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# Making Acute Tests More Ecologically Relevant: Cadmium Bioaccumulation and Toxicity in an Estuarine Clam under Various Salinities Modeled in a Toxicokinetic−Toxicodynamic Framework

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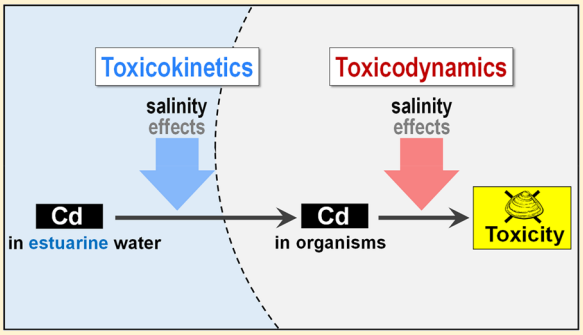
Supporting Information

**ABSTRACT:** Salinity has considerable effects on the toxicity of metals in estuarine waters. The effects of salinity are manifold, making it difficult to summarize for risk assessments. In this study, we separated and quantified the multiple effects of salinity on cadmium (Cd) in a toxicokinetic−toxicodynamic framework. The estuarine clam, *Potamocorbula laevis*, was used as a model organism. Cd bioaccumulation was measured using a stable-isotope-tracer technique; in parallel, toxicity tests were conducted. With the increase of salinity from 5 to 30, Cd uptake decreased monotonically. In contrast, the intrinsic sensitivity of organisms, measured by the toxicodynamic parameters, reached its minimum at intermediate salinities (i.e., 10 to 20). The overall salinity effects were dominated by the effects on Cd bioaccumulation; therefore, Cd toxicity decreased monotonically with the increases of salinity. The model developed in this study could provide predictions of no-effect concentration (1.7 to 34.9 $\mu$g $L^{-1}$, end point mortality) and the median lethal concentration ($LC_{50}$) of Cd at different salinities. In conclusion, we developed a framework for quantifying the multiple effects of salinity and a method for estimating no-effect concentration from acute toxicity tests, which can be used for better assessments of metal risks in estuarine waters.




## 1. INTRODUCTION

In estuarine waters, the bioavailability of contaminants, especially metals, are subject to dramatic influences of salinity.[1−3] The effects of salinity are manifold, including effects on chemical speciation, uptake processes, and organism physiology. These effects may diverge in directions and thus make the observed effects of salinity complex. For example, elevated salinity may decrease metal uptake but increase the sensitivity of organisms to the bioaccumulated metals.[4,5] The relative importance of these effects may be different in different combinations of organisms and metals and thus lead to the various trends of the salinity effects reported in the literature.[1,6] Assessing metal ecological risks in estuarine waters requires a quantitative understanding of the effects of salinity extrapolatable among different biological species. However, toxicity statistics (e.g., median lethal concentration or $LC_{50}$) fitted from conventional toxicity tests are a lump of the complex (e.g., geochemical, biological, physiological) effects of salinity, which makes it difficult to achieve such clear understanding. A new framework is needed to separately quantify the multiple effects of salinity.

Currently, ecological risk assessments and the development of water quality criteria are highly dependent on the toxicity data from acute tests.[7] Acute tests have a number of advantages including simple in design, time saving, and cost-effective.[8] In addition, acute tests measure only mortality (or immobilization), cause less handling stress to testing organisms, and thus usually show smaller variation among studies than chronic tests.[9] However, extrapolating acute values to chronic values is challenging; the current procedures like applying acute-to-chronic ratios or uncertainty factors can hardly be considered scientifically sound.[7,9] Moreover, acute values are highly dependent on the duration of exposure,[10,11] further making them difficult to be translated into ecological effects. It is thus desirable if something similar to chronic values or ecologically relevant values can be directly estimated from acute toxicity tests. The no-effect concentration (NEC) as a model parameter estimated from adequately designed acute toxicity tests may be such a candidate.[10,12] It should be noted that NEC (see the definition in section 3.5.2) is different from the no-observed effect concentration (NOEC), which is one of the exposure concentrations used and is dependent on the exposure time.[12]

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To serve the two purposes described above, i.e., delineating the multiple effects of salinity and estimating NEC from acute toxicity tests, a toxicokinetic−toxicodynamic (TK−TD) model was used in this study. TK−TD model is a flexible framework for studying metal bioaccumulation and toxicity. The TK module relates metal bioaccumulation to metal exposure by simulating the metal uptake and elimination processes; the TD module relates toxicity to the concentration of bioaccumulated metals.[13,14] Therefore, TK−TD model in principle is a suitable framework for separating the multiple effects of salinity. Previously, a TK−TD model has been successfully applied to explain the complex effects of salinity on the toxicity of Cu.[4] In the TK−TD model, a threshold concentration of metal is assumed to exist in the organisms, below which no lethal effect is expected to occur. NEC is the metal concentration in water corresponding to the internal threshold and can be estimated using the model.[12,14]

In this study, we used the clam *Portamacorbula laevis* as a model organism to study the effects of salinity on the bioaccumulation and toxicity of cadmium (Cd) under the framework of a TK-TD model. Our major objectives include: (1) developing a framework for separately quantifying the multiple effects of salinity to facilitate the better understanding of salinity effects; and (2) developing a method for estimating NEC from acute toxicity tests. The clam *P. laevis* is a euryhaline species that can survive salinities ranging from 3 to 40. It is widespread in estuarine and coastal waters of East Asia and is a dominant species of many estuaries of South China, where it is harvested as a feed for crab aquaculture and thus acts as a potential vector for transferring contaminants to humans. Cd is a priority pollutant that poses high risks to aquatic ecosystems and human health.[6,15] Moreover, Cd tends to accumulate in mollusks to higher concentrations than in other major groups of species, e.g., crustaceans and fishes.[16] Cd concentrations in impacted estuarine waters can frequently be higher than 2 to 3 $\mu$g $L^{-1}$.[6] The framework and methods developed in this study are expected to be applicable to other contaminants and biological species.

## 2. MATERIALS AND METHODS

**2.1. Organisms and Materials.** The clam *P. laevis* was collected from the Jiulong River estuary (24.469917° N, 117.930944° E), Fujian Province, China. Individuals of shell length between 1 and 2 cm were used for the experiments. In the laboratory, the clams were progressively acclimated to the experimental salinities for at least 2 weeks. Seawater of different salinities were prepared by diluting the 0.22-$\mu$m filtered seawater (salinity around 30) with deionized water (18.2 MΩ·cm). Salinity (unitless) was determined based on the practical salinity scale and was calculated from the conductivity and temperature measured with the conductivity meter (Cond 6+, OAKTON). The pH of seawater was typically around 8.0.

All experiments were conducted at the temperature of 22 ± 1 °C with a light/dark cycle of 14 h: 10 h. Exposure containers (polypropylene) were cleaned by soaking in 2% $HNO_3$ and were rinsed with deionized water before use. The stable isotope $^{113}$Cd (ISOFLEX, San Francisco, California, U.S.A.) dissolved in 5% $HNO_3$ was spiked into exposure seawater for the bioaccumulation experiments. Cadmium chloride (Sigma-Aldrich) was used for toxicity tests.

**2.2. Cd Bioaccumulation.** Two series of Cd bioaccumulation experiments were conducted, including: (1) Cd uptake and elimination at different salinities; and (2) Cd uptake at different Cd concentrations (see Table S1, experiment No. 1−3 of the Supporting Information, SI). Exposure seawater spiked with $^{113}$Cd was freshly prepared and equilibrated overnight before the start of uptake experiments. Seawater pH was checked and adjusted to 8.0 if necessary by adding microvolumes of 2 mol $L^{-1}$ NaOH. Mortality of clams was negligible in the bioaccumulation experiments.

*2.2.1. Cd Uptake and Elimination at Different Salinities.* Cd uptake and elimination was investigated at seven salinities, including 5, 8, 10, 15, 20, 25, and 30, using $^{113}$Cd as a tracer. The clams were exposed to 5 $\mu$g $L^{-1}$ of $^{113}$Cd for 12 h and then depurated for 300 h in clean seawater of corresponding salinities. An exposure duration approximates 12 h usually is suitable to allow enough isotope tracers being accumulated for reliable chemical analysis.[4,17] During the uptake period, clams were not fed and exposure medium was not renewed; during the depuration period, clams were fed with algae and the seawater was renewed daily. Three replicate beakers were used for each salinity; each beaker contained 600 mL of exposure seawater and 28 clams at the beginning of exposure. Clams were sampled every 3 h during the exposure period and were sampled at intervals of 12 to 48 h during the depuration period. When being sampled, the clams were rinsed immediately with 1 mmol $L^{-1}$ of EDTA (pH 8) to stop the Cd biouptake and to remove surface adsorbed Cd (only for uptake period). Soft tissue was further rinsed twice with EDTA and deionized water and subsequently freeze-dried, weighted, and digested in concentrated $HNO_3$. More information on the preparation of biological and water samples for metal analysis is provided in the SI.

