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Synergistic toxic effects of zinc pyrithione and copper to three marine species: Implications on setting appropriate water quality criteria

Vivien W.W. Bao [a,*], Kenneth M.Y. Leung [a,*], Kevin W.H. Kwok [a], Amy Q. Zhang [a], Gilbert C.S. Lui [b]

[a] The Swire Institute of Marine Science, Division of Ecology and Biodiversity, School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong, China
[b] Department of Statistics and Actuarial Science, The University of Hong Kong, Pokfulam, Hong Kong, China

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ABSTRACT

Zinc pyrithione (ZnPT) is widely applied in conjunction with copper (Cu) in antifouling paints as a substitute for tributyltin. The combined effects of ZnPT and Cu on marine organisms, however, have not been fully investigated. This study examined the toxicities of ZnPT alone and in combination with Cu to the diatom *Thalassiosira pseudonana*, polychaete larvae *Hydroides elegans* and amphipod *Elasmopus rapax*. Importantly, ZnPT and Cu resulted in a strong synergistic effect with isobologram interaction parameter $\lambda > 1$ for all test species. The combined toxicity of ZnPT and Cu was successfully modelled using the non-parametric response surface and its contour. Such synergistic effects may be partly due to the formation of copper pyrithione. It is, therefore, inadequate to assess the ecological risk of ZnPT to marine organisms solely based on the toxicity data generated from the biocide alone. To better protect precious marine resources, it is advocated to develop appropriate water quality criteria for ZnPT with the consideration of its compelling synergistic effects with Cu at environmentally realistic concentrations.

1. Introduction

Since the partial ban on the application of organotin biocides, especially tributyltin (TBT) in antifouling paints for small marine vessels (<25 m in length; Readman et al., 2002), a new generation of surrogate antifouling biocides such as zinc pyrithione (ZnPT), copper pyrithione (CuPT), Irgarol 1051, diuron and Sea-nine 211 have been increasingly used (Yebra et al., 2004). These surrogate biocides are often applied in conjunction with copper (Cu) compounds such as cuprous oxide, Cu thyocyanate or metallic Cu to control Cu-resistant fouling organisms (Voulvoulis et al., 2002). Today, Cu contamination in coastal marine environments is universal; and one main source of Cu to the marine environment is Cu-containing antifouling coatings on ship hulls (Srinivasan and Swain, 2007). As a consequence of the restricted use of TBT, the use of Cu-based antifouling biocides has been dramatically increased leading to elevated levels of Cu in coastal waters and sediments (Schiff et al., 2007). This problem is evident in many coastal areas, particularly busy ports such as those in the United Kingdom where the waterborne Cu level could be as high as 20 μg/L (Matthiessen et al., 1999) and marinas of the San Diego region where the dissolved Cu concentrations in the surface water samples reached an average of 8.5 μg/L (Schiff et al., 2007).

Different biocides often coexist with Cu in the marine environment, especially in coastal areas with heavy Cu contamination. Understanding the interactions between different biocides (i.e. additive, synergistic or antagonistic effect) is necessary in ecotoxicology and ecological risk assessment (Hertzberg and Macdonell, 2002). Therefore, consideration solely based on the toxicity of the surrogate biocide alone is insufficient to determine its ultimate environmental impacts. The combined effect of Cu and the surrogate biocides should be of great concern, especially in the coastal areas with high shipping activities (i.e. higher chances for being contaminated with both Cu and other biocides).

Zinc pyrithione (zinc complex of 2-mercaptopyridine-1-oxide; ZnPT), one of the most popular surrogate antifouling biocides, has long been widely used as algaecides, bactericides and fungicides and is well known for its application in antidandruff shampoos (Yebra et al., 2004). It was introduced to the market as a substitute of organotin biocides by Arch Chemicals (Norwalk, CT, USA) in 1991, and is currently viewed as the top prospect for replacing TBT in antifouling paints (Doose et al., 2004). ZnPT-based antifouling products are widely applied on yachts and large ocean-going vessels in Europe, Japan and South Korea, and ZnPT is also one of the most commonly used products in the United Kingdom on small boats (<25 m in length; Thomas, 1999).

ZnPT was found to be highly toxic to aquatic plants and animals (Turley et al., 2000), but it was assumed to be environmentally

* Corresponding authors. Tel.: +852 2809 2179; fax: +852 2809 2197 (V.W.W. Bao).
E-mail addresses: weiwei.bao@gmail.com (V.W.W. Bao), kmyleung@hkucc.hku.hk (K.M.Y. Leung).

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neutral because it could easily photo-degrade to less toxic compounds (Turley et al., 2000, 2005). However, Bellas (2005) demonstrated that the toxicity of ZnPT only decreased but did not disappear after exposure to direct sunlight. ZnPT is also suggested to persist in the marine environment where the influence of the light is limited such as waters and sediments shaded under parking vessels in marinas and harbours (Maraldo and Dahllöf, 2004), and it may accumulate in the sediment as manganese pyrithione and CuPT (Galvin et al., 1998).

Although ZnPT is largely used with Cu in antifouling paintings worldwide, the combined effects of ZnPT and Cu to marine organisms are still largely unknown, except those studies on the bioluminescent bacteria *Vibrio fischeri* (Zhou et al., 2006), the sea bream *Pagrus major* and the toy shrimp *Heptacarpus futilirostris* (Mochida et al., 2006), as well as the diatom *Chaetoceros gracilis* (Koutsaftis and Aoyama, 2006). There is a paucity of data on the toxicity of ZnPT alone or in combination with Cu to marine species.

The objective of this study was to investigate the acute toxicities of ZnPT alone and in combination with Cu ($CuSO_4$) to three marine species including the diatom *Thalassiosira pseudonana*, polychaete tubeworm *Hydroides elegans* and amphipod *Elasmopus rapax*. The results are essential for evaluating the combined toxicity of these two biocides to marine organisms. We also applied and compared two different approaches, namely the isobologram and the non-parametric response surface methods (Gessner, 1995; Greco et al., 1995), for modelling the binary mixture toxicity of ZnPT and Cu.

