Acute and chronic toxicities of zinc pyrithione alone and in combination with copper to the marine copepod *Tigriopus japonicus*

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**A B S T R A C T**

Zinc pyrithione (ZnPT) is a widely used booster biocide in combination with copper (Cu) in antifouling paints as a substitute for tributyltin. The co-occurrence of ZnPT and Cu in coastal marine environments is therefore very common, and may pose a higher risk to marine organisms if they can result in synergistic toxicity. This study comprehensively investigated the combined toxicity of ZnPT and Cu, on the marine copepod *Tigriopus japonicus*, for the first time, based on both 96-h acute toxicity tests using adult copepods and chronic full-life cycle tests (21 d) using nauplii <24-h old. As ZnPT has been reported to be easily trans-chelated to copper pyrithione (CuPT) in the presence of Cu, the acute toxicities of CuPT alone and in combination with Cu on adult copepods were also assessed. Our results showed that ZnPT and Cu exhibited a strong synergistic toxic effect on the copepod in both acute and chronic tests. During the acute test, the mortalities of adult copepods increased dramatically even with an addition of Cu at concentrations as low as 1–2 μg/L compared with those exposed to ZnPT alone. Severe chronic toxicities were further observed in the copepods exposed to ZnPT–Cu mixtures, including a significant increase of naupliar mortality, postponing of development from naupliar to copepodid and from copepodid to adult stage, and a significant decrease of intrinsic population growth when compared with those of copepods exposed to ZnPT or Cu alone. Such synergistic effects might be partly attributable to the formation of CuPT by the trans-chelation of ZnPT and Cu, because CuPT was found to be more toxic than ZnPT based on the acute toxicity results. Mixtures of CuPT and Cu also led to synergistic toxic effects to the copepod, in particular at high Cu concentrations. A novel non-parametric response surface model was applied and it proved to be a powerful method for analysing and predicting the acute binary mixture toxicities of the booster biocides (i.e., ZnPT and CuPT) and Cu on the copepod. To better protect precious marine resources, it is necessary to revise and tighten existing water quality criteria for biocides, such as ZnPT and CuPT, to account for their synergistic effects with Cu at environmentally realistic levels.

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1. Introduction

Tributyltin (TBT) has restricted application on ship hulls since the early 1980s due to its severe impacts to marine life, and a global complete ban of TBT in antifouling paints has entered into force from 2008 (IMO, 2001; Yebra et al., 2004; Fent, 2006). A new generation of booster biocides such as zinc pyrithione (ZnPT), copper pyrithione (CuPT), Irgarol 1051, diuron, and Sea-Nine 211, has been increasingly used as TBT substitutes globally, and they are usually applied in combination with Cu in which the percentage of booster biocides is usually around 5%, while the percentage of Cu can be up to 40% or more (Lassen et al., 2001; Yebra et al., 2004; HK AFCD, 2008). Elevated concentrations of many of these biocides (e.g., Irgarol, diuron, Sea-Nine 211) and their degradation products have been detected worldwide in coastal areas such as marinas and harbours as a consequence of their increased use (Konstantinou and Albanis, 2004). There is an increasing concern over the environmental occurrence, fate, toxicity and persistence of these biocides (Evans et al., 2000; Konstantinou and Albanis, 2004). Though progresses have been made on aforementioned aspects (Thomas, 2009), most relevant studies are focused on single biocides, and there is still a lack of data on the mixture toxicities of these biocides (Howell and Evans, 2009).

Another main concern is that dramatically increased application of Cu-based antifouling biocides has led to elevated Cu concentrations in coastal waters and sediments around the world (Schiff et al., 2007), particularly in coastal areas with busy

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harbours. The mean dissolved Cu concentration was reported to be 8.8 μg/L (range: 0.5–75 μg/L) in the Essex/Suffolk estuaries in UK between 1992 and 1996 (Matthiessen et al., 1999), 8.5 μg/L in marinas of the San Diego region, USA (Schiff et al., 2007), and 11.23 μg/L (range: 1.61–48.52 μg/L) in beaches of Acapulco, Mexico (Jonathan et al., 2011). Along the coastal waters in Perth, Western Australia, Department of Water (2009) found that the total Cu concentration in water ranged from <1 to a maximum of 12,000 μg/L, and exceeded the ANZECC guideline (1.3 μg/L) in six out of eight study sites, while Cu concentration in sediment ranged from 1.5 to 17,900 μg/g dry weight (dw), and exceeded the ANZECC guideline (65 μg/g dw) in three out of the eight study sites. Likewise, sediment Cu concentration was found to range from 5.8 to 846 μg/g dw with a median concentration at 60.6 μg/g dw in the Toulon Bay, France (Tessier et al., 2011), range from 256 to 279 μg/g dw in three out of the four study sites in eastern Aegean coastal waters, East Mediterranean (Onen et al., 2011), and range from 47 to 286 μg/g dw with a mean concentration at 117 μg/g dw in Sydney estuary, Australia (Birch et al., 2014).

As both booster biocides and Cu are the main components of most of the Cu-based antifouling products, their co-existence in the marine environment is expected to be not unusual especially in marinas and harbours with high boating activity and low water exchange rate. The booster biocides and Cu may interact with each other, leading to additional, synergistic or antagonistic combined toxic effect to marine organisms (Tessier et al., 1999), and understanding these interactions is necessary to determine the ultimate environmental impacts of the booster biocides on marine ecosytems (Hertzberg and MacDonell, 2002). For example, mixtures of Irgarol 1051 and Cu have been shown to have an additive toxicity to a marine copepod (Bao et al., 2013). However, there is a paucity of data on the combined toxicity of other commonly used booster biocides (e.g. ZnPT) and Cu to marine species, and almost all relevant documented studies are merely based on acute tests (Mochida et al., 2006; Zhou et al., 2006; Koutsasitis and Aoyama, 2007; Bao et al., 2008).

