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Revealing the complex effects of salinity on copper toxicity in an estuarine clam *Potamocorbula laevis* with a toxicokinetic-toxicodynamic model☆

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ABSTRACT

The effects of salinity on metal toxicity are complex: not only affecting metal bioaccumulation, but also altering the physiology and sensitivity of organisms. In this study, we used a toxicokinetic-toxicodynamic (TK-TD) model to separate and quantify the dual effects of salinity on copper (Cu) toxicity in a euryhaline clam *Potamocorbula laevis*. The toxicokinetics of Cu was determined using the stable isotope ^{65}Cu as a tracer at concentrations (10–500 μg L^{-1}) realistic to contaminated environments and at salinities ranging from 5 to 30. At low Cu concentrations (ca. 10 μg L^{-1}), Cu bioaccumulation decreased monotonically with salinity, and the uptake rate constant (k_u, 0.546 L g^{-1} h^{-1} to 0.213 L g^{-1} h^{-1}) fitted well with an empirical equation, $k_u = 1/(1.35 + 0.116 \cdot \text{Salinity})$, by treating salinity as a pseudo-competitor. The median lethal concentrations (LC_{50}s) of Cu were 269, 224, and 192 μg L^{-1} at salinity 5, 15, and 30, respectively. At high Cu concentrations (ca. 500 μg L^{-1}), elevating salinity were much less effective in decreasing Cu bioaccumulation; whereas Cu toxicity increased with salinity. The increased toxicity could be explained by the increases in Cu killing rates (k_ks), which were estimated to be 0.44–2.08 mg μg^{-1} h^{-1} and were presumably due to the osmotic stress caused by the deviation from the optimal salinity of the clams. The other toxicodynamic parameter, internal threshold concentration (C_{IT}), ranged from 79 to 133 μg^{-1} g^{-1} and showed no clear trend with salinity.

1. Introduction

Salinity has long been known to modify the toxicity of various pollutants, especially metals, to estuarine and coastal organisms (Grosell et al., 2007; Hall and Anderson, 1995), but such effects are complex. In most studies, metal toxicity decreased with increasing salinity, whereas opposite or more complicated trends were also observed (Grosell et al., 2007; Hall and Anderson, 1995). Such complexity makes it difficult to generalize the effects of salinity, which hinders the efficient use of available toxicity data in the management of ecological risks of metals. Salinity not only affects the geochemistry of metals, but also affects the physiology of organisms and alters their sensitivity to metal exposure (Grosell et al., 2007; Jones et al., 1976; Pinho and Bianchini, 2010). Estuarine animals have different strategies in response to salinity variation in their environment. Osmoregulators actively regulate salt concentration in their body fluids and maintain a stable internal osmolality; whereas osmoconformers cannot adjust water content in their tissues and keep isosmotic to their surroundings. To tease out the complex interactions underlying the effects of salinity, a mechanistic model is urgently needed, based on which the net effects of salinity can be quantitatively predicted.

One practical framework for predicting the effects of water chemistry on metal toxicity in freshwaters is the biotic ligand model (BLM) (De Schamphelaere and Janssen, 2002; Di Toro et al.,

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2001; USEPA, 2007); however, this model may not be applicable to estuarine waters in its current form. According to the BLM, metal toxicity is determined by the amount of metals bound to biotic ligands on the surface of organisms. Most frequently, the free ion of metals is considered as the most toxic species. The complexation of metal ions by inorganic or organic ligands and the competition of major cations (e.g., Na^+, Ca^{2+}) for binding biotic ligands can both mitigate the metal toxicity. Therefore, metal toxicity is predicted to become lower with increasing salinity due to the higher complexation and competition effects, but apparently such prediction disagrees with some of the literature data (Deruytter et al., 2015; Hall and Anderson, 1995). One major reason for the limited success of the current BLM in explaining the effects of salinity is its neglect of the change of physiology and intrinsic sensitivity of organisms across a salinity range (de Polo and Scrimshaw, 2012; Grosell et al., 2007).

The toxicokinetic-toxicodynamic (TK-TD) model provides an excellent framework to simulate the effects of salinity on the intrinsic sensitivity of organisms and can be used to improve the performance of BLM when applied in estuarine waters (Arnold et al., 2005). In previous studies, the change in sensitivity of organisms to metals caused by variations in temperature and exposure history was quantitatively explained using the TK-TD model (Heugens et al., 2003; Tan and Wang, 2012). In the TK-TD framework, metal toxicity can be predicted in two steps (Jager et al., 2011; Tan and Wang, 2012). The TK module first translates metal exposure into metal bioaccumulation by simulating the processes including uptake, internal distribution, and elimination. The TD module then translates the bioaccumulation into the prediction of toxic effects (e.g., mortality of organisms). Therefore, the complex effects of salinity can be separated and quantified by the TK-TD model, with a final prediction of the net effects. Time is introduced as a variable in the TK-TD model and thus enable it to deal with fluctuations in exposure concentrations, which are commonly seen in both toxicity tests and real environment. Moreover, the parameter values of such kinetic models can be used beyond the particular exposure duration under which they were estimated and hence are more useful.

In the present study, a euryhaline clam *Potamocorbula laevis* (Hinds), was selected as the model organism to investigate the complex effects of salinity on Cu toxicity in the TK-TD framework. In estuarine waters, Cu imposes high ecological risks due to its high toxicity (USEPA, 2004) and widespread pollution (Bryan and Langston, 1992; Teasdale et al., 2003; Weng and Wang, 2014). Cu is a disruptor of Na^+ exchange, acid-base balance and nitrogen excretion, all of which can lead to dysfunction of ion regulation and osmoregulation and cause toxicity in aquatic organisms (Lee et al., 2010). Therefore, Cu toxicity is expected to be highly sensitive to salinity variation which may cause osmotic stress. We choose to work on *P. laevis* because it is widespread in East Asia and is one of the dominant benthic species in many estuarine waters. In our study, the stable isotope ^{65}Cu was used as a tracer to measure the toxicokinetics of Cu at different salinities. Toxicity tests of Cu were also conducted at the same salinities. The complex effects of salinity were separately quantified by relating the estimated TK and TD parameters to salinity.

2. Material and methods

2.1. Test organisms and materials

The clam *P. laevis* was collected from Jiulong River Estuary, Fujian Province, China (24 °28′11.7″N, 117 °55′51.4″E). It is a euryhaline species, and inhabits waters of the salinity range of 2–30. Salinity at the sampling site varies between 5 and 28 due to freshwater inflow and tide (Wang and Wang, 2016). In the laboratory, the clams were acclimated progressively to desired salinities for at least one week before experiments, and were fed the green algae (*Chlorella* sp.) every two days. Clams used in the experiments were typically 1.0–1.5 cm in shell length, and had the background Cu concentration of 27.0 ± 14.8 μg g^{-1} (dry weight basis; $n = 58$).

The seawater for experiments was collected near the mouth of Jiulong River (24 °26′3.6″N, 118 ° 5′10.5″E), with a typical salinity of 30. In the laboratory, the water was first filtered through glass fiber filters (Whatman GF/C) and then through a 0.22 μm polypropylene filter (Calyx Capsule). The desired salinity was achieved by diluting the filtered seawater with MilliQ water (18.2 MΩ cm). All the uptake experiments and toxicity tests described below were conducted in acid washed polypropylene containers. The freshly prepared test solutions were equilibrated overnight before the start of experiments. The pH of test solutions was checked at the beginning of the equilibration and adjusted to 8.0 (original pH of the filtered seawater) if necessary by adding drops of 2 mol L^{-1} NaOH or HNO_3. In real estuarine waters pH usually increases with salinity. In this study pH was adjusted to the same value to minimize the confounding effects of pH (if any) and to observe the effects of salinity *per se*. In all experiments, the temperature was 22 ± 1 °C, and the light regime was 14:10 h light: dark.

