigate the effect of Ca on the Cd subcellular distribution in *N. diversicolor*, a low (2 mM) and a high (10 mM) Ca concentrations were tested. Subcellular Cd accumulation was determined after 1 week exposure to simulated sea water containing 10 μM Cd ion. Two polychaetes were briefly thawed, weighed, placed in a glass tube, and each tube was added with cold 20 mM TRIS buffer solution (pH 7.6, Fisher Scientific, Houston, TX) at a 1:10 tissue to buffer ratio. The samples were thereafter homogenized with a polytron homogenizer for 30 s. The homogenized tissue from each treatment was then transferred to centrifuge tubes.

Homogenates were then centrifuged immediately using Optima™ L-80XP Preparative Ultracentrifuge (Beckman Coulter) and subjected to initial steps of the fractionation procedure of Wallace and Luoma (2003). A total of three different subcellular fractions were obtained. Briefly, the homogenized tissue was centrifuged at $1450\times g$, 15 min, 4°C in a Sorvall RC 28S ultra centrifuge. The supernatant (S1) (containing the cytosol) was removed as well as the pellet (P1) (containing tissue fragments and cellular debris). Both the pellet and the supernatant were each put into a new centrifugation tube. The supernatant was transferred by autopipette. The pellet was re-suspended and digested in 2 ml solution of 1 M NaOH. The re-suspended pellet 1 was centrifuged at $5000\times g$ for 10 min. This produced supernatant (S2) which contained metal associated cellular debris. The pellet (P2) consisted of metal rich granules. The two phases were each transferred to a labeled glass tube and frozen for storage. It is important to recognize that these fractions are operationally defined and that their compositions are rarely verified. All fractions were digested in concentrated HNO_3 at 105°C for 12–15 h prior to metal analysis by inductively coupled plasma–mass spectrometry (ICP-MS) (see below). The distribution of metal among different fractions was compared as percentage of total mass of metal content.

Metal analysis

Before metal analysis, freeze-dried worm tissue samples were weighed into microwave digestion bombs, followed by digestion in a concentrated HNO_3 solution (70% pro-analyzed JT Baker, Deventer, The Netherlands) with a Mars5 destruction microwave oven (CEM, Matthews, NC, USA) for 1 h at 630 W. Total metal concentrations were analyzed by inductively coupled plasma–mass spectrometry (ICP-MS) with a Perkin-Elmer SciEx ELAN 6000 (Bodensee, Germany). GBW08571 Marine muscle tissue (State Bureau of Technical Supervision, People's Republic of China) was employed as certified reference materials for worm analyses. Measured concentrations did not deviate more than 7% from the reported certified concentrations for Cd.

Statistics and calculations

Data of metal concentrations in polychaetes are expressed as mean ± standard deviation ($n = 3$). Data analysis was performed with SPSS (Version 10.0) statistical software (Statistical Graphics Corp., Princeton, NJ). One way variance analysis (ANOVA) was conducted on the data for testing the significant difference among the treatments, and significant difference was defined at $p < 0.05$.

Results

Effect of Ca on the Cd and Ca accumulation in *N. diversicolor*

The uptake of Cd and Ca by *N. diversicolor* with 10 μM Cd^{2+} ions and different concentrations of Ca ions is shown in Fig. 1. The supply of Ca ions could significantly increase Ca concentration in the worms. The concentrations of Ca in the tissue of *N. diversicolor* increased from 220 to 416 μg g^{-1} dry weight with increasing Ca concentrations from 2.0 to 10.0 mM in simulated sea water.

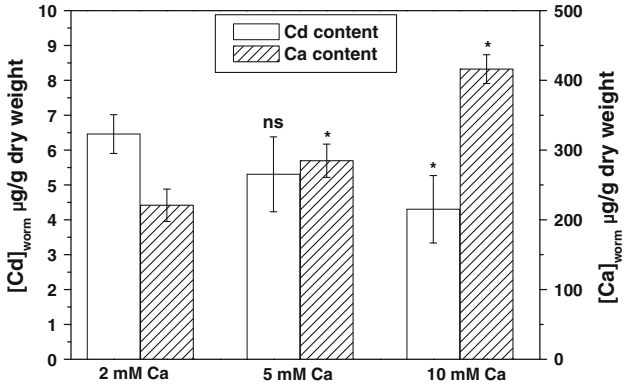

Fig. 1 Cd and Ca accumulation in the polychaete *N. diversicolor* after 1 week exposure in the solutions containing 10 µM $CdCl_2$ at three different Ca concentrations. Values are means ± S.D. ($n = 3$). *Significant difference in metal accumulation from controls (2 mM Ca, one-way ANOVA, *ns* no significant, $p < 0.05$)