ZnPT and CuPT are both metal pyrithiones, and were first introduced into the market as antifouling booster biocides in 1990s. ZnPT-based antifouling paints have been intensively applied worldwide, especially in Europe and South Korea (Thomas, 1999), and in Japan, where ZnPT and CuPT are the two most frequently used biocides in antifouling products (Okamura and Mieno, 2006). ZnPT can easily transchelate with Cu into CuPT; a partial transchelation of ZnPT into CuPT was detected in seawater with naturally present Cu, and a total transchelation of ZnPT into CuPT was detected when ZnPT was released from Cu-based antifouling paints (Grunnett and Dahlöf, 2005). Thus, the environmental fate and persistence of ZnPT are closely related to those of CuPT in the marine environment. Both ZnPT and CuPT were found to be highly toxic to aquatic autotrophic species (e.g. microalgae and cyanobacteria) and animals (Turley et al., 2000; Bao et al., 2011, 2012). However, up to now there are relatively few data on the toxicity of both metal pyrithiones to aquatic organisms. ZnPT and CuPT were marked as environmentally neutral and non-persistent in the aquatic environment as they can undergo fast photodegradation to form less toxic compounds in seawater under direct sunlight (half-life <2 min; Turley et al., 2000, 2005; Bellas, 2005). However, both metal pyrithiones are suggested to be persistent in marine environments where light is limited such as waters and sediments shaded under parked vessels in marinas and harbours, or in water columns with high turbidity; and ZnPT may accumulate in the sediment as CuPT and manganese pyrithione (MnPT) (Galvin et al., 1998; Maraldo and Dahlöf, 2004). A field experiment reported a half-life of 209 min at a depth of 0.5 m for CuPT, and photodegradation was absent at 1 m or more below surface after 1-h exposure to penetrating sunlight (Grunnett and Dahlöf, 2005). Marcheselli et al. (2010b) also found that half of the initial ZnPT quantity remained after 48-h light exposure (indirect sunlight plus laboratory fluorescent lamps). Notably, CuPT had been detected in marine sediment samples from Vietnam (range: <2–420 μg/kg-dry wt.) and Japan (range: 8–22 μg/kg-dry wt.) (Harino et al., 2006, 2007). A total pyrithione concentration of 105 nm was detected in water samples collected from Mersey estuary, UK indicating the possible existence of pyrithiones like ZnPT and CuPT in marine water columns (Mackie et al., 2004). Marcheselli et al. (2010b) reported a basal level of ZnPT and ionized pyrithione (PT-·) in the mussels Mytilus galloprovincialis inside a busy harbour of Italy, indicating that the level of ZnPT in the harbour is high enough to induce a detectable accumulation in native organisms, and the study also showed a rapid accumulation of ZnPT in the mussel in laboratory experiment, suggesting that ZnPT has potentials to accumulate in the trophic chain.

Tigrionus japonicus (Copepod, Harpacticidae) is a common intertidal rocky shore species that has a wide geographical distribution, mainly found along the Western Pacific coast. T. japonicus was selected for this study as it is ecologically important, highly abundant and small in size; while it has a short generation time and high fecundity, it can be easily cultured under laboratory conditions (Raisuddin et al., 2007). It is also a good candidate for chronic toxicity tests (e.g., life cycle test) due to its distinct post-embryonic developmental stages (6 naupliar stages, 5 copepodid stages and an adult stage), dimorphic sex and high fecundity of females with multiple broods of eggs developed sequentially after a single mating event (Ito, 1970; Raisuddin et al., 2007; Kwok et al., 2009). Additionally, toxicity tests conducted with this copepod had shown a good reproducibility (Kwok et al., 2008).

In order to investigate the combined toxicity of ZnPT and Cu, this study investigated both the acute (with mortality as the endpoint) and chronic (life cycle test with larval mortality, developmental time and intrinsic rate of increase as endpoints) toxicities of ZnPT alone and in combination with Cu on the marine copepod T. japonicus. The acute toxicities of CuPT alone and in combination with Cu on the adult copepod were also examined to elucidate the combined toxicity of ZnPT and Cu. Response surface models have proven to be successful to fit all experimental data in a single model and clearly visualizing all combined toxicity data in a three dimensional concentration response surface (Gessner, 1995; Bao et al., 2008, 2013). We applied and compared three different response surface approaches, namely the Loewe parametric response surface (CARS; Greco et al., 1995), response additive response surface (RARS; Bao et al., 2013), and the non-parametric response surface (NPRS; Gessner, 1995; Greco et al., 1995) models to describe and predict the combined acute toxicity of binary mixtures of individual biocides and Cu.

2. Materials and methods

Standard 96-h semi-static acute aquatic toxicity tests of ZnPT and Cu alone on adult copepods T. japonicus were first conducted in order to determine the median lethal concentration (LC50) for each biocide (please refer to the details of the test conditions in Section 2.3). Suitable ZnPT and Cu concentrations were chosen according to their LC50 values and pilot study results for the binary mixture acute toxicity test of the two biocides on the adult copepods, and for the binary mixture chronic toxicity test of the two biocides using newly hatched copepod nauplii less than 24-h old (Table 1). In order to investigate whether the combined toxicity of ZnPT and Cu was simply due to the formation of CuPT by the transchelation of ZnPT and Cu, the acute toxicities of CuPT alone and in combination with Cu to the adult copepod were also investigated (Table 1). For a better comparison of the toxicities between the
Table 1
The nominal concentrations (µg/L) selected for the acute toxicity tests for the binary mixtures of ZnPT–Cu, and CuPT–Cu, and those selected for the chronic toxicity test for the binary mixtures of ZnPT–Cu to the copepod Tigriopus japonicus (ZnPT: zinc pyrithione; CuPT: copper pyrithione).

<table>
<thead>
<tr>
<th>Biocide (B)</th>
<th>Acute B (µg/L)</th>
<th>Cu (µg/L)</th>
<th>Chronic B (µg/L)</th>
<th>Cu (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnPT</td>
<td>0 (C)</td>
<td>0</td>
<td>0 (C)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0 (SC, 40 ppm)</td>
<td>0</td>
<td>0 (SC, 5 ppm)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20, 50, 100, 200, 300, 400</td>
<td>100, 200, 300, 400, 600, 800</td>
<td>5, 10, 20, 50</td>
<td>5, 10, 20</td>
</tr>
<tr>
<td></td>
<td>20, 50, 100, 100</td>
<td>10</td>
<td>20, 50, 10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>50, 100</td>
<td>2</td>
<td>50, 20, 50</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10, 20, 50, 100</td>
<td>10</td>
<td>100, 50, 20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5, 10, 15, 20</td>
<td>10</td>
<td>200</td>
<td>20</td>
</tr>
<tr>
<td>CuPT</td>
<td>0 (C)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (SC, 80 ppm)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5, 10, 20, 30, 40, 60, 80</td>
<td>100, 200, 300, 400, 600, 800</td>
<td>5, 10, 20, 50</td>
<td>5, 10, 20</td>
</tr>
<tr>
<td></td>
<td>10, 20, 30, 40</td>
<td>10</td>
<td>20</td>
<td>10</td>
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<td></td>
<td>5, 10, 20, 30</td>
<td>20</td>
<td>50</td>
<td>50</td>
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<tr>
<td></td>
<td>1, 2, 5, 10</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1, 2, 5, 10</td>
<td>100</td>
<td></td>
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</tr>
</tbody>
</table>

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

single biocides and binary mixtures, the toxicity of each individual biocide (i.e., ZnPT, CuPT and CuSO₄) alone was assessed again simultaneously with the binary mixture toxicity tests. All concentrations were nominal. Fully aerated 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 tests. Tests were carried out in an environmental chamber (Sanyo Gallenkamp, Loughborough, UK) at 25 ± 1 °C with 12 h:12 h light:dark photoperiod and white illumination of 1000–2000 lux (Tenmars Lux/FC light meter TM201, Tai Wan), while the test solutions were kept at salinity 33 ± 0.5‰ and pH 8.1–8.4 throughout the test.