2.2. Cu uptake and Elimination

The uptake and elimination of Cu in the clam *P. laevis* was determined using the stable isotope ^{65}Cu (purchased from Trace Science International, Canada) as a tracer at seven salinities, i.e., 5, 8, 10, 15, 20, 25 and 30. The clams were exposed for 12 h to seawater spiked with 10 μg L^{-1} ^{65}Cu (*see* Table S1 for measured concentrations) and without the addition of food; afterwards, the clams were depurated for another 60 h in non-spiked seawater of corresponding salinities. The Cu concentration used here was commonly found in Jiulong River Estuary (which was influenced by industrial effluent release) (Weng and Wang, 2014) and other contaminated estuaries (Bryan and Langston, 1992; Teasdale et al., 2003). At each salinity, three replicated beakers were prepared, each containing 20 clams of similar size (1.0–1.5 cm) in 600 ml of exposure solution. Before the start of exposure, another six clams were collected to determine the initial ^{65}Cu content. Two clams and 3 mL of water were sampled every 3 h from each test beaker. The water samples from each three replicated beakers were pooled and acidified by adding 90 μL of ~7.3 mol L^{-1} HNO_3 (prepared by mixing one volume of concentrated HNO_3 and one volume of deionized water). During the subsequent 60 h depuration, clams were sampled every 12 h. The sampled clams were dissected immediately after being rinsed with 1 mmol L^{-1} EDTA solution (pH adjusted to 8.0), which was to stop the Cu uptake and to remove Cu adsorbed to the surface of clams by chelation (Hassler et al., 2004). The clam soft tissues were further rinsed twice with 1 mmol L^{-1} EDTA and then twice with MilliQ water to minimize surfaced adsorbed ^{65}Cu. The soft tissues were placed individually in clean ziplock bags, freeze dried, and then weighted to the nearest 0.1 mg. Dried soft tissues were digested in 15-mL polyethylene centrifuge tubes containing 0.5–1 ml concentrated HNO_3 at 80 °C for 10 h. The standard reference material (SRM 1566b, oyster tissue) was digested under the same conditions.

2.3. Toxicity tests

Two series of 72 h toxicity tests were conducted. In the first series, clams were exposed to five Cu concentrations and a control

(i.e., filtered seawater) while the salinity was held constant at 5, 15 or 30. Due to logistic issues, the three tests were conducted on different dates with different batches of clams. The five nominal Cu concentrations were 63, 125, 250, 500 and 1000 μg L^{-1}. In the second series, clams were challenged with a high concentration of Cu (nominal 500 μg L^{-1}, *see* Table S2 for measured concentrations) at seven different salinities, ranging from 5 to 30. A control beaker with clams in filtered seawater was used for each salinity. Clams were not fed during the toxicity testing period. The tests were considered valid only when the survivorship was ≥90% in all controls. In both tests, three replicated beakers, each containing 10 clams in 600 mL of test solution, were used for each treatment. The mortality of clams was examined every 6 h–12 h, and all the dead clams except those morphologically disintegrated were collected for Cu content analysis. A clam was considered dead if its valves were open (or opened) and did not close when prodded gently with a plastic spoon. The alive clams after 72 h exposure were also sampled. Water samples were taken every 24 h to monitor the decrease of Cu concentration. The clam and water samples were processed following the methods described above.

2.4. Chemical analysis

The Cu content in clam and water samples was analyzed using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x). The samples collected in the ^{65}Cu uptake experiments were analyzed for both isotopes of Cu, i.e., ^{63}Cu and ^{65}Cu, while the samples collected in the toxicity tests were measured for total Cu. Germanium (^{72}Ge) was used as an internal standard to correct sample matrix effects and instrument drift. A quality control sample was analyzed after every 10 to 20 measurements, and the instrument was recalibrated if the recovery of any analyte fell outside 90–110%. The recovery of Cu from SRM 1566b based on the measurements of ^{63}Cu or ^{65}Cu was both within 10% deviation from the certified values (71.6 ± 1.6 μg g^{-1}). All the Cu concentrations in clam tissues were expressed on dry weight basis.

Water samples for dissolved organic carbon (DOC) analysis were acidified to pH < 2 with H_3PO_4 and stored in amber glass vials at 4 °C. The concentration of DOC was determined using a TOC analyzer (TOC-Vcph, Shimadzu). DOC standards were prepared with potassium hydrogen phthalate. A deep seawater reference (Batch 10–2010, 41–44 μM DOC) and a low carbon reference water (1 μM DOC) obtained from the Hansell CRM Program (Hansell, 2005) were measured to ensure instrument performance.

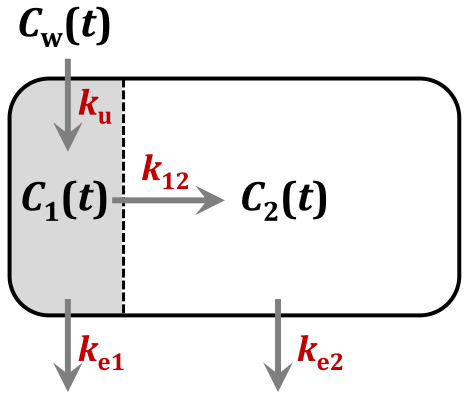

Fig. 1. The two-compartment toxicokinetic model for simulating Cu uptake and elimination in the clam *P. leavis*. See equations (1)–(3) for the meaning of parameters.

2.5. TK-TD model

The uptake and elimination of ^{65}Cu in *P. laevis* were simulated with a two-compartment TK model (*see* Fig. 1) (Tan and Wang, 2012):

$$C_{int}(t) = C_1(t) + C_2(t) \quad (1)$$

$$\frac{dC_1(t)}{dt} = k_u \cdot C_w(t) - (k_{12} + k_{e1}) \cdot C_1(t) \quad (2)$$

$$\frac{dC_2(t)}{dt} = k_{12} \cdot C_1(t) - k_{e2} \cdot C_2(t) \quad (3)$$

where $C_{int}(t)$, $C_1(t)$ and $C_2(t)$ are the concentrations of newly accumulated ^{65}Cu in the whole clam and those distributed in compartment one and two (μg g^{-1}), respectively. Because it was not possible to estimate the body mass represented by the two compartments, which were just empirical compartments and did not correspond to actual tissues or organs of the clam, $C_1(t)$ and $C_2(t)$ were normalized to the weight of the whole clam. $C_w(t)$ is the concentration of spiked ^{65}Cu in the tested solution (μg L^{-1}); k_u is the uptake rate constant (L g^{-1} h^{-1}); k_{12} is the transfer rate constant of ^{65}Cu from compartment one to compartment two (h^{-1}); k_{e1} and k_{e2} are the elimination rate constants of ^{65}Cu from compartment one and two, respectively (h^{-1}).

The survivorship of clams during the toxicity tests was modeled using the TD model (Ashauer and Brown, 2008; Tan and Wang, 2012):

$$\frac{dH(t)}{dt} = \begin{cases} k_k \times (C_{int}(t) - C_{IT}) + h_0 & if\ C_{int}(t) > C_{IT} \\ h_0 & otherwise \end{cases} \quad (4)$$

$$S(t) = e^{-H(t)} \quad (5)$$

$$S_0(t) = e^{-h_0 \times t} \quad (6)$$

where $H(t)$ is the hazard caused by internal Cu exposure (dimensionless); k_k is the killing rate (g μg^{-1} h^{-1}, or grams of clam killed by per μg of internalized Cu per hour); C_{IT} is the internal threshold concentration (μg g^{-1}); h_0 is the background hazard rate (h^{-1}); $S(t)$ is the probability of an organism surviving to time t; $S_0(t)$ is the survival probability of organisms in the negative control. The concept of the model is that when the concentration of bioaccumulated Cu exceeds the threshold (C_{IT}), the hazard caused by Cu exposure [$H(t)$] begins to accumulate. The rate of hazard accumulation (i.e., hazard rate) is proportional (constant k_k) to the excessively accumulated Cu beyond the threshold, i.e., [$C_{int}(t)$-C_{IT}].

The TK-TD model was implemented in the software ModelMaker 4 (FamilyGenetix Ltd. Oxford, UK). The TK parameters and the standard deviations were estimated using the Marquardt algorithm, and the TD parameters were estimated using the maximum likelihood method with the likelihood function described by Jager et al. (2011). The 95% confidence intervals were defined by parameter values sampled in the Monte Carlo method that satisfy a likelihood ratio test at the 5% level (Ashauer et al., 2015). The two-compartment TK-TD model can be simplified into a one-compartment model at the expense of losing some accuracy (*see* Online Supplement for details).

2.6. Data and statistical analysis

The ^{65}Cu in clam tissues had two origins, one was the uptake of spiked ^{65}Cu while the other was the background ^{65}Cu already

present before the exposure. The background ^{65}Cu existed in a constant ratio to the non-spiked isotope ^{63}Cu. Therefore, the concentration of the newly accumulated ^{65}Cu by the clams ($[^{65}Cu_{new}]$) was calculated following the equation (Croteau et al., 2004; Zhong et al., 2012):

$$[^{65}Cu_{new}] = ([Cu_{65}] - [Cu_{63}]) \cdot {}^{65}F \quad (7)$$

where $[Cu_{65}]$ and $[Cu_{63}]$ are the apparent (not the real) *total* Cu concentrations in clam tissues, which were inferred from the calibration curve built using the isotope ^{65}Cu and ^{63}Cu, respectively; ^{65}F is the isotopic abundance of ^{65}Cu in the calibration standards (Agilent, part number 5183–4688). Consequently, $[Cu_{65}] \cdot {}^{65}F$ is the total concentration of ^{65}Cu in samples; $[Cu_{63}] \cdot {}^{65}F$ is the concentration of background ^{65}Cu. Cu in the calibration standard has the natural isotopic composition; and ^{65}F is the natural abundance, i.e., 0.3085.