2. Materials and methods

Standard toxicity tests of ZnPT and Cu alone were first conducted for each species, and the median lethal concentrations (LC50, for the polychaete *H. elegans* and the amphipod *E. rapax*) or median effect concentrations (EC50, for the diatom *T. pseudonana*) of ZnPT and Cu alone were determined based on the test results. Suitable ZnPT and Cu concentrations were chosen according to their LC50 or EC50 values for the binary mixture toxicity tests of the two biocides. For better comparison of the toxicities between the single biocide and binary mixture, the toxicities of ZnPT or Cu alone to the three test organisms were assessed again at the same time with the binary mixture toxicity tests. All concentrations were nominal. The concentrations selected for the toxicity tests of ZnPT and Cu alone, and the binary toxicity of ZnPT and Cu, as well as the number of replicates for each of the treatments are shown in Table 1. Filtered artificial seawater (FAS; sea salt: Tropic Marine, Germany; salinity 33 ± 0.5‰, pH 8.1–8.4, filtered through 0.45 μm membrane filter) was used for all the toxicity tests. Tests were carried out at 25 ± 1 °C while the test solutions were kept at salinity 33 ± 0.5‰ and pH 8.1–8.4 throughout the test.

Table 1
The nominal concentrations selected for the toxicity tests of ZnPT or Cu alone and the binary toxicity tests of these two biocides, as well as the number (No.) of replicates for each of the treatments, to *Thalassiosira pseudonana*, *Hydroides elegans* and *Elasmopus rapax*

Test species	ZnPT (μg/L)	Cu (μg/L)	No. of replicates
T. pseudonana	0 (C)	0	3
	0 (SC, 0.8 ppm)	0	3
	2, 4, 8	0	3
	0	10, 50, 200, 500, 800	3
		1000	5
		1300, 1800	2
	2, 4, 8	10	3
	2, 4, 8	50	3
	2, 4, 8	200	3
H. elegans	0 (C)	0	3
	0 (SC, 0.6 ppm)	0	3
	2, 3, 4, 5, 6	0	3
	0	60, 90, 125, 150,180	3
	1.3	12.5, 62.5, 125	5
	2.6	12.5, 62.5, 125	5
	0.2, 0.4, 1, 2, 3	42	5
	0.2, 0.4, 1, 2, 3	84	5
E. rapax	0 (C)	0	3
	0 (SC, 10 ppm)	0	5
	5, 10, 20, 50, 100	0	3
	0	10, 20, 50, 200	3
		100	6
	5, 10, 15, 20	10	3
	5, 10, 20	20	3

C, seawater control; SC, solvent control, with carrier solvent DMSO at ppm (v/v) level.

2.1. Chemical preparation

A stock solution of ZnPT (10 g/L) was made by dissolving ZnPT (approx 95%; Sigma, USA) in dimethyl sulfoxide (DMSO; ACS reagent, ⩾99.9%; Sigma, USA). A stock solution of Cu (1 g Cu/L) was prepared by dissolving copper(II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$, formula weight 249.7; purity ⩾99.5%; BDH Chemicals Ltd. Pode, England) in distilled water. The stock solution was further diluted in FAS in volumetric flasks to obtain working solutions at designated nominal concentrations before dosing.

2.2. Test organisms

A pure *T. pseudonana* culture was obtained from the Department of Biology and Chemistry, City University of Hong Kong, Hong Kong and kept at controlled laboratory conditions with 16 h:8 h light:dark photoperiod in autoclaved f/2-Si medium (Guillard's medium for diatoms, Guillard and Ryther, 1962). Adults of *H. elegans* were collected from a fish farm at Sam Pui Chau, Sai Kung, Hong Kong and acclimated in an aquarium tank with mild water flow and aeration. They were fed with concentrated phytoplankton (Phytoplex, Kent Marine, USA) and diatom *Skeletonema costatum* on a daily basis. Juveniles of *E. rapax* were collected from the aquarium of the Swire Institute of Marine Science, Cape d'Aguilar, Hong Kong and acclimated in the laboratory for 24 h before the acute toxicity test.

2.3. Algal growth inhibition test

Cell concentration of a fresh *T. pseudonana* culture at exponential growth phase (<1 week old) was determined using a haemocytometer (Neubauer Improved, Precicolor HGB, Germany). According to the result, an appropriate amount of diatom culture was added to each 10 mL autoclaved test vial (with autoclaved plastic lids) with 5 mL autoclaved f/2-Si median to obtain an initial algal concentration of 10^4 cells/mL. Different levels of ZnPT or Cu (or both) were then dosed into the vials, which were then randomly positioned on a titer plate shaker (Lab-line, Melrose Park, USA) at 40 rpm under 16 h:8 h light:dark photoperiod (light intensity: ~920 lux; measured by Lux/Fc light meter, Tenmars, China) for 96 h. Then 200 μl solutions from each test vial were pipetted into a 96-well microplate (Nunc, Denmark) and fixed with 66.7 μl HCl (1 M) for subsequent counting using the haemocytometer.

2.4. Acute toxicity test for polychaete larvae

Trochophore larvae of *H. elegans* were used to assess toxicity of ZnPT and Cu independently and combined, using 48 h static acute toxicity tests which were conducted in 25 compartment square petri dish (Sterilin, UK; each compartment had a volume of ~6.5 mL).

Established procedures by Qiu and Qian (1997) were followed to obtain the trochophore larvae. Each replicate consisted of 20 larvae. Mortality of larvae was determined at the end of the 48 h test.

2.5. Acute toxicity test for the amphipod juveniles

To get a general idea about the size of the amphipod used for the experiment, 30 acclimated juvenile *E. rapax* were randomly selected for a linear measurement from the proximal end of the first thoracic segment to the distal end of the fourth thoracic segment using a stereo microscope fitted with an ocular reticle (Hacker and Steneck, 1990). The measured length ranged from 0.36 to 0.85 mm with an average of 0.54 mm (95% C.I.: 0.50–0.58 mm). Each replicate consisted of 10 individuals in 100 mL FAS. Test solutions were renewed once at 48 h. Mortality was monitored every 24 h by observing reaction of amphipods to mild agitation with a glass pipette under a stereo microscope.