2.1. Test organism

Adults of T. japonicus were collected from high shore rock pools of the Cape D’Aguilar Marine Reserve, Hong Kong. The collected copepods were kept in FAS at room temperature (25 ± 2 °C) with 12 h:12 h light:dark photoperiod and fed with the diatom Thalassiosira pseudonana for at least 2 weeks before experimentation.

2.2. Test chemicals

Stock solutions of ZnPT (10 g/L) and CuPT (1 g/L) were prepared by dissolving ZnPT (approx 95%; Sigma, USA), CuPT (≥97%; Zhejiang Hongbo Chemical Co., Ltd., China) in pure dimethyl sulfoxide (DMSO; ACS reagent, ≥99.9%; Sigma, USA), respectively and stored in amber glass bottles in the dark at room temperature. A stock solution of Cu (1 g Cu/L) was prepared by dissolving copper (II) sulphate pentahydrate (CuSO₄·5H₂O, formula weight 249.7; purity ≥ 99.5%; BDH Chemicals Ltd., Poole, England) in distilled water. Working solutions at designated nominal mixture concentrations were obtained by diluting the stock solutions with FAS in volumetric flasks before dosing.

2.3. Acute toxicity tests with adult copepod

The acute toxicities of ZnPT and CuPT alone and in combination with Cu to the adult T. japonicus were assessed using 96-h semi-static acute aquatic toxicity tests (Kwok and Leung, 2005). The copepods were acclimated in 50 mL glass beakers (10 females and 10 males in each beaker) with 25 mL FAS for 24 h before tests. After acclimation, the copepods were dosed with 25 mL FAS containing designated biocide combinations (Table 1). There were three replicates for each treatment. The copepods were then incubated in the environmental chamber for 96 h. Test solutions were renewed once after 48 h. Dead copepods were defined as the ursome at a right angle to the prosome (Finney, 1979) and counted once every 24 h until the end of exposure. Surviving copepods were also counted after 96-h exposure. The copepods were not fed during the entire acclimation and test period.

2.4. Chronic toxicity tests with copepod nauplii

The chronic toxicities of ZnPT alone and in combination with Cu to the copepods were studied using a full-life cycle approach (Kwok et al., 2009). Female copepods with egg sacs were selected and each of them was kept in 25 compartment square petri dishes (Sterilin, UK) with 4 mL FAS together with food supply (diatom T. pseudonana at 10⁵ cells/mL) for 24 h before experimentation. Then newly hatched nauplii (i.e. <24-h old) were picked out and mixed in a petri dish with 25 mL FAS. Each randomly selected healthy nauplii (i.e., those could swim actively) was allocated to a small glass test tube (5 mL in total volume) with 2 mL FAS with designated combination of the biocide mixture and 10⁵ cells/mL of T. pseudonana as food supply. There were 20 nauplii for the seawater control, and 13 nauplii for each of the other treatments for the ZnPT and Cu combination test. Test solutions with food supply were renewed every 2 d. Survivorship and development of the nauplii (i.e., stage identification based on Itô, 1970 and Raisuddin et al., 2007: 6 nauplius stages, 5 copepodid stages and adult stage) were closely monitored once everyday. In each compartment, once a female copepod reached the adult stage, one randomly selected adult male of the same treatment was transferred to the same compartment with the adult female to allow them mating for a 24-h period. Then the male was removed from the compartment and returned back to its original test tube. Reproduction in terms of the number of nauplii per each brood was monitored for each of the females. For a female, if there was no sign of an egg sac within 3 d after mating, another male was
assigned to the female for mating, in order to check whether there was a random failure of mating, or the failure occurred due to the toxicity effect. The whole test lasted 21 d.

2.5. Data analysis for acute toxicity tests

The Hill’s slope (m), and lethal concentrations at 10% and 50% (LC10 and LC50, respectively) and their 95% confidence intervals (CI) of single bioicides on adult T. japonicus were estimated by fitting a sigmoidal concentration–response non-linear regression (the Hill’s model, Eq. (1), Greco et al., 1995). GraphPad Prism version 5.00, GraphPad Software, CA). A significant difference between LC10s or LC50s was defined as non-overlapping of 95% C.I.s.

\[ E_i = \frac{E_{con} \cdot C_{conc}^{m_i}}{C_{conc}^{m_i} + LC50^{m_i}} \]  

where \( E_i \): survival rate of component i; \( E_{con} \): survival rate of T. japonicus at control treatment, \( C_{conc} \): concentration of component i; LC50; 96-h LC50 of component i alone on T. japonicus; \( m_i \): Hill’s slope of component i based on the concentration–survival curve.

Concentration addition (CA) and response addition (RA) are two commonly used null reference models in assessment of mixture responses to known non-interactive components. CA concerns mixtures of chemicals with similar toxic mode of action (TMoA), usually with similar concentration effect slopes (i.e., Hill’s slopes \( m \) in this case); and RA is used to predict the combined effect of toxicants with dissimilar TMoA (Greco et al., 1995; de Zwart and Posthuma, 2005). de Zwart and Posthuma (2005) suggested that consideration of the quantitative difference in the expected combined effect using both CA and RA models would be more helpful than selecting one by chance, because appropriate selection of either CA or RA as a reference model depends on the similarity of the TMoA of the mixture components, which is typically ambiguous information.

Two parametric response surface models, Loewe parametric response surface (i.e., concentration additive response surface; CARS, Greco et al., 1990, 1995) model and response additive response surface (RARS) model which were developed based on CA and RA model respectively, were used to analyze the joint acute toxicity data based on binary mixtures. If both CARS and RARS models failed to predict the acute toxicity of biocide–Cu binary mixture on T. japonicus, a non-parametric response surface model (NPRS, Sühnel, 1990) and its contour were used to analyze the data. For each model, modelled mortalities were compared with the observed mortalities (average data of each treatment) with a linear regression between the two (GraphPad Prism version 5.00) in order to check the appropriateness and predictability of the model for each binary mixture.

2.5.1. Loewe parametric response surface (CARS) model

Survivorship data (96 h, percentage) on the acute toxicity of the antifouling biocide and Cu alone and in combination on adult T. japonicus were analyzed using a parametric response surface model (Eq. (2), Greco et al., 1990, 1995).

\[ 1 = \frac{C_{conc1}}{LC50_1 \left( \frac{E}{k_{conc1}} \right)^{1/m_1}} + \frac{C_{conc2}}{LC50_2 \left( \frac{E}{k_{conc2}} \right)^{1/m_2}} + \alpha C_{conc1} C_{conc2} \left( \frac{E}{k_{conc1} + k_{conc2}} \right)^{1/2(m_1 + m_2)} \]  

where \( C_{conc1} \): concentration of chemical 1; \( C_{conc2} \): concentration of chemical 2; LC501: LC50 of chemical 1 to the copepod; LC502: LC50 of chemical 2 to the copepod; \( E \): survival rate of the copepod at the combination with \( C_{conc1} \) of chemical 1 and \( C_{conc2} \) of chemical 2; \( E_{con} \): survival rate of the copepod at control treatment; \( m_1 \): Hill’s slope of chemical 1 alone based on the concentration–survival curve; \( m_2 \): Hill’s slope of chemical 2 alone based on the concentration–survival curve; \( \alpha \): interaction parameter.