Cd uptake rates were also measured at a higher and lethal Cd concentration (nominal 550 $\mu$g $L^{-1}$) and at the seven salinities. The same protocol was followed except that the experiment was ended after the 12-h exposure without the subsequent depuration. This high Cd concentration was the same as that used in the toxicity tests described below.

*2.2.2. Cd Uptake at Different Cd Concentrations.* Cd uptake rates were further measured at six different Cd concentrations, including 1, 3, 10, 50, 200, and 1000 $\mu$g $L^{-1}$. This experiment was conducted in parallel at three salinities, i.e., 5, 15, and 25. Three replicates were used in each case; each replicate beaker held 6 clams in 150 mL of exposure seawater. Two clams were sampled from each replicate for Cd analysis after 0 h, 6 and 12 h of exposure.

**2.3. Toxicity Testing.** Cd toxicity at the different salinities (i.e., 5, 8, 10, 15, 20, 25, and 30) was investigated by exposing *P. laevis* to a lethal Cd concentration of 550 $\mu$g $L^{-1}$ for 100 h. This Cd concentration was chosen based on a preliminary range-finding experiment to ensure substantial mortality of clams at all salinities, which is favorable for reliable model fitting. Three replicates and one control (i.e., filtered seawater without Cd) were used for each salinity. Ten clams held in 600 mL of test seawater were used for each replicate. Clams were not fed during the test, which lasted for 100 h; test solutions were renewed one time after 48 h of exposure. Mortality of clams was checked and recorded at intervals of 8 to 14 h. Clams were considered dead if their valves were open and did not close when prodded with a plastic Pasteur pipet. The dead clams were immediately removed from the test solution. Tests were considered valid only when survival was no less than 90% in all controls. Ten mL water samples were collected from each treatment every 24 h to check Cd concentrations.



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**2.4. Chemical Analysis and Data Analysis.** Concentrations of Cd and Cd isotopes (i.e., $^{112}$Cd and $^{113}$Cd) were measured using an inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x). The concentration of newly accumulated $^{113}$Cd in clams was calculated by subtracting the background $^{113}$Cd, inferred from the concentration of $^{112}$Cd, from the total concentration of $^{113}$Cd (see Figure S1 for details on the calculation). Before analysis, the biological and water samples were diluted with deionized water or 2% $HNO_3$ to achieve a final Cd concentration between 0.1 $\mu$g L$^{-1}$ and 100 $\mu$g L$^{-1}$, $HNO_3$ around 2%, and salinity lower than 1. The internal standard $^{115}$In was used to correct any potential matrix effects of samples and drift of the instrument. A quality control sample was measured every 10 to 20 samples. The recovery of Cd from the standard reference material (SRM 1566b, oyster tissue) was within 10% deviation from the certified value (2.48 $\mu$g Cd g$^{-1}$). Measured Cd concentrations in exposure seawater were used for data analysis (see Table S2−S5 for measured concentrations). Cd concentrations in clams were expressed on dry weight basis. The speciation of Cd at different salinities was calculated using the software Visual MINTEQ 3.0 (see Figure S2 for details).

**2.5. Toxicokinetic−Toxicodynamic Modeling.** *2.5.1. Toxicokinetics.* A one-compartment toxicokinetic model was used to describe the Cd uptake and elimination processes. The concentration of Cd in clams is a balance between Cd uptake and elimination (and growth dilution):[4,18]

$$\frac{dC_{int}(t)}{dt} = J_{in}(t) - (k_e + g) \times C_{int}(t) \quad (1)$$

where $C_{int}(t)$ ($\mu$g g$^{-1}$) is the concentration of internalized Cd in clams at time $t$; $J_{in}(t)$ ($\mu$g g$^{-1}$ d$^{-1}$) is the uptake rate of Cd; $k_e$ (d$^{-1}$) is the elimination rate constant; $g$ (d$^{-1}$) is the growth rate constant. $J_{in}(t)$ is dependent on the exposed Cd concentration [$C_w(t)$, $\mu$g L$^{-1}$]. When $C_w$ remains relatively constant or when $C_w$ varies within the low-concentration range, $J_{in}$ can be related to $C_w$ with an uptake rate constant $k_u$ (L g$^{-1}$ d$^{-1}$):

$$J_{in}(t) = k_u \cdot C_w(t) \quad (2)$$

*2.5.2. Toxicodynamics.* In Cd toxicity tests, the mortality of clams was presumably due to the bioaccumulation of Cd. The instantaneous risk of mortality is defined as the hazard rate $\left[\frac{dH(t)}{dt}, \text{d}^{-1}\right]$. When the bioaccumulated Cd ($C_{int}$, $\mu$g g$^{-1}$) exceeded an internal threshold concentration ($C_{IT}$, $\mu$g g$^{-1}$), the hazard rate would be higher than the background hazard rate ($h_0$, d$^{-1}$) and be proportional to the excessively accumulated Cd:[4,13]

$$\frac{dH(t)}{dt} = k_k \times (C_{int}(t) - C_{IT}) + h_0 \quad (3)$$

where $k_k$ (g $\mu$g$^{-1}$ d$^{-1}$) is the killing rate, standing for grams of clam killed by one $\mu$g of excessively internalized Cd per hour; $h_0$ was set to zero in this study because of negligible mortality in the controls. The survivorship of the clams, i.e., $S(t)$, can thus be calculated as follows:[4,13,19]

$$S(t) = e^{-H(t)} \quad (4)$$

*2.5.3. Estimation of Model Parameters.* The toxicokinetic parameters, including $k_u$ and $k_e$, were estimated by fitting eqs 1 and 2 to the Cd uptake and elimination data. The growth rate constant (i.e., $g$) of clams was calculated through their weight change during the experiment (see Figure S3 for details). The toxicodynamic parameters, including $C_{IT}$ and $k_k$, were estimated by fitting eqs 3 and 4 to the survivorship data from the toxicity tests. The model was implemented in the software OpenModel (version 2.4.2) developed by Neil Crout at Nottingham University. The Marquardt algorithm was used for estimating parameter values and standard deviations.

## 3. RESULTS AND DISCUSSION

**3.1. Cd Toxicokinetics.** The uptake and elimination of $^{113}$Cd in the clam *P. laevis* can be well described by the one-compartment toxicokinetic model (Figure 1). During the 0.5-d exposure, the concentration of $^{113}$Cd increased linearly in the clams. During the following 12.5-d of depuration, $^{113}$Cd in clams did not decrease but surprisingly increased slightly. The increase was due to the gradual loss of weight of the clams, which in effect concentrated the bioaccumulated $^{113}$Cd (Figure S3). The growth rate constants were −0.036 d$^{-1}$ to −0.052 d$^{-1}$.

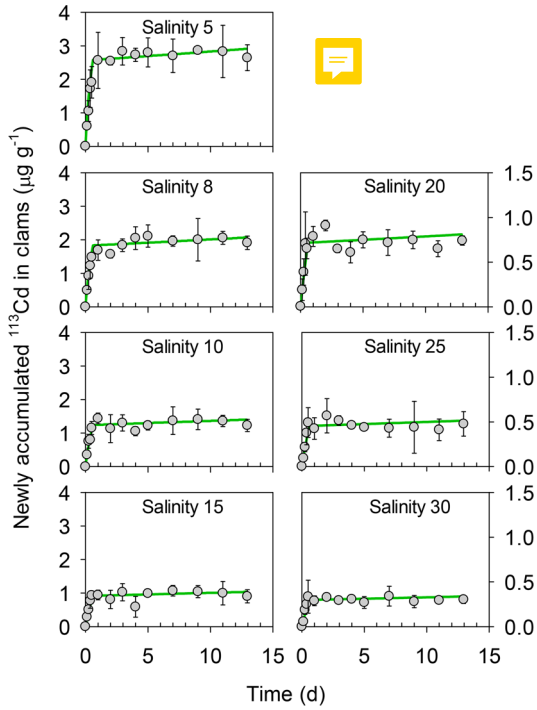


**Figure 1.** Accumulation and depuration of $^{113}$Cd in the calm *P. laevis* at different salinities. The clams were exposed to 5 $\mu$g L$^{-1}$ of $^{113}$Cd during the exposure period (0.5 d) and then depurated in clean seawater afterward. The points are measured values (mean ± standard deviation, $n$ = 3), and the curves are model fits. See Table 1 for model parameters.