The speciation of Cu at different salinities was calculated using a geochemical modeling software Visual MINTEQ 3.0 (*see* Online Supplement for details). The median lethal concentrations (LC_{50}) were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977) and the "tsk" package in the software R 3.1.3. The LC_{50} calculations were based on the time-weighted average Cu concentrations in tested solutions.

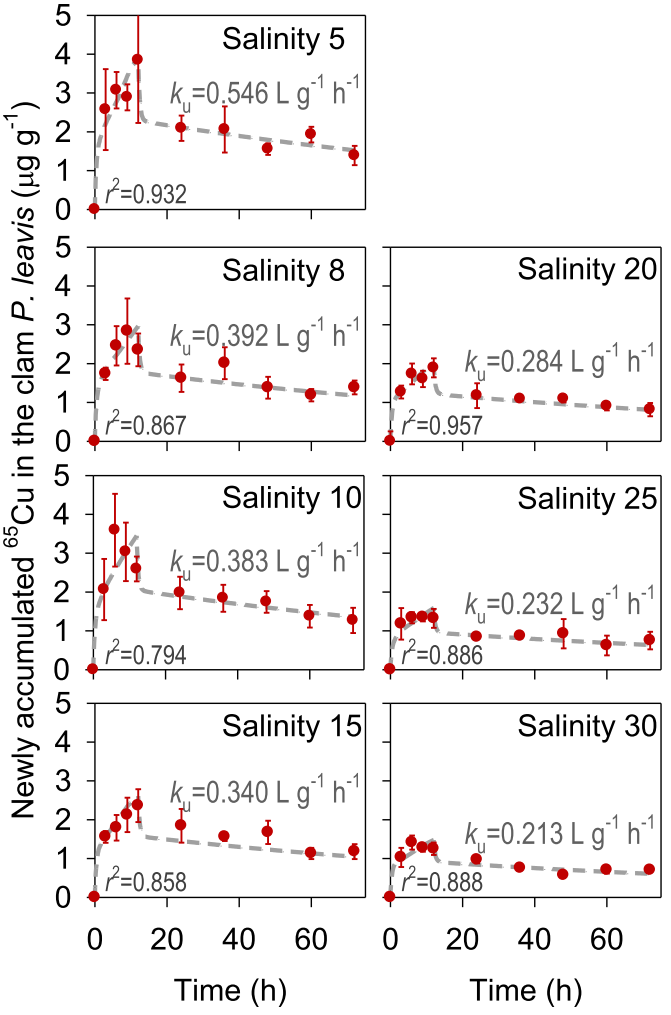

Fig. 2. The concentration of newly accumulated ^{65}Cu in the clam *P. leavis* during the 12-h exposure followed by 60-h depuration at the salinities ranging from 5 to 30. The dots are measured values; and the error bars represent standard deviation ($n = 3$). The dashed lines are model fits; and the best-fit parameter values are listed in Table 1. The goodness of fit is indicated by r^2 of the 1:1 regression line between measured and modeled data. The nominal ^{65}Cu concentrations used in the exposure were 10 μg L^{-1}; the measured ^{65}Cu concentrations ranged from 6 to 9 μg L^{-1} and are listed in Table S1.

3. Results and discussion

3.1. Cu toxicokinetics

The accumulation and elimination of Cu by the clam *P. leavis* was fitted by a two-compartment TK model (Fig. 2). It should be noted that ^{65}Cu was measured in the whole body of clams; the two compartments were identified for adequate curve fittings and were not necessarily representative of any real organs or tissues. During the 12 h exposure, ^{65}Cu was first accumulated by the clams rapidly, followed by a slower accumulation. The elimination of ^{65}Cu from the clams also showed a two-phase pattern, i.e., a rapid initial loss followed by steady decrease. The curve shapes for the ^{65}Cu uptake and elimination were in accordance with each other, indicating a very fast Cu turnover in the clams, more specifically, in compartment one. The estimated k_{e1} and k_{12} of Cu were 2.11 h^{-1} and 0.114 h^{-1}, respectively (Table 1), thus 95% (i.e., $\frac{k_{e1}}{k_{12}+k_{e1}} \cdot 100\%$) of the newly incorporated Cu was rapidly released back into the environment, and only 5% was further transferred to compartment two. In compartment two, k_{e2} was 0.0068 h^{-1} (or 0.16 d^{-1}), corresponding to a biological half-life of 4.3 d.

The elimination of Cu from the slow compartment (i.e., compartment two) of the clam *P. leavis* (k_{e2} = 0.16 d^{-1}) was much faster than those observed for other trace elements in marine bivalves, which fell within a narrow range of 0.01–0.03 d^{-1} (Reinfelder et al., 1998; Wang and Fisher, 1997). Similar high k_es of Cu were found in scallop *Chlamys nobilis* (0.148 d^{-1}), clam *Ruditapes philippinarum* (0.147 d^{-1}), and green mussel *Perna viridis* (0.131 d^{-1}) (Pan and Wang, 2009). In comparison, smaller Cu k_es were observed in black mussel *Septifer virgatus* (0.095 d^{-1}) and the strong Cu accumulator, oyster *Saccostrea cucullata* (0.032 d^{-1}) (Pan and Wang, 2009). In the clam *Potamocorbula amurensis*, a species closely related to *P. leavis*, much lower k_es of other metals were observed, i.e., 0.011 d^{-1}, 0.027 d^{-1} and 0.048 d^{-1} for Cd, Zn and Cr, respectively (Lee et al., 1998).

Compared to other marine bivalves, *P. leavis* was among the species with a relatively high k_u of Cu, i.e., 0.213–0.546 L g^{-1} h^{-1} (Table 1). For example, Pan and Wang (2009) determined the k_us of Cu using a radiotracer (^{67}Cu) technique in five marine bivalves at the salinity of 30, with a k_u ranging from 0.053 to 0.326 L g^{-1} h^{-1}. In the mussel *P. viridis*, Cu k_u was 0.083 L g^{-1} h^{-1} at the salinity of 33 (Zhong et al., 2012). However, it should be noted that the method for estimating k_u was slightly different between this study and the previous ones (Pan and Wang, 2009; Wang et al., 1996; Zhong et al., 2012). Specifically, we considered the possible efflux of newly incorporated metals when estimating k_u, and thus quantified the unidirectional uptake of metal. In comparison, it was assumed that the efflux was negligible during short period of exposures in previous studies (e.g., 1–8 h).

The k_us of Cu were determined at a measured Cu concentration of 6–9 μg L^{-1}, which were within the linear range of the Michaelis-Menten curve (*see* insets of Fig. S2B). Therefore, the k_u values can be extrapolated to lower Cu concentrations and predict Cu uptake in less contaminated waters. At a much higher Cu concentration (i.e., nominal 500 μg L^{-1}, measured concentrations listed in Table S2), k_us were also estimated based on the Cu accumulation in clams (*see* Fig. S4) by assuming the same values of other TK parameters (k_{12}, k_{e1}, k_{e2}, *see* Table 1). The k_us measured at high Cu levels were substantially lower and more comparable at different salinities

Table 1
The best-fit values and estimated uncertainties of the model parameters. k_u-10 and k_u-500 are the uptake rate constant (k_u) of Cu determined at the nominal Cu concentrations of 10 and 500 μg L^{-1}, respectively. The same k_{12}, k_{e1} and k_{e2} are assumed for different salinities. See Fig. 1 for the model and equations (1)–(6) for definition of parameters. Values of the toxicokinetic parameters are mean ± standard deviation. Values in the parenthesis are 95% confidence intervals.

Salinity	k_u-10 (L g^{-1} h^{-1})	k_u-500 (L g^{-1} h^{-1})	k_{12} (h^{-1})	k_{e1} (h^{-1})	k_{e2} (h^{-1})	C_{IT} (μg g^{-1})	k_k (10^{-3} g μg^{-1} h^{-1})
5	0.546 ± 0.138	0.132 ± 0.042	0.114 ± 0.015	2.11 ± 0.51	0.0068 ± 0.0018	133 (122, 140)	0.58 (0.29, 1.01)
8	0.392 ± 0.097	0.096 ± 0.026				97 (87, 104)	0.63 (0.36, 1.04)
10	0.383 ± 0.097	0.125 ± 0.031				111 (99, 120)	0.44 (0.20, 0.82)
15	0.340 ± 0.087	0.098 ± 0.007				92 (84, 99)	0.91 (0.55, 1.41)
20	0.284 ± 0.074	0.095 ± 0.021				79 (71, 86)	0.91 (0.54, 1.40)
25	0.232 ± 0.061	0.113 ± 0.036				89 (83, 94)	1.29 (0.92, 1.89)
30	0.213 ± 0.057	0.111 ± 0.031				96 (90, 101)	2.08 (1.35, 3.02)

(0.095–0.125 L g^{-1} h^{-1}, Table 1).