The interaction parameter \( \alpha \) was estimated by minimizing the sum of squared residuals using Matlab (version 5.30.0620(a) R11, The MathWorks Inc., USA). The \( \alpha \) indicated the toxicity interaction of the two substances. When \( \alpha = 0 \), the interaction is concentration additive; when \( \alpha > 0 \), the interaction is synergistic and when \( \alpha < 0 \), the interaction is antagonistic. Response surface and its contour plot were generated with MESH and CONTOUR functions in Matlab respectively. 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, and vice versa (Greco et al., 1995).

2.5.2. Response additive response surface (RARS) model

Similar as the CARS model, survivorship data (96 h, percentage) on the acute toxicity of the antifouling biocide and Cu alone and in combination on adult T. japonicus were used for construction of the RARS model (Eqs. (3) and (4)).

\[ P_i = 1 - \frac{E_{con} C_{conc i}^{m_i}}{C_{conc i}^{m_i} + LC50_i^{m_i}} \]  

\[ P = P_1 + P_2 - p_1 P_1 P_2 \]  

where \( P_i \): mortality rate of component i; \( E_{con} \): survival rate of the copepod at control treatment, \( C_{conc i} \): concentration of component i; LC50i: 96-h LC50 of component i alone on the copepod; \( m_i \): Hill’s slope of component i based on the concentration–survival curve. \( P \): expected mortality rate of binary mixture; \( \rho \): interaction parameter.

Eq. (4) was derived from the routine RA reference model by the addition of an interaction parameter \( \rho \). The interaction parameter \( \rho \) was estimated by minimizing the sum of squared residuals using Matlab. The \( \rho \) indicated the toxicity interaction of the two substances. When \( \rho = 1 \), the interaction is response additive; when \( \rho > 1 \), the interaction is antagonistic and when \( \rho < 1 \), the interaction is synergistic. Response surface and its contour plot were generated with MESH and CONTOUR functions in Matlab, respectively.

2.5.3. Nonparametric response surface (NPRS) model

The NPRS and its contours were constructed using the average data of each treatment, and computed using the PROC G3GRID with spline and PROC GCONTOUR procedures in SAS 9.1.3, SAS Institute Inc., Cary, NC (Sühnel, 1990; Greco et al., 1995). The experimental mortality data were fitted by a bivariate (concentrations of Cu and antifouling biocide) fifth-degree polynomial spline while the smoothness of spline was maintained by a polynomial up to order three, i.e. the function,

\[ p_i = \sum_{j=0}^{5} \sum_{k=0}^{j} q_{jk} \left( x_{Cu}^j \cdot x_{AFB}^k \right) \]  

which is estimated by the least squares approach subject to the constraints of smoothness; where \( x_{Cu}^j \) and \( x_{AFB}^k \) are concentrations of Cu and antifouling biocide respectively, \( p_i \) is the control rate for T. japonicus 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 described in Akima (1978) and Wahba (1979). Once the unknown coefficients \( q_{jk} \)'s were estimated by PROC G3GRID, the fitted mortality rates could be calculated by making use of Eq. (5) with the given values \( x_{Cu}^j \) and \( x_{AFB}^k \) automatically generated by this SAS procedure. The horizontal lines connecting the edges of the response surfaces at 10%, 30%, 50%, 70% and 90% of mortality levels were presented in contour plots.
2.6. Data analysis of chronic toxicity tests

Duration of larval development (developmental time of nauplii less than 24 h old to copepodid stage 1 [Dn] and to adult stage [Dt]), and the intrinsic rate of increase \( (r_m) \) were used as population toxicity endpoints. Data of both Dn and Dt failed to pass the Levene’s homogeneity test of variance (SPSS version 13.0, Chicago, IL, USA). Dn (or Dt) under different dose combinations were, therefore, compared using non-parametric Kruskal–Wallis tests. Similarly, both the life span and stage data failed to pass the Levene’s homogeneity test of variance, and were compared using Kruskal–Wallis tests.

The \( r_m \) (intrinsic rate of increase) is an estimate of the intrinsic population growth rate (Walthall and Stark, 1997). When \( r_m = 0 \), it represents a stable population; when it is negative, it represents a declining population and vice versa (Walthall and Stark, 1997; Kwok et al., 2008).

3. Results

3.1. Acute toxicities of single antifouling biocides

For adult copepods, the 96-h LC50 s were estimated as 175.5 and 418.1 \( \mu g/L \) for ZnPT and Cu, and 32.7 and 899.5 \( \mu g/L \) for CuPT and Cu, respectively in two different batches of acute tests (Table 2), indicating some variation in Cu toxicity among different tests. The toxicity of these three biocides, as a single substance, to the adult T. japonicus followed a decreasing order as: CuPT > ZnPT > Cu (Table 2), significant difference was considered as non-overlapping of 95% C.I.).

3.2. Combined acute toxicity of ZnPT and Cu

Mixtures of ZnPT and Cu clearly showed a very strong synergistic effect on adult T. japonicus in the 96-h acute test (Fig. 1). Although the mortality was very low at 24 h across all treatments, all copepods died after 48-h exposure to a combination of ZnPT at 15 \( \mu g/L \) or 20 \( \mu g/L \) with Cu at 50 \( \mu g/L \), 100 \( \mu g/L \), or 200 \( \mu g/L \), while less than 10% mortality was caused by ZnPT or Cu alone at those concentrations. Notably, the 96-h mortality for copepods exposed to 100 \( \mu g/L \) ZnPT alone was 22.1 ± 2.2% (mean ± SEM, so as follows) but this figure sharply increased to 59.4 ± 4.7% when only 1 \( \mu g/L \) Cu was added; the mortality in treatment with 50 \( \mu g/L \) ZnPT alone was 12.0 ± 4.2% and it dramatically increased to 85.4 ± 3.2% with an addition of 2 \( \mu g/L \) Cu. Similarly, the mortality could increase dramatically after an addition of a small amount of ZnPT at high Cu levels. For instance, the 96-h mortality increased from 1.8 ± 1.8% to 42.3 ± 13.1% and from 5.0 ± 2.8% to 56.7 ± 13.0% after the addition of 5 \( \mu g/L \) ZnPT to 100 \( \mu g/L \) and 200 \( \mu g/L \) Cu respectively, while less than 10% mortality was caused by ZnPT or Cu alone at those concentrations. The 96-h LC50 (95% C.I.) of ZnPT to adults of T. japonicus decreased from 175.5 to 29.0, 28.7, 25.8, 14.4, 6.7, 5.2, and 4.9 \( \mu g/L \) with an addition of Cu at 2, 5, 10, 20, 50, 100, and 200 \( \mu g/L \) respectively, while Cu alone at those concentrations led to ≤5% mortality in 96 h (Table 3). At 2 \( \mu g/L \), the mortality rate at the lowest ZnPT concentration (50 \( \mu g/L \)) was 85.4 ± 3.2%.