The uptake rate constant $k_u$ of Cd ranged between 0.149 L g$^{-1}$ d$^{-1}$ and 1.484 L g$^{-1}$ d$^{-1}$ at the low Cd level (~5 $\mu$g L$^{-1}$) (Table 1). At the high Cd level (~550 $\mu$g L$^{-1}$), Cd $k_u$ was lower and ranged between 0.057 L g$^{-1}$ d$^{-1}$ and 0.323 L g$^{-1}$ d$^{-1}$. The value of $k_u$ is a measure of Cd bioavailability and is thus dependent on salinity and Cd concentration among various other factors. Cd uptake in the clam *P. laevis* is relatively fast among different clam species, but considerably slower than in oysters and scallops.[20−23]



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**Table 1. Best-Fit Values of the Model Parameters**[a]

| salinity | $k_u(5)$ (L $g^{-1}$ $d^{-1}$) | $k_u(550)$ (L $g^{-1}$ $d^{-1}$) | $C_{IT}$ ($\mu$g $g^{-1}$) | $k_k$ (mg $\mu g^{-1}$ $h^{-1}$) |
|---|---|---|---|---|
| 5 | 1.484 ± 0.045 | 0.323 ± 0.012 | 23 ± 9 | 0.122 ± 0.005 |
| 8 | 1.058 ± 0.033 | 0.252 ± 0.012 | 79 ± 11 | 0.101 ± 0.008 |
| 10 | 0.654 ± 0.022 | 0.226 ± 0.012 | 68 ± 14 | 0.064 ± 0.006 |
| 15 | 0.487 ± 0.018 | 0.144 ± 0.012 | 124 ± 16 | 0.117 ± 0.020 |
| 20 | 0.347 ± 0.015 | 0.116 ± 0.011 | 161 ± 16 | 0.184 ± 0.073 |
| 25 | 0.214 ± 0.014 | 0.089 ± 0.011 | 114 ± 16 | 0.163 ± 0.037 |
| 30 | 0.149 ± 0.015 | 0.057 ± 0.012 | 82 ± 19 | 0.302 ± 0.125 |

[a]See eqs 1–4 for definition of parameters. $k_u(5)$ and $k_u(550)$ are uptake rate constants measured at nominal Cd concentration of 5 and 550 $\mu$g $L^{-1}$, respectively. The same $k_e$ value was assumed for different salinities and was estimated to be (0.0382 ± 0.0032) $d^{-1}$. Values are mean ± standard deviation.

A constant value of the elimination rate constant $k_e$ (i.e., 0.0382 $d^{-1}$) could fit well Cd depuration at different salinities (Figure 1), suggesting that Cd elimination processes, unlike Cd uptake processes, were not substantially dependent on salinity. Cd $k_e$ of *P. laevis* is slightly higher than those measured in other marine bivalves (0.01 $d^{-1}$ to 0.03 $d^{-1}$),[17,21−23] presumably due to its smaller size and thus higher surface to volume ratio. In comparison, in the clam *P. laevis*, $k_e$ of Cu (0.16 $d^{-1}$) was much higher.[4]

**3.2. Effects of Salinity on Cd Uptake.** Cd uptake rate constant $k_u$ decreased consistently with increasing salinity both at the low and high Cd levels, with the effects more substantial at the low Cd level (Figure 2A). To aquatic organisms, the free

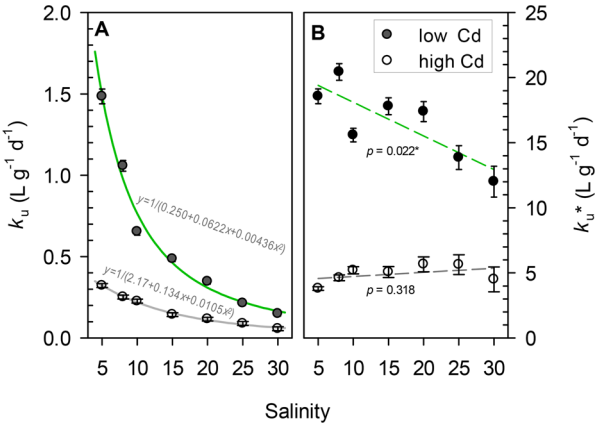


**Figure 2.** Effects of salinity on the uptake rate constant ($k_u$, panel A) and free-ion-activity-based uptake rate constant ($k_u$*, panel B) of Cd. Error bars represent standard deviation. Nominal Cd concentrations in water were 5 and 550 $\mu$g $L^{-1}$ for the low- and high-Cd experiments. The curves in panel A represent the empirical equations. The slopes of the linear regressions in panel B were −0.26 (95% confidence interval: −0.46 to −0.06) and 0.032 (95% confidence interval: −0.042 to 0.106) for the low- and high-Cd experiments, respectively.

ion of metals was usually the bioavailable species.[24,25] Calculation of Cd speciation showed that the proportion of $Cd^{2+}$ decreased from 21% to 4.3% (4.9 fold) when salinity increased from 5 to 30, whereas the complexes $CdCl^+$ (50−60%) and $CdCl_2$ (11−42%) were the major species (Figure S2). Decreases in free $Cd^{2+}$ ion concentration at higher salinities can thus be one of the possible explanations for the decreases in $k_u$.

Assuming free ion $Cd^{2+}$ as the only bioavailable species, a $Cd^{2+}$ ion-based uptake rate constant (denoted as $k_u$*, L $g^{-1}$ $d^{-1}$) can be defined by modifying eq 2:

$$k_u^* = \frac{J_{in}}{\{Cd^{2+}\}} \quad (5)$$

where $\{Cd^{2+}\}$ is the activity of $Cd^{2+}$ ion. By using $k_u$* as a measure of Cd bioavailability at different salinities, the confounding effects of complexation were removed and thereby the remaining salinity effects were revealed (Figure 2B). At the low Cd level, there was a significant decrease (1.5 fold) of $k_u$* with salinity, indicating the competition from major cations (e.g., $Na^+$, $Ca^{2+}$, $Mg^{2+}$) as predicted by the biotic ligand model.[26,27] In contrast, at the high Cd concentration, $k_u$* showed no significant relationship with salinity, indicating that competition effects became negligible at the high Cd level.

Decreases of Cd uptake at higher salinities were consistently observed for various saltwater species, including snail,[28] mussel,[28] shrimp,[29] copepod,[30] and fish.[31,32] In the clam *P. laevis*, Cu uptake also decreased at higher salinities.[4] However, the decreasing patterns of uptake as a function of salinity may not always be expected for other trace metals or metalloids (e.g., arsenic(V), chromium(III), mercury(II), and methylmercury).[32]

Complexation (by $Cl^-$) played a more important role than competition (by major cations such as $Na^+$ and $Ca^{2+}$) in the effects of salinity on Cd bioavailability in the clam *P. laevis*, decreasing Cd uptake rate by 4.9 fold (Figure S2) and 1.5-fold (Figure 2B), respectively, similar to that found in the copepod *Acartia tonsa*.[30] Alternatively, competition effects of major cations, especially $Ca^{2+}$, were clearly evidenced in a few studies. For example, in the snail *Littorina littorea* and the mussel *Mytilus edulis*, 76% and 22% of the decreases of Cd uptake rates was attributed to the elevation of Ca concentration at higher salinities, respectively.[28] In addition, in the fish *A. schlegeli*, at a constant salinity of 35, elevating Ca level from zero to full seawater level (10.5 mmol $L^{-1}$) decreased Cd uptake by 6 times.[31] Together, these results demonstrate that although inhibitory effects of salinity on Cd uptake is consistently observed, the relative contribution of the complexation and competition effects is quite variable among species.

**3.3. Cd Uptake at Different Cd Levels.** The relationship between Cd uptake rate and Cd concentration at three different salinities is shown in Figure 3. Partial saturation of the uptake mechanism occurred at high Cd concentrations; $k_u$ was thus not a constant over the wide Cd concentration range. Therefore, a power function was used to relate Cd uptake rate to Cd concentration in water:[18,23]

$$J_{in}(t) = a \times C_w(t)^b \quad (6)$$



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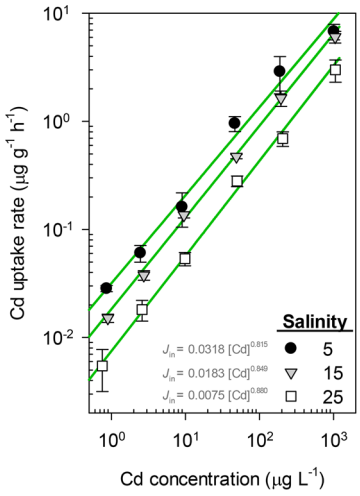


**Figure 3.** Relationship between Cd uptake rates in the clam *P. laevis* and Cd concentration in water at different salinities (i.e., 5, 15, and 25) described by power equations. The points represent measured values (mean ± standard deviation, $n$ = 3); the curves are plots of the equations.

where $a$ and $b$ are empirical constants. The value of $a$ is a measure of Cd bioavailability and decreased from 0.0318 to 0.0075 when salinity increased from 5 to 25, which is consistent with the decreases of $k_u$ at higher salinities described above (Figure 2). The values of $b$ were close to but smaller than one and ranged from 0.815 to 0.880. Alternatively, the $J_{in}(t)$ and $C_w(t)$ relationship can be fitted with a Michaelis–Menten equation. A common maximum Cd uptake rate ($J_{max}$, $\mu$g g$^{-1}$ h$^{-1}$) was used by assuming competitive inhibition of salinity components on the uptake of Cd. The common $J_{max}$ was estimated to be 8.07 $\mu$g g$^{-1}$ h$^{-1}$; the half-saturation concentrations ($K_m$s) were 348 $\mu$g L$^{-1}$, 629 $\mu$g L$^{-1}$, and 1539 $\mu$g L$^{-1}$ for salinity 5, 15, and 25, respectively (Figure S7A).

