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RESEARCH ARTICLE

Evaluation on subcellular partitioning and biodynamics of pulse copper toxicity in tilapia reveals impacts of a major environmental disturbance

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Abstract Fluctuation exposure of trace metal copper (Cu) is ubiquitous in aquatic environments. The purpose of this study was to investigate the impacts of chronically pulsed exposure on biodynamics and subcellular partitioning of Cu in freshwater tilapia (*Oreochromis mossambicus*). Long-term 28-day pulsed Cu exposure experiments were performed to explore subcellular partitioning and toxicokinetics/toxicodynamics of Cu in tilapia. Subcellular partitioning linking with a metal influx scheme was used to estimate detoxification and elimination rates. A biotic ligand model-based damage assessment model was used to take into account environmental effects and biological mechanisms of Cu toxicity. We demonstrated that the probability causing 50% of susceptibility risk in response to pulse Cu exposure in generic Taiwan aquaculture ponds was ~33% of Cu in adverse physiologically associated, metabolically active pool, implicating no significant susceptibility risk for tilapia. We suggest that our integrated ecotoxicological models linking chronic exposure measurements with subcellular partitioning can facilitate a risk assessment framework that provides a predictive tool for preventive susceptibility reduction strategies for freshwater fish exposed to pulse metal stressors.

Keywords Copper · Tilapia · Subcellular partitioning · Toxicokinetics · Toxicodynamics · Pulse exposure · Risk assessment

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Introduction

Copper (Cu) is an essential element that acts as a micronutrient at low concentrations for organisms. However, pollutions of trace metal could be persistent and cumulative, posing ecological and physiological effects in marine organisms (Luoma 1996). Acute Cu toxicity could pose inhibition of Na^+ uptake at the gills, leading to mortality resulted from disturbance in NaCl homeostasis (Paquin et al. 2002a).

In reality, the environmental metal concentration tends to be fluctuated and pulsed as a result of site-specific water chemistry conditions. When trace metals are assimilated by organisms, a series of metabolic processes and subcellular partitioning occur subsequently (Mason and Jenkins 1995; Wang and Rainbow 2005). The accumulated metals could approximately be divided into two subcellular pools, including metabolically active pool (MAP, e.g., organelles, microsomes, and heat-labile proteins) and metabolically detoxified pool (MDP, e.g., metallothionein-like proteins and metal-rich

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granules). While adverse physiological consequence is associated with metal accumulations in MAP, accumulated metals in MDP are presumed to have no toxic effects in organisms.

The concept of subcellular partitioning of metal accumulations has implicated the significance of the subcellular fate of metals (Andosch et al. 2015; Campana et al. 2015; Rosabal et al. 2012; Wallace et al. 2003). However, there is limited information for aquatic organisms exposed to pulsed metals by using subcellular partitioning in the field of ecotoxicology. The tilapia (*Oreochromis mossambicus*) is a commercially important farmed fish in Taiwan's aquaculture (Fisheries Agency 2011) that is distributed mainly in northwestern coastal regions of Taiwan. Therefore, the subcellular partitioning concept coupled with biokinetic models in response to pulsed copper (Cu) in the farmed fish tilapia was explored in this study.

The biotic ligand model (BLM) has been widely utilized to predict toxic effects of metals in aquatic organisms (Bielmyer et al. 2007; Lock et al. 2006; Niyogi et al. 2004; Smith et al. 2015; Wang et al. 2011). The BLM is derived from the free-ion activity and surface interaction models which provide that surface membranes of the gill are recognized as the biotic ligand of fish (de Schamphelaere and Janssen 2002; Lee and Landrum 2006; Morgan and Wood 2004; Paquin et al. 2002b). On the other hand, a biologically based damage assessment model (DAM) has been applied to understand the mode of actions of contaminants with both irreversible and reversible bindings (Ashauer et al. 2007; Lee et al. 2002). The DAM assumes that hazards could occur when cumulative damages reach a critical level that when initial damage overwhelms threshold damage, damages will be irreversible. The DAM provides a comprehensive framework to explore time-dependent toxicity of chemicals by incorporating with damage accumulations.

The purposes of this study were twofold: (1) to develop an integrated toxicological model by linking subcellular partitioning and ecotoxicologically based mechanistic models with the experimental data of pulsed Cu-tilapia system and (2) to provide a probabilistic risk approach to assess susceptibility risks for freshwater fish exposed to pulse metal stressors.

Materials and methods

Exposure experiments

Tilapia were cultured in the Graduate Institute of Ecology and Evolutionary Biology, China Medical University (Taichung, Taiwan). There were 45 fish used for experiment with body length of 10.09 ± 1.69 cm (mean ± SD) and body weight of 17.02 ± 9.80 g wet weight. During an acclimatization period of 14 days with a 12-h light cycle before Cu exposure experiment, tilapia were kept in the tanks of 81 L ($60 \times 30 \times 45$ cm^3) that are filled with 70.2 L tap water of Taichung. The water chemistry properties were as follows: Na^+ = 0.41 mM, K^+ = 0.07 mM, Mg^{2+} = 0.54 mM, Ca^{2+} = 1.45 mM, and dissolved oxygen = 7.5 mg L^{-1}. The water temperature and pH were 28 °C and 7.8, respectively. The adult tilapia were fed with the commercial fish feed at a ratio of 4% body weight twice a day during the pre-experimental and acclimatization periods.

The periods of Cu exposure bioassay were designed for a long-term 28 days. The experimental fish were exposed to 100 μg L^{-1} waterborne Cu as the background exposure concentration and 300 μg L^{-1} Cu as the pulsed exposure concentration. After a 14-day acclimatization period, 45 fish were equally distributed into three tanks (N = 15 per tank). The copper sulfate ($CuSO_4 \cdot 5H_2O$) (Shimada Chemical Works, Japan) stock solution was used to prepare exposure concentration with double-deionized water. To accomplish the pattern of sequential pulsed exposure, firstly, 300 μg L^{-1} Cu water was siphoned to one third of the original water level in the exposure tank and tap water was filled up to the original water level. Therefore, Cu concentration can decrease from 300 to 100 μg L^{-1}. The pulsed Cu exposure timings were occurred twice during the exposure periods at days 0.5–1.5 and 25–26 for long-term exposures, respectively (Supplementary Fig. S1). Tank water was refreshed weekly, and the water samples that were collected daily were analyzed for acquiring Cu concentrations during the exposure period. To keep water quality stable, fecal materials excreted from fish were siphoned every 6 h and forage debris removed after feeding for 1 h.