Both CARS and RARS models failed to fit the binary mixture toxicity data for the ZnPT and Cu mixture with relatively high sum of squared residuals (\( \sum R^2 \)) and low \( r^2 \) values for linear regressions between the modelled and observed mortalities (Table 4). Therefore, the non-parametric response surface (NPRS) was used to model the binary mixture toxicity and it was proven to be a powerful method to describe such data as reflected by a high \( r^2 \) value of 0.85 (single biocide data excluded; Table 4; Fig. 2). The NPRS model confirmed a strong synergistic effect between ZnPT and Cu to the copepods with downward bowed contours (Fig. 2A and B). The modelled mortality rates based on the NPRS model and observed mortality rates were listed in Table 5. It was noted that there was an overestimation of the toxicity of ZnPT at 20, 50 and 100 \( \mu g/L \) or Cu at 100 and 200 \( \mu g/L \) alone (Table 5), but the overall predictability of the NPRS model was satisfactory with a much lower \( \sum R^2 \) and a better \( r^2 \) when compared with those generated by the CARS and RARS models (Table 4; Fig. 2C).

3.3. Combined acute toxicity of CuPT and Cu

Cumulative mortalities of T. japonicus after 24, 48, 72 and 96 h of exposure to various combinations of CuPT and Cu levels were shown in Fig. 3. Although no obvious mortalities were observed at 24 h for any treatment, complicated mixture toxicities were revealed at later time points (72 and 96 h). For instance, at a high CuPT concentration of 40 \( \mu g/L \), the 96-h mortality of the treatment without Cu addition was 87.3% and this figure decreased to 40.5% and 26.7% with an addition of 5 and 10 \( \mu g/L \) Cu respectively, whereas the mortality was as low as 2.0 ± 2.0% at 100 \( \mu g/L \) Cu alone (Fig. 3). At such high levels of CuPT, addition of Cu might lead to an antagonistic effect to alleviate the CuPT toxicity to the copepod. In contrast, a reverse picture was observed at a lower CuPT concentration of 10 \( \mu g/L \); the 96-h mortality increased dramatically from 6.1% (without Cu addition) to 62.2% and 94.4% with an addition of 50 and 100 \( \mu g/L \) Cu respectively, indicating a synergistic toxicity of the mixture. Similar patterns were also observed at 48 h and 72 h, suggesting that the mixture of CuPT and Cu exhibited an antagonistic effect at low Cu levels (5, 10, 20 \( \mu g/L \)), but a strong synergistic effect at higher Cu levels (50 and 100 \( \mu g/L \)) to the copepod.

Such complicated mixture toxicity patterns were further illustrated by the 96-h LC50 values. The 96-h LC50 (95% C.I.) of CuPT alone was estimated at 32.7 (31.4–34.1) \( \mu g/L \), and it changed to 43.9 (18.8–102.7), 43.3 (32.1–58.5) and 49.1 (8.9–270.8) \( \mu g/L \) after an addition of Cu at 5, 10 and 20 \( \mu g/L \) respectively. Despite the observed increase of the 96-h LC50 of CuPT after the addition of Cu at low levels, there were no significant differences among the four LC50 values. However, the 96-h LC50 of CuPT alone (i.e. 32.7 \( \mu g/L \)) decreased dramatically to 8.6 (4.6–15.9) and 6.6 (5.7–7.6) \( \mu g/L \) after an addition of Cu at 50 and 100 \( \mu g/L \) respectively. At 100 \( \mu g/L \) Cu/L, the 96-h mortality of the copepod was only 2.0%.

Given the complexity of the mixture toxicity of CuPT and Cu, both parametric models (CARS and RARS) failed to model such data.
Table 3
Estimated 96-h-LC10 and 96-h-LC50 values (95% confidence interval, C.I., in parenthesis) of zinc pyrithione (ZnPT) and copper pyrithione (CuPT) with addition of copper (Cu) at various concentrations.

<table>
<thead>
<tr>
<th>[Cu], µg/L</th>
<th>ZnPT</th>
<th>CuPT</th>
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<tbody>
<tr>
<td></td>
<td>LC10</td>
<td>LC50</td>
</tr>
<tr>
<td>2</td>
<td>13.6 (6.5–28.3)</td>
<td>29.0 (20.2–41.7)</td>
</tr>
<tr>
<td>5</td>
<td>12.7 (6.7–24.3)</td>
<td>28.7 (20.7–39.7)</td>
</tr>
<tr>
<td>10</td>
<td>21.3 (14.1–32.1)</td>
<td>25.8 (4.7–140.8)</td>
</tr>
<tr>
<td>20</td>
<td>9.4 (7.6–11.8)</td>
<td>14.4 (12.6–16.5)</td>
</tr>
<tr>
<td>50</td>
<td>4.2 (3.4–5.3)</td>
<td>6.7 (5.8–7.7)</td>
</tr>
<tr>
<td>100</td>
<td>4.1 (3.0–5.5)</td>
<td>5.2 (4.8–5.5)</td>
</tr>
<tr>
<td>200</td>
<td>2.4 (1.6–3.7)</td>
<td>4.9 (4.2–5.7)</td>
</tr>
</tbody>
</table>

and the NPRS model was employed instead (Table 4; Fig. 2). This complex interaction between CuPT and Cu was adequately presented by the NPRS model's response surface and contours (Fig. 2D and E). Contours in the isolol contour figure (Fig. 2E) were significantly bowed downward at high Cu levels, but they became more horizontal or even bowed upward slightly at low Cu levels, indicating that there was an obvious synergistic effect between CuPT and Cu at high Cu levels (such as >50 µg/L), and thus synergistic effect became weaker at low Cu level (such as <20 µg/L) where an additive or antagonistic effect might exist.

The modelled mortality rates based on the NPRS model were listed in Table 5 for a comparison with the observed mortality rates. The modelled and actual data were in reasonably good agreement with $R^2$ of 0.79 (single biocide data excluded; Fig. 2F) while the NPRS model also gave a much lower $\sum R^2$ value when compared with the model output generated by CARS and RARS models (Table 4).