**3.4. Cd Toxicodynamics and Effects of Salinity.** The survivorship of the clam *P. laevis* when exposed to a lethal concentration of Cd (nominal 550 $\mu$g L$^{-1}$) at different salinities is shown in Figure 4. Survivorship increased with increasing salinity, indicating lower toxicity of Cd at higher salinities. A strong trend of decreasing toxicity with increasing salinity existed in the literature on a wide range of biological species. In the 33 studies reviewed by Hall and Anderson, 23 reported lower Cd toxicity at higher salinities.[1] Similarly, in a more recent review, decreases in Cd toxicity with increasing salinity were observed in 8 of the 9 species.[6]

The decreased Cd toxicity with increasing salinity is in accordance with the decreased Cd uptake (Figures 5A and 2A). It is thus tempting to speculate that the protective effects of salinity against Cd toxicity could be fully explained by the salinity effects on Cd uptake. To test this speculation, the survivorship data were further analyzed using a toxicodynamic model (eqs 3 and 4). Thereby, it was checked whether the "intrinsic sensitivity" of the clams to Cd was stable across the salinity range. Intrinsic sensitivity is defined as the sensitivity of the organisms to the internally accumulated Cd rather than Cd in the exposure medium to avoid the confounding effects of any variations in bioaccumulation processes under different exposure scenarios. The intrinsic sensitivity was measured by the two toxicodynamic parameters, the threshold internal Cd concentration ($C_{IT}$) and the killing rate ($k_k$).[4,14] The parameter $C_{IT}$ measures how much Cd the organisms can tolerate without

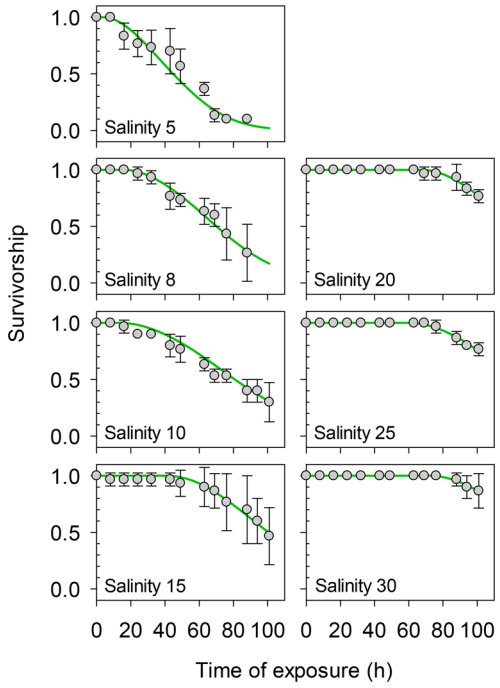


**Figure 4.** Survivorship of the clam *P. laevis* during the exposure to a high concentration of Cd (nominal: 550 $\mu$g L$^{-1}$) at different salinities. The points are observed values (mean ± standard deviation, $n$ = 3); the curves are model fits (see Table 1 for model parameter values).

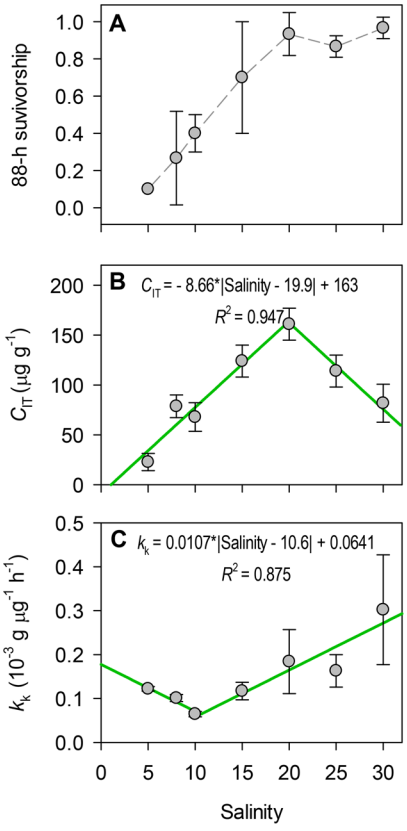


**Figure 5.** Survivorship of the clam *P. laevis* after 88 h of exposure to 550 $\mu$g L$^{-1}$ of Cd (panel A), the internal threshold concentration ($C_{IT}$, panel B) and the killing rate ($k_k$, panel C) of Cd at different salinities. Linear decrease of $C_{IT}$ and linear increase of $k_k$ was assumed when salinity deviated from the "optimal" values.



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**Table 2. TK−TD Model Parameters for Predicting Median Lethal Concentration ($LC_{50}$) and No-Effect Concentration (NEC) of Cd at Different Salinities**[a]

| model parameters or variables | equation |
|---|---|
| Cd uptake rate ($\mu$g g$^{-1}$ h$^{-1}$) | $J_{in} = a \times [Cd]^b$ |
| coefficient $a$ | $a = 0.0415 - 0.00116 \times$ salinity |
| coefficient $b$ | $b = 0.810$ |
| elimination rate constant (h$^{-1}$) | $k_e = 0.00159$ |
| internal threshold concentration ($\mu$g g$^{-1}$) | $C_{IT} = -8.66 \times \lvert\text{salinity} - 19.9\rvert + 163$ |
| killing rate (mg $\mu$g$^{-1}$ h$^{-1}$) | $k_k = 0.0107 \cdot \lvert\text{salinity} - 10.6\rvert + 0.0641$ |

[a]see Figures 6 and 7 for the predictions; see SI for the R code.

having increased risk of mortality; a lower $C_{IT}$ indicates higher intrinsic sensitivity. The parameter $k_k$ measures the potency of the bioaccumulated Cd in killing the organisms when the threshold $C_{IT}$ was exceeded; a higher $k_k$ indicates higher intrinsic sensitivity. Far from being stable, $C_{IT}$ ranged from 23 $\mu$g Cd g$^{-1}$ to 161 $\mu$g Cd g$^{-1}$; $k_k$ ranged from 0.064 mg $\mu$g$^{-1}$ h$^{-1}$ to 0.302 mg $\mu$g$^{-1}$ h$^{-1}$ (Table 1, Figure 5). Therefore, it is clearly demonstrated that in addition to the effects on Cd uptake, salinity also substantially affected the intrinsic sensitivity to Cd of the organisms.

With the increase of salinity, $C_{IT}$ first increased and then decreased; whereas the opposite trend was observed for $k_k$. Both trends indicate that organisms were more internally tolerant to Cd under the intermediate salinities, although the optimal salinity indicated by the two parameters was different, i.e., 19.9 by $C_{IT}$ and 10.6 by $k_k$ (Figure 5). If assuming a linear increase of sensitivity when the salinity deviates from the optimal value, then the relationship between $C_{IT}$ (and $k_k$) and salinity can be well described by the equations below:

$$C_{IT} = -8.66 \times |\text{salinity} - 19.9| + 163 \quad (7)$$

$$k_k = 0.0107 \times |\text{salinity} - 10.6| + 0.0641 \quad (8)$$

The least intrinsic sensitivity of the clam *P. laevis* at the intermediate salinities indicates osmotic stress superimposed on the toxic effects of Cd at the too low or too high salinities. Although *P. laevis* is a euryhaline species that can survive a wide salinity range, it has its optimal salinity range which was estimated to be around 10 to 15,[4] in agreement with what we observed in this study. When organisms were out of their optimal salinity range, they would require higher activity of membrane transporters (e.g., $Na^+/K^+$ ATPase, $Ca^{2+}$ ATPase) for ionoregulation and thus were more sensitive to the inhibitory effects of $Cd^{2+}$ on the transporters.[33] Intrinsic sensitivity to Cd was seldom measured in other species. Nonetheless, lower Cd toxicity at intermediate salinities were reported for a few species; for example, the fish *Fundulus heteroclitus*,[34] the mysid *Mysidopsis bahia*,[35] and the mysid *Neomysis integer*.[36] In these cases, the effects of salinity on the intrinsic sensitivity presumably were not masked by the salinity effects on Cd bioaccumulation as what we observed in the clam *P. laevis*.