Sampling has been done at start of the exposure (day 0) and subsequently on days 0.5, 1.5, 4, 11, 14, 18, 21, 25, 26, and 28 for long-term exposures to assess the tissues and subcellular Cu accumulations. Fish were anesthetized with benzocaine hydrochloride solution during the sampling. Weights and lengths of fish were recorded in each sampling. The dissected organ/tissue samples (including the gills and the muscle) were cleaned with double-deionized water, and later respectively stored at −20 °C for analyzing organ/tissue Cu burdens and at −80 °C for analyzing Cu concentrations in subcellular fractions until further processing.

Subcellular partitioning process

The process for determining Cu subcellular distribution was adopted and modified from methods described by Wallace et al. (2003) and Geffard et al. (2010). The tissues that were previously frozen at −80 °C were thawed on ice for preparing homogenization. Partially thawed tissues (nearly 0.1-g wet wt) were gently homogenized with a motor-driven glass tissue grinder in 10 mM Tris-HCl buffer (containing 5 mM 2-mercaptoethanol and adding 1 N sodium hydroxide (NaOH) to adjust to pH 8.6) and added in a ratio of 1:4 (tissue (g) to

buffer (mL)) to each sample. The sample was kept on ice during homogenization.

The homogenate was first centrifuged at 1500×*g*, 4 °C for 15 min. The centrifugation was repeated twice to obtain the pellet containing cellular debris (P1) and metal-rich granules and the supernatant containing cytosol (S1). P1 was resuspended in double-deionized water (the half volume of Tris-HCl buffer) and heated to 100 °C for 2 min. Subsequently, an equal volume of 1 N NaOH was added and the mixture was incubated in a water bath at 65 °C for 60 min followed by centrifugation at 10,000×*g*, 4 °C for 10 min to isolate the NaOH-resistant fraction P2 (metal-rich granules). The resulting supernatant (S2) containing cellular debris and pellet containing metal-rich granules (P2) were kept at −80 °C until chemical analysis processing. The supernatant S1 was ultracentrifuged at 100,000×*g*, 4 °C, for 60 min to obtain a pellet (P3) containing organelles and a supernatant (S3) containing cytosol fraction. Finally, the ultraclear tubes containing supernatant (S3) were heated to 80 °C for 10 min in a water bath. Upon cooling on ice for 60 min, a final centrifugation at 30,000×*g*, 4 °C, for 20 min was done to obtain a pellet P4 (heat-labile proteins) and a supernatant S4 (metallothionein-like proteins).

Finally, pellets were filled with double-deionized water to mix well. All fractions (pellets P2, P3, and P4 and supernatants S2 and S4) were solubilized with 200 μL nitric acid (HNO_3) and then stored at −80 °C until quantifications of Cu concentration in samples.

Chemical analyses

The flame atomic absorption spectrometer (Perkin Elmer AA-200, USA) was used to analyze Cu organ/tissue burdens, digested solutions from subcellular partitioning processes, and water quality. Analytical quality control of the tissue sample was achieved by digesting and analyzing identical amounts of rehydrated (90% H_2O_2) standard reference material (dogfish muscle, DORM-2; NRC-CNRC, Canada). The waterborne ion concentrations, such as total Cu, Ca^{2+}, Mg^{2+}, Na^+, and K^+, were analyzed following the standard methods based on APHA (1998). Standard solutions of ions were used to establish standard curves (Merck, Darmstadt, Germany). The 15-mL water sample with 200 μL HNO_3 was digested for 2–3 h at 95 °C, then the water characterizations were determined by inductively coupled plasma mass spectrometer (ICP-MS) (Perkin Elmer ELAN DRC ROMAN II, USA). All samples were analyzed three times. The recovery rate was 94.6 ± 3.6%, and the levels of detection were 20 μg Cu L^{-1} for the water sample and 20 μg Cu g^{-1} for the tissue sample. The experimental materials used in the exposure experiment included glassware and plastic implements. All glassware and plastic materials were cleaned by immersion in 10% Decon for 1 day, then in 25% HNO_3 for 2 days, and finally rinsed with ultrapure water and dried. All experimental processes were performed by ultraclean technique during the sample manipulations.

Subcellular partitioning fractions were digested with 1 mL 75% HNO_3, 0.5 mL 30% hydrogen peroxide (H_2O_2), and 1 mL double-deionized water at 95 °C for 5 h until the digested solution became clear. After digestion, subcellular partitioning samples were diluted to 10 mL solution with double-deionized water. The 10-mL digested solutions of subcellular partitioning samples were stored at −4 °C in the dark until they were analyzed.

Data analyses

The Cu concentrations of the whole tissue and MAP were obtained by the sum of the Cu concentration of subcellular partitioning fractions. A first-order one-compartment toxicokinetic (TK) model was used to fit the Cu concentrations in the whole tissue and MAP for tilapia exposed to pulsed Cu. The TK parameters and uptake and elimination rate constants can be estimated as

$$C_b(t) = C_{b0}e^{-k_2 t} + \frac{k_1}{k_2}C_e(t)\left(1-e^{-k_2 t}\right), \quad (1)$$

where $C_b(t)$ is the Cu accumulation in the tissue varying with time t (μg g^{-1} wet wt), C_{b0} is the initial Cu concentration in the tissue (μg g^{-1} wet wt), k_1 is the uptake rate constant (mL g^{-1} day^{-1}), k_2 is the elimination rate constant (day^{-1}), and $C_e(t)$ is the pulsed Cu concentration of waterborne exposure (μg L^{-1}) described by the unit step function as

$$C_e(t) = C_0 + C_1[U(t-T_1)-U(t-T_2) + U(t-T_3)-U(t-T_4)], \quad (2)$$

where C_0 is the initial Cu concentration in water (μg L^{-1}), C_1 is the pulsed Cu concentration (μg L^{-1}), and $U(t - T_i)$ represents the unit step function with the pulsed timings T_i. In addition, the bioconcentration factor (BCF) can be estimated by the ratio of uptake rate and elimination rate (BCF = k_1/k_2).