3.4. Combined chronic toxicity of ZnPT and Cu

The copepod nauplii exposed to Cu alone at 5, 10, 20, and 50 µg/L had survivorships ≥73% before reaching adult stage, while those exposed to ZnPT alone at 5, 10, 20 and 50 µg/L had a survivorship at 92, 64, 67, and 17%, respectively, before reaching copepodid stage 1, and 83, 56, 45, and 0%, respectively, before reaching adult stage (Fig. 4A and B). When ZnPT (10 and 20 µg/L) was mixed with Cu, survivals of nauplii before reaching copepodid stage 1 and adult stage decreased dramatically with the increase of Cu concentrations (Fig. 4A and B). All copepods died before reaching copepodid stage 1 for binary mixtures of ZnPT at 10 µg/L with Cu at 20 µg/L, and ZnPT at 20 µg/L with Cu at 10 and 20 µg/L; and furthermore, no copepods survived to adult stage for binary mixture of ZnPT at 20 µg/L and Cu at 5 µg/L. Due to the small sample size, only one replicate of survival data could be generated for each treatment.

Fig. 1. Cumulative average mortalities of Tigriopus japonicus after 24, 48, 72 and 96-h incubation under different combinations of zinc pyrithione (ZnPT) and Cu concentrations. Different shapes of data points represent different Cu levels at 0 (○), 1 (△), 2 (▲), 5 (●), 8 (∗), 10 (■), 20 (▲), 50 (▲), 100 (●) and 200 (●) µg/L.
Fig. 2. Response surfaces (A and D) and their contours (B and E) for describing the 96-h acute toxicities for binary mixtures of ZnPT–Cu, and CuPT–Cu to the adult Tigriopus japonicus respectively (ZnPT: zinc pyrithione; CuPT: copper pyrithione). The modelled mortality and the observed mortality (average data of three replicates for each treatment; single biocides data excluded) were also compared using linear regression for the mixture of ZnPT–Cu (C) and CuPT–Cu (F). The response surfaces for the mixtures of ZnPT–Cu and CuPT–Cu, were based on the non-parametric response surface (NPRS) model (see Table 4 for additional information).

Though there was only one replicate, data were combined for sigmoidal concentration–response analysis. At Cu of 0, 5, 10 and 20 μg/L, the LC50 of ZnPT to the copepod was estimated at 22.2, 10.1, 9.7 and 8.1 μg/L respectively, before reaching copepodid stage 1 and 13.5, 7.2, 9.1 and 5.0 μg/L, respectively, before reaching adult stage. The data implied that ZnPT and Cu had a synergistic toxic effect to the nauplia survival especially at high ZnPT or Cu concentrations. Within the 121 copepods that succeeded to reach adult stage, all of them except one survived till the end of the test (i.e. Day 21).

Cu alone at 5, 10, 20 or 50 μg/L did not affect the Dn and Dt significantly when compared with that of the SC, and neither did ZnPT alone at 5, 10, 20 or 50 μg/L for the Dn and at 5 or 10 μg/L for the Dt. Nevertheless, ZnPT alone at 20 μg/L delayed the Dt significantly by 21 d. When ZnPT was combined with Cu, the toxic effect on the copepodid development was more obvious. When 20 μg ZnPT/L was combined with 5 μg Cu/L, only 1 copepod (out of 12) survived to copepodid stage 1 with a delayed development, longer by 7 d, than that of the SC. At 10 μg ZnPT/L, an addition of 5 and 10 μg Cu/L significantly delayed the Dn by 1.4 and 0.9 d respectively, and delayed the Dt by 4 and 3.7 d respectively; while at 5 μg ZnPT/L, an addition of 20 μg Cu/L significantly delayed the Dn and Dt by 1.0 and 1.4 d, respectively, in contrast to that of the SC.

As mass mortality of the copepod occurred at high ZnPT concentrations (i.e. 10, 20 and 50 μg/L), an attempt was made to analyze the data on the life span of copepods which died before reaching adult stage, and the results are shown in Fig. 5. Under the binary mixtures of 10 μg ZnPT/L with 20 μg Cu/L, and 20 μg ZnPT/L with 10 and 20 μg Cu/L, copepods died before reaching adult stage had a significantly lower life span than the copepods raised under other
treatments, with mortality occurring at around nauplia stage-2 (i.e., N2; Fig. 5).

The overall influence of the mixtures on the copepod population was summarized using the intrinsic rate of increase \( r_m \) (Fig. 6). Without Cu alone, \( r_m \) values of copepods were similar in different Cu concentrations (Fig. 6). On the contrary, the \( r_m \) value of copepods exposed to ZnPT alone decreased considerably at \( \geq 10 \mu g/L \). In 5 \( \mu g \) ZnPT/L, the \( r_m \) value generally decreased with increasing Cu concentrations (Fig. 6). At 10 \( \mu g \) ZnPT/L and 10 \( \mu g \) Cu/L, the \( r_m \) value became negative, indicating a high possibility of population extinction (Walthall and Stark, 1997).

4. Discussion

4.1. Acute toxicity of single biocides

In this study, the acute toxicity of Cu alone on adult *T. japonicus* was estimated twice along with the mixture toxicity tests. The sensitivities of adult *T. japonicus* under Cu exposure in the CuPT–Cu test were much lower than the ones in the ZnPT–Cu test (i.e., the 96-h LC50 of Cu generated from the former test was significantly higher than that from the latter one; Table 2). This might be attributable to the differences between the copepod samples collected at different years, although all samples were collected at a similar time of a year (October–November, Table 2). A similar phenomenon was also observed by Jonker et al. (2004) and Kwok et al. (2008). It is therefore important to simultaneously test toxicities of chemical mixtures and individual chemicals with a view to obtaining reliable insight into combined chemical actions (Jonker et al., 2004).

Both ZnPT and CuPT are highly toxic to aquatic organisms according to the limited data in literature (Yamada, 2006; Bao et al., 2011, 2012), and their toxic mode of actions involves disruption of cellular ATP synthesis, inhibition of membrane transport, and complex binding with cellular metals and proteins (Chandler and Segel, 1978; Dinning et al., 1998; Doose et al., 2004; Dahllöf et al., 2005). In this study, CuPT showed a higher acute toxicity than ZnPT to the adult *T. japonicus*, which was in good agreement with the previous findings on the copepod with 96-h-LC50 at 170 and 30 \( \mu g/L \) (Bao et al., 2011), and 24-h-LC50 at 280 and 23 \( \mu g/L \) for ZnPT and CuPT, respectively (Onduka et al., 2007). Similar studies also showed that CuPT was more toxic than ZnPT to aquatic animal species such as the toy shrimp *Heptacarpus fulfilirostris*, the barnacle larvae *Balanus amphitrite*, the amphipod *Elastomopus rapax*, and the polychaete larvae *Hydroides elegans* with acute LC50s of ZnPT and CuPT ranging from 7.6 to 210 \( \mu g/L \) and from 2.5 to 63 \( \mu g/L \) respectively (Mochida et al., 2006; Bao et al., 2011). CuPT was also more toxic to the fish *Pagrus major* (96-h-LC50-ZnPT = 98.2 \( \mu g/L \); 96-h-LC50-CuPT = 9.3 \( \mu g/L \); Mochida et al., 2006) and *Oryzias melastigma* (96-h-LC50-ZnPT = 43 \( \mu g/L \); 96-h-LC50-CuPT = 8.2 \( \mu g/L \); Bao et al., 2011). On the other hand, ZnPT and CuPT showed similar toxicities on the growth of marine diatoms and cyanobacteria, and the toxicity of ZnPT was higher than CuPT to the sea anemone *Aiptasia sp.* (Bao et al., 2011).
4.2. Chronic toxicity of single biocides