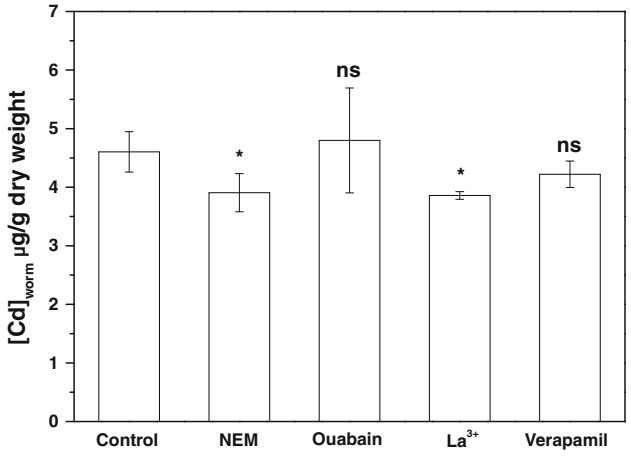

Fig. 2 Cd accumulation in the polychaete *N. diversicolor* after 1 week exposure in solutions containing 10 µM $CdCl_2$. Polychaetes were pre-exposed to La, Verapamil, Ouabain and NEM, controls in pharmaceuticals free solutions. Values are means ± S.D. ($n = 3$). *Significant differences in Cd accumulation from controls (one-way ANOVA, *ns* no significant, $p < 0.05$)

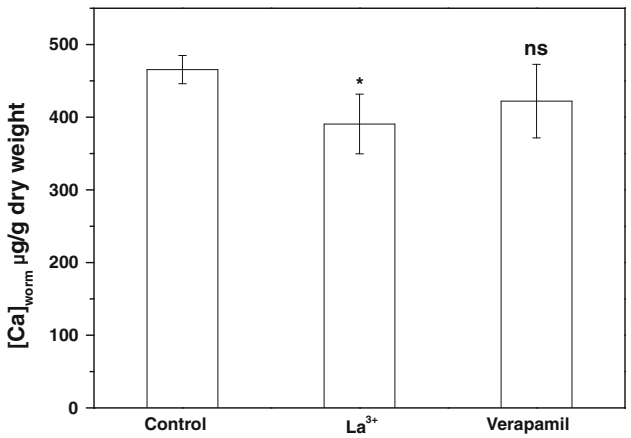

Fig. 3 Ca concentrations in the polychaete *N. diversicolor* pre-exposed to Ca channel blockers (La, Verapamil), after 1 week exposure in solutions containing 10 µM $CdCl_2$. Controls in Ca channel blockers free solutions. Values are means ± S.D. ($n = 3$). *Significant differences in Ca accumulation from controls (one-way ANOVA, *ns* no significant, $p < 0.05$)

Analysis of variance showed that the accumulation of Cd was negatively affected by the addition of Ca ions ($p < 0.05$). At 10 µM Cd, Ca significantly inhibited Cd uptake (Fig. 1), reducing Cd concentration in the worms from 6.46 µg g^{-1} dry weight at 2.0 mM Ca to 5.30 and 4.30 µg g^{-1} wet weight at 5.0 and 10.0 mM Ca, respectively (Fig. 1).

Effect of Ca ion channel blockers and metabolic inhibitors on the Cd accumulation

After exposure to 10 µM Cd^{2+}, the uptake of Cd was suppressed to a similar extent by $LaCl_3$ and NEM pre-exposure. From Fig. 2, Cd uptake was reduced by 15 and 17% when the worms were pre-exposed to NEM and La^{3+} respectively ($p < 0.05$). This was not the case for ouabain, and it caused no effect on the Cd uptake (Fig. 2). It is interesting to note that pretreatment of two Ca ion channel blockers (Verapamil and La^{3+}) did not produce consistent results on the effect of Cd^{2+} uptake. La^{3+} caused a significant decrease of the Cd^{2+} uptake, a phenomenon not seen with 20 µM verapamil pre-treatment. A measurement of the Ca concentration in worm tissue also showed a similar result (Fig. 3). The pre-exposure of La^{3+} significantly suppressed the Ca uptake, with the Ca concentration decreasing from 465 to 390 µg g^{-1} dry weight in worm tissue, while verapamil had little effect.