3.2. Effects of salinity on Cu bioaccumulation

The TK parameters k_{e1}, k_{e2} and k_{12} were assumed to be the same for clams at different salinities, and the observed differences in the accumulation of ^{65}Cu at different salinities were attributed to the effects on k_u alone (Fig. 2). In other words, we assumed that the effects of salinity on Cu toxicokinetics were simply geochemical effects occurred in the water instead of effects on the inter-compartment transfer (k_{12}) or the elimination (k_{e1}, k_{e2}) processes. These assumptions (or constraints) make it easier to interpret the model results, although they have not been strictly tested. Nevertheless, the good agreement between the model fittings and the observed time courses of ^{65}Cu accumulation implied that the assumptions were adequate (Fig. 2).

The k_us of Cu decreased monotonically from 0.546 to 0.213 L g^{-1} h^{-1} when salinity was elevated from 5 to 30 (Fig. 3). Several mechanisms in combination may have contributed to the observed effects of salinity. First, the majority (94%–98%) of Cu was complexed by dissolved organic matter (DOM) at all salinities according to the calculation by geochemical modelling (Fig. S1). Cu^{2+} and other labile inorganic species (e.g., $CuCO_3$, $CuOH^+$) comprised only a very small fraction of the total dissolved Cu, i.e., 0.31–0.88% for Cu^{2+}, 0.34–0.98% for $CuOH^+$, and 0.80–3.6% for $CuCO_3$. With the increase of salinity, the percentages of Cu^{2+}, $CuCO_3$, and $CuOH^+$ first increased and then decreased, in contrast to the monotonic decrease of k_u (Fig. 3). It should be noted that it is difficult to model Cu speciation in seawater due to the high ionic strength and the complexity in DOM composition. Therefore, the speciation provided above was just a rough estimation with considerable uncertainties. Second, the competition effects from major cations (e.g., Na^+, K^+, Ca^{2+} and Mg^{2+}) were expected to increase with salinity. Third, when salinity increased from 5 to 30, the ionic strength increased from 0.10 to 0.61 mol L^{-1}, which could decrease the activity coefficient of Cu^{2+} from 0.35 to 0.23 (based on the calculation with Davies equation) and thus its uptake rate.

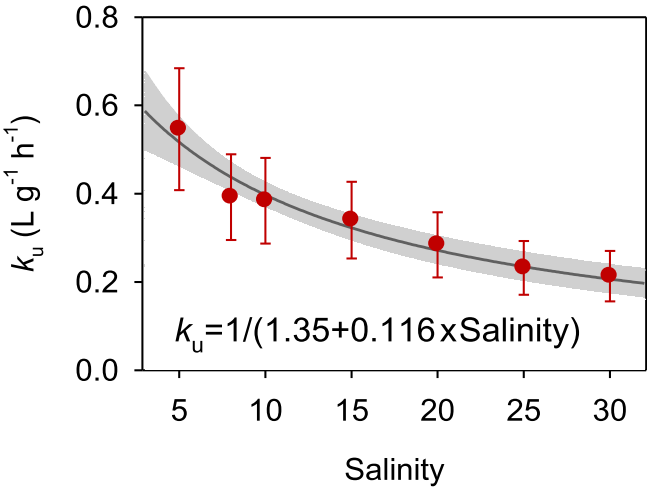

Fig. 3. The relationship between the uptake rate constant (k_u) of Cu in the clam *P. leavis* and the salinity. Salinity was treated as a pseudo-competitor which competitively inhibited Cu uptake. The shaded area is the 95% confidence interval. Error bars are standard deviation.

Although the effects of salinity on Cu uptake were manifold, they could be well described by the empirical equation below (*see* Fig. 3):

$$k_u = \frac{1}{1.35 + 0.116 \cdot \text{Salinity}} \quad (8)$$

In this equation, salinity was treated as a pseudo-competitor of Cu uptake, and the effects of salinity were considered similar to the competition effects of major cations (e.g., Ca^{2+}, Na^+) in the BLM (Slaveykova and Wilkinson, 2005). The constants in equation (8) were estimated by fitting k_us measured at different salinities with the pseudo-competition equation.

In developing BLM for freshwater organisms, the effects of different water compositions are modeled individually because their concentrations vary independently in real waters (De Schamphelaere and Janssen, 2002; Slaveykova and Wilkinson, 2005). In contrast, in estuarine waters, most of the major compositions (except HCO_3^- and DOC) vary proportionally with salinity (Bianchi, 2007). Therefore, it is possible to model the effects of salinity using a simple empirical equation like the one described above (equation (8)). One limitation of the present study is that the concentration of DOC was not deliberately controlled. DOC concentration correlated positively to salinity because the waters of different salinities were prepared by diluting the high-salinity seawater with deionized water (Table S3). To develop an estuarine BLM for predicting Cu bioaccumulation at different salinities, the effects of HCO_3^-, DOC and possibly pH may also be considered independently to improve the empirical equation.

Table 2
The 72-h median lethal concentration (LC_{50}) of Cu and 95% confidence intervals in the clam *P. leavis* determined at three different salinities (5, 15, and 30). The relationship between uptake rates (J_{in}) of Cu and Cu concentrations in exposure solution ([Cu], μg L^{-1}) are described by the Michaelis-Menten equation. See Fig. S2 for the comparison of observation and model.

Salinity	72 h LC_{50} (μg L^{-1})	J_{in} (μg g^{-1} h^{-1})
5	269 (197, 368)	$J_{in} = 50.3 \times [Cu]/(33.7 + [Cu])$
15	224 (174, 288)	$J_{in} = 50.2 \times [Cu]/(28.7 + [Cu])$
30	192 (146, 251)	$J_{in} = 47.3 \times [Cu]/(72.0 + [Cu])$

3.3. Cu toxicodynamics and the effects of salinity

In the two series of toxicity tests of different designs, Cu toxicity both appeared to increase with salinity. The first series were standard toxicity tests conducted at three different salinities (Fig. S2). Mortality of organisms was frequently monitored to construct the survivorship curves; the concentration-response at the end of the tests was plotted in the insets (Fig. S2A). The 72 h LC_{50}s of Cu were 269, 224, and 192 μg L^{-1} at salinity 5, 15, and 30, respectively, although their 95% confidence intervals overlapped (Table 2). The three tests were conducted with different batches of clams; therefore, the biological variability might have masked (partly) the effects of salinity on Cu toxicity. In order to reveal the effects of salinity more clearly, the second series of toxicity tests were conducted with one lethal Cu concentration (nominal 500 μg L^{-1}) simultaneously at seven salinities with the same batch of clams (Fig. 4). As expected, the effects of salinity became apparent, and the survivorship of clams decreased with salinity, except that the highest survivorship was observed at the salinity 10 (Fig. S3).

The survivorship of clams was fitted with the TD model. The best-fit estimates of C_{IT} and k_k were listed in Table 1. C_{IT} is the threshold Cu concentration in the clams, above which the hazard rate becomes higher than the background value, therefore measures how sensitive the organisms are. C_{IT} ranged from 79 to 133 μg g^{-1} across the investigated salinity range (Fig. 5). In comparison, k_k is the specific toxicity of the excessively accumulated Cu,