The apparent toxicity, measured as mortality of organisms, was a combination of the dual effects of salinity on both Cd bioaccumulation and intrinsic sensitivity of organisms. The former decreased monotonically with increasing salinity; the latter reached its minimum at intermediate salinities. The relative importance of the dual effects determines the trend of Cd toxicity as a function of salinity, which can be used to explain the different trends observed in the literature.[1,6] If the effects on the Cd bioaccumulation dominated, then apparent Cd toxicity would consistently decrease with the increase of salinity. Conversely, if the effects on organisms' intrinsic sensitivity dominated, then Cd toxicity would be the lowest at the intermediate salinities.

**3.5. Environmental Implications.** *3.5.1. Deriving Cd $LC_{50}$ Values for Different Salinities and Exposure Durations.* The TK−TD model developed in this study predicts survivorship of organisms as a function of time, salinity, and Cd concentration. The TK and TD parameter values at different salinities were summarized in Table 2. The $LC_{50}$ values of Cd can be calculated for different salinities and exposure durations and were presented in Figure 6. A program coded in R (version 3.4.4) is provided in the SI for the calculation.[37]

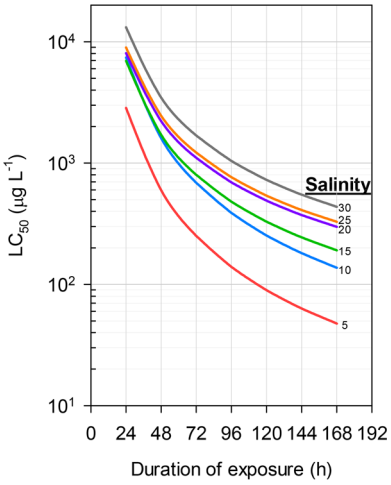


**Figure 6.** Predicted median lethal concentration ($LC_{50}$) of Cd to the clam *P. laevis* at different salinities (5 to 30) and after different exposure durations (24 to 168 h).

Conventionally, $LC_{50}$ of toxicants are derived by conducting toxicity tests for a specified duration, usually 48 or 96 h in acute toxicity tests. The duration is more an arbitrary regulatory requirement than a scientific consideration. There is no one-fit-all test duration for different combinations of species and toxicants; however, using different test durations would make it difficult to compare different studies. By using the TK-TD model, a specified exposure duration becomes irrelevant. The $LC_{50}$ values can be calculated for different durations and not limited by the duration at which the tests were actually conducted (Figure 6). As expected, $LC_{50}$ values were lower under conditions of longer exposure duration and lower salinity. The calculated 72-h $LC_{50}$ of Cd ranged from 250 $\mu$g L$^{-1}$ to 1680 $\mu$g L$^{-1}$, indicating that to the clam *P. laevis* Cd is less toxic than Cu within the range of salinity


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investigated, for which the $LC_{50}$ were 269 $\mu g\ L^{-1}$ to 192 $\mu g\ L^{-1}$.[4] When compared to other saltwater species, *P. laevis* is among the relatively Cd-sensitive species considering that the 50$^{th}$ percentile of the genus mean acute value is around 2000 $\mu g\ L^{-1}$.[6]

*3.5.2. No-Effect Concentration of Cd.* The no-effect concentration (NEC) in this study is defined as the maximum concentration in water which causes no mortality of the organisms under the (theoretical) perpetual exposure. NEC of Cd at different salinities can be derived by combining eqs 1, 3, and 6 based on the following two pieces of information: (1) when organisms are exposed to a Cd concentration equaling NEC, the maximum Cd concentration reached in the organisms should not exceed $C_{IT}$. (2) The maximum concentration in organisms is reached at the steady state, i.e., $\frac{dC_{int}(t)}{dt} = 0$ (see eq 1). Taken together, the NEC can be calculated by solving the equation below (see the SI for the R code):

$$C_{IT} = \frac{a \times NEC^{b}}{(k_e + g)} \quad (9)$$

For simplicity, the growth of the clams was ignored (i.e., setting $g = 0$) in calculating NEC, which would generate lower (and thus more conservative and protective) values of NEC. The NEC at different salinities were shown in Figure 7. The NEC increased from 1.7 $\mu g\ L^{-1}$ to 34.9 $\mu g\ L^{-1}$ when salinity increased from 5 to 30, with an inflection point at salinity 19.9, corresponding to the maximum $C_{IT}$ (Figure 5). The predicted NEC were compared to the Sea water quality standard (GB 3097−1997) of China and the saltwater Cd criteria recommended by USEPA (Figure 7). The comparison suggests that most of the standard/criteria values might not be stringent enough for protecting the clam investigated in this study especially at low salinities. The method for estimating NEC developed in this study can be applied to other organisms and contaminants; a collection of NEC values of representative species can serve as a sound basis for water quality criteria development and ecological risk assessments.

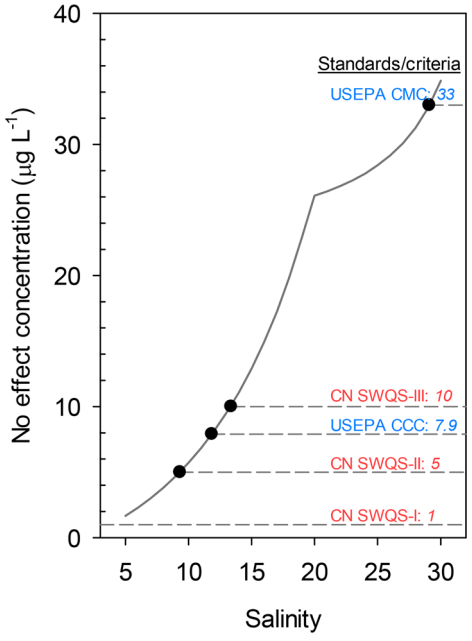


**Figure 7.** Predicted no-effect concentration (NEC) of Cd to the clam *P. laevis* at different salinities. The NEC values are compared to Seawater Quality Standard of China (CN SWQS) and the aquatic life criteria recommended by the Environmental Protection Agency, United States (USEPA). The horizontal dashed lines indicate the salinity ranges where the standard or criteria values are stringent enough to be protective based on the predicted NEC values. CN SWQS-I: 1 $\mu g\ L^{-1}$; CN SWQS-II: 5 $\mu g\ L^{-1}$; CN SWQS-III: 10 $\mu g\ L^{-1}$ (see Table S6 for details); CMC (criteria maximum concentration): 7.9 $\mu g\ L^{-1}$; and CCC (criteria continuous concentration): 33 $\mu g\ L^{-1}$.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b07095.

More details on material and methods; list of experiments with descriptions; measured Cd concentrations in exposure medium; seawater quality standard of China on Cd; explanations on analysis of stable isotope data; species distribution of Cd at different salinities; growth of clams during bioaccumulation experiment; concentration of dissolved organic carbon in exposure seawater; time-series data on Cd bioaccumulation; and Cd uptake rates described with Michaelis−Menten equations and power equations (PDF)

R code for calculating Cd bioaccumulation, NEC, and $LC_{50}$ under different salinities (TXT)

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### Notes

The authors declare no competing financial interest.

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Supporting information to:

# Making acute tests more ecologically relevant: cadmium bioaccumulation and toxicity in an estuarine clam under various salinities modeled in a toxicokinetic-toxicodynamic framework

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Number of pages: 13

Number of Tables: 6 (Table S1 to Table S6)

Number of Figures: 7 (Figure S1 to Figure S7)

## Material and Methods

### 2.1 Organisms and materials

In the laboratory, the clams were fed with the green algae *Chlorella* sp. and the seawater was refreshed every day. Seawater was collected from a clean site in Tong'an Bay (24.566944° N, 118.192472° E), Fujian Province, China. Seawater used in experiments was firstly filtered through a glass fiber filter (Whatman GF/C) and then through a 0.22-μm polypropylene cartridge filter (Calyx Capsule).

### 2.2 Cd bioaccumulation

At the beginning of exposure and each subsequent time point, two clams from each replicate were sacrificed for measuring Cd concentrations. The two clams were separately measured and the results were averaged for data analysis. Water samples were also collected for monitoring Cd concentrations in the exposure seawater. Specifically, 3 mL of water was sampled from each of the three replicated beakers; the three replicate water samples were pooled into one sample, which was then acidified and preserved by adding 90 μL of "1+1" $HNO_3$ (i.e., one volume of concentrated nitric acid mixed with one volume of deionized water, ~7.3 mol $L^{-1}$).

When being sampled, the clams were rinsed immediately with 1 mmol $L^{-1}$ of EDTA (pH 8) to stop the Cd biouptake and to remove surface adsorbed Cd. Soft tissue was dissected from each clam, further rinsed twice with 1 mmol $L^{-1}$ EDTA and deionized water, placed separately into clean polyethylene ziplock bags, and frozen at −20 ºC. The clams were subsequently freeze dried, weighted, and digested using concentrated $HNO_3$ at 80 ºC for about 8 h. The standard reference material (SRM 1566b, oyster tissue) was also digested following the same procedures.