The detoxification rate constant can be estimated by the metal influx threshold (MIT) model that was adopted and modified from Croteau and Luoma (2009). Croteau and Luoma (2009) proposed that the MIT is exceeded when metal influx equals or exceeds the combined efflux of metal loss and detoxification. Thus, once the metal influx in the tissue of aquatic organisms exceeds the threshold concentration in MAP, the mechanism of metal loss and detoxification will be triggered. However, Croteau and Luoma (2009) only considered the tissue's metal accumulation and did not take into account the internal distribution of metal in estimating detoxification rate constant.

Therefore, this study modified the original MIT model based on the concept of subcellular partitioning model (SPM) by two assumptions: (i) the metal concentration in MAP of the control group was used as the threshold active concentration because the MDP was found to accumulate metals even when organisms are exposed to very low metal concentrations and (ii) MAP is the first pool to accumulate metals during metal exposures (Wang and Rainbow 2006). Consequently, the detoxification rate constant can be estimated by metal flow effluxing to MDP from MAP. Based on the MIT model incorporated with the concept of SPM, the detoxification rate constant can be calculated as

$$k_{\mathrm{d}} = \frac{k_1 C_{\mathrm{e}}}{C_{\mathrm{b0}}} - k_{2,\mathrm{MAP}}, \tag{3}$$

where k_{d} is the detoxification rate constant (day^{-1}) and $k_{2,\mathrm{MAP}}$ is the metal elimination rate constant in MAP (day^{-1}).

For the sequential pulsed Cu exposure, due to the limitation of experimental data, the killing (k_{a}) and damage recovery rate constants (k_{r}) were obtained by directly fitting the susceptibility probability $S_{\mathrm{p}}(t)$ to the experimental data of proportion of Cu accumulated in MAP for tilapia. $S_{\mathrm{p}}(t)$ can be derived from an exponential function of cumulative hazard ($H(t)$) as (Ashauer et al. 2007; Lee et al. 2002)

$$\begin{aligned} S_{\mathrm{p}}(t) &= 1-\exp(-H(t)) \\ &= 1-\exp\left[-k_{\mathrm{k}}\frac{k_1}{k_2}C_{\mathrm{e}}(t)\left(\frac{e^{-k_{\mathrm{r}}t}-e^{-k_2 t}}{k_{\mathrm{r}}-k_2}+\frac{1-e^{-k_{\mathrm{r}}t}}{k_{\mathrm{r}}}\right)\right]. \end{aligned} \tag{4}$$

Dose-response model

A four-parameter Hill model was used in describing the relationships between Cu in the gill and the percentage of Cu in MAP as

$$E(C_{\mathrm{b}}) = E_{\min} + \frac{(E_{\max}-E_{\min})C_{\mathrm{b}}}{C_b^n + \mathrm{EC50}^n}, \tag{5}$$

where E is the effect of physiological response presented by Cu in MAP (%), C_b is the tissue's Cu burdens ($\mu\mathrm{g\ g}^{-1}$ wet wt), $E_{\min}$ and $E_{\max}$ are the minimum and maximum effects of physiological response, respectively (%), EC50 is the estimated concentration that is associated with 50% effect of the response, and n is the Hill coefficient.

Probabilistic susceptibility risk model

The dose-response profiles of the tissue's Cu burdens (C_{b}) were used as the conditional probabilities and can be expressed as $P(\%\ \mathrm{MAP}|C_{\mathrm{b}})$. This study used the distributions of the tissue's Cu burdens in tilapia exposed to waterborne Cu as the probability density functions (PDF). The susceptibility risks for the tissue can be estimated as the PDF of the tissue's Cu burden ($P(C_{\mathrm{b}})$) multiplied by the tissue-specific conditional probability of the percentage of MAP. Hence, the joint probability functions (JPF) can be used to calculate the susceptibility risk probabilities

$$P(R_{\%\mathrm{MAP}}) = P(C_{\mathrm{b}}) \times P(\%\ \mathrm{MAP}|C_{\mathrm{b}}), \tag{6}$$

where $P(R_{\%\mathrm{MAP}})$ represents the Cu susceptibility risk estimates based on the response of the percentage of Cu in MAP.

TableCurve 2D (version 5.0, AISN Software Inc., Mapleton, OR, USA) and Statistica® software (version 6.0, StatSoft, Tulsa, OK, USA) were used to perform the model fittings. Coefficient of determination (r^2) was used to describe the quality of the fit. To determine the uncertainty in all parameters, the distributions of parameters were generated by performing the Monte Carlo technique using Crystal Ball® software (version 2000.2, Decisionerring, Inc., Denver, Colorado, USA). The results showed that 10,000 iterations were sufficient to ensure the stability of results. The BLM used activities of competing, and complex ions were calculated by the WHAM (Windermere humic aqueous model) version 6 (WHAM VI, Center for Ecology and Hydrology, Lancaster, UK).

Results

Pulse Cu-tilapia subcellular partitioning

In the whole tissue and MAP of the gill, Cu accumulations varied with pulsed exposure concentrations (Fig. 1a, b). Experimental results showed that the rapid Cu accumulation was found at the first pulse of day 0.5, followed by a decline until the second pulse of day 25 (Fig. 1a, b). By the end of exposure, Cu accumulation in the tissue ($7.86 \pm 0.79\ \mu\mathrm{g\ g}^{-1}$ wet wt) (mean ± SD) showed values not obviously higher than those in the control ($6.50 \pm 0.46\ \mu\mathrm{g\ g}^{-1}$ wet wt) (Fig. 1a). In contrast with the gill, Cu accumulations of the muscle did not vary with pulsed exposure concentration significantly (Fig. 1c). Results showed that Cu accumulations in the whole tissue of the muscle and in MAP ranged from 1.9 to 3.7 and 0.5 to 1.5 $\mu\mathrm{g\ g}^{-1}$ wet wt, respectively (Fig. 1c, d).