Based on our results of the survival and developmental time of *T. japonicus*, ZnPT has a much higher chronic toxicity than Cu. Treatments with Cu at 5, 10, 20 and 50 μg/L slowed down the Dn by 0.1, 0.2, 0.1 and 0.1 d respectively in comparison to the Dn of 4.2 ± 0.1 d for the solvent control (SC), although no significant difference of the Dn was found between the above Cu treatments and the SC during the chronic test for ZnPT–Cu mixtures (Fig. 4). However, Kwok et al. (2008) showed that the development of *T. japonicus* was slowed down slightly (around 0.3 d for Dn) but significantly at 10 μg Cu/L in comparison to the seawater control with Dn of around 6 d. Such discrepancies in Cu sensitivity, in terms of developmental time, between the two studies might be associated with the differences in food supply and experimental set-ups for the copepod life cycle test, and possible dissimilarity in the physiological conditions between the two different batches of copepod populations (Kwok et al., 2008).

The lethal toxicity of ZnPT to copepod larvae (i.e., nauplii and copepodid) was much higher during the chronic test, with a LC50 of ~13.5 μg/L before reaching adult stage (Fig. 4) which was much lower than the 96-h LC50 of 175.5 μg/L for the adult copepod. ZnPT as low as 20 μg/L could adversely affect the copepod development. In other documented studies, ZnPT was found to have high chronic toxicities to aquatic animals. For instance, ZnPT was extremely toxic to embryo development of the sea urchin *Anthocidaris crassispina* with a particularly low no observed effect concentration (NOEC) at 1 × 10−11 μg/L (Kobayashi and Okamura, 2002), while the 28-d LC50 of ZnPT on juvenile rainbow trout *O. mykiss* was only 4.6 μg/L (Okamura et al., 2002). ZnPT at as low as 0.16 and 0.32 μg/L was found to affect the long-term survival and reproduction of the polychaete *Dinophilus gyrociilatus* (Marcheselli et al., 2010a).

4.3. Acute toxicity of chemical mixtures

As demonstrated in this study, the mixtures of ZnPT and Cu exhibited a strong synergistic effect on adult *T. japonicus* especially at high ZnPT or Cu concentrations. The present results are in accordance with previous studies that combined acute toxicity of ZnPT and Cu was found to be synergistic on the bioluminescent bacteria *V. fischeri* (30-min bioluminescent assay, Zhou et al., 2006), the toy shrimp *H. futilisrostris* (96-h acute test, Mochida et al., 2006), the diatom *T. pseudonana* (96-h growth inhibition test), the polychaete larvae *H. elegans* (48-h acute test) and the amphipod *E. rapax* (96-h...
acute test; Bao et al., 2008). However, the interaction of ZnPT and Cu was reported to be slightly more than additive to P. major (Mochida et al., 2006), and strictly antagonistic against the marine diatom Chaetoceros gracilis (Koutsafis and Aoyama, 2007). Both synergistic and antagonistic interactions between ZnPT and Cu were explained by the authors based on the fact that ZnPT could readily be converted to CuPT in the presence of Cu (Grunnett and Dahllöf, 2005).

Our previous study (Bao et al., 2008) also confirmed that ZnPT could transchelate with Cu to form CuPT. CuPT was usually found to be more toxic than ZnPT to aquatic organisms as discussed previously, and it is not surprising that ZnPT and Cu exert synergistic effect in most of the cases listed above, as well as in this study. It is, however, worthy to note that the formation of CuPT in the mixture of ZnPT and Cu only contributes partially to the observed synergistic effect to the copepod, and further study is needed to elucidate the underlying toxic mechanism(s).

This study uncovered a rather complicated mixture toxicity pattern of CuPT and Cu to the adult copepod that the mixture displayed additive to slight antagonistic effect at low Cu concentrations (≤20 μg/L) whereas synergistic effect was observed at high Cu concentrations (≥50 μg/L). This kind of concentration-dependent joint action was also observed by Gatidou and Thomaidis (2007), who reported an interaction of M1 (the major degradant of Irgarol) and Cu to be additive at low Cu levels, but synergistic at high Cu levels to the green algae Dunaliella tertiolecta. On the other hand, the combined effect of carbendazim and Cu on reproduction of the nematode Caenorhabditis elegans was found to be synergistic at low concentrations and antagonistic at high concentrations of the two chemicals (Jonker et al., 2004). The combined toxicity of CuPT and Cu was reported to be synergistic against the marine bioluminescent bacteria Vibrio fischeri (Zhou et al., 2006), and additive to the red sea bream P. major and toy shrimp H. futilirostris (Mochida et al., 2006). However, the conclusion of the latter study was based on averaged mortality data of all binary combinations, and the predicted mortality was estimated based on toxic unit theory rather than using concentration–response functions of the single biocides.

Fig. 5. Life span of copepods Tigriopus japonicus which died before reaching adult stage (bars, mean and SEM) and the stage (open circle, mean and SEM) of the dead copepods that were exposed to binary mixtures of zinc pyrithione (ZnPT) and Cu. Life span and stage data under different concentration combinations were compared using Kruskal–Wallis tests, and bars (or open circle) with the same letter (or number) indicate that the means are not significantly different (p > 0.05). N: nauplius stage; C: copepodid stage.

Fig. 6. Intrinsic rate of increase (r∞) of Tigriopus japonicus that were exposed to binary mixtures of zinc pyrithione (ZnPT) and Cu. ND: not determined as all copepods were dead before reaching adult stage; ND*: not determined as no copepods developed into female adult.
Table 4

Results of the comparison between the two parametric models, Loewe parametric response surface (CARS) and response additive response surface (RARS) models, for describing combined acute toxicity of the biocide mixtures of ZnPT Cu and CuPT Cu. As both parametric models failed to fit the data of the ZnPT Cu and CuPT Cu mixtures, these data were further fitted with the non-parametric response surface (NPRS) model. All datasets (A) and data on binary mixtures only (M, single biocides data excluded) were both analyzed for both CARS and NPRS models, while only M dataset was analyzed using the RARS model as single biocides data could not be analyzed with this model. Interaction parameters (α and p for CARS and RARS respectively; no parameter for NPRS), sum of squared residues ∑R², linear regression between the modelled (y) and observed (x) mortalities (average data of three replicates) were presented where appropriate.