2.6. Data analyses

LC50 (or EC50) values of ZnPT and Cu alone to each of the three species were determined by the non-linear regression of the sigmoidal dose response function (four-parameter logistic regression, GraphPad Prism version 5.00, GraphPad Software, CA). The results of combined toxicity tests were firstly analyzed using the Isobologram method (Prakash et al., 1996). ZnPT and Cu concentrations that caused the combined toxicity effect equalled to 50% of the control (e.g. 50% survivorship = 50% mortality for *H. elegans* and *E. rapax*; 50% growth of the control for *T. pseudnonana*) were fitted with the following regression function:

$$\mathrm{TU}_i^{\mathrm{Cu}} = [1 - (\mathrm{TU}_i^{\mathrm{ZnPT}})^{1/\lambda}]^{\lambda} + v_i.$$

Although the specification above may not look familiar, without consideration of error term v_i above will yield the following isobole:

$$(\mathrm{TU}_i^{\mathrm{Cu}})^{1/\lambda} + (\mathrm{TU}_i^{\mathrm{ZnPT}})^{1/\lambda} = 1,$$

where $\mathrm{TU}_i^{\mathrm{Cu}}$ and $\mathrm{TU}_i^{\mathrm{ZnPT}}$ are toxic units for Cu and ZnPT respectively, i.e., $\mathrm{TU}_i^{\mathrm{Cu}} = c_i^{\mathrm{Cu}}/\mathrm{LC50}^{\mathrm{Cu}}$ (or $\mathrm{EC50}^{\mathrm{Cu}}$) and $\mathrm{TU}_i^{\mathrm{ZnPT}} = c_i^{\mathrm{ZnPT}}/\mathrm{LC50}^{\mathrm{ZnPT}}$ (or $\mathrm{EC50}^{\mathrm{ZnPT}}$), c_i^{Cu} and c_i^{ZnPT} are the concentrations of Cu and ZnPT respectively, and LC50's (or EC50's) represent the LC50 (or EC50) of corresponding substances. The similarity parameter λ in the regression function above was estimated by the non-linear least squares approach using Matlab (The MathWorks Inc., USA). The λ of the isobole indicates the toxicity interaction of the two substances. When $\lambda = 1$, the interaction is simple additive; when $\lambda > 1$, the interaction is synergistic, vice versa.

Secondly, the non-parametric response surface and its contour were constructed using the average data of each treatment, and computed using the PROC G3Grid with (for *H. elegans* and *E. rapax*) and without spline (for *T. pseudonana*, as there was no observable difference between the surfaces plotted with and without spline) and PROC GCONTOUR procedures in SAS 9.1.3, SAS Institute Inc., Cary, NC. For the response surface with spline, the experimental observations were fitted with a bivariate fifth-degree polynomial spline while the smoothness of spline was maintained by a polynomial up to order three, i.e. the function,

$$p_i = U(x_i^{\mathrm{Cu}}, x_i^{\mathrm{ZnPT}}) = \sum_{j=0}^{5} \sum_{k=0}^{5-j} q_{jk} (x_i^{\mathrm{Cu}})^j (x_i^{\mathrm{ZnPT}})^k$$

is estimated by the least squares approach subject to the constraints of smoothness, where x_i^{Cu} and x_i^{ZnPT} are concentrations of Cu and ZnPT, respectively, p_i is the control rates for *T. pseudonana* or mortality rates for *H. elegans* and *E. rapax* and q_{jk}'s are unknown coefficients being estimated. In the cases where smoothing spline was used, the penalized least squares approach was used for estimation instead. The details of spline fitting for the response surface are referred to Akima (1978) and Wahba (1979). The horizontal lines connecting the edges of the response surfaces at 20%, 40%, 50%, 60% and 80% of control (or mortality) levels were presented in contour plots. A straight diagonal NW-SE isobol in a contour plot would be consistent with the Loewe additivity (same as the additivity line in isobologram), while a downward bowed isobol represents the Loewe synergism, vice versa (Sühnel, 1990; Greco et al., 1995).

2.7. Confirmation for the transchelation of ZnPT and Cu

In order to confirm whether the transchelation between ZnPT and Cu occurred when they encountered each other in seawater, ZnPT and Cu (as Cu^{2+} in $CuSO_4 \cdot 5H_2O$) with different ratios of molar concentrations ([ZnPT]:[Cu] = 0:0 (seawater control), 1:0, 0:1, 1:0.5, 1:1, 1:2 and 1:4, where 1 stands for a molar concentration of 315 nM; $n = 1$ for the control, and $n = 3$ for the other treatments) were mixed in FAS to have a final volume of 100 mL in a dark condition, and then extracted with 50 mL dichloromethane (DCM). After a clear separation, both the water phase and the organic phase were collected and stored under 4 °C and −20 °C, respectively until they were analyzed for Cu concentrations using an atomic-absorption spectroscopy with a furnace system (AAnalyist 800 AAS, Perkinelmer Inc.). A diluted Cu stock used for this test with a nominal concentration of 50 μg Cu/L was also analyzed at the same time to check the actual stock concentration. The underlying reasoning was that Cu of inorganic form (Cu^{2+}) would stay in the water phase, while Cu of organic form (presumably CuPT) from the transchelation of Cu and ZnPT would stay in the organic phase after reaching equilibrium.