It was observed that the percentage of Cu in MAP increased with time from 23 to 57%, whereas the percentage of Cu in MDP decreased with time from 50 to 26% during the pulsed Cu exposure in the gill (Fig. 1e, f). Results indicated that the percentage of Cu in the gill MAP sharply increased from 38 to 62% at day 26 (Fig. 1e). In contrast to the gill, Cu distributions between MAP and MDP did not change with the exposure pattern in the muscle, indicating that the percentages of Cu accumulated in MAP and MDP were 20–40 and 34–48%, respectively (Fig. 1g, h).

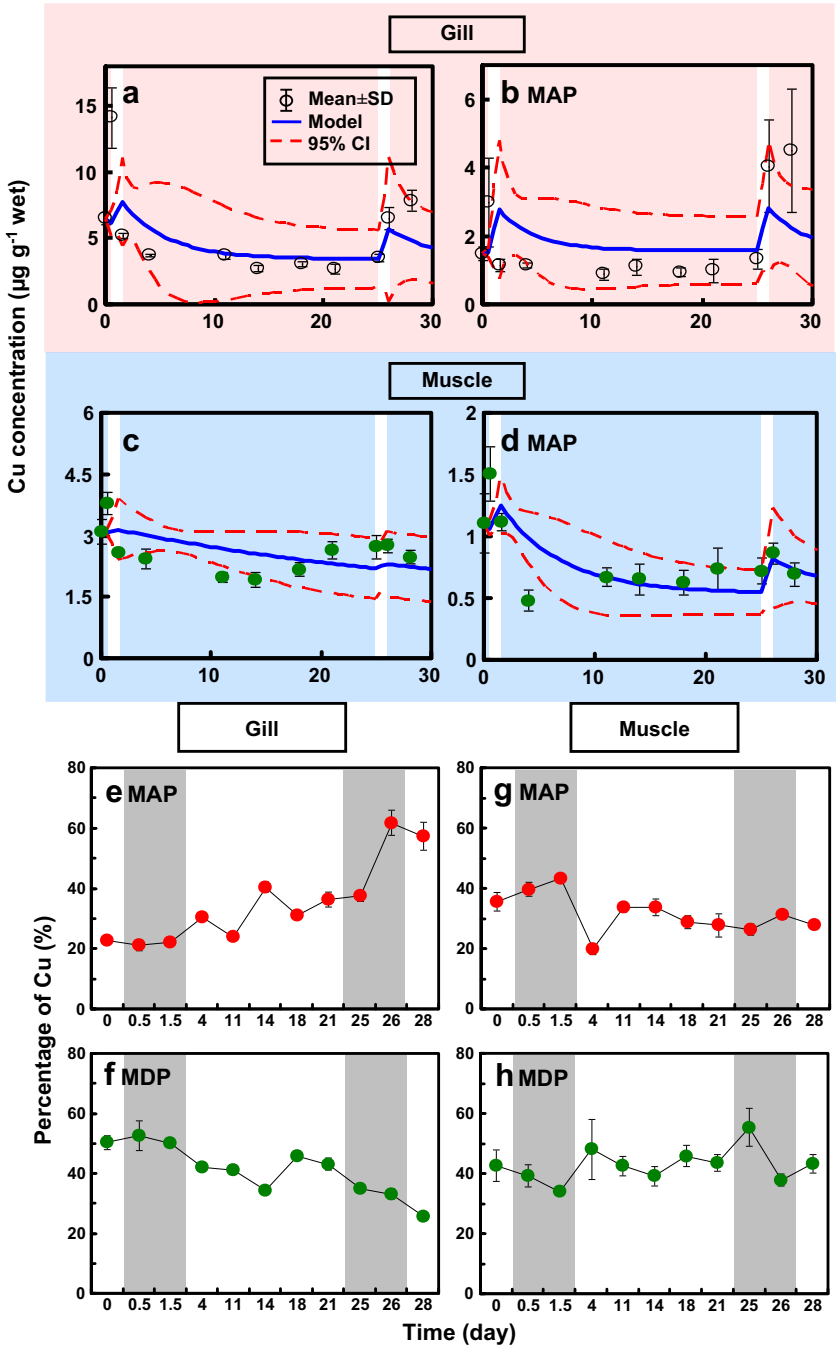

Fig. 1 Fitted TK model to experimental data of the tissues and MAP in **a**, **b** gill and **c**, **d** muscle for tilapia exposed to pulsed waterborne Cu during an exposure period of 28 days. *White shadow* represents the pulsed period. Time-varying percentages of Cu accumulation in MAP and MDP of gill (**e**, **f**) and of muscle (**g**, **h**) for tilapia exposed to waterborne pulsed Cu. All data represent mean ± SD, $n \geq 3$ for each sample of tissues of fish

For the gill, the same pattern of total Cu accumulation was found in the Cu concentration of MAP, although with lower uptake and higher excretion, in which uptake rate ($k_{1,\mathrm{MAP}}$) and elimination rate ($k_{2,\mathrm{MAP}}$) constants were calculated to be 4.86 ± 4.20 mL g^{-1} day^{-1} and 0.308 ± 0.307 day^{-1} ($r^2 = 0.20$, $p = 0.16$) (Fig. 1b, Table 1). Interestingly, in contrast with the gill, the TK parameter estimates of MAP in the muscle were relatively higher than those in the tissue (Table 1). Based on the MIT model with TK parameters (Eq. (3)), the k_d estimates were 0.33 and 0.24 day^{-1} in the gill and the muscle, respectively, where C_e used in Eq. (1) was 114 μg L^{-1} that was estimated by averaging Cu concentrations during the period of pulsed Cu exposure (Table 1).