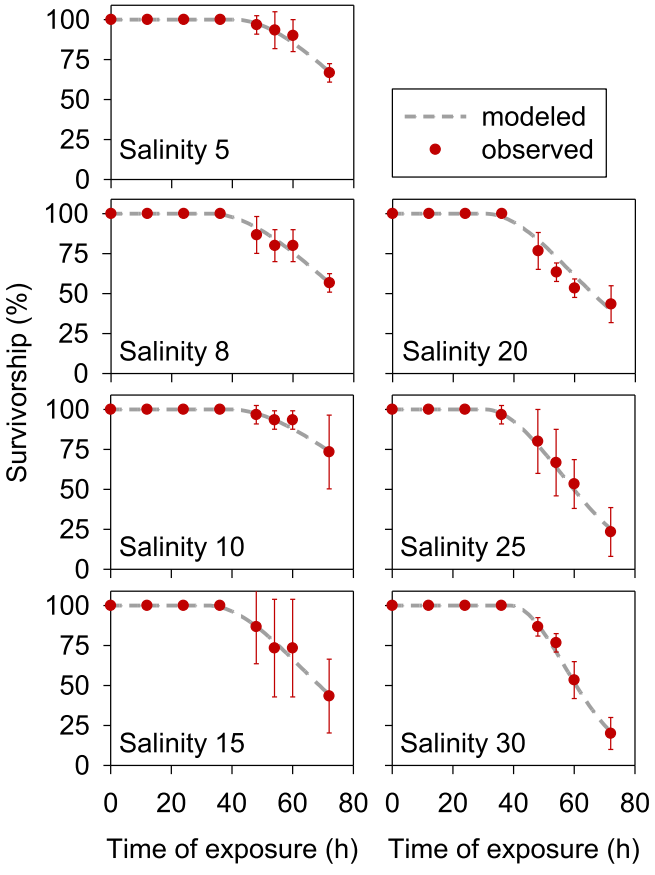

Fig. 4. The survivorship of the clam *P. leavis* during the 72-h exposure to 500 μg L^{-1} Cu (nominal) at salinities ranging from 5 to 30. The dots are the observed survivorship and the error bars are standard deviation ($n = 3$); and the dashed lines are the model fits. The best-fit parameter values are listed in Table 1. The measured Cu concentrations are listed in Table S2.

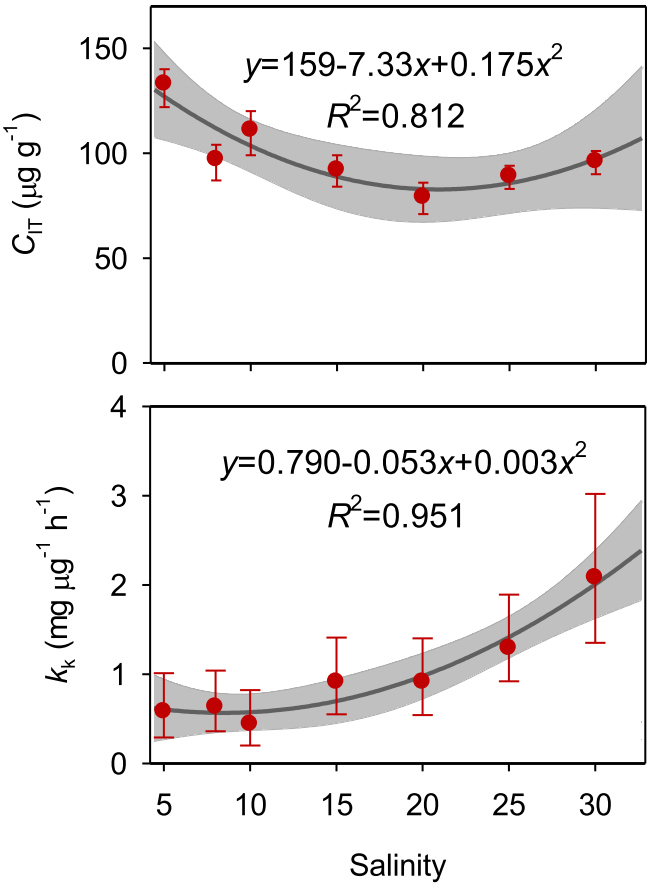

Fig. 5. Relationship between the internal threshold concentration (C_{IT}, μg g^{-1}) or the killing rate (k_k, mg μg^{-1} h^{-1}) of Cu in the clam *P. leavis* and salinity. The solid curves are quadratic regressions and the shaded areas are 95% confidence intervals. Error bars are standard deviation.

i.e., (C_{int}–C_{IT}), which measures how vulnerable the organisms would be. The k_k ranged from 0.44 to 2.08 mg μg^{-1} h^{-1} (Table 1) and increased with salinity (Fig. 5). The relationship between C_{IT} (or k_k) and salinity was described empirically by the quadratic equations (Fig. 5). The quadratic equation for C_{IT} showed a minimum around salinity 20, suggesting that the clams were most sensitive at the intermediate salinities. However, this inference is arguable due the considerable uncertainties in C_{IT} estimation.

Cu concentrations were measured in the freshly dead (<12 h) clams and the clams survived the 72 h exposure (Fig. S2 and Fig. S4). Based on the Cu concentrations in the survived clams (the first series of toxicity tests, Fig. S2B), the uptake rates of Cu were calculated using the TK model and parameter (i.e., k_{12}, k_{e1} and k_{e2}) values listed in Table 1. The relationship between uptake rate and Cu concentration can be well described by the Michaelis-Menten equation (*see* insets of Fig. S2B, Table 2). The maximum influx rate (J_{max}) ranged from 47.3 to 50.3 μg g^{-1} h^{-1} and was comparable at different salinities. In each treatment, Cu concentrations in the dead clams and living clams were similar (Fig. S2B). In the second series of toxicity tests, Cu concentrations in the dead clams ranged from 112 to 168 μg g^{-1}, and generally decreased with salinity (Fig. S4). In other words, lower tissue Cu concentrations were needed to kill the clam at higher salinities. However, it should be noted that Cu concentration measured in dead clams was different from C_{IT}, and by definition should be no lower than C_{IT} (79–133 μg g^{-1}, Table 1). Cu concentrations measured in the survived clams were similar to those in the dead ones (Fig. S4), and

showed no significant difference at different salinities, indicating that the inhibitive effects of salinity on Cu bioaccumulation was less conspicuous at high Cu concentrations. Similarly, k_u-500 s were estimated using the TK model based on Cu concentrations in these survived clams (Table 1).

The increase of Cu toxicity with salinity was uncommon in previous studies. Of the 11 studies reviewed by Hall and Anderson (1995), 7 reported decreased Cu toxicity at higher salinities, 3 reported no clear trend, and only 1 reported increased toxicity. In the last case, Cu toxicity to the mussel *Mytilus edulis* was observed to be lower at salinity 16 than at salinity 32 (Weber et al., 1992). Another case is the larvae of mussel *Mytilus galloprovincialis*, in which Cu toxicity increased with salinity within the salinity range of 23–37 (Deruytter et al., 2015). In several other studies, the lowest Cu toxicity occurred at the intermediate salinities, and both too low or too high salinity increased the sensitivity of organisms (e.g., killifish *Fundulus heteroclitus*, polychaete *Nereis diversicolor*) to Cu exposure (Grosell et al., 2007; Jones et al., 1976).

At first glance, it is surprising that Cu toxicity increased with salinity while Cu bioaccumulation decreased with salinity. One explanation is that the inhibitory effects of salinity on Cu bioaccumulation were only significant at low Cu concentrations (e.g., 10 μg L^{-1}) and were much less at higher Cu concentrations, such as 500 μg L^{-1} used in the toxicity tests. It was presumably due to the saturation of Cu uptake sites, and thus the maximum uptake rates of Cu were approached regardless of the salinity (*see* Fig. S2). An additional explanation for the seeming contradiction is the higher intrinsic sensitivity of the clams to Cu, which was represented by the higher killing rate (k_k) at higher salinities (*see* Fig. 5). The underlying physiological mechanism may be the osmotic stress caused by the deviation from the optimal salinity of the organisms. Studies on various euryhaline species showed that organisms were more tolerant to metals at their optimal salinities, at which they were subjected to minimal osmotic stress (Grosell et al., 2007; Hall and Anderson, 1995). Although the optimal salinity of *P. leavis* was not determined, we speculate that it was in the range of 10–15 based on our observation that the clams were more active in movement and feeding in this salinity range. The highest activity of the superoxide dismutase and catalase in the clam's hepatopancreas was observed at salinity 10 and 15, respectively (Lü, 2014). Taken together, the higher toxicity of Cu to *P. leavis* at higher salinities should be attributed to the associated higher osmotic stress instead of the amount of Cu accumulated.

3.4. TK-TD model and environmental implications

Interpreting and predicting Cu toxicity at different salinities is complicated by the contrasting effects of salinity on Cu bioaccumulation and intrinsic sensitivity of organisms. Our study suggested that these effects could be separated quantitatively in the framework of the TK-TD model, in which TK quantifies the effects on bioaccumulation, whereas TD quantifies the effects on physiology after eliminating the confounding effects of bioaccumulation.