3. Results

3.1. Toxicities of ZnPT and Cu alone

ZnPT alone was found to be much more toxic than Cu alone to *T. pseudonana* and *H. elegans* larvae (a significant difference was defined as non-overlapping of 95% C.I.), while the 96 h-EC50 and 48 h-LC50 values of ZnPT to these two species were both at ppb level (Table 2, Figs. 1 and 2 when [Cu] or [ZnPT] = 0 μg/L). *T. pseudonana* was quite tolerant to Cu, with a 96 h-EC50 value as high as 970 μg Cu/L (Table 2). Interestingly, Cu appeared to stimulate the growth of *T. pseudonana* at lower concentrations (50–200 μg Cu/L) and the highest growth rate was found at 200 μg Cu/L (Fig. 1A). The 72 h-LC50 values of ZnPT and Cu were not significantly different for *E. rapax*, but its 96 h-LC50 value of ZnPT was obviously lower than its 96 h-LC50 value of Cu indicating that ZnPT was more toxic to the amphipod than Cu (Table 2).

3.2. Combined toxicities of ZnPT and Cu

In general, ZnPT and Cu exhibited synergistic effects to all test species (Figs. 1–3). For example, an average 96 h growth rate of *T. pseudonana* was about 47% of the control when exposed to 2 μg/L ZnPT alone, but a binary mixture of 2 μg ZnPT/L and 10 μg

Table 2
LC50 or EC50 values (their 95% confidence intervals) of ZnPT and Cu alone to *Thalassiosira pseudonana*, *Hydroides elegans* and *Elasmopus rapax*

Species	End point	ZnPT (95% C.I.), μg/L	Cu (95% C.I.), μg/L
T. pseudonana	96 h-EC50	1.9 (1.6–2.3)	970 (870–1100)
H. elegans	48 h-LC50	4.4 (3.9–4.9)	120 (120–130)
E. rapax	24 h-LC50	>100	180 (140–240)
	48 h-LC50	>100	110 (92–130)
	72 h-LC50	70 (34–150)	84 (74–95)
	96 h-LC50	29 (19–46)	78 (68–90)

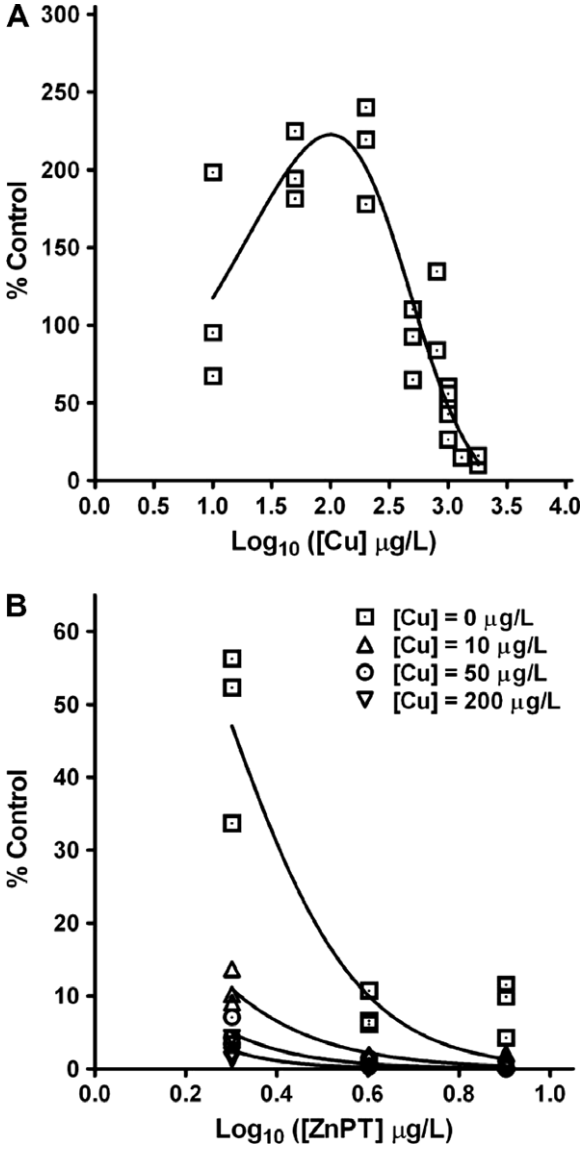

Fig. 1. Growth of *Thalassiosira pseudonana* after 96 h exposure to (A) Cu alone at different concentrations and (B) combinations of different ZnPT and Cu concentrations.

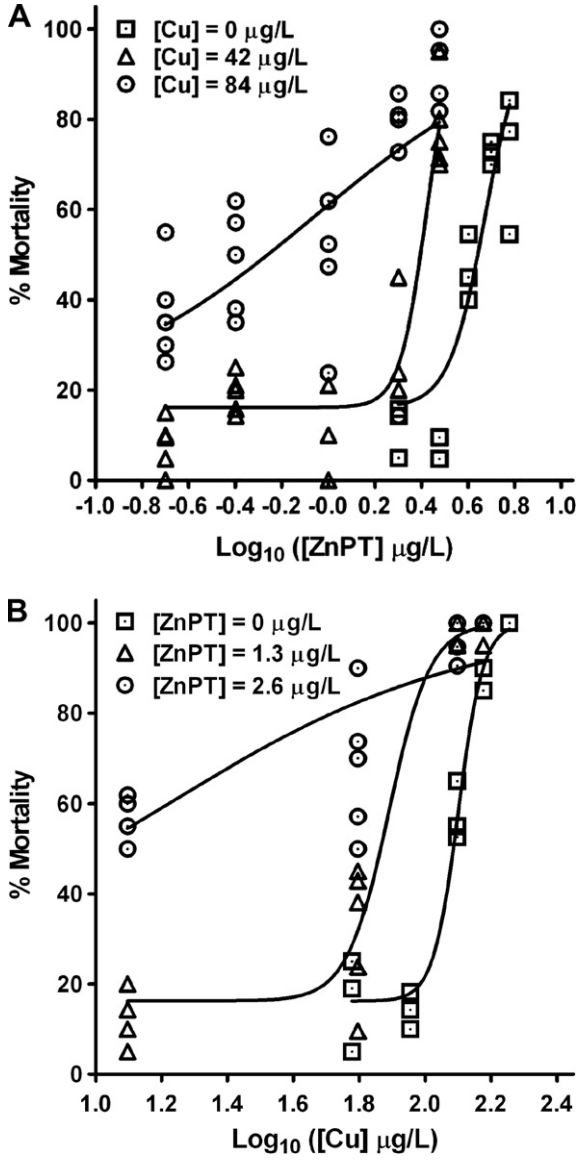

Fig. 2. Mortality of *Hydroides elegans* larvae after 48 h exposure to (A) ZnPT at different Cu concentrations and (B) Cu at different ZnPT concentrations.