Maximum Cu concentration in the gill was found at the first pulse of day 0.5 and was approximately 1.5–9.5-fold above Cu concentrations at other sampling times in most subcellular fractions (Fig. 2a–e). The Cu concentrations for all subcellular fractions increased after the second pulsed exposure except cellular debris fraction in the gill. However, increase in cellular debris fraction occurred only during the first pulse of day 0.5. For the muscle, only soluble fractions (organelles, heat-labile proteins, and metallothionein-like

Table 1 Fitted parameter estimates of toxicokinetic and metal influx threshold models for tilapia exposed to pulsed waterborne copper

	Gill	Muscle
TK model		
k_1 (mL g^{-1} day^{-1})	8.38 ± 9.65[a]	0.408 ± 1.356
k_2 (day^{-1})	0.244 ± 0.289	0.031 ± 0.061
BCF (mL g^{-1})	34.34 ± 33.39	13.16 ± 22.23
r^2	0.24	0.044
p value	0.13	0.56
MIT model		
$k_{1,MAP}$ (mL g^{-1} day^{-1})	4.86 ± 4.20	0.983 ± 0.634
$k_{2,MAP}$ (day^{-1})	0.308 ± 0.307	0.182 ± 0.112
r^2	0.20	0.39
p value	0.16	0.04
k_d (day^{-1})	0.333	0.238

[a] Mean ± standard error

proteins) showed Cu accumulation associated with the exposed concentration (Fig. 2f–j). In both the gill and the muscle, the highest Cu concentration in subcellular fraction was found in metal-rich granule fraction (Fig. 2).

For Cu percentages of total accumulation in different subcellular fractions, metal-rich granule fraction contained the relatively higher percentages of 11.5–40.4% in the gill and 13.2–36.1% in the muscle, whereas metallothionein-like protein fraction obtained minimum Cu percentages of 5.8–16.3% in the gill and 7.4–21.0% in the muscle (Fig. 3). Percentage of Cu in the metal-rich granule fraction of the gill decreased steeply from 39.7 to 11.5% during the exposure time, whereas heat-labile protein fraction contribution to total Cu accumulation increased obviously with time from 9.6 to 32.8% (Fig. 3a–e). On the other hand, there was no significant difference in Cu accumulation distribution found in response to the pulsed exposure in all subcellular fractions of the muscle (Fig. 3f–j).

MAP-based Cu susceptibility risk applications

This study used the elevated proportion of Cu in MAP of the gill that was induced by pulsed Cu exposure as the susceptible effect and fitted the equation of susceptibility probability to obtain the key physiological parameters (Eq. (4)) (Fig. 4a). However, experimental results showed that the proportion of Cu in MAP of the muscle did not increase with time. Therefore, this study cannot obtain the key parameter estimates in the muscle (Fig. 4b). The key parameter estimates of k_k and k_r were 0.085 ± 0.056 g μg^{-1} day^{-1} and 1.33×10^{-3} ± 0.06 day^{-1} in the gill, respectively ($r^2 = 0.84$, $p < 0.01$) (Fig. 4a).

Thus, we found that the gill can immediately reflect the environmental exposure tendency. Therefore, for constructing the risk assessment scheme, we used the relationships between the proportion of Cu accumulation in MAP and Cu in the gill as the dose-response profile (designed as $P(\%\ \mathrm{MAP}|C_b)$) for assessing tilapia-pulsed waterborne Cu susceptibility. The dose-response profile was described by a four-parameter Hill model with the minimum effect E_{min} = 33.20 ± 2.43% (mean ± SE), maximum effect E_{max} = 71.35 ± 16.03%, Hill coefficient n = 6.35 ± 5.13, and EC50 = 8.42 μg g^{-1} wet wt (95% CI 4.88–11.95) ($r^2 = 0.41$, $p < 0.01$) (Fig. 4c).

The Cu in aquaculture pond adopted from Lin (2009) was used as the waterborne exposure concentration. The range of Cu concentrations in water was 33.1–265.2 μg L^{-1} and can be described by a lognormal distribution of LN(56.03 μg L^{-1}, 2.20) (Fig. 5a). Figure 5b shows that the distribution of Cu burden in the gill ($P(C_b)$) was LN(1.92 μg g^{-1} wet wt, 2.20) that was obtained by multiplying waterborne Cu concentrations by the BCF of 34.34 mL g^{-1} (Table 1). Eventually, the exceedence probability of the gill's Cu susceptibility risk based on the constructed dose-response profile (Fig. 4c) can then be calculated following Eq. (6) (Fig. 5c).

It is not surprising that the results indicated that there was no significant susceptibility risk for tilapia posed by aquaculture Cu concentration in Taiwan (Fig. 5c). The probability that 50% or more of the tilapia susceptibility risks in response to Cu exposure was 33.2% (95% CI 33.2–33.3%) of Cu in MAP which had no obvious difference with the minimum effect (E_{min} = 33.20% Cu in MAP) was obtained from the dose-response profile.

Discussion

Pulse Cu subcellular partitioning in freshwater fish

Due to the limited information on effects of pulsed Cu exposure in subcellular partitioning, the estimated results were mostly based on constant metal exposures. For the tissue level, the experimental data showed that total tissue Cu concentration was positively affected by environmental Cu concentrations. The gill possessed higher Cu concentration in response to pulsed Cu exposure, which was consistent with Kamunde and MacPhail (2008). In addition, in the relationship between the tissue's Cu concentration and the percentage of Cu in MAP, Cu in MAP increased with the increasing Cu concentration in the gill, whereas the muscle showed no significant relationship, implicating that the gill is regarded as one of the target tissues of waterborne Cu toxicity. On the other hand, the muscle had relatively lower capacity of Cu accumulation, implying that it is an appropriate part of the fish tissues for human food consumption.