The effects of salinity on bioaccumulation were qualitatively similar for different metals and biological species, thus the application of a mechanistic TK model as a unifying framework is promising. In general, metal bioaccumulation decreased monotonically with salinity due to the competition and speciation effects (Blanchard and Grosell, 2005; Chong and Wang, 2001; Lee et al., 1998; Phillips, 1977; Wang et al., 1996; Wright and Zamuda, 1987; Zhang and Wang, 2007), consistent with the prediction of BLM. Therefore, BLM can be used to model the effects of salinity on the toxicokinetics of Cu (although not directly on Cu toxicity). Among the four TK parameters, k_u is the only one which is assumed to be dependent on salinity. Based on the principles of BLM, a simple equation (i.e., equation (8)) was used to model k_u at different salinities by treating salinity as a whole and as a pseudo-competitor. Although this simple BLM-based model provides good prediction in this study (Fig. 3), a more sophisticated BLM may be needed to provide more robust prediction under less controllable field conditions.

It is more difficult to predict the effects of salinity on the intrinsic sensitivity of organisms, given the inconsistent trends observed for different metals in different biological species (Grosell et al., 2007; Hall and Anderson, 1995). The two TD parameters (i.e., C_{IT} and k_k) were both used to quantify the effects of salinity, and an empirical quadratic model was currently used for relating them to salinity (Fig. 5). Mechanistic modeling of C_{IT} and k_k at different salinities requires a better understanding of the intrinsic sensitivity of organisms to salinity. Cu^{2+} is considered as an osmoregulatory toxicant and can disturb the maintenance of a Na^+ gradient between the extracellular fluid of fish and ambient water (Grosell et al., 2007). It was feasible to use the Na^+ gradient as a predictor of Cu toxicity to a fish at different salinities (Grosell et al., 2007). Although most invertebrates, including the clam *P. laevis*, are osmoconformers and do not need to maintain a Na^+ gradient, the deviation of salinity from their optimal salinity may still causes stress, which in turn increases the sensitivity of organisms to Cu. Indeed, there was a linear relationship between k_k and the deviation of salinity (Fig. S5), suggesting that it is possible to mechanistically model the effects of salinity on the intrinsic sensitivity based on the osmotic stress.

There are ongoing efforts to develop BLM for Cu toxicity in estuarine waters (Arnold et al., 2005; de Polo and Scrimshaw, 2012; Deruytter et al., 2014; Grosell et al., 2007; Pinho and Bianchini, 2010). One of the biggest challenges is to model the effects of salinity on the physiology of organisms in the BLM framework (de Polo and Scrimshaw, 2012; Grosell et al., 2007). The TK-TD model is not proposed to replace the current BLM. Instead, we propose to integrate BLM into the TK-TD model, using BLM to predict the effects of water chemistry on the toxicokinetics of metals rather than predicting toxicity directly. The physiological effects of salinity can then be separated and measured by relating the TD parameters to salinity. However, modeling the effects of salinity on the physiological processes remains a challenge, which requires a better understanding of the mechanisms of Cu toxicity. Additionally, in the TK-TD framework, time is treated as a *variable* rather than an exposure *condition* as in the BLM, therefore the model parameters are not limited to the exposure duration (e.g., 72 h) or to the specific proportion of responses (e.g., 50% mortality), to which the parameters have been calibrated. This kinetic nature of the TK-TD model makes it better suited to the dynamic estuarine environment. The real estuarine waters are surely much more complex than the simulation in this study, and many other water chemistry variables may interact with salinity in affecting metal toxicity. Nevertheless, the TK-TD model can provide a framework for organizing future studies in this area, and facilitate the better management of metal risks in estuarine systems.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.12.033.

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Online Supplement to:

Revealing the complex effects of salinity on copper toxicity in an estuarine clam *Potamocorbula laevis* with a toxicokinetic-toxicodynamic model

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Table S1 Measured concentrations of ^{65}Cu (μg L^{-1}) in the test solutions used in the uptake experiment performed at salinities ranging from 5 to 30. The nominal concentrations were 10 μg L^{-1}. Water samples were taken at the beginning of exposure and after 3, 6, 9, and 12 h of exposure.

Time	Salinity						
(h)	5	8	10	15	20	25	30
0	6.8	7.8	8.6	8.2	8.3	7.1	9.1
3	7.1	8.2	9.4	7.8	7.6	7.5	7.6
6	7.0	7.6	9.2	7.9	7.1	7.0	7.1
9	7.4	7.3	8.9	7.8	6.9	6.7	6.7
12	7.0	7.1	8.6	7.2	6.4	6.4	6.2

Table S2 Measured concentrations of Cu (μg L^{-1}) in the solutions of the toxicity tests performed at salinities ranging from 5 to 30. The nominal concentrations were 500 μg L^{-1}. Water samples were taken at the beginning of exposure and after 24, 48, and 72 h of exposure.

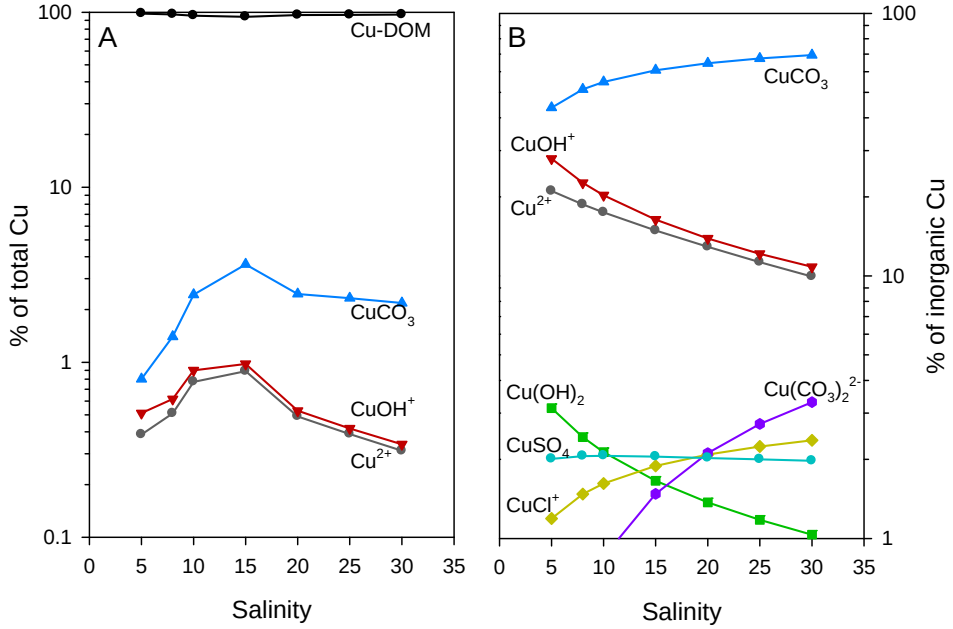
Time	Salinity						
(h)	5	8	10	15	20	25	30
0	503	494	480	479	501	473	430
24	409	426	339	380	348	325	337
48	336	360	315	369	353	306	348
72	327	384	295	385	340	353	352

Table S3 The measured concentration of dissolved organic carbon (DOC) at different salinities.

Salinity	DOC (mg L^{-1})
5	0.797
8	0.781
10	0.844
15	0.696
20	0.895
25	0.998
30	1.107

In this study, the waters of different salinity were prepared by diluting seawater of salinity 30 with deionized water; therefore, DOC concentration decreased with salinity. The results indicate that in the deionized water DOC concentration was approximately 0.8 mg L^{-1}.

Fig. S1 Effects of salinity on Cu species distribution expressed in (A) percent of total Cu and (B) percent of inorganic Cu.



Cu speciation was calculated in the geochemical modelling software Visual MINTEQ 3.0 (https://vminteq.lwr.kth.se), selecting Stockholm Humic Model (SHM) for modeling the complexation effects of dissolved organic matter (DOM). Additional assumptions on DOM properties include: (1) the ratio of active DOM to DOC was 2 (i.e., the C content of DOM was 50%), (2) 100% of active DOM was fulvic acid. Temperature and pH were set to 22 ºC and 8.0, respectively.

Cu speciation was dominated by Cu-DOM complexes (94% to 98%), and only a small portion (<10%) of Cu existed in inorganic species. The percentage of Cu^{2+}, $CuCO_3$ and $CuOH^+$ firstly increased and then decreased with salinity. The initial increase was mainly due to the increases of the competition of major cations (e.g., Na^+, Ca^{2+}) for binding DOM, which released Cu from the Cu-DOM complexes; the following decrease was mainly due to the increases in DOM concentration (*see* Table S3).

Inorganic Cu species was dominated by $CuCO_3$ (44% to 69%), which increased with salinity. Oversaturation (and thus precipitation) of $CuCO_3$ did not occur even at the highest Cu concentration (i.e., 1000 μg L^{-1}) according to the calculation. The percentage of Cu^{2+} (21% to 9.9%) decreased with salinity.