Cu/L dramatically decreased its average growth rate to 11% of the control (Fig. 1B); and the higher the Cu level, the more obvious the synergistic toxicity effect of ZnPT to *T. pseudonana*. Similar patterns could also be observed for the other two test species (Figs. 2 and 3). For *H. elegans*, addition of 42 and 84 μg/L Cu considerably reduced the 48 h-LC50 value of ZnPT from 4.4 (95% C.I.: 3.9–4.9; [Cu] = 0) μg/L to 2.5 (95% C.I.: 2.3–2.7) μg/L and 0.5 (95% C.I.: 0.3–0.7) μg/L, respectively (Fig. 2A; values are also shown in Fig. 4B as TU). Likewise, the binary mixtures of ZnPT and Cu showed a very strong synergistic effect on *E. rapax* across all exposure durations (Fig. 3). At 96 h of exposure, the average mortality of *E. rapax* exposed to a mixture of 10 μg/L ZnPT and 10 μg/L Cu was 50%, which was almost 12 times of the mortality of those exposed to 10 μg/L ZnPT alone (4% only). For *E. rapax*, addition of 10 and 20 μg/L Cu considerably reduced the 96 h-LC50 value of ZnPT from 29 (95% C.I.: 19–46; [Cu] = 0) μg/L to 11 (95% C.I.: 9.6–12) μg/L and 6.4 (95% C.I.: 5.9–7.0) μg/L, respectively (Fig. 3; values are also shown in Fig. 4C as TU).

Such synergistic effects were further confirmed with the isobolograms and their corresponding interaction parameter λ of 2.3, 1.3 and 2.2 for *T. pseudonana*, *H. elegans* and *E. rapax*, respectively (i.e. all $\lambda > 1$; Fig. 4). Remarkably, all data points on the isobolograms were distinctively below the Loewe additivity line, indicating a strong synergistic effect (Fig. 4).

The non-parametric response surface was a powerful method to model the binary mixture toxicity and could be applied to the results of all test species (Fig. 5). The isobols in the contour figures for *T. pseudonana* (Fig. 5B) and *H. elegans* (Fig. 5D) were mostly bowed downward, indicating a moderate Loewe synergism of ZnPT and Cu. The isobols in the contour figure for *T. pseudonana* bowed slightly to the right side at Cu levels from 50 to 200 μg/L, which was due to the stimulation of algal growth at these low concentrations (Fig. 5B). A stronger Loewe synergism of the mixture was observed in *E. rapax* as the isobols in the contour figure were all obviously curved downward (Fig. 5F).

3.3. Confirmation for the transchelation of ZnPT and Cu

The results clearly showed that when Cu was not in excess (the ratio of molar concentrations for [ZnPT]:[Cu] $\geqslant$ 1), all Cu added

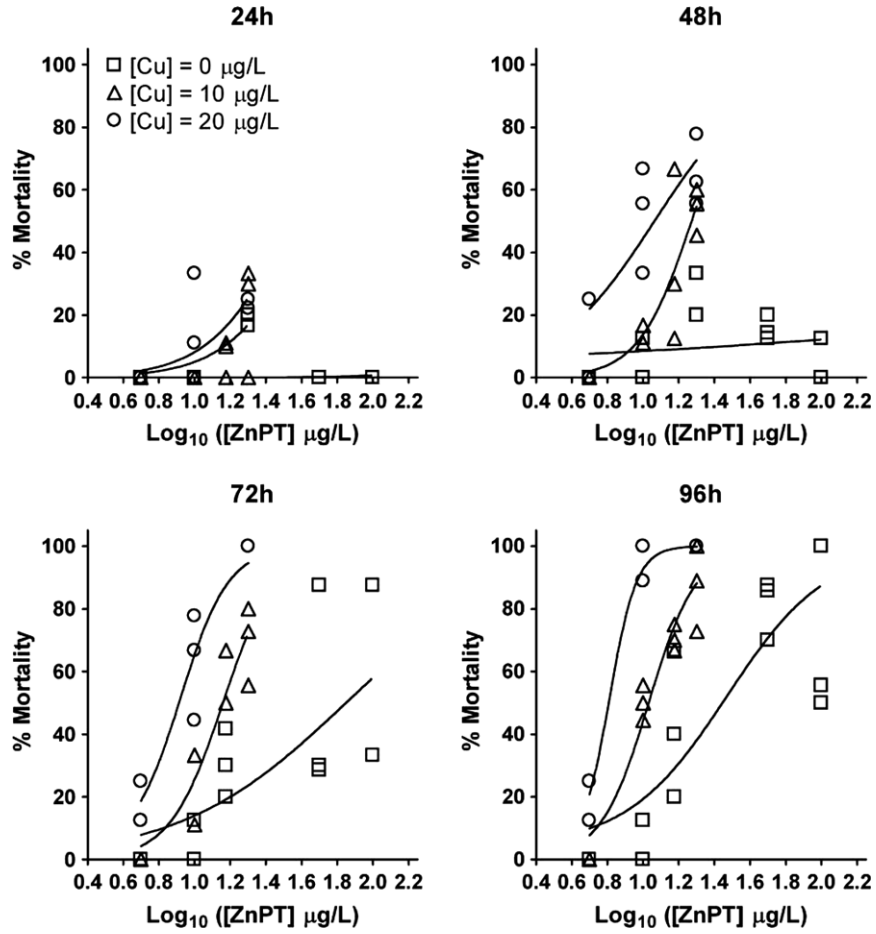

Fig. 3. Cumulative mortality of *Elasmopus rapax* exposed to different binary mixtures of ZnPT and Cu at 24 h, 48 h, 72 h and 96 h, respectively.