<table>
<thead>
<tr>
<th>Acute test</th>
<th>Data set</th>
<th>n</th>
<th>CARS model</th>
<th>RARS</th>
<th>NPRS</th>
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<tr>
<td>ZnPT + Cu</td>
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<td>126</td>
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<tr>
<td></td>
<td>M</td>
<td>87</td>
<td>217.97</td>
<td>6.40</td>
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<tr>
<td>CuPT + Cu</td>
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<td>M</td>
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<td>−21.34</td>
<td>7.19</td>
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</tr>
</tbody>
</table>

Table 5

The observed (obs) and modelled (model) mortalities of adult Tigriopus japonicus that were exposed to various binary combinations of zinc pyriproxyfen (ZnPT Cu) and copper pyriproxyfen (CuPT Cu) for the 96-h acute toxicity tests. The modelled mortalities were generated based on the non-parametric response surface (NPRS) model. A few modelled mortality rates were out of the range of 0–100%. The negative values (with *) were treated as 0, while the one >100% (with *) was treated as 100% for the calculation of the ∑R² and linear regressions between observed and modelled mortalities (Table 4, Fig. 1 C and F).

<table>
<thead>
<tr>
<th>ZnPT Cu</th>
<th>CuPT Cu</th>
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</thead>
<tbody>
<tr>
<td>[ZnPT], µg/L</td>
<td>[Cu], µg/L</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
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<td>100</td>
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Thus, the difference between the joint actions of CuPT and Cu at different Cu levels might have been overlooked in Mochida et al. (2006).

As such, the synergistic effect of ZnPT and Cu on adult T. japonicus could also be partially attributable to the synergistic effect between the CuPT formed and excessive Cu at high Cu concentrations. In addition, when Cu was in excess in the mixture of ZnPT and Cu, CuPT* (1:1 Cu:pyrithione) might also be formed (Grunnert and Dahllöf, 2005). Thus, the toxicity of the mixture of ZnPT and Cu to the test organisms could be determined by the coexistence of Cu, Zn, CuPT, CuPT* and ZnPT as well as other degradation products in the mixture solution (Grunnert and Dahllöf, 2005). The interaction between ZnPT and CuPT, for instance, had been found to be synergistic to the brine shrimp A. salina (Koutsafi and Aoyama, 2007) and the bioluminescent bacteria V. fischeri (Zhou et al., 2006).

Considering the fact that both ZnPT and CuPT are widely used as booster biocides with Cu in antifouling paints, and that ZnPT could easily be transformed to CuPT in the presence of Cu (Grunnert and Dahllöf, 2005), the strong synergistic interaction between ZnPT and Cu, as well as the possible synergistic interactions between the CuPT and Cu should be addressed when assessing the environmental risks of ZnPT and CuPT as antifouling agents.

4.4. Chronic toxicity of chemical mixtures

In agreement with the results of the acute toxicity test on adult copepods, the joint chronic toxicity of ZnPT and Cu on the survival of the copepod larvae and the growth of copepod population (as reflected by the intrinsic rate of increase, $r_m$) was also found to be synergistic, which might be attributable to the formation of more toxic CuPT due to the transchelation between ZnPT and Cu, and the further combined effect of CuPT and Cu at elevated concentrations. At the environmentally realistic level of 10 μg Cu/L, ZnPT as low as 10 μg/L significantly slowed down the copepod development, led to a mass mortality (92%) of the copepod larvae before reaching adult stage, and caused an obvious population decline (Figs. 4 and 6); while in comparison, this combination of ZnPT and Cu only caused 2% mortality on adult copepods during the 96-h acute test. Such a strong synergistic chronic effect should not be neglected while developing appropriate water quality criteria for ZnPT. More information on the chronic toxicity of CuPT alone and in combination with Cu, as well as the mixture toxicity of ZnPT, CuPT and Cu on the copepod is required to explain the synergistic phenomenon between ZnPT and Cu.

4.5. Response surface models

This study demonstrated the application of Loewe parametric response surface (CARS) model, the response addition response surface (RARS) model, and the non-parametric response surface (NPRS) model on the analysis and prediction of binary mixture toxicity of the antifouling biocides (ZnPT and CuPT) and Cu. Both CARS and RARS models are parametric, and have advantages to not only indicate the deviation of the observed binary mixture toxicity from the modelled toxicity (if no interaction occurred), but also provide a quantitative measurement of the deviation using interaction parameters (i.e. α of CARS model and $\rho$ of RARS model), which can be valuable data for risk assessment of chemical mixtures. However, both parametric (CARS and RARS) models failed to describe the joint toxicity of ZnPT–Cu and CuPT–Cu mixtures.

In the present study, the NPRS model and its contour were proven to be extremely useful to describe and model complicated mixture toxic effects like those of ZnPT–Cu and CuPT–Cu mixtures. As described previously, ZnPT can transchelate with Cu to form CuPT, and complex interactions might happen between the possibly coexisting Cu, Zn, CuPT, CuPT* and ZnPT in the mixture solution, which result in the synergistic toxicity of ZnPT–Cu mixture. That could possibly explain why ZnPT–Cu mixture toxicity data could not be fitted by the parametric CARS and RARS models which are based on a simple additive effect of two non-interactive chemicals. The results were also consistent with our previous findings that ZnPT–Cu binary mixture toxicity data of three other marine species could be fitted well by the NPRS model, but not the CARS model (Bao et al., 2008). The failure of the CARS model for CuPT–Cu mixture toxicity data might be due to the fact that the acute toxicities of CuPT and Cu alone on T. japonicus had very different Hill’s slopes (Table 2) and hence different toxic mode of actions. The NPRS model could be an extremely powerful tool for modelling mixture toxicity, especially when parametric response surface models failed to describe complicated joint toxicities. The NPRS model could also be a useful tool to reveal the concentration dependent joint action between two biocides, such as the case of CuPT and Cu mixtures in this study.

5. Conclusion

ZnPT expressed a higher chronic toxicity on the copepod T. japonicus in terms of their developmental time and population growth, when compared with the corresponding acute toxicity endpoint generated from adult copepods. The joint action of ZnPT and Cu clearly showed a strong synergistic effect on T. japonicus in both acute and chronic tests. Such a synergistic effect may be attributed to the observations that (1) CuPT can be formed via the transchelation of Zn on the ZnPT by Cu, (2) CuPT was more toxic than ZnPT to the copepod and (3) a synergistic acute effect of CuPT and Cu was also observed in this species at high Cu concentrations. The non-parametric response surface model (NPRS) was proven to be a powerful method for analyzing complicated binary mixture toxicities. Since elevated Cu concentrations in ambient water and sediment are very common in many coastal areas worldwide (Hall and Anderson, 1999; Schiff et al., 2007), good understanding of the combined toxicity of the commonly used booster biocide (e.g., ZnPT or CuPT) and Cu is prerequisite for sound ecological risk assessments of these co-existing contaminants. It is also advocated to revise and tighten existing water quality criteria for the biocides, like ZnPT and CuPT, by accounting for their compelling synergistic effects with Cu at environmentally realistic levels.

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