Effect of Ca on the intracellular Cd distribution in *N. diversicolor*

The relative distributions of Cd among subcellular fractions showed that most Cd (about 60%) in the organisms existed as fraction S1 (Fig. 4a). Cadmium bound to the tissue and cell membrane (fraction S2) was less than 35% of the total Cd, and only about 3.2% of the Cd exited in fraction P2. The presence of higher concentration of Ca in the simulated sea water did not impact the proportional distribution of Cd in three different subcellular fractions. Nevertheless, the supply of Ca ions could significantly lower Cd concentration in fraction S1 and S2 (Fig. 4b).

Discussion

To eliminate the effect of $LaCl_3$, Verapamil, Ouabain and NEM on metal complexation, the worms were pre-exposed in solutions containing these pharmaceuticals and then

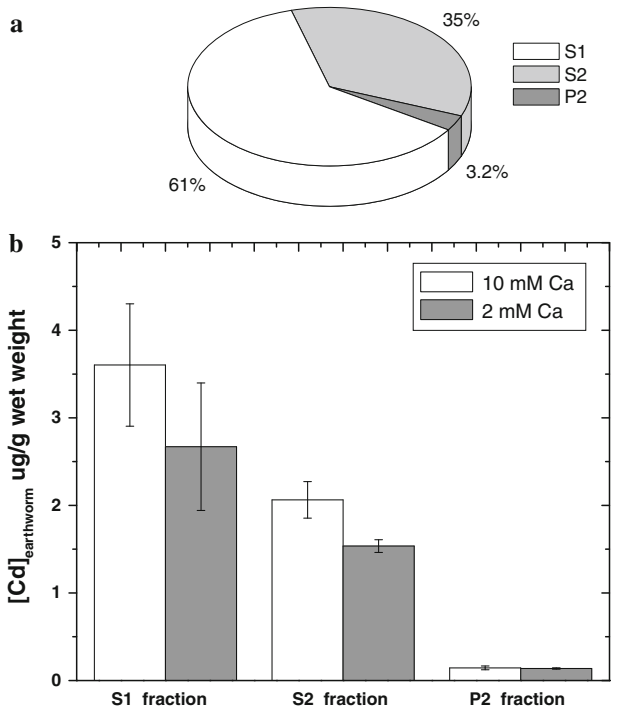

Fig. 4 Subcellular distribution of Cd (expressed as the percentage of the Cd content associated with each fraction) in the polychaete *N. diversicolor* after exposure into solutions containing 10 μM $CdCl_2$ for 1 week at a low Ca concentration (2.0 mM, **a**) and the accumulation of Cd (expressed as the actual Cd concentrations in each fraction) in the three subcellular fractions of polychaete *N. diversicolor* exposed to the solutions containing 10 μM $CdCl_2$ for 1 week at both low (2.0 mM) and high (10.0 mM) Ca concentrations (**b**). Fractions *S1*, *S2* and *P2* represent the cytosol, metal associated cellular debris, and metal rich granules, respectively

transferred to metal exposure test solutions. During exposure, there was no mortality observed in any case. The solutions were refreshed daily to minimize the possible effects of the worms on the solutions.