was transchelated with ZnPT to form Cu-complexed organic compounds (presumably CuPT), so that all Cu was only found in the organic phase (Table 3). When the ratio of molar concentrations for [ZnPT]:[Cu] was <1, Cu was in excess, and the non-transchelated portion of Cu stayed in the water phase (Table 3). The diluted Cu stock with a nominal concentration at 50 μg Cu/L was estimated to be 44.6 μg Cu/L. Based on the results, the recovery of the total Cu added in the test solutions ranged from 69% to 93% with an overall average of 78% (Table 3). Surprisingly, a low concentration of Cu (29 nM) in the organic phase was also detected in the treatment of [ZnPT]:[Cu] = 1:0 (Table 3), indicating that there was some impurity of Cu-complexed organic compounds (probably CuPT) in the chemical ZnPT purchased from Sigma (USA). If all Cu detected were assumed to be CuPT, the proportion of the impurity would account for ~9.3% by weight.

4. Discussion

ZnPT is well known to be highly toxic to aquatic organisms with a chronic NOEC (no observed effect concentration) ⩽1.5 μg/L (Turley et al., 2000). Our results also support that ZnPT is highly toxic to marine organisms as shown in the three test species with 96 h-EC50 of 1.9 μg/L, 48 h-LC50 of 4.4 μg/L and 96 h-LC50 of 29 μg/L for *Thalassiosira pseudonana*, *H. elegans* and *E. rapax*, respectively. These acute toxicity endpoints of ZnPT are comparable to the reported values for marine organisms in temperate regions. For instance, 72 h-EC50 of ZnPT to the diatom *C. gracilis* was 3.2 μg/L (Koutsaftis and Aoyama, 2006), while the 48 h-EC50 values of ZnPT, based on inhibition of embryonic development, to the sea urchin *Paracentrotus lividus* and the blue mussel *Mytilus edulis* were 2.4 μg/L and 2.5 μg/L, respectively (Bellas et al., 2005).

The current results of the combined toxicity tests clearly indicate that there is a strong synergistic effect between ZnPT and Cu to the three test species, especially *T. pseudonana* and *E. rapax*. It is important to note that such synergism was consistently observed in these two species even at low Cu concentration (i.e. 10 μg Cu/L that falls within the range of environmentally realistic Cu concentrations in coastal waters). It is postulated that such synergistic effects are partially attributable to the formation of CuPT by transchelation of ZnPT with Cu (Grunnett and Dahllöf, 2005), since CuPT has been shown to be more toxic than ZnPT to marine organisms. Mochida et al. (2006) observed that CuPT was more toxic than ZnPT to the sea bream *P. major* and the toy shrimp *H. futilirostris* (96 h-LC50 values of CuPT and ZnPT: 9.3 and 98.2 μg/L for *P. major*, and 2.5 and 120 μg/L for *H. futilirostris*, respectively). Indeed, CuPT was more toxic than ZnPT to *E. rapax* juveniles; the 96 h-LC50 of CuPT to *E. rapax* juveniles was determined to be 9.6 μg/L (95% C.I.: 8.1–11 μg/L; study by Stella Wong, unpublished data) that was considerably lower than the corresponding LC50 for ZnPT (29 μg/L). Also, CuPT was found to be more toxic to the copepod *Tigriopus japonicus* (24 h-LC50 = 41 μg/L) than ZnPT (24 h-LC50 > 500 μg/L) (Shipbuilding Research Association of Japan, 2002, cited in Yamada, 2006). Similarly, CuPT is more toxic than ZnPT to suspension-cultured fish cells CHSE-sp and juvenile rainbow trout *Oncorhynchus mykiss* (Okamura et al., 2002). In concurrence with our results, synergistic effects of the binary mixtures of ZnPT and Cu were consistently observed in the bacteria *V. fischeri* (Zhou et al., 2006), *P. major* and *H. futilirostris* (Mochida

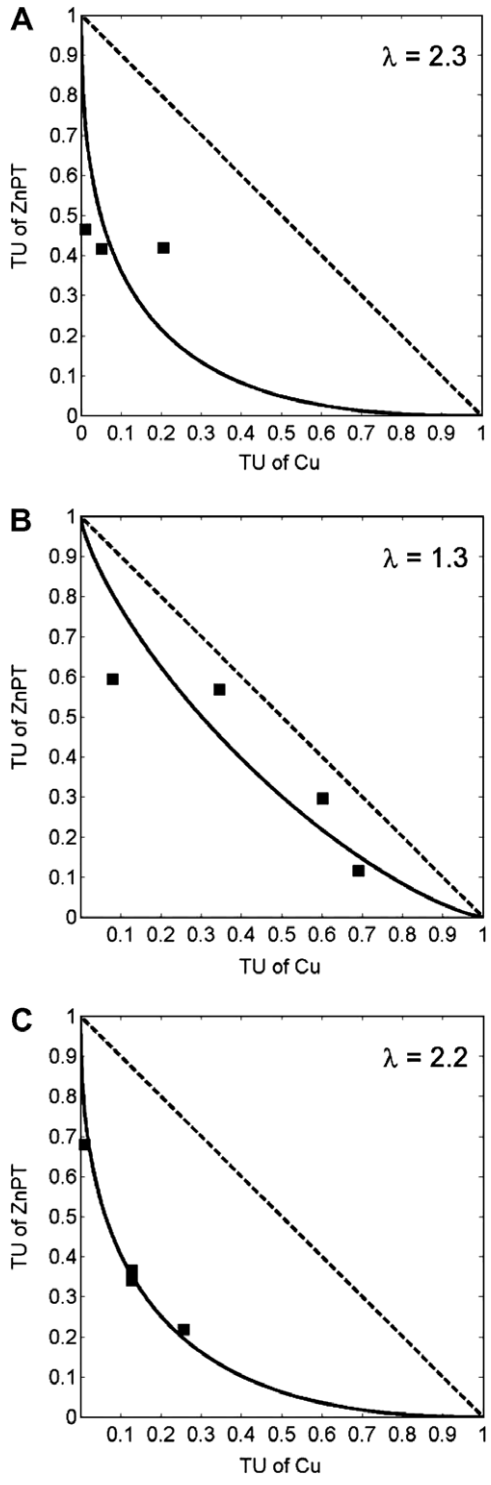

Fig. 4. Isobologram showing the combined toxicity effect of ZnPT and Cu to (A) *Thalassiosira pseudonana* (96 h algal growth), (B) *Hydroides elegans* (48 h mortality) and (C) *Elasmopus rapax* (96 h mortality). The broken line is the Loewe additivity line at 50% control level and the curve represents calculated isobole. The dark squares represent experimental data points.