As the tissue's Cu concentration increased, concentrations of Cu increased in all subcellular fractions. The metal-rich granule fraction afforded the largest average contribution to Cu accumulation of nearly 30% in the gill and muscle, indicating that

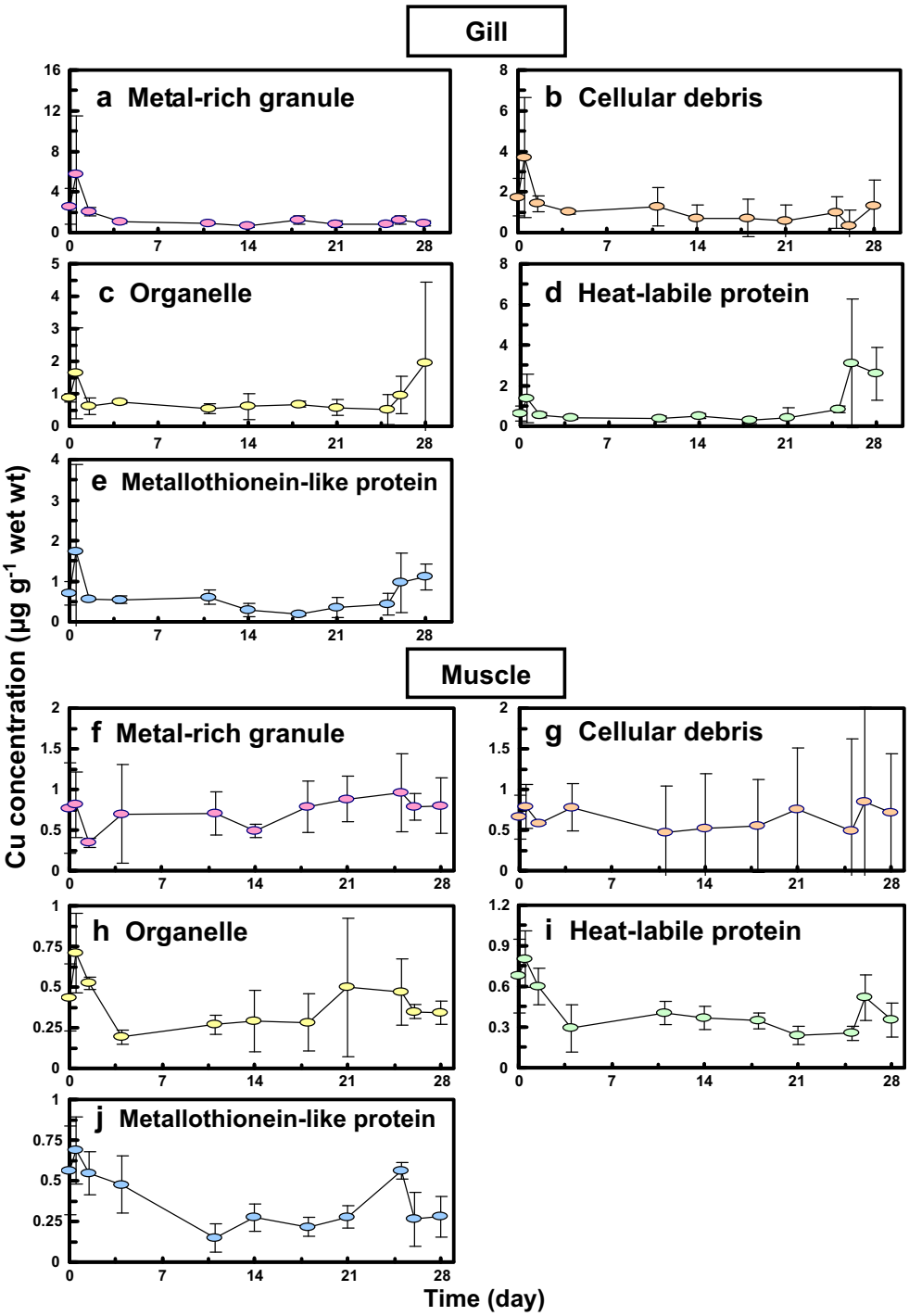

Fig. 2 Time-varying Cu accumulations in subcellular partitioning fractions of metal-rich granule, cellular debris, organelle, heat-labile protein, and metallothionein-like protein in gill (**a**–**e**) and in muscle (**f**–**j**) for tilapia exposed to waterborne pulsed Cu. All data represent mean ± SD, $n \geq 3$ for each sample of tissues of fish, except for day 0 ($n = 2$) for muscle

metal-rich granule plays an important role in detoxifying Cu in tilapia. However, Giguère et al. (2006) observed the largest contribution (~40%) of metallothionein-like protein fraction to hepatic Cu burden in yellow perch (*Perca flavescens*) exposed to environmental metals. Nevertheless, Rainbow (2002) and Vijver et al. (2004) pointed out that due to the strategies of metal handling varying among the tissues in different aquatic organisms, a more critical role of metal-rich granules in fish metal homeostasis cannot be ignored.

It has been reported that, for some invertebrate species, mineral concretions composed primarily of Ca and Mg phosphates can bind metals and play important roles in detoxifying and eliminating metals (Bonneris et al. 2005; Mason and Jenkins 1995). Moreover, Wallace et al. (1998) and Rainbow et al. (2004) indicated that metal-rich granules are more durable and less likely to be released during cellular cycling than other subcellular fractions such as heat-labile proteins and organelles.