**Table S1.** List of experiments, including their objectives, experimental conditions, and the related table and figures.

| No. | Objective | Duration | Salinity | Nominal Cd (μg $L^{-1}$) | Measured Cd | Results |
|---|---|---|---|---|---|---|
| 1 | Cd uptake and elimination kinetics at a *low* Cd concentration | 12 h (uptake) & 300 h (depuration) | 5, 8, 10, 15, 20, 25, 30 | 5 | Table S2 | Fig. 1, Fig. 2 |
| 2 | Cd uptake rate at a *high* Cd concentration | 12 h | 5, 8, 10, 15, 20, 25, 30 | 550 | Table S3 | Fig. 2, Fig. S5 |
| 3 | Cd uptake rate at a range of Cd concentrations | 12 h | 5, 15, 25 | 1, 3, 10, 50, 200, 1000 | Table S4 | Fig. 5, Fig. S6 |
| 4 | Cd toxicity at a high Cd concentration | 100 h | 5, 8, 10, 15, 20, 25, 30 | 550 | Table S5 | Fig. 3, Fig. 4 |

**Table S2.** Measured concentrations of $^{113}Cd$ (μg $L^{-1}$) in the exposure seawater of different salinities used in the accumulation experiment (No. 1 in Table S1).

| Time | Salinity | | | | | | |
|---|---|---|---|---|---|---|---|
| (h) | 5 | 8 | 10 | 15 | 20 | 25 | 30 |
| 0 | 4.9 | 4.6 | 4.4 | 4.3 | 4.6 | 4.4 | 4.2 |
| 3 | 3.0 | 3.1 | 3.3 | 3.3 | 3.7 | 3.6 | 3.6 |
| 6 | 2.3 | 2.5 | 2.7 | 2.9 | 3.3 | 3.4 | 3.5 |
| 9 | 1.9 | 2.2 | 2.5 | 2.8 | 3.1 | 3.3 | 3.3 |
| 12 | 1.7 | 2.0 | 2.4 | 2.5 | 2.9 | 3.2 | 3.2 |

**Table S3.** Measured concentrations of $^{113}Cd$ (μg $L^{-1}$) in the exposure seawater of different salinities used in the accumulation experiment (No. 2 in Table S1).

| Time | Salinity | | | | | | |
|---|---|---|---|---|---|---|---|
| (h) | 5 | 8 | 10 | 15 | 20 | 25 | 30 |
| 0 | 556 | 549 | 544 | 549 | 544 | 542 | 537 |
| 3 | 529 | 528 | 523 | 530 | 529 | 534 | 525 |
| 6 | 512 | 519 | 513 | 526 | 555 | 533 | 525 |
| 9 | 511 | 506 | 513 | 524 | 530 | 530 | 529 |
| 12 | 508 | 515 | 514 | 528 | 533 | 528 | 525 |

**Table S4.** The measured Cd concentrations in the exposure seawater for determination Cd uptake rate at a range of Cd concentrations and different salinities (No. 3 in Table S1).

| Salinity 5 | | | | | | |
|---|---|---|---|---|---|---|
| Time (h) | Nominal Cd (μg $L^{-1}$) | | | | | |
| | 1 | 3 | 10 | 50 | 200 | 1000 |
| 0 | 1.02 | 2.8 | 10.3 | 53 | 211 | 1087 |
| 6 | 0.80 | 2.3 | 8.5 | 44 | 181 | 977 |
| 12 | 0.75 | 2.2 | 8.1 | 42 | 175 | 962 |

| Salinity 15 | | | | | | |
|---|---|---|---|---|---|---|
| Time (h) | Nominal Cd (μg $L^{-1}$) | | | | | |
| | 1 | 3 | 10 | 50 | 200 | 1000 |
| 0 | 1.00 | 3.0 | 10.2 | 52 | 208 | 1090 |
| 6 | 0.87 | 2.7 | 9.4 | 47 | 189 | 1029 |
| 12 | 0.82 | 2.5 | 9.0 | 46 | 188 | 1028 |

| Salinity 25 | | | | | | |
|---|---|---|---|---|---|---|
| Time (h) | Nominal Cd (μg $L^{-1}$) | | | | | |
| | 1 | 3 | 10 | 50 | 200 | 1000 |
| 0 | 0.83 | 2.7 | 10.2 | 51 | 213 | 1080 |
| 6 | 0.76 | 2.5 | 9.7 | 49 | 203 | 1058 |
| 12 | 0.68 | 2.6 | 9.6 | 49 | 202 | 1048 |

**Table S5.** Measured concentrations of Cd (μg $L^{-1}$) in the exposure seawater of different salinities used in toxicity tests (No. 4 in Table S1).

| Time | Salinity | | | | | | |
|---|---|---|---|---|---|---|---|
| (h) | 5 | 8 | 10 | 15 | 20 | 25 | 30 |
| 0 | 522 | 532 | 528 | 529 | 530 | 526 | 531 |
| 24 | 392 | 412 | 426 | 446 | 473 | 477 | 473 |
| 48 | 367 | 386 | 390 | 416 | 439 | 453 | 463 |
| 48* | 530 | 525 | 526 | 529 | 526 | 524 | 522 |
| 88 | 513 | 490 | 468 | 480 | 491 | 504 | 496 |
| 100 | n.d. | n.d. | 468 | 472 | 488 | 502 | 498 |

* Exposure solution refreshed at this time point; n.d.: not determined.

**Table S6**. Standard values of Cd listed in "Sea water quality standard (GB 3097—1997)" of China for different environmental function areas.

| Sea water quality standard | Applicable to environmental function areas | Standard value of Cd (μg $L^{-1}$) |
|---|---|---|
| Grade I | Sea fishery waters, marine nature reserves and the protected areas for rare and endangered marine species. | 1 |
| Grade II | Aquaculture area, sea bath, sea sports or entertainment areas where people have direct exposure to sea water; industrial water in direct relation to human food. | 5 |
| Grade III & IV | General industrial water areas and costal scenic spots; Waters of port and marine development areas. | 10 |

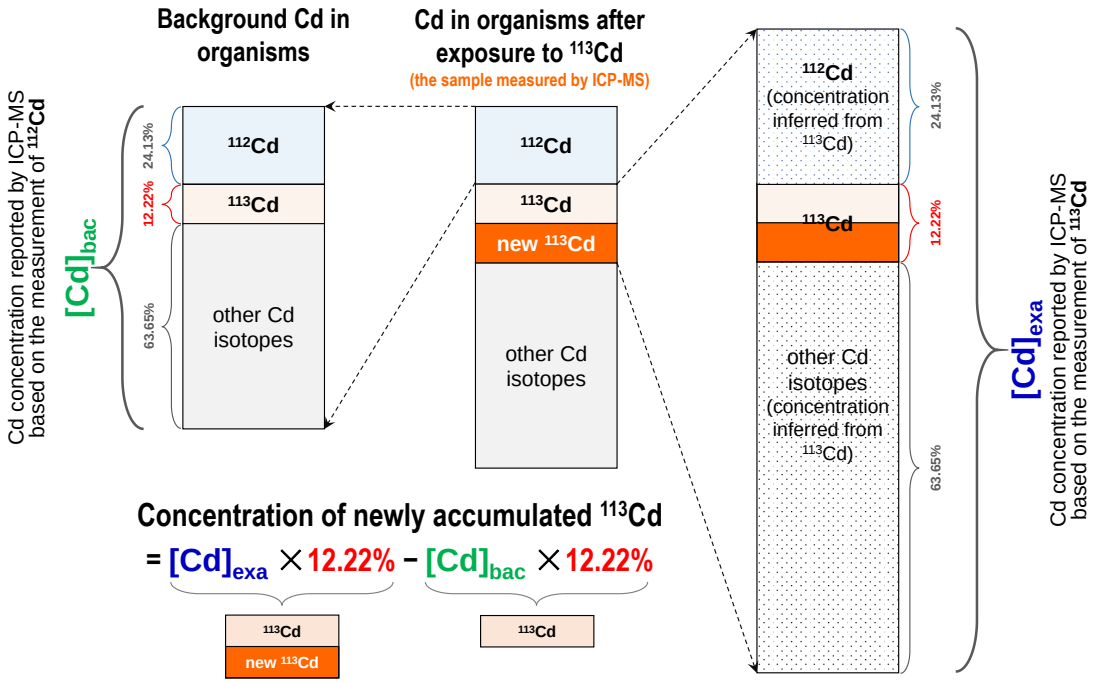


**Figure S1.** Explanation on how to analyze the ICP-MS data to calculate the concentration of newly bioaccumulated $^{113}$Cd.