Fig. S2 (A) The survivorship of the clam *P. leavis* during the 72-h exposure to nominal Cu concentrations ranging from 63 to 1000 μg L^{-1} at three different salinities, i.e., 5, 15 and 30 (the first series of toxicity tests); the insets are the relationship between the survivorship at the end of the 72-h tests and the measured Cu concentrations in test solution ([Cu], μg L^{-1}). The dots are observed values; the curves are model fits. (B) Cu concentrations in the clams that survived or died during the 72-h exposure; the insets are the relationship between calculated uptake rates of Cu (J_{in}, μg g^{-1} h^{-1}) and the Cu concentrations in test solution ([Cu], μg L^{-1}) described by Michaelis-Menten equations.

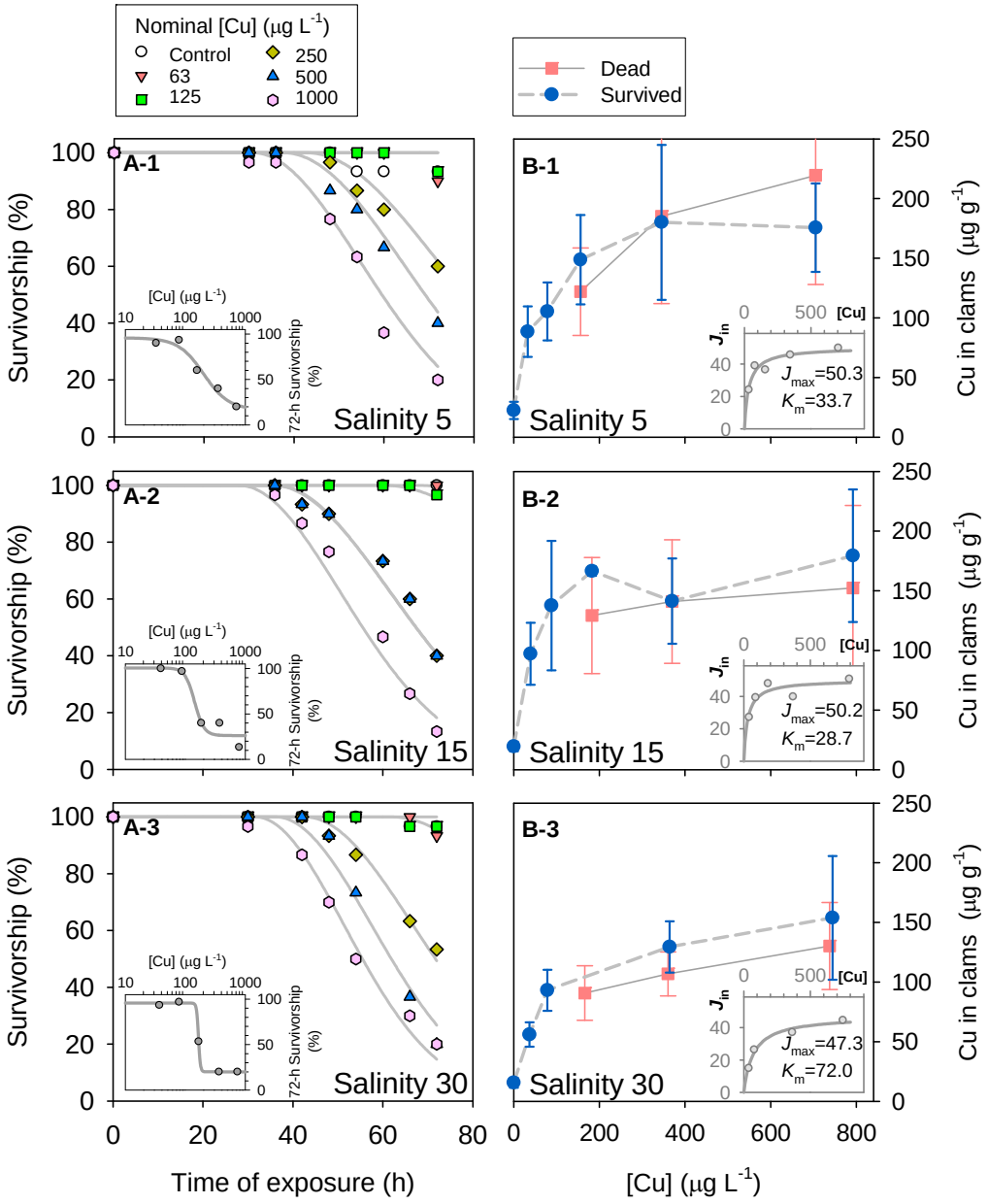


Fig. S3 The survivorship of the clam *P. leavis* during the 72-h exposure to 500 μg L^{-1} Cu (nominal) at salinities ranging from 5 to 30. This figure is a replot of Fig. 4 for a clearer comparison between salinities. The error bars (presented in Fig. 4) were omitted for simplicity.

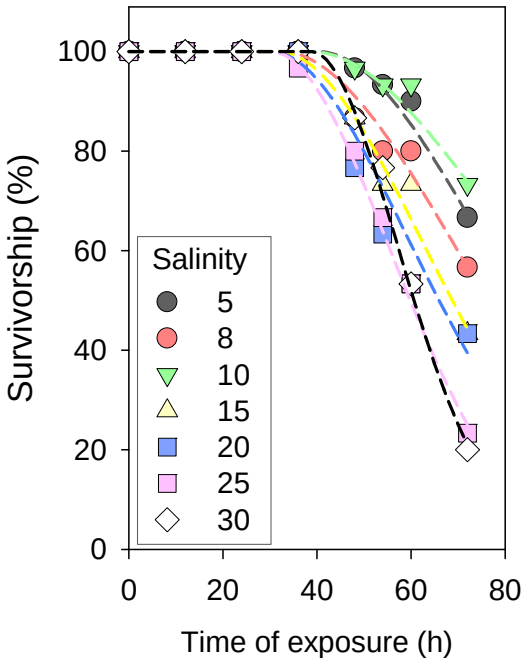


Fig. S4 Cu concentration in the clam *P. leavis* that survived or died during the 72-h exposure to 500 μg L^{-1} Cu (nominal) at salinities ranging from 5 to 30 (the second series of toxicity tests). The error bars are standard deviation (*n*= 5-15). Cu concentrations of dead clams (grey bars) sharing no common superscript letter were significantly different ($p<0.05$, Tukey HSD test).

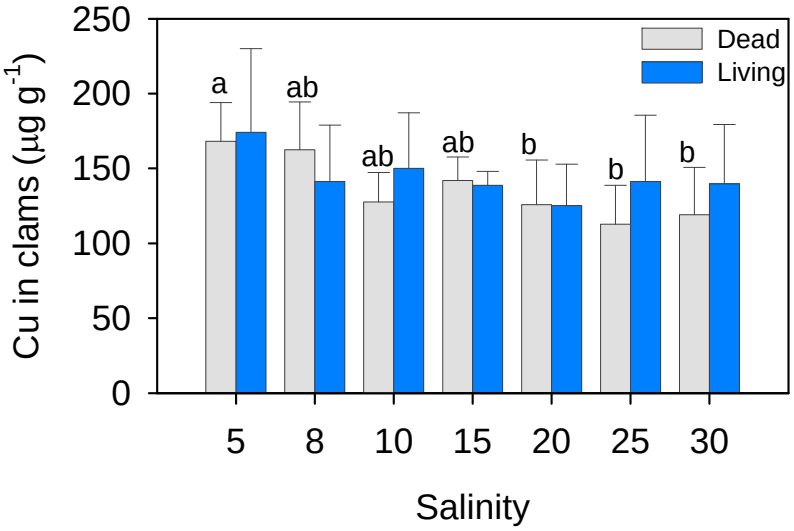
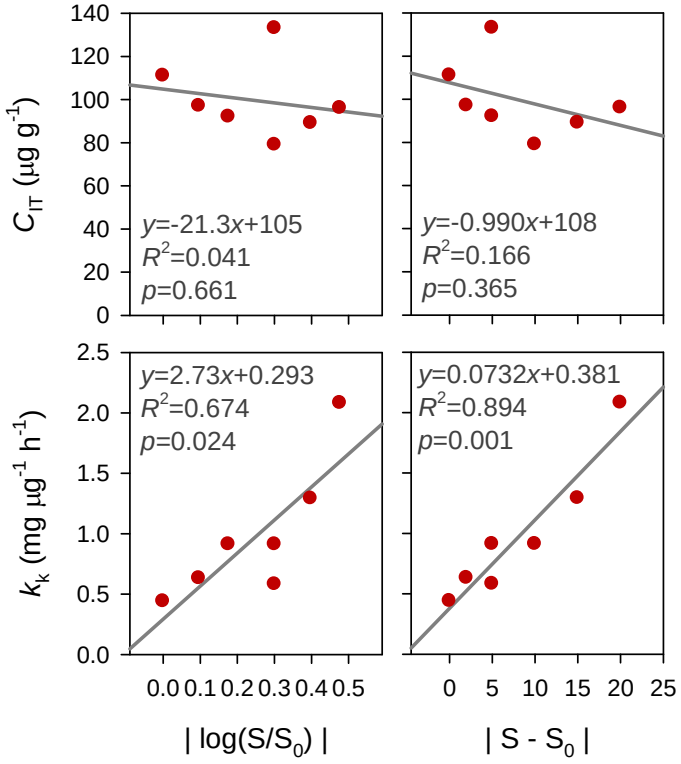


Fig. S5 Linear regression between the internal threshold concentration (C_{IT}) or killing rate (k_k) of Cu in the clam *P. leavis* and the deviation from optimal salinity.