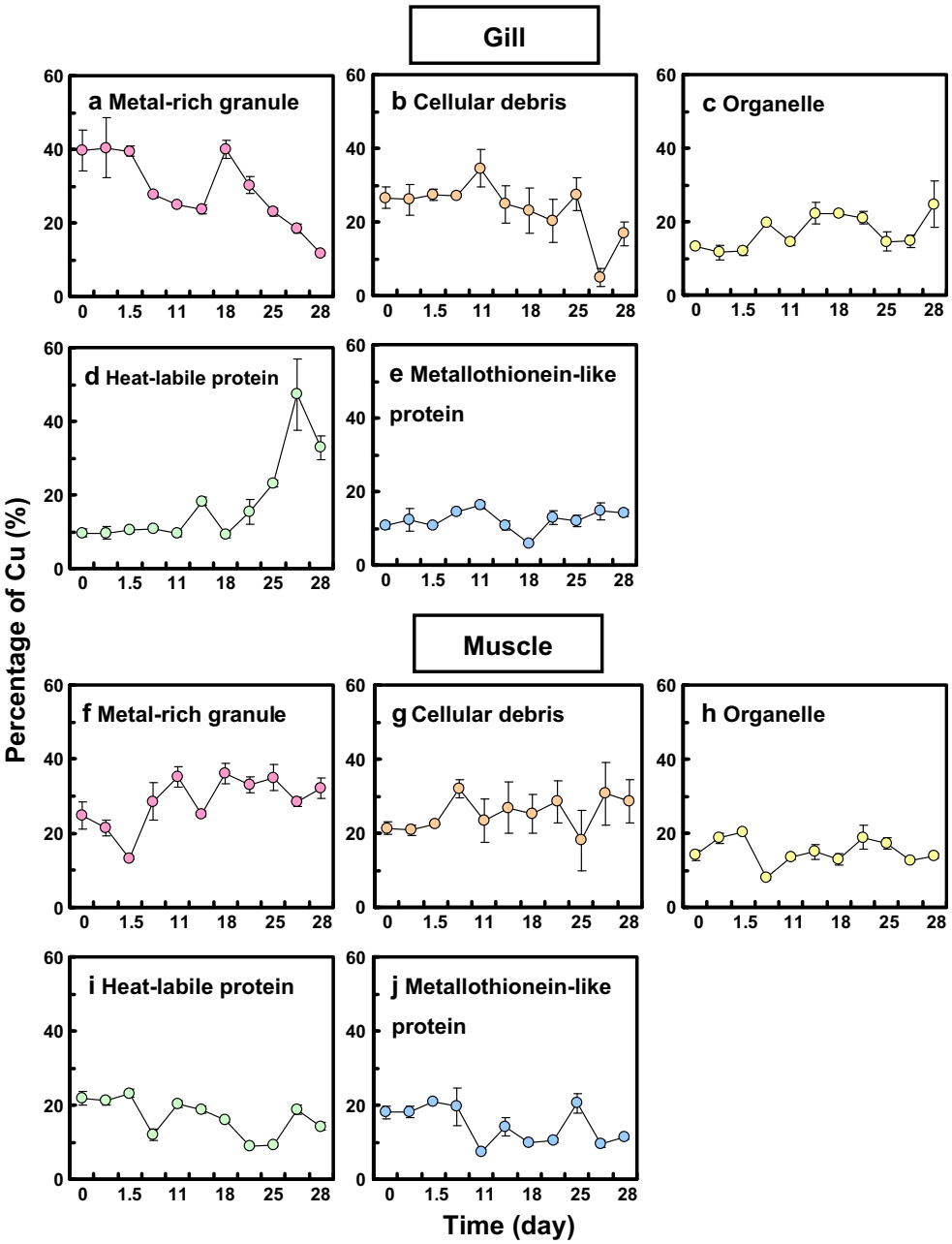

Fig. 3 Time-varying percentages of Cu accumulations in subcellular partitioning fractions of metal-rich granule, cellular debris, organelle, heat-labile protein, and metallothionein-like protein in gill (**a**–**e**) and in muscle (**f**–**j**) for tilapia exposed to waterborne pulsed Cu. All data represent mean ± SD, $n \geq 3$ for each sample of tissues of fish, except for day 0 ($n = 2$) for muscle

Although nearly one third of Cu that was accumulated in the metal-rich granule belongs to the part of MDP, the increasing proportion of Cu accumulation in MAP was observed obviously over time at the gill, notably in the second pulsed time. It may be due partly to the fact that metals bind to metal-rich granules at the beginning, and subsequently, metal-bound granules are eliminated from the cell (Gibbs et al. 1998), decreasing the fractions of metal in granules. On the other hand, accumulating Cu in MAP implies that Cu could not be completely regulated, and consequently, Cu accumulation could possibly lead to toxicity for fish exposed to the pulsed Cu concentration. The fraction of heat-labile protein attained a threefold increase in Cu concentration and increased nearly to 40% of the total tissue Cu burden in the gill. For the muscle, concurrent Cu accumulations in MAP and MDP were found over time and there was no significant change in the distribution as a consequence of pulsed Cu exposure.

Differential centrifugation has been widely used as an approach for investigating internal speciation of metals in aquatic organisms (Blanchard et al. 2009; Kamunde and MacPhail 2008; Kamunde 2009; Ng and Wood 2008; Pan and Wang 2008; Sappal et al. 2009; Serafim and Bebianno 2007). The present method divides the internal metals into different fractions of subcellular compartmentalization and classifications of MAP and MDP (Kamunde and MacPhail 2008; Rainbow 2002). There are some uncertainties that may be due to the