Cd has eight naturally occurring stable isotopes (e.g., $^{112}$Cd, $^{113}$Cd). In the Cd bioaccumulation experiments, clams were exposed only to the isotope $^{113}$Cd and no other Cd isotopes. The $^{113}$Cd detected in the clams had two origins: one was that newly accumulated from the exposure seawater, the other was that originally existed (or the background $^{113}$Cd). ICP-MS (Agilent 7700x) detects signals of specific Cd isotopes while reports concentration of the total Cd instead of that specific Cd isotope. ICP-MS calculates total Cd concentration by dividing the isotope concentration with its abundance. In our experiment, ICP-MS was calibrated with a "normal" Cd solution (Agilent, part number 5183e4688), which has natural abundance of isotopes. Therefore, if a sample has natural abundance of Cd isotopes, measuring any isotope would lead to the same reported total Cd concentration. However, if a sample was enriched with an isotope (Figure S1, middle panel), for example $^{113}$Cd, measuring $^{113}$Cd would exaggerate the real total Cd concentration (Figure S1, right panel); whereas measuring other isotopes (e.g., $^{112}$Cd) would underestimate the real total Cd concentration (Fig. S1, left panel). We use this difference to calculate the concentration of newly accumulated $^{113}$Cd.

In Figure S1, total Cd concentrations reported by measuring $^{112}$Cd and $^{113}$Cd were denoted as $[Cd]_{bac}$ and $[Cd]_{exa}$, respectively. $[Cd]_{bac}$ was the concentration of background Cd; *background* $^{113}$Cd thus was "$[Cd]_{bac} \times 12.22\%$". $[Cd]_{exa}$ was the exaggerated Cd concentration; *total* $^{113}$Cd thus was "$[Cd]_{exa} \times 12.22\%$". The concentration of newly accumulated $^{113}$Cd was the difference between the *total* and *background* $^{113}$Cd.

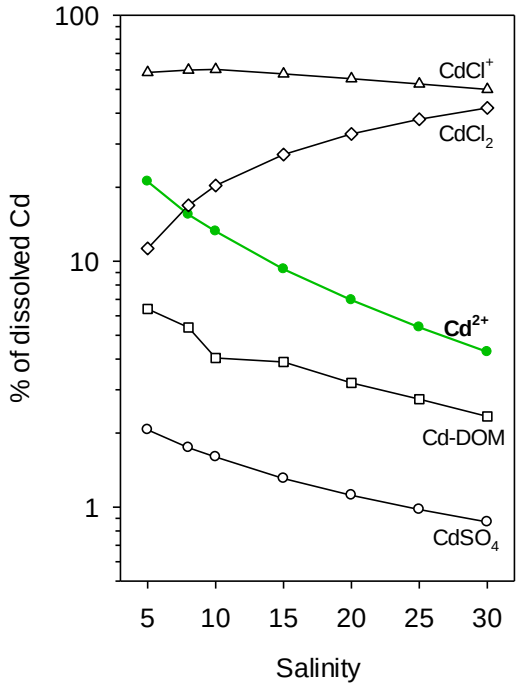


**Figure S2.** The species distribution of Cd at different salinities. Cd speciation was calculated in the software Visual MINTEQ 3.0 (https://vminteq.lwr.kth.se). Cd concentration was assumed to be 5 μg $L^{-1}$ for the calculation. Temperature and pH were set to 22 °C and 8.0, respectively. The concentrations of major ions (e.g., $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, $SO_4^{2-}$) were estimated according to the constituents of surface seawater of salinity 35 (Pilson, 2013, there Table 4.1). The Stockholm Humic Model (SHM) was selected for modeling complexation effects of the dissolved organic matter (DOM). Default settings for DOM were used, except that: (1) concentration (mg $L^{-1}$) ratio of active DOM to dissolved organic carbon (DOC) was set to 2; (2) 100% of active DOM was assumed to be fulvic acid. Measured concentrations of DOC were used for the calculation. See Figure S3 for the measured DOC concentrations at different salinities.

Pilson, M.E.Q. *An Introduction to the Chemistry of the Sea*. Second ed. 2013: Cambridge University Press.

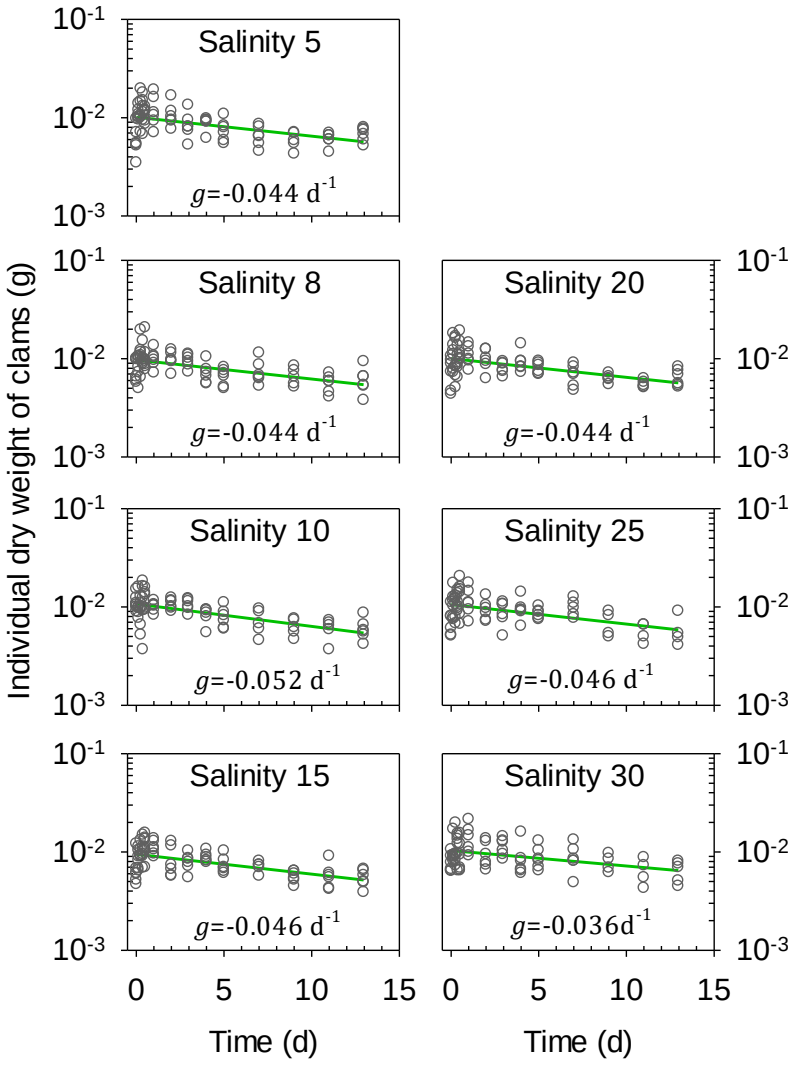


**Figure S3.** The dry weight of soft tissues of individual clams during the uptake-depuration experiment (experiment No. 1 listed in Table S1). An exponential growth model, $y(t) = y_0 \cdot e^{g \cdot t}$, was fitted to the data. Negative growth (i.e., loss of weight) was observed in all cases; the growth rate constant ($g$, $d^{-1}$) ranged from − 0.036 $d^{-1}$ to − 0.052 $d^{-1}$.

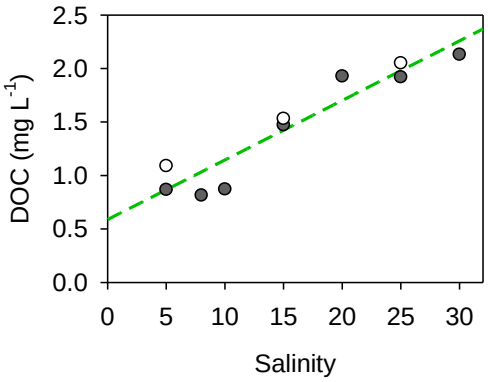


**Figure S4.** Concentration of dissolved organic carbon (DOC, mg $L^{-1}$) in the exposure seawater of different salinities used in the present study. Seawater of different salinities used in this study was prepared by diluting salinity-30 seawater with deionized water, leading a decrease of DOC from ~2 to ~1 mg C $L^{-1}$. The complexation of Cd by dissolved organic matter (DOM) is weak when compared to other metals (e.g., Cu, Hg, Ag). In our calculation, only 2.3% to 6.4% of Cd was complexed by the DOM; the percentage of complexation decreased with increasing salinity (Figure S3). This weak complexation effects should have not substantially confounded the trend of the salinity effects.