S: salinity of test solution; S_0: the speculated optimal salinity, which was assigned to be 10‰ to achieve the best fittings.

The deviation from optimal salinity was measured in two ways: (1) the absolute value of $\log(S/S_0)$, the concept of which was borrowed from Grosell et al. (2007); (2) the absolute value of the difference between S and S_0. Slightly higher R^2 was obtained in the second way.

Simplification of the Two-compartment Model into a One-compartment Model

The design of the two compartment model was slightly different from the one used conventionally (Redeker and Blust, 2004; Tan and Wang, 2012). Specifically, the back transfer of metal from compartment two to compartment one (k_{21}) was replaced by the direct elimination of metal from compartment two (k_{e2}). Biologically there is no evidence to support or refute this modification. However, mathematically there are two reasons: (1) the model fitting becomes easier and more robust; and (2) the model can be simplified into a one compartment model more easily (*see* the details below).

After a short-term exposure, substantial proportion of the Cu exists in the fast compartment (Fig. S5A). Therefore, to simulate Cu bioaccumulation in a short term-exposure, the fast compartment should not be neglected, and the two-compartment model is necessary.

After a long-term exposure, e.g., the exposure experienced by organisms living in polluted waters, the majority of Cu exists in the slow compartment (Fig. S5B). The difference between metal concentrations in compartment one and the whole organism is small, i.e., $C_{\mathrm{int}}(t) \approx C_2(t)$. Therefore, the two-compartment model can be simplified to a one-compartment model by neglecting the contribution of $C_1(t)$ to $C_{\mathrm{int}}(t)$. Considering that only a fraction (i.e., $\frac{k_{12}}{k_{12}+k_{e1}}$) of incorporated Cu is further transferred into compartment two, the uptake rate constant of the one compartment model (k'_{u}, L g^{-1} h^{-1}) can be calculated as

$$k'_{\mathrm{u}} = k_{\mathrm{u}} \cdot \frac{k_{12}}{k_{12}+k_{e1}} \quad \ldots\ldots \text{(S1)}$$

The efflux rate constant of the one-compartment model (k'_{e}, h^{-1}) is equivalent to that of the slow compartment, i.e., k_{e2}:

$$k'_{\mathrm{e}} = k_{e2} \quad \ldots\ldots \text{(S2)}$$

The variation of Cu concentration in the organism ($C'_{\mathrm{int}}(t)$, μg g^{-1}) can thus be described as

$$\frac{dC'_{\mathrm{int}}(t)}{dt} = k'_{\mathrm{u}} \cdot C_{\mathrm{w}}(t) - k'_{\mathrm{e}} \cdot C'_{\mathrm{int}}(t) \quad \ldots\ldots \text{(S3)}$$

Cu concentration in the compartment one (i.e., the fast compartment) can reach steady-state very quickly, and the steady-state concentration is

$$C_1^{ss} = \frac{k_{\mathrm{u}} \cdot \overline{C_{\mathrm{w}}}}{k_{12}+k_{e1}} \quad \ldots\ldots \text{(S4)}$$

where $\overline{C_{\mathrm{w}}}$ is the average value of the $C_{\mathrm{w}}(t)$.

In the two-compartment model, when the internal threshold concentration (C_{IT}) is reached, Cu concentration in the fast compartment should be very close to C_1^{ss}; and thus Cu concentration in the slow compartment is estimated to be ($C_{\mathrm{IT}} - C_1^{ss}$), which can be considered as the internal threshold concentration in the one-compartment model (C'_{IT}) :

$$C'_{\mathrm{IT}} = C_{\mathrm{IT}} - C_1^{ss} \quad \ldots\ldots \text{(S5)}$$

In short, four parameters are needed for the one-compartment TK-TD model, i.e., k'_{u}, k'_{e}, C'_{IT}, and k_{k}; they can be calculated based on the parameters of the two-compartment model (Table S4).

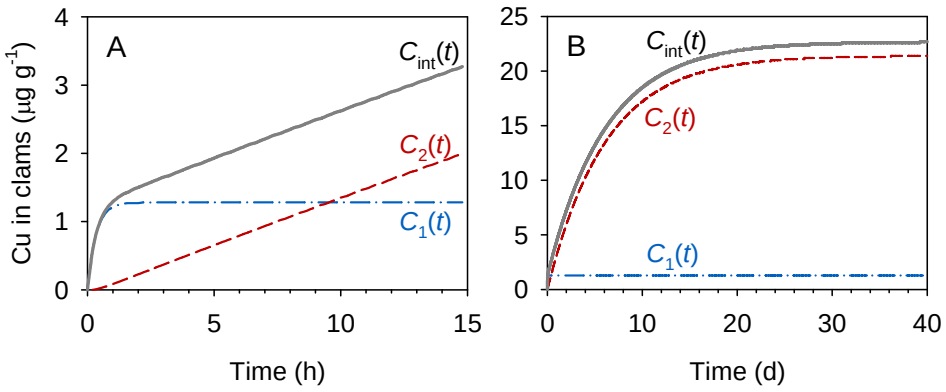
Table S4 Comparison of the model parameters of the one- and two-compartment model.

Model parameters	Unit	Two-compartment model	One-compartment model
Uptake rate constant	L g^{-1} h^{-1}	k_{u}	$k'_{\mathrm{u}} = k_{\mathrm{u}} \cdot \frac{k_{12}}{k_{12} + k_{\mathrm{e1}}}$
Inter-compartment transfer rate constant	h^{-1}	k_{12}	n.a.
Efflux rate constant (fast compartment)	h^{-1}	k_{e1}	n.a.
Efflux rate constant (slow compartment)	h^{-1}	k_{e2}	$k'_{\mathrm{e}} = k_{\mathrm{e2}}$
Internal threshold concentration	µg g^{-1}	C_{IT}	$C'_{\mathrm{IT}} = C_{\mathrm{IT}} - \frac{k_{\mathrm{u}} \cdot \overline{C_{\mathrm{w}}}}{k_{12} + k_{\mathrm{e1}}}$
Killing rate	g μg^{-1} h^{-1}	k_{k}	k_{k}

n.a.= not applicable

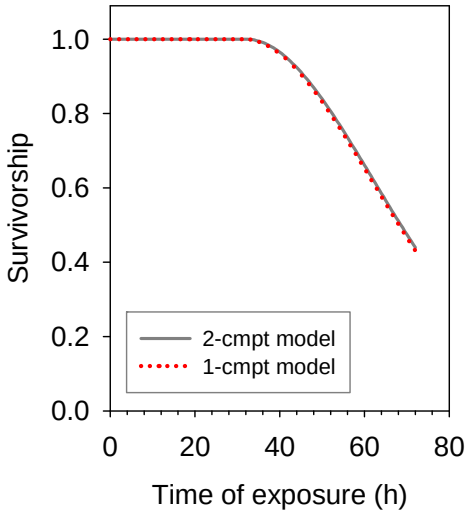
For estimating the model parameters using short-term exposures, the two-compartment model is necessary for accurate parameter estimation. Afterwards, the model can be simplified into the one-compartment model (according to Table S4) to predict Cu bioaccumulation and toxicity.

Fig. S6 The accumulation and distribution of Cu in the clam *P. leavis* simulated using the two-compartment toxicokinetic model. The parameter values for salinity 20 were used for the simulation (*see* Table 1). Cu concentration was assumed to be 10 μg L^{-1}.



(A) Short-term exposure: compartment one and two had comparable concentrations of Cu; (B) long-term exposure: Cu was predominantly distributed in compartment two.

Fig. S7 The survivorship of the clam *P. laevis* predicted by the two- and one-compartment model. The clams were assumed to be exposed to 400 μg L^{-1} of Cu at salinity 20. The predictions of the two models were nearly identical.



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