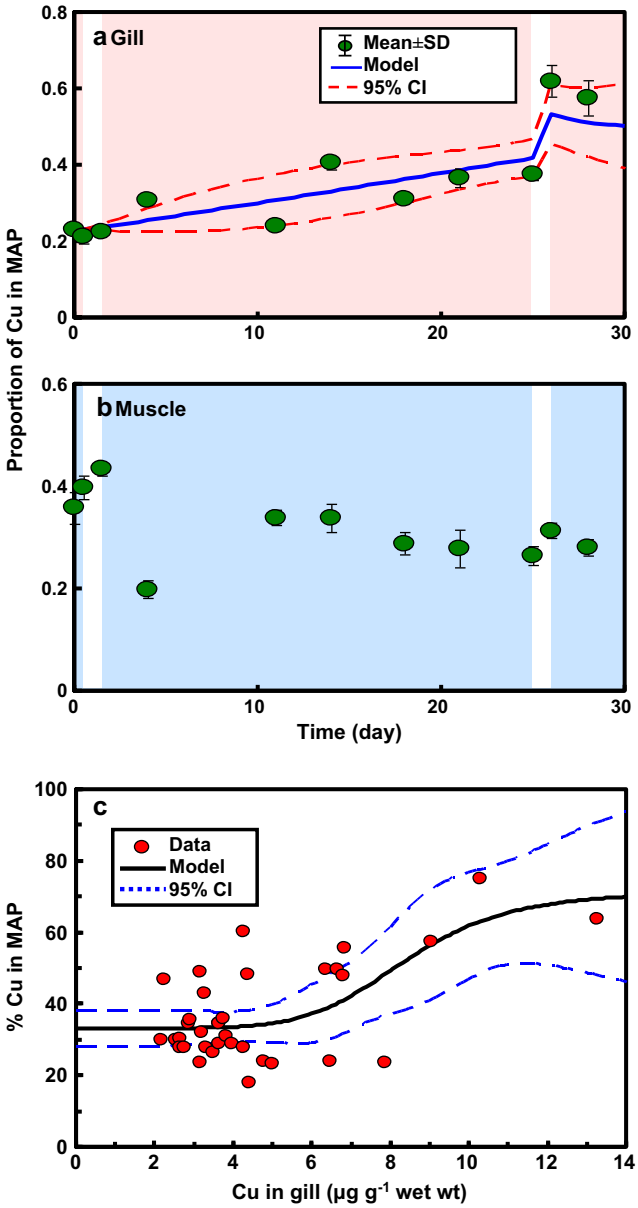

Fig. 4 **a** Fitting susceptibility function to experimental data in gill and **b** origin experimental data in muscle for tilapia exposed to pulsed waterborne Cu of 28 days. *White shadow* represents the pulsed period. **c** Hill fitting model describing the relationships between the percentages of Cu in tissues of gill and in MAP

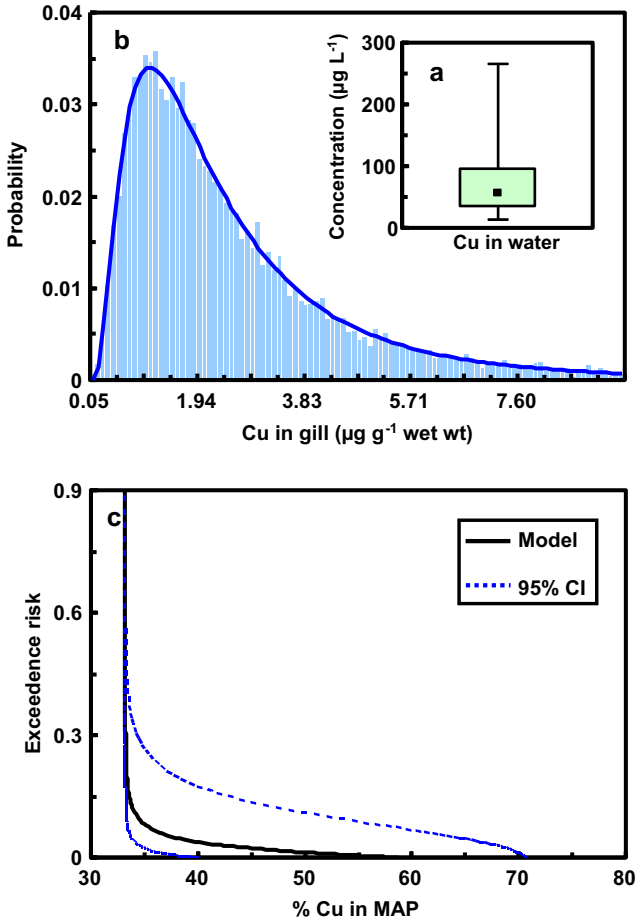

Fig. 5 **a** Copper concentrations of aquaculture pond in Taiwan showing a lognormal distribution with geometric mean of 56.03 μg L^{-1} and geometric standard deviation of 2.20. **b** The Cu concentrations of gill was estimated by multiplying waterborne Cu concentration by the bioconcentration factor (BCF). **c** Exceedence risk probability of the percentage of Cu in MAP for gill

complicated process of subcellular centrifugation, such as breakage or clumping of particles, leakage of soluble constituents from organelles, and overlap among subcellular fractions (Wallace et al. 2003). Moreover, dividing all subcellular fractions into two categories (i.e., MAP and MDP) is likely to be oversimplified in the strategy of metal accumulation.

Limitations and implications

Four key insights were provided in this study. First, the previously known toxicokinetics under pulsed exposure of Cu are combined to interpret ecophysiological responses to environmental variability. Second, the overall control design for aquatic organisms in response to pulsed toxicant exposure depending on quantitative system characteristics can be achieved by controlling pulse frequency and duration. Third, at the tissue level, pulsed waterborne Cu exposure resulted in significant Cu accumulations in the gill, whereas accumulations in the muscle were only at the first pulsed time of day 0.5. The result implies that the gill was susceptible to pulsed Cu exposure, and the muscle is not a suitable tissue to monitor environmental Cu concentrations in tilapia. Fourth, subcellular Cu distributions in the gill were characterized by the majority of accumulated Cu partitioned in MDP at the beginning of Cu exposure. Subsequently, the percentage of Cu in MAP was increased over time.

Our study established a direct link between acute/chronic exposure measurements and biodynamic models. We expect that the dynamic data along with subcellular partitioning analysis presented here could be applied for further computational

analyses such as multivariate statistics and large-scale structural or toxicokinetic models (Clements et al. 2013; Görlitz et al. 2011). We also hope that our system-level tools for mathematical analyses and modeling will facilitate future large-scale and dynamic systems biology studies not only for tilapia but also for the generic feature of model aquatic organisms.

Conclusions

We have developed a robust and integrated ecotoxicological model to predict susceptibility risks of pulse Cu exposures by incorporating subcellular partitioning with TK/TD- and BLM-based damage assessment models. This study implicates that the current risk assessment framework is readily amendable by considering pulse conditions with assistance of mechanistic models that offer a new insight into the understanding of major environmental disturbances caused by fluctuating stressors. We anticipate that this approach could be quantitatively coupled with experimental results to prevent reduction of freshwater fish under pulse metal exposures.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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