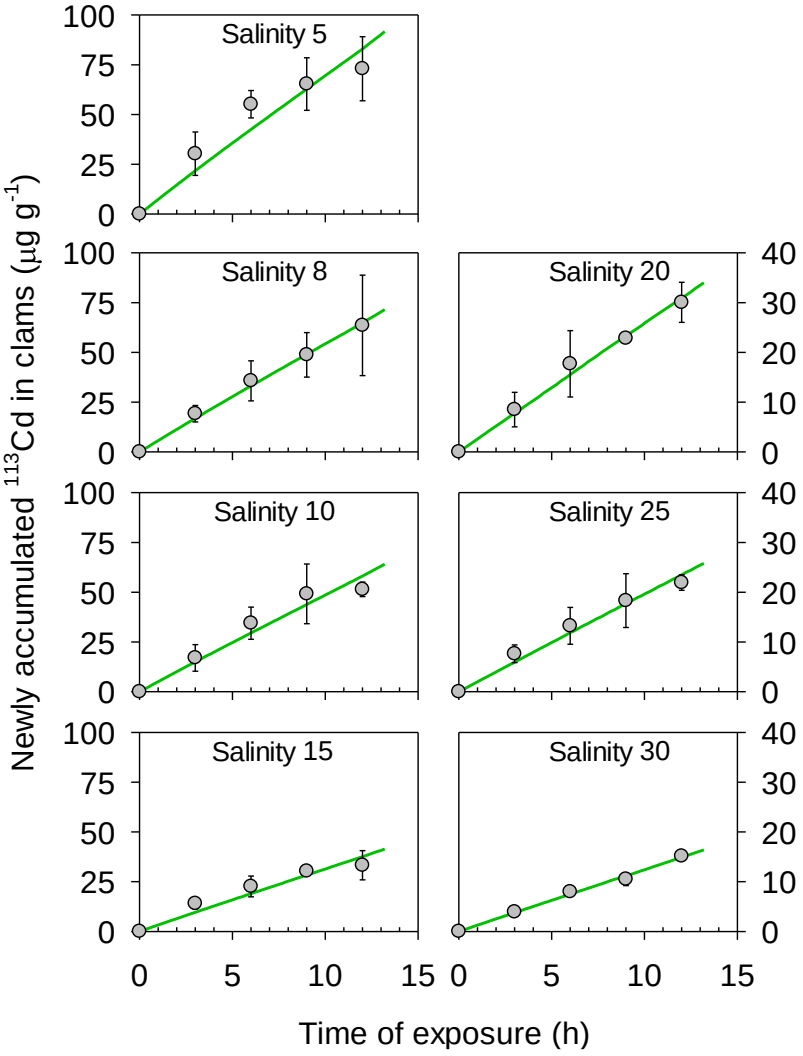


**Figure S5.** The accumulation of $^{113}$Cd in the calm *P. laevis* exposed to a high Cd concentration (nominal 550 μg $L^{-1}$) at different salinities (experiment No. 2 listed in Table S1). The points are measured values (mean ± standard deviation, $n$ = 3) and the curves are model fits. An efflux rate constant ($k_e$) of 0.0382 $d^{-1}$ was assumed for the fitting.

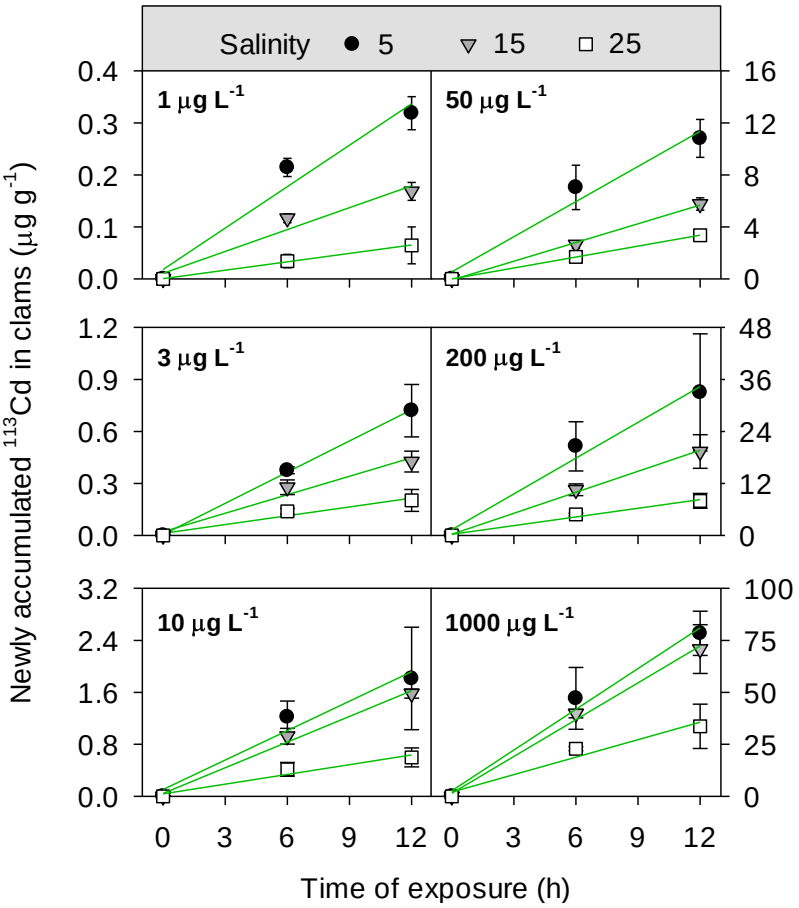


**Figure S6.** The accumulation of $^{113}$Cd in the clam *P. laevis* during the 12-h exposure to different $^{113}$Cd concentrations (nominal: 1-1000 μg $L^{-1}$) at different salinities (i.e., 5, 15 and 25) (experiment No. 3 listed in Table S1). Others as Figure S5.

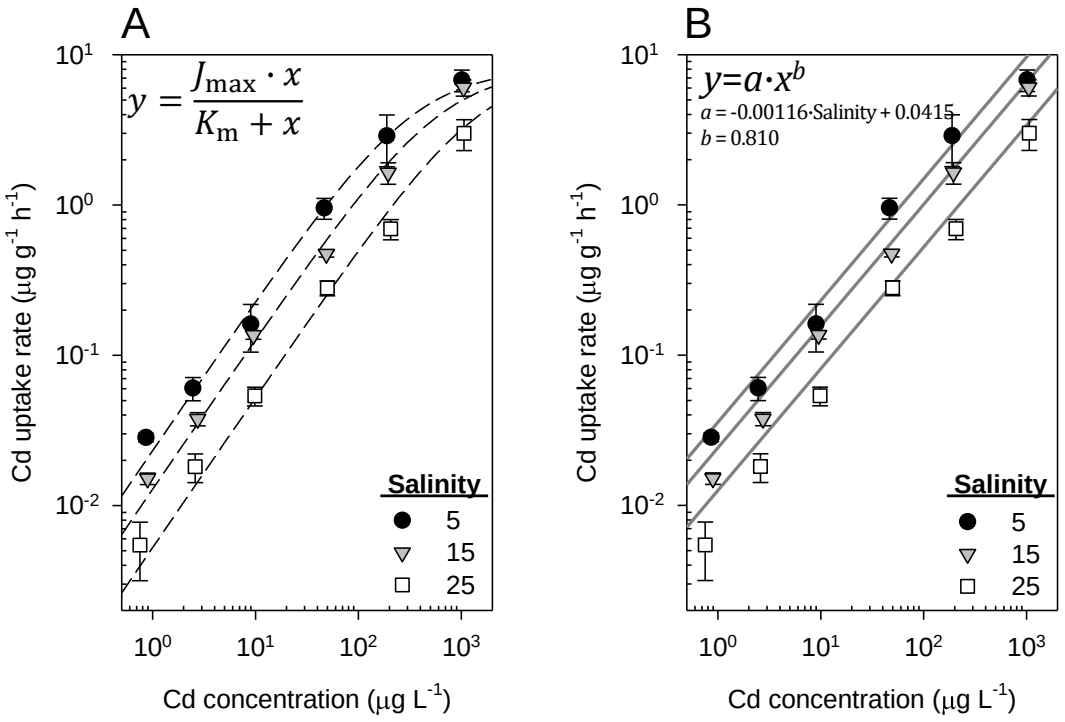


**Figure S7.** The relationship between Cd uptake rates in the clam *P. laevis* and Cd concentration in seawater. The uptake rates were measured at three different salinities (i.e., 5, 15, and 25). The relationship was described with Michaelis-Menten equations (A) or power functions (B).

A: The same $J_{max}$ was assumed for different salinities in the Michaelis-Menten fitting; $J_{max}$ = 8.07 ± 1.78 μg g$^{-1}$ h$^{-1}$; $K_m$ was 348 ± 94 μg L$^{-1}$, 629 ± 168 μg L$^{-1}$, and 1539 ± 407 μg L$^{-1}$ for salinity 5, 15, and 25, respectively.

B: The coefficients $a$ and $b$ were updated over those of Figure 3 for better prediction of survivorship in the toxicity tests (Figure 4): $b$ = 0.810; $a = -0.00116 \cdot \text{Salinity} + 0.0415$.