et al., 2006). These authors also suggested that the major reason of the synergistic effect is closely related to the formation of CuPT.

On the contrary, Koutsaftis and Aoyama (2006) reported that there was strictly antagonistic effect of combined toxicity of ZnPT and Cu to the marine algae *C. gracilis*, which was explained to be caused by the formation of CuPT through the transchelation of ZnPT and Cu and the toxicity of CuPT was believed to be less than that of ZnPT to *C. gracilis*. CuPT has been reported to be less toxic to marine diatom *S. costatum* (72 h-EC50 = 28.4 μg/L) than ZnPT (72 h-EC50 = 2.1 μg/L) (Shipbuilding Research Association of Japan, 2001, cited in Yamada, 2006). In contrast to the observations in marine animals, CuPT is less toxic to the algae *C. gracilis* and *S. costatum*, which might be because Cu is an essential element for their growth (Hall and Anderson, 1999). Our results confirm that Cu can promote the growth of *T. pseudonana* at low concentrations (50–200 μg Cu/L) but the binary mixture of ZnPT and Cu can still cause a strong synergistic effect to *T. pseudonana*. It is apparent that the binary toxic effect of ZnPT and Cu to marine algal species seems to be species specific and warrants further investigation.

Our experimental results (Table 3) further confirmed the proposed transchelation process between ZnPT and Cu that there is ligand exchange between ZnPT and Cu to form CuPT, although similar observations have been documented elsewhere (e.g., Grunnett and Dahllöf, 2005; Bones et al., 2006). Nonetheless, kinetics about the transchelation of ZnPT and Cu of different molar ratios in the natural coastal environments are expected to be much more complicated due to the existence of other ions and organic matter in the water, and the formation of $CuPT^+$ at the ratio of unity between Cu and PT when Cu is in excess (Grunnett and Dahllöf, 2005). Grunnett and Dahllöf (2005) also demonstrated that ZnPT leached from an antifouling paint containing Cu could not be detected in the leaching experiment, but instead, a high concentration of CuPT was found. The newly leached Cu is supposed to increase Cu concentration in the water near antifouling coatings, and hence in turn increase the chance of the transchelation between ZnPT and Cu.

In this study, the Cu-complexed organic impurity (presumably CuPT; ~9.3% by weight) detected in the chemical ZnPT purchased from Sigma (USA) might lead to some over-estimation for the toxicities of both ZnPT alone and the mixture of ZnPT and Cu. Given that the concentration of the impurity (29 ± 1 nM) was constant with a coefficient of variation of only 4.8%, inherent influence associated with the impurity on the toxicity of the ZnPT alone or binary mixture of ZnPT and Cu would be somewhat consistent. As such, the presence of the impurity should have a minimal effect on our results and would not change the key conclusion of the synergistic toxic effect between ZnPT and Cu to the three tested marine species.

Both isobologram and response surface approaches have their own advantages and disadvantages to interpret binary mixture toxicity data, which has been well discussed in Gessner (1995) and Greco et al. (1995). Analysis of binary mixtures using isobologram approach is relatively straight forward, and it can effectively reveal the nature of binary mixture toxicity (i.e. antagonism, addition or synergism). However, when compared with the isobologram, the response surface approach has its advantage by fitting all experimental data in a single model and clearly visualising the combined toxicities in a three dimensional does-response surface (Gessner, 1995). In a preliminary study, we attempted to make use of a parametric response surface model of Loewe synergism (or antagonistic; which is based on the general Loewe additivity model of the mixture components and the Hill's model for a single component; Greco et al., 1990, 1995) to model and assess the mixture toxicity of ZnPT and Cu, but the parametric model was failed to fit the empirical data. As ZnPT can transchelate with Cu to form CuPT and coexistence of Cu, Zn, CuPT, $CuPT^+$ and ZnPT as well as other degradation products in the mixture solution that concomitantly determine the resultant toxicity of the mixture to the test organism (Grunnett and Dahllöf, 2005), conventional parametric models primarily based on a simple additive effect are likely inappropriate. Therefore, we have applied the non-parametric response surface to model the combined toxicity of ZnPT and Cu and successfully dem-