Pretreatments with the Ca ion channel blockers and metabolic inhibitors led to different effects on the Cd uptake by *N. diversicolor*. Lanthanum, which inhibits Ca influx both via the slow Ca channel and via Na–Ca exchange, significantly inhibited the Cd uptake by *N. diversicolor* in this study. The effect of La^{3+} depends on the similarity of its ionic radius (115 Å) to that of Ca^{2+} and its higher valence than that of Ca^{2+} (Weiss 1974). La^{3+} forms a very strong bond with the Ca channel, thus preventing the passage of Ca through the ion pore. Lanthanum can also block membrane ATPase activity and ionic exchange (Kaczorowski et al. 1984). It can effectively reduce Cd influx through Ca channels in crab and fish gills (Verbost et al. 1989 and Lucu and Obersnel 1996) and many types of cells (Campbell et al. 2007). One possible explanation for the inhibition of Cd uptake is the similar radius of Cd and Ca, and Cd may be mistakenly transported through the Ca channel (Markich and Jeffree 1994; Rainbow 1995). Verapamil, a specific Ca ion channel blocker, inactivates the channel by interacting with a specific receptor domain found on a large membrane-spanning protein that constitutes a substantial portion of the calcium channel (Triggle 1992). Verapamil can inhibit Ca influx via the slow Ca ion channel, however, showed no effect on the Cd uptake by *N. diversicolor*. The indistinctive effect of Ca channel blockers verapamil on Ca uptake in *N. diversicolor* is consistent with observation in oyster gills (Roesijadi and Unger 1993). Roesijadi and Unger (1993) demonstrated that the uptake rate of Cd was inhibited by 52–70% when the excised gills of oysters (*Crassostrea virginica*) were exposed to verapamil, but at a much higher concentration (100–250 μM) than those used in our study. Therefore, the La^{3+} sensitive Ca ion channels appear to play, at least partly, a role in the transport of Cd^{2+} across the membrane of *N. diversicolor*.

Nitrogen-ethylmaleimide, which can specifically block the proteins and small SH containing compounds (e.g., glutathione), has previously been found to reduce the Cd uptake by Caco-2 cells (Aduayom et al. 2003) and Zn uptake by isolated rat renal basolateral membrane vesicles (Kaur et al. 2006), indicating that their transport was mediated by proteins or small molecular SH-containing compounds. In our study, the use of NEM as a thiol blocker, which acts by binding with proteins and low-molecular-weight SH-containing compounds such as glutathione (Bobilya et al. 1992), assumes a specific and irreversible interaction with thiol residues on proteins. This hypothesis is supported by our result that an inhibitory effect on Cd uptake was observed when the polychaete *N. diversicolor* was pretreated in NEM (Fig. 2). Nevertheless, it is also important to note that NEM could also theoretically bind to cysteine residues on channels and affect ATP driven transport in a non-specific manner. Because ouabain, an inhibitor of Na^+–K^+ co-transporter and Na^+/K^+ ATPase, did not affect metal uptake, it is apparent that the Na^+/K^+ pump is not involved in Cd uptake in *N. diversicolor*, which is consistent with findings for oysters (Roesijadi and Unger 1993) and mammalian endothelial cells (Bobilya et al. 1992).

It has been demonstrated that polychaete *N. diversicolor* possess strategies for the detoxification of heavy metals (Mouneyrac et al. 2003; Zhou et al. 2003). In the present study, a large portion of Cd was found in fraction S1, with less Cd in other subcellular fractions and probably enhancing the protection of metal-sensitive sites, such as fraction P2. This metal distribution pattern is in agreement with that found in invertebrate earthworms (Li et al. 2008b; Vijver et al. 2006). Based on the fractionation procedure used in the present study (Wallace and Luoma 2003), S1 fraction theoretically contains most of the metal-binding proteins, such as metallothionein (MT), which was induced

rapidly by the organism upon short-term exposure (within 2–4 days) to metals (Liu and Wang 2011; Shi and Wang 2005; Yang and Thompson 1996). The role of MT in the sequestration and detoxification of certain metals, such as Cd, has been well documented in marine invertebrates (Pan and Wang 2008; Wallace and Luoma 2003). It should be noted that S1 fraction would contain a wide range of other soluble and membrane-bound ligands that may be poisoned by non-specific Cd binding. The partitioning of Cd in S1 fraction between potential detoxification sites (e.g., the heat stable protein fraction) and potential target sites should be evaluated in future study, as it is most important for organism to protect against metal damage.

Conclusions

The uptake of Cd in the polychaete *N. diversicolor* was suppressed significantly by Ca. Further investigation demonstrates that Ca channels and the SH-mediated transport process were involved in the uptake of Cd in *N. diversicolor*. Our study also highlights the difference of Cd subcellular distribution patterns with low and high Ca supply level. The present study contributes to further understanding metal uptake mechanisms and the interactions of Cd and Ca uptake and effects in polychaetes.

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