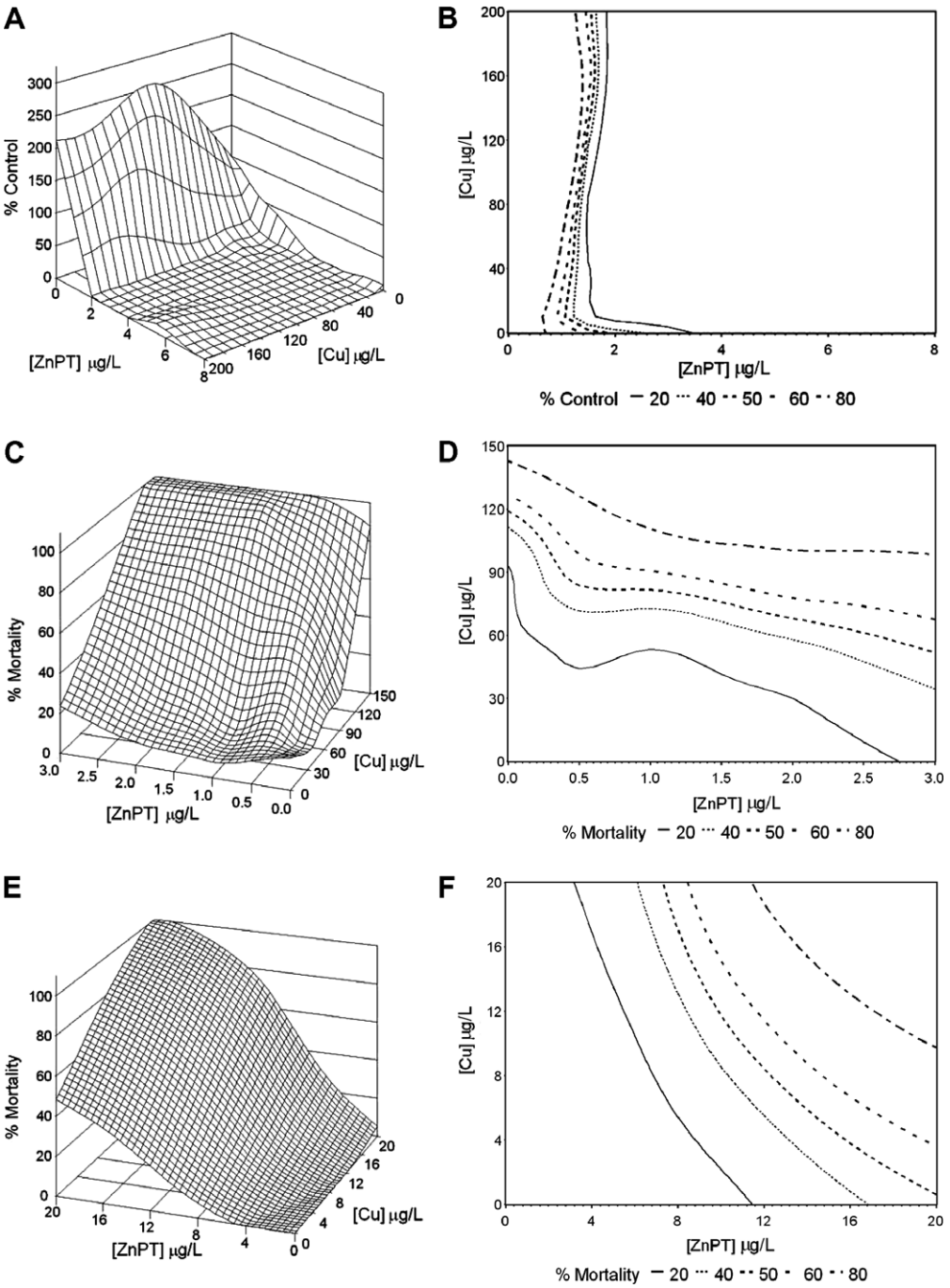

Fig. 5. Non-parametric response surfaces and contours for describing the mixture toxicities of Cu and ZnPT for *Thalassiosira pseudonana* (A and B, without spline), *Hydroides elegans* (C and D, with spline) and *Elasmopus rapax* (E and F, with spline). In contour plots (B, D and F), a straight diagonal NW–SE isobol in a contour plot would be consistent with the Loewe additivity, while a downward bowed isobol represents the Loewe synergism, vice versa.

Table 3
Proportions of Cu in the water and organic phases respectively after the extraction of seawater added with different combinations of ZnPT and Cu (i.e. different ratios of molar concentrations; $n = 1$ for the control; $n = 3$ for the other treatments; average ± 1SD)

Treatment	Cu in water phase (%)	Cu in organic phase (%)	Total Cu (nM)	Recovery of Cu added (%)
Control	ND	ND	0	NA
[ZnPT]:[Cu] = 1:0	0 ± 0	100 ± 0	29 ± 1	NA
[ZnPT]:[Cu] = 0:1	100 ± 0	0 ± 0	240 ± 142	75 ± 40
[ZnPT]:[Cu] = 1:0.5	0 ± 0	100 ± 0	159 ± 18	93 ± 8
[ZnPT]:[Cu] = 1:1	0 ± 0	100 ± 0	253 ± 20	80 ± 3
[ZnPT]:[Cu] = 1:2	35 ± 1	65 ± 1	415 ± 15	69 ± 2
[ZnPT]:[Cu] = 1:4	79 ± 15	21 ± 15	837 ± 198	72 ± 15

Based on a final volume of 100 mL, the total Cu concentration measured in both media is also presented, alongside the recovery rate of added Cu in each treatment.
ND, not detected; NA, not applicable as there was no addition of Cu in this treatment group.

onstrated that this non-parametric method is able to describe and model the synergistic toxic effects of ZnPT and Cu mixtures to all the three species. Like the parametric methods, the contour generated from this non-parametric model can be used to classify the type of combined toxic effects and used as a predictive tool for forecasting the mixture toxicity.

5. Conclusions

Elevated Cu concentrations in ambient water and sediment are very common in many coastal areas worldwide (Hall and Anderson, 1999; Schiff et al., 2007). Based on the present results and those from Mochida et al. (2006) and Zhou et al. (2006), Cu at environmentally realistic concentrations (i.e. 1–10 μg/L) can lead to a dramatic increase of ZnPT toxicity to marine organisms such as the diatom *T. pseudonana*, polychaete larvae *H. elegans* and amphipod *E. rapax*. In the presence of Cu, it is anticipated that predicted no effect concentrations (PNECs) of ZnPT will be considerably reduced (or tightened) in relation to the ambient Cu concentration. Thus, ecological risk assessments of ZnPT solely based on the ecotoxicity of ZnPT alone are probably inadequate, especially for the coastal areas with heavy Cu contamination. In order to better protect precious marine resources, it is advocated to revisit and evaluate the current water quality criteria for ZnPT with the consideration of its compelling synergistic effects with Cu.

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